Effects of different cytokinins on *in vitro* multiplication of *Prunus* 'Capdeboscq' rootstocks

Aparecido Lima da Silva*; Marcelo Rogalski and Miguel Pedro Guerra
Universidade Federal de Santa Catarina, Departamento de Fitotecnia, Caixa Postal 476, CEP 88040-900, Florianópolis, SC, Brasil. (* Corresponding Author. E-mail: alsilva@cca.ufsc.br)

ABSTRACT

Rootstocks for peach trees are obtained in Brazil from seeds without genetic uniformity or health guarantee. Micropropagation techniques supply genetically uniform shoots but the *in vitro* culture protocols must be optimized. Cytokinins are essential for breaking the apex dormancy and inducing auxiliary shoot proliferation that influence the success of *in vitro* multiplication. The objective of this study was to assess the effect of different cytokinins *in vitro* multiplication rate, and on the survival rate of apical and axyllary buds in the establishment of *Prunus* 'Capdeboscq' rootstocks. Apical and axyllary buds were inoculated in test tubes containing 10mL Lepoivre culture medium, supplemented with BAP (0.5 mg L^{-1}). After two subcultures of the *in vitro* establishment process, 1-2 cm nodal segments were treated with kinetin, isopentenyladenine and BAP, at concentrations of 0.1; 0.5; 1.0 and 2.0 mg L^{-1}. At the *in vitro* establishment phase, stem tips presented greater survival, less contamination and no hyperhidricity. BAP gave the highest *in vitro* multiplication rate, with 25.9 shoots/explant at the 1.5 mg L^{-1}. The different cytokinins did not result in differences in shoot height. The best results for the number of shoots greater than 20 mm were obtained with BAP at 1.0 mg L^{-1}.

KEY WORDS: Peach tree, rootstock, hyperhydricity, *in vitro* propagation.

INTRODUCTION

Most rootstocks used in peach and plum tree cutting production in Brazil are *Prunus* (*Prunus persicus* (L.) Batsch), from the Pelotas Experimental Station, obtained by open pollination from a crossing between the 'Lake City´ cultivar and a local selection called 'Intermediário´ (Finardi, 1998).

Cutting production technology for peach trees is based on grafting on rootstocks obtained from seeds from the preserves industry. In this process, the varieties are mixed, resulting in unequal plants, early death and principally lack of genetic identity (Fachinello, 2000).

Fachinello (2000) stated that the sustainability of the fruit production sector depends on implanting certification programs and using cuttings with proven genetic-sanitary quality. Micropropagation techniques have been routinely used for clone mass of fruit tree species that provide mother trees and cuttings with high genetic and sanitary quality (Sansavini, 1989; Damiano and Palombi, 2000). These techniques have been used in fruit cropping since the end of the 1970s, first for crops such as strawberries and blackberries, and later for several woody fruit trees, including peach tree rootstocks (Zimmerman and Debergh, 1991) and plum trees in general (Quoirin et al., 1977; Ambrozic-Turk et al., 1991; Pérez-Tornero and Burgos, 2000).

General protocols for micropropagation of several fruit tree species have been used, but the *in vitro* conditions must be optimized for most of them, especially for the woody species (Damiano and Palombi, 2000). Plant growth regulators are very important among determining conditions. The cytokinins are essential in the multiplication phase to break bud dormancy and induce proliferation of the auxiliary shoots.

During the micropropagation of *Prunus sp*, the cytokinins and the concentration are the most important factors for the multiplication rate and shoot elongation (Leontiev-Orlov et al., 2000a) along with the genotype dependent responses (Leontiev-Orlov et al., 2000b; Pérez-Tornero and Bugos, 2000) and with hyperhydricity (Ambrozic-Turk et al., 1991; Bouza et al., 1992).

The present study aimed at assessing the *in vitro* multiplication rate of the *Prunus* 'Capdeboscq' rootstock in response to different cytokinins supplemented in the culture medium.

MATERIAL AND METHODS

Plant Material

Explants were excised from stock plants of *Prunus* 'Capdeboscq' originated at the Epagri Experimental Station, Videira, SC, and maintained in a greenhouse at the Plant Science Department, CCA, UFSC.

In vitro establishment

Actively growing shoots were collected, divided into segments with three or four shoots, and disinfected. These shoots were then washed with water and detergent (10 drops L^{-1} Tween 20), agitated for one minute in 70% ethanol and for 15 minutes in sodium hypochloride (1.25%), and finally washed three times with distilled water autoclaved in a flow chamber.

Stem and axyllary buds were inoculated in test tubes (25 x 150 mm) containing 10 mL culture medium consisting of the Lepoivre salts (Quoirin et al., 1977) supplemented with sucrose (20 $g.L^{-1}$), agar (7 g L^{-1}), BAP - 6 benzylaminopurine (0,5 mg L^{-1}). The pH of the culture medium was adjusted to 5.2-5.3 prior to autoclaving at 121 ^{o}C for 15 minutes.

After 30 days , the cultures were assessed for survival rate, hyperhydricity and contamination. A randomized complete block experimental design was used, with five stem tips and side shoots per replication and five replications per treatment.

In vitro multiplication

To assess the *in vitro* multiplication, three cytokinins were tested: 6-furfurylaminopurine (KIN), N^{6}-[2-Isopentenyl]adenine (2iP) e 6-benzylaminopurine (BAP) at concentrations of 0.1; 0.5; 1.0 and 2.0 mg L^{-1}. After two subcultures, every 21 days, 1-2 cm nodal segments with the apical buds removed were inoculated in the modified Lepoivre culture medium described in the establishment phase.

The experiment was arranged in a completely randomized block design with five explants per replication and five replications per treatment. The number of shoots per explant, mean shoot height (mm) and the number of shoots greater than 20 mm was recorded after 30 days of cultivation, and submitted to a statistical analysis.

In vitro culture conditions

Plant material was kept in a growth chamber at 25±2 ^{o}C 16 hour photoperiod and 40-45 $mmol.m^{-2}.s^{-1}$ light intensity supplied by cold white fluorescent lamps.

Statistical analysis

The total mean data for the *in vitro* establishment process and the effect of the cytokinins and their concentrations were submitted to the analysis of variance (ANOVA) and to the SNK means separation test (5%). Data on the effect of the concentration of each cytokinins were submitted to polynomial regression analysis, according to Sokal and Rohlf (1995).

RESULTS AND DISCUSSION

In vitro establishment

The types of explant used affected the survival and contamination rates significantly (Figure 1). The apical buds presented greater survival rate (52%) and lower contamination rate (12%) compared to the axyllary buds which presented 20% survival and 28% contamination. These results can be considered consistent for *in vitro* establishment of *Prunus* and are in according with the survival rates of 27% to 93% obtained by Hammerschlag (1982) in the *in vitro* establishment of 11 peach tree cultivars.

The contamination values observed in the present study are similar to those reported for the *Prunus* genus by Pevalek-Kozlina and Jelaska (1987) ,who observed significant genotype effects, matrix plant age, explant type and culture medium composition. There was no significant difference among apical and axyllary buds for the physiological disturbance known as 'hyperhydricity; however, the apical buds did not present hyperhydricity, whereas this disturbance occurred in 8% of the explant axyllary buds.

The most widely used method for *in vitro* establishment is based on the use of nodal segments in peach trees. This technique normally results in a high contamination rate, and, in some cases, the merystem culture has to be used to solve or alleviate the problem (Rodrigues et al., 1999; Pérez-Tornero et al., 1999).

The process used in this study, which involved keeping stock plants in a greenhouse with systematic

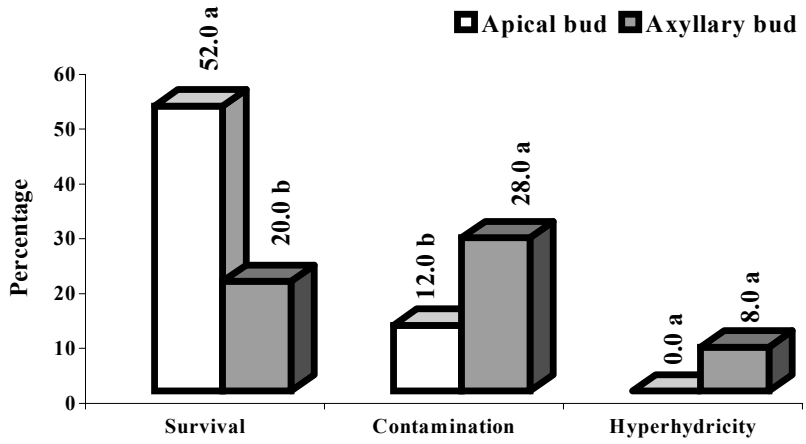

Figure 1. Effect of explant type on the survival, contamination and hyperhydricity in the *in vitro* establishment of 'Capdeboscq' rootstock, after 30 days in Lepoivre's culture medium supplemented with BAP (0.5 mg L^{-1}). UFSC, Florianópolis-SC, 2002.

pathogen control, allowed the successful *in vitro* establishment of the 'Capdeboscq' peach tree cultures (Figure 5). This methodology has greatly reduced explant contamination and death rates in the *in vitro* establishment of different fruit tree species.

Cytokinin effect on *in vitro* multiplication

The number of shoots per explant was significantly affected by the type and level of cytokinin, and the cytokinin x concentration interaction. The cytokinins used showed different responses to shoot number. BAP was the most efficient cytokinin in the proliferation of axyllary buds. The highest bud number (24.3) was obtained in response to the level of 1.0 mg L^{-1} of BAP (Table 1) with no statistical differences among the values obtained with the level of 2.0 mg L^{-1} (23.2 buds/explant). Superior results in response to BAP for shoot induction compared to other cytokinins have been reported in different *Prunus* species (Ambrozic-Turk et al., 1991; Leontiev-Orlov et al., 2000a).

The greatest number of shoots obtained by adjusting the regression equation was 25.9 shoots per explant in response to the concentration of 1.5 mg L^{-1} BAP (Figure 2). These results are similar to those observed by Arena and Caso (1992) who obtained a variation of 5.0 and 37.0 shoots per explant in *Prunus* rootstocks using BAP at concentrations of 0.5 and 1.0 mg L^{-1}. Zimmerman and Scorza (1994) reported that BAP at a 2.3 mg L^{-1} concentration resulted in a greater shoot multiplication rate for three peach genotypes.

However, Hammerschlag et al. (1987) obtained a greater peach *in vitro* multiplication rate, with a superior BAP concentration (4.0 mg L^{-1}); however, tip necrosis occurred. These authors reported that the reduction in the concentration to 2.0 mg L^{-1} BAP allowed normal shoot growth.

Mean shoot height was affected only by the concentration factor. Thus the mean height of the shoots was reduced from 9.4 to 7.0 mm with increased concentrations of the different cytokinins used (Table 1), and this effect was observed for the different cytokinins (Figure 3).

These results are similar to those obtained by Leontiev-Orlov et al. (2000a, 2000b) and Pérez-Tornero et al. (2000) who observed that increasing levels of cytokinins inhibited shoot elongation in plum trees. Leontiev-Orlov et al. (2000a) observed that BAP concentrations greater than 0.1 mg L^{-1} inhibited shoot growth in plum trees. However, for these authors, the use of different 2iP concentrations (0.1 to 1.5 mg L^{-1}) increased shoot height, but with a reduction in the multiplication rate. However, *Prunus mume* mean shoot size was not influenced by BAP concentrations (Harada and Murai, 1996).

Type of cytokinin only affected the mean total of shoots greater than 20 mm. Culture media supplemented with BAP resulted in values significantly greater to those with KIN or 2iP (Table 1). The best results for the number of shoots greater than 20 mm was obtained by the level of 0.8 mg L^{-1} BAP with the presence of 1.39 shoots per explant (Figure 4). These results are similar to those reported

Table 1. Effect of three cytokinins (KIN, 2iP and BAP) and four concentrations (0.1, 0.5, 1.0 and 2.0 mg L^{-1}) on the number of shoots, shoot height (mm) and number of shoots >20 (mm) of *Prunus* 'Capdebosq' rootstock cultured *in vitro* after 28 days in culture (means of five replicates). UFSC, Florianópolis-SC, 2002 [1/].

	Concentrations (mg L^{-1})				
Cytokinins	0.1	0.5	1.0	2.0	
		Number of shoots			Means*
KIN	6.3 c	7.8 c	10.0 c	10.7 c	8.7 B
2iP	7.5 c	8.0 c	8.0 c	9.3 c	8.2 B
BAP	8.3 c	16.3 b	24.3 a	23.2 a	18.0 A
Means**	7.4 c	10.7 b	14.1 a	14.4 a	
CV (%)			21.7		
		Shoot height (mm)			Means[(r
KIN	9.9	8.6	7.4	7.9	8.4 A
2iP	9.4	9.0	8.0	7.5	8.4 A
BAP	8.8	7.6	6.9	5.8	7.2 A
Means*	9.4	8.4 ab	7.4 b	7.0 b	
CV (%)			25.7		
		Number of shoots >20 (mm)			Means*
KIN	0.8	0.9	0.9	0.7	0.8 B
2iP	0.7	0.6	0.7	0.8	0.7 B
BAP	1.2	1.3	1.4	0.9	1.2 A
Means[(ns)]	0.9 a	0.9 a	1.0 a	0.8 a	
CV (%)			53.2		

[1/] Means with the same letter within a same row (capital letter) or a same column and interaction among factors: cytokinins x concentrations (minuscule letter) are not significantly different according to the SNK test ([(ns)] non significant differences at 0.05 level of probability, * significant at P<0.05, ** significant at P<0.01).

by Arena and Caso (1992) , who observed the greatest number of shoots superior to 15 mm in response to 0.5 mg L^{-1} BAP concentration.

BAP has been the most used cytokinin for *Prunus in vitro* multiplication (Pérez-Tornero and Burgos, 2000). The great activity of this plant growth regulator may be related to its chemical structure (Taiz and Zeiger, 1998). However, high BAP concentrations can induce reduction in shoot height and increase in the hyperhydricity rates in plum trees (Ambrozic-Turk et al., 1991; Bouza et al., 1992; Harada and Murai, 1996; Leontiev-Orlov et al., 2000a, 2000b).

Furthermore, the optimum concentration of a cytokinin is highly dependent on the genotype. Pérez-Tornero et al. (2000) and Leontiev-Orlov et al. (2000b) reported that there is a highly significant effect between the BAP concentration and the genotype in the *in vitro* multiplication of different *Prunus* species. Other important factors in multiplication are the saline composition of the medium and the plant growth regulators used (Rogalski et al., 1999; Leontiev-Orlove et al., 2000a; Pérez-Tornero and Burgos, 2000).

CONCLUSIONS

Results obtained by the present study led to the conclusion that the methodology developed for *in vitro* establishment by stem tips and side shoots was efficient for the micropropagation of the *Prunus* 'Capdeboscq' rootstock and that BAP was the most efficient cytokinin for *in vitro* multiplication, with a mean regenerative rate of 25.9 shoots per explant, at the 1.5 mg L^{-1} concentration. It was also observed, in the range tested, that increased concentration of cytokinins inhibited shoot elongation.

RESUMO

Efeito de diferentes citocininas na multiplicação *in vitro* do porta-enxerto de *prunus* 'Capdeboscq'

No Brasil, os porta-enxertos para prunáceas são obtidos de sementes, sem uniformidade genética e garantia sanitária. Técnicas de micropropagação permitem a obtenção de mudas geneticamente uniformes, porém, torna-se necessário otimizar os

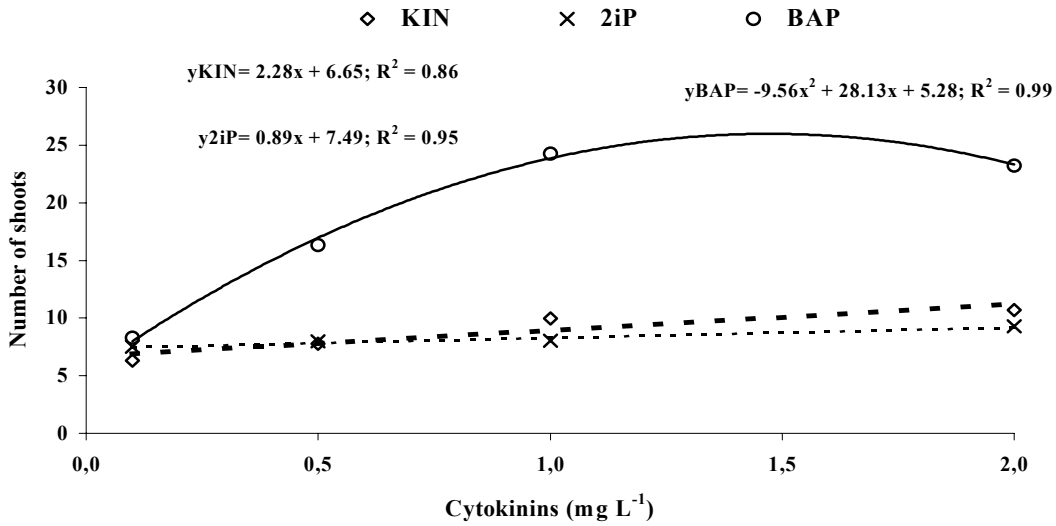

Figure 2. Effect of cytokynins on the shoot regeneration rate on the *in vitro* multiplication of *Prunus* 'Capdeboscq' rootstock, after 28 days in Lepoivre's culture medium. UFSC, Florianópolis-SC, 2002.

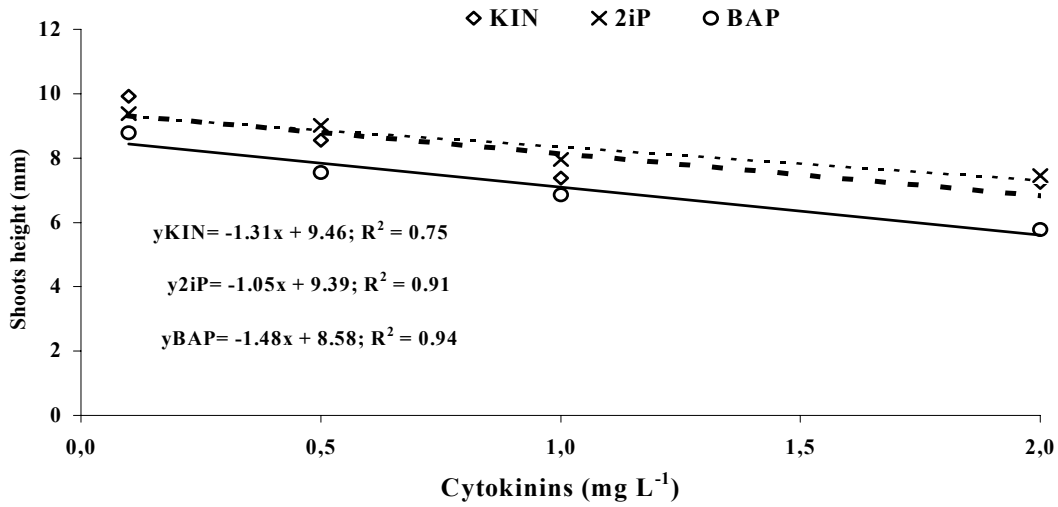

Figure 3. Effect of different cytokynins in the height of shoots (mm) in the *in vitro* multiplication of *Prunus* 'Capdeboscq' rootstock after 28 days in Lepoivre's culture medium. UFSC, Florianópolis-SC, 2002.

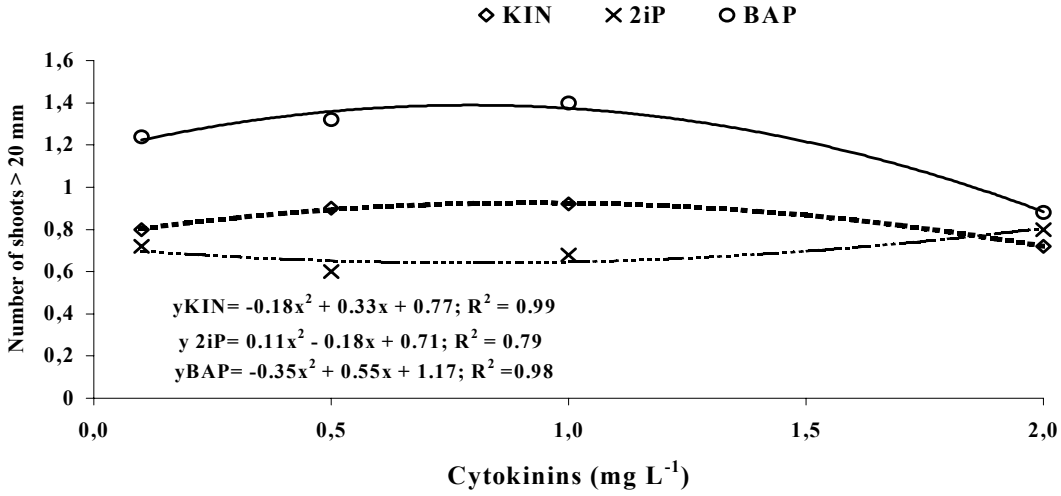

Figure 4. Effect of cytokynins on the shoot number greater than 20 mm on the *in vitro* multiplication of *Prunus* 'Capdeboscq' rootstock, after 28 days in Lepoivre's culture medium. UFSC, Florianópolis-SC, 2002.

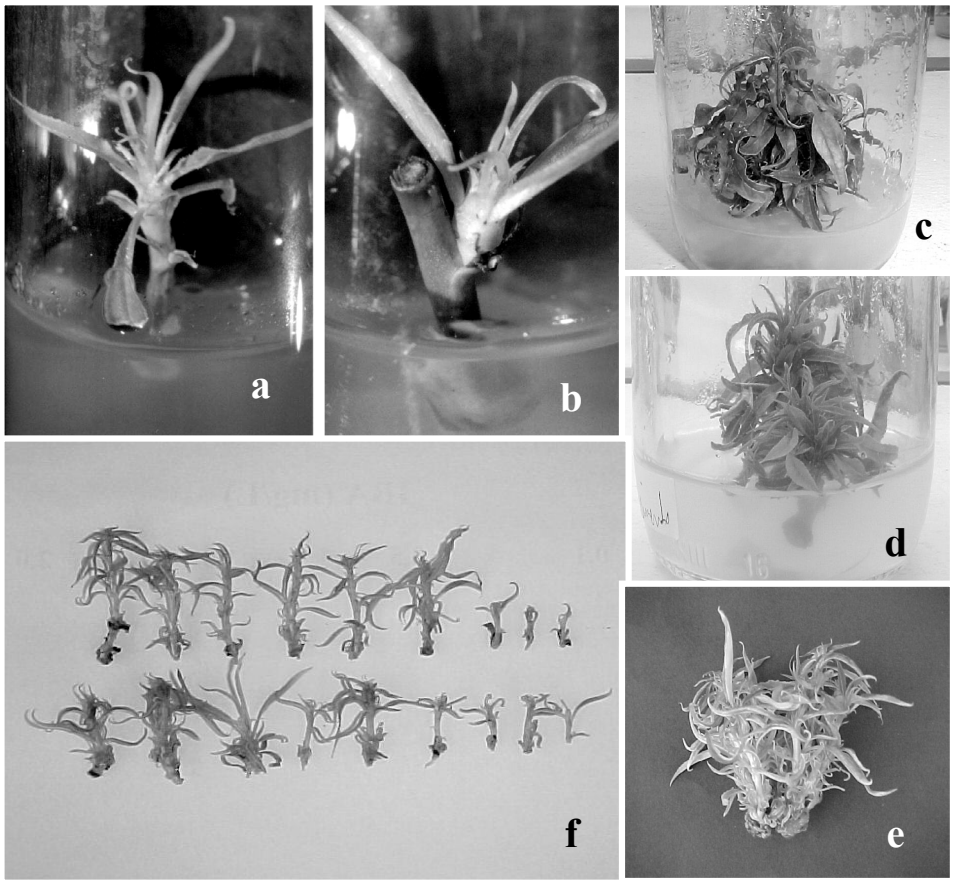

Figure 5. Morphogenetic features in the *in vitro* culture of *Prunus* 'Capdeboscq' rootstock. a) stem bud after15 days in culture. b) axylary bud after 15 days in culture. c) first subculture after 21 days. d) second subculture after 15 days. e) culture maintained in Lepoivre's medium supplemented with BAP (0.5 mg L^{-1}), after 28 days *in vitro*. f) same culture in details. UFSC, Florianópolis-SC, 2002.

protocolos de cultura *in vitro*. As citocininas são indispensáveis para a quebra de dominância apical e para a indução de proliferação de gemas axilares, influenciando no sucesso da multiplicação *in vitro*. O objetivo deste estudo foi avaliar o efeito de diferentes citocininas na taxa de multiplicação *in vitro*, bem como o índice de sobrevivência de ápices caulinares e gemas laterais no estabelecimento do porta-enxerto de *Prunus sp.* 'Capdeboscq'. Ápices caulinares e gemas laterais foram inoculados em tubos de ensaio contendo 10mL de meio de cultura de Lepoivre, suplementado com BAP (0.5 mg L^{-1}). Após duas subculturas do processo de estabelecimento *in vitro*, segmentos nodais com 1-2 cm foram submetidos aos tratamentos com KIN, 2iP e BAP, nas concentrações de 0.1; 0.5; 1,0 e 2.0 mg L^{-1}. Na fase de estabelecimento *in vitro*, ápices caulinares apresentaram maior sobrevivência, menor contaminação e ausência de hiperhidricidade. BAP proporcionou as maiores taxas de multiplicação *in vitro*, com 25.9 brotos/explante na concentração de 1.5 mg L^{-1}. As diferentes citocininas não resultaram em diferenças quanto à altura dos brotos. Para o número de brotos superior a 20 mm, os melhores resultados foram obtidos com BAP na concentração de 1.0 mg L^{-1}.

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