Notas Científicas

In vitro organogenesis from internodal segments of adult sweet orange plants

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Abstract – The objective of this work was to optimize in vitro plant regeneration via organogenesis from tissues of adult 'Hamlin', 'Pêra', and 'Valência' sweet orange plants. Explants were grown in EME culture medium with different concentrations of 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA), at 27ºC in the absence of light for 50 days, followed by a 16-hour photoperiod for 20 days. Regeneration was assessed 50 and 70 days after in vitro culture. Organogenesis in cultivars Hamlin and Valência was promoted by EME supplemented with BAP, while NAA showed no apparent effect.

Index terms: *Citrus sinensis*, adventitious buds, biotechnology, plant growth regulators.

Organogênese in vitro a partir de segmentos internodais derivados de plantas adultas de laranja doce

Resumo – O objetivo deste trabalho foi otimizar a regeneração in vitro via organogênese a partir de tecidos de plantas adultas das laranjas doces 'Hamlin', 'Pêra' e 'Valência'. Os explantes foram cultivados em meio EME com diferentes concentrações de 6-benzilaminopurina (BAP) e ácido naftalenoacético (ANA), a 27ºC sem luz por 50 dias, seguido de fotoperíodo de 16 horas por 20 dias. A regeneração foi avaliada 50 e 70 dias após o cultivo in vitro. A organogênese nas cultivares Hamlin e Valência foi favorecida pelo uso de EME suplementado com BAP, enquanto ANA não apresentou efeito aparente.

Termos para indexação: *Citrus sinensis*, gemas adventícias, biotecnologia, reguladores vegetais.

In vitro regeneration and genetic transformation of citrus plants are generally successful. However, this process is usually limited to the use of juvenile plant tissue, such as embryogenic cells (Hidaka et al., 1990), epicotyl segments from seedlings germinated in vitro (Cervera et al., 2000), and internodal segments from juvenile plants grown in greenhouses (Peña et al., 1997). Plants regenerated from these explants exhibit juvenile traits, which are undesirable for seedling production and genetic improvement.

Tissues derived from adult plants are not normally used for in vitro culture, mainly because of high levels of contamination, low morphogenetic capacity related to progressive repression or inactivation of gene activity during plant development, and low rooting of the shoots obtained (Almeida et al., 2003). However, transgenic plants obtained from tissues of adult citrus plants have been reported in a few studies using 'Pineapple' (Cervera et al., 1998) and 'Hamlin' (Almeida et al., 2003) sweet oranges [*Citrus sinensis* (L.) Osbeck]. Due to the great difficulty in achieving in vitro citrus regeneration from adult plant tissue, different types and concentrations of plant growth regulators have been used to optimize the protocol for each cultivar.

Despite this difficulty, the development of an efficient protocol for this type of tissue would be extremely advantageous, since the plants obtained would not exhibit juvenile traits, allowing for a faster assessment and selection.

The objective of this work was to optimize in vitro plant regeneration via organogenesis from tissues of adult 'Hamlin', 'Pêra', and 'Valência' sweet orange plants.

The experiments were carried out at Laboratório de Biotecnologia de Plantas Hortícolas of Escola Superior de Agricultura Luiz de Queiroz, in Piracicaba, SP, Brazil,

from August to October 2008. Fifteen-month old certified nursery trees of 'Hamlin', 'Pêra', and 'Valência' sweet oranges, grafted onto Rangpur lime [*Citrus limonia* (L.) Osbeck], were used. The trees were grown in 12-L plastic pots and kept in greenhouse. New buds formed on the lateral branches were collected and sterilized in a commercial solution of 2.5% sodium hypochlorite diluted with distilled water to 30% for 20 min, then they were washed three times in sterile water. Internodal segments (0.8–1.0 cm long) were prepared and used as explants to induce in vitro organogenesis.

The explants were grown horizontally in Petri dishes (90x15 mm) containing 20 mL of EME culture medium (Grosser & Gmitter Junior,1990), supplemented with 6-benzylaminopurine (BAP) (0.0, 1.5, 3.0, and 4.5 mg L^{-1}) and naphthaleneacetic acid (NAA) (0.0 and 0.3 mg L^{-1}), and were kept in the dark at 27°C for 50 days. During this period, the explants were transferred to new culture media every two weeks. Subsequently, the explants were transferred to Magenta boxes with the same culture medium and placed in a growth room with a 16-hour photoperiod (40 µmol m^{-2} s^{-1}) at 27°C, in which they remained for another 20 days. All culture media were supplemented with 50 g L^{-1} of sucrose, 0.8% agar, with pH adjusted to 5.8 prior to autoclaving (121°C for 20 min).

A completely randomized block design was used, with eight treatments and six replicates. Each replicate consisted of a Petri dish containing six internodal segments.

Regeneration was assessed by the percentage of explants with buds after 50 days of incubation in the dark and by counting the number of shoots per explant after 20 days under a 16-hour photoperiod. Data were subjected to nonparametric analysis of variance, and means were compared by the Tukey-Kramer test at 5% probability.

In vitro plant regeneration via organogenesis was successful for cultivars Hamlin and Valência. After approximately 30 days, early callus formation was observed at the distal region where the explants were cut. Calli differentiation led to the formation of adventitious buds after 50 days of in vitro culture in the absence of light. These results indicate that the development of the explants obtained from adult plant tissue occurred indirectly, which is in accordance with Almeida et al. (2003).

Adventitious bud development, leading to the formation of shoots, occurred after 20 days in a 16-hour photoperiod. Although this photoperiod is important for adventitious bud development and shoot formation, the initial absence of light during culturing improved organogenesis, as previously reported (Almeida et al., 2003). The other buds only developed after the material was subjected to the 16-hour photoperiod (Table 1).

The presence of the cytokinin (BAP) was essential for the development of adventitious buds in all cultivars studied (Table 1), with optimum concentrations depending on the cultivar. The best results, expressed as the percentage of explants with buds and the number of shoots per explant, were recorded for cultivars Hamlin and Valência, using high concentrations of BAP.

Table 1. In vitro organogenesis of adult plant internodal segments of three sweet orange (*Citrus sinensis*) cultivars according to different concentrations of BAP and NAA[1].

BAP and NAA concentration (mg L^{-1})	Responsive explants (%)[2]			Number of shoots per explant[3]		
	'Hamlin'	'Pêra'	'Valência'	'Hamlin'	'Pêra'	'Valência'
0.0 + 0.0	0.00b	0.00a	0.00c	0.16abc	0.31a	0.19bc
0.0 + 0.3	0.00b	0.00a	0.00c	0.07bc	0.09a	0.03bc
1.5 + 0.0	0.00b	4.17a	0.00c	0.00c	0.24a	0.22bc
1.5 + 0.3	0.00b	4.17a	0.00c	0.05bc	0.05a	0.00c
3.0 + 0.0	4.17b	6.25a	35.42a	0.33bc	0.86a	1.93a
3.0 + 0.3	0.00b	0.00a	43.75a	0.17bc	0.06a	1.39a
4.5 + 0.0	41.37a	7.50a	6.25bc	0.95a	0.75a	0.17bc
4.5 + 0.3	14.58ab	2.50a	27.50ab	0.76ab	0.79a	1.20a
Standard deviation	18.8	22.5	6.9	3.1	2.1	2.3
CV (%)	27.88	31.56	37.45	27.43	35.03	37.20

[1]Means followed by equal letters, in the columns, do not differ by the Tukey-Kramer test at 5% probability. [2]Total of 36 explants used in the treatment; measurements taken after 50 days of cultivation in the absence of light. [3]Measurements taken after 70 days of cultivation (50 days of cultivation in the absence of light plus 20 days of cultivation under a 16-hour photoperiod). BAP, 6-benzylaminopurine; NAA, naphthaleneacetic acid.

'Pêra' sweet orange explants showed low percentage of organogenesis for in vitro regeneration under the same conditions.

The addition of NAA was not necessary for organogenesis in this study. This result differs from those previously obtained by Almeida et al. (2003).

The use of cytokinins and auxins (NAA) as supplements in the culture medium has led to inconsistent results in *Citrus* spp., as shown by adventitious bud induction. According to Almeida et al. (2003), cytokinin has greater influence on the regeneration of explants than auxin, since, even in the absence of NAA, there was a high percentage of explants with buds, which was also confirmed in the present study.

Studies involving internodal segments obtained from adult plant tissues of 'Pêra', 'Valência', and 'Bahia' sweet oranges and 'Rangpur' lime showed increased regeneration with an increased dose of BAP, regardless of the cultivar or the NAA concentration. Even though the use of BAP and NAA was required for maximum bud production in all evaluated cultivars, the optimum ratios for BAP/NAA concentration varied for each one (Oliveira et al., 2010).

Cervera et al. (2008) also found a higher number of buds per explant from adult plant tissues of 'Clementine' tangerine, using BAP as supplement. Kobayashi et al. (2003), studying in vitro organogenesis of 'Pêra' sweet orange, observed up to 54% explant regeneration using lower concentrations of BAP in association with gibberellic acid in the medium.

Due to the lack of consistent results on the use of plant growth regulators in the culture medium (Moreira-Dias et al., 2000; Silva et al., 2005), their benefit in stimulating organogenesis remains unclear. Other growth factors may have a greater effect in optimizing organogenesis from adult explants (Oliveira et al., 2010).

The concentration of BAP in the EME medium has a variable effect on in vitro organogenesis of internodal segments from adult plants tissue of 'Hamlin' and 'Valência' sweet oranges, while NAA has no apparent effect.

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