Notas Científicas

Tissue culture parameters in sweet orange cultivars

Amanda Ferreira da Silva Mendes[1], Luciana Cardoso Cidade[1], Gabrielle Novais Manzoli[1], Wagner Campos Otoni[2], Walter dos Santos Soares Filho[3] and Marcio Gilberto Cardoso Costa[1]

[1]Universidade Estadual de Santa Cruz, Departamento de Ciências Biológicas, Rodovia Ilhéus-Itabuna, Km 16, CEP 45662-000 Ilhéus, BA, Brazil. E-mail: amandaf.mendes@gmail.com, lccidade@yahoo.com.br, gabymanzoli@yahoo.com.br, mcosta@labbi.uesc.br [2]Universidade Federal de Viçosa, Departamento de Biologia Vegetal, Avenida P.H. Rolfs, s/nº, CEP 36570-000 Viçosa, MG, Brazil. E-mail: wotoni@ufv.br [3]Embrapa Mandioca e Fruticultura Tropical, Caixa Postal 007, CEP 44380-000 Cruz das Almas, BA, Brazil. E-mail: wsoares@cnpmf.embrapa.br

Abstract – The objective of this work was to establish tissue culture parameters for gene transfer in sweet orange cultivars. Epicotyl explants with different ages were cultured with 6-benzylaminopurine (BAP), kanamycin and hygromycin. Shoots were cultured with alpha-naphthaleneacetic acid (NAA) alone or in combination with indole-3-butyric acid (IBA). The requirement of BAP for shoot development was genotype-specific. Epicotyl explants from 35-day-old seedlings produced significantly more shoots per explant in 'Pêra'. Kanamycin inhibited shoot regeneration for the most cultivars. The percentage of shoots that produced roots in 'Pêra' was significantly higher in medium with NAA and IBA than with NAA alone.

Index terms: *Citrus sinensis*, biotechnology, gene transfer, morphogenesis, regeneration.

Parâmetros de cultura de tecidos em cultivares de laranjeira-doce

Resumo – O objetivo deste trabalho foi estabelecer parâmetros de cultura de tecidos para transferência gênica em cultivares de laranjeira-doce. Explantes de epicótilo com idades diferentes foram cultivadas com 6-benzilaminopurina (BAP), canamicina e higromicina. Os brotos foram cultivados só com ácido alfa-naftalenoacético (ANA), ou ANA em combinação com ácido indolbutírico (IBA). O requerimento de BAP para o desenvolvimento de broto foi genótipo-específico. Explantes de epicótilo de plântulas com 35 dias de idade produziram mais brotos por explante em 'Pêra'. A canamicina inibiu a regeneração de broto, na maioria das cultivares. A percentagem de brotos que produziram raízes em 'Pêra' foi significativamente maior em meio com ANA e IBA do que só com ANA.

Termos para indexação: *Citrus sinensis*, biotecnologia, transferência gênica, morfogênese, regeneração.

A prerequisite for successful gene transfer to plants is the establishment of a suitable system of in vitro regeneration and recovering of whole plants from transformed cells. In *Citrus*, the morphogenic response of in vitro cultures depends on genotype, explant type and orientation, composition of culture medium, and conditions of incubation (Durán-Vila et al., 1989; Ghorbel et al., 1998; García-Luis et al., 1999; Bordon et al., 2000; Costa et al., 2004). Epicotyl segments from in vitro germinated seedlings have a higher morphogenic ability than other tissues used as explant source. The supplementation of the growth regulator 6-benzylaminopurine (BAP) in the shoot regeneration medium is considered an absolute requirement, although BAP concentration for optimal shoot regeneration varies and is considered to be genotype-specific (Bordon et al., 2000).

Another parameter that influences the morphogenic response of plant material cultured in vitro is the age of the tissue or organ which is used as explant source (George, 1993). In general, maturation and aging seem to be responsible for the explant regenerative potential decline in plant tissue culture.

Currently, co-cultivation of epicotyl explants with *Agrobacterium* spp. is the most efficient method for producing transgenic *Citrus* plants (Domínguez et al., 2004). However, it is very difficulty to obtain whole transgenic plants from sour orange and certain mandarin and sweet orange varieties. Recalcitrance is mainly due to difficulties to regenerate shoots only from transformed cells, and at the same time avoid the recovery of escapes (untransformed regenerants) and the low rooting efficiency of the regenerated shoots. The use of alternative selective agents – such as the antibiotic hygromycin (Costa et al., 2002) and the positive selection system based in mannose assimilation by transformed cells (Boscariol et al., 2003) – seems to be a promising strategy to circumvent the problem of escapes. Micrografting of the regenerated shoots was necessary in most cases, for the recovery of transgenic *Citrus* plants (Peña et al., 1995).

The objective of this work was to establish tissue culture parameters suitable for gene transfer in sweet orange cultivars.

Seeds of *Citrus sinensis* (L.) Osbeck – cultivars Hamlin, Midsweet, Pêra and Pineapple – were collected at the Citrus Germplasm Collection of Embrapa Mandioca e Fruticultura Tropical, in Cruz das Almas, Bahia, Brazil. Preparation of epicotyl explants from etiolated seedlings, in culture media, and conditions of incubation were as described by Costa et al. (2004).

The effects of BAP concentration (0, 0.5, 1, 2, and 4 mg L^{-1}) and seedling age (35, 42, 49, 56, and 63 days) on regeneration efficiency of the sweet oranges were evaluated. The antibiotics kanamycin (0, 25, 50, 75, and 100 mg L^{-1}) and hygromycin (0, 5, 7.5, 10, and 15 mg L^{-1}) were also tested, in order to identify the most suitable concentration for selecting transformed shoots. Regeneration frequency of the segments or mean number of shoots per explant was evaluated. Regeneration frequency was calculated as the number of segments producing buds/shoots per total number of segments cultured.

Elongated shoots (0.5 cm) of 'Pêra' and 'Pineapple' were, then, individualized and transferred to rooting medium (Costa et al., 2002), supplemented with either 1 mg L^{-1} NAA (alpha-naphthaleneacetic acid) or 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} IBA (indole-3-butyric acid). Rooting frequency, mean number of roots per shoot and average root length were evaluated.

The experimental design was a completely randomized one, with 5–10 explants per replicate, five replicates per treatment, with at least one repetition. Data were evaluated at 45 days of incubation. The statistical analysis was performed with the software BIOESTAT (Universidade Federal do Pará, Brazil). Data of rooting frequencies (%) were arc sin $x^{0.5}$ transformed prior to statistical analysis. ANOVA (analysis of variance) was applied and, for means comparison, Bonferroni's test was used at 5% probability.

Although the pattern of bud differentiation was similar in all sweet oranges, either through a process of direct organogenesis (at 1 mg L^{-1} BAP or lower concentration) or by indirect organogenesis (up to 2 mg L^{-1} BAP), the requirement of BAP for maximum shoot development was genotype-specific (Figure 1). In 'Hamlin', the supplementation of the cytokinin had no promotive effect on shoot organogenesis, compared to the control (0 mg L^{-1} BAP). In 'Midsweet', there was a significant increase on shoot organogenesis from 0 to 1 mg L^{-1} BAP. In 'Pêra', BAP stimulated maximum shoot regeneration in a range of concentrations from 0.5 to 1 mg L^{-1}, while in 'Pineapple' the maximum shoot regeneration was observed in a range of concentrations from 1 to 2 mg L^{-1} BAP.

Epicotyl explants from 35-day-old seedlings produced significantly more shoots per explant (1.57) in 'Pêra'. However, in 'Pineapple', no significant difference

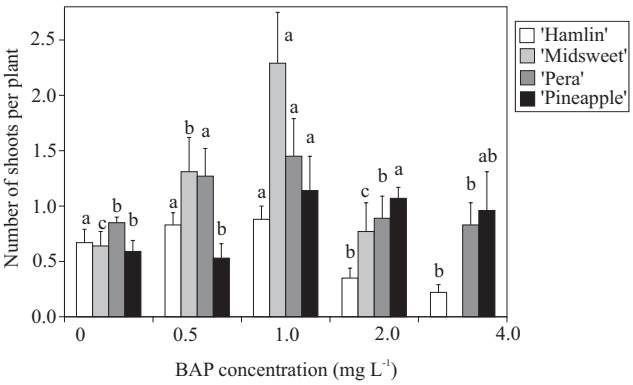

Figure 1. Effect of BAP concentration on the mean number of shoots per explant of epicotyl explants from different sweet orange cultivars. Explants were cultivated on basal shoot regeneration medium supplemented with different concentrations of BAP. Data are from two independent experiments. Means with the same letters, for each cultivar, do not differ by Bonferroni's test at 5% probability. Mean±SE.

was observed in the mean number of shoots per explant, among seedlings of different ages. In this case, younger epicotyl explants should be preferentially used, since it was reported that they give higher transformation rates than older epicotyl explants (Bond & Roose, 1998).

Bud and shoot regeneration were completely inhibited in a range of concentrations from 25 to 100 mg L^{-1} kanamycin for most sweet oranges tested ('Hamlin', 'Midsweet', and 'Pineapple'). However, such inhibition was only achieved in 100 mg L^{-1} kanamycin for 'Pêra'. As to hygromycin, bud and shoot regeneration were completely inhibited in a range of concentrations from 7.5 to 15 mg L^{-1} for 'Pineapple'. 'Pêra', however, was more tolerant to the antibiotic, since such inhibition was only observed in a range of concentrations from 10 to 15 mg L^{-1}. In epicotyl explants of grapefruit (*C. paradisi*), concentrations of 5 mg L^{-1} hygromycin or higher completely inhibited the shoot formation (Costa et al., 2002). To our knowledge, this is the first time that large differences in sensitivity to the antibiotics kanamycin and hygromycin were observed within a *Citrus* species.

The percentage of adventitious shoots that produced roots in 'Pêra' was significantly higher on rooting medium containing the combination NAA and IBA than NAA alone (Table 1). In 'Pineapple', such difference was not significant. Most interestingly, it was observed in both sweet oranges that whereas 1 mg L^{-1} NAA favored significantly higher numbers of roots per shoot than the combination of 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} IBA, the opposite result was obtained for root length. NAA at 1 mg L^{-1} caused considerable callus production at the base of the shoots, and inhibited further root growth. Together, these results demonstrate that NAA alone is very important for root differentiation in sweet orange, but its combination with IBA is essential for root elongation.

Table 1. Effect of alpha-naphthaleneacetic acid (NAA, 1 mg L^{-1}) and NAA (0.5 mg L^{-1}) + indole-3-butyric acid (IBA, 0.5 mg L^{-1}) on rooting response of adventitious shoots from 'Pêra' and 'Pineapple' sweet oranges[(1)].

Cultivar	Rooting frequency (%)[(2)]		Average number of roots per shoot		Average root length (mm)	
	NAA	NAA + IBA	NAA	NAA + IBA	NAA	NAA + IBA
Pêra	78.3b	91.7a	4.5a	3.2b	8.8b	17.5a
Pineapple	94.0[ns]	96.0	4.3a	2.2b	20.9b	28.3a

[(1)]Means followed by the same letter, for each cultivar, do not differ by Bonferroni's test at 5% probability; data from two independent experiments. [(2)]Data of rooting frequency (%) were transformed (arc sin $x^{0.5}$) prior to statistical analysis.

Sweet orange cultivars evaluated had slightly different requirements for optimal in vitro shoot regeneration and complete bud and shoot inhibition by the selective antibiotics kanamycin and hygromycin. The combination of NAA and IBA in the rooting medium provides a viable alternative to micrografting.

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