In vitro organogenesis from adult tissue of 'Bahia' sweet orange (*Citrus sinensis* L. Osbeck)

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***RESUMEN ESPAÑOL**, p. 371*

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Abstract — Introduction. Plant regeneration from *in vitro* culture of adult citrus tissue may help in early evaluation of important horticultural characteristics of genetic material from breeding programs, which incorporate biotechnological tools. Our research aimed to optimize *in vitro* culture conditions that could lead to the maximum plant regeneration through organogenesis of 'Bahia' sweet orange (*Citrus sinensis* L. Osbeck) in explants (internodes and foliar disc segments) collected from adult plants grown under greenhouse conditions. **Materials and methods**. Organogenesis was induced in internodes on DBA3 culture media supplemented with BAP at (0.0, 1.0, 2.0 and 3.0) $mg{\cdot}L^{-1}$ combined with NAA (0.5 $mg{\cdot}L^{-1}$), and in foliar discs on culture media with BAP at (0.0, 0.5, 1.0, 1.5 and 2.0) $mg{\cdot}L^{-1}$ combined or not with NAA (0.5 $mg.L^{-1}$). **Results and discussion**. The concentrations of (1.0, 2.0 or 3.0) $mg{\cdot}L^{-1}$ of BAP combined with 0.5 $mg{\cdot}L^{-1}$ of NAA induced *in vitro* organogenesis in internodes of 'Bahia' sweet orange. The use of foliar discs was not efficient for adventitious organogenesis.

Brazil / *Citrus sinensis* / micropropagation / *in vitro* regeneration / explants / culture media / organogenesis

Organogenèse *in vitro* de tissu de plants adultes d'oranger 'Bahia' (*Citrus sinensis* L. Osbeck).

Résumé — Introduction. La régénération de plants adultes d'agrumes par culture *in vitro* de tissus peut aider à l'évaluation précoce d'importantes caractéristiques horticoles de matériel issu de programmes d'amélioration génétique utilisant des outils de biotechnologie. Nos recherches ont cherché à optimiser les conditions de culture *in vitr*o qui pourraient mener à une régénération maximale de plants par organogenèse d'oranger 'Bahia' (*C. sinensis* L. Osbeck) à partir d'explants (segments d'entre-nœuds et disques foliaires) prélevés sur des plantes adultes cultivées sous serre. **Matériel et méthodes**. Une organogenèse a été induite à partir d'explants d'entre-nœuds sur des milieux de culture DBA3 additionnés de BAP à (0,0, 1,0, 2,0 et 3,0) $mg{\cdot}L^{-1}$, combinée à de l'ANA à 0,5 $mg{\cdot}L^{-1}$, et à partir d'explants de disques foliaires sur milieux de culture avec BAP (0,0, 0,5, 1,0, 1,5 et 2.0) $mg{\cdot}L^{-1}$, combinée ou pas avec de l'ANA (0,5 $mg{\cdot}L^{-1}$). **Résultats et discussion**. Les concentrations à (1,0, 2,0 ou 3.0) $mg{\cdot}L^{-1}$ de BAP combinée à 0.5 $mg{\cdot}L^{-1}$ d'ANA ont induit une organogenèse *in vitro* pour les explants d'entre-nœuds d'oranger 'Bahia'. L'utilisation des explants de disques foliaires n'a pas été efficace pour induire une organogenèse adventice.

Brésil / *Citrus sinensis* / micropropagation / régénération *in vitro* / explant / milieu de culture / organogenèse

1. Introduction

The development of citrus programs has become important in order to expand the genetic basis as well as optimize the use of germplasm banks. However, several difficulties related to citrus reproductive biology have limited traditional breeding programs, such as nucellar polyembryony, high heterozygosity and a long juvenile period [1, 2].

On the other hand, multiple aspects of biotechnology with adequate strategies for cytological, morphological and physiological studies may help to improve desirable characteristics. Among these tools, genetic transformation is considered a very promising technique to be integrated in conventional plant breeding programs because it allows the introduction of genes from other plants or organisms into the plant genome [3, 4]. This leads to the production in a short time of a modified cultivar with specific traits [5]. However, the success of the regeneration of transgenic plants depends on various conditions that include an efficient *in vitro* plant regeneration system.

In vitro plant regeneration of citrus may occur through organogenesis with the use of non-meristematic juvenile tissues such as epicotyl or internode segments [6]. One of the very few events of adventitious bud induction from non-meristematic adult tissue has been reported in citrus from internode segments of plants cultivated in a greenhouse [7, 8]. The organogenesis process includes cellular de-differentiation and re-differentiation and is dependent on the meristematic activity in mature differentiated cells or in a non-organized callus tissue [9]. Callus development may be followed by organ differentiation. In this case, the process is called indirect organogenesis.

The use of adult tissue for *in vitro* plant regeneration has not been successful, mainly because of reduced or lost morphogenetic capacity [10] and high contamination rates [11]. However, citrus genetic transformation from adult tissue may represent an important event for breeding programs, considering that the regenerated plants would not have juvenile characteristics, allowing faster evaluation and selection, leading to the possibility of releasing a new cultivar in a shorter period of time [8].

The objective of our research was to establish optimal *in vitro* conditions to assure maximum plant regeneration through adventitious organogenesis from adult tissue of 'Bahia' sweet orange (*Citrus sinensis* L. Osbeck).

2. Materials and methods

Plants of 'Bahia' sweet orange (*Citrus sinensis* L. Osbeck) budded on 'Rangpur' lime (*Citrus limonia* Osbeck) were constantly pruned and kept in a greenhouse. New flush growth (about 20-cm-long shoots) from the main stem was collected and used as an explant source.

Internode segments [(0.5 to 0.8) cm] were excised and disinfested in a 2:1 solution of commercial sodium hypochlorite (2.5%) and distilled water, in a shaker, for 25 min. Explants were then rinsed four times in distilled and sterile water.

Leaf discs (1-cm diameter) were also excised and prepared. In this case, explants were treated in a 1:2 solution of commercial sodium hypochlorite (2.5%) and distilled water, for 20 min. Different concentration and disinfestation times were used in order to adjust the disinfestation procedure and reduce tissue damage in this explant type. Explants were then rinsed four times in distilled and sterile water.

High explant contamination associated with low cell totipotency has been considered as the most limiting factor to the use of adult tissue explants [12]. Several preliminary experiments were carried out in order to reduce explant contamination (data not shown). An adequate and low contamination level was observed when 500 $mg \cdot L^{-1}$ of cefotaxime were added to the culture media, for both explants, to control endogenous contamination.

Internodal segments were horizontally cultured in Petri dishes with 20 mL of DBA3 medium (basal MT medium [13] supplemented with 20 $mL \cdot L^{-1}$ coconut water [14]), supplemented with (0.0, 1.0, 2.0 or 3.0) $mg \cdot L^{-1}$ of BAP combined with 0.5 $mg \cdot L^{-1}$ of NAA and 25 $g \cdot L^{-1}$ of sucrose, and 0.8% of agar. Experiments were conducted under a completely

Table I.
In vitro organogenesis in adult tissue (50 internode segments) of 'Bahia' sweet orange (*Citrus sinensis* L. Osbeck) related to BAP concentrations combined with 0.5 mg·L^{-1} NAA.

BAP concentrations added to 0.5 (mg·L^{-1}) NAA (mg·L^{-1})	Explants with calli (%)	Explants with calli and buds (%)	Number of buds per explant with calli
0.0	0	0	0
1.0	12	83.3	1.5
2.0	6	66.7	6.0
3.0	44	45.5	2.4

randomized design, with five replications (Petri dish), with 10 explants each.

Foliar discs were vertically cultured in flasks with 20 mL of DBA3 medium. Preliminary experiments were carried out to evaluate the isolated effect of BAP concentrations at (0.0, 0.5, 1.0, 1.5 or 2.0) mg·L^{-1}, and the effect of BAP at (0.0, 0.5, 1.0, 1.5 or 2.0) mg·L^{-1} combined with 0.5 mg·L^{-1} of NAA. Experiments were conducted under a completely randomized design, with 10 replications (flasks), with seven leaf discs each.

Explants were incubated in the dark for 60 days, at (27 ± 2) °C, and then under a 16-h photoperiod (40 µmol·m^{-2}·s^{-1}). After that, explants were evaluated for number of explants with calli, number of explants with calli that had bud induction, and number of buds per explant.

Regenerated shoots were micrografted onto *in vitro* germinated 'Ranpgpur' lime (*Citrus limonia* L. Osbeck) seedlings. Rootstock plants were decapitated up to 1/3 of their top, and then an "inverted T" incision was done to receive the shoot apex of the regenerated material. The percentage of bud take was recorded after 30 days.

3. Results and discussion

The addition of growth regulators was important for morphogenetic response and organogenesis induction in 'Bahia' sweet orange (*table I*, *figures 1–3*). The development of calli mass was observed at the internode

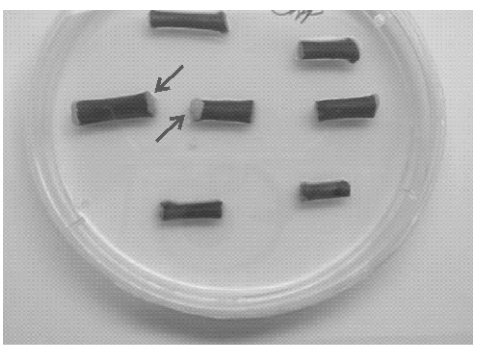

Figure 1.
In vitro organogenesis, with a BAP combined with ANA medium, in internode segments with calli of adult tissue of 'Bahia' sweet orange.

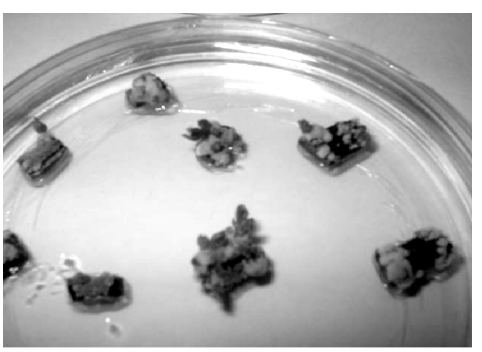

Figure 2.
Adventitious bud development developed in internode segments of adult tissue of 'Bahia' sweet orange.

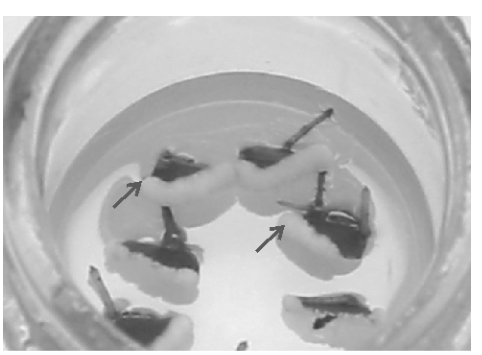

Figure 3.
Leaf discs with calli of 'Bahia' sweet orange obtained by *in vitro* organogenesis with a BAP combined with ANA medium for adult tissue.

segment explant edge (*figures 1, 2*) on culture media with (1.0, 2.0 or 3.0) mg·L^{-1} of BAP associated with 0.5 mg·L^{-1} of NAA. BAP at 3.0 mg·L^{-1} led to the best response in calli induction (44%) as well as the number of buds. These buds had well-developed leaf primodia.

On the other hand, although BAP at 2.0 mg·L^{-1} induced a lower explant response, the greatest number of buds (6.0) was observed at this concentration (*table I*). Research on organogenesis in internodal segments of adult tissue in 'Tahiti' lime revealed that the addition of 3.0 mg·L^{-1} of BAP on culture media led to bud induction in 28.5% of explants with an average of 2.5 buds per explant [15]. Organogenesis studies in adult tissue of 'Valencia', 'Natal', 'Pera' and 'Hamlin' sweet oranges occurred with 1.0 mg·L^{-1} of BAP combined with 0.5 mg·L^{-1} of NAA [8]. Bud induction was also reported in internodal segments from adult tissue of 'Pineapple' sweet orange, and flowering occurred 14 months after plant regeneration [7]. The influence of the genotype (species or cultivar) on *in vitro* organogenesis has been previously reported [16, 17]. This fact leads to the conclusion that the optimization of *in vitro* protocols is necessary [18].

The developed buds regenerated from this experiment were micrografted onto 'Rangpur' lime *in vitro* seedlings with 55% of bud take. However, the micrografted plants did not survive during acclimation, suggesting the need for other experiments to optimize plant acclimation of *in vitro* plants regenerated from adult tissue.

Organogenesis response from leaf discs has been difficult in citrus, in which only one report of this type of regeneration has been achieved in 'Hamlin' sweet orange at low rates [8]. In our experiment, a high percentage of explant response (calli induction) was observed for all plant regulator concentrations (data not show). When BAP concentrations were combined with the presence of NAA, besides calli induction (*figure 3*), root formation was also recorded. Therefore, other experiments may be developed to optimize adventitious organogenesis from this explant type.

4. Conclusions

Concentrations of (1.0, 2.0 or 3.0) mg·L^{-1} of BAP combined with 0.5 mg·L^{-1} of NAA are adequate for *in vitro* organogenesis in internode segments of adult tissue from 'Bahia' sweet orange.

Foliar discs are not adequate explants for adventitious organogenesis induction.

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Organogénesis *in vitro* a partir de tejidos adultos de naranja dulce (*Citrus sinensis* L. Osbeck) cv. 'Bahia'.

Resumen — Introducción. La regeneración de plantas mediante el cultivo *in vitro* de tejidos adultos de cítricos permite una evaluación temprana de importantes características hortícolas del material genético proveniente de programas de mejoramiento que incorporan herramientas biotecnológicas. Esta investigación tuvo por objetivo optimizar las condiciones de cultivo *in vitro* que permitan obtener la máxima regeneración de plantas mediante organogénesis en lo cultivar de naranja dulce (*Citrus sinensis* L. Osbeck) 'Bahia', en explantes (entrenudos y discos foliares) obtenidos de plantas adultas cultivadas en invernadero. **Material y métodos**. La organogénesis fue inducida en los explantes de entrenudos en el medio de cultivo DBA3, suplementado con benciladenina fosfato (BAP) [(0.0, 1.0, 2.0, 3.0) $mg{\cdot}L^{-1}$], combinado con 0.5 $mg{\cdot}L^{-1}$ de ácido naftalenacético (ANA). En los discos foliares la organogénesis fue inducida en un medio de cultivo con BAP [(0.0, 0.5, 1.0, 1.5, 2.0) $mg{\cdot}L^{-1}$], con o sin la adición de 0.5 $mg{\cdot}L^{-1}$ de ANA. **Resultados y discusión**. Las concentraciones de (1.0, 2.0 o 3.0) $mg{\cdot}L^{-1}$ de BAP, combinada con 0.5 $mg{\cdot}L^{-1}$ de ANA indujeron la organogénesis *in vitro* en los entrenudos de naranja dulce cv. 'Bahia'. Los discos foliares no fueron eficaces para lograr la organogénesis adventicia.

Brasil / *Citrus sinensis* / micropropagación / regeneración *in vitro* / explantes / medio de cultivo / organogénesis