Regeneration of cotyledonary nodes from the recalcitrant melon cultivar ‘Gaúcho’

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There is lack of suitable explants for efficient shoot proliferation in recalcitrant melon cultivars restricts genetic manipulation for crop improvement. In this study, we aimed to stimulate the regeneration capability of cells of melon (Cucumis melo L.) explants in order to increase shoot proliferation efficiency during regeneration. The media of seeds germination containing different concentrations of benzylaminopurine (BAP: 0.5, 1.0, and 1.5 mg L⁻¹) can stimulate the shoot proliferation potential of cotyledonary nodes was described. The highest number of shoots per explant was obtained when the seeds and cotyledonary nodes were cultured on Murashige and Skoog media containing 1.0 and 0.5 mg L⁻¹ BAP, respectively, and the regeneration process occurred through organogenesis.

Key words: Cucumis melo L., organogenesis, cytokinin, explants.

INTRODUCTION

Plants belonging to the family Cucurbitaceae are commonly known as cucurbits; among the major cucurbits, Cucumis melo L. has one of the highest polymorphic fruit types and botanical varieties because of the genetic diversity in this species (Mliki et al., 2001). Many varieties of both cultivated and wild melons are found worldwide (Chovelon et al., 2011). On the basis of total production and harvestable area, cantaloupes and other melons (e.g., C. melo L.) are among the most important cultivated cucurbits in the world (Nunez-Palenius et al., 2008). In Brazil, mostly in the southeast, cultivated melons belong to the saccharinus variety and have medium- to big-sized fruits that are round or oblong with white or yellow and very sweet flesh (Nunez-Palenius et al., 2008). The cultivar ‘Gaúcho’ is cultivated extensively, even though it produces perishable fruits with a shelf life of 4 to 6 days after harvest (Pinho et al., 2010). Traditional hybridization techniques have been used for melon breeding; however, there is an increasing demand for new cultivars with a wide variety of characteristics, such as high levels of disease resistance and enhanced flavor and sweetness (Chovelon et al., 2011).

Although several methods have been reported in the literature for plant regeneration with different explants (Chaturvedi and Bhatnagar, 2001; Curuk et al., 2003; Akasaka-Kennedy et al., 2004; Comlekcioglu et al., 2009), the efficiency of the techniques for melons has been usually low and highly dependent on the genotype (Galperin et al., 2003; Nunez-Palenius et al., 2008;
Chovelon et al., 2011) and the explant source (Guis et al., 2000; Pinho et al., 2010). Besides, it is very common that induced buds do not develop into normal shoots or plantlets (Stipp et al., 2001). Consequently, environmental and hormonal requirements for melon regeneration are poorly understood, and the development of simple procedures to regenerate and transform melon genotypes is necessary (Chovelon et al., 2011).

In the present study, melon cultivar ‘Gaúcho’ a new source of explant (cotyledonal nodes) and its response to stimulation of the regeneration rate and formation of normal shoots was investigated.

MATERIALS AND METHODS

Seeds of C. melo L. ‘Gaúcho’ were surface-sterilized in 1% (w/v) sodium hypochlorite and 0.01% Tween-20 for 20 min after removing the integument. Then, the solution was decanted, and the seeds were rinsed three times with sterile water. The seeds were placed for germination on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing (in mg L⁻¹) sucrose 30,000; 1-mol L⁻¹; thiamine-HCl 1.0; pyridoxine-HCl 0.05; nicotinic acid 0.05; glycine 2.0; bacto agar 7.000 (Difco Laboratories), supplemented with 0.0, 0.5, 1.0, and 1.5 mg L⁻¹ of benzylaminopurine (BAP) for seven days, and cultured at 23 ± 2°C with a 16-h photoperiod.

After seven days, cotyledonal nodes of seedlings containing shoot apical meristem cells (explant length, 3 to 5 mm) were obtained by removing the epicotyl, hypocotyl, and cotyledons and placed on three different regeneration media: (1) basal MS medium containing 0.25 mg L⁻¹ BAP and 0.3 mg L⁻¹ abscisic acid (ABA), (2) basal MS medium containing 0.5 mg L⁻¹ BAP and 0.3 mg L⁻¹ ABA, or (3) basal MS medium containing 0.9 mg L⁻¹ BAP and 0.3 mg L⁻¹ ABA. After 30 days on the regeneration media, the apical shoots and new axillary buds were cut off, and the cotyledonal nodes were placed back on the same regeneration media for a further 15 days. After 45 days on the regeneration media, newly regenerated shoots were separated and transferred to the elongation and rooting medium (basal MS medium without growth regulators) for 4 to 5 weeks. The pH was adjusted to 5.8 before autoclaving (120°C, 20 min). All steps were performed using the same growth conditions (temperature and flux density of photons) as described above. The shoots that developed healthy roots in vitro were transferred to a greenhouse and planted in pots with a mixture of equal parts (v/v) of soil and vermiculite for growth.

The experimental design for the regeneration experiment was completely randomized with three replicates (3 Petri dishes) and each replicate of 10 explants, totaling 30 explants by treatment. The results were assessed using analysis of variance (ANOVA), and the mean values were compared using Duncan’s test at 1% error probability with the statistical software WinStat (Machado and Conceição, 2007).

Histological analyses of the regeneration process were performed by observing the cotyledonal nodes 35 days after transferance to the regeneration medium. The cotyledonal nodes were immersed in a fixative (paraformal–glutaraldehyde–buffer phosphate at 1:1:8 v/v/v) for 24 h, rinsed twice with phosphate buffer for 1 h, rinsed with distilled water, dehydrated using an ethanol series (30, 50, 70, 90, and 95%), and embedded in methacrylate resin (LEICA HISTORESIN). Transverse sections (thickness, 5 µm) were serially cut with a retraction microtome, collected on microscope slides, stained with 0.05% toluidine blue (Sakai, 1973), diluted in phosphate–citrate buffer titrated to pH 4.5 (McIlvaine, 1921), and mounted in Entellan synthetic resin (Merck). Images were captured digitally by using a Leica DM LB microscope with a video camera attached to a PC and IM50 image analysis software.

RESULTS

Seven-day-old seedlings growing in media containing BAP showed abnormal growth, enlarged cotyledons, and roots swollen at the tip with none or only a few small lateral roots (Figure 1A, B, and C). In contrast, seedlings growing on media without BAP were normal, with well-developed hypocotyls and roots (Figure 1D). In addition, the cotyledonal nodes appeared to be more swollen in the seedlings that developed on BAP-supplemented media (Figure 1E).

Cotyledonal nodes on all regeneration media showed apical shoot growth (apical dominance behavior) after 30 days; a single growing axis was observed, with none or some leaf primordia. Explants from all germination media when transferred to regeneration media containing 0.25 mg L⁻¹ BAP showed similar growth of the apical shoot without callus formation. However, apical shoot growth became less pronounced, with an increase in BAP concentration during the regeneration process (Figure 2A, B, and C). Increase in BAP concentration during germination, combined with higher levels of this growth regulator during regeneration, induced the formation of calli and a significant reduction in shoot development. In addition, more leaf primordia and poor shoot formation were observed after these treatments (Figure 2D to I).

Removal of apical shoots and cultivation of those tissues for a further 15 days in the same regeneration media appeared to have the most promising effect in inhibiting apical dominance and inducing the development of multiple well-formed axillary shoots (Table 1 and Figure 3A). A higher number of well-formed axillary shoots (100% normal shoots) were obtained from explants excised from seedlings that grew in media containing 1.0 mg L⁻¹ BAP and regenerated in media containing 0.5 mg L⁻¹ BAP (5.5 shoots/explant). Furthermore, the average percentage of regenerating explants was high (94%) after considering all treatments (Table 1).

Subculturing regenerated shoots on the elongation and rooting medium (Figure 3B) without growth regulators had a positive response on shoot growth and root generation (100% rooting). Then, in vitro plants were transferred to the substrate and acclimatized under greenhouse conditions (Figure 3C).

The histological analyses showed that adventitious shoots developed directly from the epidermal and subepidermal layers of the explants at 5 days after the removal of the apical shoots (Figure 4A to E). Initial development of adventitious shoots with only one leaf primordium was verified on MS medium without growth regulators, and the shoots were regenerated on media
Figure 1. Seven-days-old melon seedlings germinated for seven days. A-C: Germination on MS basal medium containing BAP (0.5, 1.0 and 1.5 mg L⁻¹, respectively). D: Germination on MS basal medium without growth regulator. E: Cotyledonary node explant removed prepared from seedling germinated on MS basal medium containing BAP. Bars = 1 cm.

Figure 2. Propagules from cotyledonary nodes explant cultivated for 30 days on regeneration medium. A to C: Germination on 0.5 mg L⁻¹ BAP and regeneration on 0.25, 0.5 and 0.9 mg L⁻¹ BAP respectively. D to F: Germination on 1.0 mg L⁻¹ BAP and regeneration on 0.25, 0.5 and 0.9 mg L⁻¹ BAP, respectively. G to I: Germination on 1.5 mg L⁻¹ BAP and regeneration on 0.25, 0.5 and 0.9 mg L⁻¹ BAP, respectively. Bars = 1 cm.
Table 1. Percentage of regeneration and number of axillary shoots per explants of melon cotyledonary nodes after 45 days in the regeneration media.

<table>
<thead>
<tr>
<th>BAP concentration (mg.L⁻¹) during seed germination</th>
<th>BAP concentration (mg.L⁻¹) during regeneration</th>
<th>% regeneration</th>
<th>Number shoots/exp</th>
<th>% regeneration</th>
<th>Number shoots/exp</th>
<th>% regeneration</th>
<th>Number shoots/exp</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>0.25</td>
<td>60.0</td>
<td>C 2.1ᵃ</td>
<td>83.0</td>
<td>C 0.5ᵇ</td>
<td>93.3</td>
<td>B 2.2ᵃ</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>100.0</td>
<td>D 1.3ᵇ</td>
<td>93.3</td>
<td>C 0.8ᶜ</td>
<td>100.0</td>
<td>B 2.1ᵇ</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>100.0</td>
<td>A 4.1ᶜ</td>
<td>100.0</td>
<td>A 5.5ᵃ</td>
<td>100.0</td>
<td>A 4.6ᵇ</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>100.0</td>
<td>B 2.9ᵇ</td>
<td>100.0</td>
<td>A 3.4ᵇ</td>
<td>100.0</td>
<td>B 2.4ᶜ</td>
</tr>
</tbody>
</table>

Means of treatment were compared using Duncan’s test. Results for the same BAP concentration during regeneration do not differ from each other (p < 0.01) if the upper case letter on the left side of the means are the same. Results for same BAP concentration during seedling germination do not differ from each other (p < 0.01) if the lower case letter on the right side of the means are the same.

Figure 3. A to B: Shoots and melon plants, cv. ‘Gaucho’ from cotyledonary nodes cultivated in vitro. A: shoots formation with 1.0 and 0.5 mg L⁻¹ of BAP on germination and regeneration media, respectively; B: Shoot growing and root formation Growth and rooting of shoots in medium without growth regulators; C: Acclimatization of melon plants in a greenhouse. Bars= 1 cm.

containing 0.25 mg L⁻¹ BAP (Figure 4A and C). Adventitious shoots with shoot apical meristems and leaf primordia were observed on the regenerated medium with 1.0 mg L⁻¹ BAP (Figure 4B, D, and E).

DISCUSSION

The addition of BAP during seed germination has a positive influence on the regeneration rate. The best hormonal concentrations for the production of new shoots were 1.0 and 0.5 mg L⁻¹ BAP during the germination and regeneration phases, respectively (Table 1), with 5.5 shoots per explant. Furthermore, the percentage of regenerating explants ranged from 60 to 100% (average for all treatments, 94%) of the cotyledonary nodes inoculated in the regeneration media, determining a high efficiency in the final production process of in vitro plants and hence in acclimatization. These results are much higher than those obtained by Pinho et al. (2010) who used the same cultivar and another explant (cotyledons from newly germinated seeds) and achieved only a regeneration rate of 29.66%, even when the seeds were germinated on MS medium containing BAP.

The histological analyses confirmed the development of shoot apical meristems and leaf primordia from cotyledonary nodes (Figure 4), and the adventitious shoots were more developed when formed in germination media with a low BAP concentration (Figure 2). Thus, the addition of BAP in the culture media during the phases of seed germination and regeneration must have affected the internal hormonal balance of the explants (Wang et al., 2011), enabling the breaking of apical dominance and determining an increase in the number of healthy shoots by organogenesis (Figure 4). Krug et al. (2005) had studied the cotyledon explants of watermelon and observed the formation of protuberances that did not develop into adventitious buds.
Figure 4. *In vitro* organogenesis from cotyledonary nodes of melon, cv. Gaúcho, after the main axis had been removed and regenerated for a further 5 days cultured for 35 days on regeneration medium. A. Longitudinal section showing initial development of shoot (arrow) on basal medium MS without growth regulators and regenerated on 0.25 mg L\(^{-1}\) BAP. B to E. Shoots (arrows) formed directly at the explant (Ex); B. Transverse section of explant showing shoot regeneration (germinated on 1.0 mg L\(^{-1}\) BAP and regenerated on 0.25 mg L\(^{-1}\) BAP); C. Germinated on MS basal medium without hormone and regenerated on 0.5 mg L\(^{-1}\) BAP; D to E. Germinated on 1.0 mg L\(^{-1}\) BAP and regenerated on 0.5 mg L\(^{-1}\) BAP. Bars = 200 µm.

In our study, the concentrations of BAP added during germination did not affect the frequency or speed of seed germination, but altered the morphology of the seedlings (Figures 1 and 2). Auxins and cytokinins have been reported as the key hormones that control the cellular architecture of the primary root and the initiation of new lateral roots. The use of BAP during seed germination determined an impairment of growth and development of seedlings and roots, as observed in *Arabidopsis thaliana* (Cary et al., 1995; Muraro et al., 2013).

The positive effect of BAP on the regeneration of shoots has been mostly reported in melons (Ntui et al., 2009; Pinho et al., 2010) and other species such as watermelon (Chaturvedi and Bhatnagar, 2001; Krug et al., 2005), and cucumber (Selvaraj et al., 2007). However, few studies have been performed on the positive effect of pre-treatments before the regeneration stage with growth regulators. Kintzios et al. (2002) reported the positive effect of pre-treatments with growth regulators in melons to improve embryogenesis, suggesting that hormone treatments may help cells to enter organogenic differentiation pathways.

Plants that show apical dominance, such as melons have preferentially a single growing axis and few lateral
branches. Therefore, exogenous cytokinin treatments could impair apical dominance and help in the regeneration process of the cotyledonal nodes. Our observations showed that the BAP concentrations used in the regeneration step did not completely prevent apical dominance (Table 1). As expected, the inhibition of apical dominance increased with an increase in the concentration of BAP until a limit was reached; then, the cells lost their ability to produce healthy shoots (Figures 2 and 3).

A major issue for the production of new melon cultivars with new characteristics is their different tissue responses during the regeneration process and the low number of regenerated plants. A new explant source such as the cotyledonal node that responds more effectively could be used with increased efficiency in genetic transformation programs for melons. To achieve this, a better understanding of the regeneration process in different tissues of recalcitrant species such as melons is essential.

Conflict of Interest

The authors have not declared any conflict of interest.

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