Short communication

Induction of somatic embryogenesis from young, fully expanded leaves of chilli pepper (Capsicum annuum L.): effect of leaf position, illumination and explant pretreatment with high cytokinin concentrations

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Accepted 25 October 1999

Abstract

The effect of the explant position on the donor plant, illumination and explant pretreatment with high cytokinin concentrations on the induction, proliferation and development of somatic embryos from young, fully expanded leaves of chilli pepper (Capsicum annuum L.) was investigated. Explants were cultured either directly on a solid Murashige and Skoog medium supplemented with 9 μM 2,4-dichlorophenoxyacetic acid + 12.9 μM 6-benzyladenine or incubated for 24 h in a liquid MS medium containing the cytokinin at a tenfold concentration (129 μM) and then transferred to the solid MS medium. Globular embryo proliferation depended on the leaf position on the donor plant: fewer embryos were derived from the third leaf (counting from the base of the shoot) than from the first two leaves. The initial pretreatment of pepper explants with increased 6-benzyladenine concentrations significantly reduced the overall proliferation of somatic embryos without affecting the percentage of globular embryos which further developed into the torpedo-shape stage and germinated. Depending on the leaf position, somatic embryo induction was significantly affected by the initial culture incubation under illumination or in darkness. Heart- and torpedo-shaped embryos could be observed only on callus pieces initially incubated for 3 weeks in darkness. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Benzyladenine; 2,4-Dichlorophenoxyacetic acid; Leaf explant; Globular embryo proliferation; Chilli pepper

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1. Introduction

Somatic embryogenesis leads to the formation of embryos from somatic (sporophytic) tissues, such as leaves and cotyledons. In recent years, a number of investigators have developed methods in order to increase the efficiency of this process for chilli pepper micropropagation (Harini and Lakshmi Sita, 1993; Kintzios et al., 1998). Direct induction of somatic embryogenesis in Capsicum annuum has been demonstrated on immature zygotic embryos (Harini and Lakshmi Sita, 1993; Binzel et al., 1996) on MS medium, supplemented with 2,4-D, CW and high sucrose (at least 8% (w/v)) and embryo germination ensued on a medium supplemented with 1 mg l⁻¹ GA₃. In our laboratory we have previously studied the effect of light on the induction of callus and somatic embryogenesis from pepper leaf explants (cv. ‘Colombo’) derived from 20-days-old in vitro grown seedlings. Somatic embryos induced from leaf segments (derived from the first leaf) on a solid MS medium supplemented with 9 µM 2,4-D + 12.9 µM 6-BA were able to develop further to the cotyledonal stage, maturate and finally germinate, at a rate of approximately 18 germinating embryos per 100 initially induced globular embryos (Kintzios et al., 1998). Significantly, more globular somatic embryos were induced when cultures were initially incubated in darkness (for 3 weeks) and then transferred under illumination (250 µmol m⁻² s⁻¹) than when they were directly incubated under illumination during a total period of 4 weeks after culture initiation. In another experiment, we found that the addition of both an auxin (2,4-D) and a cytokinin (BA) at high concentrations was associated with increased embryo proliferation and embryo development to the torpedo-shaped stage (Kintzios et al., in preparation). However, application of 2,4-D at a higher concentration (22.6 µM) than BA affected negatively proembryo proliferation, possibly due to a toxic effect of the excessive auxin (2,4-D is a known herbicide and a powerful suppressant of organogenesis) (Dodds and Roberts, 1995). We considered it of interest to test whether the application of BA at a higher concentration to leaf explants obtained from different positions in seed-derived plants grown ex vitro would promote somatic embryogenesis, especially when cytokinins have been previously associated with increased in vitro embryogenic differentiation of this species (Nielsen and Ulvskov, 1992). The effect of 3 week incubation in darkness on somatic embryogenesis of the same type of explants was also evaluated.
2. Materials and methods

Experiment source and preparation: Donor pepper seedlings (cv. ‘Colombo’) (3–5 days-old) were potted into a 1:1 peat:perlite mixture and grown in the glasshouse (26 ± 2°C, 16/8 h photoperiod, 250 µmol m⁻² s⁻¹ from ‘cool-white’ fluorescent lamps) until explant removal. Explants were derived from the median region of fully expanded, green leaves of different positions (first, second and third node from the base) on non-flowering donor plants. Leaves were excised from each plant, surface sterilized for 10 min in 0.2% sodium hypochlorite solution, containing 2% Tween-80, and finally rinsed four times in sterile distilled water.

Callus induction: Callus was induced by inoculating 1 cm long leaf segments (abaxial side down) on ‘standard induction medium’, e.g., MS basal medium with 8% (w/v) sucrose, as suggested by Harini and Lakshmi Sita (1993), supplemented with 12.9 µM BA and 9 µM 2,4-D and solidified with 0.8% (w/v) agar-agar. Media were adjusted to pH 5.8 using 1 N NaOH or 1 N HCl, autoclaved at 121°C for 20 min and poured into glass conical flasks (100 ml of medium per flask, five explants per flask). Explants were incubated in the light for 2 weeks. Unless otherwise indicated, incubation conditions were 200 µmol m⁻² s⁻¹, with a 16/8 h photoperiod, from cool-white fluorescent lamps and 25°C temperature.

Illumination treatments: In order to investigate the effect of light and leaf position on the induction of somatic embryogenesis, callus cultures were incubated either (i) initially in darkness (for 3 weeks), and then transferred to standard illumination conditions for another week or (ii) kept under continuous illumination (24 h per day) for a total of 4 weeks. Thus, cultures were exposed to dark incubation periods of different duration (0 or 3 weeks). A total of 45 leaf explants were used per treatment.

Plant growth regulator treatments: In order to investigate the effect of explant pretreatment with increased (tenfold) cytokinin concentration, explants were initially cultured in a liquid MS (Murashige and Skoog, 1962) basal medium supplemented with 3% (w/v) sucrose and 129 µM BA and 9 µM 2,4-D for 24 h and then transferred onto solid ‘standard induction medium’. After callus induction, inoculated flasks were incubated initially in darkness (for 3 weeks), and then transferred to standard incubation conditions for one additional week. Forty-five explants were used per treatment.

Somatic embryo development, maturation and germination: After 4 weeks in culture, callus tissues with somatic embryos at the globular stage, which were induced on ‘standard induction medium’ were transferred onto modified ‘standard induction medium’ containing 3% sucrose for further embryo development under standard incubation conditions. Embryos at the torpedo-shaped stage germinated on the same medium.

Data analysis: Numbers of globular embryos per mm² of explant surface were recorded 6 weeks after culture initiation. Five explants from each (leaf position) ×
(pretreatment) combination as well as each (leaf position) × (duration of incubation in darkness) combination were assayed each time. Callus and embryo morphology was visually recorded. Results were assessed by a standard analysis of variance for a randomized complete block design, using GBS-STAT software.

3. Results and discussion

Induction of embryogenic callus: Formation of a yellowish friable embryogenic callus tissue was observed on virtually 100% of leaf explants within 2 weeks after culture initiation on ‘standard induction medium’.

3.1. Induction and proliferation of somatic embryos

3.1.1. Effects of illumination and leaf position

Depending on the position of the leaves on the donor plants, the relative length of the incubation period under illumination significantly affected somatic embryo induction and proliferation (Table 1, Fig. 1). Generally, more somatic globular embryos were induced when cultures were initially incubated in darkness for 3 weeks and the number of embryos produced decreased as the leaf position increased. Thus, fewer embryos were derived from the third leaf than from the first two leaves. When cultures were incubated directly under illumination (with a 16/8 h photoperiod), very small differences were observed in globular embryo production between the second and the third leaf, which responded better than the first leaf.

Several investigators have reported that incubation of cell cultures under a low PPFD or in the darkness may be preferable for shoot induction and somatic embryogenesis from some species, such as cucumber (Cade et al., 1988; Colijn-Hooymans et al., 1988), melon (Kintzios and Taravira, 1997) and rose (Kintzios

Table 1
Analysis of variance of somatic globular embryo production from pepper leaf explants on MS + 8% (w/v) sucrose + 12.9 μM BA + 9 μM 2,4-D in response to the different leaf position and the duration of the incubation period under illumination

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf position, A</td>
<td>2</td>
<td>2151.084***</td>
</tr>
<tr>
<td>Length of illumination period, B</td>
<td>1</td>
<td>462.7063*</td>
</tr>
<tr>
<td>A × B</td>
<td>2</td>
<td>581.8612**</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

*p Significant at p < 0.05.

** Significant at p < 0.01.

*** Significant at p < 0.0001.
et al., 1999). Concerning the effect of the relative length of the incubation under illumination or in darkness, Gray et al. (1993) observed that 1 week of initial culture of melon quiescent seed cotyledons in darkness, followed by a 16 h light/8 h dark regime, produced 26% more responding explants than two or more weeks in darkness or with no dark period at all (i.e., continuous light), but 1 and 2 weeks of darkness resulted in a similar number of embryos per explant.

3.1.2. Effects of explant pretreatment with high cytokinin (BA) concentrations and leaf position

The initial exposure of leaf explants to tenfold concentrations of BA, together with the position of the leaf on the donor plant affected very significantly (p < 0.0001) somatic embryo induction (Table 2, Fig. 2). In the first and third leaves, embryo proliferation after explant pretreatment was inferior to the direct incubation on ‘standard induction medium’, while the opposite was true for the second leaf. The higher number of embryos per callus was observed on non-

Table 2

Analysis of variance of somatic globular embryo production from pepper leaf explants on MS + 8% (w/v) sucrose + 9 μM 2,4-D + 12.9 μM BA in response to the different leaf position and the explant pretreatment with a tenfold (129 μM) concentration of BA

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf position, A</td>
<td>2</td>
<td>7315.6088*</td>
</tr>
<tr>
<td>Explant pretreatment, B</td>
<td>1</td>
<td>4762.0733*</td>
</tr>
<tr>
<td>A × B</td>
<td>2</td>
<td>976.8136*</td>
</tr>
<tr>
<td>Error</td>
<td>42</td>
<td>59.2812</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at p < 0.0001.
pretreated explants derived from the first leaf and pretreated explants from the second leaf. Embryo proliferation declined with increasing leaf position on the donor plant, indeed, for both pretreated and control explants, fewer embryos were derived from the third leaf than from the first two leaves.

The initial explant pretreatment with a very high cytokinin concentration (129 μM BA and subsequent subculture onto 'standard induction medium' was associated with an elongation of the globular somatic embryos on explants derived from the third leaf, e.g., they reached an average length of 1.5 mm. The bigger embryo size could be related to the very small amount of embryos obtained in this treatment. However, embryos induced on first leaf explants retained their globular shape but were smaller (0.02–0.05 mm) than those directly induced on standard induction medium (0.1–0.2 mm long, irrespective of leaf position). A similar reduction in size was observed on embryos derived from the second leaf (0.075 mm). Surprisingly, none of these embryos, irrespectively of their size, gave rise to fully developed embryos.

Under the experimental conditions of the present study, the initial pretreatment of pepper explants with increased BA concentrations did not improve the induction of somatic embryogenesis from fully expanded leaves. Cytokinins and light can elicit similar morphogenic and biochemical responses in a wide range of plant species or otherwise interact in the control of in vitro dedifferentiation and redifferentiation processes (Ivanova et al., 1994; Thomas et al., 1997; Karnachuk and Gvozdeva, 1998). Baum et al. (1991) provided evidence that different parts of the plant (e.g., leaves at different positions) respond in vitro to different concentrations of cytokinin, indicating that a general gradient of decreasing cytokinin levels along the plant axis from roots to leaves might be expected. We have previously demonstrated (Kintzios et al., 1996) that explant dedifferentiation from mature pepper leaves was significantly more intense when higher leaves were used as the explant source and when higher absolute BA concentrations

Fig. 2. Relative somatic globular embryo production (globular embryos per mm² explant surface) from pepper leaf explants on MS + 8% (w/v) sucrose + 12.9 μM BA + 9 μM 2,4-D in response to different leaf position and explant pretreatment with a tenfold (129 μM) concentration of BA (dotted bars = no explant pretreatment, and black bars = explant pretreatment with 129 μM BA).
were used. The increased proliferation of the unorganized callus tissue might have had a negative effect on the expression of somatic embryogenesis from higher leaves, although the fact that this material is ontogenetically older could also play a role. It is also possible that excessive growth regulator concentrations might be inhibitory for the expression of somatic embryogenesis in pepper, which could explain the poor response of the first leaves (with a presumably higher endogenous cytokinin content) under continuous light. This effect has been demonstrated for cytokinins in some plant species, such as *Cucurbita pepo* (Jelaska, 1986) and *Cucumis melo* (Oridate and Oosawa, 1986).

3.2. Further embryo development, maturation and germination

Globular somatic embryos which were subcultured on modified fresh ‘standard induction medium’ (3% sucrose) were able to further develop until heart- and torpedo-shaped forms (1–2 and 3–5 mm long, respectively) appeared. Embryo germination (at a rate of 1.1 regenerated plants per 100 embryos subcultured), indicated by the elongation of shoot structures form each embryo and the subsequent root initiation, took place on the same medium. Further development of globular somatic embryos to the cotyledonal and torpedo-shaped stage and their germination was not affected by leaf position or the explant pretreatment with BA. Although, cultures were exposed to the light (with a 16/8 h photoperiod) during this phase, fully developed embryos could be derived only from callus pieces initially incubated for 3 weeks in darkness.

In conclusion, further experiments are necessary for an optimal regulation of pepper somatic embryogenesis by means of a specific light regime and BA concentration. Leaf explants derived from ex vitro grown plants are still less responsive than in vitro derived explants (Kintzios et al., 1998). Future experiments planned to be conducted in our laboratory include the analysis of endogenous hormones in relation to exogenous growth regulators and different chromatic light effects, as well as the substitution of 2,4-D for other auxins (such as IAA and indole-3-butyric acid).

References


