Axillary shoot proliferation in cultures of explants from mature
*Juniperus oxycedrus* trees

M. P. GÓMEZ and J. SEGURA

Department of Plant Biology, Faculty of Pharmacy, University of Valencia, Avinguda Vicent Andrés Estellés s/n, 46100-Burjasot (Valencia), Spain

Received October 17, 1994

Summary We developed procedures for the micropropagation of *Juniperus oxycedrus* L. using shoot apices or nodal segments from mature plants. Of the media and explants examined, best culture establishment was obtained with shoot apices cultured on modified Schenk and Hildebrandt medium (SH medium) without growth regulators; however, shoot multiplication was only achieved when shoot apices isolated from shoots grown on SH medium without growth regulators were subcultured on SH medium containing 0.5 µM benzyladenine. None of the auxins and methods tested for root induction provided satisfactory results.

Keywords: auxins, benzyladenine, cytokinins, growth regulators, indoleacetic acid, indolebutyric acid, kinetin, naphthaleneacetic acid, tissue culture.

Introduction

Micropropagation by axillary shoot proliferation has been reported for many hardwood species (Bonga and von Aderkas 1992). In contrast, axillary shoot proliferation in conifers still presents technical difficulties (Boulay 1987, Thorpe et al. 1991). However, there is much interest in developing techniques for axillary shoot proliferation in conifers as a means of facilitating micropropagation of trees that are old enough to have demonstrated their superior value. We have developed a system for micropropagating *Juniperus oxycedrus* L. (Cupressaceae) from apical and nodal explants of mature trees. *Juniperus oxycedrus* was chosen for this study because the species has potential for reforestation of degraded Mediterranean areas (Ceballos and Ruiz 1971).

Materials and methods

Plant material

Terminal shoots from lateral branches (5 cm length) were collected from 30-year-old *J. oxycedrus* trees growing in their natural habitat (La Eliana, Valencia, Spain) and kept at 4 to 6 °C for 1–2 days. The shoots were surface sterilized by immersion in 1% NaOCl containing 0.01% Tween-20 for 15 min, followed by four 5-min washes with sterile distilled water. Subsequently, shoots apices (2 mm length) or nodal segments (with 2–3 axillary buds) were excised from the terminal shoots and cultured on different nutrient media.

Media and culture conditions

The basal media tested were: Medium A with MS macronutrients (Murashige and Skoog 1962), Medium B with SH macronutrients (Schenk and Hildebrandt 1972), Medium C with the macronutrients of Gressoff and Doy (1972), and Medium D with the macronutrients of Heller (1953) supplemented with 1 mM (NH₄)₂SO₄. In some experiments, Medium A was used with MS macronutrients reduced to 1/3 of their original concentration (A/3). All media included MS micronutrients, vitamins as described by Vieitez et al. (1985), and 30 g l⁻¹ sucrose. Solidified nutrient medium was prepared by adding 0.7% Difco Bacto agar. All media were adjusted to pH 5.8. Growth regulators were added to media before autoclaving for 20 min at 120 °C (1 × 10⁵ Pa). Explants were cultured either in 150 × 25 mm glass tubes each containing 20 ml of agar-solidified nutrient medium, or in continuously agitated (50 rpm) 125-ml Erlenmeyer flasks each containing 30 ml of liquid medium. Vessels were closed with polypropylene closures (Bellico).

Unless otherwise specified, cultures were maintained for 60 days in a growth chamber at 26 ± 2 °C and a 16-h photoperiod with light supplied by Sylvania (GTE Gro-lux, F36W/GRO, Germany) fluorescent tubes (80 µmol m⁻² s⁻¹ irradiance at culture level). In all experiments, cultures were examined for sprouting percentage, number of shoots per explant and shoot length. Twelve explants were cultured per treatment, and all experiments were repeated at least twice.

Culture establishment

To study the effect of nutrient medium on culture establishment, apical explants were cultured on agar-solidified Medium A, B, C or D supplemented with benzyladenine (BA; 0, 0.05 or 0.5 µM). The effect of 0.05 µM indoleacetic acid (IAA) on culture establishment was examined in apical explants cultured on Medium B supplemented with 0, 0.05, 0.5, 5 or 10 µM BA.

To study the influence of harvest time on culture establishment, apical explants from branches collected in spring, summer, autumn and winter were cultured on agar-solidified Medium B supplemented with 0, 0.05 or 0.5 µM BA.
Shoot multiplication

Shoot apices (0.5–0.8 cm) isolated from shoots grown for 2 months on Medium B without growth regulators were transferred to the same medium supplemented with BA (0, 0.05 or 0.5 µM) alone or in combination with 0.05 µM kinetin (KIN). In another experiment, explants were initially maintained for 1, 2, 6, 24 or 48 h in liquid Medium B supplemented with 50 µM BA. After each induction time, tissues were transferred to solid basal Medium B. Total culture period was 60 days.

Root induction

Shoots (1 cm) excised from clumps that had been cultured on agar-solidified Medium B without growth regulators were cultured for 30 days on solidified Medium B supplemented with naphthaleneacetic acid (NAA), IAA or indolebutyric acid (IBA) (0.0, 0.5, 2.5, 5.0, 10.0, 12.5 or 25.0 µM), or combinations of NAA + IAA, NAA + IBA or IAA + IBA (2.5 and 12.5 µM), then transferred to the same medium without auxins. In a second experiment, shoots were dipped in sterile aqueous solutions (pH 5.6) of IAA, NAA or IBA (50, 100, 250 or 500 µM) for 10 min and then cultured on solidified Medium B without growth regulators. Rooting percentages were recorded after 60 days.

Statistical analysis

Significance of the treatment effects was determined by analysis of variance (Statgraphics, Version 6, Manugistics, Rockville, MD, USA), employing a completely random design. Percentage data were subjected to arcsin transformation before analysis. Variations among treatment means were analyzed by Tukey’s (1953) procedure.

Results and discussion

Culture establishment

A comparison of apical and nodal explants cultured on Medium A/3 with different concentrations of BA (0, 5, 10, 20, 30 or 40 µM) indicated that apical explants were more responsive to BA than nodal explants. The presence of BA in the culture medium inhibited sprouting percentage, the mean number of shoots per explant and shoot length. Concentrations of BA higher than 10 µM caused necrosis of the explants. The use of BA concentrations less than 10 µM or the substitution of BA for KIN (0.5 or 5.0 µM) did not improve the morphogenetic capacity of the explants. None of these treatments was effective in promoting axillary bud multiplication (data not shown).

Neither nutrient media nor BA concentration significantly affected sprouting percentages (99–100%) of apical explants after 60 days. Although the type of nutrient medium significantly influenced the mean number of shoots formed per apical explant, the proliferation rates were low, ranging from 1.4 to 2.2 shoots per explant (Table 1). The length of the regenerated shoots depended on the nutrient medium and BA concentration. Maximal shoot elongation occurred on Medium B without BA (Table 1).

The presence of 0.05 µM IAA, alone or in combination with BA, in the induction medium significantly reduced sprouting percentage, number of shoots per explant and shoot elongation of the explants (data not shown). In other conifers, the presence of auxins also reduces cytokinin-induced axillary bud proliferation (Lin et al. 1991), although a low concentration of auxin can promote growth of axillary shoots by counteracting the inhibitory effects of high cytokinin concentrations on shoot elongation (see Nehra and Kartha 1994). Bud cultures in conifers have also been successfully established on media without

Table 1. Effects of nutrient media and BA concentration on shoot proliferation from shoottips of mature Juniperus oxycedrus. Culture time was 60 days. Data are mean values from 12 replications; A, B, C and D are the different culture media.

<table>
<thead>
<tr>
<th>BA (µM)</th>
<th>Sprouting percentage</th>
<th>No. shoots/explant</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A B C D</td>
<td>A B C D</td>
<td>A B C D</td>
</tr>
<tr>
<td>0</td>
<td>100 100 100 100</td>
<td>1.6 1.8 1.7 2.0</td>
<td>0.8 bcd 2.0 e 0.9 cd 1.0 d</td>
</tr>
<tr>
<td>0.05</td>
<td>98 100 100 100</td>
<td>1.6 2.0 1.8 1.7</td>
<td>0.4 ab 0.5 abc 0.4 ab 0.4 ab</td>
</tr>
<tr>
<td>0.5</td>
<td>98 100 100 100</td>
<td>1.4 2.2 2.0 1.6</td>
<td>0.3 a 0.4 ab 0.3 a 0.3 a</td>
</tr>
<tr>
<td>Mean²</td>
<td>1.5 a 2.0 b 1.8 ab 1.7 ab</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of variance

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Mean squares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sprouting percentage</td>
</tr>
<tr>
<td>Nutrient medium (A)</td>
<td>3</td>
<td>225.0 ns³</td>
</tr>
<tr>
<td>BA concentration (B)</td>
<td>2</td>
<td>56.2 ns</td>
</tr>
<tr>
<td>A × B</td>
<td>6</td>
<td>56.2 ns</td>
</tr>
<tr>
<td>Error</td>
<td>132</td>
<td>112.5</td>
</tr>
</tbody>
</table>

¹ Interaction of nutrient medium with BA; values followed by the same letter are not significantly different according to Tukey’s test at P = 0.05.
² Effect of nutrient medium; values followed by the same letter are not significantly different according to Tukey’s test at P = 0.05.
³ ns = Not significant.
⁴ * = Significant at P = 0.01.

Because time of explant collection can influence in vitro propagation of some conifers (von Arnold 1981, Bonga 1987, Laliberté and Lalonde 1988, Chesick et al. 1990, Lin et al. 1991), we examined the proliferative capacity of shoot apices collected in spring, summer, autumn and winter, and cultured on agar-solidified Medium B with or without BA (0.05 and 0.5 µM). Sprouting percentages (100%) and the mean number of shoots per explant (1.6 to 2) were not significantly influenced by time of collection, but shoot length was greater (1.9 cm) on media without BA than on media containing BA (data not shown).

**Shoot multiplication**

Apical buds, isolated from shoots grown on solidified basal Medium B, were subcultured on the same medium supplemented with BA, KIN or combinations of both cytokinins. After 60 days of culture, sprouting percentage was 100%. Furthermore, subculture favored axillary bud development and the formation of multiple shoot cultures. The highest number of shoots per explant was obtained when the medium was supplemented with 0.5 µM BA, and the greatest shoot length was obtained when the medium was supplemented with 0.5 µM BA and 0.05 µM KIN (Table 2).

Exposure to liquid Medium B containing 50.0 µM BA prior to culture on solidified medium without BA did not increase the morphogenetic response of the explants. Proliferation rates were less in explants cultured in liquid medium than on solidified medium with BA (data not shown). There was also a progressive loss of morphogenetic potential and an increase in hyperhydricity of the explants with increasing exposure to BA. However, pulse treatments of cytokinins have improved caulogenesis in other conifers (Bornman 1983, von Arnold and Tillberg 1987, Jang and Tainter 1991).

Table 2. Shoot multiplication from shoot tips of mature Juniperus oxycedrus cultured on Medium B with cytokinins. Culture time was 60 days. Data are mean values from 12 replications.

<table>
<thead>
<tr>
<th>Cytokinins (µM)</th>
<th>No. shoots/explant</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>KIN</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>1.5 a</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0</td>
<td>1.3 bc</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0</td>
<td>0.9 a</td>
</tr>
<tr>
<td>0.05</td>
<td>0.05</td>
<td>1.2 ab</td>
</tr>
<tr>
<td>0.5</td>
<td>0.05</td>
<td>1.6 c</td>
</tr>
</tbody>
</table>

**Analysis of variance**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Mean squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. shoots/explant</td>
<td>4</td>
<td>59.9*²</td>
</tr>
<tr>
<td>Shoot length</td>
<td>55</td>
<td>0.9*</td>
</tr>
<tr>
<td>Cytokinins</td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td>0.1</td>
</tr>
</tbody>
</table>

1 For each parameter, values followed by the same letter are not significantly different according to Tukey’s test at P = 0.05.
2 * = Significant at P = 0.01.

**Rooting and plant development**

None of the auxins or auxin combinations tested promoted satisfactory rooting. The frequency of rooted shoots (data not shown) ranged from 7 to 10%, and there were no significant differences between the auxins or auxin combinations tested. Some treatments, especially those including NAA, induced basal callus formation. In a previous study (Gómez and Segura 1994), we found high rooting percentages with adventitious shoots regenerated from leaves of mature J. oxycedrus suggesting that the method of micropropagation affects the rooting capability of the regenerated shoots. The high rhizogenic potential of adventitious shoots regenerated from leaves may be associated with some rejuvenation process, because an inverse relation between rooting capacity and age has been observed in many woody species (Németh 1986, Evers et al. 1988).

Micropropagation of adult Juniperus oxycedrus plants was accomplished by axillary shoot multiplication. The procedure may be useful for clonal propagation of elite genotypes, but only after improvements in the rooting of proliferating shoots have been achieved.

**Acknowledgments**

This work was supported by the Diputación de Valencia and the Conselleria de Cultura, Educación y Ciencia de la Generalidad Valenciana, grant to P.G.

**References**


