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SCIENCE DIRECT
Scientia Horticulturae 107 (2005) 64–69
SCIENTIA HORTICULTURAE
www.elsevier.com/locate/scihorti

Micropropagation of *Vitis thunbergii* Sieb. et Zucc., a medicinal herb, through high-frequency shoot tip culture

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Received 7 September 2004; received in revised form 18 March 2005; accepted 4 May 2005

Abstract

This study describes a protocol for rapid and large-scale in vitro propagation of the valuable medicinal herb *Vitis thunbergii* Sieb. et Zucc.. Culture conditions influencing shoot proliferation and rooting of the two clones (three- and five-lobed) were examined. Three medium formulations, Murashige-Skoog (MS), Woody Plant Medium (WPM) and Nitsch and Nitsch (NN) medium, were tested for growth of shoot tip culture, and WPM was found to have a superior proliferation rate. The chlorophyll content of leaves was highest in those cultured on NN, followed by those on MS and WPM medium. WPM medium supplemented with 0.5 mg l^{-1} 6-benzyladenine (BA) displayed the highest proliferation rate (15–19 nodes or 3–4 shoots per explant). The rooting was optimized using MS medium supplemented with 0.5 mg l^{-1} napthaleneacetic (NAA) with eight roots, 3 cm long after 1 month of culture. High frequency callus formation was observed in the basal end of explants cultured on NAA-containing medium. Following acclimatization, rooting plantlets were transferred to the plastic house with a 95% survival rate.

Keywords: *Vitis thunbergii*; Medium formulation; Cytokinin; Chlorophyll; Auxin

1. Introduction

Vitis thunbergii, a species indigenous to East Asia, is a perennial medicinal herb. A traditional Chinese medicine, this plant is widely used to relieve arthritis, eye irritation and hepatitis (Kan, 1977). Furthermore, the whole plants are commercially used in medicinal wines and beverages. During the past 10 years, several natural pharmaceutical components have been identified, including resveratrol polymers (Chen, 2001), caffeic acid, quercetin-3-galactoside, quercitrin, rutin, narcissin and luteolin-7-*o*-glucoside (Lin, 1997). Studies have showed that resveratrol prevents coronary heart disease and possesses anti-inflammatory, anti-neoplastic, anti-oxidase and anti-fungal activities (Stein et al., 1999).

A low survival rate by stem cuttings in *V. thunbergii* restricts its mass propagation via conventional methods (M.W. Chang, personal communication). Therefore, an efficient in vitro propagation system for producing this plant is required to further clarify its potential medicinal values and germplasm conservation. Successful micropropagation using axillary bud culture (Lee and Wetzstein, 1990; Heloir et al., 1997; Gustavsson and Stanys, 2000) or somatic embryogenesis (Salunkhe et al., 1999; Motoike et al., 2001) of various species and cultivars of *Vitis* is well documented. Gustavsson and Stanys (2000) reported the plants derived from tissue culture as having superior field performance to those derived from stem cutting in terms of survival rate, fruit yield, rhizome production and total plant weight in ''Sonna'' lingonberry (Vaccinium vitis-idaea L.). However, no in vitro study of *V. thunbergii* has ever been performed. This work demonstrated an in vitro proliferation system of *V. thunbergii* via high frequency shoot tip culture. The culture medium was optimized for propagation and high ex vitro survival rate was achieved.

Abbreviations: MS, Murashige-Skoog; WPM, Woody Plant Medium; NN, Nitsch and Nitsch Medium; BA, 6benzylaminopurine; 2-ip, 2-isopentyl; NAA, napthaleneacetic acid; IBA, indole-3-butyric acid; IAA, indoleacetic acid; FW, fresh weight; PGR-free, plant growth regulator-free

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doi:10.1016/j.scienta.2005.05.014

2. Materials and methods

Nodal explants of two clones (three- and five-lobed) with axillary buds were collected from 2-year-old *V. thunbergii* grown in a plastic house at the Miaoli District Agricultural Research and Extension Station. Following removing of the leaves, the excised nodals (0.5–1 cm long) were disinfested in 0.8% (w/v) sodium hypochlorite with 0.1% Tween 20 for 8 min, then rinsed three times with sterile distilled water. The sterilized single nodal explants were cultured on induction medium.

Shoot tips of in vitro grown plantlets after 1 month of culture were used as the starting materials. The shoot tips were excised and cultured on Woody Plant Medium (WPM, Lloyd and McCown, 1980) with 0.5 mg l^{-1} BA, 2% sucrose, and 100 mg l^{-1} myo-inositol and 0.8% agar. Unless otherwise specified, the medium pH was adjusted to 5.7 before autoclaving at 121 °C, 1.2 kg cm^{-2} for 15 min. The cultures were maintained at 25 ± 2 °C under a 16/8 h (light/dark) photoperiod with light provided by cool-white fluorescent tube with an intensity of 80 μmol $m^{-2}s^{-1}$.

Three medium formulations were tested to determine the extent, which they promoted shoot proliferation and development. These media comprised Nitsch and Nitsch (NN, Nitsch and Nitsch, 1969), Murashige and Skoog (MS, Murashige and Skoog, 1962) and WPM (Lloyd and McCown, 1980), all supplemented with 0.5 mg l^{-1} BA, 2% sucrose, 40 mg l^{-1} adenine sulfate, 170 mg l^{-1} NaH_2PO_4 and 100 mg l^{-1} myo-inositol and 0.8% agar. Three types of cytokinin (BA, kinetin and 2-isopentyl (2-ip), all supplemented 0.5 mg l^{-1}) were added to the WPM medium in a series of experiments. The index FW increase ((final FW − initial FW)/initial FW), plant height, leaves, shoots and nodes per explant (indicator of proliferation rate), rooting percentage, and chlorophyll content were examined after 1 month of culture.

Shoot tips with two leaves cultured on WPM medium were used as explants for the rooting trial. To optimize root induction, different medium formulations and concentrations (half-strength MS, MS, half-strength WPM and WPM) were tested and all of which were supplemented with 0.5 mg l^{-1} indole-3-butyric acid (IBA). Three rooting agents (NAA, IBA or indoleacetic acid (IAA)) at a concentration of 0.5 mg l^{-1} were supplemented into the MS medium to test their root induction ability. The index FW increase, plant height, root length, number, rooting and callusing percentage were counted after 1 month of culture.

For chlorophyll estimation, 0.5–1 g of detached leaves were extracted in 80% (v/v) acetone according to the method of Arnon (1967). Absorbance at 645, 663, and 652 nm were recorded, and the extinction coefficients were used to determine the chlorophyll a, b, and total chlorophyll content of the tissue.

Healthy plantlets (2–3 cm in height, 2–4 roots and 7–8 leaves) were transferred to plug with perlite/peatmoss (v/v 1:2) mixture. During the fist week of transfer, the plantlets were covered with a transparent plastic film to maintain high humidity and then fertilized at weekly intervals. The survival rate was examined 1 month after transfer.

A complete randomized block (CRD) design was used for all experiments. Five explants were inoculated per glass vessel (containing 25 ml of culture medium), and each treatment involved seven vessels. Data taken in percentage (e.g. % rooting or % callusing) was subjected to arcsin transformation before analysis and converted back into percentage form for presentation in tables (Snedecor and Cochran, 1968). The data were analyzed using SAS Version 6.12 (SAS, Raleigh, NC). Significant differences were assessed using Duncan's multiple range test at $P < 0.05$.

3. Results

3.1. Effect of medium formulations on shoot growth and development

Medium formulation displayed a strong effect on the fresh weight, height, leaves, shoots and nodes per explant in the two clones of *V. thunbergii* (Table 1). Comparing the three formulations reveals that the proliferation rate of the WPM medium was superior to the MS or NN mediums, which induced an average of 1.3 to 1.9 shoots, 7.5 to 11.2 nodes and 8 to 12 leaves after 1 month of culture (Table 1). Three-lobed clones exhibited a higher multiplication rate than five-lobed ones (Table 1). However, the FW increase and plant height cultured in MS medium was signficantly higher than for those cultured in WPM or NN medium (Table 1). The leaves of explants cultured on MS medium

Table 1
Influence of medium formulation on the propagation of *Vitis thunbergii*

	Three-lobed[a]						Five-lobed					
	Index FW increase[b]	Height (cm)	Leaves per explant	Shoots per explant	Nodes per explant	% Rooting	Index FW increase	Height (cm)	Leaves per explant	Shoots per explant	Nodes per explant	% Rooting
MS	23.0 a	2.8 a	8.0 b	1.2 b	7.3 b	37.1 a	12.4 b	2.9 a	6.5 b	1.0 b	6.5 b	20.0 a
WPM	12.8 b	1.9 b	12.4 a	1.9 a	11.2 a	0 b	6.9 a	2.1 b	7.8 a	1.3 a	7.5 a	17.1 a
NN	11.6 b	1.4 c	6.9 b	1.2 b	6.2 b	17.1 ab	6.1 b	1.4 c	5.3 c	1.1 b	4.9 c	11.4 a

[a] Each column represented the means of seven replicates each within five explants per treatment. Within each treatment, values with the same letter are similar ($P < 0.05$) according to Ducan's multiple range test for each clone.
[b] Index FW increase: (final FW − initial FW)/initial FW.