In Vitro Plant Regeneration from Leaf-derived Callus in Goldenseal (*Hydrastis canadensis*)

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Abstract

***Hydrastis canadensis*, also known as goldenseal, is among the top 10 selling herbs in the United States, and is used for enhancing general body immunity. Due the increasing demand for wild-crafted goldenseal, plant has been listed in Convention on International Trade in Endangered Species (CITES) Appendix II list as an endangered species. An in vitro propagation protocol for rapidly producing goldenseal plantlets from disk tissue of young leaves was developed. Leaf explants were inoculated on MS medium supplemented with various concentrations of NAA and TDZ for production of callus. Two-month-old calli were sub-cultured on MS media containing cytokinins (BA, Kinetin, TDZ) in different concentrations for shoot initiation. The optimum level of callus induction and maintenance was 5.3 μM NAA in combination with 2.2 μM of TDZ. Shoot multiplication was achieved on MS medium with 2.2 μM TDZ. The alkaloid profile of micropropagated plantlets was similar to the profile of mother plants. These results suggest that our in vitro propagation protocol will produce a positive impact in the conservation of *H. canadensis*.**

INTRODUCTION

Native Americans used goldenseal (*Hydrastis canadensis* L.) to treat sore eyes and mouths, colds, flus and also as a dye (Duke, 2001). *H. canadensis* was widely used in the United States during the late 19th century and early 20th century for the treatment of gonorrhea, cystitis, urinary tract inflammations, lowering of blood pressure, sedation, and constipation. The pharmaceutical companies that sold these extracts were Eli Lilly, Bristol-Myers Squibb, and Parke-Davis (Hobbs, 2001). The popularity of goldenseal increased in the 1980s with the unfounded belief that consumption of the rhizome would prevent detection of drug residues (Tyler, 1982). The rationale for the traditional use of *H. canadensis* in treatments of bacterial infections has been confirmed by the recent report of Schazzochio et al. (2001).

The over-exploitation of *canadensis* reduced the number of specimens on the rich and moist soils of deciduous forest from southern New England, to southern Wisconsin, south to Arkansas and northern Mississippi. The species has been listed in CITES Appendix II and designated as threatened in Canada and in the United States with highest priority for protection (Bannerman, 1997; CITES, 1997). Tissue culture techniques have been reported for conservation and propagation of several endangered medicinal plants (Babu et al., 1992; Sharma et al., 1993; Purohit et al., 1994; Hosoki et al.; 1995, Sudha and Seeni, 1996). This technique may rapidly increase the number of goldenseal propagules for cultivation as well as aid in the replacement of natural populations. In the present study, we report organogenesis and plant regeneration from leaf-tissue-derived callus and the effects of auxins on alkaloids such as berberine and hydrastine.

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MATERIALS AND METHODS

Plants were purchased from Richters Herbs (Goodwood, Ontario, Canada) in pots and maintained in a controlled environment at 12 C° with a 16 h photoperiod under fluorescent lights. Two to three day old whorl leaves were cut in disk and surface disinfected as follows: 1 % NaOCl and 0.1 % Tween 20 for 10 minutes, followed by three 5 minute washings, with sterile distilled water. Disinfected explants were inoculated on Murashige and Skoog's (MS) medium (1962) containing 3 % (w/v) sucrose, 0.8 % (w/v) Type E agar (Sigma Chemical Co. St. Louis) supplemented with different concentrations of α-naphthalene acetic acid (NAA) and thidiazuron (TDZ) for callus induction and maintenance (Table 1). The pH of the medium was adjusted to 5.8. The sterile medium was dispensed (25 ml) in glass culture vessels (4 cm diameter x 9.5 cm high, baby food jars with magenta B caps). Cultures were incubated at 25 C^o by 16 h photoperiod under fluorescent light with a photon flux of approximately 52 -µmol s^{-1} m^{-2}.

For shoot initiation, two months old callus were transferred to half and full strength MS salts media containing different concentrations of cytokinins N^6-benzyl-adenine (BA), kinetin (Kin) and TDZ (Table 1). Multiple shoots were noticed after 15 days. Sub-culturing for shoot elongation was done every 30 days. Activated charcoal (500 mg/L) was added to avoid cytotoxicity of compounds released to the media. Shoots were transferred to rooting media consisting of MS salts supplemented with indole-3-acetic acid (IAA), NAA, or indole-3-butyric acid (IBA) at two different concentrations (Table 3). Well-rooted shoots were rinsed with sterile water to remove residual rooting medium and transferred to planting tray kits with 6 cells and a plastic dome and base tray (Wal-Mart Stores, Inc.). For plantlet acclimatization to soil, the trays were incubated at 25 C^o by 16 h photoperiod under fluorescent light, relative humidity was 80 % in the trays and the soil mixture was Miracle-Gro potting soil.

Fully developed plantlets 5 cm tall were harvested, separated into plant parts (leaves, roots), freeze-dried and ground to a fine powder using a mortar and pestle, then extracted with methanol and analyzed by reverse phase HPLC according to Abourashed and Khan (2001). In addition, five plants that supplied explants for the micropropagation protocol, were also separated into plant parts and extracted. SPSS 9.0 performed all statistical analyses of growth and alkaloid content of the tested media for Windows, 1998 using ANOVA procedure and the T-test for the mean separation (SPSS, 1998).

RESULTS AND DISCUSSION

Callus induction was noted at the cut ends, as the explants began to whorl within 15-20 days and enlarged three to four fold within four weeks after culture initiation. Calli were produced in tested media that was supplemented with NAA in combination with TDZ. The callus proliferation and maintenance was dependent on the presence of both auxin and cytokinin in the medium. Cell growth, however was maximum in MS media supplemented with 5.3 µM NAA in combination with 2.2 µM TDZ.

The average shoot height and number of shoots induced per culture were the same in both half and full strength MS salt media supplemented with different cytokinins although the percentage of cultures with shoots was lower in half strength. Regarding overall plantlet development with vigorous growth, the optimal organogenesis response was achieved after four weeks in cultures supplemented with 2.2 µM TDZ (Table 2). Typically, a juvenile goldenseal has a single leaf with a long petiole, upon maturity, two leaves and a flower are present. These features, however, are not always consistent, in vitro regenerated cultures often produced one leaf, as well as clustered leaves in pairs (Fig.1).

Root induction was observed in all tested media supplemented with IAA (1.1-2.8 µM), IBA (1.0-2.1 µM) or NAA (1.1-2.7 µM) but more roots per culture were produced on NAA media. Plantlets on 2.8 µM of IAA showed the highest hydrastine content although cultures on MS media supplemented with 2.7 µM NAA yielded more hydrastine, considering that drug yield consist of biomass growth with drug content (Table 3). The content among treatments for in vitro berberine production, were similar regarding roots and leaves.

Comparing berberine content among in vitro plantlets and mother plant, no significant difference was found in leaves. Roots of mother plant however yielded more berberine than roots of in vitro plantlets (Fig 2). For hydrastine production, the content in leaves of mother plants were two fold greater than in cultures and this may be a function of plant age. Herbal products containing goldenseal are standardized by hydrastine and berberine content. Results from HPLC analysis confirm that the regenerated plants produce, as expected, hydrastine and berberine the same as the parent stock from which it was cultured (Fig. 2).

CONCLUSION

This micropropagation procedure can be used to regenerate large numbers of plants in a short period of time with a 100 % survival of rooted plantlets transplanted to soil after 4 weeks according to our observations. The procedure may also be used in a breeding program for in vitro selection and storage of desirable genotypes. In addition, this procedure may supply propagules for the herbal industry as well as speed up the restoration of the natural populations which may allow for the removal of goldenseal from the CITES list in the near future.

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Literature Cited

Abourashed, E.A. and Khan, I.A. 2001. High-performance liquid chromatography determination of hydrastine and berberine in dietary supplements containing goldenseal. J. Pharm. Sci. 90(7):817-822.

Babu, K.N., Sansudeen, K. and Ratnambal, M.J. 1992. In vitro regeneration from leaf-derived callus in ginger (*Zingiber officinale* Rosc.). Plant Cell, Tiss. and Org. Cult. 29:71-74.

Bannerman, J. 1997. Goldenseal in world trade: Pressures and potentials. HerbalGram. 41:51-52.

CITES. 1997. Proposal for the inclusion of Hydrastis canadensis. Appendix II, CITES.

Duke, J. 2001. Handbook of Medicinal Herbs, CRC Press LLC, Boca Raton, Fl.p. 238-239.

Hobbs, C. 2001. History of Medicines 1 Goldenseal in Early American Medical Botany. Health World Online-Herbal Medicine.

Hosoki, T., Mochida, M., Sakamoto, T. and Ohta, K. 1995. In vitro propagation of white stokesia (*Stokesia laevis* Green var. alba hort.) by leaf and root culture. J. Japan. Soc. Hort. Sci. 64 (2):375 –380.

Murashige, M. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiological Plant. 15:473-497.

Purohit, S.D., Dave, A. and Kukda, G. 1994. Micropropagation of safed musli (*Chlorophytum borivilianum*) a rare Indian medicinal herb. Plant Cell, Tiss. and Org. Cult. 39:93-96.

Schazzocchio, F., Cometa, M.F., Tomassini, L., Palmery, M. 2001. Antibacterial activity of Hydrastis canadensis extract and its major isolated alkaloids. Planta Med. 67:561 563.

Sharma, N., Chandel, K.P.S. and Paul, A. 1993. In vitro propagation of *Gentiana kurroo*-an Indigenous threatened plant of medicinal importance. Plant Cell, Tiss. and Org. Cult.. 34:307-309.

SPSS 9.0 for Windows. 1998. Copyright © SPSS, Inc., Chicago, Illinois.

Sudha, C.G. and Seeni, S. 1996. In vitro propagation of *Rauwolfia micrantha*, a rare medicinal plant. Plant Cell, Tiss. and Org. Cult. 44:243-248.

Tyler, V.E. 1982. The honest herbal – A sensible guide to use of herbs and related remedies. George F. Stockley, Philadelphia . p. 263.

Tables

Table 1. Effect of NAA and TDZ on leaf tissue explants of *Hydrastis canadensis* for production of callus.

NAA (μM)	TDZ (μM)	Amt. of callus	Friability	Cultures † with callus (%)
0.5	2.2	++	+	92
2.7	1.1	++	+	45
5.3	2.2	+++	++	92

\+ = 0- 0.9 g
++ = 1.0 to 1.9 g
+++ = 2.0 to 3.0 g
† (%) of cultures represent 10 or more replicates per treatment.

Table 2. Effect of cytokinins on shoot differentiation from *Hydrastis canadensis* calli derived from leaf section.

Cytokinins	Conc. μM	No. of shoots	Height of shoots (cm)	(%) of cultures with shoots
BAP	4.4	9.0	1.5	100
Kin	4.6	7.4	1.1	100
TDZ	4.5	7.6	1.2	100
	2.2	0.4	1.4	100
	0.2	6.2	1.4	80
MS/2+TDZ	4.5	7.3	1.1	88
Significance Media		0.55	0.91	

Data represent means of five or more replicates per treatment.

Table 3. The effect of auxins on rooting of *Hydrastis canadensis* and alkaloid content.

Auxins (μM)	Root Number †	Root Length (cm)	Cultures Dry weight (g)	Roots Hydrastine (%)‡	Roots Berberine (%)	Leaves Hydrastine (%)	Leaves Berberine (%)	Media Hydrastine (%)	Media Berberine (%)
IAA 1.1	1.00 ± 2.34	2.95 ± 0.74	1.01 ± 0.36	1.01±0.08	1.41±0.21	0.54±0.24	1.75±0.45	0.005±0.003	0.11± 0.02
2.8	1.43 ± 2.85	2.75 ± 0.64	0.84 ± 0.18	1.82±0.92	1.45±0.81	0.40±0.10	2.10±0.66	0.006±0.002	0.17± 0.05
IBA 1.0	1.28 ± 2.61	5.45 ± 2.17	0.68 ± 0.15	0.90± 0.56	1.18 ±0.38	0.58±0.49	1.60± 0.50	0.01 ± 0.001	0.08 ± 0.03
2.1	1.18 ± 2.14	3.85 ± 0.99	0.98 ± 0.26	0.92± 0.72	1.25 ±0.33	0.33±0.10	1.35± 0.17	0.01± 0.009	0.16 ± 0.12
NAA 1.1	3.86 ± 4.18	2.24 ± 0.68	1.03 ± 0.27	0.67± 0.04	1.15±0.23	0.26±0.01	1.53± 0.32	0.006±0.003	0.10 ± 0.03
2.7	3.67 ± 4.27	2.78 ± 1.15	2.92 ± 0.15	0.67± 0.09	1.13±0.14	0.46±0.60	1.82± 0.18	0.008±0.004	0.14 ± 0.05
Significance Media	0.000	0.010	0.390	0.085	0.780	0.850	0.389	0.560	0.45

† Root number is a mean of roots produced in ten or more cultures. Each sample was analyzed by HPLC in three consecutive runs.
‡ (%) Drug content of each alkaloid is a mean of four cultures extracted by methanol.

Figures

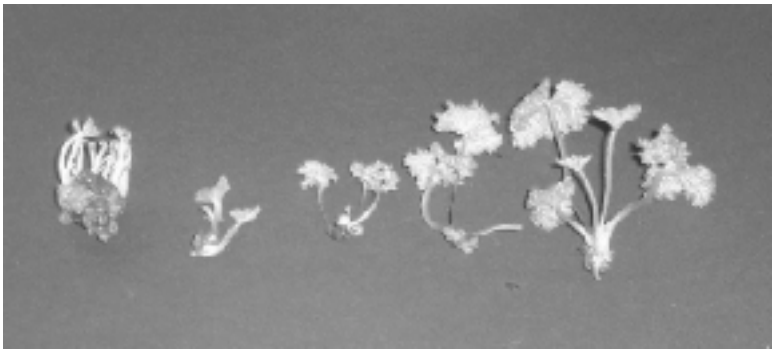

Fig. 1. Shoot induction of *Hydrastis canadensis.*

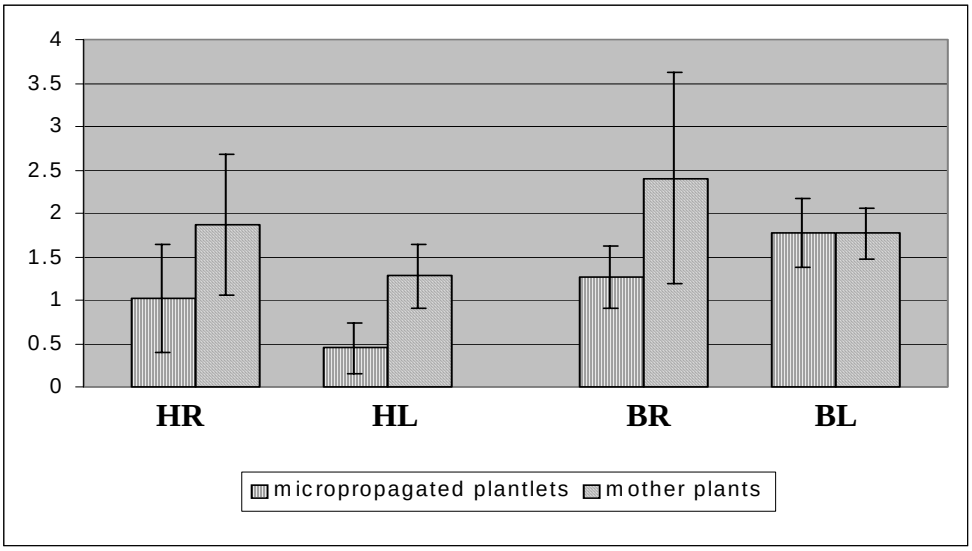

Fig. 2. Hydrastine content in roots of micropropagated goldenseal plantlets and mother plants (HR), leaves of micropropagated plantlets and mother plants (HL), berberine content in root of micropropagated plantlets and mother plants (BR) and leaves of micropropagated plantlets and mother plants (BL).