Plant Cell, Tissue and Organ Culture (2006) 84: 223–225
DOI 10.1007/s11240-005-9011-0

Research note

Direct rhizogenesis and establishment of fast growing normal root organ culture of *Withania somnifera* Dunal

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Received 23 December 2003; accepted in revised form 9 August 2005

Key words: alkaloids, Ashwagandha, bubble column reactor, untransformed root-organ culture, withanolides

Abstract

Direct rooting from leaf explants of *Withania somnifera* was achieved on half strength Murashige and Skoog's medium supplemented with 15 g l^{-1} sucrose, and different concentrations of growth regulators. Basal medium supplemented with 2.85 μM indoleacetic acid and 9.85 μM indolebutyric acid achieved maximum number of roots with 100% response. The roots were cultured on MS liquid medium for the establishment of root-organ culture with the same plant growth regulators and incubated on an orbital shaker at 80 rpm at 25 ± 2 °C. A root biomass of 6.15 ± 0.17 g was obtained after 5 weeks. When 1 g roots were inoculated to 2.5 l bubble column reactor, 47 g roots were obtained after 6 weeks. The concentration of alkaloids was increased as compared to field grown roots. The maximum concentration of withanolides (10 mg g^{-1} dry weight) was obtained in the bioreactor.

Abbreviations: IAA – indoleacetic acid; IBA – indolebutyric acid; MS Medium – Murashige and Skoog's medium; NAA – 1-naphthaleneacetic acid

Withania somnifera Dunal (Ashwagandha in Sanskrit) is an evergreen tomentose shrub, belonging to the family Solanaceae. It grows in wild and is also cultivated in several areas of India (Kulkarni et al., 1996). The roots and leaves of Ashwagandha contain various alkaloids, viz., withanolides (Nittala and Lavie, 1981; Atta et al., 1991) and Withaferins (Devi et al., 2000). The withanolides are steroidal and bear resemblance, both in action and appearance to the active ginsenosides of Asian ginseng. Within Indian traditional medicine, this drug is highly esteemed for being able to impart long life, youthful vigour and intellectual power (Saraf and Bhide, 1983). In approximately 66% of the medicinal plants used in traditional medicine, roots are the principal source for drug preparation (Kamboj, 2000).The development of a fast growing root culture system would offer unique opportunities for producing root drugs in the laboratory without having to depend on field cultivation (Sudha and Seeni, 2001). In the present study, we report the production of withanolides by culture of *Withania somnifera* roots in flasks as well as in bioreactors.

Seedlings of *Withania somnifera* were raised in a 1:1 mixture of sand and soil in earthen pots. Axillary leaves (0.5–0.9 cm) taken from the first node of 10–15-day-old plantlets, were surface sterilized with 1% (w/v) mercuric chloride (Hi Media) for 1 min and washed three to four times with double distilled water. The leaves were cut horizontally into two halves and inoculated on half strength MS (Murashige and Skoog, 1962) medium supplemented with 15 g l^{-1} sucrose. Various concentrations and combinations of IAA, IBA and NAA were added. The pH of the medium was adjusted to 5.6 ± 0.2 before sterilization. Fifteen explants were taken per treatment and

each treatment was replicated thrice. After 3 weeks of initiation, roots were separated from the explant aseptically and four segments per flask (1.0–2.0 cm in length and 200 mg fresh weight) were subcultured into 25 ml aliquots of half-strength MS medium supplemented with 2.85 μM IAA and 9.85 μM IBA in 150 ml Erlenmeyer flasks. The cultures were kept under continuous agitation at 80 rpm in an orbital shaker (Orbitek) and incubated at 25 ± 2 °C.

A bubble column reactor was set up in a 2.5 l culture vessel (Incel Tech, France). The reactor vessel was autoclaved for 15 min at 121 °C with 1000 ml half-strength MS liquid medium supplemented with 15 g l^{-1} sucrose, 2.85 μM IAA and 9.85 μM IBA. The pH of the medium was adjusted to 5.6 ± 0.2 before autoclaving. For air exchange, 0.2 μm PTFE filters (Sartorius AG., Germany) were used. Approximately 2 l min^{-1} air flow using sparger was maintained using an oil free air compressor. One gram mass of root was inoculated into the reactor and temperature was maintained at 25 ± 2 °C.

Root cultures were dried overnight in an oven at 55 °C. Dried powdered material (200 mg) was extracted with 2 ml methanol by sonication (20 KHz, Bendalin Electronics, Germany) for 20 min at room temperature. Methanolic extracts were evaporated to dryness at 45 °C in a vacuum oven. For analysis, the remainder was redissolved in 500 μl HPLC grade methanol (Qualigens) and transferred to a polypropylene microcentrifuge (Eppendorf) tube, vortexed for 10 s and centrifuged for 5 min at 3000 × *g*. After centrifugation, the clear supernatant was used for the analysis.

The analytical HPLC experiments were performed with a Merk High Performance Liquid Chromatography equipped with variable wavelength detector operating at 225 nm (L-7400). Separations were carried out with C18 (5 μm) Kromasil column with methanol:water (65:35) as an eluent at a flow rate of 2 ml min^{-1} (Burton et al., 1984).

Leaf explants inoculated on full strength MS medium produced vast amount of callus, which hindered the initiation of roots. Therefore, the experiments were carried out on half strength MS medium with different concentrations and combinations of auxins. Growth of the roots was checked on the basis of number of roots per explant and the morphology.

Among the various concentrations and combinations of auxins tested, in a medium with 2.85 μM IAA and 9.85 μM IBA direct rooting was observed in 100% of the explants within 8–10 days of inoculation with a mean number of roots of 9.2 ± 0.2. In other combinations, the response was good, but callus formation occurred after 15 days of inoculation (Table 1). For further study, the roots were transferred to half-strength MS liquid medium with the same concentration of plant growth regulators. From 200 mg inoculum 6.15 ± 0.2 g fresh weight was obtained after 5 weeks under the shake flask conditions. The roots were subcultured regularly by inoculating growing root tips (1–1.5 cm) in the optimal medium at 4 weeks interval.

The roots grown in shake flask were thicker and more sturdy compared with hairy roots (Lee et al., 1999; Sudo et al., 2002). The preliminary studies showed that the roots could grow in

Table 1. Effect of various plant growth regulators in half strength MS medium on root induction from leaf explants of *Withania somnifera*

IAA (μM)	IBA (μM)	NAA (μM)	Mean number of roots*	Response (%)	Morphology of roots
–	2.46	2.68	7.2 ± 0.4	87	R + C
2.85	9.85	–	9.2 ± 0.2	100	R
2.85	–	1.07	0.5 ± 0.1	33	R + C
2.85	–	–	1.2 ± 0.1	47	R + C
–	2.46	–	0.2 ± 0.1	27	R + C

R, Roots; C, Callus.
*For each combination 15 replicates were used.
± Standard error of the mean.

Figure 1. Roots of *Withania somnifera* growing in 2.5 l bubble column reactor.

vigorous aeration. Growing roots (1.0 g fresh weight) from the second subculture of root-organ culture were inoculated aseptically in to bubble column reactor containing 1000 ml half-strength liquid MS medium having same concentrations of auxins. Profuse root growth was observed in the reactor. The lateral roots were responsible for rapid growth. After 3–4 weeks, the lateral roots were elongated up to 8–10 cm in length and formed a mat of roots. During this period, several parts of these secondary lateral roots were converted to a brown and woody form. These roots were thick and brittle. After 6 weeks, 47 g roots was harvested from the bioreactor (Figure 1).

The alkaloid profile of these *in vitro* roots was analyzed and compared by HPLC analysis with *in vivo* grown roots as well as a standard sample obtained from Himalayan Drug Company, Banglore, India. HPLC analysis of *in vivo* root extracts yielded a peak with a retention time of 2.25 min with a withanolide content of 3 mg g^{-1} dry weight. The extracts from *in vitro* grown roots contained 4 mg withanolide per g dry weight. The maximum yield (10 mg g^{-1} dry weight) was obtained in the bubble column reactor. Co-chromatography of extracts was also performed with authentic samples. This is the first report to show that withanolides are produced *in vitro* by untransformed root cultures of *Withania somnifera*. Withanolides are important chemical constituents as they act as rasayana in Indian traditional medicines. Therefore, large-scale cultivation of *Withania somnifera* roots in bioreactor might be an alternative source to fulfill the global demands for this high value bioactive molecule.

Acknowledgements

The authors thank Mr. N.G.V. Rao, PDKV Akola and Ms. Talmale, Insecticide Testing Lab, Amravati for their kind co-operation during Sonication and HPLC analysis.

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