



Root cultures and *in vitro* production of alkannin in *arnebia hispidissima* (lehm). dc.

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ABSTRACT

Alkannin, a red-purple dye and bioactive compound found in the roots of *Arnebia hispidissima*. It has antibiotic and anti-inflammatory properties and also used in cosmetic and textile industries. The present communication demonstrates the establishment of root cultures of *A. hispidissima* with the aim of optimizing the production of alkannin. The maximum number of adventitious roots (4.2 per culture bottle) could be initiated from the *in vitro* regenerated shoots on half strength MS medium containing 1.0 mg L⁻¹ Indole-3 butyric acid (IBA). Highest alkannin content and profuse rooting was also recorded on this media combination. Production of alkannin was influenced by the different culture media. The present investigation may be applicable in designing systems for the large-scale cultivation of *A. hispidissima* roots for the production of alkannin.

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INTRODUCTION

The production of secondary metabolites through plants in natural conditions is limited by several environmental, ecological and climatic conditions (Praveen and Murthy 2011). Moreover, the production of secondary metabolites is tissue-specific and also depends upon the age of plants (Nasim *et al.*, 2010). In recent years, plant cell, tissue and organ culture technology has been efficiently utilized in the production of secondary metabolites (Shekhawat and Shekhawat 2011, Rathore and Shekhawat 2009, Piekoszewska *et al.*, 2010).

Arnebia hispidissima (family Boraginaceae) is an important medicinal plant of Indian Thar desert. It grows generally in sandy soil of Rajasthan and has great ability to tolerate hot, cold and drought stress. The alkannin, a red dye, are root-specific secondary metabolites of *A. hispidissima* (Sharma *et al.*, 2009). The alkannin has recently attracted the interest of researchers due to its anti-inflammatory, antimicrobial, antitumor activities and wound-healing properties. In addition, it has wide applications as a colorant in food, cosmetic and textiles industries (Pal and Chaudhury 2010).

In comparison with the field grown plants, the production of several biologically active compounds through *in vitro* culture system has many advantages like cell/organ culture having a higher rate of metabolism than field-grown plants and thus cell/organ can proliferate at higher growth rate. In addition, cell, tissue and organ culture are not limited by environmental or ecological

conditions and can be easily controlled and monitored (Praveen and Murthy 2011).

Adventitious roots have been successfully induced in many plant species and cultured for the production of high value secondary metabolites of pharmaceutical and industrial importance (Murthy *et al.*, 2008). Such root cultures constitute a good biological material for stable commercial production of higher secondary metabolites without foreign genes under sterile conditions. Adventitious roots once established, can be grown in a medium with low inoculum with a faster growth rate (Sivakumar 2006).

Owing to the increasing human and livestock populations, the status of wild plants have been affected, particularly those used in herbal medicine. Plant tissue and organ culture is a useful tool for the production of bioactive compounds (Singh *et al.*, 2009, Praveen *et al.*, 2010). The objective of the present investigation is to provide efficient protocol and starter inoculum for commercial production of alkannin (red pigments) in root cultures to the pharmaceutical industries.

MATERIALS AND METHODS

The plants of *A. hispidissima* were procured from sandy regions of Rajasthan (the Indian desert) during October-March. Various types of explants like shoot segments, roots segments, axillary and terminal shoot buds were used as explants for the establishment of cultures. Explants were treated with 0.1% (w/v) Bavistin (a systemic fungicide) for 10 min. Surface sterilization was carried out with 0.1% (w/v) HgCl₂ for 5-6 min after a

brief rinse in 90% alcohol for 40-50 seconds under aseptic condition in laminar air hood. These were finally rinsed 8-10 times with sterile distilled water to remove adhered HgCl₂ from the surface of explants.

First for the shoots formation, explants were cultured on agar gelled MS (Murashige and Skoog 1962) medium containing either 6-Benzylaminopurine (BAP) or Kinetin (Kn) (0.0-5.0 mg L⁻¹) alone or in combination of BAP or Kn (0.1-2.00 mg L⁻¹) with Indole-3 acetic acid (IAA) (0.1-1.0 mg L⁻¹). For the root induction, *in vitro* regenerated shoots were excised and subcultured to full and half-strength MS medium supplemented with different concentrations (0.0-2.0 mg L⁻¹) of auxins (IAA, IBA or α -Naphthalene Acetic Acid, NAA) and 200 mg L⁻¹ activated charcoal. Cultures were maintained in a growth room at 25±2°C with a 12 h photoperiod at a photosynthetic photon flux density (PPFD) of 40–50 μ mol m⁻² s⁻¹ from cool white fluorescent tubes.

For multiplication of roots in cultures, roots were inoculated on half-strength salts of MS medium supplemented with 0.1 to 5.0 mg L⁻¹ each of IBA, NAA and IAA individually. The concentration of sucrose was reduced to half (1.5%). To find out the optimum conditions of light and temperature, the cultures were kept under 30 to 70 μ mol m⁻² s⁻¹ PPFD and 20 to 28°C respectively. Experiments were also conducted to test the effects of various media combinations for the proliferation of roots and alkannin synthesis in cultures. These were MS medium (full and half strength), Woody Plants (WP) medium (Lloyd and McCown 1980), Whites medium (White 1963), B5 medium (Gamborg *et al.*, 1968), MG-5 and M9 media (Fujita *et al.*, 1981). These media were also supplemented with 0.1 to 5.0 mg L⁻¹ each of IBA, NAA and IAA individually.

The roots collected from cultures were oven-dried at 35–40°C for 24 h. After drying, roots were grounded in a mortar, weighed and dye extracted in chloroform at the room temperature. The extracted dye solution was filtered through Whatman paper. The organic phase was washed thrice with distilled water, dehydrated with MgSO₄ and evaporated *in vacuo*. The residues were weighed and used for the dye analysis. Chloroform soluble alkannin dye was separated on pre-silica coated aluminum sheets (Silica Gel 60, F.254) by TLC.

The observations were taken after every three weeks of inoculation. The experiments were repeated thrice with ten replicates per treatment. The data were subjected to statistical analysis.

RESULTS AND DISCUSSION

The market potential for plant-derived drugs and dyestuffs in the world is increasing. There has been shift in the consumer's preference away from chemical to plant-derived medicines (Kamboj 2000). The pharmaceutical companies are continuously seeking new compounds for medicines to improve their competitiveness (Tyler 1999). Tissue culture technology has been applied extensively for production of red

pigments from cell, tissue and organ cultures (Yu *et al.*, 1997, Sim and Chang 1997).

Roots of field grown plants proved to be recalcitrant explant. Cultures could not be initiated from such explants. Therefore, the *in vitro* produced shoots (Fig. 1A) were rooted on half strength of MS medium because successful rooting in regenerated shoots is the prerequisite to facilitate adventitious root cultures. In the present study, the role of different auxins (IAA, IBA and NAA) in the root induction was tested. Among different concentrations of three auxins tested, IBA (1.0 mg L⁻¹) was found to be better for the rooting in shoots (Table 1, Fig. 1B). Auxins especially IBA is used widely to induce adventitious roots in many woody and herbaceous plant species (Rathore and Shekhawat 2009, Rathore *et al.*, 2010). The present study clearly indicates that half-strength MS medium was adequate for the root induction. Relatively low salt concentration in the medium is known to enhance rooting of shoots (Rai *et al.*, 2010, Shekhawat *et al.*, 2011).

Table 1 Effect of auxins and their concentrations on roots induction from shoots of *A. hispidissima* and alkannin synthesis on half-strength MS medium + 200 mg L⁻¹ activated charcoal

Auxin (mg L ⁻¹)	% of response	Number of roots± SD	Alkannin synthesis* (red pigments)
Control 0.00	0.00	0.00±0.00	0
IAA			
0.10	49	2.4±0.83	0
0.50	57	3.2±0.77	+
1.00	66	1.2±0.89	+
2.00	58	0.8±0.40	+
IBA			
0.10	71	2.4±0.44	+
0.50	93	3.6±0.73	++
1.00	100	4.2±0.54	+++
2.00	96	2.8±0.33	+++
NAA			
0.10	62	1.0±0.62	0
0.50	76	2.2±0.11	+
1.00	71	2.6±0.42	++
2.00	64	1.4±0.31	+

*Note: Density of red color spots on TLC- 0 = No spot, + = Light spots, ++ = Medium spots, +++ = Dark spots.

The *in vitro* produced roots were used as inoculum for root proliferation and alkannin production on half strength agar-gelled MS medium with 1.0 mg L⁻¹ IBA. Inoculated roots were produced bunch of red colored roots after 2-3 weeks in the dark. Surprisingly, this media combination was found to be the most suitable for root cultures as well as pigment production. Increased concentrations of IBA (above 1.0 mg L⁻¹) caused callusing from the roots. Under optimum temperature (22±2°C), if the root cultures were kept in the dark the pigmentation was increased (Fig. 1C). Under higher PPFD (50-60 μ mol m⁻² s⁻¹) only embedded roots produced pigments. Roots, which were not submerged in the medium first elongated and then started alkannin production at the sites where the roots came in contact of the medium surface. Alkannin formation occurred only in the outer layers (bark) of the roots tissue. The inner area/part of roots remains white. The agar-

gelled medium turned dark red colored due to the synthesis of red color alkannin in the roots and their release in the medium.

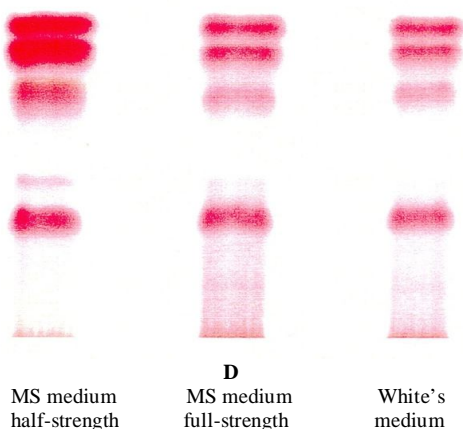
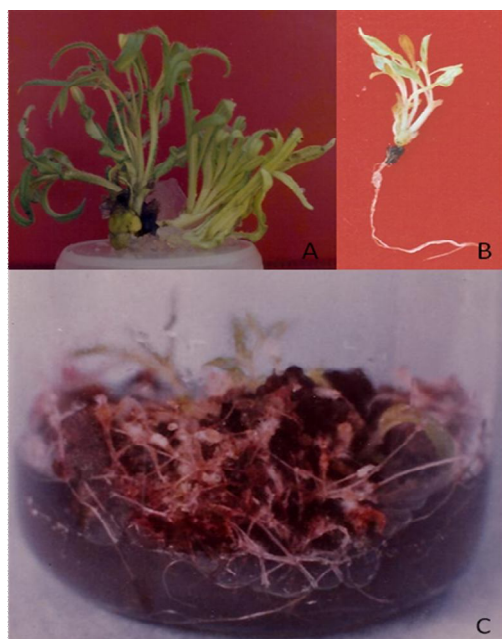


Fig. 1 A *In vitro* regenerated multiple shoots of *A. hispidissima*, B. Rooted shoot on half strength MS medium + 1.0 mg L⁻¹ IBA, C. Proliferated roots with alkannin (red color). D. TLC profile of alkannin derivatives synthesized in root cultures on MS medium (full and half strength) and White's medium.

Media	Effect on root proliferation*	Effect on alkannin synthesis**
MS medium (full strength)	+	++
MS medium (half strength)	+++	+++
WP medium	++	0
White's medium	+	+
B5 medium	+	0
MG-5 medium	0	0
M9 medium	0	0

*Note: Intensity of root multiplication- 0 = No proliferation, + = Low proliferation, ++ = medium proliferation, +++ = higher proliferation.

**Note: Density of red color spots on TLC- 0 = No spot, + = Light spots, ++ = Medium spots, +++ = Dark spots.

Present study clearly indicates that the production of alkannin was influenced by the different culture media. MG-5 and M-9 media were used by Fujita *et al.*, (1981) to synthesize shikonin (red dye) in liquid cultures of *Lithospermum erythrorhizon*, but no dye was synthesized on these media combinations in present investigation. In our previous study the maximum alkannin production was achieved on M-9 medium in cell cultures (Shekhawat and Shekhawat 2011). Contrary to our previous findings no alkannin synthesis was reported on this media combination in present investigation. The maximum amount of alkannin was synthesized on half strength MS medium (Table 2). Some amount of alkannin was also produced in White's medium but it was less than full strength of MS medium (Fig. 1D).

The roots could be multiplied only on MS (full and half strength salts), WP, White's, and B5 media combinations. The numbers of roots were proliferated better on half strength MS medium than other media combinations used. Similar results were also obtained by Baskaran and Jayabalan (2009) and Phulwaria *et al.*, (2011) in root induction and multiplication in several plant species. *In vitro* alkannin production has also been reported in *Alkanna tinctoria* by Mita *et al.*, (1994) on MS media combinations only. The protocol developed in the present study for *A. hispidissima* root culture can be used for commercial production of alkannin. The dyes produced have potential applications in the pharmaceutical industry, cosmetics and commercial/natural dye industry.

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