In vitro Withanolide Production by *Withania somnifera* L. Cultures

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In vitro multiple shoots, root, callus and cell suspension cultures of *Withania somnifera* exhibited the potentiality to produce pharmacologically active withanolides. Multiple shoots cultures exhibited an increase in withanolide A accumulation compared to shoots of the mother plant. *In vitro* generated root cultures as well as callus and suspension cultures also produced withanolides albeit at lower levels.

Key words: Cell Cultures, *Withania somnifera*, Withanolide

Introduction

Withania somnifera (L.) Dunal of the Solanaceae family is a highly reputed plant of the Indian traditional system of medicines (Kaileh *et al.*, 2007). Medicinal properties of the plant have been attributed to the presence of a group of steroidal lactones, known as withanolides present in leaves and roots (Sangwan *et al.*, 2004). Due to immense medicinal properties of withanolides and reported lower levels of some of them in plants, several efforts have been made to produce them under culture conditions (Kulkarni *et al.*, 1996; Sabir *et al.*, 2007). Callus induction, root organ culture, plantlet regeneration, and withanolide production in multiple shoots and roots have been reported (Rani *et al.*, 2003; Ray and Jha, 2001; Sabir *et al.,* 2007; Wadegaonkar *et al.*, 2006), however, efforts to detect withanolides in callus and suspension cultures have not been successful so far. We report here the *in vitro* synthesis of bioactive withanolides from multiple shoots, and root cultures from direct rhizogenesis of leaves, callus tissue and single cell suspension cultures of *W. somnifera.* To our knowledge, this is the first report where withanolides have been detected from callus, suspension cultures and other plant tissues in qualitative and quantitative terms.

Experimental

Surface-sterilized nodal segments of *W. somnifera* were inoculated on MS medium supplemented with BAP (0.5, 1, 2 and 4 mg l^{-1}) kinetin, and 3% (w/v) sucrose, and solidified with 0.8% (w/v) agar. For rhizogenesis, sterile leaves from *in vitro* generated multiple shoots were inoculated on MS medium supplemented with four different auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D), alone in 1, 2 and 4 mg l^{-1} concentrations, as well as in combination with IBA at two concentrations (2 and 4 mg l^{-1}). Callus induction and proliferation from leaves were obtained by inoculating sterile leaves on MS medium supplemented with different concentrations (0.5, 1, 2, 3, 4, and 5 mg l^{-1}) of 2,4-D and kinetin. Suspension cultures were induced by using fast-growing, friable cells of callus in liquid MS medium, devoid of gelling agent, supplemented with 2,4-D (0.5, 1, 2, 3, 4, and 5 mg l^{-1}) and kinetin in a shaker rotating at 100 rpm at 25 °C.

Fresh tissue and cultures were subjected to withanolide extraction and analysis (Sabir *et al.*, 2007; Sangwan *et al.*, 2004, 2005). The extract was dissolved in methanol and analyzed by TLC and HPLC. Authentic withanolides isolated at our laboratory were used for quantitative and qualitative comparisons (Misra *et al.*, 2005).

Results

The results revealed that BAP at 1 mg l^{-1} in combination with kinetin at 1 mg l^{-1} showed maximum proliferation [(21.8 ± 1.24) shoots/explant of (2.58 ± 0.563) cm in length)]. Our previous results had also shown that comparatively BAP was better than kinetin for promoting shoot multiplication

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Table I. Whithanolide production from *in vitro* tissue of *W. somnifera*.

Type of tissue	Age	Withanolide A [mg/g DW][a]	Withaferin A [mg/g DW][a]	Withanone [mg/g DW][a]	Total withanolides [mg/g DW][b]
Shoots of field-grown mother plant (control)	1 year	0.259 ± 0.049	15.234 ± 0.196	ND	15.50
In vitro generated multiple shoots from axillary buds	6 weeks	1.167 ± 0.39	2.48 ± 0.377	ND	3.647
Shoots from micropropagated plant (in field)	8 months	0.207 ± 0.05	10.273 ± 0.12	ND	10.48
Roots from field plant (control)	1 year	0.996 ± 0.066	ND	0.89 ± 0.18	1.89
In vitro roots from sterile leaves (rhizogenesis)	8 weeks	0.112 ± 0.03	ND	0.066 ± 0.04	0.178
Callus cultured from leaves	4 weeks	Tr	0.46 ± 0.003	ND	0.46
Suspension culture from callus (freshly induced, one time subcultured)	2 weeks	0.020 ± 0.002	Tr	ND	0.020
Suspension culture (well established, 4 times subcultured)	4 weeks	Tr	0.141 ± 0.013	ND	0.141

[a] Data are presented as the mean value of triplicate measurements with standard deviation.
[b] Data are presented as the sum value derived from the mean individual values.
Tr, traces. ND, not detected.

(Sabir *et al.*, 2007) and their proliferation from nodal segments and inclusion of kinetin enhanced the number of shoots in a smaller time period. Among the auxins, IBA when supplied alone showed rhizogenesis at all concentrations (1 to 4 mg l^{-1}). IAA and NAA poorly induced roots with short length and calli, while 2,4-D failed to induce roots at all concentrations tested. Best root induction with profuse branching and root hairs was observed with IBA at 4 mg l^{-1} in combination with IAA at 2 mg l^{-1}. Fast callus induction and proliferation were observed with 3 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of kinetin whereas at higher concentration of kinetin with 2,4-D very compact, dark brown coloured and slow growing calli were induced. Suspension cultures showed fast and plentiful growth on similar 2,4-D (3 mg l^{-1}) and kinetin (0.5 mg l^{-1}) concentration.

There was a marked increase in withanolide A and decrease in withaferin A production in the *in vitro* shoot cultures compared to the shoots of field-grown plants (Table I). This observation matches with our hypothesis that the withanolide A synthesis is probably linked to roots and their development, and shoots have higher detectable levels of withanolide A only when roots are not developed or under specific environmental conditions (Sangwan *et al.*, unpublished data). Micropropagated shoot cultures, transferred to the field after rooting and acclimatization, showed withanolide contents nearly equivalent to the mother plant. Although *in vitro* cultured roots had lower levels of withanolide A and withanone compared to the mother plant, the qualitative HPLC pattern was similar and did not show the peak of withaferin A (Fig. 1) that was also absent in the roots of the mother plant. These withanolide-producing root cultures, directly induced by rhizogenesis of leaves, exhibited the ability to grow independently of the ex-plant leaf in cultures, and therefore can be an alternative manoeuvreable resource of withanolide production. Production of withanolides in unorganized tissues of callus and suspension cultures has always been disappointing. Interestingly, callus had the presence of withaferin A while the suspension cultures that of withanolide A (Table I). In fact, mature suspension cultures with four passages of subculturing did show the presence of withaferin A (Table I). Some of the elicitor-treated callus and suspension cultures also produced both withanolides (unpublished results). The above *in vitro* tissue system also provides an opportunity to understand the withanolide biosynthetic pathway in convenient *in vitro* tissues, organ and cell cul-

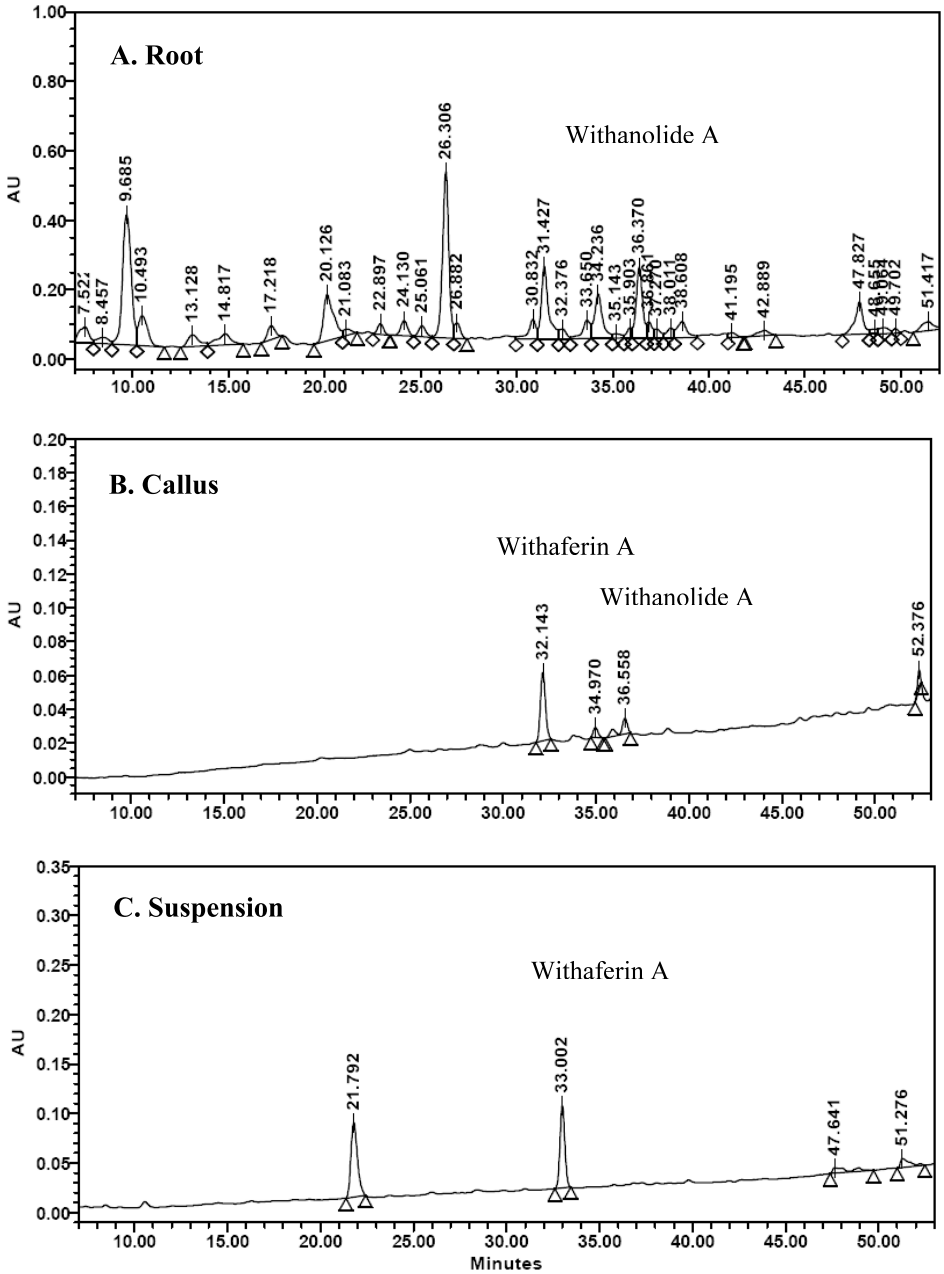

Fig. 1. HPLC profile of extracts from (A) root organ culture, (B) induced callus, and (C) established suspension culture with peaks of withaferin A and withanolide A.

tures. ^{14}C-labelled studies using acetate have revealed the biogeneration of withanolide A from the *in vitro* grown shoots and shooty teratomas in *W. somnifera* (Sangwan *et al*., 2007). The present study reports that the *in vitro* tissue systems of *W. somnifera* have the potential for withanolide synthesis that could be explored for large-scale production of valuable bioactive withanolides.

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