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BRIEF COMMUNICATION

Withanolide production by *in vitro* cultures of *Withania somnifera* and its association with differentiation

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Abstract

Withanolides – steroidal lactones, isolated from various *Solanaceous* plants have received considerable attention due to their potential biological activities. Five selected withanolides (withanone, withaferin A, withanolide A, withanolide B, withanolide E) were identified by HPLC-UV (DAD) - positive ion electrospray ionization mass spectroscopy in *Withania somnifera* (L.) Dunal cv. WSR plants and tissues cultured *in vitro* at different developmental phases. Cultures were established from five explants on Murashige and Skoog's medium supplemented with different plant growth regulators. Results suggest that production of withanolides is closely associated with morphological differentiation.

Additional key words: callus culture, plant tissue culture, *Solanaceae*, steroidal lactones.

Indian ginseng [*Withania somnifera* (L.) Dunal] is an important perennial medicinal plant (Ray and Gupta 1994, Singh and Kumar 1998). It is reported to have antimicrobial, antitumor, radiosensitising, antioxidant, adaptogenic, antistress and immunomodulatory activities which have been attributed to the presence of withanolides (Budhiraja *et al*. 2000). The use of plant cell cultures for the production of bioactive secondary metabolites is an attractive alternative approach (Nath and Buragohain 2005, Langhansová *et al*. 2005). Tissue culture studies in *Withania somnifera* have been undertaken on various aspects including production of withanolides *in vitro*. Attempts to produce withanolides through tissue culture have been reported earlier (Yu *et al*. 1974, Heble 1985, Roja *et al*. 1991, Banerjee *et al*. 1994, Vitali *et al*. 1996, Ray *et al*. 1996, Furmanova *et al*. 2001, Ray and Jha 2001). In the present communication we report the production of five selected withanolides during *in vitro* pathway of morphogenesis leading to plant regeneration in selected cultivar, WSR of *W. somnifera*.

Cultures were initiated utilizing shoot tips, nodal meristems and leaf segments collected from mature *Withania somnifera* (L.) Dunal cv. WSR plants growing in the medicinal plant garden at Regional Research Laboratory, Jammu. They were dipped in 1 % (v/v) solution of *Tween-20* (*Sigma-Aldrich*, St. Louis, USA) for 5 min, followed by washing under running tap water for 1 h. Explants were aseptically treated with *Dithane M-45* (0.05 % m/v) for 4 min followed by mercuric chloride (0.1 % m/v) for 2 min. Following repeated washes with sterile distilled water, the explants were cut into segments (3 ~ 4 mm) and cultured onto Murashige and Skoog (1962) (MS) or Gamborg *et al*. (1968) (B_5) basal media supplemented with 3 % (m/v) sucrose and 6-benzyl-aminopurine (BAP), α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KN) in varied combinations and concentrations. The pH of the medium was adjusted to 5.8 with 1 M HCl or 1 M KOH prior to autoclaving (121 °C, 20 min). The

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Abbreviations: B_5 - Gamborg's (1968) medium; BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; HPLC - high pressure liquid chromatography; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KN - kinetin; MS medium - Murashige and Skoog (1962) medium; NAA - α-naphthalene acetic acid; NMR - nuclear magnetic resonance; TLC - thin layer chromatography.
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cultures were subcultured every 4 weeks and incubated at 25 ± 2 °C and 16-h photoperiod (cool white tubes; irradiance of 65 μmol m^{-2} s^{-1}).

For isolation of withanolides 5 g of air-dried and lyophilized plant parts or *in vitro* cultured tissues of *Withania somnifera* were percolated four times with ethanol:water (1:1) at 25 ± 2 °C. The aqueous ethanolic extracts were concentrated by evaporation at reduced pressure and temperature (50 ± 5 ºC). The concentrate was extracted with chloroform and solubles dried under reduced pressure to yield total withanolide residue. These residues were analysed qualitatively by thin layer chromatography (TLC) and the constituents of the extract after purification by column chromatography identified with the help of ^{1}H, ^{13}C NMR and mass spectral data. Finally, the constituents were quantified by mass spectroscopy using *LC-MS* on *LC 1100* series *Agilent Instrument*s (Bremen, Germany) coupled to a Bruker ion trap mass spectrometer (*Esquire 3000*, Bremen, Germany) according to Khajuria *et al*. (2004). TLC analysis was conducted by spotting extracts of samples on pre-coated silica gel plates, developing the chromatograms with solvent system $CHCl_3$:MeOH (96:4) for 2 h and visualizing the spots obtained by spraying of vanillin reagent [vanillin:boric acid:methanol:sulphuric acid in the ratio of 0.5 g:50 g: 500 cm^3:10 cm^3 (50 %)].

Standard error (SE) of arithmetic mean with regard to withanolide content was calculated for individual treatments using the formula of Snedecor and Cochran (1968).

Callus cultures initiated from shoot tips (seedlings, well-grown plants, *in vitro* shoots), nodal meristems and leaf segments of mature plants when subcultured, developed friable, creamish brown undifferentiated callus on Gamborg's - B_5 medium containing 10 μM NAA, and nodular brownish green organogenetic callus interspersed with shoot primordia on MS medium containing 10 μM BAP + 1 μM IBA. Multiple shoot culture lines initiated from leaf and shoot tip explants produced highly proliferative shoot cultures on MS medium containing 0.4 μM IAA + 0.4 μM BAP. Excised shoots regenerated from various explants could be easily rooted in B_5 medium containing 1 μM IBA to produce complete plantlets.

Table 1. Withanolide contents [μg g^{-1}(d.m.)] in different parts of 270-d-old mature donor plants *Withania somnifera*. WS1 - withanolide A; WS2 - withanone; WS3 - withaferin A; WS4 - withanolide B, WS5 - withanolide E; A - absent. Extraction was carried out in EtOH:H_2O 1:1 (RT). Mean of 6 replicates ± SE.

Withanolides	Leaf	Shoot	Root
WS1	0.06 ± 0.008	0.16 ± 0.017	1.02 ± 0.018
WS2	1.62 ± 0.007	0.73 ± 0.018	0.71 ± 0.008
WS3	4.01 ± 0.029	0.21 ± 0.029	0.42 ± 0.028
WS4	0.11 ± 0.010	0.14 ± 0.004	0.10 ± 0.018
WS5	A	0.04 ± 0.023	A

TLC and HPLC analysis of undifferentiated and partially differentiated calli showing adventitious shoot bud primordia, multiple shoot cultures and regenerated plantlets revealed the synthesis of withanolides (withanone, withaferin A, withanolide A, withanolide B, withanolide E) *in vitro* (Tables 1, 2). Withanolide A and withanone were dominant fractions traced in both the types of calli. Withanolide B started appearing, as the calli initiated organogenesis. Withaferin A and withanolide E were absent in both the cultures. Multiple shoot cultures synthesized higher levels of withanolides and exhibited a uniform profile, which was comparable to that of donor plant (leaf) tissues. Withaferin A was more pronounced in shoot cultures as they began to differentiate into leaves. Withanolide E was absent in shoot cultures. Withanolide productivity among 8-wk-old regenerated plantlets from various explants showed subtle differences. The plantlets regenerated from leaf explants produced all the five withanolides, although those from *in vitro* shoot tip explants contained the highest amount of withaferin A (0.81 μg g^{-1}) and withanolide B (0.4 μg g^{-1}). The highest content of withanolide A (0.6 μg g^{-1}) and withanone (1.6 μg g^{-1}) were detected in plantlets regenerated from nodal segments. Withaferin A was absent in plantlets derived from seedlings and shoot tips of mature plants.

Tissue cultures having different morphology analyzed for withanolide production showed that the inherent biosynthetic capability of the donor plant was retained in cultures and they produced withanolides *in vitro*. The present data reveal the varied withanolide content and composition in various culture lines initiated from various explants. This suggests the importance of explants selection on production of withanolides. Culture lines established from leaf explants accumulated the highest level of withanolides and those from shoot tips of well-grown plants produced the lowest level. According to Wiermann (1981) the varied capacity to synthesize secondary metabolites by *in vitro* cultures has been attributed to differences in morphological nature of explants utilized to initiate tissue culture lines. Donor tissues have been an important role to play in biochemistry of subsequent cultures lines (Nigra *et al*. 1987, Banerjee *et al*. 1993, Mischenko *et al*. 1999).

The production of withanone and withanolide A by undifferentiated callus cultures and cell suspensions induced using the natural variations of cells (different explants) and PGRs is noteworthy. Earlier, Yu *et al*. (1974) reported that the undifferentiated callus and cell suspensions initiated from germinating seeds subjected to phytochemical investigation, failed to synthesize withanolides. Subsequently, Heble (1985) and Roja *et al.* (1991) reconfirmed the observations made by Yu *et al*. (1974) that cell cultures and callus cultures of *W. somnifera* failed to synthesize withanolides. Several factors such as, the difference in chemotype utilized as

Table 2 Withanolide contents [μg g^{-1}(d.m.)] of various cultures and micropropagated plants of *Withania somnifera* genotype WSR (age 30 d). Explants were collected from E-1 - shoot tips of seedlings, E-2 - shoot tips of *in vitro* plants, E-3 - shoot tips of well-grown plants, E-4 - mature nodes, E-5 - leaves; WS1 - withanolide A; WS2 - withanone; WS3 - withaferin A; WS4 - withanolide B, WS5 - withanolide E; A - Absent. Extraction was carried out in EtOH:H_2O 1:1 (RT). Mean of 6 replicates ± SE.

Culture type	Explants	WS1	WS2	WS3	WS4	WS5
Undifferentiated callus	E-1	0.10 ± 0.024	0.03 ± 0.014	A	A	A
	E-2	0.14 ± 0.029	0.07 ± 0.008	A	A	A
	E-3	0.10 ± 0.029	0.03 ± 0.010	A	A	A
	E-4	0.48 ± 0.004	0.07 ± 0.003	A	A	A
	E-5	0.06 ± 0.007	0.02 ± 0.007	A	A	A
Organogenic callus	E-1	0.16 ± 0.011	0.22 ± 0.025	A	0.07 ± 0.030	A
	E-2	0.14 ± 0.019	0.19 ± 0.021	A	0.15 ± 0.004	A
	E-3	0.16 ± 0.030	0.22 ± 0.032	A	0.07 ± 0.015	A
	E-4	0.04 ± 0.032	0.13 ± 0.018	A	0.12 ± 0.032	A
	E-5	0.26 ± 0.016	0.39 ± 0.012	A	0.17 ± 0.037	A
Multiple shoots	E-1	0.10 ± 0.018	0.07 ± 0.035	0.15 ± 0.007	0.06 ± 0.006	A
	E-2	2.59 ± 0.010	0.44 ± 0.032	0.14 ± 0.022	0.16 ± 0.031	A
	E-3	0.10 ± 0.007	0.07 ± 0.010	0.15 ± 0.034	0.06 ± 0.025	A
	E-4	0.38 ± 0.022	0.11 ± 0.022	0.15 ± 0.023	0.10 ± 0.034	A
	E-5	0.42 ± 0.023	0.42 ± 0.035	0.06 ± 0.006	0.02 ± 0.026	A
In vitro plantlets	E-1	0.07 ± 0.004	0.04 ± 0.022	A	0.01 ± 0.004	A
	E-2	0.04 ± 0.006	0.32 ± 0.014	0.81 ± 0.020	0.40 ± 0.029	A
	E-3	0.07 ± 0.008	0.14 ± 0.012	A	0.07 ± 0.022	A
	E-4	0.60 ± 0.010	1.61 ± 0.031	0.59 ± 0.010	0.14 ± 0.018	A
	E-5	0.38 ± 0.007	0.24 ± 0.012	0.08 ± 0.009	0.06 ± 0.032	0.05 ± 0.006

source for initiation of callus and suspension cultures, the difference in parts of the plant from which the undifferentiated callus cultures were initiated, and culture conditions such as, basal media combination and PGR types and concentrations utilized to establish callus and suspension cultures might have contributed for withanolide production by undifferentiated callus cultures in the present case. A shift towards organ differentiation (adventitious shoot buds/multiple shoots) resulted in improved potential of the cultures to synthesize withanolides. Metabolic differences between undifferentiated, shoot cultures and regenerated plants for production of withanolides noticed during present study suggest that degree of differentiation plays an important role in the synthesis of withanolides. This positive correlation between withanolide synthesis and morphological differentiation noticed during present study suggest that synthesis is regulated in tissue specific way and organogenesis might be a key regulatory factor which stimulates production of withanolides *in vitro*. Differentiated cultures produce more conventional product profile than their corresponding callus and suspension cultures. As such these results allow proposing that morphological differentiation (green structures) are needed for expression of some of the withanolides. The detection of higher content and spectrum closer to donor plant by green differentiated cultures also points out that enzymes responsible for biogenesis of withanolides *in vitro* might be optimally operative in these morphologically differentiated cultures. Possibly certain key enzymes in biosynthetic pathway of detected withanolides might be chloroplast associated which could be one of the factors for better withanolide formation by these cultures

In earlier studies, production of withanone, withanolide G, withanolide I, withanolide E and trace amount of 4-β-hydroxy withanolide E has been reported in shoot cultures established in *Withania somnifera* utilizing nodal meristems and internodes segments and pattern of withanolides in the shoot cultures was different from that of the leaves. Shoot cultures did not contain withaferin A, the major component shown by the donor plant tissues (Heble 1985). Roja *et al.* (1991) have reported the detection of trace amounts of withanolide D by shoot cultures. Vitali *et al.* (1996) have reported withanolide J as major withanolide by multiple shoot cultures produced *in vitro*. Ray and Jha (2001) have detected withaferin A and withanolide D in shoot cultures established *in vitro*. However, multiple shoot cultures established during present study showed the presence of withanone, withanolide A, withaferin A, and withanolide B. The potentialities of cultured tissues of *W. somnifera* suggest the importance of donor tissue and degree of differentiation in withanolide synthesis. Keeping this as the basis, we are investigating withanolide metabolism at cellular and molecular level in order to broaden the knowledge base for metabolic engineering of withanolide biosynthesis.

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