

Withanolide A Biogenesis in *in Vitro* Shoot Cultures of Ashwagandha (*Withania somnifera* DUNAL), a Main Medicinal Plant in Ayurveda

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Multiple shoot cultures of two experimental lines of *Withania somnifera* plants (*RS-Selection-1* and *RS-Selection-2*) were established using nodal segments as explants. The hormonal combinations of benzyl adenine and kinetin not only influenced their morphogenetic response but also differentially modulated the level of biogenesis of withanolide A in the *in vitro* shoots of the two lines. Interestingly, withanolide-A, that was hardly detectable in the aerial parts of field-grown *Withania somnifera* (explant source), accumulated considerably in the *in vitro* shoot cultures of the plant. The productivity of withanolide A in the cultures varied considerably (ca. 10-fold, 0.014 to 0.14 mg per gram fresh weight) with the change in the hormone composition of the culture media as well as genotype used as source of the explant. The shoot culture of *RS-Selection-1* raised at 1.00 ppm of BAP and 0.50 ppm of kinetin displayed the highest concentration of withanolide A in the green shoots of 0.238 g per 100 g dry weight tissue. This was a more analytical concentration keeping in view the isolation yields so far reported from the dried roots of the field-grown plant (ca. 0.015 g per 100 g dry weight), even if isolation losses are considered during purification. The enhanced *de novo* biogenesis of withanolide A in shoot cultures was corroborated with radiolabel incorporation studies using [2-¹⁴C] acetate as a precursor. Production of withaferin A was also found in the *in vitro* shoot cultures. As this compound is a predominant withanolide of native shoots as well and has been already reported to be accumulated in *in vitro* shoot cultures, its biogenesis observed in these shoot cultures is not discussed in detail.

Key words *Withania somnifera*; Ashwagandha; withanolide; withanolide A; bioactive phytochemical; neuroactive withanolide

Ashwagandha (*Withania somnifera* DUNAL, Solanaceae) is one of the most reputed Indian medicinal plants.^{1,2} Its ginseng-like health-promoting effects have earned it the popular name of Indian ginseng. The plant has been used in the traditional Indian system of medicine as a tonic and antistress supplement. Pharmacologic activities of the plant include physiologic and metabolic restoration, antiarthritic, antiaging, nerve tonic, cognitive function improvement in geriatric states, and recovery from neurodegenerative disorders like convulsions, tardive dyskinesia, etc.^{3,4} *In vitro* and *in vivo* molecular pharmacologic investigations have elucidated the association of these activities with specific secondary metabolites known as withanolides present in the plant.^{5–12}

Withanolides are C₂₈-steroidal lactones of triterpene ancestry, based on an intact or rearranged ergostane frame and chemically called 22-hydroxy ergostane-26-oic acid 26,22-lactone. Biogenesis of withanolides appears to be highly restricted to a few genera¹³ and *W. somnifera* produces the largest number of withanolides in diversified functional groups and regio/stereo-forms, some of which possess specific therapeutic significance.¹ Withaferin A and withanone (Fig. 1a) are the customary major withanolides of the plant whereas the amount of withanolide A (5 α ,20 α -dihydroxy-6 α ,7 α -epoxy-1-oxowitha-2,24-dienolide) (Fig. 1a) is usually very low.⁸ Recently, withanolide A has attracted interest due to its strong neuropharmacologic properties of promoting

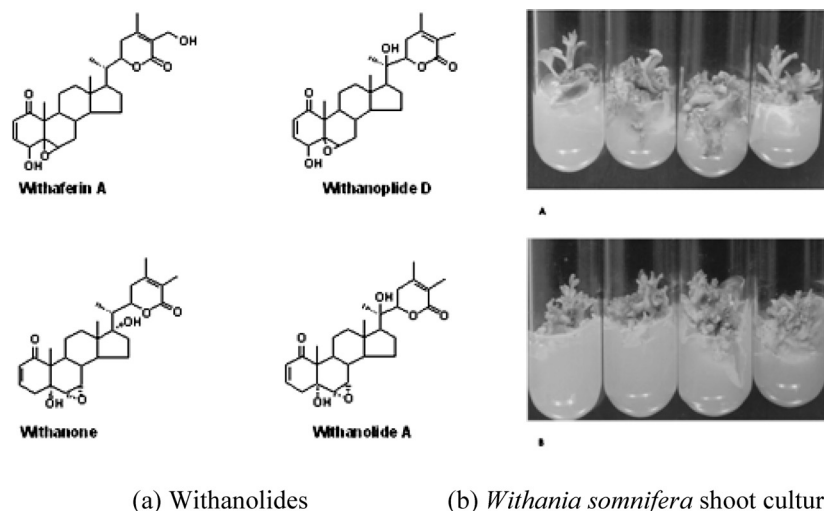


Fig. 1. (a) Withanolide A and Some Other Important Withanolides of *Withania somnifera* and (b) *in Vitro* Shoot Cultures of *RS-Selection-1* (A), and *RS-Selection-2* (B)

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neurite outgrowth and synaptic reconstruction.^{9–11)} Therefore it could be potentially useful in neurologic disorders like Alzheimer's disease and Parkinson's disease, convulsions, cognitive function impairment, *etc.*

In the native plant, withanolide A represents a very minor proportion of the withanolide complement and occurs mainly in the roots. Therefore alternative exploration of condition-optimized cultures for efficient *in vitro* biogenesis of such withanolides, that are pharmacologically promising but are severely limited in production, is important. Although withanolides like withanone, withaferin A, and withanolide D have been shown to be present in several organogenic cultures including hairy roots^{14–20)} but synthesis of withanolide A has so far not been reported from *in vitro* cultures. In this study, we demonstrated that biogenesis of withanolide A can occur in shoot cultures at levels matching those in native roots. This is the first report on withanolide A production and biogenesis in cultures.

Results and Discussion

Withanolide A is one of the most promising withanolides of *W. somnifera*. Molecular evidences of its ability to induce nerve development and improve nervous system function have been reported.^{7,11)} However, its usual very low levels in the natural plant^{8,21,22)} along with restriction of its occurrence mainly in the roots in *W. somnifera* require attempts to explore its production in *in vitro* cultures. Because of the root-specific production of withanolide A, root cultures, particu-

larly hairy roots, that can grow rapidly in simple media and can be easily upscaled in a bioreactor form the first choice for production *in vitro*. However, despite several reports on withanolide profiles (withaferin A, withanolide D, *etc.*) of *Agrobacterium rhizogene*-transformed hairy roots of *W. somnifera*, the presence of withanolide A has not been detected in those cultures.^{14,16)} Withanolides reported in these hairy root cultures are those predominantly produced by the aerial parts. We explored withanolide A biogenesis in *W. somnifera* shoot cultures, the tissue culture counterparts of the aerial parts of the native plant.

This study reports the production of withanolide A in *in vitro* shoot cultures of two experimental lines of *W. somnifera* (Ashwagandha)-*RS Selection-1 (RS-Sel-1)* and *RS Selection-2 (RS-Sel-2)* (Fig. 1b, Fig. 2). To our knowledge, this is the first report on finding withanolide A in *in vitro* shoot cultures. The presence of other withanolides (like withaferin A) observed in these cultures was similar to that in previous reports^{17–20)} and is not discussed in detail.

Since withanolide A was not available commercially, it was isolated from the roots of *W. somnifera* and structurally validated to serve as an authentic marker. Withanolide A was recovered as a pool of fractions from a silica gel column eluted with ethyl acetate : *n*-hexane in a ratio of 2 : 3 and crystallized-recrystallized to purity (white crystals). The compound on acetylation with pyridine and acetic anhydride did not change, suggesting the lack of any primary and secondary hydroxyl groups. IR (in KBr) results (cm^{-1}) of the

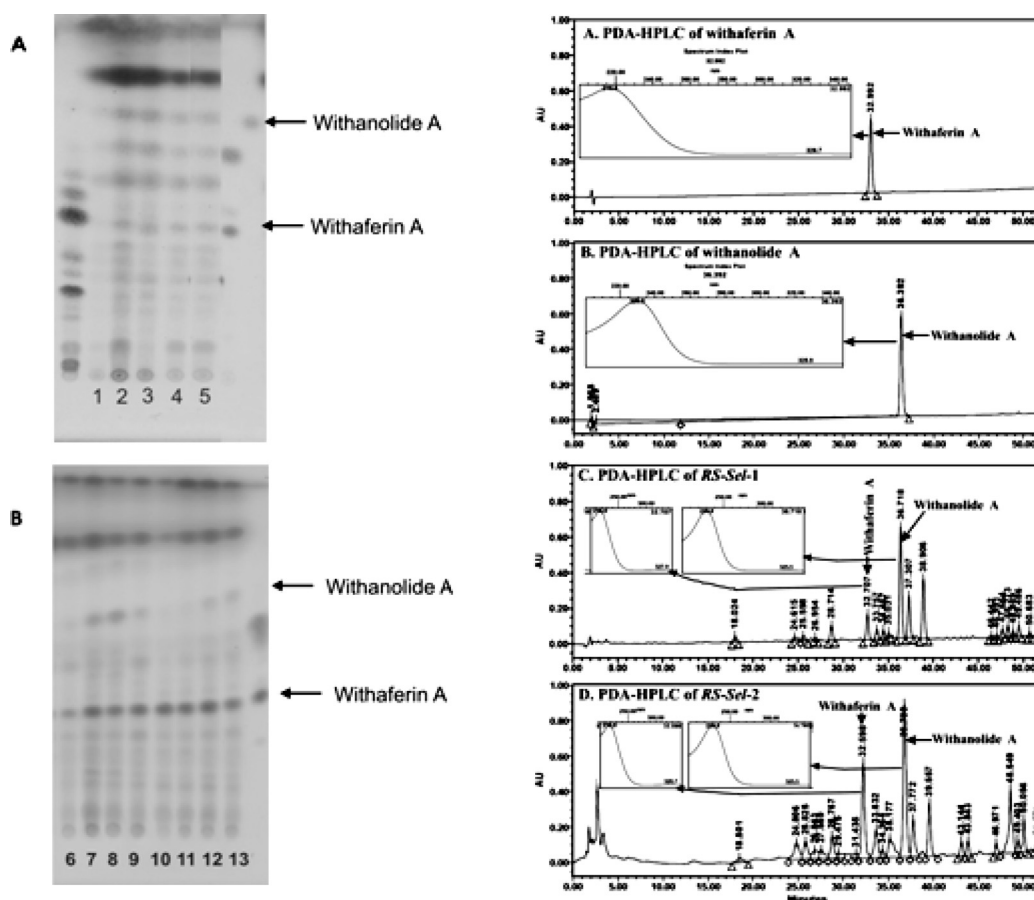


Fig. 2. TLC (Left Panel) and HPLC (Right Panel) of Shoot Culture Extract of *Withania somnifera* for Analysis of Withaferin A and Withanolide A

TLC (lane numbers show profiles of individual shoot cultures in the set): A, *RS-Selection-2*; B, *RS-Selection-1*; PDA-HPLC (insets are online UV spectra of the specified withanolide): A, standard withaferin A; B, standard withanolide A; C, representative chromatogram of *RS-Selection-1*; D, representative chromatogram of *RS-Selection-2*.

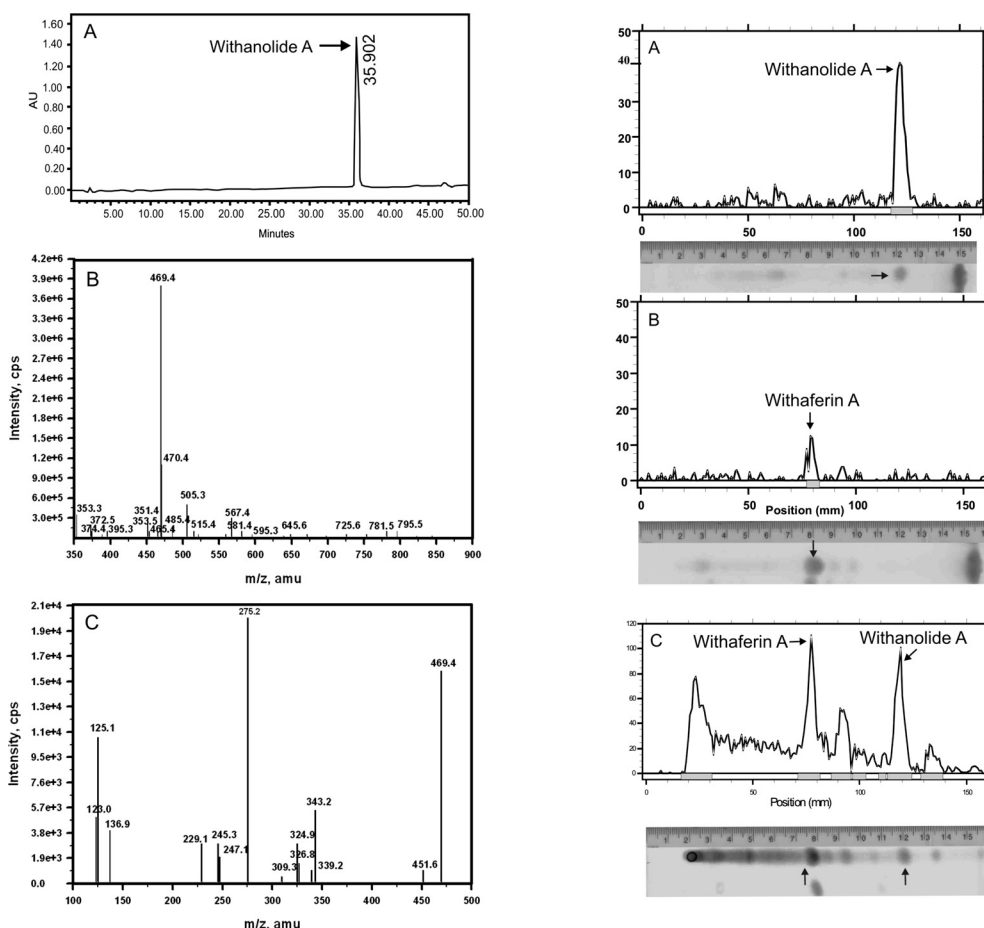


Fig. 3. (Left Panel) HPLC-MS of Withanolide A Isolated from *Withania somnifera* Roots: A, HPLC; B, MS Spectra; C, MS-MS Spectra and (Right Panel) Radio-TLC of Withaferin A and Withanolide A from Radioactive (^{14}C) Precursor (Acetate) Fed Cultured Shoots of *W. somnifera*: A, Peak (\rightarrow) of Authentic Radiolabeled Withanolide A; B, Peak (\rightarrow) of Authentic Radiolabeled Withaferin A; C, Withanolide Extract from Radioactive Acetate-Fed *in Vitro* Cultured Shoots

TLC plate at the bottom of each radio-TLC depicts separate lane (on the same radio-TLC) that had co-run unlabeled authentic withanolide and was developed chromogenically with anisaldehyde reagent after the radio scan. The positions of radioactivity peaks and chromo-spots of authentic sample on the TLC plate matched, corroborating the chemical identity of withanolide biosynthesized from radiolabeled acetate.

compound were characteristic: 3450 (for $-\text{OH}$), 2925, 2885, 1710 (α,β -unsaturated- δ -lactone), 1680 (α,β -unsaturated six-membered ketone), 1460, 1375, 1290, 1134, 1020 (epoxy function), and 900. FAB-MS (6 kV, mA) gave $[\text{M}+\text{Na}]^+$ at m/z 493 followed by $[\text{M}]^+$ at m/z 470, $[\text{M}-\text{H}_2\text{O}]^+$ at m/z 452, $[\text{M}-2\text{H}_2\text{O}]^+$ at m/z 434, and $[\text{M}-3\text{H}_2\text{O}]^+$ at m/z 416. Further, ^1H -, ^{13}C -NMR (data not shown) interpretations resulted in identification of the compound as $5\alpha,20\alpha$ (*R*)-dihydroxy- $6\alpha,7\alpha$ -epoxy-1-oxowitha-2,24-dienolide (withanolide A) that was validated by compliance of the spectral values reported for the compound in the literature.^{21,22} HPLC chromatogram and MS and MS-MS fragmentation data of authentic withanolide A are given in Fig. 3.

Shoot cultures were initiated using explants from the two selected experimental lines (*RS-Sel-1* and *RS-Sel-2*) of *W. somnifera* in the presence of different plant growth regulators on MS medium (Fig. 1b). The morphogenetic response of the two lines was different under the culture conditions. *RS-Sel-1* produced 55 ± 3.0 , 48 ± 7.0 , 15 ± 3.0 and 58 ± 5.0 shoots per explant under the hormonal regimens (ppm of BAP and kinetin) of 1.0/0.0, 1.0/0.10, 1.0/0.50, and 1.0/1.0, respectively. The corresponding values of shoots produced per explant by *RS-Sel-2* were much lower (26 ± 8.0 , 16 ± 1.0 ,

34 ± 1.5 and 17 ± 2.5 shoots per explant, respectively). In the case of *RS-Sel-2*, a prominent "solid tissue mass" (shooty teratoma) was noticed at the base of the shoot cultures under all the hormonal combinations. However, in *RS-Sel-1* it was formed only with BAP 1.00 ppm + kinetin 0.50 ppm.

In both experimental lines, withanolide A was hardly detectable analytically in the aerial tissue of the plants. However, its production could be induced in the *in vitro* shoot cultures and the extent of induction varied quantitatively (*ca.* 10-fold, 0.014 to 0.14 mg per gram fresh weight) depending upon the hormone combination used as well as genotype (Table 1). TLC profiles of withanolides and HPLC chromatograms of the shoot cultures of *RS-Sel-1* and *RS-Sel-2* are shown in Fig. 2. The shoot culture of *RS-Sel-1* raised with BAP 1.00 ppm and kinetin 0.50 ppm displayed the highest concentration of withanolide A (14.3 mg per 100 g fresh weight and 238 mg per 100 g dry weight, *i.e.*, 0.24%) in the green shoots. Comparatively, the analytical concentration obtained with the green shoot cultures (0.24% dry weight basis) was higher than the isolation yields ($<0.02\%$) reported so far from the dried roots of field-grown plants,^{8,21,22} even if substantial isolation losses are considered on the preparative scale. In the "solid mass/shooty teratoma" of the cultures, the

Table 1. Accumulation of Withanolide A in 65-d-Old *In Vitro* Shoot Cultures of the Two Experimental Lines of *Withania somnifera*

Hormonal combinations BAP+kinetin (mg/l)		<i>W. somnifera</i> experimental line			
BAP (mg/l)	Kinetin (mg/l)	<i>RS-Sel-1</i>		<i>RS-Sel-2</i>	
		Withanolide-A level (mg/gfw)		Withanolide-A level (mg/gfw)	
		Green shoot	Solid mass	Green shoot	Solid mass
1.00	0.00	0.053±0.011	n.f.	Traces	0.029±0.005
1.00	0.10	0.016±0.003	n.f.	0.014±0.003	0.016±0.003
1.00	0.50	0.143±0.029	0.037±0.006	n.f.	0.010±0.002
1.00	1.00	0.059±0.008	n.f.	0.023±0.005	0.011±0.002

n.f., not formed.

highest concentration of withanolide A production (3.7 mg per 100 g fresh weight; 46.2 mg per 100 g dry weight) was also noted in the shoot culture of *RS-Sel-1* raised with BAP 1.00 ppm and kinetin 0.50 ppm. On a comparative basis, *RS-Sel-1* had greater biogenesis/accumulation of withanolide A than *RS-Sel-2*.

The *in vitro* biosynthetic studies using a radiolabeled primary precursor (acetate) revealed a clear peak of radioactivity on the radiochromatogram which matched the position of the coloaded unlabeled withanolide A on the chromatogram on the same plate (Fig. 3). The microscale isolation and column chromatography purification of the withanolide extract from the radioactivity fed shoot cultures resulted in the isolation of almost pure radiolabeled withanolide A and its co-TLC followed by comparative radiometric analysis on the radio-TLC analyzer further ascertained that the pertinent radioactivity peak in the radiochromatogram detected in the extract from radioactivity-fed shoot cultures was withanolide A (data not shown). Thus the *in vitro* shoot cultures could *de novo* biosynthesize withanolide A, corroborating the observed production of withanolide A by the cultures. The cultures also produced withaferin A, the usual predominant withanolide of the aerial parts of *W. somnifera*. Withaferin A levels were 0.051±0.004, 0.012±0.002, 0.349±0.028, and 0.019±0.002 mg per gram fresh weight (mg/gfw) in the shoot cultures of *RS-Sel-1* and 0.025±0.001, 0.057±0.005, 0.106±0.009 and 0.127±0.007 mg/gfw in the shoot cultures of *RS-Sel-2* raised under the BAP/kinetin regimens (ppm) of 1.0/0.0, 1.0/0.10, 1.0/0.50, and 1.0/1.0, respectively. The shooty teratomas of *RS-Sel-2* had 0.07 to 0.08 mg/gfw of withaferin A. Shoot culture production of withaferin A has been previously reported,¹⁷⁾ and the data suggest that its biogenesis in the cultures was strongly modulated by the hormonal balance/ratio in the media. Notably, the concentrations of withaferin A in the cultured shoots were lower than those *in planta*.

The present study confirms that: i) *in vitro* shoot culture biosystems could be alternative to field plant root harvesting (labor intensive, counterconservative for the herb) for production of the therapeutically valuable withanolide A; ii) the shoot cultures, being amenable to fine-tuning, provide a method for ensuring consistency and quality standards by minimizing phytochemical variability for the and herb/phytochemical, an important issue for phytopharmaceuticals^{1,23)}, and iii) the cultures may constitute an appropriate system for biosynthetic/functional genomic studies of withanolide A

(also being easily maneuverable, it allows understanding of the metabolic modulation of biosynthesis).

Experimental

Plant Materials For isolation of withanolide A, roots were harvested from *W. somnifera* DUNAL (Ashwagandha) raised at the experimental farm of the Central Institute of Medicinal and Aromatic Plants, Lucknow, following standard agronomic practices. The roots were shade-dried before use. The resource explants for tissue culture were obtained from the designated experimental lines (*RS-Sel-1* and *RS-Sel-2*) maintained at the farm. The lines are deposited at the National Gene Bank, CIMAP, India, as *RS-NMITLI-II.AH* and *RS-NMITLI-II.CC*, respectively.

Chemicals All biochemicals and media were purchased from Sigma Chemical Co. (U.S.A.) or Hi Media (India). All other chemicals, solvents, reagents and precoated TLC plates were from E. Merck. Authentic withaferin A was the same as isolated and reported previously.^{1,2)} [²⁻¹⁴C] Sodium acetate (1222 MBq mmol⁻¹) was obtained from the Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre, Trombay, Mumbai, India. Pure withanolide A was isolated from the roots of field-grown plants and structurally confirmed as described here.

Isolation and Structural Characterization of Marker Withanolide A Dried powdered roots (1 kg) were extracted with methanol in a percolator, and the methanol extract was repeatedly washed with *n*-hexane to remove pigments. The depigmented methanol extract was concentrated under reduced pressure, added to water, and extracted three times with chloroform. The chloroform extract was concentrated to dryness under reduced pressure and subjected to silica-gel column chromatography. The column was eluted with a stepwise gradient of increasing (5 to 10% each step) ethyl acetate concentration in hexane starting ethyl acetate to hexane from 25 : 75. The fractions were scanned for TLC, and fractions with matching profiles were pooled, concentrated, and further purified with column chromatography and repeated crystallization. The purified phytochemicals were subjected to structural determination based on melting point, IR, NMR, and mass spectroscopy. The molecular identity of one of the constituents (white crystals in ethyl acetate, mp 282 to 283 °C, copiously detected in the ethyl acetate : *n*-hexane 40 : 60 pool from the column) thus purified was found to be withanolide A as inferred from spectral (IR, ¹H-, ¹³C-NMR, and MS) data. The IR spectrum was recorded on a Shimadzu IR-408 spectrometer. ¹H-NMR spectra were recorded on Bruker AV-300 FT NMR machine at 300 MHz and chemical shifts were recorded in d-units with TMS as internal standard. ¹³C-NMR spectra were recorded on the same instrument at 75 MHz. The FAB mass spectrum was recorded on a JEOL SX 102/DA-6000 mass spectrometer coupled to a data system using argon/xenon (6 kV, 100 mA) as the FAB gas.

In Vitro Shoot Cultures Master multiple shoot cultures of the experimental lines were developed from axillary buds as explants on agar-solidified basal medium supplemented with BAP 1.00 ppm and kinetin 0.5 ppm. Nodal segments from these *in vitro* culture generated shoots were used as explants for further culture experiments using different hormonal combinations (Table 1) with MS agar as the basal medium. The cultures were grown under a 16 h light/8 h dark cycle at 20±3 °C, with light intensity of 35±2 μmol m⁻² s⁻¹. The nodal segment explants developed into multiple shoots and had a hard mass at the base (shooty teratoma). Morphogenetic responses were compared as number of shoots per explant. Accumulation of withanolide A and withaferin A was analyzed in the differentiating shoots

when they had attained a height of 2.0 to 2.5 cm and in shooty teratomas (wherever formed in the cultures) after dissecting the two morpho-forms of the cultures. Qualitative and quantitative detection of withanolide A and withaferin A was carried out through TLC and HPLC, respectively, using withanolide A and withaferin A as marker withanolides.

Withanolide Isolation from *in Vitro* Shoot Cultures Withanolide isolation was carried out in four volumes of 50% methanol followed by defatting and depigmentation three times with an equal volume of *n*-hexane and recovering withanolides from the defatted methanolic solution into chloroform by liquid-liquid partitioning (equal volume, three times), essentially as described earlier.¹¹ Chloroform phases were pooled, evaporated to dryness at 25 °C, and redissolved in a known volume of methanol just prior to TLC and HPLC analysis.

TLC and HPLC of Withanolide Extracts Qualitative withanolide profiling was done through TLC while quantitation was carried out through HPLC. For TLC, silica gel G-60 plates were sample spotted, developed in a solvent system consisting of chloroform : ethyl acetate : methanol : benzene (74 : 4 : 8 : 24), and development was done with anisaldehyde reagent (0.5 g anisaldehyde in a mixture of acetone 20 ml, water 80 ml and 60% perchloric acid 10 ml) with heating at 110 °C. Authentic withanolides including withaferin A, withanone, and withanolide A (isolated as above) were used as markers for co-TLC. Quantification of withanolide A in the samples was carried out through HPLC on a reverse-phase RP-C₁₈ column (Nova-Pak, 4 μm, 3.9×150 mm, Waters) using water (A) and methanol (B), each containing 0.1% acetic acid, as solvent (gradient A : B, 60 : 40 to 25 : 75, 0 to 40 min; 10 : 90, 40 to 50 min) at a flow rate of 0.6 ml min⁻¹ and online UV detector (PDA) at 227 nm. The column temperature was maintained at 27 °C during the run. Authentic withanolides (withaferin A and withanolide A) were used to ascertain their discrete resolution from each other under these conditions. For computation of withanolide concentration in the samples, a calibration curve of concentration *versus* detector response (peak area) was developed for withanolide A using different injection volumes (≥5 μl) of its standard solution (1.0 mg ml⁻¹) in methanol, as reported previously.¹¹

Radiolabeled Precursor-Aided Biosynthetic Studies in Shoot Cultures Individual shoots (1 to 1.5 cm) were dissected from the above multiple shoot cultures and subcultured (6 to 8 pieces per conical flask) in the above medium containing [2-¹⁴C]acetate (74 kBq; 50 ml) and allowed to grow under the same *in vitro* environmental conditions as above for 70 d. The radiolabel-fed shoots were harvested and processed for preparation of withanolide extract as above. The extract was subjected to radio-TLC analysis. For radio-TLC analysis, the TLC plates (as above) were loaded with 10 μl of the extract. An aliquot (5 μl) of unlabeled withanolide A and withaferin A (1.0 mg ml⁻¹) were also loaded for co-chromatography as markers. TLC was performed as above, and adherent solvent allowed to evaporate and then scanned for radioactivity along the spotted lanes on a radio-TLC analyzer (BioScan AR-2000) using a 10 mm collimator and P-10 gas at a flow rate of 21 min⁻¹. The results and radiochromatograms were developed using Win-scan software (ver. 3.09 and 2D software). After the radioactivity scan of the plate lanes, the plate was sprayed with anisaldehyde reagent and color-devel-

oped as above. The radiochromatogram and chromo-chromatograms of the plates were matched for the withanolide A and withaferin A positions in the former.

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