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Withanolide A is inherently de novo biosynthesized in roots of the medicinal plant Ashwagandha (*Withania somnifera*)

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Ashwagandha (*Withania somnifera* Dunal., Solanaceae) is one of the most reputed medicinal plants of Ayurveda, the traditional medical system. Several of its traditionally proclaimed medicinal properties have been corroborated by recent molecular pharmacological investigations and have been shown to be associated with its specific secondary metabolites known as withanolides, the novel group of ergostane skeletal phytosteroids named after the plant. Withanolides are structurally distinct from tropane/nortropane alkaloids (usually found in Solanaceae plants) and are produced only by a few genera within Solanaceae. *W. somnifera* contains many structurally diverse withanolides in its leaves as well as roots. To date, there has been little biosynthetic or metabolism-related research on withanolides. It is thought that withanolides are synthesized in leaves and transported to roots like the tropane alkaloids, a group of bioactive secondary metabolites in Solanaceae members known to be synthesized in roots and transported to leaves for storage. To examine this, we have studied incorporation of ^{14}C from [2-^{14}C]-acetate and [U-^{14}C]-glucose into withanolide A in the in vitro cultured normal roots as well as native/orphan roots of *W. somnifera*. Analysis of products by thin layer chromatography revealed that these primary metabolites were incorporated into withanolide A, demonstrating that root-contained withanolide A is de novo synthesized within roots from primary isoprenogenic precursors. Therefore, withanolides are synthesized in different parts of the plant (through operation of the complete metabolic pathway) rather than imported.

Introduction

Ayurveda and traditional Chinese medicines are the key knowledge resources driving phytomolecule-based drug development programs world over. Ashwagandha (*Withania somnifera* Dunal., Solanaceae) is one of the most reputed medicinal plant of Ayurveda. The herb forms essential constituent or whole of more than 100 traditional medicines. The well-descript pharmacological activities of the plant include physiological and metabolic restoration, anti-arthritic, anti-aging, cognitive function improvement in geriatric states and recovery from neurodegenerative disorders (Lal et al. 2006, Misra et al. 2005, Sangwan et al. 2004a). In vitro and in vivo molecular pharmacological investigations have elucidated association of these activities of the herb with its specific secondary metabolites known as withanolides

Abbreviations – FAB, fast atom bombardment; FW, fresh weight; MS, Murashige and Skoog; Radio-TLC, radioactivity scanned TLC; TCS, three cycle subcultured.

(Iuvone et al. 2003, Kaileh et al. 2007, Kuboyama et al. 2005, Widodo et al. 2007, Zhao et al. 2002), a class of phytosteroids named after *W. somnifera*.

Withanolides, chemically nomenclatured as 22-hydroxy ergostane-26-oic acid 26, 22-δ-lactones, are C_{28}-steroidal lactones based on an intact or rearranged ergostane frame through appropriate oxidations at C-22 and C-26 to form a δ-lactone ring (Fig. 1). Production of withanolides in plants appears to be restricted to only a few genera within Solanaceae with their most prodigal amounts and structurally diversified forms found in *W. somnifera* (Misra et al. 2005, Sangwan et al. 2005). Major withanolides, like withaferin A and withanolide A (Fig. 1), of the plant have been demonstrated to possess significant and specific therapeutic action in carcinogenesis, Parkinson's disease and Alzheimer's disease (Choudhary et al. 2005, Jayaprakasam et al. 2003, Kaileh et al. 2007, Kuboyama et al. 2005, Matsuda et al. 2001, Su et al. 2004).

Regarding secondary metabolites, Solanaceae plants are principally recognized as producers of tropane alkaloids – alkaloids that have also been isolated from *W. somnifera* – in fact, long before the isolation of withanolides from this plant (Khanna et al. 1961). These alkaloids include *N*-methylpyrrolinium-derived nicotine alkaloids, tropine-derived true tropane alkaloids, and pseudotropine-derived nortropane alkaloids, also called calystegines (De Luca and St Pierre 2000, Drager 2006, Goossens and Rischer 2007, Griffin and Lin 2000). Biosynthetic route for these alkaloids originates from the putrescine pathway with formation of *N*-methyl putrescine from putrescine under the catalytic action of a characteristic *N*-methyltransferase (Teuber et al. 2007). Several of the enzymes and genes of the alkaloid biosynthetic pathways have been characterized in a number of Solanaceae plants of both food and medicinal value (Facchini 2001, Kaiser et al. 2006). Biosynthesis of this group of secondary metabolites (nicotine and tropane/nortropane alkaloids) in Solanaceae occurs in roots from where they are transported to leaves for accumulation, although an exception to this concept has been recently reported (Kaiser et al. 2006). However, withanolides (secondary metabolites of triterpenoid origin) predominantly found in roots and leaves of *W. somnifera* and certain other medicinal herbs of Solanaceae, have rarely been investigated for their biosynthesis.

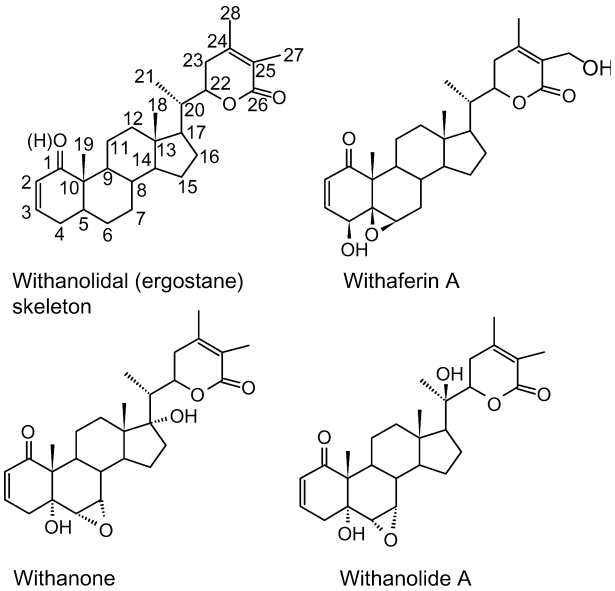

Fig. 1. Withanolide skeleton and some of the important withanolides of *W. somnifera*.

Both roots and leaves are prescribed for medicinal uses in the traditional systems of medicine (Kaileh et al. 2007, Sangwan et al. 2005), parallel to these also being the withanolide richest tissues of the plant. Phytochemical isolation data reported in the literature support significant qualitative overlap of withanolides in plant leaves and roots, despite the dearth of comparative metabolomic profiling data (Sangwan et al. 2005). The functional aspects of withanolides in plants are currently unknown owing to the lack of relevant biosynthetic research (Madina et al. 2007a, 2007b, Sharma et al. 2007, Sangwan et al. 2004b). Root withanolides are possibly imported from leaves in view of: (1) the significant match in the characteristics of withanolides from the two tissues, (2) the gradient of their tissue concentrations (i.e. higher in leaves and lower in roots), (3) the existence of glucowithanolides (withanosides) in roots and their detection in leaves, indicating transformation-facilitated transportability in glycosylated forms, (4) corollary of space in the sites of biosynthesis and accumulation of secondary metabolites in the Solanaceae family, e.g. tropane alkaloids are biosynthesized in roots and transported to leaves (Mishra et al. 1996), and (5) radiotracer studies demonstrating the withanolide biogenic capacity of plant twigs, using 24-methylene cholesterol as a precursor (Glotter 1991). Specific withanolides, such as withanolide D, have been detected in *Agrobacterium rhizogenes*-transformed hairy roots (Ray et al. 1996). However, these findings do not unambiguously signify de novo biosynthesis in natural roots, because (1) withanolide D is predominantly localized in the shoot rather than root, and is found only in shoots of specific chemotypes of *W. somnifera* like Israeli *Chemotype II* and (2) metabolic expression in hairy roots is considerably divergent from that in their native counterparts.

Therefore, we carried out biosynthetic studies in the in vitro cultured normal roots as well as native/orphan roots of *W. somnifera* and have recruited withanolide A

as the benchmark withanolide because it occurs in the plant without any chemotype-specific restriction. The studies unequivocally demonstrate that root-contained withanolide A is independently and inherently de novo synthesized within the roots of the plant from primary isoprenogenic precursors. The observations connote redundant biosynthesis of withanolides in different parts of the plant unlike tropane alkaloids.

Materials and methods

General

All chemicals and solvents were of analytical grade procured from E. Merck. Radiochemicals [2-^{14}C]acetate (1222 MBq mmol^{-1}) and [U-^{14}C]glucose (518 MBq mmol^{-1}) were purchased from Board of Radio Isotopes (Bhabha Atomic Research Centre, Mumbai, India).

Plant material

W. somnifera plants, experimental line RS-NMITLI-II.AH, were raised at experimental farm of the Central Institute of Medicinal and Aromatic Plants (Lucknow, India), and these plants were used for isolation of the marker withanolide A from the roots, native (orphan) root biosynthetic studies and explant culture (axillary buds) for the in vitro studies.

Isolation, purification, and spectral characterization of withanolide A

Withanolide A was isolated and purified as white crystals in ethyl acetate according to previously reported methods (Sangwan et al. 2007) and subjected to structure determination through melting point characterization (282–283°C), IR spectroscopy, NMR spectroscopy and mass spectroscopy. IR spectrum was recorded on a Shimadzu IR-408 spectrometer. ^{1}H-NMR spectra were recorded on a Bruker AV-300 FT-NMR system at 300 MHz, and chemical shifts were recorded in d-units using tetramethylsilane as an internal standard. ^{13}C-NMR spectra were recorded on the same instrument at 75 MHz. The fast atom bombardment (FAB) mass spectrum was recorded on a JEOL SX 102/DA-6000 Mass Spectrometer coupled to Data System using argon/xenon (6 kV, 100 mA) as the gas for FAB. The crystal's molecular identity as withanolide A was ascertained by matching its spectral information with previous reports (Anjaneyulu and Rao 1997, Subramanian et al. 1971).

Development of in vitro shoot cultures

Sterile, in vitro shoot cultures of *W. somnifera* were established with axillary buds as explants. The explant was washed in tap water and rinsed with distilled water (3×), steeped in 1% Lavolene (30 min) and rinsed with distilled water (3×), followed by immersion in 0.1% $HgCl_2$ and rinsing with sterile distilled water (3×), and finally, steeped in 70% ethanol (5 s) and rinsed thoroughly with sterile distilled water (2×). The sterilized explant was cultured in vitro for 45 days in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 0.5 mg l^{-1} kinetin and 1.0 mg l^{-1} 6-benzyl aminopurine to obtain multiple-shoot cultures. The in vitro culture conditions were 20 ± 3°C, 16 h/8 h light/dark cycles, and light intensity (35 ± 2 μmol m^{-2} s^{-1}).

Induction of roots in the in vitro shoots

The individual shoots from the multiple-shoot cultures were dissected out and subcultured in MS medium without hormones for 30 days, under the same conditions as above. Sufficient roots were available for sampling as explants at the end of this period for in vitro culturing of normal roots.

Subculturing of in vitro roots

Root-segment (1–2 cm) explants from the rooted in vitro shoots were cultured in MS medium without hormones for 60 days, under the same conditions as above. The in vitro grown normal roots were subjected to three such cycles of subculturing, and the in vitro roots from the last cycle were referred to as three-cycle-subcultured (TCS) normal roots and used for withanolide A analysis and biosynthetic (radiotracer) studies.

Isolation of withanolides from in vitro cultured roots

The TCS roots [0.5 g fresh weight (FW)] were harvested, and powdered in liquid N_2; the powder was extracted with 50% methanol (3 × 20 ml) and filtered. The filtrate was defatted with an equal volume of *n*-hexane (twice), and the defatted extract was partitioned with equal volumes of chloroform (three times). The chloroform fractions were collected and pooled, evaporated to dryness, dissolved in 50 μl methanol, and then subjected to withanolide analysis by TLC and HPLC, using withanolide A as the tracker molecule.

TLC of withanolide extract from cultured roots

TLC analysis was carried out on precoated (analytical) Silica Gel 60 (20 × 20 cm) plates (E. Merck) by loading 6 μl of the methanolic solution of the sample and 5 μl of the marker withanolide A (1.0 mg ml^{-1}). The plates

were subjected to chromatography and developed as already described (Sangwan et al. 2007).

HPLC of withanolides from cultured roots

HPLC analysis was carried out on a LC-10 system (Shimadzu, Kyoto, Japan) using a reverse-phase C_{18} column (4 μm, 3.9 × 150 mm; Nova-Pack, Waters, Milford, MA) and a solvent system (0.6 ml min^{-1}) comprised of methanol and water (each containing 0.1% acetic acid) in the gradient mode −45:55 to 65:35 (45 min). Detection was done at 227 nm using an online UV detector (SPD-10A) and the chromatogram reports were generated through integrated software (Class-LC10, version 1.63). 10 μl of the withanolide sample (methanolic solution) or 5 μl of marker withanolide was injected for each run.

Radiotracer studies in repeat-cycle subcultured roots

The explants from TCS roots were subcultured in MS medium containing [2-^{14}C]acetate (185 KBq (100 ml)$^{-1}$) in culture tubes (six tubes, 16.25 ml medium each), under the same conditions as above. After 120 days of in vitro culture in the radiolabel-containing medium, the roots were harvested and subjected to withanolide extraction as described above, followed by radio-TLC analysis of the extract.

Radiotracer studies in native (orphan) roots

Native roots (secondary, 0.5-cm diameter) were harvested (6.0 g FW) from the field-grown plants, washed with distilled water (3×), cut into 50-mm-long segments. The segments were held vertically in a test tube (three root segments of 3.0 g FW per test tube) and fed at the bottom end with distilled water (0.5 ml) containing 37 KBq radioactive precursor, either [2-^{14}C]acetate or [U-^{14}C]glucose, at ambient temperature. After the labeled-precursor-containing water was taken up by the root segments (12 h), 0.5 ml distilled water was added every 12 h. At the end of the feeding period (120 h), the root segments were subjected to withanolide extraction, and the resultant extract (in 300 μl methanol) was used for radio-TLC analysis.

Radio-TLC analysis

Precoated TLC plates were loaded with 10 μl of the root extract. A 5-μl aliquot of unlabelled withanolide A (1.0 mg ml^{-1}) was also chromatographed simultaneously as a marker. The plates were subjected to chromatography as described above, the adhering solvent was evaporated off, and subsequently, the plates were scanned for radioactivity along the spotted lanes using AR-2000 radio-TLC analyzer (BioScan, Washington DC) using 10 mm collimator and P-10 gas (90% Ar-10% methane) at a flow rate of 2 l min^{-1}. The radiochromatograms were developed using the WINSCAN (BioScan) software (version 3.09 and 2D). For the radio-TLC of the extract from the labeled-precursor-fed cultured roots, an authentic radiolabeled withanolide A that was isolated previously from a shoot culture fed with [2-^{14}C]acetate and purified as earlier (Sangwan et al. 2007), was also used as withanolide A migration marker on the TLC plate.

All the experiments were carried out in triplicate and subjected to statistical analysis. Standard deviation was ≤10% of the mean values.

Results and discussion

Plants show immense qualitative and quantitative variability in their secondary metabolism. The domains of variability include: the chemical class(es) of the secondary metabolites produced (such as alkaloids, terpenoids, flavanoids and lignans); functional, conjugational and multimeric variants within the same class of compounds; and the different types of the cells, tissues and organs involved in the production and/or storage of each class of secondary metabolites/specific phytochemical entity(ies). Among these compounds, alkaloids form the most well-studied group with respect to their sites of synthesis, transport and storage. The results of these studies suggest the lack of a unified theory for their site(s) of synthesis. The following instances represent the differences in the biosynthesis mode: nicotine and tropane/nortropane alkaloids of the Solanaceae plants are synthesized in the roots and transported to the aerial parts for storage, whereas the monoterpene-indole alkaloids of *Rauwolfia* and *Catharanthus* are synthesized in both the root and the leaf, the benzylisoquinoline alkaloids of *Papaver* are synthesized in the metaphloem (of both root and shoot) and stored in the laticifers and capsules, and the quinolizidine alkaloids in *Lupinus* are synthesized in the leaves and transported to roots (De Luca and St Pierre 2000, Drager 2006, Goossen and Rischer 2007, Lee et al. 2007).

In addition to tropane and its congener alkaloids (nicotine and calystegines) for which Solanaceae is renowned, a few genera of the family also amass another distinct class of novel secondary metabolites called withanolides, the ergostaneskeleton-based phytosteroids (Fig. 1) named after *W. somnifera*. These secondary metabolites with triterpenoid ancestry occur most predominantly in the leaves and roots of *W. somnifera*; withaferin A, withanone, withanolide D, and withanolide

A are some of the major withanolides of the plant (Sangwan et al. 2007). Quantitative variations notwithstanding, withanolide A is usually encountered in both the leaves and the roots of the plant (Sangwan et al. 2007). However, whether roots can de novo synthesize its withanolides (withanolide A) is a debatable point. Information on the biosynthetic competence of roots for brassinosteroids (the well known growth regulators of steroidal nature) has become available only recently (Kim et al. 2005). Therefore, this radiotracer and bioanalytical study of withanolide A biosynthesis in the native (orphan) roots and *in vitro*-cultured normal roots (Fig. 2A–D) of *W. somnifera* was focused on discerning the independent de novo withanogenic competence of the normal plant roots.

Withanolide A was extracted from *W. somnifera* roots through preparative-scale repeated silica gel column chromatography. The purity of the crystallized withanolide A was assessed by HPLC (Fig. 3A), and its identity was ascertained through mass spectral analysis including mass spectra and tandem mass spectra (MS-MS) in a Perkin Elmer API-2000 spectrometer (Fig. 3C–D), IR,

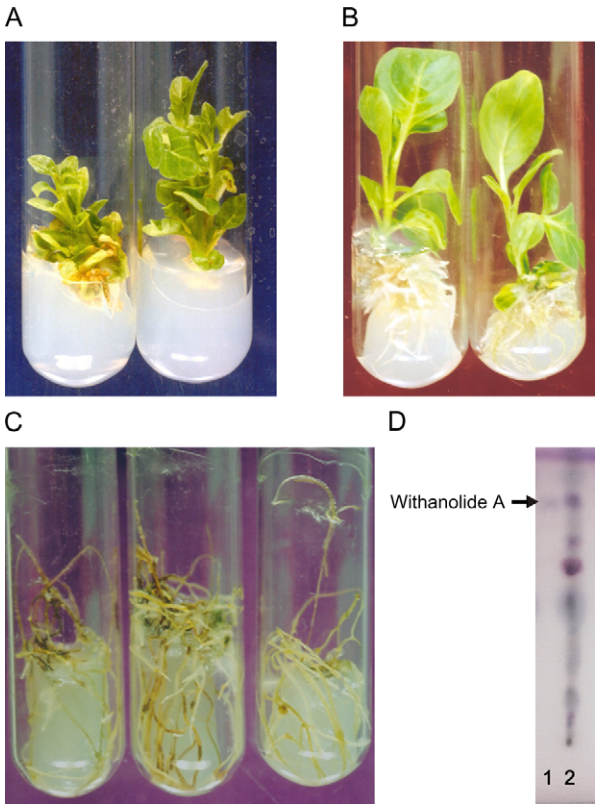

Fig. 2. In vitro cultures of *W. somnifera*: (A) multiple shoots; (B) in vitro shoots with rooting induced; (C) in vitro cultured normal roots (TCS); (D) TLC-detected withanolide A in TCS roots (lane 1, authentic withanolide A; lane 2, withanolide extract of TCS root).

and ^{1}H and ^{13}C NMR (data not provided), and the subsequent matching of the results with previous data. This sample was used for the biosynthetic studies as a tracking molecular marker.

In vitro shoot induction was carried out with the axillary bud as the explant. The in vitro shoots developed within 45 days in MS medium under the optimum hormone combination (0.5 mg l^{-1} kinetin and 1.0 mg l^{-1} 6-benzyl aminopurine; Fig. 2A). The shoots, after subculturing in the same MS medium without hormone, attained significant rooting in 30 days (Fig. 2B). These in vitro roots were used as explants for in vitro culture of roots raised in hormone-free MS medium. The roots were subjected to three such cycles of subculturing to negate the chances of carryover of any significant amount of innate withanolide A from the initial root explants. The in vitro roots after the last subculturing were referred as three-cycle-subcultured (TCS) roots (Fig. 2C), and these were subjected to withanolide extraction followed by TLC monitoring for withanolide A content. The TLC analysis clearly demonstrated the presence of withanolide A in the extract prepared from the in vitro TCS normal roots of the plant (Fig. 2D). The results were confirmed and quantified by HPLC analysis (Fig. 3B) of the extract. This presence of withanolide A suggested that it was most probably locally synthesized in the cultured roots during their independent growth *in vitro*, as chances of the withanolide being carried over in detectable amounts (from the original root explant after three subculturing cycles) were little. Nevertheless, to completely exclude such a possibility, the cultured roots were subjected to radiotracer biosynthetic studies using [2-^{14}C]-labeled primary precursor (acetate).

The cultured roots (TCS) were grown for 120 days in a culture medium containing [2-^{14}C] acetate for 120 days, extracted for withanolides, and the extract used for the TLC resolution of its constituents. When these TLC plates were subjected to radioactivity scanning on a radio-TLC analyzer, a conspicuous peak of radioactivity was detected (Fig. 4A, B). The radioactivity peak matched the position of the authentic withanolide A (visualized by chromodecoration of the same plate with anisaldehyde spray reagent) and the authentic, radiolabeled withanolide A (Fig. 4A, B). Thus, the observed ^{14}C-radiolabel incorporation from [2-^{14}C]acetate into withanolide A corroborate the hypothesis that the in vitro-cultured normal roots of *W. somnifera* possess the complete set of enzymes required for the biosynthesis of withanolide A from acetyl CoA. Similar radiotracer studies were carried out on native and orphan roots using both [2-^{14}C] acetate (Fig. 4C) and [U-^{14}C] glucose (Fig. 4D) as the exogenous carbon sources, to observe the in planta situation. Roots from the normal field-grown plant were fed with either

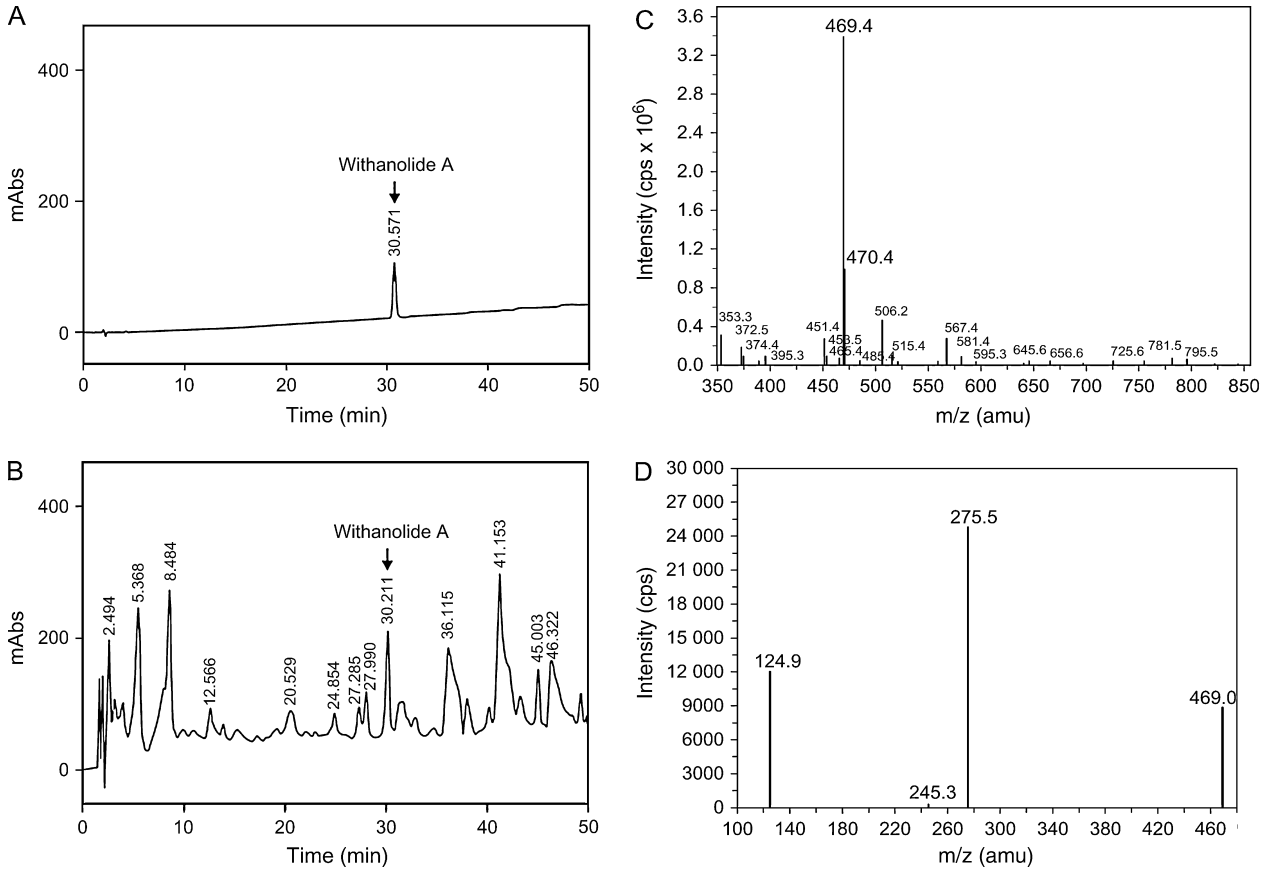

Fig. 3. HPLC and mass spectral analysis of withanolide A: (A) HPLC of authentic withanolide A; (B) HPLC of withanolide extract from TCS root, with withanolide A peak (→); (C) mass spectrum of withanolide A isolated from *W. somnifera* roots; (D) MS-MS of the withanolide A. cps, counts per scan; mAbs, milliabsorbance.

[2-^{14}C] acetate or [U-^{14}C] glucose for 120 h at room temperature, which was followed by withanolide extraction and monitoring of radioactivity in withanolide A, as was carried out for the extract from radioactivity-fed TCS in vitro roots. It was observed that native and orphan roots could biosynthesize ^{14}C-radiolabeled withanolide-A from both the isoprenogenic primary precursors (Fig. 4). These results reiterate that the in planta biosynthesis of withanolide A by the roots takes place ab initio.

On a relative basis, incorporation of the label from glucose was less efficient compared with that from acetate. This difference could be because of several possibilities, which affect the incorporation singly or in combination: (1) acetate is metabolically closer to the mevalonate pathway of isoprenogenesis, (2) label from glucose can be much widely shared through the use of glycolytic intermediates (originating from the supplied glucose) in various other biosynthetic pathways, (3) although glycolytic intermediates also cater to the alternate1-deoxy-D-xylulose-5-phosphate pathway of isoprenogenesis, their participation in withanolide biogenesis is still a disputed point (Sangwan et al. 2004b), and (4) stringently controlled metabolism of glucose through glycolysis, although its higher label density (all carbons of glucose were labeled) and better biocompatibility should favor its metabolic use for withanolide A biogenesis.

In summation, the data unequivocally illustrate that roots possess a complete and independent biosynthetic pathway for the production of withanolide A from primary metabolites. Because withanolide A, by its structural features and functional group characteristics (Fig. 1), represents a *matured* (substantially hydroxylated/oxidoreduced structure) withanolide rather than an upstream intermediate of the pathway, the above conclusion may be implicitly reiterated for the full contingent of withanolides of plant roots. This observation, in conjunction with the elucidation of withanolide biosynthesis in both leaf (Chaurasiya et al. 2007) and shoot cultures (Sangwan et al. 2007), indicates the redundant biosynthesis of withanolides in different parts of *W. somnifera* (Ashwagandha), unlike the biosynthesis of

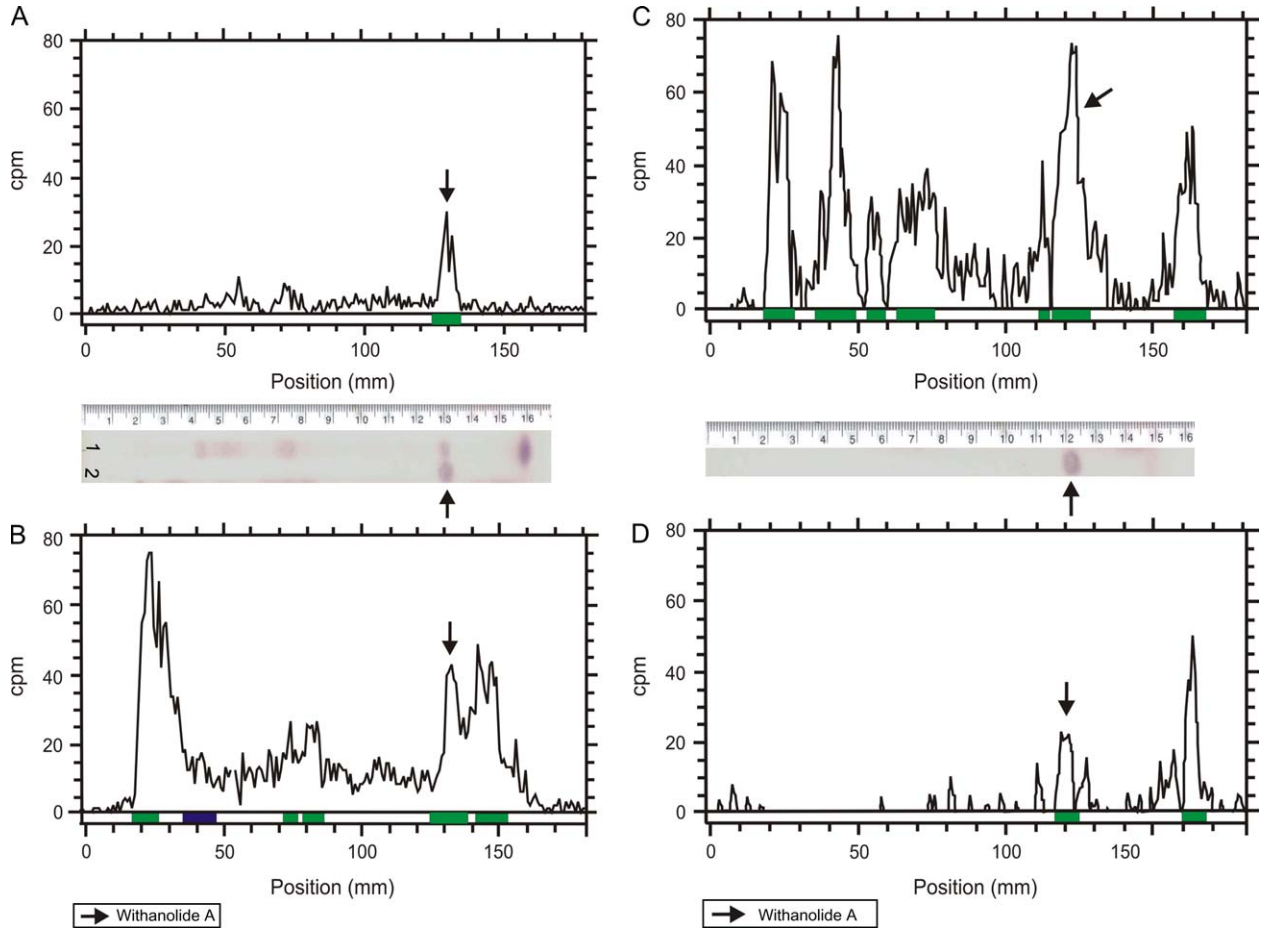

Fig. 4. Radio-TLC analysis of the extracts from radioactive [^{14}C] precursor-fed *in vitro* cultured normal roots and native (orphan) roots of *W. somnifera*: A, peak (→) of the authentic radiolabeled withanolide A; (B) withanolide extract from [2-^{14}C] acetate-fed *in vitro* cultured roots (AB panel: the plate with the ruler shows the same plate that had a sample of simultaneously run unlabeled authentic withanolide A and was developed chromogenically with anisaldehyde reagents after radioscan. Lane 1, (sample) the same lane as previously radioscanned; lane 2 unlabeled withanolide A (with spot indication →). (C) Withanolide extract of [2-^{14}C]acetate-fed native/orphan root (radiolabeled withanolide A peak →). (D) Withanolide extract of [U-^{14}C]glucose-fed native/orphan root – (C, D panel: the plate with the ruler shows the same plate that had a sample of simultaneously run unlabelled authentic withanolide A and was developed (after the radioscan) with anisaldehyde reagents.. The peak radioscan (→) and the standard withanolide A spot positions on the TLC plate matched in both the sets (cultured roots and native/orphan roots) of radiotracer studies indicating their same chemical identity.

tropane alkaloids in plants belonging to Solanaceae. The later compounds are produced near the tip of the root (with enzyme-specific localization in the pericycle, endodermis or cortex) and transported to leaf for accumulation (Facchini 2001). Nevertheless, as an exception, recently (Teuber et al. 2007), potato sprouts have been shown to actively synthesize pseudotropine-derived nortropanes (calystegines), reminding that conclusions from one plant to another, even within the same biosynthetic pathway in the same family of compounds, should be drawn cautiously.

Withanolide A is one of the most promising phytopharmaceuticals because of its recently reported impressive pharmacological properties: (1) induction of neurite regeneration and synaptic reconstruction (Kuboyama et al. 2005, Zhao et al. 2002) is important in dealing neurological disorders particularly Alzheimer's and Parkinson's diseases and (2) strong inhibition of carcinogenesis (Kinghorn et al. 2004). Any specific function of withanolides for the plant is unknown. Our preliminary observations that some *W. somnifera* accessions, which lack a specific major withanolide, show clearly altered flowering and fruiting behaviors and growth pattern (including a dwarf phenotype), indicate that withanolides might act as growth regulators *per se* or may manifest growth modulatory effect by virtue of their strongly sharing the metabolic pathway of biosynthetic origin with brassinosteroids (Fig. 5). However, there are no firm experimental data for this argument. Our ongoing work on identifying the enzymes and genes involved in the

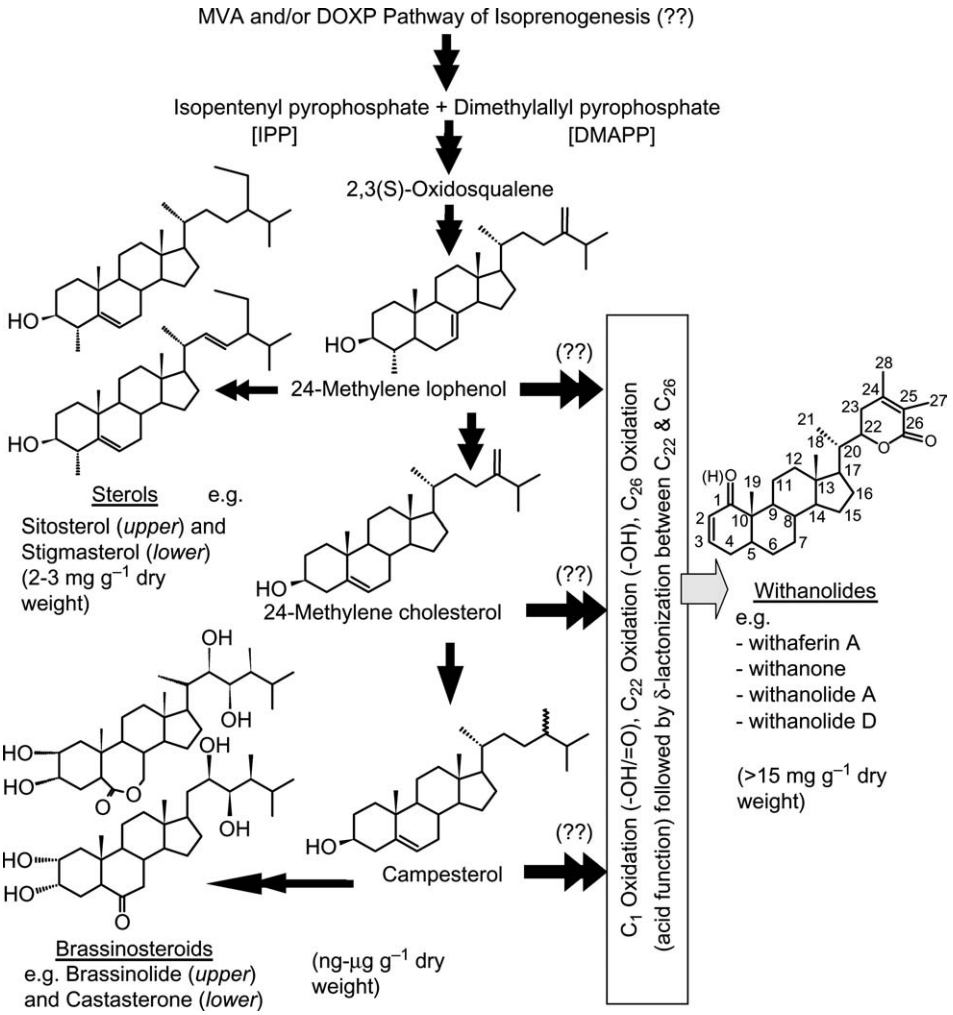

Fig. 5. Putative biosynthetic origin of withanolides in relation to the phytosterol and brassinosteroid metabolism.

putative position-specific hydroxylation (cytochrome P_{450}) in a metabolic model drawn from *Withania*-root chemoinformatics, wherein withanolide B occupies an anaplerotic position for diverse metabolic transformations into withanolide A, withanolide R, withanone and 27 hydroxywithanolide B, is expected to provide more insights into the questions raised in this study.

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