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SHORT COMMUNICATION

Leaf Ontogenic Phase-Related Dynamics of Withaferin A and Withanone Biogenesis in Ashwagandha (*Withania somnifera* Dunal.) - An Important Medicinal Herb

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***Withania somnifera* (Dunal), a medicinally important plant, is extensively used in traditional Indian herbal preparations as well as for modern nutraceutical and functional food supplements. Its characteristic phytogenic molecules are modified steroidal lactones called withanolides. Withaferin A is a predominant constituent and is pharmacologically active. Here, we studied the dynamics of biogenesis and accumulation of withaferin A and withanone in *Withania* leaves at various developmental phases (very young, young, mature, and senescent). HPLC analysis was conducted to determine the amassed quantities of these phytochemicals, while their *de novo* biosynthesizing capacity was examined via incorporation studies with a radiolabeled primary precursor, [2-^{14}C]-acetate. *De novo* biogenesis and accumulation of withanolides was most active in young leaves. Here, we also discuss developmental patterns and secondary metabolism in relation to eco-physiology and phytopharmaceutical variability.**

Keywords: Ashwagandha, withaferin A, *Withania somnifera* leaf ontogeny, withanolide biosynthesis, withanone

Ashwagandha (*Withania somnifera* Dunal; Solanaceae) is one of the most reputable sources for traditional Indian systems of medicine (TISM), particularly Ayurveda. Its health-promoting effects have earned it a reputation as the Indian ginseng. This herb is used in more than 100 formulations of TISM. Trade has recently surged for the pure phytochemicals as well as their enriched extracts because of the popularity of nutraceuticals and activity-screening for bioprospection. Rapidly growing industrial demand for this herb has led to a shift in resourcing, from wild habitats to cultivated fields. In India, *W. somnifera* is widely distributed, particularly in the central-western provinces. Its ethno-botanical health properties include adaptogenic, anti-sedative, and anti-convulsive attributes, as well as the provision of relief and recovery from neurological disorders and the treatment of geriatric debilities, arthritis, stress, and behavior-related problems (Schliebs et al., 1973; Ray and Gupta, 1994; Dhuley, 2000). Phytochemically, the plant accumulates a unique group of steroidal secondary metabolites, i.e., the withanolides. Theses triterpenoid-ancestry C_{28} phytosteroids are based on an ergostane skeleton with a carbonyl group at C_1 and a side chain with δ-lactone ring, formed by appropriate oxidation at C_{22} and C_{26} (Fig. 1). Molecular pharmacology studies have demonstrated linkages of the stated therapeutic actions to one or more withanolides from this herb (Sangwan et al., 2004; Tohda et al., 2005; Ichikawa et al., 2006; Kaileh et al., 2007).

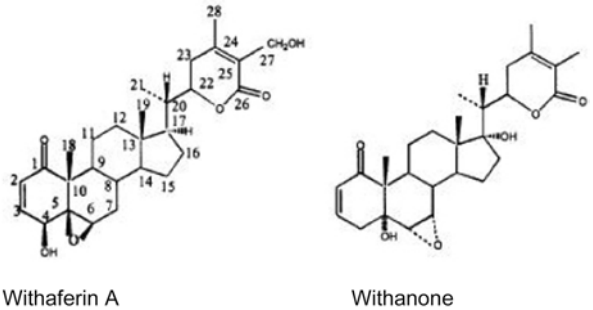

Figure 1. Withaferin A and withanone, two major withanolides from *Withania somnifera* leaves.

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Two withanolides -- withaferin A and withanone -- are accumulated in prodigious amounts in *Withania* leaves (Fig. 1). However, no information is available about the developmental physiology for their biogenesis. Understanding the ontogenic dependence of withanolide biogenesis is highly relevant for several reasons: 1) to learn about the chemo-ecophysiology for the production and accumulation of these secondary molecules, 2) for optimal harvesting of the bioactive phytochemical(s) from this crop, 3) to define the parameters for quality management of these herbal nutraceutical and therapeutic products, and 4) to determine the comparative genomics of withanogenesis. Therefore, we studied the patterns of accumulation for withaferin A and withanone in *W. somnifera* by examining their TLC profiles and HPLC quantitation, using leaf samples from five ontogenic stages -- very young, young, pre-mature, mature, and senescent. Their sequestration patterns were compared with actual biosynthetic patterns, as discerned by radio-TLC analysis of withanolide extracts prepared from the leaves at defined ontogenic phases, prior to feeding with [2-^{14}C]-acetate as the isoprenogenic precursor. The levels of radioactive incorporation were considered indices of *de novo* biosynthesis.

MATERIALS AND METHODS

Chemicals and Plant Material

All biochemicals were purchased from Sigma (USA). Solvents, reagents, and pre-coated TLC plates were obtained

from Merck (Germany). Authentic withaferin A and withanone were isolated from field-grown plants of *W. somnifera* (Ashwagandha), and their identities were spectrally ascertained according to our previous methods (Misra et al., 2005; Lal et al., 2006). [2-^{14}C]-acetate (1222.4 MBq mmol^{-1}) was obtained from the Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Centre, Trombay, Mumbai, India. Experimental line Ashwa-1/RSS-1) was grown per standard agronomic practices at the experimental farm of the Central Institute of Medicinal and Aromatic Plants, Lucknow.

Measurements of Fresh Weight (FW) and Dry Weight (DW)

Leaves were harvested at different developmental stages (very young, young, pre-mature, mature, and senescent) and weighed immediately to obtain their fresh weight (FW). To determine their dry weight (DW), samples were oven-dried at 80°C to a constant weight. Moisture content (%) was then computed from these FW and DW data. To express those values on a per-leaf basis, weights were divided by the total number of leaves harvested at each stage. For our sodium [2-^{14}C]-acetate incorporation studies, leaves were transferred to open-mouthed tubes containing 0.5 mL aqueous solutions (pH 6.8 to 7.0) of [2-^{14}C]-acetate (185 kBq, 0.151 µmol). For the specific stages, each tube contained four very young leaves, three young leaves, two pre-mature leaves, one mature leaf, or two senescent leaves).

Withanolide Extraction

At each developmental stage, fresh/wet leaves were ground to powder in liquid nitrogen and extracted overnight with 20 mL of methanol:water (25:75, v:v) at room temperature, followed by filtration (Sangwan et al., 2005). The filtrate was saved and the residue was further extracted (2 x 20 mL) at 12-h intervals. These aliquots were then pooled, filtered, and extracted with n-hexane (3 x 60 mL). The n-hexane fraction was discarded while the methanol-water fraction was further fractionated twice with an equal volume of chloroform. Those chloroform fractions were pooled together and completely dried. They were then dissolved in HPLC-grade methanol (200 µL g^{-1} FW for radio-TLC analysis or 2 mL g^{-1} DW for HPLC), then filtered through a sample clarification kit (organic) and subjected to either HPLC or TLC/radio-TLC in order to profile the contents and biogenesis of withaferin A and withanone.

TLC Resolution and Detection of Withanolides

The withanolides in our leaf extracts were resolved by TLC, using pre-coated plates (Silica Gel 60; 20 x 20 cm) that were loaded with 5 µL of the methanol-dissolved extracts. The plates were run in a solvent system consisting of $CHCl_3$:EtOAc:MeOH:C_6H_6 (70:4:8:24, v:v). Authentic withanolides (withaferin A and withanone) were also loaded (5 µL of 1 mg mL^{-1} methanolic solution) and run on the same TLC plates for R_f matching. Afterward, the TLC-resolved withanolides were detected chromogenically by spraying the plates with anisaldehyde reagent (prepared by dissolving 0.5 g anisaldehyde in 20 mL acetone, 80 mL water, and 10 mL of 60% perchloric acid). The plates were developed at 110°C for 15 min and visualized/photo-documented in visible light.

HPLC Analysis

HPLC analysis of the withanolide extracts was performed on a Waters modular system (Milford, USA) that comprised a quaternary pump (Model 600E), pump controller (Model 600E), auto-sampler (Model 717 plus), photodiode array (PDA) detector (Model 996), temperature controller module, and an Empower/Millennium chromatography manager. We used a reverse-phase (RP) Nova-Pak C_{18} column (4 µm, 3.9 x 150 mm; Waters, USA) that was subjected to binary gradient elution essentially as earlier (Chaurasiya et al., 2007). The two solvents included water containing 0.1% acetic acid (A) or methanol containing 0.1% acetic acid (B). A timed gradient programming of this solvent system was conducted at 27°C, as follows: start at 60% A, changing to 40% at 30 min; solvent composition on hold for next 2 min, followed by change to 25% A at 45 min; then changing to 5% A at 54 min, at a flow rate of 0.6 mL min^{-1}; then changing to 0% A at 55 min; and finally being held until the run time reached 60 min, at a flow rate of 1.0 mL min^{-1}. All gradient segments were linear (Curve Type 6; Empower; Waters). The PDA wavelength scan ranged from 190 to 350 nm, and the chromatograms were developed at 227 nm. Withanolide contents in our samples were computed using pre-developed calibration curves (regression from means of at least triplicate analyses) for the marker withanolides under the same conditions.

[2-^{14}C]-Acetate Feeding of Leaves

Leaves of *W. somnifera* were harvested at different stages via oblique excision at the bases of their petioles. They were immediately transferred to a beaker containing water so that their cut ends remained vertically dipped. The samples were brought to the lab where each set was transferred to an open-mouthed tube containing an aqueous solution (0.5 mL; pH 6.8 to 7.0) of [2-^{14}C]-acetate (185 kBq, 0.151 µmol). During this entire procedure, the leaves remained upright, with their petioles dipped in the radioactive acetate solution, to ensure vascular uptake of the isoprenogenic (withanogenic) precursor. The tubes were kept under fluorescent lamps (light intensity 35 ± 2 mmol m^{-2} s^{-1}) in a BOD incubator maintained at 27°C. After the precursor was taken up, successive additions (4 x 0.5 mL) of half-strength Hoagland's solution were made to maintain the leaves in a nutrient medium throughout the 50-h chase period. Afterward, the leaves were removed and subjected to withanolide extraction and TLC resolution (as above). This was followed by an determination of ^{14}C radioactivity in the withaferin A and withanone, as measured with a radio-TLC analyzer (AR-2000; Bio-Scan, USA).

Radiometric (Radio-TLC) Analysis of Withanolide Biogenesis

TLC resolution of the withanolide extracts from leaves fed with ^{14}C radio-labeled acetate was carried out as above. The plates were also loaded with non-radioactive authentic

withanolides that were chromogenically detected, a process that occurred after the radioactivity scan for matching their position (R_f) with the peaks of the samples. To detect radioactivity after the run, we first evaporated the adhering solvent from the plates, then scanned across the entire run length of each radioactive sample lane, using the radio-TLC analyzer, a 10-mm collimator, and P-10 gas at a flow rate of 2 L min^{-1}. The results and radio chromatograms were developed using Winscan software (Version 3.09 and 2D). Subsequent to this radioactivity-scan, the plates were chromo-decorated using the anisaldehyde spray reagent as above. The radio-chromatogram and chromo-chromatograms of the plates were matched for the withaferin A and withanone on the TLC plates. Incorporation of the ^{14}C label was estimated using the Winscan software, and expressed in terms of counts within the withanolide spot regions. All determinations were made in triplicate and the data were presented as means ± SD.

RESULTS AND DISCUSSION

Biogenesis of plant withanolides appears to be highly restricted to a few genera of Solanaceae; amongst them, *W. somnifera* produces the largest number of withanolides, showing diversified functional groups and regio/stereo-forms (Ray and Gupta, 1994). Its roots and leaves are prescribed for medicinal uses in Ayurveda, and both organ types are rich in withanolides (Misra et al., 2005; Lal et al., 2006). Here, we observed that the leaves could significantly incorporate [2-^{14}C]-acetate, unambiguously demonstrating that *at least* the leaf tissue of *Withania* possesses the complete pathway for withanolide biosynthesis. Furthermore, the leaf ontogeny *versus* dynamics of biogenesis and accumulation of withaferin A and withanone were examined in order to define the developmental productivity of this bioresource, and to discern the best leaf stage for investigating withanomics.

Leaf fresh and dry weights at designated physiological stages are given in Figure 2. Physiologically, the stages of very young, young, pre-mature, mature, and senescent could be construed as the phases of primordia, rapid expansion (~25% enlargement), attainment of 50% expansion, cessation of leaf expansion, and metabolic diminution/termination, respectively. Both FW and DW increased almost linearly up to maturity, but were then substantially reduced. Their respective moisture contents were 79.9, 80.8, 80.2, 84.0, and 75.6%, respectively, which implies a lack of marked alteration except for that decline during senescence.

The developmental profiles for accumulations of withaferin A and withanone suggest that the per-leaf contents of both increased almost linearly, reaching a maximum at maturity (Fig. 2). Notably, leaf senescence was accompanied by a tremendous decrease (~80%) in their contents. On a unit dry-mass basis, levels were maximal in the young leaf tissue (Fig. 3). The pre-mature and mature tissues retained significant concentrations of withaferin A (~82 and 66%,

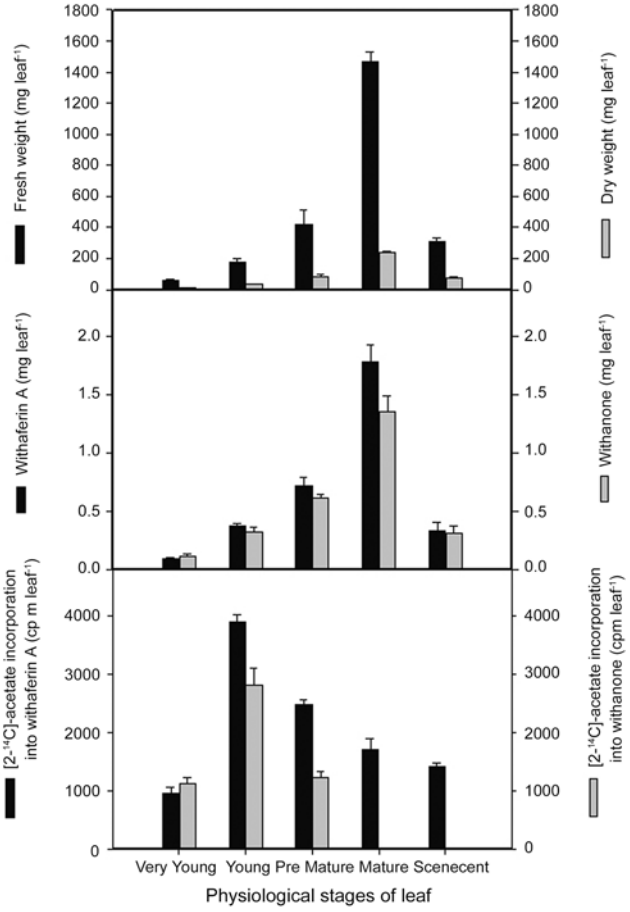

Figure 2. Fresh weight (FW), dry weight (DW), contents (mg $leaf^{-1}$) of withaferin A and withanone, and efficiency of biogenesis (^{14}C-acetate incorporation; cpm $leaf^{-1}$) in *W. somnifera* leaves at different physiological stages of ontogeny.

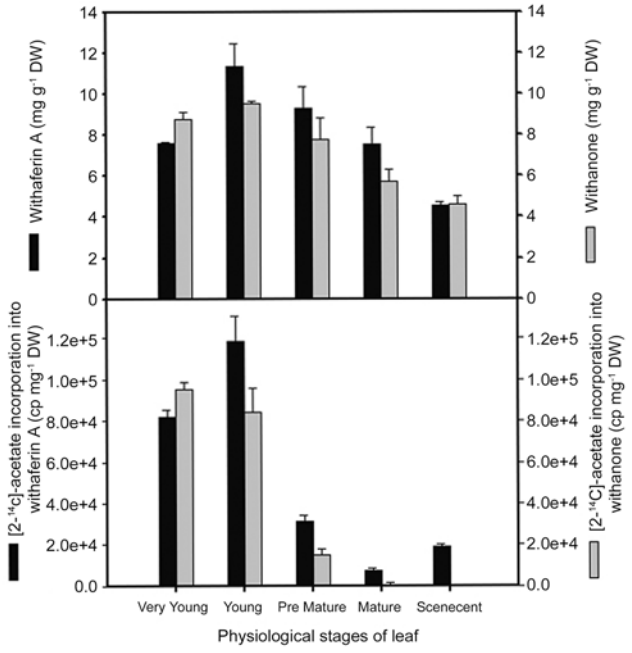

Figure 3. Tissue concentration (mg g^{-1}DW) of withaferin A and withanone, and efficiency of biogenesis (^{14}C-acetate incorporation; cpm g^{-1} DW), in *W. somnifera* leaves at different physiological stages of ontogeny.

respectively) and withanone (82 and 60%, respectively). In comparison, senescence was accompanied by a large decline in concentrations (>60 and 50% for withaferin A and withanone, respectively). We also compared the ontogenic profile of withanolide accumulation with biogenetic capacity, which was measured in terms of the level of radio-labeled incorporation of [2-^{14}C]-acetate. Withanolide biogenesis was quite significant, even in the primordial (very young) leaf, but the capacity for biosynthesis of withaferin A and withanone was maximal in the young leaf (Fig. 2). Production declined by the pre-mature stage (about 64 and 43% for withaferin A and withanone, respectively, on a per-leaf basis), but was still quite substantial (Fig. 2). Likewise, on a unit-weight basis (Fig. 3), radio-labeled incorporation was highest in the young leaf before slowing considerably thereafter. Figure 4 provides a comparative view of the radio-chromatograms for withanolide extracts when leaves at different ontogenic stages were fed with the radio-labeled precursor. The relatively diminutive peaks of radioactive withaferin A and withanone synthesized from the exogenously supplied precursor beyond pre-maturity demonstrates the meager biogenetic potential of that tissue during those later stages.

The ontogenic patterns of biogenesis and accumulation of withanolides are interesting from the perspectives of phyto-functionality, ecology, and phytopharmacology. Our results clearly suggest that the process gets set *ab initio* during leaf development in *W. somnifera*, and is maximized in the young leaf, i.e., long before this growth ceases. This leads to the attainment of maximum accumulations by the time of leaf maturity (full expansion). The degenerative (senescent) phase of ontogeny seems to be a catabolic *fait accompli*, perhaps for the relocation/mobilization of withanolide carbon from eco/defense functioning in terminal-stage tissue. Keeping in view a putative defense role for secondary metabolites, a number of hypotheses try to explain the scheduling of their production by the foliage in relation to growth. For example, the optimal defense theory considers the risk of attack/damage in the tissue *versus* the costs of biosynthesis. In contrast, various resource-based theories assume that this biosynthesis is constrained by the external availability of resources and an internal trade-off in allocations between growth and defense (Riipi et al., 2002). A growing leaf can be a good model for assessing resource availability as well as the need for growth/differentiation and defense from the standpoint of competition or compromise. Rapidly growing (very young to young) leaves are

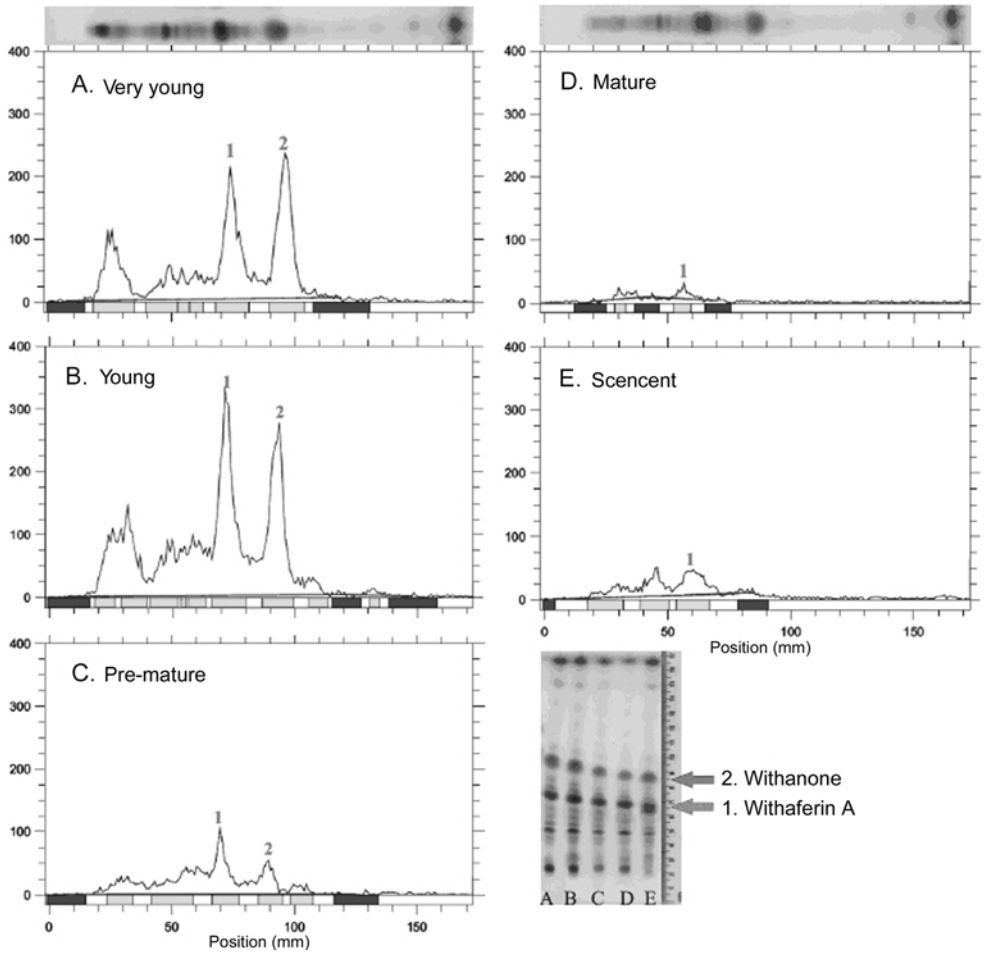

Figure 4. Radio-chromatograms demonstrating incorporation of [^{14}C] from radio-labeled precursor, [2-^{14}C]-acetate, into withaferin A and withanone at specified ontogenic stages. Figure also shows representative TLC profile of leaf withanolides at these stages. Identities of radioactivity peaks were ascertained by matching peak position with chromo-developed spots of withaferin A and withanone on the same plate, after radiometric scanning.

nearly carbon-heterotrophic or marginally autotrophic, and are anabolically most active for expansion and development, i.e., periods when they demand the most predominant primary metabolism. Nevertheless, the observed maximal rates of biogenesis and accumulation of withanolides during early leaf growth implies a stringent ecological requirement for developmental resources to be shared substantially with withanolide biogenesis even during the most active stage. Although withanolides could be recruited for several other functions besides defense, their active biogenesis may be related to the fact that the youngest leaves also may be the most susceptible to adversities (biotic and abiotic) against which they must be protected.

This essentiality as an ontogenic aspect of secondary metabolism, rather than as a compromise in growth (via discounted resource allocation), has been corroborated through various ecological studies of risk *versus* defense. Their resultant observations have included the following: 1) essential oils are rapidly synthesized and accumulated during the most active growth phase of leaves from some aromatic plants (Singh et al., 1989; Sangwan et al., 2001); 2) tropane alkaloids (exported from the roots, the site of their biosynthesis) are significantly amassed in the very young leaves of *Duboisia myopoirodes* during active growth (Mishra and Sangwan, 1996; Mishra et al., 1998); 3) total phenolics content per leaf increases steadily during the most active phase of birch leaf development (Riipi et al., 2002); 4) the neogenesis of secondary metabolite secretory structures, e.g., trichomes with volatile oils, is most significant during the early phase of leaf growth (Shanker et al., 1999; Sangwan et al., 2001; Sharma et al., 2003); 5) the synthesis of some lower terpenoids is relatively precocious before the cessation of mesophyll differentiation (Gershenzon and Croteau, 1991; Moura et al., 2005); and, finally, 6) alkaloids accumulate early in flower development for species such as *Zeyheria montana* (Machado et al., 2006). In fact, phytogenic secondary metabolite profiles as modulated by environmental factors is an important issue requiring much more representative studies than those reported so far (Woo et al., 2004; Kim et al., 2006; Choi et al., 2007).

From a phytopharmacological perspective, mature leaves of *Withania* plants have traditionally been prescribed for certain health gains/therapeutic actions. This preference corresponded to the physiological stage at which withanolide levels were believed to be maximal and stabilized prior to a cessation or near-complete arrest of this metabolism at the onset of leaf senescence. Our results also suggest significant potential for the biotechnological modulation of withanolide production *in planta*.

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