



# *Withania somnifera*: Advances and Implementation of Molecular and Tissue Culture Techniques to Enhance Its Application

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*Withania somnifera*, commonly known as Ashwagandha an important medicinal plant largely used in Ayurvedic and indigenous medicine for over 3,000 years. Being a medicinal plant, dried powder, crude extract as well as purified metabolites of the plant has shown promising therapeutic properties. Withanolides are the principal metabolites, responsible for the medicinal properties of the plant. Availability and amount of particular withanolides differ with tissue type and chemotype and its importance leads to identification characterization of several genes/ enzymes related to withanolide biosynthetic pathway. The modulation in withanolides can be achieved by controlling the environmental conditions like, different tissue culture techniques, altered media compositions, use of elicitors, etc. Among all the *in vitro* techniques, hairy root culture proved its importance at industrial scale, which also gets benefits due to more accumulation (amount and number) of withanolides in roots tissues of *W. somnifera*. Use of media composition and elicitors further enhances the amount of withanolides in hairy roots. Another important modern day technique used for accumulation of desired secondary metabolites is modulating the gene expression by altering environmental conditions (use of different media composition, elicitors, etc.) or through genetic engineering. Knowing the significance of the gene and the key enzymatic step of the pathway, modulation in withanolide contents can be achieved upto required amount in therapeutic industry. To accomplish maximum productivity through genetic engineering different means of *Withania* transformation methods have been developed to obtain maximum transformation efficiency. These standardized transformation procedures have been used to overexpress/silence desired gene in *W. somnifera* to understand the outcome and succeed with enhanced metabolic production for the ultimate benefit of human race.

**Keywords:** *Withania somnifera*, Ashwagandha, metabolites, withanolides, tissue culture, differentiation, and transformation

## INTRODUCTION

*Withania somnifera* (Ashwagandha; Solanaceae family) is one of the most recognized and studied medicinal plants due to its wide distribution all around the world. *W. somnifera* has been used for over 3,000 years in indigenous medicine (Ayurvedic) system (Scartezzini and Speroni, 2000; Kumar and Kalonia, 2007; Tuli and Sangwan, 2009; Singh et al., 2015b). Several studies collectively provide metabolic insight of more than 200 primary and secondary metabolic components of *W. somnifera*. Significance of *Withania* in therapeutic world has been recognized due to maximum accumulation and diversified form of withanolide. All the identified variants of withanolides became interesting for researchers due to their beneficial effects for human body (Figure 1A; Kumar et al., 2007; Kulkarni and Dhir, 2008; Sharada et al., 2008; Mirjalili et al., 2009; Singh et al., 2010; Dar et al., 2015).

Biosynthesis of metabolites could be improved effectively through genetic engineering, which requires full information of all the genes/enzymes involved in biosynthetic pathway. Using the limited reports available on genes as well as enzymes of *W. somnifera*, researchers have proposed possible metabolic pathway for the synthesis of different withanolides (Figure 1B; Senthil et al., 2010; Dhar et al., 2013; Sabir et al., 2013). Genes, enzymes as well as metabolites of respective metabolic pathway show differential pattern of expression according to the plant part, age, season, and other environmental factors. Optimization of various tissue culture techniques become very important to explore *W. somnifera* at different aspects, as plants obtained from fields are not enough for all *in vitro* studies. Therefore, efficient tissue culture techniques like, micropropagation, regeneration, organogenesis, hairy root production, etc. have been established. Also, development of transgenic plants has been considered as the most economical way to improve the yield of therapeutic metabolites on large scale.

Present review recognizes the importance of *W. somnifera* and discuss in detail genes/enzymes involved in the biosynthesis of secondary metabolites. The review also includes the significance of *in vitro* techniques in order to modulate the productivity of *W. somnifera* according to the desired final product. Suitable combinations of these findings create a very cooperative setting to modulate expression profile of various genes using different circumstances, results in synthesis of various secondary metabolites of *W. somnifera*.

## PROPOSED PATHWAYS FOR BIOSYNTHESIS OF WITHANOLIDES: MEDICINAL COMPONENT OF *W. SOMNIFERA*

Withanolide biosynthesis involves the key upstream metabolic step of isoprenogenesis using isoprenoid as precursor. Isoprenogenesis is known to proceed through

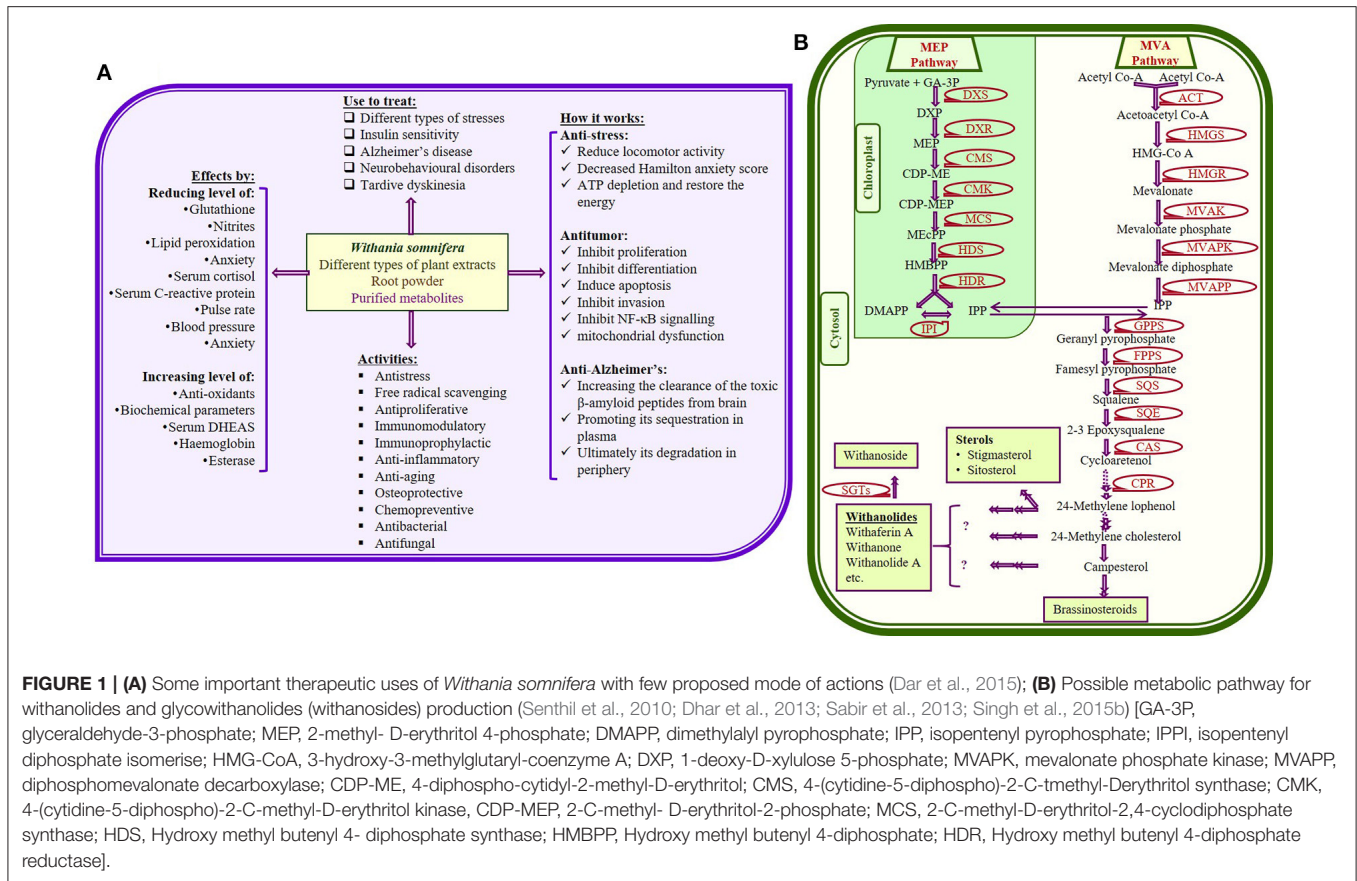
two different independent pathways; mevalonic acid (MVA) and methylerythritol phosphate (MEP; also called deoxyxylulose pathway, DOXP) pathway (Chaurasiya et al., 2007; Sangwan et al., 2007). These pathways occur in cytosol and plastid, respectively and ultimately synthesizes the 30 carbon compound (triterpenoids), 24-methylene cholesterol (Figure 1B). Till date, complete information of whole withanolide biosynthesis pathway is not available. However, combination of several studies provide an overview of pathway illustrating several enzymatic steps (Mirjalili et al., 2009; Senthil et al., 2010; Chaurasiya et al., 2012; Gupta et al., 2013a,b, 2015; Dhar et al., 2015). Enzymatic steps of MVA and MEP pathways has been prescribed through the first transcriptome analysis of the plant (Senthil et al., 2010), which keeps improving with advancement in techniques (Gupta et al., 2013b, 2015; Senthil et al., 2015). These analyses reveal numbers of tissue specific unique sequences, differentially expressed genes related to biosynthesis of secondary metabolites.

## Genes Involved in Biosynthesis of Withanolides

Genes involved in biosynthesis of withanolides are  $\Delta$ 14-sterol reductase (EC 1.3.1.70), 1-deoxy-D-xylulose-5-phosphate reducto-isomerase/reductase (DXR; EC 1.1.1.267), 1-deoxy-D-xylulose-5-phosphate synthase (DXS; EC 2.2.1.7), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MEcPP synthase, IspF, EC 4.6.1.12), 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (EC 2.7.7.60), 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGR; EC 1.1.1.34), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (EC 2.7.1.148), 4-hydroxy-3-methylbut-2-enyldiphosphate reductase (EC 1.17.1.2), 4-hydroxy-3-methylbut-2-enyldiphosphate synthase (HMB-PPS, IspG, EC 1.17.7.1), acetyl-CoA acetyltransferase (ACT, EC 2.3.1.9), C-5-sterol desaturase (C5SD, EC 1.14.19.20), cycloartenol C-24 methyltransferase (EC 2.1.1.142), cycloartenol synthase (CAS; EC 5.4.99.8), cycloecalenol cycloisomerase (EC 5.5.1.9), cytochrome-P450s reductase (CPR, EC 1.6.2.4), farnesyl diphosphate synthase (FPPS, EC 2.5.1.10), geranyl diphosphate synthase (GPPS, EC 2.5.1.1), geranyl-geranyl diphosphate synthase (GGPPS, EC 2.5.1.29), glycosyltransferases (GT, EC 2.4.-), hydroxymethyl glutaryl-CoA synthase (HMGS, EC 2.3.3.10), isopentenyl diphosphate isomerase (IPPI, EC 5.3.3.2), methyltransferase (MT, EC 2.1.1.-), mevalonate diphosphosphate decarboxylase (EC 4.1.1.33), mevalonate kinase (MVAK, EC 2.7.1.36), obtusifoliol 14-demethylase (EC 1.14.13.70), phosphomevalonate kinase (EC 2.7.4.2), squalene synthase (SQS, EC 2.5.1.21), squalene monooxygenase/epoxidase (SQE, 1.14.14.17), sterol  $\Delta$ 7 reductase (DWF, EC 1.3.1.21), etc. (Senthil et al., 2010, 2015; Gupta et al., 2013b, 2015).

To understand the interactions of various molecular network in entirety, Dhar et al. (2015) and Singh et al. (2015b) summarized the available information of some *in vitro* studies with respect to regulation of pathway genes required for withanolide accumulation.

**Abbreviations:** SA, salicylic acid; MeJA, methyl jasmonate; MI, mechanical injury; ABA, abscisic acid; JA, jasmonic acid; *Ws*, *Withania somnifera*; GA3, gibberellic acid; YE, yeast extract.



**FIGURE 1 | (A)** Some important therapeutic uses of *Withania somnifera* with few proposed mode of actions (Dar et al., 2015); **(B)** Possible metabolic pathway for withanolides and glycowithanolides (withanosides) production (Senthil et al., 2010; Dhar et al., 2013; Sabir et al., 2013; Singh et al., 2015b) [GA-3P, glyceraldehyde-3-phosphate; MEP, 2-methyl- D-erythritol 4-phosphate; DMAPP, dimethylalyl pyrophosphate; IPP, isopentenyl pyrophosphate; IPPI, isopentenyl diphosphate isomerase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; DXP, 1-deoxy-D-xylulose 5-phosphate; MVAPK, mevalonate phosphate kinase; MVAPP, diphosphomevalonate decarboxylase; CDP-ME, 4-diphospho-cytidyl-2-methyl-D-erythritol; CMS, 4-(cytidine-5-diphospho)-2-C-tmethyl-D-erythritol synthase; CMK, 4-(cytidine-5-diphospho)-2-C-methyl-D-erythritol kinase, CDP-MEP, 2-C-methyl- D-erythritol-2-phosphate; MCS, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; HDS, Hydroxy methyl butenyl 4- diphosphate synthase; HMBPP, Hydroxy methyl butenyl 4-diphosphate; HDR, Hydroxy methyl butenyl 4-diphosphate reductase].

## Few Important Catalytic Conversions of Proposed Pathways

Among a number of enzymes, SQS and SQE are considered as an important enzymes in the biosynthesis of triterpenoids. Considering this, Gupta et al. (2012) characterize isoforms of SQS gene, while, Razdan et al. (2013) perform characterization and promoter analysis of SQE gene from *W. somnifera*. To confirm the functional activity, both studies also involved the cloning, expression and purification of gens/enzymes in *E. coli*. Genes encoding DXS, DXR and HMGR enzymes expressed their importance by catalyzing the key regulatory step of the isoprenoid biosynthesis. These genes revealed tissue specific, chemotype specific and modulated expression while exposed to SA, MeJA, as well as MI (Akhtar et al., 2013; Gupta et al., 2013c).

Few members of sterol-GT (*SGT*) gene family of *W. somnifera*, have been recognized and characterized (Sharma et al., 2007; Madina et al., 2007a,b; Chaturvedi et al., 2012). *SGTs* are responsible for diversified glycosylation of sterols (including withanolides). The identified *SGTs* expressed different level of expression in different tissues as well as under different stress conditions, to prove their physiological importance (Sharma et al., 2007; Chaturvedi et al., 2011, 2012). Purified *SGTs* showed broad substrate specificity for sugar acceptor but not for the sugar donor (Madina et al., 2007a,b). Similar pattern of glycosylation was observed by Singh et al. (2013)

during functional characterization of flavonoid-GT gene from *W. somnifera*.

## Variation in Gene Expression Pattern According to Tissue and Stress Conditions

Relation among few pathway genes, withanolides accumulation with morphogenic transition has been studied by Sabir et al. (2013). *In vitro* tissues belongs to different stages of organogenesis (rhizogenesis and shoot organogenesis) were used for the experiment. Accumulation of major withanolides and expression of HMGR, FPP synthase (FPPS), SQS, SQE, cycloartenol synthase (CAS), GTs were analyzed at different morphogenic transition states.

Detailed study on four-CYP450 has been performed by Srivastava et al. (2015) to illustrate involvement of these enzyme in some specialized secondary metabolite (withanolides). The expression profiles of these CYPs showed chemotype-specific and tissue-specific variation, as well as variation in response to physiological and developmental factors. To expand the understanding of expression of genes in relation to withanolide biosynthetic pathway, Pal et al. (2016) perform experiments with different concentrations of fertilizers on fresh twigs of *W. somnifera*. Treated twigs related to highest accumulation of withaferin-A has been selected to analyse expression pattern of CYPs, allene oxide cyclases (AOCs) and few other pathway related genes.

## TISSUE CULTURE STUDIES ON WITHANIA SOMNIFERA

### Seed Germination in *W. somnifera*

Numerous, campylotropous, whitish, disk shaped seeds are found inside red or orange colored fruit (berry) of *W. somnifera*. Earlier reports mentioned high dormancy with poor seed viability (Khanna et al., 2013; Viji et al., 2013), also seeds of *W. somnifera* showed low and erratic germination with heterogeneous seedlings (Vashistha et al., 2010) having higher mortality rate of seedlings under field conditions (Khanna et al., 2013). The problems with seed germination of *W. somnifera* (*in vitro* and in field) guided the researchers toward finding of simple techniques with optimized conditions, in order to get faster and more germination rate. These conditions include nutrient medium, light conditions and condition of seeds, etc. These findings will help nursery workers and poor farmers interested in developing mass planting stock.

Soaking of seeds in water, diluted sodium hypochlorite, nitrate solutions (of potassium, ammonium, cobalt, sodium, calcium and zinc), has been suggested to soften the hard seed coat of *W. somnifera* (Kattimani and Reddy, 2001; Vashistha et al., 2010). Improved germination has been observed at  $25 \pm 2^\circ\text{C}$  and 16-h-light/8-h-dark photoperiod with the light intensity of 3,000 lux (Kambizi et al., 2006; Khanna et al., 2013; Viji et al., 2013). In addition to these conditions, incision on seed coat and few pre-incubation conditions (dark or  $15^\circ\text{C}$ ) increases the germination percentage (Pandey et al., 2013; Viji et al., 2013; Kumar et al., 2016).

### Regeneration and Multiplication of *W. somnifera*

Seedlings, embryos, cotyledon, epicotyl, hypocotyl, petiole, leaves, nodes, internodes, stem, shoot tips and roots have been used in different experiments for callus induction, adventitious root induction, regeneration, differentiation, flower induction, and fruit setting (Sharada et al., 2008; Supe et al., 2011; Singh et al., 2017). Composition of gelling matrix was standardized for encapsulation of shoot tips of *W. somnifera* along with optimization of media composition (or soilrite) for conversion of encapsulated shoot tips into plantlets (Singh et al., 2006). Most studies with optimized *in vitro* tissue culture conditions of *W. somnifera* have been briefly summarized recently by Singh et al. (2017).

## ACCUMULATION OF WITHANOLIDES IN DIFFERENT TYPES OF *IN VITRO* CULTURE

The ultimate goal of different studies on *W. somnifera* is to provide maximum and better plant material for therapeutic purpose. These involves standardization of phytochemical analysis of different types of tissues obtained from different region and accession of *W. somnifera*, for accumulation of therapeutic metabolites (Table 1). On the basis of difference in available withanolides, *W. somnifera* has been divided into various chemotypes (accessions). Differences in chemo-profile of some

selected chemotypes have been documented in several studies (Dhar et al., 2006; Kumar et al., 2007; Scartezzini et al., 2007; Bhatia et al., 2013).

Variation persist in accumulation of withanolides due to plant parts, developmental stages (Praveen and Murthy, 2010; Dhar et al., 2013), plant part obtained from different types of cultures (Sharada et al., 2007; Singh et al., 2017) of *W. somnifera*. These studies establish relationship between morphology/condition of plant tissue and withanolide contents. Sivanandhan et al., 2012a,b, 2013b,c, 2014a,b, 2015a; Singh et al., 2017) used *in vitro* grown plants in different studies to develop adventitious roots, multiple shoots, shoot suspension culture, cell suspension culture, flowers, and fruits using different growth conditions. These developed tissues were harvested to extract different combinations of withanolides.

Based on different studies, Singh et al. (2017) summarized effects of *in vitro* conditions on accumulation of withanolides. These studies involving organ and callus culture, cell suspension culture and *Agrobacterium tumefaciens* as well as *A. rhizogene* mediated transformation. Different conditions of these techniques resulted in modulated accumulation of different withanolides, some of which related to modulated gene expression pattern.

### Hairy Root Culture of *W. somnifera* and Withanolide Accumulation

Hairy root cultures are a promising approach of bioprocess engineering for large scale production of valuable plant secondary metabolites. There are several reports available in order to modulate quantity of withanolides in hairy roots culture using *A. rhizogenes* mediated transformation (Pawar and Maheshwari, 2004; Bandyopadhyay et al., 2007; Murthy et al., 2008; Saravanakumar et al., 2012; Sivanandhan et al., 2013a, 2015b). It has been reported that different factors like carbohydrates (Doma et al., 2012), inorganic supplements (Praveen and Murthy, 2013), seaweed extracts (*Gracilaria edulis* and *Sargassum wightii*; Sivanandhan et al., 2015b), hormones, elicitation (like, chitosan, JA, SA; Chaudhuri et al., 2009; Doma et al., 2012; Sivanandhan et al., 2013a), etc. modulate biogenesis of withanolides in hairy root cultures.

Difference in hairy root emergence was observed illustrating resistance or susceptibility of *W. somnifera* toward different strains of *A. rhizogenes* (Pawar and Maheshwari, 2004; Bandyopadhyay et al., 2007; Saravanakumar et al., 2012) as well as transformation efficiency of different explants used for the experiment (Murthy et al., 2008; Saravanakumar et al., 2012). Leaves proved to be more appropriate for infection by different strains of *A. rhizogene*, since used as explant in various studies (Ray et al., 1996; Bandyopadhyay et al., 2007; Chaudhuri et al., 2009; Doma et al., 2012; Saravanakumar et al., 2012; Praveen and Murthy, 2013; Sil et al., 2015; Thilip et al., 2015). Recently, Pandey et al. (2015) induced hairy root from leaf explants of *W. somnifera* expressing *sterol glucosyltransferase* gene (clone-4) using *A. rhizogenes*. The transgenic hairy roots were observed to accumulate higher amount of withanolide-A when subjected to elicitation (salicylic acid and methyl jasmonate).

**TABLE 1** | Different conditions/situation in order to accumulate therapeutically important metabolites of *W. somnifera*.

	Condition	Plant Part	Special treatment/condition/method/identification	Metabolite extracted	References
Standardization/ Identification of metabolic-analytical technique/ metabolite	<i>In situ</i>	Root; stem; leaf	HPLC for determination of withanolides	WS-1; WS-5	Ganzera et al., 2003
		Whole plant	cholinesterase inhibiting withanolides	2-new; 4- known withanolides	Choudhary et al., 2004
		Leaves	Sulfated and oxygenated withanolides	4-new; 6-known withanolides	Misra et al., 2005
		Dried roots/ leaves	HPLC and AFLP findings to relate different (15) accessions	WS-1; WS-2; WS-3; WS-7; WS-9; WSs; PG	Dhar et al., 2006
		Roots	Rare dimeric withanolide (ashwagandhanolide) Two new withanolides (TLC; NMR)	WS-1; WS-3; WS-7; WS-8; WS-14 2-new and 7-known withanolides	Subbaraju et al., 2006 Misra et al., 2008
		Leaves, roots	More reliable HPLC to determine broad range of withanolides	9- withanolides	Chaurasiya et al., 2008
		Various genotypes	HPTLC for determination of withanolides	WS-1; WS-3; WS-10	Srivastava et al., 2008
		Leaves, roots	NMR and HPLC and GC-MS for metabolic fingerprinting	48 to 62 primary/ secondary metabolite	Chatterjee et al., 2010
		Whole plant/ plant parts	Distribution in various organs	WS-3	Praveen et al., 2010
		Roots, fruits, leaves	Phenolic acids	5-phenolics; 3-flavonoids; few unknown	Alam et al., 2011
		Leaves, roots	HR-MAS-NMR to establish metabolic mapping (4 chemotypes)	41 metabolites	Bharti et al., 2011
		Leaves, stems, roots	Metabolomic characterization (NMR) from different (6) regions	Primary and secondary metabolites	Namdeo et al., 2011
		Roots	Different species	21 bioactive compounds	Kumar et al., 2011
		Metabolic/ phytochemical profiling	<i>In situ</i>	Fruits	Developmental stages of fruit (NMR; COSYDQF; TOCSY; HSQC) Fruits (LC-HRMS and LC-MS/MS) Chemotype (4) variations (GC-MS and NMR)
Leaves, roots	Clustering of accessions (25) based on phenotypic and chemotypic analysis			WS-1; WS-2; WS-3	Kumar et al., 2007
	Relation between transcript and metabolic profile in two morpho-chemovariant accessions			WS-1; WS-2; WS-3	Dhar et al., 2013
Different plant parts	Growth dependent variation in few metabolites (2 cultivars)			WS-1; WS-2; WS-3; squalene	Dhar et al., 2016
Media/soil/elicitor treatment/ variation	<i>In vitro; In situ</i>	Leaves	Nitsch and Nitsch-(NN) media + BAP + IBA	WS-1	Furmanowa et al., 2001
		Parts of seedlings	MS/ B5 basal media + (different combinations of plant hormones)	WS-1; WS-2; WS-3; WS-4; WS-6	Sharada et al., 2007
		Leaves, stem, roots	MS + BAP, IAA	WS-1; WS-3; WS-10	Dewir et al., 2010
	<i>In vitro</i>	Leaves, roots, seedling	Sandy loam soil; MS	WS-1	Johny et al., 2015
		Multiple shoots, teratoma	MS + BAP + Kinetin	WS-1; WS-3	Sangwan et al., 2007
		Multiple shoots	MS + BAP+/ IAA+/ IBA+/ NAA+/ 2,4-D	Glycowithanolides; withanolides	Ahuja et al., 2009

(Continued)

**TABLE 1** | Continued

Condition	Plant Part	Special treatment/condition/method/identification	Metabolite extracted	References
	Adventitious roots	MS + IBA + IAA	WS-3	Wasnik et al., 2009
		MS + 2,4-D/ IAA/ IBA/ NAA; B5 NN; N6	WS-3	Praveen and Murthy, 2010
	Adventitious roots from semi-friable callus of leaves	MS + 2,4-D + kinetin, MS + IBA + IAA	WS-1; WS-2; WS-3; WS-4; WS-10; WS-12; WS-13	Sivanandhan et al., 2012a
		MS + 2,4-D + kinetin, MS + IBA + NAA, Elicitors	WS-1; WS-3; WS-4; WS-12; WS-13	Sivanandhan et al., 2012b
	Plantlet	Hoagland + MeJA; SA	WS-1; WS-3	Rana et al., 2013
	Callus culture	MS + 2,4 D + kinetin	WS-1; WS-3	Chakraborty et al., 2013
	Adventitious root culture	MS + sucrose + IBA; different concentrations/ types of sugars; different pH	WS-3	Murthy and Praveen, 2013
	Cell suspension culture	MS + 40% <i>Gracilaria edulis</i> extract for 24 h	WS-1; WS-2; WS-3; WS-4	Sivanandhan et al., 2013b
	Multiple shoot cultures	MS + BAP + spermidine	WS-1; WS-2; WS-3; WS-4	Sivanandhan et al., 2013c
	Cell suspension culture	MS + kinetin + L-glutamine + sucrose + CaCl <sub>2</sub> / NH <sub>4</sub> Cl/ chitosan/ cholesterol/ MA/ squalene	WS-1; WS-2; WS-3; WS-4; WS-11; WS-12; WS-13	Sivanandhan et al., 2014a
	Shoot suspension culture	MS + <i>Gracilaria edulis</i> / <i>Sargassum wightii</i>	WS-1; WS-2; WS-3; WS-4	Sivanandhan et al., 2014b
	Flowers, fruits	MS + BAP + IAA, sucrose, L-glutamine, adenine sulfate, nitrates of NH <sub>4</sub> <sup>+</sup> , K <sup>+</sup> , Na <sup>+</sup>	WS-1; WS-2; WS-3; WS-4	Sivanandhan et al., 2015c
	<i>In situ</i>			
	Whole plant/ plant parts	Different vermicomposts	WS-1; WS-5	Raja and Veerakumari, 2013
	Leaves, roots	Organic composition of soil (bioaugmented organic + gypsum)	WS-1; WS-2; WS-3	Gupta et al., 2016
		SA; MeJA; MI (4 chemotypes)	WS-3	Gupta et al., 2011
	<i>In vitro</i>			
	<i>in vitro</i> culture	MeJA; GA3; YE	WS-1; WS-2; WS-3	Dhar et al., 2014
	Plantlet	MeJA; SA; GA3	WS-1; WS-2; WS-3	Rana et al., 2014
		MeJA; SA; 2,4-D; YE	WS-1; WS-3	Razdan et al., 2016
	<i>In vitro</i>			
	Hairy roots	LBA 9402 -pRi 1855; stem, leaves	WS-5	Ray et al., 1996
		MTCC 2364, MTCC532; stem, hypocotyle, leaves	Not mentioned	Pawar and Maheshwari, 2004
		LBA 9402; A4-pRiA4; leaves	WS-1; WS-5	Bandyopadhyay et al., 2007
		R1601- pRiA4b; different parts of seedling	WS-3	Murthy et al., 2008
		LBA9402/ A4 ± synthetic crypt gene; leaves	WS-1	Chaudhuri et al., 2009
		15834; leaves	WS-1; WS-3	Doma et al., 2012
		ATCC 15834, R1000, K599; leaves, petiole, internodes	WS-1	Saravanakumar et al., 2012
		R1601; cotyledonary leaves	WS-3	Praveen and Murthy, 2013
		R1000; leaves	WS-1; WS-2; WS-3	Sivanandhan et al., 2013a
		A4 ± SGT; leaves	WS-3	Pandey et al., 2015
		LBA9402 ± β-cryptogein gene; leaves	WS-1; WS-3	Sil et al., 2015

Strain-plasmid ± gene; tissue used for infection of *A. rhizogene*

(Continued)

TABLE 1 | Continued

	Condition	Plant Part	Special treatment/condition/method/identification	Metabolite extracted	References
<i>A. tumefaciens</i>			leaves	WS-1; WS-2; WS-3; WS-4	Sivanandhan et al., 2015b
			R1000, MTCC 2364, MTCC 532; leaves	WS-1; WS-3	Thilip et al., 2015
	<i>In vitro</i>	Teratoma	Nopaline:C58; octopine:Ach5, disarmed:LBA 4404; leaves	WS-1; WS-5	Ray and Jha, 1999
		Plantlet	GV3102 - pIG121Hm ±CAS gene/ pGSA1131 ±RNAi; leaves	Total withanolide	Mishra et al., 2016
	<i>In situ</i>	Leaves	GV3102- pBI121 ± WsSQS; leaves	WS-3	Grover et al., 2013
			Agroinfiltration (GV2260- pCAMBIA ±WsSQS) ± Microprojectile; leaves	WS-1; WS-2; WS-3; WS-4	Patel et al., 2014, 2015
			LBA4404/GV3102 - pFGC1008/pBI121/TRV2/ ±SGT gene/s; leaves	WS-1; WS-2; WS-3; WS-13	Saema et al., 2015, 2016; Singh et al., 2016
			LBA4404-pCAMBIA; leaves	WS-1; WS-2; WS-3; WS-4	Sivanandhan et al., 2015a

HPLC, high performance liquid chromatography; HPTLC, High performance thin layer chromatography; TLC, Thin layer chromatography; LC-MS, Liquid chromatography-mass spectrometry; NMR, Nuclear magnetic resonance; GC-MS, Gas chromatography mass spectrometry; FAB, Fast atom bombardment; HRMS, high resolution mass spectroscopy; COSYDQF, Two-dimensional (2D) phase-sensitive double quantum filtered correlation spectroscopy; TOCSY, Total correlation spectroscopy; HSQC,  $^1\text{H}$ - $^{13}\text{C}$  hetero nuclear single quantum correlation; HR-MAS-NMR, High Resolution Magic Angle Spinning-NMR; PCA, principal component analysis; HCA, hierarchical clustering analysis; MA, mevalonic acid; WS-1, withaferin A; WS-2, withanone; WS-3, Withanolide-A; WS-4, Withanolide-B; WS-5, Withanolide-D; WS-6, withanolide-E; WS-7, 27-hydroxywithanone; WS-8, 20-deoxywithanolide A; WS-9, withastramonolide; WS-10, 12-deoxywithastramonolide; WS-11, 12 deoxy withastramonolide; WS-12, withanoside-IV; WS-13, withanoside-V; WS-14, ashwagandhanolide; WSs, withanosides; PG, physagulin.

## A. tumefaciens Mediated Transformation and Its Application to Modulated Withanolide Biosynthesis

Numerous studies have helped in developing efficient methods for regeneration of *W. somnifera*, while only few reports are available for genetic transformation for this medicinal plant (Singh et al., 2017). Altered expressions of genes related to biosynthetic pathway, ultimately modulate quantity of plant secondary metabolites, which are of therapeutic importance. Ray and Jha (1999) infected leaves of *in vitro* grown plants (two genotypes) with wild type nopaline and octopine strains of *A. tumefaciens*. Different types of galls obtained due to different levels of virulence on the two genotypes. Two principle withanolides, withanolide D and withaferin A extracted from shooty teratoma cultures in higher amount, while, withanolide D alone was detected in rooty teratomas.

Pandey et al. (2010) performed successful *A. tumefaciens* mediated transformation with 1.67 efficiency using non-virulent strain. Leaves excised from 1-5-nodes of both *in situ* and *in vitro* grown 30 to 90-day-old seedlings of different accessions of *W. somnifera* were used for the study. LBA4404 containing the binary vector pIG121Hm showed more gus expression in second and third leaves of 75 day old seedlings. Leaf explants ultrasonicated at 47 KHz  $\pm$  6% for 10 s showed higher gus expression as compared to directly infected explants. The protocol was used to analyse *in vivo* enzymatic action of one SGT (*WsSGTL1*) of *W. somnifera* by Saema et al. (2015, 2016). RNAi silencing (Saema et al., 2015) as well as

overexpression (Saema et al., 2016) of *WsSGTL1* gene has been achieved in transgenic *W. somnifera*. As expected, reduction in the level of glycosylated products observed in transgenic with silenced *WsSGTL1* transcript. However, transgenics with overexpressing *WsSGTL1* showed early and enhanced growth, increased production of glycoesters, and glycowithanolides. These transgenics displayed biotic (*Spodoptera litura*) and abiotic (cold) stress tolerance as well as recovery after cold stress along with improved photosynthetic performance.

Patel et al. (2014) established *A. tumefaciens* mediated transformation, microprojectile bombardment and microprojectile bombardment assisted agroinfection. Apical and nodal explants obtained from multiplied culture after *in vitro* seed germination were used as explants. Modified vector pCAMBIA1301 used to confirm transgene expression. Pre-cultured explants were bombarded and immediately infected with *A. tumefaciens* for microprojectile bombardment assisted agroinfection. The transformation efficiencies achieved were 3.86, 3.62, and 8.71%, through *A. tumefaciens* mediated, microprojectile bombardment and with the combination of both, respectively.

The protocol (Patel et al., 2014) used to overexpress of *WsSQS* in *W. somnifera* (Patel et al., 2015). Grover et al. (2013) also transformed leaves and shoots of 4-6-weeks old seedlings with *A. tumefaciens* (GV3101 harboring pBI121H) containing *SQS* from *W. somnifera*. Transgenics were confirmed with enhance expression of *WsSQS* transcript and its enzymatic activity. Higher amount of different withanolides observed in transgenics to

prove the involvement of SQS with enhanced withanolide biosynthesis.

Nodal explants of 3-month old field grown plants were used to develop transformation protocol of *W. somnifera* by Sivanandhan et al. (2015a) with 10% efficiency. These explants were found as an ideal tissue for the production of higher number of multiple shoots, hence adopted for the production transgenics. Explants were precultured (6-days) to obtain maximum transformation efficiency using *Agrobacterium* suspension (strain LBA4404 harboring pCAMIBA2301) at 0.2 OD<sub>600</sub>. The transformation frequency increased significantly with wounded nodal explants subjected to a sonication (10 s, longer treatment affected the viability of regenerating cells). Maximum transformation efficiency of 10.6% was observed by Mishra et al. (2015) using nodal explants infected with *A. tumefaciens* strain GV3101 harboring pIG121Hm. Explants were pre-cultured on MS supplemented with TDZ for 2 days and infected with *Agrobacterium* (0.2 OD<sub>600</sub>) for 20 min and co-cultivated for 48 h at 22°C.

Virus induced gene silencing methods was adopted by several researchers to achieve fast and efficient characterization of genes related to withanolide biosynthesis. Using this technology, successful silencing of SQS (Singh et al., 2015a), *WsDWF-5* (Gupta et al., 2015) and three-*WsSGTLs* genes (Singh et al., 2016) were achieved in *W. somnifera*. *Ws-SQS* silenced plants revealed positive and negative effects on expression of upstream and downstream pathway genes, which ultimately reduces the accumulation of phytosterols. Silencing of *WsDWF-5* was observed with reduced accumulation of withanolide while, 3-*WsSGTLs* gene silencing found associated with enhanced level of different withanolides and reduced level of glycowithanolides. Increased expression of other upstream genes of withanolide biosynthesis pathway also relates with the suppressed activity of *WsSGTLs*, which leads to reduced tolerance toward biotic stress.

## CONCLUSION

*W. somnifera* is of great importance in lots of medical conditions due to abundance of diversified therapeutic secondary

## REFERENCES

- Ahuja, A., Kaur, D., Sharada, M., Kumar, A., Suri, K. A., and Dutt, P. (2009). Glycowithanolides accumulation in *in vitro* shoot cultures of Indian ginseng (*Withania somnifera* Dunal). *Nat. Prod. Commun.* 4, 479–482.
- Akhtar, N., Gupta, P., Sangwan, N. S., Sangwan, R. S., and Trivedi, P. K. (2013). Cloning and functional characterization of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from *Withania somnifera*: an important medicinal plant. *Protoplasma* 250, 613–622. doi: 10.1007/s00709-012-0450-2
- Alam, N., Hossain, M., Khalil, M. I., Moniruzzaman, M., Sulaiman, S. A., and Gan, S. H. (2011). High catechin concentrations detected in *Withania somnifera* (ashwagandha) by high performance liquid chromatography analysis. *BMC Complement. Altern. Med.* 11:65. doi: 10.1186/1472-6882-11-65
- Bandyopadhyay, M., Jha, S., and Tepfer, D. (2007). Changes in morphological phenotypes and withanolide composition of Ri-transformed roots of *Withania somnifera*. *Plant Cell Rep.* 26, 599–609. doi: 10.1007/s00299-006-0260-0

metabolites (withanolides). Significance of the plant leads researchers to identify the best suitable way to enhance plant productivity according to increasing demands. In order to complete the requirement, complete information related to metabolites, their biosynthesis (pathway genes/enzymes) and effect of different factors (composition of soil/media, elicitors etc.) is essential. Under the influence of significance of biosynthetic pathway, related genes/enzymes and external factors, this review describes all analyzed combinations of molecular and/or *in vitro* techniques that modifies the accumulation of desired metabolites. Several environmental factors like, soil/media composition, different types of elicitors/stresses etc. affect the withanolide biosynthesis by regulation of gene expression pattern. A lot of investigations included in this review that analyse withanolide accumulation through different types of *in vitro* culture techniques, like, micropropagation, organogenesis, hairy root production etc. Combination of optimized *in vitro* techniques and information of pathway gene/enzyme are of great interest these days. Such combination of genetic transformation and optimized *in vitro* conditions provides much better productivity in terms of metabolite accumulation. The present review describes that there are a lot more combinations available and need to utilize in order to achieve best productivity, to make it easily accessible for the progress of medical industry.

## AUTHOR CONTRIBUTIONS

VP and WA collected literature and wrote the manuscript, PM and NA critically evaluated the manuscript. All authors approved the manuscript.

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- Bharti, S. K., Bhatia, A., Tewari, S. K., Sidhu, O. P., and Roy, R. (2011). Application of HR-MAS NMR spectroscopy for studying chemotype variations of *Withania somnifera* (L.) Dunal. *Magn. Reson. Chem.* 49, 659–667. doi: 10.1002/mrc.2817
- Bhatia, A., Bharti, S. K., Tewari, S. K., Sidhu, O. P., and Roy, R. (2013). Metabolic profiling for studying chemotype variations in *Withania somnifera* (L.) Dunal fruits using GC-MS and NMR spectroscopy. *Phytochemistry* 93, 105–115. doi: 10.1016/j.phytochem.2013.03.013
- Bolledulla, J., Fitch, W., Vareed, S. K., and Nair, M. G. (2012). Identification of metabolites in *Withania somnifera* fruits by liquid chromatography and high-resolution mass spectrometry. *Rapid Commun. Mass Spectrom.* 26, 1277–1290. doi: 10.1002/rcm.6221
- Chakraborty, N., Banerjee, D., Ghosh, M., Pradhan, P., Gupta, N. S., Acharya, K., et al. (2013). Influence of plant growth regulators on callus mediated regeneration and secondary metabolites synthesis in *Withania somnifera* (L.) Dunal. *Physiol. Mol. Biol. Plants* 19, 117–125. doi: 10.1007/s12298-012-0146-2
- Chatterjee, S., Srivastava, S., Khalid, A., Singh, N., Sangwan, R. S., Sidhu, O. P., et al. (2010). Comprehensive metabolic fingerprinting of



- Withania somnifera* leaf and root extracts. *Phytochemistry* 71, 1085–1094. doi: 10.1016/j.phytochem.2010.04.001
- Chaturvedi, P., Mishra, M., Akhtar, N., Gupta, P., Mishra, P., and Tuli, R. (2012). Sterol glycosyltransferases-identification of members of gene family and their role in stress in *Withania somnifera*. *Mol. Biol. Rep.* 39, 9755–9764. doi: 10.1007/s11033-012-1841-3
- Chaturvedi, P., Misra, P., and Tuli, R. (2011). Sterol glycosyltransferases—the enzymes that modify sterols. *Appl. Biochem. Biotechnol.* 165, 47–68. doi: 10.1007/s12010-011-9232-0
- Chaudhuri, K., Das, S., Bandyopadhyay, M., Zalar, A., Kollmann, A., Jha, S., et al. (2009). Transgenic mimicry of pathogen attack stimulates growth and secondary metabolite accumulation. *Transgenic Res.* 18, 121–134. doi: 10.1007/s11248-008-9201-8
- Chaurasiya, N. D., Gupta, V. K., and Sangwan, R. S. (2007). Leaf ontogenic phase-related dynamics of withaferin A and withanone biogenesis in Ashwagandha (*Withania somnifera* Dunal.) – An important medicinal herb. *J. Plant Biol.* 50, 508–513. doi: 10.1007/BF03030691
- Chaurasiya, N. D., Sangwan, N. S., Sabir, F., Misra, L., and Sangwan, R. S. (2012). Withanolide biosynthesis recruits both mevalonate and DOXP pathways of isoprenogenesis in ashwagandha *Withania somnifera* L. (*Dunal*). *Plant Cell Rep.* 31, 1889–1897. doi: 10.1007/s00299-012-1302-4
- Chaurasiya, N. D., Uniyal, G. C., Lal, P., Misra, L., Sangwan, N. S., Tuli, R., et al. (2008). Analysis of withanolides in root and leaf of *Withania somnifera* by HPLC with photodiode array and evaporative light scattering detection. *Phytochem. Anal.* 19, 148–154. doi: 10.1002/pca.1029
- Choudhary, M. I., Yousuf, S., Nawaz, S. A., and Ahmed, S., Atta-ur-Rahman (2004). Cholinesterase inhibiting withanolides from *Withania somnifera*. *Chem. Pharm. Bull.* 52, 1358–1361. doi: 10.1248/cpb.52.1358
- Dar, N. J., Hamid, A., and Ahmad, M. (2015). Pharmacologic overview of *Withania somnifera*, the Indian Ginseng. *Cell. Mol. Life Sci.* 72, 4445–4460. doi: 10.1007/s00018-015-2012-1
- Dewir, Y. H., Chakrabarty, D., Lee, S. H., Hahn, E. J., and Paek, K. Y. (2010). Indirect regeneration of *Withania somnifera* and comparative analysis of withanolides in *in vitro* and greenhouse grown plants. *Biol. Plantarum.* 54, 357–360. doi: 10.1007/s10535-010-0063-6
- Dhar, N., Rana, S., Bhat, W. W., Razdan, S., Pandith, S. A., Khan, S., et al. (2013). Dynamics of withanolide biosynthesis in relation to temporal expression pattern of metabolic genes in *Withania somnifera* (L.) Dunal, a comparative study in two morpho-chemovariants. *Mol. Biol. Rep.* 40, 7007–7016. doi: 10.1007/s11033-013-2820-z
- Dhar, N., Rana, S., Razdan, S., Bhat, W. W., Hussain, A., Dhar, R. S., et al. (2014). Cloning and functional characterization of three branch point oxidosqualene cyclases from *Withania somnifera* (L.) dunal. *J. Biol. Chem.* 289, 17249–17267. doi: 10.1074/jbc.M114.571919
- Dhar, N., Razdan, S., Rana, S., Bhat, W. W., Vishwakarma, R., and Lattoo, S. K. (2015). Decade of molecular understanding of withanolide biosynthesis and *in vitro* studies in *Withania somnifera* (L.) Dunal, prospects and perspectives for pathway engineering. *Front. Plant Sci.* 6:1031. doi: 10.3389/fpls.2015.01031
- Dhar, R. S., Khan, S., Khajuria, R. K., and Bedi, Y. S. (2016). Dynamics of squalene content in different tissues of Ashwagandha (*Withania somnifera* L. Dunal) during its growth phases. *Ind. Crops Prod.* 84, 375–380. doi: 10.1016/j.indcrop.2016.02.003
- Dhar, R. S., Verma, V., Suri, K. A., Sangwan, R. S., Satti, N. K., Kumar, A., et al. (2006). Phytochemical and genetic analysis in selected chemotypes of *Withania somnifera*. *Phytochemistry* 67, 2269–2276. doi: 10.1016/j.phytochem.2006.07.014
- Doma, M., Abhayankar, G., Reddy, V. D., and Kavi Kishor, P. B. (2012). Carbohydrate and elicitor enhanced withanolide (withaferin A and withanolide A) accumulation in hairy root cultures of *Withania somnifera* (L.). *Indian J. Exp. Biol.* 50, 484–490.
- Furmanowa, M., Gajdzis-Kuls, D., Ruzkowska, J., Czarnocki, Z., Obidoska, G., Sadowska, A., et al. (2001). *In vitro* propagation of *Withania somnifera* and isolation of withanolides with immunosuppressive activity. *Planta Med.* 67, 146–149. doi: 10.1055/s-2001-11494
- Ganzera, M., Choudhary, M. I., and Khan, I. A. (2003). Quantitative HPLC analysis of withanolides in *Withania somnifera*. *Fitoterapia* 74, 68–76. doi: 10.1016/S0367-326X(02)00325-8
- Grover, A., Samuel, G., Bisaria, V. S., and Sundar, D. (2013). Enhanced withanolide production by overexpression of squalene synthase in *Withania somnifera*. *J. Biosci. Bioeng.* 115, 680–685. doi: 10.1016/j.jbiosc.2012.12.011
- Gupta, M., Srivastava, P. K., Shikha, N. A., and Tewari, S. K. (2016). Use of a bioaugmented organic soil amendment in combination with gypsum for *Withania somnifera* growth on sodic soil. *Pedosphere* 26, 299–309. doi: 10.1016/S1002-0160(15)60044-3
- Gupta, N., Sharma, P., Santosh Kumar, R. J., Vishwakarma, R. K., and Khan, B. M. (2012). Functional characterization and differential expression studies of squalene synthase from *Withania somnifera*. *Mol. Biol. Rep.* 39, 8803–8812. doi: 10.1007/s11033-012-1743-4
- Gupta, P., Agarwal, A. V., Akhtar, N., Sangwan, R. S., Singh, S. P., and Trivedi, P. K. (2013c). Cloning and characterization of 2-C-methyl-D-erythritol-4-phosphate pathway genes for isoprenoid biosynthesis from Indian ginseng, *Withania somnifera*. *Protoplasma* 250, 285–295. doi: 10.1007/s00709-012-0410-x
- Gupta, P., Agarwal, A. V., Akhtar, N., Sangwan, R. S., Singh, S. P., and Trivedi, P. K. (2013a). Cloning and characterization of 2-C-methyl-D-erythritol-4-phosphate pathway genes for isoprenoid biosynthesis from Indian ginseng, *Withania somnifera*. *Protoplasma* 250, 285–295. doi: 10.1007/s00709-012-0410-x
- Gupta, P., Akhtar, N., Tewari, S. K., Sangwan, R. S., and Trivedi, P. K. (2011). Differential expression of farnesyl diphosphate synthase gene from *Withania somnifera* in different chemotypes and in response to elicitors. *Plant Growth Regul.* 65, 93–100. doi: 10.1007/s10725-011-9578-x
- Gupta, P., Goel, R., Agarwal, A. V., Asif, M. H., Sangwan, N. S., Sangwan, R. S., et al. (2015). Comparative transcriptome analysis of different chemotypes elucidates withanolide biosynthesis pathway from medicinal plant *Withania somnifera*. *Sci. Rep.* 5:18611. doi: 10.1038/srep18611
- Gupta, P., Goel, R., Pathak, S., Srivastava, A., Singh, S. P., Sangwan, R. S., et al. (2013b). *De novo* assembly, functional annotation and comparative analysis of *Withania somnifera* leaf and root transcriptomes to identify putative genes involved in the withanolides biosynthesis. *PLoS ONE* 8:e62714. doi: 10.1371/journal.pone.0062714
- Johny, L., Conlan, X., Cahill, D., and Adholeya, A. (2015). *In vitro* and *in situ* screening systems for morphological and phytochemical analysis of *Withania somnifera* germplasm. *Plant Cell Tissue Organ Cult.* 120, 1191–1202. doi: 10.1007/s11240-014-0673-3
- Kambizi, L., Adebola, P. O., and Afolayan, A. J. (2006). Effects of temperature, pre-chilling and light on seed germination of *Withania somnifera*; a high value medicinal plant. *South Afr. J. Bot.* 72, 11–14. doi: 10.1016/j.sajb.2005.03.001
- Kattimani, K. N., and Reddy, Y. N. (2001). A note on influence of pre-sowing seed treatments on growth and root yield of Ashwagandha. *Karnataka J. Agri. Sci.* 14, 846–848.
- Khanna, P. K., Kumar, A., Chandra, R., and Verma, V. (2013). Germination behaviour of seeds of *Withania somnifera* (L.) Dunal, a high value medicinal plant. *Physiol. Mol. Biol. Plants* 19, 449–454. doi: 10.1007/s12298-013-0169-3
- Kulkarni, S. K., and Dhir, A. (2008). *Withania somnifera*: an Indian ginseng. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32, 1093–1105. doi: 10.1016/j.pnpbp.2007.09.011
- Kumar, A., and Kalonia, H. (2007). Protective effect of *Withania somnifera* Dunal on the behavioral and biochemical alterations in sleep-disturbed mice (Grid over water suspended method). *Indian J. Exp. Biol.* 45, 524–528.
- Kumar, A., Kaul, M. K., Bhan, M. K., Khanna, P. K., and Suri, K. A. (2007). Morphological and chemical variation in 25 collections of the Indian medicinal plant, *Withania somnifera* (L.) Dunal (Solanaceae). *Genet. Res. Crop Evol.* 54, 655–660. doi: 10.1007/s10722-006-9129-x
- Kumar, B., Yadav, R., Singh, S. C., and Singh, H. P. (2016). Seed germination behaviour of *Withania* spp. under different temperature regimes. *J. Crop Improv.* 30, 287–292. doi: 10.1080/15427528.2016.1151849
- Kumar, M. S., Vinothkumar, D., Kumar, S., Aslam, A., and Shajahan, A. (2011). The phytochemical constituents of *Withania somnifera* and *Withania obtusifolia* by GCMS analysis. *Int. J. Pharm. Phytochem. Res.* 3, 31–34.
- Madina, B. R., Sharma, L. K., Chaturvedi, P., Sangwan, R. S., and Tuli, R. (2007a). Purification and physico-kinetic characterization of 3beta-hydroxy specific sterol glucosyltransferase from *Withania somnifera* (L) and its stress response. *Biochim. Biophys. Acta* 1774, 392–402. doi: 10.1016/j.bbapap.2006.12.009
- Madina, B. R., Sharma, L. K., Chaturvedi, P., Sangwan, R. S., and Tuli, R. (2007b). Purification and characterization of a novel glucosyltransferase specific to 27beta-hydroxy steroidal lactones from *Withania somnifera*



- Sharma, L. K., Madina, B. R., Chaturvedi, P., Sangwan, R. S., and Tuli, R. (2007). Molecular cloning and characterization of one member of 3beta-hydroxy sterol glucosyltransferase gene family in *Withania somnifera*. *Arch. Biochem. Biophys.* 460, 48–55. doi: 10.1016/j.abb.2007.01.024
- Sidhu, O. P., Annarao, S., Chatterjee, S., Tuli, R., Roy, R., and Khetrpal, C. L. (2011). Metabolic alterations of *Withania somnifera* (L.) Dunal fruits at different developmental stages by NMR spectroscopy. *Phytochem. Anal.* 22, 492–502. doi: 10.1002/pca.1307
- Sil, B., Mukherjee, C., Jha, S., and Mitra, A. (2015). Metabolic shift from withasteroid formation to phenylpropanoid accumulation in cryptogem-cotransformed hairy roots of *Withania somnifera* (L.) Dunal. *Protoplasma* 252, 1097–1110. doi: 10.1007/s00709-014-0743-8
- Singh, A. K., Dwivedi, V., Rai, A., Pal, S., Reddy, S. G., Rao, D. K., et al. (2015a). Virus-induced gene silencing of *Withania somnifera* squalene synthase negatively regulates sterol and defence-related genes resulting in reduced withanolides and biotic stress tolerance. *Plant Biotechnol. J.* 13, 1287–1299. doi: 10.1111/pbi.12347
- Singh, A. K., Varshney, R., Sharma, M., Agarwal, S. S., and Bansal, K. C. (2006). Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. *J. Plant Physiol.* 163, 220–223. doi: 10.1016/j.jplph.2005.06.013
- Singh, G., Sharma, P. K., Dudhe, R., and Singh, S. (2010). Biological activities of *Withania somnifera*. *Ann. Biol. Res.* 1, 56–63.
- Singh, G., Tiwari, M., Singh, S. P., Singh, S., Trivedi, P. K., and Misra, P. (2016). Silencing of sterol glucosyltransferases modulates the withanolide biosynthesis and leads to compromised basal immunity of *Withania somnifera*. *Sci. Rep.* 6:25562. doi: 10.1038/srep25562
- Singh, P., Guleri, R., Angurala, A., Kaur, K., Kaur, K., Kaul, S. C., et al. (2017). Addressing challenges to enhance the bioactives of *Withania somnifera* through organ, tissue, and cell culture based approaches. *Biomed Res. Int.* 2017:3278494. doi: 10.1155/2017/3278494
- Singh, P., Guleri, R., Singh, V., Kaur, G., Kataria, H., Singh, B., et al. (2015b). Biotechnological interventions in *Withania somnifera* (L.) Dunal. *Biotechnol. Genet. Eng. Rev.* 19, 1–20. doi: 10.1080/02648725.2015.1020467
- Singh, S., Rishi, K., Vishwakarma, R. J., Kumar, S., Sonawane, P. D., and Ruby, K. B. M. (2013). Functional characterization of a flavonoid glucosyltransferase gene from *Withania somnifera* (Ashwagandha). *Appl. Biochem. Biotechnol.* 170, 729–741. doi: 10.1007/s12010-013-0230-2
- Sivanandhan, G., Arun, M., Mayavan, S., Rajesh, M., Jeyaraj, M., Dev, G. K., et al. (2012a). Optimization of elicitation conditions with methyl jasmonate and salicylic acid to improve the productivity of withanolides in the adventitious root culture of *Withania somnifera* (L.) Dunal. *Appl. Biochem. Biotechnol.* 168, 681–696. doi: 10.1007/s12010-012-9809-2
- Sivanandhan, G., Arun, M., Mayavan, S., Rajesh, M., Mariashibu, T. S., Manickavasagam, M., et al. (2012b). Chitosan enhances withanolides production in adventitious root cultures of *Withania somnifera* (L.) Dunal. *Ind. Crops Prod.* 37, 124–129. doi: 10.1016/j.indcrop.2011.11.022
- Sivanandhan, G., Arunachalam, C., Selvaraj, N., Sulaiman, A. A., Lim, Y. P., and Ganapathi, A. (2015b). Expression of important pathway genes involved in withanolides biosynthesis in hairy root culture of *Withania somnifera* upon treatment with *Gracilaria edulis* and *Sargassum wightii*. *Plant Physiol. Biochem.* 91, 61–64. doi: 10.1016/j.plaphy.2015.04.007
- Sivanandhan, G., Dev, G. K., Jeyaraj, M., Rajesh, M., Arjunan, A., Muthuselvam, M., et al. (2013a). Increased production of withanolide A, withanone, and withaferin A in hairy root cultures of *Withania somnifera* (L.) Dunal elicited with methyl jasmonate and salicylic acid. *Plant Cell Tissue Organ Cult.* 114, 121–129. doi: 10.1007/s11240-013-0297-z
- Sivanandhan, G., Dev, G. K., Jeyaraj, M., Rajesh, M., Muthuselvam, M., Selvaraj, N., et al. (2013b). A promising approach on biomass accumulation and withanolides production in cell suspension culture of *Withania somnifera* (L.) Dunal. *Protoplasma* 250, 885–898. doi: 10.1007/s00709-012-0471-x
- Sivanandhan, G., Kapil Dev, G., Theboral, J., Selvaraj, N., Ganapathi, A., and Manickavasagam, M. (2015a). Sonication, vacuum infiltration and thiol compounds enhance the *Agrobacterium*-mediated transformation frequency of *Withania somnifera* (L.) Dunal. *PLoS ONE* 10:e0124693. doi: 10.1371/journal.pone.0124693
- Sivanandhan, G., Rajesh, M., Arun, M., Jeyaraj, M., Dev, G. K., Arjunan, A., et al. (2013c). Effect of culture conditions, cytokinins, methyl jasmonate and salicylic acid on the biomass accumulation and production of withanolides in multiple shoot culture of *Withania somnifera* (L.) Dunal using liquid culture. *Acta Physiol. Plant* 35, 715–728. doi: 10.1007/s11738-012-1112-x
- Sivanandhan, G., Selvaraj, N., Ganapathi, A., and Manickavasagam, M. (2014a). Enhanced biosynthesis of withanolides by elicitation and precursor feeding in cell suspension culture of *Withania somnifera* (L.) Dunal in shake-flask culture and bioreactor. *PLoS ONE* 9:e104005. doi: 10.1371/journal.pone.0104005
- Sivanandhan, G., Selvaraj, N., Ganapathi, A., and Manickavasagam, M. (2014b). Improved production of withanolides in shoot suspension culture of *Withania somnifera* (L.) Dunal by seaweed extracts. *Plant Cell Tissue Organ Cult.* 119, 221–225. doi: 10.1007/s11240-014-0521-5
- Sivanandhan, G., Theboral, J., Dev, G. K., Selvaraj, N., Manickavasagam, M., and Ganapathi, A. (2015c). Effect of carbon and nitrogen sources on *in vitro* flower and fruit formation and withanolides production in *Withania somnifera* (L.) Dunal. *Indian J. Exp. Biol.* 53, 177–183.
- Srivastava, P., Tiwari, N., Yadav, A. K., Kumar, V., Shanker, K., Verma, R. K., et al. (2008). Simultaneous quantification of withanolides in *Withania somnifera* by a validated high-performance thin-layer chromatographic method. *J. AOAC Int.* 91, 1154–1161.
- Srivastava, S., Sangwan, R. S., Tripathi, S., Mishra, B., Narnoliya, L. K., Misra, L. N., et al. (2015). Light and auxin responsive cytochrome P450s from *Withania somnifera* Dunal: cloning, expression and molecular modelling of two pairs of homologue genes with differential regulation. *Protoplasma* 252, 1421–1437. doi: 10.1007/s00709-015-0766-9
- Subbaraju, G. V., Vanisree, M., Rao, C. V., Sivaramakrishna, C., Sridhar, P., Jayaprakasam, B., et al. (2006). Ashwagandhanolide, a bioactive dimeric thiowithanolide isolated from the roots of *Withania somnifera*. *J. Nat. Prod.* 69, 1790–1792. doi: 10.1021/np060147p
- Supe, U., Dhote, F., and Roymon, M. G. (2011). A review on micropropagation of *Withania somnifera*—A medicinal plant. *J. Agricult. Technol.* 7, 1475–1483.
- Thilip, C., Raju, C. S., Varutharaju, K., Aslam, A., and Shajahan, A. (2015). Improved *Agrobacterium rhizogenes*-mediated hairy root culture system of *Withania somnifera* (L.) Dunal using sonication and heat treatment. *3 Biotech* 5, 949–956. doi: 10.1007/s13205-015-0297-2
- Tuli, R., and Sangwan, R. S. (2009). *Ashwagandha (Withania somnifera): A model Indian Medicinal Plant (1st Edn.)*. New Delhi: CSIR-National Botanical Research Institute.
- Vashistha, R. K., Chaturvedi, A. K., Butola, J. S., and Nautiyal, M. C. (2010). Evaluation of emergence and vigour of Ashwagandha (*Withania somnifera* Dunal) seedlings under the influence of Sodium Hypochlorite (NaHClO). *Biol. Forum- An Intl. J.* 2, 30–34.
- Viji, M. O., Mathew, M. M., and Parvatham, R. (2013). Effects of light intensity and imbibition frequency of *in vivo* and *in vitro* propagated seeds of *Withania somnifera* (L.) Poshita on germination. *Int. J. Curr. Microbiol. App. Sci.* 2, 354–360.
- Wasnik, N. G., Muthusamy, M., Chellappan, S., Vaidhyanathan, V., Pulla, R., Senthil, K., et al. (2009). Establishment of *in vitro* root cultures and analysis of secondary metabolites in Indian Ginseng-*Withania somnifera*. *Korean J. Plant Res.* 22, 584–591.

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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