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 insights 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 withanolides. 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., 2015; 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 filed is not enough for all in vitro studies. Therefore, efficient tissue culture techniques like, micropropogation, 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 proceeds through two different independent pathways; mevalonic acid (MVA) and methylenerythritol 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 Δ14-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 1.17.1.1), 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), cycloecualenol 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 5.3.2.2), methyltransferase (MT, EC 2.1.1.), mevalonate diphosphate decarboxylase (EC 4.1.1.33), mevalonate kinase (MVAK, EC 2.7.1.36), obtusifoliol 14-demethylase (EC 1.4.13.70), phosphomevalonate kinase (EC 2.7.4.2), squalene synthase (SQS, EC 2.5.1.21), squalene monoxygenase/epoxidase (SQE, 1.14.14.17), sterol Δ7 reductase (DFW, 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.
Few Important Catalytic Conversions of Proposed Pathways

Among a number of enzymes, SQS and SQE are considered as 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 genes/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 ± 2°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°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 phychochemical 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 biogeneration 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; Philip 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 |
|-----------|------------|---------------------------------------------------|----------------------|------------|
| **In situ** | 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) | WS-1; WS-3; WS-7; WS-8; WS-14 | Subbaraju et al., 2006 |
| | Leaves, roots | More reliable HPLC to determine broad range of withanolides | 9-known 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 metabolites | 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 |
| | Fruits | Developmental stages of fruit (NMR; COSYDQF; TOCSY; HSQC) | 17 metabolites | Sidhu et al., 2011 |
| | | Fruits (LC-HRMS and LC-MS/MS) | 62 metabolites | Bolleddula et al., 2012 |
| | | Chemotype (4) variations (GC–MS and NMR) | 82 metabolites | Bhatia et al., 2013 |
| | 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**

| Condition | Plant Part | Special treatment/condition/method/identification | Metabolite extracted | References |
|-----------|------------|---------------------------------------------------|----------------------|------------|
| **In vitro; In situ** | 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 |
| | 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 |
| **In vitro** | Multiple shoots | MS + BAP+/ IAA+/ IBA+/ NAA+/ 2,4-D | Glycowithanolides; withanolides | Ahuja et al., 2009 |

(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/ BS NN; N6 | WS-3                                             |                      | Praveen and Murthy, 2010            |
| Adventitious roots   | 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., 2012            |
| from semi-friable    | WS-3                              |                                                   |                      |                                    |
| Callus of leaves     | MS + 2,4-D + kinetin, MS + IBA + NAA, Elicitors | WS-1; WS-2; WS-3; WS-4; WS-10; WS-12; WS-13        |                      |                                    |
| Plantlet             | Hoagland + MeJA; SA               | WS-1; WS-3                                       |                      | Rana et al., 2013                   |
| Adventitious root    | MS + sucrose + IBA; different     | WS-3                                             |                      | Murthy and Praveen, 2013            |
| culture              | concentrations/ types of sugars;  |                                                   |                      |                                    |
|                      | different pH                      |                                                   |                      |                                    |
| Cell suspension      | MS + 40% Gracilaria edulis extract| WS-1; WS-2; WS-3; WS-4                           |                      | Sivanandhan et al., 2013            |
| culture              | for 24 h                           |                                                   |                      |                                    |
| Multiple shoot       | MS + BAP + spermidine             | WS-1; WS-2; WS-3; WS-4                           |                      | Sivanandhan et al., 2013            |
| cultures             |                                    |                                                   |                      |                                    |
| Cell suspension      | MS + kinetin + L-glutamine +      | WS-1; WS-2; WS-3; WS-4; WS-11; WS-12; WS-13       |                      |                                    |
| culture              | sucrose + CaCl₂/ NH₄Cl/ chitosan/  |                                                   |                      |                                    |
|                      | cholesterol/ MA/ squalene         |                                                   |                      |                                    |
| Shoot suspension     | MS + Gracilaria edulis / Sargassum| WS-1; WS-2; WS-3; WS-4                           |                      | Sivanandhan et al., 2014            |
| culture              | wightii                           |                                                   |                      |                                    |
| Flowers, fruits      | MS + BAP + IAA, sucrose, L-glutamine, adenine sulfate, nitrates of NH₄⁺, K⁺, Na⁺ | WS-1; WS-2; WS-3; WS-4 |                      | Sivanandhan et al., 2014b           |
| Whole plant/ plant   | Different vermicomposts            | WS-1; WS-5                                       |                      | Raja and Veerakumari, 2013          |
| parts                |                                    |                                                   |                      |                                    |
| Leaves, roots        | Organic composition of soil (bioaugmented organic + gypsum) | WS-1; WS-2; WS-3 |                      | Gupta et al., 2016                  |
|                      | SA; MeJA; MI (4 chemotypes)       |                                                   |                      |                                    |
| Plantlet             | MeJA; GA3; YE                     | WS-1; WS-2; WS-3                                 |                      | Rana et al., 2014                   |
|                      | MeJA; SA; GA3                     | WS-1; WS-2; WS-3                                 |                      |                                    |
|                      | MeJA; SA; 2,4-D; YE               | WS-1; WS-3                                       |                      | Razdan et al., 2016                 |
| in vitro             |                                    |                                                   |                      |                                    |
| Hairy roots          | LBA 9402 -pRI 1855; stem, leaves  | WS-5                                             |                      | Ray et al., 1996                    |
|                      | MTCC 2364, MTCC532; stem,         | Not mentioned                                    |                      | Pawar and Maheshwari, 2004          |
|                      | hypocotylle, leaves               |                                                   |                      | Bandyopadhyay et al., 2007          |
|                      | LBA 9402; A4-pRI A4; leaves       | WS-1; WS-5                                       |                      |                                    |
|                      | R1601-pRI A4b; different parts of | WS-3                                             |                      | Murthy et al., 2008                 |
|                      | seedling                          |                                                   |                      |                                    |
|                      | LBA9402/ A4 ± synthetic crypt gene; leaves | WS-1 |                      | Chaudhuri et al., 2009              |
|                      | 15834; leaves                     | WS-1; WS-3                                       |                      |                                    |
|                      | ATCC 15834, R1000, K599; leaves,  | WS-1                                             |                      | Doma et al., 2012                   |
|                      | petiole, internodes               |                                                   |                      | 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;      | WS-1; WS-3                                       |                      | Sil et al., 2015                    |
|                      | leaves                            |                                                   |                      |                                    |

(Continued)
TABLE 1 | Continued

| Condition | Plant Part | Special treatment/condition/method/identification | Metabolite extracted | References |
|-----------|------------|---------------------------------------------------|----------------------|------------|
| In vitro  | Teratoma    | Nopaline:Cs58; octopine:Ach5, disarmed: LBA 4404; leaves | WS-1; WS-5           | Ray and Jha, 1999 |
|           | Plantlet    | GV3102- pGl121Hm ± CAS gene/ pGSA1131 ± RNAi; leaves | Total withanolide    | Mishra et al., 2016 |
| In situ   | Leaves      | GV3102- pBI121 ± WsQS; leaves                      | WS-3                 | Grover et al., 2013 |
|           |             | Agronifitration (GV2260- pCAMBIA ± WsQS) ± Microprojectile; leaves | WS-1; WS-2; WS-3; WS-13 | 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 spectrometry; COSYDQF, Two-dimensional (2D) phase-sensitive double quantum filtered correlation spectroscopy; TOCSY, Total correlation spectroscopy; HSQC, H–13C hetero nuclei 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, withafarin A; WS-2, withanine; WS-3, Withanolide-A; WS-4, Withanolide-B; WS-5, Withanolide-D; WS-6, withanone-E; WS-7, 27-hydroxywithanolide; WS-8, 20-deoxywithanolide A; WS-9, withaamanalide; WS-10, 12-deoxywithaamanalide; WS-11, 12 deoxy withanamalide; WS-12, withanolide IV; WS-13, withanosides; WS-14, ashwagandhanoids; 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 ± 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 WsSQS gene has been achieved in transgenic *W. somnifera*. As expected, reduction in the level of glycosylated products observed in transgenic with silenced WsSQS transcript. However, transgens with overexpressing WsSQS showed early and enhanced growth, increased production of glycerosterols, 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 enhanced 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 explant 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 pCAMBIA2301) at 0.2 OD₆₀₀. 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 OD₆₀₀) 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 affects on expression of upstream and downstream pathway genes, which ultimately reduces the accumulation of phytoestrogens. 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 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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