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Review Article

IN VITRO AND OMICS TECHNOLOGIES OPENS A NEW AVENUE FOR DECIPHERING WITHANOLIDE METABOLISM IN *WITHANIA SOMNIFERA*

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ABSTRACT

Withania somnifera, commonly known as Ashwagandha or winter cherry, is a principal medicinal plant that has been used in Ayurvedic and native medicine. In view of its varied therapeutic prospective, it has also been the subject of considerable modern scientific attention. Ashwagandha roots are an integral of over 200 formulations in Ayurveda, Siddha and Unani medicine, which are used in the treatment of various physiological disorders. The major chemical constituents of this plant, the withanolides, are a group of naturally occurring C28-steroidal lactones. It has been extensively investigated in terms of chemistry and bioactivity profiling. However, there exists only very little fragmentary evidence about the dynamics of withanolide biosynthesis. This review examines different *in vitro* approaches that had been carried out over past decade of years and newly developed omics technologies for the large scale production of withanolides as well as for the analysis of genes associated with withanolide biosynthesis.

Keywords: *Withania somnifera, Withanolides, in vitro cultures, Genomics, Transcriptomics, In silico, Proteomics, Metabolomics*

INTRODUCTION

Withania somnifera (L.) Dunal commonly known as Ashwagandha/ Indian ginseng/winter cherry, is one of the most esteemed medicinal plants used in Indian Ayurveda for over 3000 y [1]. It is used as herbal medicine in various forms (decoctions, infusions, ointments, powder and syrup) in different parts of the world [2, 3] for all age groups of patients without any side effects even during pregnancy.

The medical importance of *W. somnifera* is mainly because of the presence of steroidal lactones namely "withanolides" [4-8]. The pharmaceutically important compounds are withanolide A, withanolide B, withaferin A, and withanone (major constituents) and 12-deoxy withastramonolide, withanoside IV and withanoside V (minor constituents). Each withanolide is having a wide array of therapeutic values.

The annual requirement of dried plant material for withanolide production in India has been estimated to about 9,127 tons as against the annual production of about 5,905 tons [9]. A major bottleneck in the biosynthesis of withanolides depends on the plant's tissue type and growth conditions in natural habitats as the commercial products are entirely derived from field-grown plants [10]. This ultimately leads to difficulties in the compositional standardization of *Withania* formulations and its commercial exploitation.

To meet the current Ashwagandha worldwide market requirement, *in vitro* cultures could provide an alternative to field-grown plant for the production of therapeutically valuable compounds and thereby suggests that *in vitro* cultures can be applied for secondary metabolite production in cell/organ culture system [7]. As roots contain a number of therapeutically applicable withanolides, mass cultivation of roots *in vitro* will be an effective technique for the production of these secondary metabolites on a trade scale. Therefore, the development of a fast-growing root system would offer unique opportunities for producing root drugs in tissue culture without depending on field cultivation, which is prone to show variables [11].

A few attempts have already been made for in vitro root culture of W. somnifera. Several authors [12-15] induced direct rooting from leaf explants. Even though works on producing adventitious root cultures of W. somnifera with higher content of withanolides using different strategies are available, until a date, not more than one report is available on its mass cultivation. Hence, it is essential to develop an efficient protocol for mass cultivation of adventitious roots in a superior variety with the potential to be utilized in the production of withanolides applicable for large-scale high-throughput processing in the industry.

The metabolic constituents, particularly secondary metabolites are reported to differ with the variety, tissue type and sometimes with growth conditions [16]. Such variations often lead to poor reproducibility of promoting properties of various commercial Withania preparations [17,18]. This causes difficulties in the compositional standardization of herbal formulations and the commercial exploitation of this plant. A recent review [19] narrates cases where multi-component *W. somnifera* extracts showed better medicinal efficiency than the purified compounds. Hence, a comprehensive phytochemical fingerprinting needs to be carried out on the plant material to be used for health benefits [20-22]. The comprehensive chemical analysis is required not only to establish a correlation between complex chemical mixtures and molecular pharmacology but also to understand complex cellular processes and biochemical pathways via metabolite-to-gene network [23].

Although this plant has been well characterized in terms of phytochemical profiles as well as pharmaceutical activities, only very little information about the genes responsible for biosynthesis of these compounds is available to date. Recently, various biochemical and molecular studies have been commenced to elucidate the biosynthetic pathway for various withanolides in *W. somnifera* [6]. Putatively, withanolides (C-30) are synthesized via both mevalonate (MVA) and non-mevalonate-1-deoxy-D-xylulose 5-phosphate/2-Cmethyl-D-erythritol 4-phosphate (DOXP) pathways through cyclization of 2,3-oxidosqualene to cycloartenol; wherein 24-methylene cholesterol is the first branching point towards the biosynthesis of various withanosteroids.

Production of withanolides includes a series of desaturation, hydroxylation, epoxidations, cyclization, chain elongation, and glycosylation steps. In plants, MVA is the general precursor of variously identified isoprenoids, such as sterols, plant growth regulators, and terpenoids [24, 25]. It has been recently reported that, in *W. somnifera*, both MVA and DOXP pathway participate in the biosynthesis of the withanolide [26].

An attempt to engineer the efficient production of secondary metabolites requires an understanding of their biosynthetic pathway(s), and our present knowledge of withanolide biosynthesis is limited to only a few genes involved in the pathway. Over the past decade, many attempts have been made in tissue culture field to manifest the tissue-specific accumulation of the legendary withanolides. Very recently, Senthil *et al.* [27] reported first large-scale transcriptome profiling of *in vitro* tissues for *W. somnifera* and provides a comparative expression profiling of pathway genes involved in withanolide biosynthesis and their potential biological activity. This panorama propels us to write this review that presents a characteristic overview of studies done related to *in vitro* and molecular ideas engaged in *W. somnifera* to understand the regulation of withanolide production.

Why withanolides

Much of ashwagandha's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide A [28] of which, withaferin being the dominant metabolite of leaf tissue and withanolide A, reported to be accumulated more in root tissues [29] (fig. 1). The withanolides serve as important hormone precursors that can convert into human physiologic hormones as needed. According to the literature, Ashwagandha is thought to be amphoteric; i.e., it can help regulate important physiologic processes. The principle behind this theory is that when there is an excess of a certain hormone, the plant-based hormone precursor occupies cell membrane receptor sites so the actual hormone cannot attach and exert its effect. If the hormone level is low, the plant-based hormone exerts a small effect [30]. Withanolides have been researched in a variety of clinical examinations for their numerous therapeutic activities including cancer and immune functioning [31]. Withanolide B, withaferin A and with anyone also have remarkable activities in physiological and metabolic restoration, anti-arthritic, anti-aging, anti-cancer, cognitive function improved in geriatric states and recovery from neurodegenerative disorders [32]. Withanolide A is considered as a good candidate for neurodegenerative diseases and potentiating humoral and cell-mediated Th1 immunity [33-35].

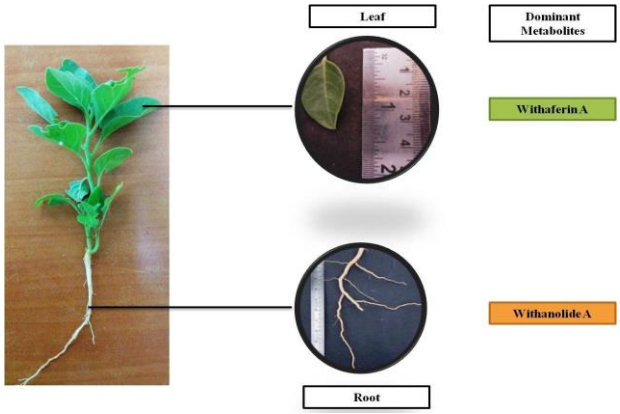

Fig. 1: Dominant metabolites among different organs of *Withania somnifera*

Withaferin A is reported to induce apoptosis through Reactive Oxygen Spices (ROS) generation mediating modulation of both intrinsic and extrinsic apoptosis signaling cascades together with abrogation of NF-kB functions [34], as an anti-inflammatory agent, to protect cardiovascular systems, as an anti-cancer agent, antioxidant and antiplatelet activity [36].

The protective effect of W. somnifera in epilepsy is considered to be through GABAergic modulation. The profound anticonvulsant activity of W. somnifera root extract as reported in various animal models is hypothesized to be through GABAA receptors [37]. In another study, the antiparkinson effects of W. somnifera extract was evaluated using 6-hydroxy dopamine (6-OHDA)-induced Parkinson's-like effect in rats [38]. Meena and Lakshmi [39] demonstrated the antiepileptic activity of leaf extracts of Ashwagandha. Anti-aging property of W. somnifera was studied by [40] and [41], who proved that W. somnifera root powder at a dosage of 3 grams daily for one year, significantly improve hemoglobin, red blood cell count, hair melanin, and seated statue. In another study conducted by [42] provided evidence that root and leaf extracts of W. somnifera significantly reduces experimental and biochemical indicators of stress without adverse effects in humans. The available scientific data thus support the conclusion that Ashwagandha is a real potent regenerative tonic due to its multiple pharmacological actions like anti-stress, neuroprotective, antitumor, anti-arthritic, analgesic and anti-inflammatory, etc.

Biotechnological approaches for augmentation of withanolides in *Withania somnifera*

Inevitable *In vitro* cultures

For commercial withanolide production, field grown plant material has generally been used but as per the literature, the quality of these products may be highly affected by different environmental conditions, pollutants, and fungi, bacteria, viruses and insects, which can result in a heavy loss in yield and alter the medicinal content of the plant. Moreover, these methods are time-consuming, laborious, and they are not able to encounter the current Ashwagandha global market requirement [8]. To try to overcome these problems, many attempts were made during the last decades to evaluate the possibility of producing withanolides by *in vitro* plant cell and organ cultures [43, 44].

At the international level, there has been an ever-increasing demand for roots of W. somnifera in larger quantities [8]. Attempts to produce withanolides through tissue culture have been reported by many authors [45-50]. Rani et al. [51] were the first to report on somatic embryogenesis from calli obtained from axillary shoots, internodal segments, root and cotyledonary leaf segments. Sivanesan and Murugesan [52] developed an efficient protocol for high-frequency plant regeneration from leaf explants of W. somnifera on Murashige and Skoog (MS) medium supplemented with 6-Benzyl amino purine (BAP), Kinetin (Kn) and Naphthalene acetic acid (NAA). Synthetic seed production and subsequent conversion of encapsulated shoot tips into plantlets have been reported by [53]. Direct regeneration from apical bud explants [54], somatic embryogenesis and plantlet regeneration from leaf explants [55], direct shoot regeneration using petiole and leaf explants [56] and direct and indirect organogenesis from nodal explants [57] of Indian ginseng have also been reported.

- **Adventitious root cultures and production of secondary metabolites**

Withanolide A was reported to be de novo synthesized within root tissues [58], hence, studies were focused on tissue specific synthesis of withanolides under *in vitro* conditions. Compared to cell cultures, adventitious roots were reported to show higher stability in their growing environment and synthesize cosmic amounts of secondary metabolites into their intercellular spaces, which can be more easily extracted, and can be grown in a phytohormone amended medium with low inoculum but a high growth rate [59]. Wasnik *et al.*, [60] established a protocol for large-scale cultivation of *in vitro* adventitious root (fig.2) cultures of *W. somnifera* in a bubble column bioreactor.

Alteration in the media composition enhanced the accumulation of withanolides

Carbon source

Plant cell cultures are usually grown heterotrophically using simple sugars as a carbon source and inorganic supply of other nutrients. The concentration of sucrose has been shown to affect the productivity of secondary metabolite-accumulating cultures. Sucrose is the most common choice in tissue culture media as it is reported to be the main sugar that can translocate in the phloem of many plants. Doma and co-workers [61] analyzed the influence of different concentrations of carbon sources on hairy root cultures of *W. somnifera* on enhancing withanolide A and withaferin A accumulation.

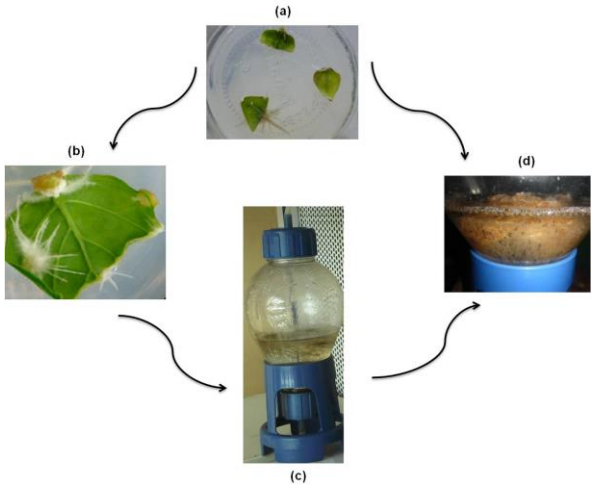

Fig. 2: Induction of *in vitro* adventitious root cultures of *W. somnifera* and its mass cultivation [60]. (a) Leaf explants inoculated in rooting medium. (b) Induction of adventitious roots from leaf explants. (c) Multiplication of *in vitro* roots in bubble column bioreactor. (d) Mass cultivation of *in vitro* roots

The authors concluded that 1733 µg/g dry weight of Withaferin A accumulated in MS basal medium supplemented with 4% sucrose and added that significantly higher amount of Withanolide A and withaferin A were accumulated (890 and 886 µg/g dry weight respectively) only in medium supplemented with 3% sucrose. Similarly, Murthy *et al.* [62] reported that only low amounts of Withanolide A (157.4 µg/g dry weight) were accumulated in hairy root cultures in MS basal medium supplemented with 4% sucrose concentration. Sivanandhan *et al.* [8] reported that the hairy roots of *W. somnifera* grown in half MS liquid medium supplemented with sucrose (4%) stimulated higher production of withaferin A (2.21 mg/g DW) and withanone (2.41 mg/g DW) on the 40th day of culture, followed by a combination of sucrose and glucose enriched medium.

Nitrate levels

Nitrogen concentration was reported to affect the level of proteinaceous or amino acid products in cell suspension cultures. The plant tissue culture medium such as MS, LS or B5 has both nitrate and ammonium as sources of nitrogen. However, the ratio of the ammonium/nitrate–nitrogen and overall concentration of total nitrogen have been shown markedly to influence the production of secondary plant products. Nagella and Murthy [63] observed that the nitrate and ammonium ions have different effects on primary and secondary metabolism in plant cell and tissue cultures and concluded that cell suspensions of *W. somnifera* have shown that both the biomass and secondary metabolite accumulation were influenced by the concentration and composition of macro elements and the ammonia–nitrate ratio. In their experiment, maximum biomass growth (110.45 g l-1 FW and 9.29 g l-1 DW) was achieved at in cell suspension medium supplemented with an $NH4^+/NO3^-$ in the concentration of 7.19/18.80, while withanolide A production was greatest (3.96 mg g-1 DW) in medium supplemented with an $NH4^+/NO3^-$ in the concentration of 14.38/37.60 mM.

Growth regulators

The concentration of growth regulator is often a crucial factor in secondary product accumulation [64, 65]. The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alter both the growth and the product formation in cultured plant cells [66] dramatically. Phytohormones, specifically auxin plays an essential role in regulating root development, and it has been shown to be intimately involved in the process of adventitious rooting. Auxin, Indoleacetic acid (IAA) was shown to be involved in the rooting process by Thimann and Went as far back as 1934 [67], and a second 'synthetic' auxin indole-3-butyric acid (IBA) also promoted rooting [68]. Adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species that are difficult to root [69, 70].

Ray and Jha [50] showed the accumulation of withanolides such as withaferin A and withanolide D in micro shorts in MS liquid medium supplemented with BAP and coconut water. Ahuja *et al.* [71] studied the accumulation of glycol withanolides. Mir *et al.* [72] reported that Withaferin A was produced in relatively high amounts (1.30 % and 1.10 % DW) in shoots of *W. somnifera* cultured in half and full strength MS liquid media respectively enriched with 0.5 µM BAP as compared to natural field grown plants (0.85 % DW).

Sivanandhan *et al.* [7] recorded that the polyamines along with plant growth regulators enhanced the withanolides production in *in vitro*-raised plants when compared to field grown parent plants. Shukla and co-workers [73] concluded that half strength liquid MS medium enriched with IAA in both 100 and 200 ppm concentration significantly elevated total alkaloid and withanolide content in hairy root cultures of *W. somnifera* when compared with control untreated root cultures. Thirugnanasambantham and co-workers [74] reported that IBA is an effective inducer of lateral root formation when compared to IAA. Accumulation of withanolide A and the biomass increases as the concentration of IBA increased to 1 mg/l (2576±0.37 µg/g DW and 12.89±0.25 g/dL respectively) and added that IAA at lower concentration favors relatively high accumulation of withanolide A (1147±0.77 µg/g DW) in 30 d old *in vitro* adventitious root cultures of *W. somnifera.*

Elicitation

It is well established in recent years that application of elicitors in root cultures can upgrade the secondary metabolite production in plant cell/organ culture. Secondary pathways are triggered in response to stress. Elicitors can be abiotic or biotic and act individually or in combination to turn on the biosynthesis of molecules that may only be produced in small amounts or may even produce new compounds [75]. Abiotic elicitors such as methyl jasmonate (MJ) and salicylic acid (SA) are used in combination with the culture for the highest level of secondary metabolite production.

Recently, Sivanandhan *et al.* [76] reported that when hairy roots of *W. somnifera* with an initial inoculum mass of 5 g fresh weight elicited separately with methyl jasmonate (MJ) and salicylic acid (SA) at various concentrations for different exposure times after 30 d of culture lead to an enhanced production of biomass (32.68 g FW and 5.54 g DW; 1.23-fold higher), withanolide A (132.44 mg/g DW; 58-fold higher), withanone (84.35 mg/g DW; 46-fold higher), and withaferin A (70.72 mg/g DW; 42-fold higher) from 40 d-old culture. Production of secondary metabolites in plant tissue, cell cultures, and adventitious root cultures was reported to be enhanced through elicitation with SA as in *Glycyrrhiza glabra* and *W. somnifera*, respectively [77,8]. Ciddi [78] reported 50-fold enhancement of withaferin A production (25 mg/l) using salacin as an elicitor in cell suspension culture of *W. somnifera*.

In a study conducted by Sivanandhan *et al.* [8], exposure to 30-day-old adventitious root cultures to 150 µM SA for 4 h as elicitor resulted in the production of 64.65 mg g^{-1} dry weight (DW) withanolide A (48-fold), 33.74 mg g^{-1} DW withanolide B (29-fold), 17.47 mg g^{-1} DW withaferin A (20-fold), 42.88 mg g^{-1} DW withanone (37-fold), 5.34 mg g^{-1} DW 12-deoxy withastramonolide (nine fold), 7.23 mg g^{-1} DW withanoside V (sevenfold), and 9.45 mg g^{-1} DW withanoside IV (nine-fold) after 10 d of elicitation (40th day of culture) when compared to untreated cultures [8].

UV-B irradiation

Kalidhasan *et al.* [79] reported that Ultraviolet (UV-B) enhanced radiation have triggered higher level of withaferin A synthesis than that of control root sample and suggested a possible enhancement of the enzymes necessary for the biosynthesis of withaferin A. Same team workers have concluded that along with withaferin A, some other UV absorbing compounds have also accumulated in higher concentration in the field grown root samples. These results suggested that *W.*

somnifera could possess possibly more precursors for the formation of phenolic compounds or UV absorbing compounds.

Thus, the above-mentioned techniques and approaches carried out in *in vitro* cultures enhances the tissue-specific accumulation of therapeutically significant withanolides in *W. somnifera*.

Understanding withanolide biosynthetic pathway

Understanding the steps involved in withanolide biosynthesis is essential for metabolic engineering of this plant to increase withanolide production. Withanolides are biosynthesized through the isoprenoid pathway, probably via both the mevalonate and nonmevalonate pathways [80] (fig. 3). The head-to-tail condensation of isopentenyl pyrophosphate (IPP) leads to the formation of farnesyl diphosphate (FPP) which is the main precursor for triterpenoids [81]. A key intermediate compound, 24-methylenecholesterol is an immediate precursor for biosynthesis of different withanolides, 24-methylene cholesterol is the first branching point towards the biosynthesis of different withanolides through a series of desaturation, hydroxylation, epoxidation, cyclization, chain elongation, and glycosylation steps [82, 83].

In plants, MVA is the general precursor of variously identified isoprenoids, such as sterols, plant growth regulators, and terpenoids [24, 25]. It has been recently reported that, in *W. somnifera*, both MVA and DOXP pathway participate in the biosynthesis of the withanolide and plastidic activity regulate this cross-talk to a varying level [83].

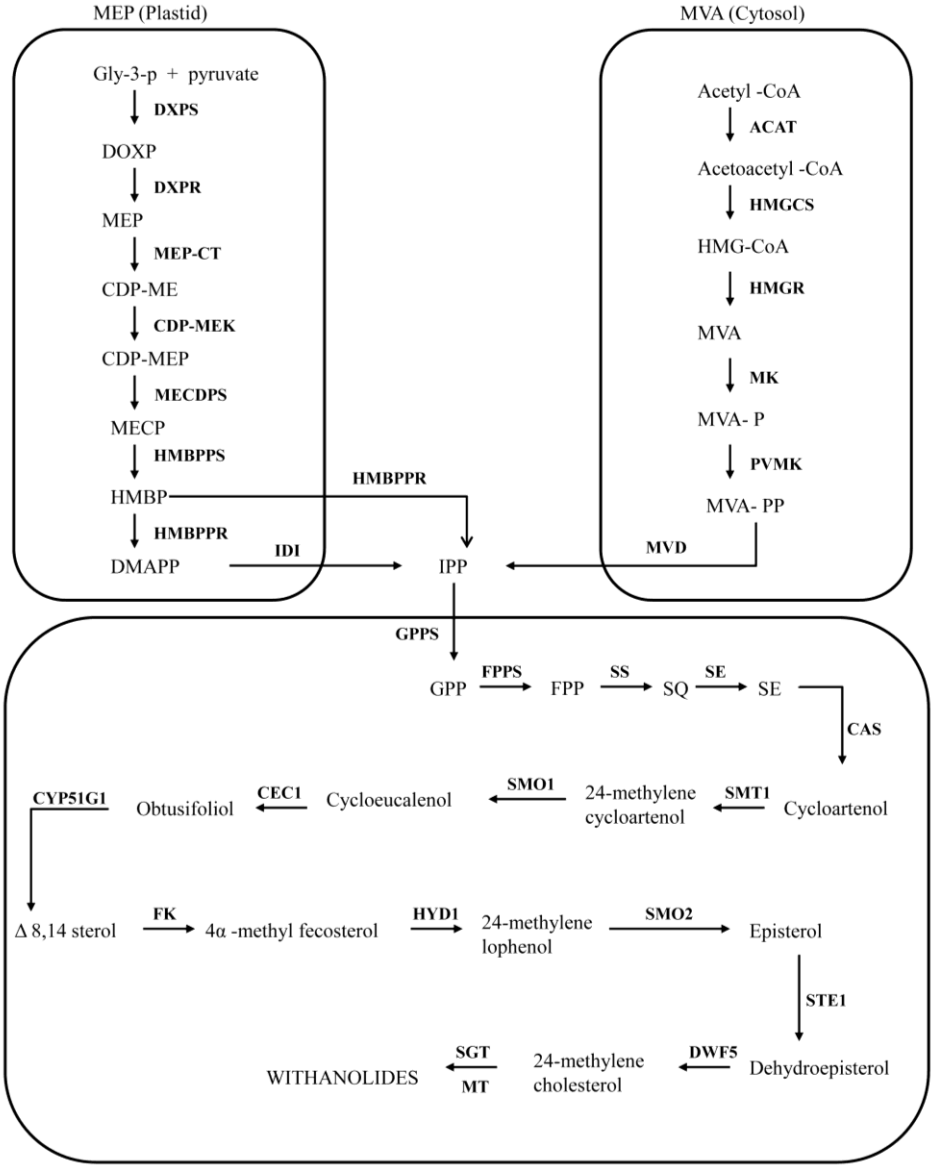

Fig. 3: Putative biosynthetic pathway of withanolide. 1-Deoxy-D-xylulose-5-phosphate synthase DXPS; 1-Deoxy-D-xylulose-5-phosphate reductoisomerase DXPR; 2-C-Methyl-D-erythritol 4-phosphate cytidylyltransferase MEP-CT; 4-(Cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase CDP-MEK; 2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase MECDPS; (E)-4-Hydroxy-3-methylbut-2-enyl-diphosphate synthase HMBPPS; 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase HMBPPR; Isopentenyl-diphosphate delta-isomerase IDI; Acetyl-CoA C-acetyltransferase ACAT; Hydroxymethylglutaryl-CoA synthase HMGCS; Hydroxymethylglutaryl-CoA reductase HMGR; Hydroxymethylglutaryl-CoA reductase HMGR; Mevalonate kinase MK; Phosphomevalonate kinase PVMK; Diphosphomevalonate decarboxylase MVD; Geranyl diphosphate synthase GPPS; Farnesyl diphosphate synthase FPPS; Squalene synthase SS; Squalene monooxygenase SE; Cycloartenol synthase CAS; Sterol 24-C-methyltransferase SMT1; Methyl sterol monooxygenase/Sterol-4a-methyl oxidase 2 SMO1/SMO2; Cycloeucalenol cycloisomerase CEC1; Obtusifoliol 14-demethylase CPY51G; Delta 14-sterol reductase FK; C-7,8 Sterol isomerase HYD1; C-5 Sterol desaturase STE1; 7-Dehydro cholesterol reductase DWF5; Sterol glycosyltransferases SGT; Methyltransferases MT

Elucidation of genes involved in withanolide biosynthetic pathway

3-Hydroxy-3-methylgutary coenzyme A reductase (HMGR)

In MVA pathway, 3-Hydroxy-3-methylgutary coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes the NAD (P) H-dependent reduction of HMG-CoA to mevalonate, the first committed step in the isoprenoid pathway, which produces the largest group of contemporary natural products [84]. This enzyme is located in the endoplasmic reticulum. According to Hemmerlin *et al.* [84], plant HMGR is known to be controlled by a number of developmental and environmental signals like phytohormones, calcium, calmodulin, light, wounding, elicitor treatment and pathogen attack. Akhtar *et al.* [85] reported that the expression level of *WsHMGR* (*WsHMGR1*) is the maximum in the flower followed by root tissue in *W. somnifera*. Recently, Senthil *et al.* [27] analyzed the expression pattern of HMGR in *in vitro* root and leaf cultures of *W. somnifera.* The authors observed that *HMGR* expression was higher in 30-day-old *in vitro* adventitious root cultures, whereas, in leaf tissue, the expression of *HMGR* was observed to be much lower.

Farnesyl diphosphate synthase (FPPS)

In these pathways, farnesyl diphosphate (FPP), which is synthesized by catalytic action of the enzyme farnesyl diphosphate synthase (FPPS), serves as a substrate for first committed reaction of several branched pathways [86] leading to the synthesis of compounds that are essential for plant growth and development as well as of pharmaceutical interest [87]. FPPS is one of the key enzymes [86] for isoprenoid biosynthesis which synthesizes sesquiterpene precursors for several classes of essential metabolites, including sterols, dolichols, ubiquinones and carotenoids as well as substrates for farnesylation and geranylgeranylation of proteins. This also plays an important role in commencing steps of triterpenoid precursor synthesis catering to withanolide biosynthesis.

FPPS catalyzed reaction occurs in two consecutive steps; condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) to form 10-C intermediate geranyl diphosphate (GPP) and condensation of GPP with another molecule of IPP which results into 15-C FPP [87]. This gene has been characterized from variety of plant species including Arabidopsis [88], Artemisia [89], Hevea [90], maize [91], rice [92] and Taxus media [93].

Gupta et al. [6] isolated and characterized the gene encoding FPPS from W. somnifera. The authors also analyzed the expression pattern of FPPS in field grown young leaf, mature leaf, flower, fruit (green berry), stem and root tissues of W. somnifera and concluded that WsFPPS transcripts levels were significantly higher in young leaf than in mature. The same team reported that higher level of WsFPPS in flowers. On the contrary, lower expression of WsFPPS was reported to be recorded in roots. Recently, Thirugnanasambantham et al. [74] analyzes the expression of FPPS in field grown root and leaf tissues at different developmental stages of W. somnifera and concluded that WsFPPS expression levels were higher in leaves than the root tissues.

Sabir *et al.* [80] compared the expression levels of FPPS between *in vitro* and field grown tissues and observed that WsFPPS slightly upregulated in *in vitro* shoots in comparison to *in vitro* roots.

Squalene synthase (SQS)

Squalene synthase (SQS: EC 2.5.1.21) is an important regulatory enzyme of cholesterol biosynthetic pathway. It is a bifunctional enzyme which catalyzes the condensation of two molecules of farnesyl pyrophosphate (FPP) in a head-to-head manner to form pre-squalene diphosphate (PSPP) and then converts the PSPP to squalene in the presence of NADPH and Mg^{2+}. As studied in engineered yeasts [94, 95] down-regulation of the squalene synthase in the sterol biosynthetic pathway leads to the accumulation of FPP, which is redirected away from this pathway and toward the synthesis of other commercially important isoprenoids. Gupta *et al.* [6] analyzed the expression levels of WsSQS using Quantitative real time–polymerase chain reaction (qRT-PCR) and demonstrated that WsSQS has a tissue-specific expression with highest expression in leaves and lowest in roots. The similar results were found with the expression of some other genes of the same pathway [96, 6]. On the contrary, Bhat *et al.* [97] reported that expression of squalene synthase (WsSQS) was seen in all tested tissues, including roots, stem and leaves with the highest level of expression in leaves.

Squalene epoxidase (SE)

The biosynthesis of the withanolide pathway up to squalene (catalyzed by SS) is an anaerobic process [98]. Squalene epoxidase (SE) catalyzes first step of oxygenation in this pathway and steps after squalene epoxidation are shared by sterol/brassinosteroids biosynthetic pathway [58]. SE (EC. 1.14.99.7) is one of the rate-limiting enzymes in the biosynthesis of triterpenoids, catalyzing the stereospecific epoxidation of squalene to 2,3-oxidosqualene. It requires the participation of flavoprotein NADPH-cytochrome P-450 reductase (E. C. 1.6.2.4) [98] and functions as a rate-limiting step in the sterol and triterpenoids biosynthesis [99]. A perusal of literature reveals that SE is a microsomal protein and also present in lipid droplets, but only ER-associated protein has been found to be active [100]. In addition to 2, 3-oxidosqualene, SE activity can result in the formation of 6, 7-oxidosqualene, 10, 11-oxidosqualene and dioxide squalene [101]. Being a rate limiting enzyme [99], overexpression of SE may have an important role in the regulation of phytosterols and steroidal lactones in *W. somnifera*.

Senthil *et al.* [27] very recently analyzed the expression of important pathway genes involved in withanolide biosynthesis. The authors reported that among the selected pathway genes, an expression level of SE was significantly higher in *in vitro* root tissues of *Withania somnifera.* Sabir *et al.* [80] also reported that higher expression of *SE* transcripts in field grown root than shoot tissues of *W. somnifera.* Gupta *et al.* [102] made the first attempt of cloning and characterization of this gene from *W. somnifera*. Thirugnanasambantham *et al.* [74] also analyzed expression levels of *SE* in field grown leaf and root tissues at different developmental stages of *W. somnifera* and concluded that expression levels of *SE* were significantly higher in field grown root tissue at yellow berry stage than leaf tissue and concluded that expression levels of *WsSE* exhibit a direct correlation with that of withanolide biosynthesis.

Cycloartenol synthase (CAS)

Cycloartenol and lanosterol are important membrane constituents that can serve as precursors to steroid hormones. It is formed from (S)-squalene-2, 3-epoxide by a cyclization reaction catalyzed by cycloartenol synthase (EC 5.4.99.8.). CAS performs the important function of breaking 11 bonds and forming 11 new ones to transform 2, 3-epoxysqualene to the plant sterol precursor cycloartenol [103].

It is presumed that cycloartenol bifurcation takes place for the biogenesis of sterol and withanolides in *W. somnifera.* Probably because of this division of cycloartenol, Dhar *et al.* [104] reported that WsCAS expression was the maximum and on the rise with each advancing phenol phase to generate a reservoir of cycloartenol which may get channelized towards the two routes leading to the biosynthesis of phytosterols and withanolides. The same team of workers identified that WsCAS exist in a copy number of two, thereby indicating the separate role of each copy of WsCAS in sterol and withanolide biosynthesis. The duplicate copy number of WsCAS is reported to be plausibly a trigger for higher expression.

It has been well documented that CAS plays an essential role in the plant cell viability and in the regulation of triterpenoid biosynthesis [105]. The differences in the biosynthesis of sterols between higher plants and yeast/mammals are generally accepted to begin at the cyclization step of 2, 3-oxidosqualene, a common precursor. Phytosterols, such as campesterol and sitosterol, are biosynthesized via cycloartenol and catalyzed by cycloartenol synthase (CAS) in higher plants [106]. Senthil *et al.* [27] reported that *CAS* genes exhibited higher expression at 45 d of growth in both leaf and root tissue under *in vitro* condition. Thirugnanasambantham *et al.* [74] expression of gene encoding cycloartenol synthase exhibited higher levels in both field grown leaf and root tissues, throughout the different developmental stages of *W. somnifera*.

Glucosyltransferase (GT)

Glycosylation of secondary plant products, such as flavonoids, coumarins, terpenoids, and cyanohydrins, is generally catalyzed by Plant secondary product glycosyltransferase (PSPGs) [107], which belong to family-1 glycosyltransferases, catalyzing glycosyl transfer (GT) from nucleoside diphosphate-activated sugars (donor) to aglycon substrate (acceptor) molecules. The activated sugar form is typically uridine diphosphate (UDP)-glucose, but UDP-galactose, UDP-glucuronide, UDP-xylulose, and UDP-rhamnose are also reported. Glycosylation not only stabilizes the products but also modulates their physiological activities and governs intracellular distribution [108].

In plants, sterols are biosynthesized by mevalonate and non-mevalonate pathways. They occur in highly diversified skeletal and structural forms that are finally glycosylated. Some of these (e. g. sitosterol, stigmasterol, brassinosteroids) are ubiquitous in plants whilst others (e. g. withanolides, limonoids) are highly restricted in occurrence. Earlier reports from Myxoamoeba and human fibroblastoma cell lines showed the activation of glucosyltransferase and the production of sterol glucoside following heat stress. The glucosides have been reported to induce the signal transduction pathway, leading to the synthesis of heat shock proteins during heat stress [109,110] in animal cells.

Madina *et al.* [82] discussed that expression of sterol glucosyltransferases was enhanced in leaves of *W. somnifera* following the application of salicylic acid. GTs that use UDPactivated sugars as donors and various types of small molecules as acceptors are called UDP-glycosyltransferases (UGTs) and represent family 1GTs. Such UGTs are present commonly in plants and animals but have been reported in a few cases only in microorganisms. In higher plants, UGT catalyzed glycosylation constitutes a prominent terminal modification in the biosynthesis of secondary metabolites and generates diverse natural glycosides [111]

In higher plants, secondary metabolites are often converted to their glycoconjugates, which are then accumulated and compartmentalized in vacuoles. These glycosylation reactions are catalyzed by glycosyltransferases (GTases) [112]. In general, glycosylation is the last step in the biosynthesis of secondary metabolites [113]. A vast variety of glycosyltransferase genes have been identified thus far, which are currently classified on the basis of their phylogenetics into 70 families [111].

Cytochrome P450 reductase (CPR)

CPRs (EC 1.6.2.4) are membrane-bound proteins localized to the ER, contain an N-terminal positioned Flavin mononucleotide (FMN) binding domain linked to NADPH binding domain *via* Flavine Adenine Dinucleotide (FAD) domain [114]. CPR shuttles electrons derived from NADPH through FAD and FMN domains into the heme iron centre of the various P450 enzymes and thus confront the high demand of electron supply during biotic and abiotic stress or differential expression at various stages of plant development [114]. It has been earlier demonstrated that CPR1 and CPR2 from different plant species have different specific activities and most of them have been assayed using a microsomal fraction or truncated polypeptide (without membrane anchor) [115]. Rana *et al.* [116] suggested that Cytochrome P450 reductase is the most imperative redox partner of multiple P450s involved in primary and secondary metabolite biosynthesis. Dhar and co-workers [104] cloned and functionally characterized two paralogs of NADPH-cytochrome P450 reductase (WsCPR1 and WsCPR2) and studied the expression pattern of the same in tissues of *W. somnifera* cultures in the field during different developmental stages.

The authors concluded that WsCPR2 showed a slight increase along the developmental phases and added that this expression level might be possibly implicating its role in the biosynthesis of withanolides. Rana *et al.* [116] conducted Quantitative real-time PCR to analyze the expression of *WsCPR1* and *WsCPR2* in various tissues of *W. somnifera* and concluded that both genes were widely expressed in leaves, stalks, roots, flowers and berries with the relatively higher expression level of *WsCPR2* in comparison to *WsCPR1*.

Transcriptome analysis

Next-generation sequencing (NGS) technology for transcriptome (RNA-seq) dispenses a new resolution for both obtaining gene sequences and quantifying transcriptome of any organism. In recent years, the RNA-seq has been an influential method for distinguishing genes involved in important secondary metabolite pathways such as biosynthesis of ginsenosides in *Panax ginseng* [117,118], carotenoids in *Momordica cochinchinensis* [119], flavonoids, theanine and caffeine in tea (*Camellia sinensis*), Initial efforts have been made to generate expressed sequence tags (ESTs) from *in vitro* tissues of *W. somnifera* [120].

Complete transcriptome analysis of *W. somnifera* leaf and root tissue was performed using next-generation sequencing in order to gain insights into withanolide biosynthesis pathways and their regulations [102]. A total number of 47,885 and 54,123 unigenes generated from leaf and root tissues, respectively, have been annotated using TAIR10 protein database (http://www. arabidopsis. org; Tair10), NCBI protein database NR (http://www. ncbi. nlm. nih. gov). Based on the sequence homology, the unigenes were categorized into 45 functional groups. On the basis of the annotation, the genes encoding enzymes involved in the biosynthesis of triterpenoid backbone (including MVA and MEP pathways) were identified from both leaf and root libraries. Apart from these, a number of methyltransferases, cytochrome P450s, glycosyltransferase, and transcription factors have also been identified and reported by [102]

Dasgupta *et al.* [121] analyze the salicylic acid induced leaf transcriptome of *W. somnifera* using Illumina Genome Analyzer. A total of 45.6 million reads was generated, and the *de novo* assembly yielded 73,523 transcript contig with average transcript contig length of 1620 bp were reported. A total of 71,062 transcripts was annotated and 53,424 of them were assigned GO terms. Mapping of transcript contigs to biological pathways revealed the presence of 182 pathways. Seventeen genes representing 12 pathogenesis-related (PR) families were mined from the transcriptome data and their pattern of expression post 17 and 36 h of salicylic acid treatment were documented. The authors reported that significant up-regulation of all families of PR genes by 36 h post-treatment.

Very recently, Senthil et al. [27] had used RNA-seq for large-scale transcriptome profiling and generated a comprehensive Transcriptome for W. somnifera by assembling the transcriptomes of in vitro adventitious root and leaf tissues from the millions of short sequence reads generated by Illumina. The authors obtained a total of 177,156 assembled transcripts with an average unigene length of 1,033 bp. About 13% of the transcripts were reported to be unique to in vitro adventitious roots, but no such transcripts were observed in in vitro-grown leaves. Annotations including functional annotation, Gene encoding enzymes involved in withanolide biosynthesis were identified using Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

***In silico* analysis**

Application of in silico tools can significantly improve the detection of genes and variation. In silico drug design or the computer-aided drug design (CADD) play a significant role in all stages of drug development from the preclinical discovery stage to late stage of clinical development. Recently, few in silico studies were carried out demonstrating the role of withanolides as a potent drug. High-throughput docking has become increasingly important in the context of compound drug identification [122-124].

Aishwarya and Santhi [125] described the docking of 26 withaferins and 14 withanolides from *W. somnifera* into the three-dimensional structure of PknG of *M. tuberculosis* using GLIDE. The authors concluded that among the withanolides, withanolide E, F and D and Withaferin-diacetate 2 phenoxy ethyl carbonate was identified as potential inhibitors of PknG. This data provides the molecular insights to the consideration of Withanolides as potential candidates against the PknG target in *M. tuberculosis*. In another study conducted by Santhi and Aishwarya [126] alkaloids namely withasomine, cuseohygrine and ana hygrine from *W. somnifera* were

docked deeply within the binding pocket region forming interaction with binding site residues of both 5-LOX and COX (anti-inflammatory drug target proteins) of humans. The authors concluded among the alkaloids: withasomine, cuseohygrine and anahygrine showed better inhibitory activity to both 5-LOX and COX, whereas, the other three alkaloids showing anti-inflammatory activity are anahygrine, 3-tropyltgloate and tropinine.

Vaishnavi *et al.* [127] examined the docking efficacy of Withaferin A (WA) and Withanone against four target proteins, namely, mortalin, p53, p21 and Nrf2. The authors demonstrated that Withaferin A that binds strongly to the selected targets acts as a strong cytotoxic agent both in normal and cancer cells. Withanone, on the other hand, has a weak binding to the targets; it showed milder cytotoxicity towards cancer cells and was safe for normal cells. This study revealed an important insight to the use of Withaferin A and Withanone for cancer treatment and development of new anti-cancer phytochemical cocktails.

Prabhakaran *et al.* [128] extensively used graphical software (MOLSOFT) for the identification of the binding energy of selected Withanolides like Withaferin-A, Withanolide-D from *W. somnifera* and to screen the phytoconstituents that will dock/bind to the active sites of COX-2 enzyme. The authors concluded that among the two constituents, withaferin A possesses great activity on COX-2 followed by withanolide-D. Also added that Withanolides may have a direct action on cox-2 enzyme by binding to the Cys-907, Cys-910, Cys-962 and Cys-964 residue.

Recently, Saha *et al.* [129] carried out molecular docking studies to find the potentiality of Withaferin A, a key metabolite of *W. somnifera*, as an inhibitor of vascular endothelial growth factor (VEGF). Molecular Docking calculations were carried out on Withaferin A/Bevacizumab-VEGF protein model. The authors concluded that Withaferin A showed favorable binding with VEGF, and the results were highly comparable with the commercially available drug Bevacizumab. Bikadi *et al.* [130] demonstrated direct covalent binding of Withaferin A to Cys303 of tubulin in MCF-7 cells. Further added that WA-binding pocket is located on the surface of tubulin and characterized by a hydrophobic floor, a hydrophobic wall, and a charge-balanced hydrophilic entrance.

Proteomics

To have a better understanding of the processes that occur in *W. somnifera*, proteome analyses were initiated on seeds and leaf tissues by Gupta and Co-workers [6], From these analyses, 70 individual proteins from seeds and 74 from leaves were identified by protein sequence database interrogation and were cataloged accordingly to different protein functions. Senthil and co-workers [131] investigated comparative protein changes between the root tissues cultivated *in vitro* and from the field. The authors recorded a similarity in protein spots in both *in vitro* and *in vivo* root samples and concluded that *in vitro* roots that are developed independently of shoot organs appear to have a similar developmental process as that of *in vivo* roots.

Metabolomics

The comprehensive chemical analysis is required not only to establish a correlation between complex chemical mixtures and molecular pharmacology but also to understand complex cellular processes and biochemical pathways via metabolite-to-gene network [23]. Chatterjee *et al.* [132] carried out metabolic profiling of crude extracts of leaf and root of Withania somnifera using Nuclear Magnetic Resonance (NMR) and chromatographic (High-Performance Liquid Chromatography (HPLC) and Gas Chromatography–Mass Spectrometry GC–MS)) techniques. A total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots were unambiguously identified. Senthil *et al.* [133] compared the total metabolome profiling between *in vitro* and field grown root tissues of *Withania somnifera* using GC-MS technique. The authors reported that total of 29 metabolites was identified in *in vitro* cultured and field-grown roots by GC-MS analysis. The metabolites included alcohols, organic acids, purine, pyrimidine, sugars, and putrescine. In their study, vanillic acid was only observed in the *in vitro* cultured root samples. Very recently, Thirugnanasambantham *et al.* [134] compared the metabolite profiles between leaf tissues of *W. somnifera* cultured in *in vitro* and field grown conditions. The authors highlighted that *in vitro* leaf cultures able to accumulate metabolites in a similar fashion as that of field grown tissues and can be utilized as alternative resources to field-grown leaves for the production of useful metabolites such as γ-aminobutyric acid and putrescine. These results highlighted the potentiality of *in vitro* roots as an alternative to field-grown roots.

CONCLUSION

Until date only very less information is available on structural and functional aspects of enzymes involved in withanolide biosynthetic pathway of *Withania somnifera*. This review gives a clear understanding of various technical approaches carried out in *W. somnifera* and emphasized the worldwide achievements associated with understanding the synthesis and regulation of pharmaceutically important secondary metabolites. These new technologies will serve to extend and enhance the continued usefulness of *in vitro* cultured plants as renewable sources of medicinal compounds.

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CONFLICT OF INTERESTS

We declare that we have no conflicts of interest in the authorship or publication of this contribution.

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