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Efficient *Agrobacterium rhizogenes* mediated hairy root culture system for withanolide production from *Withania somnifera* (L.) dunal

Abstract

Withania somnifera (L.) Dunal is an important medicinal plant with many pharmaceutical and therapeutic uses ranging from immunomodulation to anti-cancer property. Field cultivation of *W. somnifera* is time consuming and it not able to meet current global demands. For commercial production of withanolides, field grown plant materials have generally been used but the quality and uniformity in the levels of active constituents are highly affected by genotype and environmental conditions. *In vitro* culture methods are the best alternative and have possible ways to enhance commercial production of withanolides. In this review, we summarize our recent findings on characterization and screening of wild accessions of *W. somnifera* collected from Tamil Nadu state. Further, an improved *Agrobacterium rhizogenes* mediated hairy root culture system was developed and this system providing an efficient tool attaining better transformation efficiency and useful for commercial *in vitro* production of withanolides.

Keywords: *withania somnifera*, class based stratification matrix, physical leaf traits, withanolides, *agrobacterium rhizogenes*, hairy roots

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Abbreviations: MVA, mevalonic acid; MEP, methylerythritol phosphate; SAAT, sonication assisted *agrobacterium* mediated transformation

Introduction

Withania somnifera also known as Ashwagandha, Indian ginseng and winter cherry is an important medicinal plant in the traditional medicinal system of India.[1] Pharmacological activities of this plant include physiological and metabolic restoration, anti-arthritic, anti-aging, nerve tonic, cognitive function improvement in geriatric states and recovery from neurodegenerative disorders.[2,3] The herb forms an essential constituent or whole of more than 100 traditional medicine formulations. The pharmacological activities of plant are primarily due to the presence of specialized steroidal lactones, known as withanolides.[4,5] Withanolides are built on an ergostane skeleton through appropriate oxidations at C-22 and C-26 to form a δ lactone ring. Both leaves and roots of the plant are used as drug owing to the presence of these phytochemicals.[6] Withaferin A and withanolide A (Figure 1) are the major withanolides present in *W. somnifera* and are putatively produced by mevalonate and downstream of triterpenoid pathway through cyclization of 2,3-oxidosqualene to cycloartenol.[7] Chemically, withanolides are 30 carbon compounds called triterpenoids. Triterpenoid backbone, like other terpenoid compounds is biosynthesized by metabolic pathway requiring isoprene units as precursors. Therefore, isoprenogenesis could be one of the key upstream metabolic process governing fluxes of isoprene units for synthesis of metabolic intermediates of triterpenoid pathway committed to withanolides biosynthesis.[8] Dual autonomous pathways for the isoprenoid precursor biosynthesis co-exist in plant cell including the classical cytosolic Mevalonic acid (MVA) pathway and the alternative route, plastidial Methylerythritol phosphate (MEP) pathway.[9]

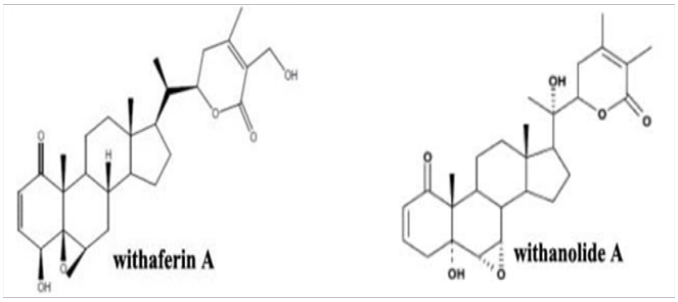

Figure 1 Structure of withaferin A and withanolide A.

Since *W. somnifera* is a cross pollinated plant, the withanolide content varies from plant to plant and thus stable production of withanolide is a major goal using metabolic engineering approach. Elite cell lines isolated from natural population can be genetically more stable and suitable for large scale production of bioactive compounds. Various *in vitro* culture methods, including callus culture,[10] adventitious shoot culture[11] and cell culture systems[12] have been adapted for the production of therapeutically valuable withanolide compounds from *W. somnifera*. Hairy roots are considered a very good system for continuous synthesis of valuable metabolic compounds in an aseptic condition in the absence of expensive growth regulators in the culture medium.[13,14] For this reason, *A. rhizogenes*-mediated hairy root culture has been utilized in several pharmaceutical important plants for the production of secondary metabolites.[15–17] However, *A. rhizogenes* mediated hairy root induction in *W. somnifera* is limited due to the lack of efficient hairy root induction procedure.[18–20] Alternative approaches for an efficient hairy root induction are valuable for large-scale production of withanolides.

Various efforts have been made to overcome the problems associated with host/tissue to increase the number of infected sites,

such as use of super virulent *Agrobacterium* strains and the addition of some compounds to the co-cultivation medium. Recently, Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) attracts much attention in several plant species.[21] It has been successfully applied for hairy root production in *Papaver somniferum*[15] and *Verbascum xanthophoeniceum.*[22] It holds great promise for the enhancement of hairy root production. The advantage of this method is that the cavitations caused by sonication cause thousands of micro-wounds on the surface of the explants. These micro-wounds permit *Agrobacterium* to penetrate deeper and more completely throughout the explant than conventional wounding, increasing the probability of infection to host cells.[21] Further, transformation efficiency in *Agrobacterium* was also improved in several plant species by the use of heat treatment.[23–25] Even though several reports on *in vitro* methods, report on successful and efficient production of withanolide using *in vitro* pathway are still limited. In this review, we have highlighted our recent findings on collection, characterization and screening of *W. somnifera* accessions from natural population and development of an efficient *Agrobacterium* mediated hairy root culture protocol for the selected elite chemo type.

Germplasm evaluation and screening of promising chemo type

We made an attempt to develop a simple and novel method for rapid evaluation of germplasm by studying physical leaf traits and total Withaferin-A content.[26] A total of 15 wild accessions of *W. somnifera* were collected from various regions of Tamil Nadu state, India. Seeds collected from these accessions were germinated and raised in uniform soil and environmental conditions. The physical leaf traits showed significant variation in all 15 accessions. All the accessions showed uniform level of leaf dry matter content except accessions ACCN03, ACCN10 and ACCN11.[26] In other words, stratification of leaf dry matter content was poor when comparing other traits studied. Observations revealed highly significant differences for all the characters studied, indicating that there was considerable genetic diversity among the accessions. Variation among individuals is equally influenced by the environment as well as genetic effects.[27] The 15 accessions from different regions of Tamil Nadu grown in a single location were subjected to the same environmental condition. Hence, the observed variations could be largely due to genetic variation. The evaluation of *W. somnifera* germplasm showed a large variation in the quantitative traits between the accessions.[26]

The distribution of *W. somnifera* in Tamil Nadu is governed by its collection and scarce cultivation. There are limited reports on wild accessions of *W. somnifera* from India and their phenetic relation.[28–30] These studies have attempted to describe the patterns of distribution of chemo types and morphotypes. However, our study reveals the uniqueness of the accessions which were collected from Tamil Nadu, from where there are no reports on this neglected, underutilized crop.[30] Genetic distances were also calculated for each pair of accessions to determine the extent of divergence. A Fitch-Margoliash cladogram was generated using these genetic distances for graphical portrayal of genetic divergence. The cladogram revealed three groups, viz. accessions ACCN02, ACCN08, ACCN05, ACCN06, ACCN15, ACCN14, ACCN07 and ACCN03 in one cluster, accessions ACCN12, ACCN10 and ACCN09 in a second group and accessions ACCN13, ACCN11, ACCN04 and ACCN01 distinct from one other and not in any cluster. The first branch of the cladogram separated ACCN13 and the second branch included the above-mentioned groupings.[26]

Among all the investigated accessions, the hyper Withaferin-A accessions (ACCN06, ACCN12 and ACCN13) could be clustered into a single group only in the cladogram based on physical leaf traits. This was not the same in other cladograms. In the cladogram based on the matrix compiled from all traits, only accessions ACCN06 and ACCN13 could be placed in one group, with 12 segregated into another cluster (Figure 2). In the RAPD-based clustering, accessions ACCN06 and ACCN13 were found to be closely related, along with accession ACCN12 in a neighboring cluster.[26] This indicates the influence of genomic traits on the Withaferin-A content. The correlations between the pairs of matrices indicate that the influence of each trait on the expression of the overall phenetic relation is highly variable. On a par with or better than the molecular markers, the physical leaf traits provided the best and most cost-effective trait set for germplasm evaluation, as evidenced by the grouping of the promising accessions ACCN06, ACCN12 and ACCN13, which have been selected for further studies.[26]

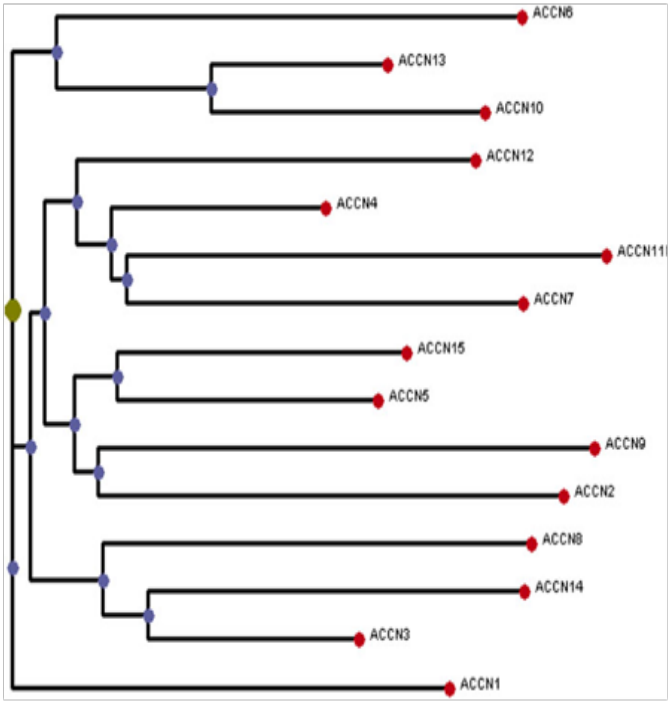

Figure 2 Cladogram for various *W. somnifera* accessions collected from Tamil Nadu, India.

Agrobacterium rhizogenes mediated hairy root culture

We standardized an efficient protocol for improve the transformation efficiency of *A. rhizogenes* for hairy root induction in *W. somnifera* ACCN-06 by means of sonication and heat treatments.[31] Leaf explants were co-cultivated with *A. rhizogenes* strains R1000, MTCC 2364 and MTCC 532 on hormone free MS medium in the dark for 2 days. Among the three strains *A. rhizogenes* R1000 showed the highest transformation rates (50.6%). MTCC 2364 was less efficient (29.3%) than R1000, but much more efficient than MTCC 532 (18.6%).[31] R1000 strain was widely used for hairy root induction in many plants such as *Torenia fournieri,*[32] *Gentiana macrophylla*,[33] *Centella asiatica*[34] and *Agastache rugosa*.[35] This intrinsic capacity of *Ri* plasmid must be higher in R1000 when compared to other strains as inferred by other reports also.[36] For the first time we analyzed in *W. somnifera*, the transformation efficiency of explants by *Agrobacterium*

with various temperature of heat treatment (39, 41, 43 or 45°C) and various time duration (3, 5, 7 or 10min). Using different heating times at 41°C heat treatment for 5 min was increased the transformation frequency (76.0%) of hairy root induction. Heating at various temperatures decreased in the hairy root induction frequency.[31]

A. rhizogenes-mediated hairy root induction has been documented earlier in *W. somnifera* by several authors.[37–39] No reports on transformation with SAAT method are available for hairy root induction in *W. somnifera*. We investigated the effect of sonication on transformation efficiency by sonication of leaf explants in *Agrobacterium* suspension for different time durations (5, 10, 15 or 20s). Among the different time duration analyzed, 15s sonication followed by 41°C for 5min heat treatment showed better transformation efficiency (93.3%) after 3 weeks.[31] We successfully transformed via SAAT method, which proved the high reliability of SAAT technology for the transformation of *W. somnifera*. Molecular evidence for transformed status of hairy roots was obtained by PCR analysis. The presence of *rol* B gene fragment and *rol* C gene fragment was confirmed using specific primers. All the transformed roots contained the target bands: 423bp corresponding to the *rol* B gene fragment. Amplification product was not detected in DNA from non-transformed roots.[31]

Withanolides content of hairy roots was analyzed through HPLC system. The maximum Withaferin A (6.17mg g L^{-1} DW) and withanolide A (3.82mg g L^{-1} DW) content were attained in hairy root biomass after 35 days of culture which were identified with peaks corresponding to key markers.[31] Our protocol showed the presence of withanolides in hairy root culture of the Indian *W. somnifera* ACCN 06 transformed using *A. rhizogenes* (Figure 3). Similarly, Murthy et al.[18] also reported the presence of withanolides in transformed hairy root cultures of Indian *W. somnifera*.

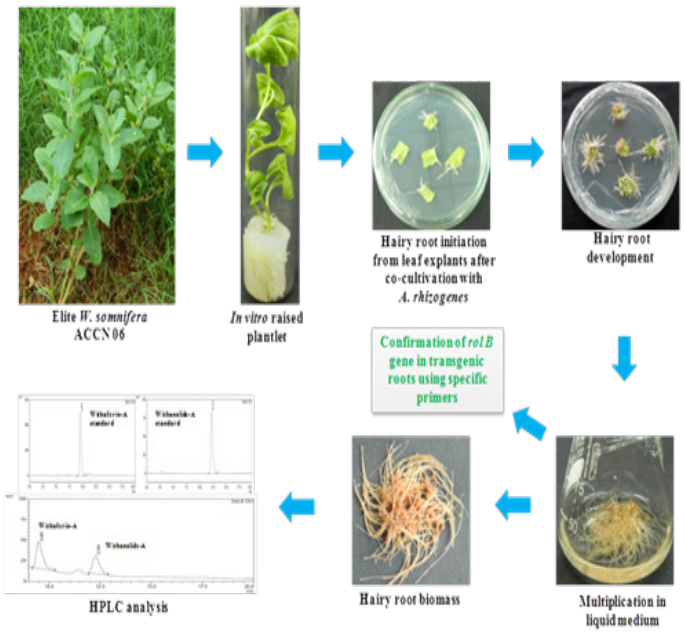

Figure 3 Schematic representation of withanolide production in hairy root culture of *W. somnifera.*

Conclusion

In *W. somnifera,* the physical leaf traits are found to be possible indicators of the quantity of the bioactive compound. This is further supported by the clustering of hyper-Withaferin A accessions in the phylogram constructed using physical leaf traits. Phytochemical screening of Withaferin-A content shows that all the accessions collected from Tamil Nadu contained this bioactive compound at easily detectable levels. Three accessions (ACCN06, ACCN12 and ACCN13) showed highest Withaferin-A content. The highest percentage of hairy root induction was obtained with *A. rhizogenes* strain R1000 into *W. somnifera* ACCN06 through 15s sonication and heat treatment at 41°C for 5min. These protocols offer a new avenue in large scale production of bioactive compounds from *W. somnifera*.

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Conflict of interest

The author declares no conflict of interest.

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