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Genetic Transformation using *Agrobacterium rhizogenes* for the Production of Valuable Anti-Cancer Compound, Withaferin-A from *Withania somnifera* (L.) Dunal

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Abstract

Withania somnifera (L.) Dunal is an important ayurvedic medicinal plant and its primary bioactive compound, withaferin-A possess several pharmaceutical and therapeutic values ranging from immunomodulation to anti-cancer property. For commercial production of withaferin-A, 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. Plant tissue culture methods are best alternative way to enhance commercial production of withaferin-A. In this present study, 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: *Ashwagandha*; Physical leaf traits; Withaferin-A; *Agrobacterium rhizogenes*; Sonication; Hairy roots.

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Introduction

Ashwagandha (*Withania somnifera* (L.) Dunal), commonly known as **Amukkiran kizhangu** is an important medicinal plant in the traditional medicinal system of Indian phamacopea. 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 [1]. Withaferin-A (Figure 1) is the major withanolides present in *W. somnifera*. Chemically, withaferin-A is 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 flux of isoprene units for synthesis of metabolic intermediates of triterpenoid pathway committed to withanolides biosynthesis.

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, adventitious shoot culture and cell culture systems [2] 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 [3]. However, *A. rhizogenes* mediated hairy root induction in *W. somnifera* is

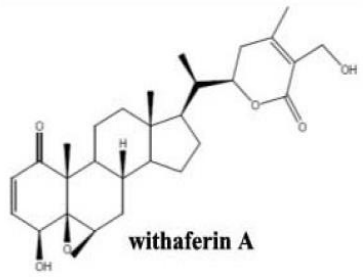

Figure 1: Structure of anticancer compound withaferin-A

limited due to the lack of efficient hairy root induction procedure [4]. Alternative approaches for an efficient hairy root induction are valuable for large-scale production of withanolides.

In this article, we have highlighted our recent research works on development of an efficient *Agrobacterium* mediated hairy root culture protocol for production of withaferin-A from the selected elite chemotype of *W. somnifera*.

Plant collection and screening

In this study, an attempt was made to develop a simple and novel method for rapid evaluation of germplasm by studying physical leaf traits and total withaferin-A content. 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. 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 [5].

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. These studies have attempted to describe the patterns of distribution of chemotypes 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 [6].

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 [5].

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. In the RAPD-based clustering, accessions ACCN06 and ACCN13 were found to be closely related, along with accession ACCN12 in a neighboring cluster [5].

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 ACCN06, ACCN12, and ACCN13, which have been selected for further studies [5].

Genetic transformation using bacterial culture of *A. rhizogenes*

This study was conducted to improve the transformation efficiency of *A. rhizogenes* for hairy root induction in *W. somnifera* ACCN-06 by means of sonication and heat treatment. 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 (18.6%) [7].

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 10 min). Using different heating times at 41 °C heat treatment for 5 min was increased the transformation frequency (76.0%) of hairy root induction. 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 20 s). Among the different time duration analyzed, 15 s sonication followed by 41 °C for 5 min heat treatment showed better transformation efficiency (93.3%) after 3 weeks [7]. We successfully transformed via SAAT method, which proved the high reliability of SAAT technology for the transformation of *W. somnifera*.

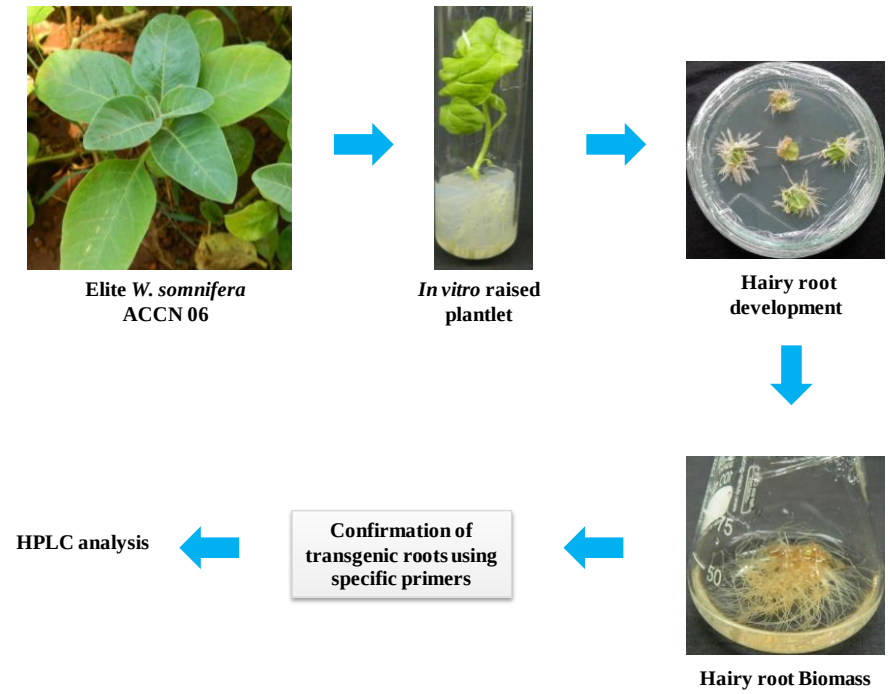

Figure 2: Flow chart of withaferin-A production in hairy root culture of *W. somnifera*.

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: 423 bp corresponding to the *rol* B gene fragment. Amplification product was not detected in DNA from non-transformed roots [7].

Withanolides content of hairy roots was analyzed through HPLC system. The maximum withaferin A (6.17 mg g L^{-1} DW) and withanolide A (3.82 mg 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 [7]. The present study found the presence of withaferin-A in transforming hairy root culture of the Indian *W. somnifera* produced using *A. rhizogenes*.

Summary

Phytochemical screening of withaferin-A content shows that all the accessions of *W. somnifera* collected from Tamil Nadu contained this bioactive compound. Accession ACCN06 showed highest withaferin-A content. The highest percentage of hairy root induction was obtained with *A. rhizogenes* strain R1000 into *W. somnifera* ACCN06 through 15 s sonication and heat treatment at 41 °C for 5 min.

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Conflict of interest

The authors declare that they have no conflict of interest.

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