

Genetic transformation in ashwagandha (*Withania somnifera* (L.) Dunal) for hairy root induction and enhancement of secondary metabolites

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Abstract

Genetic transformation was carried out in ashwagandha (*Withania somnifera* (L.) Dunal) using three different *Agrobacterium rhizogenes* strains viz., A4, ATCC 15834 and MTCC 2364, for inducing hairy roots. The explants such as hypocotyls, cotyledonary segments, leaf segments, shoot tips and nodal segments were used for genetic transformation. A4 and ATCC 15834 strains produced successful transformation and hairy (transformed) roots were induced from leaf segments and shoot tips. A4 strain produced transformation by direct inoculation of bacteria from single cell colonies as well as in the suspension form, but ATCC 15834 produced transformation only in the suspension form. Among the liquid media tested, half MS was found to be superior in promoting hairy root growth. The transformation was confirmed by PCR and dot blot analysis. A Thin Layer Chromatographic method was employed for withanolide estimation. The spot corresponding to withaferin A was observed under UV at 254 nm. Field root possessed more withaferin A followed by hairy roots and *in vitro* roots contained the least. Enhancement of secondary metabolite production was attempted through addition of osmoregulator, precursor feeding and elicitation. Withaferin A content in the hairy root biomass and the culture medium were estimated. The biotic elicitor *Aspergillus* homogenate (250 and 500 µl /125 ml) had a positive influence in the enhancement of secondary metabolites.

Key words : Genetic transformation, Ashwagandha, Hairy root

Introduction

Plants produce an array of secondary metabolites that find applications in pharmaceuticals, agrochemicals, flavours and fragrances. Advancements in genetic engineering have opened up new avenues to understand and produce precious products from the plants. *Withania somnifera* (L.) Dunal (Family: Solanaceae) commonly known as ashwagandha or Indian ginseng is a highly valued medicinal plant of the Indian system of medicine with a wide spectrum of biological activities to its credit (Zhao et al., 2002). It is an important herb in Ayurveda and has been in use for over 3000 years to treat human ailments (Sharma et al., 2011). Classically known for rejuvenative benefits, it is

the subject of considerable modern scientific attention. Although the leaves and fruit of ashwagandha are therapeutic, most of the herbal medicines available are derived from the roots. Ashwagandha is used for the treatment of anemia, anorexia, asthma, arthritis, bronchitis, carcinoma, edema, infertility, leucoderma, memory loss, paralysis, rheumatism, nervous exhaustion etc. (Umadevi et al., 2012). The medicinal properties of ashwagandha have been attributed to its chemical constituents mainly alkaloids and steroidal lactones (primarily of the withanolide class). It possesses anti-inflammatory, antioxidant, anticancer, immunomodulatory properties and many pharmacologically important chemicals and alkaloids (Naidu et al., 2003).

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Recent developments leading to the production of rapidly growing, genetically and biosynthetically stable organized hairy root cultures following the genetic transformation of plants with *A. rhizogenes* strains may revolutionize certain areas of plant cell biotechnology. The hairy roots are capable of fast growth on a hormone free medium. This system has the following wide-ranging applications: from production of secondary metabolites to foreign proteins, from restructuring of plant phenotype to the study of rhizosphere biology, from phytoremediation to molecular farming, ecology and evolution (Eapen and Mitra, 2001).

The inherent problem of slow growth rate of conventional root cultures can be overcome successfully by hairy root induction. Moreover, elicitation and modification of the culture conditions offer an interesting option to enhance secondary metabolite production. It would be a significant contribution to mankind if the bioactive compounds in ashwagandha could be successfully obtained through hairy root cultures. The study was undertaken to genetically transform ashwagandha for hairy root induction by inoculating seedling explants with *A. rhizogenes* and to establish hairy root cultures for production and enhancement of secondary metabolites.

Materials and Methods

Stock plants of ashwagandha were collected from Seed Farm, Mannuthy, Thrissur. Transformation was attempted from different explants i.e., hypocotyls segments, cotyledonary segments, shoot tips, leaf segments and nodal segments of ashwagandha. Hypocotyl segments and cotyledonary segments were excised from 10-15 days old *in vitro* seedlings germinated on half MS medium containing 2.5 per cent sucrose, whereas leaf segments, shoot tips and nodal segments were obtained from 2-2 1/2 months grown *in vitro* seedlings. The explants were pre-cultured on Murashige and Skoog's (MS) solid medium (Murashige and Skoog, 1962) in petri plates for two

days prior to their infection with bacteria.

The explants were infected by two different inoculation methods, Direct Inoculation Method (DM) and Suspension culture Inoculation Method (SM) using three *A. rhizogenes* strains namely A4, ATCC 15834 and MTCC 2364, which were initially cultured in Yeast Extract Broth (YEB) medium at $26 \pm 2^\circ\text{C}$. Bacterium from isolated single cell colonies was used as the inoculum in DM. The explants were trimmed properly with a sterile blade and ten pricks were made on each explant using hypodermic injection needle dipped in the inoculum. In SM, the explants were cut into suitable size and immersed in *Agrobacterium* suspension ($\text{O.D}_{600} \sim 1.0$) for 5 minutes with intermittent gentle agitation after prior pricking. The infected explants were blotted dry and were co-cultured in growth regulator free MS solid medium in petri plates under dark photoperiod at $26 \pm 2^\circ\text{C}$ for two days. The explants were then washed three times and transferred to solid MS containing 500 mg l⁻¹ cefotaxime for the elimination of bacteria and cultured at $26 \pm 2^\circ\text{C}$ under diffused light.

The adventitious roots that emerged from the explants one to three weeks after infection were excised and cultured in solid MS medium containing 250 mg l⁻¹ cefotaxime. The established root cultures which showed rapid growth, hairiness, lateral branching and plagiotropic growth habit were initially washed in liquid MS and then randomly cut into small pieces of 2.0-4.0 cm length. The root pieces which comprised both the root segments and root tips weighing ~ 0.5 g were transferred to 125 ml half MS liquid medium without antibiotics in 250 ml conical flask. The cultures were incubated in orbital shakers at 110 rpm under diffused light and dark condition for rapid multiplication. To study the effect of different culture media on the growth of hairy roots, the established hairy root segments were cultured in MS and half MS with 3.0 per cent sucrose and B5 with 3.0 and 2.0 per cent sucrose under dark photoperiod.

Transformation was checked by Opine, PCR and Dot blot analysis of *rolB* and *rolC* genes. Opine analysis was done according to the modified procedure given by Dessaux et al. (1991). The presence of agropinic acid, mannopine and mannopinic acid in hairy roots was analyzed by High Voltage Paper Electrophoresis at 400V/cm for 45 min. For PCR and dot blot analysis the DNA was isolated from the roots following modified Doyle and Doyle (1987) method. The *E. coli* strains containing pLJ1 cosmid with kanamycin resistance was used as the positive control. Cosmids were isolated from *E. coli* using alkaline mini prep procedure as given by Birnboim and Doly (1979). For amplifying *rolB* gene, the primer set RolB F2R2 (RolB F2 5'- gAAgCCTgCTgCAGTAAACC-3') (RolB R2 5'- TTCAGCAGCAGgATCAACAC-3') was used and the primer set RolC F1R1 (RolC F1 5'- TTAGCCgATTgCAAACCTgCTC-3') (RolC R1 5'- ATggCTgAAgACgACCTgTgTT-3') was used for amplifying *rolC* gene. The PCR products obtained from pLJ1 cosmid, using RolB F2R2 primer set (205 bp) and RolC F1R1 primer set (520 bp) was used as probes separately in dot blot analysis.

A quantitative Thin Layer Chromatography (TLC) method was used for the estimation of withanolides namely withaferin A. The ethanol extracts were spotted with standard withaferin A on Silica gel F_{254} Plates and analyzed in solvent system CHCl_3 : CH_3OH (9.8: 0.2). The spots were visualized using spray reagent vanillin (0.05 g), boric acid (1.0 g) Conc. H_2SO_4 (2.0 ml) and methanol (100 ml). A TLC densitometry technique was used for the quantification of withaferin A.

The hairy roots obtained from A4 derived root clones were subjected to enhancement studies using techniques such as addition of osmoregulator, precursor feeding and elicitation. Twenty day old hairy roots were cultured separately in half MS media supplemented with osmoregulator polyethylene glycol (PEG 6000) 2.0 and 5.0 per cent at pH 5.7 and precursor methionine 1.0 mM and

2.0 mM for eight days. The hairy root culture and the media were collected and the withaferin A content was analysed by TLC. Biotic elicitors used were *Aspergillus niger* homogenate and yeast extract. The mycelial mass of *Aspergillus niger* was grown for seven days in 50 ml LB, filtered through muslin cloth, dispersed in 40 ml distilled water and homogenized. The homogenate was autoclaved and added at the rate of 250 μl and 500 μl per 125 ml half MS media in 250 ml conical flask. Twenty five day old root culture was inoculated in the above media and was incubated in rotary shaker at 110 rpm for 72 hrs. Hairy roots were also inoculated in half MS liquid medium supplemented with 2.5 g l^{-1} and 5.0 g l^{-1} yeast extract to elicit the cultures. All the experiments were carried out in three biological replicates and the average values are given in Tables and Figures. Statistical significance was calculated by students t-test using GraphPad Prism 6.

Results and Discussion

Among the different explants tested, leaf segments of ashwagandha were found to be the best explant for efficient transformation followed by shoot tip and other explants like hypocotyl segments, cotyledonary segments and nodal segments failed to produce any successful transformation. The hairy roots were induced more from the petiolar region in the case of leaf segments and in the absence of petiole, hairy roots were produced from the proximal cut edges. From the shoot tips, hairy roots were produced from and around the basal portion only and no roots developed from the leaves attached to shoots. The age, hormonal balance and differentiation status of these tissues would have favoured effective transformation (Nin et al., 1997). The bacterial inoculum used affects the transformation frequencies. The transformation efficiency of different *A. rhizogenes* strains with respect to leaf segment and shoot tip in response to DM and SM is given in Fig. 1 and 2. When the bacterial colonies were used as the inoculum (DM), only A4 strain produced transformation, and no

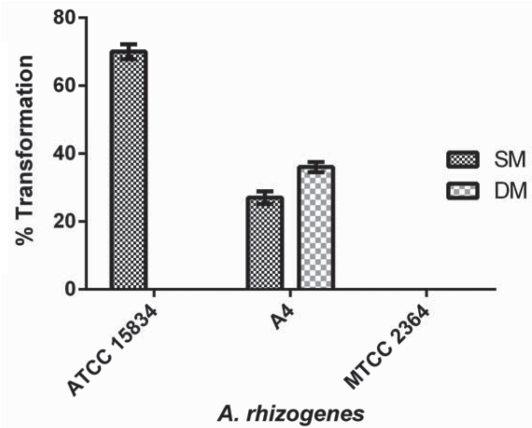


Figure 1. Transformation efficiency of *A.rhizogenes* strains with respect to leaf segment in response to DM and SM

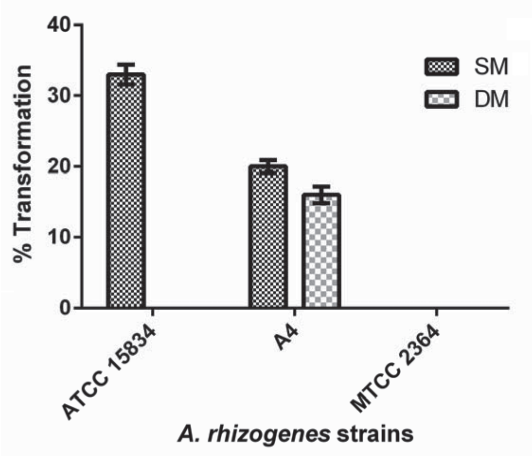


Figure 2. Transformation efficiency of *A.rhizogenes* strains with respect to shoot tip in response to DM and SM

transformation was produced by ATCC 15834 and MTCC 2364. On using bacterial suspension as the inoculum (SM), both A4 and ATCC 15834 produced successful transformation. The motile strains of *Agrobacterium* exhibit virulence only in liquid medium but mutant strains (non motile) exhibit virulence when inoculated directly on wounds (Hawes et al., 1988). Here the superior performance of ATCC 15834 in suspension form than single cell colonies may be attributed to two reasons, firstly ATCC 15834, being motile, the strain can take advantage of the liquid medium to facilitate better attachment to the wounded cells than they get when

applied as colonies. Secondly the optimum concentration of bacteria (ATCC 15834) for producing successful transformation might be present in bacterial suspension compared to colonies. But the A4 strain could exhibit virulence both when inoculated as suspension or as single cell colonies. The greater concentration of bacteria in the colonies of A4 might have favoured the transformation (Patena et al., 1988). The strain MTCC 2364 was found to be avirulent irrespective of the nature of the inoculum and hence failed to produce any transformation.

The strain ATCC 15834 showed highest efficiency (70%) in transforming the plant tissues, followed by A4 strain (36%). The strain MTCC 2364 failed to produce any successful transformation. In general 9-20 days was taken for hairy root induction. The mean of hairy roots per transformed leaf segment was 3.33 and from the shoot tip the mean was 2.66. The hairy roots induced by ATCC 15834 was initially creamy white in colour, relatively thick with high root hairs compared to that of A4 strain which produced relatively thin white hairy roots with comparatively less root hairs (Fig. 3). Hairy roots show morphological variations depending upon the interaction nature of plant cell phenotype and strain of the bacterium and show differences in root thickness, degree of branching and amount of hairy

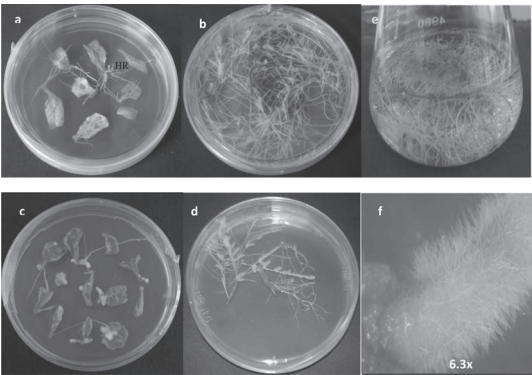


Figure 3. Emergence and establishment of hairy roots induced by A4 (a, b) and ATCC 15834 (c, d) ; Culturing of hairy roots in 1/2MS liquid medium (e) Microscopic view of hairy roots (f)

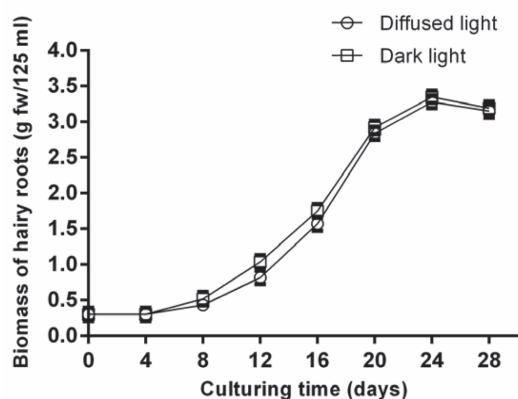


Figure 4. Growth kinetics of *W. somnifera* hairyroots under diffused light and dark

root production (Bapat and Ganapathi, 2005). The hairy roots showed fast growth with high lateral branching in hormone free basal media. Most of the hairy roots exhibited plagiotropic growth habit and some showed reduced geotropism (Sevon and Oksman-Caldentey, 2002). The hairy roots exhibited a sigmoid growth pattern. From the growth curve of hairy roots (Fig. 4), it was observed that the root growth was nil in the first four days and was very slow up to 10 days. However hairy roots grew faster and subsequently lots of lateral branches were produced within 12-15 days of incubation and the growth was much faster in the next 15-20 days. After 20 days of culture, the growth rate of hairy roots began to slow down, but the biomass of hairy roots still increased until 25 days of culture. After 25 days, the hairy roots gradually changed colour from creamish white to brown and the biomass began to decrease thereafter (Xu et al., 2004). The growth of the roots was initially slow under dark photoperiod. However by the end of 25 days root produced almost equal (3.28 g/FW/125 ml) biomass compared to roots incubated under diffused light (3.33 FW/125 ml). The hairy roots induced by A4 strain showed faster growth compared to ATCC 15834, producing more biomass.

The culture medium was found to have a significant effect on ashwagandha hairy root growth (Fig. 5).

Among the four liquid media tested, half MS was found to be superior for promoting hairy root growth followed by MS, B5 with 2.0 per cent sucrose and B5 with 3.0 per cent sucrose respectively. In B5 with 3.0 per cent sucrose, the roots showed callusing in addition to bulging and the growth was found to be very poor in this culture medium. In half MS media, roots were of normal thickness, characterized by very fast growth and high lateral branching. Hairy roots of each species or specifically each root clone have particular optimum concentrations of sucrose and mineral ions for producing maximum biomass (Oksman-Caldentey et al., 1994).

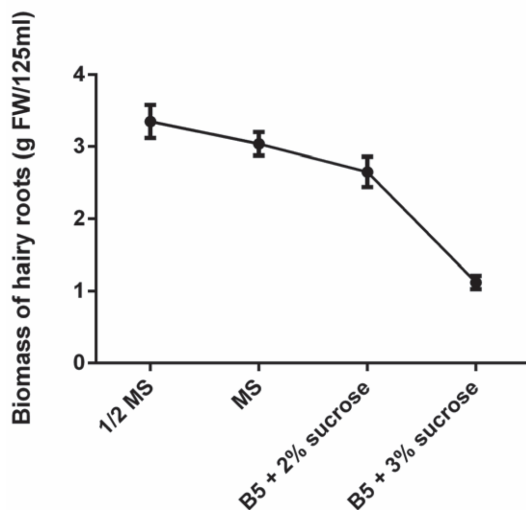


Figure 5. Effect of culture media on the growth of hairy roots

The confirmation of transformation by HVPE was unsuccessful here because of the presence of interfering substances that showed positive reaction to silver nitrate staining (Yoshimatsu et al., 2003). It was noticed that, both transformed and non transformed roots produced spots at positions corresponding to agropinic acid and no spot was produced at positions corresponding to mannopine and mannopinic acid after silver staining. Polymerase Chain Reaction was used to demonstrate the presence of TL-DNA with *rol B* and *rol C* genes in the transformed roots (Fig. 6). Two fragments of length 205 and 520 bp corresponding to *rol B* and *rol C* gene respectively

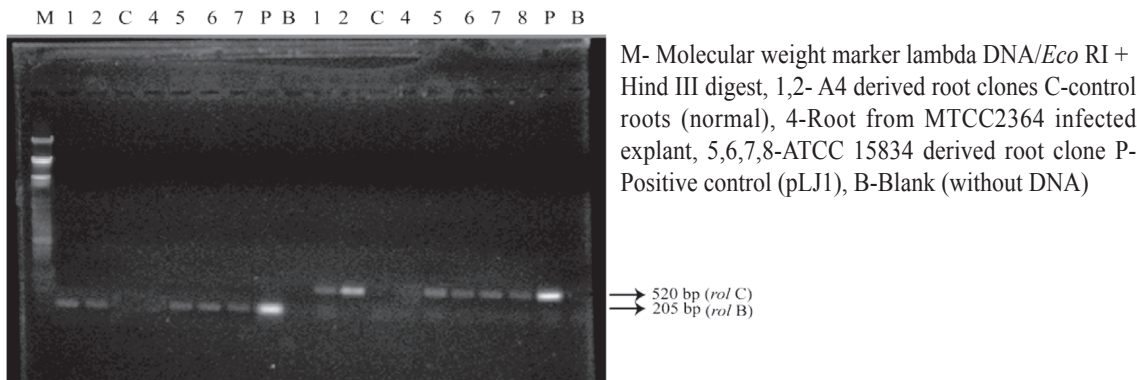


Figure 6. PCR analysis of hairy roots for *rol C* and *rol B* genes

were amplified from the hairy root cultures of A4 and ATCC 15834 and cosmid pLJ1 but not from untransformed roots (control, MTCC 2364 and blank). These results indicated that the *rol B* and *rol C* genes from the Ri plasmid of *A. rhizogenes* A4 and ATCC 15834 were successfully integrated into the genome of ashwagandha hairy roots. The roots produced from MTCC 2364 infected explants were non-transformed.

Transformation was further confirmed by dot blot analysis (Fig. 7). On using RolB F2R2 and RolC F1R1 primer sets derived probes of pLJ1 cosmid separately, positive signal of radioactivity was obtained from A4 and ATCC 15834 induced hairy roots, whereas root produced by MTCC 2364 infected explants and control roots showed no

signal. This confirmed the presence of TL-DNA with *rol B* and *rol C* genes in A4 and ATCC 15834 induced hairy roots.

The estimation of withaferin A content of fresh root samples including roots from field grown plants, non transformed *in vitro* roots and transformed hairy roots were carried out. The spot corresponding to withaferin A standard was initially magenta and on further charring, the colour turned to bluish violet (Rf 0.56). The amounts of withaferin A content in different fresh root samples obtained by using TLC densitometry analysis are given in the Table 1.

The addition of osmoregulant PEG (6000g) and precursor was found affecting the hairy root growth in no way. However they failed to elicit a positive

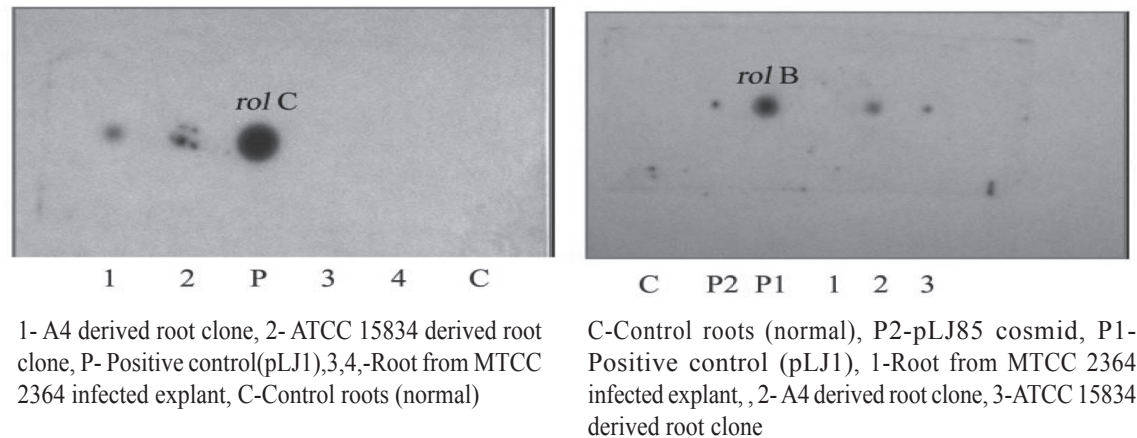


Figure7. Dot blot analysis of hairy roots for *rol C* and *rol B* genes

Table 1. Amount of withaferin A in different samples – TLC densitometry analysis

Sl. No.	Sample	Values in mg g ⁻¹
1	Roots of field grown plants	0.258 (0.025)
2	Normal <i>in vitro</i> roots	0.174 (0.017)
3	Hairy roots	0.189 (0.019)

% value is given in paranthesis

response in the biosynthesis of withaferin A in root cultures. The addition of biotic elicitor *Aspergillus* homogenate elicited a positive response on biosynthesis of withaferin A in the hairy roots of ashwagandha (Table 2). *Aspergillus* homogenate at the rate of 250 and 500 µl /125 ml produced 2.25 and 1.77 times increase (0.436 mg g⁻¹ and 0.334 mg g⁻¹) respectively in withaferin A content over control hairy roots (0.189 mg g⁻¹). The metabolite was found to be released into the media as well. The withaferin A contents in the respective media samples were 0.088 g ml⁻¹ and 0.078 g ml⁻¹. The oligosaccharides liberated from the cell wall of *Aspergillus* were the best elicitors that could have increased the cell permeability facilitating enhanced secondary metabolite production (Sevon and Oksman Caldentey, 2002). The hairy root cultures showed reduction in the accumulation of withaferin A content on adding yeast extract at the rate of 2.5 and 5.0 g l⁻¹.

Table 2. Quantitative estimation of withaferin A content in elicitor treated hairy root samples

Culture	Sample name	Withaferin A content (mg g ⁻¹)
Root biomass	S _{AH} -250	0.436 (0.043)
	S _{AH} -500	0.334 (0.033)
	S _{Control} (28days)	0.189 (0.019)
	S _{YE} - 2.5	0.086 (0.008)
	S _{YE} - 5.0	0.043 (0.004)
	S _{control} (20 days)	0.174 (0.017)
Media	M _{AH} - 250	0.088 µg ml ⁻¹
	M _{AH} -500	0.078 µg ml ⁻¹
	Control	0.000 µg ml ⁻¹

(AH-Aspergillus homogenate, YE-Yeast extract, % value is given in paranthesis)

To conclude, the hairy roots of ashwagandha offer a promising system for the synthesis of withanolides and elicitation is one of the best ways to trigger withanolide production. In this context, development of fast growing root culture system offers unique opportunities for providing root drugs in the laboratory, without resorting to field cultivation.

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