African Journal of Biotechnology Vol. 11(98), pp. 16412-16420, 6 December, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3867
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Development and optimization of hairy root culture systems in *Withania somnifera* (L.) Dunal for withaferin-A production

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Accepted 2 April, 2012

Transformation of *Withania somnifera* was carried out by using three *Agrobacterium rhizogenes* strains (ATCC 15834, R1000 and K599) for hairy root induction. Induction of hairy root was carried out in leaf, petiole and internodal explants. Hairy root induction was successful only in ATCC 15834 and R1000. The highest frequency of hairy root was obtained in petiole explants (64%) infected with R1000 and it resulted in five distinct morpho-types (callus (fragile), callus (hard), callus + hairy roots, hairy roots and callusing roots). The frequency of R1000 transformation was increased up to 93.2% by the addition of acetosyringone during various steps of infection. Molecular identification through PCR analysis of *rolC* confirmed the presence of *Ri* T-DNA. The half strength Murashige and Skoog (MS) liquid medium was found to be the best medium that supports the high root biomass accumulation than the other tested medium types (MS full strength, Gamborg B5 medium (B5) full strength and B5 half strength). High performance liquid chromatography (HPLC) analysis of hairy roots revealed the accumulation of withaferin-A (72.3 mg/g dw). This study reports the influence of *Agrobacterium* strains, explant types and acetosyringone in the hairy root induction of *W. somnifera.*

Key words: *Withania somnifera,* hairy root, *Agrobacterium rhizogenes,* hairy root morpho-types.

INTRODUCTION

Withania somnifera (Ashwagandha) is one of the most versatile plants used in traditional Indian medicine system (Ayurveda). It is highly reputed as 'Indian ginseng' and is a member of plants generally regarded as safe (GRAS) (Jayaprakasam et al., 2003), which is cultivated in India for their medicinal purposes. The roots and leaves of Ashwagandha contain various alkaloids, viz., withanolides and withaferins (Atta et al., 1991). Studies show that the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, antitumour, astringent and more recently, to treat ulcers, bacterial infection, venom toxins and senile dementia. Clinical trials and animal research supports the use for anxiety, cognitive and neurological disorders, inflammation, hyperlipidemia and Parkinson's disease (Gupta and Rana, 2007). *In vivo* research on Ashwagandha as an antitumor agent confirms its usefulness in slowing tumor growth and increasing survival time. Recently, the ability of withaferin-A in the inhibition of human breast cancer cells growth *in vivo* through G2 and mitotic arrest has been reported (Silvia et al., 2008). Presently, withanolides have been commercially obtained by solvent extraction of roots of the plants collected from the natural sources. Low yield, genotypic and chemotypic variations, heterogeneity in content, long gestation period (4 to 5 years) between planting and harvesting and uneconomical chemical synthesis are major constrains in industrial withanolide production. Continuous collections of this plant from the natural habitats brought this precious species under the category of threatened species by the International Union for Conservation of

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Nature and Natural Resources (Kavitra et al., 2000; Supe et al., 2006).

Until now, there has been no commercial process as an alternative for root-derived compounds, except in case of utilizing hairy root culture systems. Unfortunately, although many important metabolites were found in cell-culture, those cell cultures usually have a strong tendency to be genetically and biochemically unstable and are often synthesized at very low levels of useful secondary metabolites. This low concentration of certain secondary metabolites is largely due to developmental stages of these production cells, which divide and grow rapidly but without undergoing differentiation. Compared with suspension cell culture, transformed hairy root is highly differentiated and can produce plant materials that are rich in secondary metabolites. This elevated level of secondary metabolites and the rapid growth of transfected hairy roots are just the features of a successful production model for useful phytochemicals.

Transgenic hairy root cultures have revolutionized the role of plant tissue culture in secondary metabolite production. They are unique in their genetic and biosynthetic stability, faster in growth, and more easily maintained. Using this methodology, a wide range of chemical compounds has been synthesized (Shanks and Morgan, 1999; Giri and Narasu, 2000). The greatest advantage of hairy roots is that their cultures often exhibit approximately the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Kim et al., 2002). In view of the aforesaid, the present study was designed to generate hairy root system of *W. somnifera* using *Agrobacterium rhizogenes* strains ATCC 15834, R1000 and K599 and optimize the culture conditions for high root biomass productivity and withaferin-A production.

MATERIALS AND METHODS

Preparation of explants

From four to five weeks old axenically propagated *W. somnifera* plants, leaf discs (9 mm diameter) were cut using a cork-borer, nodal and intermodal explants were also collected from the same plant.

Preparation of *A. rhizogenes* culture for infection

The *A. rhizogenes* (ATCC 15834, R1000 and K599) stored at -80°C were first activated by inoculation on solid YMB media and cultured at 28°C for two days. Then, the bacteria were transferred into liquid YMB media by using a sterile bacterial loop and cultured on an orbital shaker at 280 rpm and 28°C overnight. To study this influence, 50 µM acetosyringone was added to the following steps in the transformation:

i) Activation of bacterial culture through addition of 100 µM AS an hour prior to infection.
ii) Addition of 100 µM AS in co-cultivating MS medium.
iii) Activation of bacterial culture (i) and addition in co-cultivating medium (ii).

Infection and co-cultivation

The explants were immersed in prepared bacterial suspension and swirled for various times (10 and 20 min) in different experiments. Infected explants were blotted on Whatman No. 1 paper to remove excess of *Agrobacterium* culture and placed directly on MS basal medium. Co-cultivation was carried out for two days at 16/8 h light/dark condition. After two days co-culture with different strains of *A. rhizogenes* (ATCC 15834, R1000 and K599) on hormone-free MS basal media, the explants were washed five times with sterile water to remove superficial bacteria and then were transferred to MS basal medium containing cefotaxime (250 mg/l) to further eliminate excess bacterial growth.

Cultivation of hairy roots

After hairy roots initiated from the edges of the explants and grew to nearly 2 to 5 cm long, they were excised and subcultured into solid MS basal medium for two months. Then they were transferred to shake flasks in 20 ml liquid medium for 28 days unless described otherwise. Each flask was inoculated with 3 to 5 cm long root.

Determination of root biomass

The roots were separated from the media by using a stainless steel sieve. Their fresh weights were determined after they were washed with distilled water and the excess surface water blotted away. Dry weights were recorded after the roots were dried at 60°C till constant weight is recorded.

Extraction of withaferin-A

Two hundred millgram (200 mg) dry weight (DW) of root tissue was taken for analysis. The tissues were ground and extracted overnight in methanol (5 times w/v) on a rotary shaker at 26°C and 100 rpm. The procedure was repeated three times and the methanolic extracts were pooled together. The extracts were filtered through Whatman No.1 filter paper and diluted with deionised water (1 methanol: 4 water). The resultant solution was extracted with 3 volumes of chloroform. Chloroform layer was separated from other layers through a separating funnel. The chloroform extract was dried and the residue was dissolved in 1 ml methanol, filtered through a 40 µM nylon filter and 20 µl was used for HPLC analysis. The standard of withaferin-A dissolved in methanol at strength of 0.5 mg/l and 20 µl was used for HPLC analysis (modified from Roja et al., 1991).

Polymerase chain reaction (PCR)

Genomic DNA was extracted using CTAB method (Doyle and Doyle, 1987) from each of the hairy root lines as well as from control non-transformed roots. PCR primers specific for the amplification of the 557 bp fragment of the *rolC* gene were used. A 50 µl PCR mix contained 200 ng of DNA, 10 pmoles primers, 200 µM dNTP mix, 1U of Taq DNA polymerase, 1X PCR buffer and 2 mM $MgCl_2$. PCR conditions were 94°C for 5 min, 42 cycles of 94°C for 1 min, 52.5°C for 1.5 min and 72°C for 2 min, and a final extension at 72°C for 10 min. The sequences of the primers used in the PCR are as follows:

Forward primer 5'ATGGATCCCAAATTGCTATTCCCCC ACGA3'
and Reverse primer 5'TTAGGCTTCTTTCATTCGGTTTACTG CAGC 3'

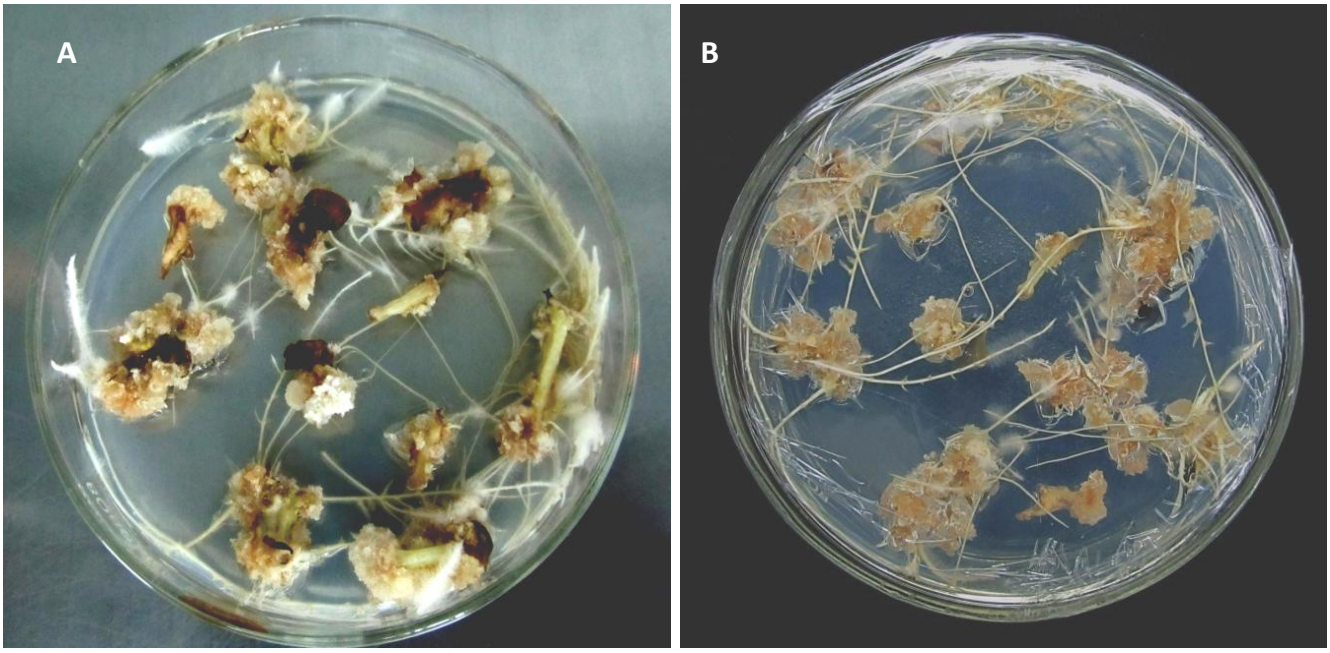

Figure 1. R1000 induced hairy roots in petiole explants of *W. somnifera*, a) front view and b) back view.

RESULTS AND DISCUSSION

Induction of hairy roots

After co-cultured with agrobacteria for three days in Petri dishes with MS medium containing cefotaxime, hairy roots began to develop from the brink of the infected explants (Figure 1). When they grew to nearly 2 cm in length, hairy roots were excised from explants and transferred to new MS medium without any auxin. After one month, roots that did not survive were normal roots, whereas roots that survived were true hairy roots. Hairy roots are capable of synthesizing endogenous auxin, thus require no supplemental auxin. The autotrophy in auxin production reflects the expression of genes within the T-DNA of the Ri plasmid from *A. rhizogenes*. The survived roots displayed the typical phenotypes of hairy roots, such as rapid growth, highly branched, plagiotropism and auxin autotrophy.

Effect of explant type on hairy root transformation

Among the different explants tested (leaf, petiole and internode), only the petiole explants induced hairy roots at greater percentage (64%) whereas the internodal segments and leaf were comparatively lesser than the petiole explants (Figure 2; Table 1). Juvenility and nature of explant influence the *Agrobacterium* mediated transformation process (Yonemitsu et al., 1990; Trypsteen et al., 1991). Nin et al. (1997) have reported that specificity of *Agrobacterium* transformation is closely connected with the age and hormonal balance of the host tissue. Potrykus (1990) stated that wound response was the most important factor for the successful transformation. He reported that explants with pronounced wound response develop larger populations of wound adjacent competent cells for regeneration and transformation. It is quite evident that different explants vary in their wound response, that is, produce the number of competent cells for transformation. The explant cells differ in their DNA synthesis and cell division ability due to the difference in physiological maturity of the cells. The present observation that petiole explants induced hairy roots may be due to their ability to produce greater number of wound adjacent competent cells for regeneration and transformation.

Frequency of *Agrobacterium rhizogenes* transformation

The strains of *A. rhizogenes* varied greatly in their ability to induce hairy roots, and their frequency of transformation ranged from 0 to 64%. R1000 had the highest induction frequency in petiole explants (64%) compared to leaf (42.5%) and internode (37.7%), while K599 did not shown infection in any of the explants studied. The infectivity of other strain (ATCC 15834) fell between the two strains (Table 1). The differential efficiency of various *A. rhizogenes* strains in promoting the induction, growth and secondary metabolite production of hairy roots has been reported in various plants. The strains of *A. rhizogenes* affected growth rate,

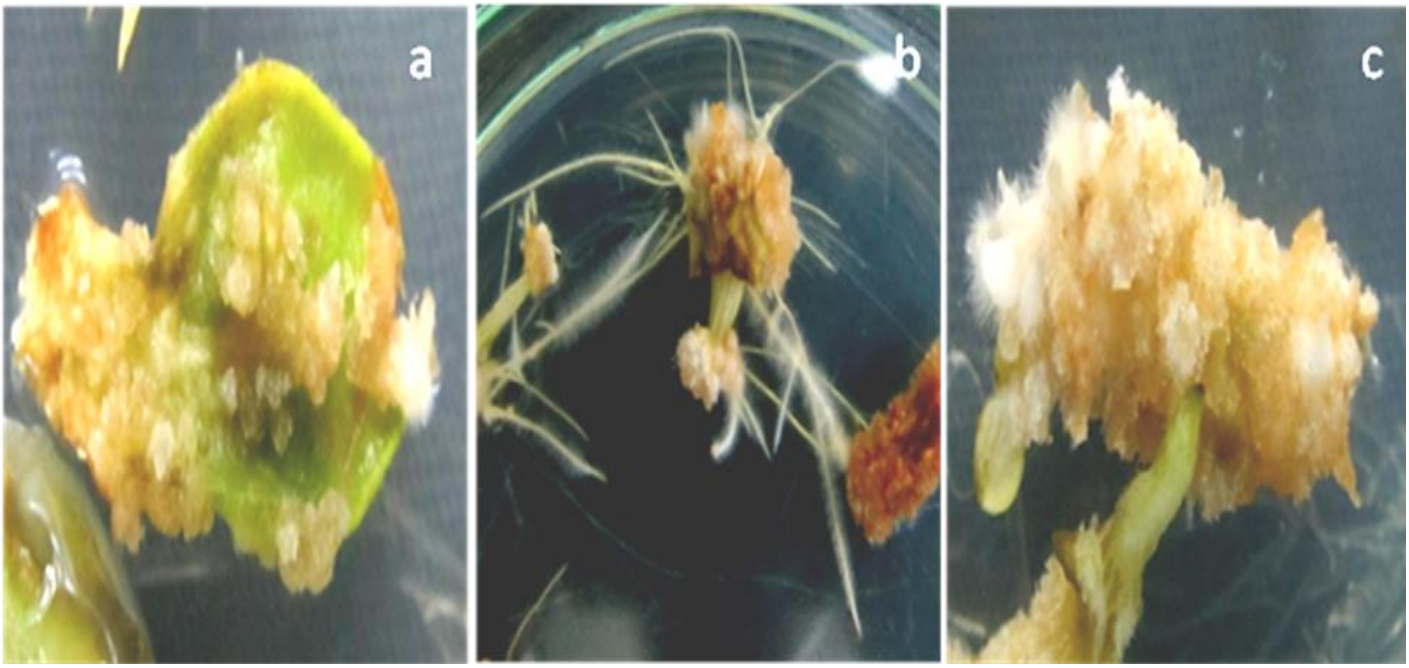

Figure 2. a) R1000 induced morphogenetic response in leaf explants; b) R1000 induced morphogenetic response in petiole explants and c) R1000 induced morphogenetic response in internodal explants.

Table 1. Comparison of different strains of *Agrobacterium rhizogenes* in their ability to induce hairy roots on various explants of *W. somnifera*.

Agrobacterium **strain**	**Infection frequency**					
	Experiment I			**Experiment II**		
	Leaf	**Petiole**	**Internode**	**Leaf**	**Petiole**	**Internode**
ATCC 15834	18.9% (7/37)	29.7% (11/37)	16.6% (5/30)	23.4% (11/47)	28.6% (10/35)	15.6% (5/32)
R1000	42.5% (20/47)	64.0% (32/50)	36.1% (17/47)	40.0% (16/40)	62.8% (22/35)	37.7% (17/45)
K599	0 (0/35)	0 (0/47)	0 (0/40)	0 (0/37)	0 (0/35)	0 (0/45)

saponin production and the ratio of different astragalosides in transgenic root cultures of *Astragalus mongholicus* (Ionkova et al., 1997). *Agrobacterium* strain also influenced the development, growth rate and tropane alkaloids production in transformed root cultures of *Hyoscyamus muticus* (Vanhala et al., 1998). Hairy root cultures of *Gentiana macrophylla* were established by infecting with four *A. rhizogenes* strains and each hairy root lines showed different response regarding growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures (Tiwari et al., 2007). Clearly, the selection of an effective *Agrobacterium* strain for the production of transformed root cultures is highly dependent on the plant species, and must be determined empirically. The frequency of transformation is calculated by the number of explants induced with hairy roots divided by the total number of explants used for transformation.

Effect of acetosyringone on R1000 transformation frequency

Factors including *Agrobacterium* strains, age and differentiation status of plant tissue, coculture time, and activation factor, that is, acetosyringone, are often considered in order to increase frequency of transformation (Luo et al., 2004). Acetosyringone is one of the phenolic inducers of the virulence genes of agrobacteria; the addition of the chemical into cultures of *A. tumefaciens* was found to increase transformation rate in *Arabidopsis thaliana* and *Atropa belladonna* (Mathews et al., 1990; Sheikholeslam and Weeks, 1987). Therefore, acetosyringone (AS) was added into various steps of R1000 transformation to increase the frequency of hairy root induction from *W. somnifera* petiole explants:

i) Activation of bacterial culture through addition of 100

µM AS an hour prior to infection.
ii) Addition of 100 µM AS in co-cultivating MS medium
iii) Activation of bacterial culture (i) and addition in co-cultivating medium (ii)

Addition of AS in both steps i and ii has notably increased the transformation frequency (Figure 2) compared to the control (without addition of AS in any steps in the transformation). Studies showed that the promoting effect of acetosyringone on *A. rhizogenes* mediated transformation in *Brassica napus* (Boulter et al., 1990), *Salvia miltiorrhiza* (Hu and Alfermann, 1993), and *Artemisia annua* (Giri et al., 2001).

Hairy root morpho-types

Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone free medium. The transformed root is highly differentiated and can cause stable and extensive production of secondary metabolites. But, the majority of the petiole explant infected with R1000 strain showed morphological variability, that is, certain explants failed to produce any roots, instead, it produces only callus after infection; whereas, some of the explants produced roots only after very short callusing phase, and most of the explants successfully produced normal hairy roots. Among the hairy roots, few hairy root lines produced callus in the roots (Figure 3). The growth rates of transformed roots are known to vary greatly between species, but differences are also observed between different root clones of the same species (Mano et al., 1989). The alteration of the phenotype of transgenic tissue is more likely related to the position of the T-DNA in the plant genome and its subsequent expression (Zhu et al., 2001) and it has been reported by many authors in various plants. The development of callus may be attributed to TR-DNA insertion since it harbours the *aux* genes and its expression could result in additional auxin production and callus formation in transformed root lines (Robins et al., 1991; Jung et al., 1995). The callusing root cultures could be due to the expression of the *aux1* gene as reported earlier in carrot, *Hyoscyamus muticus* and *Cucumis sativus* (Guivarc'h et al., 1999). In *Catharanthus roseus*, transformed root cultures showing *rol*A and B^{-}/*ags*$^{+}$ insertions were also correlated with callusing, indicating a role for the TR-DNA in the callus morphology (Batra et al., 2004). The callusing root phenotype (harbouring both the TL and TR-DNA segments) could depend on the number of inserts of TL and TR segments, since both segments can be transferred independently (Villaine and Casse-Delbart, 1987) (Figure 4).

Standardization of medium type and strength for hairy root culture

Many reports described the effects of medium composition on the growth rate of hairy roots. Nutritional factors might affect the number of lateral branches per unit length or the average cross-sectional dimensions of the roots (Hilton and Rhodes., 1990). Medium types (MS and B5) and strength (full strength and half strength) displayed significant effect on both fresh and dry weight of *W. somnifera* hairy roots (Figure 5).

Half strength B5 medium was the weakest medium for the growth of *W. somnifera* hairy roots. Both the fresh and dry masses of hairy roots grown in half strength B5 medium were significantly lower than that in half strength MS and full strength B5, MS medium. MS medium was the best medium among the four media studied, in which the dry mass of hairy roots attained 0.46 g per flask after 30 days (Figure 5).

As demonstrated by Saenz-Carbonell et al. (1997), hairy roots are less susceptible to manipulation by changes in medium composition than callus and cell suspension cultures. Half strength MS has shown increased fresh and dry weight of roots; the content of nitrogen could be an important factor that affected the growth of *W. somnifera* hairy roots. Since full strength MS and B5 contained more nitrogen, approximately 22.5 and 26.8 mM than their half strength counterpart, these observed results are in accordance with Wysokinska and Rózga (1998) in *Paulownia tomentosa*, where the hairy roots did not grow in full strength B5 and MS media. When medium was diluted to half strength, either B5 or MS, they grew rapidly. But for some species, the medium strength seemed not to influence the growth of hairy roots. As reported by Giri et al. (2001), hairy roots of *Artemisia annua* grew well on 1.0, 0.5 and 0.25 strength MS media (Figure 6).

Different impact patterns of the changes of medium strength on the synthetic secondary metabolites were found in different species. As the medium strength decreased to half strength, solasodine level in *Solanum mauritianum* hairy roots was lowered but ajmaline and ajmalicine levels in *Pauvolfia micrantha* were increased (Drewes and van Staden, 1995; Sudha et al., 2003).

Molecular identification and quantification of withaferin-A

To confirm the integration of T-DNA from the *A. rhizogenes* into the hairy root genomic DNA, DNA from hairy roots were subjected to PCR analysis. PCR was used to demonstrate that the T-DNA from the Ri plasmid of *A. rhizogenes* was present in *W. somnifera* hairy roots. The PCR results showed that all the hairy root lines contained *rolC* gene which was a part of T-DNA of Ri plasmid of *A. rhizogenes* (Figure 7).

In the present study, HPLC was used to assess the hairy roots *W. somnifera* for the production withaferin-A in dry roots (Figure 8). The amount of withaferin-A was 72.3 mg/g of dw of hairy roots.

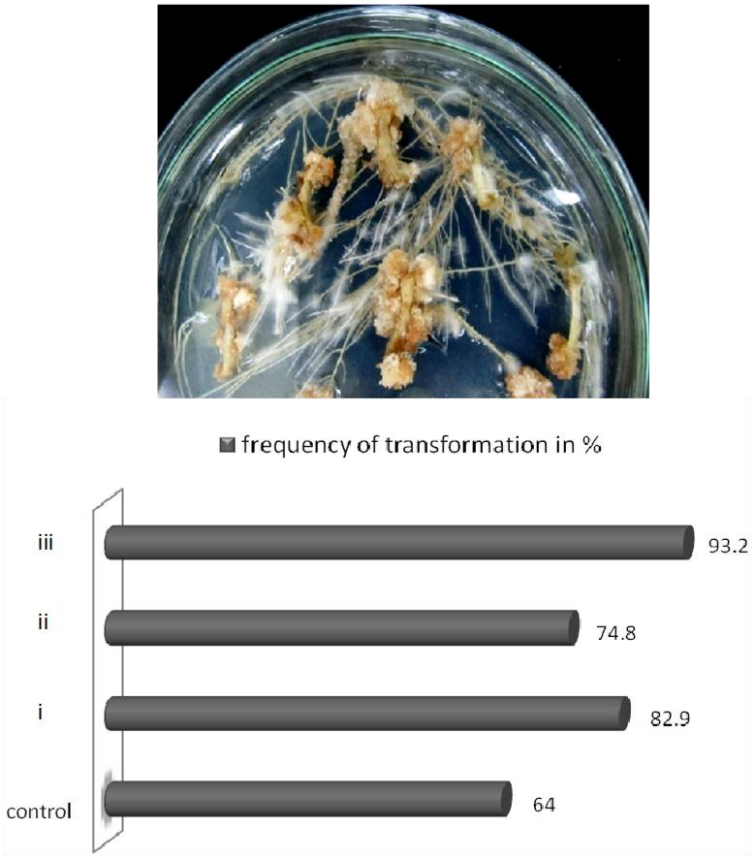

Figure 3. Influence of AS on the frequency of *A. rhizogenes* R1000 transformation.

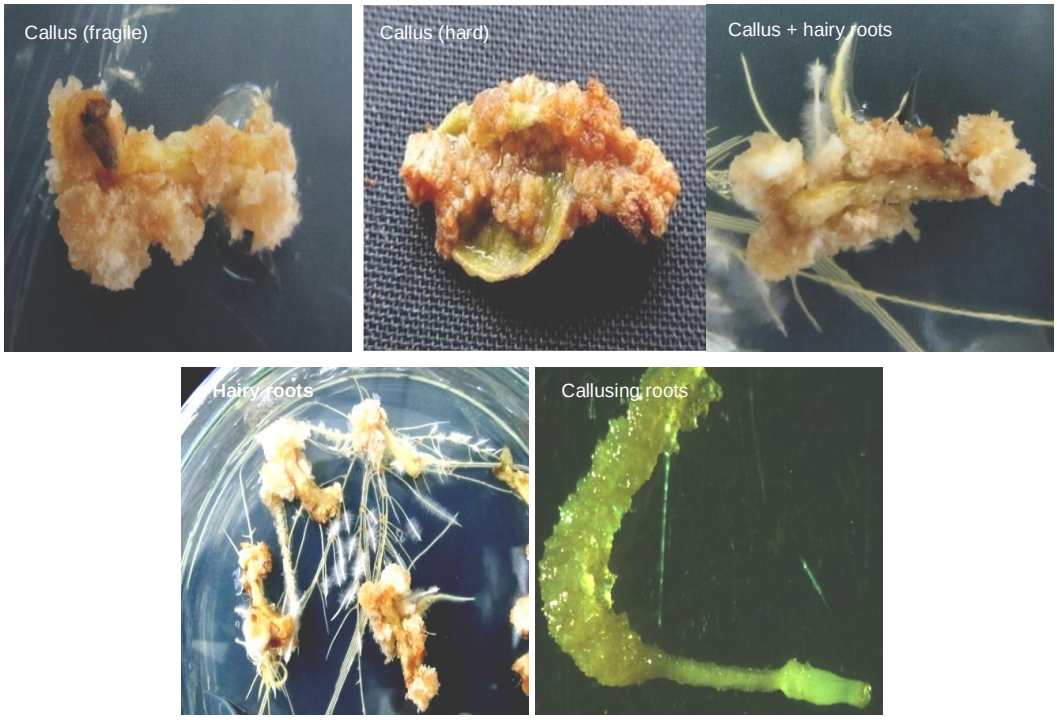

Figure 4. *A. rhizogenes* R1000 induced hairy root morpho-types in *W. somnifera*.

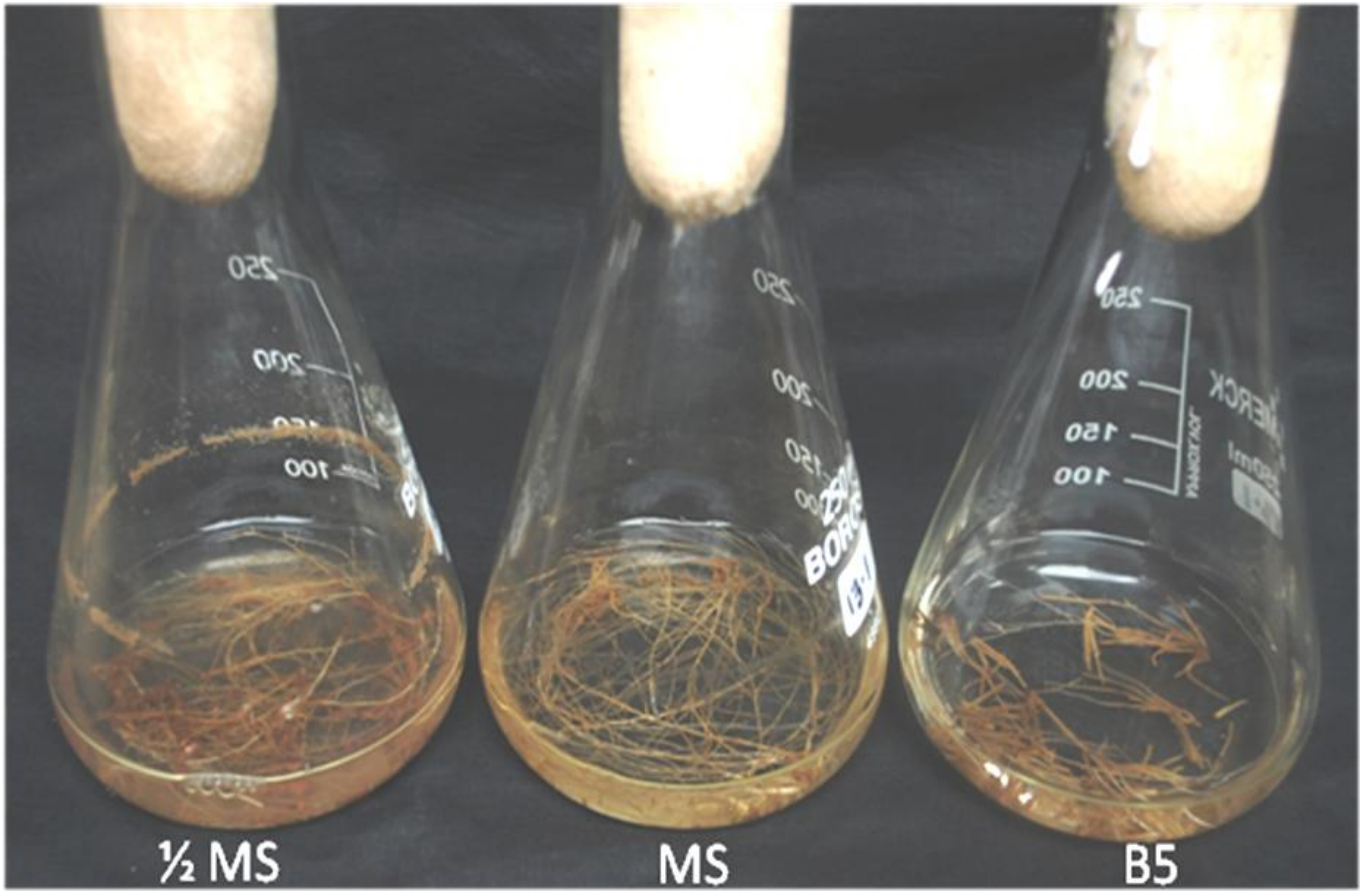

Figure 5. Influence of medium strength and type on *W. somnifera* hairy root culture.

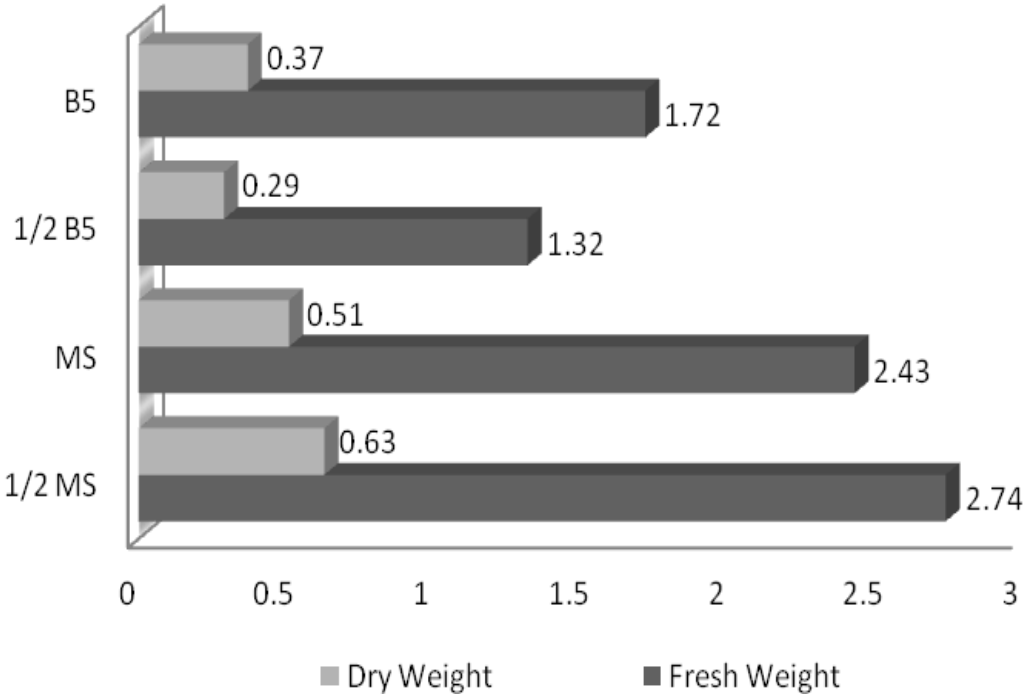

Figure 6. Influence of medium strength and type on *W. somnifera* hairy root biomass.

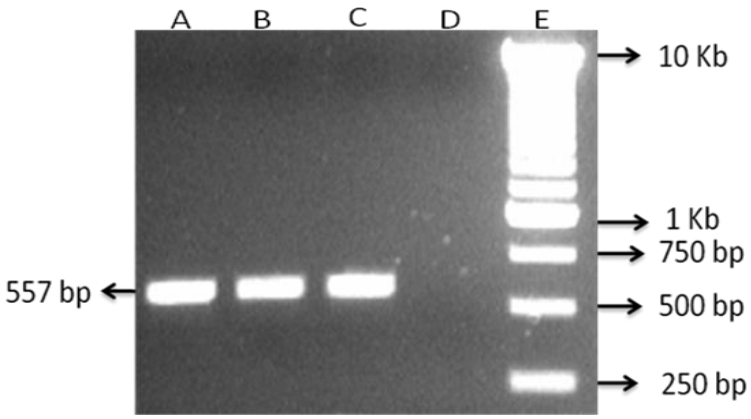

Figure 7. PCR analysis of *A. rhizogenes* R1000 induced hairy root lines. Lanes A, B and C: R1000 induced hairy root lines; lane D: Negative control (IBA induced adventitious roots); lane E: 500 bp ladder.

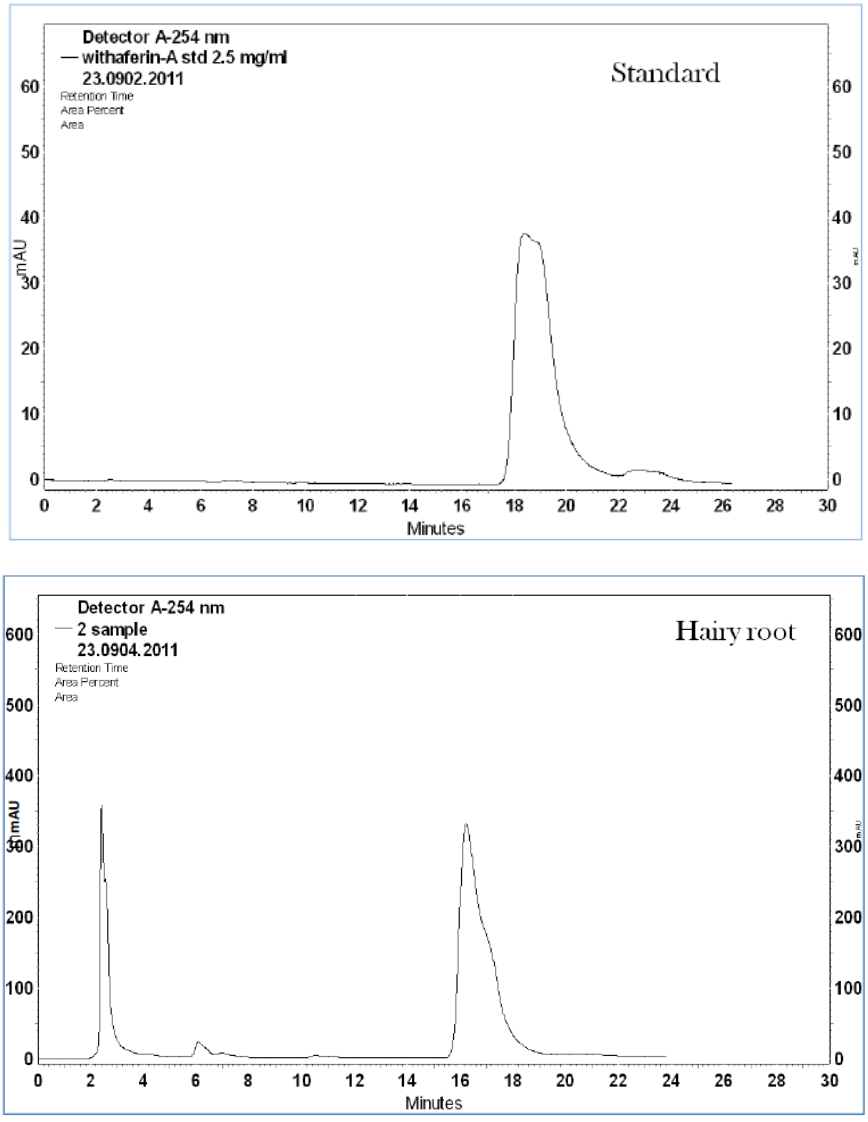

Figure 8. HPLC analysis of withaferin-A.

Conclusion

This paper demonstrates that the ability of three *A. rhizogenes* and type of explants are influential in terms of hairy root induction and growth *in vitro* in *W. somnifera*. Unraveling this potential role of *Agrobacterium* strain and explant type in hairy root induction is not only of great scientific interest, but is also of substantial relevance, as it would allow the rational manipulation of hairy root biomass production in large scale industrial applications for *W. somnifera*.

ACKNOWLEDGEMENTS

The authors are thankful to the Secretary, Principal and Management Committee members of National College and Jamal Mohamed College for their constant support and encouragement.

REFERENCES

Atta-ur-Rahman, Jamal A, Choundhary MI, Asif E (1991). Two withanolides from *Withania somnifera*. Phytochemistry 30(11):3824-3826.

Batra J, Dutta A, Singh D, Kumar S, Sen J (2004). Growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration. Plant Cell Rep. 23:148-154. DOI: 10.1007/s00299-004-0815-x

Boulter ME, Croy E, Simpson P, Shields R, Croy RRD, Shirsat AH (1990). Transformation of *Brassica napus* L. oilseed rape using *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*: a comparison. Plant Sci. 70:91-100.

Doyle JJ, Doyle JL (1987). Isolation of DNA from fresh plant tissue. Focus 12:13-15.

Drewes FE, Van Staden J (1995). Initiation of and solasodine production in hairy root cultures of *Solanum mauritianum* Scop. Plant Growth Regul. 17:27-31.

Giri A, Narasu ML (2000). Transgenic hairy roots: Recent trends and applications. Biotechnol. Adv. 18:1-22.

Giri A, Ravindra ST, Dhingra V, Narasu ML (2001). Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*. Curr. Sci. 81:378-382.

Guivarc'h A, Boccara M, Prouteau M, Chriqui D (1999). Instability of phenotype and gene expression in long-term culture of carrot hairy root clones. Plant Cell Rep. 19:43-50. DOI:10.1007/s002990050708.

Gupta GL, Rana AC (2007). *Withania somnifera* (Ashwagandha): a review. Pharmacog. Rev. 1:129-136.

Hilton MG, Rhodes MJC (1990). Growth and hyoscyamine production of hairy root cultures of *Datura stramonium* in modified stirred tank reactor. Appl. Microbiol. Biotechnol. 33:132-138.

Hu Z, Alfermann AW (1993). Deterpenoid production in hairy root cultures of *Salvia miltiorrhiza*. Phytochemistry 32:699-703.

Ionkova I, Kartnig T, Alfermann W (1997). Cycloartane saponin production in hairy root cultures of *Astragalus mongholicus*. Phytochemistry 45:1597-1600.

Jayaprakasam B, Zhang YJ, Seeram NP, Nair MG (2003). Growth inhibition of human tumor cell lines by withanolides from *Withania somnifera* leaves. Life Sci. 74:125-134.

Jung KH Kawah SS, Choi CY, Liu JR (1995). An interchangable system of transformed root and cell suspension cultures of *Catharanthus roseus* for indole alkaloid production. Plant Cell Rep. 15:51-54. DOI: 10.1007/BF01690252.

Kavidra NT, Neelesh CS, Vaibhav T, Brahma D (2000). Micropropagation of *Centella asiatica* (L) a valuable medicinal herb. Plant Cell Tissue Org. Cult. 62:175-179.

Kim YJ, Weathers PJ, Wyslouzil BE (2002). The growth of *Artemisia annua* hairy roots in liquid and gas phase reactors. Biotechnol. Bioeng. 80:454-464.

Luo C, Peng Z, Pu L (2004). Transformation of medicinal plants mediated by *Agrobacterium rhizogenes*. Biotechnology 14:58-61.

Mano Y, Ohkawa H, Yamada Y (1989). Production of tropane alkaloid by hairy root cultures of *Duboisia leichhardtii* transformed by *Agrobacterium rhizogenes*. Plant Sci. 59:191-201.

Mathews H, Bharathan N, Litz RE, Narayanan KR, Rao PS, Bhatia CR (1990). The promotion of Agrobacterium mediated transformation in *Atropa belladonna* L. by acetosyringone. J. Plant Physiol. 136:404-409.

Nin S, Bennici A, Roselli G, Mariotti D, Sehiff S, Magherini R (1997). *Agrobacterium* mediated transformation of *Artemisia absinthium* L. (wormwood) and production of secondary metabolites. Plant Cell Rep. 16:725-730.

Potrykus I (1990). Gene transfer to plants: Assessment and perspectives. Physiol. Plant 19:206.

Robins RJ, Bent FG, Rhodes MJC (1991). Studies on the biosynthesis of tropane alkaloids by *Datura stramonium* L. Transformed root cultures. the relationship between morphological integrity and alkaloid biosynthesis. Planta 3(185): 385-390 DOI: 10.1007/BF00201061

Sáenz-Carbonell LA, Maldonado-Mendoza IE, Moreno-Valenzula O, Ciau-Uitz R, López-Meyer M, Oropeza C, Loyola-Vargas VM (1997). Effect of the medium pH on the release of secondary metabolites from roots of *Datura stramonium, Catharanthus roseus*, and *Targetes patula* cultured *in vitro*. Appl. Biochem. Biotechnol. 38:257-267.

Shanks JV, Morgan J (1999). Plant 'hairy root' culture. Curr. Opin. Biotechnol. 10(2):151-155.

Sheikholeslam SN, Weeks DP (1987). Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. Plant Mol. Biol. 8:291-298.

Silvia DS, Eun-Ryeong H, Renaud W, Shivendra VS (2008). Withaferin A Causes FOXO3a- and Bim-Dependent Apoptosis and Inhibits Growth of Human Breast Cancer Cells *In vivo*. Cancer Res. 68(18): doi:10.1158/0008-5472.CAN-08-1510.

Sudha CG, Reddy BO, Ravishankar GA, Seeni S (2003). Production of ajmalicine and ajmaline in hairy root cultures of *Rauvolfia micrantha* Hook f., a rare and endemic medicinal plant. Biotechnol. Lett. 25:631-636.

Supe U, Dhote F, Roymon MG (2006). *In vitro* plant regeneration of *Withania somnifera*. Plant Tissue Cult. Biotechnol. 16(2):111-115.

Tiwari RK, Trivedi M, Guang ZC, Guo GQ, Zheng GC (2007). Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes* : growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. Plant Cell Rep. 26:199-210.

Trypsteen M, Van Lijsebettens M, Van Severen R, Van Montagu M (1991). *Agrobacterium rhizogenes* mediated transformation of *Echinacea purpurea.* Plant Cell Rep. 10:85-89.

Vanhala L, Eeva M, Lapinjoki S, Hiltunen R, Oksman-Caldentey K (1998). Effect of growth regulators on transformed root cultures of *Hyoscyamus muticus*. J. Plant Physiol. 153:475-481.

Villaine F, Casse-Delbart F (1987). Independant induction of transformed roots by the TL and TR regions of the Ri plasmid of agropine type *Agrobacterium rhizogenes*. Mol. Gen. Genet. 206:17-23. DOI: 10.1007/BF00326531.

Wysokiińska H, Rózga M (1998). Establishment of transformed root cultures of *Paulownia tomentosa* for verbascoside production. J. Plant Physiol. 152:78-83.

Yonemitsu H, Shimomura K, Satake M, Mochida S, Tanaka M, Endo T, Kaji A (1990). Lobeline production by hairy root culture of *Lobelia inflate* L. Plant Cell Rep. 9:307-310.

Zhu ZH, Alhman A, Li XY, Wealander MW (2001). Transformation of the apple root stockM.9/29 with the *rolB* gene and its influence on rooting and growth. J. Hortic. Sci. Biotechnol. 76:758-763.