

Full Length Research Paper

An efficient hairy root culture system for *Withania somnifera* (L.) Dunal

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***Withania somnifera* is an important aromatic medicinal plant and possesses wide array of pharmacological properties. In the present investigation, an improved version of hairy root culture system was developed by optimizing various transformation parameters such as type of explant, concentration of acetosyringone, *Agrobacterium* types and co-cultivation period. Between the leaf and cotyledon explants and two *Agrobacterium rhizogenes* strains (R1000 and A4) tested, leaf explants infected with R1000 and cocultured for five days on MS basal half strength medium in the presence of acetosyringone (100 µM) attained a higher frequency (88%) of hairy root induction. By adopting this protocol, we could utilize the hairy root culture for industrial scale production of withanolides.**

Key words: Leaf explant, *Agrobacterium rhizogenes*, *Withania somnifera*, co-cultivation period, acetosyringone.

INTRODUCTION

Withania somnifera (L.) Dunal (*Solanaceae*), commonly known as 'ashwagandha' and "Indian ginseng" is a highly valued medicinal plant in Indian Ayurvedic and African traditional systems. Major withanolides like withanolide A and withaferin A present in *W. somnifera* have been demonstrated to possess specific therapeutic action against carcinogenesis, Parkinson's disease and Alzheimer's disease (Mishra et al., 2000). The requirement of dried plant material for withanolides drugs production in India has been estimated as about 9127-tonnes as against the annual production of about 5905-

tonnes (Sharada et al., 2008). Moreover, field cultivation is time consuming, laborious and it is not able to meet the current Ashwagandha global market requirement (Sivanandhan et al., 2012b; 2013a): First, the plant-to-plant variation in secondary metabolites yield along with quality and second the long growing period (4-5 years) between planting and harvesting. To improve commercial cultivation of *W. somnifera*, biological advances must be made that should either increase the yield or reduce the time gap and assure quality (Banerjee et al., 1994).

To enhance commercial prospects for production of

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withanolides, an alternative choice could be the use of plant cell/organ cultures. Hairy root cultures offer many advantages over conventional cell culture systems for secondary metabolites production (Georgiev et al., 2007; Sivanandhan et al., 2013b). Hairy roots are adventitious roots derived from cells transformed by the root-inducing plasmid of *A. rhizogenes* and they grow in the absence of phytohormones. The hairy root harbors the T-DNA segment of plasmid Ri within its genome (Tepfer, 1990). Rao and Ravishankar (2002) suggested *A. rhizogenes*-mediated hairy root formation as the valuable tool for the biosynthesis of secondary metabolites, metabolic engineering studies and biotechnological production of root-derived compounds.

A. rhizogenes-mediated transformation has been previously reported in *W. somnifera* using various explants (leaf, cotyledon, cotyledonary node, hypocotyls, stem, and root) and various strains (A4, LBA9402, MTCC 2364, MTCC 532, ATCC 15834, LMG 150, A2/83, A20/83, R1000 and R1601) by various authors (Banerjee et al., 1994; Ray et al., 1996; Pawar and Maheswari, 2004; Kumar et al., 2005; Bandyopadhyay et al., 2007; Murthy et al., 2008; Praveen and Murthy, 2011; Sivanandhan et al., 2012a; Sivanandhan et al., 2013b). In the present study, we documented a higher number of hairy roots and transformation frequency upon inclusion of AS and MES buffer in the co-cultivation medium by optimizing different factors in *W. somnifera*. Hence, in the present study, two agropine type *A. rhizogenes* strains (R1000 and A4) have been selected to induce hairy roots in the Kolli Hills genotype of *W. somnifera*.

MATERIALS AND METHODS

Plant material

The explants, leaf and cotyledon were prepared as per our earlier report (Sivanandhan et al., 2013b).

Agrobacterium rhizogenes-transformation

A single colony of the wild type *Agrobacterium rhizogenes* strains R1000 and A4 were selected and cultured in LB medium (30 ml; Himedia, Mumbai, India) in darkness at 28°C for 12 h at 180 rpm. The bacterial cells were pelleted by centrifugation followed by washing twice with liquid half strength MS medium (Murashige and Skoog, 1962). The suspension was employed for *A. rhizogenes* infection.

Standardization of transformation parameters

In order to induce the hairy roots with *A. rhizogenes*, we tested two types of explants, namely leaves and cotyledons. The explants such as leaf and cotyledon were pricked with sterile hypodermic needle (0.63×25 mm; Dispovan, Haryana, India Ltd.) and the explants were immersed in the bacterial suspension culture (OD 600≥1) for 15 min and blotted on sterile tissue paper for 10 min. After dryness, the explants were placed in half strength MS medium supplemented with different concentrations of acetosyringone (AS)

(0, 50, 100, 150, and 200 µM; Sigma, St. Louis, USA), 3% (w/v) sucrose (SRL, Mumbai, India), and 0.2% (w/v) phytigel (Sigma, St. Louis, USA), at 23 ± 2°C in dark conditions. These explants were co-cultivated for different days (3, 5 and 7 days). After the co-cultivation period, the explants were washed first with sterilized-distilled water followed by half strength MS medium, which contained 300 mg/l cefotaxime (Alkime Laboratory, Mumbai, India) and transferred to 30 ml half strength MS medium supplemented with 3% (w/v) sucrose, 0.2% (w/v) phytigel, and 300 mg/l cefotaxime. After 12-18 days of culture, the transformed roots were appeared at the wounded sites of the explants. The induced transformed roots of more than 1-2 cm in length were excised from the explants and transferred to 30 ml half strength MS medium supplemented with 3% (w/v) sucrose, 0.2% (w/v) phytigel and 300 mg/l cefotaxime until the residual bacteria have been completely killed.

Statistical analysis

All the experiments were set up in a completely randomized design and the data were subjected to Duncan's multiple range test using SPSS software version 11.5. The efficiency of hairy root formation was calculated as the percent of respective explants showed hairy root induction out of total number of explants inoculated in a particular treatment.

RESULTS AND DISCUSSION

Effect of *Agrobacterium* strains on hairy root induction

The used bacterial strains had significant transformation efficiency on leaf and cotyledon explants in *W. somnifera*. In total of 150 leaf explants infected by R1000, an average of 132 leaf explants produced 28.2 number of hairy roots/leaf explant of at least 3 cm in length after 12 days (Table 1). In total of 150 cotyledon explants infected by R1000, an average of 96 cotyledon explants produced 17.6 number of hairy roots/cotyledon of at least 2 cm in length after 16 days. In the case of A4 strain, 150 leaf explants out of 118.7 explants produced 24.3 hairy roots/explant of at least 2 cm in length after 14 days of culture. In total of 150 cotyledon explants infected by A4, an average of 58.3 cotyledon explants produced 14.5 number of hairy roots/cotyledon of at least 1.5 cm in length after 18 days (Table 1 and Figure 1). Among different bacterial strains tested, R1000 was the most effective strain and stimulated rooting efficiency of 88 and 64% for leaf and cotyledon explants, respectively. A4 strain induced rooting efficiency of 79 and 38% for leaf and cotyledon explants, respectively. Murthy et al. (2008) reported that transformation response for cotyledon and leaf explants of *W. somnifera* showed 3 and 40%, respectively with R1601 strain. In our study, the cotyledon and leaf explants showed 64 and 88% of transformation efficiency, respectively. Banerjee et al. (1994) obtained 0.9 and 0.7% of transformation response in leaf explants using A4 and LBA9402, respectively. Pawar and Maheswari, (2004) showed that 20% of leaf explants produced hairy root induction in *W. somnifera*

Table 1. Transformation frequency of hairy roots induced from explants by *Agrobacterium rhizogenes* strains in *W. somnifera* on MS medium supplemented with 100 μ M AS at 5 day co-cultivation.

Bacterial strains-explants	Total number of explants infected	Number of transformed explants	Transformation frequency (%)	After induction of hairy root (days)
R1000				
Leaf	150	132.7 \pm 0.24 ^a	88.4 ^a	12
Cotyledon	150	96.3 \pm 0.21 ^c	64.2 ^c	16
A4				
Leaf	150	118.7 \pm 0.26 ^b	79.0 ^b	14
Cotyledon	150	58.3 \pm 0.22 ^d	38.8 ^d	18

Values represent the mean \pm standard error of three replicates. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.

using both MTCC 2364 and MTCC 532. Bandyopadhyay et al. (2007) reported that LBA 9402 and A4 showed equal effect in leaf explants (85%) and observed three morphological phenotypes of transformed roots. Kumar et al. (2005) used various bacterial strains-ATCC 15834, LMG 150, A2/83 and A20/83 for hairy root induction but, they failed to report the effect of bacterial strains in hairy root induction since their study was aimed to test the antioxidant activity in transformed hairy roots. In the present study, the results demonstrated that *W. somnifera* is more susceptible to strain R1000 than strain A4. It has been reported that the virulence of *A. rhizogenes* strain varies among plant hosts (Bush and Pueppke, 1991) and that the transformation efficiency of host species can vary between different bacterial strains (Godwin et al., 1991). Similar result was found in *Gentiana macrophyll* hairy root culture. In *G. macrophyll* species, the R1000 strain showed 32% of transformation frequency in matured leaf explant compared to 53% of transformation frequency in stem explants (Tiwari et al., 2007). It was observed from the present study that most hairy roots emerged from the wounded sites of midrib of leaf and cotyledon explants infected by both strains used. It was hypothesized that the cell contains high level of auxin and sucrose are ideal target for hairy root induction (Nilsson and Olsson, 1997). Since the phloem cells, positioned deep in plant organs are supposed to have high sucrose and IAA, they could be the target of *A. rhizogenes*. This might be the possible explanation of observation. Therefore, the *Agrobacterium* strain plays an important role in hairy root induction depending on explants in *W. somnifera*. This study strongly suggests that R1000 was a more effective strain in hairy root induction from 45-day-old leaf explants.

Effect of acetosyringone on hairy root induction

During induction of transformed roots, every individual hairy root was counted to evaluate the effect of aceto-

syringone concentration. For activation of 0-200 μ M AS in co-cultivation medium, the transformed hairy roots formed from the wounded regions of leaf and cotyledon explants were observed on 12-18 days after co-cultivation. This indicates that, AS contributes more crucial enhancement in root formation during co-culture of *A. rhizogenes* with the explants (Figure 2). AS enriched medium had a major effect on hairy root induction in leaf and cotyledon explants infected with R1000 strain. Addition of 100 μ M AS in co-cultivation medium resulted to two-fold higher number of hairy roots. Among leaf and cotyledon explants infected by R1000 strain, the leaf explants produced 28.2 number of hairy roots/explant in 5 day co-cultivation period when 100 μ M AS was added to the co-cultivation medium (Figure 2). Whereas in cotyledon explant, they produced 17.6 number of hairy roots/explant in 5 day co-cultivation period with the addition of 100 μ M AS to co-cultivation medium. Similarly, inclusion of AS to co-cultivation medium caused dramatic improvement in the number of responding leaf and cotyledon explants infected with A4 strain. Addition of AS (100 μ M) resulted higher number of hairy roots from leaf explants than cotyledon explants infected by A4 strain. The leaf explants produced 24.3 number of hairy roots/explant in 5 day co-cultivation period whereas an average of 14.5 number of hairy roots/explant was produced in 5 day co-cultivation period in cotyledon explants (Figure 2). When addition of AS in the co-cultivation medium increased beyond 100 μ M, there was reduction in growth of hairy roots from leaf and cotyledon explants of *W. somnifera*. When compared to the effect of AS in infectivity of R1000 and A4 strains, the R1000 strain showed best results in leaf explants when the co-cultivation medium contained 100 μ M AS. None of the authors have reported the effect of acetosyringone in hairy root induction in *W. somnifera* whereas, Bandyopadhyay et al. (2007) added 200 μ M acetosyringone in bacterial culture prior to infection. Moreover number of studies put forth the effect of acetosyringone for higher hairy root induction (Kim et al.,

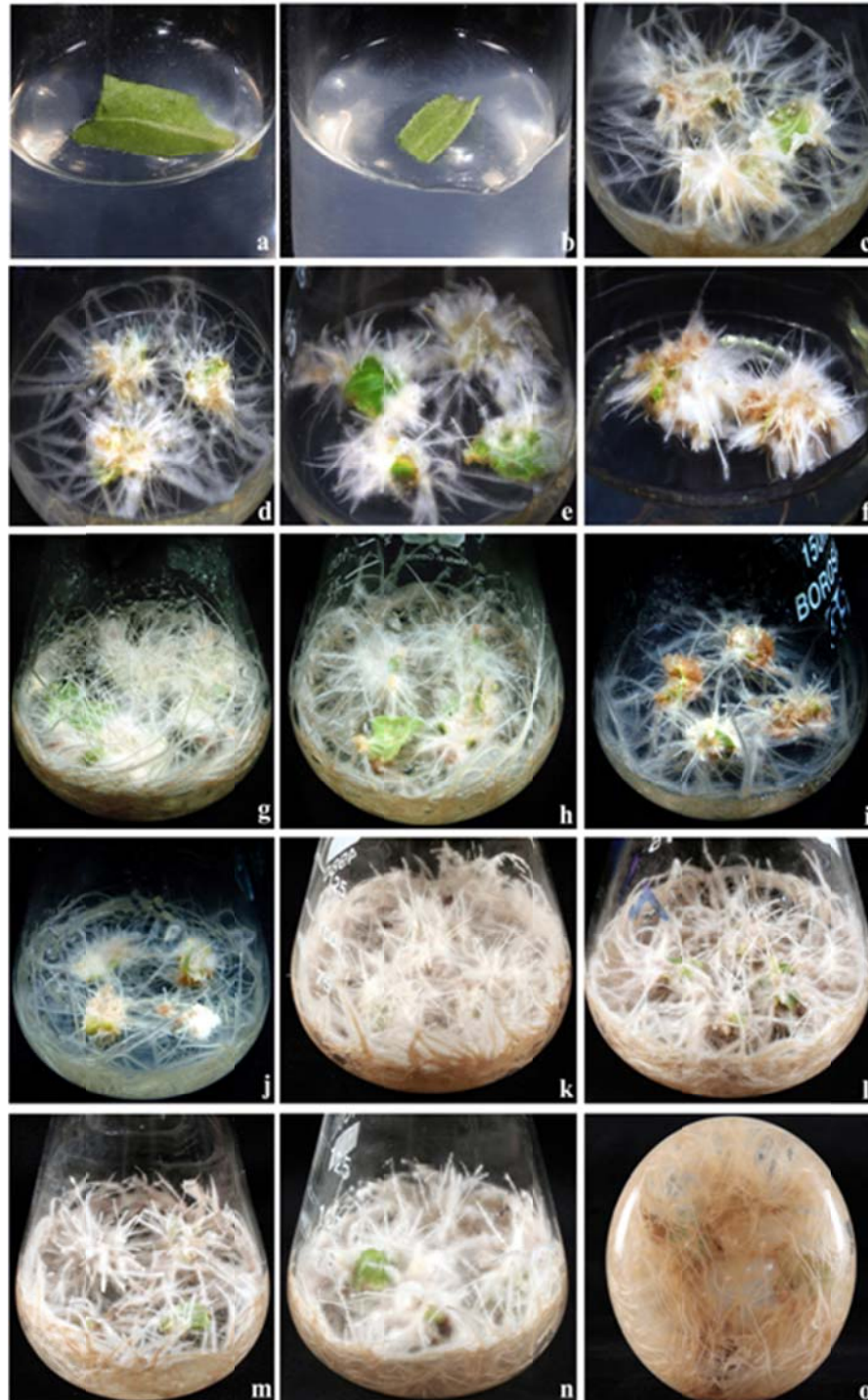


Figure 1. High frequency of hairy root induction from leaf and cotyledon explants of *W. somnifera*. **a and b.** Leaf and cotyledon explants placed on co-cultivation medium supplemented with 3 % sucrose containing 100 μ M acetosyringone. **c and d.** Hairy root induction from leaf and cotyledon explants infected with R1000 strain after 12 days. **e & f** Hairy root induction from leaf and cotyledon explants infected with A4 strain after 12 days. **g & h** Hairy root formation from leaf and cotyledon explants infected with R1000 strain after 16 days. **i and j.** Hairy root formation from leaf and cotyledon explants infected with A4 strain after 16 days. **k and l.** Hairy root formation from leaf and cotyledon explants infected with R1000 strain after 18 days. **m and n.** Hairy root formation from leaf and cotyledon explants infected with A4 strain after 18 days. **o.** Bottom view of hairy root formation from leaf explant infected with R1000 after 12 days.

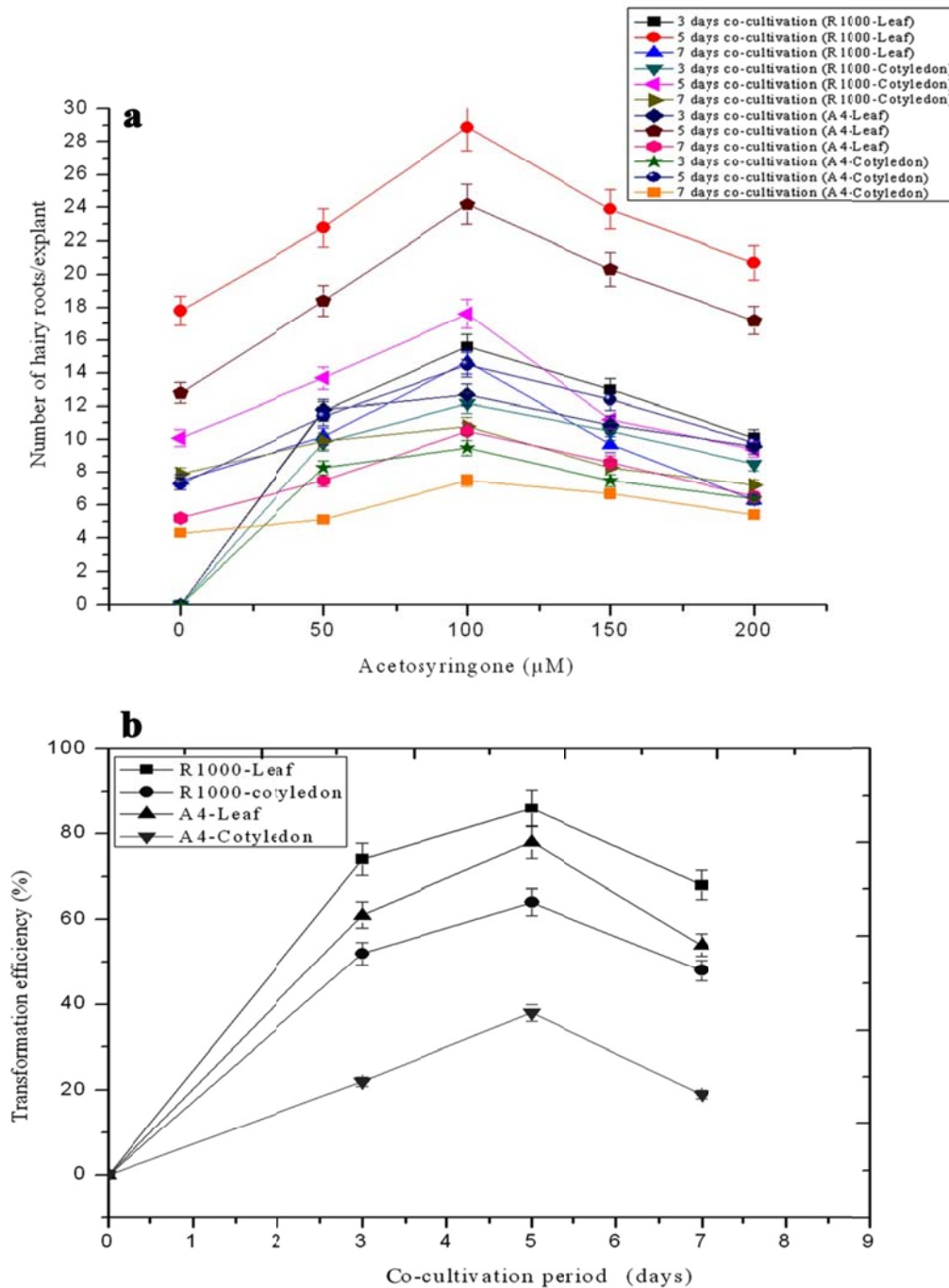


Figure 2. Effect of AS and co-cultivation periods on hairy root induction in *W. somnifera*. **a)** AS supplementation in co-cultivation medium in hairy roots induction on both explants of *W. somnifera* infected by R1000 and A4 strains. **b)** Co-cultivation period on transformation efficiency in hairy roots formation infected by both R1000 and A4 in leaf and cotyledon explants of *W. somnifera*. Values represent the mean \pm standard error.

2007). Acetosyringone, reported as a virulence inducer to *Agrobacterium*, promotes *Agrobacterium* mediated infection of plants (Stachel et al., 1985). It is widely used as an effective enhancer for *Agrobacterium* mediated plant transformation. A number of plant species, their

mediating *Agrobacterium* transformation, and an exogenous supplement of AS have been reported as applied through pre-treating the explants (Sunikumar et al., 1999) or *Agrobacterium* culture (Gelvin and Liu, 1994). Moreover, by inclusion of AS in the culture medium

(Godwin et al., 1991) and combining the pretreatment of explants and *Agrobacterium* culture (Boase et al., 1998) had also been found to enhance the efficiency of *Agrobacterium*-mediated transformation. Thus, we demonstrated that 100 μ M acetosyringone along with optimal co-cultivation days are essential for rapid induction of hairy roots from 45-day-old leaf explants of *W. somnifera*.

Effect of co-cultivation on hairy root induction

A co-cultivation for 5 days increased the efficiency of the leaf explants with hairy root formation, as it was not observed to obtain a higher number of hairy roots than remaining co-cultivation period. A 5 day co-cultivation period induced 28.2 and 24.3 number of hairy roots/leaf explants infected with R1000 and A4 strains, respectively. In the case of cotyledon explant, the co-culture for 5 days produced 17.6 and 14.5 number of hairy roots/explants infected with R1000 and A4 strains, respectively (Figure 2). Prolonged co-cultivation to 7 days resulted to growth of the bacteria around the both explants which led to explant necrosis. Shorter co-cultivation period may result to an unsuccessful gene transfer from strain to explant. So, the transformation efficiency may differ due to different co-cultivation periods (Figure 2). Of different co-cultivation period tested, 5 day co-cultivation time duration showed dramatic improvement in hairy root induction from the leaf and cotyledon explants, infected with R1000 and A4 strains in *W. somnifera*. A co-cultivation period for 2 and 3 days was found to be suitable for hairy root induction in *W. somnifera*, as reported by Pawar and Maheswari, (2004), Murthy et al. (2008), Banerjee et al. (1994), Kumar et al. (2005) and Bandyopadhyay et al. (2007), but Ray et al. (1996) did not mention the co-cultivation duration, whereas in the present study, 5 day co-cultivation period was found to be superior and abundantly increased the efficiency of hairy root induction (Figure 2). These variations in the requirement for a definite co-cultivation period may originate in the specificity of the plant tissue, the *Agrobacterium* strain or the medium used for bacterial culture and co-cultivation. Co-cultivation is an important phase in transfer of gene from bacteria to plant. But co-cultivation period produced successful transformants on depending upon the plant species and time duration. For R1000 strain, transformation efficiency increased in line with co-cultivation duration and stability on 5th day of culture. During the co-cultivation period, many factors influence the efficiency of the genetic transformation process. After *Agrobacterium* infection, the plant tissues and bacteria are cultivated for a few days. Important events occur during co-cultivation; plant cells and bacteria divide further, and T-DNA is transferred from the bacteria to the plant cells (Kim et al., 2007). The length of the co-cultivation period also influences transformation

efficiency (Tao and Li, 2006). These results confirm that the optimization of co-cultivation period is critical for higher hairy root induction in *W. somnifera*.

Effect of explants on hairy root induction

Among the various explants tested, only leaf was a greater explant to the infection of both strains. The leaf explant produced higher number of hairy roots by being infected with both the strains R1000 and A4. But the cotyledon explants showed lower number of hairy roots by being infected with both the strains when compared to leaf explants. The leaf explants infected by R1000 and A4 strains produced 28.2 and 24.3 number of hairy roots/explants, respectively and the cotyledon explants infected by R1000 and A4 strains produced 17.6 and 14.5 numbers of hairy roots, respectively (Figure 2). Most of the authors reported that leaf explants only showed the best response for hairy root induction in *W. somnifera*, but Murthy et al. (2008) obtained lower transformation efficiency in cotyledon explants (3%) whereas in the present study, cotyledon explants also produced hairy roots almost equal to leaf explants. Kang et al. (2006) showed the importance of explant choice by producing more hairy roots of *Aralia elata* on the root segment than on the petiole explant. It has been reported in several previous studies that the morphological patterns and hairy root production characteristics of infected tissues differed substantially (Ottani et al., 1990). These differences in hairy root production may depend upon differences between species, plant organs, or sites of infection. The choices of explants for hairy root induction after infection with *A. rhizogenes* constitute the most salient of the integrated factors for a successful hairy root transformation. Plant transformation efficiency differs significantly according to the source of the explant (Alpizar et al., 2006). Therefore, leaf explants possibly influence the rate of hairy root induction than the cotyledon explants by optimizing different factors such as co-cultivation, acetosyringone and bacterial strain in *W. somnifera*.

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Conflict of Interests

The author(s) have not declared any conflict of interest.

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