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Successful Production of Hairy Root of *Valeriana sisymbriifolium* by *Agrobacterium rhizogenes*

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Abstract: The successful induction and proliferation of hairy-roots of *Valeriana sisymbriifolium* explants through the transferece of the *rolA* gene by *Agrobacterium rhizogenes* (strain 15834) is reported. Hypocotyls, petiole and leaf explants (0.5-2 cm) were cut from the young plants after sprouting of the sterilized seeds on hormone-free Murashige-Skoog basal medium. The addition of 100 μM acetosyringone to YMB growth medium for *Agrobacterium rhizogenes* was shown to enhance the inducing ability of the bacterium. Successful and stable transfer of *rolA* gene was illustrated by PCR using both forward and reverse primers of the gene.

Key words: *Agrobacterium rhizogenes*, hairy-roots, *rolA* gene, *Valeriana sisymbriifolium*

INTRODUCTION

Chemical synthesis of some desired natural plant compounds is simply not feasible and gradual destruction of the natural habitats of herbs and plants have encouraged scientists to go for the mass production of such compounds by means of biotechnological techniques.

Some interesting secondary metabolites of plants are synthesized in the roots where they are stored, excreted from or transmitted to other organs. The cell culture of the roots has been successful in some cases (Mulabagal and Tsay, 2004) but it is not considered as a generic approach mainly because of the genetic instability, slow growth and low efficiency of the cell cultures (Ming *et al*., 2003). Therefore, the advent of the hairy root technique was welcomed by plant biotechnologists to address the cell culture limitations. Hairy root is produced as a result of transferring root inductive genes into the plant cells/tissue by means of a gene-gun or the gram-negative bacterium called *Agrobacterium rhizogenes* (AgR) under *in vitro* condition (Giri and Narasu, 2000). Hairy roots have shown considerable genetic and biosynthetic stability. Fast growth, simple maintenance and vast biosynthetic ability are advantages that make hairy roots efficient sources for plant metabolites production. Already several successful reports of such have been presented in the literature (Yazaki *et al*., 1998; Zárate, 1999; Krolicka *et al*., 2001).

Valeriana is a medicinal plant used mainly for its tranquilizing, antispasmodic and sedative effects. Valerian, a compound which is obtained from this plant, was proven to be effective for treatment of neuropsychiatric disorders. Preclinical studies suggest that valerian has sedative and muscle-relaxant effects (Krystal and Ressler, 2001). Frernandez and colleagues have demonstrated the tranquilizing and sedative properties of the flavone glycoside linarin extracted from Valerian (Fernandez *et al*., 2004). However, the field of research looks young if the discovery of more substances like valtrate, valeranone, hydroxypinoresinol, valerenic acid, valerianic acid, hesperidin, tamariscene and even alkaloids in the root are come into account (Hromadkova *et al*., 2002; Bent *et al*., 2006).

Valeriana sisymbriifolium is the most famous species of valeriana in Iran. In spite of its natural abundance in the North-West region of the country and that it is cultivated for medicinal purposes, the only report on the species is limited to a rather recent survey on the volatile oils of the herb (Javidnia *et al*., 2006). Considering the importance of the subject, this research was devoted to the study of *V. sisymbriifolium* for hairy roots production by AgR. It is noteworthy to mention that the successful hairy roots production of only one species of *Valeriana sambucifolia*, has been reported and studied in detail so far (Granicher *et al*., 1992). Interestingly, the research has shown the existence of derivatives in the hairy root essential oil which is not observed in the natural root of the species (Granicher *et al*., 1995). What adds to the importance of this research is the fact that one particular species of a family might be able to produce substances not observed in the other species, such as, the novel substances found in the roots of *V. prionophylla* (Piccinelli *et al*., 2004). Furthermore, this capability can be enhanced by the impact of biodiversity. These considerations necessitate the study of biotechnological potential of each individual species of a valuable family like Valerina.

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MATERIALS AND METHODS

Chemicals, plant material and bacterial strain: All of the chemicals used in this research were taken from authentic samples mainly purchased from the Merck distributor. The *V. sisymbriifolium* seeds were obtained from the Medicinal Plants Research Institute of Jahad Danenshgahi in Tehran. The sterilized seeds of *V. sisymbriifolium* germinated on solid hormone-free Murashige and Skoog (MS) basal medium (pH 5.8, sucrose 30 g L^{-1}, agar 8 g L^{-1}) in darkness at 25±1°C. The resulting plantlets were subcultured on the same medium and maintained under the conventional conditions of the laboratory green house.

AgR (ATCC 15834) was provided by the Bank of Microbes at the National Research Center for Genetic Engineering and Biotechnology (NIGEB, Tehran, Iran). Mono clones of AgR were proliferated in Yeast Manitol Broth (YMB) medium with or without 100 μM acetosyringone for 24 h on a shaker at 150 rpm and 28°C.

Plant explants infection: Hypocotyls, leaf and petiole explants of 0.5, 1 and 2 cm long were cut from the two to three-week old *V. sisymbriifolium* plantlets. No specific physical or chemical treatment was carried out on the explants prior to the bacterial infection. So, the explants were immersed in 50 mL of the AgR broth culture and swirled for 20 min. To remove extra bacteria, the explants were blotted on sterile filter paper and subsequently transferred onto a hormone-free MS medium and kept in darkness at 25±1°C. The explants were not exposed to antibiotic at this stage. After 3 days, the explants were transferred onto fresh hormone-free MS medium containing 500 mg L^{-1} of cefataxime to remove the bacteria. Explants were sub-cultured on the same medium every 48 h the antibiotic concentration being decreased in each subsequent subculture to 400, 200 and 100 mg L^{-1} and eventually eliminated from the medium altogether.

The resulting hairy roots were cultured in liquid MS medium containing 100 mg L^{-1} cefataxime and 20 g L^{-1} sucrose in darkness on a shaker (90 rpm) at 25±1°C. Sub-cultures were prepared every 10 days. A few samples of the natural root of the plant were cultivated under the same conditions. The biomass of the natural and the transformed roots was measured as follows. Root samples were primarily dried by a hair dryer and then precisely weighed. They were, then, maintained in an oven at 37°C and reweighed intermittently until no further weight loss was observed. The final weight of the dried biomass was recorded.

***rolA* gene detection:** The extraction of DNA from leaf, root and hairy root samples was carried out using the Dellaporta method (Dellaporta *et al*., 1983). The conventional alkaline lysis method was used for extraction of the bacterial plasmid. Concentration of the extracted DNA was calculated by recording the optical density of the sample at 260 nm using a Beckman DU-70 spectrophotometer.

PCR experiments were carried out with a set of forward and reverse primers in a Touchgene Gradient thermocycler. The primers for extending a 248 fragment of 720 nucleotides *rolA* gene were designed by Oligo 5 software and checked through the Blast search. The primers consisted of 22 and 23 nucleotides with the sequences of 5'-CGTTGTCGGAATGGCCCAGACC-3' and 5'-CGTAGGTCTGAATATTCCGGTCC-3' for the Forward and Reverse, respectively.

The extracted bacterial plasmid DNA (140 ng) and the plant genomic DNA (120 ng) in 25 μL was used in each PCR. The reaction mixture contained 30 pmole of each primer, dNTP (2 mM), $MgCl_2$ (1.5 mM) and DMSO (5% v/v). The extension reaction was carried out in the presence of 1 unit of Taq polymerase. The initial denaturation of the DNA strands was performed at 95°C for 10 min followed by 35 cycles of 1 min denaturation at the same temperature. Annealing and extension were done at 55 and 72°C, respectively, for 1 min. Final extension was performed at 72°C for 10 min.

The electrophoresis of the PCR products were performed on 1.2% agarose gel (0.5x TBE buffer) applying a constant voltage of 100 Volts. The gel was subsequently stained in 0.8 μg mL^{-1} ethidium bromide solution and examined under UV light.

RESULTS AND DISCUSSION

AgR (ATCC 15834) has the Root induction (Ri) plasmid that induces hairy roots in plants. The advent of hairy roots can be caused by just one or a combination *rolA*, B, C genes which exist in T-DNA part of Ri plasmid. It has been shown that the corresponding protein of *rolA* affects the Gibberellins metabolism (Mukundan *et al*., 1997; Xu *et al*., 2004).

Wounded plants secrete acidic latex containing different phenolic compounds that are able to stimulate the AgR Vir genes. The most famous and effective Vir genes inducers are acetosyringone and alpha-acetosyringone which rarely exist in intact plants. Therefore, freshly cut leaf, petiole and hypocotyls explants of *V. sisymbriifolium* plantlets were exposed to AgR and sub-cultured as explained in the experimental section. The best response was observed in the explants which had been wounded 24 h before exposure. It is assumed that the latex secreted during this period facilitates the induction process (Giri and Narasu, 2000).

Antibiotic was applied to the culture medium of the infected explants after three days of infection resulting in the formation of hairy roots on the leaf and petiole explants within 8 to 10 days after the infection. Longer co-cultivation periods did not improve the results. Similar observations about the length of the co-cultivation period have been reported by the others (Niu *et al*., 2000; Tao and Li, 2006). Hypocotyls did not respond to the treatment as well as the leaf and petiole explants. The observation is in agreement with the fact that plant transformation efficiency differs significantly according to the source of the explant (Shi and Kintzios, 2003). However, the result can not be compared with the *V. sambucifolia* as Granicher *et al*. (1992) have not reported the source of the used explants in their experiments.

Surveying the acetosyringone effect: In contrast to the work of Granicher *et al*. (1992) on *V. sambucifolia*, the effect of acetosyringone on the induction ability of AgR was also studied. Experiments of this research showed that the number of the *V. sisymbriifolium* explants transformed by the AgR doubled when 100 μM acetosyringone was added to the YMB liquid medium in which the bacterium was proliferated (Table 1). This result supports the scientific literature which suggest that adding certain phenolic compounds into the proliferation medium of AgR increases the hairy roots induction power of the bacterium (Giri and Narasu, 2000).

The proof of *rolA* gene transformation: There are three distinct steps to prove successful transformation of *V. sisymbriifolium* by AgR. First, the existence of the Ri gene in 15834 strain had to be confirmed. Hence, the specific primers for the *rolA* gene was synthesized as explained in the experimental section. Strain 15834 DNA was successfully extracted and amplified by the synthesized primers. Considering the *rolA* gene sequence and the primers used, a 248 bp fragment was expected to extend (Fig. 1).

In the second and third steps, it has to be shown that the *rolA* gene has been transferred into the transformed *V. sisymbriifolium* explants while it is not seen in the wild species. Therefore, the genomic DNA of both the wild type and transformed root were extracted and subjected to the *rolA* gene amplification in the presence of the synthesized primers by the PCR method described. Results showed that a 248 bp fragment was extended in the transformed *V. sisymbriifolium* hairy roots and confirmed the absence of *rolA* gene homologous fragment in the wild plant genome (Fig. 2).

Table 1: Parameters A-D represent the comparative numbers of *V. sisymbriifolia* explants which developed hairy root after getting infected by AgR which was proliferated in the presence of 0, 50, 100 and 150 mM acetosyringone, respectively. Parameters F and G represent the comparative biomass of the natural and transformed hairy roots, subcultured in liquid MS for one month

Parameters	A	B	C	D	F	G
Amount in percent	100	144	202	197	100	327

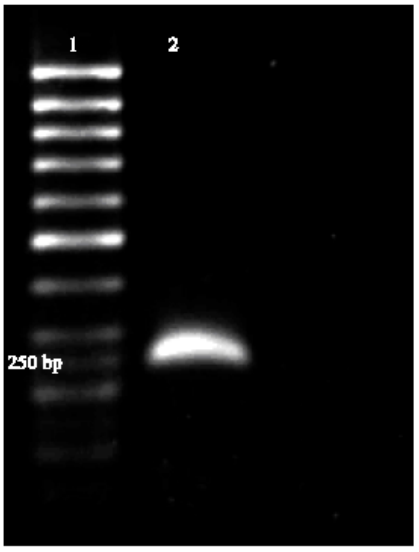

Fig. 1: Bands of (1) DNA marker and the (2) resulting band of *rolA* gene amplification

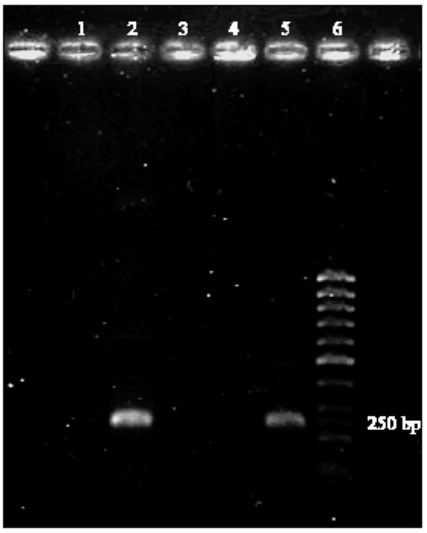

Fig. 2: The PCR amplification of *rolA* gene of (1) the blank PCR sample, (2) the AgR (Strain 15834) DNA, (3) the extracted DNA from the leaf of the wild *V. sisymbriifolia*, (4) the extracted DNA from the root of the wild *V. sisymbriifolia*, (5) the extracted DNA from the hairy root of the transformed *V. sisymbriifolia* and (6) the DNA marker

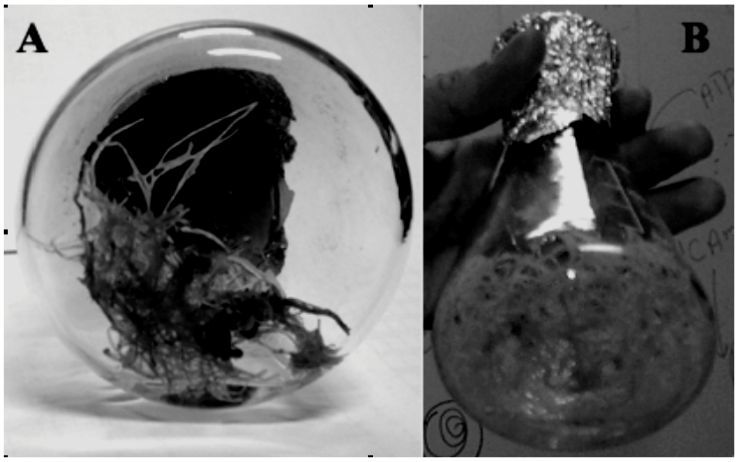

Fig. 3: The appearance of the (A) natural root and the (B) transformed root of *V. sisymbriifolium* by AgR after three sub-cultures under identical conditions described in the experimental section

Biomass of the transformed root: Measuring the dried biomass of the natural and transformed roots after three sub-cultures under the same condition shows that the transformed samples had produced more than 300% biomass in comparison with the natural samples (Table 1). Figure 3 shows the natural appearance of the samples after three sub-cultures.

As mentioned earlier, the only report on the hairy root of valeriana belongs to *V. sambucifolia* which has been directed mainly toward the analysis of the effective substances (Granicher *et al*., 1992). As a result, little attention was paid to the technical aspects of the hairy root production. Considering the fact that one particular species of a family might respond better than others to this method of rooting, it is difficult to compare the results of *V. sisymbriifolium* with the ones of *V. sambucifolia*. However, results of this work indicate that the method can be easily modified for the large scale production of the *V. sisymbriifolium* hairy roots provided a survey of the biosynthetic capabilities of the transformed roots is performed in advance. The known compounds can be traced by the introduced HPLC methods (Goppel and Franz, 2004; Silva *et al*., 2002) and advanced mass spectroscopy methods can be used for scanning possible novel substances.

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