

Virulence of Different Strains of *Agrobacterium rhizogenes* on Genetic Transformation of Four *Hyoscyamus* Species

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Abstract: Different species of the genus *Hyoscyamus* are rich sources of tropane alkaloids, which are widely used in medicine and the demand for them is continuous. In the last two decades there has been considerable interest in the production of these compounds by genetically transformed root cultures because unlike cell suspension, hairy root cultures display stable production of tropane alkaloids which is often comparable to, or even greater than, plant roots. A method of the induction of hairy roots of four *Hyoscyamus* species (*H. arachnoideus* Pojark., *H. kurdicus* Bornm., *H. reticulatus* L. and *H. squarrosus* Griff.) was established in this study by infecting leaf discs with five strains of *Agrobacterium rhizogenes* (1724, 2659, 15834, A4 and LBA 9402) in two media; MS and B5. The number of individual formed roots and transformation frequency were used as measurements of virulence. Four best strains; 15834, 2659, LBA 9402 and 1724 for hairy root induction in *H. arachnoideus*, *H. kurdicus*, *H. reticulatus* and *H. squarrosus* leaves, respectively were determined. In addition, the two type of media (MS and B5) used for transformation has no influence in this respect.

Key words: *Agrobacterium rhizogenes* % Hairy roots % *Hyoscyamus* species % Tropane alkaloid

INTRODUCTION

The soil-borne bacterium *Agrobacterium rhizogenes* is the causative agent of hairy root disease in dicotyledonous plants. This disease results from the transfer and integration of transferred-DNA (T-DNA) of root-inducing (Ri) plasmid to the plant genome [1]. The transfer of T-DNA is mediated by virulence genes, which form the *vir* region of the Ri-plasmid and the bacterial *chv* genes [2]. The transformed roots can be excised and grown *in vitro* as hairy root cultures. Hairy roots have several properties that have promoted their use for plant biotechnological applications. Their fast growth, genetic and biosynthetic stability, low doubling time, ease of maintenance and have the ability to synthesize a range of chemical compounds that makes them a suitable system for *in vitro* production of secondary metabolites [2]. To date, hairy root cultures of many dicotyledonous and monocotyledonous plants have been established and found to accumulate the same metabolites as natural roots [3-4]. In addition, this type of culture is known to produce a spectrum of secondary metabolites that are not present

in the parent plant [5]. Hairy roots are often able to regenerate whole viable plants and maintain their genetic stability during continuous subculturing and plant regeneration. Moreover, transgenic root systems possess a tremendous potential for introducing additional genes along with the Ri plasmid of *A. rhizogenes* for alteration of metabolic pathways and production of useful metabolites or compounds of interest [2]. These differentiated cultures are also a valuable tool to study the biochemical properties and the gene expression profile of metabolic pathways.

Hyoscyamine and scopolamine are medicinally important tropane alkaloids. Because of possessing anticholinergic and central nervous system activities, they are widely used in medicine (e.g. in ophthalmology, cardiology and gastroenterology) [6] and there are no other classes of compounds that could substituted for these plant-derived drugs [7]. Industrially, tropane alkaloids are produced exclusively by Solanaceae plants of the genera such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* and the demand for them is continuous [8]. During the past two decades considerable

efforts have been made to develop an economically feasible *in vitro* production of these compounds. Unfortunately, cell cultures of different Solanaceous species have shown low tropane alkaloid production, mainly due to lack of differentiation [9-10]. Root cultures can produce hyoscyamine and scopolamine at higher levels than cell cultures or even parent roots, since these alkaloids are synthesized specifically in plant roots [11-13]. In this aspect, hairy roots have several advantages over natural ones, among which their high growth rates in hormone-free media and their genetic and biochemical stability.

To the best of our knowledge, no investigation has been carried out to evaluate the formation, growth and tropane alkaloid production in hairy roots of *H. arachnoideus* Pojark., *H. kurdicus* Bornm., *H. reticulatus* L. and *H. squarrosus* Griff. that are growing wild in Iran. Therefore, this study was conducted to determine the best condition for hairy root formation in some *Hyoscyamus* species by infecting leaf explants with five strains of *A. rhizogenes* (1724, 2659, 15834, A4 and LBA 9402) in two different media MS [14] and B5 [15].

MATERIALS AND METHODS

Plant Material: The mature seeds of growing wild plants of *Hyoscyamus arachnoideus*, *H. kurdicus*, *H. reticulatus* and *H. squarrosus* were collected from different parts of Iran including Mashhad, Kurdistan, Hamadan and Birjand, respectively for each species. Dormancy of seeds was broken by immersing them in 100 ppm GA3 solution for 24 h. The seeds were germinated in washed sand in a growth chamber under 16 h daily light periods and then irrigated using half-strength Hoagland's solution [16]. Afterwards, seedlings with 3-4 leaves were transferred to soil in pots where they grew into normal healthy plants in greenhouse condition.

Bacterial Strains: *A. rhizogenes* strains 1724, 2659 and 15834 (gifts from Dr. M. Karimi, Gent, Belgium), along with A4 and LBA 9402 (obtained from Ferdowsi University of Mashhad, Iran) were used to determine the transformation efficiency. The bacteria were maintained on YMB [17] solid media as required. Prior to infection, the bacterial strains were grown for 48 h in 20 ml YMB liquid medium at 28°C on a rotary shaker at 100 rpm in the dark.

Induction of Hairy Roots: The leaves from four-week-old greenhouse-grown seedlings were surface sterilized for 10 min in 1% sodium hypochlorite solution to which

2-3 drops of Tween 20 were added and thoroughly rinsed with sterile distilled water. The sterilized leaves were cut into about 1–2 cm² length pieces and pre-cultured for 24 h on solid and growth regulator-free MS and/or B5 medium, supplemented with 3% sucrose and 0.8% agar. Then, each explant was immersed in bacterial suspension separately for 5 min. The explants were blotted dry on sterile filter-paper to remove excess bacteria and placed back on their original culture plates. In addition, many explants were immersed in sterile distilled water and incubated in the same conditions as control. After 2 days of co-culture at 28°C in the dark, the explants were transferred onto hormone-free media containing 500 mg/l cefotaxime to eliminate bacteria and then incubated at 25±2°C under 16 h light and 8 h dark photoperiod. The frequency of genetic transformation was investigated during a five week period. Only the main roots and not lateral branches which formed were designated as individuals.

Statistical Analysis: MSTAT-C software (MSTAT, Michigan, USA) was used to analyze the data of this experiment. The frequency of hairy root formation was the mean of four independent experiments (±S.E.) with a minimum of 20 explants per treatment.

RESULTS AND DISCUSSION

Five strains of *A. rhizogenes* were utilized to infect leaf explants of four *Hyoscyamus* species wildy growing in Iran. Visible roots were formed after 10-21 days at the site of bacterial inoculation of leaf discs. The hairy roots were formed mainly on the midrib of the infected leaves and they differed morphologically depending on the type of bacteria. No roots formation was observed in control explants.

Different types of *A. rhizogenes* had great influence on the induction of hairy roots. In contrast, MS and B5 media used for the transformation have no influence in all tested systems. The transformation efficiency of LBA 9402 and 1724 were more efficient than that of 2659, A4 and 15834 for *H. reticulatus* transformation (Fig. 1). In this species, LBA 9402 produced the maximal number of hairy roots per explant (10.25), followed by 1724 strain (Table 1). For induction of hairy roots in *H. kurdicus* leaves, 2659 had the highest transformation frequency with 86.6% and LBA 9402 had the lowest transformation frequency with 65.6% (Fig. 2). The number of roots per explants was higher with the strains A4 (9.25) and 2659 (8.13) than with the other ones (Table 1). In *H. squarrosus*, the best root

Table 1: Effect of various strains of *A. rhizogenes* on the number of hairy roots obtained from different *Hyoscyamus* species explants after five weeks of culture

Strain	<i>H. arachnoideus</i>	<i>H. kurdicus</i>	<i>H. reticulates</i>	<i>H. squarrosus</i>
15834	5.38±0.65	6.63±0.94	6.13±0.40	6.25±0.59
1724	3.63±0.38	7.25±0.92	9.38±0.38	8.25±0.70
2659	4.13±0.44	8.13±0.88	7.75±0.65	5.88±0.48
A4	2.75±0.37	9.25±0.49	7.50±0.50	7.75±0.53
LBA 9402	4.63±0.46	5.25±0.53	10.25±0.90	3.88±0.48
Control	-	-	-	-

Values represented the mean ± S.E. of a minimum of twenty explants

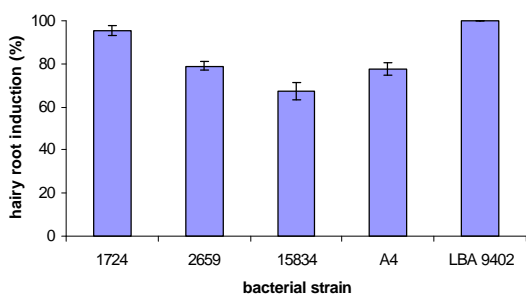


Fig. 1: Frequency of hairy root induction on leaf explants of *H. reticulates* infected with different strains of *A. rhizogenes*

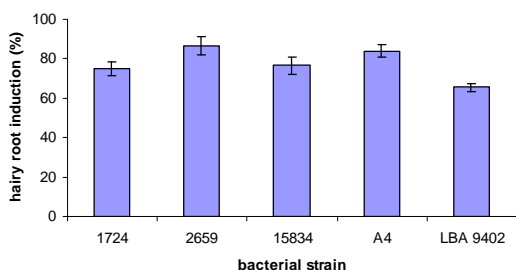


Fig. 2: Frequency of hairy root induction on leaf explants of *H. kurdicus* infected with different strains of *A. rhizogenes*

formation response was obtained with the bacteria in the order: 1724, A4, 2659, 15834 and LBA 9402 (Fig. 3). Similarly, more roots were obtained with leaves infected by 1724 and A4 strains (Table 1). In addition, among the various bacterial strains examined in this study, 15834 and 2659 were found to be the most virulent *A. rhizogenes* strains for hairy root formation in 89.0% and 67.1% of the leaf explants of *H. arachnoideus*, respectively (Fig. 4). 15834 induced 5.38 roots per explants, while only 2.75 roots were obtained in leaves inoculated by A4 strain (Table 1).

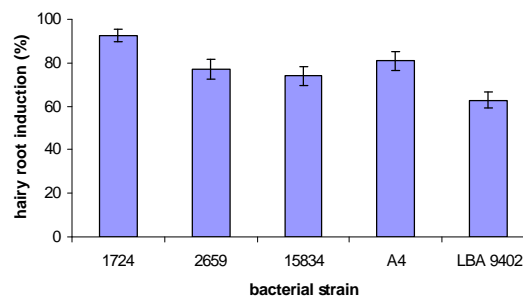


Fig. 3: Frequency of hairy root induction on leaf explants of *H. squarrosus* infected with different strains of *A. rhizogenes*

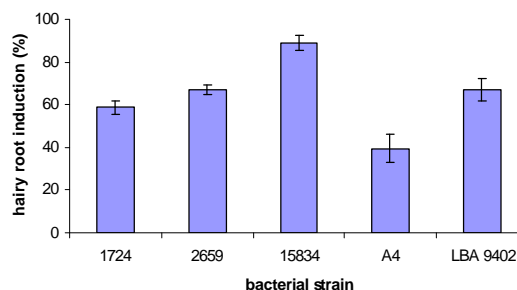


Fig. 4: Frequency of hairy root induction on leaf explants of *H. arachnoideus* infected with different strains of *A. rhizogenes*.

Soil-borne pathogens of genus *Agrobacterium* are able to transfer part of their DNA, the T-DNA carried on a large plasmid, to the genome of a host plant cell. *A. rhizogenes* is the causal agent of hairy root disease in plants and has been used for the production of hairy root cultures from a large number of plants. Potential of hairy roots for production of secondary metabolites has been recognized since last two decades [18]. The main advantage of using hairy root cultures is their ability to grow in defined basal media without supplementation of phytohormones. Due to their differentiated nature, they show genetic stability and tend to produce high levels of secondary metabolites characteristic of the species.

The genetic transformation mediated by *Agrobacterium* is affected by explant genotype and structure, chemical and physical factors, bacterial strains and signal molecules [19]. Different strains of *A. rhizogenes* vary in their transforming ability [20-22]; this was confirmed in our study that bacterial strains used in the experiment demonstrated the significant differences in virulence. Strain specificity observed in the present study agrees with the hypothesis that, the ability for infection of the different *A. rhizogenes* strains in a given

species was different [11, 23]. The difference in virulence and morphology could be explained by the plasmids harboured by bacterial strains [24]. In addition, extensive morphological variation in individual hairy root cultures can be possibly due to differential expression of T-DNA genes present in the transformed roots, variable copy numbers of T-DNA inserts and positional integration effects of the T-DNA in the host genome [25].

CONCLUSION

A systematic study using different strains of *A. rhizogenes* was carried out to determine the best condition for hairy root formation in four species of the genus *Hyoscyamus* in two different culture media. The data presented confirm the results of previous investigations that different bacterial strains had various hairy roots generating capacity. The best strains for induction of hairy root in *H. arachnoideus*, *H. kurdicus*, *H. reticulatus* and *H. squarrosus* leaf explants were determined; 15834, 2659, LBA 9402 and 1724, respectively. However, further research is needed to evaluate the growth and tropane alkaloid production from obtained hairy roots.

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