Influence of different strains of Agrobacterium rhizogenes, culture medium, age and type of explant on hairy root induction in Echinacea angustifolia

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ABSTRACT

Hairy roots culture is a potential system for secondary metabolite production in medicinal plants. In this research, hairy root induction in Echinacea *angustifolia* was established using Agrobacterium rhizogenes. The effects of different A. rhizogenes strains (AR15834, A4, K599 and MSU440) in two solid growth regulator-free culture media, half strength MS salts with B5 vitamins and WPM, were evaluated on hairy root induction in 60-day-old explants. The highest percentage of root induction was obtained by AR15834 strain in the WPM medium (46.6%). Also, the effects of age (20, 40 and 60 day-old) and type (leaf, leaf lamina and petiole) of explant on hairy root induction were investigated by AR15834 strain in the WPM medium. The high frequency of hairy root induction was obtained in leaf explants (52.4%) and 40 day-old leaf explants (62.3%).Transgenic hairy roots were confirmed by PCR analysis with specific primers of rolB gene.

Keywords: Agrobacterium rhizogenes, Echinacea angustifolia, Hairy roots, Explant.

INTRODUCTION

Echinacea is a genus of herbaceous perennial plants in the family Asteraceae. Three species of *Echinacea* are generally used medicinally containing E. *purpurea* Moench (roots and tops), E. *angustifolia* DC (roots) and E. *pallida* (roots) (Perry *et al.*, 2001). *Echinacea* has a long history of medicinal use for a variety of conditions in North American and *Echinacea* products are among the best-selling herbal medicines in several developed countries (Lucchesini *et al*., 2009; Percival, 2000). E. *angustifolia,* commonly known as Narrow-leaved purple coneflower, is an herbaceous medicinal plant used by all Indians of the Great Plains to treat a wide range of ailments, from venomous bites and stings, to infectious or inflammatory conditions such as cold and flu, toothaches, cough, sore eyes, and rheumatism (Barnes *et al*., 2005; Kindscher, 1989).

Due to the long growth cycle and the limitation of environment, drug production of E. *angustifolia* is difficult. Different strategies have been employed to develop the production of secondary metabolites in *in vitro* systems including the use of genetically transformed roots (Bourgaud *et al*., 2001). The hairy root culture system is a potential system for the production of secondary metabolites, especially pharmaceuticals. *Agrobacterium rhizogenes* is the natural agent of hairy roots formation in

plants. Soil-borne pathogens of genus *Agrobacterium* can transfer parts of their DNA, the T-DNA carried on a large plasmid, to the genome of a host plant cell. This bacterium has been used for the production of hairy roots in different plants (Doran, 1997). The produced secondary metabolites by hairy roots arising from the infection of plant material by A. *rhizogenes* are the same as those generally synthesized in intact parent roots, with similar or higher yields (Sevo´n and Oksman-Caldentey, 2002). The fast growth in hormone-free culture medium, genetic and biochemical stability, low doubling time, eases of maintenance and ability to produce different secondary metabolites offers some advantages for hairy roots culture (Chandra and Chandra, 2011; Georgiev *et al*., 2007; Sevo´n and Oksman-Caldentey, 2002). Moreover, their ability to secondary metabolites synthesize could be higher than intact plant (Dehghan *et al*., 2012), cell suspension and callus cultures (Chandra and Chandra, 2011). Since hairy root culture is less expensive, less laborious, required a less growth period and an ecofriendly method, it might be a suitable alternative for important secondary metabolite production. The use of hairy root cultures as an alternative will not only reduce the dependence of the pharmaceutical industry on natural habitats but also ensure the quality of raw materials which are affected by various factors (Huang *et al.*, 2014). Several factors affect the rate of A. *rhizogenes* mediated transformation in plants such as genotype, bacterial strain, signal molecules and culture medium (Tao and Li 2006).

Since only few studies have focused on hairy root induction in E. *angustifolia*, the objective of the present study was to provide information on the establishment of hairy roots in this medicinal plant and investigate the effects of different A. r*hizogenes* strains, culture media, age and type of explants on hairy root induction.

MATERIALS AND METHODS

The seeds of *Echinacea angustifolia* were obtained from Pakanbazr Co. (http://www. Pakanbazr.com). The seeds were washed with tap water and were surface sterilized for 1 min in 70% ethanol. Then the seeds were washed with sterile distilled water, and were immersed in a 5.25% (w/v) sodium hypochlorite solution containing 2-3 drops of Tween 20 per 50 ml for 20 min and finally rinsed three times (5 min for each time) with sterile distilled water. Explant cultures were done in jam jars (5.5 cm in diameter, 8 cm in height, and 250 ml in volume) containing 50 ml hormone-free Murashige and Skoog (MS) medium supplemented with 3%(w/v) sucrose and 0.6% (w/v) agar-agar (Merck). The pH of the medium was adjusted to 5.8 by the addition of KOH or HCl prior to autoclaving. Cultures were kept at 25 ± 2ºC under a 16/8 h (day/night) photoperiod provided by cool white fluorescent lamp with a light intensity of 4000 lux. In this research, three experiments were carried out. Four A. rhizogenes strains including AR15834, A4, K599 and MSU440 were used for hairy root induction in E. *angustifolia* explants. A. rhizogenes were grown to mid-log phase (A600 = 0.6) at 26 ºC on an incubator shaker in the liquid Luria–Bertani medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl, pH 7.0). The bacterial cells were centrifuged (2500 rpm, 10 min) and resuspended at a cell density of A600 = 1.0 in the two liquid inoculation media containing half strength MS salts with B5 vitamins and WPM supplemented with 30 g/l glucose. In the first experiment, leaves were excised from 60-day-old seedlings and used as explants for the induction of hairy roots. The explants were immersed in the bacterial suspension for 10 min and then were blotted dry on a sterile filter paper to remove excess Agrobacterium. In addition, some explants were immersed in sterile distilled water and incubated in the same conditions as control. Then explants incubated in the dark at 25ºC on agar-agar solidified half strength MS salts with B5 vitamins and WPM media supplemented with 30 g/l sucrose. After 3-4 days of co-cultivation with bacteria, the leaf explants were transferred to the hormone-free solid media (half strength MS salts with B5 vitamins and WPM) containing 30 g/l sucrose and 300 mg/l cefotaxime. The process was repeated four times at 4-day intervals to ensure that no bacterial cell colony survived (Abdoli *et al*., 2013). In two other experiments, the effects of explant type (leaf, leaf lamina and petiole) and explant age (20, 40 and 60-days-old) were examined on hairy root induction, by the use of the best treatment determined in the previous experiment (strain AR15834 and WPM medium). In all experiments 3 replications were used and each replication consisted of a plastic

Petri dish (100×15 mm) including 10 explants. Overall, hairy roots appeared during 20-25 days after inoculation and the mean percentage of hairy root induction was measured.

DNA extraction and PCR analysis

After the development of hairy roots, for molecular confirmation, DNA extraction was performed using the cetyltrimethyl ammonium bromide (CTAB) method (Khanuja *et al.*, 1999) and PCR analysis was carried out with specific primers of rolB gene according to the procedure of Chaudhuri et al. (2005). The sequences of the primers used in the PCR were as follows:

FrolB: 5′-GCTCTTGCAGTGCTAGATTT-3′

RrolB: 5′-GAAGGTGCAAGCTACCTCTC-3′

Amplified products were detected by ultraviolet light after electrophoresis in a 1.2 % agarose gel stained with ethidium bromide. In this study, DNA from A. rhizogenes strains served as the positive control and seedling roots were used as the negative control.

The statistical analysis was performed according to the V11.5 SPSS system. The first experiment was carried out as a factorial experiment with two factors (bacterial strain and culture medium) on the basis of a completely randomized design. The other experiments were conducted as a completely randomized design. Mean and standard errors were used throughout, and the statistical significance between the mean values was assessed applying a Duncan's multiple range test. A probability of $P<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Visible hairy roots were formed after 20-25 days at the site of bacterial inoculation of explants (Figure.1). No root formation was observed in the control explants. In the present study, to confirm the integration of T-DNA from the A. *rhizogenes* into the hairy roots, rolB gene was tested using rolB-specific primers (Figure. 2). It is already known that rolB has a central role in transformation (White *et al*., 1985). PCR results showed the presence of diagnostic bands with a size of approximately 423bp that was related to specific reproduction of *rol*B gene, so the transgenic hairy roots were confirmed (Figure. 2).

The results of the first experiment showed that the interaction between strain of A. *rhizogenes* and culture medium had a significant influence on the induction of hairy roots (Table 1). The AR15834 and A4 strains in WPM medium were more efficient for hairy root induction. The AR15834 strain produced the highest percentage of rooted explants (46.6%), followed by A4 strain in WPM medium (23.3%). The lowest percentage of rooted explants (2%) was observed in the interaction of K599 strain and half strength MS medium salts with B5 vitamins (Figure. 3).

Table 1. Analysis of variance for effects of culture medium and A. rhizogenes strains on the percentage of rooted explants in E. angustifolia

Source of variation	Degree of freedom	Mean of squares
Culture medium (A)	1	651*
Strain (B)	3	1317.7*
(A) × (B)	3	151*
Error	16	19.7

* Significant at 5% probability level

Figure 1. Hairy roots obtained by A. rhizogenes in E. angustifolia in WPM medium, 40-45 days after inoculation.

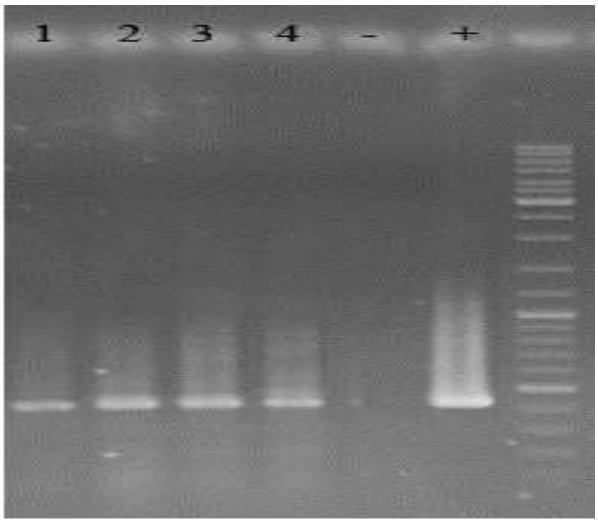


Figure 2. Confirmation of integrated Ri T-DNA in transformed roots of E. angustifolia by PCR analysis with rolB primers. Lanes 1-4: transformed hairy roots by A. rhizogenes, C -: non-transformed root, C +: plasmid.

Influence of A. *rhizogenes* strain on hairy root induction frequency has been documented earlier in several plant species (Sujatha *et al.,* 2013). Plasmid harboured by bacterial strains could be one of reasons of the differences in virulence (Nguyen *et al.,* 1992). Also, the different percentages of rooted explants could be explained possibly by differential expression of T-DNA genes present in the explants, variable copy numbers of T-DNA inserts and positional integration effects of the T-DNA in the host genome (Cho *et al.,* 1998). Moreover, compatibility between A. *rhizogenes* and host plant tissue to the T-DNA, phytohormone production and juvenility of host tissues are important factors in inoculation and hairy root production (Huang *et al.,* 1991). Some studies have shown the effects of the medium composition and culture conditions on the hairy roots induction (Sauerwein *et al.,* 1991; Giri and Narasu, 2000; Mehrotra *et al.,* 2008).

In the present research it was found that the most effective treatment to induce hairy roots in this medicinal plant was a combination of AR15834 strain and WPM medium, so it was used in the next two experiments. *In Rhaponticum carthamoides,* hairy roots were induced from leaf explants transformated by *A. rhizogenes*, strains A4 and ATCC 15834 and the best response (43%) was achieved by infection with A4 strain in WPM medium under periodic light (Skala *et al.,* 2015). In Hypericum perforatum higher susceptibility of explants was detected with strain 15834 (Bivadi *et al.,* 2014). Various efforts have been made to overcome problems associated with host/tissue to increase the number of infection sites, such as use of supervirulent *Agrobacterium* strains and some other factors (Thilip *et al.,* 2015).

In the experiments 2 and 3, the effects of type and age of the explants on hairy root induction were significant (Table 2). Different explants showed various degrees of susceptibility to infection with *A. rhizogenes*. The results showed that the explants obtained from 40-day-old seedlings responded better than those of 20 and 60-days-old explants to *A. rhizogenes*. The highest frequency of rooted explants (62.3%) was obtained from the 40-days – old explants (Figure.4a).Also the results showed that the leaf, leaf lamina and petioles had the significant differences for hairy root regeneration and leaves were better than other explants (Figure.4b).

Some studies showed that the explant age and type can have many effects on the abundance production of hairy roots because of their effects on physiological properties of cells (Pirian *et al*., 2012). A high percentage of hairy root production in leaf explants in the present study may be related to the more competence for transformation and more sensitivity of these explants to *A. rhizogenes* than other explants, which this sensitivity depends on the physiological status of tissues (Mehrotra *et al.,* 2008; Pawarand Maheshwari, 2004; Shi and

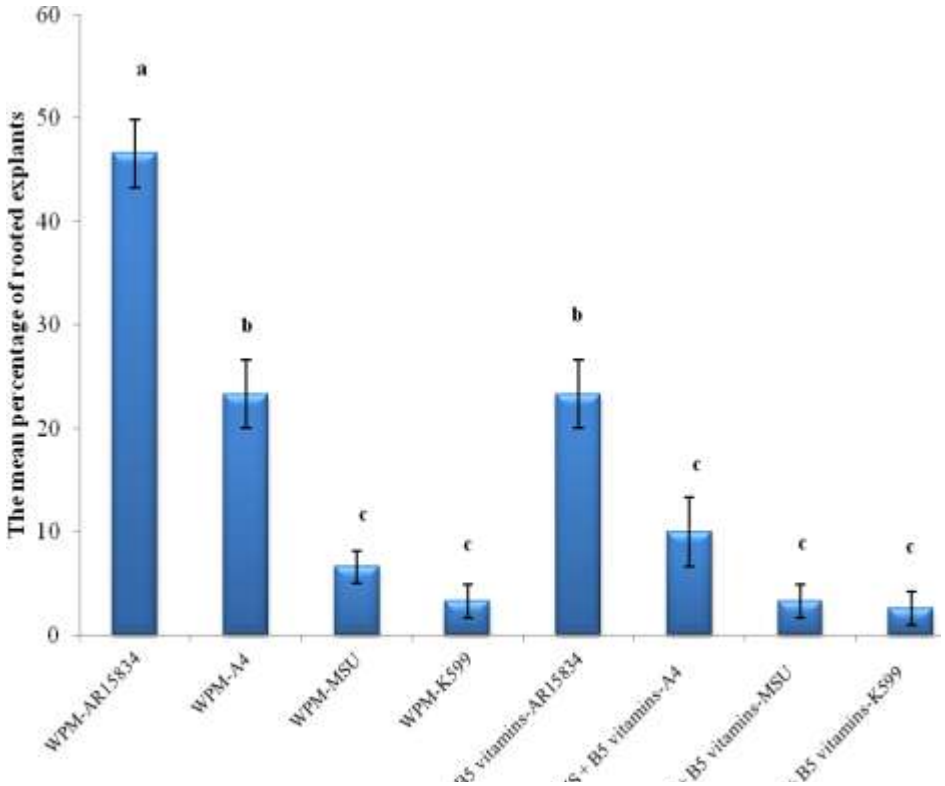


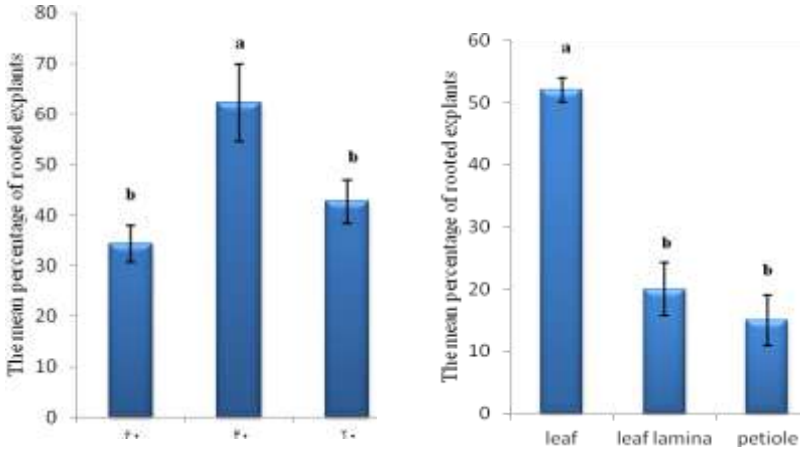
Figure 3. Interaction between different strains of A. rhizogenes and hairy root induction media for hairy root production. Different letters on bars refers to significant differences (P<0.05).

Table 2. Analysis of variance for the effects of type and age of explant on the percentage of rooted explants in E. angustifolia.

S.O.V.	d.f.	M.S.
Age of explant	2	1377.7*
Error	6	58.3

S.O.V.	d.f.	M.S.
Type of explant	2	2400*
Error	4	70.8

* Significant at 5% probability level



a) Explant age b) Explant type

Figure 4. Effects of age (a) and type (b) of explant on the percentage of rooted explants. Mean comparison were done by Duncan's multiple range test. Different letters on bars refers to significant differences (P<0.05).

Kintzios, 2003). Effect of explant on hairy root induction has recently also been observed in *Withania somnifera*.Leaf explants had a higher frequency of hairy root induction (88.4 %) than the cotyledon explant (64.2 %) when these explants were co-cultivated with *A. rhizogenes* strain R1000 (Sivanandhan *et al.,* 2014). The cotyledons, radicles and hypocotyls were also the most suitable explants for the maximum hairy root induction in *Capsicum* species (Nursuria *et al*., 2014). In another study, influence of strain MTCC8196 on the percentage of hairy root production in node, leaf, petiole and stem explants in *Centella asiatica* was investigated and it was found that, the highest induced hairy roots was obtained by nodal explants followed by leaves and petioles and root explants had no reaction to induce hairy roots (Gandi and Giri, 2012). It was also reported that the highest transformation efficiency (93.3 %) was obtained when the leaf explants of *Withania somnifera* were subjected to sonication and heat treatment during transformation with *A. rhizogenes* (Thilip *et al.,* 2015). In fact the nature of explants influence the *Agrobacterium* mediated transformation process (Trypsteen *et al.,* 1991; Yonemitsu *et al.,* 1990). Also, specificity of *Agrobacterium* transformation is related with the age and hormonal balance of the host tissue (Nin *et al.,* 1997). Wound response may be the main factor for the successful transformation. Explant cells difference in their DNA synthesis and cell division ability may be due to the difference in physiological maturity of the cells. Hairy root induction in leaves may be due to their ability to produce a greater number of wounds adjacent to the competent cells for regeneration and transformation (Potrykus, 1990).

In conclusion, the in *vitro* hairy roots were regenerated in *E. angustifolia*. The data showed that the different bacterial strains, culture media and explants had various hairy roots generating capacity in this medicinal plant. The best hairy root induction in *E. angustifolia* was obtained in the interaction between WPM medium and AR15834 strain. Also hairy root induction in this plant was influenced by different physiological conditions of the explants. In future studies, it will be necessary to optimize the hairy roots induction in this plant to identify an efficient hairy roots production protocol for important secondary metabolites production.

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