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Establishment of Inula helenium hairy root culture with the use of Agrobacterium rhizogenes

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ABSTRACT: Elecampane (Inula helenium L, composite) is one of the medicinal plants whose roots have secondary metabolites such as Inulin, helenin and sesquiterpene lactones. In this research, hairy root culture of I. helenium was established by inoculation of the leaf and stem explants with Agrobacterium rhizogenes strains (AR15834 and A4). The emergence of the hairy roots was observed 7-10 days after inoculation on the wounded sites of the explants. The hairy roots transformation was confirmed by polymerase chain reaction (PCR) analysis with the use of rolB specific primers. The highest transformation frequency was obtained (80%) in the stem explants with AR15834. The growth rate of the hairy roots was 10-fold as compared to control root in the same condition.

Keywords: Inula. helenium; Agrobacterium rhizogenes; Hairy root culture; RolB.

Abbreviations: PCR-polymerase chain reaction; MS-Murashige and Skoog.

INTRODUCTION

Inula helenium L. (Compositae family) is one of the perennial plants widely found in Europe and East Asia. Roots of I. helenium plant, were collected in the autumn from the two or three year old ones. The roots contain up to 5% of essential oil with eudesmane-type sesquiterpene lactones (mainly alantolactone and isoalantolactone), thymol derivatives, triterpenes, sterol and up to 44% of the polysaccharide inulin (Blaschek et al., 1998). Inula helenium roots have been used in the folk medicine against a variety of ailments including asthma, cough, bronchitis, lung disorders, tuberculosis, indigestion, chronic enterogastritis, infectious and helminthic diseases. Recent pharmacological studies have demonstrated that the extract of I. helenium possesses anti-tumor, anti-bacterial and insecticidal activities (Huo et al., 2009). Its oil is one of the richest sources of sesquiter-penoid lactones, which have strong anthelminthic activity and the potential to induce detoxifying enzymes (Livermore, 2002; Jeon et al., 2005). There are a lot of applications of inulin in food and pharmaceutical industries, due to its special properties such as very low caloric value, sweetening power and solubility in water (Lopez-Molina et al., 2005). Some interesting secondary metabolites of plants are synthesized in the roots where they are stored or excreted from. The root culture has been successfully established 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, great efforts have been focused on transformed hairy roots (Kim et al., 2002). Hairy root cultures have been proposed as an alternative method of producing plant secondary metabolites because of their genetic and biochemical stability, rapid growth rate and a capability to synthesize secondary products at levels comparable to that of the original plants (Sevon and Oksman-Caldentey, 2002). Rahnama et al. (2008) studied hairy root induction in Silybum marianum with A. rhizogenes AR15834 and reported Silymarin production in hairy root culture. Hairy roots are induced by the incorporation of a bacterial-derived segment of DNA transferred (T-DNA) into the chromosome of the plant cell. The expression of genes encoded within the T-DNA promotes the development and production of roots at the site of infection on most dicotyledonous plants (Venna and Taylor, 2007). The most widely studied Agrobacterim rhizogenes strains, which belong to the group of agropine strains, have two T-DNA regions on their Ri plasmid called TL-DNA and TR-DNA. The TR-DNA carries the genes responsible for opine synthesis (De Paolis et al., 1985), and sequences homologous to auxin synthesis genes (iaa MandiaaH) of Agrobacterium tumefaciens T-DNA (Huffman et al., 1984; Jouanin, 1984). Several loci on the TL-DNA of Ri plasmids have been shown to be essential for hairy root induction (so-called rol genes for root oncogenic loci). The TL-DNA of the agropine-type Ri plasmid (AR15834 or A_4) consists of at least four loci, rolA, B, C, and D

(White et al., 1985; Estramareix et al., 1986; Slightom et al., 1986). The greatest advantage of the 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). An important feature of A. rhizogenes-induced roots is their unique ability to grow in vitro in the absence of exogenous plant growth regulators (Rao and Ravishankar, 2002). The cultures have turned out to be a valuable tool with which to study the biochemical properties and the gene expression profile of metabolic pathways; Moreover, the cultures can be used to elucidate the intermediates and key enzymes involved in the biosynthesis of the secondary metabolites (Hu and Du, 2006). To succeed in establishing a hairy root culture system for a certain plant species, several essential conditions should be taken into consideration. These conditions include the bacterial strain of A. rhizogenes, explants appropriate, a proper antibiotic to eliminate redundant bacteria after cocultivation, and a suitable culture medium (Zhou et al., 1998).

MATERIALS AND METHODS

Plant material

Inula helenium seeds were collected from medicinal plants garden of Bu Ali Sina University (Hamedan, Iran). The seeds were surface-sterilized when rinsed with 5% Tween 20 for 15 min and later sterilized with 70% ethanol for 5s and subsequently with 5% sodium hypocholorite for 10 min. The seeds were cultured on semi solid hormone-free MS (Murashige and Skoog, 1962) medium and incubated at 25±2°C for a photoperiod of 16h light.

Bacterial strains

Agrobacterium rhizogenes wild strains A4 and AR15834 were used in the present study. The strains were kindly provided by Dr. T. Hasanloo, the Agricultural Biotechnology Research Institute karaj, Iran. Prior to infection mono clones of strains, A4 and AR15834 were grown individually to mid-log phase (Optical density at 600nm OD_{600}=0.6) in liquid Luria-Bertani (LB) medium with rifampicine at 28°C on a rotary shaker at 110 rpm.

Induction of hairy root cultures

Leaf and stem explants obtained from different ages (3 weeks and 6 months old) in vitro grown plant were used for inoculation with A. rhizogenes strains. The explants were pricked with sterile Scalpel and then immersed in overnight culture of bacteria suspension (OD_{600}=0.6) for 10 min and then blotted dry on sterile filter paper. Explants were pricked with sterile scalpel, dipped in sterile distilled water, served as control, and incubated in the same medium. After 48 h, the explants were transferred on fresh MS medium supplemented with 0.5 g l^{-1} cefotaxime in order to eliminate bacteria. This activity was repeated 4-5 times and during sub-culturing the antibiotic level gradually reduced. Determination of the transformation frequency response of the leaf and stem explants to bacterial infection in terms of the hairy root emergence was observed. The transformed frequency was determined as follows.

$$TF = \frac{\text{The number of explants showing hairy root emergence}}{\text{Total no. of explants}} \times 100$$

Hairy root lines were cultured by transferring 3-4 cm long root pieces to hormone-free Ms liquid medium with 3% sucrose at 25±2°C on a rotary shaker (110 rpm) in 16-h photoperiod and sub-cultured every 2 weeks.

Confirmation of transformed hairy roots

Having been obtained, bacteria free cultures were sub-cultured on MS medium without antibiotics. Genomic DNA was extracted from both transformed and normal roots according CTAB method (Cai et al., 1997). Isolated DNA was analyzed by PCR for rolB gene (forward primer 5'-ATGGATCCCAAATTGCTATTCCCCACGA-3'and reverse primer 5'-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3'). For amplification, initial denaturation was performed at 94°C for 5 min, followed by 37 cycles of amplification (each comprising 1 min. at 94°C , 1 min. at 58°C and 1 min. at 72°C) and a final extension at 72°C for 7 min. The amplified products were separated on a 1.2% (w/v) agarose gel and detected under UV light.

Measuring the dried biomass

The two-month hairy roots were harvested from the liquid medium and washed twice, with the use of doubled distilled water, then blotted filter paper to remove excess water. Dry weight was measured by drying the fresh hairy roots in the room temperature for 48h. Statistical significance for the dry weight was calculated by the t- test.

RESULTS AND DISCUSSION

Hairy roots could emerge from inoculated explants at the wounded sites 7-10 days after infection with Agrobacterium strains (Fig 1). No hairy roots were formed on control explants.

The highest transformation frequency (80%) was observed in AR15834 strain following inoculation with stem explants at the 3-week age (Table 1). The transformation frequency (hairy root induction) was influenced by bacterial strain, the type and the age of explants, and a proper antibiotic to eliminate extra bacteria after inoculation (Hu and Du., 2006). The two A. rhizogenes strains examined, revealed differences in transformation ability. The efficiency of transformation differs regarding different bacterial strains (Giri et al., 2001). Marked differences in the ability of A. rhizogenes strains inducing hairy roots have been reported by several researchers (Kittipongpatana et al., 1998). Maximum transformation frequency (TF) was recorded in stem explants with both strains.

A number of previous studies showed that the type and the age of the explants had a great influence on the hairy root induction since the age of the explants is a major factor that alters the physiological properties of the cells (Dupre et al., 2000). The inoculation explants with A_4 died after the emergence of the hairy roots, probably because of inappropriate antibiotic used for eliminating extra bacteria on the tissue. The experiment was continued by AR15834 and TF decreased during 6 months in the stem and leaf explants of the inoculation with AR15834 in 13 and 19, respectively. The transformed roots obtained from AR15834 or A4 showed no morphological difference. These roots growing rapidly, showed plagiotropic growth and were highly branched on phytohormone-free medium. From this, it might be concluded the transformation is based on the typical morphologies of the hairy root. The sub-cultured roots were the hairy roots genetically transformed by Ri plasmid, but that should be confirmed at the molecular level.

To be sure that the rolB gene was not amplified from AR15834, the following procedure was performed: The hairy root cultures were maintained on a medium with antibiotics for several subcultures, and then they were grown on a medium without antibiotics for several sub-cultures. Later, hairy root cultures were homogenized and suspensions obtained were placed on LB medium. The medium was incubated for 7 days, and A. rhizogenes was never found on the medium. So, we could make sure that there was no A. rhizogenes in the plant tissue. With the use of primers designed according to the sequence of the rolB in the TL-DNA, the PCR was successfully amplified rolB gene from the genomes of the hairy roots and not in the DNA isolated from the untransformed roots (Fig 2). This finding indicated that the rolB genes from the Ri plasmid of A. rhizogenes were integrated into the genome of I. helenium hairy roots.

Dried biomass of the normal and transformed roots was measured after 2 months of sub-culturing under the same conditions. Production of biomass in the hairy root was more than that in the normal root (Table 2). Akbarian et al (2011) tested different A. rhizogenes strains to investigate the ability for transformation of Trigonella foenum and obtained the higher growth index in hairy roots induced by strains, 15834 and 9126.

CONCLUSIONS

Our results indicated, for the first time, an efficient A. rhizogenes-mediated transformation protocol for the establishment of I. helenium hairy root cultures. It is necessary to evaluate the metabolites production in these hairy roots. Hairy root cultures receive more attention as biological matrices for producing valuable metabolite because they have several interesting trails, such as their rapid growth, easy culture in hormone free medium and especially their ability to synthesize useful metabolites even higher than that of plant roots.

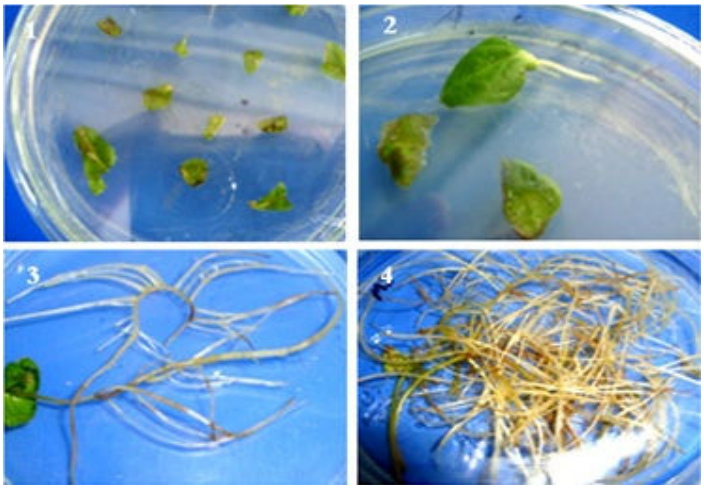

Figure 1. Stepes of I. helenium hairy root induction in leaf explants via A. rhizogenes

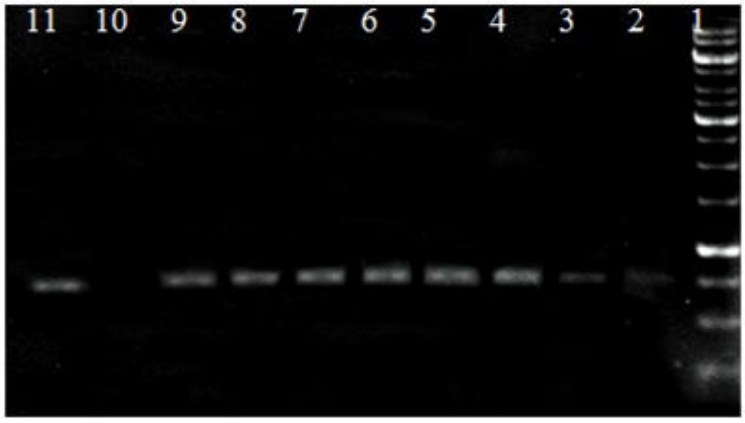

Figure 2. PCR analysis of hairy roots for rolB. 1: Molecular weight marker, 2 – 9: hairy roots, 11: positive control (Plasmid DNA from AR15834), 10 Negative control (normal root)

Table 1. Influence of A. rhizogenes strains (A_4 and AR15834) and source of explants (leaf and stem) in frequency of the transformatio

Transformation frequency		
Explants	strains	
	A_4	AR15834
Leaf	15	30
Stem	71	80

Table 2. Dry weight (DW) produced by I. helenium hairy root and normal root in MS liquid medium

Type of root	DW
Normal root	328/6a
Type of root	30/93b

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