Culture conditions for induction of hairy roots in medicinal plant *Coleus blumei*

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Abstract

Hairy root induction in plants has affected by many factors including type, age of explant and bacterial strain. In the present study, the effects of bacterial strains (A4, C58, 15834 and GM), two concentrations (OD600= 0.5 and 1) and two explants (leaves and stems) in hairy root induction of *C. blumei* were investigated. According to the results of this study, the four strains showed different abilities to induce hairy root. The highest percent of induced roots (85%) was seen in explant treated with C58 strain, at OD600= 0.5 and the lowest induced roots (5%) by using of GM strain at OD600 = 1 and stem explants observed. The highest number of hairy roots per explant (12.94) has shown in leaf explant of leaf inoculated with C58 strain at (OD600 = 0.5) and the lowest number of hairy roots (1/1) was obtained from stem segments treated with GM strain at (OD600 = 1). According to the results of the analysis of variance, the effects of double reciprocal of strain in the concentration and strain in the explant was significant at a 1% probability level. Polymerase Chain Reaction (PCR) analysis using the role gene was performed for identification of the transformed hairy roots. In the lines of transgenic hairy roots, a sharp band of 304-bp was amplified, but no such amplicon was observed in the untransformed root (negative control) sample. The results of this study indicated that the type of bacteria strain, explant type, and bacterial concentration are important factors in hairy roots induction.

Keywords: bacterial strain; bacterial concentration; *Coleus blume*i; explant

Hossein Khah, S., E. Dorani Uliaie and **A. Samadi.** 2019. ' Culture conditions for induction of hairy roots in medicinal plant *Coleus blumei*'. *Iranian Journal of Plant Physiology* 9(3), 2849-2855.

Introduction

Plants as well as in vitro plant cell or tissue cultures have served as resources for preservatives, natural pigments, flavors, enzymes, bio-based fuels and plastics, cosmetics, and bioactive compounds (Mary, 2005). Rosmarinic acid has antioxidant properties (Herodez et al., 2003) and anticancer activities (Scheckel et al., 2008) as well as broad industrial applications. In plants, RA biosynthesis takes place through the flavonoids biosynthesis pathway. Flavonoids are classified as antioxidant compounds that play a role in creating the colour in plants; they protect the plant against attacks from pathogens and exposure to UV radiation and also act as an indicator on the conditions due to tension and scavenging of free radicals (Hamilton et al., 2003). Many biotechnological strategies have been hypothesized and experimented for enhanced production of secondary metabolites from medicinal plants. Some of these include a

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Received: October, 2018
Accepted: March, 2019

screening of high yielding cell lines, media modification, precursor feeding, elicitation, large scale cultivation in a bioreactor system, hairy root culture, plant cell immobilization, biotransformation and others (Namdeo, et al, 2002, Vanishree et al., 2004).

Agrobacterium rhizogenes infects wounds of many plant species and the infections are characterized by the production of adventitious roots with numerous hairy root. The *A. rhizogenes* has caused characterized hairy roots by fast growth on hormone-free medium, lack of geotropism, abundant lateral branching, and genetic stability. Recent progress in the scaling-up of hairy root cultures makes this system an attractive tool for industrial production of plant secondary metabolites (Guillon et al., 2006 a, b).

Coleus blumei is susceptible to Agrobacterium infection (Bauer et al., 2002). Transformed callus cultures of *C. blumei* have been established and shown faster growth and higher RA accumulation than normal calli and intact plants (Bauer et al. 2004).

The medicinal importance of RA and biotechnological strategies of hairy roots has caused that optimization of important factors for the induction of hairy root in *C. blumei* be important and necessary for the present study.

Materials and Methods

Explants preparation

C. blumei seedlings were prepared in a potted form and propagated using the cuttings method in the laboratory. The explants were separated from greenhouse plants and were thoroughly washed with water for 10 minutes, and surface-sterilized by immersing in 70% (v/v) ethanol for 90 seconds then the explants were washed in sterile distilled water for 5 minutes. In the next step, the explants were immersed in 1.8%(v/v) sodium hypochlorite (commercial bleach) for 10 minutes and were stirred repeatedly. Finally, sterilized explants were washed three times with sterile distilled water for 5, 10 and 15 minutes, respectively.

Activation of bacterial strains

Four wild-type strains, 'A4, C58, 15834 and GM' of *A. rhizogenes*, were kindly provided by the National Institute of Genetic Engineering and Biotechnology, Iran. For preparing of bacterial suspension for each strain, a single bacterial colony was cultured into liquid LB medium (Bertani, 1952) then supplemented with 50 mg/l rifampicin and maintained at 28 °C for 48h on a rotary shaker at 100 rpm. The transformation procedure was down as described previously (Samadi et al., 2012). The overnight culture bacterial was centrifuged at 3,500rpm for 10 min and to form a bacterial deposit at the bottom of the falcon, the bacterial pellet was re-suspended in liquid MS medium (Murashige and Skoog, 1962) in pH 5.5. The final density of bacterial suspension was diluted with LB medium to 0.4-0.6 OD (optical density at 600 nm) that two density of bacterial suspension (0.5 and 1) were used for the infection.

Inoculation of explants and induction of hairy roots

Prepared explants immersed in bacterial suspension under sterile conditions for 20 minutes. Then the explants, removed from the suspension and dried by the sterilized Whatman paper. Inoculated explants were transferred to agar (7.5g/l) solidified hormone-free MS medium for co-cultivation under dark incubation at 24 ± 2°C for 2 days. The explants of *C. blumei* without bacterial inoculation were also used as a negative control. After co-cultivation, the explants washed in the liquid MS medium containing 300 mg/L Cefotaxime antibiotic and after drying on sterile Whatman paper, sub-cultured in solid MS medium with 300 mg of Cefotaxime antibiotic. All cultures placed in the growth room at 25 ± 2 °C under darkness. The subsequent subcultures were down on the same medium without antibiotics. Emerged hairy roots from the wound sites of explants (about 15 to 20 mm in length) separated from the tissue of explants and sub-cultured on solidified hormone-free MS medium and maintained in a growth chamber at 24 ± 2 °C under darkness. Rapidly hairy roots transferred to 20 ml of MS liquid medium, containing 30 g/l sucrose, in 100 ml Erlenmeyer flasks on a rotary shaker (110 rpm) at 24 ± 2 °C in the dark and sub-culturing was carried

Table 1
Analysis of variance of data on the transgenic percent of explants and hairy root number per explants in the *C. blumei*

Source of change	Degree of freedom	Average of squares	
		the transgenic percent of explants	Hairy root number per explant
Strain	3	3656.250**	37.699**
Bacterial concentration	1	2025**	8.666**
Explant	1	16256.250**	765.560**
Strain. Bacterial concentration	3	91.667^{ns}	6.033**
Strain. Explant	3	289.583^{ns}	12.721^{ns}
Concentration. Explant	1	225^{ns}	1.246^{ns}
Concentration. Strain. Explant	3	41.667 ^{ns}	1.672^{ns}
Experimental error	48	123.958	0.703
The coefficient of variation (percent)	-	29.44	15.8

** Significant at 1% probability level; * Significant at 5% probability level; ns (non-significant!)

out after every 14 days in the same medium for proliferation of hairy roots.

Analysis of hairy roots by a polymerase chain reaction

Genomic DNA extracted from both hairy roots and untransformed roots (control) by Dellaporta method (Dellaporta et al., 1983). For confirmation of the transgenic nature of hairy roots, the presence of the rol genes located on the T-DNA which are main determinants for the development of hairy roots was examined by polymerase chain reaction (PCR) analysis using corresponding gene-specific primer pairs. The Ri plasmid of *A. rhizogenes* strains 'A4, C58, 15834 and GM' was used as a positive control. The primer sequences to amplify a 100-bp portion of the rolA gene were 5´-ACGGTGAGTGTGGTTG TAGG-3´and 5´-GCCACGTGCGTATTAATCCC-3´. The primers (5'-ATGCCCGATCGAGCTCAAGT-3' and 5'-CCTGACCCAAACATCTCGGCTGCCCA-3' as forward and reverse primers, respectively were designed based on the virD gene from the not-transferred were also used to ensure transformed root tissue was free of bacterial cells. PCR reaction profiles involved: initial denaturation at 95 °C for 5 min, 35 cycles consisted of denaturation for 30 s at 95 °C, annealing for 40 s at 55 °C, an extension for 30 s at 72 °C and a further extension step for 7 min at 72 °C. Amplified DNAs has analysed by staining with ethidium bromide after electrophoresis in a 0.8% (w/v) agarose gel at 85 V for 85 min.

Statistical Analysis

The used experimental design in this study was factorial based on a completely randomized design (CRD) with four replications per treatment. Data were subjected to the analysis of variance (ANOVA) using the MSTAT computer package. Normalizing of the transgenic percent of explants was performed by angular transformation, log conversion, and angular transformation respectively. The comparison of the meanings was down by Duncan's multi-domain test at a 5% probability level. The charts were also drawn by Excel software.

Results

Establishment of hairy root cultures

According to the results of the comparison

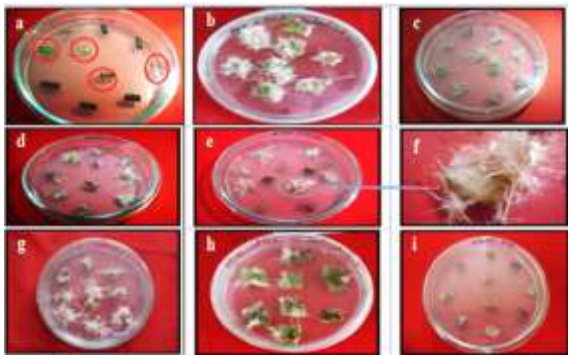

Fig. I. Hairy roots induced on different sites of leaf and stem explants in *C. blumei* one week after inoculation by *A. rhizogenes* strains; (a) the emergence of hairy roots from stem explants by C_{58} strain; (b, c, d, e, f, h, i) induction of hairy roots from leaf explants by $C_{58,}$ $A_{4,}$ GM, strain at 0.5 and 1 bacterial concentrations, respectively; (f) close view of a hairy root; (g) induction of hairy roots from leaf explants by 15834 strain at one bacterial concentration

of the means (Figs. I and II) the highest number of

hairy roots per explant, were recorded in the C58 strain with a concentration of OD_{600} = 0.5 (12.94) while the lowest number of hairy roots was related to the GM strain with a concentration of OD_{600} = 1 in stem explant (1/1).

According to the results of analysis of variance (Table 1), simple effects of strain, concentration, and explants was significant at 1% probability level, also double reciprocal effects of strain and the concentration and also strain and the explant were significant at P≤0.01. The effect of dual interaction of concentration and the explants and the triple interaction of the strain, the concentration, and the explant were not significant at P≤0.05.

Effect of explant on hairy root efficiency

Leaf and stem segments were taken from laboratory grown plants, and were infected with different strains of *A. rhizogenes*. There was a variation in the number of hairy roots of various explants and the time required for hairy roots induction (Fig. I). The results of the present study showed that leaf explants were more susceptible to infection with *A. rhizogenes* strains as 85% of the hairy roots, whereas, stem explants exhibited thin hairy roots and the lowest infection frequency (15%). Also, the leaf explants were infected by all strains, though only the C58 strain was able to transgenic in stem explants (Fig. I).

Effect of bacterial strains in hairy roots induction

According to the results of comparisons of the means, the highest transgenic percentage was found in C58 strain at OD_{600}= 0.5 at leaf explant (85%) and the lowest transgenic level was recorded in GM strain, concentration of 1 and in stem explant (5%). Based on the results of this study, all four strains had the ability to induce hairy roots, but the transgenic percentage was different. These results indicate that type of bacteria strain is an important factor for inducing various hairy roots.

Concentration of bacteria

In addition to the bacterial strain, the concentration of bacterial is also an important factor in the transgenic process. Correct contact and interaction of Agrobacterium with plant cells is a prerequisite for gene transfer through agrobacterium. Among two concentrations used, half bacteria concentration showed the highest percentage of transgenic compared to the concentration 1. The results of mean comparisons based on Duncan's multi-domain test showed that

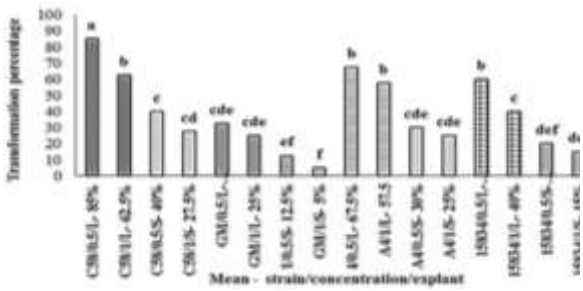

Fig. II. Comparison of transformation percentage in extracts of *C. blumei* using four strains of *A. rhizogenes* at two concentrations

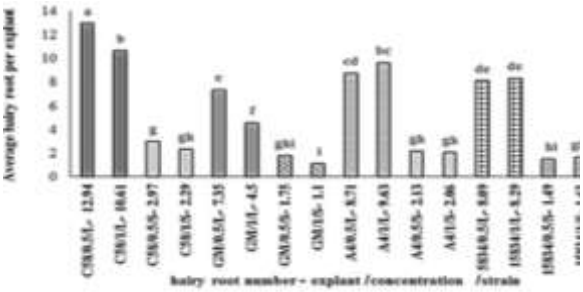

Fig. III. Comparison of mean number of hairy roots per single explant of *C. blumei* using four strains of *A. rhizogenes* at two concentrations

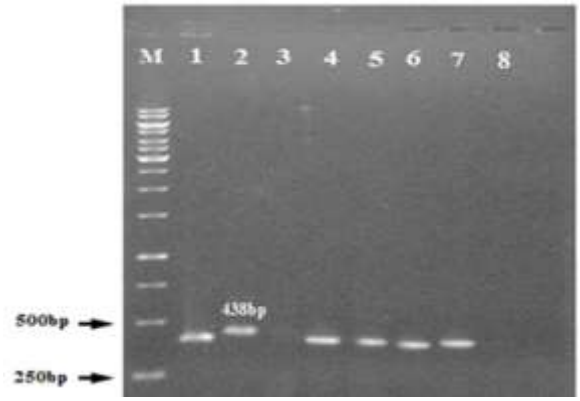

Fig. IV. PCR analysis for hairy roots lines of *C. blumei* using the *rolA* gene specific primers; M: SM0311 Gene Ruler 1 kb DNA Ladder; 1: PCR analysis using the *rolA* gene specific primer; 2: PCR analysis using the *virD* gene specific primer; 3: Wild plant leaf as first negative control; 4 to 7: Transgenic hairy roots induced on leaf explants infected by Agrobacterium; 8: Non-DNA template PCR reaction as second negative control

C58 strain at the concentration of OD_{600} = 0.5 had the highest number of hairy roots per explant (12.94 number) and the lowest number of hairy roots was recorded at concentration 1 (OD_{600} = 1), in the GM strain and in stem explant (1.1 number) was shown.

Detection of relevant transgenes in the selected hairy root lines

PCR method can be used simply for detecting T-DNA sequences in putative transformant (Palazon et al., 2003). Four loci involved in root formation have been identified in the T-DNA of the Ri plasmids and designated root loci (*rol*)A, B, C, and D (Ayala-Silva et al., 2007). In this study, the presence of the *rol A* gene in the hairy root lines was confirmed by PCR analysis (Fig. IV) using primers specific for core sequence located at the *rol*A gene. Genomic DNA was extracted and purified from both transformed and untransformed roots grown in Murashige and Skoog (MS) media. In the lines of transgenic hairy roots, a sharp band of 304-bp was amplified, but no such amplification was observed in the untransformed roots (negative control) sample (Fig. IV). The *vir*D gene, located outside the T-DNA, was diagnostic for the presence of any remaining agrobacteria in the root tissue. The negative results of PCR amplification for the *vir*D gene demonstrated that no bacterial DNA was involved in *rol*A amplification leading to false positives (Fig. IV). This result indicated that the explants of *C. blumei* are susceptible for transformation with *A. rhizogenes* strains.

Discussion

Hairy root cultures of *C. blumei* were initiated by inoculation of leaf and stem explants with four strains, A4, C58, 15834, and GM of *A. rhizogenes*. Development was evident after 8 weeks whereas no adventitious roots formed from the control explants. These roots exhibited characteristics typical of transformed roots, that is rapid growth, extensive lateral branch, root covered with a mass of tiny hairy roots, and the lack of geotropism (Sevon and Oksman-Caldentey 2002). There was a variation in the number of hairy roots of various explants and the time required for hairy roots induction. Different explants respond differently to *A. rhizogenesis* to induce hairy roots. This different sensitivity of explants may be related to the physiological stage of tissue in the plant (Shahabzadeh et al., 2013). Sook Young et al., (2008) investigated rosmarinic acid production in hairy root cultures of *Agastache rugosa* Kuntze showing that the frequency of bacteria at stem explants was higher than leaf explants. Each species has different cell wall structure, physiological status, and marker molecules, which may lead to a difference in the ability to form hairy roots in different species (Kuzovkina and Schneider, 2006). Also, differences in physiological condition, DNA synthesis and cell division in different tissues may be the reason for different ability of plants to produce hairy roots (Pirian et al., 2012). These results indicate that type of bacteria strain is an important factor for inducing various hairy roots. Naderian et al. (2012) investigated the effect of different strains of *A. rhizogenes* in *Datura innoxia* and found that A4 strain had the highest transgenic with 86.66%, which indicates the different effects of various strains on the ability to induce hairy roots. Different *A. rhizogenes* strains have various responses and rooting ability and finally a wide variation of accumulation of secondary metabolites. The selection of an effective agrobacterium strain is highly responsible for the production of transformed root cultures on types of plant species and it could be determined empirically (Lee et al., 2010). The probable cause of this phenomenon can be the difference in the plasmids of each strain (Nguyen et al., 1992; Vanhala et al., 1995). Correct contact and interaction of agrobacterium with plant cells is a prerequisite for gene transfer through agrobacterium. Reducing the concentration of bacteria causes non-transgenic growth and increasing its concentration will destroy the explants after the stage of the co-cultivation (Kumar et al., 1991). According to the results of Pirian et al. (2012), the highest transgenic percentage was observed in leaf explants inoculated with 15834 strain at a concentration of 0.6. Bacterial concentration plays an important role in transformation systems. The results of Shuai et al. (2016) showed that treatment of petioles with *A. rhizogenes* suspension at an OD600 of 0.2, 0.4, and 0.6 generated the steadily

increasing transformation efficiency although denser *A. rhizogenes* suspensions (OD600= 0.8 and 1.0) resulted in the contamination of the explant by *A. rhizogenes* itself. Exposure to high levels of bacteria can cause irreversible physiological perturbation resulting in browning of tissues and unsuccessful recovery of transformed cells (Sreeramanan et al., 2008).

Conclusion

Hairy root culture is a valuable alternative approach for the production of RA from *C. blumei.* We found that *A. rhizogenes* strain C58 was best for induction of hairy roots in *C. blumei.* All four strains induced hairy root formation but at different levels. Based on our study, we conclude that different explants, bacterium strains, and various bacterial concentration are important factors for improvement of culture condition and rosmarinic acid production.

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