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EFFECT OF *Agrobacterium rhizogenes* ON HAIRY ROOTS INDUCTION IN FENNEL (*Foeniculum vulgare* MILLER)

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Fennel

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ABSTRACT

This study was conducted to access the effects of seven *Agrobacterium rhizogenes* strains i.e. A4, T4, MSU, TCC15834, 2656, Gm1 and R1000, on the hairy root generation from various explants i.e. hypocotyl, cotyledon, root, stem and leaf of Fennel ecotypes (Bonab, Izmir, Esfahan and Moghan). Results of study revealed that induction of hairy root varies with the *A. rhizogenes* strains and used explants. Among the various tested combinations, highest root induction was recorded from hypocotyl explants using A4 and TCC 15834 (70 and 67%, respectively) strains of *A. rhizogenes*. Response of explant toward hairy root induction and number of the hairy roots per explant was different and decreased from hypocotyl, leaf and cotyledonary explants, respectively. Among the various tested *A. rhizogenes* strain, TCC 15834 capable to induced hairy Roots development in all ecotypes but most effectively in Izmir (36.66 ± 0.57) and it was followed by Bonab (32 ± 0.44), Esfahan (30 ± 0.99) and Moghan (16.66 ± 0.79). All transformed hairy root were successfully confirmed by PCR using rolB primers. Further hairy root induction could be proved as an alternative approach for the production of secondary metabolites and its increasing use as model system in metabolic engineering.

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1 Introduction

Fennel (*Foeniculum vulgare* Miller) is an important herbal plant growing in Mediterranean regions and widely used as a vegetable and medicinal crop (Jamshidi et al., 2012). This plant is well known for its anti-inflammatory, antispasmodic, antiseptic, carminative, diuretic and analgesic effect. Its anti-ulcer and anti-oxidant properties were also well reported by researcher. Further, it is widely used to gastrointestinal and neurological disorders (Birdane et al., 2007; Delaram et al., 2011). The medicinal benefit of fennel is mainly because of essential oils and aromatic compounds which are produced in secretory canals present in all organs of this tree. Among various studied aromatic and volatile compounds, anethole is the main alkaloid and the primary component of its volatile oil (Hnnanlt et al., 1989). Obtaining medicinally important compounds from the wild or cultivated plants is limited due to difficulties in cultivating under field conditions, risk of extinction due to over exploiting plant from natural habits and geopolitical limitation (Verpoorte et al., 2002). In order to overcome these problems, many attempts have been made over last few decades, possibility of producing medicinally important plant compounds through plant tissue or organ cultures is one of the alternate to avoid above mentioned such barriers (Berlin, 1986; Alfermann & Petersen, 1995). However, in most of the cases, medicinally effective compounds were undetectable or were accumulated at low levels in the in-vitro cultures. Therefore several strategies such as screening and selection of high yield producing cultivars or ecotypes, elicitation and culture of differentiated tissues were developed. In most of the cases several problems were encountered which prevented the development of an economically valuable commercialization of the biotechnologically produced compounds (Verpoorte et al., 2002; Hu & Du, 2006).

A. rhizogenes mediated hairy root production is a valuable tool to overcome the difficulties associated with the in-vitro plant organ cultures and metabolic engineering (Ravishankar & RamachandraRao, 2000; Hu & Du, 2006). Further, it also considered as a beneficial tool in obtaining fast growing organs with extensive branching and capability of producing the main metabolites of the mother plant or even new metabolites that neither detected in the mother plant nor in other kinds of in vitro cultures (Nader et al., 2006). So far, in vitro studies conducted on the production of the fennel secondary metabolites are mainly based on the callus or cell suspension cultures (Paupardin, 1976; Garcia-Rodriguez et al., 1978). However, plant cell suspension cultures for the production of secondary metabolites have been hampered by several limitations such as low yields of desired compounds, expensive culturing process, application of phytohormones, heterogeneous cell types, lack of storage tissue and products easily degraded by the enzymes released in the media (Davies & Deroles, 2014). Hairy root cultures have been induced in many plant species which leading to the in vitro production of numerous plant secondary metabolites and pharmacologically

active compounds (Bensaddek et al., 2008; Ooi et al., 2013; Nagella et al., 2013; Sharafi et al., 2014; Thwe et al., 2016; Yao et al., 2016). Genetic engineering is a modern tool which regulates the production of secondary metabolism. In order to introduce exogenous genes into plants for increasing the amount of target compounds or to gain insight into the functions of genes, the transformation conditions must first be optimized. However, the success of hairy root induction in plants species depends on various parameters such as the plant species, ecotypes age of the plant tissue (Sevon & Oksman-Caldentey, 2002), strain of *A. rhizogenes* and the density of the bacterial suspension (Park & Facchini, 2000). To the best of our knowledge, several species of the fennel family have been transformed by *A. rhizogenes* for producing hairy roots in the past (Baranski, 2008), but this is the first report considering the effect of different factors on *A. rhizogenes* transformation of *F. vulgar*. Present study has been focused on the developing an efficient protocol for induction of high frequency hairy root in fennel.

2 Materials and Methods

2.1 Plant material, culture media and conditions

Seeds of fennel ecotypes (Bonab, Izmir, Esfahan and Moghan) were obtained from a local supplier. Healthy seeds were scrutinized by physical inspection and selected seed were taken for surface sterilization. For surface sterilization, seeds were washed under tap water for 5 minutes, and it was followed by the application of 3% sodium hypochlorite solution containing 0.1% Tween-20 for 15min and rinsed three times with sterile distilled water. Finally seeds were sterilized with 70% ethanol for 2 minutes and rinsed again several times with sterile water. Ten sterilized seeds were cultured in individual Petri dishes containing half-strength of MS media (1.5% sucrose and 5 g/l agar). Seeds were left to germinate under a 16/8 h (light/dark) photoperiod for 3-4 weeks. Various explants cotyledon, hypocotyl, root (of one-week-old seedlings) leaf and stem (of two-weeks-old plants) about 1 cm were excised and used as starting material for co-cultivation with *A. rhizogenes*.

2.2 *A. rhizogenes* strains and Preparation of Inoculum

Various strains of *A. rhizogenes* including A4, T4, MSU, TCC15834, 2656, Gm1 and R1000 harboring a Ri plasmid that involved in the root induction process were provided by Dr. A. Mirshamsi (Department of Plant Breeding and Biotechnology, University of Mashhad, Iran). Single colony of the *A. rhizogenes* were cultured in 25 ml of LB medium and incubated at 28°C in the dark on a rotary shaker at 120 rpm. Bacterial cultures were allowed to grow until the OD₆₀₀ of 0.3 was obtained. Bacterial suspensions were centrifuged (3000 rpm. for 10 min) and obtained pellets were re-suspended in freshly prepared MS liquid medium to obtain the best optical densities (OD₆₀₀) 0.6 (tested among OD₆₀₀ 0.2- 0.8) for inoculation to explants.

Figure 1. *A. rhizogenes* mediated transformation of *F. vulgare* with different *Agrobacterium rhizogenes* strains for one month

hairy root induction after 2 weeks of inoculation in leaf explants (a), hypocotyl explants (b) and cotyledon explant (c), callus formation in stem explants (d), Three-week-old *C* transformed root cultures in MS liquid medium (e) and hairy roots induction morphology after one month of inoculation (f)

2.3 Induction of hairy root culture

For induction of hairy root culture, each plant part (hypocotyl, cotyledon, root, stem and leaf) were cut in to 1 cm length and immersed in 10 mL of the bacterial suspension that prepared 20 min before the inoculation (best time tested among 10- 50 min). For each explants minimum 30 plant tissue were immersed with bacterial suspension. These inoculated explants were cultured in Petri dishes containing basal MS medium without hormones (5 g/l agar) in the dark for two days. Later on these explants were transferred to fresh MS medium containing 0.5 g/l Cefotaxime. Bacteria-free roots were subcultured in 150 ml Erlenmeyer flask containing MS medium (hormone-free) and were used as controls (Figure 1, e).

2.4 Statistical analysis

Three fennel ecotypes were evaluated with seven *A. rhizogenes* strains and five explants in a factorial arrangement based on completely randomized design. Each treatment consisted 10 explants per Petri dish with three replicates. Two parameters viz. induction of hairy root and number of hairy root were measured after one month and analyzed using a two-way analysis of variance (ANOVA) with SAS 9.0 and standard deviation and mean values expressed as mean $\pm$ SD.

2.5 PCR analysis

Genomic DNA from putative transformed hairy roots and normal roots was extracted by the cetyl-trimethylammonium bromide

(CTAB) (Murray &Thompson, 1980). Ri plasmid DNA was extracted from all strains using the SDS/alkalinolysis and used as positive control. For conforming of transgenic roots, polymerase chain reaction (PCR) were used to amplification of *rolB* gene for randomly chosen events among all explants specific using 5' – GCTCTTGCA GTGCTAGATTT-3' and 5' -GAA GGT GCA AGC TAC CTC TC-3' as the forward and reverse primers, respectively.

3 Results and Discussion

3.1 Establishment of hairy roots

In the present study, seven strains of *A. rhizogenes* (A4, T4, Msu, R1000, 2656, Gm1 and TCC 15834) and five explants (hypocotyl, cotyledon, root, stem and leaf) were tested for their ability to induce hairy root on four fennel ecotypes (Bonab, Izmir, Esfahan and Moghan). During study, putative hairy roots were obtained from leaf (Figure 1, a), hypocotyl (Figure 1, b) and cotyledon (Figure 1, c) after 14 days of co-cultivation, but root and stem explants were dead followed by the necrosis of the tissue in all fennel ecotypes. No hairy roots or callus were formed in control explants. Also very few number of explants showed callus-like morphology (Figure 1, d) as reported by others (Moyano et al., 1999). When the hairy roots grew to 2-3 cm in length, they were excised and subcultured in plant-hormone-free MS liquid medium (figure 1, f). Callus formation was reported lowest in the cases of *A. rhizogenes* strain A4, T4, MSU and TCC15834; these strains were also found most effective strains for hairy root induction for all fennel ecotypes. However, other strains (R1000, 2656 and

Gm1) did not induce any hairy root. Therefore in this study *A. rhizogenes* strains A4, T4, MSU and TCC15834 and explants leaf, hypocotyl and cotyledon explants were found effective in the hairy root generation. The hairy roots were formed from the wounded site of *A. rhizogenes* infected explants (Figure1). These results are in accordance with the findings of Porter & Hector (1991) and Winans (1992) those who reported that the wounded sites are a common location for hairy root induction since they are used as a genetic transfer point for *A. rhizogenes*. The induced hairy roots of four fennel ecotypes were similar in terms of structure, coloration, and growth pattern (figure1, e).

3.2 Effect of *A. rhizogenes* strains, ecotype and types of explant on hairy root induction

In this experiment two parameters *A. rhizogenes* strain and various plant parts were measured to evaluate the hairy root induction efficiencies of fennel ecotypes. Analysis of variance (ANOVA) showed a significant difference among various ecotypes, explants and strains and their interactions for induction of hairy root percentage in fennel ($P < 0.01$) (Table 1). All the strains of *A. rhizogenes* led to hairy root induction after 3–4 weeks of culture. However these *A. rhizogenes* strains were not equally efficient in hairy root induction percentage. Strain A4 conferred the highest induction percentage for all three explants examined and produced 50, 53 and 70% hairy root induction from leaf, cotyledon and hypocotyl, respectively (Figure 2). This hairy root induction percentage was followed by the strain TCC 15834, T4 and strain MSU respectively (Figure 2). Figure 2 indicates that strains A4 and ATCC 15834 were more competent than the other strains for hairy root induction in each explant type. Similarly, Arafa et al. (2015) reported that A4 was the best *A. rhizogenes* strain for transformed root induction in *Nepeta cataria*. In another report, the young leaves of *Przewalskia tangutica* showed highest sensitivity to A4 for the effectiveness (100%) in the induction of hairy roots (Lan & Quan, 2010). Further, ATCC 15834 has been also reported as a most widely used *A. rhizogenes* strain for strong root induction ability (Kochan et al., 2013; Kumar et al., 2014). Variation in hairy root induction among different bacterial strains could possibly attribute to disparity in virulence (Porter & Hector, 1991).

In addition, types of explants also influenced the hairy root induction percentage in all strains (table 1). In present study, hypocotyls were reported more efficient with all strains of *A. rhizogenes* in hairy root production as compared to leaf and cotyledon (Figure 2). Similar results were reported by Lee et al. (2004) when these researchers conducted similar study in *Taraxacum platycarpum*. Findings of this study are contradictory to the findings of Hasnat et al. (2008) and Setamam et al. (2014) those who have reported higher hairy root induction from the cotyledonary regions of the *Capsicum species*. Previous findings indicated that *A. rhizogenes* strain and type of explants significantly influenced the induction of hairy root (Chaudhuri et al., 2005; Danphitsanuparn et al., 2012; Sudha et al., 2012).

Table 1 Mean squares of hairy root induction and hairy root induction frequency per single explant in fennel after one month

Source	df	Mean Square	
		Hairy root induction	Hairy root induction frequency
Ecotype	3	661.583*	45.21 ^{ns}
Explant	2	2747.299**	6048.58**
Strain	3	22078.694**	389.54**
Ecotype * Explant * Strain	18	438.046*	63.762 ^{ns}
Ecotype * Explant	6	772.104*	46.120 ^{ns}
Explant * Strain	6	1038.215**	112.287**
Ecotype * Strain	9	56.645 ^{ns}	89.464*
Error	96	207.264	38.299

ns, not significant; *, ** significant at 0.05 and 0.01 probability level.

Figure 2 Effect of different *A. rhizogenes* strains on different explants for Percentages of hairy root induction per total explants (n = 30) of *F. vulgare* after 1 month.

Figure 3 Effect of different *A. rhizogenes* strains on different ecotypes for Percentages of hairy root induction per total explants (n = 30) of *F. vulgare* after 1 month.

Significant differences were observed between different ecotypes for hairy root induction percentage (table1). Results of this study showed that the hairy root induction percentage in Bonab were highest as compared to the other fennel ecotypes (Figure 3).

Table 2 Effect of *Agrobacterium* strain, ecotype and explant on number of induced Hairy root per single explants (mean $\pm$ standard deviation) in *F.vulgare* after 1 month

Ecotypes	Explant type	Strains			
		TCC 15834	A4	MSU	T4
Bonab	Leaf	27.66 $\pm$ 0.57	16.66 $\pm$ 0.52	13.33 $\pm$ 0.57	14.66 $\pm$ 0.53
	Cotyledon	8.66 $\pm$ 0.45	9.66 $\pm$ 0.47	3.66 $\pm$ 0.81	3.66 $\pm$ 0.96
	Hypocotyl	32.14 $\pm$ 0.44	25.33 $\pm$ 0.54	16.21 $\pm$ 0.73	25.33 $\pm$ 0.50
Izmir	Leaf	36.66 $\pm$ 0.57	17.12 $\pm$ 0.9	14.66 $\pm$ 0.53	24.34 $\pm$ 0.96
	Cotyledon	7.32 $\pm$ 0.62	9.66 $\pm$ 0.75	4.66 $\pm$ 0.93	4.33 $\pm$ 0.82
	Hypocotyl	32.66 $\pm$ 0.95	10.66 $\pm$ 0.98	20.33 $\pm$ 0.42	23.41 $\pm$ 0.52
Esfahan	Leaf	26 $\pm$ 0.35	30.15 $\pm$ 0.99	16.41 $\pm$ 0.86	15.22 $\pm$ 0.79
	Cotyledon	7.33 $\pm$ 0.75	9.51 $\pm$ 0.65	4.85 $\pm$ 0.51	4.66 $\pm$ 0.67
	Hypocotyl	24.66 $\pm$ 0.67	29.33 $\pm$ 0.35	12.33 $\pm$ 0.65	22.18 $\pm$ 0.25
Moghan	Leaf	16.66 $\pm$ 0.79	13.66 $\pm$ 0.46	10.66 $\pm$ 0.97	5.33 $\pm$ 0.67
	Cotyledon	7.46 $\pm$ 0.83	8.84 $\pm$ 0.52	6.36 $\pm$ 0.64	4.33 $\pm$ 0.74
	Hypocotyl	15.69 $\pm$ 0.45	15.05 $\pm$ 0.56	9.19 $\pm$ 0.44	10.30 $\pm$ 0.56

Interaction between explants and ecotypes revealed a significant relationship between the hypocotyl and all ecotypes except Moghan and had high root induction percentage range from 59–74% (Figure 3). The leaf and cotyledons of all fennel ecotypes except Moghan showed hairy root induction between 37% and 56%. Leaf explants of Moghan ecotypes had the highest induction rate at 58% as compared to the leaf explants of other ecotypes

(Figures 3). The mean percentages for interaction between all ecotypes and A4 and ATCC 15834 strains were high at 66–83% hairy root induction (Figure 4). These results of this study demonstrate that explant type and ecotype play an important role in hairy root induction. This may be because of the variation in the gene expression in various ecotypes (Winans, 1992; Konstantin et al., 2006). In this study, number of hairy roots in single explant

Figure 4 Effect of different explants on different ecotypes for Percentages of hairy root induction per total explants (n = 30) of *F. vulgare* after 1 month.

expressed the frequency of hairy root induction. The results of study suggested that explant types and strains of *A. rhizogenes* had significant differences in the number of hairy root induction (Table 1). Also the interactions between strains, explant and ecotypes were reported significant. The highest number of hairy roots was achieved for leaf and hypocotyl explants with ATCC 15834 strain in all ecotypes that flowed by A4 (Table 2). The cotyledonary explants showed least hairy root number in all strains and ecotypes. ATCC 15834 strain had the highest number of hairy roots for hypocotyl and leaf in all ecotypes (Table 2). Explant and strain specificity observed in the present study are in the agreement with the findings Sudha et al. (2003) and Setamam et al. (2014), those who reported that hypocotyl explants infected with strain ATCC 15834 gave maximum number of roots in *Rauvolfia micrantha* and *C. frutescens* respectively. However, ATCC 15834 showed the highest root induction in Izmir ecotype in leaf (37%) and hypocotyl (33%) explant (Table 2). Results showed that the hairy root induction number was high in the genotype Bonab with bacterial strain ATCC 15834 for hypocotyle and leaf explants with 33 and 28 respectively. While highest hairy root number was recorded for the ecotype Esfahan with leaf and hypocotyl explants along with bacterial strain A4 at 31 (Table 2). Among the tested ecotypes, lowest hairy root number was reported from the ecotype Moghan inoculated with bacterial strain ATCC 15834 and A4 (Table 2). Several reports are available in family Apiaceae for their ability of producing hairy root; some species are potential candidates benefiting from this technique (Baranski, 2008).

3.3 Confirmation of transgenic roots by PCR

Total genomic DNA was extracted from normal roots, transformed hairy roots (explants were randomly selected from all ecotypes and bacterial strains treatments), and TpRi plasmid DNA was isolated from each *A. rhizogenes* strains as positive control for PCR analysis. PCR analysis was conducted using forward (FrolB) and reverse (RrolB) primers to confirm hairy roots. PCR successfully amplified rolB from the genomic DNA of all hairy root lines and bacterial plasmids (Figure 5). No amplification was detected in negative control C (-) as well as in the genomic DNA of non-transformed normal roots of *F. vulgare* ecotypes (lanes B, I, E and Mo) (Figure 5).

Conclusion

This is the first attempt to assess the effect of different *A. rhizogenes* strains and explant types on the induction of hairy root in *F. vulgare* from different ecotypes. Finding of this study suggest that A4 and ATCC 15834 were given the optimal hairy roots in fennel. Further, A4 had higher efficiency of root induction as compared with ATCC 15834, MSU and T4, while ATCC 15834 showed more hairy root induction number compared with A4, MSU and T4. Different

Fig 5 PCR amplification of 423 bp fragment of rolB gene using hairy root derived genomic DNA for all strains of *Agrobacterium rhizogenes*. Lane M 100–500 bp molecular weight marker; Lane C⁺ A4, TCC 15834, Msu and T4 *Agrobacterium rhizogenes* DNA (positive control), Lane C⁻ DNA from non-transformed roots (negative control); Lane B, I, E and Mo hairy root lines DNA of Bonab, Izmir, Esfahan and Moghan ecotypes, respectively

ecotypes showed almost similar results. However for each ecotype, suitable strain and explant was introduced for optimal hairy root induction. In ascending order, the most suitable explant types for maximum hairy root induction were hypocotyls, leaves and cotyledons. In present study transgenic hairy roots need to be further investigated for their potential as source of enhanced production of commercially important secondary metabolites and also for increasing specific medicinal products content by manipulation of the culture medium as well as precursor additions or elicitation (next paper). Also hairy root culture improved understanding of the biochemical pathways leading specific medicinal products synthesis will, in turn, be exploitable in manipulating and maximizing product synthesis under tissue culture and, ultimately, bioreactor conditions.

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