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RESEARCH ARTICLE

Efficient genetic transformation and regeneration system from hairy root of *Origanum vulgare*

Peyman Habibi[1,2] · **Maria Fatima Grossi de Sa**[1,2,3] · **André Luís Lopes da Silva**[1] · **Abdullah Makhzoum**[4] · **Jefferson da Luz Costa**[1] · **Ivo Albertto Borghetti**[1] · **Carlos Ricardo Soccol**[2]

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Abstract *Origanum vulgare L* is commonly known as a wild marjoram and winter sweet which has been used in the traditional medicine due to its therapeutic effects as stimulant, anticancer, antioxidant, antibacterial, anti-inflammatory and many other diseases. A reliable gene transfer system via *Agrobacterium rhizogenes* and plant regeneration via hairy roots was established in *O. vulgare* for the first time. The frequency of induced hairy roots was different by modification of the co-cultivation medium elements after infection by *Agrobacterium rhizogenes* strains K599 and ATCC15834. High transformation frequency (91.3 %) was achieved by co-cultivation of explants with *A. rhizogenes* on modified (MS) medium. The frequency of calli induction with an 81.5 % was achieved from hairy roots on MS medium with 0.25 mg/L^{-1} 2,4-D. For shoot induction, initiated calli was transferred into a medium containing various concentrations of BA (0.1, 0.25, 0.5, 0.75 and 1 mg/L^{-1}). The frequency of shoot generation (85.18 %) was achieved in medium fortified with 0.25 mg/L^{-1} of BA. Shoots were placed on MS medium with 0.25 mg/l IBA for root induction. Roots appeared and induction rate was achieved after 15 days.

✉ Abdullah Makhzoum
abmakhzoum@gmail.com

✉ Carlos Ricardo Soccol
soccol@ufpr.br; carlosricardo.soccol@gmail.com

[1] Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, Curitiba, Paraná, Brazil

[2] Embrapa Genetic Resources and Biotechnology, Brasilia, DF, Brazil

[3] Catholic University of Brasília, Brasília, DF, Brazil

[4] Department of Biology and the Biotron, The University of Western Ontario, London, ON N6A 5B7, Canada

Keywords *Origanum vulgare* · *Agrobacterium rhizogenes* · Hairy roots · Calli · Regeneration

Abbreviations

2,4-D	2,4 dichlorophenoxyacetic acid
BA	6- Benzyladenine
NAA	α-Naphthalene Acetic Acid
IBA	Indol-3- Butyric Acid

Introduction

Origanum vulgare L. belongs to the family of Lamiaceae and well-known for its essential oils, including phenolic monoterpenoids which are used as food flavors. This species is commonly known as a wild marjoram/winter sweet and has been utilized in medicine due to its anti-inflammatory, anticancer and antibacterial effects (Béjaoui et al. 2013; de Souza et al. 2016; Govindarajan et al. 2016; Pahlavan et al. 2013). Therefore, large-scale production of secondary metabolites of this plant using cell culture might open up a new avenue for the commercialization of its valuable metabolites for pharmaceutical industry. Recently, various methods have been employed to control plant sources for secondary metabolites production at commercial scale, including callus cultures, cell suspension culture, root cultures, organogenesis and embryogenesis (Paul et al. 2011). Hairy root system is one of the interesting aspects of plant cell cultures which provide an effective understanding of the physical and chemical requirements, growth and development of plant cells. Basically, hairy roots are induced through the integration of *rol* genes of *Agrobacterium rhizogenes* in the chromosome region of plant cell cultures. (Georgiev et al. 2010). When various susceptible parts of plants, such as the leaf, shoot and root, are wounded,

they excrete simple phenolic substances, such as acetosyringone which subsequently promote induction of virulence (vir) genes which are involved in the excision and transfer of the T-DNA fragment. This process results in generation of hairy roots at the wounded site (Makhzoum et al. 2013). Additionally, this platform has been investigated in plant secondary metabolism (Harfi et al. 2015; Makhzoum et al. 2015, 2011) as well plant molecular pharming and the production of heterologous recombinant pharmaceutical proteins and enzymes (Makhzoum et al. 2014; Moustafa et al. 2015) and phytoremediation. Since the hairy root platform presenting added advantages, such as, genetic stability, fast growing specially on hormone free medium, it can help in the development of affordable cost production by increasing of yield and ease of management, they could be considered as an attractive system to substitute current plant cell culture systems as the last ones are criticized for their genetic instability and low production rate (Georgiev et al. 2012). Hairy roots induction by *Agrobacterium*-mediated transformation opens up new windows for various fundamental applications in plant genetic engineering. This interesting system could allow modifications and production of phytomolecules that cannot realistically produce via chemical semi-synthesis. Interestingly, hairy root platform can offer a reliable protocol for regeneration in plant transformations as improvement in in vitro regeneration systems and feasible stable genetic transformation systems represent the main obstacle for successful plant genetic modifications (Al-Shalabia et al. 2014; Chandra and Chandra 2011; Yang et al. 2013; Zhang et al. 2007). In this context regeneration of whole plant via hairy roots has already been achieved in some species including: *Ipomoea batatas* and *Ipomoea trichocarpa* (Otani et al. 1993, 1996), *Brassica campestris* and *B. oleracea* (Christey et al. 1997), *Crotalaria juncea* (Ohara et al. 2000), *Panax ginseng* (Yang and Choi 2000) etc. Considering the importance of the subject, we highlight a reliable system for *A. rhizogenes*-mediated *O. vulgare* transformation to investigate the effect of medium composition modifications on hairy root induction frequency. Here we report for the first time the hairy root induction and the regeneration of the medicinal plant *O. vulgare* by *A. rhizogenes* as well as optimization of medium composition.

Material and methods

Plant materials and tissue culture

Seeds of *O. vulgare* were washed with running tap water for 20 min and were surface sterilized with 70 % ethanol for 1 min and subsequently suspended on 2 % sodium hypochlorite for 12 min. Thereafter, seeds were washed 3–4 times with sterile distilled water. Half strength MS (Murashige and Skoog 1962) medium without plant growth regulator, containing 30 gL^{-1} of sucrose was used for seed germination. Before seed culture, the medium was adjusted to pH 5.7 and autoclaved under 1.06 kg/cm^2 pressure at 121 °C for 15 min. Cultures were incubated and maintained at 26 ± 1 °C and for 16 h photoperiod.

A. rhizogenes preparation

Strains of *ATCC15834* and *K599* were inoculated in YEB agar medium (Vervliet et al. 1975) containing 5 g/L beef extract, 0.5 g/L MgCl2, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 15 $g.L^{-1}$ agar and pH 7.2 to an optical density of 0.6, at 28 °C, 120 rpm on a shaker incubator for 24 h. The strains were pelleted by centrifugation for 15 min at 3000 rpm and resuspended to a cell density of OD_{600} = 0.8 in a co-cultivation medium that consisted of MS with salts and vitamins, supplemented with 50 mg/L sucrose.

Induction of hairy roots and culture conditions

In order to induce hairy roots, leaves were isolated from 20 to 25 day old plants and were cut into 2–3 cm segments and randomly wounded by using a sterile needle and immersed in the *Agrobacterium* suspensions and swirled in liquid inoculation medium for10 min. The explants were blotted to dry on sterile filter paper and then transferred to a co-cultivation medium in the dark. In this experiment, the medium used for co-cultivation contained MS salts, vitamins, 30 gL^{-1} sucrose, 100 μM acetosyringone and 7 gL^{-1} agar (designated as Medium 1). This medium was finally modified by removing the following salts: KH_2PO_4 (co-cultivation medium 2); KH_2PO_4, $CaCl_2$ (co-cultivation medium 3); KH_2PO_4, NH_4NO_3, KNO_3 (co-cultivation medium 4); KH_2PO_4, NH_4NO_3, KNO_3, $CaCl_2$ (co-cultivation medium 5); KNO_3, $CaCl_2$, KH_2PO_4, Na_2EDTA, NH_4NO_3, $MgSO_4$, $FeSO_4$, vitamins and microelements (co-cultivation medium 6). Approximately, 40 to 50 leaf explants were inoculated with *A. rhizogenes* for each treatment in each experiment in dark conditions for 2 days at 28 C. After 2 days of co-cultivation, the explants transferred to MS media supplemented with 300 mgL^{-1} cefotaxime® to remove the *Agrobacterium* for 14 days. Hairy roots of *O. vulgare* were produced after 2 weeks and cultivated in hormone-free medium, containing 30 gL^{-1} of sucrose supplemented with 200 mgL^{-1} cefotaxime to establish axenic transformed root cultures for 15 days. The amount of cefotaxime in the culture medium was gradually decreased to 50 mgL^{-1} after 60 days. Induced hairy roots were subcultured every week. The selection of induced hairy roots was performed based on fast growth and shape and then they brought onto a 100 mL MS liquid medium in 250 ml flask and

kept at 110 rpm for further research. These conditions were kept for control explants but except inoculation with A. *rhizogenes*.

Callus induction from hairy roots

Induced hairy roots (5 g) were cut into segments and cultured on solid callus induction media. The callus induction medium contained MS micro and macro- nutrients which was supplemented with different levels (0.0, 0.1, 0.5, 0.75 and 1.0 mg/L) of 2,4-D.

Shoot regeneration from callus derived from hairy roots

The induced callus in the previous step was excised into 2–3 cm segments and transferred onto MS solid medium supplemented with 3 % sucrose, 0.1, 0.25, 0.5, 0.75, and 1 mg/L BA and pH was adjusted to 5.8. After 3 weeks, the regenerated shoots (2–2.5 cm) were excised and transferred to a root induction medium (MS medium supplemented with 0.5 mg/L IBA).

Polymerase chain reaction analysis

Extraction of genomic DNA from hairy and normal roots (negative control) of *O. vulgare* was performed by DNA extraction kit (Qiagen, Germany) based on manufacturer's instructions. The plasmid DNA of A. *rhizogenes* strain was also extracted with the plasmid isolation kit (QIAGEN, Germany) according to manufacture instruction. The DNA samples were run to PCR amplification with specific primers for the confirmation of the *rolB* gene (forward primer 5'ATGGATCC CAAATTGCTATTCCCCCACGA-3' and reverse primer 5'TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3') according to (Ahmadi Moghadam et al. 2013) under the following conditions: 94° for 7 min, followed by hot start at 94 °C for 10 min and amplified during 30 cycles at 94 °C for 45 s, 52 °C for 55 s, 72 °C for 1 min and followed by a final extension step at 72 °C for 5 min. The electrophoresis of the PCR products was performed on 1.2 % agarose gel under a constant voltage of 80 V.

Southern hybridization

Rapid genomic DNA extraction of transgenic hairy roots and non-transgenic roots of *O. vulgare* was carried out by DNA extraction kit based on manufacturer's instructions and then 20 μg of extracted DNA was digested by XbaI (Fermentas Co., Germany) and the separation of digested DNA was performed by 0.8 % agarose gel and finally shifted to a nylon membrane. Probe was prepared based on *rolB* sequence, using the DIG DNA labeling Kit (Roche Co., Germany). Prehybridization, hybridization, washing and detection were performed based on the DIG Labeling and Detection System instruction manual of (Roche Co., Germany).

Statistical analysis

The experimental design used in our experiments was completely randomized. So, each treatment was consisted of six replications and nine explants that were cultured in each Petri dish. The data was submitted to the analysis of variance (ANOVA) and to normality analysis for the Lilliefors test. Regression analysis was used to study Quantitative data while qualitative data were analyzed by the Scott-Knott test. All statistical analysis were performed at a level of $P < 0.05$. The SOC software was employed to perform all statistical analyses at a level of $P < 0.05$ and variables from percentages were transformed to arcsin x /100.

Results and discussion

The effect of bacterial strains and media compositions

Agrobacterium rhizogenes strains: 15834 and K599 were investigated to check their ability for transformation. Both strains were capable to induce hairy roots 2 weeks after inoculation directly from wounded sites (Fig. 1a). Wounded shoot explants were significantly receptive to transformation by *A. rhizogenes*. The highest rate of infection (91.3 %) was gained by two strains in leaves explants during 4 weeks on medium 5 whereas medium1 has shown the lowest rate of hairy root induction (34.6 %) via two strains (Fig. 2).

Callus induction from hairy roots

We attempted to induce callus from hairy roots explants by using different concentrations of 2–4-D (Fig. 1b). After 2 weeks, the highest frequency (81.5 %) of callus induction was achieved from hairy roots on MS medium containing 0.25 mg/L 2,4-D, and the lowest induction frequency (38.3 %) was achieved on a medium containing 1 mg/L 2,4-D as shown in (Fig. 3).

Shoot regeneration from callus raised from hairy roots

In this present, we could not obtain shoot induction directly from hairy roots without an intervening callus phase (data not shown). In order to establish shoot regeneration, segments of the induced callus from a hairy root clone were transferred to MS medium in different concentrations of BA. Shoot formation of *O. vulgare* was initiated from callus (Fig. 1c). The frequency of shoot induction in media with different concentrations of BA was different after 3 weeks of cultivation. The maximum frequency (85.18 %) of shoot formation was

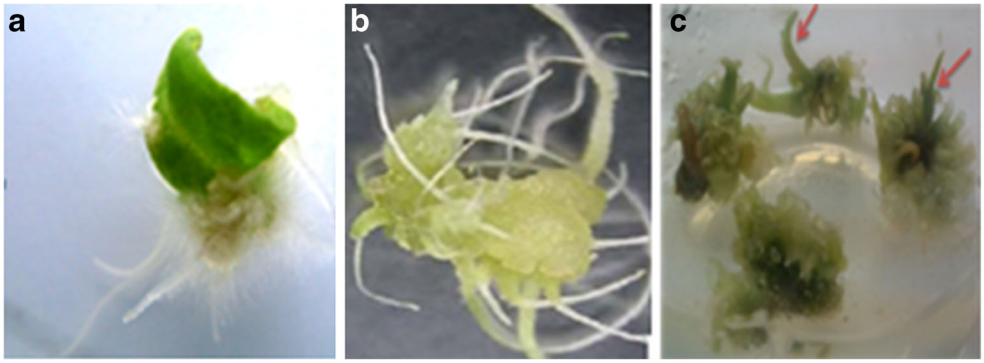

Fig. 1 **a** Hairy root induction of *O. vulgare*, **b** callus induction from a hairy root of *O. vulgare* **c** induction of an adventitious shoot from a hairy root of *O. vulgare*

achieved in a medium with 0.25 mg/l BA and the lowest frequency (24.69 %) with a concentration of 1 mg/L BA was detected (Fig. 4).

Molecular analysis of transgenic hairy roots

Selected hairy root samples were subjected to PCR analysis. PCR results showed the presence of the *rolB* gene in transformed clones (Fig. 5). The integration of the *rolB* gene was further confirmed by Southern-blot analysis and also confirmed the insertion of T-DNA into hairy root genome, since non transformed roots showed the integration signal (Fig. 6).

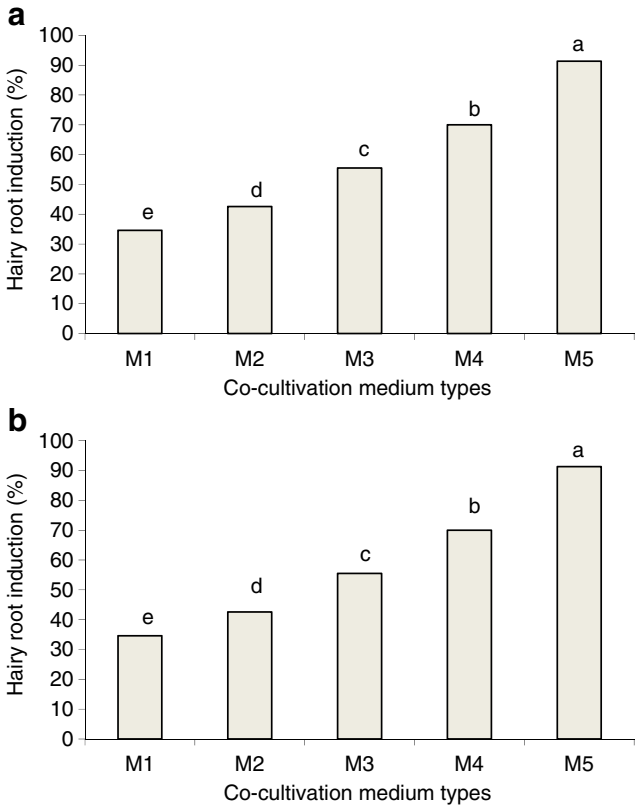

Fig. 2 The effect of different co-cultivation media on percentage of hairy root induction in *O. vulgare* by strain of ATCC15834 (**a**) and the effect of different co-cultivation media on percentage of hairy root induction in *O. vulgare* by strain of K599 (**b**)

These results represent a sustainable conjugation of TL-DNA of *A. rhizogenes* into hairy root chromosomal genome (Jouanin et al. 1987; White et al. 1985).

The efficiency of the genetic transformation of a plant species via *Agrobacterium* is associated with the type of the culture medium, temperature, co-culture time, genotype, and *Agrobacterium* strain (Lopes da Silva et al. 2013). Previously, the capability of various strains of *A. rhizogenes* has been reported to influence induction and development of hairy roots. *A. rhizogenes* strains ATCC15834 and K599 belong to the agropine-type Ri-plasmids, which are very similar as a group (Sevón et al. 2002). Trypsteen et al. (1991) showed that the overall process of T-DNA integration in an agropine type is very similar (Trypsteen et al. 1991). Tinland (1996) showed that the age and differentiation status of plant tissue and the level of tissue differentiation can affect hairy root induction after inoculation with *A. rhizogenes* (Tinland 1996). Porter (1991) believes that the capability of *A. rhizogenes* to transform plant species rely on the strain, but Lee et al. (2010) concluded that the effectiveness of *Agrobacterium* strain to induce hairy roots potentially rely on plant species (Lee et al. 2010; Porter 1991). Removing KH_2PO_4 and $CaCl_2$ or NH_4NO_3, KH_2PO_4 and KNO_3 in medium 3 or 4, respectively, obviously promoted root induction frequency. It has been recommended that the low concentration of PO_4 may influence the expression of virG and release positive signal to facilitate infection process (Sharafi et al.

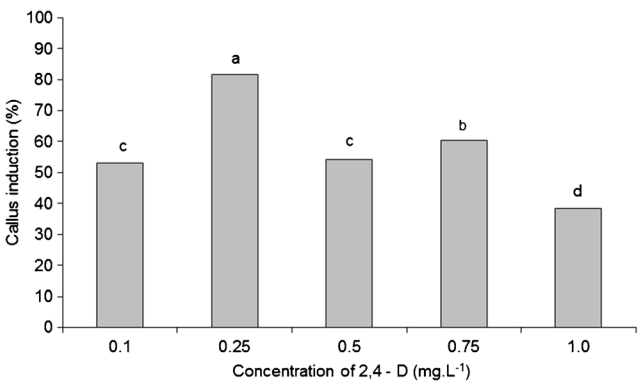

Fig. 3 Concentration of 2, 4-D in an MS medium on a frequency of adventitious callus induction in *O. vulgare*

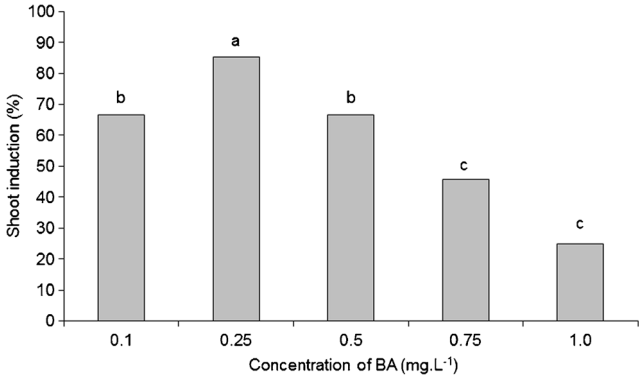

Fig. 4 Effect of different concentrations of BA in an MS medium supplemented with 0.1 mg/l NAA on frequency of adventitious shoot induction in *O. vulgare*

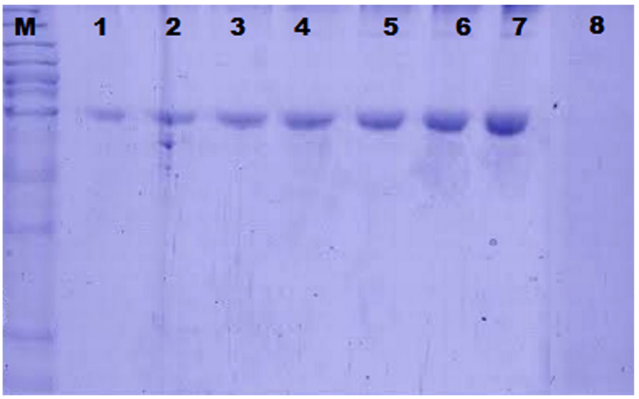

Fig. 6 Confirmation of transgenic nature of hairy roots by southern hybridization. Lanes 1–6 Genomic DNA isolated from transgenic *O.vulgare.* Lane *7 Agrobacterium rhizogenes* plasmid as a positive control, lane 8 Non-transformed plant genomic DNA as negative control

2012). Also, in comparison to the full-strength MS medium (Medium 1), in Medium 5, hairy root inductions were significantly increased by removing $CaCl_2$. Medium with high salt concentration such as, MS may divest the process of root development due to lacking of sufficient water and nutrients absorption from medium. That while a lacking mineral components medium was used for co-cultivation, a high transformation rate of *Ginkgo biloba* was achieved (Dupre et al. 2003). Thus, the concentration of components involved in inoculation and co-cultivation media may affect the frequency of transformation of *O. vulgare* explants by *A. rhizogenes.* The transformation efficiency significantly increased through a change in some major mineral components. Bacterial multiplication and hairy root formation would be influenced in a low-salt medium (Azadi et al. 2010). Key factors as the capacity of a calcium inhibitory effect and lacking PO_4 in activating virulence genes as well as the possibility of biofilm formation have also been demonstratedto influence *Agrobacterium*-mediated transformation (Danhorn et al. 2004; Flego et al. 1997; Winans 1990). However, more investigations on the effect of these components are needed to determine how gene transfer is influenced by mineral elements. These results are in agreement with the results obtained by other experiments in other plant species (Azadi et al. 2010; Hoshi et al. 2004; Sharafi et al. 2012). The induced callus via hairy root of *O. vulgar* (Fig. 1b) may be the result of its physiological state, which provides actively dividing cells. The development of a callus from hairy roots is directly related to the presence of 2,4-D, which is one of the favorable growth hormone for callus induction in most plants. The wounding during the process of cutting resulted in asynchronous cell division. This is considered to be a process of de-differentiation of organized tissue. Previously, the assessment of plant growth hormones effect on induced callus from hairy roots in *pumpkin* (*Cucurbita pepo* L.) represented inhibitory effect of low2,4-D concentration on root proliferation while exposed positive effect on influence of callus induction (Katavic and Jelaska 1991). In another study on *crownvetch* (*Coronilla varia* L.) carried out by Han and his co-workers, hairy root induction and plant regeneration was achieved by using *A. rhizogenes*. The results of the study showed that the callus induction with 100 % frequency was achieved from hairy roots in an MS medium containing 0.2 mg/L 2,4-D (Han et al. 2006). In another work done by Wang and his collaborators, the maximum frequency of callus induction from hairy root has been established in medium with 2.0 mg/L 2, 4-D and 0.5 mg/L 6-BA. The callus establishment was gained after 28 days, and transferred to an MS medium to facilitate shoot regeneration (Wang et al. 2001). Kumari and Saradhi (1992) reported that the best callus induction of *Origanum vulgare* was noted in a medium with 2, 4-D alone (Kumari and Saradhi 1992). Embryogenic studies of *Beta vulgaris L.* by callus induced from hairy roots system has been reported by Ninković et al. (2010). They concluded that the maximum callus frequency could be retrieved on MS medium supplemented with 1 mg/L thidiazuron (TDZ) and 1 mg/L 2, 4- D (Ninković et al. 2010).

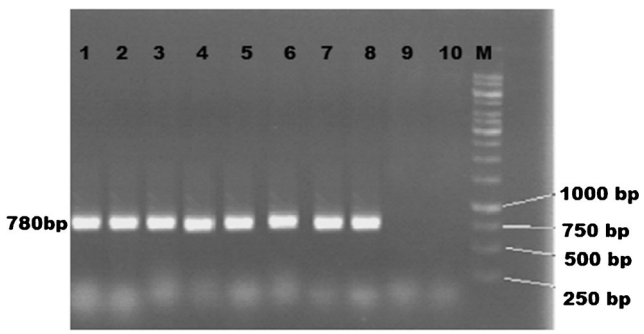

Fig. 5 PCR analysis for detection of the *rolB* gene in hairy roots lines and regenerated callus and shoot of *O.vulgare*. Lane 1:*Agrobacterium rhizogenes* DNA (positive control); Lanes 2-6 hairy root lines; Lane 7 regenerated callus from hairy root of *O.vulgare;* lane 8 regenerated shoot from hairy root of *O.vulgare;* lane 8,9 = none transformed explant (negative control); Lane M : Marker

It was shown that the best shoot induction of *O. vulgare* was obtained in a medium supplemented with BAP and NAA (Kumari and Saradhi 1992). The concentration of 0.5 mg/L BA was recommended to obtain maximum shoot regeneration from callus of *Populus tremuloides Michx* (Noh and Minocha 1986). In this case, our studies are similar and in agreement with (Han et al. 2004). They also remarked BA as an effective hormone involved in shoot regeneration. Similar results have been drawn in another study on the *bottle gourd* that explains BA is essential for shoot bud formation (Saha et al. 2007). In our experiments, shoot formation was not detected on the hormone-free medium, which is in consistence with the results obtained by *Pogostemon cablin* and *Dracocephalum kotschyi* (He-Ping et al. 2011; Sharafi et al. 2014), respectively. In summary, the insertion of native *A. rhizogenes* T-DNA into the *O. vulgare* genome worked successfully. Our data shows that regeneration is possible from hairy roots, but there is no significant difference between strains of bacteria K599 and ATCC15834 on hairy root induction of this plant. The co-cultivation of bacteria with explants in MS medium lacking macro elements is the best condition for the induction of hairy roots in *O. vulgar*. Thus, it could be concluded that macro elements significantly affect hairy root induction. Since transgenic shoots of *O. vulgare* on PGR-free medium are impoverished to generate root (Fig. 5c), they were placed on MS medium supplemented with 0.25 mg/L IBA. Root induction (90 %) appeared from the bottom of the shoots after 2 weeks. Finally, for the first time, we report hairy root induction and plant regeneration from induced hairy roots of *O. vulgare* and the results of these experiments could be used to study and scale up valuable secondary metabolites by this plant in the future. Moreover, the possibility of regenerating *O. vulgare* plants from hairy roots may allow for a higher production of secondary metabolites through genetic engineering. Therefore, the study of valuable secondary metabolite production of *O. vulgare* using hairy roots may lead to a higher production of these bioactive compounds.

Conclusion

In conclusion, the native A. *rhizogenes* T-DNA has been successfully inserted into the *O. vulgare* genome in active positioning state. In this research, we have established a well-founded protocol to induce transgenic hairy roots from *O. vulgare* and then plant regeneration from induced hairy roots was obtained by different concentrations of plant hormones. Results of this work could potentially improve the production of valuable secondary metabolites in this plant as large scale production of these secondary metabolites has been a priority in plant cell culture system and phytochemical industry.

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