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Dopamine Production in Hairy Root Cultures of *Portulaca oleracea* (Purslane) Using *Agrobacterium rhizogenes*

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ABSTRACT

An efficient transformation system for the medicinal plant *Portulaca oleracea* was established using agropine-type *Agrobacterium rhizogenes* ATCC15834. Hairy roots were obtained directly from cotyledon leaves explants seven days after inoculation with the bacteria. The highest transformation efficiency was obtained from cotyledon leaves explants, and amounted to 53.3% within two weeks. Roots grew rapidly on solid growth regulator free ½ Murashige and Skoog medium and demonstrated characteristics of transformed roots such as fast growth and high lateral branching. Successful and stable transfer of *rolB* gene was illustrated by PCR using specific primers of the gene. The hairy roots showed an ability to synthesize natural and medicinal product, dopamine. Elicitation of dopamine production in *P. oleracea* hairy roots was tested using different concentrations of methyl jasmonate (0, 100, 150, 200 µM) and salicylic acid (0, 125, 250, 500 µM), added to the hairy root cultures during the late growth phase. The results showed that the various concentrations of the methyl jasmonate significantly increased the dopamine content, but, at concentration of 100 µM, its impact was the most pronounced. Salicylic acid had no significant influence on dopamine production in hairy roots of *P. oleracea*.

Keywords: Dopamine increasing, Methyl jasmonate elicitation, Strain ATCC15834, *Portulaca oleracea*.

INTRODUCTION

In the beginning of the twenty-first century, medicinal plants are economically important pharmaceuticals used for human health. Examples of important drugs obtained from plants are morphine and codeine from *Papaver somniferum*, vincristrine and vinblastine from *Catharanthus roseus*, digoxin from *Digitalis lanata* (Hollman, 1996), and quinine and quinidine from *Cinchona spp* (Bensaddek *et al*., 2008). There are many plant-derived anticancer agents that are in clinical use these days such as vinblastine, irinotecan, topotecan, oposide, and paclitaxel (Cragg and Newman, 2005).

The isolation of medicinal compounds from extracts of wild or cultivated plants can be limited by various factors including cultivation difficulties, risk of extinction for over exploited plants, and geopolitical problems (Verpoorte *et al*., 2002). Plant tissue cultures have been suggested as a potential tool for the production of useful secondary metabolites. Thus, using this technology, secondary metabolites could be produced under controlled and reproducible conditions, independent of geographical and climatic factors (Shanks and Morgan, 1999).

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According to Verpoorte *et al*. (2002), several strategies such as screening and selection of high producing cell lines, cell immobilization, elicitation, and culture of differentiated tissues were developed; however, in most cases, secondary metabolites were undetectable or were accumulated at low levels in the cell cultures. Hairy root cultures established by *A. rhizogenes*-mediated transformation due to their rapid growth, biochemical stability, and relatively high production of secondary metabolites are widely used to produce useful compounds (Shanks and Morgan, 1999; Zolala *et al.*, 2007; Shirazi *et al.,* 2012). Genetic transformation does not impair the natural roots synthetic capacities. Therefore, hairy root systems, which can often grow vigorously and produce high levels of secondary metabolites, have been recognized as a source for production of secondary compounds from medicinal plants (Hamill *et al*., 1987; Yoshikawa and Furuya, 1987). Growth of hairy roots can be enhanced and exploited for commercial production of secondary metabolites in using different elicitors. Exposure to biotic elicitors or to stress agents (abiotic elicitors) frequently induces the synthesis of secondary metabolites in plants (Benhamou, 1996).

Portulaca oleracea is a medicinal plant found in Europe, Asia, and in Iran. The importance of this plant is in the treatment of urinary, digestive problems, and cardiovascular diseases. *P. oleracea* has a variety of pharmacological activities, including analgesic, anti-inflammatory, anti-fungal, wound healing, and hypoglycemic (Dighe *et al*., 2008). It contains plenty of bio-constituents, including catecholamines, l-noradrenalin, dopamine (Figure 1), l-dopa, α-amyrin, β-amyrin, and portuloside A. Dopamine (4-(2-aminoethyl)-1, 2-benzenediol), stimulates the nervous system and is used for treatment of Parkinson's disease, congestive heart failure, and myocardial dysfunction. Previous phytochemical studies demonstrated that *P.oleracea* contains dopamine and could be considered a source of this important secondary metabolite (Dighe *et al*., 2008). Plant tissue culture study of *P. oleracea* was performed in Iran by Safdari and Kazemitabar (2009). The main objective of the present work was to optimize and evaluate the dopamine production in hairy root cultures of *P. oleracea*.

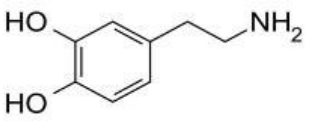

Figure 1. Dopamine (Andrés *et al*., 2012).

MATERIALS AND METHODS

Plant Material

Seeds of *P. oleracea* were collected from the Medicinal Plant Garden of the Hamedan city, Iran. Seeds were surface-sterilized in 70% ethanol for 45 seconds, then washed with sterile water several times. Afterwards, seeds were transferred to 2% sodium hypochlorite solution in a sealed bottle under sterile condition, gently agitated for 10 minutes and then rinsed three times with sterile distilled water. Next, sterilized seeds were transferred to the sterilized petri dish containing wet filter paper soaked in the ½ MS (Murashige and Skoog, 1962) liquid.

Bacterial Strain and Culture Conditions

Wild type of *A. rhizogenes* strain ATCC 15834 (harboring pRi15834) was obtained from Department of Molecular Physiology, Agricultural Biotechnology Research Institute of Iran in Karaj. The bacteria were maintained on Luria–Bertani medium (LB contains: Yeast extract 5 g L^{-1}, Tripton 10 g L^{-1}, NaCl 10 g $L^{-1,}$ and agar 15 g L^{-1}, adjusted to pH 7.0). A single bacterial colony was inoculated in10 ml of LB medium supplemented with rifampicin (50

mg 10 mL^{-1}) and the culture was placed on rotary shaker (110 rpm) at 26˚C for 24 hours.

Induction and Maintenance of *P. oleracea* Hairy Roots

Two weeks after seedling establishment in a medium containing wet filter paper soaked in the ½ MS liquid (Figure 2-a), different seedling parts including roots, stems and cotyledon leaves were isolated from *in vitro* growing seedlings and were cut (length 3 cm for roots and stem and complete cotyledon leaves with some petiole) into explants, then pre-cultured on solid, growth regulator-free 1/2 MS medium for 24 hours. Explants

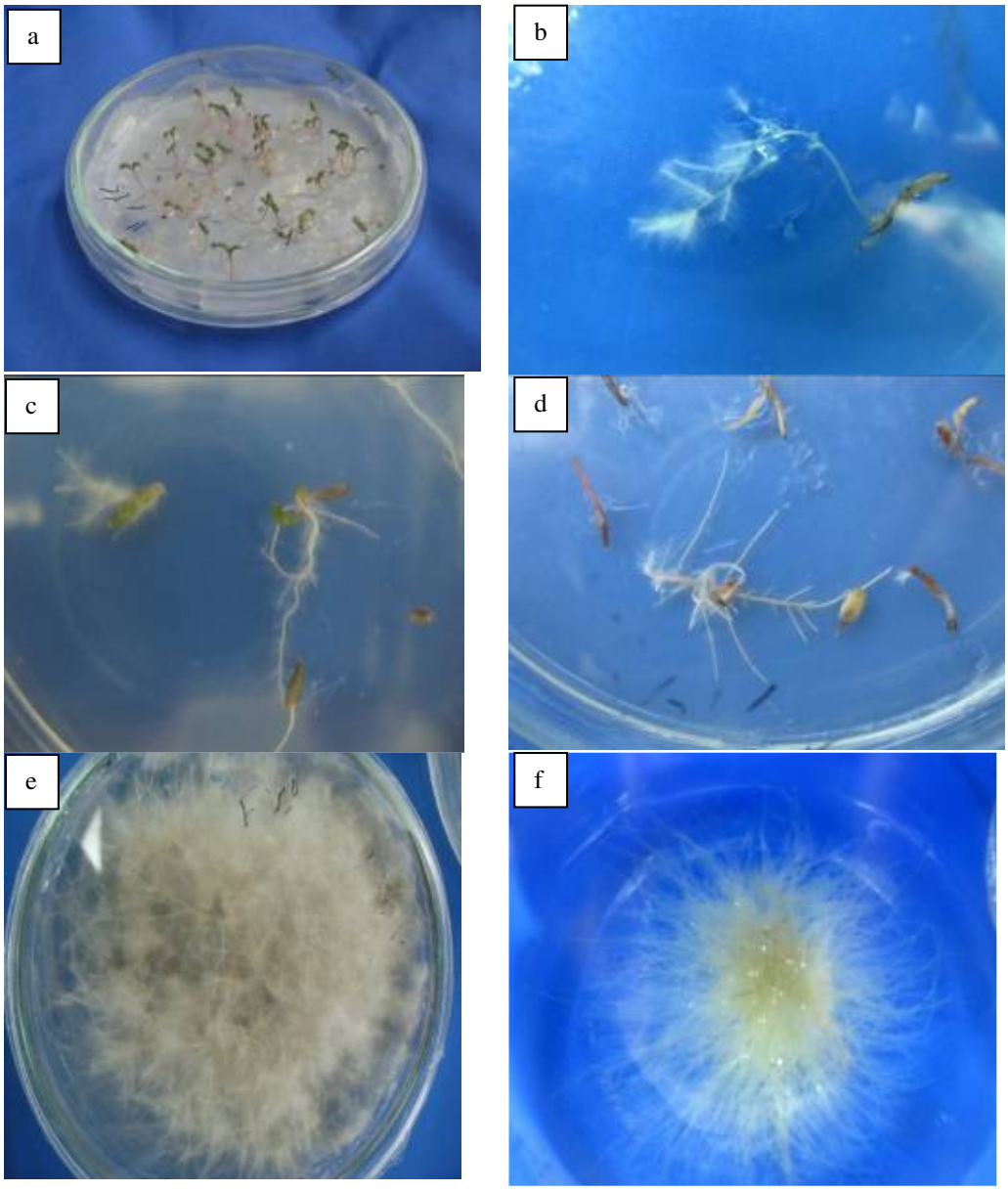

Figure 2. Hairy roots induction of *Portulaca oleracea*. (a) Germinated seedlings of *Portulaca oleracea.* (b, c, d) Hairy roots appeared from incision site; (e) The selected hairy root clones (B line) were cultured on ½ MS solid medium, (f) The typical hairy roots (B line) were cultured in ½ MS liquid medium.

(wounded surface with sterile scalpel and intact explants), were infected by dipping them into an *Agrobacterium* suspension for 10 minutes. After 2 days of co-cultivation at 24˚C in the dark, the explants were transferred onto ½ MS solid medium containing 300 mg L^{-1} cefotaxime (filter-sterile) to remove residual bacteria from explants. Controls consisted of explants treated similarly, except that they were not co-cultivated with *A. rhizogenes*. Explants were kept on ½ MS solid medium containing 300 mg L^{-1} cefotaxime in an air-conditioned chamber at 24˚C, under 16 h day^{-1} light to induce hairy roots. After 7 days of co-cultivation with bacteria, roots with a length 4-5 cm were excised from the incision site and placed on ½ MS medium for further growth. Six different root lines were established (A to F). These lines were maintained by subculturing of 3–4 cm long pieces of roots on ½ MS solid medium every 4 weeks. The hairy root cultures were also maintained in ½ MS liquid medium on a rotary shaker (110 rpm) at 25 C, under 16 h day^{-1} light. After two months of subculture (eight times), the elongating root tips were cut off and transferred to growth regulator-free ½ MS solid medium without cefotaxime. This procedure was repeated 3–4 times until no bacteria appeared. Sterile hairy root cultures were maintained at 25˚C, under 16 h day^{-1} light on growth regulator free ½ MS solid medium. Also, in this phase, effect of 10, 20, 30, and 40 minutes co-cultivation were examined.

PCR Analysis of Hairy Roots

DNA was extracted from each hairy root line and non-transformed roots originated from*, in vitro* germinated seedlings using the CTAB method (Cai *et al*., 1997). Putative transgenic roots were tested for the presence of the *rol* B gene (Dhakulkar, *et al.,* 2005) by PCR using genomic DNA, and primers (forward primer 5′-*ATGGATCCCAAATTGCTATTCCCCCACGA*-3′ and reverse primer (5′-TTAGGCTTCTTTCATTCGGTTTACTGC AGC-3′), which amplified a 780 bp fragment of the *rol B* gene. The PCR reactions were carried out in a total 50 µL volume and consisted of 200 ng of DNA, 10 µ*M* primer, 200 m*M* dNTP, 1 U of Taq DNA polymerase, 1X PCR buffer, and 2 m*M* $MgCl_2$. For amplification, the PCR parameters consisted of a denaturation step of 5 minutes at 94˚C (initial denaturation), and 35 cycles (each consisting of 1 minute at 94˚C, 1 minute at 60˚C, and 1 minute at 72˚C), followed by a final extension at 72˚C for 10 minutes). Plasmid DNA from *A. rhizogenes* strain 15834 was extracted as described by Sambrook *et al*. (1989) by alkaline lysis method was used as a positive control and non-transformed roots used as a negative control.

Gel Electrophoresis of Amplified DNA

Amplified products were separated by electrophoresis on 0.9% agarose gel in 1X Tris-acetic acid buffer (TAE) and detected by staining with ethidium bromide and visualizing under UV light using a Trans illuminator.

Media Testing for Optimization of Hairy Roots Growth

Transgenic hairy root line B was chosen for evaluation of the effect of different liquid media on root growth. MS basal medium, ½ MS, B5 medium and White's medium were investigated. Growth was measured on the basis of hairy roots dry weight determined at the end of 4-week culture.

Dopamine Extraction and Analysis by HPLC

After being air-dried, hairy root lines and seedling leaves were crushed into powder, and 100 mg of the accurately weighed samples were extracted with 5 mL of 0.1M HCl solution in an ultrasonicator for 1.5

hours. The extract was then filtered through a filter paper and a 0.45 mm filter membrane to be ready for analysis (Chen *et al*., 2003). HPLC analysis was carried out on a Knauer HPLC system (Berlin, Germany) equipped with a Eurospher C18 column (25 cm×4.6 mm) and a UV detector. The mobile phase was 0.02M KH_2 PO_4 solution (95%), Acetonitrile (5%), pH 3.0, 280 nm for detection wave length and the injection volume was 20 µL each time. This protocol was modified by the authors.

Preparation of Abiotic Elicitors

Two elicitors, namely, methyl jasmonate (MJ), filter- sterilized (with a purity of 95%) at the concentration of 0 (control), 100, 150 and 200 µM, and salicylic acid, (SA) at the concentration of 0 (control), 125, 250 and 500 µM, were applied to four-week old line B hairy root cultures to evaluate their effects on dopamine production. Ethanol (% 96) and NaOH (0.1N) were used to dissolve MJ and SA, respectively. Roots from MJ- and SA-treated and untreated control cultures were harvested 48 hour after elicitation (30 days after the culture).

Chemicals

Methyl jasmonate and salicylic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dopamine was obtained from Caspian Tamin pharmaceutical company (Gilan, Iran).

Statistical Analyses

All analytical values represent the means of three analytical replications. Excel 2007 software was used for drawing diagrams. Significance was determined by analysis of variance (ANOVA) using SAS software (version 9.1) and difference between the means were compared by Duncan's multi range test ($\acute{\alpha} \leq 0.05$).

RESULTS

Induction of Hairy Roots

Inoculation of stem, root, and cotyledons with *A. rhizogenes* strain ATCC15834 resulted in formation of roots. Two-week-old seedling explants such as root, stem, and cotyledons showed a significant difference in potential for root induction after co-cultivation with *A. rhizogenes* (Figure 3). No root emerged from the controls treatment and explants treated with scalpel. The highest frequency of root induction was achieved from cotyledon explants, 53.3% in the co-cultivation time of 20 minutes (Figure 4) and was significantly different than the frequency of induced roots on stem (23.3%) and root (13.33%) explants. On the cotyledon leaves explants, the roots were observed at the incision site with petiole, merely one or two roots with numerous branches (Figure 2, c-d). Since the high percentage of root induction was on the cotyledon leaves explants, all transgenic hairy root lines were selected from these explants. These roots were fast growing, highly branching, plagiotropic, and hairy in morphology and showed phytohormone

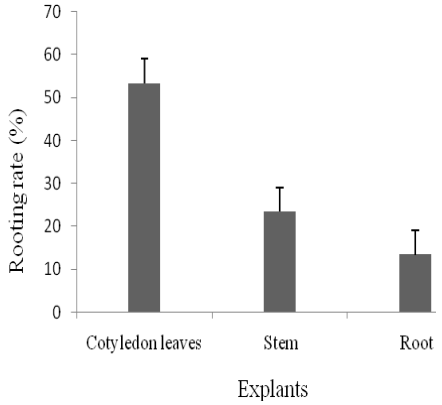

Figure 3. Effect of explant types on the rooting rate. The experiment was performed in independent triplicate and each experiment contained 8 explants. No root emerged from three control treatments. Bars indicate standard errors (n= 3).

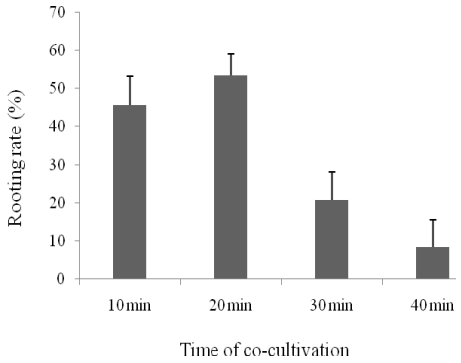

Figure 4. Effect of different co-cultivation times on the rooting rate. The experiment was performed in triplicate and each experiment contained 8 explants. Bars indicate standard errors (n= 3).

autotrophy (Figure 2, b-f).

The morphological identification of the transformed hairy roots of all six lines was confirmed by PCR using *rol* B gene specific primers. *A. rhizogenes* served as the positive control and DNA from the non-transformed seedling roots served as the negative control. All transformants showed presence of the 780 bp *rol* B amplified product. No *rol* B gene was found in the control tissue (Figure 5).

Growth performance of the transgenic hairy root line B showed maximum biomass increase on ½ MS basal medium, 165.067 mg (mg dry wt per 50 mL flask) compared to that of White medium, 81 mg (Figure 6). This medium was used for maintenance of the hairy root lines.

Production of Dopamine in Hairy Roots Cultures

Hairy roots cultivated in growth regulator-free ½ MS medium for 28 days were harvested and used for determination of dopamine content by HPLC. Dopamine was detected in crude extracts prepared from non-transformed roots as well as in hairy roots. Retention time of peak obtained with standard dopamine (Figure ٧-A) was used to identify the corresponding peaks in root extracts. In addition, the root extracts were spiked with standard dopamine to show the peaks corresponding to dopamine (Figures 7-B and -C). Peaks related to the dopamine were detected at 4.5 minutes. The production of dopamine was determined after 28 days (Table 1). The content of

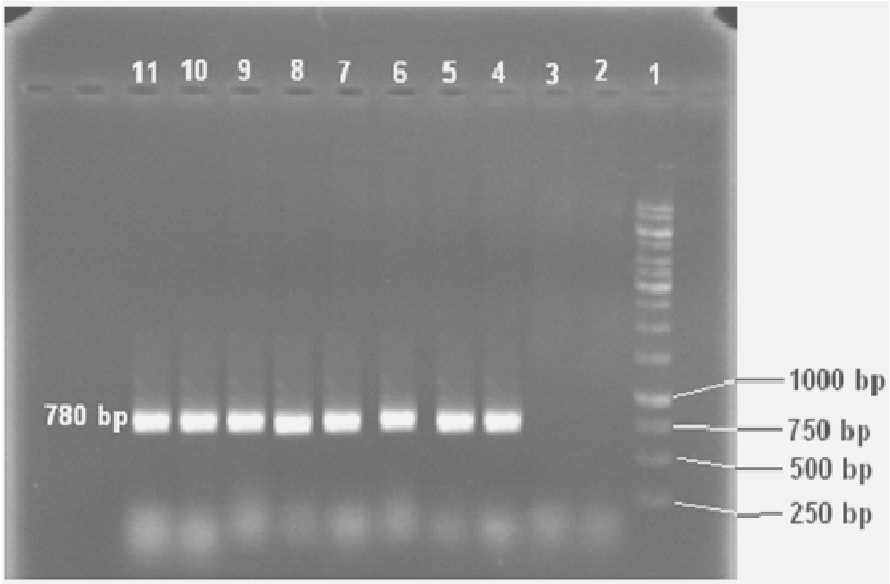

Figure 5. PCR amplification of a 780 bp fragment of the *rol* B gene using hairy root derived DNA. Lane 1= Molecular weight marker (1,000 bp ladder); Lanes 2 and 3= Non-transformed roots (negative control); Lanes 4 and 5= *Agrobacterium rhizogenes* DNA (positive control); Lane 6= A line; Lane 7= B line; Lane 8= C line; Lane 9= D line; Lane 10= E line, Lane 11= F line.

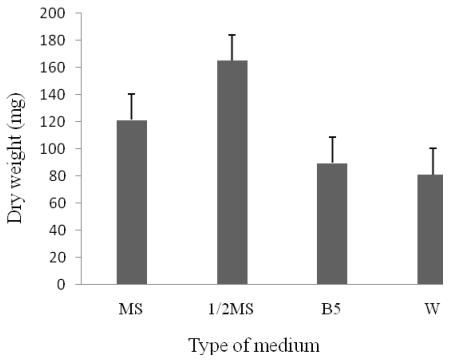

Figure 6. Growth of transformed roots (B line) of *P. oleracea* in four different liquid medium (MS, ½ MS, W, and B5) after 28 days. Bars indicate standard errors (n= 3).

dopamine in transgenic line F after 28 days of culture reached maximum level (0.9 mg g^{-1}), which was approximately 18 and 3 times higher than that of normal roots and seedling leaf, respectively.

Effect of Abiotic Elicitors on Production of Dopamine

The hairy root line B was chosen for elicitor feeding investigations due to its sustainable growth. We compared the effect of different concentrations of MJ on dopamine accumulation. The results revealed that addition of 100 µm MJ was suitable for highest accumulation of dopamine, 4.35 fold (1.21 mg g^{-1}) increases over the controls (0.28 mg g^{-1}). Although with increasing MJ concentration the amount of dopamine was decreased, but this amount was significantly higher compared to that of the control (Figure 8). SA treatment had no significant effect on the amount of dopamine compared to the control (Figure 9).

DISCUSSION

Hairy roots induced by *A. rhizogenes* have received a lot of attention from plant biotechnologists for the production of

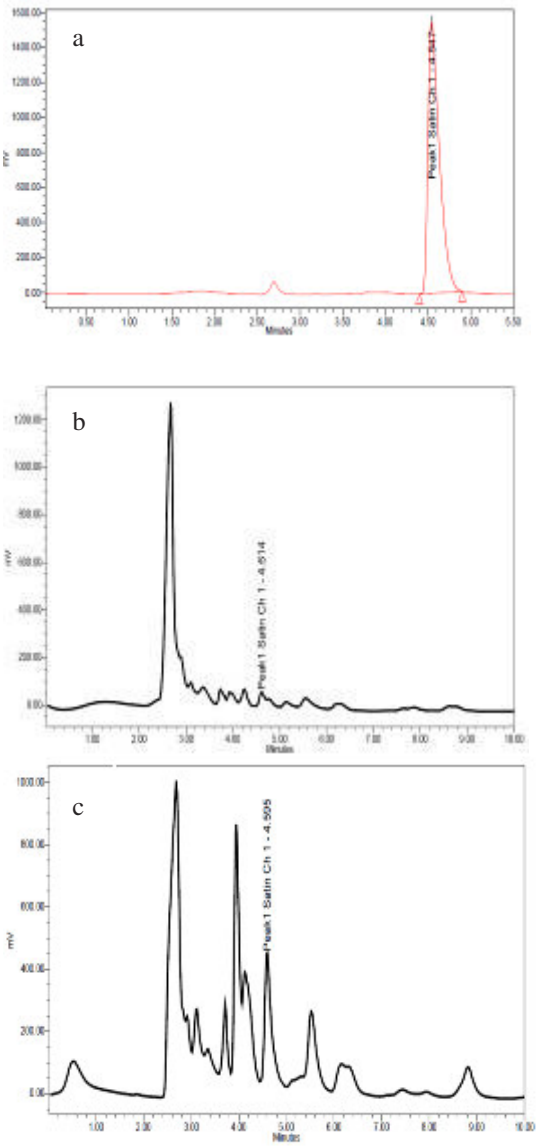

Figure 7. HPLC analysis of dopamine in hairy roots of *P. pleracea* (a-c). (a) Authentic dopamine; (b) Non- transformed Roots of *P.oleracea*, (c) Transformed roots of *P.oleracea* induced by *Agrobacterium rhizogenes* ATCC 15834.

secondary metabolites. The hairy roots could be indefinitely propagated on a synthetic medium without phytohormones (Tepfer and Tempe, 1981; Eapen and Mitra, 2001). They show genetic stability and tend to produce high levels of secondary metabolites characteristic of the original species. In our

Table 1. Dopamine content in *Portulaca oleracea* hairy roots detected by high performance liquid chromatography.

samples	Dry weight after 4 weeks (mg)[a]	Dopamine content (mg g^{-1} dry weight)
Seedling leaf	-	0.30± 0.01
Untransformed roots	18.05 ± 0.36	0.058 ± 0.006
Line A	48.5 ± 0.70	0.52 ± 0.008
Line B	160.8 ± 0.50	0.28 ± 0.006
Line C	71.83 ± 0.56	0.31 ± 0.004
Line D	88.16 ± 0.53	0.31 ± 0.002
Line E	154.6 ± 0.2	0.30 ± 0.003
Line F	120.06 ± 0.21	0.90 ± 0.004

[a] Hairy roots dry weight was determined at the end of 4 weeks culture in liquid medium. Values represent the mean±SD of three independent measurements.

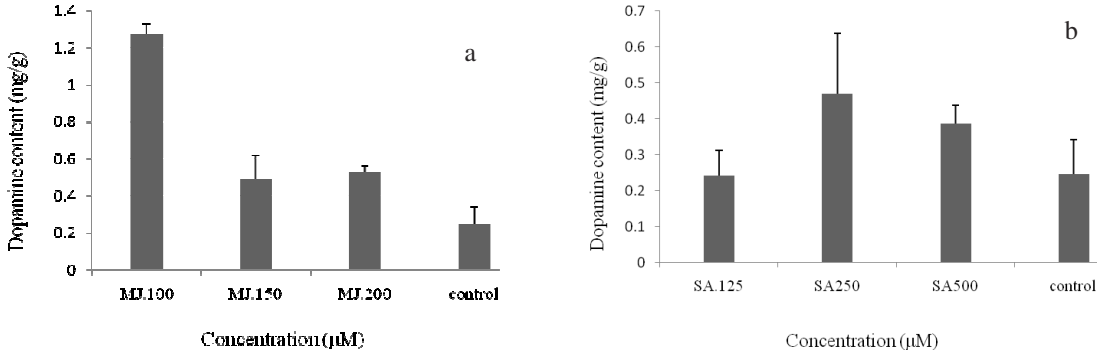

Figure 8. The effect of different concentrations of a) methyl jasmonate (MJ) b) salicylic acid (SA) on content of dopamine. Bars indicate standard errors (n= 3).

study, an efficient transformation system for *P. oleracea* using *A. rhizogenes* ATCC15834 (Pirian *et al*., 2012) was used. Our results also showed that *P. oleracea* was susceptible to ATCC15834 infection with the highest 53.3% frequency for cotyledonary explants. This higher susceptibility of cotyledons to hairy root induction, compared to stem and root explants, could be due to the fact that leaves are more competent for transformation than stem or root explants (Shi and Kintzios, 2003). Juvenility and nature of explants influence the *Agrobacterium* mediated transformation process (Yonemitsu *et al*., 1990; Trypsteen *et al*., 1991). Nin *et al*. (1997) have reported that specificity of *Agrobacterium* transformation is closely connected with the age and hormonal balance of the host tissue. Potrykus (1990) declared that wound response was the most important factor for the successful transformation. Explant cells differ in their DNA synthesis and cell division ability due to the difference in physiological maturity of the cells. The present observation that cotyledon leaves explants induced hairy roots may be due to their ability to produce greater number of wound adjacent competent cells for regeneration and transformation.

Many studies have described the effects of the medium composition and culture conditions on the growth rate of hairy roots and the production rate of secondary metabolites (Sauerwein *et al*., 1991). The composition of culture medium, e.g. the sucrose concentration, is known to influence the growth of transformed roots (Giri and Narasu, 2000). In this study, to find the

optimal medium for hairy root biomass accumulation, the hairy roots were cultured on four different media such as MS, ½ MS, B5, and W. It was observed that the maximum biomass was obtained during cultivation in the ½ MS medium. Hairy roots grew vigorously in the ½ MS liquid or solid medium and had typical features of transformed roots, such as fast growth and high lateral branching.

The results showed that *P. oleracea* hairy roots could produce dopamine, and the amounts of dopamine in some transgenic lines (F and A lines) were significantly higher than that of the normal roots and cotyledon leaves. Transgenic lines differed in biomass accumulation and dopamine content. Previous works showed that different transgenic lines were significantly different in secondary metabolite production. In *Catharanthus roseus*, hairy root clones accumulated variable amounts of ajmalicine and serpentine caused by random and multiple integrations of T-DNA. Also, it was reported that these differences in alkaloid accumulation might be due to differences in the integration sites of the T-DNA (Batra *et al*., 2004). These differences may be attributed to the variation in the T-DNA insertion, copy number, size, and location of integration of T-DNA of Ri-plasmid into the plant genome (Doran, 2002). The accumulation of secondary metabolites in plants is a part of their defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses (Zhao *et al*., 2005). Therefore, the treatment of biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in hairy root cultures. MJ has been shown to enhance the production of secondary metabolites in both cell and hairy root cultures. In cell culture, MJ treatment resulted in enhancing of biosynthesis and accumulation of paclitaxel and related taxanes in *Taxus* sp. (James and Muenduen, 2000), rosmarinic acid in *Lithospermum erythrorhizon* (Mizukami *et al.,* 1993), indole alkaloids in *Catharanthus roseus* (Xiang *et al*., 2011), and anthocyanins in *Vaccinium pahale* (Yukimune *et al*., 1996). Also, in hairy root cultures, MJ improved and increased the accumulation of secondary metabolites in many plants such as artemisinin in *Artemisia annua* (Waraporn *et al*., 2007), also paclitaxel (Syklowska-Baranek *et al.*, 2009), glycyrrhizin in *Glycyrrhiza inflata* (Winida *et al*., 2011), and andrographolide in *Andrograph*is *paniculata* (Sharmila and Subburathinam, 2013). In the present study, it was found that addition of MJ into the culture medium enhanced the production of dopamine. MJ was found to favor production of dopamine compared to SA in the present study using *P. oleracea* hairy root cultures, and the results for MJ were quite promising. Hairy roots maintained in medium supplemented with 100 µM MJ demonstrated increase in dopamine production, whereas cultivation in the media with higher concentration of MJ decreased dopamine production. It seems that dopamine production was stimulated by lower concentration of MJ such as 100 µM. Higher concentration of MJ used for elicitation (from 150 to 200 µM) resulted in loss of cell viability and demonstrating toxic effects.

CONCLUSIONS

Transgenic hairy root cultures are the ideal systems for research on metabolic engineering. In the present study, transgenic hairy root cultures of *P. oleracea* using *A. rhizogenes* ATCC15834 were established. The most suitable explants for hairy root induction proved to be cotyledon explants. And ½ MS medium was the most appropriate medium for culture of hairy roots. The hairy roots were capable of growing in the nutrient medium without growth regulators and synthesized dopamine. The effect of MJ and SA on the accumulation and biosynthesis of dopamine in hairy roots culture of *P.oleracea* was investigated. Elicitation with MJ resulted in

about 4.3 fold higher dopamine yield compared to the control hairy root cultures. Thus, it could be concluded that the use of appropriate amount of signal compounds can increase the productivity of dopamine in hairy root cultures of *P. oleracea*. Finally, hairy root techniques may be considered as a useful system for large-scale production of dopamine in cultures of *P. oleracea*.

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تولید دوپامین در ریشه های موئین ایجاد شده در گیاه خرفه (*Portulaca oleracea*) با استفاده از آگروباکتریوم رایزوژنز

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چکیده

در این پژوهش، روش کارآمد انتقال ژن با استفاده از آگروباکتریوم رایزوژنز، استرین ATCC 15834از نوع آگروپین در گیاه دارویی خرفه بهینه گردید. اولین ریشه ها به طور مستقیم بعد از هفت روز تلقیح با باکتری، از ریز نمونه های برگ های لپه ای بدست آمدند. بالاترین میزان تراریختی با استفاده از آگروباکتریوم رایزوژنز بعد از گذشت دو هفته از آلودگی، در ریز نمونه های برگ های لپه ای به مقدار ۵۳.۳ درصد به دست آمد. ریشه ها به سرعت در محیط MS ۱/۲ جامد فاقد هورمون رشد کرده و دارای ویژگی های ریشه های موئین از جمله رشد سریع و وجود ریشه های فرعی بالایی بودند. انتقال موفق و پایدار ژن rol B توسط PCR و با استفاده از پرایمرهای اختصاصی نشان داده شد. ریشه های مویین نشان دادند که توانایی تولید ترکیب طبیعی و دارویی دوپامین را دارند. استخراج دوپامین از ریشه های مویین با استفاده از غلظت های مختلف متیل جازمونات (۰، ۱۰۰، ۱۵۰ و ۲۰۰ میکرومول) و اسید سالیسلسک (۰، ۱۲۵، ۲۵۰ و ۵۰۰ میکرومول) مورد آزمایش قرار گرفت. این غلظت ها در اواخر فاز رشدی ریشه ها به محیط کشت ریشه های موئین اضافه گردیدند. نتایج نشان دادند که همه غلظت های مختلف متیل جازمونات به طور معنی داری باعث افزایش مقدار دوپامین شده ودر این اینجا بیشترین مقدار دوپامین مربوط به غلظت ۱۰۰ میکرومولار بود. هیچ کدام از تیمارهای به کار برده شده اسید سالیسلیک تاثیر معنی داری بر افزایش دوپامین در ریشه های موئین گیاه خرفه نداشتند.