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Auxin sensitivity improves production of rosmarinic acid in transformed hairy roots of *Lavandula angustifolia*

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Abstract

Hairy root culture is a promising approach to improve production of plant secondary metabolites. The genes, which are located in T-DNA of a root-inducing plasmid, regulate auxin sensitivity of hairy roots. Therefore, this study was aimed to improve the growth and rosmarinic acid production of *Lavandula angustifolia* hairy roots. Lateral branches of hairy roots were transferred to ½ MS and ½ B5 liquid media. To assess auxin sensitivity, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) with four different concentrations (0, 0.1, 0.5 and 1 mg/l) were also applied. The growth of hairy roots in ½ MS medium was two-fold higher than in ½ B5 medium. In addition, both auxins were found to significantly improve the growth of hairy roots whereas non-transformed roots stopped growing in the presence of the auxins. The highest dry weight and rosmarinic acid production of hairy roots were obtained from ½ MS medium supplemented with IBA irrespective of its concentration. As a result, the hairy roots grown in ½ MS medium supplemented with IBA produced the maximum amount of rosmarinic acid (7.98 mg/g dry weight of hairy roots). This first report of rosmarinic acid production in *L. angustifolia* hairy roots provides new insights into the auxin sensitivity of *L. angustifolia* hairy roots.

Keywords: auxin, basal media, hairy roots, rosmarinic acid, TL-DNA

Introduction

Hairy roots are known as a biotechnological approach to improve the production of plant secondary metabolites (Giri and Narasu, 2000). Molecular analysis of Ri plasmid shows that the genes located on T-DNA segment are different between *A. rhizogenes* agropin and non-agropin strains. Integration of TL-DNA containing *rol* genes into plant genome is necessary to induce hairy roots, so rol genes are present in Ri plasmid of all *A. rhizogenes* strains (Veena and Taylor, 2007). A proposed function of *rol* genes is to improve the sensitivity of hairy roots to auxins. In contrast, TR-DNA is not necessary to induce hairy roots and it is transferred to plant genome independent of TL-DNA integration. *aux*1 and *aux*2 genes located in TR-DNA are involved in the pathway of auxin biosynthesis. These findings suggest that auxin has a key role in the growth and other biological processes of hairy roots. Therefore, there is a need to investigate how hairy roots respond to auxin as it has been reported in some medicinal species (Balvanyos, Kursinszki and Szoke, 2001; Liu et al., 2002; Yang et al., 2010).

Rosmarinic acid is a natural antimicrobial and antioxidant agent and the herbs containing it are considered valuable medicinal plants. Biosynthesis and accumulation of this agent are primarily determined by a plant genotype, as it is largely found in Lamiaceae and Boraginaceae plant families (Petersen and Simmonds, 2003).

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However, developmental and environmental factors also strongly influence the production of rosmarinic acid in plant tissues (Fletcher, Slimmon, McAuley and Kott, 2005; Manukyan, 2013). Lavender (*Lavandula angustifolia*) belongs to the Lamiaceae family, and is known to be rich in valuable secondary metabolites. There are some reports concerning the production of rosmarinic acid in cell suspension culture of this plant (Pavlov, Ilieva and Panchev, 2000; Nitzsche, Tokalov, Gutzeit and Ludwig-Muller, 2004). As far as we know, rosmarinic acid production in the hairy root culture of *L. angustifolia* has not been reported to date. Therefore, this study was conducted to develop the hairy root culture of *L. angustifolia* and assess the effect of auxin sensitivity of hairy roots on their growth and rosmarinic acid production.

Materials and methods

Plant material

L. angustifolia plants were provided from local markets and the species was confirmed by the Research Center of Plant Sciences, Ferdowsi University of Mashhad. Young leaves and stems were separated and washed under running tap water for 30 min. Surface sterilization was performed by 70 % ethanol for 1 min and then 1 % sodium hypochlorite for 20 min. To remove disinfectants, the leaves were washed three times with sterile distilled water in aseptic conditions. Thereafter, explants were cut into 1 cm pieces and inoculated onto ½ MS medium for 2 days prior to transformation.

Agrobacterium strains and culture conditions

A. rhizogenes strains K599, MSU-1, R1000, ATCC 15834 and NCPPB 2656 were grown in LB broth medium on a rotary shaker at 28 °C and 120 rpm. Overnight bacterial precultures ($OD_{600\ nm}$ = 0.8–1) were collected by centrifugation at 4000×g and then resuspended in the same volume of sucrose 5 % solution containing 100 µM acetosyringone. After a 2-hour shaking, bacterial suspensions were used for the transformation of explants.

PCR Determination of agropin and non-agropin strains of the bacteria

Bacterial strains were screened by colony PCR using *rol*C gene specific primers to confirm the presence of Ri plasmid and *aux*1 gene specific primer to determine agropin and non-agropin strains (Table 1).

Transformation procedure

To improve transformation efficiency, two treatments were performed in the transformation procedure: 1) Leaf and stem segments were dipped in the bacterial suspension.

Table 1. Primers used in PCR analysis of bacterial strains and hairy roots

Primer Name	Sequence (5′–3′)	Size (bp)
*rol*C	F: CTCCTGACATCAAACTCGTC	612
	R: TGCTTCGAGTTATGGGTACA	
*aux*1	F: CTCCGATTCCTTTCCAACCG	791
	R: CGCACGTTATCCTCATACCC	
*vir*D	F: CTCATCAGGCACGCTTG	668
	R: GCGGATGCTTCAAATGG	

Then sonication assisted *Agrobacterium rhizogenes* transformation (SAArT) was performed at 45 kHz frequency for 10 minutes. Finally, the explants were transferred to ½ MS solid medium (Murashige and Skoog, 1962) supplemented with 100 µM acetosyringone without growth regulators. 2) Leaf and stem segments were infiltrated with *A. rhizogenes* strains. Then, the infected explants were transferred to ½ MS solid medium (Murashige and Skoog, 1962) supplemented with 100 µM acetosyringone and 2 g/L of 40 min-autoclaved glutamic acid into the coculture media. The explants were kept in both light and dark conditions. 3 days after cocultivation, explants were transferred to the same medium supplemented with 400 mg/l cefotaxime sodium. The following subcultures were performed by gradually decreasing the antibiotic concentration until it reached 100–200 mg/l. When the roots reached about 3-4 cm in length, they were cut off and transferred to ½ MS liquid media and subcultured every two weeks.

Genomic DNA isolation and PCR analysis of T-DNA integration pattern

Plant samples were powdered in liquid nitrogen. Then, genomic DNA of the samples was isolated using DENAzist plant DNA extraction kit (DENAzist Asia, Mashhad, Iran). PCR analysis of genomic DNA of putative hairy roots, non-transformed roots (negative control) and bacterial R1000 strain (positive control) was performed with *rol*C gene specific primers to confirm the transformation, and *vir*D gene specific primer to ensure that hairy roots were free of bacterial cells. The transformed clone was further analyzed to determine the presence of TR-DNA in hairy roots using the *aux*1 primer (Table 1). The PCR products were separated by electrophoresis in a 1 % agarose gel stained with GreenView™.

In vitro auxin treatment of hairy roots and non-transformed roots

The tips of hairy roots and non-transformed roots of in vitro grown plants (3-4 cm) were transferred to ½ MS medium supplemented with 1 mg/l IAA or IBA. ½ MS

auxin-free medium was also used as the control treatment. The number of lateral roots and the average root length was determined as the growth pattern of the roots.

Effects of basal media and auxins on the growth of hairy roots

The tips of lateral branches of hairy roots (3-4 cm) were inoculated in ½ MS and ½ B5 (Gamborg, Miller and Ojima, 1968) media containing 3 % sucrose. To assess auxin sensitivity, the media was also supplemented with 0, 0.1, 0.5 and 1 mg/l IAA or IBA. After 3 months of culture, the hairy roots were harvested and kept in a dark place at room temperature until a constant weight was achieved, and then the dry weights were measured. The factorial experiment for growth of hairy roots was conducted in a completely randomized design with three factors (different basal media, different types of auxins and their various concentrations) in three replicates. The data were subjected to analysis of variance (ANOVA) using R agricolae package (Mendiburu, 2017). Significant differences within means were determined by Duncan's multiple range test at 5 % probability.

Rosmarinic acid extraction and HPLC analysis

Dried hairy roots, leaves and roots of naturally grown *L. angustifolia* plants with three replications were ground into powder and extracted with dichloromethane using a tissue-solvent ratio of 1:10 (w/v). Each sample was sonicated for 7 min at 45 kHz and 80 % amplitude, and then kept for 6h in a dark place at room temperature. The mixture was centrifuged at 4000×g for 10 min, and then the supernatant was separated and evaporated. Afterward, the residue was dissolved in methanol and ZORBAX RX-C18 column was used as the stationary phase for HPLC analysis (Agilent 1260, USA).Detection was performed at the wavelength of 280 nm. To determine the rosmarinic acid content of the samples, the peak areas were compared with the peak area of rosmarinic acid authentic standard compound (Sigma-Aldrich) at the respective retention time.

Results and discussion

Determination of bacterial agropin and non-agropin strains

All 5 bacterial strains were verified by the presence of the *rol*C band with the expected size of 612 bp in their PCR products. Amplification of *aux*1 gene showed that the bacterial strains were different in the genetic structure of Ri plasmid T-DNA. R1000, MSU-1 and ATCC 15834 strains had the TR-DNA region and grouped into *A. rhizogenes* agropin strains, but K599 and NCBPP 2656 strains were grouped as non-agropin strains because they did not have the TR-DNA (Fig. 1a). In previous studies, R1000 and ATCC 15834 strains have been used as agropin strains (Rosic, Momcilovic, Kovacevic and Grubisic, 2006), and K599 and NCBPP 2656 have been introduced as cucumopine strains of *A. rhizogenes* (Haas, Moore, Ream and Manulis, 1995; Weller, Stead and Young, 2006). Among *A. rhizogenes* strains, agropin types are widely used in plant hairy root induction. The powerful transformation efficiency of these strains in addition to the achievement of high growing hairy roots is attributed to the presence of the TR-DNA region, especially auxin biosynthesis genes (Camilleri and Jouanin, 1991).

Determination of transformation efficiency

The results of this study revealed that different types of explants and light management strongly affect adventitious root formation in lavender, since no adventitious root was induced in light condition. In addition, stem segments did not produce any roots and turned brown one week after cocultivation. In vitro growth of *Lavandula* sp. cells has been shown to be sensitive to light condition and resulted in limited cell differentiation of these species (Goncalves and Romano, 2013).

Among the studied *A. rhizogenes* strains, K599 with 25 % frequency of adventitious root formation showed the lowest capability to induce roots in leaf explants but the other bacterial strains did not show significant differences, and the frequency of adventitious root induction was about 40–50 % of the total explants (data not shown). Growth evaluation of adventitious roots in liquid culture for two months showed that just one root clone (originated from coculture of a leaf explant with R1000 bacterial strain) developed many lateral branches, so further studies were performed on this hairy root clone.

Sonication of explants using low-frequency ultrasound produces a small wounding in the plant cell wall and improves T-DNA delivery into cells of some plant species (Georgiev, Ludwig-Muller, Alipieva and Lippert, 2011). In addition, monoterpenoids of *Lavandula* sp. essential oil possess antibacterial properties that may have an inhibitory effect on the growth of bacteria and transformation efficiency. Therefore, glutamic acid derivatives were used in the coculture media as a terpenoid adsorbent with SAArT through the transformation procedure (Sandal et al., 2007). The results showed that none of those treatments had a positive effect on the transformation efficiency. In addition, the adventitious roots did not show morphogenetic properties of hairy roots including a high growth rate and the production of numerous lateral roots. In transformation studies, plant and bacterial genotypes are the most important factors that should be considered. This could be the reason why to date, there is no report on

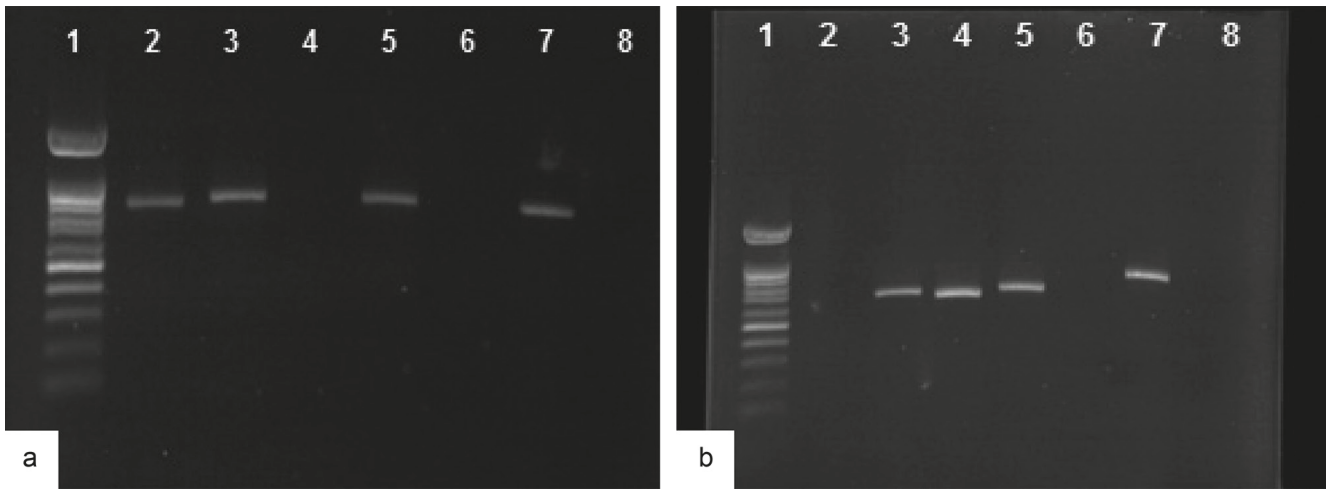

Fig. 1. a. Electrophoregram of PCR products of *rol*C, *vir*D and *aux*1genes in different samples of plants and bacteria. 1 — 100 bp plus size marker; 2 — A4; 3 — ATCC 15834; 4 — K599; 5 — MSU-1; 6 — NCBPP 2656; 7 — R1000; 8 — Negative control (water). b. PCR analysis of T-DNA integration pattern in the hairy root of *L. angustifolia*. 1 — 100 bp plus size marker; 2–4 — PCR analysis of *rol*C gene, 2 — non-transformed root, 3 — R1000 strain of *A. rhizogenes*, 4 — transformed hairy root; 5–6 — PCR analysis of *vir*D gene, 5 — R1000 strain of *A. rhizogenes*, 6 — transformed hairy root, 7–8 — PCR analysis of *aux*1 gene, 7 — R1000 strain of *A. rhizogenes*, 8 — transformed hairy root.

successful hairy root induction of some valuable medicinal plants such as *L. angustifolia*.

Molecular analysis of T-DNA integration

To investigate the integration of TL-DNA and TR-DNA, PCR amplification of *rol*C, *aux*1 and *vir*D genes was performed on the transformed hairy roots. The expected band of the *rol*C gene was observed in PCR products of the transformed hairy roots and R1000 strain of *A. rhizogenes* but it was not presented in the genomes of the non-transformed roots and deionized water as negative controls. In addition, there was no amplicon of *vir*D gene in PCR product of hairy root whereas this gene existed in Ri plasmid of R1000 strain (Fig. 1b). These results confirmed that TL-DNA was successfully transferred to *L. angustifolia* cells and the obtained hairy root was free from contamination of *A. rhizogenes* strains. Moreover, the *aux*1 gene was detected in R1000 strain but it was not transferred to the hairy root genome.

Effects of auxins on the growth of hairy roots and non-transformed roots

The growth pattern of hairy roots and non-transformed roots in auxin-containing media showed significant differences during thirty days of culture. Both IAA and IBA inhibited the growth of non-transformed roots and callus was induced along the root axis whereas hairy roots grew more rapidly than those of auxin-free media and large amounts of lue-pigmented lateral branches were observed (Fig. 2). The pigmentation had gradually decreased 3 weeks after transfer to the liquid media and stopped during successive subcultures. This result was in agreement with previous reports, which concerned the proposed function of *rol* genes as the regulator of the plant cell growth and differentiation (Blakesley and Chaldecott, 1993). *rol*B is the central member of root inducing loci and releases IAA from its glucoside conjugate (Estruch, Schell and Spena, 1991). As a result, the *N. tabacum rol*B-transferred protoplasts have shown 10^4 fold-hypersensitivity to auxin (Maurel et al., 1991). Therefore, the synergistic effects of *rol* genes resulted in the improved growth of *L. angustifolia* hairy roots in response to auxin even though TR-DNA was not presented in the genome of the hairy roots.

Effects of different basal media and auxins on the growth of hairy roots

The effect of different concentrations of IAA and IBA in combination with half-strength MS and B5 basal media on biomass production of hairy roots was evaluated. The average dry mass of hairy roots grown in ½ MS medium was two-fold higher than ½ B5 medium. In addition, ½ B5 medium induced callus formation in hairy roots while it was not observed in ½ MS medium (Fig. 3, a–h). Nutrition-dependent growth of hairy roots was also reported in some plant species such as *Cucumis melo* (Pak et al., 2009), *Arachis hypogaea* (Condori et al., 2010), and *Momordica charantia* (Thiruvengadam et al., 2014).

Both types of auxins initially stimulated the growth of hairy roots, but after two months a different trend was noticed in IAA-treated hairy roots as they stopped growing and their color gradually turned dark brown. This phenomenon finally resulted in lower biomass production of IAA-treated hairy roots compared to the auxin-free cultures (Fig. 4, c and f). These results are in agreement with those found by Balvanyos et al. (2001), whose work revealed that the linear growth of *Lobelia inflata* hairy roots in the IAA-containing medium significantly

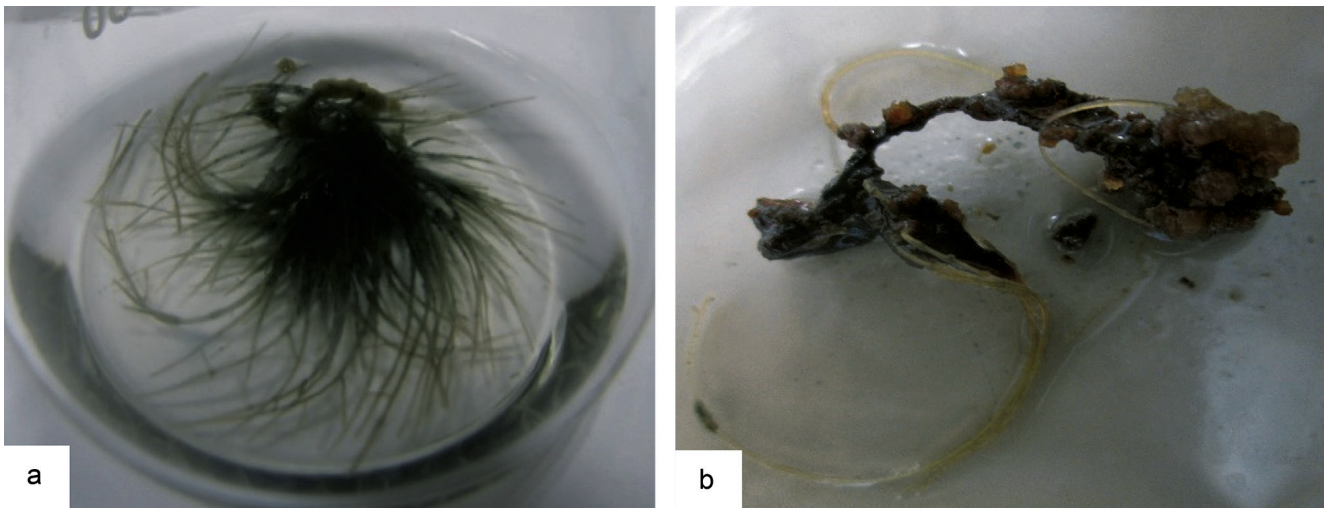

Fig. 2. The growth of roots in response to 1 mg/l IBA. a — transformed hairy root; b — non-transformed root.

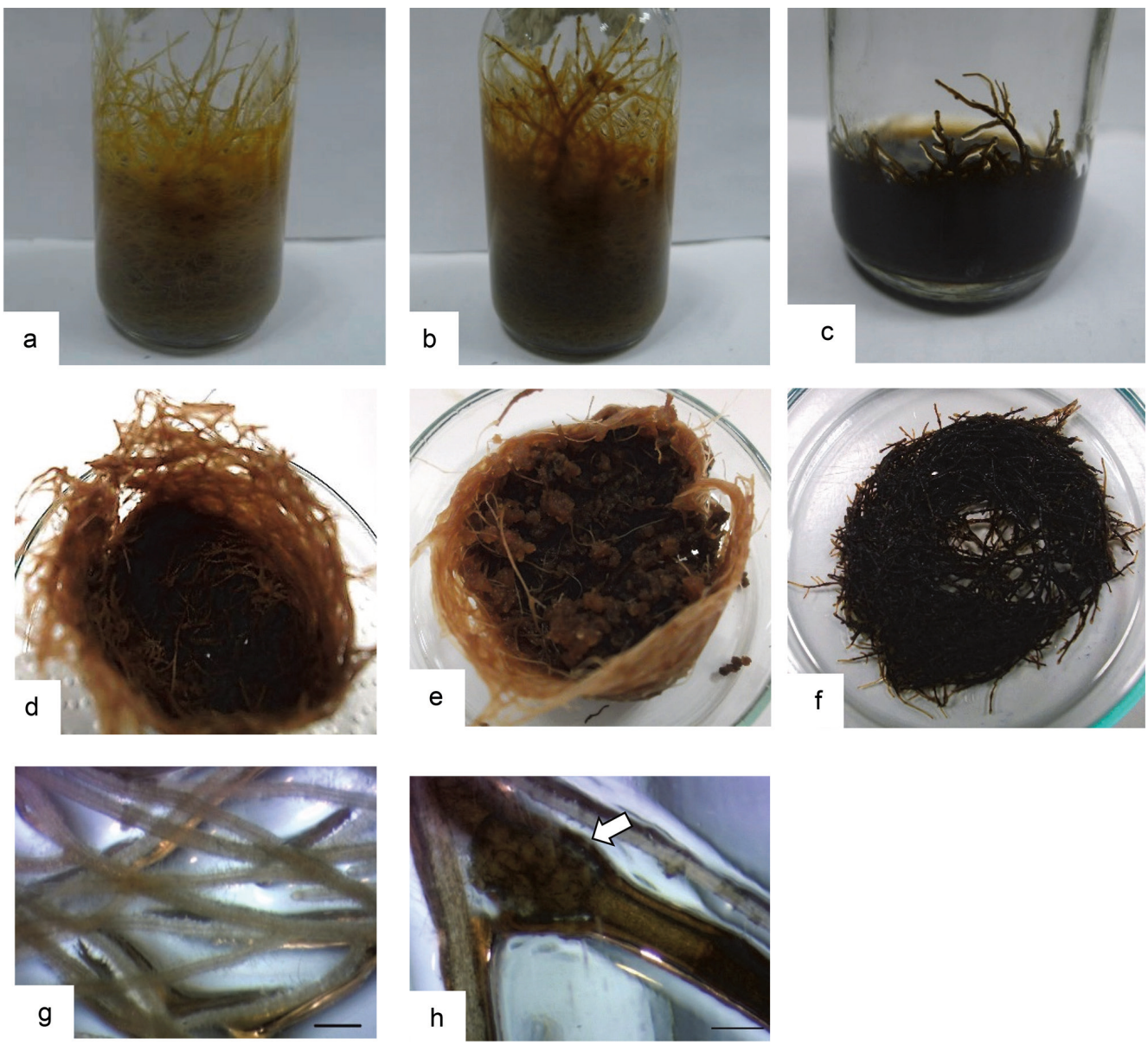

Fig. 3. Morphological characteristics of treated hairy roots; a–c — lateral view of hairy roots in the growth flasks, d–f — upper view of hairy roots and the inner space of their growth, g–h — macroscopic view of single hairy roots with callus formed along root axis (showed by arrow), a, d, g — ½ MS medium with 1 mg/l IBA, b, e, h — ½ B5 medium with 1 mg/l IBA, c, f — ½ MS medium with 1 mg/l IAA. Scale bar: 0.5 mm.

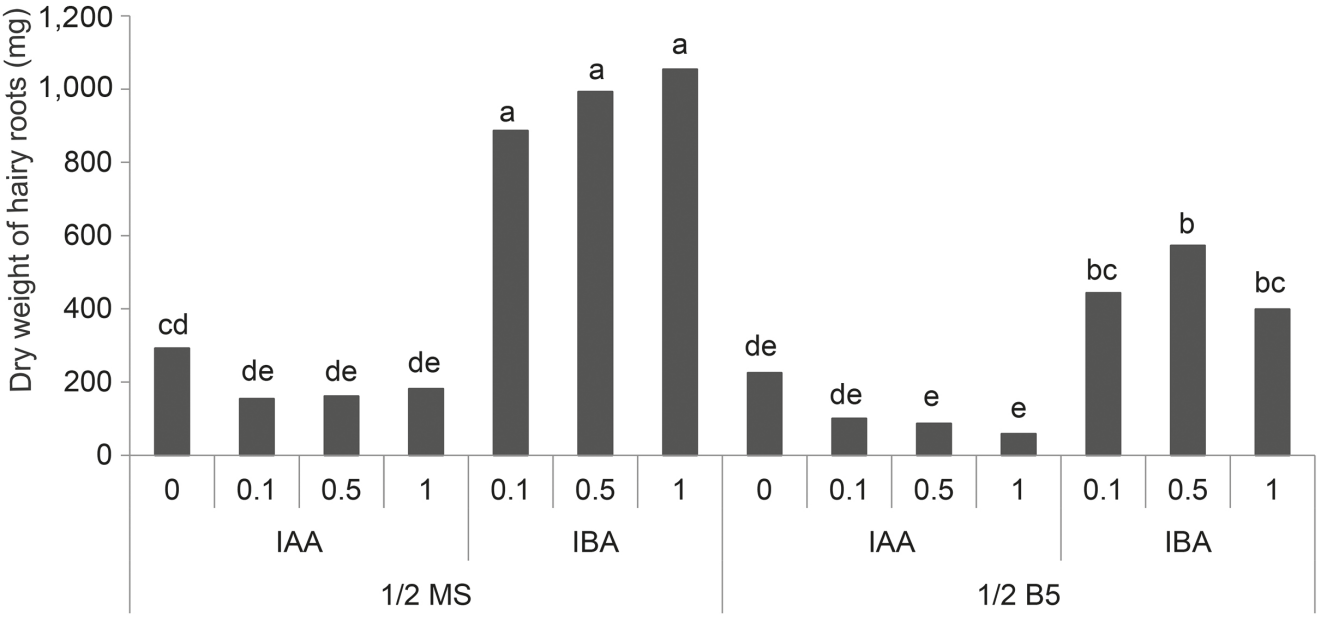

Fig. 4. Biomass production of hairy roots in response to different basal media and auxin treatments. Different letters indicate significant differences between groups at $p \leq 0.05$ according to Duncan's multiple range test.

decreased after 27 days. In contrast to the effect of different types of auxins, dry mass of hairy roots was similar in various concentrations of both IAA and IBA (Fig. 4).

Auxins play important roles in many aspects of plant development, but high concentration of auxins stimulates some abnormalities in the fundamental processes of plant cells. For instance, auxin-induced ABA inhibits the growth of both shoots and roots of plants (Hansen and Grossmann, 2000). On the other hand, regulatory mechanisms have also evolved simultaneously with the biosynthesis pathway of endogenous auxins in plants. Previous studies indicate that IBA is the storage form of IAA and acts as an important regulator of auxins, especially for IAA derivatives in a peroxisome-dependent manner (Ludwig-Muller, 2000; Simon and Petrasek, 2011). Therefore, it is reasonable to propose that the presence of IAA in a long-term hairy root culture probably disrupts IAA/IBA homeostasis and directly induces some plant inhibitory mechanisms in hairy roots of *L. angustifolia*.

Table 2. Rosmarinic acid content in hairy roots in response to different basal media and various concentrations of IBA

	IBA (mg/l)	RA* (mg/g DW)	Total RA (mg)
Hairy roots in ½ MS	0	2.21 b	0.624
	0.5	2.84 b	2.790
	1	7.98 a	8.363
Hairy roots in ½ B5	0	1.86 bc	0.416
	0.5	0.89 c	0.506
	1	0.58 c	0.232
leaves		10.2 a	
normal roots		1.94 bc	

* Different letters indicate significant differences between groups at $p \leq 0.05$ according to Duncan's multiple range test. RA: Rosmarinic acid, Total RA: RA×DW.

Effects of basal media and auxins on rosmarinic acid production of hairy roots

According to the results obtained from the growth assessment of hairy roots, the chromatographic analysis was not performed on IAA-treated cultures. Therefore, six treatments of hairy roots (two basal media in combination with three concentrations of IBA), non-transformed roots and leaves of naturally grown lavender were assayed for the content of rosmarinic acid. The results showed that the production of rosmarinic acid in the absence of IBA was not affected by different types of basal media, but interestingly, the interaction of basal media and various concentrations of IBA significantly affected the rosmarinic acid content in hairy roots. A slight decrease in the production of rosmarinic acid was observed upon increasing the concentration of IBA in ½ B5 medium, however, it was not significant, but the elevated levels of IBA greatly improved the biosynthesis of rosmarinic acid in ½ MS-cultured hairy roots. As a result, rosmarinic acid content in those hairy roots was approximately 3.5-fold higher than that of hairy roots grown in ½ MS media without any auxins (Table 2). Similar results were also reported in the hairy root culture of *Nepeta cataria*, where the production of rosmarinic acid

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in the presence of IBA was 1.5-fold higher compared to the control (Yang et al., 2010). HPLC analysis also revealed that the roots of naturally grown plants have low amount of rosmarinic acid. This indicates that the type of tissue is a determinant of rosmarinic acid production, but the best results could also be achieved by the manipulation with environmental conditions as well as with genetic background. Previous studies also showed the increased growth of hairy roots of different species such as *Scutellaria lateriflora* and *Rauvolfia micrantha* in response to IBA application, but the growth of hairy roots in these studies has been tripled at most compared to the control treatment. In this study, the total amount of RA in the presence of 1 mg/L^{-1} IBA was increased 13.4-fold higher than control (without any auxins). In conclusion, it seems possible to obtain a high yield of rosmarinic acid production from hairy root cultures using the advantages of in vitro conditions together with promising characteristics of hairy roots such as auxin hypersensitivity.

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