Journal of Medicinal Plants and By-products (2014) 2: 155-160

Original Article

Hairy Roots Formation in Four Solanaceae Species by Different Strains of *Agrobacterium rhizogenes*

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Article History: Received: 05 November 2013/Accepted in revised form: 03 September 2014

Abstract

Plants are the important sources of drugs. Secondary metabolites are responsible for therapeutic propertiesin plants. Three compounds including (-)-hyoscyamine, its racemate atropine, and scopolamine (hyoscine) are the most famous tropane alkaloids in the *Solanaceae* familly. Nowadays, attempts to developthese alkaloids in biotechnological procedures which are principally based on the hairy root cultures using *Agrobacterium rhizogenes.* In the present study, we showed the percent of induced hairy roots percent in the leaf and root explants of four plants from the *Solanaceae* family (*Atropa belladonna* L.*, Hyoscyamus niger* L.*, Datura stramonium* L. *and Datura metel* L.), that infected with the six strains of *Agrobacterium rhizogenes* (A4, A7, Ar15834, Ar9534, Ar9402, and Ar318). Hairy roots were appeared from the leaf and roots explants on ½MS medium culture. The presence of T-DNA in the supposedly transformal lines was shown by PCR. The highest transformation yield of 93% was accomplished using leaf explants of *D. metel* infected by AR15834 and A4 strains. One fastest growing clone of transforming *D. metel* roots line (induced by AR15834)was selected and the biomass of hairy and natural roots were measured and compared after 0, 2, 4, 6, 8 and 10 days. The results showed that, the fresh and dry weight of hairy roots was 4.44 and 4.92 times higher than the weights of non-transformed roots respectively after 8- 10 days. These roots are hormone-autotrophic and have the great lateral branches. Therefore, the hairy roots of *D. metel* and *D. stramonium* can be used to increase tropane alkaloids production yield in the pharmaceutical industry.

Key words: Hairy root, *Agrobacterium rhizogene,* Solanaceae, Tropane alkaloids

Introduction

Plant-derived drugs are important sources for a various types of medicines, dyes, oils, flavors and resins [1]. A large number of the extracted materials with biological activity are alkaloids. Three compounds including (-)-hyoscyamine, its racemate atropine, and scopolamine (hyoscine) are the most famous tropane alkaloids [2,3].These alkaloids found usually in several members of the *Solanaceae* family [4].These alkaloids are anticholinergic agents and used as antispasmodic, preoperative medication, sedative, narcotic and analgesic. The other use of tropane alkaloids are in asthma, motion sickness and Parkinson's disease treatment [5]. The procedures such as development of calli or cell cultures for tropane alkaloids production are not proliferous [6].Thus, attempts to develop these alkaloids in biotechnological procedures which are principally based on hairy root cultures using *Agrobacterium rhizogenes* that is a gram negative bacterium. The neoplastic roots are distinguished by high growth rate, genetic stability and lateral branching [7]. Furthermore, hairy roots are recognized to make a high yield of secondary metabolites as compared to that of

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undifferentiated plant cell suspensions [8,9]. Hence, such a transformation of explants follows a good strategy for *in vitro* tropane alkaloids production. The hairy roots attained from the transformation of the *Solanaceae* family with various *A. rhizogenes* strains produced different tropane alkaloids content, and in the several cases higher than in plants natural roots [10-12].

The *A. rhizogenes* plays a role to integrate a T-DNA from root-inducing (Ri) plasmid into plants DNA [13]. The T-DNA has a group of genes that encode the enzymes for control of auxin and cytokinin production and the new hormonal ratio can be affected on hairy root formation [13]. This study demonstrates the percent of transformation in four species of the *Solanaceae* family (*Atropa belladonna, Hyosyamus niger Datura stramonium and Datura metel*) cultures by six different *A. rhizogenes* strains. The selection of one or more species in the *Solanaceae* family with the highest transformed tissue can be used for industrial tropane alkaloids production.

Material and Methods

Plant material

The Mature seeds (Pakan Bazr, Isfahan) of four plants, *Atropa belladonna, Hyosyamus niger, Datura stramonium and Datura metel* were surface-steriled by soaking in NACLO 2% (v/v) for 5 min and then immerged in ethanol 70% (v/v) for 1 min and were washed three times with sterile water. Such treated seeds were cultured on MS medium at pH 5.8 supplemented with 30.0 g/l sucrose and 7.5 g/l agar, at a temperature of 25±1 °C and photoperiod of 16-h light:8-h dark [14].

Bacteria strains

The six strains of *Agrobacterium rhizogenes* (A4, A7, Ar15834, Ar9534, Ar9402, and Ar318), kindly provided by Dr. A. Mirzaie-asl (Bu-Ali Sina University, Hamedan, Iran), were used in all experiments. A single clone of these *Agrobacteriums* was cultured in solid LB medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl Dissolve in 1 L ddH_20), supplemented with 50mg/l rifampin, at 28 ºC. One clones of these bacteria were put in liquid LB medium with the similar condition and were maintained on a shaker in 120 rpm, until obtaining an optical density 600nm of 0.6 (OD600= 0.6) [15-16].

Transformation and creation of hairy root cultures

After germination and seedling appearance, the leaf and roots of the four selected plants were cut and 5-7 mm explants were made. These explants were placed with grown *A. rhizogenes* in 10ml of liquid LB medium (OD_{600}= 0.6). Then, for co-cultivation of explants with *Agrobacterium*, the leaf and root explants placed back on with solid ½MS medium culture plate for 24h. Then these explants transferred into MS medium contained 500mg/l cefotaximefor elimination of the bacteria. Several explants without soaking in as suspension of bacteria were used as negative control[17]. All experiments were carried out in triplicate and then analyzed variance with SPSS (version 21) software using the univariate procedure at $P < 0.05$ level.

Molecular confirmation of hairy roots

Extraction of Genomic DNA of roots was performed by the CTAB method and natural roots genomic DNA of these plants used as the negative controls [18].Plasmid DNA from *A. rhizogenes* was extracted by the Sam brook method [19].This plasmid DNA was applied as positive control. The presence of integrated T-DNA in the supposedly transformed lines was demonstrated by PCR. The PCR primers (forward/reverse) were employed for amplification cycles of *rolB* gene. The sequences of these primers were 5′-ATGGATCCCAAATTGCTATTCCCCACGA-3′ and 5′ TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3′. The optimized PCR conditions contained of 2 ng of plant DNA (with a 2 µl volume), 2.0 µl of 10× Taq buffer, 0.4 µl of 10 mM dNTPs, 0.5 µl of 5 Units/ µl Taq DNA polymerase, 2.0 µl of 50 mM $MgCl_2$, 1 µl of 1 mM from each primer, 11.1 µl of sterile ddH_2O and the total volume made 20 µl. Thirty five thermal cycles were performed by PCR where each cycle included of 94 ^oC (1 min), 58 ^oC (1 min), and 72 ^oC (1 min) [14, 20].

Biomass analysis for hairy roots of D. metel

One fastest growing clone of transforming *D. metel* roots line (induced by AR15834), among other lines was selected, because developing hairy roots in leaf explants of *D. metel* has a great percent after infected by AR15834.Therefore, 4-5cm of hairy and natural roots were cut and cultured in ½ MS liquid medium [21]. The biomass of the two different roots was measured after 0, 2, 4, 6, 8 and 10 days.

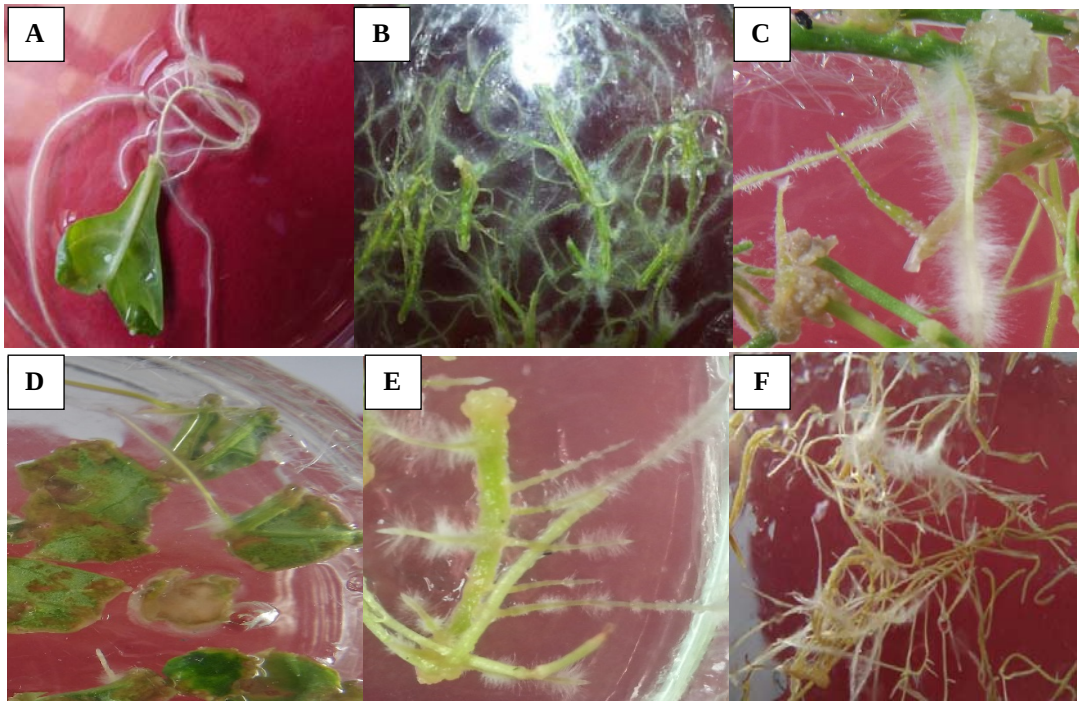

Fig. 1 Hairy Root (HR) induction in leaf and root explants of *Solanaceae* family. A, B) HR induction in leaf and root explants of *A. belladonna*. C) HR induction in root explants of *D. stramonium*. D, E) HR induction in leaf and root explants of *D. metel*. F) HR induction in root explants of *H. niger*.

Results and Discussion

The hairy roots were appeared after approximately 15-20 days from leaf and root explants of four the *Solanaceae* family (*Atropa belladonna, Hyoscyamus niger, Datura stramonium and Datura metel*) that induced by six strains of *A. rhizogenesis* (A4, A7, Ar15834, Ar9534, Ar9402, and Ar318), (Fig. 1).

The different level of transformed roots was appeared from leaf and root explants (Fig. 2 and 3). The results showed that the highest percent of transformation (93%) were observed in leaf explants of *D. metel* that induced by A4 and AR15834.For other explants, the highest percent of transformation was being in leaf explants of *A. belladonna* which induced by AR15634 and AR318 with 76% and 67% respectively, root explants of *H. niger* infected by AR9402 with 71%, leaf explants of *D. stramonium* induced by A4 and AR15834 with 78% and 70% respectively and root explants of the same plant infected by A4 with 55%. Root explants of *D. metel* induced by A4 with 75%. The leaf explants of *H. niger* were browned after infection by all strains of *A. rhizogenesis*. Królicka *et al*. reported various types of *A. rhizogenes* show remarkable difference in their infection ability [22]. Bacterial strains, explants genotype and signal molecules can have effects on the transformation of hairy roots [23]. The various transformations percent induced by different strains of *A. rhizogenes*, is explained by plasmids that carrying with these bacteria [24]. Furthermore, the diverse expression of T-DNA genes in hairy roots, integration of T-DNA genes in different location in genomic DNA of explants and several copy numbers of T-DNA integrates to these genomes are the reason of the particular variation in the formation of hairy roots [25]. Potty and Chandran, showed that the strain 15834 and A4 have a powerful growth in YEP medium and the hairy root formation by this strains was developed in the short period [26]. Therefore, they suggested that the use of these strains is the vigorous selection for hairy root formation. These strains are agropine-type and have the most power transform ability [27,28]. Pawar and Maheshwari, (2004) showed that, only leaf explants of several species in the *Solanaceae* family are induced by *A. rhizogenes* [29]. It has been reported that acetylsyringone which is released through the wounding sites of explants, changes the potential of hairy root formation. This compound causes a substantial increase in the quantity of roots transformation. Acetylsyringone is related to activation of the *vir* genes of *A. rhizogenes* and transfer T-DNA to plant cell genes [30].

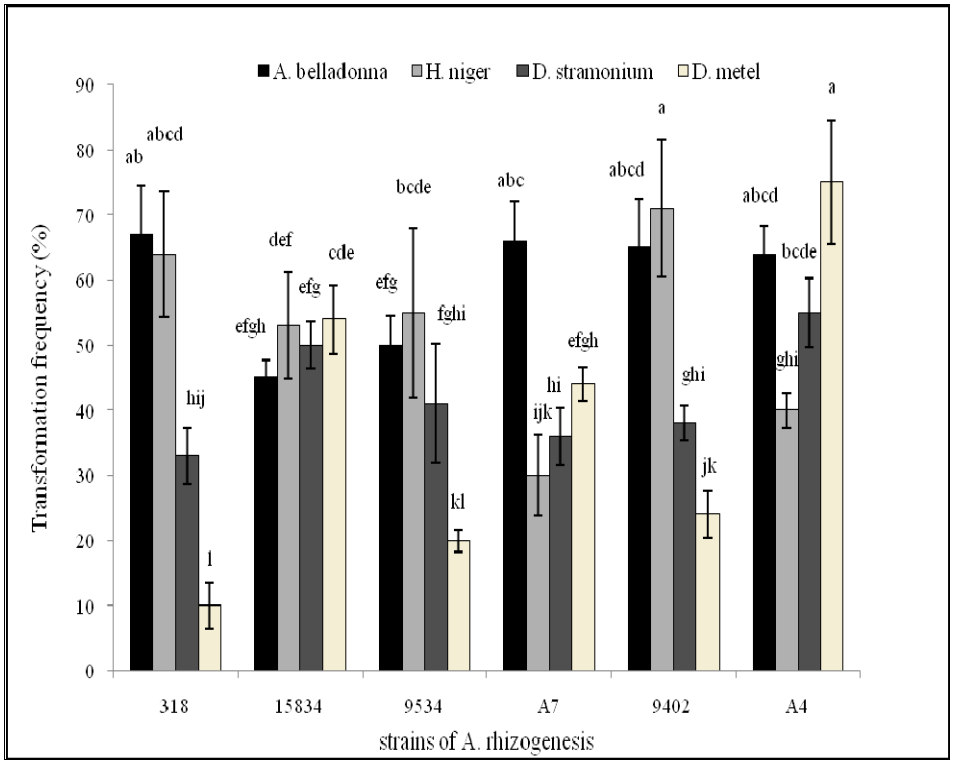

Fig. 2 The percent of transformation was compared in root explants of four species of the *Solanaceae* family (*Atropa belladonna, Hyoscyamus niger, Datura stramonium and Datura metel*) after induced by six strains of *A. rhizogenesis* (A4, A7, Ar15834, Ar9534, Ar9402, and Ar318). Different alphabets were used to show the significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

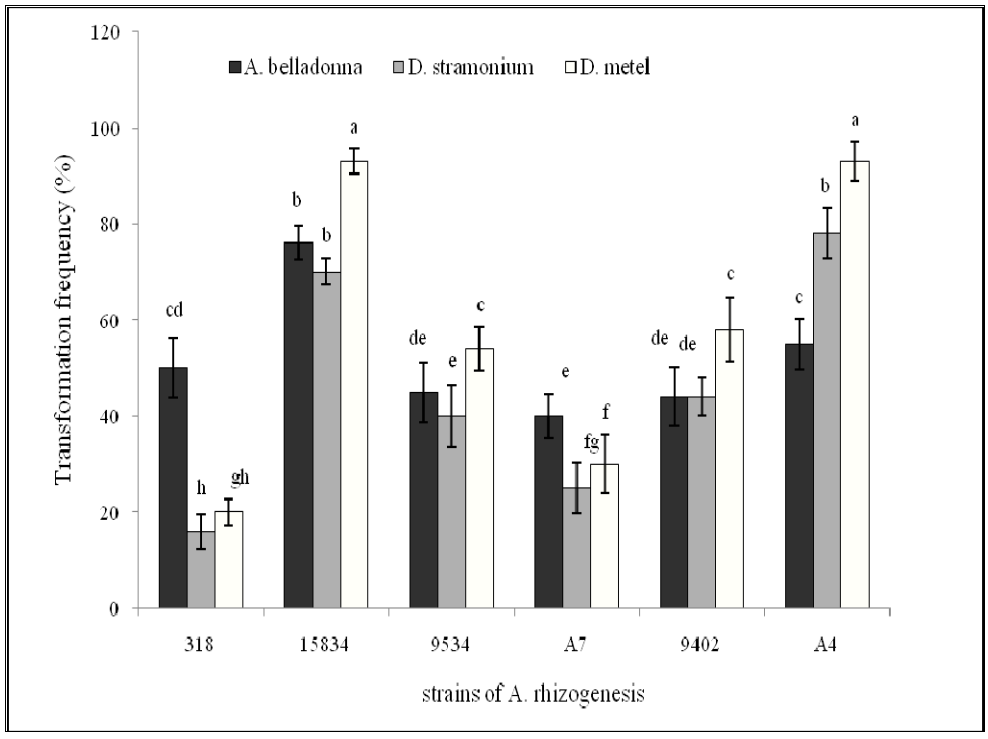

Fig. 3 The percent of transformation was compared in leaf explants of three species of the *Solanaceae* family (*Atropa belladonna, Datura stramonium and Datura metel*) after induced by six strains of *A. rhizogenesis* (A4, A7, Ar15834, Ar9534, Ar9402, and Ar318). Different alphabets were used to show the significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

The PCR confirmed the being of the *rolB* gene in the supposedly transformed lines in 780bp. The DNA of Ri-plasmid from *A. rhizogenes* served as the positive control and confirmed the existence of the *rolB* genes in 780 bp DNA fragments on gel electrophoresis, while natural roots genomic DNA was applied as the negative control that did not have *rolB* amplified product in 780 bp (Fig.4). Previously Rahnama *et al.* demonstrated these fragments on gel electrophoresis [20].

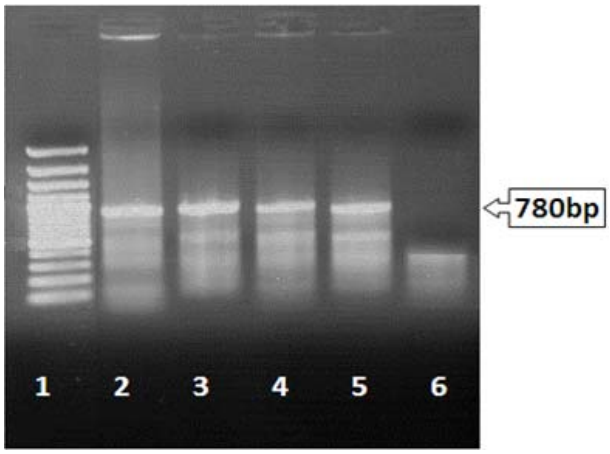

Fig. 4 PCR amplification of *rolB* gene (780bp). Lane 1: molecular weight marker (1000 bp ladder). Lane 2, 3 and 4: *rolB* gene in Hairy roots DNA. Lane 5: positive control (plasmid DNA from *A. rhizogenes*) and Lane 6: negative control of non-induced roots.

The results showed that the fresh and dry weight of hairy roots were 4.44 and 4.92 times higher than these weights in non-transformed roots respectively after 8- 10 days (Fig.5).Transformed roots are hormone-autotrophic and secrete auxin to outside and this compound is important for the roots growth[31].

The roots that transformed by *A. rhizogenes* are specified by the development of great lateral branches, the principal factor contributing to their high biomass efficiency [32] (Fig. 6).

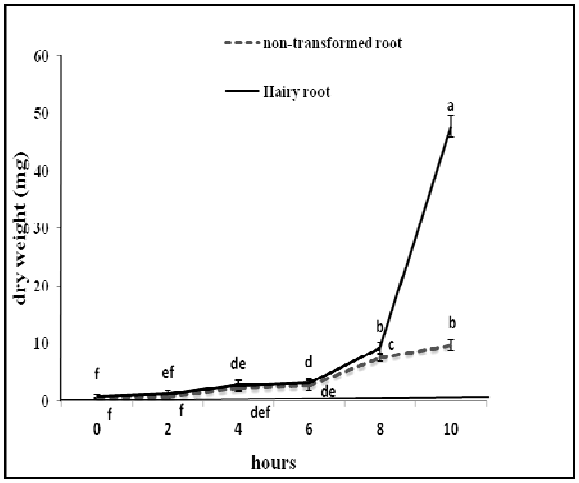

Fig. 5 Wet and dry weights (A and B respectively) of non-transformed and transformed roots of *D. metel* after induced by AR15834. Different alphabets were used to show the significant differences in mean values for each parameter using Duncan's test ($P < 0.05$).

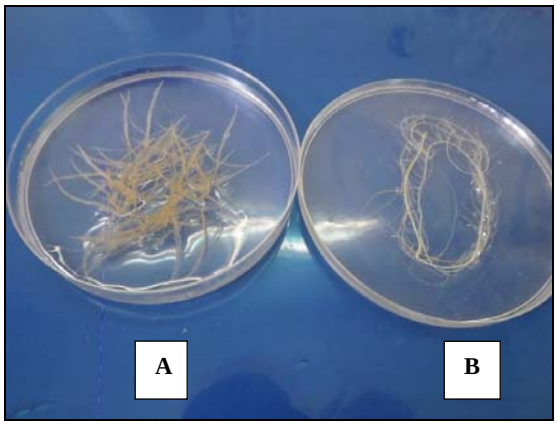

Fig. 6 Transformed or hairy roots (A) and non-transformed roots (B) of *D. metel* that induced by AR15834 after 10 days.

Conclusion

In this study, hairy roots in the *Solanaceae* family, especially in *Datura* spp. were induced by different types of *A. rhizogenes* and the strains A4 and AR15834 were the best strains for this occasion. These roots were specified by the greatest growth rate, genetic stability and fine lateral branching. Hence, the hairy roots of *D. metel* and *D. stramonium* can be used to increase the tropane alkaloids production yield in the pharmaceutical industry.

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