UDC 602.64 **doi: 10.15407/biotech9.02.055**

TARRAGON (*Artemisia dracunculus* L.) "HAIRY" ROOT CULTURE PRODUCTION

K. O. Drobot
A. M. Shakhovsky
N. A. Matvieieva

Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine, Kyiv

E-mail: katyadrobot@gmail.com

Received 20.02.2016

This paper is devoted the biotechnology development of *Artemisia dracunculus* L. genetic transformation. We obtained the transgenic *A. dracunculus* "hairy" root culture using *A. rhizogenes* A4-mediated transformation. The conditions of tarragon's genetic transformation were optimized. It was shown that leaves of *in vitro* cultivated plants were the optimal type of explants. The transgenic root formation frequency was up to 20% in case of leaves usage. The time of explants cocultivation with *Agrobacterium* suspension was found to be an important factor of biotechnology which affects the frequency of transgenic root growth. Transgenic root lines differed in morphological features and growth rate. Specific mass increase varied from 17 to 32 times after 3 weeks cultivation on 1/2 Murashige-Skoog medium.

Key words: *Artemisia dracunculus* L., genetic transformation, *Agrobacterium rhizogenes* A4, "hairy" root culture.

Biotechnological approaches are widely used to create plants with new properties and to obtain biologically active substances (BAS). At the moment such approaches are a topical trend of modern scientific researches in field of biology. Genetic engineering is one of the biotechnology approaches for construction of plants with new properties. Transformation method using soil bacteria *Agrobacterium rhizogenes* can be used to create transgenic roots which produce BAS. Regarding this the use of medicinal plants as an object for genetic transformation is of special interest. *Artemisia* spp plants may be used for achieving this goal.

Artemisia genus belongs to Asteraceae family and includes more than 500 plant species [1]. Tarragon — *Artemisia dracunculus* L. — is a well-known perennial plant, which is widespread throughout the world and also grows in Ukrainian steppe and forest steppe ecoregions [2]. The use of *A. dracunculus* was mentioned in ancient Greece, but some historicans considered that Asia is a real tarragon's origin. At present, there are two well-described cultivars (Russian and French [3]) of *A. dracunculus*, which differ in physiology, botanical features and phytochemical profile. French tarragon (2n = 36) is a sterile chromosomal derivative of Russian tarragon (2n = 90). The last one can produce viable seeds, instead of French tarragon, which doesn't produce seeds [4].

Tarragon is widely used in folk medicine as well as in cosmetics and cuisine due to high essential oil content. It is also extremely popular in French cuisine as a spice and is also used for vinegar preparation. Light licoric or basil-, anise-like flavor could be referred to tarragon's culinary benefits [5].

Flavonoids, coumarins, phenilpropanoids, terpenes determine antimicrobial, antiviral, antifungal and antioxidant activities of *A. dracunculus*. Such a broad spectrum of biological activities could cause tarragon`s use in pharmaceutical industry for treatment of diseases such as inflammation [6], hepatitis [7] and different kind of infections (bacterial, viral) [8, 9]. Leaves of *A. dracunculus* accumulate artemisinin up to 0.27% [10]. Recent studies of *A. dracunculus* were devoted to plant micropropagation [11], medicine compounds accumulation [12] and artemisinin synthesis in particular [10].

"Hairy" root culture is a type of plant tissue culture, which is considered to be an alternative way for producing valuable plant derived compounds [13]. It could be obtained

via *Agrobacterium rhizogenes*-mediated transformation. *A. rhizogenes* is a soil bacteria which naturally cause hairy-root disease in plants owing to the presence of root-inducing plasmids. Transgenic hairy roots have been obtained in more than 89 different taxa, representing 79 species from 55 genera and 27 families using *A. rhizogenes*-mediated transformation [14]. This amount is rising from year to year.

"Hairy" root culture is characterized by unlimited and fast growth without use of exogenous hormones; it is unpretending to the growth conditions and doesn't require lighting, so the roots can be cultivated in bioreactors [15]. In contrast to cell culture, "hairy" root culture is characterized by high genetic stability. Establishment of transgenic roots in some cases takes only a few weeks [16]. This implies that the use of "hairy" root culture could attract particular attention and especially for study of tarragon`s transformation and BAS production possibility. *A. rhizogenes*-mediated genetic transformation was conducted for plants of some *Artemisia* representatives: *A. annua* [17 18], *A. dubia* and *A. indica* [19], *A. aucheri* [20], *A. vulgaris* [21], *A. absinthium* [22]. Transgenic root cultures obtained were used for producing BAS in bioreactors, especially for artemisinin content increasing. Nevertheless genetic transformation of tarragon has not been carried out yet. Establishment of biotechnology of genetic transformation of *A. dracunculus* using *A. rhizogenes* A4 wild strain was the aim of our work.

Materials and Methods

A. dracunculus were introducted in *in vitro* culture using seeds surface sterilization method. 14-days-old seedlings which were cultured on hormone-free half-strength Murashige-Skoog (½ MS) [23] basal medium were used for genetic transformation experiments.

A. rhizogenes A4 wild strain was inoculated to the liquid LB medium (10 g/l casein hydrolyzed, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0) and was left to grow overnight at 28 °C using rotation shaker SpeedVac Savant AES 2010 (Labconco, USA). Leaves, hypocotyls, stems and roots of 14-day tarragon seedlings were used as the explants for genetic transformation. Explants were sliced and cocultivated during 30 min with an overnight bacterial suspension. Then they were transferred to the agar-solidified ½ MS medium (20 g/l sucrose, pH 5.7) supplemented with 600 mg/l cefotaxime for agrobacteria elimination. After cocultivation explants were placed onto ½ MS agar-solidified basal medium and were grown for 2–7 days. Well-grown roots formed on the explants after genetic transformation were excised from the explants and transferred to individual Petri dishes for further "hairy" root growth. The root formation frequency was calculated as a number of root producing explants in percents.

Extraction of DNA was carried out according to CTAB-method. The presence of *rolB* genes in established "hairy" roots was determined using PCR analysis on Mastercycle personal 5332 amplifier (Eppendorf). Amplification was carried out in following conditions: primary denaturation — 94 °C, 3 min, 30 cycles of amplification (94 °C, 30 s — 56 °C, 30 s –72 °C, 45 s), final polymerization —72 °C, 3 min. Products of reaction were separated in 1.5% agarose gel. O'GeneRuler 1 kb Plus DNA Ladder #1163 was used for sizing of *rolB* genes. Primers 5′-atggatcccaaattgctattccttccacga-3′ and 5′-ttaggcttctttcttcaggtttactgcagc-3′ (Size of amplified fragment is 780 b.p.) were used to confirm the presence of *rol B* gene in *A. dracunculus* "hairy" root lines.

Transgenic roots were subcultured every two weeks. Subcultivation was conducted at 24 °C and 16 h/d photoperiod. Growth rate of transgenic root lines was studied after 3 week of cultivation of excised root tips on ½ MS medium at the same conditions.

Results and Discussion

The results of our experiments approved that the time of explants cocultivating with *Agrobacterium* suspension is an important factor for transgenic root formation. The optimal time of explant cultivation on the medium without cefotaxim for agrobacterial genes transfer into plant cells appeared to be four days. Prolongation of this term has led to explants death, while reducing of this wasn't successful for root obtaining. Roots have formed on the 7-th day after cocultivation with bacterial suspension on leaf explants. "Hairy" roots weren't obtained using hypocotyls, stems and roots. Time of cocultivating of these explants with Agrobacterium suspension didn't effect transformation frequency. After two weeks of cultivation hypocotyls, stems and roots have lost capacity to grow and became dark. So the using of leaves as explants and transformational conditions mentioned above

a *b* *c*

Fig. 1. A. dracunculus in vitro **cultivated plant and "hairy" root culture:**
***A. dracunculus* 14-days seedling (*a*); initiation of "hairy" root growth (*b*);**
***A. dracunculus* "hairy" root culture (*c*)**

were successful for *A. dracunculus* "hairy" roots induction.

So the using of leaves as explants and transformational conditions mentioned above were successful for *A. dracunculus* «hairy» roots induction.

The amplified products of 780 bp for rolB gene was determined using PCR analysis in all *A. dracunculus* "hairy" root lines obtained, as it shown in Fig 2.

The root formation frequency was found to be up to 20%. The root number per explant reached up to 5. It is known that frequency of transformation usually depends upon *Agrobacterium* strain, plant species and explant type [24]. For example Sujatha et al. [21] conducted transformation of *A. vulgaris* using four *Agrobacterium* strains: A4GUS, R1000, R1601and ATCC15834 and three explant

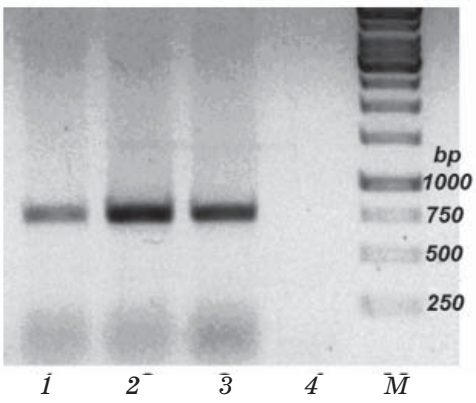

Fig. 2. **Electrophoregram which demonstrates the products of PCR analysis of "hairy" root clones transformed using *A. rhizogenes* A4 strain:**
marker for sizing DNA fragments (*M*);
DNA of *A .dracunculus* transgenic roots (*1, 2*);
bacterial DNA (*3*); genomic DNAs
of non-transformed roots (*4*)
Results of typical experiment are presented

types: shoot tip, leaf and node. The authors proved that *A. rhizogenes* A4 GUS strain is more competent than other strains and the highest transformation rates were observed in leaf explant (92.6%). Giri et al. demonstrated that the ability of *A. annua* transformation by four different *A. rhizogenes* strains (LBA 9402, 9340, 9365, 15834 and A4) was also found to be different. The best root formation response (up to 100%) was observed for LBA 9402 strain, the worst one — 75% in case of using A4 strain [17]. Leaves often are the most susceptible to genetic transformation type of explant of another *Artemisia* species plants (*A. dubia* and *A. indica* (100%), *A. aucheri* (93%), *A. absinthium*(57,1%). High root formation frequency of *A. vulgaris* after *A. rhizogenes* transformation (up to 100%) we observed earlier [25]. The data cited demonstrate significant species-depended difference in transformational frequency among *Artemisia* representatives. Obviously the low transformation frequency of tarragon plants in our experiment was caused by species-specific susceptibility of this plant. It is necessary to take into account the growth rate as well as target compounds content in order to estimate metabolic activity and capacity to synthesize BAS in "hairy" roots. It is an important step for selecting the most productive "hairy" root lines. High growth rate allow to obtain more valuable substances in short time. Transgenic lines that are notable for high growth rate and target compounds content can be an alternative source of plant-derived valuable substances. In our investigation specific mass increase varied from 17 to 32 times afrer 30 days growth on ½ MS medium. So, biomass increase among different "hairy" root lines varied considerably in our investigation Such variability allowed to select the most productive "hairy" root

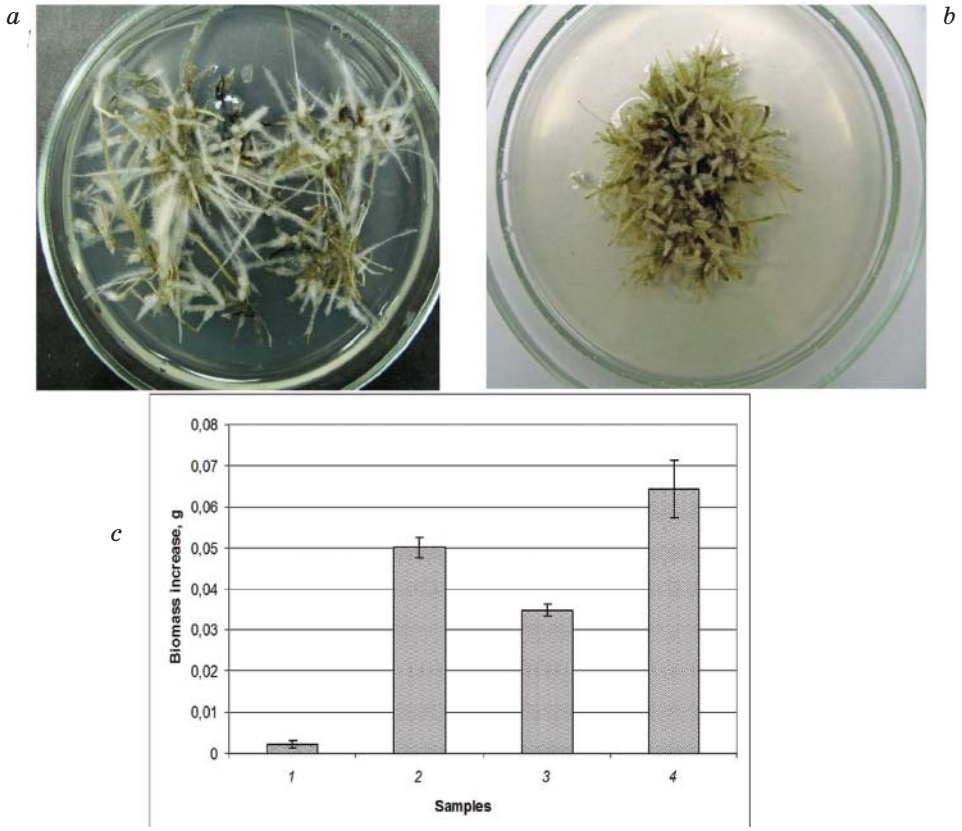

Fig. 3. ***A. dracunculus*** **"hairy" root growth:**
a, b — differences in *A. dracunculus* "hairy" root morphology (lenghth, branching); *c* — biomass increase of *A. dracunculus* control non-transformed roots (*1*) and "hairy" root lines (*2–4*) ($P < 0.05$)

lines, particularly lines №5 and line №7 (mass increase after 30 days of cultivation on hormone-free medium was up to 25 and 32 times respectively). It should be noticed that non-transformed roots had also ability to grow on hormone-free medium. However mass increase after 30 days of cultivation was only 1,08 times and they became dark in few weeks. "Hairy" roots obtained in our experiments didn't lose the capacity to grow even after 1.5 years of in vitro cultivation.

"Hairy" root lines obtained had a typical phenotype such as excessive and non geotropic roots, high branching growth on a hormone-free culture medium. We noted also morphological alterations among different transgenic lines. They differed in water cut range, fibril growth, root thickening and root branching (Fig. 3).

This phenomenon may be explained with variability in the expression level of *rol* genes or could be related to the peculiarities (site and number) of T-DNA integration into the plant genome [26].

Thus we firstly obtained biotechnology for genetic transformation of *Artemisia dracunculus* L. and have firstly obtained *A. dracunculus* "hairy" root culture using *A. rhizogenes* A4 wild strain. Our overall results reveal that the induction of *A. dracunculus* transgenic roots by *A. rhizogenes* can be successfully established using leaves of 14-days-old seedlings. The results agree with the earlier findings in case of transformation of other *Artemisia* spp. plants and confirm that leaves are the most suitable explants for genetic transformation of plants which belong to *Artemisia* genus. The transgenic root formation frequency was up to 20%.

It was found that the time of explants cocultivating with *Agrobacterium* suspension is an important factor which affect the frequency of transgenic root growth. It was shown that the optimal period of cocultivation with *Agrobacterium rhizogenes* used to be 4 days. Thus, the use of the proposed biotechnology of *A. dracunculus Arhizogenes*-mediated genetic transformation enable to obtain "hairy" root lines which transgenic nature was confirmed by PCR analysis.

REFERENCES

1. *Bora K. S., Sharma A.* The genus *Artemisia*: A comprehensive review. *Pharm. Biol.* 2011, V. 49, P. 101–109.
2. *Boiko A. V.* Specific features of *Artemisia* L. species distribution of the flora of Ukraine. *Indust. Botany*. 2013, V. 13, P. 73–79.
3. *Abad M. J., Bedoya L. M., Apaza L., Bermejo P.* The *Artemisia* L. Genus: a review of bioactive essential oils. *Molecules*. 2012, 17 (3), 2542–2566.
4. *Sutton S., Humphries C., Hopkinson J.* Tarragon. Garden UK. *British Museum (Natural History), South Kensington, London, UK*. 1985, 110 (5), 237–240.
5. *Uhl S. R., Strauss S.* Handbook of Spices, Seasonings and Flavorings. *Lancaster*: *Technomic Publishing*. 2000, 330 p.
6. *Eidi A., Oryan S., Zaringhalam J., Rad M.* Antinociceptive and anti-inflammatory effects of the aerial parts of *Artemisia dracunculus* in mice. *Pharm Biol*. 2016, 54 (3), 549–554.
7. *Aglarova A. M.* Comparative Analysis of Secondary Metabolites of *Artemisia dracunculus* L., Russian and French cultivars (*Doctoral dissertation*). Available from ProQuest Dissertations & Theses database. 2006. (UMI No. 327681).
8. *Mohsenzadeh M.* Evaluation of antibacterial activity of selected Iranian essential oils against *Staphylococcus aureus* and *Escherichia coli* in nutrient broth medium. *Pak. J. Bio.l Sci*. 2007, 10 (20), 3693–3697.
9. *O'Mahony R., Al-Khtheeri H., Weerasekera D., Fernando N., Vaira D., Holton J., Basset C.* Bactericidal and anti-adhesive properties of culinary and medicinal plants against *Helicobacter pylori*. *World J. Gastroenterol*. 2005, 11 (47), 7499–7507.
10. *Mannan A., Ahmed I., Arshad W., Asim M. F., Qureshi R. A., Hussain I.* Survey of artemisinin production by diverse *Artemisia* species in northern Pakistan. *Malar J*. 2010, V. 9, P. 310.
11. *Fernández-Lizarazo J., Mosquera-Vásquez T.* Efficient micropropagation of french tarragon (*Artemisia dracunculus* L.). *Agronomia colombiana*. 2012, 30 (3), 335–343.
12. *Obolskiy D., Pischel I., Feistel B., Glotov N., Heinrich M. Artemisia dracunculus* L. (Tarragon): A critical review of its traditional use, chemical composition, pharmacology and safety. *J. Agric. Food Chem*. 2011, 59, 11367–11384.
13. *Srivastava S., Srivastava A. K.* Hairy root culture for massproduction of high-value secondary metabolites. *Crit. Rev. Biotechnol*. 2007, V. 27, P. 29–43.
14. *Christey M. C.* Use of Ri-mediated transformation for production of transgenic plants. *In Vitro Cell Dev. Biol. Plant*. 2001, V. 37, P. 687–700.
15. *Kim Y. J., Weathers P. J., Wyslouzil B. E.* The growth of *Artemisia annua* hairy roots in liquid and gas phase reactors. *Biotechnol. Bioeng*. 2002, V. 80, P. 454–464.
16. *Giri A. Narasu M. L.* Transgenic hairy roots: recent trends and applications. *Biotechnol. Adv*. 2000, 18 (1), 22.
17. *Weathers P. J., Bunk G., McCoy M. C.* The effect of phytohormones on growth and artemisinin production in *Artemisia* annua hairy roots. *In Vitro Cell Dev. Biol. Plant*. 2005, 41 (1), 47–53.
18. *Ahlawat S., Saxena P., Ram M., Pravej A., Tazyeen N., Anis M., Malik Z. A.* Influence of *Agrobacterium rhizogenes* on induction of hairy roots for enhanced production of artemisinin in *Artemisia annua* L. Plants. *Afr. J. Biotechnol*. 2012, 11 (35), 8684–8691.
19. *Mannan A., Shaheen N., Arshad W., Qureshi R. A., Muhammad Z., Bushra M.* Hairy roots induction and artemisinin analysis in *Artemisia dubia* and *Artemisia indica*. *Afr. J. Biotechnol*. 2008, 7 (18), 3288–3292.
20. *Sharafi A., Sohi H. H., Mirzaee H., Azadi P. In vitro* regeneration and *Agrobacterium* mediated genetic transformation of *Artemisia aucheri* Boiss. *Physiol. Mol. Biol. Plants*. 2014, 20 (4), 487–494.
21. *Sujatha G., Zdravkovic-Korac S., Calic D., Flamin G., Ranjitha Kumari B. D.* Highefficiency *Agrobacterium rhizogenes* mediated genetic Transformation in *Artemisia vulgaris*: Hairy root production and Essential oil analysis. *Industr. Crops Prod*. 2013, V. 44, P. 643–652.
22. *Nin S., Bennici G., Roselli D., Mariotti D., Schiff S., Magherini R. Agrobacterium* mediated transformation of *Artemisia absinthium* L. (wormwood) and production of secondary metabolites. *Plant Cell Rep*. 1997, 16 (10), 725–730.
23. *Murashige T., Skoog F.* A revised medium for rapid growth and bioassay with tobacco tissue culture. *Phys. Plant*. 1962, 15 (3), 473–497.
24. *Van-de-Velde W., Karimi M., Den-Herder G., Van-Montagu M., Holsters M., Goormachtig S. Agrobacterium rhizogenes*-mediated transformation of plants. *Genetic Transformation of Plants (Molecular Methods of Plant Analysis*). Jackson J. F., Linskens H. F. (Eds.). *Springer-Verlag Berlin*. 2003, P. 221–229.
25. *Drobot K. O., Shakhovsky A. M., Matvieieva N. A.* Construction of *Artemisia vulgaris* l. "hairy" root culture with human interferon alpha 2b gene. *Factors in Experimental*

Evolution of Organisms: Proceedings of the Tenth International Conference. *Chernivtsi*: *Yuriy Fedkovych Chernivtsi National University*, 2015.

26. *Schmulling T., Schell J., Spena A.* Single genes from *Agrobacterium rhizogenes* influence plant development. *The EMBO J*. 1988, 7 (9), 2621–2629.

ОТРИМАННЯ КУЛЬТУРИ «БОРОДАТИХ» КОРЕНІВ ЕСТРАГОНУ *Artemisia dracunculus* L.

К. О. Дробот
А. М. Шаховський
Н. А. Матвєєва

Інститут клітинної біології та генетичної інженерії НАН України, Київ

E-mail: katyadrobot@gmail.com

Метою роботи було розроблення біотехнології генетичної трансформації рослин естрагону *Artemisia dracunculus* L. Як вектор було використано *Agrobacterium rhizogenes* дикого штаму A4. Уперше одержано культуру «бородатих» коренів естрагону. Серед використаних експлантів (корені, стебло, листя та гіпокотилі 14-добових культивованих *in vitro* проростків естрагону) оптимальним типом виявилось листя. У разі його використання частота трансформації сягала 20%. Встановлено, що важливим біотехнологічним чинником, який впливає на частоту трансформації, є час кокультивування експлантів з агробактерією. Трансгенні лінії коренів відрізнялися за морфологією та швидкістю росту. Приріст маси «бородатих» коренів через три тижні культивування на безгормональному середовищі ½ Мурасіге-Скуга варіював від 17 до 32 разів.

Ключові слова: *Artemisia dracunculus*, генетична трансформація, *Agrobacterium rhizogenes* A4, культура «бородатих» коренів.

ПОЛУЧЕНИЕ КУЛЬТУРЫ «БОРОДАТЫХ» КОРНЕЙ ЭСТРАГОНА *Artemisia dracunculus* L.

Е. А. Дробот
А. М. Шаховский
Н. А. Матвеева

Институт клеточной биологии и генетической инженерии НАН Украины, Киев

E-mail: katyadrobot@gmail.com

Целью работы была разработка биотехнологии генетической трансформации растений эстрагона *Artemisia dracunculus* L. В качестве вектора был использован дикий штамм *Agrobacterium rhizogenes* A4. Впервые получена культура «бородатых» корней эстрагона. Среди использованных эксплантов (корни, стебли, листья и гипокотили 14-суточных *in vitro* культивируемых проростков эстрагона) оптимальным типом оказались листья. При их использовании частота трансформации достигала 20%. Установлено, что важным биотехнологическим фактором, влияющим на частоту трансформации, является время кокультивирования эксплантов с агробактериями. Трансгенные линии корней отличались по морфологии и скорости роста. Прирост массы «бородатых» корней через три недели культивирования на безгормональной среде ½ Мурасиге-Скуга варьировал от 17 до 32 раз.

Ключевые слова: *Artemisia dracunculus*, генетическая трансформация, *Agrobacterium rhizogenes* A4, культура «бородатых» корней.