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ORIGINAL ARTICLE

Efficient induction of hairy roots in *Tagetes patula* L.

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We carried out a genetic transformation of leaf explants of *Tagetes patula* L. using *Agrobacterium rhizogenes* strains (A-4b, 15834 and 8196RT). An effective way of genetic transformation of *T. patula* is the co-cultivation of sterile leaf explants with a suspension of soil agrobacteria, diluted in Murashige-Skoog medium. The duration of co-cultivation of the explants and the agrobacterial suspension, which ensured effective transformation, was 24 hours, followed by transfer to MS medium containing 20-25 g l^{-1} of sucrose. Hairy roots formed after 14 days of cultivation. The frequency of transformation was 85%. The wild *A. rhizogenes* strain 8196RT was the most productive for obtaining a stably growing culture of hairy roots.
Keywords: hairy roots; *Tagetes patula* L.; *Agrobacterium rhizogenes*; virulence; strain

Advances in biotechnology, in particular methods of cultivating plant cells, provide new opportunities for the industrial use of various plant species and the chemicals they produce. These new technologies expand and increase the usefulness of plants as renewable resources of valuable substances. Plant cell cultures are of considerable interest as a potential alternative to traditional agriculture for the industrial production of secondary metabolites (Balandrin et al., 1985; Dicosmo & Misawa, 1995; Mulabagal & Tsay, 2004). The main advantages of cell cultures include the synthesis of bioactive secondary metabolites in a controlled environment, regardless of climatic and soil conditions; elimination of negative biological effects that affect the production of secondary metabolites in nature (microorganisms and insects); the possibility of choosing varieties with higher production of secondary metabolites; automation of cell growth control and regulation of metabolic processes, which leads to lower costs and increased production (Mulabagal & Tsay, 2004).

Suspension and callus cultures, as well as the culture of hairy roots are the main methods of growing *in vitro* plant cells. The latter is of great interest as an alternative method of obtaining medicinal compounds from plants. Hairy roots are called a culture of genetically transformed roots. The transformation process is carried out with gram-negative soil bacteria *Agrobacterium rhizogenes*, which live in the rhizosphere of the roots. Under certain circumstances, agrobacteria containing Ri-plasmids have the unique ability to induce intense root formation in most dicotyledonous plants. Hairy roots have a number of properties that contribute to their widespread use in various fields. Their rapid growth, short doubling times, ease of maintenance, and the ability to stably synthesize various chemical compounds make them a suitable system for the production of secondary metabolites *in vitro* (Giri et al., 2001; Chandran & Potty, 2011). Currently, effective protocols have been developed for growing hairy roots for many medicinal plants. These cultures show relatively high productivity of secondary metabolites, which are potentially important pharmaceutical products. For example, the following substances were isolated from a hairy roots culture: anthraquinone, alizarin (*Rubia peregrina* L.) (Lodhi et al. 1996), c-anolides, aferin, withaferin-A (*Withania somnifera* L.) (Saravanakumar et al., 2012), cannabinoids (*Cannabis sativa* L.) (Farag & Kayser, 2015), rosemary acid (*Solenostemon scutellarioides*) (Saleh & Thuc, 2009) and others.

Clinical microbiologists have recently been actively screening medicinal plants for antimicrobial activity in order to search for phytochemicals as potentially new therapeutic agents. Therefore, we have chosen as an object of our study one of the representatives of the genus *Tagetes*, which is a source of flavonoids and terpenes exhibiting pharmacological and insecticidal properties (Jain et al., 2012; Gupta et al., 2016). It is known that various species of this genus produce secondary metabolites, which are used as components in the production of pharmaceuticals, pesticides and aromatic additives in the food industry. For example, the volatile oils of the Mexican marigold (*T. minuta* L.) show a high inhibitory activity against a number of human, animal and plant pathogens, as well as food spoilage agents. In addition, they are used in perfumery and as an aromatic ingredient in many products (Mohamed et al., 1999). There are many reports of the biological activity of thiophenes, highly unsaturated sulfur-containing heterocycles, which are contained in the marigold. These substances act against insects and nematodes and provide stable and long-term control compared to synthetic chemicals. Thiophenes also help prevent food grain spoilage (Rao et al., 2001). *T. patula* L. also contains biologically active substances that are used in

traditional medicine, as well as in agriculture as components of bioinsecticides, biofungicides and bionematicides. Lutein is found in French marigold flowers, which is an antioxidant with important pharmacological properties (Modi et al., 2009).
It is known that many factors influence the success of a hairy root culture. These are bacterial strains (Toruan-Mathius et al., 2004; Hu & Du, 2006; Ermayanti et al., 2009; Saleh & Thuc, 2009; Chandran & Potty, 2011), genotype of plant (Toruan-Mathius et. al., 2004; Ermayanti et al., 2009; Chandran & Potty, 2011), explant (Pawar & Maheswari, 2004; Toruan-Mathius et al., 2004; Ermayanti et al., 2009; Chandran & Potty, 2011) and cultural media (Toruan-Mathius et al., 2004; John et al., 2009; Chandran & Potty, 2011; Swain et al., 2012).
The purpose of this study was to develop an effective protocol for the induction of the hairy roots of *T. patula* by selecting a virulent *Agrobacterium rhizogenes* strain.

Materials and methods

Plant Material

Mature dry seeds of *T. patula* were washed by water and surface-sterilized with 2% (v/v) lysoformin-3000 solution for 15 min, then rinsed three times in sterilized water. Seeds were placed on 25-30 ml of agar-solidified Murashige and Skoog (MS) medium in 100 ml glass bottle. The seeds were germinated in a growth room at 24 ± 1 ºC with dark conditions for 3-5 days, and transferred to grow up under 16-h light / 8-h dark photoperiod.

Explant preparation

After 5 to 8 days of germination, the cotyledons are ready to be cut. Leaves were placed on sterile filter papers to use as explants for inoculating with *Agrobacterium rhizogenes* by co-cultivation method.

Bacterial strains

Three bacterial strains were used in this study, namely A-4b, 15834 and 8196RT (obtained from Russian Academy of Sciences, Timiryazev Institute of Plant Physiology). The bacteria were maintained on YMB solid media as required. Prior to infection, the bacterial strains were grown for 24 h on YMB solid medium at 28 °C in the dark.

Induction of hairy roots

The explants were immersed in prepared bacterial suspension and swirled for 24 h at 28 °C on a rotary shaker at 100 rpm. One day later, the infected explants were rinsed with sterile distilled water and liquid medium MS. Then explants were blotted on filter paper to remove excess of *Agrobacterium* culture and placed on hormone-free MS basal media containing cefotaxime (500 mg l^{-1}) to further eliminate bacterial growth. Stepwise elimination of *Agrobacterium rhizogenes* was performed gradually by subculturing the infected explants with reduced concentration of cefotaxime (250, 125 and 60 mg l^{-1}).

Statistical analysis

In each case, 12-15 explants were used, and the experiment was performed in triplicate, and all data were processed using analysis of variance. The reliability of the differences among the mean values was tested using the Least Significant Differences ($LSD_{0,05}$). The results were statistically processed using the Microsoft Office Excel 2010 application software.

Results and Discussion

One of the key factors influencing the formation of a hairy roots culture in a laboratory experiment is the selection of the "suitable" strain of soil agrobacteria. The use of modified objects bearing alien genetic structure for roots transformation might be problematic in case that practical application of the received culture is planned. For example, if the purpose of an experiment is to create medicinal plant hairy roots which can be used as alternative raw materials in the medical and food industry, there is need to involve only wild strains of agrobacteria (Kuzovkina & Vdovitchenko, 2011). Identification of the virulence of various *A. rhizogenes* strains for receiving genetically transformed roots of the French marigold included joint short-term suspension cultivation of leaf explants and the relevant agrobacteria within 24 hours and further cultivation on the agarized MS growth medium, supplemented with 25 g l^{-1} sucrose and 500 mg l^{-1} an antibiotic.
Three strains of *A. rhizogenes* were assessed for virulence: 8196RT, 15834, and A-4b, the first two are wild, and the last one is genetically modified. All strains were obtained from the collection of the Root Metabolism Laboratory of the Institute of Plant Physiology, Russian Academy of Sciences. We evaluated different morphological traits including color changes, plagiotropic growth, abundant lateral root formation. The first signs of genetically modified roots were observed at the cut surface of infected explants after 3 to 4 days of cultivation. The success of the transformation on the 7th day of cultivation varied from 63.3 to 79.4%, depending on the strain. The modified strain A-4b showed the maximum virulence for this type of explant. When explants were infected with wild strains 5834 and 8196 RT, the frequency of rhizogenesis significantly decreased, reaching 67.8 and 63.3%, respectively (Fig. 1A). An assessment of the intensity of root formation on leaf explants also showed the advantage of the modified strain compared to wild forms. The average number of roots per an explant reliably exceeded the results of inoculation of the object with strains 15834 and 8196RT (Fig. 1B).
The frequency of rhizogenesis on the 14th day after inoculation was significantly increased with the use of 8196RT and 15834 strains compared with a seven-day culture (Fig. 1A). While the use of strain A-4в allowed us to get good results on the 7th day. Transformation success ranged from 81.7 to 84.9%. Thus, on 14th day, we did not observe significant differences between the efficiency of the studied strains. Growing roots branched well, forming a characteristic rhizogenic composition. The number of roots per explant was minimal when infected with strain 8196RT (Fig. 1B).

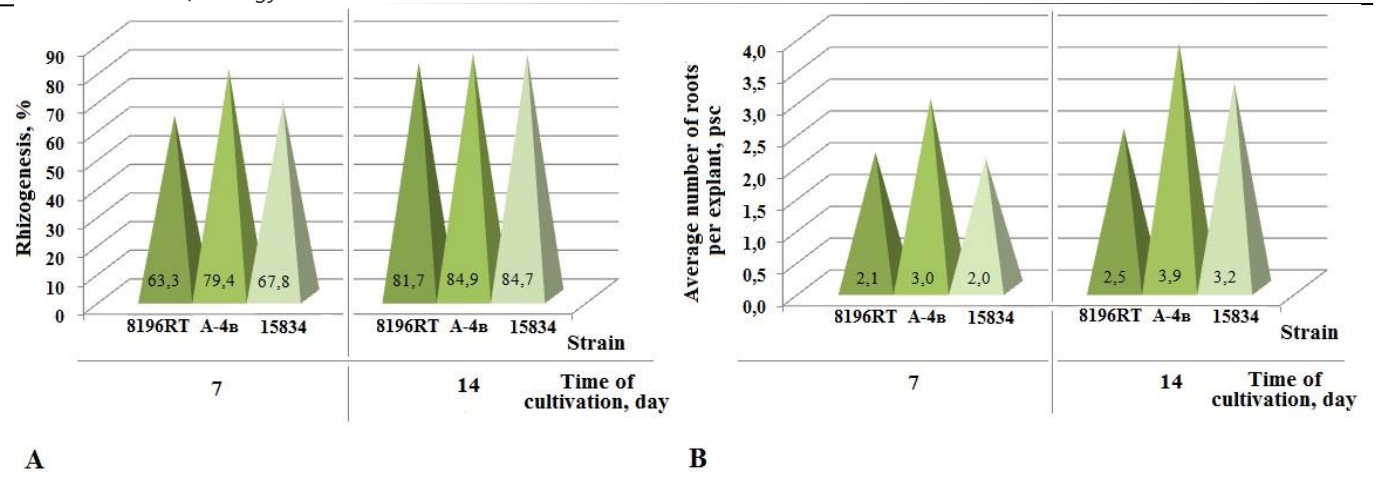

Figure 1. Genetic transformation of leaf explants of *Tagetes patula* L. with different strains of *Agrobacterium rhizogenes*: A – frequency of transformation ($LSD_{0,05}$ = 4,4); B – root formation intensity ($LSD_{0,05}$ = 0,8)

The dynamics of root growth depending on the number of passages confirms an inhibiting effect of the antibiotic on the intensity of development of genetically transformed roots, though it differs while using various strains of agrobacteria. So concentration of Klaforan with each passage decreased twice: the 1st passage – 500 mg l^{-1}, the 2nd passage – 250 mg l^{-1}, the 3rd passage – 125 mg l^{-1}, the 4th passage – 60 mg l^{-1}. This, in turn, led to an increase in the root growth intensity that was expressed in an increase in their length (Fig. 2).

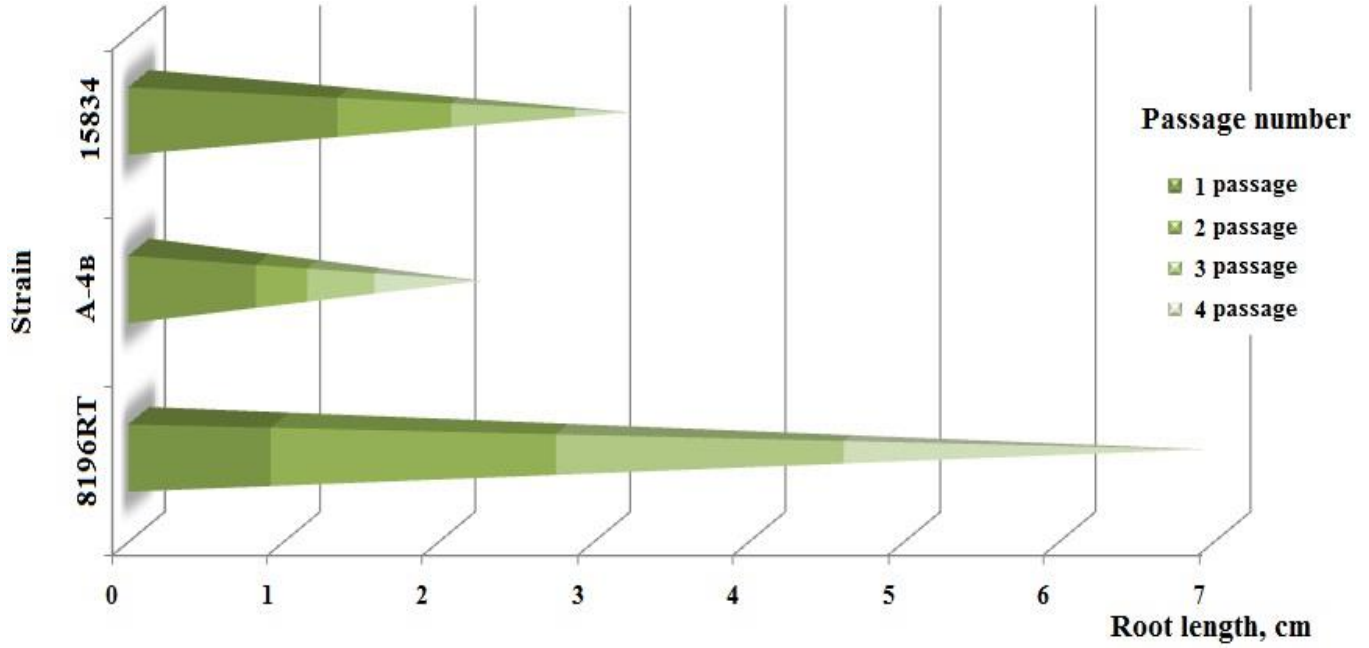

Figure 2. Length of the roots which were formed on leaf explants of *Tagetes patula* L. by genetic transformation with various strains of *Agrobacterium rhizogenes* ($LSD_{0,05}$ = 0,9)

The maximum length was found in the fourth passage when infected with a strain 8196RT. It reached more then 7cm. The weakest reaction was observed when explants were inoculatated with strain A-4b. The root length was less than 2 cm. Thus, comparative assessment of virulence of various *A. rhizogenes* strains at infection of French marigold leaf explants showed that the most effective way to receive genetically transformed roots is to use the wild strain 8196RT. The success of transformation when infected with this strain was not inferior to the virulence of strains 15834 and A4в. However, the culture was characterized by dynamic growth, which allowed us to quickly obtain a large biomass of roots. In addition, this strain, unlike A4b, is wild one, therefore it is more suitable for the production of raw materials for pharmaceuticals.

Conclusion

n effective way of genetic transformation of *T. patula* is the co-cultivation of sterile leaf explants with a suspension of *Agrobacterium rhizogenes*, diluted in Murashige-Skoog medium. The duration of co-cultivation of the explants and the agrobacterial suspension, which ensured effective transformation, was 24 hours, followed by transfer to MS medium containing 20-25 g l^{-1} of sucrose. The wild *A. rhizogenes* strain 8196RT was the most productive for obtaining a stably growing culture of hairy roots. As a result of the genetic transformation of *T. patula* leaf explants, we obtained stable cultures of hairy roots.

Conflict of interest

The authors declare that they have no conflict of interests.

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