

RESEARCH ARTICLE

Optimization of conditions for the induction of *Tagetes patula* L. hairy roots

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The *hairy root* cultures are promising sources of secondary metabolites of plants, including rare and endangered species. They possess genetic and biochemical stability, unlimited growth rate in free-hormone medium, short doubling times, high biosynthetic activity and ecological purity of plant raw materials. The *hairy root* cultures of *Tagetes patula* L. can be used to produce biologically active substances with biocidal activity. The study aimed to determine the virulent strain of *Agrobacterium rhizogenes* and the most effective period of co-cultivation of *T. patula* leaf explants with an agrobacterium to induce actively growing *hairy root* cultures. We used 3 strains (A-4b, 8196RT and 15834). The time of infection ranged from 3 to 33 hours in increments of 3 hours. We found that 24 h is the best time of infection to induce *hairy roots* with the highest transformation efficiency (92%). The wild strain *A. rhizogenes* 15834 turned out to be the most virulent when infected leaf explants of spreading marigold. This strain provided the maximum transformation effect, reaching 85.4%. We have identified 5 actively growing clones of *hairy roots* with intensive branching, the growth indices of which were 64-75. In the future, they will be transferred to a liquid medium for biomass accumulation and scaling.

Keywords: *Tagetes patula*; Leaf explants; *Agrobacterium rhizogenes*; Hairy root culture

Introduction

One of the most promising areas of modern phytobiotechnology is the use of cell cultures of higher plants to obtain biologically active substances for medicine, veterinary medicine, perfumery, agriculture, food and chemical industries (Dicosmo and Misawa, 1995; Ramachandra Rao and Ravishankar, 2002; Mulabagal and Tsay, 2004; Malik et al., 2011; Orlova et al., 2014; Isah et al., 2018; Kochkin et al., 2019; Watcharatanon et al., 2019). The validity of this approach is determined, on the one hand, by the ever-growing demand for natural compounds that are unique in structure and activity, and on the other hand, by the extremely low level of natural resources of producers, many of which are rare and endangered plant species (Verpoorte, 2002; Nosov, 2012). In addition, the relative simplicity in the extraction of the metabolites from *in vitro*-produced tissues makes the method suitable for commercial application (Kolewe et al., 2008; Gonçalves and Romano, 2018). One of the sources of secondary metabolites is the *hairy roots* culture, the growth of which is initiated by the transfer of the *Rol* gene of the soil bacterium *Agrobacterium rhizogenes* to the plant cell genome (Bensaddek et al., 2008; Ono and Tian, 2011; Vladimirov et al., 2015; Dhiman et al., 2018). The root culture obtained in this way has genetic and biochemical stability, unlimited growth rate in growth regulator-free media, short doubling times, high biosynthetic activity and responsiveness to elicitor treatments as well as ecological purity of the plant raw materials (Vdovichenko et al., 2007; Kuzovkina and Vdovichenko, 2011; Khan et al., 2018; Halder et al., 2019). Over the past decades, there was a lot of data on the development of effective protocols for inducing *hairy root* cultures that can synthesize biologically active substances (Giri et al., 2001; Saleh and Thuc, 2009; Saravanakumar et al., 2012; Farag and Kayser, 2015; Belabbassi et al., 2016; Patra and Srivastava, 2016).

The genus *Tagetes* belongs to the family Asteraceae and is used as an ornamental plant (Marotti et al., 2004). However, the chemicals that make up the plants determine the usefulness of representatives of this genus (marigolds) as sources of various classes of secondary metabolites that are used in the pharmaceutical and food industries (Giri et al., 2011; Chkhikvishvili et al., 2016). In addition, these substances can also be used as the main component of agents with biocidal activity (Mares et al., 2004; Mulabagal and Tsay, 2004; Faizi et al., 2011; Politi et al., 2016; Ayub et al., 2017; Krzyzaniak et al., 2017; Mir et al., 2019). For example, marigolds are known to be a source of thiophenes, which are a group of heterocyclic sulfur compounds, the most common of which is α -Tertieryl (Ketel, 1986). Thiophenes have allelopathic, insecticidal, nematocidal, antifungal, bactericidal and cytotoxic effects (Talov et al., 1994; Rajasekaran et al., 1999, 2004; Taha et al., 2013). The bioactive substances of natural origin are known to have some advantages, since they are obtained from renewable resources, and the selection of resistant forms occurs at a slower rate than with synthetic insecticides. Also, they show low or no toxicity to mammals and bees (Ghosh et al., 2012). It was revealed that the remains of the marigold plants prevented the germination of the seeds of some weeds, thereby determining the potential of thiophenes as natural weed control agents (Batish et al., 2007). Moreover, it was found that the number of nematodes in the soil decreased when some crops were grown using root extracts of plants of the genus *Tagetes* (Siddiqui et al., 1987). When studying the effect of the hexane extract of tricyclic thiophenes produced from genetically transformed roots of *T. patula* L., a high larvicidal activity on mosquito larvae of several species was noted (Rajasekaran et al., 2004). In addition, α -Tertieryl has a short half-life in the environment, which is 4 hours, not showing cross-resistance to other pesticides due to the new mode of action (Hasspieler et al., 1988). A culture of genetically transformed marigold roots can be an alternative source of thiophene-rich raw materials (Kyo et al., 1990; Buitelaar et al., 1991; Rajasekaran et al., 1999, 2004; Ramachandra Rao et al., 2001). A comparative analysis in different

phases of the growth of *hairy root* culture showed that the total thiophene content in most *hairy root* lines was almost 4 times higher than in wild-type roots (Gupta et al., 2016). The aim of this work was the induction in *in vitro* of rapidly growing lines of *hairy root* cultures of *T. patula* for their further use in the production of biologically active substances with biocidal activity.

Materials and Methods

To obtain aseptic plants, *T. patula* seeds were thoroughly washed in a soap solution and placed under running water for 15 minutes. Sterilization was carried out with 70% ethyl alcohol for 2 minutes, followed by disinfection in lysiformine-3000 for 15 minutes. After washing 3-4 times with sterile distilled water, the seeds were transferred into glass flasks on the agarized Murashige and Skoog (MS) base medium (Murashige & Skoog, 1962) with a sucrose concentration of 30 g l⁻¹, supplemented with 4 μM gibberellic acid. The seedlings were grown in a ICH 750L Memmert climatic chamber at a temperature of 24 ± 0.3 °C and under 16-h light/8-h dark photoperiod. The collection of *A. rhizogenes* strains was maintained on an agarized YEB medium at a temperature of +4 ± 0.3 °C, followed by transfer to fresh medium once every 3 weeks. For the genetic transformation of spreading marigold explants, we used the protocol that we developed earlier (Bychkova et al., 2018). First of all, fresh cultures of agrobacteria of 3 strains were prepared. These were A-4b, 8196RT and 15834 (from the collection of K.A. Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences, Moscow). Bacteria were cultured on YEB medium for 24 hours at a temperature of 28 ± 1 °C in the dark. Flushing of the bacterial culture was used for its joint cultivation with explants.

After the seedlings reached 10 days of age, we carried out an agrobacterial transformation by co-cultivating an overnight culture of agrobacteria and *T. patula* leaf explants damaged by a sterile needle. Flasks with plant explants and a suspension of agrobacteria were placed on a rotary shaker with a speed of 90 rpm at 26 ± 1 °C. Time intervals for co-cultivation of leaf explants and agrobacteria were evaluated by observing 10 periods. The time of contacting bacteria with explants ranged from 3 to 33 hours in increments of 3 hours. In this experiment, agrobacterial transformation was carried out by strain 15834. Further cultivation of transformed explants was done as follows. The suspension was filtered, the explants were washed with liquid MS medium and transferred to Petri dishes on a semi-solid hormone-free MS base medium with a sucrose concentration of 25 g l⁻¹. To exclude further bacterial growth, the antibiotic Klaforan (500 mg l⁻¹) was added to the nutrient medium, the concentration of which subsequently decreased with each passage to 250, 125, and 60 mg l⁻¹ (Mikhailova et al., 2017; Bychkova et al., 2018). Then we carried out the transformation of the explants with the three strains indicated above. The period of joint cultivation of leaf segments and bacteria in a liquid medium was 24 hours.

The root induction frequency was calculated by the ratio of the number of initiated root lines to the number of explants passaged onto the semi-solid medium (%). To assess the growth of root culture, the length of primary roots, as well as the number of secondary and tertiary roots, were determined. The growth activity of the cultures was evaluated by the growth index (GI), which was calculated by the difference in the length of the roots at the end and beginning of the passage using the formula:

$$GI = l_1 - l_0/l_0,$$

where l_0 is the initial root length; l_1 is the final length of the roots.

The experiment was performed in 5 replicates, 10 explants per replicate. The Least Significant Differences (LSD_{0.05}) were used to assess the reliability of the differences among the mean values.

Results and Discussion

Artificial induction of *hairy roots* in plants is technically relatively uncomplicated. Despite this, not all plants can quickly be infected with *A. rhizogenes*. There are so-called "difficult" species for which it is necessary to resort to certain "tricks", including selection of the type and age of the plant tissue, the presence or absence of explant wounding, the density of the bacterial suspension, strain genotype, infection technique, the duration of the period of co-cultivation of the explants and bacteria (Park and Facchini, 2000; Bensaddek et al., 2008; Saleh and Thuc, 2009; Sharada et al., 2015). The explants most commonly used for infection are young tissues of sterile seedlings. We infected aseptic young leaves of 10 day old seedlings, having previously injured them with a sterile needle. The duration of the plant – bacterium contact during the inoculation and the co-cultivation is known to be parameter that can be optimized. According to various sources, the duration of co-cultivation can range from several hours to several days (Vdovichenko et al., 2007; Wahyuni et al., 2017). We evaluated the effects of 10 periods of incubation of explants in suspension of strain 15834 on induction of a primary *hairy root* culture. The effectiveness of agrobacterial transformation of marigold leaf explants depending on the time of their co-cultivation with strain 15834 is shown in Figure 1.

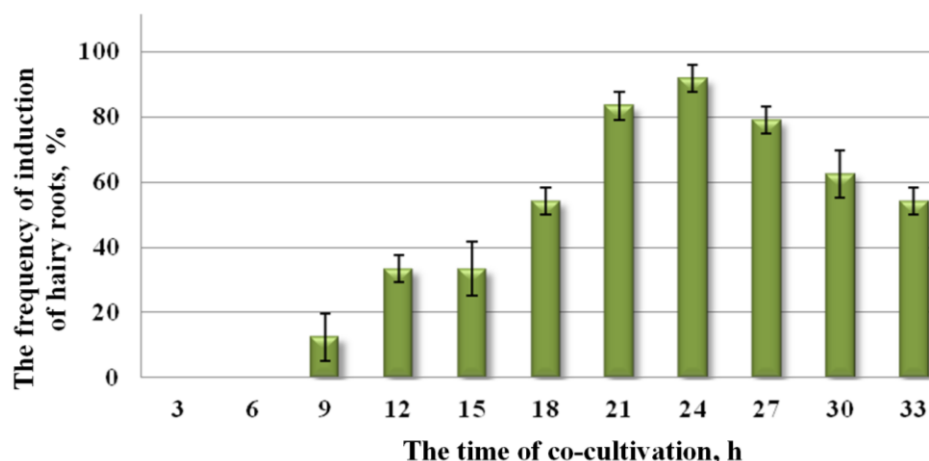


Figure 1. Frequency of induction of *hairy roots* depending on the time of co-cultivation of leaf explants of *Tagetes patula* L. with *Agrobacterium rhizogenes* strain 15834 (LSD_{0.05} = 6.2).

After the transformation and transfer of leaf explants into Petri dishes to the semi-solid medium MS, primary roots appeared after 4-5 days of incubation. The proportion of explants that form roots ranged from 0 to $91.7 \pm 4.2\%$ depending on the time of their joint cultivation with agrobacteria. A single root formation was observed after co-cultivation for 9 hours, which was about $12.5 \pm 7.2\%$. The maximum induction of hairy roots was observed at 21 and 24 hours of co-cultivation, reaching 83.3 ± 4.1 and $91.7 \pm 4.2\%$, respectively. A longer incubation of leaf explants with the agrobacterial strain did not lead to an increase of rhizogenesis, but, on the contrary, a decrease in the trait was observed. At 27-30 hours, the level of rhizogenesis was 79-62.5%. After explants were incubated in a bacterial suspension for 18 hours, their further cultivation on a semi-solid medium led to the formation of callus in 12% of cases. According to Kuzovkina and Vdovichenko (2011), callus formation quite often precedes root formation on explants. Thus, the co-cultivation of leaf explants of spreading marigold with a bacterial suspension of strain 15834 provided the maximum effect by contacting for 24 hours.

Infection of explants with a more virulent strain can significantly increase the efficiency of transformation. Therefore, for more successful infection, it is necessary to use different strains of soil agrobacteria (Giri et al., 2001; Saleh and Thuc, 2009; Lee et al., 2010). Also, if the goal of the experiment is to obtain a hairy root culture as an alternative raw material for medicine or the food industry, then it is necessary to use only wild strains. In this case, the further applying the cultures obtained with the participation of genetically modified strains can be problematic (Kuzovkina and Vdovichenko, 2011). Having determined the optimal incubation time, we carried out the transformation of explants with three strains of agrobacteria (A-4b, 8196RT and 15834). The frequency of transformation varied significantly depending on a strain genotype (Figure 2).

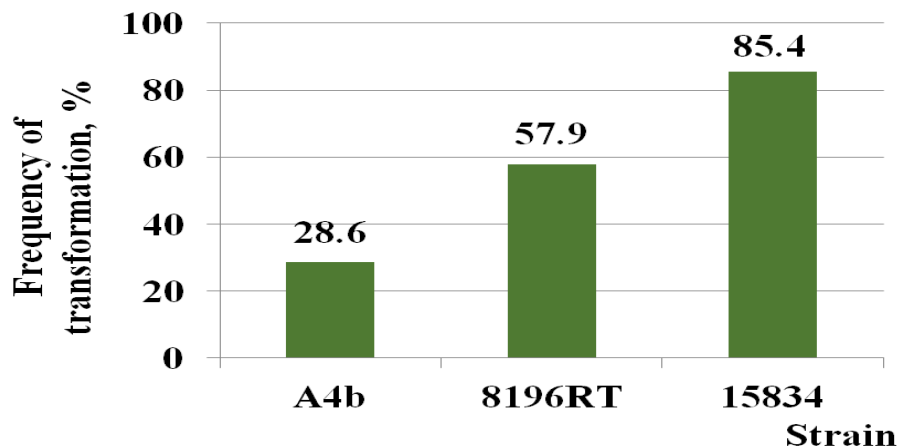


Figure 2. *In vitro* transformation of leaf explants of *Tagetes patula* L. by various strains of *Agrobacterium rhizogenes* ($LSD_{0,05} = 7.8$).

Maximum success was observed using the wild strain 15834 ($85.4 \pm 6.1\%$). The modified A4b strain appeared to be less aggressive, the effectiveness of which was 3 times lower. Strain 8196RT showed an intermediate virulence, providing a level of transformation in the range of $57.9 \pm 5.6\%$. It should be noted the different nature of the effects of strains on *T. patula* leaf explants. So 15834 caused mainly multiple rhizogenesis, and A4b, on the contrary, provided single rhizogenesis. A specific feature of the action of strain 8196RT was the induction of callusogenic processes which inhibited the formation of root cultures (Figure 3).

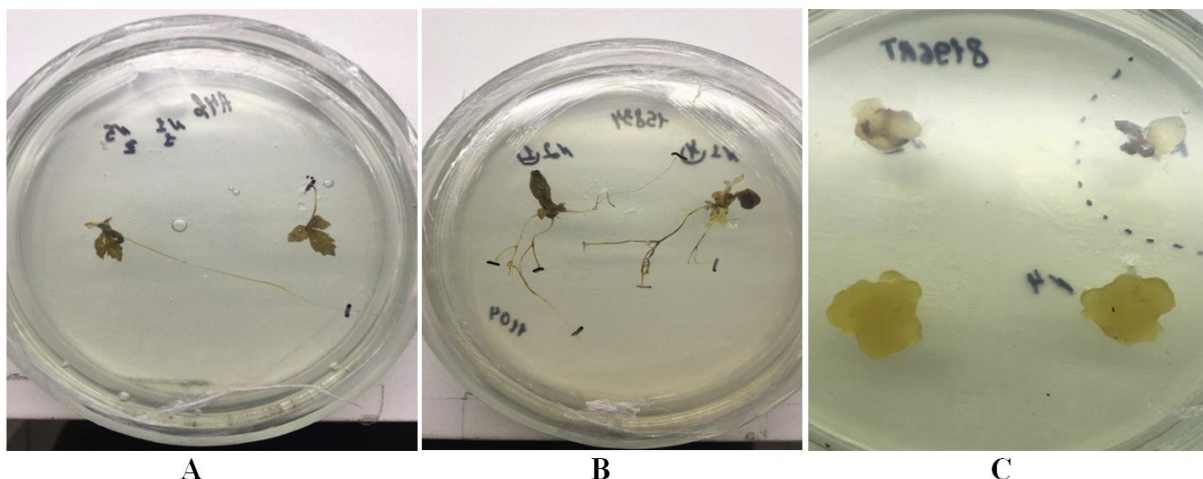


Figure 3. Different types of induction processes during transformation of *Tagetes patula* L. explants by various strains of *Agrobacterium rhizogenes*: A – Single root induction (A4b); B – Induction of several roots (15834); C – Callus induction (8196RT).

It is known that roots obtained *in vitro* culture have certain morphological characters, the assessment of which suggests that they are most likely genetically transformed (Kuzovkina & Vdovichenko, 2011; Kuluev et al., 2015). The *hairy roots* induced in our study grew well on hormone-free media, branching intensively, and had plagiotropic growth. When assessing the growth activity of individual root lines, we found that they significantly differed from each other both in the growth rate and in the type of branching. After 3 weeks, some of them formed a composition characteristic of genetically transformed roots, while other lines slowed down

growth or the growth rate was not significant (Figure 4). In total, more than 50 lines were isolated; the characteristics of the best of them are presented in Table 1.

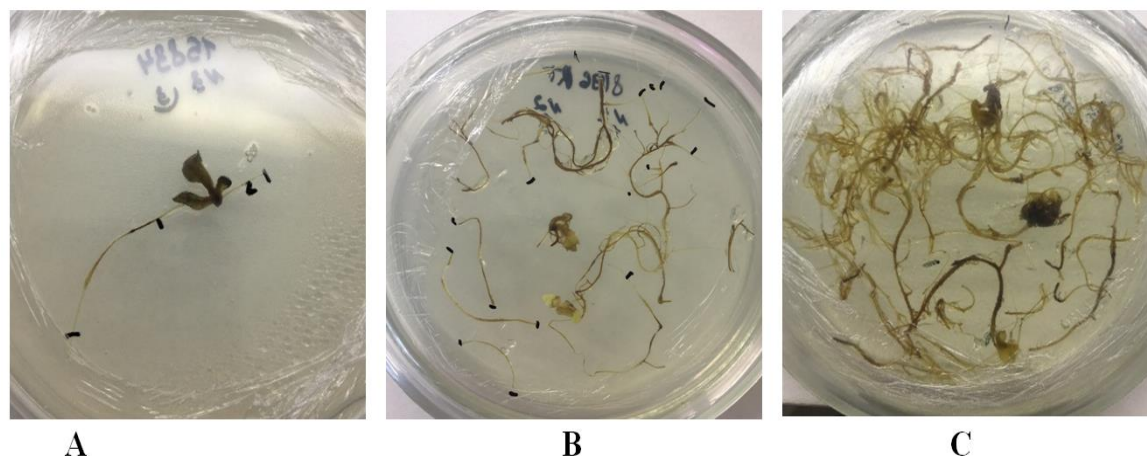


Figure 4. Various models of growth and branching of *Tagetes patula* L. genetically transformed roots :
A – Slowly growing, non-branching roots; B – Weakly branching roots; C – Fast-growing, well-branching roots.

The main indicator of the effectiveness of an agrobacterial strain is the growth rate of the formed roots, characterized by a growth index. The maximum values were shown by clones initiated by strain 15834, whose growth indices reached 75-fold values. The root growth rate induced by strains 8196RT and A4b was inferior to the lines described above. Assessments of growth of root cultures also included the length of the primary roots, as well as the number of secondary and tertiary roots. The greatest length of the primary roots was observed in the lines obtained as a result of transformation by strain 15834. The same lines were distinguished by intensive branching, as evidenced by the number of lateral roots of the second and third orders, which form the main increase in root biomass. The root cultures obtained by transformation with strain A-4b had the lowest values of the length of primary roots and a relatively small number of secondary and tertiary roots.

Table 1. Growth characteristics of *Tagetes patula* L. hairy root lines.

Hairy root line	Growth index	Length of primary roots, cm	Number of secondary roots, pcs.	Number of tertiary roots, pcs.
1-15834	75	15.2	28	> 50
2-15834	69	13.6	26	> 50
3-15834	68	13.2	19	> 40
4-8196RT	63	11.3	12	> 40
5-8196RT	64	11.0	11	32
6-8196RT	47	9.6	11	26
7-A4b	54	12.5	9	19
8-A4b	39	9.1	9	21
9-A4b	42	9.1	12	24

Conclusion

The co-cultivation of leaf explants of spreading marigold (*T. patula*) with a bacterial suspension of strain 15834 provided the maximum effect by contacting for 24 hours. The wild strain *A. rhizogenes* 15834 turned out to be the most virulent when infected marigold leaf explants. This strain provided the maximum transformation effect, reaching 85.4%. We have identified 5 actively growing clones with intensive branching, the growth indices of which were 64-75. In the future, they will be transferred to a liquid medium for biomass accumulation and scaling.

Conflict of Interest

The authors declare that they have no conflict of interests.

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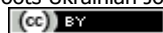
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