IMAM KHOMEINI INTERNATIONAL UNIVERSITY
IJGPB
Iranian Journal of Genetics and Plant Breeding
Print ISSN : 2251-9610
Online ISSN : 2676-346X

Effects of explant type and *Agrobacterium rhizogenes* strains on hairy root induction and alizarin production in madder (*Rubia tinctorum* Ardakan)

Ebrahim Dorani[1]*, Ommolbanin Honarmand[1], Mostafa Valizadeh[1]

[1]Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.
**Corresponding author, 0000-0002-4229-6984. Email: dorani@tabrizu.ac.ir.*

ABSTRACT INFO

Research Paper

Received: 06 Nov 2023

Accepted: 20 Dec 2023

ABSTRACT

Hairy root culture of plants is a considerable way for the *in vitro* production of valuable metabolites because of genetic stability, rapid growth rate, biochemical stability, and high capacity in the synthesis of secondary metabolites. Alizarin is an anthraquinone derived from the roots of madder (*Rubia tinctorum*) and has been used since ancient times as a natural red dye and exhibits various pharmacological and biological activities including anticancer, antioxidant, and anti-microbial activity. The influence of two factors including explant (leaf, internode, cotyledon) and strains of *A. rhizogenes* (15834, 2656, MSU, R1000) was tested on hairy root production of madder. All explants produced hairy roots with acceptable frequencies but leaf explants produced the highest number of roots per explant followed by cotyledons. The highest root induction rate (100%) and the highest number of hairy roots per explant were obtained from leaf explants inoculated with 15834 and R 1000. Analysis of the PCR products showed the presence of a 403 bp amplicon related to the specific reproduction of *rolA* gene in transgenic roots. Anthraquinone production was documented in transgenic roots but there was a significant difference between roots from different bacterial strains. As a brief result, the use of suitable bacterial strains and explants were effective factors for hairy root induction in madder.

***Key words*: *Alizarin*, Anthraquinone, Madder, Secondary metabolite.**

How to cite this article:
Dorani E., Honarmand O., and Valizadeh M. (2023). Effects of explant type and *Agrobacterium rhizogenes* strains on hairy root induction and alizarin production in madder (*Rubia tinctorum* Ardakan). *Iranian Journal of Genetics and Plant Breeding,* 12(2): 1-9.
DOI: 10.30479/IJGPB.2023.19535.1361

INTRODUCTION

Plant-derived drugs represent a significant proportion of the pharmaceutical market. Plants are major sources of valuable secondary metabolites used in the pharmaceutical, cosmetic, perfumery, and food industries because of their antioxidant, antimicrobial, and biological activities and their color, flavor, and fragrance properties (Karuppusamy, 2009). Plants are a major source of pharmaceuticals and fine chemicals that are used in the food industry as biologically active additives, medicines, as well as flavorings, and food colorings.

Rubia tinctorum L, commonly known as wild madder, is a member of the Rubiaceae family. It is an herbaceous perennial plant. Methanol extract of *Rubia tinctorum* is effective in reducing body weight, improving the lipid profile, and normalizing hyperglycemia, insulin resistance, and hyperinsulinemia. (Eltamany *et al.,* 2020). This plant is the main source of several anthraquinone (AQ) derivatives such as 1-hydroxyanthraquinone in its roots which, are important compounds in the pharmaceutical industry for the treatment of kidney and bladder stones, as a laxative mixture and mild sedative (IARC, 2002; Siva *et al.,* 2011). Alizarin, a hydroxyl derivate of anthraquinone (*i.e*., 1,2-*dihydroxyanthraquinone*), derived from the roots of *Rubia tinctorum* L that has been reported to exhibit various pharmacological and biological activities including anticancer, antimalarial, antimicrobial, antifungal and antioxidant activities (Lee *et al.,* 2010).

Plant *in vitro* culture has many advantages in producing secondary metabolites including controlled culture conditions; unaffected from geographic, seasonal, or weather conditions, and safer extracts because it avoids the use of land and has a low environmental impact (Perassolo *et al.,* 2020).

The hairy root system based on *A. rhizogenes* has become popular as a reliable method of producing secondary metabolites synthesized in plant roots (Palazon *et al.*, 1997). Their fast growth, short doubling time, ease of maintenance, and ability to synthesize a range of chemical compounds and proteins offer their advantages in comparison with plant cell suspension cultures as a continuous source for the production of valuable secondary metabolites and foreign proteins. Hairy root cultures are usually able to produce the same compounds found in wild-type roots of the mother plant (Veena and Taylor, 2007).

Many studies have reported the efficiency of different strains *of A. rhizogenes* in root induction and biomass production of hairy roots from explants and secondary metabolite biosynthesis in hairy root cultures (Gutierrez *et al*., 2020; Sathasivam *et al*., 2022; Zheleznichenko *et al*., 2023). Thwe *et al.* (2016) reported that different *A. rhizogenes* strains induce different numbers and types of hairy roots and secondary metabolites. The selection of an effective Agrobacterium strain for induction of hairy root significantly depends on the plant species and genotype. The differences in virulence, morphology, and growth rate are at least partially related to the variety of Ri (root-inducing)-plasmids within each bacterial strain. No studies have been reported for the induction of hairy roots in Iranian cultivars of *R. thincturom* (Ardakan). Therefore, our study was designed to develop hairy root culture from *R. thincturom* Ardakan using different strains of *A. rhizogenes* for the production of anthraquinones, and alizarin.

MATERIALS AND METHODS

Plant material

Seeds of *R. tinctorum* Ardakan were obtained from the Yazd Agricultural Research Center. Seeds were surface-sterilized with 70% (v/v) ethanol for 1 min and 5%(v/v) sodium hypochlorite solution for 20 min, then rinsed three times with sterilized water. Nine seeds were placed in Petri dishes (100×15 mm) containing agar-solidified culture medium. The surface-sterilized seeds were cultured on the basal medium MS with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 before adding agar and then sterilized by autoclaving at 121 °C for 20 min. The seeds were germinated in a growth chamber at 25±2 °C, under a 16 h light/8 h dark photoperiod.

Agrobacterium rhizogenes and growth conditions

Selected strains of *A. rhizogenes* including 15834, R1000, 2656, and MSU were retrieved from glycerol stocks and grown overnight at 28 °C with shaking (120 rpm) in liquid Luria-Bertani medium (LB: Duchefa, Nederland), to mid-log phase (OD_{600}=0.6-0.8). *A. rhizogenes* cells were pelleted by centrifugation for 10 min (4000 rpm) and resuspended in a liquid inoculation medium (liquid MS medium). The *A. rhizogenes* cell density was adjusted to give an A_{600} of 1.0 for inoculation.

Induction and production of hairy Root

Three types of explants were used including leaf, internode, and cotyledons of in vitro grown seedlings. Explants were cut into 1 cm pieces and inoculated with bacterial suspension for 5 min then were dried

on a sterile filter paper and placed on MS medium without hormones and antibiotics for co-cultivation and incubated at 25±2 °C under 16 h light/8 h dark photoperiod for 48 hours. After 48 hours of co-cultivation, the explant tissues were transferred to a hormone-free medium containing MS salts and vitamins, 30 g/l sucrose, 500 mg/l cefotaxime, and 8 g/l agar. The experiment was conducted as a factorial experiment with a completely randomized design (CRD) in three replications. Hairy roots were formed from the injured sites within two weeks. The number of hairy roots producing explants and the number of hairy roots per explant were calculated for all treatments within three weeks. The hairy roots were detached from the explant (0.4 g D.W/l) after five weeks and transferred to an MS liquid medium, containing 30 g/l sucrose, in 250 ml flasks. Root cultures were maintained at 25±2 °C on a shaker (65 rev/min) in a growth chamber under cool white fluorescent tubes and 16 h light/8 h dark photoperiod.

After three weeks of culture (Figure 1), hairy roots were harvested and dried at 65 °C temperature for at least 28 hours. Samples were ground into a fine powder by a mortar and pestle. Samples (0.1 g) were extracted two times with 10 ml extraction solvent (5 ml ethanol (80%)+5 ml HCL (3 N)) at 70 °C in a water bath for 30 min. Finally, the supernatant was collected by centrifugation at 4000 rpm and then concentrated under reduced pressure and the residue was dissolved in 1.0 mL extraction solvent. The quantitative analysis of alizarin (the main anthraquinone of Madder) in the hairy roots was done by Bio-Rad UV/V spectrophotometers. The absorbance of alizarin was performed at 517 nm, and their content was calculated by comparing it with the calibration curves obtained with standard alizarin from (MERK).

Statistical analysis

Data were analyzed with SPSS Software and mean comparisons were done by LSD test at 0.01 significant level.

Molecular analysis of transgenic roots

After the development of hairy roots, molecular confirmation of transgenic roots was done by PCR. To ensure the complete removal of bacteria from roots, the roots were subcultured in the antibiotic-containing medium for some days. DNA extraction was performed by the method of Cai *et al*. (1997). The PCR analysis of transgenic roots was done using the *rol*A gene-specific primers (F: 5′-ACGGTGAGTGTG GTTGTAGG-3′, and R: 5′- GCCACGTGCGTATTAATCCC-3′) to amplify the 430 bp fragment. The PCR was started

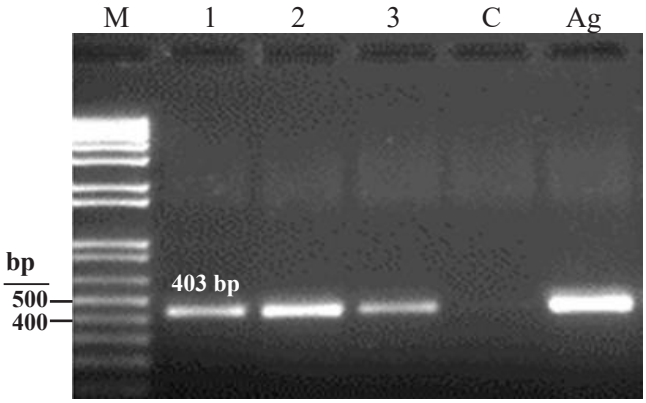

Figure 1. PCR analysis for hairy roots produced using primers *rolA*; M. DNA Marker with 1 kb size; 1–3.Positive Hairy roots induced by *Agrobacterium rhizogenes* (containing vectors); C. control root (containing no vectors) as negative control; Ag. Sample plasmid from *Agrobacterium rhizogenes* as *the* positive control.

in initial denaturation at 94 °C for 5 min, and then 30 cycles were done at the denaturation for 30 s at 94 °C, annealing for 50 s at 58 °C, extension for 30 s at 72 °C, and finally, extension remained for 5 min at 72 °C.

RESULTS

In vitro establishment of *R. tinctorum* Ardakan cultures was carried out successfully with 58% matured seed germinated in MS medium supplemented with B5 vitamin without any plant growth regulator. Hairy roots have been produced from the wounded young, fully expended leaves, cotyledons, and internode explants (derived from 2-week-old seedlings). Results of PCR analysis with specific primers for *rolA* gene confirmed the presence of T-DNA in transgenic hairy roots (samples were selected randomly from hairy roots). The 403 bp fragment amplified in all hairy root clones (Figure 1).

Statistical analysis showed a significant difference in the percentage of root induction and the average number of hairy roots produced for all strains of *A. rhizogenes* at 99% confidence level. There was no significant difference between the 15834 and R1000 strains in transformation efficiency (Figures 2 and 3). This indicated that these two strains were equally effective at inducing hairy roots in the leaf and cotyledon explants of *R. tinctorum* Ardakan. The hairy roots were generated from the leaf explants infected with 15834 and R1000 strains after 7 days of co-cultivation. Induction of hairy roots by MSU and 2656 occurred with delay after 10-12 days from co-cultivation. The hairy roots from the internode

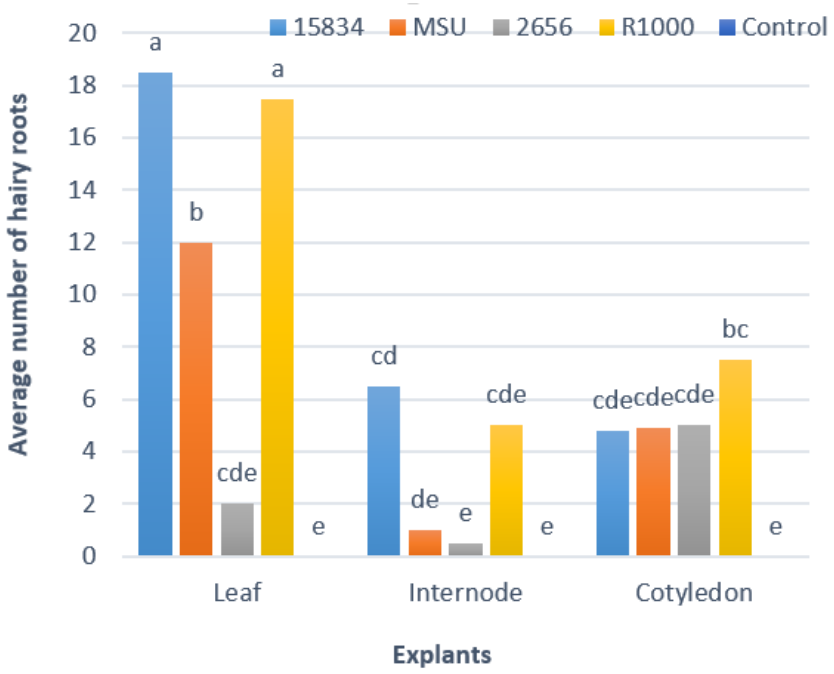

Figure 2. Comparative analysis of the interaction between three explants and four bacteria strains on the average number of hairy roots generated in the *Rubia tinctorum* Ardakan in MS medium supplemented with B5 vitamin under 16 h light/8 h dark photoperiod at 35 days of culture. The untransformed root culture serves as the control. The mean difference is significant at the 0.01 level. Adjustment for multiple comparisons: Least Significant Difference.

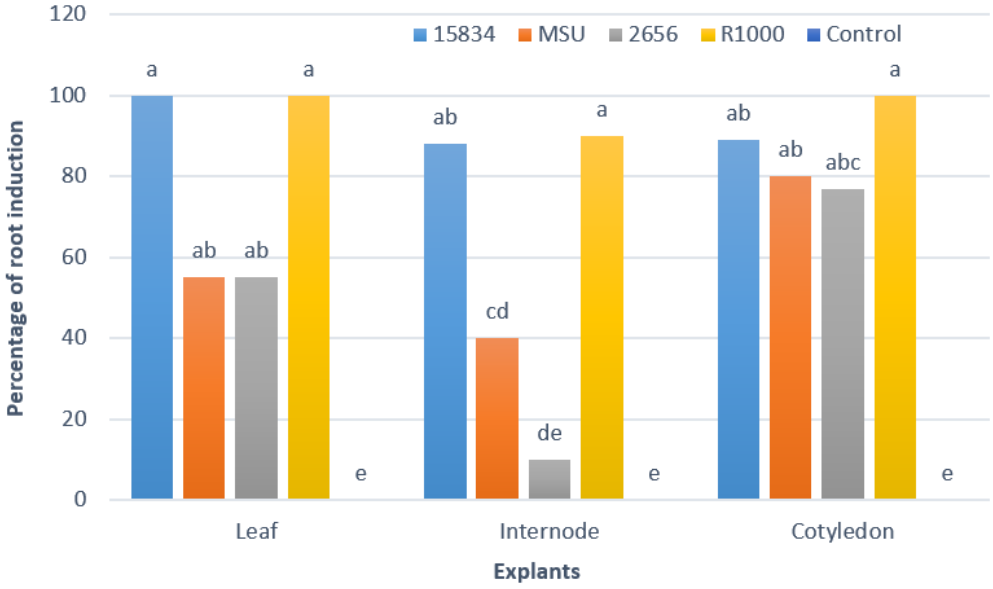

Figure 3. Comparative analysis of the interaction between explants and bacteria strains on the percentage of root induction in the *Rubiatinctorum* Ardakan in MS medium supplemented with B5 vitamin under 16 h light/8 h dark photoperiod at 35 days of culture. The untransformed root culture serves as the control. The mean difference is significant at the 0.01 level. Adjustment for multiple comparisons: Least Significant Difference.

explants infected with all strains of *A. rhizogenes* were generated after 19 days. In fact, the internode explants induced hairy roots at a much lower rate. From the aspect of transformation frequency, both 15834 and R1000 exhibited higher transformation frequencies with all explants (89.33–100 present) at $P<0.01$. The transformation frequencies for the rest of the strains ranged between 11.06% and 88.33% However, in

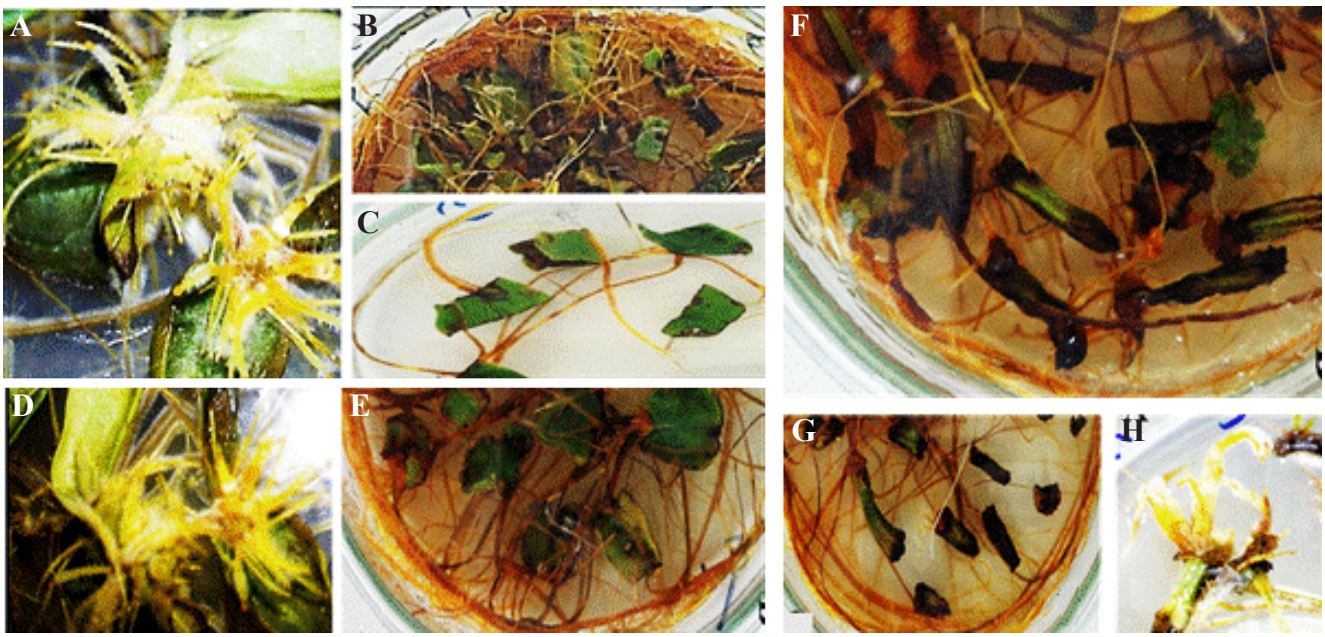

Figure 4. Hairy root induction of the leaf explants of *Rubia tinctorum* Ardakan by *A. rhizogenes* **A:** strain 15834, **B:** strain R1000 and **C:** strain 2656; Hairy roots induction of the cotyledon explants by *A. rhizogenes* **D:** strain 15834 and **E:** strainR1000; Hairy roots induction of the internode explants by *A. rhizogenes* **F:** strain R1000, **G:** strain 15834 and **H:** strain MSU, on solidified full strength MS medium under 16 h light /8 h dark photoperiod at day 35.

the comparison within the group effect, there were significant differences between all tested strains.

There were significant variations among explants, *A. rhizogenes* strains, and interaction between strains and explant at a level of $p<0.01$. Hairy roots induced by 15834 and R1000 strains exhibited better growth as compared to the other hairy root lines, at 35 days of culture under 16 h light/8 h dark photoperiod (Figures 4A-4C).

For the average number of generated hairy roots, there were significant differences among all *A. rhizogenes* strains treated with the explants, under solid full-strength MS medium culture conditions, $P<0.01$ (Figure 2). Leaf explants incubated with the R1000 and 15834 produced more hairy roots than the other strains, but roots derived from 2656 infected explants exhibited the lowest number of roots. MSU and 2556 give lower efficiency with leaf and internodes but had a good performance with cotyledons. It could be concluded that cotyledon explants were more competent for transformation by *A. rhizogenesis*.

The hairy roots generated by 15834 and MSU strains were highly branched while the hairy roots generated by 2656 and R1000 were much longer, with less branching (Figure 4). The color of the hairy roots produced by the leaf explants was brighter than the other explants (cotyledon and internode). Figures 4A-4H showed the different morphology of the hairy roots induced by all of the *A. rhizogenes* strains and all of the explants on MS medium under 16 h light/8 h dark photoperiod at day 35.

Hairy roots from leaf explants were transferred to an MS liquid medium for three weeks. The dry weight of the roots increased from the original inoculum level of 0.4 g/l to 8.2-13.8 g/l in three weeks (Figure 5). Alizarine production ranged from 2.6 to 3.12 mg g-1 D.W. in different strains. Among the four different hairy root lines and adventitious wild-type roots, R1000 led to the highest amount of alizarin (3.45 mg/g D.W.) and. in contrast, the lowest production of alizarin (2.61 mg/g D.W.) was found in roots from 2656 strain (Figure 6).

DISCUSSION

Plant metabolites and functional compounds have attracted interest because of their valuable pharmaceutical properties and various useful applications in the medical field (Banerjee *et al.,* 2012; Kaliyan and Agastian, 2015). *Rubia thincturum* known as madder is a rich source of anthraquinones responsible for its traditional, phytochemical, and pharmacological activities.

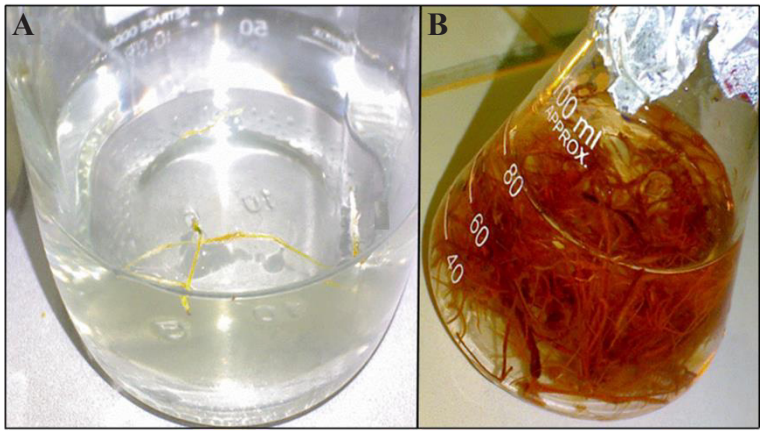

Figure 5. Fast growth of hairy roots from leaf explants of *Rubia tinctorum* Ardakan by *A. rhizogenes* strain 15834 that isolated and transferred on liquid half-strength MS medium under 16 h light /8 h dark photoperiod, **A:** at the first days after transfer on liquid half-strength MS medium, **B:** On the twenty-first after transfer on liquid half-strength MS medium.

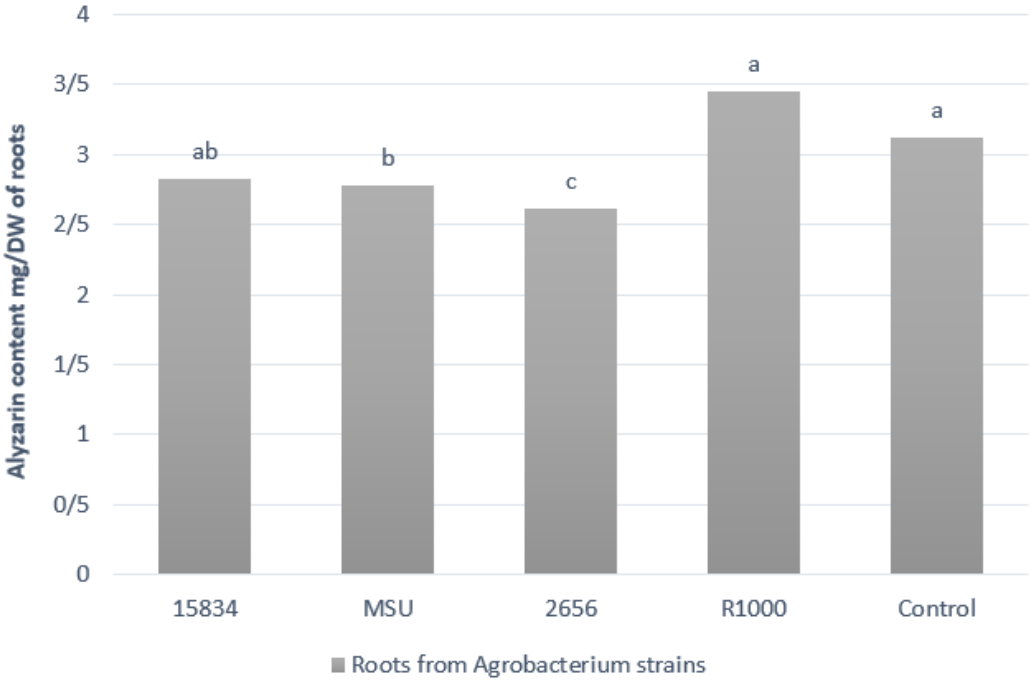

Figure 6. Alizarin content (gr/l) of hairy roots from different strains grown in half-strength liquid MS medium supplemented with B5 vitamin after 21 days of culture under 16 h light/8 h dark photoperiod that, Comparison of mean was done by LSD with non-hairy roots (different letter on bars refers to significant differences) at p=0.01.

In this study, we used three types of explants and four strains of *A. rhizogenes* (15834, 2656, MSU, and R1000) for establishing *R. tinctorum* hairy root cultures. All strains were able to produce hairy roots. Inoculation of all three explants with 15834 and R1000 strain indicated a high frequency of transformation (100%) and along with a higher number of hairy roots (18.56 and 17.5) per explant. The study conducted by Gulhan and Taskin (1999) showed that *A. rhizogenes* strain 2628 induced only callus formation on the cut surface of cotyledonary explants while 15834, R1000 and 9365 strains produced hairy roots on the same explants. The number of roots per explant was in highest in leaf explants except when inoculated with 2656. It can be explained by the cutting edge of this explant compared to others because hairy roots originate from injured cells around the cutting edge. According to research conducted by Lee *et al.* (2010), the growth and anthraquinone (alizarin) production in each hairy root of *R. akane* from infection by five

different *A. rhizogenes* strains were investigated. The highest production of alizarin (4.3 mg/g D.W.) was found in R1601.

As the various strains of *Agrobacterium* have different gene arrangements in their root-inducing plasmid they have different capacities for the induction of roots (Gutierrez-Valdes *et al.,* 2020). The transformation efficiency of various *A. rhizogenes* strains (ATCC 13333, ATCC 15834, A4, R1000, R1200, and R1601) has been examined in *Ocimum basilicum* by Sathasivam *et al.* (2022). They observed a significant difference among the six strains in terms of hairy root production. Our results showed two strains out of four were efficient in transformation and increasing the number of roots per explant.

Different explants have different abilities in terms of hairy root production because they have diverse contents of indigenous hormones that have affected the production of hairy roots (Li and Wang, 2021). Some studies have shown that the explant type affected the abundance of hairy roots due to the physiological properties of the cells (Pirian *et al.,* 2012; Miao *et al.,* 2021).

Pirian *et al.* (2012) examined four strains of *Agrobacterium rhizogenes* (15834, 9534, 318, and A 4) on the production of hairy root and showed that 15834 was the most efficient and among used explants (leaves with petiole, leaves without petiole, stems and roots) leaves with petiole, produced the highest rate of roots showing consistency with our finding.

The influence of *A. rhizogenes* strain on hairy root induction frequency has been documented earlier by many researchers. (Lee *et al.,* 2010; Tariverdizadeh *et al.,* 2018; Abadi *et al.,* 2020; Sathasivam *et al.,* 2022; Naderian *et al.,* 2022). Sathasivam *et al.* (2022) examined the transformation efficiency of various *A. rhizogenes* strains (ATCC 13333, ATCC 15834, A4, R1000, R1200, and R1601) for transgenic hairy roots induction in *Ocimum basilicum*. According to their results, R1601 was found to be one of the most promising strains for mass production of hairy roots in terms of transformation efficiency (94%) and the number and length of hairy roots. Gulhan and Taskin (1999) examined the effect of *Agrobacterium rhizogenes* strains (15834, 2628, 9365, and R1000) on the cotyledon explant in some *Rubia tinctorum* L. (4 different regions of Turkey) on the initiation of the hairy root, and found that the Strain 15834 was the best, and the strain R1000 was the second (Gulhan and Taskin, 1999).

Alizarin analysis showed that there was a significant difference between alizarin values obtained from transgenic roots produced by different strains of *Agrobacterium* (Figure 6). Roots from R1000 and 15834 were in the same group as the wild-type root but roots from MSU and 2656 produced lower alizarin.

Research on some species including *R. tinctorum* should focus on the induction of hairy roots, the selection of superior clones, the optimization of the culture media, and physical parameters that affect the accumulation of biomass and anthraquinone (Murthy *et al.,* 2023). We investigated the ability of some Agrobacterium strains and explants types for enhancement of root biomass and secondary alizarine production in madder. Our results showed the leaf explant in combination with the R1000 and 15834 was the best in point of root induction and anthraquinone production. Previously, other studies have also reported the differential efficiency of various *A. rhizogenes* strains in promoting the induction, growth, and secondary metabolite production of hairy roots. Different *A. rhizogenes* strains affected growth rate, saponin production, and the ratio of different astragalosides in transgenic root cultures of *Astragalus mongholicus* Bge. (Yonkova, 1997). The strain of *Agrobacterium* also influenced the tropane alkaloids production in transformed root cultures of *H. muticus* (Matius, 2000). Hairy root cultures of *Gentiana macrophylla* were established by infecting with four *A. rhizogenes* strains and each hairy root line showed a different response regarding the growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures (Tiwari, 2007). Clearly, the selection of an effective *Agrobacterium* strain for the production of transformed root cultures is highly dependent on the plant species and must be determined empirically (Sathasivam *et al*., 2022).

CONCLUSION

Hairy roots are a unique *in vitro* system to produce valuable secondary metabolites in many plants. The present study describes the successful genetic transformation and establishment of a highly productive hairy root culture of *Rubia tinctorum* Ardakan. The results obtained in this study indicate that the use of different bacterial strains and various explants were effective factors for hairy root induction and *R. tinctorum* hairy roots culture can be a valuable alternative approach for the production of anthraquinone (Alizarin).

ACKNOWLEDGMENTS

The authors would like to thank the University of Tabriz for their grant and technical support of this research.

REFERENCES

Abadi M., Ganjeali A., Lahouti M., and Moshtaghi N. (2020). Influence of different *Agrobacterium rhizogenes* strains on hairy roots induction and secondary metabolites production in *Ocimum basilicum* L. *Journal of Horticultural Science*, 34: 273-284.

Banerjee, S., Singh, S., and Rahman, L. U. (2012). Biotransformation studies using hairy root cultures: A review. *Biotechnology Advance*, 30: 461-468.

Cai D., Kleine M., Kifle S., Horloff H. J., Sandal N. N., Marcker K. A., Lankhorst R. M. K., Salentijn E. M. J., Lange W., Stiekema W., Wyss V., Grundler F. M. W, and Jung C. (1997). Positional cloning of a gene for nematode resistance in sugar beet. *Science*, 275: 832-834.

Eltamany E. E., Nafie M. S., Khodeer D. M., El-Tanahy A. H. H., Abdel-Kader M. S., Badr J. M., and Abdelhameed, R. F. A. (2020). *Rubia tinctorum* root extracts: chemical profile and management of type II diabetes mellitus. *The Royal Society of Chemistry,* 10: 24159-24168.

Gulhan E. A., and Taskin K. M. (1999). *Agrobacterium rhizogenes* mediated hairy root formation in some *Rubia tinctorum* L. *American Journal of Botany*, 23: 373-377.

Gutierrez-Valdes N., Häkkinen S. T., Lemasson C., Guillet M., Oksman-Caldentey K. M., Ritala A., and Cardon F. (2020). Hairy root cultures-a versatile tool with multiple applications. *Front in Plant Science*, 11: 33-39.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2002). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, World Health Organization, Vol. 82, pp. 590.

Ionkova I., Kartnig T., and Alfermann W. (1997). Cycloartane saponin production in hairy root cultures of *Astragalus mongholicus*. *Phytochemistry*, 45: 1597-1600.

Kaliyan B. K., and Agastian P. (2015). *In vitro* regeneration of a rare antidiabetic plant *Epaltes divaricata* L. *South Indian Journal of Biological Sciences*, 1: 52-59.

Karuppusamy S. (2009). A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. *Journal of Medicinal Plants Research*, 3(13): 1222-1239.

Lee S. Y., Kim S. G., Son W. S., Kim Y. K., Park N. I., and Park S. U. (2010). Influence of different strains of *Agrobacterium rhizogenes* on hairy root induction and production of alizarin and purpurin in *Rubiaakane nakai*. *Romanian Biotechnological Letters*, 15: 5405-5409.

Li C., and Wang M. (2021). Application of hairy root culture for bioactive compounds production in medicinal plants. *Current Pharmaceutial Biotechnolgy*, 22(5): 592-608.

Mateus L., Ceerkaoui S. Christen P., and Oksmam K. M. (2000). Simultaneous determination of scopolamine, hyoscyamine and littorine in plants and different hairy root clones of *Hyoscyamus muticus* by micellar electrokinetic chromatography. *Phytochemistry*, 54: 517-523.

Miao Y., Hu Y., Yi S., Zhang X., and Tan N. (2021). Establishment of hairy root culture of *Rubia yunnanensis* Diels: Production of Rubiaceae-type cyclopeptides and quinones. *Journal of Biotechnology*, 341: 21-29.

Murashige T., and Skoog A. (1962). Revised medium for rapid growth and Bio Assays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 473-497.

Murthy H. N., Joseph K. S., Paek K. Y., and Park S. Y. (2022). Anthraquinone production from cell and organ cultures of Rubia species: An overview. *Metabolites*, 13: 39-47.

Naderian P., Moshtaghi N., Bagheri A., and Malekzadeh-Shafaroudi S. (2022). Influence of different *Agrobacterium rhizogenes* strains on hairy roots induction and secondary metabolites production in *Datura innoxia* Mill. *Journal of Medicinal Plants*, 21: 50-64.

Palazon J., Pinol M. T., Cusido R. M., Morales C., and Bonfill M. (1997). Application of transformed root technology to the production of bioactive metabolites. *Recent development in Plant Physiology*, 1: 125-143.

Perassolo M., Cardilllo A. B., Busto V. D., Rivie S., Cerezo J., and Talou J. R. (2020). Elicitation as an essential strategy for enhancing anthraquinone accumulation in hairy root cultures of *Rubia tinctorum*. In book: Hairy Root Cultures Based Applications, 133-152.

Pirian K., Piri K. H., and Ghiyasvand T. (2012). Hairy roots induction from *Portulaca oleracea* using *Agrobacterium rhizogenes* to Noradrenaline,s production. *International Research Journal of Applied and Basic Sciences*, 3(3): 642-649.

Rao R. S., and Ravishankar G. A. (2002). Plant tissue cultures; chemical factories of secondary metabolites. *Biotechnology Advances*, 20: 101-153.

Sathasivam R., Choi M., Radhakrishnan R., Kwon H., Yoon J., Yang S. H., Kim J. K., Chung Y. S., and Park S. U. (2022). Effects of various *agrobacterium rhizogenes* strains on hairy root induction and analyses of primary and secondary metabolites in *Ocimum basilicum. Frontiers in Plant Science*, 13: 983776.

Tariverdizadeh N., Mohebodini1 M., Chamani1 E., and Ebadi A. (2018). Effects of explant age and strain of *Agrobacterium rhizogenes* on hairy root induction in Fenugreek (*Trigonella foenum–* graecum L.). *Iranian Journal of Genetics and Plant Breeding*, 7(1): 50-58.

Tariverdizadeh N., Mohebodini M., Chamani E., and Ebadi A. (2018). Influence of *grobacterium rhizogenes* strains on hairy roots induction in *Trigonella foenum-graecum* L. and secondary metabolites production*. Journal of Plant Molecular Breeding,* 6: 53-60.

Thwe A., Valan Arasu M., Li X., Park C. H., Kim S. J., Al-Dhabi N. A., et al. (2016). Effect of different *Agrobacterium rhizogenes* strains on hairy root induction and phenylpropanoid biosynthesis in tartary

buckwheat (*Fagopyrum tataricum* gaertn). *Frontiers in Microbiology*, 7: 1-10

Tiwari R. K., Trivedi M., Guang Z. C. Guo G. Q., and Zheng G. C. (2007). Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. *Plant Cell Reports*, 26: 199-210.

Zheleznichenko T., Voronkova M., Asbaganov S., Kukushkina T., Filippova E., Protsenko M., Mazurkova N., and Novikova T. (2023). Impact of different *Agrobacterium rhizogenes* strains on secondary metabolites accumulation in *Nitraria schoberi* L. hairy roots and antiviral activity of their extracts against influenza virus of subtypes A (H5N1) and A (H3N2*). In Vitro Cellular and Developmental Biology*, 59: 378-392.