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HAIRY ROOT CULTURES AS A POTENTIAL TOOL FOR THE BIOSYNTHESIS OF ACTIVE COMPOUNDS IN *CATHARANTHUS ROSEUS*

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Abstract

Catharanthus roseus (L.) G. Don is a medicinal plant that produces terpenoid indole alkaloids (TIAs) of immense value. Vinblastine (VBL) and vincristine (VCR) are both anticancer drugs. Hairy root cultures (HRCs) originated from leaf, stem, and root explants of *C. roseus* using *Agrobacterium rhizogenes*. The binary plasmid pBI121-containing *A. rhizogenes* strains (A4 and 15834) was utilized. A4-infected leaf explants converted hairy roots more effectively than strain 15834 after 60 days. In 90-day HRCs, the frequency of VCR and VBL was higher. After 60 days of cultivation, A4 strain-converted leaf-derived HRC enhances VCR and VBL content, as well as fresh or dry biomass weight. The PCR fingerprints were confirmed. Our findings showed that HRCs might be used as a promising technique for hairy root biomass and biotechnological synthesis of VBL and VCR from *C. roseus* medicinal plants.

Key words: Periwinkle, Terpenoid indole alkaloids, Hairy root cultures (HRCs), Growth kinetic, Agrobacterium rhizogenes, PCR

Introduction

Catharanthus roseus is a beautiful flowering perennial herb that is always in bloom as an ornamental plant. It is known as Madagascar periwinkle (Guerriero *et al*., 2018; Chaturvedi *et al*., 2022) The medicinal plant *Catharanthus roseus* (L.) G. Don (Apocynaceae) possesses over 50 indole alkaloids (IAs; VCR/VBL). This plant produces substantial ajmalicine and serpentine. VCR/VBL treats cancer, hypertension, diabetes, and menstruation (Chaturvedi *et al*., 2022). Wild perennials produce little terpenoid indole alkaloids (TIAs). TIAs need 1,000 kg of dried leaves per gram (Noble, 1990). Mass-producing alkaloids is costly and complicated (Taha *et al*., 2014). Vindoline and catharanthine may semi-synthesize alkaloids (VCR and VBL) (Ishikawa *et al*., 2009). Bioactive phytoconstituents' medicinal alkaloids support tissue and cell culture (Rawat *et al*., 2019). Cell suspensions and hair root cultures tested alkaloid augmentation (Rawat *et al*., 2019). Cheap VBL and HRC alkaloids. TIAs synthesis in cell suspension or HRCs cannot create these high-value chemicals (Zhao *et al*., 2013). Standardized tissue culture conditions created transgenic Catharanthus strains. Sonication and co-cultivation durations influenced *C. roseus* leaf explant direct bud organogenesis (Verma & Mathur, 2011). Catharanthus transgenesis optimized (Verma & Mathur, 2011). Callus culture indirect regeneration increases chromosomal alterations (Ali *et al*., 2017). Plant genetics and secondary metabolic chemical production are concerns. Low-content medicinal plants with high pharmacological components need reliable, cost-effective transformation. This herb relieves hypertension, rheumatism, menstrual irregularities, dyspepsia, and other significant disorders (Chaturvedi *et al*., 2022).

Agrobacterium rhizogenes, a Gram-negative bacterium, causes hairy root development in dicotyledonous plants (Chaturvedi *et al*., 2022). Rhizogenes are linked to plant genetic change. This natural-vector pathogen alters hairy roots and enhances secondary metabolite production (Qin *et al*., 2022). Hairy roots produced significant biomass in a free hormonal medium (Makhzoum *et al*., 2013). Hairy roots generate substantial levels of bioactive chemicals, making them a potential and reliable method for secondary metabolites biosynthesis (Mujib *et al*., 2012; Malik *et al*., 2016, Elateeq *et al*., 2022; Abdelkawy *et al*., 2023). Due to gene stability, HRCs benefit from helpful secondary metabolites. Catharanthine, ajmalicine, and serpentine are produced by *C. roseus'* hairy roots (Hanafy *et al*., 2016). Plants provide many important natural compounds, but commercial production is difficult. Organic synthesis is too costly for industrial products, and biosynthetic routes are typically too complicated to transfer to a microbe. Hairy root cultures provide genetic and biochemical stability, quick growth, and a hormone-free culture medium for non-agricultural natural product manufacturing. Metabolic engineering and synthetic biology methods to design hairy roots and bioreactor technologies are close to commercialization (Morey & Peebles, 2022). In the current study, HRCs were established using two strains of *A. rhizogenes* from leaf, stem, and root explants of *C. roseus*, and the content and accumulated VCR and VBL in the transformed hairy roots were evaluated.

Methods

Plant materials: *C. roseus* seeds were obtained from the Horticulture Research Institute (HRI), Agricultural Research Centre (ARC), Giza, Egypt (longitude

31°12'24.2"E latitude 30°01'13.4" N). Seeds were sterilized with 70% EtOH for 30 sec. before being transferred to a solution of 25% Clorox (including 5.5% NaOCl) for 15 min. After that, the seeds were washed in sterilized distilled water (SDW). Seeds germination was done on basal Murashige and Skoog, pH 5.8, media for two weeks under 25±2°C and photoperiod (16hr, white fluorescent lamp, 35 µmol s/m^2). The seedling is employed as a plant source to provide leaf (4-5 mm), stem (1 cm), and root (1cm) explants.

Growth of *A. rhizogenes* strains: Two *A. rhizogenes* strains (A4 and 15834, VTT Bio and Chemical processes, Plant Biotechnology Group) were utilized for transformation. Bacteria (both strains) were cultured in liquid Luria Bertani medium (LB) provided with yeast extract (5 g/l), NaCl (10 g/l), tryptone (10 g/l), and agar (7 g/l). The bacteria strains were cultured for two days at 27°C. The developing bacteria colonies were suspended in a liquid LB medium and cultured overnight at 27°C in darkness on shaker equipment at 100 rpm. A spectrophotometer was used to measure the optical density **(**OD) to 0.5 at 600 nm.

Establishment of HRCs: Sterilized leaf, stem, and root explants of two-week-old seedlings of *C. roseus* were submerged in a culture of the two *A. rhizogenes* strains supplemented with 200 uM acetosyringone for 20 min. Explants were blotted and dried with sterile filter paper (Whatman No. 1), then put on solidified free MS media at 26±2°C for 48 hr. to eliminate excess germs. Following the co-cultivation period, the explants were washed five times in SDW to remove excess bacteria before being sub-cultured on fresh MS medium containing 50 mg/l kanamycin and 400 mg/l cefotaxime and then incubated at 25±2°C under complete darkness. Hairy roots originated from the infected sites during three weeks of incubation. The isolated hairy roots were transferred to sterile flasks containing liquid basal MS media and incubated at 27°C on a shaker at 60 rpm and photoperiod (16 hr, white fluorescent lamp, 35 µmol s/m^2).

Measurement of hairy roots cultures

1- The transformation effectiveness (%) was measured as the total number of explants that induced hairy roots divided by the total number of cultivated explants× 100.
2- Fresh (FW) and dry (DW) weights of hairy roots derived from leaf stem or root explants (mg/jar).

Growth kinetic of HRCs: For growth kinetics, the HRCs ~ 50 mg/jar were subcultured for three subcultures intervals of 30, 60, and 90 days. FW and DW of the hairy roots (mg/jar) were determined and TIAs (VBL and VCR) contents were evaluated compared to the wild plant.

Confirmation of *A. rhizogenes* strains using Polymerase Chain Reaction (PCR) technique: For analysis, DNA was extracted from non-transformed leaves of *C. roseus* as a negative control, *Agrobacterium* strains as a positive control, and from initiated hairy roots. Total DNA was isolated from the two *A. rhizogenes* strains (A4, and 15834) using an *In vitro* gene Kit (Cat. No.CS11301). DNA was isolated also from hairy roots and plant leaves according to (Dellaporta *et al*., 1983). The PCR was performed with the following primers: 5´GCTCTTGCAGTGCTAG ATTT-3´(forward primer) and 5´GAAGGTGCAAGCTACCTCTC-3´(reverse primer). The primers were used to amplify a 423-bp fragment of the *rol* B gene as described by (Hazaa *et al*., 2006) were used to amplify 20 bp fragments of the rol A gene. In the PCR amplification, the PCR mixture was prepared (2.5 µl 10xbuffer, 2.5 mMMgCl2, 2.5mM of each dNTP) before 1 µl (20 ng) from each of the primer pairs and 0.2-unit Taq DNA polymerase (Alliance Bio Cat. No. M010TP20) were added to the mixture plus 2 µl from the DNA (20 ng) and d.d H_2O to a final volume of 25 µl. The thermal conditions of the reaction set as follows; 94°C for 3 min for DNA denaturation, 30 repeated cycles of 94°C for 1 min, 55°C for 2 min for annealing, and 72°C for 2 min for the extension. The PCR products were analyzed using 0.8% agarose gel in 1x TAE buffer (Tris-Acetate EDTA electrophoresis buffer), 40mM Tris, 20mM acetic acid, 1mM EDTA) and stained with ethidium bromide (10 µl/ml) for 10 min. Bands were visualized by examination under a UV trans-illuminator and photographed using a Fuji film digital camera fine pix s9100/ fine pix s9600.

Identification of Indole alkaloids by HPLC analysis: Fresh samples of HRCs of *C. roseus* (1.0 g) were ground in liquid nitrogen. The frozen dry material was homogenized with 1 ml methanol for an hour in a sonicate bath according to (Hong *et al*., 2013). The extracts were centrifuged (15,000 rpm for 5 min. at 25±2°C) and the supernatant was collected. VCR and VBL were determined by an aliquot of 100 µl injection volume and were separated using high-performance liquid chromatography (HPLC). The mobile phase was acetonitrile: Na_3PO_4 buffer (5 mM/l) 45:55% at pH 5.9). The flow rate was 0.7 ml/min and the detection wavelength was 220 nm. The standard solutions of VCR and VBL (Sigma, USA) were dissolved in 1 ml MeOH and chromatographed. Alkaloids were detected and estimated based on their retention times and the concentration of the standards. The standard curves for VCR and VBL (Fig. 1) were derived at 254 nm and 280 nm, respectively.

Statistical analysis

The experiment was performed in a complete randomized design (CRD). Every treatment had 3 replicates; each replicate had 5 culture vessels. The analysis of variance (ANOVA) of data was performed using (Snedecor and Cochran., 1989) method. Computation was done using the COSTAT computer package (CoHort software Monterey, California, USA), and means were compared according to Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. The results are present as the means ± standard error (SE).

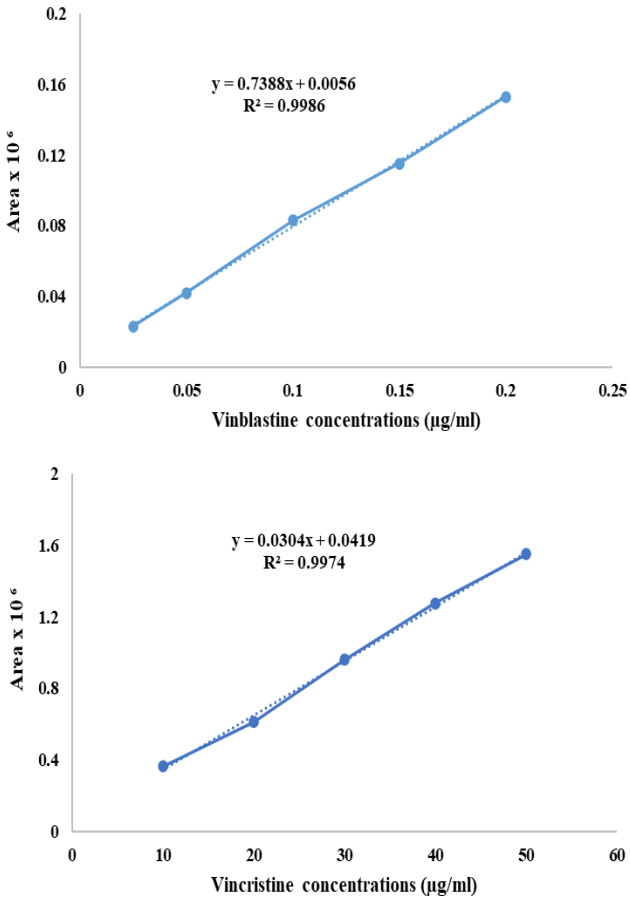

Fig. 1. The standard curves of vinblastine (VBL) and vincristine (VCR) alkaloids (µg/ml).

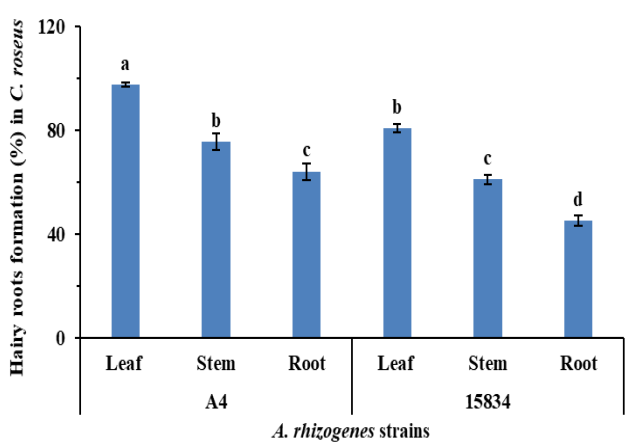

Fig. 2. Transformation efficiency of hairy root formation in different explants (leaf, stem, and root) of *C. roseus* infected with A4 and 15834 strains of *A. rhizogenes*. Bars represent ±SE (n=3). Columns with different letters are statistically different ($p \leq 0.05$) according to DMRT.

Results

1-Transformation efficiency of hairy roots formation: The results showed the efficiency of two *A. rhizogenes* strains (A4 and 15834) in the *C. roseus* transformation. Hairy roots emerged from all explants inoculated with the two studied strains, while the infected leaf explants induced the highest hairy roots compared to other tested explants. Data in (Fig. 2) show the highest percentages of hairy root transformation with A4 strain compared with 15834 one. Leaf explants were more pronounced than stem and root explants. The highest significant percentage ($p \leq 0.05$) of hairy root transformation (97.33%) was recorded for leaf explants infected with the A4 strain. No statistical differences were recorded between leaves infected with the 15834 strain (80.67%) and stem explants infected with the A4 strain (75.33%). Root explants displayed the lowest significant percentages of hairy root transformation, especially when infected with 15834 strain (45%).

2-Fresh and dry weights of transformed hairy roots: In general, the biomass FW and DW of hairy roots transformed with the A4 strain were higher than that obtained by infection with 15834 strain (Fig. 3). Leaf explants produced hairy roots having high biomass compared with stem and root explants. The topmost significant FW and DW of the transformed hairy roots (87.23 and 7.96mg/jar, respectively) were recorded for leaf explants infected with the A4 strain (Fig. 3A and B). Leaf explants treated with 15834 also highly reported FW and DW without significant differences when compared with stem explants infected with A4 strain (67.8 and 75.23mg/jar for FW; 5.62 and 5.63mg/jar for DW, respectively). Hairy roots transformed with A4 or 15834 using root explants showed the minimum significant FW and DW.

3-Growth kinetic of hairy roots after 30, 60, and 90 days of subculture: In the current experiment, a 50 mg FW/jar of hairy roots induced from the leaf, stem, and root explants of *C. roseus* infected with either A4 or 15834 strains were subcultured on a growth medium aiming to monitor the growth kinetics of hairy roots during 30, 60, or 90 days of subculture. The data in (Fig. 4) show FWs and DWs of hairy roots after 30 days of subculture. The maximum significant biomass of HRCs was noticed for leaf-derived hairy roots for both *A. rhizogenes* strains. The highest significant FW and DW (106.27 and 9.52 mg/jar, respectively) were scored for HRCs established from leaf explants infected with the A4 strain, followed by HRCs of the 15834-infected leaf (95.70 and 8.46 mg/jar, respectively). The biomass FW and DW of the HRCs were reduced significantly when root explants were employed to produce hairy roots *via* both strains. The same trend of 30 days culture was also observed for 60- and 90-day cultures (Figs. 5 and 6). After 60 days of culture, the maxima FW and DW of HRCs were 339.26 and 29.36 mg/jar, respectively, when leaf explants were infected with the A4 strain (Fig. 5A and B). HRCs originated from stems infected with A4 and leaves infected with 15834 had the same significant values of biomass FW (189.20 and 218.23mg/jar, respectively) and DW (15.39 and 18.39, mg/jar, respectively). Root-derived HRCs for both strains as well as stem-derived HRC for 15834 recorded the minimum significant values of biomass weights. Similarly, after 90 days of culture, HRCs established from A4-infected leaf recorded the highest significant biomass production (Fig. 6), herein, the values were 775.70 and 62.46mg/jar for FW and DW, respectively, followed by HRCs of leaf transformed with 15834. A4-mediated cultures produced higher biomass than 15834-mediated hairy roots. The lowest significant production of hairy root biomass FW for the 90-day culture was observed

for root-derived hairy root transformed by 15834 strain (195.10 mg/jar). However, the biomass DW reached the lowest values for cultures of root-derived hairy roots with A4 and 15834 strains as well as for HRCs established through the 15834-infected stem (21.02, 17.08, and 21.91mg/jar, respectively) without statistical differences between them. The best strain for obtaining maximum FW and DW was the A4 strain compared to the 15834 one. The optimum period for cultivation is 60 days from the subculture. The competence explant was a leaf explant compared to stem or root explants. As shown in (Fig. 7) hairy roots were induced from leaf explants of *C. roseus* with A4 strain.

4-Identification of indole alkaloids by HPLC analysis: The changes in the terpenoid indole alkaloids; VBL and VCR were evaluated by HPLC after 30, 45, 60, and 90 days of hairy roots cultivation (Figs. 9 and 10). The values of VBL or VCR are expressed as a relative percentage for those in the intact plant. The percentages of VBL or VCR in the wild plant of *C. roseus* were 0.047% and 0.033%, respectively. Data in (Fig. 9) illustrate that the greatest relative percentages of VBL were 4.61, 4.15, 3.89, 3.72, 1.93, and 1.27% with hairy roots derived from leaf, stem, and root explants treated with A4 followed by 15834 strains, respectively after 90 days of cultivation. However, the highest value of kinetic growth index was shown after 60 days with all explants treated with either A4 strain or 15834 strains. Concerning the relative percentage of VCR to wild plants, the highest percentages were 4.06 and 3.17% with leaf explants treated with the A4 strain. Stem explants recorded 2.54, and 2.29% for A4 and 15834strains, respectively (Fig. 10). The lowest relative percentage was found for root-derived HRCs which recorded 1.51 and 1.25%for 15834 and A4 strains, respectively. The cultivation for 90 days responded to the maximum relative percentage of either VBL or VCR, the cultivation for 60 days is more efficient.

Overall, the implementation of *C. roseus* leaf in hairy root production is more efficient and economical for biomass, VBL, and VCR production, especially A4-mediated HRCs compared to 15348. The cultivation of HRCs for 60 days is more suitable for biomass fresh or dry weight production, as well as enhancement of VCR or VBL production.

Regarding, PCR analysis resulted in the successful amplification of *rolB* products in the two hairy root clones analyzed as shown in (Fig. 8).

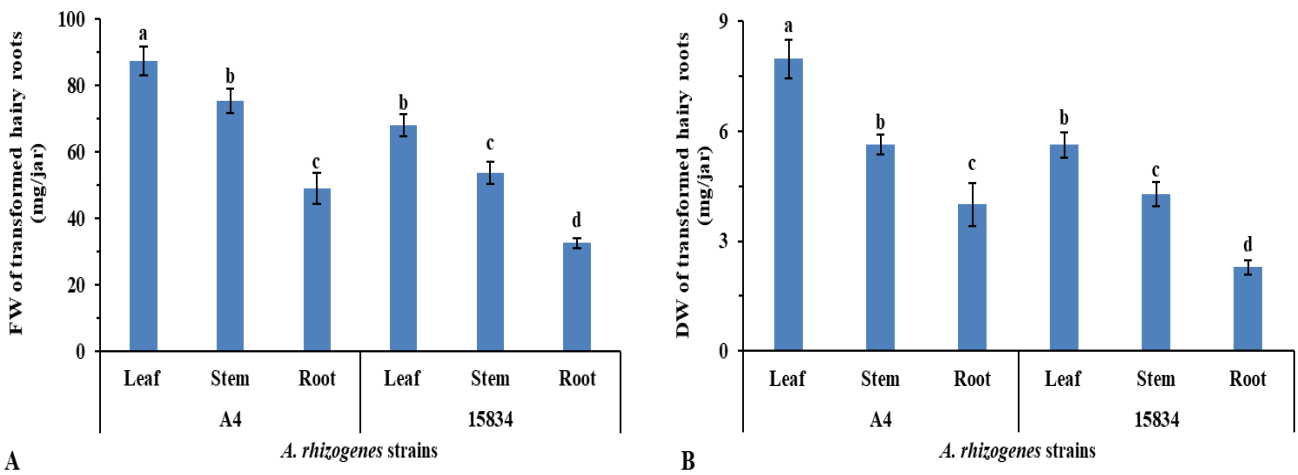

Fig. 3. Effects of two strains of *A. rhizogenes* (A4 and 15834) on hairy roots FW (A) and DW (B) (mg/jar) induced from different explants (leaf, stem, and root) of *C. roseus*. Bars represent ±SE (n=3). Columns with different letters are statistically different ($p \leq 0.05$) according to DMRT

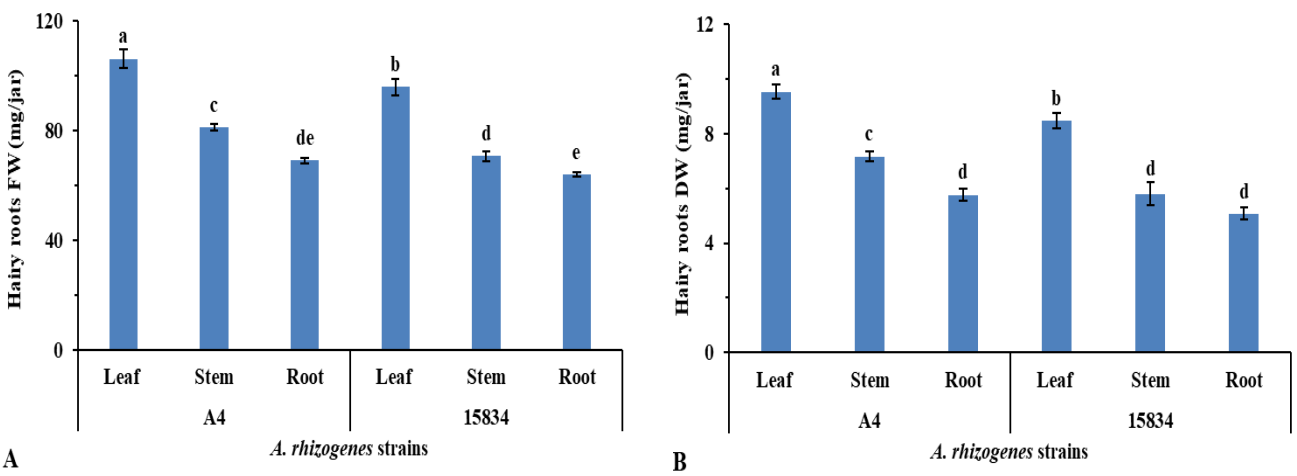

Fig. 4. Effects of two *A. rhizogenes* strains (A4 and 15834) on the growth kinetics of hairy roots FW (A) and DW (B) (mg/jar) produced from different explants (leaf, stem, and root) of *C. roseus* after 30 days of culture. The initial FW of the inoculum was 50 mg. Bars represent ±SE (n=3). Columns with different letters are statistically different ($p \leq 0.05$) according to DMRT.

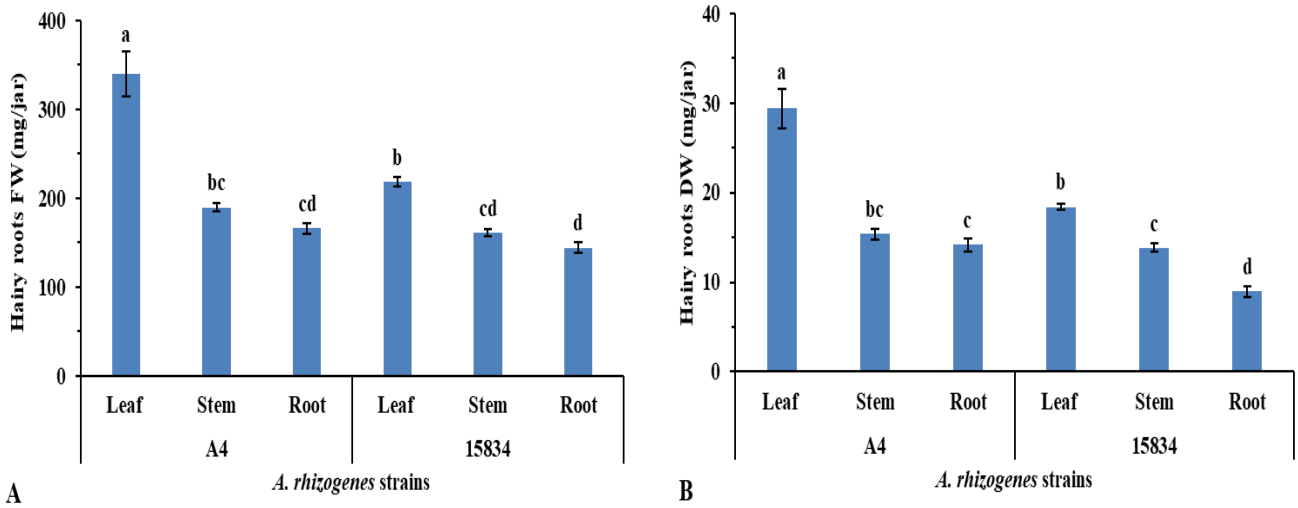

Fig. 5. Effects of two *A. rhizogenes* strains (A4 and 15834) on the growth kinetics of hairy roots FW (A) and DW (B) (mg/jar) produced from different explants (leaf, stem, and root) of *C. roseus* after 60 days of culture. The initial FW of the inoculum was 50 mg. Bars represent ±SE (n=3). Columns with different letters are statistically different ($p \leq 0.05$) according to DMRT.

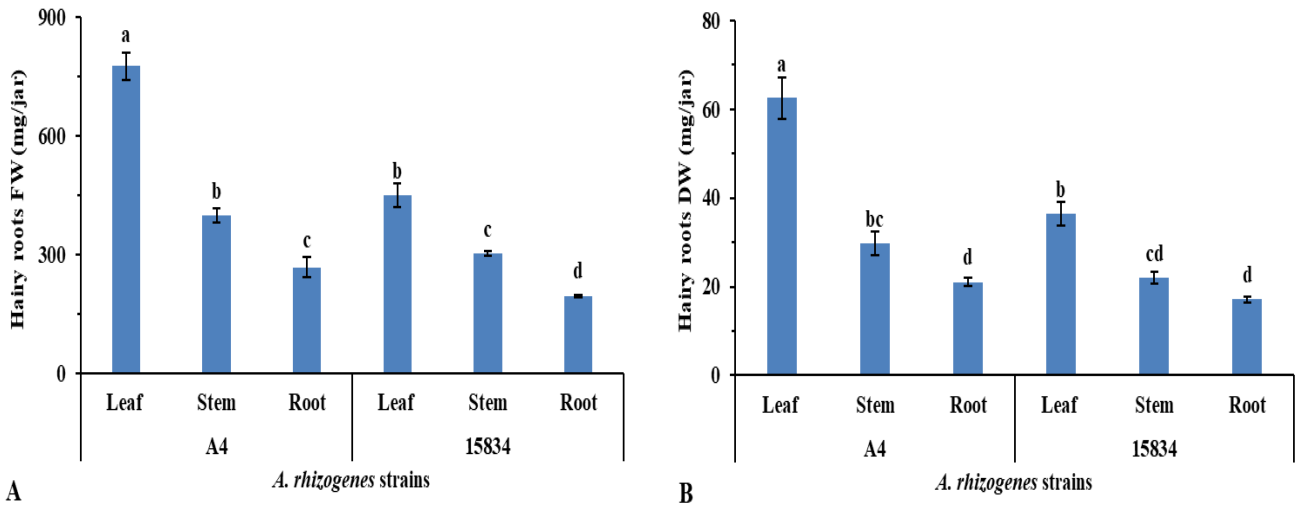

Fig. 6. Effects of two *A. rhizogenes* strains (A4 and 15834) on the growth kinetics of hairy roots FW (A) and DW (B) (mg/jar) produced from different explants (leaf, stem, and root) of *C. roseus* after 90 days of culture. The initial FW of the inoculum was 50 mg. Bars represent ±SE (n=3). Columns with different letters are statistically different ($P \leq 0.05$) according to DMRT.

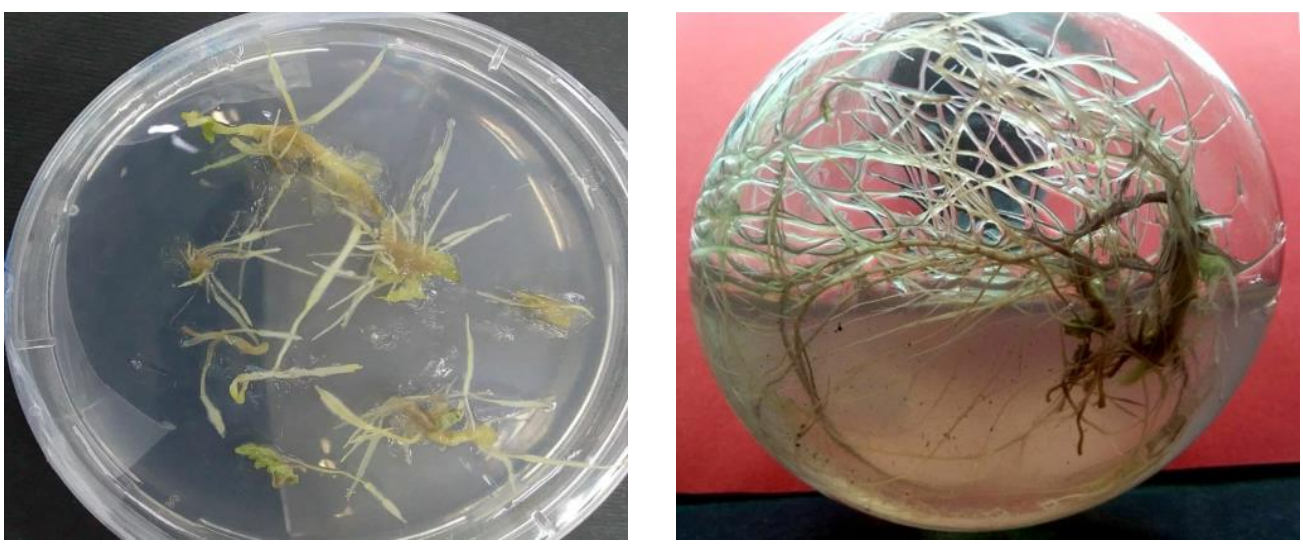

Fig. 7. Hairy root cultures (HRCs) of *C. roseus* induced from leaf explants infected with the A4 strain of *A. rhizogenes.*

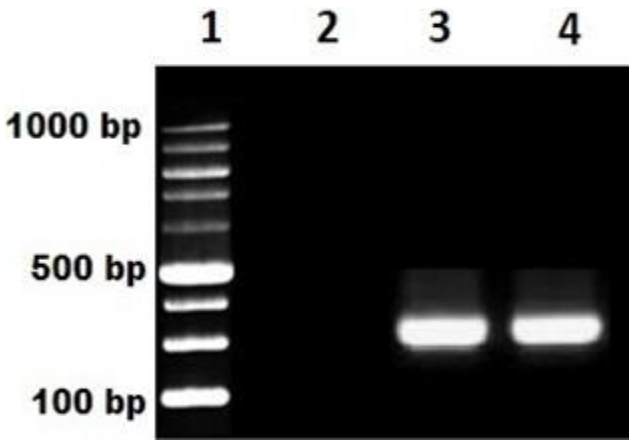

Fig. 8. Lane 1, 1Kb Ladder DNA, 2: Non-Transgenic leaf, 3: Hairy Root (A4), 4: Hairy Root (15834).

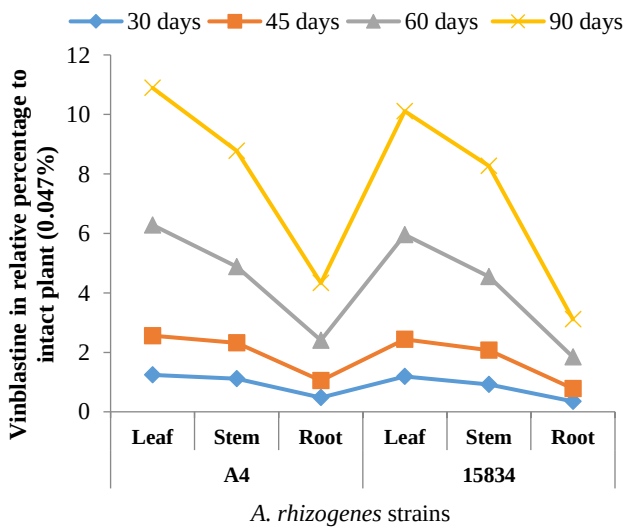

Fig. 9. The relative percentage of VBL to intact plants after 30, 45, 60, and 90 days of cultivation of different explants (leaves, stems, and roots) of *C. roseus* treated with *A. rhizogenes* A4 or 15834 strains.

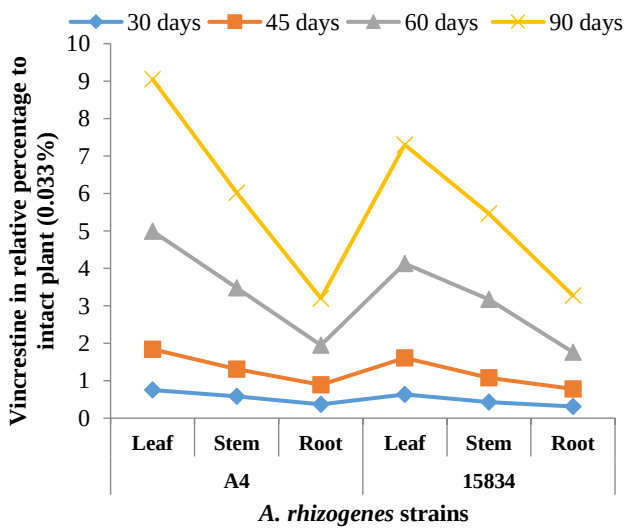

Fig. 10. The relative percentage of VCR to intact plant after 30, 45, 60, and 90 days of cultivation of different explants (leaves, stems, and roots) of *C. roseus* treated with *A. rhizogenes* A4 or 15834 strains.

Discussion

A. rhizogenes strain A4 and 15834mediated transformations of HRCs in *C. roseus* are efficient for the production of biomass fresh and dry weight after 60 days of cultivation. Similarly, Hanafy *et al.* worked on transformed HRCs of *C. roseus* with *A. rhizogenes* K599 carrying p35SGPGUS + plasmid and found that the infected leaves explants induced the high hairy root initials (Hanafy *et al*., 2016). Different *Agrobacterium* strains (R1601, LBA9402, and R1000) have affected the growth in hairy roots of *Morus alba*. *A. rhizogenes* (LBA9402) was the most effective strain for the transformed roots culture (Park *et al*., 2017). As a result, obtaining a homogenous culture of hairy root identical to a cell suspension is impossible. Batra *et al*. showed that the HRCs of *C. roseus* originated from leaf explants infected with *A. rhizogenes*, an atropine-type A4 strain. They separated and classified the hairy roots into four groups based on the number of lateral roots, branches, and growth patterns (Batra *et al*., 2004). PCR analysis resulted in the successful amplification of *rol*B products in the two hairy root clones analyzed (Fig. 8). The PCR products were absent in reactions performed with DNA isolated from normal plant roots (Lane 2). Thus, it has been confirmed at the molecular level that the T-DNA of *Ri* plasmid had been expressed into the genome of the *C. roseus* plant. Additionally, the *rol* ABC hairy root of *C. roseus* showed the development and morphology of a wild-type hairy root pattern on a solid medium. However, they are very adaptable to liquid media and do not develop calli while being grown (Hong *et al*., 2006). Furthermore, a culture of hairy roots caused by *A. rhizogenes* infection is an ideal strategy for synthesizing the secondary bio-molecules by the plant cells due to their chemical and genetic stability (Park *et al*., 2017; Qin *et al*., 2022). Secondary metabolites include TIAs are naturally nitrogenous organic compounds (Debnath *et al*., 2018). *C.roseus* is the source of TIAs (VBL and VCR) exhibiting anticancer and antihypertensive properties. Subsequently, it is vital to the pharmaceutical industry. The high demand for TIAs and limited production in wild plants highlight the importance of the new biotechnological techniques (Acharjee *et al*., 2022). Therefore, it is interesting to examine the effectiveness of different *A. rhizogenes* strains on the induction of HRCs and the accumulation of high-value secondary metabolites compounds (VCR and VBL).

In the current study, the *A. rhizogenes* strain A4 was more efficient than 15834 for the production of VCR and VBL in HRCs of *C. roseus* originating from leaf explants after 60 days of cultivation. In agreement with the obtained results, (He *et al*., 2023) clearly showed that HR formation of *T. baccata* was successfully achieved by natural transformation using the *A. rhizogenes* A4 strain, with a transformation efficiency of 14.3% generating one HR. On the other hand, the production of indole alkaloids is quite complicated with several involved enzymes (Benyammi *et al*., 2016). Hughes *et al.* found that increased tryptophan availability could promote alkaloid accumulation during the exponential phase of the HRCs growth. Despite the

more than 300-times rise in tryptamine and tryptophan, they discovered that the concentration of TIAs had little to no change, except leukerinin, which increased by 81% following three days of the induction period. They concluded that TIA levels are closely regulated (Hughes *et al*., 2004). The tri-terpenes content in the hairy roots culture of *Panax ginseng* was increased by over-expressing farnesyl-pyrophosphate synthase and mevalonate,5-pyrophosphate decarboxylase enzymes (Kim *et al*., 2014). Likewise, metabolic engineering with the maize (C1) transcription factor increased the contents of diterpenoids in the hairy roots culture of *Salvia miltiorrhriza*. Additionally, metabolic engineering using the maize transcription factor (C1) in the hairy roots culture of *S. miltiorrhriza* improved the endogenous concentration of diterpenoids. Moreover, HRCs of *Papaver bracteatum* increased morphinan alkaloids *via* the over-expression of *Codeinonreductase* implicated in their synthesis pathways (Zhao *et al*., 2015). The synthesis of TIAs in the transgenic HRCs of *C. roseus* depends on the physical status and strength of the culture medium. On the other side, catharanthine, VCR, and VBL alkaloids could be extracted from the liquid culture (Hanafy *et al*., 2016).

Conclusion

This study proved that *A. rhizogenes* mediated the transformation of *C. roseus* hairy root cultures, depending on a comparison of two *A. rhizogenes* strains (A4 and 15834) on three studied explants (leaf, stem, and root) for different induction periods. Both strains enhanced the hairy root formation and the production of biomass, VCR, and VBL depending on the type of explants and the incubation period. Transformation of leaf explants with A4 strain and incubation for 60 days after subculture achieved the best performance. The findings obtained here revealed the possibility of using the HRCs of *C. roseus* as a promising technique for the production of hairy root biomass and high value phytoconstituents.

References

Acharjee, S., R. Kumar and N. Kumar. 2022. Role of plant biotechnology in enhancement of alkaloid production from cell culture system of *Catharanthus roseus*: A medicinal plant with potent anti-tumor properties. *Ind. Crops Prod.*, 176: 114298.

Ali, M., A. Mujib, D. Tonk and N. Zafar. 2017. Plant regeneration through somatic embryogenesis and genome size analysis of *Coriandrum sativum* L. *Protoplasma*, 254: 343-352.

Abdelkawy, A.M., S.O. Alshammari and H.A. Hussein. 2023. Effect of silver nanoparticles on tropane alkaloid production of transgenic hairy root cultures of *Hyoscyamus muticus* L. and their antimicrobial activity. *Sci. Rep*., 13: 10397.

Batra, J., A. Dutta, D. Singh, S. Kumar and J. Sen. 2004. Growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration. *Plant Cell Rep*., 23:148-154.

Benyammi, R., C. Paris, M. Khelifi-Slaoui, D. Zaoui, O. Belabbassi, N. Bakiri, M.M. Aci, B. Harfi, S. Malik, A. Makhzoum, S. Desobry and L. Khelifi. 2016. Screening and kinetic studies of catharanthine and ajmalicine accumulation and their correlation with growth biomass in *Catharanthus roseus* hairy roots. *Pharm. Biol.*, 54: 2033-2043.

Chaturvedi, V., S. Goyal, M. Mukim, M. Meghani, F. Patwekar, M. Patwekar, S.K. Khan and G.N. Sharma. 2022. A comprehensive review on *Catharanthus roseus* L. (G.) Don: Clinical Pharmacology, Ethnopharmacology and Phytochemistry. *J. Pharmacol. Res. Dev.*, 4: 17-36.

Debnath, B., W.S. Singh, M. Das, S. Goswami, M.K. Singh, D. Maiti and K. Manna. 2018. Role of plant alkaloids on human health: A review of biological activities. *Mater. Today Chem.*, 9: 56-72.

Dellaporta, S.L., J. Wood and J.B. Hicks. 1983. A plant DNA minipreparation: Version II. *Plant Mol. Biol. Report.*, 1: 19-21.

Elateeq, A.A., A.M.M. Gabr, A.M. Abdelkawy, N.M. Toaima, H.A. Bosila, M.M. Zarad, H.S. Ebrahim, J. Jiao, H. Pan, S. Ullah, Y. Fu. 2022. Establishment of *Gypsophila paniculata* root culture for biomass, saponin and flavonoid production. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 50(4): 12886.

Guerriero, G., R. Berni, J.A. Muñoz-Sanchez, F. Apone, E.M. Abdel-Salam, A.A. Qahtan, A.A. Alatar, C. Cantini, G. Cai, J.F. Hausman, K.S. Siddiqui, S.M.T. Hernández-Sotomayor and M. Faisal. 2018. Production of plant secondary metabolites: Examples, tips and suggestions for biotechnologists. *Genes*, 9(6): 309.

Hanafy, M.S., M.A. Matter, M.S. Asker and M.R. Rady. 2016. Production of indole alkaloids in hairy root cultures of *Catharanthus roseus* L. and their antimicrobial activity. *S. Afr. J. Bot.*, 105: 9-18.

Hazaa, M.M., K.A. El-Dougdoug and S. Abo El-Maaty. 2006. Eradication of banana viruses from naturally infected banana plants 2. Production of certified banana plants and virus tested. *J. App. Sci. Res.*, 2(10): 714-722.

He, J., J.P.A. Plácido, I. Pateraki, S. Kampranis, B.T. Favero and H. Lütken. 2023. Hairy root induction of *Taxus baccata* L. by natural transformation with *Rhizobium rhizogenes*. *Horticulturae*, 9(1): 4.

Hong, B., W.J. Li, A.H. Song and C.J. Zhao. 2013. Determination of indole alkaloids and highly volatile compounds in rauvolfia verticillata by HPLC-UV and GC-MS. *J. Chromatogr. Sci.*, 51: 926-930.

Hong, S.B., C.A.M. Peebles, J. V. Shanks, K.Y. San and S.I. Gibson. 2006. Terpenoid indole alkaloid production by *Catharanthus roseus* hairy roots induced by *Agrobacterium tumefaciens* harboring rol ABC genes. *Biotechnol. Bioeng.*, 93: 386-390.

Hughes, E.H., S.B. Hong, S.I. Gibson, J. V. Shanks and K.Y. San. 2004. Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid levels. *Biotechnol. Bioeng.*, 86: 718-727.

Ishikawa, H., D.A. Colby, S. Seto, P. Va, A. Tam, H. Kakei, T.J. Rayl, I. Hwang and D.L. Boger. 2009. Total synthesis of vinblastine, vincristine, related natural products, and key structural analogues. *J. Amer. Chem. Soc.*, 131: 4904-4916.

Kim, Y.K., Y.B. Kim, M.R. Uddin, S. Lee, S.U. Kim and S.U. Park. 2014. Enhanced triterpene accumulation in *Panax ginseng* hairy roots overexpressing mevalonate-5-pyrophosphate decarboxylase and farnesyl pyrophosphate synthase. *ACS Synth. Biol.*, 3: 773-779.

Makhzoum, A.B., P. Sharma, M.A. Bernards and J. Trémouillaux-Guiller. 2013. Hairy roots: An ideal platform for transgenic plant production and other promising applications. *Phytochemicals, Plant Growth, and the Environment*. Springer New York. pp. 95-142.

Malik, S., S. Bhushan, M. Sharma and P.S. Ahuja. 2016. Biotechnological approaches to the production of shikonins: A critical review with recent updates. *Crit. Rev. Biotechnol.*, 36: 327-340.

Morey, K.J. and C.A.M. Peebles. 2022. Hairy roots: An untapped potential for production of plant products. *Front. Plant Sci.*, 13: 1:17.

Mujib, A., A. Ilah, J. Aslam, S. Fatima, Z.H. Siddiqui and M. Maqsood. 2012. *Catharanthus roseus* alkaloids: Application of biotechnology for improving yield. *Plant Growth Regul.*, 682 68: 111-127.

Noble, R.L. 1990. The discovery of the vinca alkaloids - Chemotherapeutic agents against cancer. *Biochemistry and Cell Biology*. NRC Research Press Ottawa, Canada. pp.1344-1351.

Park, C.H., S. Zhao, H.J. Yeo, Y.E. Park, T.B. Baska, M.V. Arasu, N.A. Al-Dhabi and S.U. Park. 2017. Comparison of different strains of *Agrobacterium rhizogenes* for hairy root induction and betulin and betulinic acid production in *Morus alba*. *Nat. Prod. Commun.*, 12: 479-482.

Qin, S., Y. Liu, J. Yan, S. Lin, W. Zhang and B. Wang. 2022. An optimized tobacco hairy root induction system for functional analysis of nicotine biosynthesis-related genes. *Agronomy*, 12: 348.

Rawat, J.M., A. Bhandari, M. Raturi and B. Rawat. 2019. *Agrobacterium rhizogenes* mediated hairy root cultures: A promising approach for production of useful metabolites. *New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Secondary Metabolites Biochemistry and Applications*. Elsevier. pp. 103-118.

Snedecor, G.W. and W.G. Cochran. 1989. Statistical Methods. The Iowa State University Press U.S.A.

Taha, H.S., K.A. Shams, N.M. Nazif and M.M. Seif-El-Nasr. 2014. *In vitro* studies on Egyptian *Catharanthus roseus* (L.) G.Don V: Impact of stirred reactor physical factors on achievement of cells proliferation and vincristine and vinblastine accumulation. *Res. J. Pharm. Biol. Chem. Sci.*, 5: 330-340.

Verma, P. and A.K. Mathur. 2011. Agrobacterium tumefaciens-mediated transgenic plant production via direct shoot bud organogenesis from pre-plasmolyzed leaf explants of *Catharanthus roseus*. *Biotechnol. Lett.*, 33: 1053-1060.

Zhao, L., G.W. Sander and J. V. Shanks. 2013. Perspectives of the metabolic engineering of terpenoid indole alkaloids in *Catharanthus roseus* Hairy Roots. *Adv. Biochem. Eng. Biotechnol.*, 134: 23-54.

Zhao, S., J. Zhang, R. Tan, L. Yang and X. Zheng. 2015. Enhancing diterpenoid concentration in *Salvia miltiorrhiza* hairy roots through pathway engineering with maize C1 transcription factor. *J. Exp. Bot.*, 66: 7211-7226.

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