

Full Length Research Paper

Hairy roots production of transgenic *Catharanthus roseus* L. plants with *Agrobacterium rhizogenes* under *in vitro* conditions

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Hypocotyl explants of *Catharanthus roseus* L. gave rise to callus at a frequency of 90% when cultured on Murashige and Skoog (MS) basal medium supplemented BA (31.1 μM) and NAA (5.4 μM) on dark conditions. The calli (8 weeks old) were placed onto half strength liquid MS medium that had been polluted by bacterial clones of *Agrobacterium rhizogenes*. Transgenic of calli regenerated hairy roots after 10 days of inoculation and they have grown after 8 weeks. Hairy roots of *in vitro* *C. roseus* L. plant which produces several important terpenoid indole alkaloid by infection with *A. rhizogenes*. Transformed hairy root increased and produced alkaloids, including vinblastin and vincristine, which are extracted and analyzed by High Performance Liquid Chromatography (HPLC) and used for anticancer and antimicrobial researches.

Key words: *Catharanthus roseus*, *Agrobacterium rhizogenes*, genetic transformation, hairy roots.

INTRODUCTION

An exceedingly large population relies on pharmaceuticals derived from plants. It is estimated that 75% of the world's population is dependant upon plant-derived pharmaceuticals. The need for methods of increasing the production of plant-derived pharmaceuticals cost-effectively and with environmental consideration is becoming more important. Of particular interest are the pharmaceutically valuable alkaloids from the *Catharanthus roseus* (Gaines 2004). *C. roseus* (L.) Don. (Madagascar periwinkle) is a perennial tropical/subtropical plant belonging to the family *Apocynaceae* that produces more than 100 terpenoid indole alkaloids (TIAs) including two commercially important powerful cytotoxic dimeric alkaloids (vinblastine and vincristine) used in cancer chemotherapy (Magnotta et al., 2006; Moreno et al., 1995; Jaleel et al., 2007, 2008) and some other pharmaceutical compounds from

this plant, e.g., ajmalicine (anti-hypertensive) and serpentine (sedative) are also of economical importance and roots of this plant are the main source of alkaloid ajmalicine (Jaleel et al., 2006). These two drugs are produced in small yields within the plant, which makes them expensive to produce commercially (Gaines, 2004; Taha et al., 2008). An increase in production of these pharmaceutical through hairy root culture will result in greater accessibility and affordability of product (Gaines, 2004). The distribution and accumulation of the alkaloids in *C. roseus* were studied by Reda (1978).

Metabolic engineering of this plant by genetic transformation may lead to enhancement of the production of specific alkaloid compounds at the whole-plants level (Choi et al., 2004; David et al., 1984). During the past two decades, we have witnessed a significant increase in the number of reports on the successful *Agrobacterium rhizogenes*-mediated genetic transformation of various plant species, variants and cultivars (Estrella et al., 2005).

In this study, production and growth hairy roots of transgenic callus with *A. rhizogenes* was investigated. The purpose of this research is production of hairy roots from transgenic callus that they have a high level of alkaloids than usual plants.

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Abbreviations: NAA, α -Naphthalen acetic acid; BA, 6-benzyladenine; MS, Murashige and Skoog.

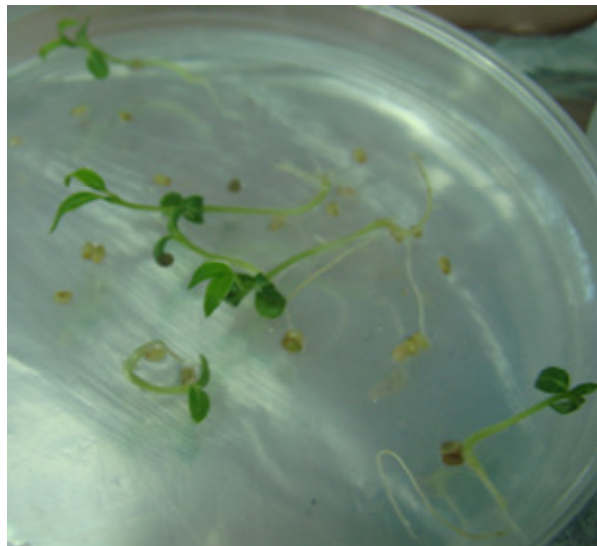


Figure 1. seedlings from *C. roseus* L. seeds On MS medium free hormone.

MATERIALS AND METHODS

Plant materials

Seed culture

Seeds of *C. roseus* were obtained from Research Center Mahallat and Shahre Rey. Seeds surface were sterilized under aseptic conditions of laminar flow hood, using 5% NaOCl (hypochlorite) for 30 min and were washed by sterile water for 3 - 4 times. Seeds to germinate into seedling were cultured on solid MS medium (Murashige and Skoog, 1962). The media was dispensed (25 ml) into each plastic petri dish (9 mm). All cultures were maintained under light (approximately 3 W/m² from cool-white fluorescent lamps with a 16/8 h light dark photoperiod) at temperature 25°C.

Production of callus

Hipocotyl explants that were produced from 15-day-old per winkle seedlings, approximately were cut into 0.7 - 1 cm long explants and cultured on MS agar medium supplemented with 6-Benzyladenine (31.1 µM) and α-Naphthalen acetic acid (5.4 µM). Each treatment consisted of 4 explants per dish with 20 replicates. All cultures were placed on dark conditions at 25 - 30°C for 8 - 12 weeks.

Different media preparation

MS medium: The basal medium used throughout the experiments consisted of MS inorganic salts, 100 mg l⁻¹ myo-inositol, 0.4 mg l⁻¹ thiamine, 3% sucrose, and 4 g l⁻¹ agar. The pH of the different culture media (seed culture, callus production and hairy roots formation) were adjusted at 5.8 using 0.1 N NaOH or HCL, then autoclaved at 121°C and the pressure was 1.2 Kg. cm² for 20 min. (Taha et al., 2008).

Agrobacterium medium: LB agar medium was used as a complete medium for *A. rhizogenes* strain. It consists of (g l⁻¹): 5 g glucose, 10 g Bacto-peptone, 10 g Bacto-yeast extract, 5 g NaCl, 7 g agar and 960 ml water. The pH was adjusted at 7 - 7.5, after autoclaving the medium was dispensed (15 ml) into each plastic petri dish.

Bacteria culture

A. rhizogenes RI000 (that were cultured into LB agar medium) was used for inoculation with callus explants. The bacteria for growth and multiplication were cultured into LB agar medium.

Callus inoculation with bacteria

The bacteria were inoculated into half strength liquid MS basal medium before callus explants were placed into the tube. After 30 min of incubation, callus explants were placed onto solid MS basal medium and co-cultivated. After 48 h of inoculation, callus explants were rinsed three times with half strength liquid MS basal medium supplemented with 800 mg l⁻¹ cefataxime.

Hairy roots culture medium

For hairy roots production, callus explants (after inoculate), were transferred onto solid MS basal medium (free hormone) with 400 mg l⁻¹ cefataxime and placed on dark conditions.

RESULTS AND DISCUSSION

Seed growth

Sterile seeds of perwinkle plant (that were placed onto MS medium) were germinated after 5 days of culture and the seedling (approximately 35 - 45 mm long) was produced after 15 days of culture (Figure 1).

Callus formation

Hipocotyl explants of *C. roseus* seedlings formed callus

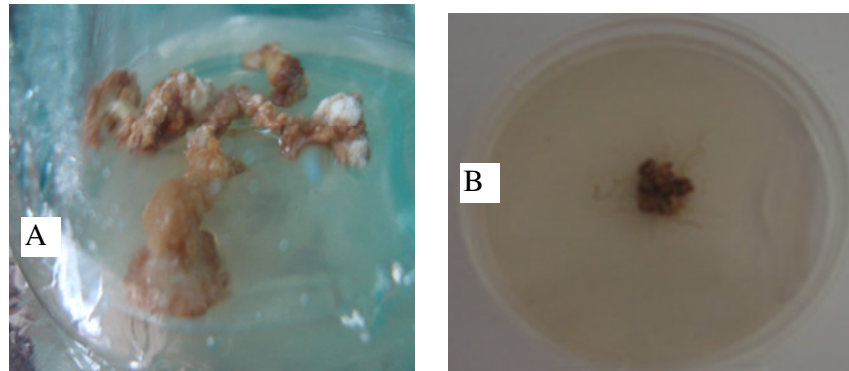


Figure 2. (A) Non-transgenic calli (B) transgenic calli on MS medium free hormone.

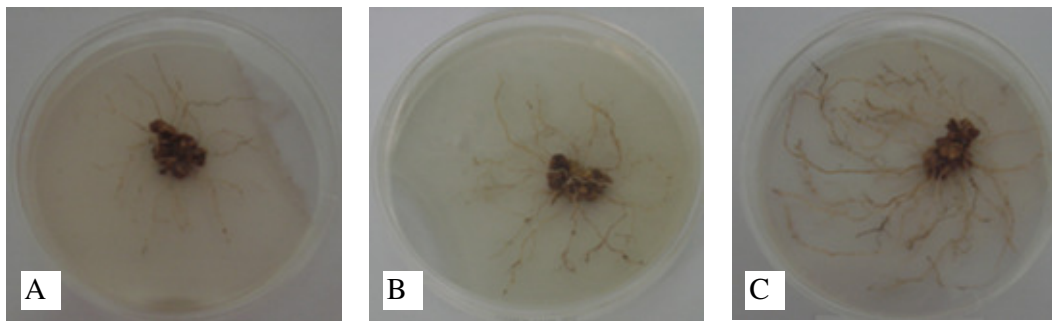


Figure 3. Hairy root regenerated from transgenic calli (A) 20 days (B) 40 days (C) 60 days after inoculation with *A. rhizogenes*.

on the cut edges after 7 - 10 days of culture. The callus of growth during 8 weeks.

Hairy roots process

Callus-explants cultures maintained on MS basal medium (free-hormones) formed hairy roots on after 10 days of inoculation. The hairy roots had a suitable growth and 8 - 9 cm long after 8 weeks (Figure 2).

Investigation of morphology of natural and transgenic callus

After gene transformation, morphology investigation of periwinkle plant callus has shown that cultural media of callus has not changed after gene transformation and it has been that same as MS, as well as, cultural media of non-transgenic callus has been contented with plant growth regulators (NAA and BA), But there was no rooting in non-transgenic callus, Although, transgenic callus has been cultured in media without hormone, They did rhizogenesis and produced hairy roots (Figure 3). The

observations have demonstrated that gene transferring is *A. rhizogenes* to plant, because after trans-fragment T (T-DNA) from plasmid of *Agrobacterium* to gene plant that plants derived from hairy roots retained the Ri TI-DNA (Choi et al., 2004) and it makes phyto-hormones (auxin and cytokinin) itself and it does not need hormone, again. The mechanism had been shown in plant through hairy roots production with suitable growth in periwinkle plant callus after gene transformation. Hairy roots increased during culture on MS medium free hormone (Figure 4). Alkaloids produced from hairy roots regenerated, extracted with soluble and analyzed by high performance liquid chromatography (HPLC). The important alkaloids were vincristin and vinblastin that were used for antimicrobial and anticancer researches. The soil-borne plant pathogen *A. rhizogenes* causes production of transformed hairy roots (is a pathological syndrome of dicotyledonous plants) at the infection site in plants (Giri et al., 2001; Choi et al., 2004; Tanaka et al., 2004; Wang et al., 2001), Hairy roots are characterized by high growth rate and genetic stability (Choi et al., 2004). In recent years production of transgenic plants of *C. roseus* by *A. rhizogenes* has been developed (Zarat and Verpoorte, 2007; O'keefe et al., 1997; Taha et al., 2008; Hughes et al.,

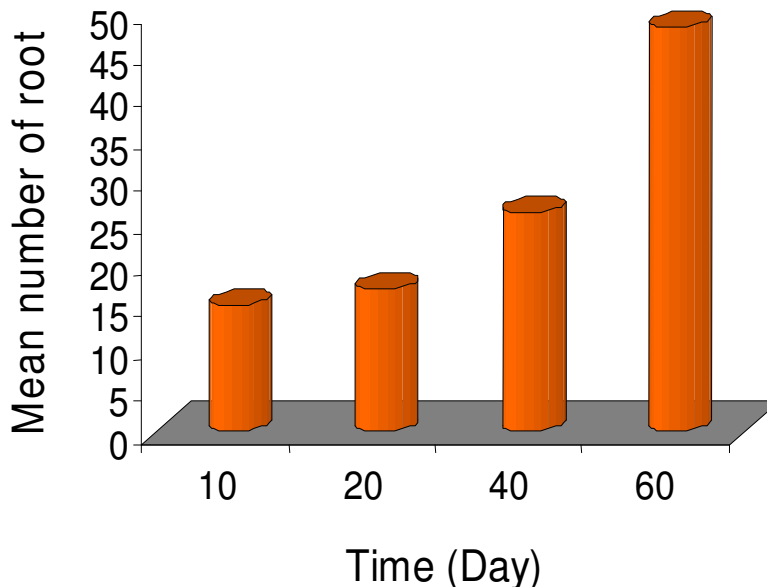


Figure 4. Mean number of hairy root regenerated during culture period.

2004; Brown et al., 2009; Guillon et al., 2002; Tanaka et al., 1994).

REFERENCES

- Choi P, Kim Y, Choi K, Chong H, Choi D, Liu J (2004). Plant regeneration from hairy root cultures transformed by infection with *Agrobacterium rhizogeneses* in *Catharanthus roseus*, *Plant Cell Rep.*, 22: 828-831.
- David C, Chilton MD, Tempe J (1984). Conservation of T-DNA in plants regenerated from hairy root cultures. *Biotech.*, 2: 73-76.
- Gaines JL (2004). Increasing alkaloid production from *Catharanthus roseus* suspensions through methyl jasmonate elicitation. *Pharm. Eng. J.*, 24: 24-35.
- Guillon S, Gantet P, Trémouillaux-Guiller J, Thiersault M, Rideau M (2002). Hairy Roots of *Catharanthus roseus*: Efficient routes to monomeric indole alkaloid. *J. Chromatogr.*, 955: 87-93.
- Herrera-Estrella L, Simpson J, Martinez-Trujillo M (2005). Transgenic plants: An historical perspective, *Methods Mol. Biol.*, 286: 3-32.
- Hughes EH, Hong SB, Gibson SI, Shanks JV, San KY (2004). Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine, *Metab Eng.*, 6: 268-276.
- Jaleel CA, Sankar B, Sridharan R, Panneerselvam R (2008). Soil salinity alters growth, chlorophyll contents and secondary metabolite accumulation in *Catharanthus roseus*, *Turk. J. Biol.*, 32: 79-83.
- Jaleel CA, Gopi R, Kishorekumar A, Manivannan P, Sankar B, Panneerselvam R (2008). Interactive effects of triadimefon and salt stress on antioxidative status and ajmalicine accumulation in *Catharanthus roseus*, *Acta Physiologiae Plantarum*, 30: 287-292.
- Jaleel CA, Gopi R, Sankar B, Gomathinayagam M, Panneerselvam R (2008). Differential responses in water use efficiency in two varieties of *Catharanthus roseus* under drought stress, *Comptes Rendus Biologies*, 331: 42-47.
- Jaleel CA, Gopi R, Sankar B, Manivannan P, Kishorekumar A, Sridharan R, Panneerselvam R (2007). Alterations in germination, seedling vigour, lipid peroxidation and proline metabolism in *Catharanthus roseus* seedlings under salt stress, *South Afr. J. Bot.*, 73: 190-195.
- Jaleel CA, Gopi R, Alagulakshmanan M, Panneerselvam R (2006). Triadimefon induced changes in the antioxidant metabolism and ajmalicine production in *Catharanthus roseus* (L.) G. Don., *Plant Sci.*, 171: 271-276.
- Jaleel CA, Gopi R, Manivannan P, Panneerselvam R (2007). Responses of antioxidant defense system of *Catharanthus roseus* (L.) G. Don. to paclobutrazol treatment under salinity, *Acta Physiologiae Plantarum*, 29: 205-209.
- Jaleel CA, Gopi R, Manivannan P, Kishorekumar A, Sankar B, Panneerselvam R. (2006b). Paclobutrazol influences vegetative growth and floral characteristics of *Catharanthus roseus* (L.) G. Don., *Indian J. Appl. Pure Biol.*, 2: 369-372.
- Jaleel CA, Gopi R, Panneerselvam R (2008). Growth and photosynthetic pigments responses of two varieties of *Catharanthus roseus* to triadimefon treatment, *Comptes Rendus Biologies*, 331: 272-277.
- Jaleel CA, Manivannan P, Sankar B, Kishorekumar A, Panneerselvam R, (2008). Salt stress mitigation by calcium chloride in *Phyllanthus amarus*, *Acta Botanica Croatica*, 67: 53-62.
- Jaleel CA, Gopi R, Manivannan P, Panneerselvam R (2008). Soil salinity alters the morphology in *Catharanthus roseus*; Effects on endogenous mineral constituents, *Eur-Asian J. BioSci.*, 2: 18-25.
- Magnotta M, Murata J, Chen V, De-Luca A (2006). Identification of a low vindoline accumulating cultivar of *Catharanthus roseus* (L.) G. Don. by alkaloid and enzymatic profiling, *Phytochem.*, 67: 1758-1764.
- Moreno PRH, Van Der Heijden R, Verpoorte R (1994). Elicitor-mediated induction of isochlorismate synthase and accumulation of 2,3-dihydroxybenzoic acid in *Catharanthus roseus* cell suspension and shoot cultures. *Plant Cell Rep.*, 14: 188-191.
- O'keefe B, Mahady R, Ahady GB, Gills JJ, Beecher CW, Schilling AB (1997). Stable vindoline production in transformed cell cultures of *Catharanthus roseus*, *J. Nat. Prod.*, 60: 261-264.
- Reda F (1978). Distribution and accumulation of alkaloids in *C. roseus* G. Don during development, *Pharmazie*, 33: 223-233.
- Taha HS, Salah EM, Ola I, El-Hamshary M, Nahla S, Naglaa A, Nazif M, El-Bahr M, Medhat M, El-Nasr S (2008). *In vitro* studies on Egyptian *Catharanthus roseus* (L.) G. Don: III. Effects of extra tryptophan decarboxylase and strictosidine synthase genes copies in indole alkaloid production, *J. Cell Mol. Biol.*, 2: 18-23.
- Taha HS, El-Bahr MK, Seif-El-Nasr MM (2008). *In vitro* studies on Egyptian *Catharanthus roseus* (L.) G. Don.: 1- Calli production, direct

- shootlets regeneration and alkaloids determination. J. Appl. Sci. Res., 4: 1017-1022.
- Tanaka N, Suzuki K, Yamashita I, (2004). Evidence of horizontal gene transfer from *Agrobacterium rhizogenes* to tobacco plants during the evolutionary process, Endocytobiosis Cell Res., 15: 151-162.
- Tanaka N, Takao M, Matsomoto T (1994). *Agrobacterium rhizogeneses*-mediated transformation and regeneration of vinca minor L. Plant Tissue Cult. lett., 11: 191-198.
- Wang Y, Wang JB, Luo D, Jia JF (2001). Regeneration of plants from callus cultures of roots induced by *Agrobacterium rhizogenes* on *Alhagi pseudoa*.
- Zarate R, Verpoorte R (2007). Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Don., J. Phytochem. Rev., 6: 475-491. Ihagi, Cell Research. 11: 279-284.