Scientia Horticulturae 119 (2009) 325–329

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Screening of vincristine yield in *ex vitro* and *in vitro* somatic embryos derived plantlets of *Catharanthus roseus* L. (G) Don.

Junaid Aslam [a,b], Abdul Mujib [a,*], Seikh A. Nasim [a], Maheshwar Prasad Sharma [a]

[a] *Cellular Differentiation and Molecular Genetics Section, Department of Botany, Hamdard University, New Delhi 110062, India*
[b] *Plant Tissue Culture Laboratory, Dubai Pharmacy College, Al-Muhaisanah 1, Al-Mizhar, P.O. Box 19964, Dubai, United Arab Emirates (U.A.E.)*

ARTICLE INFO

Article history:
Received 13 April 2008
Received in revised form 11 August 2008
Accepted 15 August 2008

Keywords:
Apocynaceae
Catharanthus roseus
Embryogenic callus
High performance liquid chromatography
Somatic embryogenesis
Vincristine

ABSTRACT

Catharanthus roseus L. (G.) Don. (Apocynaceae) is an important dicotyledonous medicinal plant as it is the sole source of vincristine and vinblastine that are used against a variety of cancers. Quantitative estimation of vincristine was carried out using high performance liquid chromatography (HPLC) in various *in vitro* grown tissues; calluses (embryogenic and non-embryogenic callus), embryogenic stages (proliferated, matured and germinated embryos), somatic embryo derived plantlets (leaf, root and whole plant) and leaves of *ex vitro* developed plantlets. The yield in those *in vitro* and *ex vitro*-developed tissues was monitored for 30 weeks. Except at an early lag period, vincristine production was detected up to 20–25 weeks old plant samples. Vincristine content was very high in leaf callus and germinated embryos. Leaves of *in vitro* raised plants showed higher amount of vincristine when compared to *ex vitro*-developed leaves of similar age. Vincristine production was tissue specific and age dependent that was discussed in detail in this present communication.

1. Introduction

The genus *Catharanthus* comprises of eight species, which are subshrubs (30–90 cm long), generally upright or decumbent, produce white latex and a strong pungent smell when damaged (Anonymous, 1992). The *C. roseus* has a pantropic distribution, naturalized in continental Africa, America, Asia, Australia, South Europe and on some islands in the Pacific Ocean. It is a rich source of alkaloids; more than 130 different compounds have so far been reported (Verpoorte et al., 1993; Van der Heijden et al., 2004), in which vincristine (Fig. 1) is highly important. Vincristine is mainly used to treat against acute leukemia, Hodgkin's disease, rhabdomyosarcomas, neuroblastoma and other lymphomas (Mukherjee et al., 2001; Van der Heijden et al., 2004). Traditionally, *C. roseus* is propagated through seed, which leads to genetic segregation and decline in uniform yield of dimeric alkaloid (Venkataramaiah et al., 1980; Chand and Singh, 1999, 2004). Structurally, vincristine and vinblastine (another anticancerous compound, present in the same plant) are bisindole alkaloids with vindoline attached to tetracyclic indole, carbomethoxyvelnamine. These two compounds are very similar in structure and display nearly the same kind of action by inhibiting cell division at metaphase. Isolation of this substance from wild plant is impeded by low productivity, is therefore very expensive. Thus, synthetic preparations have been attempted to produce these alkaloids in several laboratories at an affordable rate. Although total synthesis of vinblastine has been achieved in a number of cases synthesis of vincristine has not been accomplished except a recent report of stereo-selecting coupling of demethyl vindoline with an eleven membered carbomethoxyverbanamine precursor (Kuboyama et al., 2004). This *de novo* synthesis of vincristine was described to be the first synthesis of its kind, which was not dependent on derivatization of vinblastine or on semi-synthesis with vindoline—the other two ways of syntheses, previously reported (Kuboyama et al., 2004). As the yield is not always good, a comprehensive multidisciplinary approach has long been attempted in order to improve the content (Verpoorte et al., 1993; Moreno et al., 1995; Van der Heijden et al., 1989, 2004). In plant tissue culture, different tissues of this plant have been used to establish cultures and the content of dimeric alkaloid was analyzed (Hirata et al., 1990; Batra et al., 2005), but the yield of this compound is still not very high. The technique of somatic embryogenesis has been used and reported in a wide range of plant species (Thorpe, 1995; Mujib and Samaj, 2006) but the same embryonic tissue has never been utilized in alkaloid enrichment programme as somatic embryogenesis report in *C. roseus* is relatively new (Junaid et al., 2006, 2007a,b). In the present study we quantified the level of vincristine from various *in vitro* raised tissues and a comparison was made with the plants grown

* Corresponding author. Tel.: +91 11 26059683x5542; fax: +91 11 26059663.
E-mail address: amujib3@yahoo.co.in (A. Mujib).

0304-4238/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2008.08.018

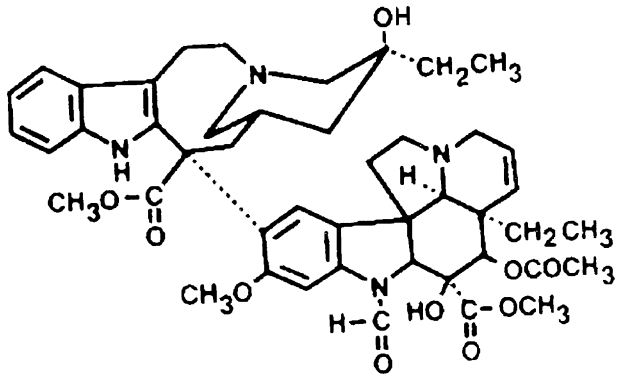

Fig. 1. Chemical structure of vincristine.

ex vitro. This protocol may be used in pharmaceutical industries to synthesize enhanced level of vincristine.

2. Materials and methods

2.1. Plant material, sterilization, in vitro seed germination and culture condition

Catharanthus roseus L. (G). Don. was collected from the Jamia Hamdard (Hamdard University, New Delhi) herbal garden, was identified by one of our experts (Dr. M.P. Sharma, Plant taxonomist, Department of Botany) and used as experimental material. The voucher specimen has been deposited in the Herbarium of the same Department, Faculty of Science, Jamia Hamdard (Hamdard University, New Delhi, India).

Various explants (leaf, nodal segments and root) and unripe fruits were used as explants. The surface sterilization of explants and the method of *in vitro* seed germination were discussed earlier (Junaid et al., 2006). Germination medium (Murashige and Skoog, 1962) contained all the components of MS and 3% sucrose but without organic compounds and plant growth regulators. Germinated seedlings were grown until they reached 2 cm in length; the hypocotyls were later excised for culture.

The medium pH was adjusted to 5.8. The medium was sterilized in an autoclave for 15 min at 121 °C. Cultures were incubated at 25 ± 2 °C under 16h photoperiod with cool white fluorescent illumination (100 μmol m^{-2} s^{-1} PFD).

2.2. Induction of callus

The initial procedure of establishment of callus was carried out as described earlier (Junaid et al., 2006). The explants were cultivated on MS supplemented with optimized concentration of 2,4-D (6.96 μM).

2.3. Somatic embryo (SE) initiation and proliferation

The embryogenic callus was cultured on medium added with 3% sucrose and optimized concentrations of 5.37 μM NAA + 6.72 μM BAP for embryo initiation and further proliferation (Junaid et al., 2006). Half of the proliferated culture was processed for alkaloid extraction and the rest of the embryos were maintained in medium for maturation.

2.4. SE maturation and germination

SE maturation was made in optimized medium that was discussed at length in earlier report (Junaid et al., 2006). The medium basically amended with 3% maltose and optimized 2.60 μM GA_3. Part of the matured embryos was oven dried for extraction and the other half was cultured for germination/plantlet conversion.

Matured SEs were cultured on MS that was added with optimized level of BAP (2.24 μM) for ensuing quick germination (Junaid et al., 2006). Plantlets, roots, etc. developed from *in vitro* germinated embryos were separately dried for alkaloid extraction purpose.

2.5. Quantification of vincristine by HPLC and sample preparation

The content of vincristine in field-grown plants and *in vitro* cultures were estimated using vincristine (Sigma–Aldrich) as standard.

One gram each of various harvested *in vitro* cultural sample and field grown *Catharanthus* plant were dried separately at 45 °C for a week and pulverized in a mortar and pestle. Dried material was shaken in 20 ml methanol for 24 h, evaporated up to 2 ml and then 20 ml of (0.5)N H_2SO_4 (Hi-media Lab., India) was added. Solution was made alkaline by adding 25% ammonium hydroxide (Hi-media Lab., India) solution (pH 9–12) and finally extracted three times with chloroform (Hi-media Lab., India) and evaporated up to dryness. The left residue was reconstituted in 4 ml methanol (Hi-media Lab., India). The methanol soluble fraction of each sample was further diluted to 1:10 and processed for HPLC.

2.6. Standard vincristine and standard curve

Two milligram of vincristine sulphate with over 97% purity available from Sigma–Aldrich (St. Louis, U.S.A.) was processed in the same way similar to sample preparation. It was prepared in methanol and 1 ml of this solution was diluted to 10 ml with methanol to get 100 μg^{-ml} solutions, the solution was used for application on TLC plate for preparation of standard plot. The UV spectrum of standard vincristine solution in methanol (50 mg^{-ml}) was recorded using a UV spectrophotometer (UV-1601, Shimadzu, Japan) for authentication. The λ_{max} (220 nm) obtained was matched with that as reported for standard vincristine (Chu et al., 1996).

Stock solution 1 mg^{-ml} of vincristine was prepared in methanol. From the stock solution, dilution of 2–20 μl was taken in triplicate and analyzed independently by HPLC and a standard curve was plotted between concentration and peak area. The injected quantities showed good linearity. The data of peak area *vs.* vincristine concentration were treated by linear least-square regression and the regression equation thus obtained from standard curve was used to estimate vincristine in different samples.

2.7. High performance liquid chromatography (HPLC) and quantification of vincristine in different samples

For HPLC analysis, A Merck LiChro CART C18 column (125 mm × 4 mm 5 μm) and the solvent systems (acetonitrile and methanol; 3:1) were used at a constant flow rate of 1 ml^{-min} with 2300 pressure. A PDA detector was employed for detection of peaks, set at wavelength of 220 nm and bandwidth of 5. All the analyses were performed with three replicates.

Samples of *in vitro* culture and field grown plant, 20 μl each, were applied in triplicate for quantification of vincristine. The alkaloid was quantified by using regression equation of calibration curve.

2.8. Statistical analysis

The influence of various days' intervals at vincristine yield was analyzed by one-way analysis of variance (ANOVAs). Values are