

Somatic Embryos in *Catharanthus roseus*: a Scanning Electron Microscopic Study

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Abstract

Catharanthus roseus (L.) G. Don is an important medicinal plant as it contains several anti-cancerous compounds, like vinblastine and vincristine. Plant tissue culture technology (organogenesis and embryogenesis) has currently been used in fast mass propagating raw materials for secondary metabolite synthesis. In this present communication, scanning electron microscopic (SEM) study of somatic embryos was conducted and discussed. The embryogenic callus was first induced from hypocotyls of *in vitro* germinated seeds on which somatic embryos, differentiated in numbers, particularly on 2,4-D (1.0 mg/L) Murashige and Skoog (MS) was medium. To understand more about the regeneration method and *in vitro* formed embryos SEM was performed. The SEM study revealed normal somatic embryo origin and development from globular to heart-, torpedo- and then into cotyledonary-stage of embryos. At early stage, the embryos were clustered together in a callus mass and could not easily be detached from the parental tissue. The embryos were often long cylindrical structure with or without typical notch at the tip. Secondary embryos were also formed on primary embryo structure. The advanced cotyledonary embryos showed prominent root- and shoot axis, which germinated into plantlets. The morphology, structure and other details of somatic embryos at various stages were presented.

Keywords: electron microscopy, embryogenic callus, Madagascar periwinkle, scanning somatic embryo

Introduction

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) is an important species of the *Catharanthus* genus, native and endemic in Madagascar. The species has long been cultivated as herbal medicine and ornamental as well (Gupta *et al.*, 2007). In traditional medicine, the plant extracts have been used to treat numerous diseases including diabetes, malaria and Hodgkin's disorder (Mukherjee *et al.*, 2001). The primary compounds like vinblastine and vincristine, extracted from the plant are used in the treatment of leukemia, however, *in vivo* grown tissues contain a very low level of drugs (0.0005% dry weight basis) (Van der Heijden *et al.*, 2004). Cell and molecular biological studies have currently been employed to improve alkaloid yield and, in some cases, the *in vitro* grown tissues showed enhanced yield of alkaloids (Junaid *et al.*, 2009; 2010). Several key factors controlling the biosynthesis of alkaloids have also been optimized (Moreno *et al.*, 1995; Mujib *et al.*, 2002; Mujib *et al.*, 2012). In plant biotechnology, various plant parts have been used in establishing culture for plant regeneration by various methods, the information on micropropagation via somatic embryogenesis in *C. roseus* is however, relatively new (Dhandapani *et al.*, 2008; Junaid *et al.*, 2006; 2007). The advantages of somatic embryogenesis over other methods is its unlimited potentiality in producing clones, to be used as medicinal raw material for secondary product synthesis

(Mujib and Samaj, 2006). Although there is a risk of somaclonal variation in indirect embryogenesis, particularly developed from long term culture (Hao and Deng, 2002; Mujib *et al.*, 2013), the technique has often been used in several biotechnological researches including transgenic programs (Davletova *et al.*, 2001; Rommens *et al.*, 2004; Walter, 2004). Even though somatic embryogenesis plays an important role in producing somaclones in numbers (Azad *et al.*, 2009; Wang *et al.*, 2006; Yan *et al.*, 2010) the ontogeny, structure, development and conversion of plantlets have not always been studied in detail, as the whole process of *in vitro* embryogeny is quite complex (Vooková and Kormutak, 2006). In *C. roseus*, somatic embryos were obtained and it closely mimics zygotic embryogenesis, i.e. by producing all forms of embryo stages (globular, heart, torpedo and cotyledonary), which finally germinated into plantlets (Junaid *et al.*, 2006). Ultra-structural studies, involving transmission electron microscopy (TEM) and scanning electron microscopy (SEM), have often been used to investigate the ontogeny, structure and development of embryos in various studied plant materials (Bradley *et al.*, 2001; Ovečka and Bobák, 1999), in this plant no such investigation has been carried out yet. In this communication, SEM investigation was conducted and the origin of embryo generation from callus was presented, describing the morphology/structure, the growth and development of *in vitro* embryos in *C. roseus*.

Materials and methods

Plant Material

Catharanthus roseus (L.) G. Don fruits were collected from herbal garden, Jamia Hamdard (Hamdard University), New Delhi and were used as experimental material; the plant was earlier authenticated for *in vitro* studies (Junaid *et al.*, 2006).

Surface sterilization and *in vitro* seed germination

Surface sterilization was carried out following Junaid *et al.* (2006) method. In brief, the seeds were isolated from fruits inside a laminar hood, treated with 0.5% mercuric chloride for 2 minutes, followed by 1.0% (w/v) H₂O₂ for 3 minutes, and finally rinsed with sterilized double distilled water. These surface-disinfested seeds (20-25) were placed in GA-7 Magenta vessels (Sigma, St. Louis, MI, USA) containing 50 ml MS (Murashige and Skoog, 1962) medium without organic compounds and plant growth regulators. The medium was adjusted to pH 5.7 and sterilized at 121 °C for 15 minutes.

Embryogenic callus induction

Germinating seedlings were grown until they attained a height of 2 cm, later these seedlings were removed from the culture vessels. Hypocotyls (6-8 mm) of 5 to 7 days old seedlings were excised as explants and placed on MS medium supplemented with optimized concentrations of 2, 4-D (1.0 mg/L) for the induction of callus.

The induced calluses were sub-cultured at a regular interval of 4 weeks. The callus later transformed into embryogenic callus on medium supplemented with same concentration of 2, 4-D (1.0 mg/L) or in medium amended with NAA (1.0 mg/L) and BAP (1.5 mg/L). The entire embryo induction process was described earlier and was followed (Junaid *et al.*, 2006).

Scanning Electron Microscopy (SEM)

For SEM, the embryo bearing embryogenic callus was fixed in 2% glutaraldehyde, adjusted to pH 6.8 in 0.1M phosphate buffer for 24 h at 4 °C. The tissue was washed in buffer, postfixed for 2 h in similarly buffered 1% osmium tetroxide, dehydrated in a graded ethanol series and finally coated with gold palladium.

The prepared samples were examined and photographed in a LEO 435 VP (Zeiss, Oberkochen, Germany) scanning electron microscope, operating at 15-25 kV.

Results and discussion

Embryogenic callus induction

Hypocotyls of *in vitro* germinated seeds were used as explant on 2,4-D added MS medium, which induced white to yellowish callus within 8-10 days of incubation. The callus growth was prolific, which later turned into embryogenic callus within 4 weeks of culture.

The embryogenic callus was white, granular, friable, fast growing, and started to differentiate somatic embryos (Figs. 1A and 1B) within 4 - 6 weeks of culture.

The importance of auxins especially 2,4-D in inducing callus and in triggering embryogenesis has been reported in a

number of studied plants (Gaj, 2004; Mujib *et al.*, 2006). In the case of this plant as well, 2,4-D was very efficient in producing callus and embryos; and this synthetic auxin was earlier observed to be very important in several investigated plants during callus induction and early embryogenesis stages (Pasternak *et al.*, 2002; Su *et al.*, 2009).

SEM study of embryo bearing embryogenic callus revealed different forms of somatic embryos appeared on callus surfaces that are presented in Fig. 2 (A-E). The embryos were developed as a heterogeneous mixture, where globular embryos were low in number. The somatic embryos were smooth surfaced, often clustered together in a common mass and could not easily be detached from the parental tissue. SEM of callus also revealed that several somatic embryos were long cylindrical structures devoid of unique notches with constricted base firmly attached to callus (Figs. 2A and 2C).

Heart shaped embryos were maximum in number with typical notch at the tip (Fig. 3 A-D).

In several embryogenic cultures, secondary embryos were also formed at basal end of heart- and torpedo embryos. Torpedoes were less in number, compared to the cotyledonary embryos. The cotyledonary embryos showed distinct shoot end with well-defined cotyledonary leaf primordia (flanked at the two sides) and root axis (Fig. 3 E). Beside embryos' normal forms and structure, SEM study also revealed embryos of various aberrant morphological types (not shown).

Somatic embryos obtained from *in vitro* grown tissues had been reported in a number of plant genera (Bajaj, 1995; Mujib and Samaj, 2006; Pasternak *et al.*, 2002; Raghavan, 1986; Thorpe, 1995) but the incident has been relatively new in *C. roseus*.

The formation of somatic embryos is one of the important methods of *in vitro* regeneration in *C. roseus* and having the potential of producing unlimited clones with functional root and shoot system in contrast to organogenesis where shoots and roots were induced separately on two different media. The culture of plant tissues via somatic embryos on a large scale presented the possibility of production of synthetically coated seeds/synthetic seed (Gray *et al.*, 1995; Mehpara *et al.*, 2012; Rai *et al.*, 2009).

In the present study, embryogenic callus induced from hypocotyl of *in vitro* germinated seeds were used for SEM study, which helped describe embryo morphology and structure more efficiently; it also indicates about embryos' origin i.e. of being single cell or from pro-embryogenic mass of cells.

The present study agreed with previous similar observation, reported by other workers (Brown *et al.*, 1995; Corredoira *et al.*, 2003). Schlogl *et al.* (2012) reported that the embryogenic program expression could subsequently be monitored on transformation of pro-embryogenic clusters into first visible globular embryos. Rodríguez *et al.* (1995) studied different somatic embryonic stages (globular, heart, torpedo and cotyledonary) in sugarcane by SEM, in which globular embryos, with lateral notch and scutellum, were observed. In this study, advanced somatic embryos appeared to have well organized shoot meristem with root primordial ends, which later turned into leaves and roots respectively.

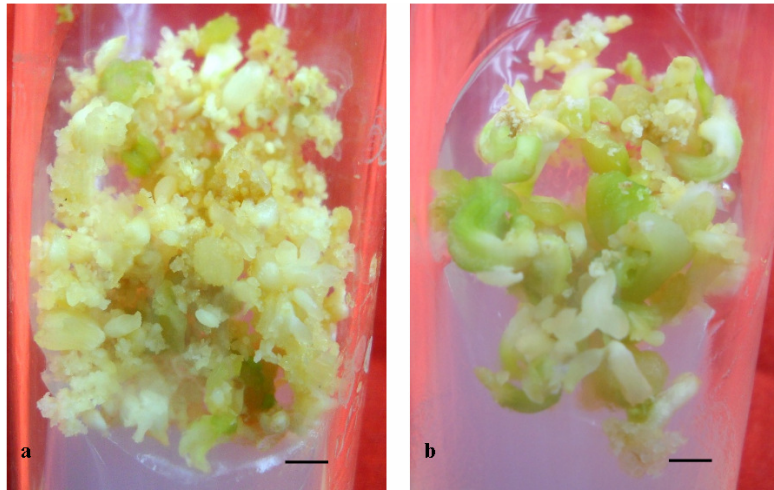


Fig. 1a. and 1b. Morphological view of 8-10 weeks old cultures showing somatic embryos (torpedo, cotyledonary) with secondary embryos in *C. roseus* (bar a, b: 0.5cm)

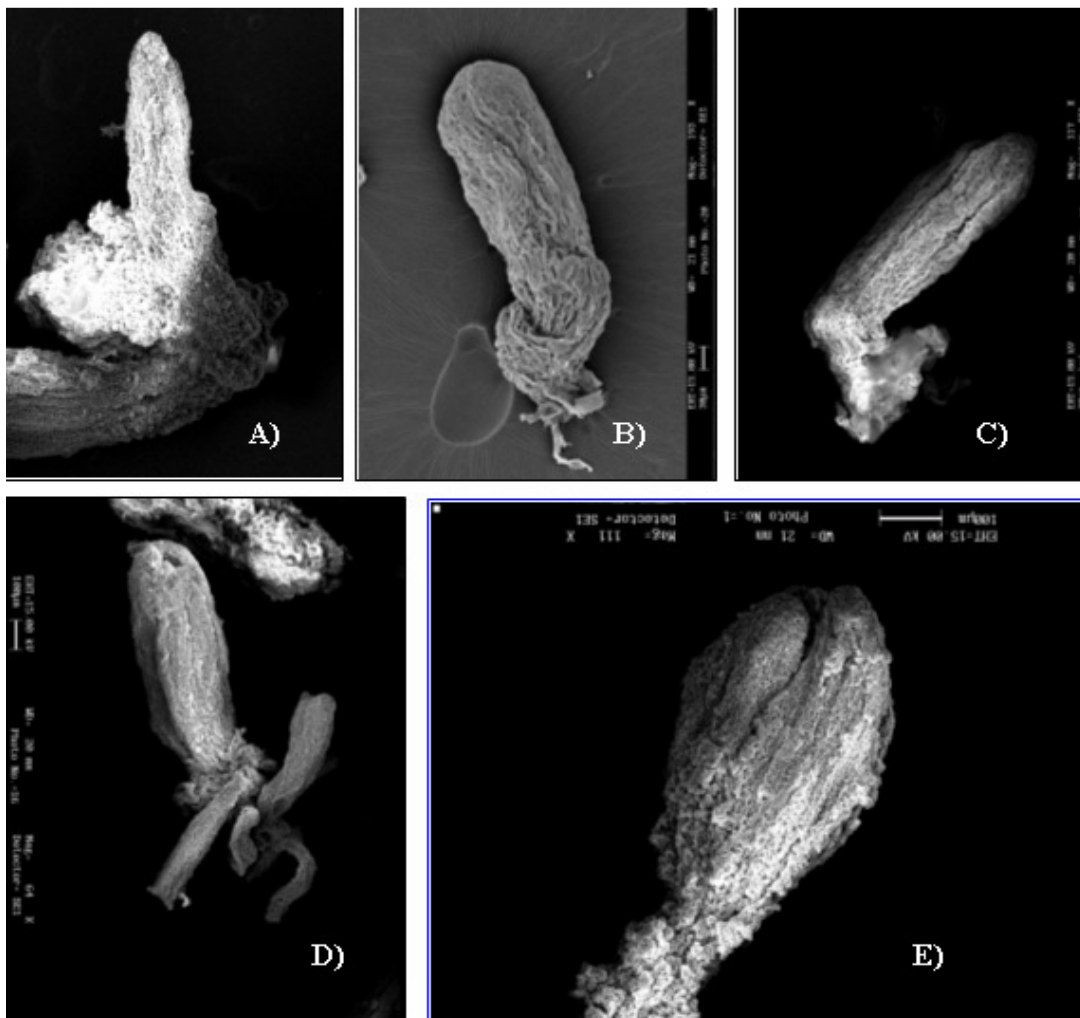


Fig. 2. SEM of somatic embryos at various stages of development; A, B, C) Cylindrical somatic embryos without any furrows at the apex; D) A budding embryo; E) An embryo showing a developing furrow at the apex and a small root end

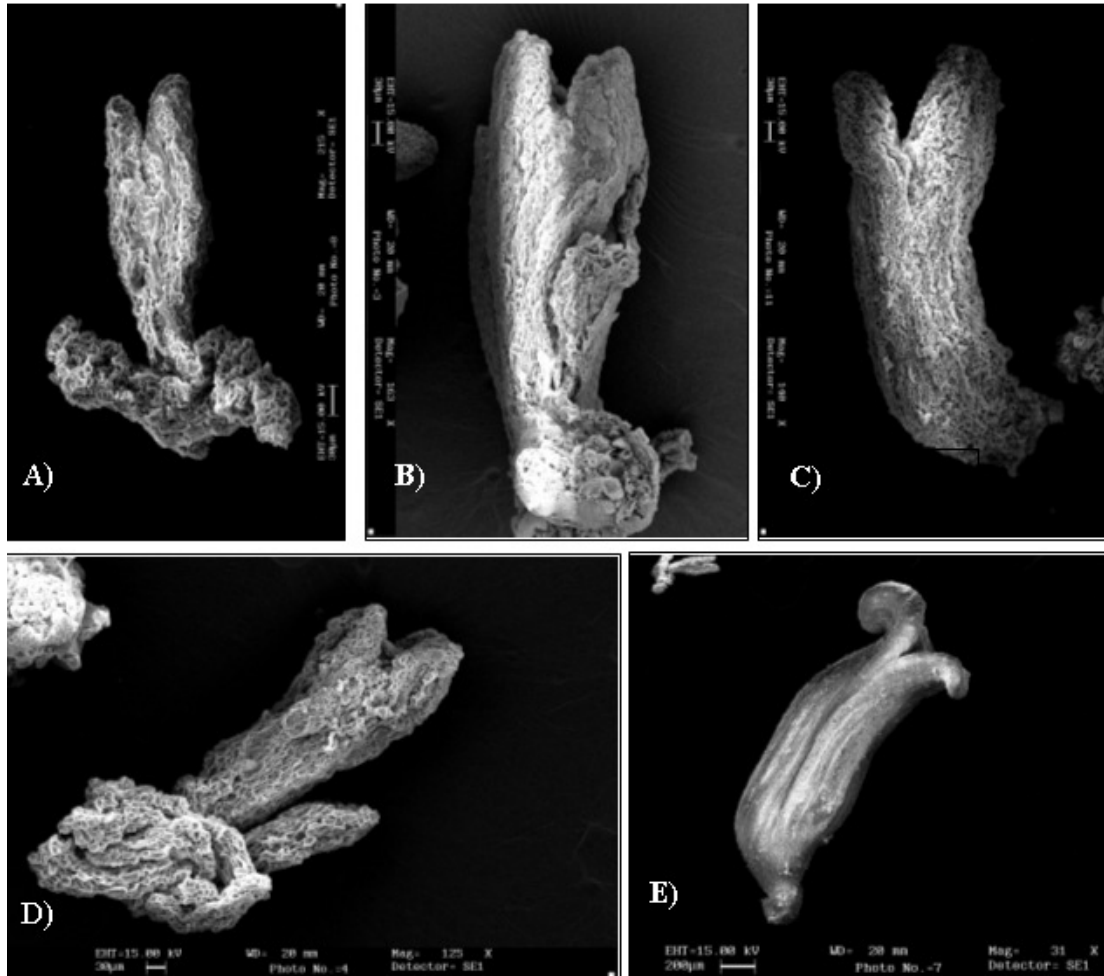


Fig. 3. SEM of somatic embryos at advanced stages; A, B, C) Heart shaped somatic embryos at different growing stages; D) A heart shaped embryo along with a small cylindrical embryo, developed from the callus surface; E) A cotyledonary somatic embryo showing two leaf primordia and a rudimentary root axis

The SEM of safflower (*Carthamus tinctorius* L.) also described the morphology of globular to cotyledonary embryos more efficiently and the embryo development was noted to be asynchronous in nature (Mandal *et al.*, 2006). In *Drosera spathulata* (Bobak *et al.*, 2006) and *Actinidia deliciosa*, SEM was used to study different developing stages of somatic embryos (Popielarska, 2006). Recently, SEM was conducted in *Heliconia chartacea* for studying embryogenic callus, developed *in vitro* (Ulisses *et al.*, 2010). Therefore, the application of SEM along with TEM, histology and morphological investigation are very important and efficient in understanding embryo forms and in achieving successes following post embryo germination processes.

Conclusions

The present work describes the ontogeny and structures of somatic embryo in *C. roseus* through SEM. It helps in revealing origin, development of advanced embryo from its early stage. The SEM-observed embryo types, normal progression towards maturity or even morphologically aberrant (often-observed) somatic embryos will determine the success of *in vitro* plant regeneration, to be used as an alternative and sustainable method of propagation.

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