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Morphological Anomalies in Somatic Embryo Structure in *Catharanthus roseus*: Improving Embryo Germination by Amending Plant Growth Regulators, Activated Charcoal and Sucrose Level

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Authors' contributions

Authors Dipti and SF performed the experiments, made the statistical analysis. Author AM conceived the idea, wrote and edit the manuscript, drafted by author SF. All authors read and approved the final manuscript

Original Research Article

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ABSTRACT

The present study describes the incidence of somatic embryo (SE) irregularities in *Catharanthus roseus* (L.) G. Don. In this ornamental and anti-cancerous medicinal plant, SEs were developed at a very high rate from hypocotyl callus on MS medium, amended with 1.0 mg l^{-1} naphthalene acetic acid (NAA) and 1.5 mg l^{-1}benzylaminopurine (BA). But at high (1.5 mg l^{-1}) plant growth regulators (PGRs) levels severe embryo developmental abnormalities were caused. The major aberrant SE types have been described in the present communication. In several cases, the *in vitro* raised SEs had underdeveloped root ends or with aborted root axis. These irregularities in embryo structure appreciably reduced embryo germination rate and plantlet recovery. Here, we evaluated the effect of various PGRs, activated charcoal (AC), and various levels of sucrose in order to improve SE quality and germination. Medium added with 4.0 % sucrose improved rooting ability and growth. The addition of AC improved plantlet conversion significantly by promoting rooting.

Keywords: Catharanthus roseus; amended PGRs; embryo irregularity; embryo germination.

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1. INTRODUCTION

Catharanthus roseus(L.) G. Donis an important ornamental plant. It belongs to the family Apocynaceae; many of the genera including *Catharanthus* are used for medicinal as well as ornamental purposes. The species tolerates dryness and can even normally grow in nutritionally deficient conditions. *Catharanthus*is also planted for its long flowering period, in tropical conditions it blossoms throughout the year while in warm temperate climates the flowering extends from spring to the late autumn. Like many other Apocynaceous plants, *Catharanthus* is a rich source of a variety of alkaloids of which vincristine and vinblastine are highly important. These alkaloids are used to treat against several cancers like leukemia, Hodgkin's diseases, neuroblastoma and other lymphomas [1]. *C. roseus* is sexually propagated that tends to show genetic heterozygosity and synthesizes nonuniform yield of dimeric alkaloid. The *in vitro* techniques have long been attempted in order to improve the content involving several *in vtiro* plant tissues including SEs. The capacity for somatic embryogenesis is a remarkable property of plant cells. It is the process by which somatic cells develop into embryo through characteristic morphological stages (globular, heart, torpedo and cotyledonary) leading to the complete plantlet. Somatic embryogenesis offers an alternative and efficient means of plant multiplication, this technique shows unlimited potential in producing clones to be used for secondary metabolite production [2] and it can also produce transgenic *en masse* [3]. The early embryos or embryo mother cells are exceptionally suitable for transformation via *Agrobacterium tumificians* [4], microinjection [5], and particle bombardment [6]. Similarly, the embryogenic cultures can effectively be cryopreserved with liquid nitrogen without any loss of viability - an efficient way of maintaining germplasm [7]. The ontogeny is very similar to zygotic embryos (if not identical) as they pass through the same developmental stages which exists in seed, forming regular embryo structure with shoot and root axis. Somatic embryogenesis has been reported in a wide range of plants [8,9] and these embryos are described as bipolar, but several aberrant embryo forms also do exist in the same embryogenic population [10,11]; these irregularities often reduce the rate of conversion of SEs into complete plantlets. The frequency of occurrence of such abnormal forms of embryos is often high enough to be in contrast to the uniformity seen in zygotic embryos. The number of unusual SE formation however varies and depends on various factors such as organic, inorganic and PGRs supplements. In *C. roseus*, although SE induction was achieved [12], the process has been plagued with poor rate of embryo germination and the studies providing information for improving embryo germination are not enough as yet.

In the present study, we described morphological irregularities in embryo structure in *C. roseus*. The use of PGRs, AC and sucrose concentrations in improving embryo quality and germination were also discussed.

2. MATERIALS AND METHODS

2.1 Plant Material and Sterilization

Mature unripe fruits of *Catharanthus roseus* were collected from plants grown in Jamia Hamdard (Hamdard University, New Delhi) herbal garden. Seeds were isolated and surface disinfested with 0.5% $HgCl_2$ for 2 min and finally rinsed four times with sterilized double distilled water. Seeds were placed in conical flask containing 50 ml of MS medium [13] without organic compounds and PGRs. Germinated seedlings were grown and the explants i.e. hypocotyl, leaf and stem were excised and cultured on MS. The detail procedure of surface sterilization was described earlier [12].

2.2 Embryogenic Callus, SE Initiation and Embryo Multiplication

For induction of embryogenic callus, the explants were placed on MS supplemented with 3.0 % (w/v) sucrose, added with various auxins (2, 4-D, CPA, NAA) and was solidified with 8 g l^{-1} agar. As described earlier [12] the hypocotyl produced embryogenic callus, calli originated from other explants were non-embryogenic. Friable embryogenic callus was cultured on solid MS amended with optimized concentration of NAA (1.0 mg l^{-1}) and various concentrations of BA (0.5-2.0 mg l^{-1}). The somatic embryos (SE) were visible within 2-3 weeks of culture, which proliferated rapidly on fresh nutrient medium. The embryogenic callus was maintained with periodic sub-culturing at an interval of four weeks. The number of SEs, frequency of embryogenesis and occurrence of abnormal embryo structures were noted accordingly.

2.3 Identification of Normal and Aberrant SE

New embryogenic callus (40-50 mg) was induced from established cultures that showed steady growth, was carefully separated and cultured for current sets of experiments. The frequency and the extent of normal and abnormal SE development of all morphological types were examined from each population. Data were recorded every 4 weeks of culture. All the SEs larger than 2 mm were counted and classified.

2.4 Germination of SE and Conversion into Plants

Advanced cotyledonary SEs were collected after 8 weeks of culture and transferred to MS germination medium amended separately with different PGRs namely GA_3 (1.0 and 2.0 mg l^{-1}), ABA (1.0 and 2.0 mg l^{-1}), 10% (w/v) AC and sucrose concentration (1.0 – 6.0 %), without any post maturation treatment. The germination medium was of the same as that of induction and proliferation medium except the PGR part. The root emergence ability (rooting percentage and length of roots) was noted at various treatments after 8 weeks of culture. Unless mentioned otherwise, all the media were supplemented with 3.0 % (w/v) sucrose, and were solidified with 8 g l^{-1} agar. The pH of medium was adjusted to 5.7 before autoclaving at 121 °C for 15 min. Different stages of cultures were incubated at 25 ± 2°C under a light intensity of 100 □m m^{-2} s^{-1} provided by cool white fluorescent lamps with 16 h photoperiod.

2.5 Statistical Analysis

For SE abnormality study, 6-10 SEs was cultured in each treatment, for confirmation, each experiment was repeated three times with 5 replicates. The influence of AC, GA_3, ABA and sucrose on SE morphology and development was studied using ANOVA followed by a least significant difference (LSD) test to compare the means. Within each column, values with common letters are not significantly different at $P < 0.05$ level according to LSD test.

3. RESULTS

3.1 Embryogenic and Non-Embryogenic Callus

The embryogenic callus was induced from hypocotyls on MS medium within two weeks of culture, 1.0 mg l^{-1} 2, 4-D being the most effective treatment in producing friable embryogenic

callus (Fig. 1A) that were maintained well on medium added with other PGRs as well. The leaf, root and stem explants also produced calli but was turned out to be non-embryogenic. The embryogenic callus was white yellow, friable and grew very fast on fresh nutrient medium upon regular sub-culturing.

3.2 Induction and Mass Proliferation of SEs

The embryogenic callus was placed on medium containing various PGRs for producing SEs. Immediately after transfer, the calli rapidly differentiated into variable number of SEs (Fig. 1B). The medium amended with NAA and BA was noted to be very effective for embryo proliferation, 1.0 mg l^{-1} NAA and 1.5 mg l^{-1} BA being the best treatment on which a maximum number of embryos were formed (Table 1). At higher concentrations, the embryogenic culture appeared to become necrotic and after 7^{th} week the callus browning was more pronounced. The SEs were produced in masses and the population (Fig.1C) contained all i.e. globular, heart, torpedo and cotyledonary stages of embryos. The medium added with NAA alone (without BA) was effective at early stages and produced only globular, heart or rarely torpedo-staged embryos without much progress to mature-staged embryos (torpedo or beyond), even the embryo number was relatively less.

3.3 Formation of SEs of Unusual Morphology

Along with typical bipolar SEs, the proliferation medium witnessed a variety of embryo structures, which were different from normal SEs. Although it was difficult to categorize those structures into distinct classes, based on predominant nature and frequency we classed them into following four types (B, C, D and E) while A is normal (Fig. 1D). All the SEs larger than 2 mm were counted and placed into categories: A - Single SEs, showing normal bipolar structures with root and shoot axis; B - Multiple, fused embryos, looked to be one morphogenetic unit; C - SEs showing shoot axis without root end; D - SEs showing root axis without shoot primordium; E - Callused embryos with aberrant morphology.

The NAA and BA level seems to control the normal development of SEs in culture which is summarized in Table 2. Embryogenic tissues were similarly grown on medium supplemented with various NAA and BA combinations, of which 1.0 mg l^{-1} NAA and 0.5 mg l^{-1} BA produced SEs with lower abnormality compared to medium with higher levels of NAA and BA. The embryos kept on medium without PGRs, however, improved embryo quality. Both 2, 4-D and p-chlorophenoxyacetic acid (CPA) considered to be 'strong auxin' induced more embryo abnormalities at increasingly higher concentrations. NAA and BA were also separately tested and we noted that the irregularities were even more in BA supplemented medium (Fig. 2). This unpredictable SEs morphology with nonsynchronous germination behaviour, in fact, limits the application of embryogeny in getting maximum number of somaclones. As the SEs did not have developed root axes always and in cases where the root ends were aborted during culturing process an alternative *in vitro* approach was adopted to raise roots afresh before transplantation (Fig. 1E). In this study, selected mature SEs were transferred to germination medium. Various MS-based medium amended with IBA and NAA, with or without AC, GA_3, ABA, different sucrose levels (1.0 – 6.0 %) were used. No significant difference in rooting ability was observed on half and full strength MS medium. The addition of 10 % (w/v) AC into the medium, however, significantly improved embryo germination and rooting. The use of two root promoting PGRs (IBA and NAA) were also evaluated. We noted that both the PGRs enhanced embryo germination, but 1.0 mg l^{-1} IBA was more active compared to the higher concentration (2.0 mg l^{-1}), this effect was even more enhanced in AC

supplemented medium (Table 3), where maximum rooting (81.99 %) was observed (Fig. 1F). The addition of GA_3 and ABA also showed similar promotive effect and use of AC into this medium improved germination further, maximum being on 1.0 mg l^{-1} in both cases.

In this study we also observed differences in root emergence when embryos were cultured on varied sucrose concentrations with or without AC. The highest rooting percentage was observed on medium supplemented with 4.0 % sucrose. The addition of AC improved embryo germination and rooting frequency. A combination of 4.0 % sucrose and 10% AC showed the best result (91.71 %) (Table 4, Fig.1G) in which maximum root growth was observed. The appearance of radical/ root emergence was noted within 2-3 weeks time and subsequently epicotyls started to appear; finally a fully developed plantlet was formed. The plantlet conversion frequency however varied and no morphological differences were noted between somatic emblings and plantlets when grown fully in *in vitro* cultural conditions.

Table 1. Mean number of SEs in *C. roseus*, MS medium contained optimized concentration of NAA (1.0 mg l^{-1}) and varied concentration of BA; 40-50 mg of embryogenic callus was cultured and data were scored after 8th weeks of culture.

BA (mg l^{-1})	No of SEs / culture	Stages of SE			
		Globular	Heart	Torpedo	Cotyledonary
0.0	18.42 ± 1.43^{d}	11.15 ± 0.39^{d}	6.13 ± 2.09^{c}	1.14 ± 1.21^{d}	0.0^{c}
0.5	36.49 ± 3.65^{c}	21.52 ± 1.71^{c}	10.88 ± 1.21^{b}	4.09 ± 0.21^{c}	0.0^{c}
1.0	66.85 ± 2.44^{b}	42.20 ± 2.04^{b}	15.33 ± 0.72^{a}	7.18 ± 0.68^{b}	2.14 ± 0.21^{b}
1.5	92.66 ± 2.32^{a}	53.21 ± 3.05^{a}	16.75 ± 0.34^{a}	14.91 ± 0.19^{a}	7.79 ± 0.21^{a}
2.0	35.79 ± 1.54^{c}	21.36 ± 1.53^{c}	6.77 ± 0.52^{c}	5.61 ± 0.36^{b}	2.05 ± 0.74^{b}

Values represent the mean ± standard error of three replicates. Within each column, values are followed by the same superscript letter are not significantly different at p = 0.05 level according to LSD test.

Table 2. Embryo proliferation medium containing various levels of NAA and BA showing SE abnormality. Data were scored after 8th weeks of culture.

PGR (mg l^{-1})		Abnormality%
NAA	BA	
0	0	32.16 ± 2.13^{e}
0.5	0.5	37.34 ± 2.23^{e}
0.5	1.0	44.54 ± 1.21^{d}
0.5	1.5	61.67 ± 3.26^{b}
1.0	0.5	34.80 ± 0.15^{e}
1.0	1.0	73.13 ± 1.63^{a}
1.0	1.5	64.58 ± 3.57^{b}
1.5	0.5	37.43 ± 2.64^{e}
1.5	1.0	54.30 ± 2.34^{c}
1.5	1.5	76.87 ± 2.86^{a}

Values represent the mean ± standard error of three replicates.Within each column, values are followed by the same superscript letter are not significantly different at p = 0.05 level according to LSD test.

Table 3. Effect of various PGRs and AC (10%) on germination (rooting) of SE. The SEs were cultured on half- and full strength MS medium. Data were scored after 8th weeks of culture

Treatment	Rooting %	Root length (mm)
Half MS	11.50 ± 2.27[f]	3.17 ± 0.11[d]
Full MS	13.63 ± 2.25[f]	3.40 ± 0.41[d]
MS + AC	46.4 ± 3.94[d]	6.27 ±0.43[b]
MS + IBA (1.0 mg l^{-1})	72.69 ± 1.96[ab]	8.44 ± 0.10[a]
MS + IBA (2.0 mg l^{-1})	43.94 ± 0.31[d]	6.35 ± 0.37[b]
MS + IBA (1.0 mg l^{-1}) + AC	81.99 ± 1.11[a]	9.10 ± 0.90[a]
MS + NAA (1.0 mg l^{-1})	42.64 ± 1.26[d]	6.20 ± 0.23[b]
MS + NAA (2.0 mg l^{-1})	38.47 ± 0.86[e]	5.08 ± 0.12[c]
MS + NAA (1.0 mg l^{-1}) + AC	67.53 ± 4.02[b]	7.23 ± 0.04[b]
MS + GA_3(1.0 mg l^{-1})	77.77 ± 3.86[a]	8.65 ± 1.51[a]
MS + GA_3(2.0 mg l^{-1})	68.45 ± 2.25[b]	6.58±1.15[b]
MS + GA_3(1.0 mg l^{-1}) +AC	81.38± 2.95[a]	8.75 ± 1.85[a]
MS + ABA (1.0 mg l^{-1})	66.55 ± 3.65[b]	6.56 ±1.65[b]
MS + ABA (2.0 mg l^{-1})	43.45 ± 2.56[d]	5.95±0.95[b]
MS + ABA (1.0 mg l^{-1}) + AC	81.58 ± 1.65[a]	9.25± 1.86[a]

Values represent the mean ± standard error of three replicates.Within each column, values are followed by the same superscript letter are not significantly different at p = 0.05 level according to LSD test.

Table 4. Effect of various sucrose concentrations (with/without AC) on radicle elongation, SEs were placed on optimized MS medium added with 1.0 mg l^{-1} IBA. Data were scored after 8th weeks of culture.

Sucrose concentration (%)	Without AC		With AC	
	Rooting %	Root length (mm)	Rooting %	Root length (mm)
1.0	0.0[e]	0.0[f]	0.0[f]	0.0[f]
2.0	39.59 ± 1.61[d]	6.13 ± 0.05[e]	40.41 ± 0.94[e]	5.80 ± 0.22[e]
3.0	52.69 ± 1.96[c]	8.44 ± 0.10[d]	81.99 ± 1.11[b]	9.10 ± 0.90[c]
4.0	76.91 ± 1.98[a]	19.27 ± 4.01[a]	91.71 ± 4.20[a]	15.50 ± 1.04[a]
5.0	64.70 ± 2.11[b]	14.05 ± 1.60[b]	71.26 ± 2.22[c]	12.16 ± 0.28[b]
6.0	60.34 ± 0.56[b]	12.11 ± 0.05[c]	63.00 ± 4.58[d]	7.14 ± 0.39[d]

Values represent the mean ± standard error of three replicates.Within each column, values are followed by the same superscript letter are not significantly different at p = 0.05 level according to LSD test.

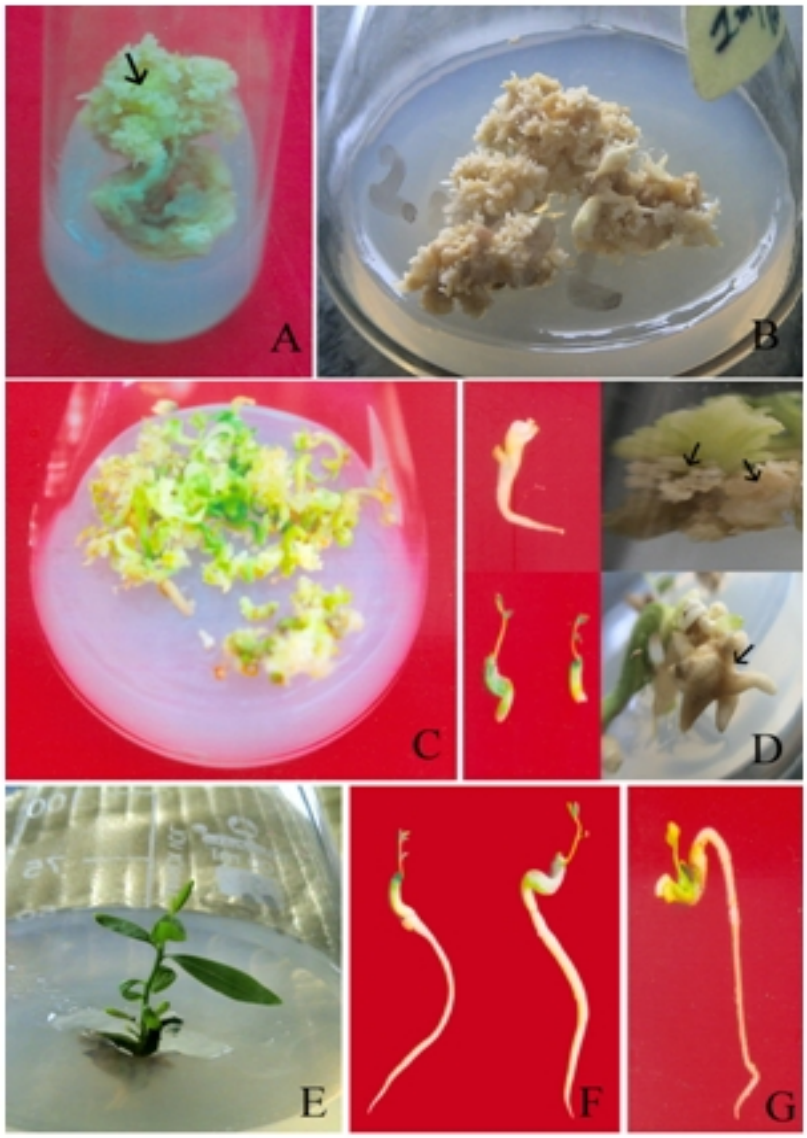

Fig. 1. Somatic embryogenesis, embryo irregularities and plant regeneration in *C. roseus.* A) Hypocotyl derived friable embryogenic callus (arrowhead), B) Heterogeneous mass of somatic embryos, developed on MS added with 1.0 mg l^{-1} NAA and 0.5 mg l^{-1} BA, C) Somatic embryos at later stages of development, D) Embryo showing root axis without shoot primordium, top left; callused embryo with aberrant morphology (arrowheads), top right; embryo showing shoot axis without root end, bottom left; and fused embryos (arrowhead), bottom right, E) Complete plantlet grown on *in vitro* condition, F) Tiny plantlet developed from somatic embryo on germination medium, added with 1.0 mg l^{-1} IBA and AC (10%), G) Germinated somatic embryo on 4% sucrose + 1.0 mg l^{-1} IBA + AC (10%) added medium.

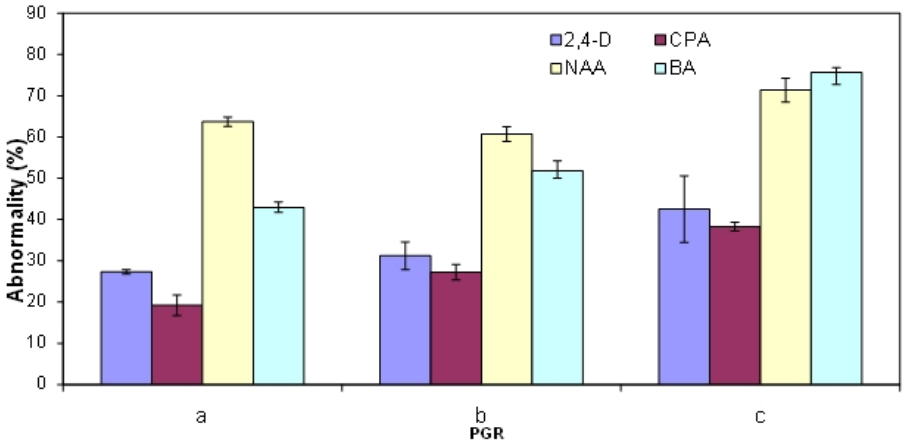

Fig. 2. Influence of PGRs (NAA, 2,4-D,CPA and BA) on abnormality percentage of embryos in *C. roseus*.

4. DISCUSSION

The process of embryogenesis occurs in two ways, SEs arise either directly on somatic explants or on intermediate callus in which cells somehow acquire embryo competence. In *C. roseus*the development is indirect i.e. it occurs through intervening callus stage. It seems from the present observations that by manipulating the balance of PGRs SEs development in *C. roseus*can effectively be regulated. These results also reconfirmed that the requirement of PGRs are different at various stages of embryo differentiation [14,15]. The unusual abnormalities developed in cultures particularly during embryo's normal progression are often affected by the imbalances of the culture medium and physical environment [16,17,18,19]. It may also arise as *in vitro* raised embryos lack suspensor and nourishing tissue endosperm. These are clearly the two main structural elements, which play a key role in bringing about successful progression of embryos during the embryogenic process.

The SEs with less developed radicles were often noted in culture, which reduced germination ability [20,21]. The same incidence was noted in *C. roseus*and the addition of IBA (1.0 mg l^{-1}) to medium significantly improved rooting of the germinating SEs. The use of IBA has been widely applied to improve/induce roots in tissue culture raised plants [22,23]. Similarly, IBA treatments were also applied to SEs for fast root elongation and growth before transplantation [24]. The rooting of plants and SE's development under the influence of IBA and AC have also been reported in many plants [25,26,27]. In our experiments with *Catharanthus*, we noted that the addition of AC to the IBA- supplemented medium had a significant influence on germination. Beside IBA, embryo germination efficiency was promoted by culturing embryo on medium supplemented with ABA and GA_3. In this medicinal plant, we observed that the presence of GA_3 and ABA was very essential for germination of somatic embryo in addition to AC. The response is consistent with several other reports that included ABA and polyethylene glycol (PEG) in maturation and germination medium [28,29]. The use of these compounds was used earlier in several studied plantsfor the same purpose i.e SE maturation and germination time [30,31]. The promotive effect of AC on SE development may be related to a water stress condition or it

may function as absorbent for residual toxic matters leaching to the medium [32]. The morphology and quality of SE has often been influenced by GA_3 in medium [33]. In *C. roseus*, on transfer to GA_3 containing medium, the SE turned chlorophyllous and green; and this may be due to over synthesis and accumulation of photosynthetic storage or fat reserve, essential for fast maturation and germination of embryos. The quality of embryo is an important consideration for the optimization of somatic embryogenesis protocol and this is primarily depend on embryo morphology and biochemical similarity to zygotic embryos [34].

We used external sucrose as a source of photosynthate and tested various concentrations on rooting in *C. roseus*and we noted maximum rooting on medium amended with 3.0 – 4.0 % sucrose. Addition of lower and higher sucrose concentration resulted in poor rate of root formation. Earlier, various carbon sources including sucrose have been noted to be very promotive in *in vitro*embryogenic systems [35]. Although the physiology is not yet known in detail, the rapid uptake of PGRs and active transport across the plasma membrane may trigger cellular machinery in inducing faster root growth [36]. Rapid SEs maturation with 3.0 % sucrose was reported in *Castenia sativa* [37]. Vookova and Kormutak [26] reported that a combination of IBA, AC and sucrose improved SE germination in *Abiesnumidica.* The enhanced rooting in *Catharanthus*improved survivability when plants were transferred to outdoor condition. However, the understanding about the quality of SE and the technical processes require to improve embryo quality are still found inadequate and more research is needed in this direction.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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