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ORIGINAL ARTICLE

Somatic embryo mediated mass production of *Catharanthus roseus* in culture vessel (bioreactor) – A comparative study

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Abstract The purpose of this study was to evaluate and compare the use of liquid and solid Murashige and Skoog (MS) medium in different culture vessels for mass production of *Catharanthus roseus*, an important source of anticancerous compounds, vincristine and vinblastine. Three media conditions i.e. agar-solidified medium (S), liquid medium in agitated conical flask (L) and growtek bioreactor (B) were used. Rapid propagation was achieved through in vitro somatic embryogenesis pathway. The process of embryogenesis has been categorized into induction, proliferation, maturation and germination stages. All in vitro embryogenesis stages were conducted by withdrawing spent liquid medium and by adding fresh MS medium. In optimized 4.52 μM 2,4-D added MS, the callus biomass growth was low in solid (1.65 g) compared to liquid medium in agitated conical flask (1.95 g) and in bioreactor (2.11 g). The number of normal somatic embryos was more in solid medium (99.75/50 mg of callus mass) compared to liquid medium used in conical flask (83.25/callus mass) and growtek bioreactor (84.88/callus mass). The in vitro raised embryos maturated in GA_3 (2.60 μM) added medium; and in bioreactor the embryo growth was high, a maximum length of 9.82 mm was observed at the end of four weeks. These embryos germinated into seedlings in BAP (2.22 μM) added medium and the embryo germination ability was more (59.41%) in bioreactor compared to liquid medium in conical flask (55.5%). Shoot length (11.25 mm) was also high in bioreactor compared to agitated conical flask. The liquid medium used in agitated conical flask and bioreactor increased seedling production efficiency, at the same time it also reduced

Abbreviations: BA, N6-benzyladenine; 2,4-D, 2,4-Dichlorophenoxyacetic acid; GA_3, gibberellic acid; NAA, naphthalene acetic acid; MS, Murashige and Skoog (1962) medium; S, agar-solidified medium; L, liquid medium in agitated conical flask; B, growtek bioreactor

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plant recovery time. The embryo generated plants grew normally in outdoor conditions. The exploitation of medium to large culture vessel or bioreactor may make the process more efficient in getting large number of *Catharanthus* plant as it is the only source of anti-cancerous alkaloids, vincristine and vinblastine.

1. Introduction

Catharanthus roseus belongs to the family Apocynaceae and is an immensely important medicinal plant. The plant is widely distributed and naturalized in Africa, America, Asia, Australia, Southern Europe and some parts of Pacific islands. It produces large numbers of phyto-compounds like terpenoid indole alkaloids, phenolics, anthocyanins, fatty acids, steroids, proteins, enzymes, etc., several alkaloids are anti-cancerous in nature (Van der Heijden et al., 2004). Vincristine and vinblastine are the two most powerful anti-cancerous compounds, extracted from leaves (Magnotta et al., 2006). Vincristine and vinblastine are used to treat against acute leukemia, Hodgkin's disease, rhabdomyosarcomas, neuroblastoma and other forms of lymphomas (Mukherjee et al., 2001; Van der Heijden et al., 2004). These important secondary phyto-compounds are isolated by extraction from whole plants or specific tissues. Plants obtained through conventional methods are observed to be inadequate as a sole source of raw materials. In vitro rapid propagation of plants/cultures not only complements or adds options of resources, but will also protect natural important flora from over-exploitation. It offers other important advantages such as continuous availability of biochemicals, optimized production of compounds or even with enhanced yields (Paek et al., 2005). In recent years, various plant parts are used and cultural conditions are optimized in order to obtain cell lines with enriched yield of alkaloids in *Catharanthus* (Van der Heijden et al., 1989; Van der Heijden et al., 2004). The yield of these two compounds (vincristine and vinblastine) is however, very low and needs to be enriched for commercialization. Continuous cultivation of plant biomass in agitated liquid medium in a variety of bioreactors may consolidate the process by improving the culture growth several times (Schlatmann et al., 1994; Mujib et al., 1995; Das et al., 1999; Satdive et al., 2003). In last two decades, there have been sincere efforts to automate micropropagation by making simple, cost-effective bioreactors and several liquid media based reactors have been referred that include stirred tank-, bubble column-, balloon type bubble-, and air lift bioreactors (Ziv, 2010). Temporary immersion system has also been practised in micropropagation to reduce hyperhydricity of shoots, often noted in submerged liquid conditions (Afreen, 2006; Ducos et al., 2009; Adelberg and Fári, 2010). The immersion cycle, nutrient volume and the type of reactors are noted to be critical during shoot proliferation stage of micropropagation (Snyman et al., 2011). For successful automation, large culture vessels, fermenters and bioreactors with low shear stress have been reported to be very efficient for fast growth of suspension and alkaloid yield (Zhao et al., 2001; Valluri, 2009). There are many low shear-stress bioreactors such as bubble-column and airlift bioreactor, which induce enhanced growth of tissue biomass (Moreno et al., 1995; Ziv, 2010). Recently, a mist bioreactor employing a disposable bag as culture chamber was used to propagate plantlets (Fei and Weathers, 2014). These mist bioreactors of varied structural configurations improved shoot proliferation and somatic embryo formation (Towler et al., 2006). The use of a disposable plastic bag as culture chamber offers a cost effective way for propagating plantlets in vitro with full control over nutrient feeding and ventilation (Liu et al., 2009). Growtek is a low cost plant tissue culture autoclavable container or bioreactor. It has a side tube with silicon membrane that permits replenishment of nutrient medium without disturbing much the cultivated tissues. It also has a floating explant holder which keeps the cultured tissue in constant touch with liquid medium. It can be kept as static and agitated conditions. Owing to several advantages, the plant tissues are often cultivated and harvested in liquid medium as the cells efficiently and easily uptake inorganic, organic nutrients and precursor molecules and facilitate fast, enriched accumulation of alkaloids (Junaid et al., 2009; Zhao et al., 2009; Mujib et al., 2012). In this present study, the authors discussed the role and comparative performances of solid and liquid MS medium on mass cloning of *C. roseus* in agitated conical flask and growtek bioreactor by using somatic embryogenesis method of plant regeneration.

2. Materials and methods

2.1. Seed germination and other cultural conditions

Seeds of *Catharanthus roseus* (L.) G. Don. cv. Nirmal were used in this study, the detailed process of embryogenesis was described earlier (Junaid et al., 2006) and was followed. In brief, the seeds were obtained from outdoor grown plants of Jamia Hamdard (Hamdard University) herbal garden. The surface disinfected seeds were placed in a conical flask containing 50 ml of MS solid medium (Murashige and Skoog, 1962) with no growth regulator. Germinated seedlings were grown until they had attained 2–4 cm length. Various parts (stem, leaf, and hypocotyl) were used and inoculated in test tubes (Borosil, India) as explants. For embryogenic callus initiation, the MS medium was supplemented with 4.52 μM 2,4-Dichlorophenoxyacetic acid (2,4-D). For somatic embryo proliferation, the same medium was added with naphthalene acetic acid, NAA (5.37 μM) with various N6-benzyladenine (BAP) concentrations (2.22–8.90 μM). Maturation and germination medium contained optimized level of 2.60 μM gibberellic acid (GA_3) and 2.24 μM BAP respectively.

The influence of liquid (L) medium in a conical flask was studied by using 25 ml liquid medium in a 250 ml conical flask, agitated on a rotary shaker at 120 rpm. The liquid medium was also used in growtek bioreactor (B) (Tarson, India). The volume of the medium was 50 ml. In the case of bioreactor, the medium was drained off and fresh medium was added by the attached tube, thus regular sub-culturing was avoided. For

agar-solidified test tube culture and agitated liquid medium in a conical flask, the sub-culturing was made at four week intervals. The pH of the medium was adjusted to 5.7 before autoclaving at 121 °C for 20 min. All the cultures were incubated under a 16-h photoperiod by cool white (Fluorescent F40 T12/CW/EG) lamp at a photon flux density of 100 μmol m^{-2} s^{-1} at 25 °C light/20 °C dark temperature.

2.2. Statistical analysis

The data collected on different parameters were subjected to statistical analysis to determine the degree of authenticity. The data on the effects of plant growth regulators on different stages of embryogenesis were analyzed by one-way analysis of variance (ANOVAs). Values are means of five replicates from two experiments, and the presented mean values were separated using Duncan's multiple range test (DMRT) at $P \leqslant 0.05$.

3. Results

All the plant parts responded well in culture and produced callus on auxin supplemented solid MS medium. The maximum callus induction percentage was noted on 4.52 μM 2,4-D added medium (data not shown). Higher levels of auxin inhibited callus induction and subsequent callus growth. The hypocotyl callus was friable, light yellow, proliferated well on medium and this solid medium induced callus was used for further course of investigation. The callus biomass growth was however, relatively low in solid compared to liquid MS medium. In 4.52 μM 2,4-D added MS medium the callus biomass in solid was 1.65 g while the biomass was 1.95 g in a agitated liquid conical flask and in growtek bioreactor the callus attained a mass weight of 2.11 g after 45 days of incubation (Fig. 1). The hypocotyl induced callus later transformed into embryogenic callus (EC) (Fig. 2a). The calli induced from other plant parts were non-embryogenic (NEC), which were compact, hard nodular structure and grew slowly.

Within a three-four week time the EC rapidly differentiated into embryos particularly in NAA supplemented medium (Fig. 2b). The same level of other tested auxins (2,4-D, CPA) was observed to be less productive. The embryogenic culture seemed to be a mix of heterogeneous embryos, composing of four different types (globular, heart, torpedo and cotyledonary) at variable numbers. The embryo numbers increased with time. The addition of BAP in NAA amended medium improved embryo number further. Table 1 summarizes and compares the importance and efficiency of culture vessels in promoting embryo number. It is clear that the numbers of normal somatic embryos were more in solid medium (99.75/50 mg of callus mass) compared to agitated liquid medium in a conical flask (83.25/callus mass). The use of growtek bioreactor did not have much influence in promoting embryo number (84.88/callus mass).

3.1. Somatic embryo maturation

Advanced cotyledonary somatic embryos were cultured on MS medium, added with 2.60 μM GA_3 for embryo maturation. The embryos turned green and elongated (Fig. 2c) in almost all the tested tubes and vessels, suggesting the initiation of maturation process. Embryo length was also increased and after four weeks the length reached up to 9.83 mm (Table 2). In bioreactor, the embryo growth was quite high, attained a maximum length of 9.82 mm at the end of four weeks. In agitated conical flask and bioreactor, the embryo showed germination on embryo maturation medium, although the rate was relatively poor (over 2%).

3.2. Embryo germination

Green matured somatic embryos were cultured on medium, added with 2.24 μM BAP in culture tube, agitated flask (Fig. 3a) and in bioreactor (Fig. 3b and c) to identify the right embryo germination environment. Embryos were germinated in almost all the vessels (Table 3), but compared to culture tubes, liquid medium in agitated conical flask showed more germination (55.5%); in bioreactor, the germination ability was even better (59.41%). Germinated embryos had well developed shoot and root ends (Fig. 4a). Shoot (10.0 mm) and root (7.67 mm) lengths were more in conical flask containing liquid medium compared to solid. Shoot length (11.25 mm) was higher in bioreactor compared to agitated conical flask. In solid medium, the embryos were often aggregated, laterally fused and showed seedlings with multiple shoots. The

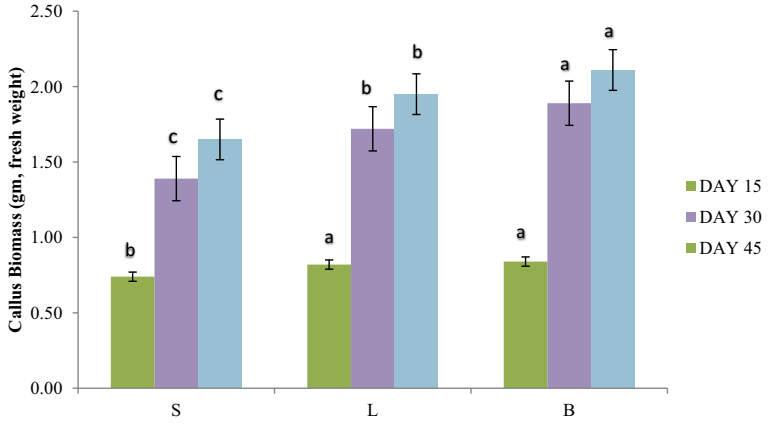

Figure 1 Callus biomass growth in different cultural environments. MS medium contained 4.52 μM 2,4-D (100 mg i.e. 0.1 g embryogenic callus was used).

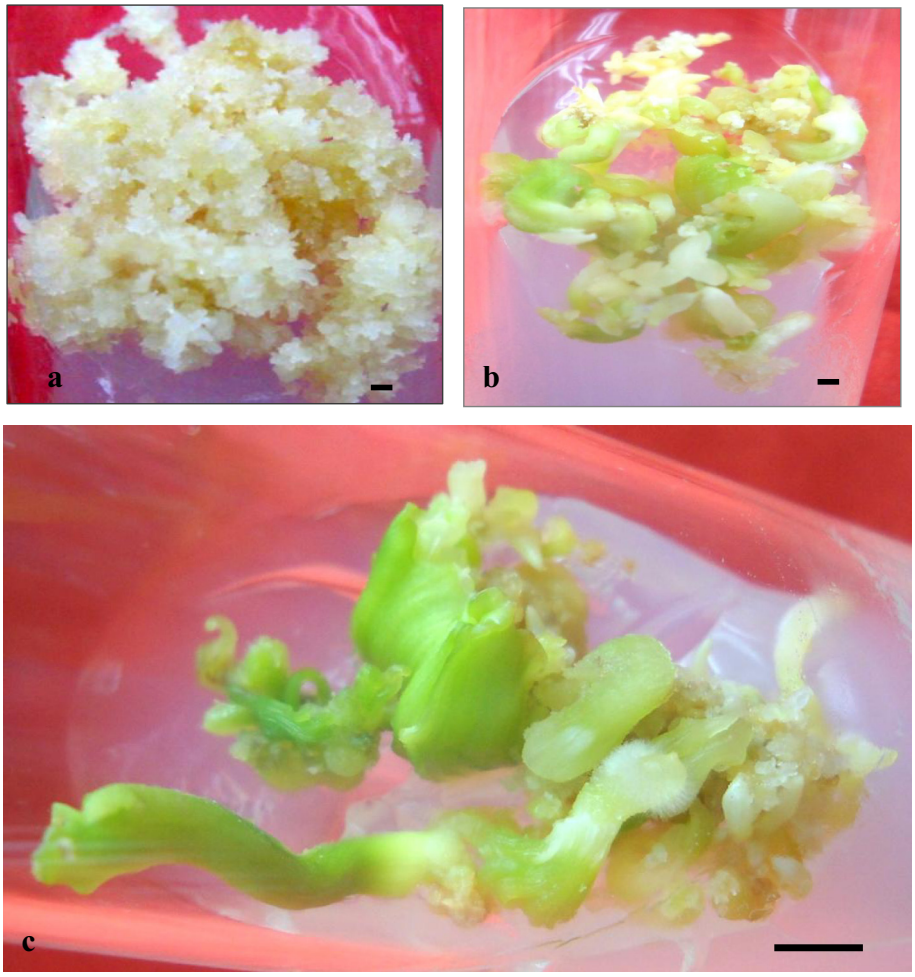

Figure 2 (a) Embryogenic tissue developed from hypocotyl (bar = 1 mm), (b) somatic embryo formed in solid MS medium (bar = 1 mm), and (c) matured embryo in solid MS medium (bar = 5 mm).

Table 1 The number of somatic embryo in MS proliferation medium, added with NAA (5.37 μM) and various BAP concentrations (40–50 mg embryogenic callus was used and data were scored after 7th week of culture).

BAP (μM)	S	L	B
2.22	38.65 ± 3.42d	36.52 ± 2.68d	35.42 ± 2.75d
4.40	82.51 ± 3.69b	62.96 ± 3.26b	65.25 ± 7.85b
6.62	99.75 ± 2.27a	83.25 ± 2.20a	84.88 ± 2.19a
8.90	65.52 ± 6.42c	52.46 ± 5.55c	50.25 ± 6.18c

Values are mean numbers ± standard errors of three replicates of two experiments. Within each column, means followed by the same letter are not significantly different at $P \leqslant 0.05$ according to DMRT. S: agar-solidified medium: L: liquid medium in agitated conical flask, B: growtek bioreactor.

Table 2 Maturation of somatic embryos in MS medium added with 2.60 μM GA_3, data were scored after 4 weeks of culture.

Culture	Initial length	After 2 weeks	After 4 weeks	Germination%
S	5.65 ± 0.08b	6.93 ± 0.22b	8.80 ± 0.08b	0.00 ± 0.0b
L	5.68 ± 0.05b	7.88 ± 0.23a	9.83 ± 0.11a	2.58 ± 0.33a
B	5.73 ± 0.04a	7.70 ± 0.18a	9.82 ± 0.22a	2.67 ± 0.26a

Values (mm) are means ± standard errors of three replicates with six embryos per treatment. Within each column, means followed by the same letter are not significantly different at $P \leqslant 0.05$ according to Duncan's multiple range test. S: agar-solidified medium: L: liquid medium in agitated conical flask, B: growtek bioreactor.

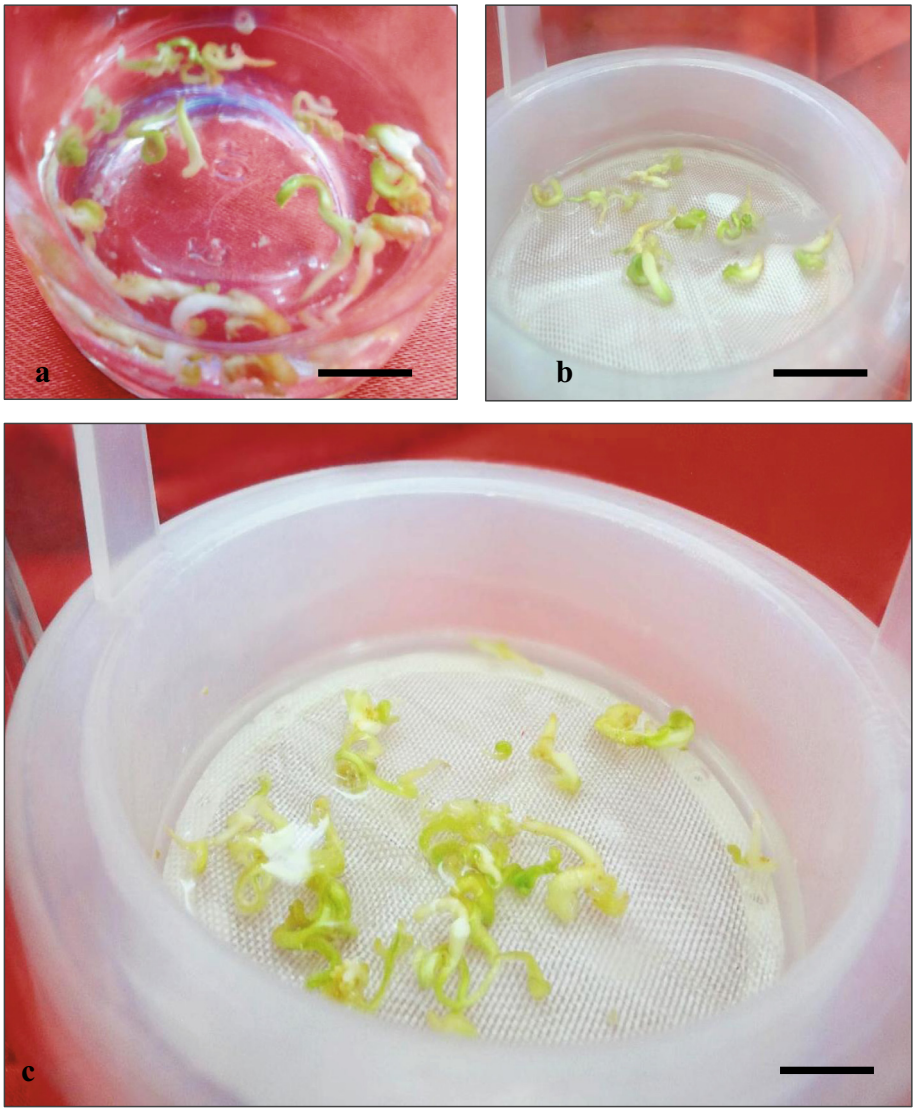

Figure 3 (a) Mature somatic embryos cultivated in agitated liquid medium in agitated conical flask (bar = 5 mm) (b and c) embryos at different stages, grown in growtek bioreactor (bar = 5 mm).

Table 3 Somatic embryo germination (plantlet conversion) in BAP (2.22 μM) added MS medium. Data were scored after 6 weeks of culture.

	S	L	B
Germination%	38.89 ± 5.56c	55.55 ± 14.70b	59.41 ± 5.56a
SL	3.33 ± 0.10b	10.00 ± 0.29a	11.25 ± 0.31a
RL	4.37 ± 0.61b	7.67 ± 1.45a	7.69 ± 1.35a

Values are means ± standard errors of three replicates with six embryos per treatment. Within each row, means followed by the same letter are not significantly different at $P \leqslant 0.05$ according to Duncan's multiple range test. RL, Root length (mm) of embryo; SL, shoot length (mm) of embryo. S: agar-solidified medium: L: liquid medium in agitated conical flask, B: growtek bioreactor.

incidence of secondary somatic embryos on primary embryos was also very common in solid medium compared to liquid and bioreactor conditions that restricted the growth of primary embryos toward maturity. Embryos produced in liquid medium or in bioreactor were far more isolated, generated normal seedlings (Fig. 4b and c), very analogous to zygotic seedlings, which grew normally in outdoor condition. The recovery time (Table 4) for obtaining somatic seedlings from embryogenic callus on solid medium was 135–150 days. The plantlet recovery period was reduced to a minimum of

Figure 4 (a) Somatic embryos, isolated from growtek bioreactor showing shoot and root ends (bar = 5 mm), (b) Regenerated plantlet (bar = 5 mm), and (c) Plantlet before transfer to outdoor condition (bar = 5 mm).

Table 4 Plantlet recovery time (day) in different culture environments.

Trial	S	L	B
1st	135.12 ± 8.65c	115.00 ± 8.25b	112.65 ± 6.85a
2nd	142.26 ± 7.45b	118.25 ± 10.12a	116.25 ± 8.51a
3rd	150.00 ± 10.62c	125.25 ± 8.65b	120.52 ± 8.62a

Values are means ± standard errors of three replicates of three experiments. Within each row, means followed by the same letter are not significantly different at $P \leqslant 0.05$ according to Duncan's multiple range test. S: agar-solidified medium: L: liquid medium in agitated conical flask, B: growtek bioreactor.

115 days in liquid medium and in growtek bioreactor the period further lowered to 112 days. The regenerated plants were healthy and grew normally in outdoor conditions.

4. Discussion

In our earlier reports of *C. roseus* the incidence of somatic embryogenesis from hypocotyl embryogenic callus was discussed in solid MS medium (Junaid et al., 2006; Junaid et al., 2008). Here, the embryo formation was indirect i.e. an intervening callus stage is necessary while in others the embryos were originated directly on explants (Thorpe, 1995; Mujib et al., 1998). Besides solid and liquid medium, the use of liquid overlaying also improved in vitro embryogenesis process significantly in several investigated plants including *C. roseus* (Zahid et al., 2011). The present communication described the comparative embryogenic responses in solid and liquid medium in agitated conical flasks and in growtek bioreactors. Liquid medium has been used over solid in several

plant genera as it has the ability to improve shoot proliferation, somatic embryogenesis, in vitro rooting, micro-tuberization, other morphogenetic growth and development processes (Fennel and Ascough, 2004; Hvoslef-Eide and Preil, 2005; Pullman et al., 2009). The use of liquid medium has other advantages as replenishment of nutrient medium is easy, the numbers of regular sub-culturing may be minimized or escaped; it also reduces the unit cost as there is no requirement of gelling agent (Gupta and Timmis, 2005; Rizvi et al., 2007). Liquid medium used as merged or partial immersion has often been considered temporary as a thin film of medium is retained in the tissue, does not hinder gaseous exchange and the growing tissues use nutrient mist efficiently (Berthouly and Etienne, 2005). However, in liquid condition many of the in vitro raised shoots showed vitrification as the plants are sensitive to liquid media; and the survival of plants (upon transfer of shoots outdoor) is low as regenerated shoots are vulnerable to outdoor environmental stresses (Robert et al., 2006; Shaik et al., 2010). In *Pyrus*, the use of liquid media in the form of liquid overlay was also tested for fast proliferation of shoots (Bell and Reed, 2002); in rubber, the overlaying technique enhanced embryogenic protoplast culture (Sushamakumari et al., 2000). In *C. roseus*, we observed that the solid medium was more efficient in promoting embryo number compared to liquid, used either in agitated conical flask or in bioreactor. In the present study, liquid medium amended with GA_3 facilitated fast embryo maturation in culture. In the same maturation medium, the embryos also germinated at a very low rate. Advanced mature somatic embryos germinated better in liquid medium, especially in bioreactor (59.41%), next best condition was agitated conical flask where germination percentage was 55.5%. The improvement in somatic embryo maturation and germination was partly attributed to the increased availability of diffused oxygen in liquid medium in agitated conical flask and in bioreactor or by better nutrient uptake by the cells of developing embryos. It is known that the bioreactor transfers oxygen (provided by agitation and impeller devices) from gaseous to the liquid stage, makes oxygen available to cultivating tissues or cells and promotes in vitro biomass growth (Leathers et al., 1995; Sajc et al., 2000). The optimum presence of several gases like O_2 and CO_2 is very critical for somatic embryo as the requirement varies at different stages of embryo growth (Shimazu and Kurata, 1999; Jeong et al., 2006; Takamura et al., 2010; Rosnow et al., 2011). Similar to the above observations, improved plant regeneration, growth and development were earlier noted in different vessels utilizing liquid media in several studied plants (Te-chato and Lim, 1999; Nitayadatpat and Te-chato, 2005). In solid medium, in vitro growth has often been poor as gelling agents like agar reduces water potential in medium (Ghashghaie et al., 1991) and in some cases several macro-and micro elements adhere to agar, therefore are non or less available to growing cells/tissues (Scholten and Pierik, 1998). In this present observation, we noted that the use of liquid medium in agitated conical flask and bioreactor shortened 'embryo-plantlet' recovery time by a fair margin compared to agar solidified medium. This reduction of time with fast propagation would be very productive for secondary metabolite synthesis in medicinally important plant genera. The established protocol may also offer advantages in raising transgenics plants *en masse* at fast pace.

5. Conclusion

Callus was initiated profusely in solid MS medium, amended with 4.52 μM 2,4-D, which grew well in liquid medium. Somatic embryo formation and proliferation were more in solid medium compared to liquid. The embryos matured fast in both solid and liquid MS mediums, amended with 2.60 μM GA_3. Somatic embryo germination was quite high and fast in 2.22 μM BAP added liquid medium particularly in bioreactor and conical flask that lowered 'plant recovery time' (callus to plantlet) compared to solid medium.

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