Mono- and dicotyledonous plants continuously show in vitro responses by undergoing callusing and subsequent plant regeneration although there are few initial limitations (Tripathi and Tripathi 2003). We selected C. roseus, a dicotyledonous plant for our experimental material. In this current study, MS medium was used for successful establishment of culture. Various explants were used and all the tested explants showed responses in producing callus when cultured in vitro. The developmental status like age of the explants influence culture establishment and only young tissue such as hypocotyls responded well in C. roseus. In the present study, the used explants produced two types of callus, one is non-embryogenic and the other is embryogenic callus tissues. The embryogenic callus was only induced from hypocotyls in which auxin (1.0 mg l$^{-1}$ 2, 4-D) was found very effective. The competence for embryogenic callus induction may be the result of varying auxin sensitivity of these cells (Von Arnold et al. 2002). Two mechanisms appear to be important for in vitro formation of embryogenic cells: asymmetric cell division and control of cell elongation (Emons 1994). Asymmetric cell division was reported to be promoted by PGRs that alter cell polarity by interfering pH gradient or in influencing the electrical field around cells (Smith and Krikorian 1990). The ability to control cell expansion, on the other, was noted to be accomplished by association with polysaccharides and corresponding hydrolytic enzymes of the cell wall (Fry 1995).

The PGRs in media generally play a key role in different morphogenesis programme, however, the required concentrations varied significantly from one explants to other. It was also observed that among three auxins (2, 4-D, NAA and IAA) tested 2, 4-D was very effective for callus induction. This is due to the reason that 2, 4-D is much more stable and less inactivated during culture processes than the other auxins (Covington and Harmer 2007). The requirement of auxin or other PGRs for the initiation of somatic embryogenesis is largely determined by the developmental stage of the explant tissue and usually embryogenic callus is formed in medium, added with auxin (Von Arnold et al. 2002). In C. roseus, BA in combination with NAA at lower concentration (0.5-1.0 mg l$^{-1}$) had profound influence on initiation of embryos, and for long term maintenance and growth of tissues. Plant species which are capable of expressing their embryogenic potential regardless of the explant include Daucus carota and Medicago sativa, while for many other species embryonal or highly juvenile tissue was suggested to be used as explants only (Von Arnold et al. 2002).

In our study, continuous presence of 2, 4-D blocks the development of embryo, however, the addition of NAA into the medium facilitated embryo development significantly, the
observation is similar to the findings made by Pasternak et al. (2002). The extent of embryo differentiation which takes place in presence of auxin varies in different species and it was noted in most cases that the auxin is taken up very rapidly from the culture medium (Nishimura et al. 2009). Embryogenic cultures of some species and some genotypes are sub cultured for prolonged period on medium containing PGRs, and still retain their full embryogenic potential i.e., the capacity in producing somatic embryos continuously, which later develop into plants, while in others the embryogenic ability gradually diminishes with time (Von Arnold et al. 2002).

The pattern of developmental response of cultured tissue in some cases is epigenetically determined and is influenced by the stage of development of the plant, the nature of the explant etc. (Litz and Gray 1995). Hirata et al. (1994) proved that PGRs are of importance for the growth and morphological differentiation of tissues, leading to the formation and development of shoots in *C. roseus*. PGRs are also responsible for the diversification of alkaloids and even it increases the yield (Junaid et al. 2010). PGR like BA and NAA added to the MS medium was noted to potently stimulated the formation of shoots, whereas 2, 4-D suppressed their differentiation and formation (Yuan and Hu 1994) and similar such responses were also noticed in our present study. In *C. roseus*, we found the development of secondary/adventive embryos on primary somatic embryo. The development of secondary embryos on primary embryos is however, common and observed in several investigated plant genera (Iancheva et al. 2001; Barbulova et al. 2002).

As the embryos progress towards maturity (maturation and germination stage) the somatic embryos undergo various morphological and biochemical changes (George et al. 2008). The storage organs such as cotyledons often expand with deposition of storage reserves, show extra acquisition of desiccation tolerance and even demonstrate repression of germination (Thomas 1993). The accumulated storage products in somatic embryos are very similar to zygotic embryos and exhibit almost the same characteristics as those of zygotic counterpart (Merkele et al. 1995). In contrast to early stage embryos (globular and heart shaped embryos), the maturing embryos of *C. roseus* were quite long, green and chlorophyllous, identified as a good morphological marker of embryo maturity.

In the present investigation, the protoplast was isolated from embryogenic callus by using different enzyme mixtures. Later, protoplasts were cultured and regenerated into plantlets via somatic embryogenesis. The embryogenic cell suspensions, often noted to be the best source
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for protoplasts with a very high morphogenic potential (Fiuk and Rybczynski 2007). We also used embryogenic suspension as source of tissue that provided good yield of protoplasts. In fact, protoplasts can be derived from any type of plant cells (Sheen 2001). However, several factors influence the success of plant regeneration from protoplast, which includes culture medium, nitrogen source, culture technique like the size of aggregates, time of sampling, enzyme treatment and their duration (Davey et al. 2005b).

In our study, different combinations of digestive enzymes were tested for protoplast yield and viability, and we noted that the cocktail of cellulase (2.0%) + pectinase (1.0%) + macerozyme (0.02%) + driselase (0.50%) along with osmotica was the optimum treatment for protoplast yield and viability. It was suggested earlier that the different combinations of digestion enzymes are important that needs some optimization for better protoplast yields (Te-Chato et al. 2005). The mixtures of enzyme in digestion solution has been reported to be an important factor on yield and viability of protoplasts in many other studied plant species such as *Artemisia judaica* L., *Echinops spinosissimus* Turra etc. (Pan et al. 2003). In our experiments, inclusion of driselase into enzyme mixture increased protoplast yield to significant level, similar to Loh and Rao (1985) observation; Koh et al. (1988) also reported that the inclusion of 0.5% Driselase into the enzyme mixture of 1.5% Cellulase Onozuka R-10 and 0.5% Macerozyme R-10 increased the yield of mesophyll protoplasts in *Aranda* hybrids. Digestion time is also an important criterion, in *Grevillea*, an incubation time of 10 h produced over 80% of viable protoplasts, in our experiment, 12 h incubation time showed maximum protoplast yield and viability. The prolonged incubation period however, decreased the yield and viability of protoplasts because of over digestion (Zhu et al. 2005).

The various osmotica and their concentrations used in enzyme solution significantly affect the yield and viability of protoplasts (Davey et al. 2005b). The osmotica used in protoplast study are sorbitol, mannitol, sucrose, CaCl$_2$.2H$_2$O, KCl, etc. In *C. roseus*, 0.6 M sorbitol with 100 mM CaCl$_2$.2H$_2$O showed good yield of protoplast with improved viability. In *Aponogeton madagascariensis*, the separate use of mannitol, glucose and sucrose in enzyme mixture produced a poor number of protoplasts when compared with sorbitol used conditions (Christina and Arunika 2010). Sorbitol as a carbohydrate seemed to provide the most suitable osmotic pressure in culture and allowed the cells to remain intact (Pongchawee et al. 2007), while mannitol level was effective in generating maximum yield of protoplasts in plants like poplar (Park and Son 1992), Rorippa (Mandal and Sikdar 2003), Betula (Wakita et al. 2005) and Ipomoea (Guo et al. 2007), all these responses are very similar to our present findings.
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After protoplast isolation, the culture was cultivated in medium amended with different PGRs. In our study, culture medium amended with different PGR combinations showed diverse response; 0.5 mg l\(^{-1}\) NAA + 0.5 mg l\(^{-1}\) 2, 4-D was observed to be the best treatment in which maximum number of micro-colonies were formed, followed by 0.25 mg l\(^{-1}\) NAA + 0.25 mg l\(^{-1}\) 2, 4-D. Dietert et al. (1982) also reported that a low concentration of 2, 4-D improved callus growth in protoplast culture of genus *Brassica*. Better cell division and callus proliferation were noted in high 2, 4-D added medium in some other reports (Kohlenbach et al. 1982). In saffron, it was noted that different auxin-cytokinin combinations exerted a key influence on regeneration of plantlets (Babak et al. 2007). Ebrahemzadeh and Karamian (2001) reported that the treatment of NAA-BA was more effective on regeneration of germinated embryos when a variety of PGRs combinations were studied and compared. The same auxin-cytokinin combinations promoted plant regeneration from protoplast-derived calli in several plant systems (Borgato et al. 2007).

In *C. roseus*, the combination of NAA and BA had no major influence at initial stage of growth of micro colonies; however, both improved responses at proliferation stage. A low level of PGR use in medium lowers the cost and may help to reduce the accumulation of 2, 4-D or other PGRs in culture during protoplast isolation and post-culturing time; in some cases the gradual accumulation of 2, 4-D exhibited a negative effect on somatic embryo formation and regeneration (Negrutiu et al. 1979). In our study, after culturing the protoplasts on optimum medium composition, the micro-callus and callus were formed, which slowly overcame the stress, imposed by wall degrading enzymes and regained normal growth as evidenced with gradual increase of biomass. In *Muscari armeniacum* Leichtl. ex Bak, the highest plating efficiency (10.9%) was obtained in 1.0 mg l\(^{-1}\) NAA and 1.0 mg l\(^{-1}\) BA added medium (Nakano et al. 2005). In our investigation, the combination of 0.5 mg l\(^{-1}\) NAA and 0.5 mg l\(^{-1}\) 2, 4- D produced maximum mean number of colonies per petriplate from protoplasts whereas in *Cryptocoryne wendtii* De Wit. colony formation was observed after 30 days of protoplast culture in MS, supplemented with 0.2 mg l\(^{-1}\) 2, 4-D, 1 mg l\(^{-1}\) NAA, 0.5 mg l\(^{-1}\) Zeatin, 0.3 M mannitol, and 0.15 M sucrose (Pongchawee et al. 2007).

Somatic embryo production from protoplast derived callus was greatly inhibited on BA-containing MS medium as in the case of embryos developed from normal callus which was used as a protoplast source (Suzuki and Nakano 2001) and these results are in confirmation with our present observations. Plant regeneration has been found possible when callus and cell suspension were used as source of protoplast isolation and culture (Pauk et al. 1994). We
also developed a protocol from ‘protoplast to plant’ by using embryogenic suspension (derived from hypocotyls-callus) as source of protoplasts. Hypocotyl derived embryogenic suspension was earlier used for protoplast isolation studies in plants like sugar beet where it offered better source for the regeneration of plants (Dovzhenko and Koop 2001) as compared to guard cells-derived protoplasts (Hall et al. 1996). Plant regeneration from protoplasts of orchids was first reported on Phalaenopsis (Ichihashi and Shigemura 2002) by culturing embryogenic callus-derived protoplasts in medium supplemented with coconut water and 2, 4-D.

As digestion of cells with enzyme mixture and osmotica created a cellular stress, we investigated various biochemical attributes and different antioxidant enzyme (CAT, SOD, APX and GR) activities in protoplast raised tissues and compared with normal tissues developed in vitro. The protein content was observed to be high in PDEC than in NEC; and this protein level also increased with time. Similar response i.e., enhanced level of protein was noticed in NaCl-stress callus cultures in rice (Priya et al. 2011). Sugar content was also high in enzyme treated callus than in the normal callus; however, the content gradually decreased towards proliferation stage. In TN5-M6 rice, soluble sugar content was high during callus induction stage, this enhanced level was reported to be developed by the over-uptake of sugar from the medium (Wen-Lii and Li-Fei 2002). In a majority of plants, salt stress alters gene expression with increased synthesis of osmo-protectors and osmo-regulators (Demiral and Turkan 2006). In other plants, there is an increased accumulation of amino acids and amines such as proline and B-alanine in their tissues in response to salt stress (Gobinathan et al. 2009). In our investigation, increased protein content was noted in PDEC than in NEC and the level gradually increased with time.

In the current study, it was noted that the specific activities of enzymes increased when suspension culture was treated with wall degrading enzymes and the same was observed in two investigated species of Grevillae in which CAT activity increased significantly in tissue after treatment with cell wall digestion enzymes, being the most in leaf tissue treated with cellulysin + cellulase in G. ilicifolia, and with cellulysin + macerase in G. arenaria (Kennedy and De Filipps 2004). The enzyme CAT, which catalyzes the disproportionation of hydrogen peroxide to dioxygen and water, is found in all aerobic microorganisms, plant and animal cells. It was reported that the CAT activity was activated in S. pombe cells that were exposed to hydrogen peroxide and the activity was regulated at the mRNA level (Mutoh et al. 1995). In C. roseus, we found increased activity of CAT in PDEC than in the NEC. The increased
activities of antioxidant enzymes upon salt stress are often related to the enhanced protection given to cells in order to tolerate adverse stress situation (Mittova et al. 2004).

In barley root, increase of APX activity was relatively low compared with that of SOD and CAT under NaCl-treatment (Sang et al. 2005). In our experiment, we also found increased activity of APX in PDEC than in the NEC but was low compared with SOD and CAT. The SOD activity was also high in PDEC than in the NEC. The increased enzyme activity in stress tissues including in *C. roseus* is primarily due to exposure of plant cells in digestion mixtures containing various enzymes and salts. The GR activity was low similar to CAT, SOD and APX but the activity was higher in PDEC when compared to NEC. Yung and Don (1975) earlier observed about 8 times high enzyme activity in cells than in the stress-free protoplasts. The significant increase in SOD, CAT, APX and GR activities is due to reactive oxygen species (ROS) formed after the plant cells were treated with enzymes and salts.

As a promising alternative to produce plant secondary metabolites, plant cell culture technology has many advantages over traditional field cultivation and chemical synthesis, particularly for compounds that are either derived from slow growing plants or difficult to be synthesized with chemical methods (Zhao and Verpoorte 2007). In addition, compounds from tissue cultures are more easily purified because of the simple extraction processes and also the absence of contaminants such as pigments, thus consequently, reducing the production and processing costs (Bourgand et al. 2001). In *C. roseus*, the same cell culture technologies with various culturing conditions aiming at higher production of alkaloids have been used for last few decades (Xu and Dong 2005b). The present study was carried out to investigate the effect of yeast extract (YE), a biotic elicitor on VB and VC yield in protoplast derived tissues and in protoplast regenerated plantlets in *C. roseus*. We noted that the yield of VB and VC was improved on addition of YE particularly at T3 treatment. There are several similar reports on YE-induced synthesis of triterpenoids/other secondary metabolites in cell and tissue cultures of plants like *Scutellaria baicalensis* (Yoon et al. 2000), *Panax ginseng* (Lu et al. 2001), *Centella asiatica* (Kim et al. 2007), *Angelica gigas* (Rhee et al. 2010) and *Pueraria candollei* (Korsangruang et al. 2010).

It is quite well known that the plant’s response to a pathogen is based on its ability to recognize signature molecules produced by the pathogen (Nuernberger 1999). These molecules are called elicitors and are primarily proteins and cell wall-derived oligosaccharides (Mahalingam and Fedoroff 2003). Elicitors are usually capable in inducing
various modes of plant defense including the production of ROS (reactive oxygen species), the hypersensitive response and the synthesis of phytoalexins, antimicrobial secondary compounds and other elements of defensive nature (Montesano et al. 2003). Elicitors have been successfully used for the production of secondary metabolites in cell cultures of different species such as *Thalictrum rugosum* (Brodelius et al. 1989), *Tagetes patula* (Buitelaar et al. 1992), *C. roseus* (Vazquez–Flota et al. 1994), *Taxus* sp. (Ciddi et al. 1995) and many others.

Many valuable bioactive compounds like azadirachtin, artemisinin and tanshinones accumulation has been reported to be successfully stimulated by YE elicitors (Putalun et al. 2007, Prakash and Srivastava 2008; Zhao et al. 2010). However, the mechanism of elicitation is noted to be diverse in plants and in most studied cases an ‘elicitor-receptor interaction’ is the basis after which a rapid array of biochemical responses occur (Radman et al. 2003). Rodriguez et al. (2003) showed that methyl jasmonate, a chemical inducer of secondary metabolism, promoted tabersonine biosynthesis in hairy root cultures of *C. roseus*.

The addition of YE also influenced the cultivated cells by altering synthesis of biochemical products like protein, proline and sugar as was observed from our present investigation. It was recently noted that the total soluble protein content was increased in *Euphorbia pekinensis* in response to biotic elicitor application (Gao et al. 2011). In this study, the total soluble protein content increased with an increase in YE concentration upto T3 at different developmental stages, so did proline and sugar. Beside acting as an osmo-protectant, proline also serves as a sink for energy to regulate redox potentials as a hydroxyl radical scavenger (Sharma and Dietz 2006), it also protects macromolecules against denaturation and helps in reducing acidity in the cell (Kishor et al. 2005). Extra proline accumulation has recently been reported in response to biotic stress that antagonizes GABA-induced quenching of quorum-sensing in *Agrobacterium tumefaciens* (Haudecoeur et al. 2009). Sugar levels, however, do not follow a static model and was reported to vary with genotype and with imposed stress level (Morsy et al. 2007).

The activities of POD, SOD, and CAT are usually used to evaluate physiological and biochemical responses of plants to biotic and abiotic stresses, and in studies of plant systemic acquired resistance system (Tanabe et al. 2008). The activity of CAT was increased after elicitor treatment, which suggests the possible origin and involvement of H$_2$O$_2$ in cultivated tissues (Gao et al. 2011). In our study of *C. roseus*, the enzyme activity was also increased
with application of YE, a biotic elicitor. The tissues of PDEC origin showed increased enzymatic activity than in NEC. The enzyme activity increased further on addition of YE and it may be due to over production of ROS. The major ROS scavenging mechanisms of plants include SOD, APX and CAT (Mittler 2002). The enzyme activity generally becomes stronger as the stress increases, or increases at the begini ng and then decreases by the end of stress treatment (Ren et al. 2002). In Rosea cultivar, increased antioxidative enzyme activity scavenged ROS successfully, prevented oxidative injury of membrane and enhanced oxidative stress tolerance in plants (Hassanein 2000). Glutathione reductase (GR) is a key enzyme of the ascorbate-glutathione cycle that also protected cells against oxidative damage and maintained a high GSH/GSSG ratio (Foyer and Noctor 2005). Elicitation, which does change enzyme levels was more effective in enhancing overall flux to indole alkaloids from hairy roots (Rijhwani and Shanks 1998).

Catharanthine production in C. roseus was reported to be high on optimized cultivation conditions (Smith et al. 1987a). Use of immobilization techniques (Facchini and DiCosmo 1991) and application of different elicitors like homogenates of fungal mycelium (Eliert et al. 1986) or non-biotic elicitors such as vanadium (Tallevi and DiCosmo 1988) also improved the yield. Moreno et al. (1996) studied the effect of elicitation on different metabolic pathways involved in secondary metabolism of C. roseus cell suspension cultures. Namdeo et al. (2007) reported that for successful protocol development involving precursor feeding lies in identification of cheapest by product of the other processes which can be converted to desired secondary metabolites by selected plant cell line. Junaid et al. (2008) reported efficient C. roseus embryogenesis (proliferation and maturation) in moderate levels of L-glutamine and L-asparagine. Whitmer et al. (2002) reported that supplying tryptamine or tryptophan along with iridoid precursors to transgenic cell lines S1 of C. roseus produced enhanced level of alkaloid accumulation. Morgan and Shanks (2000) studied the effect of addition of terpenoid and tryptophan branches to the culture medium and its effect on metabolic flux leading to indole alkaloids in C. roseus. In our experiments biotic elicitor (YE) was used in medium that effectively enhanced VB and VC yield, but the influence is treatment-specific, effective up to T3 i.e. 1.5 mg L⁻¹.

PDEC also showed a higher level of alkaloid as compared to NEC, the yield of VB and VC was even more in YE added medium. The perception of YE was earlier reported to be involved in stimulating the induction of TIA biosynthetic genes including strictosidine synthase (STR) and tryptophan decarboxylase, TDC (Pauw et al. 2004). A semi-synthetic
method of chemical or enzymatic coupling of parent monomeric indole alkaloids vindoline and catharanthine in forming VB was successfully exploited in previous studies (Goodbody et al. 1988; Kutney 1990) in which peroxidase was involved in coupling step of reaction (Smith et al. 2003). In this present study, we observed that on application of YE, the peroxidase activity was increased and that may enhance the VB and VC level in culture. The study of this nature is important as the plant is the only source of expensive and important chemotherapeutic agents with activity against several kinds of cancer (Schmeller and Wink 1998) and the alkaloids are present in a minute amounts (around 0.0005% DW).

The production of rosmaric acid (RA) and related phenolic compounds in *Salvia miltiorrhiza* hairy root cultures was similarly stimulated by YE and Ag+, an abiotic elicitor (Qiong et al. 2006). The stimulation of RA accumulation by biotic and abiotic elicitors (YE and MJ) has also been observed in cell cultures of other investigated species such as *Orthosiphon aristatus* (Mizukam et al. 1992), *Coleus blumei* (Szabo et al. 1999). In *L. erythrorhizon* suspension cultures too, addition of YE rapidly and transiently enhanced RA accumulation (Ogata et al. 2004). Sanchez-Sampedro et al. (2005) reported about three-fold production of silymarin after YE treatment in *Silybum marianum*. YE of 0.5 g L\(^{-1}\) was found to be optimum for elicitation in *Gymnema sylvestre* in which over 5.25 folds of gymnemic acid production was noted within 20 days of culture (Veerashree et al. 2012). YE is found to activate genes responsible for terpenoids indole alkaloids in *C. roseus* cells (Memelink and Gantet 2007). Over 113.3% increase in total phenolic content over control was reported in YE treated cells in *Astragalus chrysochlorus* (Ozgur and Sule 2009). In our study, we found enhancement of alkaloid yield on treatment with YE, and 1.5 g L\(^{-1}\) was observed to be the optimum treatment for VB and VC enrichment. In *E. californica* cells, the production of total alkaloids was 180% higher in medium, added with YE and MJ than that of YE alone (Farber et al. 2003).

YE is known to contain several components like chitin, *N*-acetylglucosamine oligomers, β-glucan, glycopeptides and ergosterol (Boller 1995) but the specific fractions that trigger the synthesis of alkaloids is still not elucidated properly. The peptide (Menke et al. 1999a) and polysaccharide (Zhao et al. 2011) fraction, present in YE has been suggested to be the molecules of stimulating nature. It is known that the protoplasts and protoplasts obtained tissue themselves suffer several stress conditions, provided by treatment with wall degrading enzymes, presence of PGRs in medium and by excess influx/efflux of required ions (George et al. 2008a). The addition of YE in medium in which protoplast or post-protoplast derived
tissues were cultivated may raise the stress level further (as was evidenced from increasing levels of biochemical stress markers like proline and enzymes) that may trigger in enhancing the level of VB and VC in cultured tissues.

Somatic embryogenesis acts as an alternative to organogenesis for regeneration of plants. In this present investigation, induced somatic embryos were encapsulated by using sodium alginate and calcium chloride. Encapsulated embryos were preserved at low temperatures and their viability was assessed later on. In *Catharanthus*, different combinations of sodium alginate and calcium chloride were used to efficiently optimize the encapsulation method and their conversion. Maximum encapsulation was seen by treating the embryos with 2.5% sodium alginate and 100mM calcium chloride. In plant systems like *Morus alba* (Patnaik et al. 1995) and *Hyoscyamus muticus* L. (Pandey and Chand 2005) 3.0% sodium alginate and 70-80mM calcium chloride were used for encapsulation that showed maximum conversion capacity. A 2% and 4% solution of sodium alginate upon complexation with 100 mM calcium chloride produced clear transparent firm capsules with an ion exchange duration of 30 min in *Flickingeria nodosa* (Dalz) Seidenf (Nagananda et al. 2011).

It has been shown that the percentage of sodium alginate employed for maximum conversion ability depends on plant species under investigation (Redenbaugh et al. 1986). We observed that a low percentage of sodium alginate resulted in the formation of tender and weak beads that had comparatively low conversion capacity. The presence of high percentage of sodium alginate resulted in the formation of hard beads that had a low conversion frequency in conformity with observation of Mohanraj et al. (2009). Several authors observed similar results in other investigated species like Papaya (Castillo et al. 1998) in which uniform beads were formed on 2.5% sodium alginate added medium.

The concentration of the complexing agent calcium chloride showed a significant impact on conversion capacity in this present case. A 15 min exposure to calcium chloride induced firm, round and uniform beads. It is known that the capsule hardiness depends upon optimal ion exchange of Na\(^+\) and Ca\(^{2+}\), however, this may vary with different propagules as well as with different plant species (Rai et al. 2009). It was also observed that the nutrient supplemented to the alginate matrix reduced the viscosity and the ability of the gel to form solid beads (Daud et al. 2008). We noticed that polymer concentration, alginate viscosity, calcium concentration and exposure time are fundamental in determining a successful encapsulation technique.
The present study also revealed the influence of PGRs on the conversion of embryos. We noticed highest conversion rate when the matrix was supplied with 0.25 mg l\(^{-1}\) NAA + 0.25 mg l\(^{-1}\) BA. The combination of NAA and BA also showed increased number of regenerated shoots in *Taxus baccata* (Abbasin et al. 2010), tea roses (Noriega and Sondhal 1991), but in other cases like tomato, the same combination produced increased callus instead (Harish et al. 2010). Contrary to this, BA alone was noted to be very effective in producing shoots in several studied plants like *Hibiscus sabdariffa* L. (Gomez-Levya et al. 2008).

It has been reported that the encapsulated somatic embryos may survive up to 4 weeks when stored at 4\(^{0}\)C and no loss in conversion ability was observed (Lulsdorf et al. 1993). Similar results were also reported in Olive (Micheli et al. 1998) and in apple rootstock (Sicurani et al. 2001). In our study, synthetic seeds kept at 4\(^{0}\)C showed quality storage with higher resistance than the naked embryos, which showed maximum conversion. An increase in temperature resulted in desiccation of embryos and in loss of conversion rate.

The release from cold-stratification of normal seeds has been demonstrated in different *Bunium persicum* populations (Sharifi and Pouresmael 2006). Encapsulated somatic embryos of Olive stored at 2 or 4\(^{0}\)C for 2-3 months showed high conversion rates of 61% (Micheli et al. 1998). It has been suggested that several compounds such as amino acids, ammonium compounds, sugars, sugar alcohols are possibly produced by cultivated plant cells in response to stress (osmotic -, salt-, low temperature stress and desiccation etc.) provide protection to cells (Hincha et al. 2006). It is, however, very difficult to explain the mechanism by which a compound or group of compounds acting together while giving tolerance to cells. In this present investigation, it appeared that the added alginate and sugar in capsule might be involved as functional compound in making cells tolerant to freezing. Nieves et al. (2001) observed that hydrated synthetic seeds were difficult to store at room temperature because of depleted nutritive reservoir and lack of quiescence that lowered conversion percentage. In *alfalfa*, at 4\(^{0}\)C even, the storage life of synthetic seeds was quite short (Redenbaugh et al. 1987), and the conversion rate was poor in *Asparagus cooperi* (Ghosh and Sen, 1994) and in *Eucalyptus citrisdora* (Muralitharan and Mascarenhas 1995). However, in *Santalum album*, the encapsulated embryos retained their conversion (18%) ability for over 6 weeks or more after storage at 14\(^{0}\)C (Rao et al. 1993).

In our present study, synthetic seeds showed highest frequency of conversion after 30 days of storage which was, however, reduced when encapsulated embryos were kept for extended
period of time indicating that with increased storage periods conversion frequency is reduced. In normal seeds also, the conversion frequency decreases with time as was found in Bunium persicum (Sharma and Sharma 2010). The decline in conversion from encapsulated embryo into plants may be related to both oxygen deficiency in the gel bead and its rapid drying (Swamy et al. 2009). The exploitation of stable and regenerative embryogenic masses is an attractive tool for the fast production of clonal plants including transgenics where synthetic seed technology can preserve those mother tissues. In C. roseus, encapsulation technology is therefore important to conserve important cell lines in which plantlets may be produced from beads after short to medium term storage.