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Plant Regeneration from Encapsulated Adventitious Shoot Buds and Somatic Embryos of Tylophora Indica- An Effective Way of Mass Propagation and Germplasm Exchange

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Abstract

An efficient procedure for plant regeneration from alginate/ chitosan encapsulated adventitious shoot buds and somatic embryos of *Tylophora indica*- an endangered medicinal plants has been developed. Adventitious shoot buds were induced from mature leaf explants on Murashige and Skoog's (MS) medium supplemented with 6- benzyladenine (22µM) and adenine sulphate (1.35 µM), where 50-60 shoots/explant were formed in 90% of cultures. Embryogenic callus was obtained from leaf explants on MS medium supplemented with 2, 4-dichlorophenoxy acetic acid (9.74µM) and 3% sucrose and various stages of embryo development were observed in the cultures. Somatic embryos and shoot buds thus formed *in vitro* were encapsulated using sodium alginate (2%) or chitosan (0.1%) to form artificial seeds which could be stored at low temperature (4°C) for more than 90 days. The viability of these seeds was checked after different periods of storage and the regenerated plantlets were successfully acclimatized and established in the field conditions with 95% survival rate.

Keywords*: Tylophora indica, synthetic seeds, adventitious shoot buds, alginate, somatic embryos.*

Abbreviation*: BMS: Basal Murashige and Skoog's medium, NAA- Naphthalene acetic acid, K – Kinetin, 2,4- D- 2,4-dichlorophenoxy acetic acid, BA – 6 benzyladenine, TPP- Tripolyphosphate.*

Introduction

Tylophora indica (Burm. f.) Merrill is an important medicinal plant of family Asclepiadaceae which is widely used in traditional medicine for the treatment of bronchial asthma, bronchitis, rheumatism, inflammations, dermatitis and allergies [1, 2]. The leaves and roots of this plant contain active alkaloids like tylophorine, tylophorinine and anticancerous tylophorinidine which exhibit various pharmacological and biological activities [3, 4]. The conventional propagation of T. indica occurs through seeds but seeds are too small and have low seed viability and germination. The plant is not amenable to vegetative propagation through cutting or grafting thus limiting multiplication of desired cultivars. Due to the shortage of high quality planting material, commercial cultivation of this valuable plant T. indica is uncommon and its overexploitation and indiscriminate use have rendered the species highly vulnerable to extinction. It is, therefore, imperative to adopt in vitro methods which can be effectively used for large scale multiplication and conservation of this endangered species.

Plant regeneration through somatic embryogenesis is considered to be more efficient and a preferred pathway than organogenesis as it offers better potential for large scale multiplication. There are only a few reports on the induction of somatic embryos from the leaf explants of Tylophora indica [5-8]. These reports are, however, of preliminary nature and no histological studies or detailed microscopic studies have been carried out to corroborate embryogenesis. However, in the present investigation, stereozoom microscopy and detailed histological studies were carried out to study various developmental stages of embryo like globular, heart shaped, torpedo and cotyledonary in the cultures to ascertain somatic embryogenesis. In recent years, encapsulation technology has drawn much attention for the production of artificial seeds as it helps in minimizing the cost of micropropagated plants and can be useful for the conservation, exchange and distribution of germplasm of elite and endangered plants. Mostly somatic embryos have been used for the production of synthetic seeds, however, in recent years, the possibility of encapsulating vegetative propagules such as axillary buds, shoot tips and nodal segments as an alternative for somatic embryos has also been explored [9 -14].

In T. indica, there is only one report of synthetic seed production by encapsulating somatic embryos in sodium alginate and maximum frequency (65%) for conversion of encapsulated seeds into plantlets was investigated for 6 weeks only [7]. There are no reports on the storability and morphogenetic response of these seeds beyond 6 weeks. In the present investigation, we describe for the first time the successful encapsulation of in vitro raised adventitious shoot buds for the development of synthetic seeds. In T. indica, synthetic seed technology will offer many advantages and opportunities since it is a cross pollinated plant having low seed set and germination viability. Hence, this investigation is aimed to develop an efficient protocol for plant regeneration through successful encapsulation of T. indica and also to evaluate the regenerative response of these synthetic seeds after different periods of storage.

Material and Methods

Explant Preparation and Culture Medium

Leaves were collected from 2 years old mature field grown healthy plants of *T. indica* maintained at Thapar University Campus, Patiala. Explants were washed thoroughly under running tap water for 30 minutes followed by their immersion in teepol solution (1% v/v) for five minutes and subsequent washings with tap water. The explants were then treated with bevistin (0.1% w/v) for 10-12 minutes followed by repeated washings with water. Thereafter the explants were surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride for 3-4 minutes inside the laminar chamber, followed by 3-4 washings in sterile distilled water to remove all traces of mercuric chloride. Fresh cuts were given to the segments after sterilisation to remove dead portions and explants of 4-5 mm in size were cut.

The excised explants were cultured on MS [15] medium supplemented with various growth regulators like NAA, 2,4-D, IAA, IBA, K, BA and adenine sulphate either alone or in various combinations, 2- 3% sucrose and 1% agar with pH adjusted to 5.8 before the addition of agar. Culture bottles containing media were autoclaved at 121^{o}C for 15 lbs/inch^{2} for 15 minutes. All the inoculated cultures were incubated in growth room in controlled conditions at a temperature of 25 ± 2^{o}C, 16 hr light/8 hr dark photoperiod and continuous illumination provided by cool white fluorescent tubes at 50µmolm^{-2}s^{-1}. Thirty replicates were used for each treatment and each experiment was repeated thrice.

Induction of Adventitious Buds and Somatic Embryos

De novo adventitious shoot bud formation from leaf explants occurred on Murashige and Skoog's medium (MS) supplemented with BA (22µM) either alone or in conjunction with adenine sulphate (1.35 µM). Embryogenic but slow growing callus was obtained on MS medium supplemented with 2, 4-D (9.74 µM) with 3% sucrose. Formation of fast growing, green and compact callus was observed on α- Naphthalene acetic acid (29.4 µM) and Kn (4.65 µM) supplemented medium containing 2% sucrose but the callus remained non-embryogenic even after repeated subculturing. Callus, thus, formed was transferred to 2, 4-D (9.74-19.48 µM) supplemented MS medium augmented with 3% sucrose for the induction of somatic embryos.

Histological Studies

Histological studies of embryogenic calli were carried out to study various stages of embryo development in the cultures. The callus was fixed in FAA (Formalin: Acetic acid: Alcohol 5: 5: 90 v/v) for 48 hrs. It was then passed through different grades of tertiary butyl alcohol for dehydration followed by infiltration in molten paraffin wax and microtome sections were cut. The sections were deparaffinized and stained with saffranin and fast green followed by mounting in Canada balsam and the slides were kept in oven for 24 hours for drying. Microscopic examination of the slides was carried out thereafter.

Encapsulation and Preparation of Artificial Seeds

For artificial seed production, somatic embryos and shoot buds were subjected to encapsulation with sodium alginate by dropping method [16]. Encapsulation was done using sodium alginate as the gelling agent in the range of 1-4% (w/v) whereas 100 mM of calcium chloride was used as complexing agent. Encapsulation was done by mixing somatic embryos or shoot buds with sodium alginate and were added as drops into pre-chilled calcium chloride solution and kept as such for 20-30 minutes to obtain polymerization of sodium alginate.

Somatic embryos and shoot buds were also encapsulated in chitosan complex to form synthetic seeds. Chitosan solution was prepared in the range of 0.1% - 1% in 0.1N acetic acid. Somatic embryos and shoot buds were mixed with chitosan solution and then gently dropped into 0.1% Tripolyphosphate solution (TPP). Encapsulated

beads (5mm in diameter) were then collected on a sterile filter paper in a petridish and were stored at 4º C for further use. In order to retrieve plantlets after different periods of storage (0, 20, 30, 40, 60 and 90 days), encapsulated somatic embryos and shoot buds were cultured on growth regulator free 1% MS basal medium.

Acclimatization and Establishment of Plants in Soil

For acclimatization, plantlets with well developed roots were carefully rescued from culture media covered with perforated polythene covers and were kept for a period of 15 days under growth room conditions. They were covered with perforated plastic bags and were kept for a period of 15 days under growth room conditions. Plantlets were then transferred to polybags containing sterile potting mixture of soil: vermicompost (1:1) and were maintained inside the growth room for another 4 weeks. Plantlets were watered periodically and monitored. The hardened plantlets in plastic bags were then shifted to the green house for 2 weeks before their final transfer to full sunlight outdoor for the normal growth.

Results

Leaf explants (4-5mm in size) were cultured on variously supplemented MS medium for *de novo* adventitious shoot formation. MS medium supplemented with BA (22µM) and adenine sulphate (1.35 µM) produced the greatest number of shoot buds directly from the explant. Nodular meristemoids differentiated from the cut surface of the leaf lamina after 8-10 days of culturing (Fig.1a) and within 3 weeks the entire segment was covered with these meristemoids. Eventually these nodular meristemoids developed into green leafy shoots in 90% of cultures after 5 weeks (Fig. 1b). Repeated subculturing accelerated the formation of shoots in large numbers (50-60/culture) without any decline in their proliferation.

Fast growing, green and compact callus was obtained from the leaf explants on NAA (29.4 µM) + Kn (4.65 µM) supplemented medium. Initiation of callus occurred either at the cut ends or along the entire surface of leaf segment and within 3-4 weeks the whole explant turned into a mass of callus. The callus, however, remained non-embryogenic even after repeated subculturing. For induction of embryogenesis, the callus, thus, formed was transferred to different concentrations of 2, 4-D (9.74-19.48µM) and sucrose (2, 3 & 4%). Best results occurred on 19.48 µM 2, 4-D and 3% sucrose which yielded highest number of somatic embryos in about 95% cultures. Embryogenic but slow growing callus could be obtained directly when leaf explants were cultured on MS medium supplemented with 2, 4-D (9.74 µM) and 3% sucrose. Stereozoom microscopy revealed various stages of embryo development in the callus cultures. Numerous prominent globular shaped embryos were seen after 4 weeks (Fig. 1c) which passed through various developmental stages like heart shaped, torpedo and cotyledonary (Figures 1d, e & f). Cotyledonary embryos thus formed developed into tiny plantlets with distinct roots and shoot axis (Fig. 1g). Different stages of embryogenesis were also studied histologically to confirm the formation of somatic embryos in the cultures. The early stages showed differentiation of embryogenic cells with prominent nuclei and dense cytoplasm. These embryogenic cells divided further and various stages of embryo formation like globular, torpedo, heart and cotyledonary were seen in the cultures histologically (Fig. 1h, i & j).

Somatic embryos and shoot buds formed under *in vitro* were used for the construction of synthetic seeds by encapsulating them either in sodium alginate or chitosan. Seeds were constructed by encapsulating somatic embryos mostly at early cotyledonary stage and shoot buds (3-4mm in size) in different concentrations of sodium alginate (1-4%) and calcium chloride (100 mM) as the gel matrix. Sodium alginate at a concentration of 2% was found to be most appropriate for encapsulation resulting in the formation of clear, transparent isodiametric beads approximately 5mm in diameter (Fig. 2a). Magnified somatic embryos and shoot bud encapsulated beads are shown in Figures 2b and c. Lower concentrations (1 %, 1.5 %) of sodium alginate resulted in the formation of fragile beads and higher concentrations (3 %, 3.5 %, 4%) favoured the formation of hard beads which had a marked effect on the germination or conversion process later on. Somatic embryos and shoot buds were also encapsulated in chitosan, where best complex was formed using 0.1% chitosan with 0.1% TPP solution. Chitosan is a polycationic in acidic medium and can interact with negatively charged species like TPP and leads to the formation of biocompatible cross linked chitosan beads. The encapsulated beads were collected on a sterile filter paper in a petridish (Fig. 2d) which was sealed with a parafilm and stored in refrigerator at 4°C. The frequency of conversion of these synthetic seeds into plantlets was evaluated after different storage periods of 0, 20, 30, 40, 60 and 90 days at 4°C. The highest conversion rates for encapsulated somatic embryos and shoot buds was 95 and 98% after 0 day of storage which decreased to 80 and 85%

respectively after 20 days. For retrieving plantlets, the encapsulated shoot buds were cultured on Basal MS medium supplemented with 2% sucrose. The alginate coat dissolved after 3-4 days followed by sprouting of shoot bud (Fig. 2e) which elongated further (Fig. 2f) and formation of complete plantlets with well developed root and shoot system was observed within 18- 20 days (Fig.2g). Numerous well developed plantlets with normal phenotype were formed from shoot bud encapsulated seeds (Fig. 2h). Germination of somatic embryo encapsulated beads occurred after 7-8 days of culturing on basal MS medium where emergence of radical occurred first (Fig. 2i) and complete plantlet was formed within 14-15 days (Fig. 2j). A number of plantlets formed from encapsulated somatic embryos are shown in figure 2k.

A gradual reduction in the conversion rates and plantlet regeneration from these synthetic seeds was observed with increase in the storage period. Synthetic seeds could be stored at 4º C for more than 90 days but there was a gradual reduction in the conversion percentage and plantlet regeneration which decreased to 10-15% after 90 days. The % response for conversion of these synthetic seeds stored for 0, 20, 30, 40, 60 & 90 days is depicted in Figure 3. Somatic embryos and shoot buds encapsulated in chitosan complex were transferred to basal MS medium to check the survival percentage. The seeds however failed to show any germination.

The rooted microshoots (5-6 cm) were on successfully hardened off, initially on the moist cotton (Fig. 2l) for 2 weeks before their transfer to the potting mixture of (1:1) soil: vermicompost (Fig. 2m) maintained in the growth room under high humidity for another 4 weeks. Thereafter, the hardened plantlets in plastic bags were shifted to green house for 2 weeks before their final transfer to natural soil under full sun (Fig.2n). By this time plants have become sturdy, developed an efficient root system, formed new leaves and became phytosynthetically active (Fig. 2n). The acclimatized plants with 95% survival rate were successfully established in the field conditions and showed uniform growth and true to type morphology.

Discussion

The objective of present investigation was to develop an efficient protocol for plant regeneration in *Tylophora indica* from synthetic seeds produced from somatic embryos and adventitious buds and also to evaluate the regenerative response of theses synthetic seeds after different periods of storage at 4º C. The present study reports the first successful attempt to encapsulate the adventitious shoot buds formed from the leaf explants in *Tylophora indica* for the development of synthetic seeds. Faisal and Anis 2007 [17} reported *in vitro* propagation of *Tylophora indica* from encapsulated nodal segments. However use of adventitious shoot buds in the present report clearly demonstrates that adventitious shoots buds are more efficient as a material in synthetic seed technology since a large number of synthetic seeds can be constructed. In the present study, large number adventitious buds (50-60 in number) could be induced from a small piece of leaf tissue and further the regenerative potential did not decline even with repeated subculturing. Hence, production of synthetic seeds via adventitious shoot buds offers a potent, low cost, high volume propagation system for *Tylophora indica*. In recent years, non embryogenic vegetative propagules have been successfully encapsulated in several important plants specially as an alternative to somatic embryos and especially in those plants where somatic embryogenesis has not been documented [18 -21].

For callus induction, synergistic action of NAA (29.4 µM) and kinetin (4.65 µM) gave the best results leading to the formation of green, compact and fast growing callus from the leaf explant. The callus however remained non-embryogenic even after repeated subculturing. For the induction of somatic embryos, the callus was transferred to MS medium supplemented with different combinations of 2, 4-D (9.74- 29.4 µM**)** and sucrose (2-4%). MS medium supplemented with 2, 4-D (19.48 µM) and 3% sucrose produced the highest number of somatic embryos. Embryogenic callus could also be obtained directly by culturing the leaf explants on MS medium supplemented with 2, 4-D (9.74 µM) and 3% sucrose. The callus thus formed was comparatively slow growing. Chandrasekhar *et al*. 2006 [7] reported 2, 4 D (1.5 µM) along with TDZ (0.5 µM) to be most effective in inducing somatic embryos from the leaf callus of *Tylophora indica*. Jayanthi and Mandal 2001 [6] found 2ip to produce maximum number of somatic embryos from the leaf callus of *Tylophora indica* whereas Sahai *et al.* 2010 reported high frequency somatic embryogenesis on BA supplemented MS medium. Role of 2, 4 –D either alone or in combination with other plant growth regulators for induction of somatic embryogenesis is well documented in many plants like *Citrus sp* [22], *Podophyllum peltatum* [23] and *Dalbergia sissoo* [14].

In the present study, *in vitro* formed somatic embryos and adventitious shoot buds were subjected to encapsulation to form synthetic seeds using different concentrations of Na alginate and $CaCl_2$ as gel matrix. Out of various concentrations of Na alginate tested, 2% sodium alginate and 100mM $CaCl_2$ was the best for the formation of Ca alginate beads. Encapsulation of somatic embryos and vegetative propagules using different concentrations of sodium alginate ranging from 1.5% to 6% has been reported in different plant species [24- 26].The present results are in conformity with the report of Chandrasekhar *et al*. 2006 who obtained best results in *Tylophora indica* by encapsulating somatic embryos with 2% sodium alginate which resulted in 65% germination after 6 weeks. Synthetic seeds developed by encapsulation of somatic embryos and adventitious shoot buds could be stored upto 90 days although the percentage germination and conversion of these seeds into plantlets decreased gradually with increase in the storage duration at 4º C. The seeds were checked for their viability and germination into plantlets after 0, 20, 30, 40, 60 and 90 days on BMS medium with 2% sucrose. Encapsulated shoot buds showed 98% conversion or germination after 0 day, 85% after 20 days, 75% after 30 days, 60%, 40% and 20 % after 40, 60 and 90 days respectively. Conversion of encapsulated somatic embryos was 95%, 80%, 70%, 55%, 30% and 15% after storage at 0, 20, 30, 40, 60 and 90 days respectively. Similarly decline in conversion frequency on storage of synthetic seeds is reported in a number of plants. In *Spilanthes acmella* encapsulated shoot tips stored at 4º C showed 50% conversion or germination after 60 days [21]. In *Dalbergia sissoo*, conversion percentage of encapsulated somatic embryo was 29.4% and 16.1% after 15 and 45 days respectively [14], whereas in cork oak germination rate declined from 53% (after 2 months) to 33% after 5 months of storage [24].

Rooted microshoots thus formed were transferred to moist soil and incubated at 25 ± 2°C for 15 days, followed by acclimatization in soil: vermicompost potting mixture. The plantlets thus formed were shifted to green house, where they are thriving very well with 90% survival rate. It is therefore concluded that the present synthetic seed technology can be successfully employed for low cost, large scale production and short term storage of desirable elite genotypes of *Tylophora indica.*

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Appendixes: Figures

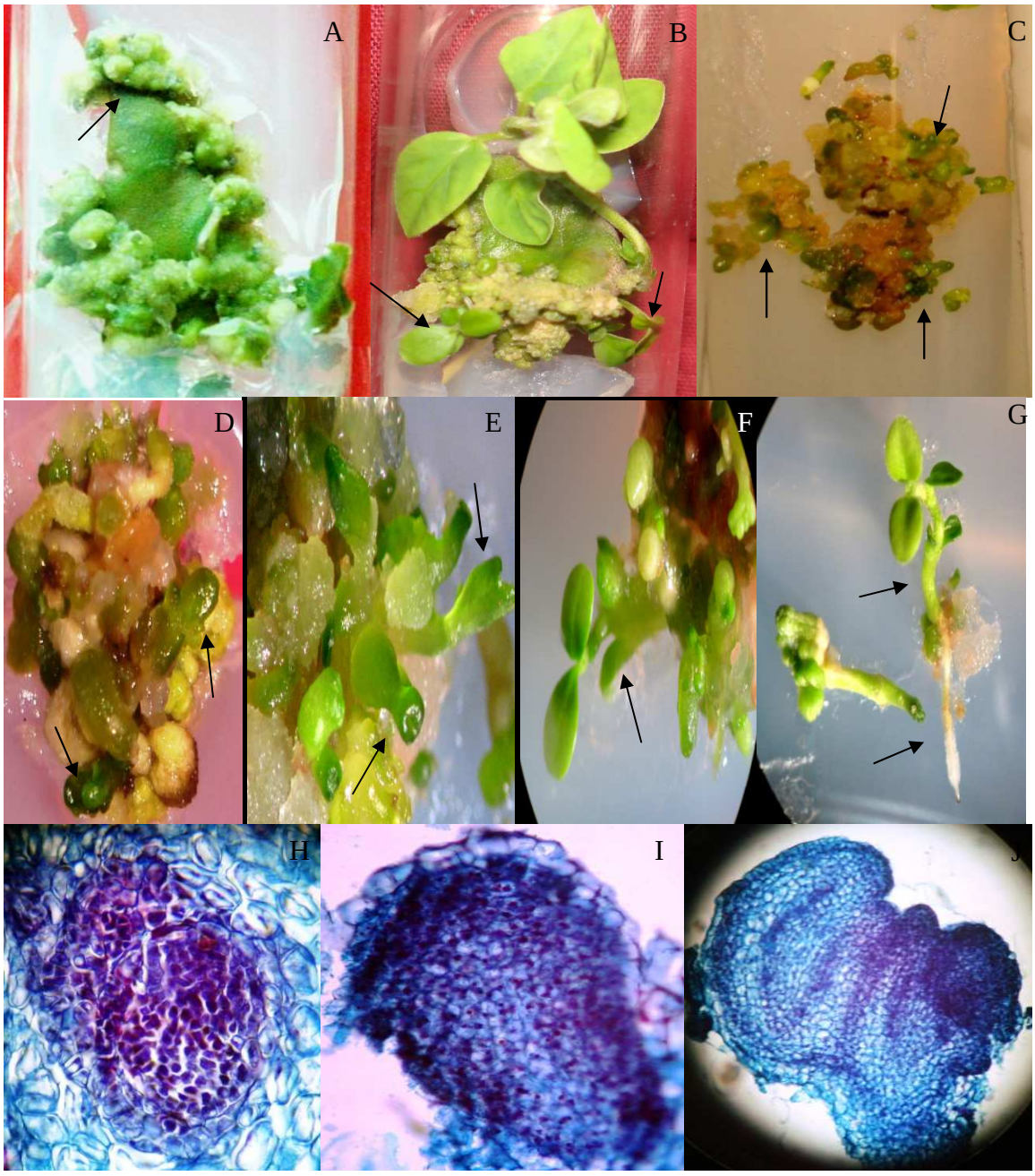

Figure1 a Formation of nodular meristemoids from leaf explants on BA (8.8 µM) and adenine sulphate (1.35 µM) after 8-10 days **b** Differentiation of green leafy shoots from meristemoids after 5 weeks of culturing **c** Formation of numerous prominent somatic embryos formed on 2, 4- D (9.74 µM) with 3% sucrose **d, e & f** Stereozoom photographs of different developmental stages of somatic embryos at globular, heart, torpedo and cotyledonary stages X 80 **g** Development of tiny plantlets from cotyledonary embryos showing well defined root and shoots **h, i & j** Histological observations showing globular, torpedo and cotyledonary stages during somatic embryogenesis X 400

Figure2 a Artificial seeds formed by encapsulation with sodium alginate **b & c** Encapsulated somatic embryos and shoot buds on a magnified scale **d** Chitosan encapsulated somatic embryos and shoot buds **e** Sprouting of shoot bud from encapsulated bead **f** Further elongation of shoot bud on BMS medium **g** Plantlets derived from shoot bud encapsulated beads after 15-20 days **h** Numerous well developed plantlets from encapsulated shoot buds **i** Emergence of radical from somatic embryo encapsulated bead **j** Formation of a plantlet with distinct root and shoot axis from somatic embryo encapsulated bead **k** Formation of large number of well developed plantlets

l Hardening of plantlet on moist cotton **m** Plant transferred to soil : vermicompost potting mixture in the growth room **n** Acclimatized plantlet transferred to plastic bag **o** A well developed plantlet transferred to full sunlight outdoor for the normal growth.

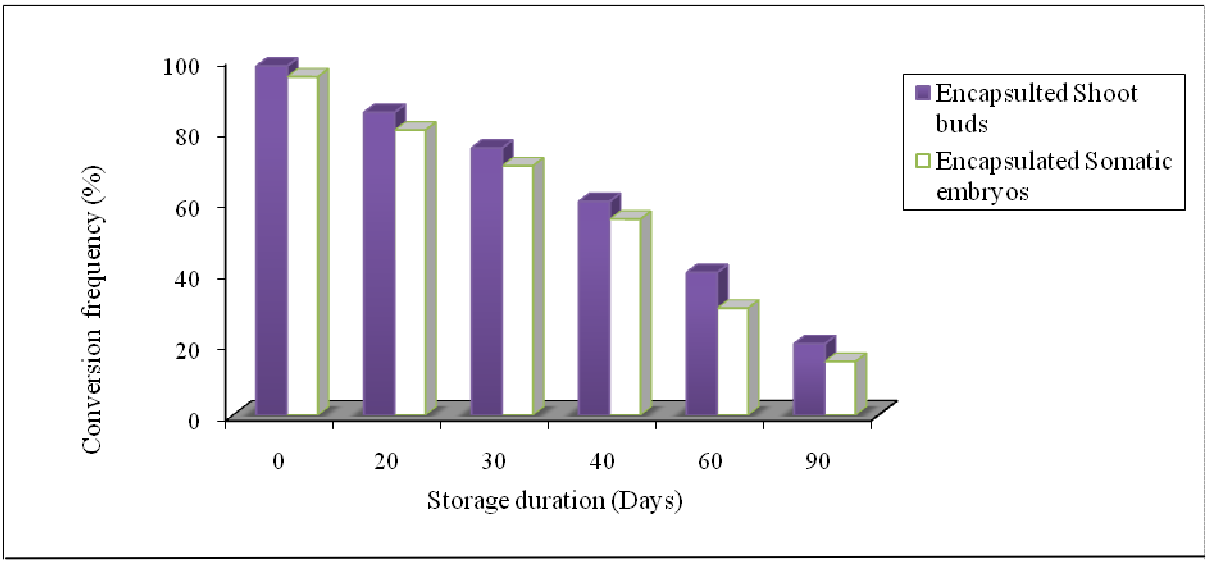

Figure 3 Percentage Conversion of Encapsulated Seeds after Different Storage Periods