SYNTHETIC SEED PRODUCTION

6.1. Introduction

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under in vitro or ex vitro conditions and that retain this potential also after storage (Capuano et al., 1998). Earlier, synthetic seeds were referred only to the somatic embryos that were of economic use in crop production and plant delivery to the field or greenhouse (Gray and Purohit, 1991; Janick, et al., 1993). In the recent past, however, other micropropagules like shoot buds, shoot tips, organogenic or embyrogenic calli, etc. have also been employed in the production of synthetic seeds. Thus, the concept of synthetic seeds has been set free from its bonds to somatic embryogenesis and links the term not only to its use (storage and sowing) and product (plantlet) but also to other techniques of micropropagation like organogenesis and enhanced axillary bud proliferation system. Implementation of synthetic seed technology requires manipulation of in vitro culture systems for large-scale production of viable materials that are able to convert into plants for encapsulation.

Although results of intensive researches in the field of synthetic seed technology seem promising for propagating a number of plant species, practical implementation of the technology is constrained due to the following main reasons:

- Limited production of viable micropropagules useful in synthetic seed production.
- Anomalous and asynchronous development of somatic embryos.
- Improper maturation of the somatic embryos that makes them inefficient for germination and conversion into normal plants.
- Lack of dormancy and stress tolerance in somatic embryos that limit the storage of synthetic seeds.
- Poor conversion of even apparently normally matured somatic embryos and other micropropagules into plantlets that limit the value of the synthetic seeds and ultimately the technology itself.
In the present attempt, synthetic seed production strategies were developed for the two study species, *Acacia caesia* and *Acalypha fruticosa*. For the former species, *in vitro* nodal segments and leaf callus derived somatic embryos were encapsulated and for the later species, *in vitro* leaf derived callus and *in vitro* node were encapsulated. The germination efficiency of the seeds in terms of callus formation, and shooting and rooting attributes as influenced by the concentration of sodium alginate in encapsulation matrix, and storage period and temperature was also studied by standardizing the MS medium.

### 6.2. Materials and methods

The immature part of leaf and nodal segments were collected from the young plants of the two study species, *Acacia caesia* and *Acalypha fruticosa* raised in the greenhouse for the production of *in vitro* explants. They were sterilized with 0.1% (w/v) HgCl$_2$ for 3 min and washed thoroughly with sterile distilled water.

For the species, *Acacia caesia*, the leaf discs were cultured by using standardized MS medium which contained TDZ and NAA at 1.5 and 0.3 mg/l respectively for the production of somatic embryos. The nodal explants were collected from *in vitro* derived multiple shoots of this species. For the other species, *Acalypha fruticosa*, the leaf discs were cultured by using standardized MS medium which contained BAP and NAA at 2.0 and 1.2 mg/l respectively for callus induction. The nodal explants for this species were derived from *in vitro* cultures. The pH of the medium was adjusted to 5.7 – 5.8 prior to autoclaving at 121°C for 20 min. Cultures were maintained at a temperature of 25 ± 2°C under 16 h light (2000 lux)/18h dark photoperiod and subcultured every 4 weeks. The callus, nodes and somatic embryos proliferated on this medium were used for the production of synthetic seeds of the respective study species.

Sodium alginate solution at the concentrations of 2, 3, 4, 5 and 6 % (w/v) were prepared in full strength MS medium with 2 % sucrose (w/v). In each concentration, each numbering of 50 *in vitro* leaf derived callus, leaf callus derived somatic embryos and *in vitro* node of the two study species were immersed for few seconds. Later, the micropropagules in alginate medium were picked up by tweezers and dropped into a sterile solution of 100mM calcium chloride. The drops each containing a single micropropagule were placed in this solution for half an hour to allow polymerization.
Calcium alginate beads containing the micropropagules were retrieved from the solution and rinsed twice with autoclaved distilled water to remove the traces of calcium chloride. The beads were then transferred to sterile filter paper in Petri dishes. Blot dried beads were stored in Petri dishes sealed with parafilm for 2, 4 and 6 months at refrigerator (4°C) and plant tissue culture room (25°C).

After storage, the encapsulated beads were cultured in regeneration medium and incubated in a culture room maintained at 25 ± 2°C. Various regeneration media tested were supplemented with different concentrations of various growth regulators. Frequency of callusing, number of shoots/explant and shoot length were measured to know the regenerating efficiency of synthetic seed. The percentage of shoot emergence from the encapsulated beads was calculated after 3-4 weeks of incubations.

The subculturing for shooting behaviors and subsequent rooting behaviors were made only for the beads which showed higher conversion frequency. For shooting, the emerged shoots were subcultured onto the MS medium supplemented with various combinations and concentrations of growth regulators. The shooting characters such as per cent shooting, number of shoots and shoot length were observed. For rooting, developed shoots were transferred to MS medium with various combinations and concentrations of growth regulators. The rooting attributes like rooting percentage, root length and number of roots were observed.

The well developed healthy plantlets were removed from the culture flasks and were thoroughly washed in running tap water to remove the adhering nutrient medium completely without causing damage to roots. Then the plants were soaked in 1% (w/v) fungicide, methyl-3 benzimidizole carbamate (Bavistin) solution for 10-15 minutes and transferred to small plastic pots filled with various types of sterilized potting media as detailed below:

<table>
<thead>
<tr>
<th>Composition</th>
<th>Proportion of components (v/v)</th>
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<tbody>
<tr>
<td>Red soil + sand</td>
<td>1:1</td>
</tr>
<tr>
<td>Vermiculate + sand + forest litter</td>
<td>1:1:1</td>
</tr>
<tr>
<td>Decomposed coir waste + perlite + compost</td>
<td>1:1:1</td>
</tr>
<tr>
<td>Vermicompost + soil</td>
<td>1:1</td>
</tr>
<tr>
<td>Red soil + sand + vermicompost</td>
<td>1:1:1</td>
</tr>
<tr>
<td>Vermiculate + coir waste + forest litter</td>
<td>1:1:1</td>
</tr>
<tr>
<td>Garden soil + sand + vermicompost</td>
<td>1:1:1</td>
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</tbody>
</table>
Twenty five plantlets per potting mixture were tested and growth rates were calculated after 30 days of hardening. During hardening process, initially the plantlets were kept under shade net present inside the shade house for 10 days. Then they were gradually transferred to 75% shade house and maintained for 2 weeks followed by the exposure of them in the natural environmental conditions.

6.3. Results

6.3.1. Effect of different concentrations of sodium alginate on conversion frequency

In the present study, the explants such as in vitro leaf derived callus, leaf callus derived somatic embryos and in vitro node were used for encapsulation. The polymerizing ability of sodium alginate at different concentrations (2, 3, 4, 5 and 6%) varied markedly for the two study species (Tables 15 and 23). Very firm, clear, isodiametric beads of uniform size and shape were achieved using 4% sodium alginate solution and 100mM calcium chloride (Figs.16 and 23 and Plates IXa, IXb, Xa, Xb, XIa, XIb, XIIa and XIIb). Concentrations of sodium alginate, lower than 4% were not suitable as the beads were too soft to handle, while at higher concentrations (5 and 6%), were too viscous, harder and hindered the emergence of shoots.

For the species, Acacia caesia, among the two in vitro explants, the conversion frequency was highest for the beads contained nodal segments (78%) when immersed with 4% sodium alginate. In addition, it has been noted that the conversion frequency was decreased with the increase of sodium alginate concentration (37% at 6%). For the in vitro leaf callus derived somatic embryos, the conversion frequency was higher (70%) at 4% sodium alginate concentration, while it was decreased with the increase of sodium alginate concentration (35% at 6%). Based on the response, for further subculturing experiments, the beads containing in vitro node and leaf callus derived somatic embryos were used.

For the other species, Acalypha fruticosa, among the two in vitro explants, the conversion frequency was higher for the leaf derived callus segments (79%) when immersed with 4% sodium alginate. In addition, it has been noted that the conversion frequency was decreased with the increase of sodium alginate concentration (38% at
6%). Where as, the in vitro derived nodal segments registered higher conversion frequency (74%) at 4% sodium alginate concentration, while it was decreasing with the increase of sodium alginate concentration (34% at 6%). Based on the response, for further subculturing experiments, the beads containing in vitro leaf derived callus and in vitro nodal segments were used.

6.3.2. Effect of storage period and temperature on shoot emergence from encapsulated beads

For the species, Acacia caesia, the shoot emergence from in vitro derived encapsulated node is directly depending on storage period and temperature (Tables 16). It has been observed that 4 months old in vitro derived nodal explants encapsulated beads recorded high emergence of shoots, ranging between 45.24 and 75.00% when compared to the 2 and 6 months old beads (42.43 – 72.10% and 40.32 – 68.57% respectively). On the other hand, it has been noted that 2 months old in vitro leaf callus derived somatic embryo encapsulated beads produced higher emergence of shoots (38.15 – 70.00) in comparison with that of 4 and 6 months old beads (40.31 – 68.43 and 30.54 – 60.23 respectively). The suitable temperature determined for the storage of in vitro explants encapsulated beads for 2, 4 and 6 months durations is 25°C.

For the other species, Acalypha fruticosa, the shoot emergence from in vitro leaf derived callus encapsulated seed is directly depending on storage period and temperature (Tables 24). It has been observed that 4 months old in vitro callus encapsulated beads recorded high emergence of shoots ranged between 46.00 and 77.34% when compared to that of 2 and 6 months old beads (43.45 – 74.62% and 41.25 – 68.11% respectively). On the other hand, it has been noted that 2 months old in vitro node encapsulated beads produced higher emergence of shoots (44.21 – 71.10) in comparison with that of 4 and 6 months old beads (42.00 – 69.38 and 33.19 – 62.00 respectively). The suitable temperature for storage of in vitro explants encapsulated beads for 2, 4 and 6 months durations is determined to be 25°C.

6.3.3. Effect of various growth regulators on bud sprouting and regeneration of encapsulated beads

After storage in proper conditions, the synthetic seeds prepared from in vitro leaf derived callus, leaf callus derived somatic embryos and in vitro nodal segments for the
study species, *Acacia caesia* and *Acalypha fruticosa* were cultured on MS medium with different growth regulators to determine the optimum combinations and concentrations of growth regulators for effective regeneration. The study reported the following results:

The effect of various combinations and concentrations of certain growth regulators on the shooting attributes of *in vitro* nodal segment encapsulated synthetic seeds of the study species, *A. caesia* is exhibited in Table 17 and Fig. 17. The higher amount of shooting (77%) was observed in the MS medium contained TDZ and NAA at 1.0 and 0.5 mg/l respectively, followed by 69.43% in TDZ and NAA at 1.5 and 0.5 mg/l respectively and 68.55% in the combination of TDZ and Kn at 2.5 and 0.2 mg/l respectively. The number of shoots produced per explant was well pronounced (10 shoots/explant) in the MS medium fortified with TDZ and NAA at 1.0 and 0.5 mg/l respectively. Similarly, the shoot length was also higher (5.5cm) in the same concentrations and combinations of TDZ and NAA. The other combinations and concentrations were also showed satisfactory response with respect to shooting attributes for this species (Plate IXc).

The proliferation attributes of synthetic seeds prepared from *in vitro* leaf callus derived somatic embryos of the study species are presented in Table 18 and Fig. 18. Higher frequency of shoot formation (73%) was noted while the beads were cultured onto the MS medium fortified with the growth regulators, TDZ and IBA at 2.5 and 0.3 mg/l respectively (Plate Xc), while the lowest frequency of shoot formation (30.24%) was observed in the MS medium containing the IBA alone at 0.5 mg/l. The number of shoots produced was significantly higher (8 shoots/explant) in the MS medium supplemented with TDZ and IBA at 2.5 and 0.3 mg/l respectively. On the other hand, the growth regulators, IBA alone at 0.5 mg/l resulted very lower number of shoot formation (1.78 shoots/explant). The highest shoot length (5.1cm) was measured while subcultured onto the MS medium containing the same combination and concentration of TDZ and IBA. The lowest shoot length (1.9cm) was measured while culturing the beads onto the medium containing IBA alone at 0.5 mg/l.

The variation in proliferation level of *in vitro* leaf derived callus encapsulated synthetic seeds of the study species, *Acalypha fruticosa* as influenced by different combinations and concentrations of growth regulators is given in Table 25 and Fig. 24.
The higher amount of shooting (80%) was observed in the MS medium contained BAP and Kn at 2.0 and 0.5 mg/l respectively, followed by 75.00% in BAP and Kn at 1.5 and 0.5 mg/l respectively and 72.16% in the combination of BAP and NAA at 3.0 and 0.5 mg/l respectively. The number of shoots produced per explant was well pronounced (12 shoots/explant) in the MS medium fortified with BAP and Kn at 2.0 and 0.5 mg/l respectively. Similarly, the shoot length was also higher (5.8cm) in the same concentrations of BAP and Kn. The other combinations and concentrations were showed less influence over the shooting attributes (Plate XIc).

The proliferation attributes of synthetic seeds prepared from in vitro nodal segment encapsulation for this study species are presented in Table 26 and Fig. 25. The higher frequency of shoot formation (75%) was noted to be present while the beads were cultured onto the MS medium fortified with the growth regulators, BAP and GA3 at 2.0 and 0.3 mg/l respectively (Table 26 and Plate XIIc). Lowest frequency of shoot formation (15.11%) was observed on the MS medium containing the BAP alone at 0.5 mg/l. The number of shoots produced was significantly higher (10 shoots/explant) in the MS medium supplemented with BAP and GA3 at 2.0 and 0.3 mg/l respectively. On the other hand, the growth regulators, BAP alone at 0.5 mg/l, resulted very low degree of shoot formation (1.36 shoots/explant). The highest shoot length (5.5cm) was measured while subcultured onto the MS medium containing the BAP and GA3 at 2.0 and 0.3 mg/l respectively. The lowest shoot length (1.3cm) was measured when culturing the beads onto the MS medium containing BAP alone at 0.5 mg/l.

6.3.4. Rooting and acclimatization of plantlets regenerated from encapsulated beads

The data on rooting ability of in vitro derived nodal explant encapsulated synthetic seed of the study species, Acacia caesia are presented in Table 19 and Fig. 19. The rooting frequency was significantly higher (70%) in the MS medium supplemented with IBA alone at 1.5 mg/l, followed by 66.68% in the MS medium with IBA alone at 2.0 mg/l. The number of roots produced was higher (8.00 roots/shoot) in the MS medium containing IBA alone at 1.5 mg/l. The root length was also greater (6.8 cm) while the shoots were subcultured onto the MS medium containing IBA alone at 1.5 mg/l (Plate IXd). The auxins, IAA and NAA in combination with IBA have also
satisfactory response on the rooting characters of the in vitro derived node encapsulated synthetic seeds of the study species, *Acacia caesia*.

The rooting attributes of in vitro leaf callus derived somatic embryo encapsulated synthetic seeds of *A. caesia* are given in Table 20 Fig. 20. The rooting frequency was significantly higher (67%) while the shoots were cultured onto the MS medium with IBA and IAA at 2.0 and 0.8 mg/l respectively. The IBA and IAA at 2.5 and 1.0 mg/l respectively were also better for rooting frequency (62.48%). The number of roots produced was higher (7.38 roots/shoot) in the MS medium containing IBA and IAA at 2.0 and 0.8 mg/l respectively. The root length was measured to be greater (6.0 cm) during subculturing of shoots onto the MS medium containing the same concentration and combination of growth regulators, IBA and IAA (Plate Xd). The results showed that the growth regulators, IBA and IAA at the concentration of 2.0 and 0.8 mg/l respectively are found to be more favourable for rooting performance.

The rooting attributes of in vitro leaf derived callus encapsulated synthetic seeds of other study species, *Acalypha fruticosa* are given in Table 27 Fig. 26. The rooting frequency was significantly higher (78%) while the shoots were cultured onto the MS medium with IBA and IAA at 2.0 and 0.2 mg/l respectively. The IBA and IAA at 1.5 and 0.2 mg/l respectively were also better for rooting frequency (75.49%). The number of roots produced was higher (9.00 roots/shoot) in the MS medium containing IBA and IAA at 2.0 and 0.2 mg/l respectively. The root length was measured to be higher (7.4 cm) during subculturing of shoots onto the MS medium containing IBA and IAA at 2.0 and 0.2 mg/l respectively (Plate XId). The results showed that the growth regulator, IBA and IAA at the concentration of 2.0 and 0.2 mg/l is found to be more favourable for rooting performance.

The data on rooting characters of in vitro derived nodal explants encapsulated synthetic seeds of the study species, *A. fruticosa* are presented in Table 28 and Fig. 27. The rooting frequency was significantly higher (72%) in the MS medium supplemented with IBA and NAA at 2.0 and 1.2 mg/l respectively, followed by 63.76% in the MS medium with IBA and NAA at 1.5 and 0.9 mg/l respectively. The number of roots produced was higher (8.00 roots/shoot) in the MS medium containing IBA and NAA at 2.0 and 1.2 mg/l respectively. The root length was also higher (6.5 cm) while the shoots
are subcultured onto the MS medium containing the IBA and NAA at 2.0 and 1.2 mg/l respectively (Plate XIID). The auxins, IAA in combination with IBA have considerable effect on the rooting characters of the *in vitro* derived node encapsulated synthetic seeds.

Hardening experiments were conducted for the *in vitro* derived node encapsulated beads and *in vitro* leaf callus derived somatic embryo encapsulated beads derived plantlets of *Acacia caesia* by using various hardening media to determine the survivability rate of plantlets (Tables 21 and 22). For the *in vitro* derived nodal segment encapsulated beads derived plantlets, the survivability rate was significantly higher (64%) in the hardening medium composed by garden soil, sand and vermicompost in the ratio of 1:1:1 by volume followed by the hardening medium consisting of decomposed coir waste, perlite and compost in the ratio of 1:1:1 by volume (56%) (Table 21, Fig. 21 and Plate IXe).

Of the five hardening media with different components attempted, the vermiculate, coir waste and forest litter in the ratio of 1:1:1 by volume recorded higher (60%) percentage of plantlets survivability for the *in vitro* leaf callus derived somatic embryo encapsulated beads under greenhouse conditions (Table 22, Fig. 22 and Plate Xe). The combination of decomposed coir waste, perlite and compost in the ratio of 1:1:1 by volume was also found to be a suitable hardening medium which registered 52% of plantlet survivability for these beads.

Acclimatization data by hardening experiments for the plantlets of the study species, *Acalypha fruticosa* is given Tables 29 and 30. For the *in vitro* leaf derived callus encapsulated beads derived plantlets, the survivability rate was significantly higher (68%) in the hardening medium composed by garden soil, sand and vermicompost in the ratio of 1:1:1 by volume followed by the hardening medium consisting of decomposed coir waste, perlite and compost in the ratio of 1:1:1 by volume (60%) (Fig. 28 and Plate XIe).

Of the five hardening media with different components attempted, the vermiculate, coir waste and forest litter in the ratio of 1:1:1 by volume recorded higher (60) percentage of plantlet survivability for the *in vitro* derived nodal segment encapsulated beads derived plantlets for this species under greenhouse conditions (Fig.
29 and Plate XIIe). The combination of decomposed coir waste, perlite and compost in the ratio of 1:1:1 by volume was also found to be a suitable hardening medium which registered 56 per cent of plantlet survivability for these beads.

6.4. Discussion

The concept of synthetic seed has been developed as a result of an increased understanding of the phenomenon of somatic embryogenesis and method of encapsulation. An imbalance between the efficiency of in vitro propagation and the delivery of regenerants possess certain limitations on the practical application of tissue culture technologies. In this connection, the use of “synseeds” (encapsulated propagules/’somatic seeds”) as an efficient storage and delivery system is being increasingly realized. Initially synthetic seed preparations were limited to somatic embryos (Kitto and Janik, 1982) only. In recent times, in addition to using somatic embryos, research has also been focused on the use of unipolar vegetative propagules for this purpose (Rao et al., 1996). Production of synthetic seeds using vegetative propagules such as shoot tips and axillary nodes have been reported by many workers (Bapat et al., 1987; Ahuja et al., 1989; Mathur et al., 1989; Fukai et al., 1994; Sajina et al., 1997; Adriani et al., 2000; Gangopadhyay et al., 2005; Singh et al., 2010).

Sodium alginate is a copolymer composed of D-mannuronic acid and L-glucuronic acid units and has been extensively studied because of its biocompatibility, biodegradability and its capability to form hydrogels in the presence of divalent cations. The ridge structure and large pore size of these gels, which are insoluble in water, make them useful for the encapsulation of live cells of plants. Polymer concentration, degree of viscosity of the alginate used, CaCl₂ concentration and curing time are important parameters determining the permeability, resistance and hardness of the resulting beads and the subsequent successes of the encapsulation method (Block, 2003).

In the present study, the polymerizing ability of sodium alginate at different concentrations markedly varied when used to encapsulate the explants. The influence of optimum concentration (4%) of sodium alginate on bead quality and shoot emergence is in agreement with the earlier reports (Mathur et al., 1989; Ghosh and Sen, 1994; Castillo et al., 1998; Jaydip Mandal et al., 2000; Kumaraswamy et al., 2009).
The decline in the germination percentage among the synthetic seeds for a period of 2 – 6 months may be due to inhibited respiration of plant tissue by alginate leading to loss of viability (Redenbaugh et al., 1987). The best storage temperature determined to be is 25°C for the storage period of 2, 4 and 6 months old in vitro explants encapsulated beads of two study species. The morphology and growth of regenerated shoots are not affected at 25°C. However, at 4° C the emerged shoots exhibited slow growth with necrotic and vitrification symptoms. After 6 months of storage, the per cent frequency of conversion was reduced along with death and decay of the encapsulated explants. This might be due to the cracks and dehydration of the beads.

For the in vitro derived node encapsulated synthetic seeds of the study species, Acacia caesia, the cytokinin, TDZ and the synthetic auxin, NAA in the MS medium significantly increased the frequency of shoot formation. On the other hand, the enhancement of number of shoots and shoot length produced per explant were highly influenced by these two growth regulators. George (1993) explained that the regeneration potentiality of artificial seed culture can vary, according to type and concentration of growth regulators, the type and storage period of encapsulated material and the species from which it is derived. Danso and Ford Lloyd (2003) observed that positive influence of cytokinin and auxin in the shooting ability of node encapsulated synthetic seed in the species, Manihot esculenta.

In vitro leaf callus derived somatic embryo encapsulated synthetic seeds produced high amount of shoots, shoot length and other shooting attributes during culturing onto the MS medium containing the cytokinin, TDZ and IBA. However, in the absence of TDZ, the individual treatment with IBA reduced the shooting performance. It indicates that TDZ is the most required hormone and in combination with the auxin, IBA it acted still well for shoot formation. It is of common fact that cytokinin induced somatic embryos in many species (Hutchinson et al., 1996; Tabassum et al., 2010). Similarly, for many woody and medicinal plant species, cytokinins like TDZ also promoted the germination of artificial seeds (Bates et al., 1992; Lu, 1993; Huetteman and Preece, 1993; Mc Kently, 1995).

The rooting performance of the in vitro derived node encapsulated synthetic seed was generally better in the MS medium supplemented with the auxin IBA alone or in
combination with IAA or NAA for the species, *Acacia caesia*. The frequency of rooting, number of shoots rooted and the length of the root were highly influenced by the auxin, IBA alone in the MS medium. Sharma *et al.* (1999) pointed out that the response of synthetic seed for the root formation to auxin treatment is species specific and explant specific in many species.

The rooting ability of the *in vitro* leaf callus derived somatic embryo encapsulated synthetic seeds of *A. caesia* was well pronounced while subcultured the shoots onto the MS medium enriched with the auxins, IBA and IAA. It indicates that the combination of the auxins, IBA and IAA is most appropriate for the rooting attributes. As observed in *in vitro* derived node encapsulated synthetic seeds, auxins influenced largely for rooting performance. However, the concentration of these auxins is determined to be most essential with respect to rooting of *Acacia caesia*. Induction of roots from the synthetic seeds by the auxins in the MS medium has already been well documented for many species (Bharati, 2002; Kumaraswamy *et al.*, 2009).

For the other study species, *Acalypha fruticosa*, generally the response of leaf callus encapsulated synthetic seed for shoot formation was highly influenced by the cytokinins, BAP and Kn at different concentrations in the MS medium. The percentage of seed responded for shoot formation and the number of shoots produced per explant were highly influenced by these two growth regulators. Kumaraswamy *et al.* (2009) also observed the positive influence of cytokinin in the shooting ability of leaf callus encapsulated synthetic seeds of the species, *Pogostemon cablin*. It is of common fact that the cytokinin is determined to be one of the important growth hormones for shoot formation of synthetic seeds encapsulated by any propagules in many species. The exogenous application of BAP with kinetin has major role in the process of shoot formation (Sankhla *et al.*, 1994; Zhou *et al.*, 1994; Mondal *et al.*, 1998; Mondal *et al.*, 2002). Similarly, these two growth regulators determined to have major influence over producing lengthy shoot system also for the leaf callus encapsulated synthetic seed of this species.

*In vitro* derived node encapsulated synthetic seeds produced high amount of shoots, greater shoot length and other shooting attributes during culturing onto the MS medium containing the cytokinin, BAP and gibberlic acid (GA$_3$). However, in the
absence of GA$_3$, the individual treatment with BAP reduced the shooting performance. It indicates that GA$_3$ is the most required hormone and in combination with the cytokinin, BAP it acted well for shoot formation. It is of common fact that gibberellins are well known germination stimulants for the seeds of many species both at natural and artificial levels (Corns, 1960). Similarly, for many weed and medicinal plant species, cytokinins like BAP also promoted the germination of natural and artificial seeds (Taylorson, 1979). The combinations of gibberlins and cytokinins for the enhancement of germination of seeds of certain species have already been well documented (Egley, 1972; Khan and Samimy, 1982; Jung et al., 2004). Bekhert (2006) also reported the most crucial role of these two growth hormones in the shooting processes of synthetic seeds of garlic plant and he explained the species specific requirement of growth hormones for shooting attributes.

In the species, A. fruticosa, the leaf callus encapsulated synthetic seed performed well for the rooting attributes *viz.*, rooting frequency, root number and root length, while subcultured onto the MS medium contained the auxin, IBA and IAA. However, the other auxin, NAA has least effect on rooting behavior of synthetic seeds of this species. This fact indicates that the concentration of auxin, IBA at higher level is most appropriate for the rooting attributes which showed hormone specificity of this species for root formation from the callus encapsulated synthetic seeds. Many early works have been carried out to show the importance of auxins in the root formation from the synthetic seeds (Ganapathi et al., 1992; Afza et al., 1996; Kulkarni et al., 1997). Faisal and Anis (2007) reported that the presence of high amount of auxins in MS medium generally promoted the rooting performance of synthetic seeds in many species.

The rooting performance of the *in vitro* derived node encapsulated synthetic seed of A. fruticosa was generally well in the MS medium containing the auxins, IBA and NAA at higher concentrations in MS medium. Freire et al. (2002) also observed similar kind of results in rooting behaviours as influenced by these auxins in the cultivars of rocha and pear. Nower et al. (2007) explained that the enhanced content of auxins in the full strength MS medium induced root formation in *Pyrus communis*. The induction of roots from the synthetic seeds as influenced by auxins in MS medium has already been well documented in many species (Bapat, 1993; Ganapathi et al., 2001; Amiri, 2002; Thobunaluepop, 2009).
Hardening is a crucial step prior to transplantation of plant to soil. The well developed plantlets of the study species were acclimatized in various potting media. The hardening medium for higher survivability of plantlets was varied according to the beads derived from different propagules of the two study species, Acacia caesia and Acalypha fruticosa. The in vitro derived node encapsulated beads derived plantlets of the study species, Acacia caesia showed better response in the hardening medium encomposed by garden soil, sand and vermicompost, while the in vitro leaf callus derived somatic embryo encapsulated beads derived plantlets of this species responded well in the medium containing vermiculate, coirwaste and forest litter. It may be due to the presence of suitable physical and chemical conditions of respective potting media for the best survivability of plantlets of this species. Hence, it is recommended that these specific hardening media for this study species can be used before transplanting the plantlets to the natural habitats for their better survival in the field.

For the other species, Acalypha fruticosa, the in vitro rooted plantlets were acclimatized in various potting media. The hardening medium for higher survivability of plantlets was varied according to the plantlets derived from different explants encapsulated. The in vitro leaf callus encapsulated beads derived plantlets showed better response in the hardening medium encomposed by garden soil, sand and vermicompost, while the in vitro derived node encapsulated beads derived plantlets responded well in the medium containing vermiculate, coirwaste and forest litter. It may be due to the presence of suitable physical and chemical conditions of respective potting media for the survivability of the study species. Hence, it is recommended that these specific hardening media for this study species can be used before transplanting the plantlets of this species to the natural communities for their better survival in the field.

For the study species, A. caesia and A. fruticosa, the leaf callus derived somatic embryos, the leaf derived callus and in vitro derived node were used as encapsulation materials which showed high morphogenetic potential and well succeeded in germination and survivability. Therefore to meet the future demand, these parts can be explored for the production of the synthetic seeds.