Influence of Explant Selection and Culture Conditions on Organogenesis and Germplasm Conservation in *Bacopa monnieri* (L.) Wettst.

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Abstract

Investigations were conducted to optimize in vitro culture conditions for multiple shoot induction and use of nodal segments for germplasm conservation in *Bacopa monnieri*. Explants derived from leaf and internode tissues of the field-grown and in vitro cultured plants were cultured on the MS medium supplemented with four concentrations and combination of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic (2,4-D) plant growth regulators. Sucrose (30 g/L) and agar (7 g/L) were kept constant in all experiments. The conditions investigated for the total number of shoots produced were the number of explants from one leaf in transverse and longitudinal plane, wounding/incisions on explants, and the effect of plant growth regulators. Internode explants were used as either whole with incisions or cut into two halves longitudinally and wounded. Highest number of shoot regeneration occurred from leaf explants which were cut in half and cultured in medium containing 0.5 mg/L BAP + 0.5 mg/L 2,4-D under 16 h photoperiod. Shoot induction occurred only at the cut / wounded surfaces of explants. Furthermore, the optimum shoot regeneration from internode explants also occurred at 0.5 mg/L BAP + 0.5 mg/L 2,4-D, but when the internode explants were kept intact and wounded followed by culturing under 16 h light. Preliminary studies suggest that nodal explants can be used for germplasm conservation/transportation purpose by encapsulating them in sodium alginate.

INTRODUCTION

Bacopa monnieri (L.) Wettst. (Scrophulariaceae family) is a well known medicinal plant that has been used in Ayurved, the traditional system of Indian medicine. *Bacopa monnieri* also referred to as *Bacopa monniera* in some texts, water hyssop, brahmi or nirbrahmi is found growing naturally in the tropics in shallow waters and marshes. It is a small, weak, creeping and succulent herb (Fig. 1A). Approximately 85% of traditional medicine involves the use of plant extracts (Vieira and Skorupa, 1993).

Bacopa monnieri is traditionally used as a brain tonic to enhance memory development, learning and concentration (Mukherjee and Dey, 1966). It is also used to treat asthma, epilepsy, hoarseness, spleen enlargement, snake bite, rheumatism, leprosy, eczema and ring worm, and as a diuretic, aperitive and cardiotonic (Basu and Walia, 1944). *Bacopa* possesses anti-inflammatory, analgesic and antipyretic activities (Vohora et al., 1997). It has been reported that *Bacopa* extracts may have anticancer properties, possibly due to its inhibition of DNA replication in cancer cell lines in vitro (Elangovan et al., 1995). Bacosides, the bioactive phytochemical in Bacopa, aid in the repair of damaged neurons by enhancing kinase activity, neuronal synthesis, restoration of synaptic activity, and ultimately nerve impulse transmission (Singh and Dhawan, 1997).

The estimated annual requirement of *Bacopa* in India is around 12,700 tons of dry biomass (Ahmad, 1993), and is met solely from natural populations. Due to the lack of proper cultivation practices, destruction of plant habitats, and the illegal and indiscriminate collection of plants from their natural habitats, many medicinal plants are

threatened or endangered (Nalawade et al., 2003). Due to such practices, *B. monnieri* is now listed as a threatened species (Pandey et al., 1993). Therefore, development of protocols for a rapid clonal multiplication of this important herb has become imperative (Tiwari et al., 1998, 2001). Tissue culture techniques could therefore prove valuable in maintaining natural populations and providing a constant supply of disease free plants to the pharmaceutical industry. Various factors like type of explant, incubation temperature and light/dark condition, and plant growth regulators, have been studied relative to their influence on organogenesis (Compton, 1999; Gu and Zhang, 2005). Further, since *Bacopa* plants have a creeping habit and the main stem grows at the soil surface, this causes accumulation of heavy load of contaminating bacteria and fungi on the plant, and therefore establishing initial clean cultures becomes a challenge. Furthermore, metabolic engineering is emerging as one of the important approaches to improve and modify secondary metabolite contents of medicinal and aromatic plants (Mann et al., 2000; Ye et al., 2000) and thus, the development of protocols for plant regeneration will assist all these initiatives.

Considering all this, the purpose of this research was to develop rapid and efficient protocols for the micropropagation of *Bacopa*. Here we report our results on tissue selection, explant preparation and wounding, and plant growth regulator response individually and in combination on organogenesis in *Bacopa.* We also present our preliminary findings on the encapsulation of nodes in order to develop synthetic seeds of *Bacopa* in the sodium alginate based matrix and the possibility of storing germplasm at 4–8°C for 30 days and their growth response at 25°C after cold storage.

MATERIALS AND METHODS

Plant Material and Sterilization

Actively growing, healthy shoots bearing 3–4 nodes were cut from plants growing in the greenhouse at the Fort Valley State University (Fig. 1A). Shoots were immediately placed into a flask containing double distilled water and a drop of liquid detergent. This plant material was washed under running tap water for 2 h. The washed plant material was then sterilized in 10% solution of Clorox bleach (Clorox, Oakland, CA) for 12 min, followed by three washes with autoclaved double distilled water. The plant material was then placed in a 1% plant preservative mixture (PPM) solution (Plant Cell Technology, Inc., Washington D.C.) for 60 min prior to inoculation. In vitro cultures, thus established, were used as donor plants for all studies reported in here. Elongated in vitro raised shoots were used for making explants and were placed on the experimental culture medium without sterilization.

Culture Media and Conditions

Studies for shoot bud induction were conducted using modified MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar. Four concentrations each of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic (2,4-D), including control, were incorporated to this basal medium either alone or in combination (Table 1). In this study, three concentrations of BAP (0.1, 0.5 and 1.0 mg/L), three concentrations of 2,4 D (0.1, 0.5 and 1.0 mg/L), and three combination treatments of BAP and 2,4 D (0.1+0.1, 0.5+0.5 and 1.0+1.0 mg/L, respectively) were tried to study organogenesis. Culture medium, agar and plant growth regulators used in this investigation were purchased from Caisson Laboratories, Logan, UT. Media were prepared in distilled water and the pH of media was adjusted to 5.8 before autoclaving at 121°C for 20 min. The sterilized media were then dispensed into sterile plastic petri dishes (100 mm x 15 mm, Fisher Scientific, USA) and allowed to solidify in the laminar flow hood at room temperature for 24 h. After inoculation, all cultures were transferred to a 16 h photoperiod (50 $\mu E\ m^{-2}\ s^{-1}$) at 24 ±2°C for five weeks.

Explant Selection

In vitro cultured plants were used to prepare explants as depicted in Fig. 1B (a-g). The leaf explants were cut transversely as well as longitudinally in two and three pieces, whereas one-cm-long internode explants which were trimmed at both ends were either used as whole with incisions throughout the surface, or were again cut in to half with incisions made in both halves. Shoot induction was investigated by number of explants generated from one leaf or internode with respect to the role of wounding on organogenesis, and plane of explants cutting (transverse vs. longitudinal).

Rooting, Acclimatization and Transfer to Soil

Four to five-cm-long shoots were cut and transferred to rooting medium in a test tube consisting of half strength MS basal medium supplemented with 1.0 mg/L indole-butyric acid (IBA). These cultures were incubated at 24 ±2°C for two weeks. Once four to five roots are visible, plants were removed from the test tube and the agar from roots was washed off carefully. Plants were kept under slow running tap water to remove traces of agar from the roots. These plants with clean roots were then transferred to eight inch plastic pots containing potting mixture (Pro-Mix 'BX', Cassco, Montgomery, AL), and placed in the mist chamber for acclimatization. After one week, the plants were removed from the mist chamber and maintained in the greenhouse for an additional four weeks prior to transferring to the field.

Encapsulation

Sterile nodes measuring 5 mm in length were encapsulated in 3% sodium alginate that was complexed with 80 mM calcium chloride to make synthetic seeds (synseeds). Complete protocols for the encapsulation process have been described by Joshee et al. (2007). Synseeds were stored at 4–8°C for 30 days, following which they were taken out of the cold storage and were placed into petri dishes containing basal MS media. The cultures were placed in the culture room under a 16 h photoperiod (50 μE m^{-2} s^{-1}) at 24 ±2°C.

Statistics

All data were subjected to statistical analysis using ANOVA (SAS, 1996). Means separations were by Duncan's Multiple Range Test.

RESULTS

The goal of this study was to develop protocols for an efficient in vitro multiple shoot induction for *Bacopa monnieri*. We also present details for shoot induction, explant rooting, acclimatization and transfer of regenerated plantlets to the soil. A preliminary research on the encapsulation of nodal segments to develop synseeds and their potential to regenerate after cold storage has also been discussed.

Plant Growth Regulators

Two sets of experiments were conducted for this study using leaf explants. In the first experiment, intact leaf with incisions was used (Fig. 2) while another study was carried out using leaf explants cut transversely or longitudinally into either two or three segments to see the effect on multiple shoot count (Table 2). In control explants, two shoots regenerated at the cut end of intact leaf explants (Fig. 1C). Increasing concentrations of BAP had a positive effect on the number of shoots produced in both experiments though the number of shoots produced was higher in the second set of experiment. As the BAP concentration increased from 0.1 to 0.5 to 1.0 mg/L, shoot count averages varied from 13 to 13 to 6, respectively, in the leaf explants that were cut transversely in half and cultured under constant light throughout (Fig. 2). With increasing concentrations, 2,4-D exhibited inhibitory role on shoot induction since 2,4-D concentrations increased from 0.1 to 0.5 to 1.0, number of shoots counted were 6, 1 and 1, respectively (Fig. 2). We found that a combination of BAP and 2,4-D (0.5+0.5 mg/L) was

superior in comparison to other concentrations investigated. This combination of BAP and 2,4-D, on an average slightly increased shoot regeneration to 14 (Figs. 1D and 2). In the second set of experiments, pattern of shoot induction response was more or less similar but the number of shoots in BAP and combination treatment was higher than the first experiment where intact leaf was used as an explant (Table 2).

Wounding / Number of Times Explant Cut

Wounding is essential in increasing shoot regeneration from any explant. Shoot buds were observed only at the cut ends of the explants. Stabbing the explants increased shoot bud formation (Figs. 1F, 3B and D). The number of times a leaf explant was cut also affected the number of shoots it developed (Table 2, Fig. 4A). Increasing the number of times the leaf explant was cut (i.e., in half or in thirds), showed the potential to increase shoot regeneration (Table 2). However, the results do not show a clearly significant relationship but only indicates that cutting leaves into thirds instead of halves has the potential to increase shoot bud regeneration. Furthermore, increasing the extent of wounding of internode explants by cutting them longitudinally in half had a detrimental effect. All internode explants that were cut in half longitudinally died within first three weeks of culture.

Plane of Cut of Leaf Explant

The present study shows that leaf explants cut transversely, on average had higher shoot counts. However, the difference between shoot regeneration in explants cut transversely and longitudinally is small and is not statistically significant.

Encapsulation of Nodal Segments

Nodal segments trapped in a drop of 3% sodium alginate were complexed with 80 mM $CaCl_2$ for 30 min. In 30 min time, alginate beads were formed and hardened, and were then transferred to sterile distilled water for washing. After washing, 50–70 synseeds were placed on the basal MS medium in a petri dish (Fig. 4B). After 30 days, 15–20 synseeds were transferred to fresh basal MS medium plates in the culture room under 16 h photoperiod at 24 ±2°C. After two to three weeks in culture, encapsulation matrix ruptured and plantlets developed from node axils striking their own roots (Fig. 4C).

DISCUSSION

Plant growth regulators (PGR) are essential in optimizing shoot regeneration. Earlier research has illustrated the importance of PGR for adventitious shoot bud induction in *Bacopa monnieri* (Shirvastava and Rajani, 1999) and other plants (Clog et al., 1990; Stamp et al., 1990). In the study presented here, a combination of cytokinin BAP and the auxin 2,4-D, each at a concentration of 0.5 mg/L, gave optimum shoot regeneration in *Bacopa monnieri*. The combination of cytokinin and auxin seems to have a synergistic effect as cytokinins enhance cell division, stimulate axillary bud and adventitious shoot proliferation, activate RNA synthesis, and stimulate protein and enzyme activity. Auxins regulate cell elongation, tissue swelling and shoot expansion, and thus, the combination of auxin(s) and cytokinin(s) promote higher frequency of shoot regeneration. The synergistic influence of auxins and cytokinins on optimizing shoot regeneration has been reported in several species, such as *Kigelia pinnata* (Thomas and Puthur, 2004), *Catalpa ovata* (Lisowska and Wysokinska, 2000), *Photomorphe umbellate* (Pereira et al., 2000), *Hypericum perforatum* (Pretto and Santarém, 2000), *Acacia mangium* (Xie and Hong, 2001), and *Echinacea purpurea* (Koroch et al., 2002).

Shoot bud formation was observed at the wounded surfaces of the explant (Figs. 1F, 3B and D). Similar findings were reported in another study on *Bacopa monnieri* (Tiwari et al., 2001). The shoot buds originated at a small distance from the cut ends. Increasing the number of times a leaf explant was cut, on an average increased the shoot regeneration. This is generally because cutting the explant into more pieces increases the

cut surface area thus resulting in larger site(s) for activity. This research paper demonstrates that the greater number of cuts equates with the higher shoot bud regeneration potential (Table 2; Fig. 3B and D, and Fig. 4A). However, care is needed at the same time not to have too many cuts and also to ensure clean precise cuts to reduce tissue damage and eventual tissue death.

Internode explants that were wounded but left intact, exhibited good shoot regeneration potential (Figs. 1F and 3D). Intact internode with wounding in control treatment stayed green and alive with little swelling on the surface of the explants (Fig. 1E). At the same time cut end of the internode induced many shoot buds on the medium with BAP (Fig. 3C). However, when the internodes were cut longitudinally in half or wounded as well as cut longitudinally in half, the explant tissue died across all treatments. This was due to too much wounding/cutting which ultimately crushed the tissue and led it to death.

On an average slightly higher shoot counts occurred when leaf explants were cut transversely compared to those which were cut longitudinally (Table 2). When the leaf explants were cut longitudinally, the cut may have been made through the mechanical tissue (the xylem and phloem) that usually results in less number of shoot inductions.

In conclusion, a successful tissue culture procedure has been identified for *Bacopa monnieri*, in regards to optimizing shoot regeneration from leaf and internode explants. The efficient rooting of regenerated shoots and the survival of plantlets in the soil are the important final steps for successful micropropagation. This method has the potential to lower the pressure on natural populations, to help rebuild wild populations, and to provide a continuous, year-round supply of guaranteed healthy plants to the pharmaceutical industry. Future experiments are planned to identify better composition of encapsulating matrix in order to support longer preservation at lower temperatures.

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Tables

Table 1. Concentrations and combinations of plant growth regulators used.

Treatment	BAP Concentration (mg/L)	2,4-D Concentration (mg/L)
Control	0.0	0.0
T1	0.1	0.0
T2	0.5	0.0
T3	1.0	0.0
T4	0.0	0.1
T5	0.0	0.5
T6	0.0	1.0
T7	0.1	0.1
T8	0.5	0.5
T9	1.0	1.0

Table 2. Shoot bud regeneration as affected by the size and plane of cutting in the leaf explants. The values represent the mean (±SE) from six leaf explants for each treatment.

	Leaf Transverse Cut		Leaf Longitudinal Cut	
Treatment	Two explants/leaf	Three explants/leaf	Two explants/leaf	Three explants/leaf
Control	4.7 ±1.2	0	6.0 ±1.0	
0.1B	12.3 ±3.8	31.0 ±2.1	5.0 ±0.6	30.0 ±2.9
0.5B	13.0 ±3.8	29.3 ±6.4	5.7 ±0.7	27.7 ±5.8
1.0B	24.7 ±4.0	6.7 ±0.9	3.5 ±0.5	5.3 ±0.3
0.1D	6.2 ±1.2	0	0	0
0.5D	0	2.0 ±0.0	0	0
1.0D	0	0	8.0 ±2.0	0
0.1B+0.1D	4.3 ±1.2	0	3.0 ±0.6	0
0.5B+0.5D	51.0 ±5.6	0	19.7 ±2.2	0
1.0B+1.0D	0	0	0	0

Figures

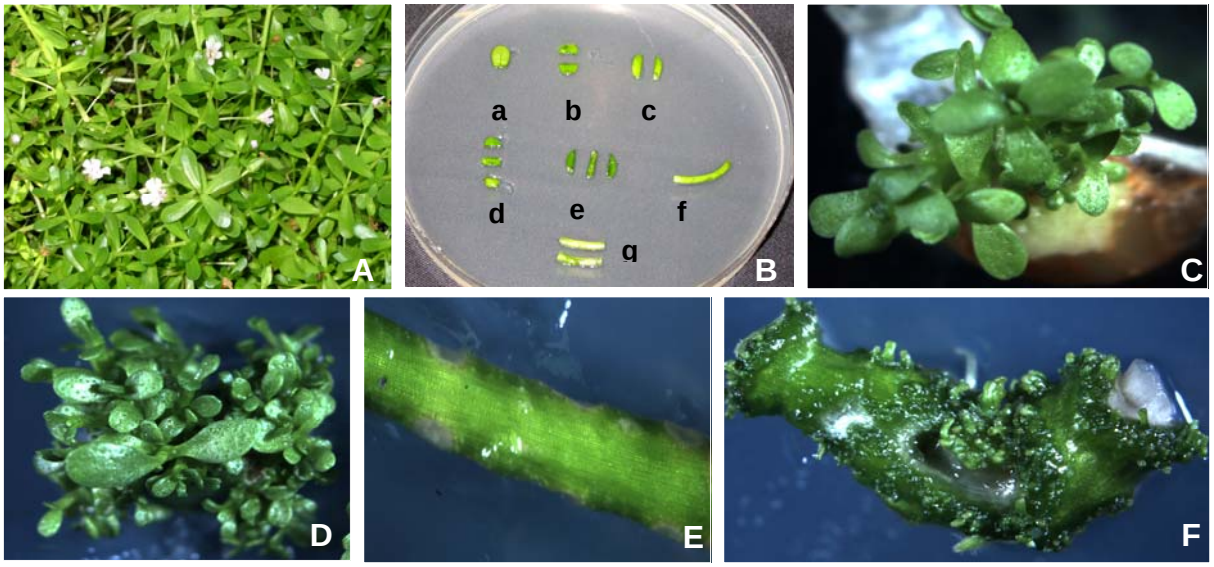

Fig. 1. (A). Natural patch of *Bacopa monnieri* at FVSU. (B). Various explants used in the study, a. intact leaf, b. leaf T.S. two explants, c. leaf, L.S. two explants, d. leaf T.S. three explants, e. leaf L.S. three explants, f. internode, intact with stabs, g. internode, split into two explants. (C). Shoot induction on control leaf explant. (D). Optimal shoot induction on leaf explants cultured on MS+ 0.5 mg/L BAP + 0.5 mg/L 2,4-D, (E). Shoot induction on control internode explant. (F). Optimal shoot induction on internode explant cultured on MS + 0.5 mg/L BAP + 0.5 mg/L 2,4-D.

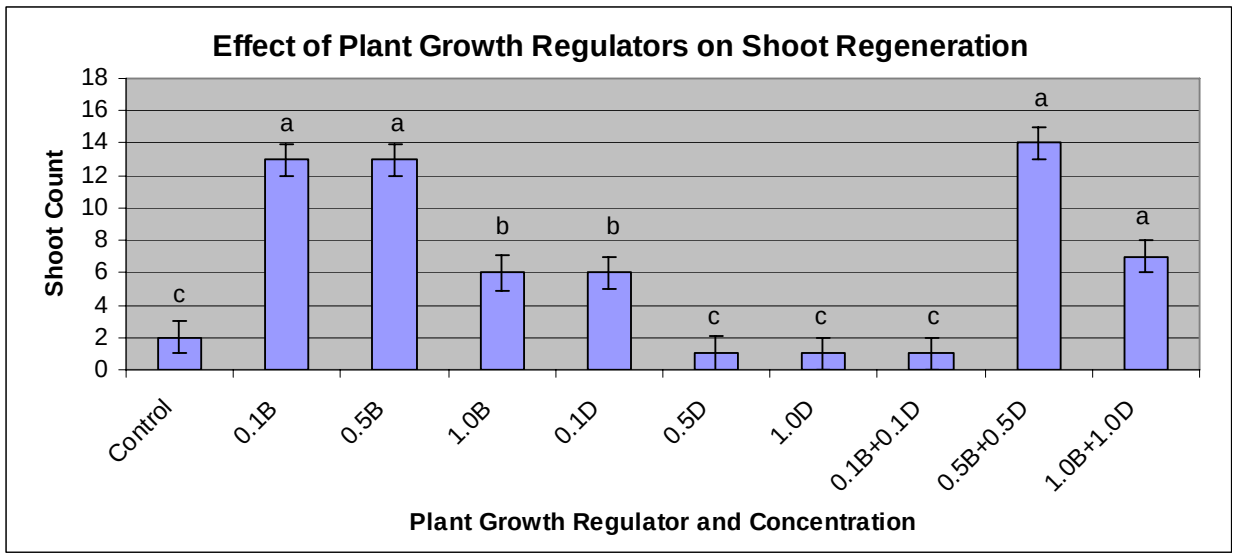

Fig. 2. Effect of plant growth regulators on shoot regeneration in *B. monnieri.* The values represent the mean (±SE) from 30 explants for each treatment. Data were analyzed using a MIXED model of SAS. Means (± SE) followed by the same lowercase letters are not significantly different (P≤0.05).

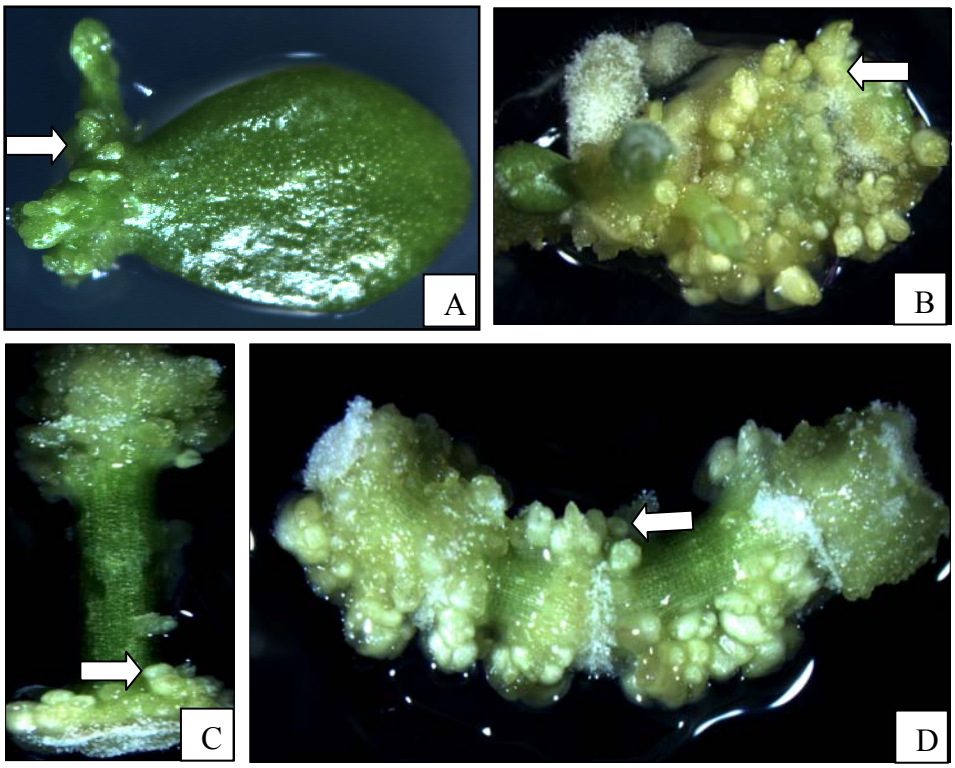

Fig. 3. Wounding is essential for shoot bud induction (MS + 1 mg/L BAP). Shoot buds form on the cut ends / wounded sites of explants. Increasing the amount of wounded areas increases shoot bud formation. (A) and (C) are leaf and internode explants with one and two cut ends respectively. (B) & (D) are similar explants with incisions all over. Arrows indicate shoot buds.

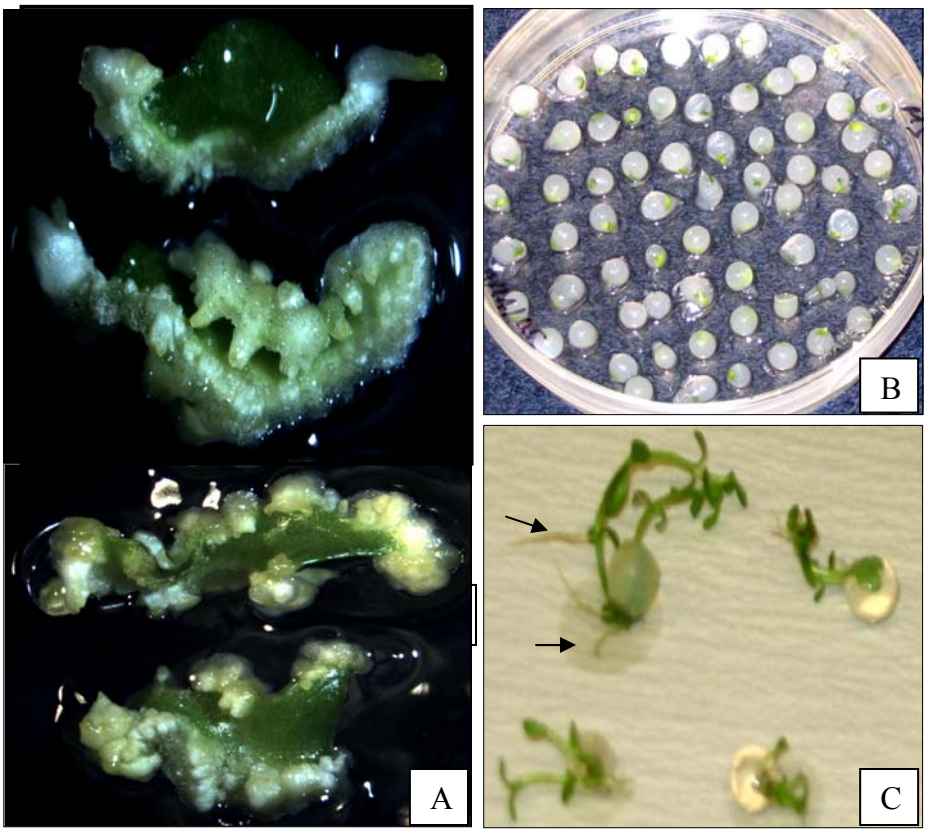

Fig. 4. (A). Increasing the number of explants from one leaf increases total number of shoot bud formation. This leaf has been cut transversely to produce four explants and all four explants have numerous shoot buds. (B). Encapsulation of nodal segments in sodium alginate (C). Growth of individual plants from encapsulated nodal segments after 30 days storage at 4–8ºC. Arrows indicate newly formed roots.