Synthetic Seed Production in *Chirita zeylanica* as a Conservation Method

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Abstract
Chirita zeylanica is an endemic flowering herb in Sri Lanka that possesses a greater potential of being an ornamental pot plant. Currently, the plant is subjected to extinction due to collecting plants from its natural habitats. Therefore, there should be a reliable conservation method and also, there should be an efficient way to produce large number of plants in a short period of time. In the present study, production of synthetic seeds was attempted from this species. The structure of the beads and the percentage of germination from encapsulated shoot tips were influenced by the concentration of sodium alginate and the growth regulators used. It was found that among the concentrations tested, 4%°sodium alginate produced optimal beads with firm, clear, round and uniform size, and were convenient for handling. It was also observed that the beads produced with 4% sodium alginate and, growth regulator combination of 2.0 mg/L BAP and 0.2 mg/L NAA showed the highest percentage of germination (80%) after 4 weeks from the establishment. The shoot tips of the beads remained green after storage at 4 °C for a period of 8 weeks. However, all the beads turned to brown color after 4 weeks from the establishment. The findings suggested that the encapsulation method for micro-shoots could be used as a conservation method for *Chirita zeylanica* after developing a procedure to acclimatize the beads for growth under room temperature before establishment.

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Introduction

Chirita zeylanica is an endemic herb with 10–35 cm height which belongs to family Gesneriaceae. It grows in undisturbed montane forests of the moist and intermediate zones at elevations above c. 750 (Dassanayake, 1987). It possesses a greater potential of being an ornamental pot plant due to its beautiful flowers. Currently the plant is subjected to extinction due to collecting it from its natural habitats.

Therefore, *Chirita zeylanica* is named as a threatened plant in the IUCN Red Data Book published in 2012.To commercialize *Chirita zeylanica* as a pot plant, there should be reliable conservation methods. Also, there should be an efficient way to produce large number of plants in short period of time for commercial level production. Micropropagation is an ideal option for obtaining large number of plants. However, when using in-vitro cultures, sub culturing should be done within short intervals, thus making it less-efficient as a conservation method.

Artificial seed production technology is currently considered as an effective and efficient alternative method of propagation of several commercially important agronomic and horticultural crops. Synthetic seeds are small in size and can store for a certain period of time. Therefore, synthetic seeds can be considered as one of the most efficient ways for conservation and commercialization of *Chirita zeylanica*.

The present work was conducted with the main objective of developing a methodology for synthetic seed production in *Chirita zeylanica* and the specific objectives were; to find the best sodium alginate concentration that gives best structure and germination percentage for the beads, to find the best growth regulator combination and to find the storage and germination ability of the synthetic seeds produced.

Materials and Methods

Micro shoots were obtained from *In vitro* grown *Chirita zeylanica* plants maintained under optimum physical conditions (temperature of 26 ± 2 °C, 75% relative humidity and 16 hour photo period) in a culture room. MS medium was used with 3.0mg/L BAP as culture maintenance medium. Encapsulation matrix consisted of sodium alginate solution at two concentrations (2and 4%) was prepared in MS basal medium solution (pH 5.7) added with 100mg/L Myo-inositol and 30g/Lsucrose.

Two concentrations were selected based on the results of previous studies on different plant species. For hardening process 100mM(14.7g/L) $CaCl_2.2H_2O$ 5 Solution was prepared by dissolving $CaCl_2.2H_2O$ in distilled water.

Separated shoot tips were individually dipped for a few seconds in sodium alginate solution. Then a single shoot tip and alginate mixture was picked up by sterile 2 mm stainless steel spoon. Single coated shoot tip was then dropped into $CaCl_2.2H_2O$ solution. After 30 minutes, the beads were washed with sterilized distilled water.

The best sodium alginate concentration which gives the best structure for the beads was selected by giving marks out of 100 for each bead by observing the characters such as size, shape, strength, whether the shoot was fully covered or not and on easiness for preparation and handling. Collected data were statistically analyzed using Mann-Whitney Test. Thirty replicates were used in each concentration.

Best results were obtained with 4% sodium alginate solution and for the next experiment beads were prepared with 4% sodium alginate solution containing four different growth regulator combinations (Table1). Concentrations for the growth regulators were decided based on the previous studies done on micropropagation of *Chirita zeylanica* and also synthetic seed production of other plant species.

Table 1: Growth regulator combinations used with sodium alginate solutions for the production of synthetic seeds

Treatment	BAP (mg/L)	NAA (mg/L)
T1	2	0.2
T2	2	-
T3	-	0.2
T4	-	-

The encapsulated beads of two sodium alginate solutions and four different growth regulator combinations were stored in a refrigerator at low temperature (4 ± 1°C). The survival rates for germination after 0, 2, 4, 6 and 8 weeks were recorded. For germination purpose, the beads were maintained under the culture room conditions of temperature at 26±2°C, 75% relative humidity and 16hour photo period under fluorescent illumination.

MS medium containing 3mg/L BAP was used as the substrate. Five replicates were tested at each time. The experiments were conducted using completely randomized design. Data collected were statistically analyzed using ANOVA and the means were separated by Duncan's Multiple Range Test (DMRT).

Results and Discussion

According to the Mann-Whitney Test, the beads Prepared with 4% sodium alginate solution showed a significantly higher median than the beads prepared with 2% sodium alginate solution. The beads prepared with 2% sodium alginate solution were hard to prepare, hard to handle, very fragile when handling with the forceps, formed without a proper shape and their size was also not equal. Plant material was also not covered properly. The beads prepared with 4% sodium alginate solution were easy to prepare compared to that with 2% sodium alginate solution. Damages did not happen to the beads when handling with the forceps and the beads were considerably hard. Round shaped beads were obtained and the beads were equal in size. Plant material was properly coated.

It has been reported that the concentration of sodium alginate needed for encapsulation of somatic embryos or micro shoots varies depending on species (Redenbaugh *et al.*, 1986). The hardness of the beads or capsules mainly depends on the number of sodium ions exchanged with calcium ions. 4% sodium alginate solution contains hither number of sodium ions than 2% sodium alginate solution. Hence, the beads prepared with 4% sodium alginate solution were much harder than beads prepared with 2% sodium alginate solution. The beads prepared with 2% and 4% sodium alginate solutions were established in an MS medium containing 3 mg/L BAP for germination just after preparation and after the storage periods of 2, 4, 6 and 8 weeks.

Beads established just after preparation were started to germinate after one week from the establishment. At the 4th week from the establishment, 52% of beads (prepared with 4% sodium alginate solution) were germinated whereas only 26% was germinated from the beads prepared with 2% sodium alginate solution. There was a significant difference in germination percentages ($p < 0.05$).

In stored beads, the green color of the shoot tip was still remained when taken out of the storage even after eight weeks of storage period. After establishment, the shoot tips of the stored beads turned to brownish green color within few days (2–4 days) and gradually turned to brown color. After 4 weeks, all the shoot tips (100%) turned to brown color. The beads prepared with 4% sodium alginate solution containing four different growth regulator combinations were established in an MS media containing 3mg/L

BAP for germination just after preparation and after the storage periods of 2, 4, 6 and 8 weeks. Beads established just after preparation started to germinate one week after the establishment. At the 4th week of the establishment, 80% of beads were germinated from T1 whereas 56%, 66% and 52% beads were germinated from T2, T3 and T4, respectively. The germination percentage of the T1 was significantly different from the other treatments at the 4th week of the establishment ($p < 0.05$).

In previous studies, sodium alginate (encapsulation matrix) has been prepared with different solutions, i. e., either distilled water only, distilled water with hormones or MS solution with hormones (Daud *et al.*, 2008). The beads can potentially serve as a reservoir for nutrients that may aid the survival and speed up growth. In plant tissue culture, auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the direction of morphogenesis (George *et al.*, 2008). In this study, the best results were observed in the treatment containing both auxin and cytokinin. The higher performance may be due to the same reason.

In the experiment, the stored beads were gradually turned in to brown color after establishment. But the shoot tips were still green when they were taken out from the storage. Therefore, it can be assumed that the beads were still viable when taking out from the storage and the viability has been lost after the establishment. The beads established without storage (just after preparation) were germinated without any problem. Therefore the medium had no effect on the viability of the beads. The problem may be in the acclimatization of the beads to the growth room temperature when taking out from the storage. The sudden temperature change may be a stress condition for the shoot tips. For this reason, the artificial seed matrix should be supplemented with nutrients successive in storage. Application of stress preventing chemicals to the encapsulation matrix may be a better option to prevent browning after the establishment of the stored beads.

Synthetic seeds of *Chirita zeylanica* prepared with 4% sodium alginate concentration and growth regulator combination of BAP 2 mg/L and NAA 0.2 mg/L give the best structure and best germination percentage without storage. When storing, further studies are needed for the acclimatization of the beads for the growth at room temperature.

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