

Encapsulated Embryogenic Callus of *Clitoria ternatea* L. for Regeneration and Conservation

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Abstract—Encapsulated embryogenic callus of *Clitoria ternatea* L. were successfully created from leaf explants within 3 weeks after germination on Murashige and Skoog (MS) media. The seeds were initially washed with tap water and teepol, then the seeds were sterilised with 99% (v/v) sodium hypochlorite solution for 1 minute and rinsed with distilled water three times. In a laminar flow cabinet, the seeds were dipped in 70% (v/v) ethanol for 1 minute and blotted with sterilised tissue. The 3 mm² leaf explants were encapsulated with 3% alginate (w/v) which were supplemented with various concentrations (0.5-2.5 mg l⁻¹) and combinations of NAA, BAP and adenine. The optimum concentration for the formation of encapsulation matrix was 3.0% sodium alginate (NaC₆H₇O₆). Encapsulated beads were soaked in 100 mM calcium chloride dehydrate (CaCl₂·2H₂O) solution for 30 minutes. No suitable beads were formed with low concentration (1-2%) of sodium alginate. Within 10 minutes soaking in calcium chloride dehydrate, clear and bead formation with no definite shape was observed. While, within 20 minutes in calcium chloride dehydrate, clear beads, solid and round in shape was observed, however, inside the bead was still in liquid condition. In the present study, the rate of germination of synthetic seeds were slightly decreased from 100% to 77% after 60 days of storage at 4°C. Embryogenic tissue from leaf explants of *Clitoria ternatea* was distinguished by double staining method with bright red of acetocarmine. This technology is an alternative and supplementary method for regeneration, mass propagation and conservation of this medicinal, attractive ornamental and also forage crop for future uses and exploitation.

Index Terms—BAP, encapsulated embryogenic callus, *In vitro* regeneration, NAA.

I. INTRODUCTION

Clitoria ternatea L. is a climber plant that usually grows in various garden hedges. *Clitoria ternatea* L. or its common name including butterfly-pea, blue-pea, cordofan-pea and Asian pigeon wings, belongs to the family Fabaceae. The United State Development Agency (USDA) intends to conserve *Clitoria ternatea* L. along with other 16 leguminous

species with potentially useful phytochemicals [1]. It is a strongly persistent, a herbaceous perennial legume with stems fine twining, sparsely pubescent, suberect at base, 0.5-3 m long [2]. In addition, *Clitoria ternatea* L. is a tropical twining herb, growing wild and also in gardens, bearing white or ink blue flowers resemble a conch-shell. It is widely planted as an ornamental on fencerows. Originally it is selected as a cover crop, but now used for short and medium-term pastures and as green manure, cover crops and protein bank.

Clitoria ternatea contains flavonoids such as quercetin, kaemferol, robinin and Clitorin. It also contains starch, tannin, resin and anthocyanins [3] and several glycosides including malvidin-3-β-glycoside, deiphinidin-3-β-glycoside [4]. In Malaysia, petals of *Clitoria ternatea* L. or commonly known as 'Bunga Talang' are consumed to make 'Nasi Kerabu' which is blue in colour (natural food dye), and a famous local dish in Malaysia.

The extract of *Clitoria ternatea* L. was found to have anxiolytic, antidepressant, anticonvulsant and antistress properties [5]. According to the traditional system of medicine 'Aparajita' is considered as a 'Medhya' drug to improve intelligence and enhance memory function [6]. It is also used in the treatment of chronic bronchitis, dropsy, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumors. In Ayurveda Indian medicine, the roots are most widely used and are bitter, refrigerant, laxative, intellect promoting, diuretic, anthelmintic and as tonic. This root is useful in dementia, burning sensation, inflammation and asthma. The seeds are cathartic, while the leaves are used in otalgia and hepatopathy. Besides, the roots, stems and flowers are recommended for the treatment of snakebite and scorpionsting. The phytochemical investigations revealed the presence of saponins, carbohydrates, alkaloids, proteins, anthroquinones and phytosterols. It is used as diuretics, antihelmintic, antidiabetic, antipyretic and brain tonic [7], [8].

In vitro propagation is a viable alternative for a species which is difficult to regenerate by conventional methods; where populations have decreased due to over exploitation by destructive harvesting and can effectively be used to meet the growing demand for clonally uniform elite plants. Therefore, the measures to develop micropropagation protocols for elite stocks of *Clitoria ternatea* L. are urgently needed. The development of encapsulated or synthetic seed technology brings up a new prospect in agriculture and floriculture industry. The aims of this paper are to produce artificial seeds and to investigate the ability of the synthetic seeds to regenerate within 90 days of storage at low temperature (4 ± 1°C).

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II. MATERIALS AND METHODS

A. Production of Synthetic Seeds of *Clitoria Ternatea* L.

1) Explant sources

Leaf explants of *Clitoria ternatea* L. obtained from 3-week-old seedling were cultured on MS medium supplemented with different concentrations of NAA (0.5-2.5 mg l⁻¹NAA), added with 0.5 mg l⁻¹BAP and 40 mg l⁻¹ adenine. The cultures were incubated and maintained at 25±1°C with 16 hours light and 8 hours dark. Thirty replicates for each treatment were prepared for three times repetition. Illumination was at 1000 lux and relative humidity was 90–100%.

2) Preparation of 3% (w/v) sodium alginate solution (NaC₆H₇O₆)

The standard method for preparation of capsule matrix was followed [9]. To prepare 3% (w/v) sodium alginate solution in 100 ml MS basal medium without calcium chloride dehydrate (CaCl₂·2H₂O), 1g sodium alginate powder was dissolved gradually. Sucrose (3.0 g) and hormones (NAA, BAP and adenine) were added. Media pH was adjusted to 5.8. This solution was autoclaved for 20 minutes at 121°C and 104kpa.

3) Preparation of calcium chloride dehydrate solution (CaCl₂·2H₂O)

Calcium chloride dehydrate solution was used as a complexation agent. To prepare 75mM (w/v) calcium chloride dehydrate in 100 ml distilled water, 1.47 g CaCl₂·2H₂O was dissolved gradually. This solution was autoclaved for 20 minutes at 121°C and 104kpa.

4) Encapsulation techniques and bead formation

The leaf explants were encapsulated (hardened) by allowing them to remain in CaCl₂·2H₂O solution for 30 minutes. These beads were taken out and transferred into the sterile distilled water to wash out the excess CaCl₂·2H₂O solution and were blotted with sterile tissue paper. The beads were then cultured on solid MS media at 25±1°C under 16 hours light. Thirty replicates for each treatment were prepared for three experiments.

5) Low temperature storage

Steriled encapsulated beads of *Clitoria ternatea* L. were stored on solid MS basal medium under dark condition (wrapped with aluminium foil) at low temperature (4±1°C). The survival rates were recorded for 90 days. Thirty replicates for each treatment were prepared for three experiments.

B. Identification of Embryonic Callus Using Double Staining Method

1) Preparation of 2% acetocarmine

Glacial acetic acid (45 ml) was added to 55 ml distilled water in a conical flask (45% acid solution). 2 g of carmine were weighed out and added to 45% acid solution. The flask was placed on a hot plate in the fume hood and boiled gently for 5 minutes. The cooled solution was filtered using Whatman filter paper and stored at room temperature.

2) Preparation of 0.5% evan's blue

Evan's Blue powder (0.5 g) was added to 100 ml distilled water in a conical flask. The flask was then capped and

swirled by hand to mix the solution.

3) Double staining method

Callus (3 mm²) was placed on a glass slide. A few drops of 2% acetocarmine was added to the callus. The callus then was gently divided into very small pieces in the acetocarmine using a needle. The slide was held with forceps and heated over a low flame for a few seconds. Then, the callus was washed 2 to 3 times with distilled water and all liquid were removed. Two or three drops of Evan's Blue were added to acetocarmine stained cells and after 30 seconds the stained cells were washed 2 to 3 times with water and all the water was removed. One or two drops of glycerol was added to the stained cells to prevent the preparation from drying. The specimen was then observed under the microscope.

4) Acclimatization

After six months in cultures (*in vitro*), all regenerated plantlets (complete with roots and shoots) were transferred to different substrates (sterilised and non-sterilised black garden soil) for further growth and development.

5) Data analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p < 0.05$.

III. RESULTS AND DISCUSSION

The current study resulted in compact green callus formation from leaf explants cultured on MS media supplemented with various combinations and concentrations of NAA, 0.5 mg/l BAP and 40 mg/l adenine (Table II). The 3 mm² leaf explants were excised from 3-week-old germinating seedlings on MS basal media. The highest (0.83 + 0.17 g) of dry callus formation per explant was obtained on solid MS media supplemented with combination of 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine, after 2 weeks. The shoots formed after 12 weeks. In previous study [10], reported that the highest frequency of productive shoot regeneration (red calyx, 8.5%) was obtained within 8 weeks from explants of immature inflorescence cultured on MS medium supplemented with 1.0 mg/l benzyl amino purine (BA) and 1.0 mg/l 1-naphthalene acetic acid (NAA), added with 40 mg/l adenine and 3% sucrose. The current result was followed by 0.31 + 0.62 g dry weight of callus per explant on solid MS basal media after 4 weeks. Whilst, the lowest (0.15 + 0.77 g) was obtained on MS media supplemented with combinations of 2.5 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine after 8 weeks. [11] reported the micropropagation of *Clitoria ternatea* L. on DKW medium with maximum number of shoots was achieved in DKW medium containing 1 mg/l BAP and the maximum number of roots multiplication was achieved in DKW medium containing 2.0 mg/l NAA.

Based on Table II, combination of 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine was added to 3% sodium alginate solution to produce synthetic seed of *Clitoria ternatea*. Initially, embryogenic tissue from leaf explants was distinguished by double staining method (Fig. 1). Basically,

all embryogenic cells had large nuclei and dense cytoplasm. These nuclei stained intense bright red with acetocarmine. According to [12] genetic improvement through transgenic technology is impeded due to non-availability of efficient regeneration system in many grain legumes. Embryogenic system offers an ideal tool for *in vitro* production and selection of transgenic plants. However, only after 3 months, the compact green callus changed to shoots formation. According to [13], the inclusion of cytokinins and auxin caused swelling at the bases of explants over 6-10 days of culture and the addition of a cytokinins and auxin to a medium was essential to induce axillary shoot proliferation. The concentration and type of cytokinin together with auxin used significantly affected the number of shoot, number of nodes and length of shoot regeneration. Based on [14], identified that the proliferation of shoots was achieved on MS medium supplemented with various concentrations of 6-benzyladenine (BA), Kinetin (Kin) and 2-isopentenyl adenine (2-iP) either singly or in combination with α -naphthalene acetic acid (NAA). According to [15], optimum embryogenic callus (75 %) was induced from cotyledonary explants on [16] Murashige and Skoog, (MS) medium supplemented with 2 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), followed by subculturing the callus on MS medium supplemented with 2 mg/l 6-benzyladenine (BA) and 0.5 mg/l α -naphthalene acetic acid (NAA).

The current study shows that different concentrations of sodium alginate ($\text{NaC}_6\text{H}_7\text{O}_6$) and soaking duration of calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) influenced the bead formation of *Clitoria ternatea*. With sterile condition, leaf explants encapsulated using sodium alginate solution for beads formation with ideal texture with uniform, isodiametric shape and size. The optimum concentration for the formation of encapsulation matrix was 3.0% sodium alginate ($\text{NaC}_6\text{H}_7\text{O}_6$). Encapsulated explants were soaked in calcium 100 mM chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) solution for 30 minutes (Fig. 2). No suitable beads were formed with low concentration (1-2 %) of sodium alginate. No definite shape of beads were formed within 10 minutes soaking in calcium chloride dehydrate. While, after 20 minutes in calcium chloride dehydrate, beads were clear, solid and round at outside, however, inside bead was still with liquid condition which caused problems when the beads were cultured on solid MS basal media, the beads became shrunk and explants died. The optimum soaking period was 30 minutes in calcium chloride dehydrate with high concentration of 3% sodium alginate which formed very hard beads but perfect round shape. Then, the beads (containing sodium alginate supplemented with 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine) were cultured for 4 weeks on the same MS solid media (MS medium supplemented with 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine) as the basis. The callus formed after 2 weeks and shoots were formed after 12 weeks. Finally, the beads were transferred to MS basal medium for another 4 weeks for root formation.

The survival rate of synthetic seeds on MS media (viability for every 15 days of storage) was shown in Table II. The current study resulted in the highest survival rate of synthetic

seeds of *Clitoria ternatea* (100 %) on MS media supplemented with combination of 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine compared to 92% on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l NAA [14]. However, the survival rate was decreased to the lowest (53.33 ± 1.22 %) after 90 days, while [15] reported the synthetic seeds kept at 4°C showed 86 % viability even after 5 months of storage. In addition, according to [17], after breaking dormancy at $4 \pm 1^\circ\text{C}$ for 6 weeks, most of seedlings from somatic embryos developed into healthy plants in culture room at $24 \pm 1^\circ\text{C}$.

TABLE I: THE CALLUS FORMATION OF *CLITORIA TERNATEA* L. FROM LEAF EXPLANTS ON SOLID MS MEDIA SUPPLEMENTED WITH DIFFERENT COMBINATIONS OF NAA, BAP AND ADENINE AT $25 \pm 10^\circ\text{C}$ WITH 16 HOURS LIGHT AND 8 HOURS DARK, WITH 1000 LUX INTENSITY OF LIGHT

MS + Hormones (mg l ⁻¹)	Dry weight, g (mean \pm SE)	Observation
MSO	0.31 ± 0.62^a	Compact green callus formation after 4 weeks
MS+0.5 mg/l NAA+0.5 mg/l BA+40 mg/l adenine	$0.27 \pm 0.43^{a,b}$	Compact green callus formation after 6 weeks
MS+1.0 mg/l NAA+0.5 mg/l BA+40 mg/l adenine	0.83 ± 0.17^a	Compact green callus formation after 2 week
MS+1.5 mg/l NAA+0.5 mg/l BA+40 mg/l adenine	0.20 ± 0.32^b	Compact green callus formation after 7 weeks
MS+2.0 mg/l NAA+0.5 mg/l BA+40 mg/l adenine	0.25 ± 0.12^b	Compact green callus formation after 6 weeks
MS+2.5 mg/l NAA+0.5 mg/l BA+40 mg/l adenine	0.15 ± 0.77^c	Compact green callus formation after 8 weeks

*Each value represents the mean \pm SE of 30 replicates. The mean with different letters in the same column differ significantly at $p \leq 0.05$.

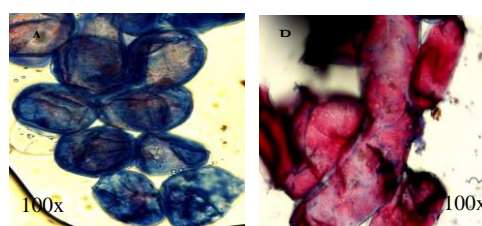


Fig. 1. Embryogenic calli from leaf explants from double staining method observed with camera lens as the calli stained intense bright red with acetocarmine, A) Non-embryogenic cell with blue nucleus at magnification 100x, B) clearer somatic embryogenesis cell with red nucleus at magnification 100x.

The resulting plantlets were then subsequently transferred to soil and acclimatized on different substrates (sterilized and non-sterilized black garden soil) for further growth and development (Table III and Fig. 3). From previous studies, [18] were successful in sowing *Hibiscus moscheutos* directly into a greenhouse. The current study only shows positive response on encapsulated leaf explants on solid MS media supplemented with 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine. Unfortunately, encapsulated beads became shrunk and contaminated on sterilized and non-sterilized black garden soil. According to [19], *in vivo* growth of encapsulated

axillary buds of mulberry (*Morusindica* L.) supplemented with additional fungicide to the alginate beads prevented contamination of the buds and increased survival of the buds when sown in soil. [20] also observed that addition of fungicide (0.1% carbendazim) and bacteriocide (0.1% streptomycin) into the encapsulating gel reduced the incidence of fungal and bacterial contamination to a minimum level and nearly 95% of the beads remained healthy. After six months being acclimatized, vivid blue colour of flower were formed the same as mother plant (Fig. 3).

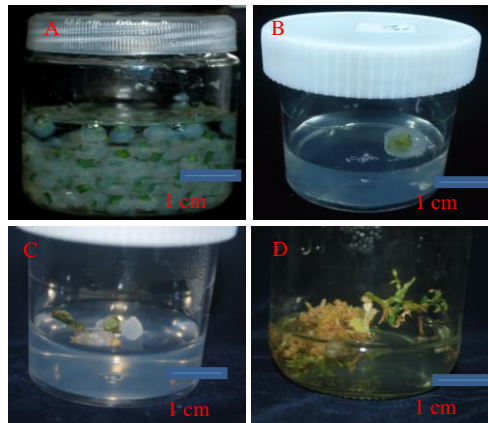


Fig. 2. Sodium alginate encapsulated leaf explants being stored at $4\pm 1^\circ\text{C}$, A) encapsulated leaf soaked in calcium chloride for 30 minutes, B) cloudy, solid and round bead of encapsulated callus, C) Surviving encapsulated or synthetic seeds after 90 days in storage, D) multiple shoots formation and elongation.

TABLE II: EFFECT OF STORAGE PERIOD ON GERMINATION RATE OF *CLITORIA TERNATEA* L. AT $4\pm 1^\circ\text{C}$ (SODIUM ALGINATE ENCAPSULATED) IN 90 DAYS

Encapsulated method ($4\pm 1^\circ\text{C}$)		
Period of storage (day)	No. of survival (mean \pm SE)	Survival rate (%)
0	30.00 \pm 0.00a	100.00 \pm 0.00a
15	28.81 \pm 0.32a	98.73 \pm 0.51a
30	27.61 \pm 1.10b	93.32 \pm 0.51a
45	26.42 \pm 0.63b	88.04 \pm 1.02c
60	23.12 \pm 1.32c	77.03 \pm 1.01b
75	20.31 \pm 0.20c	60.03 \pm 1.52c
90	16.34 \pm 0.82d	53.33 \pm 1.22c

*Each value represents the mean \pm SE of 30 replicates. The mean with different letters in the same column differ significantly at $p\leq 0.05$.

Biotechnology has emerged as a strong tool in mass propagation and improvement of all plant species. Clonal multiplication is production of true to type plants in large number, in a short period of time. It offers a method to increase valuable genotype rapidly and expedite the release of large numbers of plantlets. To fulfil the increasing demand of this potent medicinal plant, *in vitro* culture is an alternative method for conservation of this diminishing plant population [21] through production of artificial seeds [22].

Interestingly, synthetic seeds that was stored prior to being cultured showed significantly higher conversion frequency than synthetic seeds directly sown on MS basal media (control). Higher percentage of survival was also recorded when the synthetic seeds were stored prior to being cultured on MS basal media compared to the control. It was also observed that storage period of 60 days was the most optimum to ensure a high plantlet conversion rate and survival percentage. Shoot formation was also the highest from

synthetic seeds stored for 30 days. This was clearly demonstrated in Table I.

TABLE III: SYNTHETIC SEEDS GERMINATION OF *CLITORIA TERNATEA* ON DIFFERENT SOWING SUBSTRATES

Sowing substrates		Treatment	Observation
Solid and Murashige and Skoog media (MS)		Supplemented with 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine	Encapsulated beads regenerated to complete plantlets
Sterilised garden soil	black	Moistened with distilled water	Encapsulated beads became shrunk and the explant died
Non-sterilised garden soil	black	Moistened with distilled water	Encapsulated beads became shrunk and contaminated



Fig. 3. A) 6-month-old plantlet of *Clitoria ternatea* L. with formation of primary roots on MS basal medium, B) Shoots elongation after two months being acclimatized, C) Vivid blue flowers formation after six months being acclimatized, D) Morphological of leaf, flower and fruit were similar to mother plant.

Table III indicates various media or substrates tested for germination of the synthetic seeds. The optimum substrate was MS basal medium (Fig. 1 and Fig. 2). After four weeks, the microshoots elongated and eventually undergone complete regeneration. Other sowing substrates were shown to produce poorer results when compared to MS basal media. Substrates moistened with only distilled water resulted in shrinking of the synthetic seeds, while substrates moistened with liquid MS without sucrose resulted in fungal contamination. Finally, the plantlets regenerated from synthetic seeds sowed on MS basal media were successfully acclimatized in the garden.

IV. CONCLUSION

In the present study, production of encapsulated or synthetic seeds was attempted from *Clitoria ternatea* L. and managed to survive for 90 days after storage at 4°C . The highest survival rate of synthetic seeds of *Clitoria ternatea* was 100% on MS media supplemented with combination of 1.0 mg/l NAA, 0.5 mg/l BA and 40 mg/l adenine. While, the lowest ($53.33 \pm 1.22\%$) after 90 days.

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