Synthetic Seeds Production and Regeneration of *Oxalis triangularis* for Mass Propagation and Conservation

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Abstract—*Oxalis triangularis* is an attractive ornamental plant propagated by using bulbs and has no natural viable seeds. It is an ideal decorative plant for growing in pots and other containers. The plant is not widely found in Malaysia and mostly obtained from Thailand. Hence, it is necessary to propagate this plant through tissue culture system for large scale production. In the present study, production of synthetic seeds was attempted from this species and the synthetic seeds managed to survive after 7 and 30 days after storage at 4°C. The rate of synthetic seeds conversion to complete plants after 7 days was 96.67% with 4.57 mean shoot numbers, while after 30 days of storage, the conversion rate slightly decreased to 90% with only 3.97 shoots formation per bead. The present work reports and discusses the potential of tissue culture technique as an alternative method for mass propagation and conservation of this interesting and attractive ornamental plant for future uses and exploitation.

Index Terms—*In vitro* propagation, BAP, NAA, synthetic seeds.

I. INTRODUCTION

Oxalis triangularis, commonly known as “False Shamrock”, is an edible perennial plant belonging to the Oxalidaceae family. It is native to Brazil. *Oxalis triangularis* grow from bulbs, and their propagation is obtained by division of the bulbs. Like other bulbous plants, *Oxalis triangularis* goes through dormancy period on a regular basis. Somatic embryos and synthetic seeds (embryos or micro shoots encapsulated in artificial endosperms) hold great potential for large scale clonal propagation of superior genotypes of heterogeneous plants [1], [2]. They have also been used in commercial plant production and for the multiplication of parental genotypes in large scale hybrid seed production [3], [4].

In Malaysia despite popular demand, this species is usually imported from Thailand, hence there is a necessity to mass propagate this species. Nowadays, mass propagation by using tissue culture method is quite common, the current work reports and discusses the production of artificial seeds from micro shoots derived from stem explants of this species. The subsequent *in vitro* regeneration was also achieved at high rates and the regenerated plantlets were successfully acclimatized. The development of encapsulated

or synthetic seed technology brings up a new prospect in agriculture and floriculture industry. Production of synthetic seeds is effective and acts as an important alternative method of propagation in commercially important plants, which do not produce seeds. Plants could be produced in large scale with high volumes. Consequently, genetic uniformity and stability of the plants could be maintained. Due to the sterility, it could be easily transported and has potential for long term storage without losing viability. The aims of this paper are to produce artificial seeds and to investigate the ability of the synthetic seeds to regenerate after 7 and 30 days of storage at low temperature ($4 \pm 1^\circ\text{C}$).

II. MATERIALS AND METHODS

A. Explant Sources and Encapsulation Matrix

Micro shoots of *Oxalis triangularis* were induced from stem explants cultured on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP. The stem cultures were maintained in the culture room at $25 \pm 1^\circ\text{C}$ for 16 hours light and 8 hours dark. Illumination was at 1000 lux and relative humidity was 90 – 100%. The standard method for preparation of capsule matrix was followed [5].

B. Final Stage Preparation of 3% (w/v) Sodium Alginate Solution ($\text{NaC}_6\text{H}_7\text{O}_6$)

To prepare 3% (w/v) sodium alginate solution in 100 ml MS basal medium without calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 1g sodium alginate powder was dissolved gradually. Sucrose (3.0 g) and hormones (1.0 mg/l NAA and 1.5 mg/l BAP) were added. Media pH was adjusted to 5.8. This solution was autoclaved for 20 minutes at 121°C and 104kpa.

C. Preparation of Calcium Chloride Dehydrate Solution ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)

Calcium chloride dehydrate solution was used as a complexing agent. To prepare 75 mM (w/v) calcium chloride dehydrate in 100 ml distilled water, 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was dissolved gradually. This solution was autoclaved for 20 minutes at 121°C and 104kpa.

D. Encapsulation Techniques and Bead Formation

They were then, being dropped one by one in the $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution. The micro shoots were allowed to get encapsulated (hardened) by allowing them to remain in $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution for 30 minutes. These beads were taken out and transferred into sterile distilled water to wash out the excess $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution and were blotted with sterile tissue paper.

Manuscript received on March 5, 2013; revised May 6, 2013. This work was supported by University of Malaya Research Grants (RP025/2012A and PV025/2011B).

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E. Low Temperature Storage

Sterile encapsulated beads of *Oxalis triangularis* were stored under dark condition at low temperature ($4 \pm 1^\circ\text{C}$). The survival rates for germination after 7 and 30 days were recorded. Thirty replicates were used in each treatment.

F. Data Analysis

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

III. RESULTS

Synthetic seeds were created by encapsulation of micro shoots of *O. triangularis* in sodium alginate solution (Fig. 1a). The micro shoots were derived from stem explants of this species after being cultured for one month on MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP. Table 1 shows the germination of synthetic seeds on MS basal media after 0, 7 and 30 days of storage. Even after 30 days of storage, the artificial seeds retained the ability to germinate and gave high regeneration rate (90 %) and the percentage of survival rate was also high (77-86 %) as compared to control. Mean number of shoots formed was the highest after 7 days of storage. Root formation was observed in all treatments.

TABLE I: GERMINATION OF SYNTHETIC SEEDS OF *OXALIS TRIANGULARIS* AFTER BEING STORED AT DIFFERENT DURATIONS

Storage duration (Days)	Conversion frequency (%)	Percentage of survival (%)	No. of shoots (Mean $\pm$ SE)	Root formation (%)
0 (control)	60.00	66.67	$2.10 \pm 0.6a$	$100.00 \pm 0.0a$
7	96.67	86.21	$4.57 \pm 0.7b$	$100.00 \pm 0.0a$
30	90.00	77.78	$3.97 \pm 0.7b$	$100.00 \pm 0.0a$

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

TABLE II: SYNTHETIC SEEDS GERMINATION OF *OXALIS TRIANGULARIS* IN DIFFERENT SOWING SUBSTRATES

Sowing substrate	Treatment	Observation
MS basal medium	Without hormone	Encapsulated beads regenerated into complete plantlets.
Black soil (non sterile)	Moistened with distilled water	Encapsulated beads started to shrink after a few days and eventually contaminated.
	Moistened with liquid MS without sucrose	All encapsulated beads were contaminated with fungal infection.
Black soil (sterile)	Moistened with distilled water	Encapsulated beads showed no response and ultimately shrink.
	Moistened with liquid MS without sucrose	All encapsulated beads were contaminated with fungal infection.

Each medium was represented by 20 replicates.

Interestingly, synthetic seeds that were stored prior to being cultured showed significantly higher conversion frequency than synthetic seeds directly sowed 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 7 days was most optimum to ensure a high plantlet conversion rate and survival percentage. Shooting was also the highest from

synthetic seeds stored for 7 days. This was clearly demonstrated in Table I.

Table II indicates various media or substrates tested for germination of the synthetic seeds. The optimum substrate was MS basal medium (Fig. 1b and Fig. 2). After four weeks, the micro shoots elongated and eventually undergo 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.

Fig. 1. (a) Encapsulated beads of *Oxalis triangularis* before being stored at low temperature ($4^\circ\text{C} \pm 1^\circ\text{C}$), (b) Shoots emerged from the encapsulated micro shoots of *Oxalis triangularis* after 7 days being cultured on MS basal medium.

IV. DISCUSSION

Creation and production of artificial seeds have been reported in many species [5], [6] - [9]. From Tables I and II, the observations after 7 days of encapsulated beads being germinated on the substrates both in black soil and sterile black soil gave negative results which failed to survive and finally the synthetic seeds died. On black soil substrate moistened with distilled water, the encapsulated beads during the first 7 days already showed shrinking sign where

they failed to germinate and eventually contaminated with fungal infection. While synthetic seeds of *Oxalis triangularis* which were sown on sterile black soil moistened with distilled water did not show any sign of contamination, no germination response was observed. However, the beads eventually shrunk. Both synthetic seeds of *Oxalis triangularis* sown in black soil and sterile black soil moistened with liquid MS without sucrose gave similar observations whereby, all encapsulated beads were contaminated with fungal infection after seven days.

Fig. 2. (a) Development of micro shoots of *Oxalis triangularis* from synthetic seeds after 4 weeks being cultured on MS basal medium, (b) Regenerated plantlet of *O. triangularis* after successful acclimatization.

Further studies were also carried out to compare synthetic seeds of *Oxalis triangularis* sown directly on MS basal medium, black soil and sterile black soil (Table II). The results showed that conversion frequency of synthetic seeds of *Oxalis triangularis* into complete plantlets were better under *in vitro* conditions compared to *in vivo* conditions. Synthetic seeds of *Oxalis triangularis* have the capability to respond when cultured on MS basal medium resulted in a high rate of plantlets regeneration. More than 60% successful regeneration from synthetic seeds of *Oxalis triangularis* was observed (Table II). Successful plant regeneration from synthetic seeds of *Oxalis triangularis* on MS basal medium was probably because the beads were

cultured under aseptic conditions. Therefore, probability for the beads to get contaminated was much lower compared to seeds directly sown on *in vivo* substrates.

The potential of synthetic seeds to be sown directly on *in vivo* substrates has received limited attention. From previous studies, Preece and West [10] were successful in sowing *Hibiscus moscheutos* directly into a greenhouse hydroponic system, both with and without light pretreatment. Bapat and Rao [11] conducted research on *in vivo* growth of encapsulated axillary buds of mulberry (*Morusindica* L.) and reported that addition of a fungicide to the alginate beads prevented contamination of the bud and increased survival of the buds when sown in soil. Ramakrishnappa [12] also observed that addition of fungicide (0.1% carbendazim) and bactericide (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. Researches need to be done to improve germination percentage of synthetic seeds when directly sown on non-sterile environment, such as in greenhouse or directly in the field. The present study proved that artificial seeds and tissue culture methods can be useful for plant multiplication and conservation.

V. CONCLUSION

In the present study, production of synthetic seeds was attempted from this species and the synthetic seeds managed to survive after 7 and 30 days after storage at 4°C. The rate of synthetic seeds conversion to complete plants after 7 days was 96.67% with 4.57 mean shoot numbers, while after 30 days of storage, the conversion rate slightly decreased to 90% with only 3.97 shoots formation per bead.

ACKNOWLEDGMENT

The authors would like to thank University of Malaya, Kuala Lumpur, Malaysia, for the Research Grant RP025/2012A and Postgraduate Research Grant PV025/2011B.

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