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In vitro Development of Cauliflower Synthetic Seeds and Development of Plantlets *In vivo*

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Abstract

Synthetic seeds of **c**auliflower cv. Chillout were developed by encapsulating mature somatic embryos in neutral gel media. Somatic embryos were obtained by optimizing callus and cell suspension cultures of cauliflower. Friable, yellowish embryogenic calli were obtained on MS supplemented with 2 mg/l 2,4-D and 0.5 mg/l BAP using hypocotyl as explants, while calli were regenerated in media consisting of 5 mg/l BAP, 2 mg/l Kn and 6 mg/l GA_3. Somatic embryogenesis was induced in cell suspension culture where auxins were removed in successive steps triggering conversion of globular cells into the heart, torpedo stage (71%) and finally into cotyledonary/somatic embryos (28%). The mature somatic embryos were encapsulated by mixing mature cell suspension with sodium alginate and calcium chloride mixture (1 : 4). Developed synthetic seeds germinated into complete plantlets when placed in neutral gel media. Germination efficiency of synthetic seeds decreased to about 50 per cent after 12 weeks of storage at 4ºC followed by a rapid decrease to zero per cent after 16 weeks. It was also observed that cauliflower plantlets from synthetic seeds survived successfully when transferred to soil demonstrating that cauliflower synthetic seeds is a promising step towards their *in vivo* direct use.

Introduction

Cauliflower (*Brassica oleracea* var. *botrytis*) belonging to Brassicaceae is one of the most cultivated vegetables in the world. In addition, it has also been recently found to be useful in the prevention of cancer. The growers are facing several problems in the production of a uniform and quality crop of cauliflower. One of

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the major problems in cauliflower cultivation is the quality seeds production. Cauliflower is an open pollinated crop and there are technical challenges to producing a reliable self incompatible inbred lines. So, there is a strong demand for improved seed biotechnology resulting in efficient and stable regeneration methodology. An effective protocol was designed for the production of suitable cauliflower propagules from fractionated and graded curd. These propagules were considered suitable for encapsulation in sodium alginate for synthetic seed production (Kieffer et al. 2001). The use of micro shoots has been reported to be successful for the production of synthetic seeds in a variety of plant species *viz. Picrorhiza kurrooa* (Mishra et al. 2010).

The major objective of this present study was to establish an efficient callus induction protocol from hypocotyls for cell suspension to produce cauliflower synthetic seeds. The assessment of germination viability of synthetic seeds and the establishment of plantlets from synthetic seeds *in vivo* was another aim of this study.

Materials and Methods

Cauliflower seeds of cv. Chillout were placed on a sterile moist filter paper in Petri plates and incubated in the dark at 25 ± 2°C for germination. Before plating, seeds were surface sterilized with 0.1% mercuric chloride solution for 5 min followed by washing with sterile distilled water three times, 5 min each under aseptic condition. The hypocotyl explants from 5 - 7 days old seedlings were used for callus formation. The hypocotyls were cut into 0.3 - 0.6 cm long pieces and placed horizontally in callus induction MS supplemented with 2 mg/l 2,4-D, 0.5 mg/l BAP, 3% sucrose and solidified with phytagel**;** the pH of the medium was adjusted at 5.7 - 5.8 before autoclaving. Pro-embryogenic callus was obtained approximately after 15 days with regular sub-culturing at 7 days interval. Initially the cultures were maintained in the dark for 3 - 5 days and subsequently placed under 16/8 hrs photoperiod.

To check the shoot regeneration response of cauliflower, the established embryogenic calli were placed in various shoot regeneration media, each supplemented with different hormonal supplements. The best combination of regeneration media was 4 - 6 mg/l BAP, 2 - 4 mg/l Kn and 4 - 6 mg/l GA_3 fortified with MS vitamins (30 µM adenine sulphate, 3 µM thiamine HCl and 580 µM NaH_2PO_4).

The cell suspension of the embryogenic callus was established in the callus medium initially for one week, followed by subculturing it in the same medium without 2,4-D. For this purpose, 250 - 300 mg of calli were placed in 30 ml of cell suspension media and placed on a rotary shaker at 90 rpm under 16 hrs

photoperiod at 22 ± 2°C. Cells were regularly observed under the microscope (Olympus U-PMTVC 3B15937, Japan). For synchronization to achieve uniform cell suspension and to remove dead cell clumps, cells were sieved through a stainless sieve of 150 μm pore size mesh. Further, the cell suspension containing mature torpedo shaped somatic embryos were used for synthetic seed formation. The cell suspension containing embryos and sodium alginate solution (3%, w/v) was mixed in the ratio of 1 : 4, and the mixture was dispensed into 100 mM calcium chloride solution ($CaCl_2$. $2H_2O$). Beads formation started as soon as the drops of sodium alginate fell into the calcium chloride solution placed on a magnetic stirrer. After half an hour, beads were removed from the calcium chloride solution followed by the coating of regeneration media and washed with Radomil Gold solution (fungicide) @ 0.25%. After half an hour beads were collected through filtration.

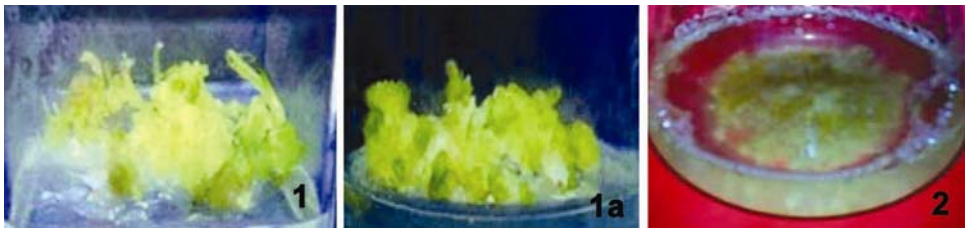
Synthetic seeds were stored at 4ºC. Their germination viability was checked after 16 weeks of their storage. For germination assessment, neutral gel consisting phytagel dissolved in autoclaved tap water was used. Complete plantlets regenerated from synthetic seeds were transferred into pot containing autoclaved compost soil under controlled conditions. The compost soil was prepared by thoroughly mixing clay, sand and compost in the ratio of 1 : 1 : 1 respectively. The plants were completely covered with plastic bag for 2 weeks to maintain the humidity and then progressively exposed to normal environmental conditions for acclimation.

Results and Discussion

Calli were developed from hypocotyls in MS containing 2,4-D and BAP. The embryogenic callus was identified by their anatomical features such as yellowish color, small globular but densely filled cytoplasmic cells. It was observed that a suitable concentration of BAP and 2,4-D is required for the induction of friable, nodular, yellowish embryogenic callus (Aly et al. 2002, Ma and Xu 2002, Hernandez et al. 2003, Nath and Bugagohain 2005). Cauliflower embryogenic callus and somatic embryo induction showed auxin **-** and cytokinin dependent response. The study revealed that the requirement of *in vitro* regeneration may vary from cultivar to cultivar (Sonia et al. 2010). In another study, it was observed that 2,4-D in combination with cytokinin (BAP), are two essential plant growth regulators for the induction of embryogenic callus and somatic embryos (Chalupa et al. 1990, Maureen et al. 1990, Haider et al. 1993, Kim et al. 2003). The influence of different concentrations of sucrose such as 20 g, 30 g, and 40 g per liter of media was also observed to influence the embryogenic potential of callus. In this study, 30 g sucrose was found to be the best for embryogenic callus

induction. These results were similar to those reported by Ganesan et al. (2007) in okra and in black iris by Shibli and Ajlouni (2000). For successful regeneration of synthetic seeds from the somatic embryos, good quality embryogenic callus is essential (Ganesan and Jayabalan 2004).

To observe the shoot regeneration response of embryogenic calli, different media were tested. In the present investigation, it was observed that the treatment of 2 mg/l Kn, 6 mg/l GA_3 combined with 5 mg/l BAP and MS vitamin, gave the optimal regeneration results from the callus in terms of the number of growing shoots as depicted in Fig. 1 and 1a. Therefore, this combination is recommended for enhanced regeneration in cauliflower cv Chillout. Cytokinin concentrations have a central role associated with sink activity and nutrient partitioning (Kuiper 1988, Kuiper et al. 1989).



Figs 1-2. Shoot regeneration from embryogenic callus (1, 1a) and cell suspention cultures (2) of Cauliflower cv. Chillout.

After the optimization of callus induction and shoot regeneration from callus, cell suspension was made in the same medium devoid of 2,4-D. The fresh established cell suspension was morphologically heterogeneous as shown in Fig. 2. Under the microscope, two types of cells were observed. The type-I cells were elongated in shape, vacuolated and larger in size without starch contents. Such cells were few in number (10 - 15%). They were discarded by sieving through 150 μm mesh. The type-II cells were globular, round in shape and smaller in size characterized by dense cytoplasm, nucleolus like bodies with rich starch grains and plastids. The type of such cells were found up to 85 - 90 per cent of the total viable cells. Both types of cells were clearly seen under the microscope (Fig. 3a & b). A similar type of cell morphology in suspension culture was reported in carrot by Fujii et al. (1989). In the present study, it was also observed that in the cell suspension cultures, the suspended cells of cauliflower passed through different stages of embryogenesis. The initial normal globular cells developed into heart-, torpedo- and cotyledonary shaped embryogenic cells. If the cell suspension cultures were sieved at a longer interval than normal duration of 7 days, the percentage of the non-embryogenic elongated cells increased

dramatically. These results are similar to those reported in cucumber cell suspension cultures (Tabassum et al. 2010).

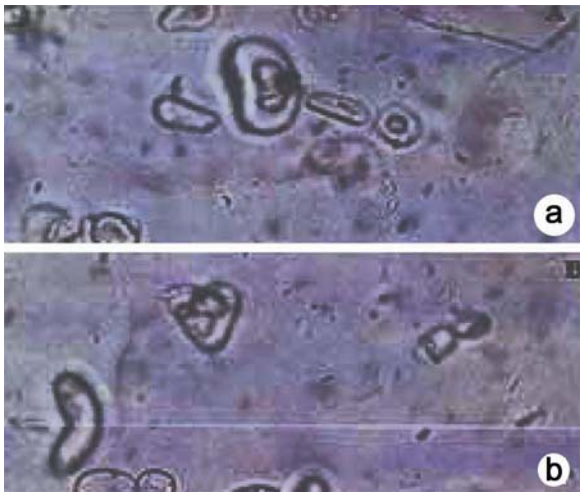


Fig. 3. Microscopic views of the suspended cells in suspension culture. (a) Two types of cells were clearly visible (i) elongated in shape vacuolated and larger in size, (ii) round in shape and smaller in size, dense cytoplasm, apparent nucleolus. (b) Globular cells turned into heart or angular shaped.

The suspended cells were synchronized according to the procedure reported by Giuliano et al. (1983). After two to three successive subculturing at weekly intervals, 84 per cent of the cells were found at the globular stage; thereafter they were transferred to modified suspension media containing BAP only. After 2 - 3 days, based on microscopic studies, 71 per cent of the total cells successfully turned into heart- and torpedo-shaped embryos. Subsequent subculturing on MS supplemented with BAP and NAA, led to uniform maturation of only 28 per cent somatic embryos (Fig. 4). The values obtained were found highly significant as revealed by ANOVA (Table 1). After synchronization, the mature somatic embryos were coated by using sodium alginate. Mature cell suspension and sodium alginate (ratio 1 : 4) were dropped into 100 mM calcium chloride solution to form synthetic seeds. For hardening, these synthetic seeds were rinsed in double distilled water, followed by immersing the seeds in MS- containing 3% sucrose, MS fortified with 5 mg/l BAP, 2 mg/l Kn and 6 mg/l GA_3 and MS vitamin. Thereafter, the seeds were incubated at gyratory shaker for 30 - 40 min. After incubation, mature embryos turned into complete seed structures as shown in Fig. 5. It is reported that sodium alginate complex with calcium chloride is most suitable for encapsulation because of viscosity, low toxicity and quick

gelatinization (Redenbaugh et al. 1993). It is also reported that in carrot 2 per cent sodium alginate solution with 75 mM calcium chloride is the best for coating the somatic embryos to form synthetic seeds (Latif et al. 2007).

Table 1. Showing the results of ANOVA.

No. of cells					
	Sum of squares	df	Mean square	F	Sig.
Between groups	4604.667	2	2302.333	56.307	0.000
Within groups	245.333	6	40.889		
Total	4850.000	8			

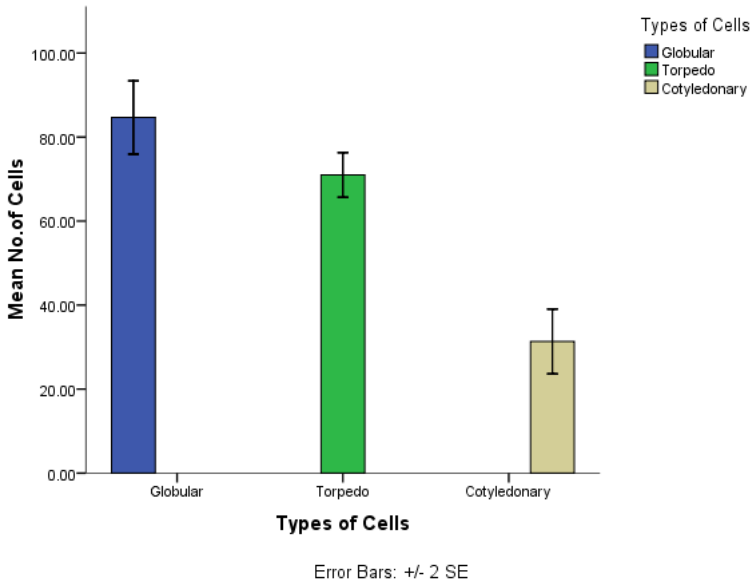


Fig. 4. Conversion of globular shaped cells to heart- and finally into cotyledonary- cells in cell suspension culture. Values were found significant as revealed by ANOVA.

The germination percentage of cauliflower synthetic seeds was found to be 83.5. It was also observed that the germination efficiency was considerably decreased with the increase of storage time as shown in Fig. 6. The germination efficiency was reduced almost to 50 per cent after 12 weeks of storage but as the storage time was prolonged to the 16th week, the rate of germination percentage was zero. Yussof et al. (2011) used encapsulation technique for production of broccoli synthetic seeds (*Brassica oleracea*) with sodium alginate and observed that 70 per cent germination of synthetic seeds. In our study, synthetic seeds

were germinated in neutral gel (Fig. 7), while encapsulations of synthetic seeds were done with nutrient mixture; calcium alginate was used to protect the synthetic seeds from microbial infections and mechanical damages during handling. Thus it showed that somatic embryo encapsulation is one of the promising methods for sowing embryos and regeneration of cauliflower into plantlets.

Shoot and root formation was observed when germinated synthetic seeds were transferred from the Petri plate to test tube containing shoot and root regeneration media. In this study, we used three different regeneration media for shoot formation and among the three combinations, excellent performance was obtained in the third combination followed by the second and the first one. The best root formation was also observed in the third combination containing 1.0 mg/l NAA in addition (Fig. 8). A similar observation was reported in broccoli regeneration from synthetic seeds (Yussof et al. 2011). In their study, George et al. (2008) reported plant propagation by means of tissue culture. They recommended multiple shoot formation in higher concentration of BAP and lower concentration of IAA.

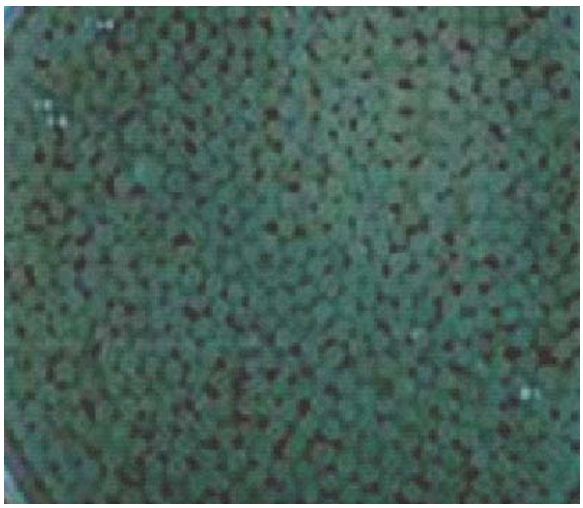


Fig. 5. Cauliflower synthetic seeds.

After regeneration in a controlled environment, the developing plantlets were transferred into pot**s** containing autoclaved compost soil. The optimal conversion rate and viability were obtained in 1 : 1 : 1 of clay, sand and compost, respectively (Fig. 9). In several earlier studies researchers investigated the possibility of sowing synthetic/artificial seeds in soil, for example, using vermiculite, sand and soil for M.26 apple rootstock (Micheli et al. 2002) and

Citrus reticulata (Antonietta et al. 2007) and the use of sand for elite indica rice (Roy and Mandal 2008). It needs to be emphasized that the optimal conditions need to be determined empirically for each species examined.

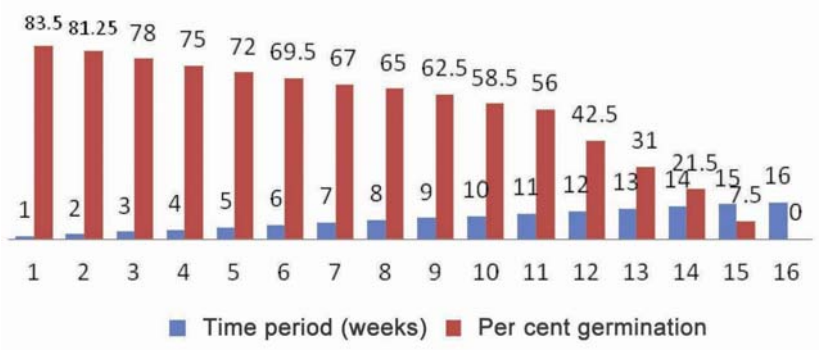
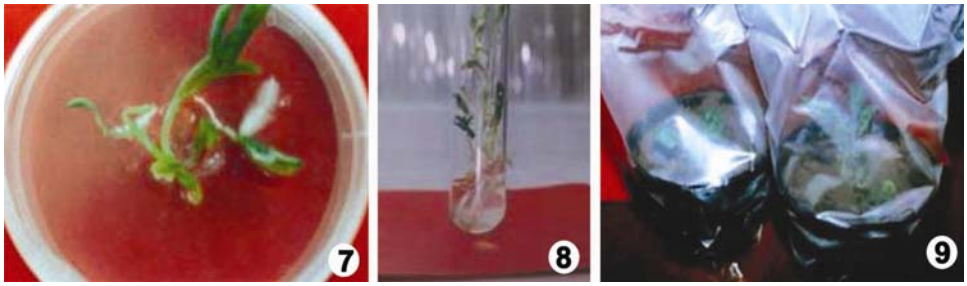


Fig. 6. Germination viability percentage of cauliflower synthetic seeds developed by encapsulating mature somatic embryos by sodium alginate.



Figs 7 - 9. 7. Cauliflower synthetic seeds germinated on neutral gel media. 8. Cauliflower plantlets with regenerated shoots and roots derived from synthetic seeds. 9. Complete cauliflower plantlets derived from synthetic seeds.

This is the first successful study of cauliflower synthetic seed production from the hypocotyl, successful germination and regeneration of synthetic seeds into complete plantlets. The main objective of the present study was to optimize an inexpensive and yet a sophisticated method for synthetic seed production of cauliflower with a prolonged period of viability capable of germinating and developing into viable plants. Synthetic seed technology seems to be a promising method in plant tissue culture industry. This method is very advantageous, especially for those plant species which do not produce seeds or for a long term storage of elite genotypes selected through traditional breeding methods or genetically engineered plants.

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