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SHORT COMMUNICATION

Production of Artificial seeds derived from encapsulated *in vitro* micro shoots of cauliflower, *Brassica oleracea* var. *botrytis*

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Abstract

A high number of micro shoots (21±2.31) of Brassica oleracea var. botrytis (cauliflower) were obtained when hypocotyl explants from 2-week-old aseptic seedlings were cultured on MS medium supplemented with 0.1 mg/L NAA and 5 mg/L BAP. Artificial or synthetic seeds were formed when the micro shoots were encapsulated in 4% (w/v) sodium alginate with 100 mM $CaCl_2$ as complexing solution. The artificial seeds took 12 days (after 7 days storage) and 14 days (after 30 days storage) to germinate on MS basal medium. The germination percentage of artificial seeds was enhanced by the inclusion of 0.3 mg/L NAA and 3.0 mg/L BAP in the encapsulation matrix after 7 and 30 days of pre-germination storage. The time taken for germination was also faster (5 days after 7 days of storage and 11 days after 30 days of storage) when MS fortified with 0.3 mg/L NAA and 3.0mg/L BAP were used. Isolated micro shoots encapsulated in MS supplemented with 0.3 mg/L NAA and 3.0mg/L BAP gave high germination percentages (70±5.43% and 63.33±4.17%) after 7 and 30 days of pre-germination storage period, respectively. Complete plant regeneration was achieved from the germination of these artificial seeds derived from the micro shoots after 3-4 weeks in culture.

Key words: Synthetic seeds, multiple shoots, hypocotyl explants, MS medium, germination.

Introduction

Plant propagation through artificial seeds broadens the horizon of plant biotechnology and agriculture [9]. The technology provides methods for preparation of seed analogues from the micropropagules such as axillary shoots, apical shoot tips, embryogenic calli, somatic embryos as well as protocorm or protocorm-like bodies [18]. Micropropagules are encased in productive coatings of gelling agents such as alginate, agar, carrageenan, gellan gum (gelrite), sodium pectate, and carboxyl methyl cellulose. Encapsulation of micro shoots and somatic embryos and subsequent retrieval of complete plantlets has been reported in a number of plant species, such as sandalwood, mulberry, banana, cardamom, sugar beet, rice and peer [3,16,1,4,2]. These evidences have shown that artificial seed production is potentially useful for the large scale propagation of superior hybrids of economically important species.
As one of the important value-added plant tissue culture products, artificial seed technology can only be successful with efficient upstream production of micropropagules as well as downstream germination protocols for high percentage of plant regeneration. Various micropropagules have been considered for artificial seed production; however, somatic embryos and axillary shoot buds were mainly favoured. Somatic embryos have been used as the micropropagules to produce artificial seeds in a vast variety of fruit and crop species, including *Arachis hypogaea* (groundnut), *Daucus carota* (carrot), *Medicago sativa* (alfalfa), *Picea abies* (Norway spruce), *Psidium guajava* (guava), and *Vitis vinifera* (grape) [8,14,10,13,5]. On the other hand, in many plant species, such as *Actinidia deliciosa* (Kiwifruit), *Malus pumila Mill* (apple), *Rubus idaeus* L. (raspberry), *Syringa vulgaris* L.

(Lilac) and *Zingiber officinale* Rosc. (ginger), the unipolar axillary shoot buds and apical shoot tips have been encapsulated to produce artificial seeds [6,7,16,3,17].
Brassica oleracea var. *botrytis* is one of the most important vegetable crops and has a high international market demand. According to Food and Agricultural Organization of the United Nation (FAO), United States per capita consumption of cauliflower is around 2 pounds (900 grams) per capita. During the 1995-2005 period, the global harvesting area of cauliflower increased by about 28% mainly as a consequence of the significant increase in China, from 219,000 ha to 363,000 ha, and in India, from 250,000 to 258,000 ha. The production of synthetic seed is useful since this species naturally produce very tiny seeds. To our knowledge, there is no published report on the production of artificial seeds from encapsulated micro shoots of cauliflower. Therefore, the aims of the present study were to evaluate the effects of different plant growth regulators (PGRs) on the multiple shoots induction using hypocotyls as explants, subsequently the *in vitro* micro shoots produced were harvested and encapsulated in sodium alginate to create artificial seeds. The storage ability and *in vitro* germination rates of artificial seeds were also evaluated.

Materials and methods

Commercial seeds of cauliflower, *B.oleracea* var. *botrytis* (YMWOO Corporation) purchased from Kuala Lumpur, Malaysia were utilized. Seeds were stored at 4 ºC until used. Cauliflower seeds were soaked in distilled water for 20 minutes with addition of 1-2 drops of Tween-20, followed by 60% (v/v) sodium hypochlorite (Clorox) solution and gently agitated for 15 minutes. The seeds were then rinsed 3 times in distilled water and were then soaked in 70% (v/v) ethanol for 30 seconds. The seeds were subsequently rinsed 3 times in sterile distilled water. Surface disinfected seeds were cultured on MS (Murashige and Skoog, 1962) basal medium [15] (~ 20ml) in sterile screwed-cap bottles. MS basal medium contained 3% (w/v) sucrose and 0.8% (w/v) agar at pH 5.8. The culture condition was maintained at 25 ± 1 ºC under a 16-h photoperiod regime of cool white fluorescent light (40µmEm^{-2}s^{-1}).

***In vitro* materials and culture medium for multiple shoots induction**

Hypocotyl segments from two-week-old seedlings were used as explants. The hypocotyls were excised into sections of 8 mm in length. Different concentrations and combinations of plant growth regulators (PGRs) (Fig. 1) were screened and tested for their effects on multiple shoots induction on hypocotyl explants. Each treatment was done in 30 replicates for the hypocotyl explants. Multiple shoots were induced from hypocotyl explants cultured on MS medium supplemented with 0.1 mg/L NAA and 5 mg/L BAP as stated above. Micro shoots with size not more than 8 mm were isolated from six-week-old multiple shoot cultures.

Sodium alginate (4% w/v) supplemented with 0.3mg/L NAA and 3mg/L BAP was prepared in calcium free liquid MS medium containing 3% sucrose with 100 mM $CaCl_2.2H_2O$ as complexing solution. Encapsulation was accomplished by gently mixing the micro shoots in sodium alginate gel. By using sterile disposable plastic pipette, the excised micro shoot was drawn up with sodium alginate and was dropped into the $CaCl_2$ solution, for 30 minutes. After hardening, the beads were soaked for 2 minutes in sterile distilled water to remove calcium chloride residues. The beads were stored at 4 ºC for 7 days and 30 days, respectively before being inoculated on germination media. For *in vitro* germination, the synthetic seeds were inoculated on MS basal medium with 3% (w/v) sucrose and 0.8% (w/v) agar. Three replicates each with 30 synthetic seeds were inoculated on germination media aseptically. The germination rate and incubation period required by germination of artificial

seeds were recorded. Germination of artificial seed was ascertained when the bead cracks with the outgrowth of micro shoot. For comparative responses as well as germination rate, interval plots were drawn by software of SPSS.

Results

Multiple shoots induction from hypocotyl explants

Frequency of forming multiple shoots and the number of multiple shoots were evaluated based on 3 independent experiments with 30 explants per treatment. Different concentrations and combinations of PGRs (NAA, BAP, kinetin and TDZ) were tested for their efficiency to induce multiple shoots from hypocotyl explants (Fig. 1).

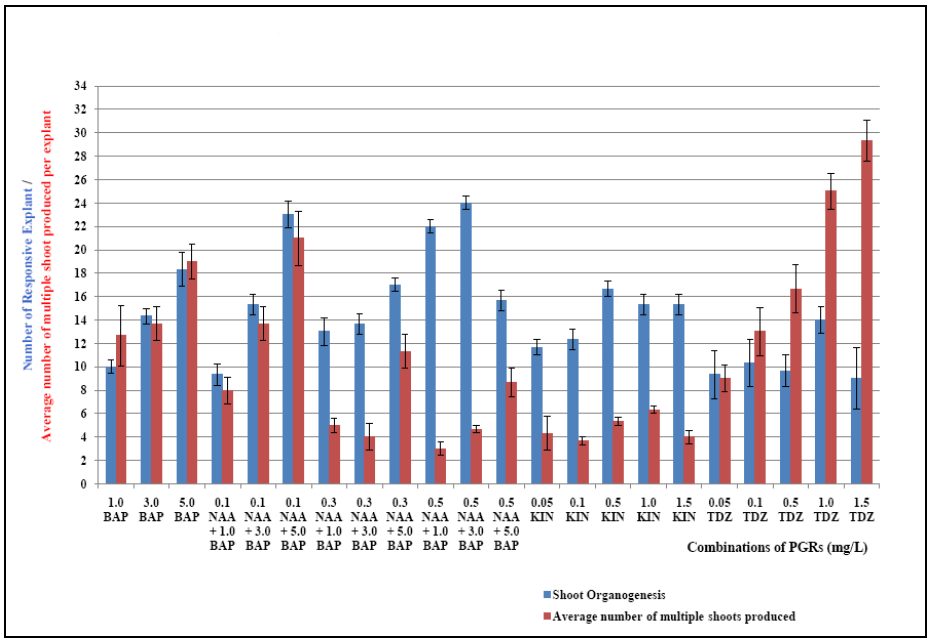

Fig 1. Responses of hypocotyl explants to different combinations of PGRs in multiple shoots induction

Multiple shoots were induced directly on hypocotyl explants when kinetin was used (Fig. 2B) while, indirect formation of multiple shoots were induced using TDZ, NAA, BAP as well as combination of NAA and BAP (Fig. 3A-D). For direct multiple shoots induction using kinetin, the shoot formation was first observed after three weeks of incubation without forming any callus on the explant surface. Best PGR treatment for direct multiple shoots induction from hypocotyl explants was MS medium supplemented with 0.5mg/L kinetin (Fig. 2B). For indirect multiple shoots induction, callus was first observed on the hypocotyl explants after two weeks of incubation. PGR treatment with 0.1 mg/L NAA and 5mg/L BAP was the best treatment to induce multiple shoots on hypocotyl explants (Fig. 1,3C). By using this treatment, the highest number of healthy and normal shoots (21±2.31 shoots per explants) was achieved (Fig. 1).

Fig 2. Synthetic seeds created by encapsulation of the micro shoots of cauliflower obtained from direct organogenesis with sodium alginate

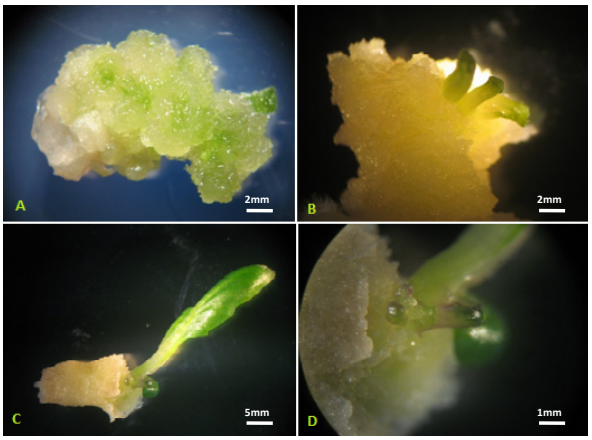

Fig 3. Indirect multiple shoots induction from hypocotyls explants of cauliflower

Apart from that, PGR treatments with different concentrations of TDZ produced abnormal multiple shoots with observed symptoms of shoot fasciations (Fig. 4A-C). The multiple shoots fused together at the leaf base, elongated and enlarged abnormally when TDZ in the concentrations range from 0.05-1.5 mg/L were used to induce indirect multiple shoots from hypocotyls explants.

Fig 4. Proliferation of abnormal (fasciation) multiple shoots derived from hypocotyl explants of cauliflower.

In vitro germination of artificial seeds after 7 days of storage

Germination rate of synthetic seeds encapsulated in MS basal media after stored at 4 ºC for 7 days was 34.43±5.5% (Fig. 5). Cracking of beads was first observed with outgrowth of micro shoot after twelve days of incubation. The artificial seeds germinated into single plantlets with 5 cm in height after 5 weeks of incubation. On the other hand, with encapsulation matrix

of MS supplemented with 0.3 mg/L NAA and 3.0 mg/L BAP, the germination rate was increased to 70±5.43% (Fig. 5). Cracking of beads was first observed with outgrowth of micro shoot after five days of incubation (Fig. 6A). The artificial seeds germinated into single plantlets with 5cm in height after 3 weeks of incubation.

***In vitro* germination of artificial seeds after 30 days of storage**

Germination rate of synthetic seeds encapsulated in MS basal media after stored at 4 ºC for 30 days was 22.33±6.37% (Fig. 5). Cracking of beads was first observed with outgrowth of micro shoot after fourteen days of incubation. The artificial seeds germinated into single plantlets with 5cm in height after 4 weeks of incubation. On the other hand, with encapsulation matrix of MS supplemented with 0.3 mg/L NAA and 3.0 mg/L BAP, the germination rate was increased to 63.33±4.17% (Fig. 5).

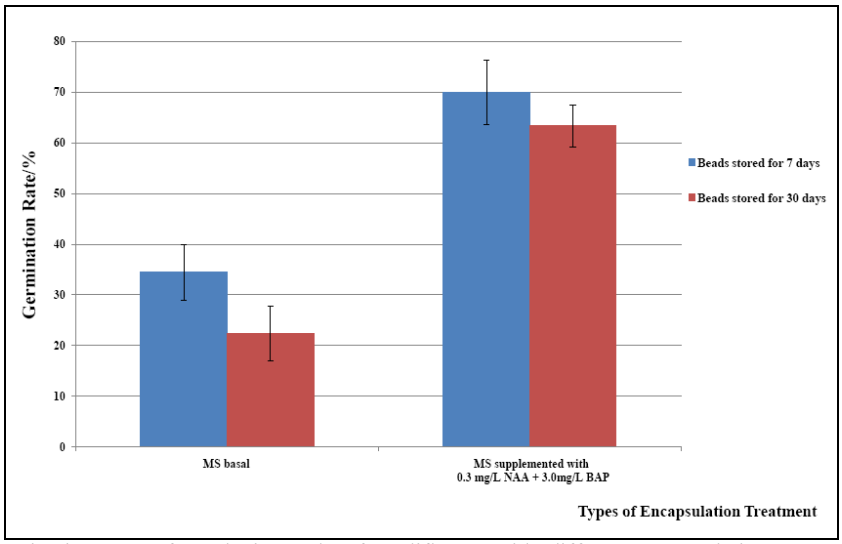

Fig 5. Germination rate of synthetic seeds of cauliflower with different encapsulation treatments and storage periods

Cracking of beads was first observed with outgrowth of micro shoot after eleven days of incubation. The artificial seeds germinated into single plantlets with 5cm in height after 4 weeks of incubation. Complete plant regeneration was achieved from the artificial seeds after 3-4 weeks of inoculation and ready to be transferred to garden soil or field.

Fig 6. The germination of synthetic seeds of cauliflower into single plantlets

Discussion

For the purpose of producing artificial seeds, an effective protocol of multiple shoots induction should be pre-developed in order to provide a large number of micro shoots for encapsulation. In this study, 2-week-old aseptic seedlings were used as explant sources. Hypocotyl is a type of juvenile explants which is still immature and undifferentiated completely in morphology. Rationale of choosing hypocotyl of seedlings as explants to initiate cultures was mainly due to the highly responsiveness of these tissues in tissue culture compared to more mature explants from adult plants such as curd, stem, root and mature leaf. For cauliflower, multiple shoots can be induced on the explants through direct or indirect shoot organogenesis, which mainly depends on the type of PGRs used. From the results, multiple shoot can be induced directly on the explants by kinetin at different concentration levels without any intervening callus phase on the hypocotyl explants (Fig. 2B).

Multiple shoot induction through indirect shoot organogenesis can be induced by using TDZ, BAP, and combinations of BAP and NAA at different concentration levels. In indirect shoot organogenesis, morphogenic callus formed and shoot buds emerged from the callus subsequently (Fig. 3A-D). In indirect shoot organogenesis, hypocotyl explants were found to be most responsive under treatment with 0.5 mg/L NAA and 3mg/L BAP, however, the average number of shoots under this treatment was only about 5 shoots per explants (Fig. 1,3A,B). Apart from that, hypocotyl explants gave rise to the highest number of shoots, (29 shoots per responsive explants) under treatment of 1.5 mg/L TDZ (Fig. 1). However, majority of the multiple shoots were abnormal in terms of shoot fasciation. The leaf bases of shoots were fused together and the leaf arisen from it was usually flatten and abnormal. Leaves were arranged abnormally with two or more typically emerged simultaneously (Fig. 4A-C). Hence, micro shoots arisen from TDZ treatment were also not suitable to be used as explants in synthetic seeds production because the fascinated shoots were abnormal in morphology and caused difficulty in separating the micro shoots.

In the present work, synthetic seeds were successfully created by encapsulating the cauliflower micro shoots derived from multiple shoot induction. Micro shoots excised from multiple shoot cultures induced by treatment of 0.1 mg/L NAA and 5 mg/L BAP (Fig. 1) were encapsulated in 4% (w/v) sodium alginate in MS medium with 100 mM $CaCl_2.2H_2O$ as complexing agent (Fig. 2C). In order to enhance the germination rate of synthetic seeds, the encapsulation matrix was supplemented with 0.3 mg/L NAA and 3.0 mg/L BAP in addition to the encapsulation matrix with only MS basal. This combination of PGRs was selected because it can efficiently induce both shoot and root formations simultaneously. The beads formed by the above described procedure were firm, radial and isodiametric in shape. The beads formed were 8 mm in diameter with spherical shape (Fig. 2C).

The synthetic seeds were inoculated onto MS basal media for germination. For synthetic seeds with encapsulation matrix supplemented with 0.3 mg/L NAA and 3.0 mg/L BAP and stored for 7 days (Fig. 2C), the outgrowth of encapsulated micro shoots was first observed on the fifth day after inoculation on germination media. The micro shoots protruded and emerged from the surface of beads (Fig. 6A). The micro shoots developed into single green shoots and subsequently plantlets on the MS media (Fig. 6B). After 3 weeks of inoculation, majority of the single plantlets reached 5cm in height (Fig. 6C). However, the period required for rooting was not uniform according to the present observation. Meanwhile, synthetic seeds with encapsulation matrix supplemented with 0.3 mg/L NAA and 3.0 mg/L BAP showed higher germination rates, i.e. 70% and 63.33% after being stored for 7 days and 30 days, respectively. The results indicated that the treatment of 0.3 mg/L NAA and 3.0 mg/L BAP was essential and has profound effect on the germination rate if compared to encapsulation matrix

with only MS basal, with germination rate as low as 34.43% and 22.33%, respectively. In addition, the *in vitro* germination rate of natural seed was 75.38% (data not shown), slightly higher than 70% in synthetic seeds which were stored for 7 days at 4 ºC prior to inoculation on MS media. This comparable results showed that the synthetic seeds technology have a high potential to provide an efficient mean to mass propagate cauliflower with elite genotype as well as an alternative method to regenerate cauliflower in transgenic studies.

Previously, encapsulation of *in vitro* derived shoot tips of *Gypsophila paniculata* in different concentrations of sodium alginate dissolved in MS basal medium and sterile distilled water were investigated by [12]. The encapsulated shoot tips were stored for 30 days and 90 days at 4 ºC and cultured on MS basal medium and MS medium containing 0.5 mg/L each of NAA and BAP. The highest frequency of shoot emergence and maximum number of shoots were recorded for beads encapsulated in sodium alginate (4% w/v) dissolved in MS which were stored for 30 days and grown on medium containing 0.5 mg/L each of NAA and BAP [12]. However, the germination rate of the synthetic seeds was not reported in their study. On the other hand, Kumar et al. [11] reported that the synthetic seeds of rice with artificial endosperm constituents of MS nutrient, sucrose (3% w/v) 0.5 mg/L IAA, 0.5 mg/L NAA, 0.5mg/L BAP and activated charcoal (1.25% w/v) gave maximum germination rate of 30% by using somatic embryos at globular stage as propagules. They also reported that the inclusion of activated charcoal had enhanced the germination to the maximum extent by increasing the diffusion of gases, nutrients and respiration of embryoids.

Artificial seeds have many other significant applications. Synthetic seeds derived from micro shoots or somatic embryos can be created from very small pieces of plant tissue (as small as 3 mm), to serve as a supplement to the already existing propagules, to increase productivity and to propagate plants with very tiny and expensive seeds. Synthetic seeds can also be used for cryopreservation of elite genotypes. Synthetic seeds consist of viable plant parts which can be stored at 4 ºC without losing the viability and thus can be germinated whenever necessary. For future work, longer storage period should be tested, ideally 6 months to one year.

Conclusion

PGR or hormones supplemented to MS medium, in the present work, 0.1mg/L NAA and 5mg/L BAP effectively induced a high number of multiple shoots on cauliflower hypocotyl explants for downstream artificial seeds production. The germination percentage of encapsulated micro shoots was affected by composition of encapsulation matrix and duration of pre-germination storage. Isolated micro shoots encapsulated in MS supplemented with 0.3mg/L NAA and 3.0mg/L BAP gave high germination percentage for both 7 and 30 days of pre-germination storage period.

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