Plant Regeneration and Synthetic Seeds Production of *Brassica oleracea* var. *italica*

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Abstract

Agricultural industry has changed tremendously, especially since the end of World War II. Food industry has blossomed due to new technology, addition of preservative usage and government policies that favoured maximizing productions. Thus, sustainable vegetable production has captured the attention of many countries worldwide while at the same time preserving environmental quality and making efficient use of non-renewable resources. *Brassica oleracea* var. *italica* or commonly known as broccoli from the family *Brassicaceae* is an important vegetable. Broccoli is a member of the cabbage family providing good source of vitamins A and C among other nutritional benefits. Broccoli explants (peduncle, stem and root) were cultured on Murashige and Skoog (MS) medium with different concentrations and various combinations of growth hormones including α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), 2,4-dichlorophenoxy acetic acid (2,4-D) and Thidiazuron (TDZ) ranging from 0.1 to 3.0 mg/L. Multiple shoots were best observed in MS media with combinations of 3.0 mg/L BAP and 1.0 mg/L NAA. Further studies including synthetic seeds production were done. Synthetic seeds were created by encapsulating multiple shoots with calcium alginate. Multiple shoots which were encapsulated with calcium alginate supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA gave the best result for germination (70%). These results showed that synthetic seeds have potential to manipulate in vitro culture systems for large scale production. The ability to produce synthetic seeds of broccoli is essential in increasing Malaysia's agricultural industry and economy.

INTRODUCTION

Brassica is an important vegetable and highly diversified group of crops grown world-wide and belong mainly to the species of *Brassica oleracea* and *B. campestris*. The *Brassica* species include kale, brussels sprouts, cabbage and cauliflower (Qin et al., 2006). Being known as the most economically important genus in the *Brassicaceae* family, they are widely used in the cuisine of many cultures and recognized as a valuable source of dietary fiber and also recently found to be useful in the prevention of cancer.

Brassica oleracea var. *italica* is one of the many valuable *Brassica* species, which is still less cultured under in vitro condition (Widiyanto and Erytrina, 2001). Some experimental results showed successful in vitro culture of *Brassica* species from hypocotyl segments, root segments, primary leaf discs, cotyledons and anther (Cao and Earle, 2003). In most *Brassica* species, the successful application of in vitro culture is mostly dependent on the genotype and the influence of growth regulators (Ravanfar et al., 2009). The addition of cytokinins, e.g., benzyladenine would enhance shoot proliferation and root formation (Arnison et al., 1990) while various concentrations of auxins, e.g., α-naphthalene acetic acid (NAA), have been evaluated for rooting of in vitro regenerated shoots of broccoli and cauliflower (Vandemoortele et al., 1999; Widiyanto and Erytrina, 2001).

Synthetic or artificial seeds have been defined as somatic embryos engineered for

use in the commercial propagation of plants (Gray and Purohit, 1991; Redenbaugh, 1993). It will help in developing a clonal propagation system that will enable the vegetative propagule to be stored for long periods of time and at the same time will enable multiplication of the plant.

The objective of this study is to evaluate the best proliferation of different explants at various combinations of plant growth regulators and select the best callus, shoot proliferation and rooting medium to produce complete plantlets of *Brassica oleracea* var. *italica*. This paper also reports on the production of synthetic seeds of *Brassica oleracea* var. *italica* via multiple shoots.

MATERIALS AND METHODS

Germination of Aseptic Seedlings

Commercial seeds of broccoli were first soaked in distilled water for 2 h. Then, the seeds were surface sterilized in 100% commercial bleach (chlorox) added with 1 drop of Tween 20 for 2 min. Sterilization was repeated with different concentrations of chlorox (90, 70, 50, 30 and 10%). Finally, the seeds were soaked in 70% ethanol for 2 min before rinsing with sterile distilled water and cultured on MS (Murashige and Skoog, 1962) medium under aseptic technique. MS basal medium containing 3% sucrose and 0.8% (w/v) agar was used in this research. All the cultured explants were kept in the culture room at 16 h light and 8 h dark photoperiod and the temperature was maintained at 24±1°C.

Induction of Plant Regeneration

Peduncles, shoots and roots explants were excised from 8-day-old broccoli aseptic seedlings. The explants were cultured on MS medium supplemented with different concentrations and combinations of hormones. The combinations include NAA+BAP, 2,4-D+BAP, NAA+TDZ and 2,4-D+TDZ. The concentration for auxin ranged from 0 to 2.0 mg/L while for cytokinin, the concentration ranged from 1.0 to 3.0 mg/L. All the cultured explants were kept in the culture room at 16 h light and 8 h dark photoperiod and the temperature was maintained at 24±1°C.

Preparation of Synthetic Seeds

Encapsulation of plant microprapagules in calcium alginate matrix was performed using the protocol described by Slade (1989), Jaiswal et al. (2000), Mamiya and Sakamoto (2001), Nower et al. (2007) and Kumar (2009). Calcium alginate (4% w/v) supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA was prepared in calcium-free liquid MS medium containing 3% sucrose. The complex solution of calcium chloride (dehydrate) was prepared by adding calcium chloride (dehydrate) into distilled water. Both gelling and complexing agent were autoclaved at 121°C, 15 psi (103 kPa) for 20 min.

Synthetic Seeds of Broccoli

For preparation of synthetic seeds of broccoli, multiple shoots were cut into smaller pieces about 5 mm in length and encapsulated with calcium alginate. The encapsulated multiple shoots were soaked in the calcium alginate for 30 min. The beads were then rinsed with distilled water 3 times to remove all excess calcium chloride. The beads were then cultured in MS medium supplemented with 3.0 mg/L NAA and 1.0 mg/L BAP.

Statistical Analysis

All the observations were recorded and analyzed using SPSS (Statistical Package for Social Science) Version 10.0 for Window (Chicago, SPSS Inc.).

RESULTS AND DISCUSSION

Different explants of broccoli have been used for regenerations, including peduncle explants (Christey and Earle, 1991), hypocotyls (Metz et al., 1995; Puddephat et al., 2001), leaf tissues (Cao and Earle, 2003), flowering stalk and hypocotyl petiole (Metz et al., 1995). In the present study, three types of plant tissues have been used i.e., peduncles, leaves and roots. Comparison between the three different types of tissues showed that peduncles explant gave a better regeneration response compared to leaf and roots explant (data not shown). This is in agreement with the study done by Christey and Earle (1991) where they concluded that the average regeneration rates of more than 75% were obtained from peduncle explants in a regeneration study of broccoli. Hence, peduncles were used for further experiments.

Multiple shoots can be induced on the explants through indirect or direct shoot organogenesis, which mainly depends on the types of plant growth regulators used. Indirect organogenesis involved the production of callus, while this stage was absent in direct organogenesis. In this study, indirect regeneration of broccoli was induced by combinations and different concentrations tested; NAA and BAP, BAP and 2,4-D, TDZ and NAA and TDZ and 2,4-D. Observations showed that most of the combinations of hormones will form calli first and only then subsequently produce multiple shoots and roots. This results in the indirect regeneration of a complete plantlet. Callus tissues are known to have the ability to produce any types of cell since they are a mass of undifferentiated cells. Productions of embryos can be induced from the callus tissues with manipulation of plant growth regulators in all suspension.

Among the many combinations of hormones tested (Fig. 1), combination of hormones TDZ and 2,4-D showed high percentage of callus production (100%) and very low ability to regenerate multiple shoots. Optimum callus production was observed from MS medium supplemented with 2.5 mg/L TDZ and 1.0 mg/L 2,4-D and MS medium supplemented with 3.0 mg/L TDZ and 2.0 mg/L 2,4-D resulted in 100% of callus production.

As observed in Figure 2, the best regeneration of multiple shoots were obtained from MS medium supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA, with the production of 7 shoots per explant after 8 weeks of culture. This is followed by MS medium supplemented with 2.0 mg/L BAP and 0.2 mg/L NAA, 1.5 mg/L TDZ and 0.2 mg/L NAA and 2.5 mg/L TDZ and 0.2 mg/L NAA with 6 shoots per explant. These results differ significantly when compared to the control (Table 1). Best roots formation was obtained in the MS medium supplemented with BAP and NAA (35%) followed by TDZ and NAA, BAP and 2,4-D and TDZ and 2,4-D (data not shown). According to George et al. (2008), BAP is effective in enhancing shoot multiplication and triggering shoot elongation. BAP also promotes differentiation of cells into shoot initials followed by the formation of shoots. One of the functions of NAA is to stimulate cell enlargement and stem growth. Thus, it can be concluded that multiple shoots can be produced with high concentrations of BAP and low concentrations of NAA.

Synthetic seeds of broccoli were successfully created by encapsulating the microshoots of broccoli in 4% (w/v) calcium alginate (Fig. 3). The beads produced were firm, radial and isodiametric in shape. Germination of synthetic seeds was observed and analyzed at two different storage times and with different encapsulation matrices, as shown in Table 2. The hormones added in the production of synthetic seeds were similar with the multiple shoots induction experiment as they are known to promote simultaneous growth of both shoots and roots. As implied in Table 2, synthetic seeds with encapsulation matrix supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA produced higher germination percentage (70% for 7 days and 66.67% for 30 days) compared to encapsulated matrix with only MS basal medium (37.78% for 7 days and 30% for 30 days). This indicates that the treatment of hormones was essential and had profound effect in the germination rate. These findings correlated with hormones added to the encapsulation matrix of rice (Kumar et al., 2005) and hormones added to the culture media of *Gypsophila paniculata*'s synthetic seeds (Rady and Hanafy, 2004).

CONCLUSIONS

Complete plant regeneration and multiple shoots induction of *Brassica oleraceae* var. *italica* was obtained from MS media supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA under 16 h of light and of 8 h dark photoperiod at temperature of 24±1°C. Successful regeneration of *Brassica oleraceae* var. *italica* via the use of synthetic seeds was also achieved.

Synthetic seeds is another method for propagation in plant tissue culture industry. This method is very advantageous especially for those plants which do not produce seeds or have expensive seeds. In the present study, encapsulation matrix of synthetic seeds matrix supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA showed high regeneration percentage (70%) compared to encapsulation matrix with only MS basal medium (34%). These results showed that synthetic seeds have potential to manipulate in vitro culture systems for large scale production. The ability to produce synthetic seeds of broccoli is essential in increasing Malaysia's agricultural industry and economy.

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Tables

Table 1. Response of peduncles explants that were cultured in different concentrations and combination of hormones under 16 h light and 8 h dark photoperiod at 24±1°C.

Treatment	BAP+NAA	BAP+2,4-D	TDZ+NAA	TDZ+2,4-D
MSO (without hormone)	-	-	-	-
1.0	-	5.0±3.0*	-	-
1.0+0.1	4.5±3.5	1.5±0.5	-	-
1.0+0.2	3.5±2.5	-	2.5±1.5	-
1.0+1.0	-	2.5±1.5	5.0±3.0*	-
1.0+2.0	3.0±1.0	-	-	-
1.5	2.5±2.5	4.0±2.0*	4.0±2.0	-
1.5+0.1	1.5±0.5	1.5±0.5	4.0±2.0	-
1.5+0.2	-	1.5±0.5	6.0±2.0*	2.5±1.5
1.5+1.0	-	-	-	-
1.5+2.0	1.5±0.5	-	2.5±1.5	-
2.0	-	2.5±1.5	2.5±1.5	2.5±1.5
2.0+0.1	-	2.5±1.5	-	2.5±1.5
2.0+0.2	6.0±4.0*	-	3.5±2.5	-
2.0+1.0	-	-	-	-
2.0+2.0	-	-	-	-
2.5	-	3.0±1.0*	-	-
2.5+0.1	4.5±3.5	1.5±0.5	-	-
2.5+0.2	-	-	6.0±4.0*	-
2.5+1.0	-	-	-	-
2.5+2.0	4.5±3.5	-	-	-
3.0	4.5±3.5	-	-	5.0±3.0
3.0+0.1	-	-	-	-
3.0+0.2	-	1.5±0.5	5.0+3.0*	-
3.0+1.0	7.0±5.0*	-	-	-
3.0+2.0	-	-	-	-

Symbol (-) indicate no response.
* The mean difference is significant at the .05 level.
[a] Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table 2. Germination of synthetic seeds with different stages and encapsulation matrices.

Storage time	Encapsulation matrix	Germination	
		Mean±S.E	Percentage (%)
7th day	MS basal medium	11.3±1.86	37.78
	Hormone (3.0 mg/L BAP and 1.0 mg/L NAA)	21.0±1.15	70.00
30th day	MS basal medium	3.0±2.31	30.00
	Hormone (3.0 mg/L BAP and 1.0 mg/L NAA)	20.0±2.31	66.67

Figures

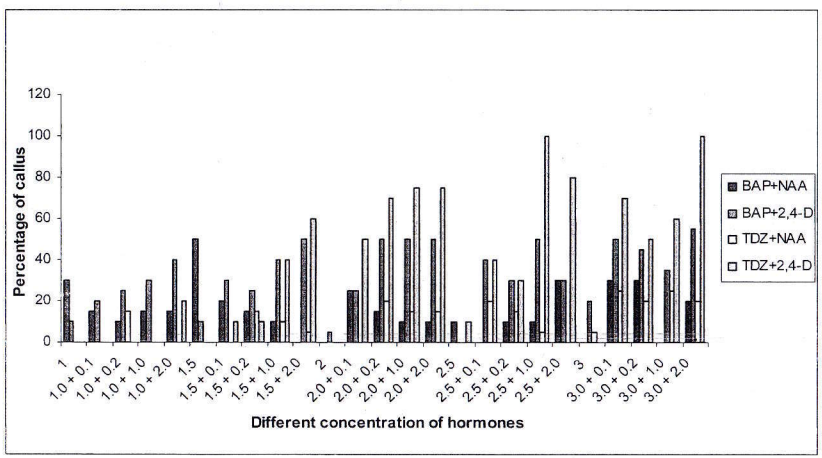

Fig. 1. Percentage of callus production on four different combinations and concentrations of hormones.

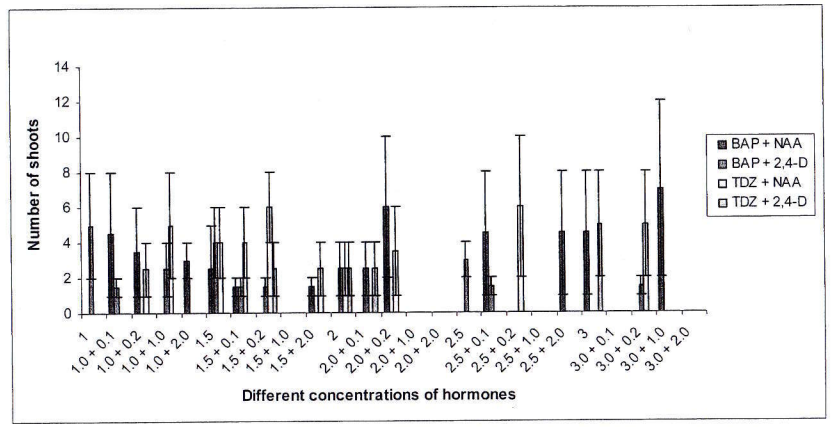

Fig. 2. Comparison of shoots regeneration in the various of combinations and concentration of hormones.

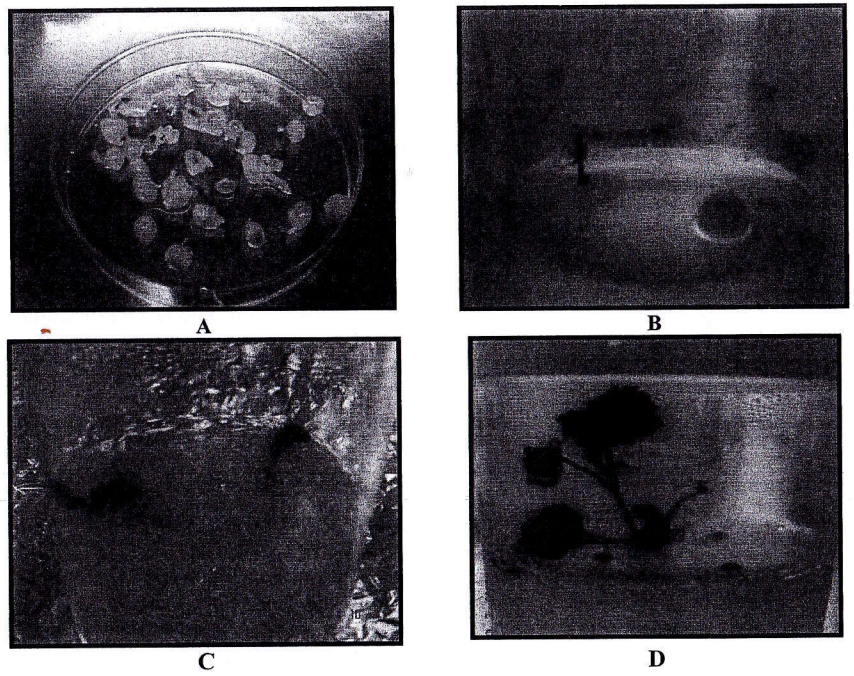

Fig. 3. Procedure of synthetic seeds. A. Synthetic seeds comprised of microshoots completely engulf by the encapsulated matrix. B. Shoots started to protrude from the bead. C. Leaf started to grow from the new shoots. D. The complete in vitro explants of broccoli produced from synthetic seeds.