



SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM HYPOCOTYL AND LEAF EXPLANTS OF *BRASSICA OLERACEA* VAR. *BOTRYTIS* (CAULIFLOWER)

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We investigated direct and indirect formation of somatic embryogenesis in *Brassica oleracea* var. *botrytis* (cauliflower), a very important vegetable crop worldwide. Direct somatic embryogenesis, which is rather rare, was achieved in culture of 2-week-old hypocotyl explants of *Brassica oleracea* var. *botrytis* on MS medium supplemented with 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5; 1.0; and 1.5 mg/l kinetin. Initial induction of embryogenic callus was achieved on MS supplemented with very low concentrations of 2,4-D (0.05 mg/l and 0.1 mg/l). Indirect somatic embryogenesis from leaf sections was obtained on MS supplemented with 0.05 or 0.1 mg/l 2,4-D. We examined various stages of somatic embryos (globular, heart, torpedo, cotyledonary). More embryos per explant were produced through the indirect pathway (23–25) than through the direct pathway (14–19). The number of embryos produced was high. There is a potential for recurrent, repeated or secondary somatic embryogenesis, possibly an unlimited source for mass propagation and ideal for synthetic seed production in this species. Plant regeneration was achieved on half-strength MS medium without any hormones.

Key words: Somatic embryogenesis, cauliflower, *Brassica oleracea* var. *botrytis*, embryogenic callus, tissue culture.

INTRODUCTION

Plant cells are totipotent, and somatic embryogenesis is evidence of totipotency (Ikeda-Iwai et al., 2003). Somatic embryogenesis is the process by which somatic cells develop into somatic embryos (Arnold et al., 2002) through characteristic embryological developments without gametic fertilization (Schumann et al., 1995). Somatic embryogenesis, with high production of regenerants, lower frequency of chimeras and low incidence of somaclonal variation (Ahloowalia, 1991), is a reliable mass propagation system in plant tissue culture. Somatic embryogenesis can be induced to occur directly or indirectly (Sharp et al., 1980) by modulating tissue culture conditions in vitro (Namasivayam, 2007). In direct somatic embryogenesis, embryos develop directly on the surface of explants; in indirect somatic embryogenesis there is an intermediary step of callus formation or cell suspension culture (William and Maheswaran, 1986). Direct or indirect somatic embryogenesis can be achieved in a plant species by manipulating the plant growth regulators and

explant types (Vikrant and Rashid, 2001; Martin and Madassery, 2005; Ali et al., 2007).

Brassica oleracea var. *botrytis*, commonly known as cauliflower, is a member of the Brassicaceae (Cruciferae) family. It is widely consumed as a vegetable and cultivated all over China and other parts of the world (Lv et al., 2005). Due to its high economic value, it has received much attention from plant biotechnologists worldwide. Genetic improvement of this species is extensively reported, including research on the breeding system (Watts, 1963), hybrid seed production (Bhalla and Nicole, 1999) and genetic transformation (Pogrebnyak et al., 2006; Lv et al., 2005; Eimert and Siegemund, 1992; David and Tempe, 1988). Several types of explants have been used as starting material to initiate in vitro culture of this species, including protoplasts (Vatsya, 1982; Jourdan et al., 1990; Frasz, 1994; Chikkala et al., 2008), leaf (Pareek and Chandra, 1978), hypocotyls (Zobayed et al., 1999; Leroy et al., 2000; Qin et al., 2006) and curd (Kieffer

Abbreviations: MS – Murashige and Skoog; 2,4-D – 2,4-dichlorophenoxy acetic acid; NAA – naphthalene acetic acid; BAP – 6-benzylaminopurine

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et al., 1995; Kieffer et al., 2001). Indirect shoot organogenesis (Qin et al., 2006; Chikkala et al., 2008) and indirect somatic embryogenesis (Pareek and Chandra, 1978; Leroy et al., 2000) of cauliflower have been studied, but we know of no reports of direct somatic embryogenesis in this species.

Here we examined the effects of different combinations and concentrations of plant growth regulators (2,4-D and kinetin) on indirect and direct somatic embryogenesis in cauliflower. We report, for the first time, the formation of somatic embryos without an intervening callus phase on hypocotyl explants derived from cauliflower seedlings. We also examined the different developmental stages of somatic embryos originated from embryogenic callus in indirect somatic embryogenesis.

MATERIALS AND METHODS

PLANT MATERIALS

This work used commercial seeds of cauliflower, *Brassica oleracea* var. *botrytis* (YMWO Corporation) purchased in Kuala Lumpur, Malaysia. They were stored at 4°C until used.

SURFACE STERILIZATION

The seeds were soaked in distilled water with 1 or 2 drops of Tween-20 for 20 min, followed by 60% (v/v) sodium hypochlorite (Chlorox) solution, gently agitated for 15 min. The seeds were then rinsed 3 times in distilled water, soaked in 70% (v/v) ethanol for 30 sec, and rinsed 3 times in sterile distilled water.

SEED GERMINATION

Surface-disinfected seeds were cultured on MS (Murashige and Skoog, 1962) basal medium (~20 ml) in sterile screw-cap bottles. MS basal medium contained 3% (w/v) sucrose and 0.88% (w/v) agar. Seeds were germinated at 25±1°C under a 16 h photoperiod (light intensity 40 µm m⁻²s⁻¹).

CULTURE MEDIUM

For all direct and indirect somatic embryogenesis, 2,4-D and kinetin at different concentrations, both singly and combined, were evaluated for their effects on indirect and direct somatic embryogenesis. 2,4-D and kinetin were dissolved in 1 M NaOH. Technical agar (0.8%, w/v) was used as solidifying agent. The pH of the medium was adjusted to 5.8 before sterilizing at 121°C and 103 kPa for 20 min. All the tissue culture media were poured into sterile screw-cap bottles (~20 ml) and stored at

25±1°C prior to use. Half-strength MS medium was used as regeneration medium for cotyledonary-stage somatic embryos.

EXPLANT PREPARATION AND CULTURE

Hypocotyls (10 mm long) and juvenile leaf segments (8 × 8 mm) derived from 2-week-old aseptic seedlings were excised and used as initial explants. The leaf surface was wounded with a scalpel before inoculation onto MS medium.

Standard tissue culture methods were used in this work. All cultures were incubated at 25±1°C under a 16 h photoperiod (light intensity 40 µm m⁻²s⁻¹). All cultures were subcultured at 2-week intervals onto fresh media.

IDENTIFICATION OF EMBRYOGENIC CALLUS

Callus (0.1 g) was placed on a glass slide and 2 or 3 drops of 2% (w/v) acetocarmine solution were dropped onto the callus. The callus was divided into small pieces and heated over a low flame for 3 sec. The slide was rinsed with distilled water to remove all liquid. Two to three drops of 0.5% (w/v) Evan's blue solution was dropped onto acetocarmine-stained cells. After 30 sec the slide was rinsed again with distilled water and all excess water was removed. One or two drops of glycerol were added to the stained cells to prevent the cells from drying.

SCANNING ELECTRON MICROSCOPIC (SEM) STUDIES

Fresh specimens of embryogenic callus were rinsed with sterile distilled water and soaked in aqueous solution of osmium tetroxide (OsO₄) at 4°C for 12 h. The specimens were then rinsed with sterile distilled water 3 times. The specimens were dehydrated sequentially in an ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, v/v), absolute ethanol and absolute acetone, 15 min for every reagent.

The specimens were further dehydrated with SPI-Dry CPD equipment. The dehydrated specimens were mounted onto aluminum stubs with conducting carbon cement (LEIT-C) and then sputter-coated with a 50 nm layer of gold (Spi-Module sputter coater). The surface micromorphology of the specimens was viewed with a JOEL JSM 6400 at 6 kV to 10 kV.

STATISTICAL ANALYSIS

All experiments followed a completely randomized design. Thirty cultures were raised for each treatment. Mean values were compared by ANOVA and Duncan's multiple range test (Duncan, 1955).

RESULTS

Generally, whitish cream and yellowish callus were observed after 2–3 weeks of culture on most of the media used. Preliminary studies had indicated that NAA and BAP induced only nonembryogenic callus which did not develop into somatic embryos. The present work showed that 2,4-D applied singly as well as in combination with kinetin was able to induce somatic embryogenesis in cauliflower. Juvenile leaf and hypocotyl explants produced callus in vitro on MS media supplemented with several concentrations of 2,4-D individually as well as combined with kinetin (Tab. 1). The part most productive of callus in cauliflower was juvenile leaf when cultured on MS supplemented with 0.05 mg/l 2,4-D. However, embryogenic callus was successfully induced only from juvenile leaf explants cultured on MS media supplemented with 0.05 mg/l and 0.1 mg/l 2,4-D; the latter concentration gave higher embryogenic callus formation. The juvenile leaf explants enlarged and callus tissue was initiated from the cut edges and the wounds on the leaf explants. The cultures were maintained at $25 \pm 1^\circ\text{C}$ under a 16 h photoperiod and subcultured every 4 weeks. Callus initiation began 2–3 weeks after inoculation and 4–6 weeks after culture establishment. Callus proliferated massively and subsequently covered the entire surface of the explants (Fig. 1a,c). The yellowish callus was later identified as embryogenic callus by double staining after the fourth week (Fig. 1b). The yellowish embryogenic callus was structurally friable (Fig. 1a,c). Scanning electron microscopy (SEM) was used to observe callus cell structure, and showed the micromorphology of the embryogenic callus surface to be nodular (Fig. 1h). Somatic embryos were observed on the callus from the sixth week onwards. Different stages of somatic embryos were observed simultaneously on the seventh week: globular (Fig. 1d), heart (Fig. 1e), torpedo (Fig. 1f) and cotyledonary (Fig. 1g). Withdrawal of 2,4-D from the media was needed for the somatic embryos to develop to maturation. Cotyledonary-stage somatic embryos were transferred to half-strength MS media and successfully converted to plantlets in the absence of 2,4-D (Fig. 3)

In this work we also evaluated combinations of 1.0 mg/l 2,4-D with different concentrations of kinetin for their effects on direct somatic embryogenesis in cauliflower. Nonmorphogenic callus was observed on hypocotyl explants cultured on MS media fortified with 1.0 mg/l 2,4-D + 0.05 mg/l kinetin as well as 1.0 mg/l 2,4-D + 0.1 mg/l kinetin. Unlike the embryogenic callus, nonmorphogenic callus was white and compact in structure (Fig. 2a). Somatic embryos formed directly on the surface of hypocotyl explants cultured on MS media with 1.0 mg/l 2,4-D + 0.5/1.0/1.5 mg/l kinetin (Tab. 1).

Adventitious somatic embryos formed directly on the hypocotyl explants without an intervening callus phase, meeting the condition of direct somatic embryogenesis. Hypocotyl explants were slightly swollen and became darkened during the first 5 weeks of culture. Initiation of somatic embryos began 4–5 weeks after inoculation, with a sparse distribution on the surface of hypocotyl explants (Fig. 2b). Six to seven weeks after culture establishment, somatic embryos proliferated extensively on the explants (Fig. 2c,d), but in these experiments we observed embryo-like structures on the hypocotyl explants, and their development ceased at the globular stage.

DISCUSSION

We found that hypocotyl and leaf explants of *Brassica oleraceae* var. *botrytis* (cauliflower) could form somatic embryos directly and indirectly when cultured on MS medium supplemented with low concentrations of 2,4-D and kinetin. In *Phyla nodiflora*, Ahmed et al. (2011) induced viable embryogenic callus on MS medium supplemented with 2,4-D and NAA with ascorbic acid; it did not occur in their control (without growth regulators). We induced abundant embryogenic callus from juvenile leaf of cauliflower on MS medium supplemented with 0.1 mg/l 2,4-D (Fig. 1a,c). Leroy et al. (2000), in contrast, found a combination of auxin and cytokinin (2,4-D and kinetin) to be effective for embryogenic callus induction in cauliflower, using hypocotyls as initial explants. Their embryogenic callus was bright green and structurally friable. Our embryogenic callus from juvenile leaf was off-white and friable. To induce direct somatic embryogenesis on hypocotyl explants of cauliflower we found it essential to add kinetin together with 2,4-D, but those somatic embryos ceased at the globular stage (Fig. 2c,d). On the other hand, the somatic embryos originated from embryogenic callus induced on 2,4-D alone developed through the globular, heart, torpedo and cotyledonary stages (Fig. 1d–g) before successfully converting to plantlets on half-strength MS. Karami (2008) found that higher concentrations of 2,4-D (2.0 mg/l) promoted embryogenic callus formation in carnation (*Dianthus caryophyllus* L.).

Indirect and direct somatic embryogenesis can both be achieved in a particular plant species by manipulating the plant growth regulators, and have been reported in *Paspalum scrobiculatum* (Vikrant and Rashid, 2001), *Quassia amara* L. (Martin and Madassery, 2005), *Phyla nodiflora* L. (Ahmed et al., 2011) and *Saccharum officinarum* (Ali et al., 2007). Recurrent, repetitive or secondary somatic embryogenesis using somatic embryos as initial explants presents a potentially unlimited source of somatic

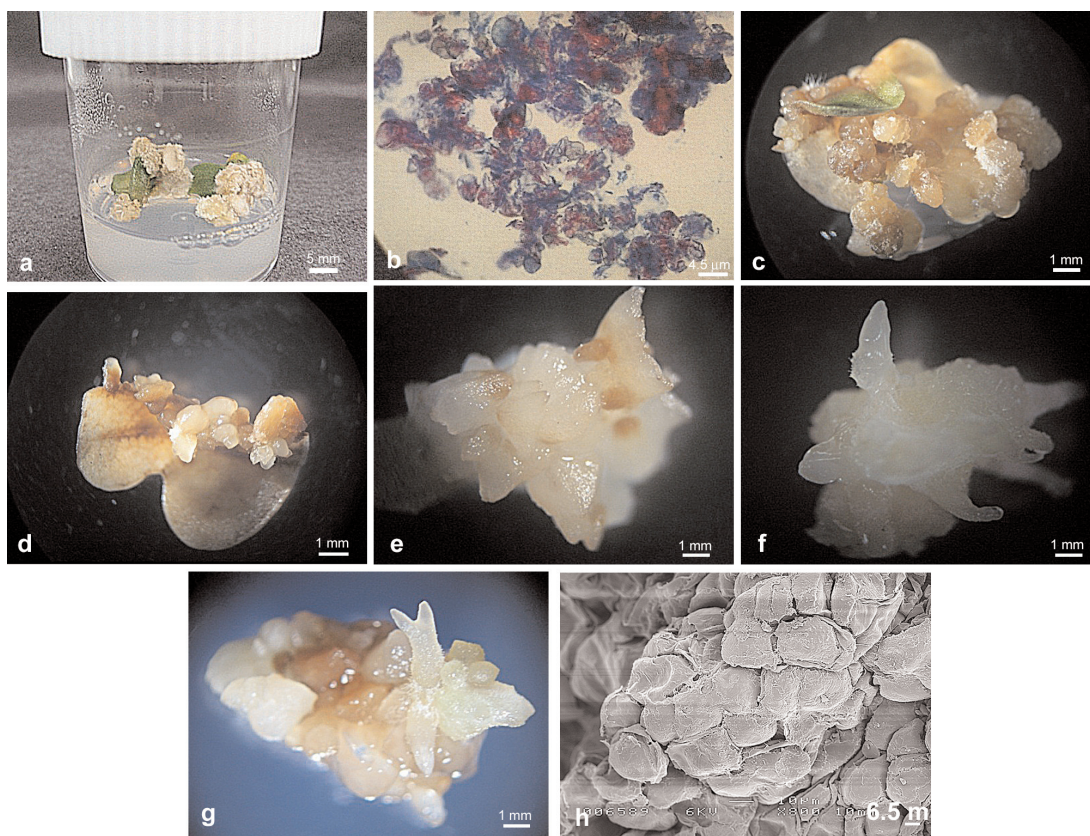


Fig. 1. Indirect somatic embryogenesis from hypocotyl explants cultured on MS medium supplemented with 0.1 mg/l 2,4-D. (a) Embryogenic callus induced on explant surface, (b) Embryogenic cells stained bright red, suspensor cells stained blue in double staining test, (c-g) Stages of somatic embryos: (c) Pre-globular, (d) Globular, (e) Heart, (f) Torpedo, (g) Cotyledonary, (h) SEM shows the micromorphology of embryogenic callus, nodular in structure.

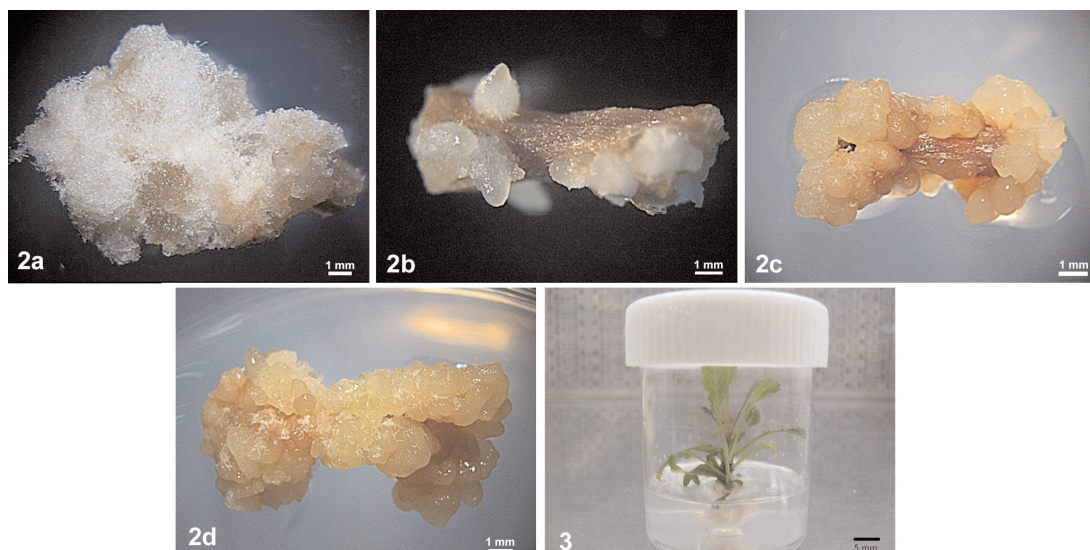


Fig. 2. Somatic embryogenesis from hypocotyl explant cultured on MS medium supplemented with 2,4-D and kinetin. (a) White and compact non-morphogenic callus formed on hypocotyl explants cultured on MS + 1.0 mg/l 2,4-D + 0.05 mg/l kinetin, (b) Somatic embryos emerged on surface of hypocotyl explant cultured on MS + 1.0 mg/l 2,4-D + 1.0 mg/l kinetin, (c) Proliferation of globular-stage somatic embryos on explant surface, (d) Somatic embryos covering the entire surface of hypocotyl explants. **Fig. 3.** Regenerated plantlet cultured on MS supplemented with 0.05 mg/l IBA after 3 weeks of incubation.

TABLE 1. Induction of indirect and direct somatic embryogenesis from hypocotyl and juvenile leaf explants of *Brassica oleracea* var. *botrytis*

2,4-D (mg/l)	Kinetin (mg/l)	Explant	Explants inducing callus (%)	Explants inducing embryogenic callus (%)	Number of embryos per explant	Mode of somatic embryogenesis
Basal medium		Hypocotyl and juvenile leaf	0	0	0	No formation
0.05	Without Kinetin	Juvenile leaf	56.7±0.6	31.1±0.5	23.3±5.6ab	Indirect
0.1			54.4±0.5	36.7±0.6	25.1±7.2a	
0.5			31.1±0.3	0	0	No formation
1.0			34.4±0.5	0	0	No formation
	0.05	Hypocotyl	32.2±0.4	0	0	No formation
	0.1		23.3±0.5	0	0	No formation
	0.5		0	0	14.3±5.7d	Direct
	1.0		0	0	19.9±8.6c	
	1.5		0	0	15.33±7.3d	

Values are means ±SE of 30 explants per treatment. Means with the same letter within columns do not differ significantly by one-way ANOVA and Duncan's multiple range test ($p < 0.05$).

embryos obtained from primary somatic embryogenesis. Recurrent somatic embryogenesis has been reported in *Dianthus caryophyllus* (Karami et al., 2007) and *Coriandrum sativum* L. (Murthy et al., 2008). Before recurrent somatic embryogenesis can be studied, an efficient and reproducible protocol for primary somatic embryogenesis must be established. Apart from providing initial explants for recurrent somatic embryogenesis, the regeneration system we have established should prove useful for transgenic studies as well as to for supplying propagules such as somatic embryos and microshoots for artificial seed production in this species.

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