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Plant Regeneration of *B. juncea* Through Plant Tissue and Protoplast Culture

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Abstract

New types of cytoplasmic male sterility in *Brassica* species would be very useful for the production of F_1 hybrid seeds. Leaves and stems of rapid cycling stock of *B. juncea* (CrGC4-3) containing Anand CMS were used as experimental materials for plant regeneration from protoplast culture. Very high plant regeneration rate (85%) was found in the Kao & Michayluk medium supplemented with 2 mg/L zeatin, 0.5 mg/L BAP, and 1 mg/L NAA when only leaf, not stem, segments were cultured. Protoplasts were isolated from leaves using mixtures of enzymes (1% Cellulycin, 0.5% Macerozyme) in 0.4 M mannitol and 50 mM $CaCl_2 \cdot 2H_2O$. Mcrocalli induced from protoplasts were transferred to the shoot regeneration medium containing 2 mg/L BAP, 2 mg/L zeatin, and 0.5 mg/L NAA. After 60 days of initial protoplast culture, regenerated plantlets were obtained, acclimatized, transplanted into the pots, and grown up to the flowering stage.

Introduction

The genus *Brassica* includes many important species such as cauliflower, broccoli, cabbage, and others being used for oil, condiment, forage, and food production (Cardi and Earle, 1997). Chinese cabbage, the major ingredient in Kimchi which is a fermented food, is the most important vegetable in Korea. In these *Brassica* species as well as in other species within the same genus, the commercial production of F_1 hybrids has gained considerable importance in the past few years. Many types of CMS were found in several *Brassica* spp. and related genera (Arumugam et al., 2000). Among these CMS sources, Ogura CMS has been most frequently used in the production of the F_1 seeds in *Brassica* vegetables (Ogura, 1968; Bannerot et al., 1977; Menczel et al., 1987; Thomas et al., 1992; Pelletier et al., 1995; Heath and Earle, 1996; Sigareva and Earle, 1997). Introduction of CMS from various sources into the *Brassica* vegetables has been done using traditional crossing experiment. But, it has not been very successful to obtain the fertile plants with CMS, since these hybrids were sterile in most cases and possessed chlorosis, being unable to make F_1 hybrids under the low temperature condition (Heath and Earle, 1996). Somatic hybridization technology using protoplast fusion methods has been widely used in *Brassica* species for the preservation and enlargement of genetic variability at the cytoplasmic level, especially providing new and improved CMS systems (Cardi and Earle, 1997).

The "Anand" cytoplasm, originally derived from *B. tournefortii* have been used as donors of nuclear and organelle genes in the protoplast fusion experiments, mainly focused on the production of somatic hybrids and cybrids with *B. nupus* (Landgren and Glimelius, 1994; Liu et al., 1995). Rapid cycling *Brassica* stocks with capacity of producing many generations per year due to their short life cycles (Williams and Hill, 1986), were developed and utilized in the production of the somatic hybrids in *Brassica* species for the exchange of valuable traits including disease resistance and CMS among species that normally

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can not be crossed sexually (Cardi and Earle, 1997; Hansen, 1998; Koh and Loh, 2000).

In this study, as the first step for the production of CMS in *Brassica* vegetables using Anand CMS system, we tested plant regeneration capability using tissue explants in stem and leaf and protoplasts of rapid cycling *B. juncea* equipped with "Anand" cytoplasmic male sterility.

Materials and Methods

Seeds of rapid cycling stocks of *B. juncea* (CrGC4-3) with Anand CMS were surface-sterilized by 1-min incubation in 70% ethyl alcohol followed by 10 min. in 50% commercial Clorox bleach. The sterilized seeds were placed on MS medium supplemented with 3% sucrose and solidified with 0.8% agar. Three-four weeks old plants grown under in vitro condition were used for the experiments.

Plant regeneration

Leaf and stem segments were used for plant regeneration experiment. Explants were cultured on MS (Murashige and Skoog, 1962) and Kao's basal medium (Kao and Michcharyluk, 1975) in combinations with plant growth regulators. Effect of different concentrations of BAP, zeatin, and NAA were examined. In each treatment, twenty explants were used in five replications. After four weeks of culture, shoot regeneration frequency was examined, and statistical analysis was performed based on PC-SAS program.

Protoplast isolation and culture

Protoplasts of *B. juncea* (CrGC4-3) was isolated from leaves of the in vitro grown seedlings for 3-4 weeks followed by dark treatment. For the protoplast isolation, leaves were cut into approximately 0.5-1 mm wide strips in a pre-plasmolysis solution containing 0.4 M Mannitol, and 0.05 M $CaCl_2 \cdot 2H_2O$. After one to three hours, the pre-plasmolysis solution was removed, and the mixtures were incubated in cell wall degrading enzymes solution [0.4 M Mannitol, 0.5% Macerozyme, 1% Cellulysin (Calbiochem-Novabiochem Corporation), and PH 5.6-5.8] for 16-20 h at 28℃, followed by shaking at 30 rpm. After incubation, the digestion mixture was filtered through 60 μm nylon sieve. The protoplasts were purified by centrifugation at 800 rpm for 10 min, and viable protoplasts were floated on the surface of the filtrate. Protoplasts were washed twice with W5 solution (18.4 g/L $CaCl_2 \cdot 2H_2O$, 9.0 g/L NaCl, 1.0 g/L glucose, 0.8 mg/L KCl, and PH 5.5-5.8), and the protoplast density was determined using a heamocytometer.

The freshly isolated protoplasts were cultured at a density of 2×10^5/mL K8P (Glimelius et al., 1986) medium supplemented with 0.2 mg/L 2,4-D, 0.5 mg/L BAP, 0.1 mg/L kinetin, and 0.1 mg/L NAA. Culture vessels containing cultured protoplasts were placed in dark at 25℃. After protoplasts began dividing, cells were transferred into semisolid 0.2% agarose (Sigma XII type) medium.

Results and Discussion

Plant regeneration

After two weeks culture of leaf and stem explants, shooting took place only on the leaf segments. For the optimum concentrations of BAP and zeatin affecting high shoot regeneration, rapid cycling clone, CrGC4-3 was tested. The highest regeneration frequency of 85% was observed when plantlets were cultured on Kaos medium supplemented with 2 mg/L zeatin, 0.5 mg/L BAP, and 1 mg/L NAA. Difference in the basal medium between Kao's and MS leads to the almost 40% difference in the shoot regeneration when other components including plant growth regulators were the same (Table 1).

As shown in this study, it was also reported that in *Brassica* species, BAP was regarded as the critical factor for shoot regeneration (Moloney et al., 1989; Hanchey et al., 1991; Ono et al., 1994; Takasaki et al., 1996; Zhang et al., 1998). In our experiment, 0.5-2 mg/L of zeatin were combined with BAP and NAA, and the highest regeneration frequency (64%), when MS was used as basal medium, was obtained in the medium supplemented with 2 mg/L zeatin, 1 mg/L BAP, and 1 mg/L NAA. This agrees with results reported by Lim et al. (1997) in that an addition of 1-2 mg/L zeatin to the combinations of BAP and NAA

Table 1. Effect of two types of basal medium and plant growth regulators on shoot regeneration

Plant Growth Regulators (mg/L)			Frequency (%)	MS	Kao's
				Frequency (%)	
Zeatin	BAP	NAA			
2.0	2.0	1.0	14	NT	NT
2.0	1.0	1.0	64	NT	NT
2.0	0.5	1.0	48	NT	NT
BAP	Zeatin	NAA			
2.0	2.0	1.0	14	NT	NT
2.0	1.0	1.0	7	64	76
2.0	0.5	1.0	2	48	85

*NT: Not Tested

stimulated the shoot regeneration to a high degree in Chinese cabbage. After two weeks of culture, regenerated shoots were differentiated from leaves. When stem tissues were used as experiment materials, however, no shoot was induced (data not shown).

Protoplast isolation and culture

The use of *in vitro* shoot cultures for obtaining year-round plant materials ensures juvenile growth and provides maximum control on the growth conditions and axenic explants materials for efficient protoplast isolation. Stability of protoplasts can be increased by cold (4-10℃) or 6-24 hours of a combination of cold/dark pretreatment as well (Féher and Dudits, 1994). We also placed plants at 4℃ for 24 h prior to protoplast isolation. Even though we did not do comparative experiments at the same time, protoplasts seemed to be more stable with pretreatment at low temperature as described by Féher and Dudits (1994). Explant types for the protoplast isolation were known to be very important (Eriksson, 1986). Since it was known that large numbers of protoplasts could be obtained from young leaves (Hansen and Earle, 1994), protoplasts were isolated from leaves of 3-4 weeks old plants for this experiment.

During enzyme treatment for the removal of cell wall and release of protoplasts, the enzyme types and their levels can influence greatly on the viability and yields of protoplasts. Protoplasts were obtained at the rate of 1.5×10^6 protoplast/g fresh weight when mixture of enzymes of 1% Cellulycin and 0.5% Macerozyme was used. The first and second cell divisions were observed in protoplasts being cultured in 2-3 days. Browning usually occurred in protoplast cultures originating from cotyledons or leaves,

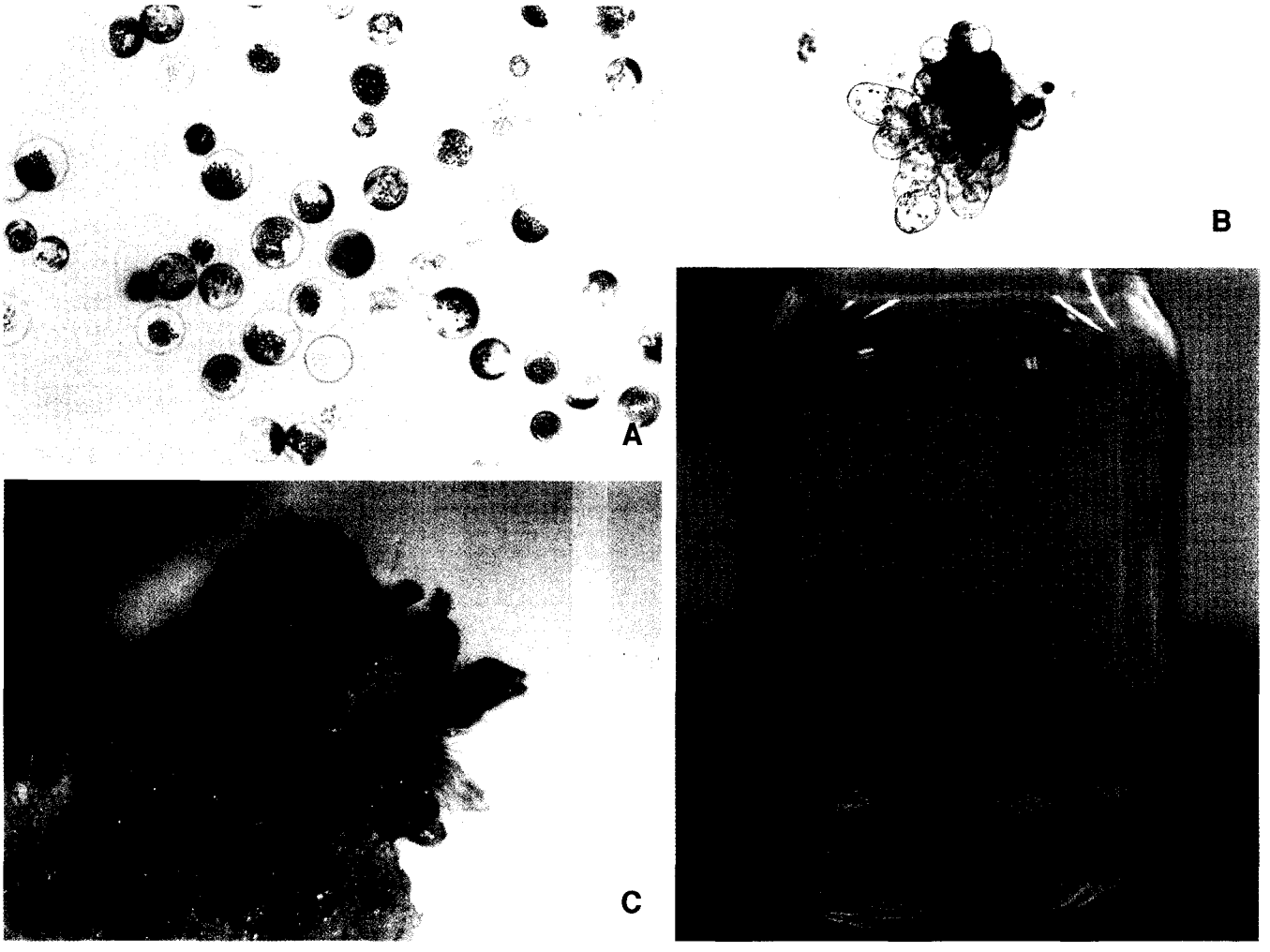

Figure 1. Plant regeneration from leaf mesophyll protoplasts. A: Isolated mesophyll protoplasts, B: Cells at division, C: Shoot regeneration from protoplasts-derived callus after 60 days of initial protoplast culture, D: Plantlets with a flower in normal morphology.

Table 2. Effect of semi-solid agarose on plating efficiency (%) protoplast culture and regeneration in *Brassica juncea.*

Culture types	Plating efficiency (%)	Formed colonies	Regeneration (%)
Liquid culture	0.02	50	25
0.2% agarose	1.1	200	43

and consequently cell growth became arrested. In this study, protoplast divided and formed colonies, which were then embedded into semisolid agarose medium to reduce browning and to improve the plating efficiency (Table 2). Browning has also been reported in mesophyll protoplasts of other *Brassica* species (Glimelius, 1984). It has been suggested that browning is due mainly to the presence of phenolic compounds produced by protoplasts, and these compounds may be toxic to cultured cells (Ulrich et al., 1980; Yamashita and Shimamoto, 1989).

After embedding of microcalli derived from protoplasts, petridishes were placed on dim light and dark conditions, respectively. The cells cultured on dim light conditions resulted in light green in color with fast growth. When calli had reached a size of 1 mm in diameter, they were transferred to regeneration medium. Macrocalli were transferred on regeneration medium supplemented with 2 mg/L BAP, 2 mg/L zeatin, and 0.5 mg/L NAA. After 60days of culture, regenerated plantlets were obtained (Figure 1). All protoplast-derived plants looked normal, and regenerated plantlets were rooted when they were transplanted into hormone-free MS medium.

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