원예과학기술지 19(3):315-319. 2001

Plant Regeneration from Cotyledon and Hypocotyl Tissues of Chinese Cabbage

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ABSTRACT The study was carried out to develop a simple and efficient system to regenerate plants from cotyledon and hypocotyl tissues of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis* cv Seoul). Among the various combinations of naphthalene acetic acid (NAA) and 6-benzyladenine (BA) tested, the best shoot induction medium for cotyledon, with 2.67 shoots per explants, contained 2.0 mg · L^{-1} NAA, 1.0 mg · L^{-1} BA and 16.7 mg · L^{-1} $AgNO_3$. The shoot induction medium with 1.0 mg · L^{-1} NAA, 5.0 mg · L^{-1} BA and 16.7 mg · L^{-1} $AgNO_3$, was best for shoot induction from hypocotyl explants, with 1.87 shoots per explants. After shoot induction, regenerated shoots were excised and rooted on rooting medium. Rooted plantlets were then hardened in the high humidity growth chamber and transplanted to pots, and then grown in the greenhouse. Regenerated plants appeared phenotypically normal and there were no changes in chromosome number.

Additional key words: *Brassica campestris* L. ssp. *pekinensis*, 6-benzyladenine (BA), chromosome number, naphthalene acetic acid (NAA), silver nitrate

Introduction

Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*), a member of the genus *Brassica*, is one of the most important vegetable for agricultural production and is widely cultivated in Asia, especially in Korea, Japan, and China.

Many members of the Brassicacea family are amenable to regenerate from cultured cells or tissue via organogenesis (Dunwell, 1981). In this family, regenerated plants have been obtained from various tissue explants: hypocotyls (Yang et al., 1991), cotyledons (Hachey et al., 1991), stems (Stringam, 1977), leaves (Radke et al., 1988), roots (Xu et al., 1982), anthers (Keller and Armstrong, 1977), microspores (Lichter, 1989) and protoplasts (Glimelius, 1984; Spangenberg et al., 1986). Organ regeneration from a variety of explants of Brassica species is largely genotype and species dependent (Dietert et al., 1982). However, Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) is known to be recalcitrant to in vitro shoot regeneration compared to other *Brassica* species (Jain et al., 1988; Murata and Orton, 1987; Narashimhulu and Chopra, 1988).

Almost all possible parameters have been investigated such as media, genotype (Jain et al., 1988), variation among different explants and the amount of growth regulators applied (Khehra and Mathias, 1992). However, the most important factor that influences in vitro organogenesis seems to be plant growth regulators. Silver nitrat is a well known inhibitor of ethylene action (Beyer, 1976) and have been shown to enhance shoot regeneration in *Brassica* (Chi and Pua, 1989; Chi et al., 1990; Sethi et al., 1990; Williams et al., 1990).

A reliable plant regeneration system is required for incorporation of agronomically useful genes via genetic transformation. One of the objectives of this study was to develop a simple, reliable and efficient system to regenerate plants from cotyledon and hypocotyl tissues of Chinese cabbage, which will be used for applying genetic engineering technology to improve Chinese cabbage cultivars. The other objectives of this study were to determine whether shoot regeneration from explants of *B. campestris* cv. Seoul was influenced by $AgNO_3$, commonly used as ethylene inhibitor, and finally to determine wherther phenotype and chromosome number of regenerants were changed.

Materials and Methods

Seeds of *Brassica campestris* ssp. *pekinensis* cv. Seoul were firstly dipped in 70% EtOH for 1 min. and rinsed three times with distilled water. Then the seeds were surface-sterilized in

※ Received for publication 1 August 2001. Accepted for publication 22 August 2001. This work was supported by grant from National Institute of Agricultural Science, Rural Development Administration and a grant, Brain Korea 21, from Ministry of Education.

0.8% sodium hypochlorite solution with 0.01% Tween 80 for 20 min. and rinsed with sterile distilled water five times. The seeds were germinated in 70×170 mm bottle containing MS basal medium (Murashige and Skoog, 1962) with vitamins, 3% sucrose, and 0.8% agar. The pH of medium was adjusted to 5.8 before autoclaving.

The effect of naphthalene acetic acid (NAA) and 6-benzyladenine (BA) of cotyledon and hypocotyl explants on shoot regeneration was examined in combinations of four levels of NAA (0, 0.5, 1.0, and 2.0 mg · L^{-1}) and four levels of BA (0, 1.0, 3.0, and 5.0 mg · L^{-1}). The effect of $AgNO_3$ treatment on shoot induction was also tested. $AgNO_3$ 16.7 mg · L^{-1} was treated to shoot induction medium containing 16 different combinations of NAA and BA and compared to non-treated medium. Cotyledons were removed to include 1−2 mm petioles from 6 days-old seedlings right before the emergence of the first leaf. Hypocotyls were cut 2−3 mm below the cotyledon, and the shoot apex and roots removed. The petri plates were cultured and kept in the condition of a 18/6 h light period and 25±2℃ temperature. Shoots induced from cotyledon and hypocotyl explants were transferred to MS agar medium with 1.0 mg · L^{-1} NAA for development of the root system.

Plates were distributed in a culture room in a completely randomized design. Data were recorded after 4 weeks for the number of shoots exceeding 5 mm in length. All experiments described above were repeated four times, and data were combined and analyzed using SAS program (SAS Insitute, 1985). F-tests were used to determine the significance of treatments and mean separations were based on least significant difference (LSD).

Rooted plantlets were rinsed in running water to remove the agar and planted peat pots containing vermiculite. Rooted plantlets were then hardened in the high humidity growth chamber and then transferred into 10 cm pots. Followed by 35 days cold treatment, regnerated plants were grown in the greenhouse for 3 months. To identify the fertility of regenerated plants, self pollination was conducted and seeds were harvested. Chromosome count was made by the Giemsa C-banding method (Ashmore and Gould, 1981) using dividing cells in actively growing root-tip of regenerated plants to analyzed ploidy level.

Results and Discussion

The effect of plant growth regulator on shoot regeneration from cotyledon or hypocotyl tissue was tested using 16 combinations of NAA and BA (Table 1 and 2). Shoots were obtained via organogenesis from cells around cut edge of the cotyledonary petioles within 4 weeks. The critical factor for shoot regen-

Table 1. Effect of NAA, BA and $AgNO_3$ on shoot induction from cotyledon explant of 'Seoul' Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*).

Plant growth regulator	Number of shoots regenerated per explant[z]							
	NAA (mg · L^{-1})							
BA	0		0.5		1.0		2.0	
(mg · L^{-1})	+[y]	-[x]	+	-	+	-	+	-
0	0.00 c[w]	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c
1	0.00 c	0.00 c	0.87 bc	0.60 ab	2.33 a	0.13 bc	2.67 a	0.33 bc
3	0.00 c	0.00 c	2.13 ab	0.27 bc	0.80 bc	0.00 c	1.40 abc	0.00 c
5	0.00 c	0.00 c	2.00 ab	0.00 c	0.80 bc	0.80 a	0.20 c	0.00 c

[z]Total number of regenerated shoots / total number of explants.
[y]The medium with $AgNO_3$ 16.7 mg · L^{-1}.
[x]The medium without $AgNO_3$.
[w]Mean separation within treatments by least significant difference, $p = 0.05$.

Table 2. Effect of NAA, BA and $AgNO_3$ on shoot induction from hypocotyl explant of 'Seoul' Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*).

Plant growth regulator	Number of shoots regenerated per explant[z]							
	NAA (mg · L^{-1})							
BA	0		0.5		1.0		2.0	
(mg · L^{-1})	+[y]	-[x]	+	-	+	-	+	-
0	0.00 c[w]	0.00 b	0.33 bc	0.00 c	0.00 c	0.00 c	0.00 c	0.00 b
1	0.00 c	0.00 b	0.00 c	0.13 a	1.27 ab	0.00 b	0.87 ab	0.00 b
3	0.00 c	0.00 b	0.00 c	0.00 b	0.13 c	0.00 b	0.53 bc	0.00 b
5	0.00 c	0.00 b	0.13 c	0.00 b	1.87 a	0.00 b	1.67 a	0.00 b

[z]Total number of regenerated shoots / total number of explants.
[y]The medium with $AgNO_3$ 16.7 mg · L^{-1}.
[x]The medium without $AgNO_3$.
[w]Mean separation within treatments by least significant difference, $p = 0.05$.

eration was the combination of BA and NAA in the medium. In the absence of BA from the medium, no induction of shoot regeneration was observed. The important role of BA for shoot differentiation in *Brassica* cotyledons has been reported previously (Moloney et al., 1989; Sharma et al., 1990; Hachey et al., 1991). On the other hand, Hachey et al. (1991) also described a requirement of NAA for the shoot regeneration from *B. campestris* cotyledons.

Our results indicated that the addition of NAA increased the frequency of shoot regeneration, callus and root formation, are consistent with results for *B. napus* (Moloney et al., 1989) and *B. juncea* (Sharma et al. 1990). The optimal concentrations of NAA and BA for shoot regeneration from cotyledon explants were 2.0 mg $\cdot L^{-1}$ and 1.0 mg $\cdot L^{-1}$, respectively (Table 1). However, 1.0 mg $\cdot L^{-1}$ NAA and 5.0 mg $\cdot L^{-1}$ BA were most effective for direct shoot induction from hypocotyl explants (Table 2).

A high frequency of shoot regeneration from cotyledon and hypocotyl explants was obtained only when $AgNO_3$ was included in culture medium. For cotyledon, the best shoot induction medium, with 2.67 shoots per explants, contained 2.0 mg $\cdot L^{-1}$ NAA, 1.0 mg $\cdot L^{-1}$ BA, and 16.7 mg $\cdot L^{-1}$ $AgNO_3$ (Table 1). The shoot induction medium with 1.0 mg $\cdot L^{-1}$ NAA, 5.0 mg $\cdot L^{-1}$ BA, and 16.7 mg $\cdot L^{-1}$ $AgNO_3$, was best for shoot induction from hypocotyl explants, with 1.87 shoots per explants (Table 2). $AgNO_3$ is another important factor in determining the rate of shoot regeneration. Silver nitrat is a well known inhibitor of ethylene action (Beyer, 1976) and since ethylene inhibits auxin transport, one of its functions may be to allow polar auxin transport to the petriole base where regeneration occurs. De Block et al., (1989) also reported that $AgNO_3$ was absolutely required for shoot recovery from transformed *Barassica napus* explants.

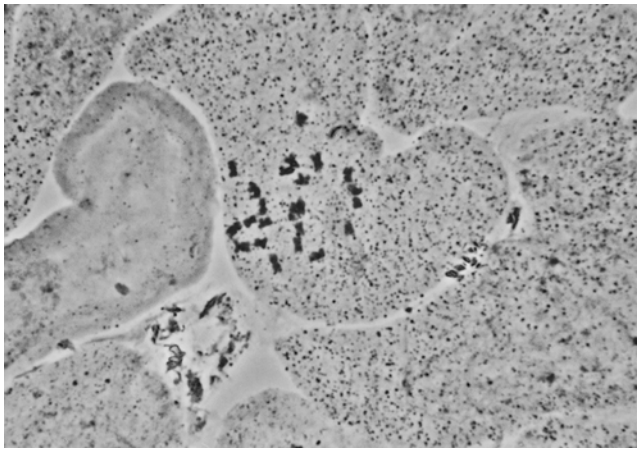

Fig. 2. Light microscope (1600x) photograph of choromosome from root tip of 'Seoul' Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*).

Although shoot regeneration can be achieved with a fair degree

Fig. 1. Major stages in Chinese cabbage regeneration. A: Shoot induction from cotyledon explants in shoot induction medium; B: Regenerated shoots rooted in rooting medium with 1.0 mg $\cdot L^{-1}$ NAA; C: Regenerated plant grown in the greenhouse for 50 days; D: Mature regenerants with flowers.

of reproducibility in some *Brassica* species, this is not the case with others and reports indicates that the C genome carries genes for shoot regeneration while the A and B genomes are inhibitory (Murata and Orton, 1987; Narasimhulu and Chopra, 1988). Chinese cabbage carries the A genome and is one of the most recalcitrant species in regeneration experiments, although good responses are obtained from some genotypes (Palmer, 1992).

Regenerated shoots were transferred to rooting media with various NAA concentrations (none, 0.5, 1.0, 2.0 and 4.0 mg · L^{-1}). Although root formation was found in growth regulator free medium, the presence of NAA was effective in promoting root formation. The presence of NAA in the medium caused the concurrent formation of roots along with shoots from the explants. The highest root production was recorded in medium containing 1.0 mg · L^{-1} NAA, where all shoots formed roots within 2 weeks (data not shown). However, in MS medium containing 0.5 and 2.0 mg · L^{-1} NAA, where also effective for root formation, the root system was qualitatively poorer than grown in 1.0 mg · L^{-1} NAA medium (data not shown).

A part of the population of rooted shoots was transferred to vermiculite, then grown in soil. These regenerated plants were not morphologically different from the source plants. Regenerated plants produced normal flowers and seeds after self-pollination (Fig. 1).

Chromosome counts by Feulgen staining demonstrated that in vitro regenerated plants had diploid (2n=20) state (Fig. 2). There were no changes in chromosome number in regenerants from cotyledon and hypocotyl explants.

Hermsen et al. (1981) reported that phenotypic variation observed in explant-derived shoots of diploids mainly concerned changes in the ploidy level and morphological changes which were probably the result of mutated genes or deletions. However, in this experiment regenerated plants showed stability of chromosome number and did not show phenotypic variation.

In this paper, we reported a simple and efficient system to regenerate plant from cotyledon and hypocotyl explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis* cv. Seoul). Plantlet regenerations from cotyledon and hypocotyl explants were dependent on the composition of plant growth regulators in the medium employed and the explants used. Silver nitrate has also been demonstrated to enhance shoot regeneration.

초 록

배추의 자엽과 배축 절편체로부터의 식물체 재분화

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본 연구는 배추(*Brassica campestris* L. ssp. *pekinensis*)의 자엽 및 하배축 조직으로부터 효율적인 식물체 재분화 체계를 확립하기 위하여 실시하였다. 다양한 조합의 식물생장조절제 NAA와 BA를 조사한 바 자엽은 NAA 2.0mg/L, BA 1.0mg/L와 $AgNO_3$ 16.7 mg/L를 첨가한 배지에서 절편당 2.67개의 가장 좋은 신초발생을 보였다. 하배축 절편체로부터는 NAA 1.0mg/L, BA 5.0mg/L와 $AgNO_3$ 16.7mg/L를 첨가하였을 때 절편당 1.87개의 가장 좋은 신초발생을 보였다. 재분화된 신초는 발근배지에서 뿌리를 유기하였으며 식물체는 습도가 유지된 생장상에서 순화를 거쳐 온실에서 재배하였다. 재분화 개체는 표현형과 염색체수에서는 이상이 없는 것으로 나타났다.

추가 주요어 : 6-benzyladenine (BA), *Brassica campestris* L. ssp. *pekinensis*, 엽색체 수, naphthalene acetic acid (NAA), silver nitrate

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