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An Improved Plant Regeneration Protocol using Cotyledonary Explants from Inbred Lines of Chinese Cabbage (*Brassica rapa* ssp. *pekinensis*)

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Abstract

We studied the effect of genotype, explant, age of explant, medium (plant growth regulators and gelling agents), and standardized an efficient regeneration protocol for inbred lines of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). Of the different concentrations of NAA and BA tested, the combination of 5 mg/L BA and 0.5 mg/L NAA gave the highest frequency of shoot regeneration. The cotyledonary explants had more shoot regeneration frequency (≥ 40%) than the hypocotyl explants. Besides, the cotyledonary explants, excised from the 4-day old seedlings, showed higher shoot regeneration (56.7%). Of the three gelling agents and their concentrations used, 16 g/L agar was found to be the best for shoot regeneration. Shoot regeneration frequency increased significantly by supplementing the medium with 4 mg/L of $AgNO_3$. MS medium devoid of NAA showed higher frequency of rooting in the regenerated shoots than the ones supplemented with NAA. Our improved regeneration protocol will be especially useful for the genetic transformation of Chinese cabbage inbred lines to develop transgenic hybrids.

Key words: Chinese cabbage, *Brassica rapa* ssp. *pekinensis*, explants, shoot regeneration

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Introduction

The genus *Brassica* is one of the genera in the subtribe Brassicinae and includes a number of widely cultivated crops. Of the different cultivated Brassica species, *B. rapa* consists of a number of vegetables such as *Pak-choi*, Chinese cabbage, etc. Chinese cabbage (*B. rapa* ssp. *pekinensis*) is one of the most widely used vegetable crops as dried, pickled or cooked. Genetic improvement of crop plants including Chinese cabbage has been confined to conventional plant breeding strategies including development of F_1 hybrids. However, recent techniques in plant genetic engineering have opened new avenues for the crop improvement by developing transgenics. Towards this endeavor, an efficient plant regeneration system is crucial. The efficacy of developing transgenics using *Agrobacterium*-mediated transformation is dependent on the frequency of infection by *Agrobacterium* as well as regeneration from the infected tissues (Zhang et al. 1998). In dicots, *Agrobacterium*-mediated genetic transformation is relatively easier than in monocots as the former have the ability to induce signal for *Agrobacterium* infection. However, it is relatively difficult to develop transgenics by *Agrobacterium*-mediated transformation in Chinese cabbage. Though there are a few reports of development of transgenics in Chinese cabbage (Christey et al. 1997; Cho et al. 2001, Cho et al. 2003), the frequency of transformation is very low.

In comparison to other *Brassica* species, *B. rapa* has since been known to be recalcitrant to tissue culture (Murat and Orton 1987). This species has the lowest regeneration

frequency among the three basic species as well as their amphidiploids (Narsimhulu and Chopra 1988). A number of studies have been carried out to increase the regeneration frequency of B. rapa and remarkable progress has been achieved. Using 4-day old cotyledons with petioles as explants, Hachey et al. (1991) reported 70% shoot regeneration frequency in oleiferous *B. rapa*. Zhang et al. (1998) standardized a protocol in *B. rapa* ssp. *pekinensis* using open-pollinating population and obtained a maximum regeneration frequency of 53.3%. However, there is a need of increasing the regeneration frequency of the inbred lines of Chinese cabbage. In order to develop transgenic hybrid cultivars, it is important to transform the inbred lines than plant from open-pollinating population. Moreover, a major limitation encountered in the *Agrobacterium*-mediated transformation of *Brassica* species .was the poor shoot regeneration potential from explants (Moloney et al. 1989).

Considering the above, this study was carried out to develop an efficient plant regeneration system in two diverse inbred lines of *B. rapa* ssp. *pekinensis* with the goal to increase the efficiency of *Agrobacterium*-mediated genetic transformation.

Materials and Methods

Culture method

Two inbred lines of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) 'Chiifu' and 'Kenshin' were used as the experiment material. Seeds of these lines were surface sterilized with 70% ethanol for 1 min, followed by rinsing in sterilized distilled water for 1 min. The seeds were then treated with 2% and 1% sodium hypochlorite solution for 20 min each, followed by thorough washing with sterilized distilled water thrice, each for 5 min. Seeds were then cultured on MS medium (Murashige and Skoog 1962) solidified with 2 g/L phytogel until cotyledons were fully expanded.

Cotyledons and hypocotyls were excised from 2 to 5 days old seedlings and cultured on the regeneration medium. After 20 days of culture, the regenerated shoots were counted. These shoots were transferred to rooting medium. All cultures were incubated at 25℃ under a 16/8 h day/night photoperiod of 30 μmol/m^2/s illumination. Shoot regenerants were allowed to root and acclimated before transferring to field.

Phytohormones

The effect of phytohormones on shoot regeneration was studied using hypocotyl and cotyledonary explants from 4 to 5 days old seedlings of inbred lines, 'Chiifu' and 'Kenshin'. To optimize the concentration of phytohormones, the explants were cultured on MS medium containing 8 g/L agar. The medium was supplemented with different concentrations of BA (0-10 mg/L) and NAA (0-5 mg/L) for hypocotyls explants. However, for cotyledonary explants, concentrations of BA and NAA were 0-5 mg/L and 0-1.0 mg/L, respectively. pH of all the media was adjusted to 5.8. The regenerated shoots were counted on the 20th day after culturing.

Age of explants

Cotyledonary explants form 2, 3, 4 and 5 days old, *in vitro* cultured, seedlings of 'Kenshin' were transferred to MS medium supplemented with 5 mg/L BA, 0.5 mg/L NAA and 8 g/L agar at pH 5.8. Data on regeneration of shoots were recorded on 20th day of culturing.

Gelling agents

Cotyledonary explants from 4-days old seedlings of 'Kenshin' were cultured on the above described medium containing different concentrations of gelling agents, *viz.*, agar (Duchefa) (8, 16 and 32 g/L), phytogel (Sigma) (2, 4 and 6 g/L) and agarose (Sigma) (5, 10 and 15 g/L). Data on number of shoots regenerated were recorded on the 20th day after culturing.

$AgNO_3$

Cotyledonary explants from 4-days old seedlings of 'Kenshin' were cultured on the above described medium containing 16 g/L of agar. The medium was supplemented with different concentrations of $AgNO_3$ (0-8 mg/L). The number of shoots regenerated was recorded on the 20th day after culturing

Rooting

Regenerated shoots were transferred to MS medium supplemented with 0.0, 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/L of NAA. The number of regenerated shoots with roots was recorded after one month of culture.

Acclimation

The rooted plantlets were transferred to pots and covered with polyethylene bags with small holes for one week in growth chamber and then the bags were removed. After two weeks of acclimation, the pots were transferred to glass house.

Results and Discussion

The data on the effect of different combinations of BA and NAA concentrations on shoot regeneration from hypocotyl explants in the two inbred lines of Chinese cabbage are given in Table 1. The highest shoot regeneration frequency (10.0%) from the hypocotyl explants of 'Chiifu' was observed in combination of 8 mg/L BA and 1 mg/L of NAA. However, this was at par with the combinations of 8 mg/L BA and 2 mg/L, NAA and 10 mg/L BA with 1 and 2 mg/L NAA. The combination of 6.0 mg/L of BA with 1 mg/L of NAA showed regeneration frequency of 2.5%. There was no regeneration in the remaining combinations. With one exception, the above combinations of BA and NAA were also effective in shoot regeneration from hypocotyls of 'Kenshi n', and the maximum frequency (12.0%) was observed in the combination of 10 mg of BA and 1 mg/L NAA, which in turn was at par with 8 mg/L of BA and 1.0 mg/L NAA.

Data on the effect of different BA and NAA concentrations on shoot regeneration from the cotyledonary explants in the two inbred lines of Chinese cabbage are presented in Table 2. A perusal of this data, in conjunction with that in Table 1, reveals that shoot regeneration frequencies from the cotyledonary explants were much higher than those from hypocotyl explants. Recently, in a study on shoot regeneration in *Brassica* species including *B. rapa*, Tang et al. (2003) used four explants and reported the highest frequency of shoot regeration from cotyledons. The combination of 5 mg/L of BA and 0.5 mg/L of NAA resulted in the highest frequencies of shoot regeneration in 'Chiifu' (42.0%) and 'Kenshin' (40.0%). In the absence of either BA or NAA, no shoot regeneration was observed in both the inbred lines, implying that these hormones are critical in shoot regeneration in Chinese cabbage. Moreover, there

Table 1. Effect of different BA and NAA concentrations on shoot regeneration from hypocotyls of two inbred lines

BA and NAA combinations		Shoot regeneration frequency (%)	
BA (mg/L)	NAA (mg/L)	Chiifu	Kenshin
4	0	0	0
	1	0	2.5±0 b
	2	0	0
	3	0	0
	4	0	0
	5	0	0
6	0	0	0
	1	2.5±0 a	2.5±0 b
	2	0	2.5±0 b
	3	0	0
	4	0	0
	5	0	0
8	0	0	0
	1	10.0±3.5 a	10.0±0 a
	2	7.5±0 a	7.5±3.5 b
	3	0	0
	4	0	0
	5	0	0
10	0	0	0
	1	7.5±0 a	12.0±1.8 a
	2	5.0±3.5 a	8.0±1.8 b
	3	0	0
	4	0	0
	5	0	0

Data based on 2 duplicates, each comprising 40 plants. The mean values were compared by DMRT. Mean±SD followed by same superscript alphabet are statistically same at $P \leq 0.05$.

Table 2. Effect of different BA and NAA concentrations on shoot regeneration from cotyledonary explants of two inbred line

Different BA and NAA combinations		Shoot regeneration frequency (%)	
BA concentration (mg/L)	NAA concentration (mg/L)	Chiifu	Kenshin
0	0	0	0
	0.1	0	0
	0.3	0	0
	0.5	0	0
	1	0	0
1	0	0	0
	0.1	0	0
	0.3	15.0±1.4 e	16.0±0.4 c
	0.5	0	0
	1	0	0
3	0	0	0
	0.1	0	0
	0.3	30.0±0 cd	34.0±4.9 a
	0.5	17.0±0.7 e	0
	1	35.0±0.7 bc	0
5	0	0	0
	0.1	27.0±2.1 b	22.0±0.7 bc
	0.3	37.0±1.4 ab	32.0±2.8 ab
	0.5	42.0±2.1 a	40.0±1.4 a
	1	0	0

Data based on 2 duplicates, each comprising 100 plants. The mean values were compared by DMRT. Mean±SD followed by same superscript alphabet are statistically same at $P \leq 0.05$.

was no response of BA upto 2 mg/L in combination with 1-5 mg/L NAA on shoot regeneration from hypocotyl explants (data not presented).

Earlier workers also underlined the importance of both BA and NAA on shoot regeneration in Chinese cabbage (Takashi et al. 1996; Zhang et al.1998). The combination of 5 mg/L of BA and 0.5 mg/L NAA, as observed in the present study, has earlier been reported to be the best for shoot regeneration from the cotyledonary explants of Chinese cabbage (Zhang et al. 1998). Hachey et al. (1991) reported that combination of 2 mg/L BA and 1 mg/L NAA was optimum for shoot regeneration in *B. campestris* (oil type). The optimal concentration of BA and NAA appears to be dependent upon the genotype. Zhang et al. (1998) reported that shoot regeneration frequency among 123 genotypes of Chinese cabbage varied from 95% to 0%.

The other parameters included in the regeneration protocol, *viz.*, age of explant, influence of gelling agents and silver nitrate, and effect of NAA on root induction in the regenerated shoots were studied in 'Kenshin'. Data on the effect of age of seedling at the time of cotyledonary explant excision, presented in Figure 1, reveal that cotyledonary explants excised from the 4-day old seedlings had the maximum shoot regeneration frequency (56.7%), whereas those excised from 2-day old seedlings had the minimum (3.3%). Of the three gelling agents along with their concentrations used, 16 g/L agar was the most responsive to enhance shoot regeneration from the cotyledonary explants of inbred line 'Kenshin' (Figure 2).

The effect of concentration of $AgNO_3$ on the shoot regeneration from the cotyledonary explants is shown in Table 3. The addition of 4 mg/L of $AgNO_3$ to the shoot regeneration medium gave the highest frequency of shoot regeneration (18.0%). In general, *in vitro* morphogenesis is inhibited by ethylene in plants. It is reported that Ag^+ inhibits ethylene action by competitively binding to ethylene receptors (Beyer 1976). The addition of ethylene inhibitors, such as $AgNO_3$ and AVG (aminoethoxyvinylglycine) are reported to improve shoot regeneration in *B. rapa* (Chi et al. 1990; Burnett et al. 1994). Chi et al. (1990) reported that $AgNO_3$ enhanced ACC (1-aminocyclopropane-1-carboxylic acid synthase) activity leading to enhanced shoot production.

In some instances, the regenerated shoots had scanty underdeveloped roots, which were inadequate for survival of the plant when transferred to soil. Therefore, the regenerated shoots were subjected to root induction. The regenerated shoots, when grown in medium containing 5 concentrations (0.25 to 2.0 mg/L) of NAA for rooting, the frequency of rooted plants was the highest (90.0%) in the medium devoid of NAA. With increase in the concentration of NAA, there was progressive decline in the frequency of rooted plants, which was minimum in 2 mg/L NAA. Rooted plants were acclimatized by transferring them in pots covered with polyethylene bags containing small holes and maintaining high humidity. More than 70% of these plants survived and were transferred to glass house. These plants were morphologically similar to the parental plants.

The highly efficient regeneration system for Chinese cabbage inbred lines, reported herein, is being employed in

Table 3. Effect of $AgNO_3$ on shoot regeneration from cotyledonary explants of inbred line 'Kenshin'

$AgNO_3$ (mg/L)	Regeneration frequency
0	15.5±0.76 b
2	16.5±0.71 ab
4	18.0±0 a
6	16.0±0 ab
8	15.5±0.78 b

Data based on 2 duplicates, each comprising 40 plants. The mean values were compared by DMRT. Mean±SD followed by same superscript alphabet are statistically same at $P \leq 0.05$.

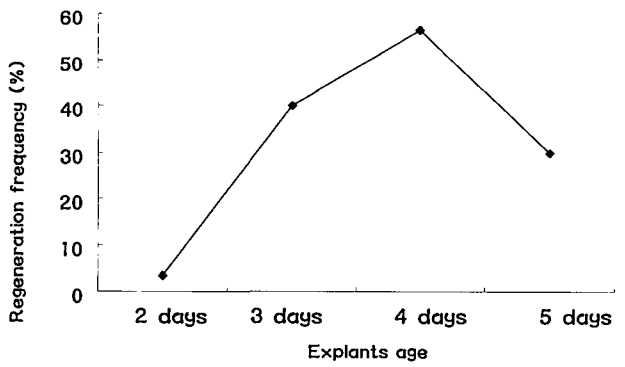

Figure 1. Effect of seeding age on shoot regeneration from cotyledonary explants of inbred line kenshin

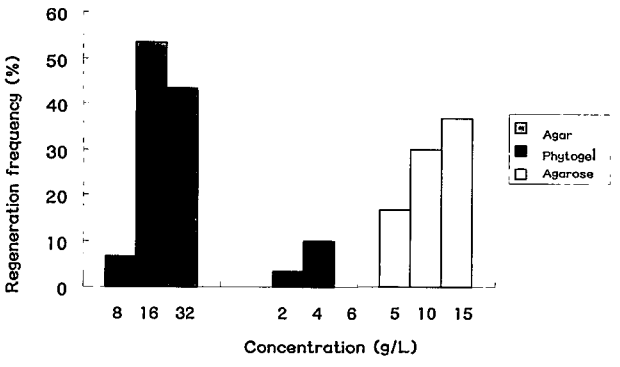

Figure 2. The influence of gelling agents on shoots regeneration frequency of inbred line kenshin from cotyledonary explants

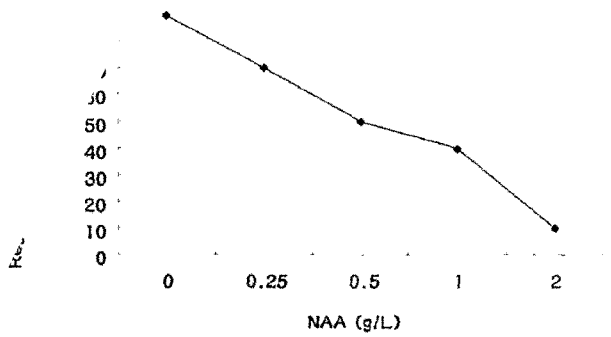

Figure 3. The influence of different NAA concentration on rooting of regenerated shoot s from cotyledonary explants of inbred line Kenshin

Agrobacterium-mediated transformation with the ultimate objective to develop transgenic hybrid cultivars of Chinese cabbage.

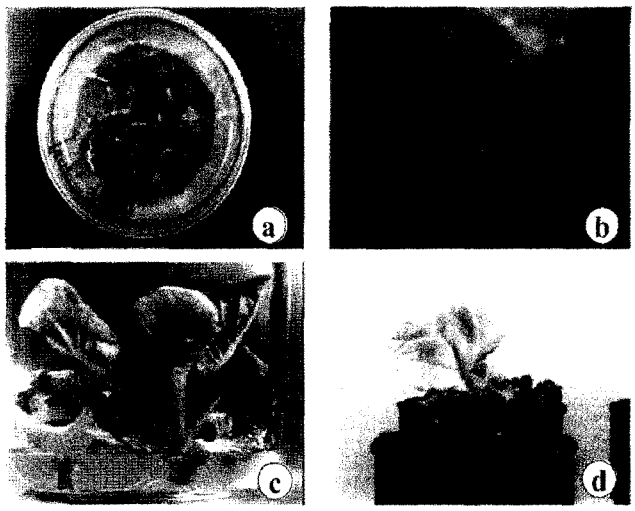

Figure 4. Stages of in vitro plantlet regeneration in Chinese cabbage, a: Regeneration of shoots from cotyledonary explants, b: Magnified view of regenerating shoots, c: Regenerated shoots in root induction medium, d: Plants transferred to pots after acclimation

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