식물생명공학회지 제36권 제1호
J Plant Biotechnol
Vol. 36, No. 1, 81-86 (2009)

High frequency plant regeneration from transverse thin cell layers in Indian mustard (*Brassica juncea* L.)

Mohammed Shafi Ullah Bhuiyan[1] · Yong Pyo Lim[2] · Sung Ran Min[3] · Kwan Sam Choi[1] · Jang R. Liu[3*]

[1]*Plant Physiology and Cell Biotechnology Laboratory, Department of Agricultural Biology, Chungnam National University, Daejeon 305-764, Korea*

[2]*Department of Horticulture, Chungnam National University, Daejeon 305-764, Korea*

[3]*Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea*

ABSTRACT An efficient and reproducible plant regeneration system was established using transverse thin cell layers (tTCLs) in five cultivars of *Brassica juncea* L. The effects of medium conditions, explant types (tTCLs of hypcotyl and cotyledonary petiole) on shoot regeneration were examined in this study. The maximum shoot regeneration frequency was obtained in Murashige and Skoog (MS) medium supplemented with 4 mg/L 6-benzylaminopurine (BA) and 0.2 mg/L 1-naphthaleneacetic acid (NAA). The hypocotyls derived tTCL explants had more shoot regeneration frequency (52%) than the cotyledonary petiole derived tTCL explants. Shoot induction was further improved by the addition of silver nitrate ($AgNO_3$) in the regeneration medium. A significant genotypic effect was also observed between the five cultivars; Rai-5 displayed higher capacities to produce shoots than other cultivars. Regenerated shoots were rooted on MS basal medium without PGRs which induced 90% of roots. The plantlets established in greenhouse conditions with 99% survival, flowered normally and set seeds. The regenerated plants were fertile and identical to source plants.

Introduction

Indian mustard (*Brassica juncea* L.) is an important oilseed crop for the South East Asian region. Its cultivation has increased tremendously during the last decade and, by now, it is a significant contributor to the world supply of vegetable oil and biofuels. In addition, it is able to produce high biomass with added economic value. Hence, increasing its production is one of the most important challenges. To this effect, researchers focused on the genetic improvements. For *B. juncea*, it is known that shoot regeneration largely depends on genotype, explant source, medium, growth hormones and physical conditions (Jain et al. 1988; Zhang et al. 1998; Tang et al. 2003; Guo et al. 2005). Different explants of *B. juncea* has been used for in vitro shoot regeneration such as mesophyll protoplasts (Chatterjee et al. 1985), hypocotyls (Sethi et al. 1990), cotyledon or leaf segments (Guo et al. 2005), petioles (Pua and Chi 1993), peduncle (Eapen and George 1996, 1997), and microspores (Prem et al. 2005). Recently, Ghnaya et al. (2008) reported shoot regeneration from transverse thin cell layer (tTCL) explants in *Brassica napus*. However, the use of transverse thin cell layer (tTCL) of hypocotyls or cotyledonary petioles for shoot regeneration in *B. juncea* has never been described which could be a model system in plant tissue culture and genetic transformation in other *Brassica* species.

The present work describes an efficient and high frequency shoot regeneration system using transverse thin cell layers (tTCLs) in *B. juncea* L. A series of experiments were carried out to investigate the influence of culture media, explant types and genotype on shoot regeneration in *B. juncea* L.

*Corresponding author Tel 042-860-4430 Fax 042-860-4608
E-mail: jrliu@kribb.re.kr

Materials and methods

Plant material

Five *Brassica juncea* L. cultivars (Bangladeshi) were used to evaluate shoot regeneration: BARI sarisha-10, BARI sarisha-11, Daulot, Rai-5, and Shambol. These five different cultivars were pure lines, genetically fixed, and were obtained by autofertilization.

Origin and preparation of tTCL explants

Seeds were surface-sterilized by immersion for 30 s in 70% ethanol (v/v in water), 10 min in 10% Chlorox (v/v in water), followed by three times 3-min rinses in sterile distilled water. The seeds were then germinated in plant culture dishes (100 × 40 mm) on MS basal solid medium containing Murashige and Skoog (1962) salts and vitamins (Duchefa Biochemie, Netherlands) and 3% (w/v) sucrose and incubated under a photoperiod of 16 h (40 μmol photon. $m^{-2}.s^{-1}$) provided by cool white fluorescent lamps with a 24/22℃ thermoperiod (light/dark). Explants (tTCLs of 0.3 - 0.5 mm) were excised from hypocotyls and cotyledonary petioles of 7-days old seedlings and placed in contact with the medium (25 ml) in 90 x 15 mm Petri dishes (50 tTCLs for each treatment). The experiment was repeated three times for each treatment.

Culture medium

tTCLs of hypocotyl and cotyledonary petiole were cultured on MS medium (comprising macronutrients, micronutrients and vitamins of Murashige and Skoog 1962) supplemented with different concentrations of BA (1 - 6 mg/L), NAA (0 - 0.4 mg/L) and 30 g/L sucrose. All media were solidified with phytoagar (6 g/L), adjusted to pH 5.7 by 0.1 N NaOH and sterilized by autoclaving at 121℃ and 1 kg cm^{-2} for 15 min.

Culture conditions

The cultures were incubated in the same conditions as previously described. The number of explants with shoot buds was scored after 3 weeks of culture and the adventitious shoots formed per explant were counted. Regenerated shoot buds were sub-cultured on MS (MS salts and vitamins) medium without growth regulator in culture dish for shoot elongation and root formation. Plantlets with well-developed roots were removed from the culture dish, washed under running tap water and transferred to pots containing soil mixture. The rooted shoots were acclimatized under a 16 h photoperiod at 40 μmol photon.$m^{-2}\cdot s^{-1}$ provided by cool white fluorescent lamps with a 24/22℃ thermoperiod (light/dark). Plastic bags with small holes were placed over the plants to maintain humidity and watered daily during the first week. Then acclimatized plants were transferred to a naturally-lighted greenhouse. The acclimatization experiments were repeated several times to check the capacity for normal flowering and fertility.

Statistical analysis

The number of explants that showed shoot formation, as well as the number of shoots per tTCL explant were counted and the frequency of shoot regeneration was calculated. All statistical analyses were done using SAS version 9.1 (SAS Institute, Carey NC, USA) and the differences among means (5% level of significance) were tested by the Dunkan's Multiple Range Test (DMRT).

Results

The optimum medium for shoot regeneration from tTCLs in *B. juncea* cv. Rai-5 was determined using various combinations of BA and NAA. The tTCLs explants (Fig. 3a) were prepared from hypocotyls and cotyledonary petioles of 7-days old axenic seedlings grown on MS basal media. Typically, the explants had swollen after 3-4 days of culture. Sometimes, a small amount of light-green callus proliferation was observed through the light microscope on the subepidermal area. Shoot regeneration occurred from tTCL explants that appeared green and formed a peripheral crown of buds after 12-14 days (Fig. 3b). Non-regenerating explants showed either rhizogenesis only or browning and necrosis after 3-7 days of culture. Results indicate that shoot regeneration ability is strongly influenced by plant growth regulators (PGRs). MS medium supplemented with BA and NAA enhanced shoot regeneration ability, while BA alone produced smaller rate of

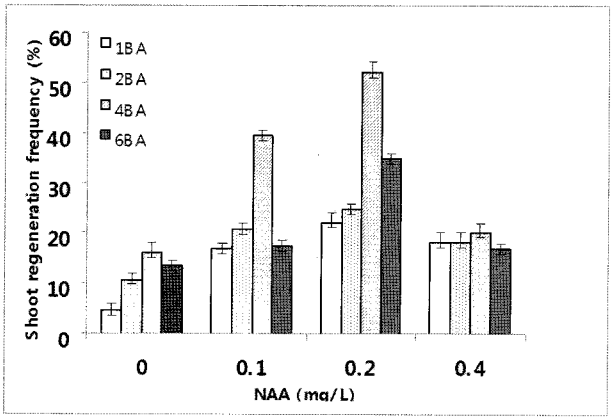

Figure 1. Effect of different combinations of PGRs (BA and NAA) on shoot regeneration from tTCL explants of hypocotyl in *Brassica juncea* cv. Rai-5. Data consist of three replicates and 50 explants were used for each replicate. *Vertical* bars represent standard deviation (SD) of the means

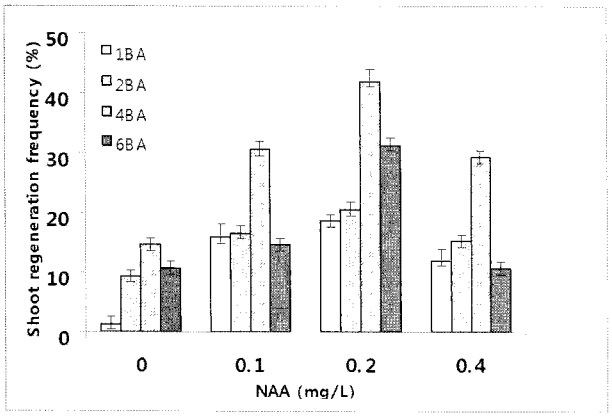

Figure 2. Effect of different combinations of PGRs (BA and NAA) on shoot regeneration from tTCL explants of cotyledonary petiole in *Brassica juncea* cv. Rai-5. Data consist of three replicates and 50 explants were used for each replicate. *Vertical bars* represent standard deviation (SD) of the means

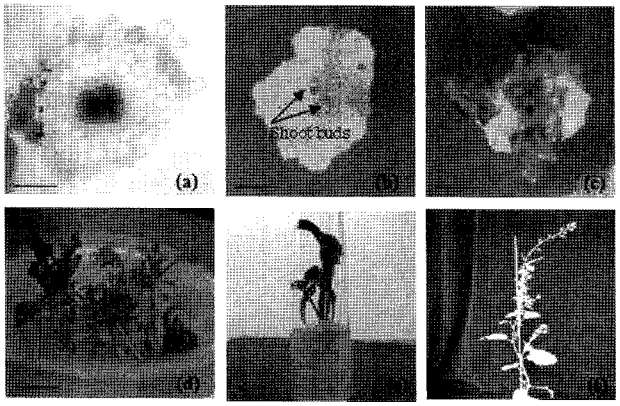

Figure 3. Plant regeneration from hypocotyls derived tTCL explants of *Brassica juncea* L. cv. Rai-5. (a) tTCL explants from hypocotyls of 7-days old seedlings, (b) callus and shoot bud initials induction from tTCL explant after 14 days of culture in MS medium supplemented with 4 mg/L BA, 0.2 mg/L NAA and 2.0 mg/L $AgNO_3$, (c) multiple shoots after 21 days of culture (d) shoot elongation on MS basal medium without any PGRs, (e) rooted plantlet on MS basal medium, (f) flowering of regenerated plant. *Scale bars* represent 50 μm (**a**), 50 mm (**b, c**), 2 cm (**d**), 3 cm (**e**) and 15 cm (**f**)

shoot regeneration (Fig. 1 & 2). Explants on MS media without PGRs had not produced any callus or shoots and become brown and died. The highest rate of shoot organogenesis 52% was obtained in MS medium supplemented with 4 mg/L BA and 0.2 mg/L NAA from hypocotyl derived tTCLs. On this medium, each explant regenerated 2.7 shoots (data not shown). The shoot regeneration rate was lowest (4.67%) in medium containing 1 mg/L BA only. The origin of tTCL explants also affected shoot regeneration, hypocotyl derived tTCLs best responded compared to cotyledonay petiole derived tTCLs. In case of cotyledonary petiole derived tTCLs explants, the highest

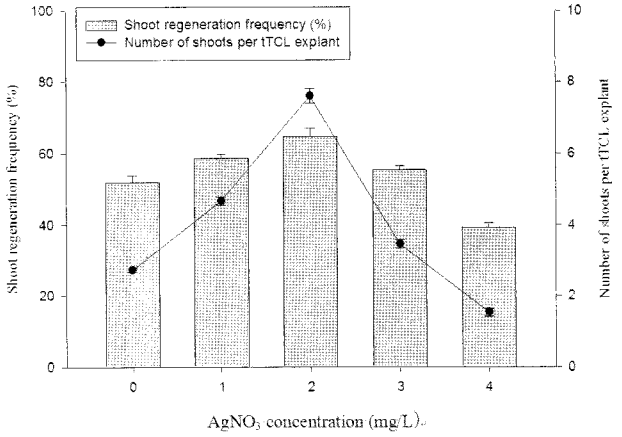

Figure 4. Effect of $AgNO_3$ concentration on shoot regeneration from tTCL explants of hypocotyl in *B. juncea* cv. Rai-5. Data consist of three replicates and 50 explants were used for each replicate. *Bar* represents SD of the means

frequency of shoot regeneration (42%) was also observed in MS medium supplemented with 4 mg/L BA and 0.2 mg/L NAA. Based on these results, this medium was chosen for investigating the effect of $AgNO_3$ on shoot regeneration. After adding different concentrations of $AgNO_3$ (0 - 4 mg/L) to regeneration medium, the shoot regeneration frequency and the number of shoots per explant improved significantly (Fig. 4). The highest shoot regeneration frequency (64.67%) and the number of shoots per explant (7.6) were achieved on a medium containing 2.0 mg/L $AgNO_3$. Shoot number was threefold more than that obtained on the medium without $AgNO_3$.

Optimized conditions (MS medium supplemented with 4 mg/L BA, 0.2 mg/L NAA and 2 mg/L $AgNO_3$) were used to investigate the shoot regeneration ability of 5 genotypes of *B. juncea* L. The shoot regeneration ability is strongly influenced by the genotypes. The frequency of shoot formation ranged from 9.33% in G5(Shambol) to 64.67% in G4(Rai-5) (Fig. 5). The number of shoots per explant ranged from 3.17 in Shambol to 7.6 in Rai-5 (Fig. 5). DMRT shows that frequency of shoot regeneration and the number of shoots per explant is significantly affected by genotype ($P \leq 0.05$).

Normal plantlets were regenerated from all induced shoots and transferred to pot soil and covered by plastic bags with holes for 7-days to maintain humidity. After that the plants were transferred to greenhouse and watered at every 2-days interval. The plants produced flowers and were fertile. The regenerated plants were also identical with the source plants and true-to-type.

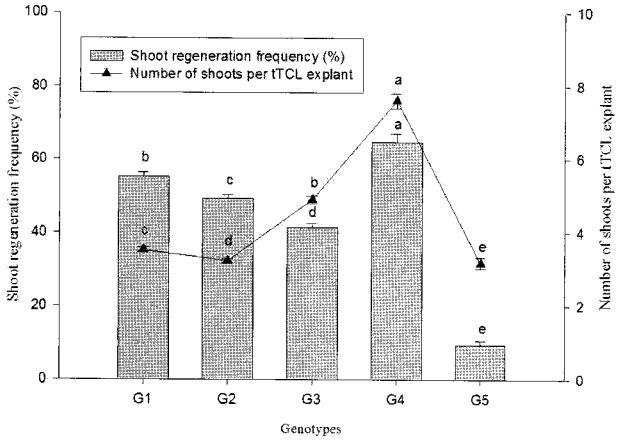

Figure 5. Effect of genotypes on shoot regeneration from tTCL explants of hypocotyl in *B. juncea*. Shoots induced on shoot regeneration medium (MS medium supplemented with 4 mg/L BA, 0.2 mg/L NAA and 2 mg/L $AgNO_3$). G1: *B. juncea* cv. BARI sarisha-10, G2: *B. juncea* cv. BARI sarisha-11, G3: *B. juncea* cv. Daulot, G4: *B. juncea* cv. Rai-5, G5: *B. juncea* cv. Shambol. Data consist of three replicates and 50 explants were used for each replicate. *Bar* represents SD of the means. Values with different letters are significantly different at $P \leq 0.05$ (DMRT)

Discussion

Thin cell layer technology was known efficient for the propagation of various plant species. This study was undertaken to achieve a high rate of regeneration in *B. juncea* L. from tTCL explants. In our experiments, all the factors evaluated (plant growth regulators, $AgNO_3$, explant types, and genotype) influenced shoot regeneration. It was demonstrated that regeneration efficiency depends significantly on medium components such as minerals, nutrients, sugar, vitamins and PGRs. Shoot formation is often enhanced by the combination of auxins and cytokinins. Tang et al. (2003) and more recently Ghnaya et al. (2008) showed that PGR concentration affected significantly the regeneration process from traditional explants as observed from tTCL explants in our study. To determine the optimum culture condition, various combinations of NAA (0, 0.1, 0.2, and 0.4 mg/L) and BA (1, 2, 4, 6 mg/L) on MS medium were studied. All concentrations of BA combined with NAA offered better results compared to the use of BA alone. A concentration of 6 mg/L BA was excessive and produced vitrification and death of shoots. In the absence of PGRs, no shoot regeneration was observed, suggesting that the presence of PGRs is the critical factor for shoot regeneration of *B. juncea* L. This agrees with results reported by Takasaki et al. (1996), who stated that BA and NAA were indispensable for shoot regeneration from *B. campestris* cotyledons.

In addition, it was found that explant types significantly affected shoot regeneration process. In *B. juncea* L. cv. Rai-5, hypocotyl tTCLs best responded compared to cotyledonary petiole tTCLs and exhibited the highest shoot regeneration rate (Fig. 1 & 2). This result compare favorably with recent studies of shoot regeneration of rapeseed (*B. napus* L.) from traditional explants (Tang et al. 2003; Akasaka-Kennedy et al. 2005), longitudinal thin cell layers (Klimaszewska and Keller 1985) and transverse thin cell layers (Ghnaya et al. 2008).

Moreover, the addition of $AgNO_3$ was significantly beneficial to shoot regeneration for *B. juncea* cv. Rai-5. The positive effect of $AgNO_3$ was consistent with previous results from the traditional explants such as cotyledons of *Brassica rapa* ssp. *oleifera* (Burnett et al. 1998), *Brassica campestris* ssp. *pekinensis* (Chi et al. 1991; Zhang et al. 1998), hypocotyls of *Brassica juncea* (Pua and Chi 1993) and *Raphanus sativus* (Pua et al. 1996), peduncle and leaf segments of *Brassica napus* (Eapen and George 1997; Akasaka-Kennedy et al. 2005). $AgNO_3$ is a potent inhibitor of ethylene action, and ethylene is considered to suppress shoot morphogenesis *in vitro*. Zhang et al. (1998) showed that $AgNO_3$ enhanced both shoot regeneration frequency and ethylene production in *B. campestris*. The increase of ethylene production by $AgNO_3$ is considered to be due to interference from ethylene perception or stress induced by Ag^+. They considered that the increase of shoot regeneration frequency by $AgNO_3$ is caused by the interruption of an ethylene signal transduction pathway. Shoot regeneration frequency and number of shoots per explant were enhanced by increasing $AgNO_3$ concentration. But the presence of excess AgNO3, especially more than 4 mg/L seems to be sensitive significantly in the enhancement of shoot regeneration and the number of Shoots per tTCL explants as well.

Shoot regeneration ability is strongly influenced by genotype as proved earlier in *B. napus*, *B. campestris* and *R. sativus* L. (Ono et al. 1994; Takasaki et al. 1996; Zhang et al. 1998; Murakami et al. 1995). More recently, Ghnaya et al. (2008) showed that shoot regeneration from tTCL was influenced by genotypes in rapeseed (*B. napus*). In our study, shoot regeneration ability from tTCL explants also influenced by genotype in *B. juncea* L. Such genotypic variability indicates the genetic control of shoot regeneration ability.

Conclusion

In the present study, an original and efficient regeneration system from tTCL explants has been developed in *Brassica juncea* L. It

produces good results with tTCL explants of all genotypes. Despite a smaller surface and a larger number of wounded cells, shoot regeneration is obtained typically 21-days after the tTCL explant initiation culture. This swift response, in agreement with the observation described previously by Tran Thanh Van (1973), due to the combined process of cell dedifferentiation and reprogramming. Typically, two months later, we observed the normal flowering of regenerated plantlets. Nonetheless, in this system, a single subculture step preceding the regenerated plant transfer into culture dish is required and no subsequent phenotypic alterations were observed in these plants at all. For further improvements, other factors could be taken into account, such as physical parameters, the age of the mother plant, the medium pH, the addition of various sugars, but also more specific factors such as tTCL explant thickness or position along the organ.

Our tTCL model could be used as a tool for fundamental regeneration studies and for crop improvement using *Agrobacterium*-mediated transformation of *Brassica juncea* L. cultivars as well as other Brassica species. Furthermore, this system can be used for *in vitro* selection, in presence of many metals, of plants which may be used in different phytoremediation process.

Acknowledgments

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-211). The authors are sincerely grateful to Mr. M.A.N. Nazim-ud-Dowla (Bangladesh Agricultural Research Institute, Gazipur, Bangladesh) for providing all *Brassica juncea* L. seeds.

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(Received March 20, 2009; Accepted March 24, 2009)