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Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by *Agrobacterium tumefaciens*

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Abstract

An efficient system for shoot regeneration and *Agrobacterium*-mediated gene transfer into *Brassica napus* was developed through the modification of the culture conditions. Different concentrations of benzyladenine (1.5, 3.0 and 4.5 mg dm^{-3}) and thidiazuron (0.0, 0.15 and 0.30 mg dm^{-3}) were evaluated for shoot regeneration of 7, 14 and 21-d-old hypocotyl explants. Maximum shoot regeneration frequency was obtained in 21-d-old explants using 4.5 mg dm^{-3} benzyladenine and 0.3 mg dm^{-3} thidiazuron. Under above culture condition, the highest percentage of shoot regeneration frequency was 200 %. *Agrobacterium*-infected explants grown on the selection medium gave rise to transgenic shoots at a frequency of 11.8 %. Transformed shoots rooted when cultured on a medium supplemented with 2 mg dm^{-3} of indolebutyric acid and 10 mg dm^{-3} kanamycin. The rooted plantlets were successfully established in the soil and developed fertile flowers and viable seeds. Evidences for transformation were confirmed by GUS assay and PCR analysis.

Additional key words: age of explant, benzyladenine, gene transfer, thidiazuron, tissue culture.

Introduction

Brassica napus L. is one of the world's most important sources of vegetable oil and protein meal. The results of plant breeding efforts have greatly improved the crop characteristics but genetic engineering offers new possibilities. Stable genetic transformation and regeneration have been accomplished in some *Brassica* species (Radke *et al.*1992, Schroder *et al.* 1994, Takasaki *et al.* 1997, Wang *et al.* 1999).

Cytokinins in general favour shoot organogenesis in cultured tissues. Thidiazuron (TDZ), a substituted phenylurea (N-phenyl N'-1,2,3-thiadiazol-5-ylurea) has been found to be more effective than 6-benzyladenine (BA) in the promotion of shoot proliferation (Kern and Meyer 1986, Singh *et al.* 2002, Dennis 2003). *In vitro* regeneration response, in terms of number of shoots produced, is an important factor in determining the success of transformation experiments. Media containing two different cytokinins may improve the number and the quality of shoots formed as compared to media with only one cytokinin (Nielsen *et al.* 1995, Tomsone and Gertnere 2003).

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Abbreviations: BA - benzyladenine; B5 medium - Gamborg medium; CaMV35S - cauliflower mosaic virus 35S promoter; CIM - callus induction medium; CTAB - cetyl trimethyl ammonium bromide; 2,4-D - 2,4-dichlorophenoxyacetic acid; GUS - β-glucuronidase; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NPTII - neomycin phosphotransferase II; RIM - root induction medium; SIM - shoot induction medium; SMM - shoot maturation medium; TDZ - thidiazuron; X-gluc - 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.
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The present study describes the organogenetic response in hypocotyl explants of three different ages, cultured with three different concentrations of thidiazuron and benzyladenine. This method can enhance the *Agrobacterium*-mediated transformation of *Brassica napus* hypocotyls, since the transformation efficiency is highly dependent on the regeneration ability.

Materials and methods

Preparation of hypocotyl sections: *Brassica napus* L. cv. PF7045/91 seeds were surface sterilized with 1.5 % sodium hypochlorite and 0.01 % Triton X-100 for 10 min. The seeds were washed 5 times in sterile distilled water and germinated in a jar containing agar-solidified 50 % Murashige and Skoog (1962, MS) medium without growth regulators at a density of 15 seeds per jar. Hypocotyls were excised from 7-, 14-, and 21-d-old seedlings grown at temperature of 25 ºC, 16-h photoperiod, and irradiance of 40 - 50 μmol m^{-2} s^{-1}. They were cut 7 - 10 mm in length, and 20 sections were plated on a precultivation medium (MS salts, 100 mg dm^{-3} myo-inositol, 1.3 mg dm^{-3} thiamine-HCl, 200 mg dm^{-3} KH_2PO_4, 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 3 % sucrose, 0.6 % agar, pH 5.8) for 48-h at 25 ºC in the dark.

Plant regeneration: The hypocotyl sections were transferred to a callus induction medium (CIM), B5 salts and vitamins (Gamborg *et al.* 1968), 1 mg dm^{-3} 2,4-D, 3 % sucrose, 0.6 % agar, pH 5.8, for 7 d. The hypocotyl sections were then transferred to a shoot induction medium (SIM), B5 salts and vitamins, BA (1.5, 3.0, 4.5 mg dm^{-3}), TDZ (0, 0.15, 0.3 mg dm^{-3}), 1 % sucrose, 0.7 % agar, pH 5.8. Sections were subsequently transferred to a fresh medium of the same composition every two weeks to obtain regenerated shoots. After 6 weeks, percent of shoot regeneration frequency was evaluated from number of regenerated shoots divided to total number of explants. The green shoots were excised from calli and placed on a shoot maturation medium (SMM), B5 salts and vitamins, 1 % sucrose, 0.6 % agar, pH 5.8. All plates were sealed with gas permeable tape and maintained at 25 ºC under 16-h photoperiod and irradiance of 40 - 50 μmol m^{-2} s^{-1}. Two weeks later, shoots were placed into jars containing a root induction medium (RIM), B5 salts and vitamins, 2 mg dm^{-3} indole-3-butyric acid (IBA), 1 % sucrose, 0.6 % agar, pH 5.8. After 4 - 5 weeks, rooted plantlets were transferred to potting soil. Plants were grown to maturity and produced fertile flowers and set seeds.

Experimental design and statistical analysis: Treatments consisted of three concentrations of BA (1.5, 3.0, 4.5 mg dm^{-3}), three concentrations of TDZ (0, 0.15, 0.3 mg dm^{-3}) and three explant ages (7, 14, 21 d). They were arranged in a factorial experiment as randomized complete block design. Each treatment combination was replicated three times. Analyses of variance were performed on the variable of interest with *ANOVA* procedures of *MSTATC* software. Frequencies of shoot regeneration were logarithmically transformed before analysis. Means of main effect levels and their interactions (both double and triple) were compared by Duncan's test. Finally, all data were changed to primary form.

Transformation procedure: Single colony of *Agrobacterium tumefaciens* strain LBA4404, carrying the binary vector pBI121 (*Clontech,* USA), was grown overnight at 28 ºC in LB medium supplemented with 50 mg dm^{-3} kanamycin. This vector contains the reporter *GUS* gene under the strong CaMV35S promoter activity. The bacterial pellet was diluted 1:2 with *Agrobacterium* infection medium (MS medium containing 1 % glucose, pH 5.2). This suspension was used as the bacterial inoculum. The 21-d-old hypocotyl sections were inoculated by immersing for 10 min in the bacterial inoculum, after which they were transferred in cocultivation medium containing MS salts, 100 mg dm^{-3} myo-inositol, 1.3 mg dm^{-3} thiamine-HCl, 200 mg dm^{-3} KH_2PO_4, 1 mg dm^{-3} 2,4-D, pH 5.2 and incubated at 25 ºC for 48-h in the dark. After cocultivation, the hypocotyl sections were transferred to CIM supplemented with 200 mg dm^{-3} cefotaxime and cultured for 7 d. They were then transferred to SIM containing 4.5 mg dm^{-3} BA and 0.3 mg dm^{-3} TDZ supplemented with 200 mg dm^{-3} cefotaxime and 10 mg dm^{-3} kanamycin. Sections were subsequently transferred to a fresh medium of the same composition every 2 weeks. After 6 weeks, green shoots were excised from calli and placed on SMM containing 150 mg dm^{-3} cefotaxime and 10 mg dm^{-3} kanamycin. After 2 weeks they were transferred to RIM supplemented with 150 mg dm^{-3} cefotaxime and 10 mg dm^{-3} kanamycin. After 4 - 5 weeks rooted plantlets were transferred to vermiculate and soil (1:1). Plants were grown to maturity and set seeds.

Histochemical GUS assay: The expression of *GUS* gene in the petiole and leaf segments of green plants was assayed. The tissues were dipped in a *GUS* assay buffer containing 50 mM phosphate buffer (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 1 mM EDTA, 0.001 % Triton X-100 and 10 mM β-mercaptoethanol and incubated overnight at 37 ºC. The chlorophyll of leaf segments was destained by rinsing in 96 % ethanol.

DNA extraction and PCR analysis: Genomic DNA was extracted from young leaves of green plants by the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thampson 1980). Two specific primers derived from *GUS* gene were used to amplify a 520 bp fragment including:
GUS 2, 5´-CCGGCATAGTTAAAGAAATCAT-3' and GUS 4, 5´-TGGTCAGTCCCT TATGTTACG-3'.

The amplification assay contained: 100 ng of DNA, 3 mM $MgCl_2$, 250 μM dNTPs, 0.25 μM of each primer, 1 × PCR buffer and 0.5 unit of Taq DNA polymerase (*Cinnagen*, Iran) in a reaction volume of 0.02 cm^3. The PCR was performed using *Perkin Elmer* (USA) thermocycler (*9600*) with the following temperature profile: 94 ºC for 4 min, followed by 30 cycles of 94 ºC for 1 min, 57 ºC for 1 min and 72 ºC for 1 min with final elongation of 72 ºC for 7 min.

Results

All hypocotyls cultivated on 2,4-D medium for one week produced extensive callus after transferring into SIM. The initial buds began to appear on the green callus four weeks after planting. The regenerated shoots were excised from calli and transferred to shoot maturation medium. The elongated shoots were grown in root induction medium and produced roots after four weeks.

Table 1. Main effects of different age of explant and concentrations of BA and TDZ on shoot regeneration frequency. Values represent the mean of three replicates. Means followed by the same letter are not significantly different (Duncan's multiple range test, P <0.05). Data were backtransformed from logarithmic average.

Age of explant [d]	BA [mg dm^{-3}]	TDZ [mg dm^{-3}]	Regeneration frequency [%]
7			80.37 c
14			107.59 b
21			129.82 a
	1.5		79.81 c
	3.0		101.30 b
	4.5		136.70 a
		0.00	83.30 c
		0.15	108.90 b
		0.30	125.60 a

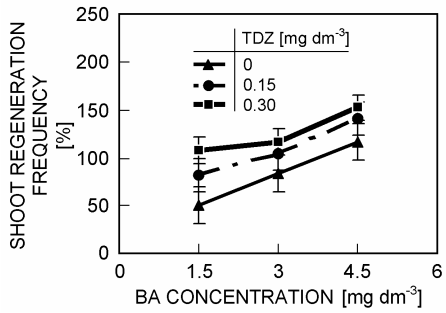

Fig. 1. Interaction of BA and TDZ concentrations on regenerated shoot frequency. Data consist of 3 replicates of 20 explants each.

Our results indicated that significant difference exist among the means of three levels of explant ages for percent of shoot regeneration frequency (P < 0.01). The highest shoot regeneration frequency (129.8 %) was obtained with 21-d-old seedlings and the least amount mean was obtained from 7-d-old explants (Table 1). Significant difference occurred among the number of regenerated shoots (P < 0.01) in their responses to varying BA concentrations. The highest number of regenerated shoot (136.7 %) resulted from 4.5 mg dm^{-3} BA (Table 1). TDZ had a significant impact on shoot regeneration frequency (P < 0.01). The maximum shoot regeneration (125.6 %) was obtained in medium containing 0.3 mg dm^{-3} TDZ. Explants formed only a few shoots when cultured on a medium without TDZ (Table 1).

The interaction between age of the explants and the concentration of BA had not a significant effect on percentage of regenerated shoots. Furthermore, no significant difference was observed in shoot regeneration frequency in response to interaction between TDZ concentration and the age of explant. Significant difference was found in the combinations of BA and TDZ. The largest frequency of shoot regeneration (152.2 %) was induced by combination of 4.5 mg dm^{-3} of BA and 0.3 mg dm^{-3} of TDZ (Fig. 1). Increasing TDZ concentration increased shoot regeneration in each BA concentration. No significant interaction of age of explants, BA and TDZ concentrations for shoot regeneration frequency was found. The largest percentage of regenerated shoots (200 %) was obtained with 21-d-old explants, 4.5 mg dm^{-3} BA and 0.3 mg dm^{-3} TDZ (Table 2).

The quality of shoots produced was clearly dependent upon the growth regulator used and the age of explants. The youngest seedlings planted on minimum concentrations of TDZ and BA produced the lowest number of shoots (35.0 %) of abnormal shape (Fig. 2*A*). They were not able to produce roots in RIM efficiently. The highest frequency of shoot regeneration (200.0 %) occurred in the oldest explants when exposed to media with high concentrations of TDZ and BA (Fig. 2*B*). Most of these shoots produced root four weeks after incubation in RIM. The rooting shoots were transferred to

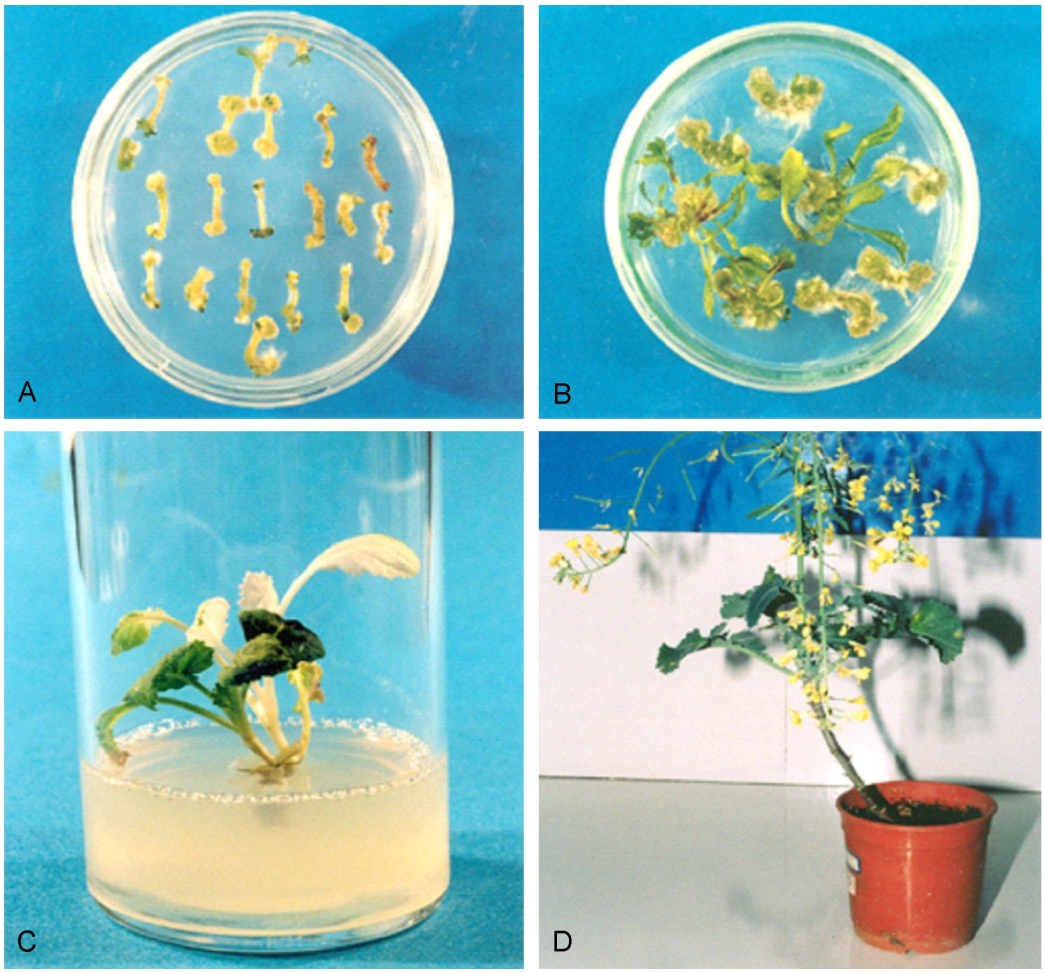

Fig. 2. Shoot regeneration from *B. napus* using TDZ, BA and different ages of the explant. *A* - Shoot regeneration from 7-d-old explants on shoot induction medium (SIM) containing 1.5 mg dm^{-3} BA without TDZ after 6 weeks. *B* - High frequency of shoot regeneration from 21-d-old explants on SIM containing 4.5 mg dm^{-3} BA and 0.3 mg dm^{-3} TDZ after 6 weeks. *C* - Transformed (green) and untransformed (white) shoot regenerated from hypocotyl on the selection medium supplemented with kanamycin. *D* - Establishment of transformed plantlets in the soil and appearance of normal flowers and seeds.

Table 2. Triple interaction effects of explant ages, BA and TDZ concentrations on shoot regeneration frequency. Values represent the mean of three replicates. Means followed by the same letter are not significantly different (Duncan's multiple range test, P <0.05). Data were backtransformed from logarithmic average.

BA [mg dm^{-3}]	TDZ [mg dm^{-3}]								
	0.0			0.15			0.30		
	7 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
1.5	35.0 g	35.0 g	80.0 def	60.0 fg	100.0 cde	83.3 def	116.7 a-e	105.0 b-e	103.3 cde
3	80.0 def	70.0 ef	100.0 cde	81.0 def	111.7 be	120.0 a-e	80.0 def	126.7 a-e	141.7 a-d
4.5	86.7 df	118.3 a-e	145.0a-d	88.3 def	140.0 a-d	195.0 ab	95.0 cde	161.7 abc	200.0a

soil and acclimatized in the growth chamber. Most of the plants were phenotypically normal and produced fertile flowers and viable seeds.

For the selection of transformed shoots, kanamycin was used as selectable marker. The transformed shoots containing *NPTII* gene had a normal dark green color and

quiet vigorous growth in selection medium supplemented with kanamycin. The untransformed shoots appeared as pale green leaflets with anthocyanin coloration on their surface (Fig. 2*C*). Green shoots were cut and transferred to RIM. Roots were formed in kanamycin resistant plants after 4 - 8 weeks. Rooted plantlets were transferred to soil and produced fertile flowers and set seeds (Fig. 2*D*). The highest transformed shoot regeneration (11.8 %) was obtained in 10 mg dm^{-3} of kanamycin.

PCR analysis using DNA from kanamycin resistant plants confirmed that most of the green plantlets contained *GUS* gene. The 520 bp fragment was detected from transformed lines, but not from control untransformed plants (Fig. 3*A*). *GUS* gene expression in kanamycin resistant plants was also investigated. The majority of transgenic lines (70 %) were found to express *GUS* gene in the leaves and petioles (Fig. 3*B*).

Discussion

We developed a regeneration method for oilseed rape hypocotyls *via* organogenesis by using thidiazuron in combination with benzyladenine. The greatest efficiency in shoot regeneration was obtained by concentrations of

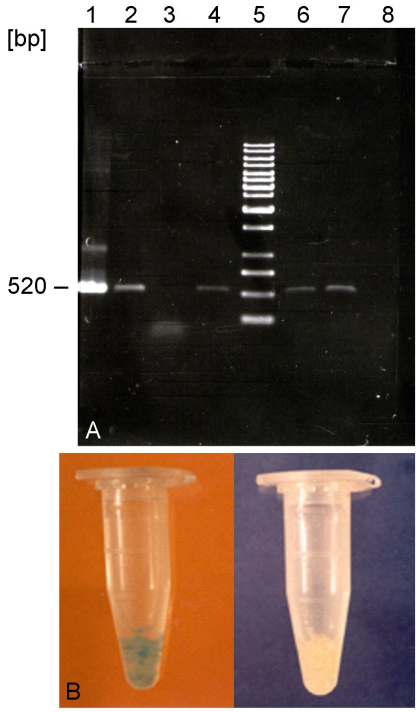

Fig. 3. Analysis of *B. napus* transgenic plants. *A* - PCR analysis of kanamycin-resistant plants showing amplification of the 520 bp DNA fragment of the *GUS* gene. *Lane 1*: positive control (pBI121); *lanes 2, 3, 4, 6 and 7*: transformed plants; *lane 8*: negative control (wild type); *lane 5*: molecular size marker 1 kb ladder. *B* - Histochemical GUS assay for transgenic (left) and untransgenic (right) petiole segments.

4.5 mg dm^{-3} BA and 0.3 mg dm^{-3} TDZ and in 21-d-old seedlings. Thidiazuron is a substituted phenylurea. Although the biochemical action of the phenylurea is not completely understood, it is believed to function in the regulation of purine cytokinin metabolism and act directly as a cytokinin or in combination with cytokinins (Mok *et al.* 1982, 2000). It has been suggested that TDZ promotes the conversion of cytokinin ribo-nucleotides to the biologically more active ribonucleo-sides in the callus tissue of *Phaseolus lunatus* L. (Capelle *et al.* 1983). The biological activity of TDZ is higher than or comparable to that of the most active adenine type cytokinins (Mok *et al.* 1987).

In our experiments, TDZ increased shoot regeneration induction. Christey *et al.* (1999) obtained high efficiency of shoot regeneration using 0.1 mg dm^{-3} TDZ and 10 mg dm^{-3} BA in *Agrobacterium rhizogenes*-mediated transformants of *B. napus*, supporting the consistency of results obtained in our experiment. Cheng *et al.* (2001) also reported more than 90 % regeneration using 4.5 mg dm^{-3} BA and 94 % regeneration with 0.5 mg dm^{-3} TDZ and 0.01 mg dm^{-3} IAA from 3-d-old hypocotyl of *B. oleracea*. Wang *et al.* (1999) used hypocotyls explants for tissue culture in *Brassica campestris*. They applied medium supplemented with 1 mg dm^{-3} TDZ, 0.2 mg dm^{-3} NAA and 7.5 mg dm^{-3} $AgNO_3$.

Although the effects of cytokinins are well known, the mechanism of cytokinin perception is still not understood. In order to identify cytokinin receptor(s), numerous cytokinin-binding proteins (CBPs) were characterized (Brault *et al.* 1999). Based on an analogy to the animal hormone system, Nielsen *et al.* (1995) proposed a model for cytokinin action in plant cells. Both BA and TDZ can bind to a receptor of cytokinin binding protein. The CBP has two different binding sites; one site binds adenine-type cytokinin naturally, while the other is able to bind phenylurea type cytokinins. Exogenously supplied BA leads to an elevated cytokinin effect which can be explained by more adenine cytokinin sites on CBP being occupied. Because an exogenous supply of TDZ has a marked effect only in the first subculture, the binding of TDZ to the phenylurea CBP site enhances the effect of BA or endogenous adenine-type cytokinin already bound to CBP. The proposed of two binding sites on one receptor may explain why the cytokinin effect of TDZ is more variable among species than the effects of adenine cytokinins. However, no cytokinin binding protein was characterized as a protein involved in cytokinin perception, and the biological interest of these proteins remains to be established (Brault *et al.* 1999).

Since the cells within a plant can have different

content of endogenous growth regulators, receptor affinity or cellular sensitivity to plant growth regulators (Kim *et al.* 1997), it is reasonable to expect that *in vitro* responses will vary with and within the species. In our experiment, variation in responses obtained in the explants of different age group is probably due to the heterogeneous developmental states of the tissues. The effect of developmental age resulted in different endogenous levels of plant growth regulators.

The advantages of the hypocotyl explant transformation and regeneration system are the ease in generating uniform sterile seedlings, and in excising and cocultivating large numbers of hypocotyl explants. Our results show that *B. napus* hypocotyl tissue is also amenable to genetic transformation. We obtained 11.8 % frequency of transformation from hypocotyl explants. Barfield and Pua (1991), Schroder *et al.* (1994) and Takasaki *et al.* (1997) obtained 5, 10, and 5 % transformed plants, respectively, supporting the high efficiency rates obtained in our experiment. The transfer system described in this method has a great potential for genetic improvement of *B. napus* by introducing genes responsible for agronomically important traits, *i.e.*, herbicide tolerance and insect resistance into the plants.

References

Barfield, D.G., Pua, E.C.: Gene transfer in plants of *Brassica juncea* using *Agrobacterium tumefaciens*-mediated transformation. - Plant Cell Rep. **10**: 308-314, 1991.

Brault, M., Caiveau, O., Pedron, J., Maldiney, R., Sotta, B., Miginiac, E.: Detection of membrane-bound cytokinin binding proteins in *Arabidopsis thaliana* cell. - Eur. J. Biochem. **260**: 512-519, 1999.

Capelle, S.C., Mok, D.W.S., Kirchner, S.C., Mok, M.C.: Effects of thidiazuron on cytokinin autonomy and metabolism of N6- (2-isopentyl)[8-14C] in callus tissue of *Phaseolus lunatus* L. - Plant Physiol. **73**: 796-802, 1983.

Cheng, P.K., Lakshmanan, P., Swarup, S.: High frequency direct shoot regeneration and continuous production of rapid-cycling *Brassica oleracea* in *in vitro*. - *In Vitro* cell. dev. Biol. Plant **37**: 592-598, 2001.

Christey, M.C., Braun, R., Kenel, F.O., Podivinsky, E.: *Agrobacterium rhizogenes*-mediated transformation of swede (*B. napus* var. *rapifera*) - In: Wratten, N., Salisbury, P.A. (ed.): Proceedings of the 10th International Rapeseed Congress. Pp. 105-109. The Regional Institute Ltd., Canberra 1999.

Dennis, T.T.: Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonary explants of mulberry. - Biol. Plant. **46**: 529-533, 2003.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension culture of soybean cells. - Exp. Cell Res. **50**: 51-158, 1968.

Kern, H.R., Meyer, M.M.: Tissue culture propagation of *Acer* × *Freemanii* using thidiazuron to stimulate shoot tip proliferation. - HortScience **21**: 1209-1210, 1986.

Kim, M.S., Schuman, C.M., Klopfenstein, N.B.: Effects of thidiazuron and benzyladenine on axillary shoot proliferation of three green ash clones. - Plant Cell Tissue Organ Cult. **48**: 45-50, 1997.

Mok, M.C., Martin, R.C., Mok, D.W.S.: Cytokinin: Biosyntesis, metabolism and perception. - *In Vitro* cell. dev. Biol. Plant **36**: 102-107, 2000.

Mok, M.C., Mok, D.W.S., Armestrong, D.G., Shuda, K., Isoyai, Y., Okamoto, T.: Cytokinin activity of N-phenyl-N-1, 2, 3-thiadiazol-5-yl urea (thidiazuron). - Phytochemistry **21**: 1509-1511, 1982.

Mok, M.C., Mok, D.W.S., Turner, J.E., Muser, C.V.: Biological and biochemical effect of cytokinin-activate phenylurea derivatives in tissue culture systems. - Hort. Sci. **22**: 1194-1197, 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Murray, M.G., Thampson, W.F.: Rapid isolation of high molecular weight plant DNA. - Nucl. Acids Res. **8**: 4321-4325, 1980.

Nielsen, J.M., Hansen, J., Brandt, K.: Synergism of thidiazuron and benzyladenine in axillary shoot formation depends on sequence of application in *Miscanthus* × *ogiformis*, 'Giganteus'. - Plant Cell Tissue Organ Cult. **41**: 165-170, 1995.

Radke, S.E., Turner, J.C., Facciotti, C.: Transformation and regeneration of *Brassica rapa* using *Agrobacterium tumefaciens*. - Plant Cell Rep. **11**: 499-505, 1992.

Schroder, M., Dixelius, C., Rahlen, L., Glimelius, K.: Transformation of *Brassica napus* by using the *aadA* gene as selectable marker and inheritance studies of the marker genes. - Physiol. Plant. **92**: 37-46, 1994.

Singh, R., Srivastava, K., Jaiswal, H.K., Amla, D.V., Singh, B.D.: High frequency multiple shoot regeneration from decapitated embryo axes of chickpea and establishment of plantlets in the open environment. - Biol. Plant. **45**: 503-508, 2002.

Takasaki, T., Hatakeyama, K., Ojima, K., Watanabe, M., Toriyama, K., Hinata, K.: Factors influencing *Agrobacterium*-mediated transformation of *Brassica rapa* L. - Breed. Sci. **47**: 127-134, 1997.

Tomsone, S., Gertnere, D.: *In vitro* shoot regeneration from flower and leaf explants in *Rhododendron*. - Biol. Plant. **46**: 463-465, 2003.

Wang, L.J., Ni, D.A., Wang, G.Y., Xia, Z.A., Xu, Z.K.: Preliminary studies on tissue culture and *Agrobacterium*-mediated transformation of *Brassica campestris* ssp. *chinensis*. - Shi Yan Sheng Wu Xue Bao **32**: 93-99, 1999.