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IMPROVED *BRASSICA NAPUS* L., REGENERATION FROM HYPOCOTYLS USING THIDIAZURON AND BENZYLADENINE AS CYTOKININ SOURCES

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Abstract

A reproducible system to produce regenerated *Brassica napus* L., plants has been developed using hypocotyl explants. Different concentrations of Benzyladenine (1.5, 3.0 and 4.5 mg l^{-1}) and Thidiazuron (0, 0.15 and 0.30 mg l^{-1}) were evaluated for shoot regeneration using 7, 14 and 21 day old hypocotyl explants. Treatments were arranged in a factorial experiment as a randomized complete block design with three replications. Significant differences were found in the interactions of Benzyladenine and Thidiazuron concentrations as well as the ages of the explants. Maximum shoot regeneration was obtained using 4.5 mg l^{-1} Benzyladenine and 0.3 mg l^{-1} Thidiazuron. Shoot regeneration was highly affected by the age of the explants. It was found that 21 day old explants were more likely to undergo shoot development than the others. Under these culture conditions, the highest percentage of shoot regeneration was 174.0% for hypocotyl explants. Regenerated shoots rooted when cultured on a root induction medium supplemented with 2 mg l^{-1} of Indolebutyric acid. Rooted plantlets were successfully established in the soil which developed normal fertile flowers and viable seeds. In light of its efficiency, this hypocotyl regeneration method could be a suitable tool for genetic transformation using *Agrobacterium.*

Introduction

Oilseed rape, *Brassica napus* L., is one of the most important crops for production of vegetable oils in the world. In the last 30 years, a great deal of efforts have been focused on improving the production and the quality of *B. napus* using both classical breeding and several tissue culture techniques. Genetic engineering can potentially be used as a method to add reproducible transformation to existing varieties (De Block *et al*., 1989; Takasaki *et al*., 1997; Wang *et al*., 1999). More precisely, the generation of transgenic plants is an integrated process which involves many different factors such as plant regeneration, the choice of regenerable explant culture conditions and transformation techniques.

In vitro cultured plant cells and tissues can be induced to differentiate into complete plants through organogenesis or somatic embryogenesis. Cytokinins, in general, favor shoot organogenesis in cultured tissues. Thidiazuron (TDZ), a substituted phenylurea (N-phenyl N'-1, 2, 3- thiadiazol–5-ylurea) is primarily used as a cotton defoliant (Eapen *et al*., 1998), and aids in rapid plant regeneration of a number of plant species (Chand *et al*., 1999; Seelye *et al*., 1994; Kanakis & Demetriou, 1993; Malik & Saxena, 1992).

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Mok *et al*., (1982) discovered the cytokinin–like activity of TDZ. The first reports described callus promoting activity of TDZ was in *Phaseolus lunatus* L., (Mok *et al*., 1982) and induction of plant regeneration in *Nicotiana tabacum* (Thomas & Katterman, 1986). Subsequently, the potential of TDZ to stimulate axillary shoot proliferation was discovered (Kern & Meyer, 1986). Furthermore, TDZ has been found to be more effective than 6-benzyladenine (BA) in the promotion of shoot proliferation (Kerns & Meyer, 1986). *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 alter the number and the quality of shoots formed as compared to media with only one cytokinin (Nielsen *et al*., 1995).

The present report describes the organogenetic response in *Brassica napus* L., hypocotyl explants of three different ages cultured with three different concentrations of Thidiazuron and/or Benzyladenine.

Materials and Methods

Preparation of hypocotyl sections: *Brassica napus* L., cv. SLM 460 was used as the experimental plant material. 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% MS salts (Murashige & Skoog, 1962) without growth regulators at a density of 15 seeds per jar. Hypocotyls were excised from 7, 14 and 21 day old seedlings grown in a growth chamber at 25 ºC under fluorescent light (16-h photoperiod, 40-50 µmol m^{-2} s^{-1}). They were cut into 7-10 mm in length and 20 sections were plated on a precultivation medium (MS salts, 100 mg l^{-1} Myo-inositol, 1.3 mg l^{-1} Thiamine-HCl, 200 mg l^{-1} KH_2PO_4, 1 mg l^{-1} 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 l^{-1} 2,4-D, 3% sucrose, 0.6% agar, pH 5.8, for 7 days. The hypocotyl sections were then transferred to a shoot induction medium (SIM), B5 salts and vitamins, BA (1.5, 3.0, 4.5 mg l^{-1}), TDZ (0, 0.15, 0.3 mg l^{-1}), 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 shoot regeneration. After 6 weeks, 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 fluorescent lights (16-h photoperiod, 40-50 µmol m^{-2} s^{-1}). Two weeks later, shoots were subcultured into jars containing a root induction medium (RIM), B5 salts and vitamins, 2 mg l^{-1} 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 they produced fertile flowers and set seeds.

Experimental design and statistical analysis: Treatments consisted of three levels of BA (1.5, 3.0, 4.5 mgl^{-1}), three levels of TDZ (0, 0.15, 0.3 mgl^{-1}) and three explant ages (7, 14, 21 days). They were arranged in a factorial experiment as randomized complete block design. Each treatment combination was replicated three times for each parameter

and analyses of variance were performed on the variable of interest with ANOVA procedures of MSTATC software. Frequencies of shoot regeneration were logarithmically transformed, and percent of regenerated hypocotyl and rooting shoots were transformed by square root before analysis. After mean comparison, all data were changed to primary form.

Fig. 1. Shoot regeneration of rapeseed (*Brassica napus* L.) using TDZ, BA and different ages of the explant. (A) Callus formation from hypocotyl explants after one week incubation in callus induction medium (CIM) containing 1 mg l^{-1} 2,4-D, and subsequent transfer into shoot induction medium (SIM). (B) Shoot regeneration from 7 day old explants on SIM containing 1.5 mg l^{-1} BA without TDZ after 6 weeks. (C) High frequency of shoot regeneration from 21 day old explants on SIM containing 0.3 mg l^{-1} TDZ and 4.5 mg l^{-1} after 6 weeks. (D) Root formation on root induction medium (RIM) supplemented with 2 mg l^{-1} IBA after 4 weeks. (E) Establishment of *B. napus* plantlets in soil and appearance of normal flowers and pods.

Results

All hypocotyls cultivated on 2,4-D medium for one week produced extensive callus after transferring into SIM (Fig. 1A). The initial buds began to appear on the green callus four weeks after culture. The regenerated shoots were excised from calli and transferred to shoot maturation medium. The elongated shoots were incubated in root induction medium which produced roots after four weeks.

The data were analyzed statistically using ANOVA (variance analysis) and Duncan's test. These tests revealed that there were significant differences ($p<0.05$) in shoot

production with the variation of TDZ, BA and the ages of explants. The three parameters evaluated consisted of percent of regenerated hypocotyls (number of hypocotyls with shoots after 6 weeks / total number of explants, RH), percent of shoot regeneration frequency (number of regenerated shoots / total number of explants, RF) and percent of rooting shoots (number of root-producing shoots / number of regenerated shoots, RS).

Our results indicated that significant differences exist among the means of the three levels of explant ages for RH and RF (p<0.01). The highest RH (26.11%) and RS (16.99%) frequencies were obtained with 21 day old seedlings and the least RH mean was obtained from 7 day old explants. The highest frequency of shoot regeneration (126.85%) occurred in 14 day old explants (Table 1).

Table 1. Effect of explant age, BA and TDZ on regenerated hypocotyls, rooting shoots per total regenerated shoots and regenerated shoots per explant.

Explant age (days)	BA (mg l^{-1})	TDZ (mg l^{-1})	Regenerated hypocotyls[1] (%)		Rooting shoots[1] (%)		Shoot regeneration frequency[2] (%)	
7			20.9	b	14.2	a	105.9	b
14			22.2	ab	13.9	a	126.9	a
21			26.1	a	17.0	a	124.6	a
	1.5		17.0	b	16.4	ab	70.6	b
	3.0		26.3	a	17.3	a	144.6	a
	4.5		25.9	a	11.4	b	142.2	a
		0.00	18.9	b	15.0	a	88.9	c
		0.15	22.0	b	13.4	a	118.1	b
		0.30	28.3	a	16.7	a	150.4	a

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).
[1]Data were backtransformed from logarithmic average.
[2]Data were backtransformed from square root average.

The cytokinins were used for *in vitro* shoot regeneration. Without the application of cytokinins, no shoots were formed under any of the conditions. Significant difference occurred among the number of RH, RF and RS (p <0.01) in their responses to varying BA concentrations. The highest number of RH (26.30%) and RS (17.29%) resulted from 3 mg l^{-1} BA. The best shoot regeneration frequency (144.6%) was obtained by 3 mg l^{-1} BA that accompanied by 4.5 mgl^{-1} BA in the same statistical group by Duncan's test (Table 1).

Shoot regeneration was also induced in TDZ-supplemented medium. TDZ had a significant impact on RH and RF (p<0.01). The largest values obtained using 0.3 mg l^{-1} TDZ for RH, RS and RF were 28.33%, 16.67% and 150.4%, respectively. Explants formed only a few shoots when cultured on a medium without TDZ (Table 1).

The age of the explants and the concentration of BA had a significant effect on RH and RF (p <0.01). The best value of the RH mean (32.78%) was recorded when 21 day old explants were cultured on a BA concentration of 3 mg l^{-1}, accompanied by 7 day old explants and 4.5 mgl^{-1} BA, in the same statistical group by Duncan's test. The highest frequency of shoot regeneration (165.6%) was obtained from 14 day old explants and 3 mg l^{-1} BA. The highest number of RS resulted when 21 day old explants were cultured on 1.5 mg l^{-1} BA (Table 2).

Table 2. Effect of explant age and BA on regenerated hypocotyls, rooting shoots per total regenerated shoots and regenerated shoots per explant.

Explant age (days)	BA (mg l^{-1})	Regenerated hypocotyls[1] (%)		Rooting shoots[1] (%)		Shoot regeneration frequency[2] (%)	
7	1.5	13.9	d	13.1	ab	47.2	e
	3.0	18.9	cd	18.3	ab	108.3	bc
	4.5	30.0	a	11.1	b	162.2	a
14	1.5	17.8	cd	13.5	ab	83.9	cd
	3.0	27.2	ab	15.6	ab	165.6	a
	4.5	21.7	bc	12.5	ab	131.1	ab
21	1.5	19.4	cd	22.4	a	80.6	d
	3.0	32.8	a	18.0	ab	160.0	a
	4.5	26.1	ab	10.5	b	133.3	ab

Values represent the mean of three replicates. Means followed by the same letter are not significantly different (Duncan's multiple range test, $p < 0.05$).
[1]Data were backtransformed from logarithmic average.
[2]Data were backtransformed from square root average.

Significant difference was observed in RH ($p<0.001$) in response to interaction between TDZ concentrations and the ages of explant. The highest percent of RH (32.8%) resulted from 21 day old explants on 0.3 mg l^{-1} TDZ. No significant difference occurred in interaction of TDZ concentrations and the ages of the explant for RS and RF, although the high concentration of TDZ produced the most RF on all three ages of the explants (Table 3). The highest amount of RS was produced by 0.3 mg l^{-1} TDZ and 21 day old explants, however, all treatments were in the same statistical group in Duncan's test (Table 3).

Significant difference was found in the interaction of BA and TDZ concentrations for RF (Table 4). The largest frequency of shoot regeneration (174.0%) was stimulated by a BA concentration of 4.5 mg l^{-1} and TDZ concentration of 0.3 mg l^{-1}. Increasing TDZ concentration made shoot regeneration to increase in BA concentration levels. No significant differences were found in the interaction of BA and TDZ concentrations for RH and RS. The largest percent of RS (18.67%) was obtained with 3 mg l^{-1} BA and 0.30 mg l^{-1} TDZ. The quality of shoot 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 (41.1%) that were abnormally shaped (Fig. 1B). They were not able to produce roots in RIM efficiently. The highest frequency of shoot regeneration (174.0%) occurred in the oldest explants when exposed to media with high concentrations of TDZ and BA (Fig. 1C). Most of the shoots produced extended root four weeks after incubation in RIM (Fig. 1D). The rooting shoots were transferred to soil and acclimatized in the growth chamber. Most of the plants were phenotypically normal and produced fertile flowers and viable seeds (Fig. 1E).

Table 3. Effect of explant age and TDZ on regenerated hypocotyls, rooting shoots per total regenerated shoots and regenerated shoots per explant.

Explant age (days)	TDZ (mg l^{-1})	Regenerated hypocotyls[1] (%)		Rooting shoots[1] (%)		Shoot regeneration frequency[2] (%)	
7	0.00	18.3	d	17.0	a	87.2	c
	0.15	20.0	cd	11.8	a	99.4	bc
	0.30	24.4	bcd	13.7	a	131.1	ab
14	0.00	18.9	d	14.3	a	94.4	bc
	0.15	20.0	cd	10.2	a	121.7	ab
	0.30	27.8	ab	17.1	a	164.4	a
21	0.00	19.4	d	13.6	a	85.0	c
	0.15	26.1	abc	18.1	a	133.3	ab
	0.30	32.8	a	19.2	a	155.6	a

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).
[1]Data were backtransformed from logarithmic average.
[2]Data were backtransformed from square root average.

Table 4. Effect of BA and TDZ on regenerated hypocotyls, rooting shoots per total regenerated shoots and regenerated shoots per explant.

BA (mg l^{-1})	TDZ (mg l^{-1})	Regenerated hypocotyls[1] (%)		Rooting shoots[1] (%)		Shoot regeneration frequency[2] (%)	
1.5	0.00	12.2	e	17.1	a	41.1	e
	0.15	16.7	de	13.6	a	66.1	d
	0.30	22.2	cd	18.4	a	104.4	c
3.0	0.00	22.8	cd	18.5	a	117.8	bc
	0.15	25.0	bc	14.7	a	143.3	abc
	0.30	31.1	ab	18.7	a	172.8	a
4.5	0.00	21.7	cd	9.2	a	107.8	bc
	0.15	24.4	bc	11.9	a	145.0	ab
	0.30	31.7	a	13.0	a	173.9	a

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).
[1]Data were backtransformed from logarithmic average.
[2]Data were backtransformed from square root average.

Discussion

A regeneration protocol for oilseed rape hypocotyls via organogenesis was developed using the strong cytokinin analogue Thidiazuron as an effective shoot inducing

growth regulator. The greatest level of efficiency in shoot regeneration was obtained by a concentration of 0.3 mg l^{-1} TDZ and 4.5 mg l^{-1} BA in 21 day old seedlings. Thidiazuron is a substituted phenylurea. Although the biochemical action of the phenylureas is not completely understood, it is believed to function in the regulation of purine cytokinin metabolism, act directly as cytokinins or in concern with cytokinins (Mok *et al*., 1982; Mok & Mok, 1985). The biological activity of TDZ is higher than or comparable to that of the most active adenine type cytokinins (Mok *et al*., 1987; Huetteman & Preece, 1993).

In the present study TDZ increased shoot regeneration induction when hypocotyl explants were exposed to 0.3 mg l^{-1} TDZ and 4.5 mg l^{-1} BA. Christey *et al*., (1999) obtained high efficiency of shoot regeneration using 0.1 mg l^{-1} TDZ and 10 mg l^{-1} BA in *Agrobacterium rhizogenes*-mediated transformation 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 l^{-1} BA and 94% regeneration with 0.5 mg l^{-1} TDZ and 0.01 mg l^{-1} IAA from 3 day old hypocotyl of *B. oleracea*.

Sango *et al*., (1996) obtained plant regeneration through organogenesis for the pea using TDZ. They showed that shoot formation was significantly influenced by TDZ and approximately 50% root formation was obtained when shoots were cultured on medium containing IBA. We obtained only 19.5% root formation in RIM supplemented by 2 mg l^{-1} IBA that most likely was due to the inhibitory effects of TDZ on root formation. Residual effects of TDZ in treated tissues can inhibit rooting capacity (Sango *et al*., 1996; George, 1993).

Media supplemented with TDZ and BA have been found to be very effective for promotion of shoot regeneration. Nielsen *et al*., (1995) described the synergistic effect of BA and TDZ. They reported that in the first subculture, shoot formation was enhanced with an increasing concentration of TDZ, whereas increasing concentration of BA influenced shoot formation in the second and third subcultures. The mode of action by which TDZ induces cytokinin-like effects is not understood very well. It has been suggested that TDZ promotes the conversion of cytokinin ribonucleotides to the biologically more active ribonucleosides in the callus tissue of *Phaseolus lunatus* L., (Capelle *et al*., 1983). Others have suggested that TDZ stimulates the synthesis of endogenous adenine-type cytokinins, or inhibits their degradation in callus tissues of *Glycine max* L., (Thomas & Katterman, 1986).

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 (CBP). The CBP has two different binding sites; one site binds adenine–type cytokinin naturally, while the other is able to bind phenylurea type cytokinins. Binding of an adenine-type cytokinin to CBP somehow induces the well-known cytokinin effects of shoot formation (Tamas, 1987) and inhibition of root formation (George, 1993).

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 proposal 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 (Nielsen *et al*., 1995).

Since the cells within a plant can have different endogenous levels of plant growth regulators and additional variation in 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 species. The various responses to the differing ages of explant in our experiment are probably due to the heterogeneous developmental states of the tissues. The effect of developmental age resulted in different endogenous levels of plant growth regulators.

In the present study we have described a method for efficient proliferation and high frequency *in vitro* regeneration of rapeseed plants. Increase in the shoot regeneration rate from hypocotyl segments is an important condition for the improvement of the processes of transformation *via Agrobacterium* (Takasaki *et al*., 1997). Several genes of agronomic value, such as herbicide or pathogen resistance, could be introduced into the rapeseed plants with greater efficiency.

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