ISSN 1392-3196
Zemdirbyste-Agriculture, vol. 96, No. 3 (2009), p. 168–175
UDK 633.854.54:631.524.01:631.811.98:632.526.32

Regeneration of adventitious shoots of linseed (*Linum usitatissimum* L.) from hypocotyl explants

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Abstract

In this study, the morphogenic ability of hypocotyl explants of linseed (*Linum usitatissimum* L.) was investigated in relation to genotype and the effect of growth regulators in the medium. Among investigated genotypes, the explants of cultivars 'Mikael' and 'Barbara' manifested the highest and the lowest adventitious bud formation frequency. The optimum ratio of growth regulators for shoot regeneration was shown to depend on the genotype. Growth regulators combinations 2.0 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA were found to be the best for shoot regeneration from hypocotyls of 'Barbara' and 'Mikael' plants, while 2.0 mg l^{-1} BAP in combination with 0.1 mg l^{-1} NAA induced efficient adventitious shoot regeneration frequency with a high number of shoots per explant from hypocotyls of 'Szaphir' plants.

Key words: adventitious shoots, cultivar, growth regulators, hypocotyls, *Linum usitatissimum* L.

Introduction

Linseed (*Linum usitatissimum* L.) is one of the oldest cultivated plants and is the only member of *Linaceae* that is of economic importance. It is a dual-purpose crop, a source of oil and fibre. Breeding of linseed even nowadays is a long and complicated process, based on interspecific hybridization and selection of the best plants, therefore the development of genetically stable lines takes a very long time – 10–12 years. In recent years, plant transformation technology has developed greatly, which has given rise to many new plant lines. Several genes of agronomic value, such as herbicide tolerance or pathogen resistance, have been introduced into the best linseed genotypes /McHughen, Holm, 1991/. Non-conventional breeding methods offer new promising strategies for the improvement of this crop but these techniques require a reproducible *in vitro* regeneration protocol. Despite the availability of various reports on linseed regeneration via indirect/direct organogenesis, the frequency of regeneration was comparatively low and it was largely dependent on the nature of the explants, the cultivars, growth regulator combinations and physical conditions of culture /Bonell, Lassaga, 2002; Blinstrubiene et al., 2004; Chen, Dribnenki, 2004; Burbulis et al., 2005/. Thus, in order to apply genetic transformation technology to linseed improvement and the creation of transgenic plants, it is necessary to evaluate and optimize the *in vitro* culture for direct regeneration of shoots.

Auxin and cytokinin commonly used for plant regeneration from linseed hypocotyls are 1-naphthylacetic acid (NAA) and 6-benzylaminopurine (BAP) /Bretagne et al., 1994; Dedicova et al., 2000; Rutkowska-Krause et al., 2003/. Recently, new cytokinins, like thidiazuron (TDZ) have been used for plant regeneration *in vitro*. Thidiazuron, a substituted urea (1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea) with cytokinin activity, has been as efficient as BAP or even better for promotion of shoot proliferation and adventitious shoot regeneration from various explants of several plants /Phippen, Simon, 2000; Tiwari et al., 2001; Faisal et al., 2005; Siddique, Anis, 2007/. TDZ exhibits the unique property of mimicking both auxin and cytokinin effects on the differentiation of cultured explants, although structurally it is different from both auxins and purine-based cytokinins /Murthy et al., 1998/.

The objective of this study was to develop an efficient protocol for high frequency adventitious shoots regeneration via direct organogenesis from hypocotyl explants of linseed.

Materials and methods

Investigation was carried out during 2007 and 2008 at the Agrobiotechnology Laboratory, Lithuanian University of Agriculture with three linseed varieties 'Barbara', 'Szaphir', 'Mikael'. Seeds were surface sterilized with 10% sodium hypochlorite for 10 min, washed with sterile water and placed for germination and growth *in vitro* on basal MS medium /Murashige, Skoog, 1962/ without growth regulators, supplemented with 10.0 g l^{-1} sucrose and 8.0 g l^{-1} agar. Seeds were incubated at +22 ± 2°C temperature, under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night). Hypocotyls were excised from 4–5 day-old seedling. Explants were placed on MS medium supplemented with different combinations of 1-naphthylacetic acid (NAA) (0–0.1 mg l^{-1}), 6-benzylaminopurine (BAP) (0–2.0 mg l^{-1}), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (TDZ) (0–2.0 mg l^{-1}) and N^6-(2-isopentyl)adenine) (2iP) (0–2.0 mg l^{-1}), 30.0 g l^{-1} sucrose and 8.0 g l^{-1} agar. Media adjusted to pH 5.5 prior to autoclaving at +115°C for 30 min. Culture media (20 ml) were dispensed into 90 mm diameter Petri dishes and sealed with parafilm. Sterilization of explants and transfer of the culture was carried out under aseptic conditions. Explants were cultivated at +22 ± 2°C temperature, under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night).

Experiments were set up in a completely randomized design and three replicates per treatment with 30 explants for each replicate were used. The percentage of bud regeneration [(number of explants with adventitious buds/total number of explants) x 100%] and the number of shoots per explant (number of adventitious shoots/number of explants forming adventitious shoots) were calculated for the explants that had been cultured for 4 weeks. The least significant differences of the results were computed using the software *Anova* /Tarakanovas, Raudonius, 2003/. Mean value and standard error (SE) for each genotype were calculated based on the number of independent replication.

Results and discussion

All the media induced shoot regeneration via direct organogenesis without forming callus. Regeneration potential of hypocotyls segments was explored on

MS medium without and with various plant growth regulators and results are summarized in Table 1.

When linseed hypocotyls explants were placed onto medium without growth regulators, adventitious buds regeneration frequency varied from 5.9% (‘Barbara’) to 82.2% (‘Mikael’). On this medium hypocotyls of cultivar ‘Mikael’ showed the significantly higher buds regeneration frequency in comparison with ‘Barbara’ and ‘Szaphir’.

Table 1. Effect of growth regulators on adventitious buds regeneration frequency from linseed hypocotyls

1 lentelė. *Augimo reguliatorių poveikis sėmeninio lino hipokotilių eksplantų pridėtinių pumpurų susiformavimo dažniui*

Growth regulators *Augimo reguliatoriai* mg l^{-1}				Cultivar / *Veislė*					
				‘Barbara’		‘Szaphir’		‘Mikael’	
				bud formation frequency / *pumpurų formavimosi dažnis*					
BAP	TDZ	2iP	NAA	No. / *vnt.*	%	No. / *vnt.*	%	No. / *vnt.*	%
0	0	0	0	5.3	5.9 ± 0.37	14.0	15.6 ± 0.64	74.0	82.2 ± 0.64
0.25	–	–	0	6.0	6.7 ± 0.64	37.7	41.9 ± 0.74	81.3	90.4 ± 0.98
0.75	–	–	0	21.0	23.3 ± 0.64	85.3	94.8 ± 1.62	89.7	99.6 ± 0.37
1.0	–	–	0	17.3	19.3 ± 0.98	79.7	88.5 ± 0.98	82.3	91.5 ± 0.98
1.0	–	–	0.025	52.3	58.2 ± 0.98	89.7	99.6 ± 0.37	90.0	100 ± 0.00
1.0	–	–	0.05	76.0	84.4 ± 1.28	90.0	100 ± 0.00	90.0	100 ± 0.00
2.0	–	–	0.1	85.0	94.4 ± 1.93	90.0	100 ± 0.00	90.0	100 ± 0.00
–	0.25	–	0	29.0	32.2 ± 0.64	83.0	91.9 ± 0.74	85.0	94.4 ± 1.28
–	0.75	–	0	39.7	44.1 ± 0.37	89.7	99.6 ± 0.37	89.7	99.6 ± 0.37
–	1.0	–	0	33.0	36.7 ± 0.64	80.0	88.9 ± 0.85	82.7	91.9 ± 0.98
–	1.0	–	0.025	63.3	70.4 ± 1.33	90.0	100 ± 0.00	90.0	100 ± 0.00
–	1.0	–	0.05	74.0	82.2 ± 1.93	90.0	100 ± 0.00	90.0	100 ± 0.00
–	2.0	–	0.1	81.0	90.4 ± 1.33	90.0	100 ± 0.00	90.0	100 ± 0.00
–	–	0.25	0	27.3	30.4 ± 0.37	38.0	42.2 ± 0.64	86.7	96.3 ± 1.34
–	–	0.75	0	41.0	45.6 ± 0.64	79.3	88.2 ± 0.98	89.7	99.6 ± 0.37
–	–	1.0	0	34.0	37.8 ± 0.64	70.7	78.5 ± 0.98	76.0	84.4 ± 0.64
–	–	1.0	0.025	54.0	60.0 ± 1.11	89.7	99.6 ± 0.37	90.0	100 ± 0.00
–	–	1.0	0.05	77.3	85.9 ± 1.34	90.0	100 ± 0.00	90.0	100 ± 0.00
–	–	2.0	0.1	85.0	94.1 ± 1.62	90.0	100 ± 0.00	90.0	100 ± 0.00

Pastaba / *Note*. Data are mean ± SE within 4 weeks of culture / *Vidutiniai duomenys* ± $S\bar{x}$ *po 4 kultivavimo savaičių.*

Explants cultured on media fortified with cytokinins alone induced buds at a higher frequency compared to the medium without growth regulators. Buds formation frequency of tested cultivars was significantly increased with increase in cytokinins concentration from 0.25 mg l^{-1} to 0.75 mg l^{-1}. Hypocotyls of ‘Szaphir’ showed the best response on medium supplemented with 0.75 mg l^{-1} TDZ, while hypocotyls of ‘Barbara’ showed similar morphogenic response on media containing TDZ or 2iP (0.75 mg l^{-1}), 44.1% and 45.6% respectively. However, for cultivar ‘Mikael’ there was no significant

difference among tested cytokinins. Further increasing cytokinins concentrations resulted in decreased bud regeneration frequency. Cytokinin concentration in the medium was probably too high and thus inhibited adventitious buds formation.

The use of NAA in combination with tested cytokinins resulted in significant increasing in bud formation frequency for cultivars ʻBarbaraʼ and ʻSzaphirʼ. The best response was obtained on medium containing 2.0 mg l^{-1} BAP with 0.1 mg l^{-1} NAA and 2.0 mg l^{-1} 2iP with 0.1 mg l^{-1} NAA (ʻBarbaraʼ), and on medium supplemented with 1.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA and 1.0 mg l^{-1} 2iP with 0.05 mg l^{-1} NAA (ʻSzaphirʼ).

In terms of the number of shoots produced per explants, the tested cultivars showed a high variation (Table 2).

In the presence of only cytokinins, the highest mean value of regenerated shoots per explant was observed on MS with 0.75 mg l^{-1} BAP (ʻSzaphirʼ), while 0.75 mg l^{-1} TDZ induced the highest number of shoots from hypocotyls of ʻBarbaraʼ and ʻMikaelʼ.

Table 2. Effect of growth regulators on the number of adventitious shoots per linseed explant

2 lentelė. *Augimo reguliatorių poveikis sėmeninio lino eksplantų pridėtinių ūglių kiekiui*

Growth regulators *Augimo reguliatoriai* mg l^{-1}				Cultivar / *Veislė*		
				ʻBarbaraʼ	ʻSzaphirʼ	ʻMikaelʼ
BAP	TDZ	2iP	NAA	Number of shoots per explant / *Ūglių kiekis eksplante*		
0	0	0	0	1.0 ± 0.58	5.0 ± 0.58	3.7 ± 0.33
0.25	–	–	0	1.3 ± 0.33	9.3 ± 0.33	5.7 ± 0.33
0.75	–	–	0	2.3 ± 0.33	15.3 ± 0.33	6.0 ± 0.58
1.0	–	–	0	2.0 ± 0.00	14.7 ± 0.33	5.7 ± 0.33
1.0	–	–	0.025	2.7 ± 0.33	16.3 ± 0.33	10.7 ± 0.88
1.0	–	–	0.05	3.3 ± 0.33	19.7 ± 0.33	12.3 ± 1.20
2.0	–	–	0.1	3.7 ± 0.33	22.0 ± 0.58	13.0 ± 1.00
–	0.25	–	0	3.7 ± 0.33	10.0 ± 0.58	6.3 ± 0.33
–	0.75	–	0	4.3 ± 0.33	12.7 ± 0.88	11.7 ± 0.58
–	1.0	–	0	4.0 ± 0.58	12.3 ± 0.33	8.0 ± 0.33
–	1.0	–	0.025	4.7 ± 0.33	13.3 ± 0.33	12.7 ± 0.33
–	1.0	–	0.05	5.0 ± 0.58	14.0 ± 0.58	13.0 ± 0.58
–	2.0	–	0.1	5.7 ± 0.33	15.0 ± 0.58	15.3 ± 0.88
–	–	0.25	0	2.3 ± 0.33	5.0 ± 0.58	4.0 ± 0.58
–	–	0.75	0	3.0 ± 0.58	8.3 ± 0.58	6.0 ± 0.33
–	–	1.0	0	2.7 ± 0.33	7.0 ± 0.33	5.3 ± 0.33
–	–	1.0	0.025	3.3 ± 0.67	9.3 ± 0.67	6.3 ± 0.58
–	–	1.0	0.05	3.7 ± 0.33	11.7 ± 0.88	7.0 ± 0.58
–	–	2.0	0.1	4.0 ± 0.58	16.0 ± 0.58	8.0 ± 0.58

Pastaba / *Note*. Data are mean ± SE within 4 weeks of culture / *Vidutiniai duomenys ± $S\bar{x}$ po 4 kultivavimo savaičių.*

Addition of NAA markedly enhanced the number of shoots per explants for all the three cultivars tested. The maximum number of shoots per explant was obtained at 1.0 mg l^{-1} BAP with 0.05 mg l^{-1} NAA and at 2.0 mg l^{-1} BAP with 0.1 mg l^{-1} NAA ('Szaphir') and at 2.0 mg l^{-1} TDZ with 0.1 mg l^{-1} NAA ('Barbara' and 'Mikael'). 6-benzylaminopurine (BAP) has been widely employed for the *in vitro* culture of linseed. It plays an important role in both the induction and regeneration of linseed via direct adventitious shoot organogenesis /Dedicova et al., 2000/ or through callus from various explants of a range of linseed germplasms /Blinstrubiene et al., 2004/. N^6-(2-isopentyl)adenine (2iP) is not very often used in tissue culture, however exzogenous 2iP has shown beneficial effect on adventitious shoot formation for some plants /Mary Mathew et al., 1999; Maity et al., 2005/. Improved regeneration using the urea-based cytokinin TDZ, rather than adenine-based cytokinins, is well documented in different plant species /Bacchetta et al., 2003; Faisal et al., 2005; Dai et al., 2007/. There are now many publications that have investigated different aspects of the effect of TDZ on the physiology of plant regeneration. One reason for great level of interest this compound has attracted is due to its unique mode of action which may involve, at least in part, the modulation of the levels of endogenous growth regulators, polar auxin transport, and accumulation of proline in TDZ-induced tissues /Murthy et al., 1995/.

In linseed it was reported that the efficiency of thidiazuron is much higher than that of the other auxin/cytokinin combinations /Dedicova et al., 2000; Mundhara, Rashid, 2006/. Bretagne et al. (1994) indicated that medium containing TDZ in combination with NAA resulted in the highest shoot regeneration frequency from linseed hypocotyls.

In the work presented here, 2.0 mg l^{-1} TDZ with 0.1 mg l^{-1} NAA were found to be the best combination for adventitious shoots regeneration from hypocotyls of cultivars 'Barbara' and 'Mikael'. However, medium supplemented with 2.0 mg l^{-1} BAP and 0.1 mg l^{-1} NAA was the most effective for cultivar 'Szaphir', providing a high shoot regeneration frequency (100%) associated with a high number of shoots (22.0) per explants. Similar results were obtained by Matt and Jehle (2005) in *Prunus avium* where highest regeneration rates were observed for cultivar 'Schneiders' on medium supplemented with BAP and indole-3-butyric acid (IBA) while all other tested cultivars showed highest regeneration with TDZ in combination with IBA. The current study indicates that there is a strong genotype effect on adventitious buds regeneration from linseed hypocotyls, and therefore specific combination of growth regulators must be designed for each genotype.

Among the many factors that may affect plant tissue culture responses, especially regeneration ability, genotypic difference is a primary one. A great genetic diversity in tissue culture responses has been reported among cultivars of many crops, such as *Lilium longiflorum* /Bacchetta et al., 2003/, *Beta vulgaris* /Zhang et al., 2004/, *Crataegus pinnatifida* /Dai et al., 2007/, *Brassica napus* /Burbulis et al., 2008/. In the present study the three genotypes exhibited different regeneration responses. Hypocotyls segments from cultivar 'Szaphir' gave the best results, maximum percentage of regeneration frequency and the number of shoots per explant reached 100% and 22.0, respectively. The two other tested cultivars had a lower organogenic response. Variability in morphogenic response and the different growth regulator requirements for

hypocotyls culture of each genotype could suggest a different endogenous hormone balance of the explants, as indicated for other plants /Zhang et al., 2004; Maity et al., 2005/.

Conclusion

In this study, the system of adventitious shoot regeneration from hypocotyls segments of three linseed cultivars was optimized by using different plant growth regulators. The highest percentage of bud regeneration frequency reached 94.4% for cultivar 'Barbara' and 100% for cultivars 'Mikael' and 'Szaphir' and the maximum numbers of shoots per explants were 5.7, 22.0 and 15.3, respectively. The procedure for adventitious shoots organogenesis from linseed hypocotyls segment could potentially be useful in linseed transformation technology.

Received 17 02 2009
Accepted 15 04 2009

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ISSN 1392-3196
Žemdirbystė-Agriculture, t. 96, Nr. 3 (2009), p. 168–175
UDK 633.854.54:631.524.01:631.811.98:632.526.32

Sėmeninio lino (*Linum usitatissimum* L.) pridėtinių ūglių regeneracija iš hipokotilių kultūros

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Santrauka

Tirtas genotipo ir egzogeninių augimo reguliatorių poveikis sėmeninio lino (*Linum usitatissimum* L.) morfogenezei *in vitro* hipokotilių kultūroje. Veislės 'Mikael' hipokotilių audiniai pridėtinius pumpurus formavo didžiausiu dažniu, o veislės 'Barbara' – mažiausiu. Nustatyta, kad optimalus augimo reguliatorių derinys maitinamojoje terpėje priklauso nuo genotipo. Augimo reguliatorių – 2,0 mg l^{-1} 1-fenil-3-(1,2,3-tiadiazol-5-il)-karbamido (TDZ) ir 0,1 mg l^{-1} α naftilacto rūgšties (NAR) – derinys skatino veislių 'Barbara' ir 'Mikael' tiesioginę ūglių regeneraciją iš hipokotilių audinių, o veislės 'Szaphir' didžiausias ir pridėtinių ūglių susiformavimo dažnis, ir eksplanto ūglių kiekis nustatytas terpėje, papildytoje 2,0 mg l^{-1} 6-benzilaminopurino (BAP) ir 0,1 mg l^{-1} NAR.

Reikšminiai žodžiai: pridėtiniai ūgliai, veislė, augimo reguliatoriai, hipokotiliai, *Linum usitatissimum* L.