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Organogenesis in callus cultures of *Linum usitatissimum* L.

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Abstract

Linseed flax cultivars 'Lirina', 'Barbara' and 'Szaphir' were tested for their regeneration capacity through adventitious shoot organogenesis from hypocotyl-derived and stem segment-derived callus. Shoots number per explant was strongly influenced by genotype, culture medium and application of growth regulators. The highest shoot number per explant was obtained from hypocotyl-derived callus of cultivar 'Szaphir' on combined MSB_5 media, supplemented by 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine. Hypocotyl-derived callus gave the better results than stem segment-derived callus. Four-week-old callus showed the maximum shoot regeneration frequency.

Key words: callus, genotype, growth regulators, linseed flax, shoot organogenesis.

Introduction

Flax belongs to the family *Linaceae*, with more than 200 species, and only one species – common flax (*Linum usitatissimum* L.) has a practical use. Common flax is a plant with a long history of cultivation and breeding in Lithuania (Bacelis 2001), although it is attracting new interest in Europe. The fibre flax cultivars are mostly grown in Lithuania, but recently the interest in oil cultivars is also increasing. The breeders of the Lithuanian University of Agriculture have started to work on breeding of linseed flax using tissue culture techniques.

The development of procedures for efficient regeneration of plants from cultured cells, tissues and organs are a prerequisite for application of *in vitro* culture techniques to plant gene manipulation for crop germplasm enhancement (Zhang et al. 2004). The capacity of cells to regenerate via different morphogenic programmes is a result of cell dedifferentiating to become competent to the stimulus. This is then followed by induction for the developmental programme and eventual development into the organ (Nhut et al. 2003). Regeneration methods frequently depend on the type of tissue used to initiate cultures, with the generation or acquisition of starting material potentially becoming a limiting factor (Koroch et al. 2003).

Flax improvement has not developed at the same rate as in other crops (mainly cereals) in recent years. Tissue culture can speed up the novel breeding which can lead to flax improvement and even to incorporation of valuable and desirable traits into flax cultivars

(resistance to fungal diseases, oil quality improvement and herbicide tolerance) through somatic hybridization and somaclonal variation (Basiran et al. 1987).

Although tissue culture of *Linum usitatissimum* L. has been carried out for more than 20 years information about what controls organogenesis and embryogenesis in this species and its cultivars is still scarce (Dedicova et al. 2000).

In the present paper we report the organogenetic response of linseed flax callus to different media, supplemented by growth regulators.

Materials and methods

The investigation was carried out with three linseed varieties: 'Lirina', 'Barbara', and 'Szaphir'. To study linseed morphogenesis, hypocotyls and stem segments were used as explants. Tissue culture protocols were the same as described previously (Blinstrubiene et al. 2004). Linseed tissue was plated on basal Murashige and Skoog (1962; MS) medium, B_5 medium (Gamborg 1975) and on combined medium MSB5 (MS macro salts and B_5 micro salts with vitamins), 0.75MSB_5 (75 % MS macro salts and B_5 micro salts with vitamins), 0.5MSB_5 (50 % MS macro salts and B_5 micro salts with vitamins) supplemented with sucrose 30 g l^{-1} and Difco-Bacto agar 6 g l^{-1}. The following growth regulator combinations were used: 1.0 mg l^{-1} kinetin + 0.1 mg l^{-1} indole-3-acetic acid (IAA), 1.0 mg l^{-1} 6-benzylaminopurine (BAP) + 0.05 mg l^{-1} α-naphtylacetic acid (NAA), 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine (2iP), 1.0 mg l^{-1} kinetin. The media pH was 5.7 ± 0.1, illumination – 5000 lx, photoperiod – 16 h, temperature 25 ± 2 °C. Each variant consisted of 50 explants and four replications were used. Induced callus were transferred to the same fresh medium every four weeks using 50 ml media into 200 ml glassware.

The morphogenic potential of tissues was evaluated by analyzing morphological parameters of the structures formed on the explants. The evaluation was based on the relative frequency of callus producing shoots (%) as well as the average number of shoots per explant.

Significant differences were evaluated using a computer program (Tarakanovas 1996) for ANOVA. The mean value and SE for each genotype were calculated.

Results

The shoots were produced spontaneously from the green soft callus of common flax with or without subculture onto fresh medium. Organogenesis of shoots from hypocotyl-derived callus that had been grown for four weeks on the culture media is shown in Table 1.

The number of shoots per explant was significantly affected by the culture media. The use of combined MSB_5 medium resulted in significantly more shoots per explant than any other medium and this was observed for all three cultivars. However, there were differences in shoot formation on MSB_5 medium supplemented with different growth regulators. Kinetin resulted in less shoot regeneration frequency in all cultivars, whereas 2iP had a stimulative effect on shoot formation in all cultivars tested. Cultivars differed significantly in the number of shoots per explant. Hypocotyl-derived callus from the cultivar 'Szaphir' gave the best results, while the cultivar 'Lirina' had the lowest organogenous response. MSB_5 medium supplemented with 2iP gave a regeneration rate of 4.67 shoots per explant for cultivar 'Szaphir'; 3.84 for 'Barbara' and 2.96 for 'Lirina'. This growth regulator appeared

to give the best regeneration frequency for all three cultivars tested.

The observed effects of medium composition on shoot organogenesis from stem segment-derived callus are summarized in Table 2.

Generally, stem segment-derived callus response to medium composition was similar

Table 1. Effect of medium composition and growth regulators on number of shoots per hypocotyl-derived callus of three linseed flax (*Linum usitatissimum* L.) cultivars. Data are means ± SE within four weeks of culture. MS, Murashige and Skoog medium; B_5, Gamborg medium; IAA, indole-3-acetic acid; BAP, 6-benzylaminopurine; NAA, naphthylacetic acid; 2iP, N^6-Δ^2-isopentenyl adenine

Geno-type	Growth regulators (mg l^{-1})	Number of shoots per explant				
		MS	B_5	MSB_5	$0.75MSB_5$	$0.5MSB_5$
'Lirina'	kinetin 1.0 + IAA 0.1	0.68 ± 0.08	1.30 ± 0.11	1.59 ± 0.14	0.34 ± 0.02	0.15 ± 0.01
'Lirina'	BAP 1.0 + NAA 0.05	1.84 ± 0.10	1.98 ± 0.12	2.52 ± 0.15	0.50 ± 0.03	0.23 ± 0.02
'Lirina'	2iP 2.0	2.39 ± 0.10	2.49 ± 0.10	2.96 ± 0.13	0.59 ± 0.03	0.29 ± 0.02
'Lirina'	kinetin 1.0	0.49 ± 0.12	1.17 ± 0.10	1.20 ± 0.11	0.26 ± 0.02	0.10 ± 0.01
'Barbara'	kinetin 1.0 + IAA 0.1	2.25 ± 0.13	2.66 ± 0.15	3.10 ± 0.18	0.75 ± 0.02	0.30 ± 0.01
'Barbara'	BAP 1.0 + NAA 0.05	2.28 ± 0.15	2.96 ± 0.17	3.24 ± 0.20	0.86 ± 0.02	0.42 ± 0.02
'Barbara'	2iP 2.0	2.60 ± 0.17	3.15 ± 0.20	3.84 ± 0.22	0.90 ± 0.03	0.49 ± 0.01
'Barbara'	kinetin 1.0	1.85 ± 0.14	2.34 ± 0.11	2.45 ± 0.10	0.64 ± 0.02	0.33 ± 0.01
'Szaphir'	kinetin 1.0 + IAA 0.1	2.39 ± 0.20	2.79 ± 0.23	3.38 ± 0.21	1.15 ± 0.10	0.60 ± 0.01
'Szaphir'	BAP 1.0 + NAA 0.05	2.67 ± 0.24	3.18 ± 0.20	3.90 ± 0.27	1.27 ± 0.11	0.68 ± 0.03
'Szaphir'	2iP 2.0	3.26 ± 0.26	3.62 ± 0.24	4.67 ± 0.30	1.53 ± 0.10	0.75 ± 0.05
'Szaphir'	kinetin 1.0	2.09 ± 0.15	2.47 ± 0.18	3.15 ± 0.20	1.09 ± 0.07	0.46 ± 0.02

Table 2. Effect of medium composition and growth regulators on number of shoots per stem segment-derived callus of three linseed flax (*Linum usitatissimum* L.) cultivars. Data are means ± SE within four weeks of culture. MS, Murashige and Skoog medium; B_5, Gamborg medium; IAA, indole-3-acetic acid; BAP, 6-benzylaminopurine; NAA, naphthylacetic acid; 2iP, N^6-Δ^2-isopentenyl adenine

Geno-type	Growth regulators (mg l^{-1})	Number of shoots per explant				
		MS	B_5	MSB_5	$0.75MSB_5$	$0.5MSB_5$
'Lirina'	kinetin 1.0 + IAA 0.1	0	1.20 ± 0.10	1.47 ± 0.14	0.26 ± 0.02	0.10 ± 0.01
'Lirina'	BAP 1.0 + NAA 0.05	1.73 ± 0.10	1.81 ± 0.16	2.00 ± 0.20	0.40 ± 0.09	0.15 ± 0.01
'Lirina'	2iP 2.0	2.24 ± 0.11	2.30 ± 0.10	2.37 ± 0.12	0.44 ± 0.08	0.20 ± 0.02
'Lirina'	kinetin 1.0	0	1.10 ± 0.10	1.15 ± 0.12	0.20 ± 0.02	0.07 ± 0.01
'Barbara'	kinetin 1.0 + IAA 0.1	1.92 ± 0.16	2.34 ± 0.20	2.79 ± 0.22	0.63 ± 0.07	0.22 ± 0.01
'Barbara'	BAP 1.0 + NAA 0.05	2.15 ± 0.18	2.51 ± 0.21	2.86 ± 0.22	0.68 ± 0.08	0.30 ± 0.04
'Barbara'	2iP 2.0	2.46 ± 0.21	2.89 ± 0.23	3.00 ± 0.21	0.75 ± 0.06	0.32 ± 0.03
'Barbara'	kinetin 1.0	1.40 ± 0.13	2.00 ± 0.16	2.19 ± 0.15	0.54 ± 0.05	0.16 ± 0.01
'Szaphir'	kinetin 1.0 + IAA 0.1	2.11 ± 0.15	2.48 ± 0.10	2.94 ± 0.16	0.85 ± 0.06	0.38 ± 0.02
'Szaphir'	BAP 1.0 + NAA 0.05	2.49 ± 0.17	2.89 ± 0.20	3.21 ± 0.23	0.92 ± 0.05	0.46 ± 0.03
'Szaphir'	2iP 2.0	2.97 ± 0.20	3.46 ± 0.24	3.76 ± 0.26	1.10 ± 0.07	0.53 ± 0.04
'Szaphir'	kinetin 1.0	1.86 ± 0.16	2.21 ± 0.18	2.65 ± 0.21	0.77 ± 0.05	0.28 ± 0.02

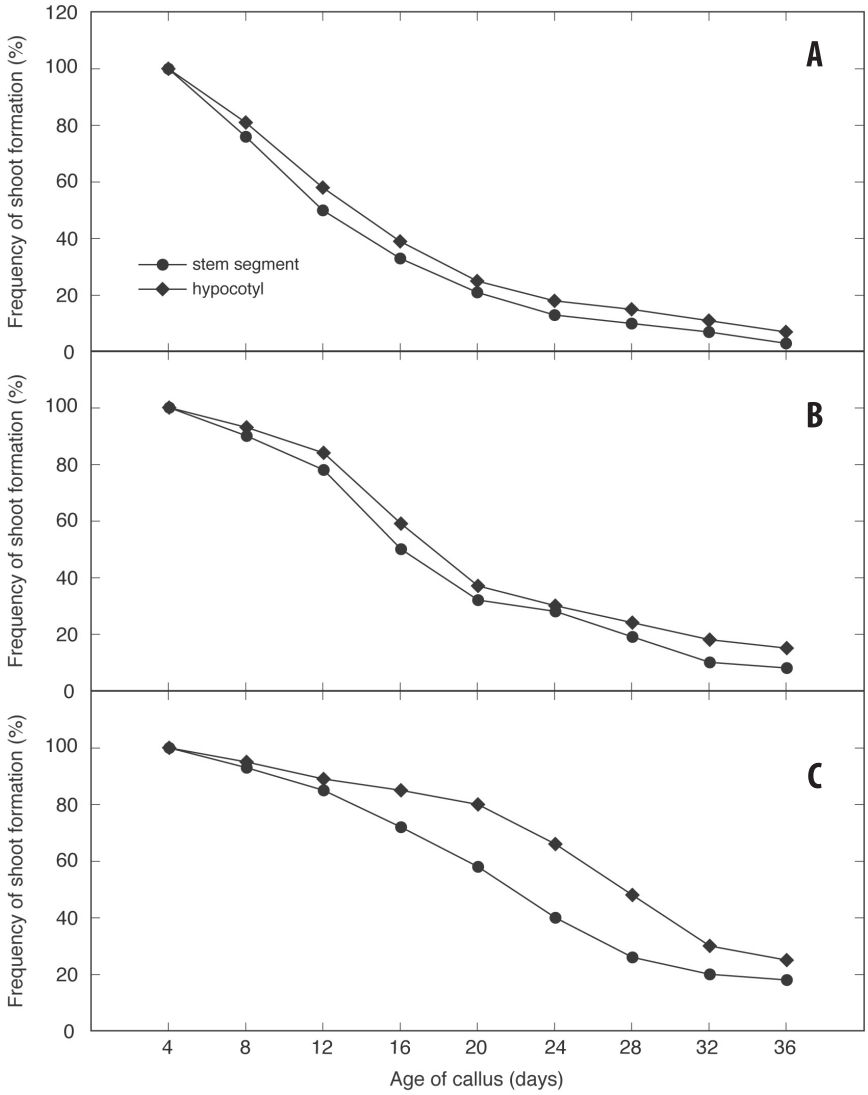

Fig. 1. Effect of callus age on frequency of shoot formation of linseed flax cultivars 'Lirina' (A), 'Barbara' (B) and 'Szaphir' (C).

to the hypocotyl-derived callus. The highest shoot number per explant was obtained on combined MSB_5 medium supplemented with 2iP.

There were significant differences in response to shoot regeneration among the tested cultivars. The number of shoots produced per stem segment-derived callus ranged from 3.76 for cultivar 'Szaphir' to 2.37 for cultivar 'Lirina' (Table 2). The results indicate that shoot regeneration ability is strongly influenced by the genotype used.

The age of callus also affected shoot regeneration in oilseed flax. Four-week-old callus showed the maximum shoot regeneration frequency (Fig. 1).

The shoot regeneration frequency decreased with an increase in callus age from 4 to 36 weeks, similarly for stem segment-derived and hypocotyls-derived callus, excepting cultivar 'Szaphir'. The organogenesis potential of hypocotyl-derived callus of 'Szaphir' declined less with age: the shoot formation frequency from twenty-weeks-old callus was only 20 % less than that from four-week-old callus. Among the tested cultivars, the morphogenic potential was most rapidly lost with age for callus of cultivar 'Lirina'.

Discussion

The process of organogenesis appears to be complex, involving multiple internal and external factors. The reinitiation of cell division, considered to be one of the key factors during regeneration, appears to be controlled differently depending on the model system. The type of first division under inductive conditions can be different (Blervacq et al. 1995), often depending on growth regulators in the culture medium and the type of the primary explant used (Dedicova et al. 2000).

Studies spanning the past 50 years have shown the effects of plant hormones on cell proliferation. However, because most hormones also provoke morphogenic effects, the cell-cycle consequences may be direct or part of the morphogenetic response. Cytokinins and auxins are indispensable for maintaining undifferentiated cells in proliferation during in vitro culture and are directly linked to cell proliferation (Dewitte, Murray 2003). Many plant species require both exogenous auxin and cytokinin in a suitable proportion in order to induce shoot formation. Chen et al. (1998) observed that a medium containing the combination of 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 1 mg l^{-1} BAP significantly improved shoot regeneration in an anther culture of flax. In another study, the maximum shoot regeneration frequency in *Brassica* species was obtained in medium supplemented with 3.0 mg l^{-1} BAP and 0.15 mg l^{-1} NAA (Tang et al. 2003). Hypocotyls of *Beta vulgaris* showed the best response of adventitious shoot regeneration in medium supplemented by BAP and NAA (Zhang et al. 2004). However, other studies have shown that shoot formation on hypocotyls of *Linum* seedlings was marginally promoted by a cytokinin (BAP) or thidiazuron (Mundhara, Rashid 2002).

Although the use of low auxin/cytokinin ratios is common for *in vitro* shoot induction of flax (Marchall, Courduries 1992; Cunha, Ferreira 1996), the development of adventitious shoots of the tested linseed cultivars seems to be determined by a low 2iP concentration. The present results suggest that kinetin, at least at concentration above 1.0 mg l^{-1}, inhibits shoots regeneration from oilseed flax callus. In fact, the kinetin in combination with 0.1 mg l^{-1} IAA had lowest inhibitory effect on the development of shoots.

A single media was employed by Blinstrubiene et al. (2004) to induce callus and regeneration from stem segments and hypocotyls. Generally there was no difficulty in the induction of callus, with the stem segments producing more callus than the hypocotyls. However, the present results show that hypocotyl-derived callus had better morphogenic ability in comparison with stem segment-derived callus. Successful shoot regeneration was found to depend on genotype and culture media. This is in agreement with the results of other published work with flax cultivars (Chen et al. 1998; Nichterlein et al. 1991).

In conclusion, this study demonstrates that hypocotyls and stem segments of linseed flax can be used for adventitious shoot organogenesis and provide a regeneration method using a convenient and abundant tissue source that will facilitate the application of

biotechnology for the continued study and improvement of this plant species.

References

Bacelis K. 2001. Achievements in fibre flax breeding. *Agriculture* 75: 206–214.

Barisan N., Armitage P., Scoot R.J., Draper J. 1987. Genetic transformation of flax (*Linum usitatissimum* L.) by *Agrobacterium tumefaciens*: Regeneration of transformed shoots via a callus phase. *Plant Cell Rep.* 6: 396–399.

Blervacq A.C., Dubois T., Dubois J., Vasseur J. 1995. First division of somatic cells in cichorium hybrids "74". *Protoplasma* 186: 163–168.

Blinstrubiene A., Burbulis N., Sliesaravicius A. 2004. Effect of genotype and medium composition on linseed (*Linum usitatissimum* L.) morphogenesis in tissue culture. *Agriculture* 86: 13–21.

Chen Y., Kenaschuk E., Dribnenki P. 1998. High frequency of plant regeneration from anther culture in flax, *Linum usitatissimum* L. *Plant Breeding* 117: 463–467.

Cunha A.C.G., Ferreira M.F. 1996. Somatic embryogenesis, organogenesis and callus growth kinetics of flax. *Plant Cell Tissue Organ Cult.* 47: 1–8.

Dedicova B., Hricova A., Samaj J., Obert B., Bobak M., Pretova A. 2000. Shoots and embryo-like structures regenerated from cultured flax (*Linum usitatissimum* L.) hypocotyl segments. *J. Plant Physiol.* 157: 327–334.

Dewitte W., Murray J.A.H. 2003. The plant cell cycle. *Annu. Rev. Plant Biol.* 54: 235–264.

Gamborg O.L. 1975. Callus and cell culture. In: *Plants tissue culture methods*, pp. 1–10.

Koroch A.R., Karteyn J., Juliani H.R., Simon J.E. 2003. *In vitro* regeneration of *Echinacea pallida* from leaf explants. *In Vitro Cell. Dev. Biol. Plant* 39: 415–418.

Marshall G., Courduries P. 1992. An assessment of somaclonal variation in linseed (*Linum usitatissimum* L.). *Ann. Appl. Biol.* 120: 501–509.

Mundhara R., Rashid A. 2002. Stimulation of shoot-bud regeneration on hypocotyl of *Linum* seedlings, on a transient withdrawal of calcium: effect of calcium, cytokinin and thidiazuron. *Plant Sci.* 162: 211-214.

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

Nhut D.T., da Silva J.A.T., Aswath C.R. 2003. The importance of the explant on regeneration in thin cell layer technology. *In Vitro Cell. Dev. Biol. Plant* 39: 266–276.

Nichterlein K., Umbach H., Friedt W. 1991. Genotypic and exogenous factors affecting shoot regeneration from anther callus of linseed (*Linum usitatissimum* L.). *Euphytica* 58: 157–164.

Tang G.X., Zhou W.J., Li H.Z., Mao B.Z., He Z.H., Yoneyama J. 2003. Medium, explant and genotype factors influencing shoot regeneration in oilseed *Brassica* spp. *J. Agron. Crop Sci.* 189: 351–358.

Tarakanovas P. 1996. *The Computer System "Selekcija" for Processing and Evaluation of Breeding-Genetical Research Results.* Akademija, Dotnuva. 76 p. (in Lithuanian)

Zhang C.L., Chen D.F., Elliot M.C., Slater A. 2004. Efficient procedure for callus induction and adventitious shoot organogenesis in sugar beet (*Beta vulgaris* L.) breeding lines. *In Vitro Cell. Dev. Biol. Plant* 40: 475–481.

Organoģenēze *Linum usitatissimum* kallusa kultūrā

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Kopsavilkums

Pārbaudīja sēklu linu šķirņu 'Lirina', 'Barbara' un 'Szaphir' reģenerācijas spēju adventīvo dzinumu veidošanā no kallusiem, kas iegūti no hipokotila un stumbra segmentiem. Uz vienu eksplantu iegūto dzinumu daudzumu ievērojami ietkmēja genotips, kultivēšanas barotne un izmantotie augšanas regulatori. Vislielāko skaitu dzinumu uz eksplantu ieguva uz hipokotila kallusa šķirnei 'Szaphir', kultivējot uz kombinētās barotnes MSB_5 2.0 mg l^{-1} N^6-Δ^2-isopentenil adenīna klātbūtnē. Hipokotila kallusu izmantošana ļāva iegūt labākus rezultātus, nekā stumbra segmentu kallusu izmantošana. Maksimālā dzinumu reģenerācijas frekvence bija četras nedēļas veciem kallusiem.