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Genotypic and exogenous factors affecting linseed ovary culture

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Abstract

The current study investigated the effect of genotype, growth regulators and type of carbohydrates on callus induction and indirect shoot regeneration in ovary culture of 8 linseed (*Linum usitatissimum* L.) cultivars. Callogenic response varied from 9.17% to 100% depending on the cultivars and medium composition interaction. The replacement of sucrose with a combination of sucrose + maltose significantly improved the callogenesis in 3 or 4 investigated cultivars, depending on growth regulators in the induction medium. The frequency of shoot formation from ovary-derived callus in 5 responsive cultivars ranged from 4.17% to 75.00%, whereas the other three cultivars tested did not exhibit any shoots. The replacement of sucrose with a sucrose + maltose combination in induction medium reduced or completely inhibited shoot formation frequency of responsive cultivars. The significantly highest mean shoot formation frequency (52.50%) was obtained from ovary-derived callus of the cultivar 'Mikael'. The analysis of variance revealed that cultivar (C), combination of growth regulators (GR), type of carbohydrates (CH) and their interaction significantly influenced callus induction and shoot formation frequency. In most cases, a higher shoot regeneration frequency was obtained when callus was from induction medium supplemented with 2 mg l^{-1} thidiazuron (TDZ) + 1 mg l^{-1} α-naphthylacetic acid (NAA) with 6% sucrose. Cytological analysis of root tips showed that 21.88% of the regenerated plants were haploids, while another group of regenerants (78.12%) were diploid and mixoploid.

Key words: carbohydrates, genotype, growth regulators, *Linum usitatissimum*, morphogenic response, ovary culture.

Introduction

Linseed (*Linum usitatissimum* L.) is one of the most economically important oilseed crops in many regions of the world. Linseed oil is widely used for healthy food production, in medicine, chemistry and technical industry as well as valuable feed for livestock (Tolkachev, Zhuchenko, 2000; Zuk et al., 2015). Consequently, nowadays linseed breeding is focused on improvement of fatty acids composition in order to create cultivars with a lowered α-linolenic acid content (Pretova, Obert, 2000). Breeding efforts are needed for further development of low linolenic acid varieties, the oil of which can be widely used as cooking oil. As linseed is highly nutritious, it is necessary to reduce its anti-nutrient components and also bio-convert its less acceptable alpha-linolenic acid (18:3ω3) into acceptable stearidonic acid (18:4ω3). More concerted efforts for development of varieties resistant to different disease like wilt, rust, powdery mildew and *Alternaria* blight are also required (Yadava et al., 2012; Pavelek et al., 2015).

Development of novel genotypes by conventional breeding methods is time consuming and complicated (Mikelsone et al., 2013); therefore the development of alternative methods for producing homozygous lines is required. Development of haploids by biotechnological methods leads to the creation of homozygous genotypes in one generation; therefore a double haploid system is an extremely valuable breeding tool (Wedzony et al., 2009). Plant regeneration *in vitro* from isolated ovary has been achieved in some crops such as onion (Kamštaitytė, Stanys, 2002), maize (Tang et al., 2006), sugar beet (Gurel et al., 2000), wheat (Sibi et al., 2001). Lately, the overall plant regeneration frequency in flax from anther has been significantly increased by the modification of medium composition (Chen, Dribnenki, 2002; Rutkowska-Krause et al., 2003; Burbulis et al., 2005) and culture conditions (Obert et al., 2004 a). However, application of ovary culture for linseed haploid production has been documented in few reports (Bartosova et al., 2003; Obert et al., 2004 b); therefore little information is available concerning the various factors that contribute to the successful gynogenesis induction. One of the most important factors for morphogenesis induction in ovary culture is a properly chosen combination of growth regulators (Chand, Sahrawat, 2007). A significant effect of the thidiazuron (TZD) on organogenesis was reported for many plants, such as *Cannabis sativa* L. (Lata

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et al., 2009), *Rubus fruticosus* L. (Vujović et al., 2010), *Cymbidium giganteum* Wall. ex Lindl. (Ghosh et al., 2014). According to Guo et al. (2011), thidiazuron has strong cytokinin-like activity and stimulates effective morphogenic responses in *in vitro* cultures. Many research groups have reported that sucrose is the best carbon source for morphogenesis induction *in vitro* from all types of explants. However, it has been found that maltose is more suitable for callus formation and plant regeneration in anther culture of barley (Finnie et al., 1989), wheat (Navarro-Alvarez et al., 1994; Redha, Talaat, 2008) and flax (Tejklova, 1998). The aim of the present study was to investigate the effect of growth regulators and type of carbohydrates on morphogenic response in linseed ovary culture.

Materials and methods

Research was carried out in 2015–2016 in Institute of Biology and Plant Biotechnology of Aleksandras Stulginskis University and Laboratory of Agrobiotechnology of Joint Research Centre.

Plant material and tissue culture. Experiments were carried out with 8 linseed (*Linum usitatissimum* L.) cultivars: 'Atalante', 'Dnepr-2', 'Indus-2', 'Lirina', 'Mikael', 'Norman', 'Valuta-1' and 'Zaltan-1'. Seeds were germinated and grown in a growth chamber with a 16 h photoperiod, temperature 18/14°C (day/night) and 75% humidity. All plants were grown in a mixture of peat, vermiculite and sand at 3:1:2 ratio in 16.5 cm pots. The plants were watered and fertilized with diluted 20:20:20 N:P:K at a rate of 4 g l^{-1} as required.

Harvested buds (3.5–4.0 mm in length) were surface sterilized in 70% ethanol for 1 min, then in 2% sodium hypochlorite for 10 min and rinsed three times with sterile distilled water. The ovaries were placed onto a plastic Petri dish containing 15 ml of modified MS (NH_4NO_3 – 165 mg l^{-1}) induction MS medium (Murashige, Skoog, 1962) solidified with 0.6% agar and incubated at 25°C in the dark. Combinations of cytokinin and auxin (2 mg l^{-1} thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea) (TDZ) + 1 mg l^{-1} α-naphthylacetic acid (NAA) and 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA) and carbohydrate (6% sucrose or 3% sucrose + 3% maltose) were selected based on the results from our previous experiments (unpublished data). Every four weeks, the calli were subcultured to fresh medium and were maintained at 27/24°C (day/night) under a 16 h photoperiod, at a light density of 50 µmol m^{-2} s^{-1}.

Ovary-derived calli with a diameter of more than 4 mm and which were produced within four weeks after ovary isolation were transferred to shoot regeneration medium contained MS mineral salts and vitamins supplemented with 1 mg l^{-1} 6-benzylaminopurine (BAP), 375 mg l^{-1} glutamine, 3% sucrose and 0.6% agar. After organogenesis induction buds were cut and transferred to a shoot elongation medium containing MS mineral salts and vitamins supplemented with 0.001 mg l^{-1} NAA, 0.01 mg l^{-1} BAP, 3% sucrose and 0.6% agar. The shoots regenerated from ovary-derived calli were transferred for rooting to MS medium without growth regulators and containing reduced concentrations of macro and micro salts by 50% and maintained at 25/22°C (day/night) under a 16 h photoperiod, at a light density of 50 µmol m^{-2} s^{-1}. Rooted plantlets were transferred to plastic pots containing sterilized soil and vermiculite at 1:1 ratio.

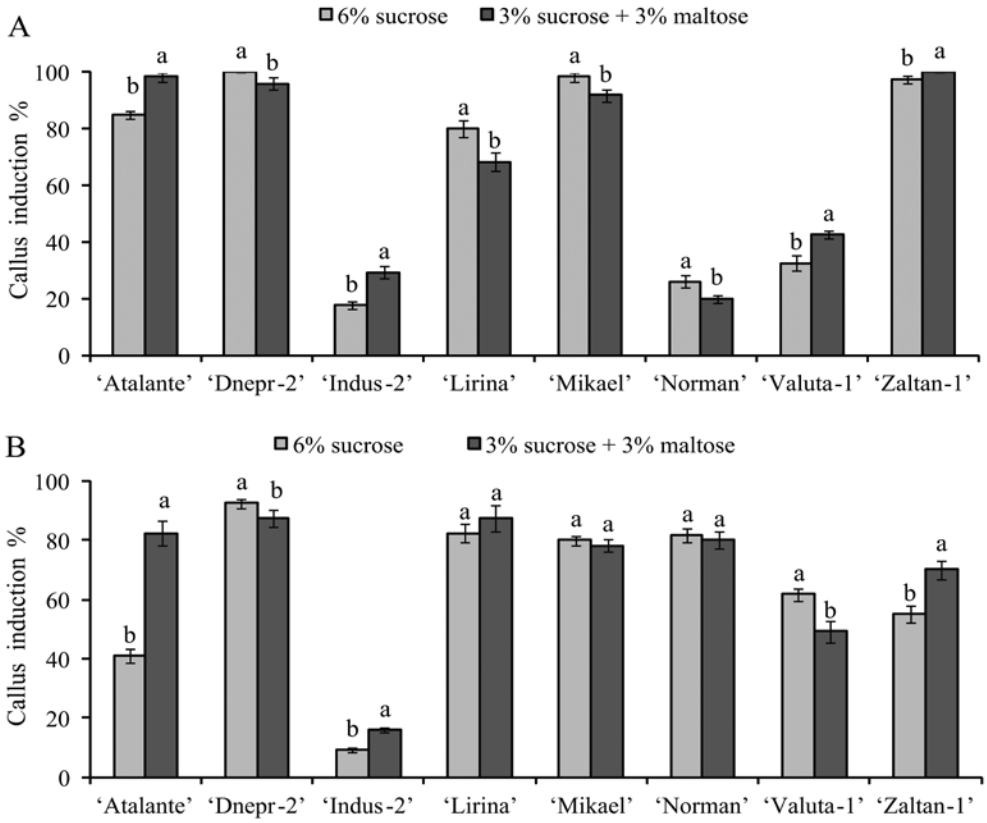
For establishment of the chromosome number in the regenerated plantlets, root tips were fixed in Carnoy solution (100% ethanol:glacial acetic acid, 3:1) for 7 days. Subsequently, samples were washed thoroughly with distilled water and treated with 1 N HCl at 60°C temperature for 10 min. Then the macerated tissue was washed again and stained with Schiff reagent as described by Bartošová et al. (2005).

Statistical analysis. Experiments were set up in a completely randomized design with three replicates per treatment and 30 explants per each replicate; each experiment was done in triplicate. The percentage of callus induction: (number of explants with callus/total number of explants) × 100%, the percentage of callus forming shoots: (number of calli with adventitious shoots/total number of calli) × 100, were calculated using software *STAT 1.55* from *SELEKCIJA* and *IRRISTAT* (Tarakanovas, Raudonius, 2003). Mean value of callus induction, shoot formation frequency and standard error (SE) for each cultivar were calculated based on the number of independent replications. All percentage values were transformed using arcsine square root (√P) (Compton, 1994) to normalize the distribution prior to analysis of variance. Effects of factors (cultivar, growth regulators combination, carbohydrate) and their interaction on shoot formation were studied by a three-way analysis of variance. For multiple comparisons, Tukey test was used.

Results and discussion

Gynogenesis in linseed predominantly involves the organogenesis pathway in which plant regeneration is achieved from unfertilised egg cell, through an intermediate callus phase. Therefore efficient production of high-quality callus is a prerequisite for achieving efficient plant regeneration. Formation of callus was observed within three weeks after isolation of ovaries. The effect of growth regulator combination and carbohydrate type on callus induction in the tested linseed cultivars is presented in Figure 1. Callogenesis response varied depending on the cultivar and medium composition interaction. The mean values of the callus formation frequency ranged from 9.17% for the cultivar 'Indus-2' on medium with 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA supplemented with 6% sucrose (Fig. 1 B) to 100% for the cultivar 'Dnepr-2' on medium with 2 mg l^{-1} TDZ + 1 mg l^{-1} NAA supplemented with 6% sucrose and for the cultivar 'Zaltan-1' on medium with 2 mg l^{-1} TDZ + 1 mg l^{-1} NAA supplemented with 3% sucrose and 3% maltose (Fig. 1 A).

In the presence of sucrose, cultivars 'Atalante', 'Mikael', 'Zaltan-1', 'Dnepr-2' and 'Indus-2' showed a higher value of responsible explants on medium with 2 mg l^{-1} TDZ and 1 mg l^{-1} NAA, while ovaries of cultivars 'Lirina', 'Valuta-1' and 'Norman' showed a better response in a medium with 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA. The replacement of sucrose with combination of sucrose + maltose in the induction medium supplemented with 2 mg l^{-1} TDZ + 1 mg l^{-1} NAA improved the callogenesis in cultivars 'Atalante', 'Valuta-1' 'Indus-2' and 'Zaltan-1'. On media with 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA the use of carbohydrates combination resulted in significant increase ($P < 0.01$) in callus formation frequency for cultivars 'Atalante', 'Zaltan-1' and 'Indus-2'. In our previous study we reported that replacement of sucrose with a combination of sucrose + maltose in induction medium supplemented with 2 mg l^{-1} BAP + 1 mg l^{-1} NAA improved the callogenesis in cultivars 'Lirina' and 'Mikael' from isolated anthers (Burbulis, Blinstrubienė, 2011). However, in the present study the



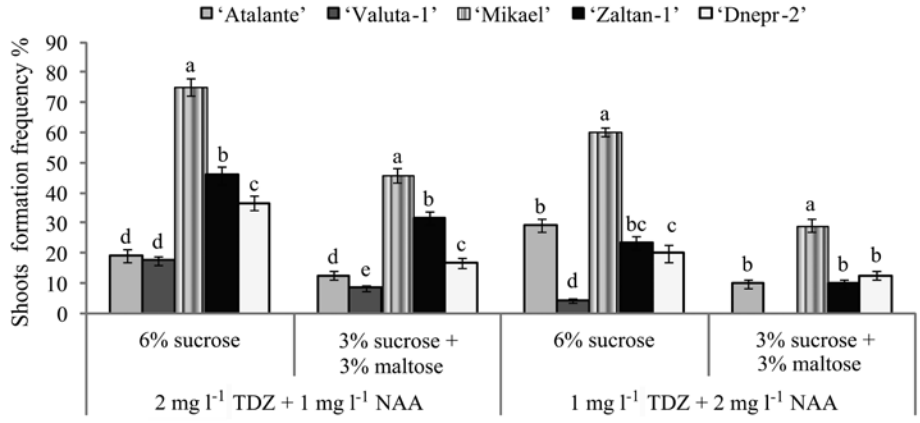
Note. A – 2 mg l^{-1} thidiazuron (TDZ) + 1 mg l^{-1} α-naphthylacetic acid (NAA); B – 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA; means ± SE within a cultivar followed by the same letter are not significantly different at $P < 0.01$.

Figure 1. Effect of growth regulator and carbohydrate on callus induction in ovary culture of eight linseed cultivars

use of carbohydrates combination on media with 2 mg l^{-1} TDZ + 1 mg l^{-1} NAA resulted in significant decrease in callus formation isolated ovaries of these cultivars. This indicated that there are differences between anther and ovary cultures response within the same genotype.

After transfer of ovary-derived callus to the shoot regeneration medium, formation of meristemic zones and adventitious buds was observed within 2–3 weeks for cultivars 'Atalante', 'Valuta-1', 'Mikael', 'Zaltan-1' and 'Dnepr-2'. Shoot formation frequency in responsive cultivars ranged from 4.17% to 75.0% for all treatments tested (Fig. 2).

It is well documented that TDZ significantly increased shoot formation frequency from somatic tissues of different plants (Lata et al., 2009; Ghosh et al., 2014) as well as in *Linum usitatissimum* (Mundhara, Rashid, 2006). Diao et al. (2009) reported positive effect of TDZ on gynogenesis in ovary culture of *Cucumis sativus*; however, optimal concentration has been shown to be strongly genotype-dependent and must be determined for each cultivar. In the present study, induction medium supplemented with 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA promoted organogenesis from ovary-derived callus of cultivar 'Atalante', while the combination 2 mg l^{-1} TDZ with



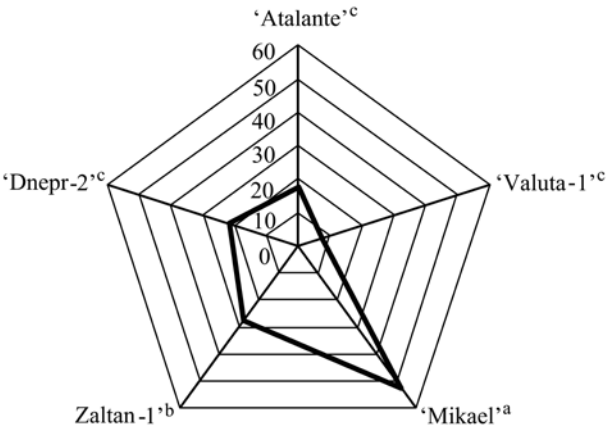
Note. TDZ – thidiazuron, NAA – α-naphthylacetic acid; means ± SE within a cultivar followed by the same letter are not significantly different at $P < 0.01$.

Figure 2. Effect of growth regulator and carbohydrate in the induction medium on shoot formation from ovary-derived callus of responsive linseed cultivars

1 mg l^{-1} NAA increased shoot regeneration frequency of cultivars 'Valuta-1', 'Mikael', 'Zaltan-1' and 'Dnepr-2'. The replacement of sucrose with a sucrose-maltose combination in induction medium supplemented with 2 mg l^{-1} TDZ + 1 mg l^{-1} NAA reduced shoot formation frequency of all responsive cultivars. When ovary-derived calli were obtained on medium supplemented with 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA, the replacement of sucrose with a combination of sucrose + maltose significantly decreased organogenesis in cultivars 'Atalante' 'Mikael', 'Zaltan-1' and 'Dnepr-2', and completely inhibited shoot formation from ovary-derived callus of cultivar 'Valuta-1'. In contrast, our previous study showed that combination 3% sucrose + 3% maltose improved the shoot formation frequency from anther-derived callus of cultivars 'Lirina' and 'Mikael' (Burbulis, Blinstrubienė, 2011).

Genotypic difference is one of the most important factors, which may affect regeneration efficiency from ovary-derived callus. The ability of callus induction and shoot regeneration can vary considerably among various species of genus or among cultivars within the same species. Most of the published reports on the linseed have noted strong genotypic effect on morphogenesis in generative tissue cultures (Pretova et al., 2006; Burbulis et al., 2007; Obert et al., 2009). In the present study, significant differences in shoot formation frequency were observed among the studied cultivars.

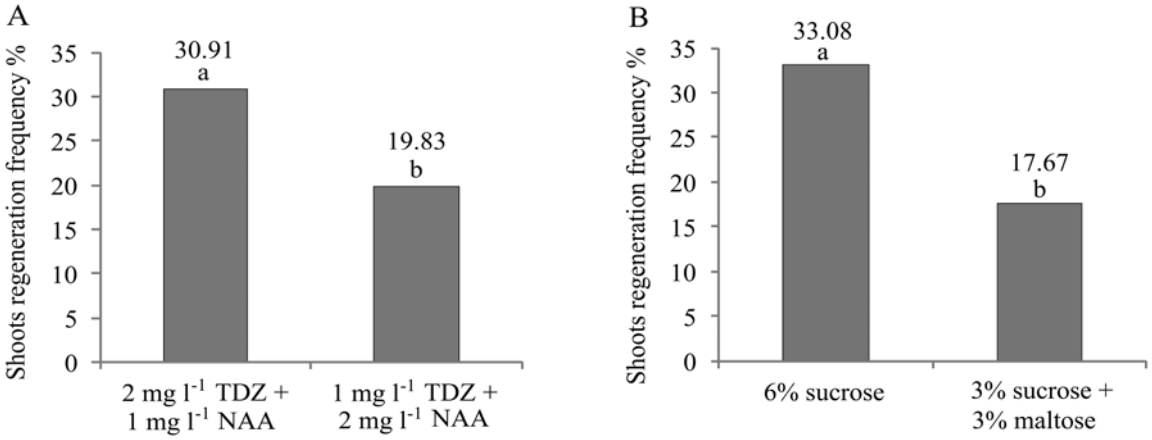
The highest mean (52.50 %) shoot formation frequency, significantly differing ($P < 0.01$) from the other cultivars, was obtained from ovary-derived callus of cultivar 'Mikael' (Fig. 3).



Note. Means within a cultivar followed by the same letter are not significantly different at $P < 0.01$.

Figure 3. Effect of cultivars on shoot regeneration from ovary-derived callus of responsive linseed cultivars

Culture medium composition is another important factor affecting plant regeneration. Our results showed that a combination of growth regulators in the induction medium had very significant effect ($P < 0.01$) on shoot regeneration frequency from ovary-derived callus in the regeneration medium. Statistical analysis did not allow considering combinations of 2 mg l^{-1} TDZ + 1 mg l^{-1} NAA and 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA as one homogenous group (Fig. 4 A).



Note. TDZ – thidiazuron, NAA – α-naphthylacetic acid; means within a growth regulators and carbohydrate combinations followed by the same letter are not significantly different at $P < 0.01$.

Figure 4. Effect of growth regulators (A) and carbohydrate (B) combinations on shoot regeneration from ovary-derived callus

The level and type of carbohydrates in the culture medium are also predetermining factors in the induction of gynogenic structures. Maltose was found to be the most effective carbohydrate for shoot induction in linseed somatic tissue culture (Millam et al., 1992). The capacity of combination 3% sucrose + 3% maltose to stimulate morphogenesis in anther culture of linseed was reported by Tejklova (1998). In the present study, the combination sucrose + maltose in the induction medium increased callus formation frequency in some cultivars (Fig. 1); however, significantly decreased shoot formation frequency from ovary-derived callus (Fig. 2). A significant effect ($P < 0.01$) of carbohydrate type in induction medium on shoot regeneration frequency from ovary-derived callus of studied linseed cultivars was observed (Fig. 4 B). The statistical analysis showed that each of the tested carbohydrate types (6% of sucrose alone and combination 3% sucrose + 3% maltose) constituted one separate homogenous group.

The analysis of variance revealed that cultivar (C), growth regulators (GR) combination, type of carbohydrate (CH) and their interaction significantly influenced callus induction and shoot formation frequency from ovary-derived callus. However, the effect of interaction GR × CH on shoot regeneration frequency was not significant (Table).

Cytological analysis of root tips showed that 21.88% of the regenerated plants were haploids, while another group of regenerants (78.12%) were diploid and mixoploid. Different ploidy level of plants regenerants

Table. Effect of different factors on callus induction and shoot formation frequency

Effect	*F*-values of callus induction frequency %	*P*-values for callus induction frequency	*F*-values of shoots formation frequency %	*P*-values for shoots formation frequency
Cultivar (C)	235.08**	< 0.001	297.68**	<0.001
Growth regulators (GR) combination	39.34**	< 0.001	199.75**	<0.001
Carbohydrate (CH)	7.88**	< 0.007	324.27**	<0.001
C × GR	89.17**	< 0.001	25.38**	<0.001
C × CH	17.32**	< 0.001	6.84**	<0.001
GR × CH	3.53 ns	0.065	1.66 ns	0.205
C × GR × CH	3.01*	0.009	4.54**	0.004

* – significant at 5% level ($P < 0.05$), ** – significant at 1% level ($P < 0.01$), ns – not significant

has also been obtained in summer squash unpollinated ovule culture (Shalaby, 2007). Obert et al. (2009) suggested that cells of flax tissue often react to *in vitro* conditions by rapid increase in the DNA amount in the course of prolonged culture; therefore it is necessary to transfer the callus to regeneration medium as soon as calli are big enough for manipulation.

Conclusions

1. For linseed (*Linum usitatissimum* L.) cultivars 'Dnepr-2 and 'Mikael' callus induction rate was the highest in the medium supplemented with 2 mg l^{-1} thidiazuron (TDZ) + 1 mg l^{-1} α-naphthylacetic acid (NAA) and 6% sucrose, while this combination of growth regulators significantly increased callogenesis in cultivars 'Atalante' and 'Indus-2' when medium was supplemented with 3% sucrose and 3% maltose. A combination of 1 mg l^{-1} TDZ + 2 mg l^{-1} NAA and 6% sucrose promoted callus induction from ovaries of cultivars 'Norman' and 'Valuta-1', while a combination of 3% sucrose and 3% maltose in this medium was more suitable for callus induction from ovary of cultivars 'Lirina' and 'Zaltan-1'.

2. Induction medium supplemented with 2 mg l^{-1} TDZ + 1 mg l^{-1} NAA and 6% sucrose gave the highest shoot formation frequency from ovary-derived callus for cultivars 'Valuta-1', 'Mikael', 'Zaltan-1' and 'Dnepr-2'.

3. The present study showed that 21.88% of plants regenerants obtained from ovary-derived callus were haploids.

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Genotipo ir egzogeninių veiksnių įtaka sėjamojo lino mezginių kultūrai

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Santrauka

Tirta genotipo, augimo reguliatorių ir angliavandenių tipo įtaka sėmeninio lino (*Linum usitatissimum* L.) aštuonių veislių kaliaus indukcijai ir netiesioginei ūglių regeneracijai izoliuotų mezginių kultūroje. Kaliaus genezė varijavo nuo 9,17 iki 100 %, priklausomai nuo genotipo ir maitinamosios terpės sąveikos. Derinys sacharozė + maltozė esmingai didino trijų arba keturių tirtų veislių kaliaus indukciją, priklausomai nuo augimo reguliatorių derinio indukcijos maitinamojoje terpėje. Penkių iš aštuonių tirtų veislių mezginių indukuotas kalius ūglius formavo 4,17–75,00 % dažniu, kitos trys ūglių neformavo. Sacharozę indukcijos terpėje pakeitus deriniu sacharozė + maltozė, ūglių susiformavimo dažnis regeneracijos terpėje esmingai sumažėjo arba buvo visiškai slopinamas. Veislės 'Mikael' izoliuotų mezginių suformuotas kalius ūglius formavo esmingai didžiausiu dažniu – 52,50 %. Dispersinė analizė parodė, kad kaliaus indukcijos ir ūglių formavimosi dažnis priklauso nuo veislės, augimo reguliatorių derinio ir angliavandenių tipo bei jų sąveikos. Daugeliu atvejų didesnis ūglių susiformavo dažnis gautas iš kaliaus, indukuoto indukcijos terpėje, papildytoje 2 mg l^{-1} 1-fenil-3-(1,2,3-tiadiazol-5-il)-karbamido (TDZ) + 1 mg l^{-1} α naftilacto rūgšties (NAR) ir 6 % sacharozės. Citologiniais tyrimais nustatyta, kad 21,88 % augalų regenerantų buvo haploidai, 78,12 % – diploidai ir miksoploidai.

Reikšminiai žodžiai: angliavandeniai, augimo reguliatoriai, genotipas, *Linum usitatissimum*, morfogeninė galia, mezginių kultūra.