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Some factors affecting callus induction in ovary culture of flax (*Linum usitatissimum* L.)

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Flax (*Linum usitatissimum* L.) is an important crop in Lithuania for the production of both oil and fibre, but no Lithuanian flax cultivar has been developed. The haploid technique is a fast and efficient tool for developing new varieties in a comparatively short time. Many research groups successfully created new flax genotypes through anther culture; however application of ovary culture for flax haploid production has been published in few reports only. The breeding program for developing a Lithuanian flax cultivar was started at the Genetic-Biotechnology Laboratory of Lithuanian University of Agriculture. The present paper reports the effect of genotype, growth regulators and the level of sucrose on callus induction in ovary culture of flax. Ovaries were cultured on a modified MS medium supplemented with three different combinations of plant growth regulators. Three levels of sucrose were evaluated. Variable callogenic responses were expressed by all of the five genotypes and their hybrids tested on different induction media. Ovaries of the genotypes 'Lirina', 'Barbara' and 'Mikael' showed the highest value of induced ovaries in a medium supplemented with 2.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA, while a combination of 1.0 mgl^{-1} BAP and 2.0 mgl^{-1} 2,4D significantly reduced the number of ovaries producing callus. The current study indicates that there is a strong effect of the genotype on callus production from ovary in flax, and therefore specific combinations of growth regulators and sucrose level must be designed for each genotype in order to elicit optimum results.

Key words: flax cultivars and hybrids, plant growth regulators, ovary culture, sucrose level

INTRODUCTION

As in androgenesis, gynonegic haploids may develop directly or indirectly via regeneration from the callus. Ovary and flower bud cultures have generally been found to be more efficient than ovule culture because of the less intrusive manipulation [1]. *In vitro* culture of unpollinated ovaries and ovules has been successfully applied in many plant species [1, 2]. Gynogenesis has not been investigated as thoroughly or with as many species as androgenesis; therefore, less information is available concerning the various factors that contribute to the successful production of haploids from the female than the male gametophyte. The major problems affecting the use of gynogenesis are the lack of established protocols for most species, poor yields, and production of diploid or mixoploid plants. The genotype of the donor plant is one of the most important factors for the induction of gynogenesis. Genotypic differences in response were demonstrated in *Allium cepa* [3, 4], *Beta vulgaris* [5–7], *Triticum durum* [8, 9]. Since each genotype shows a different response, a specific protocol must be followed for maximal efficiency. The objective of the present study was to investigate the effect of growth regulator combinations and sucrose level on callus induction in the ovary culture of flax.

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MATERIALS AND METHODS

To study flax callus induction from unfertilized ovaries *in vitro*, the following cultivars were chosen: 'Lirina', 'Barbara', 'Mikael', 'Szaphir', 'Atalante' as well as F_1 hybrid plants: hybrid No. 30 – 'Barbara' × 'Lirina'; hybrid No. 23 – 'Lirina' × 'Barbara'; hybrid No. 31 – 'Barbara' × 'Mikael'; hybrid No. 02 – 'Mikael' × 'Barbara'.

Donor plants were grown in a growth chamber at the light intensity of at least 4000 lx with a 16 / 8 h (day / night) photoperiod, temperature 22 / 18 °C (day / night) and 75% humidity. Plants were sown at a depth of 1 cm, 30 plants per pot (about 5 kg of soil in pots). All plants were grown in a 2 : 1 mixture of compost and peat, pH 5.8–6.5. The plants were watered and fertilized with 1.15 g/pot NH_4NO_3, 0.96 g/pot KH_2PO_4, 0.43 g/pot K_2SO_4, 0.37 g/pot KCl. No means against diseases and pests were used.

Buds taken from donor plants grown under controlled conditions were externally sterilized in 70% hydrous ethanol solution (1 min.) 2% natrium hypochloride (10 min.) then three times washed with sterile distilled water. Sterilization of explants and transfer of the culture were carried out under aseptic conditions. Sterile explants were cultivated in plastic Petri dishes (at 10 pcs. / dish of explants) containing 3 ml of modified MS medium (NH_4NO_3 165 mg l^{-1}) [10], solidified with 6000 mg l^{-1}Difco-Bacto agar. The medium pH was 5.7 ± 0.1. Isolated tissues and cells *in vitro* were grown in a dark cultivation

chamber for 28 days and then transferred into light and cultivated under controlled conditions: light intensity 5000 lx, photoperiod 16 / 8h (day / night), temperature 25 ± 2 °C.

Selection of the combination of growth regulators in the induction medium

Three different combinations of auxins and cytokinins on a modified MS medium were tested using genotypes 'Lirina', 'Barbara', 'Mikael', 'Szaphir', 'Atalante': 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA; 1.0 mg l^{-1} BAP + 2.0 mg l^{-1} 2,4D; 1.0 mg l^{-1} BAP + 0.05 mg l^{-1} NAA.

Evaluating the influence of the genotype, the flax cultivars 'Lirina', 'Barbara', 'Mikael' and their reciprocal hybrids No. 30, No. 23, No. 31, No. 02 were studied. Ovaries were cultivated on a modified MS medium with the following combination of cytokinins and auxins: 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA; 1.0 mg l^{-1} BAP + 2.0 mg l^{-1} 2,4 D.

Optimization of sucrose level in the induction medium

6%, 9% and 12% levels of sucrose in the modified MS medium containing 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA were investigated using the genotypes 'Lirina', 'Barbara', 'Mikael', 'Szaphir', 'Atalante'.

Evaluating the influence of the genotype, cultivars 'Lirina', 'Barbara', 'Mikael' and their reciprocal hybrids No. 30, No. 23, No. 31, No. 02 were studied. Ovaries were cultivated on a modified MS medium with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, differing in the levels of sucrose (6%, 9% and 12%).

A complete randomized design was used for all the experiments. For each treatment, 40 ovaries were cultured (10 ovaries / Petri dish; 4 replicates / treatment) and each experiment was done in triplicate. The number of ovaries producing calli was scored at 28 days after the initial inoculation. The percentage of ovaries with callus was calculated as the number of ovaries producing calli / 100 inoculated ovaries.

The data of the investigations were calculated using STAT 1.55 from SELEKCIJA [11] and ANOVA for EXEL software, vers. 2.1. The mean value and SE for each genotype were calculated based on the number of independent replications.

RESULTS AND DISCUSSION

Influence of growth regulators on callus induction

The beginning of callus formation of the test genotypes was observed within three weeks after isolation of unpollinated ovaries. Variable callogenic responses were expressed by all the five genotypes tested on the different induction media. It was documented that isolated ovaries of the genotypes 'Lirina', 'Barbara', 'Mikael' induced statistically reliably more callus in a medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA. In contrast, ovaries of 'Szaphir' showed a better response in a medium with 1.0 mg l^{-1} BAP + 2.0 mg l^{-1} 2,4 D, whereas callus formation in this genotype was strongly reduced by 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA and completely inhibited by 1.0 mg l^{-1} BAP + 0.05 mg l^{-1} NAA combinations. For the genotype 'Atalante' there was no significant difference between 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} BAP + 2.0 mg l^{-1} 2,4 D combinations in callus induction (Fig. 1).

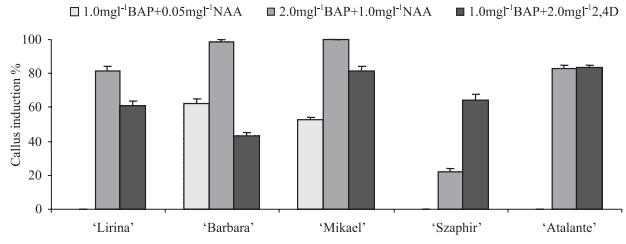

Fig. 1. Effect of plant growth regulators on callus induction in ovary culture (MS modified medium with 6% sucrose)

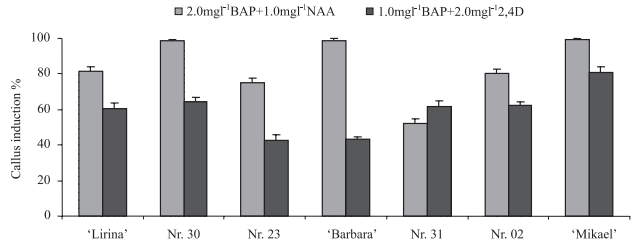

Fig. 2. Effect of plant growth regulators on callus induction in three flax genotypes and their hybrids in ovary culture (MS modified medium with 6% sucrose)

Ovaries of 'Lirina', 'Szaphir' and 'Atalante' cultured on a medium supplemented with 1.0 mg l^{-1} BAP + 0.05 mg l^{-1} NAA did not show any response even after six weeks of culture and subsequently became necrotic.

Ovaries of hybrids No. 30, Nr. 02, No. 23 induced statistically reliably more callus in a medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, while hybrid No. 31 showed a better response in a medium supplemented with 1.0 mg l^{-1} BAP + 2.0 mg l^{-1} 2,4D (Fig. 2).

All the hybrids were less responsible in comparison with parental forms in a medium with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, except hybrid No. 30 ovaries.

The medium was identified as an important factor in gynogenesis. The most commonly used basal media for recovering gynogenic haploids are MS, B5 or variations on these media. While gynogenesis has been induced in a few species without the use of growth regulators, most species have required auxins and / or cytokinins in the medium. A significant effect of the combination 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA on callus formation in flax ovary culture was reported by Obert et al. [12]. The present study shows that the combination 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA in the induction medium is more suitable for at least six of the nine flax genotypes tested.

Influence of sucrose level on the productivity of ovaries

The level of sucrose in the nutrient medium is also a predetermining factor in the induction of gynogenic structures. Our study has shown a different influence of sucrose concentrations on callus induction of in flax cultivars in the culture of isolated ovaries (Fig. 3). Increased levels of sucrose from (6% to 9%) induced dedifferentiation of ovaries of the genotype 'Lirina', however, with increasing the level of sucrose from 9% to 12% the number of ovaries producing callus significantly reduced.

A nutrient medium supplemented with 9% of sucrose reduced callus induction of isolated ovaries of the genotype

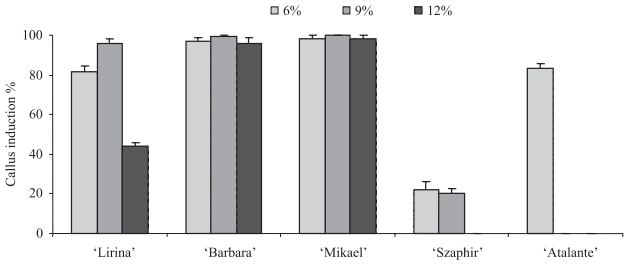

Fig. 3. Effect of sucrose on callus induction in ovary culture (MS modified medium with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA)

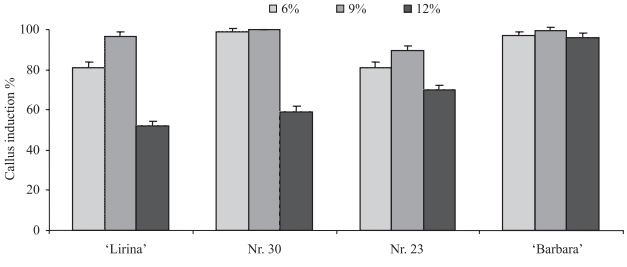

Fig. 4. Effect of sucrose on callus induction in ovary culture of cultivars 'Lirina' and 'Barbara' and their hybrids (MS modified medium with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA)

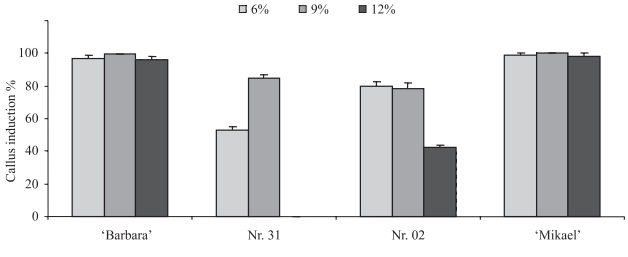

Fig. 5. Effect of sucrose on callus induction in ovary culture of cultivars 'Barbara' and 'Mikael' and their hybrids (MS modified medium with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA)

'Szaphir' and completely inhibited callus formation from ovaries of the genotype 'Atalante'. Experiments did not show any significant differences in 'Barbara' and 'Mikael' ovary callogenesis the tested sucrose concentrations in the induction medium.

The levels of sucrose to increased 9% improved the frequency of responding ovaries of hybrids No. 23 from 80.9% to 89.6% (Fig. 4). Increased levels of sucrose (from 9% to 12%) significantly reduced callus formation from ovaries of hybrids No. 23 and No. 30.

No significant differences in callus induction of hybrid No. 02 ovaries in media with 6% and 9% sucrose were determined, while 9% of sucrose significantly increased the amount of callus forming ovaries of hybrid No. 31 (Fig. 5). Increasing the level of sucrose from 9% to 12% significantly reduced the number of ovaries producing calli in hybrid No. 02 and completely inhibited callus formation from ovaries of hybrid No. 31.

For gynogenesis induction, sucrose concentration varied from 3% to 12% in a culture medium. Hovewer, higher sucrose concentrations (10%) were found to be important for gynogenic induction in onion [1] and rice (6%) [13]. The current study shows that a higher level of sucrose (9%) improves callus induction frequency in the cultivar 'Lirina' and hybrid No. 31.

Significant effects of the genotype, combination of growth regulators, sucrose level and their interaction on callus induction in the ovary culture of flax were observed, suggesting that growth regulator combinations and sucrose level in the induction medium for flax ovary culture must be modified and optimized for specific flax genotypes within each particular breeding program.

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VEIKSNIAI, LEMIANTYS LINŲ (*LINUM USITATISSIMUM* L.) KALIAUS INDUKCIJĄ MEZGINIŲ KULTŪROJE

S a n t r a u k a

Linai (*Linum usitatissimum* L.) yra svarbūs aliejiniai ir pluoštiniai augalai Lietuvoje, tačiau lig šiol nėra sukurta nė vienos lietuviškos linų veislės. Haploidija – vienas greičiausių ir efektyviausių naujų veislių kūrimo būdų. Moksliniuose leidiniuose gausu duomenų apie linų dvigubų haploidų kūrimą taikant dulkinių kultūros metodą, tačiau publikacijos apie izoliuotų mezginių panaudojimą kuriant DH linijas tėra tik kelios. Lietuvos žemės ūkio universiteto Genetikos-biotechnologijos laboratorijoje linų selekcija vykdoma *in vitro*. Tirta genotipo, augimo reguliatorių ir sacharozės koncentracijos įtaka kaliaus indukcijai linų mezginių kultūroje. Izoliuotos mezginės kultivuotos MS modifikuotoje terpėje naudojant tris skirtingus augimo reguliatorių derinius. Nustatyta, kad mezginių gebėjimas formuoti kalių priklauso nuo genotipo, kryžminimo kombinacijos bei maitinamosios terpės sudėties. Genotipų 'Lirina', 'Barbara', 'Mikael' izoliuotos mezginės statistiškai patikimai daugiau kaliaus indukavo terpėje, papildytoje 2,0 mg l^{-1} BAP + 1,0 mg l^{-1} NAR. Linų kaliaus formavimasis daugiausia priklauso nuo donorinio augalo genotipo, todėl optimalios kultivavimo sąlygos turi būti parenkamos kiekvienam konkrečiam genotipui.