

Comparison of anther culture response among *Linum usitatissimum* L. cultivars and their hybrids

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Abstract

Linseed (*Linum usitatissimum* L.) is an important crop in Lithuania for production of both oil and fibre, but a Lithuanian cultivar of linseed has not previously been developed yet. Identification of responsive genotypes and development of efficient culture protocols are a prerequisite to initiating an effective doubled haploid production system in applied breeding programme. Anther culture response in *L. usitatissimum* was studied in the hybrid populations from responsive and poor/non-responsive cultivars and their parental forms. Two different growth regulators combinations and three concentrations of sucrose were studied. Variation for anther culture response was significant both within and between the hybrids and their parents. F₁ hybrids, produced from crosses between responsive genotype 'Lirina' and poor/non-responsive genotype 'Barbara', showed dramatic increases in callus induction in comparison with the parental form on medium supplemented by 1 mg l⁻¹ 6-benzylaminopurine and 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid containing 6 % sucrose.

Key words: anther culture response, cultivars, hybrids, linseed.

Introduction

Flax (*Linum usitatissimum* L.) is an important oil crop in Europe and is also used as a protein source for animal rations.

Haploid technique is considered to be a much faster and more efficient tool for breeding new varieties in a comparatively short time. Rapid breeding techniques could help in producing new linseed varieties with characters that are adapted to the current demands of industry, which would therefore gain new markets for this crop. Another advantage of doubled haploid lines in a breeding programme is, as a result of gametic-segregation ratios and homozygosity of lines, the smaller size of population necessary to select a desired genotype (Friedt et al. 1995).

Using F₁ hybrids instead of homozygous genotypes would produce the regenerated plants with possibly novel and beneficial gene combinations. Doubled haploid plants and the somatic diploids derived from anther culture can also be distinguished by morphological, biochemical and molecular markers. Furthermore, the overall efficiency of regeneration in anther/microspore culture of F₁ hybrids has been found to be higher than of their homozygous parent genotypes in flax (Chen et al. 1998; Chen, Dribnenki 2002).

Anther culture response is controlled by the genotype of donor plants and non-genetic factors (Kurt, Evans 1998; Rutkowska-Krause, Mankowska 2002; Obert et al. 2004). While

studies on the non-genetic component have produced some dramatic increases in anther response, such factors are more difficult to fix than the genetic component. Changing the genetic component has the added advantage that it can be manipulated in a desired direction (Deaton et al. 1987). As most of the anther-culture responsive maize materials have been found in non-commercial germ plasm, it is likely that culturability can be transferred from the responsive, non-commercial, exotic maize germ plasm into elite types (Afele, Kannenberg 1990). Both nuclear and cytoplasmic factors have been shown to have an effect on wheat (*Triticum aestivum*) anther culture (Sopory, Munshi 1997).

The objectives of this study were to estimate responsibility of F_1 hybrids in anther culture in comparison with parental forms.

Materials and methods

Plant material and anther culture

The experiments were carried out with the following flax cultivars: 'Lirina', 'Barbara', 'Mikael' and their hybrids: F_1 'Barbara' \times 'Lirina', F_1 'Lirina' \times 'Barbara', F_1 'Barbara' \times 'Mikael', F_1 'Mikael' \times 'Barbara'. Seeds were germinated and grown in a growth chamber with a 16 h photoperiod, temperature (22/18 °C, day/night) and 75 % humidity. All plants were grown in mixture of peat, vermiculite and sand in a 3:1:2 ratio in 16.5 cm pots. The plants were watered and fertilized with diluted 20-20-20 (N:P₂O₅:K₂O) at the rate of 4 g l⁻¹ as required.

Flower buds (3.5 to 4.0 mm in length) were collected when the microspores were at the mid uninucleate stage previously determined by microscopic observation of anthers (0.9 to 1.1 mm in length) and stained with 1 % acetocarmine. Harvested buds 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. Five anthers from each of two buds (total 10) were inoculated onto a plastic Petri dish (35 \times 10 mm) containing 3 ml of modified (NH₄NO₃ – 165 mg l⁻¹) MS induction medium (Murashige, Skoog 1962) and incubated at 25 °C in the dark. 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 40 mmol m⁻² s⁻¹.

Detailed experiment design and data analysis

Experiment 1. Effect of plant growth regulators on callus induction. Two different combinations of auxin and cytokinin [2 mg l⁻¹ 6-benzylaminopurine (BAP) + 1 mg l⁻¹ α -naphthylacetic acid (NAA), 1 mg l⁻¹ 6-benzylaminopurine (BAP) + 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4D)] on a modified MS medium were tested. All media were supplemented with 6 % sucrose and solidified with 0.6 % agar.

Experiment 2. Effect of sucrose level on callus induction. For investigation of the effect of sucrose level anthers of tested genotypes and their hybrids were cultivated on a modified MS medium containing 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D.

A complete randomized design was used for all experiments. For each treatment 120 anthers were cultured (10 anthers per Petri dish; 12 replicates per treatment) and each experiment was done in triplicate. The number of anthers producing calli was scored at 28 days after initial inoculation. The percentage of anthers with calli was calculated as the number of anthers producing calli per 100 inoculated anthers.

The data of the investigations were analysed using the computer programmer STAT 1.55 from "SELEKCIJA" (Tarakanovas 1999) and ANOVA for EXCEL, vers. 2.1. Mean values and SE's for each genotype were calculated based on the number of independent replications.

Results

Experiment 1. Effect of plant growth regulators on callus induction

The whole androgenetic process of anther culture in this study was similar as described previously (Burbulis et al. 2005). Formation of a callus was observed within two weeks after culture initiation. Results of the effect of two tested growth regulator combination on callus induction in three flax genotypes and their hybrids are summarized in Fig. 1.

Each genotype responded differently according to the medium. Anthers of 'Lirina' showed the highest value of induced anthers on medium supplemented by 2 mg l⁻¹ BAP with 1 mg l⁻¹ NAA while the combination of 1 mg l⁻¹ BAP with 2 mg l⁻¹ 2,4D significantly reduced the number of anthers producing callus. In contrast, anthers of 'Mikael' showed a better response on medium with 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D, whereas callus formation in this genotype was strongly reduced by the 2 mg l⁻¹ BAP with 1 mg l⁻¹ NAA combination. Anthers of 'Barbara' cultured on both tested media did not show any response even after six weeks of culture and subsequently became necrotic.

For F₁ 'Barbara' × 'Lirina' hybrid, the combination of 1 mg l⁻¹ BAP with 2 mg l⁻¹ 2,4D in induction medium significantly increased callus formation in comparison with superior

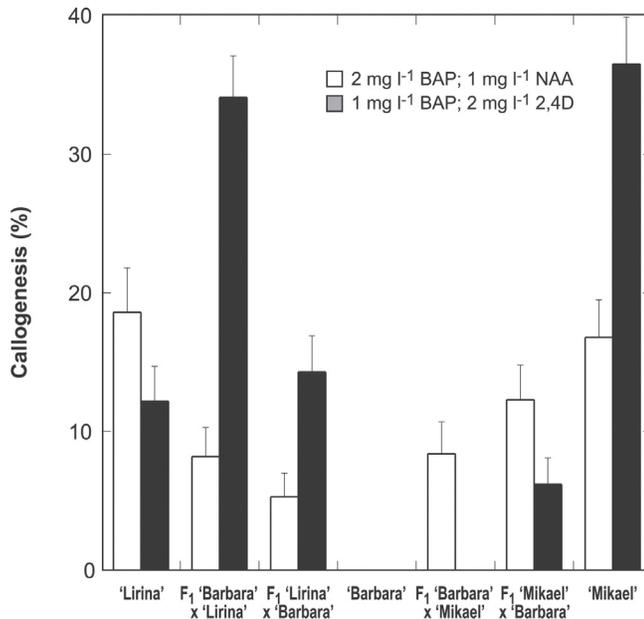


Fig. 1. Effect of plant growth regulator combinations on callus induction in three flax genotypes and their hybrids in anther culture on medium with 6 % sucrose. BAP, 6-benzylaminopurine; NAA, α -naphthylacetic acid; 2,4D; 2,4-dichlorophenoxyacetic acid.

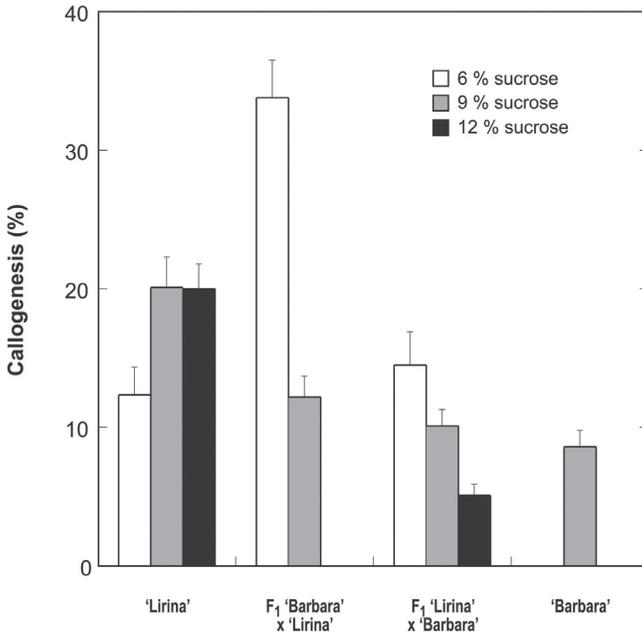


Fig. 2. Effect of sucrose level in culture medium containing growth regulators, 1 mg l⁻¹ 6-benzylaminopurine and 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid on callus induction in anther culture of cultivars 'Lirina' and 'Barbara' and their hybrids.

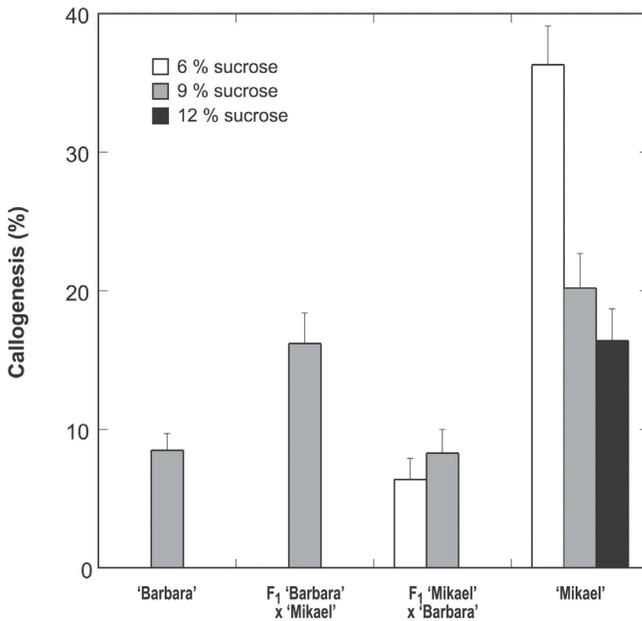


Fig. 3. Effect of sucrose level in culture medium containing growth regulators, 1 mg l⁻¹ 6-benzylaminopurine and 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid on callus induction in anther culture of cultivars 'Barbara' and 'Mikael' and their hybrids.

parent 'Lirina'. However, there was no significant difference between 'Lirina' and F_1 'Lirina' \times 'Barbara' anthers cultured on the same medium.

For F_1 'Mikael' \times 'Barbara' hybrid, the combination of 2 mg l⁻¹ BAP with 1 mg l⁻¹ NAA increased callus induction in comparison with 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D combination, however callus formation was significantly lower as compared with the superior parent 'Mikael'. The growth regulator combination 2 mg l⁻¹ BAP with 1 mg l⁻¹ NAA promoted callus development in anthers of F_1 'Barbara' \times 'Mikael', while the combination 1 mg l⁻¹ BAP with 2 mg l⁻¹ 2,4D completely inhibited callus formation from anthers of this genotype.

Although cultivar 'Barbara' was not responsive for anther culture it is important to note that F_1 hybrids from crosses 'Barbara' with 'Lirina' and 'Mikael' were more or less responsive, except F_1 'Barbara' \times 'Mikael' anthers cultured on medium supplemented by 1 mg l⁻¹ BAP with 2 mg l⁻¹ 2,4D.

Experiment 2. Effect of sucrose level on callus induction

The results of the effect of sucrose level on callus induction in linseed cultivars 'Lirina', 'Barbara' and their hybrids are presented in Fig. 2.

An increasing level of sucrose from 6 % to 9 % increased the frequency of responding anthers of 'Lirina' (from 12 % to 20 %) and 'Barbara' (from 0 % to 9 %), but significantly reduced callus formation in their hybrids. Especially sensitive to higher sucrose content was the F_1 'Barbara' \times 'Lirina' hybrid. Cultured anthers of this hybrid on medium supplemented with 9 % sucrose caused a decrease in frequency of responding anthers from 34 % to 12 % in comparison with the 6 % sucrose level.

Increasing the sucrose level from 9 % to 12 % completely inhibited callus formation in anthers of 'Barbara' and F_1 'Barbara' \times 'Lirina' hybrid, and significantly reduced callus formation in F_1 'Lirina' \times 'Barbara'. For cultivar 'Lirina' there was no significant difference between 9 % and 12 % sucrose levels in callus induction.

Callus formation from F_1 'Barbara' \times 'Mikael' hybrid was obtained only when anthers were cultured on medium with 9 % of sucrose, as well as from parent 'Barbara' (Fig. 3). However, there was no significant difference between 6 % and 9 % of sucrose for F_1 'Mikael' \times 'Barbara' hybrid.

In contrast, 9 % of sucrose significantly reduced the number of anthers producing callus in 'Mikael'.

Increasing the level of sucrose from 9 % to 12 % completely inhibited callus induction from anthers of F_1 'Barbara' \times 'Mikael' and 'Mikael' \times 'Barbara' hybrids. However, for cultivar 'Mikael' there was no significant difference between the 9 % and 12 % sucrose level.

Discussion

The presence of an appropriate concentration of growth regulators in the medium plays a critical role in callus formation in anther culture. The reports available so far on anther culture suggest that, in the majority of cases, auxin or/and cytokinin has been required as a component of the medium. Growth regulators have widely been used for callus enhancement in anther culture of flax. A significant effect of the combination of 1 mg l⁻¹ BAP and 1 mg l⁻¹ NAA on callus formation in flax anther culture has been reported by Obert et al. (2005), while higher callogenesis in flax anther in medium with 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D was observed in experiments reported by Chen et al. (1998a). In the

present study, the combination of 1 mg l⁻¹ BAP with 2 mg l⁻¹ 2,4D in induction medium produced a higher percent of calli in the cultivar 'Mikael' (37 %), F₁ 'Barbara' × 'Lirina' (34 %) and F₁ 'Lirina' × 'Barbara' (14 %). However, anther of 'Lirina', F₁ 'Barbara' × 'Mikael' and F₁ 'Mikael' × 'Barbara' showed a better response on medium supplemented with 2 mg l⁻¹ BAP and 1 mg l⁻¹ NAA.

Response to androgenesis for a number of crops including flax is known to be strongly genotype dependent and influenced by numerous exogenous factors. Our previous study has shown significant variation in callus producing ability between the genotypes. The cultivar 'Mikael' had the highest callus induction rate, while cultivar 'Barbara' was only able to produce calli in medium supplemented with 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D containing 9 % sucrose. This results corresponds with that obtained by Nichterlein et al. (1991), Chen et al. (2002), Obert et al. (2005) who found that anther induction rate of linseed varied according to the plant genotype. This difference in the level of culturability is strongly indicative of genetic components to the response rate. To confirm this, crossing experiments involving responsive ('Mikael' and 'Lirina') and poor/non-responsive ('Barbara') genotypes were made. The current study showed that the appropriate growth regulators combination for hybrids and their parental form differs. Anther of cultivar 'Lirina' showed a higher level of callogenesis on medium with 2 mg l⁻¹ BAP and 1 mg l⁻¹ NAA, while reciprocal hybrids showed a better response on medium containing 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D. In contrast, cultivar 'Mikael' showed the higher value of induced anthers on medium with 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D, whereas the combination 2 mg l⁻¹ BAP and 1 mg l⁻¹ NAA promoted callus formation in anthers of reciprocal hybrids of this genotype.

It has been documented that concentration of sucrose is also a very important factor for callus induction in anther culture. Our study results show a different influence of sucrose level on callus induction of cultivars and their hybrids. An increased concentration of sucrose (9 %) promoted higher callogenesis of the cultivars 'Lirina' and 'Barbara', while a lower sucrose level (6 %) was more suitable for their reciprocal hybrids. In contrast, 'Barbara' × 'Mikael' hybrid showed a better response in medium supplemented with 9 % of sucrose, as also did cultivar 'Barbara', while the same sucrose level significantly decreased callus induction of cultivar 'Mikael' in comparison with 6 % of sucrose.

The current study indicates that here is a strong genotype-dependent effect of growth regulators combination and sucrose level on callus production from anthers in flax, and therefore the induction medium must be designed for each genotype. F₁ hybrids, produced from crosses between responsive genotype 'Lirina' and poor/non-responsive genotype 'Barbara' showed dramatic increases in the callus induction in comparison with the parental form on medium supplemented by 1 mg l⁻¹ BAP and 2 mg l⁻¹ 2,4D containing 6 % sucrose. The high heritability for anther response estimate in our study suggests that a relatively rapid genetic gain can be made in transferring this trait from responsive to poor/non responsive germ plasm.

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Putekšņu kultūras reakcijas salīdzinājums dažādām *Linum usitatissimum* L. šķirnēm un to hibrīdiem

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Kopsavilkums

Lini (*Linum usitatissimum* L.) ir svarīga lauksaimniecības kultūra Lietuvā gan eļļas, gan šķiedru ieguvei, bet vietējās linu šķirnes vēl nav izveidotas. Kompetentu genotipu atlase un efektīvu kultivēšanas protokolu izstrādāšana ir priekšnoteikums dubulto haploīdu iegūšanas sistēmas izveidošanai praktiskās selekcijas programmā. *L. usitatissimum* putekšņu kultūras reakciju pētīja hibrīdu populācijās no reaģētspējīgām un slikti reaģētspējīgām šķirnēm un to vecāku formām. Pētījumos izmantoja divas dažādas augšanas regulatoru kombinācijas un trīs saharozes koncentrācijas. Putekšņu kultūras reakcija būtiski atšķīrās gan starp hibrīdiem, gan to vecāku formām. F₁ hibrīdi no krustojumiem starp reaģētspējīgo genotipu 'Lirina' un slikti reaģētspējīgo genotipu 'Barbara' parādīja būtisku kallusa veidošanās indukcijas pieaugumu vidē ar 1 mg l⁻¹ benzilaminopurīnu un 2 mg l⁻¹ 2,4-dihlorofenoksietilskābi salīdzinājumā ar vecāku formām.