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Effect of genotype and medium composition on flax (*Linum usitatissimum* L.) anther culture

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Abstract. Flax (*Linum usitatissimum* L.), a member of the family *Linaceae*, is an important crop in Europe for the production of both oil and fibre. Organic agriculture is based on minimizing the use of external inputs and avoiding the use of synthetic fertilizers and pesticides, therefore resistant genotypes are preferable in an organic agriculture system. Breeding flax using haploid techniques allows breeders to develop new varieties in a shorter time period. However the overall efficiency of plant regeneration is not efficient, therefore identification of responsible genotypes and improvements of protocols are prerequisites for applied breeding programs. The effect of the combination of genotype and growth regulators on callus induction and shoot regeneration in anther culture of flax was investigated. Anther culture response in nine flax cultivars was studied, and four responsible genotypes have been selected. The results suggested that specific combinations of growth regulators must be designed for each genotype. The highest rate of shoots per plant has been obtained in second subculture.

Key words: anther culture, *Linum usitatissimum* L., organogenetic ability

INTRODUCTION

Breeding of flax is practiced in many countries, including Lithuania. Breeding of flax even today 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 (Chen et al., 1998). Rapid breeding techniques could help in producing new flax lines such as those resistant to pathogen infection and/or with increased productivity.

Breeding flax using haploid techniques has the advantage of rapid development of completely homozygous lines within one generation and is an efficient means of genotypic selection. Haploid methods may offer improved efficiency to achieve breeding objectives, such as breeding for disease resistance. Anther culture has been used in breeding programs to produced flax lines resistant to *Fusarium oxysporum*-induced fungal wilt (Rutkowska-Krause et al., 2003). Anther culture is currently the most successful method of producing doubled haploid lines in flax. However, the overall efficiency of plant regeneration is not efficient for practical breeding programmes (Chen et al., 1998).

Identification of responsive genotypes and development of efficient culture protocols are prerequisites to initiate an effective doubled haploid system in applied breeding programs. The objectives of this study were: (1) to evaluate the anther culture response of nine flax cultivars; (2) to determine the effect of medium composition on callus induction and shoot regeneration in flax anther culture.

MATERIALS AND METHODS

The experiments were carried out with the following flax cultivars: 'Lirina', 'Barbara', 'Szaphir', 'Mikael', 'Norman', 'Symphonia', 'Atalante', 'Linola', 'Avangard'. Seeds were germinated and grown in the growth chamber with a 16 h photoperiod, temperatures of 18/14°C, day/night and 75% humidity. All plants were grown in a 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 ($N : P_2O_5 : K_2O$) at the rate of 4 g l^{-1} as required.

Flower buds (3.5−4.0 mm in length) were collected when the microspores were at the mid-uninucleate stage previously determined by microscopic observation of anthers (0.9−1.1 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 x 10 mm) containing 3 ml of modified MS induction medium (NH_4NO_3 – 165 mg l^{-1}, as described by Murashige & Skoog, 1962) with different combinations of cytokinin and auxin (2 mg l^{-1} 6-benzylaminopurine (BAP) + 1 mg l^{-1} 1-naphthylacetic acid (NAA), 1 mg l^{-1} 6-benzylaminopurine (BAP) + 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4D) and 1 mg l^{-1} 6-benzylaminopurine (BAP) + 2 mg l^{-1} indole-3-acetic acid (IAA)) and incubated at 25°C in the dark. All media were supplemented with 6% sucrose and solidified with 0.6% agar. Every 4 weeks the calli were subcultured in a fresh medium and were maintained at temperatures of 27/24°C day/night, under a 16 h photoperiod, and at a light intensity of 50 μmol m^{-2} s^{-1}.

A complete randomized design was used for all experiments. For each treatment 120 anthers were cultured (10 anthers/Petri dish; 12 replicates/treatment) and each experiment was replicated in triplicate. The number of anthers producing calli recorded at 28 days after initial inoculation. The percentage of anthers with calli was calculated as the number of anthers producing calli/100 inoculated anthers. The shoot regeneration medium contained MS mineral salts and vitamins supplemented with 375 mg l^{-1} glutamine, 3% sucrose, 1 mg l^{-1} BAP 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 3% sucrose, 0.001 mg l^{-1} NAA, 0.01 mg l^{-1} BAP and 0.6% agar. The percentage of callus forming shoots was calculated after each subculture.

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

RESULTS AND DISCUSSION

Efficient production of a high-quality callus is a prerequisite for achieving efficient plant regeneration via indirect organogenesis in anther culture of flax. The whole androgenetic process of anther culture in the present study was similar as has been described previously (Burbulis et al., 2005). Formation of the callus was observed within two weeks after culture initiation. The frequencies of callus induction varied among different treatments and tested genotypes. Results of the effect of growth regulator combination on callus induction in nine flax genotypes are summarized in Table 1.

Table 1. Effect of growth regulator combinations on callus induction in anther culture of nine flax genotypes.

Genotype	Percentage of anthers producing callus (%)		
	2 mg l^{-1} BAP + 1 mg l^{-1} NAA	1 mg l^{-1} BAP + 2 mg l^{-1} 2,4-D	1 mg l^{-1} BAP + 2 mg l^{-1} IAA
'Lirina'	38.6±0.30b	29.2±0.95a	41.6±0.50b
'Barbara'	31.9±0.73c	27.8±0.74a	5.5±0.27h
'Szaphir'	67.8±1.02a	9.7±0.53b	43.6±0.57a
'Mikael'	40.3±1.02b	7.5±0.46c	31.3±0.27c
'Norman'	9.7±0.74f	0d	20.0±0.46e
'Symphonia'	0g	0d	16.4±0.30f
'Atalante'	26.4±0.99d	8.6±0.30bc	14.5±0.27g
'Linola'	19.4±0.99e	27.2±0.74a	22.0±0.27d
'Avangard'	0g	11.1±0.57b	0i

Data are means ± SE within four weeks of culture. Means within a column followed by the same letter are not significantly different, indicated by Duncan's multiple-range test ($P \leq 0.01$)

The mean values of the percentage of anthers producing callus ranged from 5.5%, for the 'Barbara' cultivar on the medium with 1 mg l^{-1} BAP + 2 mg l^{-1} IAA, to 67.8% for the 'Szaphir' cultivar on medium supplemented by 2 mg l^{-1} BAP + 1 mg l^{-1} NAA.

The 'Barbara', 'Szaphir', 'Mikael' and 'Atalante' cultivars showed the highest value of induced anthers on the medium with 2 mg l^{-1} BAP and 1 mg l^{-1} NAA, whereas callus formation in these genotypes was strongly reduced by the two other growth regulator combinations. 'Linola' and 'Avangard' anthers showed the best response on medium supplemented by 1 mg l^{-1} BAP and 2 mg l^{-1} 2,4-D while the combination of 1mg l^{-1} BAP with 2 mg l^{-1} IAA promoted callus formation in anthers of the 'Lirina', 'Norman' and 'Symphonia' cultivars.

Shoot regeneration from the anther derived callus is a critical phase of the androgenetic process. One of the factors affecting the efficiency of regeneration from the anther derived callus is the plant genotype. Of nine flax genotypes examined for shoot regeneration ability, four showed shoot formation (Table 2).

Table 2. Effect of growth regulator combinations on shoot regeneration in anther culture of four flax genotypes.

Genotype	Shoot formation frequency (%)		
	2 mg l^{-1} BAP + 1 mg l^{-1} NAA	1 mg l^{-1} BAP + 2 mg l^{-1} 2,4-D	1 mg l^{-1} BAP + 2 mg l^{-1} IAA
'Lirina'	9.2±0.62b	0c	12.7±0.50a
'Barbara'	5.2±0.12c	22.0±0.49 b	0c
'Mikael'	11.7±0.40a	0c	8.0±0.07b
'Linola'	0d	24.5±0.68a	0c

Data are means ± SE within four weeks of culture. Means within a column followed by the same letter are not significantly different, indicated by Duncan's multiple-range test ($P \leq 0.01$)

The frequency of shoot formation in four responsive genotypes ranged from 5.2% in 'Barbara' to 24.5% in 'Linola', whereas none of the other tested cultivars exhibited any shoots. ANOVA and the Duncan test suggested that frequency of shoot formation is significantly affected by genotype ($P < 0.01$). This result indicates that shoot formation ability is controlled genetically. Our previous results demonstrated that anther culture response could be improved when the F_1 hybrids of recalcitrant genotypes with responsive genotypes were used as the donor plants (Burbulis & Blinstrubiene, 2006).

Growth regulator combination in the induction medium is another factor affecting shoot regeneration. In flax it was reported that the combination of 1 mg l^{-1} BAP with 2 mg l^{-1} 2,4-D in the induction medium dramatically improved organogenetic capacity of anther culture-derived callus, in subsequently significantly increased the overall efficiency of regeneration compared with the combination of 2 mg l^{-1} BAP and 1 mg l^{-1} NAA (Chen et al., 1998). The significant effect of 2,4-D on callus formation and plant regeneration has been reported in studies of anther culture in brussels sprouts (Ockendoon & McClenaghan, 1993), soybean (Rodrigues et al., 2004), cucumber (Ashok Kumar et al., 2003) and loquat (Li et al., 2008).

In the present study, the combination of 1 mg l^{-1} BAP and 2 mg l^{-1} 2,4-D in the induction medium improved shoot regeneration frequency in 'Barbara' and 'Linola', but completely inhibited shoot formation from the anther-derived callus of 'Lirina' and 'Mikael'. The cultivar 'Lirina' showed the highest shoot regeneration frequency when the callus originated from the induction medium supplemented with 1 mg l^{-1} BAP and 2 mg l^{-1} IAR while a combination of 2 mg l^{-1} BAP and 1 mg l^{-1} NAA promoted shoot development from the callus of 'Mikael'. There was no correlation between shoot regeneration and callus induction (data not shown). Gene(s) responsible for shoot regeneration appeared not to be involved in callus induction.

The number of shoots per explant sample tissue ranged from 2.33 in 'Barbara' to 14.82 in 'Mikael'. The effect of subculture passages was also evaluated. The highest rate of shoots per sample was obtained in the second subculture (Fig.1).

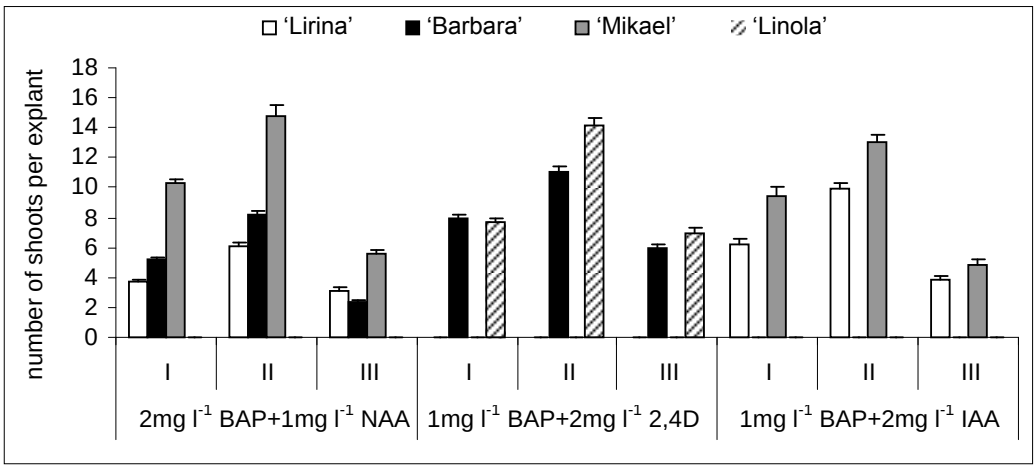

Fig. 1. The effect of subculture on shoots number per explants samples in anther culture of four flax genotypes (I – first subculture; II – second subculture; III – third subculture).

These results illustrate, that genetic background is important in callus induction and shoot regeneration of anther culture in flax, and therefore specific combinations of growth regulators must be designed for each genotype in order to elicit optimum results. Genotypic difference in anther culture response has been previously reported in flax (Chen & Dribnenki, 2002; Lassaga et al., 2004), rapeseed (Burbulis et al., 2004) and rye (Ma et al., 2004). The results of this study demonstrate that the percentage of plant regenerated from anthers of cultivars tested can reach up to 24.5% under optimum conditions. The responsive cultivars identified in this study have been used as parents with agronomically desirable genotypes to produce F_1 hybrids for doubled haploid production.

The high efficiency of plant regeneration obtained in this study should help promote the application of this technique in breeding programs. Experiments are underway to optimize the system to overcome the problem of genotype-dependent regeneration by modifying growth regulator combinations in the induction medium.

CONCLUSIONS

The current study indicates that there is a strong genotype effect on callus induction and shoot regeneration from anthers in flax, and therefore specific combinations of growth regulators must be designed for each genotype. The maximum number of shoots per explant tissue sample was obtained in the second subculture.

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