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Flax anther culture: effect of genotype, cold treatment and media

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Abstract

We report on screening of wide range of flax cultivars for androgenic response and on testing of induction conditions for flax (*Linum usitatissimum* L.) anther culture and plant regeneration. Anthers were cultured on four different media: Mo, N6, MS and N&N supplemented with various combinations of growth regulators. The induction of callus formation from cultured anthers was the highest on N6 (with cultivar PR FGL 77 – 12%) and N&N media (with cultivar Carolin – 2.8%), preferentially after cold pretreatment (7 days at 8 °C). Shoots were formed on calli derived from the microspores inside the cultured anthers on media N&N and N6 supplemented with 1 mg l^{-1} zeatin or 1 mg l^{-1} BAP + 1 mg l^{-1} NAA, respectively and elongated on MS medium supplemented with 2 mg l^{-1} zeatin. The highest number of shoots (120) was observed with cultivar Red Wing. Shoots were rooted on MS medium supplemented with 2 mg l^{-1} IAA. Our experiments resulted in total in 62% anther response and 155 plants regenerated and transferred into soil.

Abbreviations: BAP – 6-benzylaminopurine; 2,4D – 2,4-dichlorophenoxy acetic acid; Mo – medium according to Monnier (1978); MS – medium according to Murashige and Shoog (1962); NAA – naphthalenacetic acid; N & N – medium according to Nitsch and Nitsch (1986); N6 – medium according to Chu (1978)

Introduction

Flax (*Linum usitatissimum* L.) is used for fibre and oil production. The use of haploid techniques in flax breeding would enable advantages of rapid development of completely homozygous lines within one generation and an efficient mean of genotypic selection (Rajhathy, 1976). The first source of haploid plants reported in *Linum* was via polyembryony (Kappert, 1933). Anther culture is currently the most successful method of producing flax DH lines, however, the overall efficiency of plant regeneration is not yet suitable for practical breeding programs and flax is considered recalcitrant to anther culture based regeneration of dihaploid plants (Chen and Dribnenki, 2002).

Anther culture of *Linum usitatissimum* L. has been the subject of several studies (Sun and Fu, 1981; Nichterlein et al., 1991; Friedt et al., 1995; Bergmann and Friedt, 1996; Tejklova, 1996; Chen et al., 1998a, b, 1999, 2002; Pretova et al., 2000; Pretova and Obert, 2000, 2003; Rutkowska-Krause, 2003). These studies revealed the high genotypic dependence in anther response and influence of culture conditions. Efficiency of regeneration in flax anther culture in limited number of breeding lines has been improved through modification of culture conditions (Chen et al., 1998a, b). Although success with wide range of genotypes in anther culture induction was achieved (Pretova and Obert, 2000) and process of gametic embryogenesis in flax was studied in more details and compared to zygotic and somatic embryogenesis (Pretova et al., 2000; Pretova and Obert, 2003), general procedure for flax anther culture induction and dihaploid plant regeneration is not available yet.

The objective of present study was to evaluate the anther culture response of different linseed and

fiber flax genotypes, to determine the effect of donor material pretreatment, effect of different media and medium composition on callus induction and shoot regeneration and to elaborate efficient general protocol for flax (linseed and fiber flax) anther culture.

Materials and methods

Experiments were carried out with two linseed oil cultivars Szegedi 30 and Flanders, the fibre cultivars Carolin, Viking, Super, Belinka, Red Wing, and two breeding lines PR FGL 77 and San Elias 192/22.

The donor plants were grown in field conditions, at the experimental field of Institute of Plant Genetics and Biotechnology in Nitra. Flower buds were collected when microspores were in uninucleate stages. Anthers of all cultivars were analysed for the developmental stage of microspores using acetocarmine staining (Tomaskova, 1974) and for microspore viability using FDA (fluoresceindiacetate) (Widholm, 1972). When effect of cold pre-treatment was tested, the flower buds were wrapped in a moist filter paper and in an aluminium foil and stored in a refrigerator at 8 °C for 7 days.

Flower buds were surface sterilised in 70% ethanol for 1 min and then in 0.8% NaClO for 10 min and rinsed three times with sterile water. Thereafter, anthers were isolated and placed on induction media.

Induction media used in our experiments were: MS (Murashige and Skoog, 1962), N6 (Chu, 1978), Mo (Monnier, 1978) and N&N (Nitsch and Nitsch, 1986) containing 6% sucrose or 3% sucrose and 3% maltose (60 mm Petri-dishes, 10 ml medium and 50 anthers/dish). Media were supplemented with growth regulators: 2,4-D (0.5 mg l^{-1} or 2.5 mg l^{-1}), dicamba (2 mg l^{-1}), BAP (1 mg l^{-1}) + (NAA 1 mg l^{-1}), kinetin (1 mg l^{-1}, 2 mg l^{-1}, 6 mg l^{-1}), zeatin (1 mg l^{-1}, 2 mg l^{-1}, 6 mg l^{-1}) or thidiazuron (2 mg l^{-1}).

Based on the results from these experiments two combinations of growth regulators added to N6 media were selected and used in further studies (Table 2 and 3).

Anther cultures were incubated at 23 °C under a 16-h photoperiod with light source providing 8.6 W m^{-2} light intensity (Phillips TLD, PHILLIPS). Calli with a diameter of more than 3 mm were transferred onto fresh media of the same composition as induction media for shoot induction. Calli were cultured at 21–23 °C under a 16-h photoperiod and light intensity of 17.8 W m^{-2}. For cytological analyses three calli per each treatment were embedded in paraffin, cut on a rotary microtome and observed in light microscope (Axioplan II, Carl Zeiss, Jena) after staining with ferric hematoxylin.

Shoots from differentiated calli were transferred onto MS medium with 2 mg l^{-1} zeatin for shoot elongation. Shoots 4 cm long were isolated and transferred to a rooting media (MS basal or MS supplemented with 2 mg l^{-1} IAA). Experiments were replicated three times. More than 7500 isolated anthers have been used in each of three repetitions.

Results and discussion

The response of flax anther cultures is highly genotypic dependent (Chen et al., 1998a, b). It is important to reduce this problem by continual intensified research on the induction of *in vitro* androgenesis in flax anther culture.

Anther culture of flax (*Linum usitatissimum* L.) have been subject of studies with fibre flax (Sun and Fu, 1981) and linseed (Nichterlein et al., 1991; Chen et al., 1998a, b; Steiss et al., 1998; Rutkowska-Krause et al., 2003) and plant regeneration has been described (Nichterlein et al., 1991; Chen et al., 1998a, b). Microspore culture of flax was studied as well (Nichterlein and Friedt, 1993). Anther culture is presently considered as the most promising method for producing doubled haploids in flax (Steiss et al., 1998).

However, up to 50% of anther culture derived plants originated from the somatic tissues of anthers (Friedt et al., 1995), which are of no value for breeding purposes. In our studies, we used cultivation media with osmotic level (6% of sucrose or 3% of sucrose combined with 3% of maltose) not favourable for callogenesis of anther somatic tissues (Pretova and Obert, 2000). Beside this, calli were produced after anthers opened in the induction medium. These conditions ensure that calli were produced from microspores. For further proof, molecular methods have to be employed. For practical purpose the use of described princi-

ples ensures the plant origin from haploid cell only.

The overall efficiency of shoot initiation and plant regeneration was generally higher than anther efficiency since more than one callus from responding anther was generated what suggests that more microspores within one anther were induced and formed calli.

One of the critical points for successful induction in anther culture is the selection of suitable donor material. Not only the genotype, but also the physiological condition of donor plants as well as the developmental stage are crucial for haploid induction. Our present and previous results (Pretova and Obert, 2000) suggest, that the optimal stage of microspores is the mid- and late-uninuclear stage of the microspore. The viability of microspores in anthers was higher than 60% at the beginning of cultivation. Later the viability dropped dramatically – when only microspores which were able to redirect their developmental pathway to sporophytic development were able to survive.

Four different basal media (MS, Mo, N6 and N&N) for the induction of *in vitro* androgenetic response were tested. Reasons for choosing those were as follows: MS medium is the most frequently used in tissue culture, the Mo medium very well supported somatic embryo formation in flax (Pretova and Williams, 1986) and N6 and N&N media were developed for haploid induction for different plant species. Variable responses were shown by the flax genotypes cultured on different media (Table 1). Callus formation was only achieved on specific media developed for anther cultures N6 (ranges from 0.7% with genotype Szegedi 30 to 12% with cultivar PR FGL 77) and N&N (0.25% Szegedi 30 and 2.8% Carolin). Statistical analyses of responses show, that differences between media were significant ($p < 0.05$) as well as differences among genotypes were significant ($p < 0.05$). No callus formation was observed using MS and Mo media.

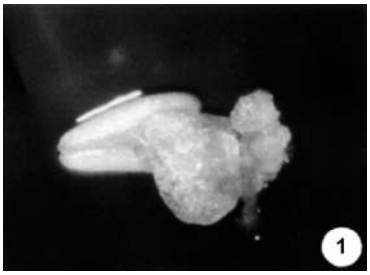

Figure 1. Responding anther of cv. Carolin with callus growing within 3 weeks in culture.

First calli appeared on anthers after 3 weeks in culture (Figure 1). Callogenesis was not synchronous and the callus formation was of different intensity depending on the cultivar and culture conditions used. The haploid origin of calli was shown by the haploid number of chromosomes in most of the calli cells (Figure 2). Cytological analyses were performed in the first subculture as

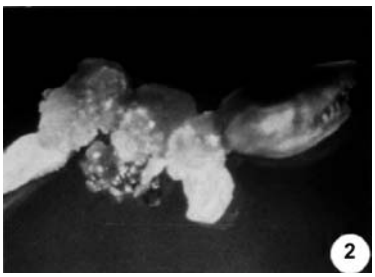

Figure 2. Shoot buds on the N6 medium supplemented with 2 mg l^{-1} zeatin and 60 g l^{-1} sucrose after 4 weeks in culture (cv. Carolin).

Table 1. Effect of different culture media on callus and shoots formation from flax anthers

	Media	No. of cultivated anthers	% of induced anthers	Shoot regeneration (%)
Carolin	N6	470	1.9 ± 0.4	70
Carolin	N&N	500	2.8 ± 1.0	42
Flanders	N6	350	1.7 ± 0.6	19
Flanders	N&N	400	0.0	0
Szegedi-30	N6	400	0.7 ± 0.3	15
Szegedi-30	N&N	500	0.3 ± 0.0	46
Viking	N6	800	3.9 ± 1.2	0
Viking	N&N	400	0.0	0

later the number of chromosome irregularities in the calli cells increased.

To improve anther culture response of recalcitrant genotypes some physiological and environmental factors can be employed that can influence the response of anther culture (Chen and Dribnenki, 2002). Usually a stress is provided by heat or cold treatment or by starvation to induce switch of developmental programme from pollen development to *in vitro* androgenesis (Touraev et al., 1997). Our better results in flax anther culture were due to pre-cultivation of the donor material at 8 °C for 7 days.

Two combinations of growth regulators added to N6 media were selected to test androgenic induction in all flax genotypes. These combinations were used for cultivation of flax anthers with and without cold pre-treatment.

The flax anthers after cold pre-treatment and fresh anthers without any pre-treatment of genotypes Super, Belinka and San Elias 192/22 gave no response (Table 2). Androgenic response was achieved after cold pre-treatment with genotypes Szegedi 30, Flanders, Red Wing and PR FGL 77. Androgenic reaction of genotype PR FGL 77 was independent of cold pre-treatment. This genotype was most suitable for induction of *in vitro* androgenesis using the system employed in this study. In case of genotype Szegedi 30, we obtained anther response using both combinations of growth regulators only when the donor material was cold pre-treated (Table 2). Flanders and Red Wing were also responsive after cold pretreatment, but only when cultivated on media supplemented with 1 mg l^{-1} NAA and 1 mg l^{-1} BAP (Table 3). Anthers of genotype Viking responded only when fresh material (without cold treatment) was cultivated on media with 1 mg l^{-1} zeatin (Table 2). Anther induction was affected by cold treatment only slightly ($p = 0.122$). In general, significantly better results on structure induction ($p < 0.05$) were obtained after pre-cultivation of donor material at 8 °C for 7 days.

Table 2. Effect of genotype and cold treatment on flax anthers cultivated on media N6 supplemented with 1 mg l^{-1} zeatin

Genotype	Cold treatment	Anther response (%)	No. of shoots per 100 anthers
Super	+, –	0	0
Belinka	+, –	0	0
San Elias 192/22	+, –	0	0
Szegedi 30	+	8	24
Szegedi 30	–	0	0
Flanders	+	0	0
Flanders	–	0	0
PR FGL 77	+	8	40
PR FGL 77	–	4	40
Red Wing	+	0	0
Red Wing	–	0	0
Viking	+	0	0
Viking	–	4	4

Comparing oilseed, fibre flax and new flax breeding lines, we can conclude that the highest anther response was achieved with breeding lines, lower was the response with oil genotypes and the lowest was with the fibre genotypes. Our results are in agreement with some previous data for induction of the androgenic process depending on the genetic predisposition of the given genotype (Nichterlein et al., 1991; Nichterlein and Friedt, 1993; Friedt et al., 1995; Bergmann and Friedt, 1996; Tejklova, 1996; Chen et al., 1998a, b; Steiss et al., 1998; Rutkowska-Krause et al., 2003).

Most of the structures obtained in our experiments were calli, which were of different colour (white yellow, yellow or yellow-green and light green) and their growth was not uniform. Within 4 weeks of cultivation on regeneration media shoot buds appeared on calli (Figure 3). Shoots regenerated from calli on media N&N and N6 supplemented with 1 mg l^{-1} zeatin or 1 mg l^{-1} BAP combined with 1 mg l^{-1} NAA. Number of calli forming shoots was highest on N6 medium with cultivar Carolin (Figure 3 and Table 1). The highest number of shoots per 100 anthers was achieved on calli derived from anthers of genotype

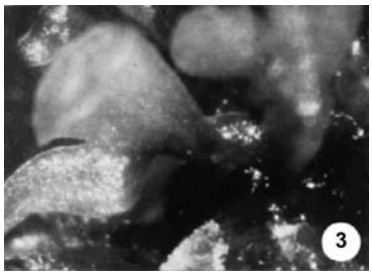

Figure 3. Shoot regeneration on N6 medium supplemented with 2 mg l^{-1} zeatin and 60 g l^{-1} sucrose after 5 weeks in culture (cv. Carolin).

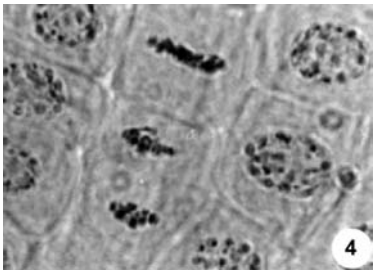

Figure 4. Haploid chromosome number ($n = 15$) in root tip cells of microspore-derived anther regenerants of cv. Carolin.

Table 3. Effect of genotype and cold treatment on flax anthers cultivated on media N6 supplemented with 1 mg l^{-1} NAA and 1 mg l^{-1}BAP

Genotype	Cold treatment	Anther response (%)	No. of shoots per 100 anthers
Super	+, –	0	0
Belinka	+, –	0	0
San Elias 192/22	+, –	0	0
Szegedi 30	+	6	80
Szegedi 30	–	0	0
Flanders	+	4	24
Flanders	–	0	0
R FGL 77	+	12	40
PR FGL 77	–	12	68
Red Wing	+	4	120
Red Wing	–	0	0
Viking	+	0	0
Viking	–	0	0

Red Wing on N6 medium (Table 3). Statistical analyses of shoot regeneration showed significant differences between cultivars ($p < 0.01$) and culture conditions ($p < 0.05$).

The most effective rooting (80%) was achieved on MS (Murashige and Skoog, 1962) basal medium with 2 mg l^{-1} IAA. In total, our experiments resulted in 62% responding anthers and a total of 155 plants regenerated and transferred into soil.

Our results form basis for further research to develop efficient protocols for doubled haploid production. Experiments on establishing reliable microspore culture in flax that may fully guarantee the microspore origin of the regenerants and experiments on the molecular assessment of microspore origin in anther culture regenerated plants are in progress.

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