Obtaining Calli and Regenerated Plants in Anther Cultures of Pea

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Abstract

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Pea (*Pisum sativum* L.) is a species for which there is no efficient method for the recovery of haploid plants yet. This research investigated the influence of various genotypes, nutrient media, and stress treatments on callus formation, embryogenesis and plant regeneration in anther cultures of pea. A wide range of pea genotypes and nutrient media was studied. Morphogenic calli were initiated on media supplemented with α-naphthaleneacetic acid (NAA), 6-benzyladenine (BA), and 2,4-dichlorophenoxyacetic acid (2,4-D) without application of stress treatments. Embryogenic calli and embryos were regenerated on media with low sucrose content in the presence of 2,4-D or indole-3-butyric acid (IBA) after cold stress (4°C) of isolated buds, alone or in combination with *in vitro* treatment of isolated anthers at higher temperatures (35–38°C). The efficiency of regeneration via shoot morphogenesis on different nutrient media and the peculiarities of regeneration from embryogenic calli were investigated. Green embryogenic calli initiated on 2,4-D were able to develop through shoot morphogenesis on a medium supplemented with BA and NAA. This process led to regeneration of hypertrophic embryos at various developmental stages. The origin of regenerated plants (i.e. from microspores or somatic anther cells) was estimated using marker alleles determining morphological traits. Almost all R_0 regenerants derived from morphogenic calli originated from anther somatic cells.

Keywords: callus; embryo; haploid plant; microspore; regeneration; stress treatment

Haploidy is a powerful method of plant breeding. Completely homozygous plants with various combinations of genes may be obtained in a short period of time. Research on pea (*Pisum sativum* L.) haploidy began in the 1960–80s. Calli, roots, shoots and embryos were obtained in anther culture (Gupta *et al*. 1972; Gupta 1975; Gosal & Bajaj 1988). More recent work attempted haploid plant recovery from cultures of isolated anthers and microspores. Calli, embryo-like structures, regenerated shoots and plants were produced in anther culture by Sidhu and Davies (2005). Multinucleate syncytia with the intact exine were observed in a culture of isolated microspores (Croser *et al*. 2005), while haploid plants were obtained by means of shock temperature and osmotic stress treatments or electroporation (Ochatt *et al*. 2009; Lulsdorf *et al*. 2011).

Initialization of plant regeneration in a callus culture of pea is a critical step. Two different strategies have been introduced: plants could be obtained via shoot organogenesis in the presence of 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA) (Hussey & Gunn 1984; Griga *et al*. 1986; Kubalákova *et al.* 1988; Ochatt *et al.* 2000) or through somatic embryogenesis and subsequent plant regeneration from developing embryos.

Somatic embryos could be produced as a result of direct embryogenesis or indirectly – through the formation of embryogenic callus tissues (Griga 1998). For instance, somatic embryos were initiated from calli of immature zygotic embryos and apical meristems on media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram (Kysely *et al.* 1987; Lehminger-Mertens & Jacobsen 1989; Stejskal & Griga 1992). Direct embryogenesis was observed in the presence of NAA or 2,4-D in the culture of immature embryos (Tetu *et al*. 1990). Picloram was used for the initiation of direct embryo-

genesis in the culture of apical meristems (Griga 1998). Embryogenesis was also initiated in a culture of isolated protoplasts (Lehminger-Mertens & Jacobsen 1989; Ochatt *et al*. 2000, 2005). Multicellular origin of somatic embryos was verified for both the direct and indirect regeneration (Griga 2002).

Regeneration of whole plants from somatic embryos was achieved on media with a low content of NAA, BA, kinetin and zeatin (Lazzeri *et al.* 1987), NAA (Lehminger-Mertens & Jacobsen 1989), or GA_3 (gibberellic acid) (Stejskal & Griga 1992). Regeneration via embryo germination occurred on media with 3 mg/l indole-3-butyric acid (IBA) and 0.5 mg/l BA (Tetu *et al*. 1990) or without growth regulators (Griga 1998, 2002). Efficient regeneration was observed as a result of massive *de novo* formation of adventitious buds and shoot organogenesis in large embryos placed onto media with thidiazuron (Griga 1998).

Pea is a species for which there is no reliable method of haploid plant recovery yet. The aim of the current research was to investigate the influence of various genotypes, nutrient media, and stress treatments on callogenesis, embryogenesis and plant regeneration from anther cultures of pea.

MATERIAL AND METHODS

Initiation of morphogenic calli. Isolated anthers of pea variety Orlovchanin and hybrid F_1 K-23-00 (LU-203-94 × Tristar) were inoculated on agar media. Plants of LU represented "multibean apical peduncle" (Uvarov 1993).

Several modifications (Np4, Np41, Np5, Np51) of culture medium N_6 (Chu 1978) supplemented with growth regulators NAA, BA, and 2,4-D were tested (Table 1). No stress treatment was applied to buds or isolated anthers. In total, 480 anthers were placed on nutrient media. Isolated anthers were cultivated *in vitro* at 25°C and a 16-hour photoperiod.

Determination of the microspore development stage was conducted according to Telmer *et al.* (1992). Propionic carmine (4%) was applied for microspore staining. An Axioscope 40 microscope (Karl Zeiss, Jena, Germany) was used in experiments. Anthers were isolated from buds containing microspores predominantly at the uninucleate developmental stage, which corresponds to the length of about 6–7 mm (Bobkov 2010).

Initiation of embryogenic calli. Anthers of pea varieties Pharaon, Zaryanka, Vizir, Stabil, F_1 hybrids K-23-00 (LU-203-94 × Tristar), 341 (Madonna × LU-268-98), and interspecific F_1 hybrid SF – *Pisum sativum* (variety Stabil) × *P. fulvum* (accession I609881) were inoculated on agar media.

Modified nutrient media MSB (Griga *et al.* 1986) contained MS salts (Murashige & Skoog 1962), vitamins B5 (Gamborg & Eveleigh 1968) and 100 mg/l of myo-inositol were supplemented with growth regulators (2,4-D or IBA, Table 1). All media tested were supplemented with low (< 15 g/l) or moderate (> 15.1 g/l) amount of sucrose. To increase osmotic pressure, maltose was added into media with low sucrose content in some variants. In total, 2700 or 2220 anthers were inoculated on media with low or moderate sucrose content, respectively.

Two subsequent levels of stress treatments were applied: isolated buds were pretreated at a low temperature (4°C), anther cultures *ab initio* at higher temperatures (from 35 to 38°C). Anthers were placed on MSBE medium (Table 1) after 18 h at 37°C, or after a combination of low temperature for 24 h, followed by heat treatment (38°C for 18 h) (cv. Stabil). Cultivar Visir was treated under two regimes: at a low temperature (4°C) for 72 h followed by heat shock (35°C for 18 h) or at a low temperature for 96 h with subsequent heat shock (35°C, 18 h). For anther isolation and further cultivation see the methodology in the above subchapter.

Plant regeneration. Regeneration from morphogenic calli was stimulated and maintained on various combinations (Table 1) of MSB media, designed for pea micropropagation (Griga *et al.* 1986). Green morphogenic calli (3–5 mm), initiated previously on modifications of media N_6 and MSB, were transferred onto MSB0 medium. Morphogenic calli derived from anthers of F_1 hybrid K-23-00 (LU-203-94 × Tristar) were then cultivated on the media MSB1, MSB2, MSB3, and XN (Table 1). Segments of callus tissue were transferred onto fresh media once a month several times. Embryogenic calli from anther cultures of Vizir variety, regenerated from the 72/18 h cold/heat treatment were transferred onto R1 medium (Table 1) designed for shoot morphogenesis. Rooting was stimulated on four variants of MSB medium (Table 1) designed for rhizogenesis (Kubalákova *et al*. 1988). Calli and regenerated plants were cultivated at 25°C and a 16-hour photoperiod. Data on the efficiency of plant regeneration on various media were statistically analysed using ANOVA.

Estimation of pea regenerant origin. The origin (either from microspores or from anther somatic cells) of regenerants R_0 and R_1, derived from the anther culture of pea F_1 hybrid K-23-00 (LU-203-94 × Tristar) was reliably determined, as somatic cells of that hybrid

has a genotype *DetdetFasfas* (*det* – determinant type of stem, *fas* – fasciated stem), whereby plants with such *DetdetFasfas* genotype are characterized by a normal stem (Uvarov 1993).

RESULTS

Initiation of morphogenic calli. In the anther culture of pea, inoculated on various agar media, non-morphogenic and green morphogenic calli were initiated (Figure 1a). In total, sixteen (3.3%) green calli were produced from 480 anthers placed on nutrient media. A small difference in the efficiency of green callus formation was observed on media Np4 and Np5 which had different content (2 or 0.2 mg/l) of both growth regulators (BA and NAA, Table 2). The efficiency of morphogenic callogenesis increased on media Np41 and Np51 which were supplemented with 0.1 mg/l of 2,4-D in comparison with Np4 and Np5. In the anther culture of F1 hybrid K-23-00, the efficiency of green callus formation reached a maximum of 10% on Np41 medium. On the other hand, no regeneration of green calli was observed in the Orlovchanin variety.

Initiation of embryogenic calli. The initiation of embryos and embryogenic calli was a rare event. It is

Table 1. The nutrient media used in experiments

Name	Composition
Morphogenic callogenesis	
Np4	N_6 with 40 g/l sucrose, 500 mg/l glutamine, 500 mg/l myo-inositol, 2 mg/l NAA, 2 mg/l BA, and 6 g/l agar
Np41	N_6 with 40 g/l sucrose, 500 mg/l glutamine, 500 mg/l myo-inositol, 2 mg/l NAA, 2 mg/l BA, 0.1 mg/l 2,4-D, and 6 g/l agar
Np5	N_6 with 40 g/l sucrose, 500 mg/l glutamine, 500 mg/l myo-inositol, 0.2 mg/l NAA, 0.2 mg/l BA, and 6 g/l agar
Np51	N_6 with 40 g/l sucrose, 500 mg/l glutamine, 500 mg/l myo-inositol, 0.2 mg/l NAA, 0.2 mg/l BA, 0.1 mg/l 2,4-D, and 6 g/l agar
Embryogenic callogenesis	
b15	MSB (MS, vitamins B5) with 15 g/l sucrose, 8 mg/l IBA, and 7 g/l agar
mmd1m	MSB with 10 g/l sucrose, 10 g/l maltose, 500 mg/l glutamine, 100 mg/l casein hydrolysate, 0.5 mg/l 2,4-D, and 6 g/l agar
mmd1	MSB with 7.6 g/l sucrose, 12.4 g/l maltose, 500 mg/l glutamine, 1 g/l casein hydrolysate, 0.5 mg/l 2,4-D, and 6 g/l agar
mmd1a	MSB with 7.6 g/l sucrose, 12.4 g/l maltose, 500 mg/l glutamine, 1 g/l casein hydrolysate, 0.05 mg/l 2,4-D, and 6 g/l agar
msf	MSB with 0.5 g/l sucrose, 0.05 mg/l 2,4-D, and 5% Ficoll
e5a	MSB with 60 g/l maltose, 5 g/l glutamine, 1 g/l casein hydrolysate, 0.5 mg/l 2,4-D, and 6 g/l agar
MSBE	MSB with 20 g/l sucrose, 50 mg/l thiamine, 500 mg/l nicotinic acid, 50 mg/l pyridoxine, 5 mg/l biotin, 50 mg/l folic acid, 100 mg/l glutamine, 100 mg/l casein hydrolysate, 0.05 mg/l 2,4-D, and 6 g/l agar
Regeneration	
MSB0	MSB with 30 g/l sucrose, 5 mg/l BA, 0.2 mg/l NAA, and 6 g/l agar
MSB1	MSB with 40 g/l sucrose, 5 mg/l BA, 0.2 mg/l NAA, and 6 g/l agar
MSB2	MSB with 40 g/l sucrose, 4 mg/l BA, 1 mg/l NAA, 5 mg/l adenine, and 6 g/l agar
MSB3	MSB with 40 g/l sucrose, 4 mg/l BA, 0.5 mg/l NAA, 10 mg/l adenine, and 6 g/l agar
XN	N_6 with 40 g/l sucrose, 4 mg/l BA, 1 mg/l NAA, 5 mg/l adenine, and 6 g/l agar
R1	MSB with 40 g/l sucrose, 4 mg/l BA, 1 mg/l NAA, and 6 g/l agar
Shoot rooting	
MR1	MSB (1/2 macrosalts content) with 30 g/l sucrose, 1 mg/l IBA, and 6 g/l agar
MR2	MSB (1/2 macrosalts content) with 30 g/l sucrose, 0.5 mg/l IBA, and 6 g/l agar
MR3	MSB (1/2 macrosalts content) with 40 g/l sucrose, 0.25 mg/l IBA, 0.25 mg/l NAA, and 6 g/l agar
MR4	MSB (1/2 macrosalts content) with 40 g/l sucrose, 0.25 mg/l IBA, 0.5 mg/l NAA, and 6 g/l agar

NAA – α-naphthaleneacetic acid; BA – 6-benzyladenine; IBA – indole-3-butyric acid; 2,4-D – 2,4-dichlorophenoxyacetic acid; MSB – MS medium with vitamins B5

of interest that green embryogenic calli and embryos were predominantly produced on media with low sucrose content. For example, isolated anthers of Pharaon variety and interspecific hybrid SF were cultivated on mmd1m medium after 8 days of cold stress treatment and translucent calli were produced. The efficiency of callus formation was 6.6–13.3%. Calli were transferred onto the same medium but without maltose. After 90 days of cultivation calli reached a size of 1.5–2.5 cm, and green globular embryos appeared on their surfaces. In the anther culture of interspecific hybrid SF globular and torpedo embryos were produced. Green microcalli (size up to 2 mm) were initiated in the anther culture of F_1 hybrid K-23-00 with the efficiency of 80% on mmd1 medium with low sugar content (Table 1). On variation of that mmd1a medium, which was characterized by low concentration (0.05 mg/l) of 2,4-D, green globular embryos were obtained from anthers of F_1 hybrid 341 (Madonna × LU-268-98). Two globular embryos (1.5–2 mm) from anthers of that hybrid were produced in msf liquid medium (Table 1).

Green-yellowish calli were obtained on b15 medium in the anther culture of Visir variety after cold stress treatment of buds for 1 day. After 60 days of cultivation five globular embryos (2.5–3 mm) appeared on the surface of one callus. In conditions of that nutrient medium two globular embryos were produced in the anther culture of Zaryanka variety after the same stress treatment.

The presence of sucrose (as the only sugar source) in nutrient media was not necessary for the emergence of initial calli (Figure 1b). Callogenesis with 20% efficiency was observed on e5a medium (Table 1) without sucrose (but substituted with maltose) from anthers of Stabil variety after cold treatment of buds for one month.

Green embryogenic calli (Figure 1c) were produced from anthers of the varieties Stabil and Vizir inoculated on MSBE medium (Table 1). They were all obtained after the use of successive cold (4°C, 24–96 h) and heat (35 or 38°C, 18 h) stress treatments.

Table 2. Efficiency of green calli formation in pea anther culture (%)

Medium	Variety, hybrid		Mean
	Orlovchanin	K-23-00	
Np4	30 (3.3)*	90 (2.2)	2.8
Np41	60 (0)	60 (10)	5
Np5	60 (3.3)	30 (3.3)	3.3
Np51	120 (1.7)	30 (6.7)	4.2

*Number of anthers inoculated (percentage of green calli)

Plant regeneration. Morphogenic calli in the presence of BA and NAA were characterized by cell proliferation and shoot morphogenesis. On average, 10.3 shoots per glass were regenerated after cultivation of tissues on MSB0 medium (control) for one month (Table 3). The number of shoots less or more than 1.5 cm in length was equal to 9.3 and 1, respectively. An increase of sucrose content in MSB1 medium led to a decrease of morphogenic activity. MSB3 medium with 10 mg/l adenine stimulated the formation of multiple shoots with stems less than 3 mm in length and an enlarged base. XN medium with 40 g/l sucrose and moderate adenine content (5 mg/l) showed the best efficiency of shoot morphogenesis (16 shoots per one tissue). In comparison with the control the number of shoots with stem length less or more 1.5 cm on XN medium increased up to 12.9 and 3.1, respectively.

Embryogenic calli placed onto R1 medium, designed for shoot morphogenesis, demonstrated some peculiarities of plant regeneration. Together with cell proliferation and shoot morphogenesis, embryos were also present in tissues (Figure 1d). Subsequently, embryos became hypertrophic (meaning enlargement in size) at globular (Figure 1e), heart and torpedo (Figure 1f) stages of development. Regeneration of hypertrophic shoots (Figure 1g) was also observed, and tissues which regenerated normal shoots were isolated.

Regenerated shoots were transferred onto media MR1, MR2, MR3, and MR4 (Table 1) for the stimulation of root formation. MR3 medium was characterized by the highest efficiency for shoot rooting (Figure 1h).

Estimation of pea regenerant origin. Almost all R_0 regenerants derived from anthers of F_1 hybrid K-23-00

Table 3. Efficiency of long-term regeneration on different media in tissue cultures of F_1 hybrid K-23-00

Medium	No. of shoots per glass	No. of shoots with stem length	
		< 1.5 (cm)	> 1.5
MSB0 (control)	10.3	9.3	1.0
MSB1	6.3*	5.3*	1.0
MSB2	8.6	7.4	1.2
MSB3	multiple shoots with stems less than 3 mm long and an enlarged base		
XN	16.0*	12.9*	3.1*
LSD_{05}	3.9	3.4	1.8

*Differences are significant at α = 0.05; MSB – MS medium with vitamins B5

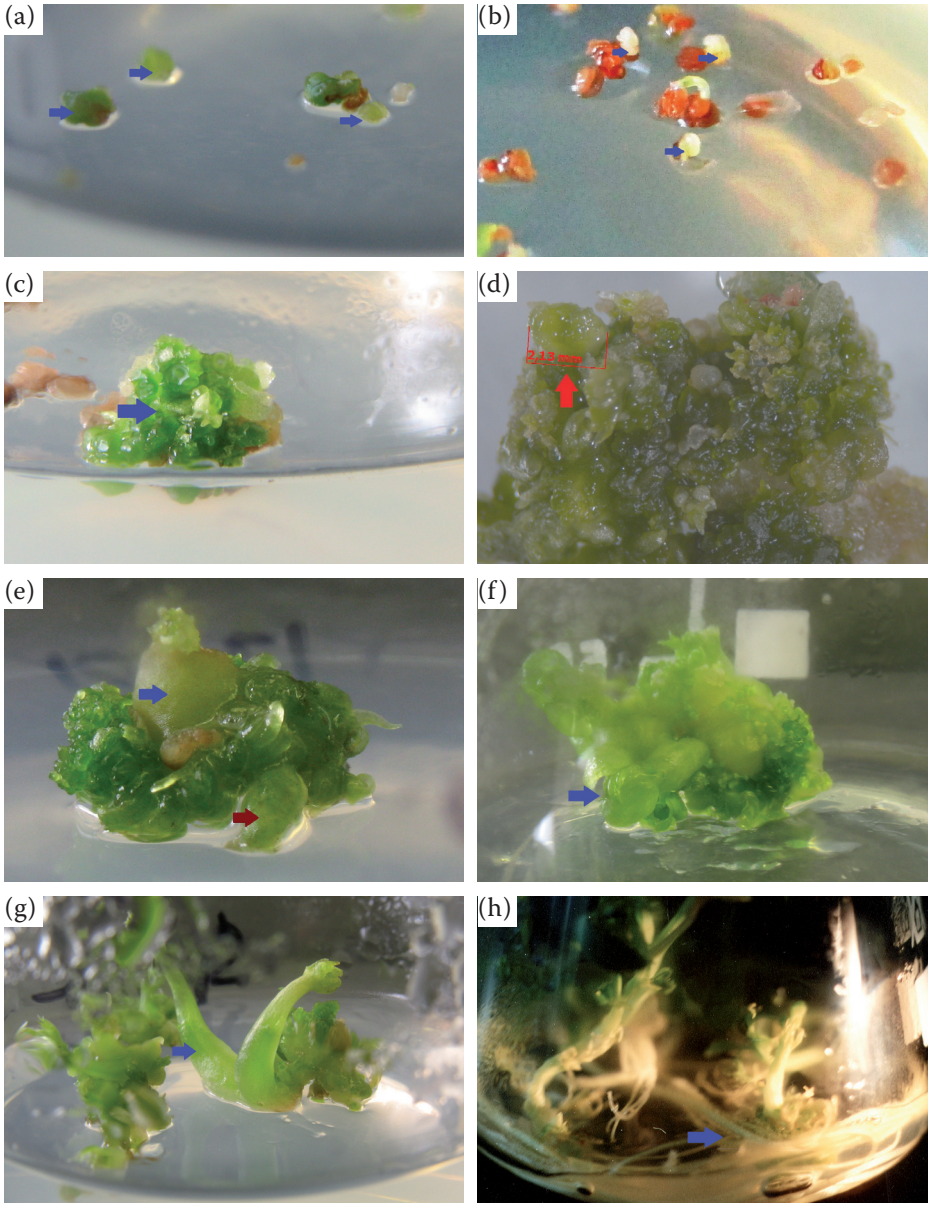

Figure 1. Green calli (arrows) on Np41 medium in the anther culture of F_1 hybrid K-23-00 (LU-203-94 × Tristar) (a); calli formation (arrows) in the anther culture of Stabil variety on e5a medium after bud pretreatment with cold for 1 month (b); green embryogenic callus (arrow) initiated in the anther culture of Vizir variety in the presence of 0.05 mg/l 2,4-D (c); shoot regeneration on the embryogenic callus of Vizir pea variety, heart-like embryo 2.13 mm (arrow) (d); hypertrophic globular-like embryo (blue arrow) and regeneration of a shoot (red arrow) on the callus tissue of Vizir pea variety (e); torpedo-like embryo (arrow) on the callus tissue of Vizir pea variety (f); regeneration of hypertrophic shoots (arrow) from the callus tissue of Vizir pea variety (g); shoot rooting (arrow) on MR3 medium supplemented with 0.25 mg/l IBA and 0.25 mg/l NAA (h)

(LU-203-94 × Tristar) had a normal stem. Plants in families of R_1 regenerants were segregated on marker alleles *Det*, *det*, *Fas*, *fas*. This result showed the heterozygosity of regenerants R_0 and their origin from anther somatic cells (genotype *DetdetFasfas*). However, regeneration of two plants R_0 with fasciated stem was observed in a single flask, and fasciated stem could be under the control of postmeiotic genotype *DetDetfasfas*.

DISCUSSION

Pea is a recalcitrant species in terms of haploid plant production. In the anther culture of pea with temperature stress treatments (4 and 37°C), calli, embryo-like structures and two regenerants were obtained (Sidhu & Davies 2005). In the culture of isolated microspores, appearance of multinuclear syncytia with the intact

exine and single regenerants were observed (Croser *et al*. 2005). In later experiments, a reduced number of haploid regenerants were produced after the application of low temperature, osmotic stresses and electroporation (Ochatt *et al*. 2009). It was also noted that the treatment of buds with cold (4°C) for more than 48 h increased the number of microcalli.

In current research nutrient media with relatively high (Np4, Np41) and low (Np5, Np51) content of growth regulators showed small differences in the efficiency of morphogenic calli formation. We proposed that some other factors like stress treatments and media composition could strongly influence the reprogramming of pea microspores to the sporophytic pathway of development. For example, in our experiments green embryogenic calli and embryos were predominantly produced on media with low sucrose content. In the anther culture of Stabil variety microcalli were initiated on the nutrient medium e5a without sucrose (but substituted with maltose).

In pea anther culture the initiation of calli often occurred from somatic cells (Bobkov 2010). We hypothesized that the use of severe temperature stress treatments and low concentration of growth regulators might lead in future to the recovery of haploid plants. In our latest experiment we used treatments of anthers with high temperature (from 35 to 38°C). Viable green embryogenic calli were produced after the treatment of isolated calli at 35°C applied to isolated anthers in conditions of nutrient medium. For the stimulation of cell division, 2,4-D at a low concentration (0.05 mg/l) was used.

Regeneration via shoot morphogenesis is an effective system which allows obtaining large numbers of regenerants for pea breeding. Reprogramming of embryogenic calli from embryogenic development to the pathway of shoot morphogenesis could be an attractive mode of regeneration. In this respect, an efficient method of regeneration was described by Griga (1998), who showed that morphologically abnormal embryos initiated in the presence of picloram were able to produce massive *de novo* regeneration on MSB medium supplemented with thidiazuron via shoot organogenesis without differentiation of the embryo apex.

In our experiment, following the transfer of embryogenic calli onto MSB medium supplemented with BA and NAA, cultivated tissues with shoot regeneration were established. In these tissues, hypertrophic embryo-like structures at various developmental stages were observed. Totipotent cells of these tissues non-programmed for embryogenic development may be recruited on the pathway of shoot morphogenesis.

There are several methods for the evaluation of regenerants, originated from microspores or anther somatic cells. For example, a haploid set of chromosomes in somatic cells of regenerants could prove their origin from microspores. Flow cytometry is a simple, quick and reliable method for indication of androgenic divisions within microspores (Ribalta *et al*. 2012). Markers could also help to evaluate the origin of regenerants independently of their cell ploidy. In the current research, morphological markers were applied to evaluate the origin of regenerants from somatic cells of anthers or microspores.

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