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In vitro responses of various explants of *Fagus sylvatica*

Abstract: There are limited published data on in vitro reproduction of *Fagus sylvatica* L. (European beech). This study was aimed to determine the efficiency of induction of somatic embryogenesis or organogenesis of beech from different types of explants in various culture conditions. Explants derived from immature, fresh seeds (collected in 2011 and 2013) and from mature seeds, stored at –10ºC and some stratified at 3ºC, were placed on induction media with various combinations of plant growth regulators: zeatin, 2,4-dichlorophenoxyacetic acid (2,4-D) and/or benzyladenine (BA). Initial cultures were kept in darkness or weak light (white fluorescent or blue-red LED). Limited success has been achieved in initiation of somatic embryogenesis. We obtained friable, yellow-white callus with characteristic PEM-like structures (cPEM-ls, from embryonic axes or fragments of immature embryos with embryonic axes), which may be an early developmental stage of embryogenic callus of *Fagus sylvatica*. This type of callus regenerated from explants incubated in darkness, mainly on WPM medium with addition of 6.8 μM zeatin or WPM and MSG media with 9.1 μM 2,4-D and 2.2 μM BA. The highest frequency of regeneration of callus with cPEM-ls was 5%. Instead, we succeeded to induce organogenesis from both immature and mature zygotic embryos and from embryonic axes. The best results were obtained for mature zygotic embryos incubated on ½WPM medium (half-strength Woody Plant Medium) with 9.1 μM 2,4-D and 2.2 μM BA. Adventitious buds were regenerated on up to 15% of the explants. The induced buds developed into shoots, enabling us to establish tissue cultures of beech. Induction of organogenesis from the tested explants was more efficient than induction of somatic embryogenesis.

Additional key words: European beech, micropropagation, culture conditions, tissue culture

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Introduction

Intensive studies of somatic embryogenesis of trees – both coniferous and deciduous species – were started in the mid-1980s in a number of research centers around the word (USA, Canada, France, Spain, New Zealand and India). Their main objective was to develop and improve this micropropagation method of economically and ecologically valuable trees. The practical aim was to provide high-quality planting material for clonal plantations, to meet the needs of the industry (wood, paper) or forestry.

Somatic embryogenesis is an attractive in vitro propagation method because of the potentially high reproduction rate and the ability to automate somatic embryo production. In the past 3 decades, somatic embryogenesis was studied intensively in both

gymnosperm and angiosperm tree species (Chalupa 1987; Durzan and Gupta 1988; Becwar et al. 1990; Chmielarz 1999; Chmielarz et al. 2005; Hazubska and Szczygieł 2003; San et al. 2007; Teixeira da Silva and Malabadi 2012). In Europe, among broadleaved trees, most attention was paid to oaks (*Quercus* spp.) (Joergensen 1988; Gingas and Lineberger 1989; Chmielarz 1999; Wilhelm 2000; Valladares et al. 2006; Corredoira et al. 2006; Vengadesan and Pijut 2009; references in Lelu-Walter et al. 2011; Blasco et al. 2013). In contrast, research on the application of this method in the micropropagation of *Fagus* is scarce (Chalupa 1987; Joergensen 1988; Vieitez et al. 1992; Dujičkova and Mala 1995; Naujoks 2003).

Fagus sylvatica (European beech) is one of the most economically important deciduous tree species in Central Europe (Cuenca et al. 2000). It is grown for timber and used for reforestation and its numerous cultivars as ornamentals (Chalupa 1996). Trees of this species rarely bear fruit abundantly (usually every 5–8 years), while the seeds are difficult to store. Despite this, propagation by seeds is the main way of reproduction of beech (Vieitez and San-José 1996). Vegetative propagation of *F. sylvatica* by conventional methods is very difficult (Vieitez and San-José 1996; Cuenca et al. 2000). The first work on propagation of beech using in vitro culture was published by Jacquiot in 1959 (Suszka 1990). Disorganized tissue was then produced from the cambium of beech, which was incapable of long-term growth. Attempts on in vitro micropropagation of beech from protoplasts were made by Ahuja (1984a). To induce the tissue culture, mesophyll protoplasts were isolated from young leaves of 70-year-old trees. Using protoplasts, that author obtained regeneration of cell walls and the newly formed cells exhibited the ability to divide. However, their further growth and differentiation of tissues were not possible. In 1985, Chalupa reported the plant regeneration in selected species of the genus *Fagus* from apical shoots.

Research on somatic embryogenesis of *F. sylvatica* was also started by Chalupa (1987), who obtained embryogenic callus from immature zygotic embryos. Joergensen (1988) used beech anthers as explants, but he achieved only one somatic embryo. Naujoks (2003) succeeded to generate embryogenic callus of this species from immature zygotic embryos of 4 lines. These embryos were capable of further development into plantlets, which were transferred to the soil. Since then, no further studies on the application of this system for micropropagation of *F. sylvatica* have been reported.

The aim of our study was to develop an effective micropropagation method of *F. sylvatica* by using several types of explants (mature and immature zygotic embryos, cotyledons, embryonic axes) in various culture conditions (medium, plant growth regulators, light) on the basis of somatic embryogenesis or organogenesis.

Materials and methods

Plant material

Five types of explants were used for induction of embryogenic callus of *Fagus sylvatica*. The first 2 types were mature zygotic embryos and/or embryonic axes isolated from seeds collected in the Kórnik Arboretum and in Gryfino Forest District (seed lot No. 2695) in 2011. Prior to the in vitro culture, the seeds were stored for up to 12 months at –10ºC. Part of the seeds from Gryfino and Kórnik was subjected to cold stratification at 3ºC for 7 weeks. The next 3 types of explants were immature zygotic embryos, embryonic axes and cotyledons taken from seeds harvested in 2011 and 2013 (from 1 and 2 trees, respectively) in the Kórnik Arboretum. The explants for induction of embryogenic callus were taken immediately after harvesting the seeds. The seeds were disinfected in 0.1% $HgCl_2$ for 2.5 min (Chmielarz 1999) or in 30% H_2O_2 for 5 or 7.5 min (Hazubska and Szczygieł 2003) or in 5% NaOCl for 20 min (Kraj and Dolnicki 2003) and subsequently rinsed 3 times in sterile distilled water and placed on the induction media. One part of the mature seeds was washed additionally with commercial disinfectants (Domestos, Harpic) before standard surface disinfection. Afterwards, the embryos and embryonic axes were halved longitudinally and the cut surface was placed on the medium to encourage callus formation.

Experiment I

Entire, mature zygotic embryos and embryonic axes both excised from stored seeds (origin: Kórnik 2011) were placed in 2012 on induction media of 2 types: half-strength WPM (Lloyd and McCown 1980) and MSG (Becwar et al. 1990). ½ WPM medium was supplemented with 6.8 μM zeatin and 13.4 μM 1-naphthaleneacetic acid (WPM A) or 9.1 μM 2,4-D and 2.2 μM BA (WPM B), while MSG medium, with 9.1 μM 2,4-D and 2.2 μM BA. Plant hormone combinations were applied according to Naujoks (2003). After two months explants were transferred on the media supplemented with cytokinin only. The explants were cultured in darkness or white fluorescent light (25 μmol m^{-2} s^{-1}; 16 h/8 h day/night photoperiod) at 22±1ºC. Twenty explants per variant were used. The experiment was carried out only once. The contamination percentage of explants and the frequency of callus and adventitious buds after 2 months of the culture were evaluated.

Experiment II

In this experiment, embryonic axes excised from stored, stratified seeds (origin: Gryfino and Kórnik, 2011) were taken as explants. Isolated embryonic axes in 2012 were cultured in the same media and conditions as in Experiment II, but the induction media were supplemented additionally (per dm^3 of the medium) with 3 cm^3 of Plant Preservative Mixture (PPM, Plant Cell Technology, Inc.). The explants were cultured in darkness or white fluorescent light (25 μmol $m^{-2}\,s^{-1}$; 16 h/8 h day/night photoperiod) at 22±1ºC. Twenty-five explants for each medium/light variant were taken. For explants from Gryfino, the experiment was carried out only once, while for explants from Kórnik, 4 times. After 2 months of culture, the contamination percentage of explants and their ability to produce callus and adventitious shoot buds were evaluated.

Experiment III

Entire mature zygotic embryos and embryonic axes, both excised from stored seeds (origin: Kórnik 2011), were placed in 2012 on the induction media supplemented with 3 cm^3 of PPM, to estimate the efficiency of surface disinfection of explants by using this sterilization agent. Media without PPM were the control variant. The explants (15 per variant) were cultured in darkness, at 22±1ºC. The experiment was carried out twice. During 2 months of culture, the contamination percentage of explants was assessed.

Experiment IV

Immature zygotic embryos, embryonic axes and cotyledons (origin: Kórnik) were collected weekly from mid-July till late August (8 weeks) in 2011. The fresh embryos collected in the first 3 weeks and embryonic axes as well as cotyledons excised from embryos collected in the following 5 weeks were placed on WPM medium supplemented with 6.8 μM zeatin or on MSG medium with 9.1 μM 2,4-D and 2.2 μM BA. Next the explants were placed in 3 lighting variants: darkness, weak white fluorescent light or blue-red LED light (25 μmol $m^{-2}\,s^{-1}$; 16 h/8 h day/night photoperiod in both cases) at 22±1ºC. About 200–220 explants for each medium/light variant from all collection dates were tested. Two months after placement of the explants on the induction media the auxin was omitted. The contamination percentage of explants and the frequency of 2 types of callus (non-embryogenic and cPEM-ls) and adventitious shoot buds after 2 months of the induction phase were evaluated.

Experiment V

Immature zygotic embryos, embryonic axes and cotyledons (origin: Kórnik) were collected weekly from late May to early September (15 weeks) in 2013. The fresh embryos collected from late May to early July (6 weeks) were transferred onto the induction media together with the immature seed coat (Fig. 1a). In the later weeks, only fragments of immature zygotic embryos with the embryonic axes were placed on the induction media because of their large size (Fig. 1b). Two media were tested: MSG with 9.1 μM 2,4-D and 2.2 μM BA and WPM with the same plant growth regulators (designated as WPM Z). The tested explants were placed in darkness and 120 explants on each collection date were tested. After 5 weeks of cultivation of the explants on the induction media, the percentage of friable, yellow-white cPEM-ls was assessed.
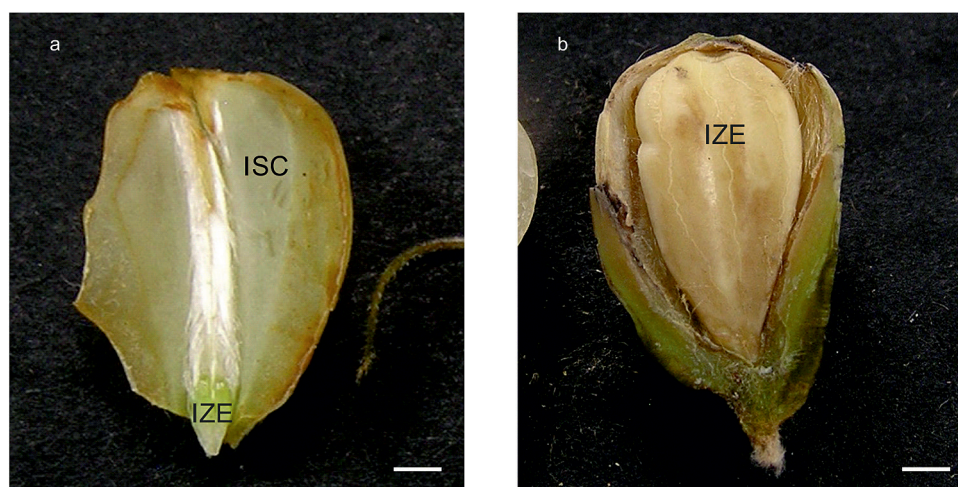

Fig.1. Immature zygotic embryos (IZE) of *Fagus sylvatica* at various stages of development, from seeds harvested in late May (a) and mid July (b) in 2013, used for induction of embryogenic callus (experiment V); ISC = immature seed coat. Bar = 1 mm

Table 1. Effects of explant type, agar media and light conditions on induction of callus and adventitious shoot buds of *Fagus sylvatica* (seed origin: Kórnik, 2011, stored seeds; experiment I in 2012)

Explant type	Medium	Light conditions	Non-embryogenic callus (%)	Adventitious shoot buds (%)
Mature zygotic embryos	½WPM A	Darkness	30.0	0.0
		White light	15.0	0.0
	½WPM B	Darkness	0.0	0.0
		White light	20.0	15.0
	MSG	Darkness	10.0	0.0
		White light	0.0	5.0
Overall mean			12.5	3.3
Embryonic axes	½WPM A	Darkness	50.0	0.0
		White light	15.0	0.0
	½WPM B	Darkness	10.0	0.0
		White light	0.0	0.0
	MSG	Darkness	10.0	0.0
		White light	0.0	0.0
Overall mean			17.0	0.0

Media: ½ WPM (half-strength WPM medium recommended by Lloyd and McCown 1980) with (A) 6.8 µM zeatin and 13.4 µM 1-naphthaleneacetic acid or (B) 9.1 µM 2,4-D and 2.2 µM BA; MSG (Becwar et al. 1990) with 9.1 µM 2,4-D and 2.2 µM BA.

Statistical analysis

Data on contamination of explants as well as frequency of induced non-embryogenic callus, callus with PEM-like structures and adventitious buds were expressed as percentage. Before statistical analysis data were converted to the Bliss degrees and afterwards analyzed using the Wilcoxon rang-sum test when the experiment was performed more than once (Experiment II and IV). Data collected in Experiment III were analyzed using one-way analysis of variance (ANOVA). Tests were conducted at the $\alpha=0.05$ level. The Statistica™10 (Statsoft Poland) package was used for data analysis.

Results

Experiment I

In this experiment, average 33.3% of mature zygotic embryos and on average 45.0% of embryonic axes was contaminated (data not shown). We observed that both types of explants from Kórnik were able to regenerate the non-embryogenic callus only with a frequency of up to 30% (mature zygotic embryos) and 50% (embryonic axes) (Table 1). The frequency of inducing this type of callus from both explant types was highest on ½WPM A medium, in darkness. In the case of mature zygotic embryos, 15% of explants placed on ½WPM B medium and 5% of explants on MSG medium, incubated in white light, regenerated adventitious buds (Table 1). In general, 12.5% of mature zygotic embryos and 17.0% of embryonic axes produced non-embryogenic callus on average. Only 3.3% of explants on average regenerated adventitious buds from hypocotyls of whole plantlets developed from mature zygotic embryos (Table 1).

Experiment II

Average contamination percentage of explants collected from stratified seeds amounted up to 36.0% for embryonic axes from Gryfino and 37.5% for those from Kórnik (data not shown). The explants produced also non-embryogenic callus only, but those from Gryfino with a much higher frequency (41.3%, versus Kórnik 8.8% on average; Table 2 and 3). The

Table 2. Induction of non-embryogenic callus and adventitious shoot buds from embryonic axes collected from stratified seeds of *Fagus sylvatica* (seed origin: Gryfino, 2011, seed lot No. 2695; experiment II in 2012). Explants were cultured on media with Plant Preservation Mixture (PPM, 3 cm³ dm⁻³)

Medium	Light conditions	Non-embryogenic callus (%)	Adventitious shoot buds (%)
½WPM A	Darkness	36.0	0.0
	White light	60.0	0.0
½WPM B	Darkness	20.0	0.0
	White light	32.0	5.0
MSG	Darkness	48.0	0.0
	White light	52.0	0.0
Overall mean		41.3	0.8

Media: ½ WPM (half-strength WPM medium recommended by Lloyd and McCown 1980) with (A) 6.8 μM zeatin and 13.4 μM 1-naphthaleneacetic acid or (B) 9.1 μM 2,4-D and 2.2 μM BA; MSG (Becwar et al. 1990) with 9.1 μM 2,4-D and 2.2 μM BA.

Table 3. Induction of non-embryogenic callus from embryonic axes collected from stratified seeds of *Fagus sylvatica* (seed origin: Kórnik, 2011; experiment II in 2012). Explants were cultured on media with Plant Preservation Mixture (PPM, 3 cm^3 dm^{-3})

Medium	Light conditions	Non-embryogenic callus (%)	p-value
½WPM A	Darkness	3.0 ± 0.4	0.3419
	White light	10.0 ± 1.1	
½WPM B	Darkness	8.0 ± 0.8	0.7959
	White light	10.0 ± 1.0	
MSG	Darkness	16.0 ± 1.5	0.2149
	White light	6.0 ± 0.9	
Overall mean		8.8 ± 4.4	

Media: ½ WPM (half-strength WPM medium recommended by Lloyd and McCown 1980) with (A) 6.8 µM zeatin and 13.4 µM 1-naphthaleneacetic acid or (B) 9.1 µM 2,4-D and 2.2 µM BA; MSG (Becwar et al. 1990) with 9.1 µM 2,4-D and 2.2 µM BA. Means and standard deviation are based on 4 replications of 25 explants each.

highest percentage of callus (60%) from explants of this type was regenerated on ½WPM A medium in white light (Table 2). Moreover, 5.0% of these explants were able to produce adventitious buds on ½WPM B medium. In total, however, adventitious buds were produced by only 0.8% of explants from Gryfino on average. Embryonic axes from Kórnik, taken from stratified seeds, proved to be less reactive, regardless of the media and light conditions applied during the induction phase, as compared to those from Gryfino (Table 3).

Experiment III

PPM applied to the induction media did not give the expected results in our experiment. Contamination percentage was not statistically significant regardless of application of this sterilization agent in both explant types (Fig. 2). However, in the case of embryonic axes we observed a reduced contamination percentage from 23.3% in the control to 6.7% in the presence of PPM.

Experiments IV and V

In 2011, entire immature zygotic embryos were initially used as explants. This type of explants was characterized by a very high contamination percentage (up to 81%, data not shown), so from the 4th date of collection, we decided to use embryonic axes and cotyledons separately. The sterilization procedure was more efficient for embryonic axes than for cotyledons (Table 4). During 2 months of incubation on induction media, we observed that both, immature zygotic embryos and embryonic axes produced non-embryogenic callus (Fig. 3), while we did not find any callus on cotyledons.

On the surface of a few calluses formed from embryonic axes excised from seeds harvested in the first 3 weeks of August, the characteristic PEM-like structures were noticed, which may be the initial phase of development of embryogenic callus (Fig. 4). In some cases, adventitious bud formation was also observed during incubation of explants on induction medium of this type. Adventitious buds were formed on the surface of the callus regenerated from embryonic
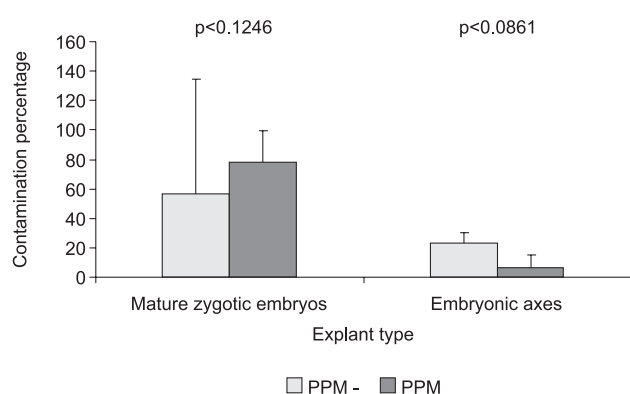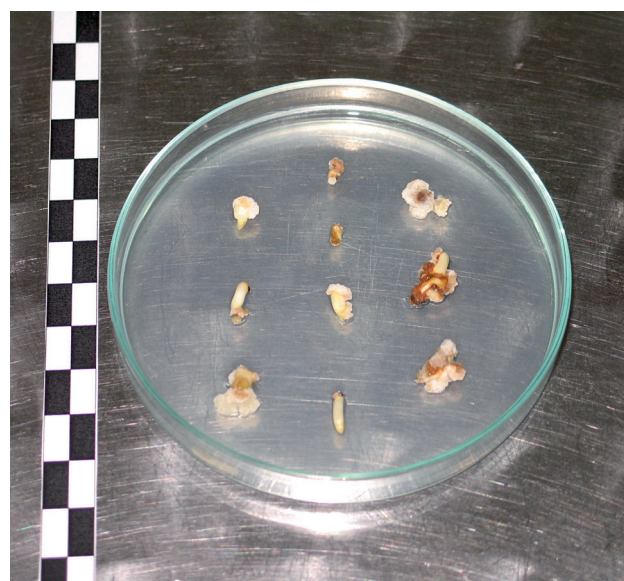

Fig. 2. Efficiency of surface disinfection of explants after application of Plant Preservative Mixture (PPM) to induction media (experiment III); PPM- – media without PPM; PPM – media with PPM

Fig. 3. Induction of non-embryogenic callus from embryonic axes of *Fagus sylvatica*

Fig. 4. Callus with proembryogenic–like structures (cPEM-ls) on the surface, derived from immature embryonic axes of *Fagus sylvatica*. Bar = 1 mm

Fig. 5. Multiplication of the shoots obtained from adventitious buds of *Fagus sylvatica* induced on callus derived from embryonic axes

axes. These buds developed into shoots capable of growth in vitro for several months (Fig. 5). Shoot cultures were proliferated on the LS medium (Chalupa and Durzan 1973), supplemented with 0.05 μM NAA and 0.44 μM BA, as recommended by Chalupa (1979).

Contamination percentage of embryonic axes incubated on WPM and MSG media in 3 variants of light conditions was 52.2% on average (data not shown). The highest potential for production of non-embryogenic callus was recorded for the explants incubated on the MSG medium in blue-red LED light (Table 5). The most limited regeneration of this type of callus was noticed in white light, regardless of the induction media. In general, only 26.0% of all explants on average were able to produce callus then. The frequency of induction of cPEM-ls was the highest in the dark and amounted to 3.1% for both induction media (Table 5). MSG medium, blue-red LED and white light were factors contributing to the development of adventitious buds from embryonic axes as explants. The frequency of adventitious bud

regeneration was up to 6.5% and 4.1% in blue-red LED and white light, respectively. Overall, very few of the tested explants produced cPEM-ls (1.1% on average) and adventitious buds (2.3% on average).

In 2013, entire immature zygotic embryos or their fragments comprising the embryonic axes were used. During 5 weeks after placement on the induction media (WPM-Z and MSG), a few explants (originating from both trees) regenerated friable, yellow-white cPEM-ls (Table 6). Explants derived from tree II produced this type of callus after collection on 8 dates, compared to 5 dates for tree I (Table 6). The maximum frequency of induction of cPEM-ls (as % of all explants collected on a specific date) amounted up to 2.5% and 5.0% for trees I and II, respectively. MSG medium proved to be the most useful, especially for explants originating from tree II (Table 6).

Discussion

In this study, we tested the in vitro response of various types of explants retrieved from immature and mature seeds of *Fagus sylvatica*. The immature seeds were used immediately after harvesting, while mature seeds were stored at –10ºC. Part of the stored mature seeds was stratified at 3ºC, to break seed dormancy. The first problem was the very high contamination percentage of explants derived from these seeds, although the seeds were sterilized in 30% H_2O_2 or 0.1% $HgCl_2$ or 5% NaOCl in accordance with the procedures given by Hazubska and Szczygieł (2003), Chmielarz (1999) and Kraj and Dolnicki (2003), respectively. We later decided to wash the mature seeds additionally with commercial disinfectants (such as Domestos and Harpic) before sterilization, but these treatments did not give better results, either (data not shown). In general, the contamination percentage of the explants ranged from 24.5% for embryonic axes derived from immature embryos (Table 4) to 81.0% for whole immature zygotic embryos (data not shown).

Table 4. Comparison of the contamination percentage of embryonic axes and cotyledons of *Fagus sylvatica* as explants excised from fresh seeds harvested in 2011 and incubated for one month on induction media (experiment IV)

Collection date	Explant type	Contamination (%)	p-value
01.08.11	Embryonic axes	24.5	0.0040
	Cotyledons	81.4	
08.08.11	Embryonic axes	40.7	0.0051
	Cotyledons	78.0	
16.08.11	Embryonic axes	34.3	0.02971
	Cotyledons	53.7	
22.08.11	Embryonic axes	26.7	0.2623
	Cotyledons	61.7	
29.08.11	Embryonic axes	70.0	0.3367
	Cotyledons	92.7	

Table 5. Induction of 2 types of callus and adventitious shoots from embryonic axes excised from fresh seeds of *Fagus sylvatica* (seed origin: Kórnik, 2011; experiment IV) in various light conditions

Medium	Light conditions	Non-embryogenic callus (%)	cPEM-ls (%)	Adventitious shoot buds (%)
WPM	Darkness	35.7	3.1	1.0
	White light	5.0	0.8	0.0
	LED	26.4	0.0	2.2
MSG	Darkness	27.3	3.1	0.0
	White light	12.2	0.0	4.1
	LED	49.4	0.0	6.5
Overall mean		26.0	1.1	2.3

cPEM-ls = callus with proembryogenic-like structures. Media: WPM (Lloyd and McCown 1980) with 6.8 μM zeatin; MSG (Becwar et al. 1990) with 9.1 μM 2,4-D and 2.2 μM BA.

Kraj and Dolnicki (2003) reported that seeds of *F. sylvatica* are difficult to disinfect. The main reasons are the microbes that usually infected the seeds and the limited possibilities of infiltration of the sterilizing agent into the folded cotyledons. $HgCl_2$ is the most commonly agent used for surface sterilization of explants of beech (Ahuja 1984b; Chalupa 1987; Kraj and Dolnicki 1998), but it is believed to be a mutagenic factor (Kraj and Dolnicki 2003).

Kraj and Dolnicki (2003) succeeded to improve the sterility of the cultures developed from embryos of beech by 30% by adding PPM (2–4 $cm^3 dm^{-3}$) to the growing medium. In our study we did not achieve satisfactory results after adding this agent to the induction media, at a concentration of 3 $cm^3 dm^{-3}$. For example, the contamination percentage of embryonic axes excised from mature zygotic embryos from Kórnik was 45.0% on average, while for axes taken from stratified seeds, 37.5% after their incubation with PPM in the medium (data not shown). We did not succeed also to improve significantly the sterility of cultures of mature zygotic embryos and embryonic axes (origin Kórnik 2011) after application of PPM to the induction media (Experiment III), although in the case of embryonic axes we observed a reduced contamination percentage in the presence of PPM (Fig. 2).

We achieved limited success in initiation of somatic embryogenesis of *Fagus sylvatica* when using embryonic axes or fragments of immature embryos with embryonic axes. From these explant types we obtained friable, yellow-white callus with characteristic PEM-like structures (cPEM-ls), which may be an early developmental stage of embryogenic callus. In some cases the induction of adventitious buds under the influence of white light from mature zygotic embryos or embryonic axes was possible. Embryonic axes taken from immature zygotic embryos treated with weak white fluorescent light or blue-red LED light (25 μmol $m^{-2}s^{-1}$) can also be a source of explants to induce the process of organogenesis (Table 5). These results are opposite to the results reported by Gingas and Lineberger (1989), who observed *Quercus rubra* embryoid regeneration through exposure of immature zygotic embryo tissues to light, inducing the process of embryogenesis.

Table 6. Induction of callus with proembryogenic-like structures (cPEM-ls) from immature zygotic embryos of *Fagus sylvatica* excised from fresh seeds (seed origin: 2 trees, Kórnik Arboretum, 2013; experiment V). The table includes only the variants where calluses with cPEM-ls were noted

Tree No.	Collection date of nuts	Successful medium	No. of cPEM-ls	cPEM-ls per 30 explants (%)	cPEM-ls per all explants (%)
I	26/06/13	WPM Z	1	3.3	0.8
	10/07/13	WPM Z	1	3.3	1.7
		MSG	1	3.3	
	17/07/13	WPM Z	1	3.3	0.8
	07/08/13	MSG	3	10.0	2.5
	04/09/13	WPM Z	1	3.6*	1.9
II	05/06/13	MSG	1	3.3	0.8
	26/06/13	MSG	6	20.0	5.0
	03/07/13	MSG	3	10.0	2.5
	10/07/13	WPM Z	2	6.7	5.0
		MSG	4	13.3	
	17/07/13	MSG	1	3.3	0.8
	24/07/13	WPM Z	1	3.3	4.2
		MSG	4	13.3	
	31/07/13	WPM Z	1	3.3	0.8
	07/08/13	MSG	3	10.0	2.5

WPM (Lloyd and McCown 1980); MSG (Becwar et al. 1990). Both media with 9.1 μM 2,4-D and 2.2 μM BA; * per 28 explants.

There are several reports concerning in vitro propagation of *Fagus sylvatica* based on organogenesis (Vieitez et al. 1993; Vieitez and San-José 1996; Cuenca et al. 2000). The regeneration of adventitious buds of this beech species was achieved from intact hypocotyls of whole plantlets obtained from embryonic axes, which were initial explants, but also from hypocotyl and cotyledon segments isolated from in vitro growing seedlings derived from embryos (Vieitez et al. 1993), and from leaf and internode explants derived from in vitro grown shoots (Vieitez and San-José 1996; Cuenca et al. 2000). Cuenca and coworkers (2000) succeeded in induction of adventitious buds from internode explants on WPM supplemented with 4.5 μM thidiazuron (TDZ) or 17.8 μM BA, although the medium containing TDZ was more efficient. In turn, Vieitez and San-José (1996) obtained the best results from proximal leaf sections cultured on the same medium but with addition of 2.9 μM indole-3-acetic acid (IAA) and 8.9 μM BA or 2.3 μM TDZ. The cited authors reported that initially bud formation capacity was enhanced by darkness, but after 10 days, the lack of light inhibited this process.

The present study on induction of embryogenic callus from various explants in different culture conditions allowed to obtain friable, yellow-white callus with characteristic PEM-like structures (cPEM-ls) and adventitious shoot buds. This type of callus regenerated from embryonic axes or fragments of immature zygotic embryos with embryonic axes excised from seeds harvested in 2011 and 2013, respectively. On the basis of morphological features (friable, yellow-white color, spherical structures), we suppose that this type of callus may be an early developmental stage of embryogenic callus of beech. Very similar structures (PEMs) were observed during induction of embryogenic tissues of *Picea* sp. (Hazubska-Przybył et al. 2008). More or less numerous PEMs appeared on the surface of callus developed from explants about 4–6 weeks after placement on induction media. Some of these structures were capable of proliferation, while some others degenerated. cPEM-ls regenerated from explants that were incubated in darkness, mainly on WPM medium supplemented with 6.8 μM zeatin or WPM and MSG media supplemented with 9.1 μM 2,4-D and 2.2 μM BA. In respect of collection date, the highest frequency of regeneration of this type of callus was 5.0%, for explants excised from seeds harvested on 26 June and 10 July 2013 from tree II. In respect of medium type, the best results were obtained on MSG medium with addition of 9.1 μM 2,4-D and 2.2 μM BA (Table 6). On this medium, 6 of 30 explants induced cPEM-ls (20%).

Induction of embryogenic callus is the output stage of somatic embryogenesis in plants. Embryogenic callus of *Fagus sylvatica* obtained by Naujoks (2003) was friable and pale yellowish. For comparison, embryogenic tissues/calluses in coniferous species are mucilaginous, white to translucent and flocculent. After staining with acetocarmine, characteristic structures are visible, composed of embryogenic regions and suspensor cells, i.e. the so-called proembryos or PEMs (Hazubska-Przybył et al. 2008). After the treatment of embryogenic callus with cytokinin in *F. sylvatica* or ABA in coniferous species, somatic embryos develop (Naujoks 2003; Hazubska and Szczygieł 2003).

Immature zygotic embryos were successfully applied as explants for induction of embryogenic callus of beech by Naujoks (2003). The beechnuts were harvested in the middle of June 2000 from 3 trees. Explants were placed on induction media of 3 types – WPM (Lloyd and McCown 1980), MSG (Becwar et al. 1990) and S (Gupta and Durzan 1987) – containing different combinations of plant growth regulators (auxins and/or cytokinins) and kept under weak white light (3–5 μE m^{-2} s^{-1}). Embryogenic lines were induced from 2 mother trees. The frequency of induction of embryogenic callus was 7.9%. The best results were noticed on the WPM medium supplemented with zeatin (1.5 mg l^{-1} = 6.8 μM), on which 5 of 30 explants regenerated embryogenic callus (16.6%). A similar result was obtained on MSG medium supplemented with 2,4-D (2 mg l^{-1} = 9.1 μM) and BA (0.5 mg l^{-1} = 2.2 μM). On this medium, 4 of 27 explants were able to regenerate embryogenic callus (14.8%).

For comparison, cPEM-ls obtained in our laboratory regenerated on similar media, but its potential for further proliferation was very limited. Naujoks (2003) reported induction of embryogenic callus from very young explants. In our study, cPEM-ls appeared also on the explants derived from seeds harvested later (in mid-July and August), when the zygotic embryos had already fully developed embryogenic axes and cotyledons (Fig. 1b).

Immature zygotic embryos were successfully used for induction of somatic embryogenesis of various species of oak, belonging to the same family as beech (*Fagaceae*). Gingas and Lineberger (1989) obtained the highest embryoid numbers of *Quercus rubra* from immature embryos cultured on modified MS (Murashige and Skoog 1962) medium, supplemented with 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BA in 16 h light. Those authors demonstrated that the embryoid regeneration efficiency was dependent on the time that elapsed after fertilization. The best results were obtained 4–7 weeks after this process. Also Chmielarz (1999) induced embryogenic callus of *Q. robur* from immature zygotic embryos excised from acorns, taken from the tree from 26 July till 1 August. Explants were cultured on MS or WPM media with addition of 0.5 mg l^{-1} 2,4-D and 0.1–1.0 mg l^{-1}

BA in darkness. Another type of explants used for successful induction of somatic embryogenesis of *Q. rubra* were immature cotyledons excised from acorns gathered in mid-July (Vengadesan and Pijut 2009). Immature cotyledon explants produced embryogenic callus when cultured on MS medium supplemented with various concentrations of 2,4-D in darkness. In our study we did not obtain induction of any callus from cotyledons, despite the attempts made in 2011. Moreover, cotyledons proved to be very difficult explants because of the high contamination percentage, which ranged from 53.7 to 92.7% (Table 4).

Attempts to induce somatic embryogenesis of *Fagus sylvatica* from various types of explants in the present study were not completely successful as yet. Although we obtained some types of callus with PEM-like structures derived from immature zygotic embryos and embryonic axes, further studies are required to confirm if the observed structures are capable of development into somatic embryos. However, in the course of this study we were able to induce adventitious shoot buds, capable of multiplication. This may indicate that induction of organogenesis in European beech is associated with fewer difficulties than the process of somatic embryogenesis.

Acknowledgments

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