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Early stages of somatic embryogenesis in root callus of grasspea (*Lathyrus sativus* L.)

Wczesne etapy somatycznej embriogenezy w kalusie korzeniowym lędźwianu siewnego (*Lathyrus sativus* L.)

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Abstract

Callus cultures from root explants of *Lathyrus sativus* L. 'Derek' were tested for their morphogenic capacity. Primary explants (fragments of roots) were cultivated on three induction media. We obtained three lines of callus tissue among which we identified two non-embryogenic lines and one embryogenic line. Callus originally cultivated on modified MS medium supplemented with 0.05 mg*L^{-1} picloram, formed embryo-like structures upon transfer to media containing 0.1 mg*L^{-1} picloram or 0.9 mg*L^{-1}2,4-D. Histological examinations confirmed embryogenity of obtained structures. Previous studies had revealed that, notwithstanding efficient callus induction and proliferation, its capacity to differentiate shoots or somatic embryos is limited. Consequently, rhizogenesis was only form of complete organogenesis obtained in our experiments. However attempts to develop the methods for indirect plant regeneration in *L. sativus* would allow creation of new genetic variations required to improvement of this species.

Keywords: auxins, embryo-like structures, grasspea, rhizogenesis

Streszczenie

Kalus uzyskany z korzeniowych eksplantatów *Lathyrus sativus* 'Derek' był badany w celu określenia zdolności morfogenetycznych. Eksplantaty pierwotne (fragmenty korzeni) uprawiane były na trzech pożywkach indukcyjnych. Uzyskano trzy linie kalusa, między którymi zidentyfikowano dwie linie nieembriogeniczne i jedną embriogeniczną. W tkance kalusowej, proliferującej początkowo na zmodyfikowanej pożywce MS z dodatkiem 0.05 mg*L^{-1} picloramu, po przeniesieniu na pożywkę zawierającą 0.1 mg*L^{-1} picloramu lub 0.9 mg*L^{-1} 2,4-D obserwowano powstawanie struktur zarodko-podobnych. Badania histologiczne potwierdziły embriogeniczność uzyskanych struktur. Wcześniejsze doświadczenia ujawniły, że pomimo wydajnej indukcji i proliferacji kalusa, jego zdolność do różnicowania pędów lub zarodków somatycznych jest ograniczona. Jedyną formą kompletnej organogenezy uzyskaną w niniejszych badaniach była ryzogeneza. Rozwinięcie metod pośredniej regeneracji roślin u *L. sativus* pozwoliłoby na stworzenie nowej zmienności genetycznej koniecznej do ulepszenia tego gatunku.

Słowa kluczowe: auksyny, lędźwian, ryzogeneza, struktury zarodko-podobne

Introduction

Global warming, constantly diminishing water resources around the world and a growing human population mean that introduction of new strategies for sustainable agriculture becomes necessary. Consequently, scientists, breeders and farmers turned their attention to the forgotten and underestimated crops that would improve food security of people (Polignano, et al., 2005). One of such plant is grasspea (*Lathyrus sativus* L.), which is distinguished by unusual resistance to drought (Campbell, et al., 1994). For that reason this species is recognised as a model plant for the needs of sustainable agriculture (Vaz Patto, et al., 2006).
An additional way to broaden the gene pool of crops, apart from mutational breeding, are biotechnological methods, including tissue culture. Tissue cultures, especially callus culture, generate a wide range of genetic variation that could be exploited in breeding programs. It is easy to select mutants with useful features, such as tolerance to drought or salinity, or resistance to disease through *in vitro* selection. The potential of somaclonal variability should be fully exploited by breeders (Jain, 2001), especially in species with limited gene pool to which grasspea also belongs. However, the development of indirect plant regeneration through callus tissue is often long and inefficient. It results, among others, from changes occurring in the cells, such as point mutations, chromosomal rearrangements, methylation of DNA or activation of transposons (Jain, 2001).
The aim of the presented study was the establishment of embryogenic and non-embryogenic callus lines of Polish grasspea cultivar and their stimulation to *in vitro* indirect regeneration (through somatic embryogenesis or organogenesis).

Material and methods

Plant material

One Polish genotype of *Lathyrus sativus* - 'Derek' was used as plant material. Seeds were kindly provided by prof. Wojciech Rybiński from Polish Academy of Sciences, Poznań, Poland. Seeds were surface sterilized in 70% ethanol for 60 s followed by immersing in 0.1% aqueous solution of mercuric chloride ($HgCl_2$) for 30 min. and rinsed five times in sterile distilled water. Then seeds were sown in 350-ml jars containing 50 ml medium composed of MS macro and microelements (Murashige and Skoog, 1962) with 20 $g*L^{-1}$ sucrose and solidified with 1 $g*L^{-1}$ agar (OXOID). They were allowed to germinate at 25±1°C on light (80 $\mu mol*m^{-2}*s^{-1}$ photosynthetic photon flux density) in a 16 h photoperiod.

Explants, culture media and conditions

21–day old seedlings were used as a source of primary explants for callus induction. Root fragments (length 2-3 mm, ø 1-2 mm) were placed horizontally on three induction media: (1) MSN, (2) MBN, (3) MMP (Table1).
In presented experiment, as secondary explants were used stabilized callus lines (at least 1 passage) from three induction media. Each line of callus was subcultured on various media according to scheme presented at Fig. 1. Media were based on MS and supplemented with different growth regulators. Aditionally, organic additives such as activated charcoal (AC) and chitosan were added to the media (Table 1). Where applicable, callus-structure were cultivated on modified MS medium containing 1 $mg*L^{-1}$ BAP. Macroscopic observations of callus colour and texture as well evaluation of tissue mass increase and frequency of various structure formation were conducted during cultivation.

Each combination was established in five replicates (Erlenmeyer flasks). Each flask contained five explants. Callus was cultivated in the light (photoperiod: 16 h day/ 8 h night, 80 µmol*m-^{2}*s-^{1} photosynthetic photon flux density) and passaged every 4-5 weeks. All media were gelled with 0.75% agar (BTL Ltd.) and contained 3% sucrose.

Table 1. Media composition
Tabela 1. Skład pożywek

Medium code	Mineral salts	Vitamins	Growth regulators (mg*L^{-1})	Supplements (g*L^{-1})
MSN	MS [a]	MS[a]	2.0 kinetin 2.0 NAA 2.0 2.4-D	-
MSN ac	MS	MS	2.0 kinetin 2.0 NAA 2.0 2.4-D	0.5 activated charcoal
MSN 1ac	MS	MS	2.0 kinetin 2.0 NAA 2.0 2.4-D	1.0 activated charcoal
MBN	MS	B_5[b]	2.0 NAA 4.0 BAP	-
MBN ac	MS	B_5	2.0 NAA 4.0 BAP	2.0 activated charcoal
MBN ch	MS	B_5	2.0 NAA 4.0 BAP	0.02 chitosan
MMP	MS	MS[c]	0.05 picloram	-
MM 0.1P	MS	MS	0.1 picloram	-
MM 0.9D	MS	MS	0.9 2.4-D	-

[a] According to Murashige and Skoog (1962)
[b] According to Gamborg et al. (1968)
[c] According to Kysely and Jacobsen (1990)

Line I (from MSN*)	→ → →	MSN MSN ac MSN 1ac
Line II (from MBN)	→ → →	MBN MBN ac MBN ch
Line III (from MMP)	→ →	MM 0.1P MM 0.9D

Figure 1. Scheme of callus lines subculturing
Rycina 1. Schemat pasażowania linii kalusa
* media composition in Table 1
* skład pożywek w Tabeli 1

Histological examination

Small representative fragments of callus tissue (approximately 2-3 mm) were fixed in glutaraldehyde (Forssmann, 1969) and then prepared according to Luft (1961). Epon blocks containing samples were sectioned horizontally and longitudinally at 1 µm thickness using an ultramicrotome (Tesla 490A). Sections were stained with 0.1% aqueous methylene blue solution, embedded in Canada balsam and observed under light microscope (Nicon ECLIPSE E400).

Results

Stabilized callus lines

In primary experiments we obtained three lines of callus tissue initiated on various media. Among them we identified two non-embryogenic lines: Line I (raising on MSN) – creamy-yellow, loose and greasy tissue with green, compact lumps; Line II (raising on MBN) - green, very compact tissue of crystalline structure and one embryogenic line: Line III (raising on MMP) – yellow, loose and granular tissue (Fig. 2a,c,d).

Callus appearance

After five weeks of cultivation on MSN, proliferating tissue of callus line I remained creamy-yellow, loose and greasy with green, compact lumps. On the other hand on media containing activated charcoal tissue structure was not changed, but the callus grew dark beige and brown, and then quickly decayed (Fig. 2b). After this time of culture, callus line II on MBN as well on medium with addition of chitosan had still compact, crystalline structure, but the tissue was mostly creamy-beige colour, not original green. However on the medium of the same composition enriched with 2.0 $g*L^{-1}$ activated charcoal, most tissues got browning and died. In turn, yellow, very lumpy callus from MMP, on media containing higher concentrations of auxins than in starting medium did not undergo modifications in terms of colour and structure, but embryo-like structures appeared in it (Fig. 2e)

Callus proliferation

After five weeks of culture, rate of tissue proliferation was highest on induction media (MSN and MBN) as well as on those with chitosan and 2,4-D alone. Mass of single callus clump on these media was 1,5 to above 7 times greater than originally (Table

1). Activated charcoal, as well as higher dose of picloram (0.1 mg*L-^{1}) significantly slowed down or almost inhibited tissue proliferation (Table 2).

Table 2. Effect of media on callus mass increase, rhizogenesis and embryo-like structures formation (different letters indicate statistically significant differences at α = 0.05)
Tabela 2. Wpływ pożywki na przyrost masy kalusa, ryzogenezę oraz powstawanie struktur zarodko-podobnych (wartości oznaczone tą samą literą nie różnią się istotnie przy α=0.05)

Medium*	Callus mass increase		Rhizogenesis		Embryo-like structures	
	%	mg	% explants with roots	roots number /explant	% explants with ELS	ELS number/ explant
MSN	773 c SE±271	168	0	-	0	-
MSNac	28 b SE±10	14	40	2,4 a	0	-
MSN1ac	40 b SE±14	18	60	5,7 b	0	-
MBN	397 c SE±190	346	45	5,0 ab	0	-
MBNac	4 a SE±1	55	0	-	0	-
MBNch	306 c SE±108	277	0	-	0	-
MM0,1P	7 a SE±3	92	24	1,5 a	72	14,6 a
MM0,9D	145bc SE±90	362	20	5,0 ab	100	36,6 b

* media composition in Table 1
* skład pożywek w Tabeli 1
different letters indicate statistically significant differences at α = 0.05
różne litery oznaczają statystycznie istotne różnice przy α = 0.05

Callus derived structures

During experiment we observed formation of two structure types in callus lines. Numerous roots (average 1.5-5.7 root/callus clump) were formed in tissue cultivated on various media. High doses of auxin and lower concentrations of activated charcoal promoted rhizogenesis, which occurred respectively in 20-45% and 40-60% of single callus clumps (Table 2).
After five weeks of cultivation, the appearance of embryo-like structures (ELSs) were observed in callus line III cultivated on media with higher doses of auxins (Fig. 2e). ELSs formation was observed in 72% of explants on medium with 0.1 mg*L^{-1} picloram and in all callus tissue fragments cultivated on medium with 0.9 mg*L^{-1} 2,4-D (Table 2). Significantly more ELS (36.6 ELSs/explant) were formed on medium containing 2,4-D than with picloram (14.6 ELSs/explant) (Table 2). However, transfer of ELSs on the medium with 1 mg*L $^{-1}$ BAP did not stimulate their further development.

Histological examination

Callus line I. Under surface of loose and greasy tissue, cells with large nuclei and dense cytoplasm were observed. Histological examination revealed also differentiation of vessels (Fig. 2f). Meristematic centres did not further develop after subculturing. Additionally, on media with AC elements of vascular tissue degenerated (Fig. 2g).

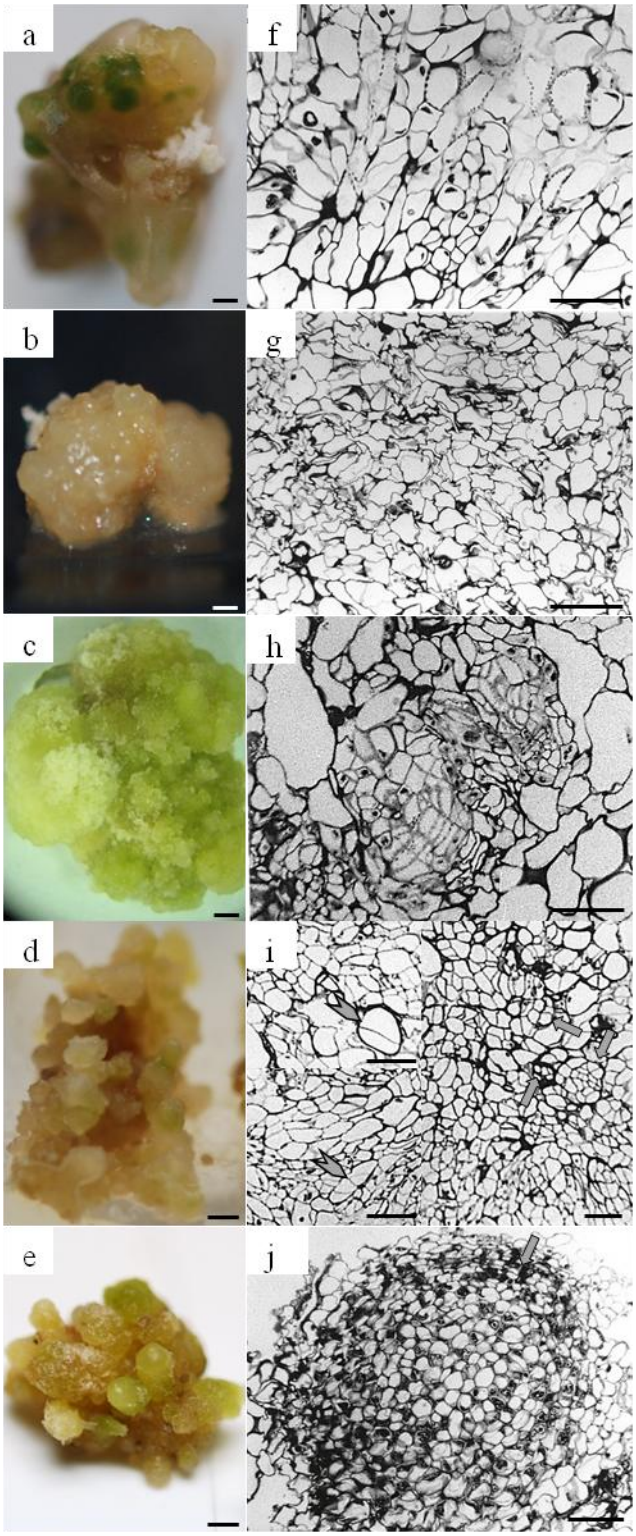

Figure 2. Callus appearance and histological preparation **a)** Non-embryogenic callus (line I), **b)** Non-embryogenic callus (line I) decaying on media with activated charcoal,

c) Non-embryogenic callus (line II), **d)** Embryogenic callus (line III), **e)** Embryo-like structures in callus line III, **f)** Meristematic cells and vascular elements in callus line I, **g)** Degenerating vessels in callus line I on media with activated charcoal, **h)** Meristematic cells and vascular elements in callus line II, **i)** Asymmetrically dividing cells (arrowheads) and radially arranged cells clusters (arrows) in callus line III, **j)** Antyclinal divisions (arrow) in subsurface of embryo-like structure. Bars = 1 mm (a,b,c,d,e); 50 μm (f,g,h,i,j)

Rycina 2. Wygląd kalusa I preparaty histologiczne **a)** Kalus nieembriogeniczny (linia I), **b)** Kalus nieembriogeniczny (linia I) zamierający na pożywce z węglem aktywowanym, **c)** Kalus nieembriogeniczny (linia II), **d)** Kalus embriogeniczny (linia III), **e)** Struktury zarodko-podobne w kalusie linii III, **f)** Komórki merystematyczne i naczynia w kalusie linii I, **g)** Degenerujące naczynia w kalusie linii I na pożywce z węglem aktywowanym, **h)** Komórki merystematyczne i naczynia w kalusie linii II, **i)** asymetrycznie dzielące się komórki (groty) i skupiska komórek o układzie radialnym (strzałki) w kalusie linii III, **j)** podziały antyklinalne (strzałka) w strukturze zarodko-podobnej. Podziałka = 1 mm (a,b,c,d,e); 50 μm (f,g,h,i,j)

Callus line II. In very compact, green tissue differentiation leading to meristematic cells and vascular elements formation was observed (Fig. 2h). Again, these group of cells did not develop after passage.

Callus line III. Histological preparations revealed asymmetrically dividing cells and radially arranged cells clusters in callus from induction medium (Fig. 2i). After successive passages (on media with 0.1 mg*L^{-1} picloram or 0.9 mg*L^{-1} 2,4-D) appearance of ELSs in this callus were confirmed (Fig. 2j). ELSs were not surrounded with the epidermis, but subsurface anticlinal divisions indicated beginnings of protoderm formation (Fig. 2j).

Discussion

In the presented studies on grasspea callus cultures proliferation of the tissue proceeded efficiently on the initial media (MSN, MBN and MMP). However during tissue subculturing its proliferation was dependent on the medium composition, especially in terms of applied growth regulators and supplements. Our previous studies had revealed that, notwithstanding efficient callus induction and proliferation, its capacity to differentiate shoots or somatic embryos is limited (Piwowarczyk and Pindel, 2009, 2010). Results presented in this paper confirm the existing view that different genotypes and even explants require tailor-growth regulators. It was also confirmed in *Lathyrus* P-24 cultivar, that organogenesis from root callus occurred only on medium supplemented with 2.0 mg*L^{-1} NAA and 0.4 mg*L kinetin^{-1} (Roy, et al., 1992). On the other hand, in order to regenerate shoots of the same cultivar from internodal and foliar callus, the addition of 2.0 mg*L^{-1} NAA and 0.5 mg*L^{-1} BAP was necessary (Roy, et al., 1991, 1993). Our study revealed that attempt to stimulate the differentiation of callus tissue by the addition of activated charcoal failed. In 'Derek' AC inhibited proliferation and caused its decaying. It is in contrast to reports supporting AC usefulness in improving cell growth and development in tissue cultures (Thomas, 2008). Most likely decaying of 'Derek' callus resulted from AC abilities to absorption of plant growth regulators (Ebert and Taylor, 1990). On the other hand it stimulated existing meristematic centres to development but in a way leading to formation of roots.

Rhizogenesis was only form of organogenesis obtained in our experiments. Sinha et al. (1983) also mention a frequent formation of roots on media with various combinations of growth regulators and in various genotypes. These researchers observed no differentiation of other organs in the tissue that produced roots, in turn in our experiment rhizogenesis did not stop formation of ELSs.

Generally, process of efficient somatic embryogenesis in cultures of Leguminosae members is limited to a few species (Lakshmanan and Taji, 2000). To our knowledge there are merely two reports referring to somatic embryogenesis in tissue culture of grasspea and only one concerns indirect somatic embryogenesis. Gharyal and Maheshwari (1983) obtained embroids in foliar callus on B5 medium containing both auxin and cytokinin. Direct somatic embryogenesis from immature leaflets and nodal segments of grasspea was achieved by Barna and Mehta (1995) who had found 2,4-D most effective in its induction. It is believed that direct somatic embryogenesis occurred from proembryonic competent cells already present in explants. Whereas, acquisition of embryogenic status by callus cells needs major cell reprogramming (Quiroz-Fgueroa, et al. 2006). In the presented study obtainment of embryo-like structures in root callus of grasspea on medium supplemented with auxins: pikloram (0.1 mg*L^{-1}) or 2,4-D (0.9 mg*L^{-1}) was a success. However, cultivation of ELSs on medium with cytokinin did not stimulate their further growth. The structure which allows to transformation of embryos beyond globular stage is protoderm (Quiroz-Fgueroa, et al. 2006). We did not observed completely formed protoderm that could be the reason of stopping histodifferentiation. The exact characteristic of somatic embryo developmental process, especially its early stages, is still uncertain (Lakshmanan and Taji, 2000, Quiroz-Fgueroa, et al. 2006). Among many factors having influence on histodifferentiation, genotype plays an important role (Li and Grabau, 1996).

Due to its unique traits, grasspea is ranked among commercially important species particularly in Indian subcontinent and Africa. In European countries, especially those with conservative attitude towards genetic modified organisms, grasspea could be used as protein components of animal fodder, replacing modified soybean. For that reason more efforts should be employed to developed protocols for efficient indirect organogenesis or somatic embryogenesis of grasspea. In the future, obtained in this way plants (somaclones) with new features could be incorporate in breeding process. Furthermore, studies on callus tissue, carried out at the cellular level, could also help us to understand mechanism regulating totipotency of cells especially in recalcitrance plant species, to which belong large-seeded legumes.

Conclusions

1. Initiation and proliferation of grasspea callus was efficient. Proliferation of tissue was dependent on the media composition, especially in terms of applied growth regulators and supplements.
2. Modified MS medium supplemented with picloram or 2,4-D was effective in initiating the first stages of somatic embryogenesis in root callus of grasspea.
3. Lower concentration of activated charcoal and higher concentrations of auxin stimulated tissue to rhizogenesis.

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