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CALLUS INDUCTION AND RHIZOGENESIS IN *LATHYRUS SATIVUS* L.

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Abstract

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Grass pea (*Lathyrus sativus* L.) is a leguminous plant distinguished by great resistance to abiotic and biotic stresses that can become a valuable source of protein feed in the nearest future. However, neglected by breeders, it needs the improvement of some disadvantages. Biotechnological techniques, including *in vitro* culture, are new and modern tools supporting plant breeding. Unfortunately, grass pea belongs to grain legumes that are well-known for their recalcitrance to *in vitro* manipulation and consequently plant regeneration.
The aim of the study was the evaluation of different factors influence on callus induction and proliferation, as well as cell differentiation and organogenesis. Callus culture were initiated from different explants of two Polish cultivars of grasspea on media supplemented with auxin and various cytokinins both on the light and in the dark.
The rate of tissue proliferation was significantly increased in both tested cultivars by light. More than 400 mg of tissue from one explant was obtained after 12 weeks of culture. The most intensive increase in callus mass was noted for internode fragments of 'Krab' (380 mg/one explant) and root fragments of 'Derek' (361 mg/one explant). On the media with the addition of thidiazuron callus tissue grew better (852 mg/one explants) than on the media with zeatin and kinetin (56–164 mg/one explant). Formation of the roots was the only type of organogenesis observed during the study. In 'Derek' callus on LP medium rhizogenesis occurred the most frequently (38%). The roots regenerated from this callus was also the most numerous (1.6) and the longest (12.5 mm).

Keywords: callus tissue, cytokinins, grass pea, light conditions, roots type of explants

INTRODUCTION

Increasing demand for plant proteins caused that some forgotten legumes plants aroused the interest of researchers as an alternative source of this nutrient. Among different species grass pea (*Lathyrus sativus* L.) enjoys a very great interest. Grass pea, often underestimated, is one of the oldest cultivated plants in the world. This species is characterized by features distinguishing it from other legumes. The uniqueness of grass pea results from the ability to grow and develop both in prolonged drought periods, as well as periodic flooding. These properties make it very popular in African and Asian countries. No less important features are the tolerance to the type and pH of the soil and the resistance to many diseases and pests. Another advantage of this plant is the high content of protein in the seeds. Protein with a very good amino acid composition because of the presence of lysine (Campbell *et al*., 1994; Vaz Patto *et al*., 2006; YAN *et al*., 2006). Long-lasting breeding negligence caused that this species still needs improvement of some negative traits like: low seed yield, susceptibility to lodging, indeterminate nature of growth and content of anti-nutritional compounds (eg. β-N-oxalyl-α-diaminopropionic acid – ODAP) in the seeds. Difficult task is additionally limited by the narrow gene-pool (Rybiński, 2003; Rybiński *et al*., 2005). Biotechnological techniques, including *in vitro* culture can be useful in achieving a desired

set of genes. Somaclonal variation induced in tissue culture can be successfully used in improvement of plants (Jain, 2001).

However, morphogenic capacity of grain legumes in tissue culture are generally poor (Sinha *et al*., 1983). Earlier studies showed that apart from the difficulties in the regeneration of shoots from callus tissue of grass pea (Gharyal and Maheshwari, 1983; Sinha *et al*., 1983) it is very difficult to get the whole plant by indirect organogenesis and it succeeded only in a few cases in this species (Barik and Kar, 2005; Roy *et al*., 1991; Roy *et al*., 1992; Zambre *et al*., 2002). Furthermore, in all reports on *Lathyrus sativus*, successful plant regeneration was very genotype-dependent.

The aim of the presented study was the evaluation of different factors influence on induction and proliferation of callus tissue as well as any morphogenic changes in callus of Polish grass pea cultivars.

MATERIAL AND METHODS

Plant material comprised two established, original Polish cultivars of grass pea: Derek and Krab. Fragments of internodes, petiols and roots of 14-day-old *in vitro* seedlings were used as primary explants and put on different media. To obtain *in vitro* plants, seeds were surface sterilized in 70% ethanol for 60 s followed by immersing in 0.1% aqueous solution of mercuric chloride ($HgCl_2$) for 25 min and rinsed five times in sterile distilled water. Then seeds were put on a basal medium composed of MS macro and microelements without vitamins (Murashige and Skoog, 1962) with 20 g/l sucrose and solidified with 10 g/l agar. Seeds were kept at 25 ± 1 °C on the light (50 µmol/m^2/s^1 photosynthetic photon flux density) and in 16/8 h photoperiod.

The media for induction and proliferation of callus tissue consisted of MS macro-, microelements and vitamins with 30 g/l sucrose and were solidified with 8 g/l agar. The media were supplemented with 0.5 mg/l naphthylacetic acid (NAA) and 1 mg/l of various natural or synthetic cytokinins: 6-(α,α-dimethylallylamino)-purine (2iP; medium code – LP) or zeatin (ZEA; LZ), 6-benzyloaminopurine (BAP; LB), thidiazuron (TDZ; LT) or kinetin (KIN; LK). For each combination five explants were placed in each of ten vessels. Explants were cultured at 25 ± 1 °C both in the dark (5 vessels) and on the light (5 vessels). Every four weeks, explants were passaged on fresh medium. After each passage percent of explants forming callus tissue and additional structures were evaluated. The mass of callus, number of additional structures and their size were also assessed. Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA) was used to carry out statistical analyses. All results were subjected to ANOVA analysis. Significant differences between means (for influence of each factor for single passage) were determined on the basis of *post hoc* Tukey's test at $P \leq 0.05$.

RESULTS

Callus Tissue Initiation and Morphology

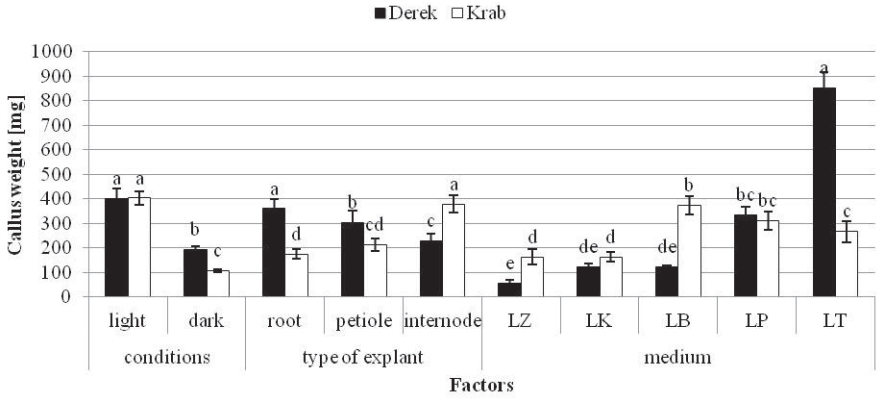
Initiation of the callus formation was observed after about 2 weeks regardless of the medium composition and the light condition of culture. Grass pea explants responded very well to the applied media and culture conditions. Callus tissue appeared on 100% of explants in the case of Derek variety (data not shown). Whereas the percentage of explants forming callus tissue were lower in Krab variety, especially in the case of root fragments cultivated in the dark. The callus morphology was described after four weeks of culture. The colour of callus was from bright green through yellow-green, green to dark green, when explants were cultivated on the light. In turn, in the dark callus was white, cream, yellow or orange. In the case of explants taken from the Krab variety browning and blackening of emerging tissue in the place of contact with the medium was observed very often. Obtained callus had compacted, crystalline or granular structure.

Callus Tissue Proliferation

In the dark callus tissue proliferated significantly better from the 'Derek' explants (Fig. 1). However, the light increased significantly the rate of callus growth in both cultivars. After 12 weeks of culture, 'Derek' callus weight was 2 times higher on the light (403 mg from one explant) than in the dark (193 mg) and weight of 'Krab' tissue was 4 times higher on the light (404 mg) than in the dark (108 mg) (Fig. 1). Among culture media tested, LT medium resulted in the most intense callus proliferation from 'Derek' explants (852 mg) and LB and LP from 'Krab' ones (374 and 312, respectively) (Fig. 1). LK and LZ media caused the weakest tissue proliferation in both cultivars. Callus weight from one explant on these media ranged from 56 to 164 mg (Fig. 1). The intensity of the callus proliferation was influenced significantly by the type of explants, as well. In 'Derek' cultivar the best explant type was a root fragment (361 mg) and the worst an internode one (230 mg). In turn, it was quite contrary in 'Krab'. Internode fragment was the best (380 mg) and root fragment the worst (176 mg) type of explant (Fig. 1).

Callus Tissue Differentiation

The roots formation was observed throughout whole experimental period in described culture. Initially direct rhizogenesis was noted but after 8 and 12 weeks of cultivation roots were formed from callus tissue (Tab. I, Fig. 2). Statistically significant impact on rhizogenesis (percentage of explants with roots, the average number and length of roots) in both genotypes had light conditions (Tab. I). The dark promoted frequency of root formation (28%), average root number (1.4) and their length (7.3 mm) in 'Derek'. Contrary, the light promoted rhizogenesis in Krab cultivar (Tab. I). After 12



1: *Callus weight increase in Derek and Krab varieties depending on different factors (light conditions, type of explants and culture medium) after 12 weeks of culture (means with the same letter are not significantly different within one factor)*

I: *Frequency of rhizogenesis, average number of roots per explants and average root length in Derek and Krab varieties depending on different factors (light conditions, type of explants and culture medium) after 12 weeks of culture (means with the same letter are not significantly different within one passage)*

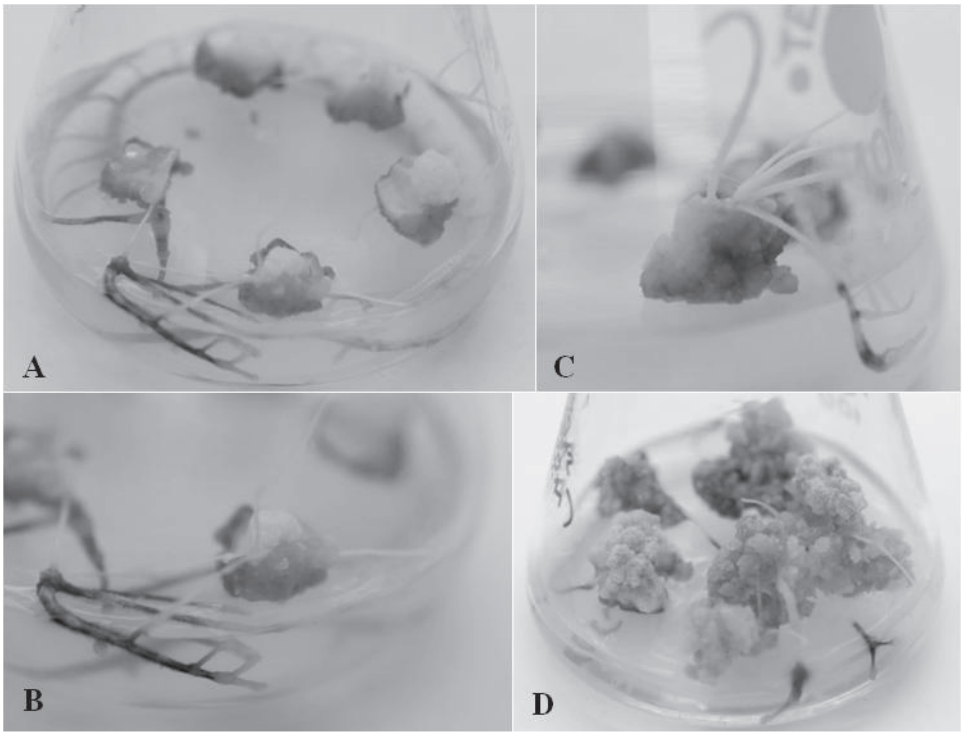
Factors		Frequency of rhizogenesis [%] ±SE		Number of roots per explant ±SE		Roots length [mm] ±SE	
		Derek	***Krab***	***Derek***	***Krab***	***Derek***	***Krab***
Conditions	light	11 ± 2 c	16 ± 4 b	0.3 ± 0.0 d	0.9 ± 0.1 b	3.2 ± 0.4 d	6 ± 0.7 b
	dark	28 ± 5 a	17 ± 3 b	1.4 ± 0.1 a	0.8 ± 0.1 c	7.3 ± 0.8 a	5.2 ± 0.6 c
Type of explant	root	21 ± 6 b	2 ± 1 e	0.7 ± 0.1 c	0.2 ± 0.1 d	2.7 ± 0.4 e	0.7 ± 0.2 f
	petiole	22 ± 5 b	11 ± 2 d	1.3 ± 0.2 b	0.7 ± 0.1 c	7.8 ± 1.0 b	7.0 ± 0.9 c
	internode	16 ± 5 c	36 ± 6 a	0.6 ± 0.1 c	1.6 ± 0.2 a	5.2 ± 0.7 d	9.2 ± 0.8 a
Culture medium	LZ	0 ± 0 f	7 ± 2 e	0 ± 0 g	0.7 ± 0.1 e	0 ± 0 g	6.5 ± 1.0 d
	LK	13 ± 7 d	5 ± 2 e	0.2 ± 0.1 f	0.3 ± 0.1 f	1.6 ± 0.6 f	4.8 ± 0.9 e
	LB	23 ± 4 c	23 ± 8 c	1.4 ± 0.2 ab	1.3 ± 0.3 bc	9.6 ± 1.4 c	4.5 ± 0.8 e
	LP	38 ± 6 a	33 ± 7 b	1.6 ± 0.1 a	1.1 ± 0.1 cd	12.5 ± 1.1 a	10.7 ± 1.3 b
	LT	23 ± 9 c	13 ± 4 d	1.0 ± 0.2 d	0.7 ± 0.1 e	2.5 ± 0.3 f	1.6 ± 0.2 f

weeks of culture, the longest roots (9.2 mm) were formed most often (36%) and most numerous (1.6) in callus from internode fragments of 'Krab'. In turn, rhizogenesis in callus from 'Derek' cultivar was the most intense when petiole fragments was used as initial explants (Tab. I). Clearly stimulating influence on the formation of roots had LP medium. LZ and LK medium had inhibitory effect on rhizogenesis in both cultivars (Tab. I). Generally, rhizogenesis occurred more often in callus of 'Derek'.

DISCUSSION

Tissue culture and especially callus culture generate a wide range of genetic variation which may be used in breeding programs. Additionally, selection of mutants with useful traits, such as tolerance to drought and salinity or resistance to disease is also much easier due to *in vitro* selection. The potential of somaclonal variation should be fully exploited by breeders (Jain, 2001), especially in species with limited gene pool like grass pea. However, the development of the intermediate plant regeneration protocols (via callus tissue) is often lengthy and inefficient. Very often it is a result of changes in the cells like point mutations, DNA methylation or transposons activation (Jain, 2001).

In the presented studies we used two Polish cultivar 'Derek' and 'Krab'. Experiments showed that inducing of callus was not difficult. Emerging of tissue was observed, regardless of the type of explant, variety, and culture conditions, on almost 100% of explants. An equally high percentage of grass pea explants forming callus (above 90%) recorded only Gharyal and Maheshwari (1980, 1983), but only on apical and lateral buds as explants cultivated on B5 media with 0.5 mg/l BAP and 2 mg/l NAA or IAA or 2,4-D. Morphology of callus depended on the light conditions of culture. Tissue formed on the light was usually green colour, which obviously resulted from the chlorophyll synthesized under such conditions. Induction of callus from various grass pea explants and further tissue growth was carried out on the light (Barik and Kar, 2005; Roy *et al*., 1991, 1992, 1993; Sinha *et al*., 1983) or in the dark for 2 to 7 days, and then on the light (Gharyal and Maheshwari, 1980, 1983; Zambre *et al*.,



2: *Rhizogenesis after 12 weeks of culture in callus originated from: A–B) internode fragments of 'Derek' cultivated in the dark on LP medium; C) petiole fragments of 'Derek' cultivated in the dark on LB medium; D) internode fragments of 'Krab' cultivated on the light on LB medium*

2002). Just as the callus induction, its proliferation proceeded without major problems. However, the rate of tissue proliferation depended on the applied growth regulators, genotypes, type of explant and light conditions. Our studies showed that light influenced positively callus proliferation. Similar light effect on the callus proliferation of different plant species noted also some other researchers (Chakravarty and Sopory, 1998; Kintzios *et al*., 1999; Moon and Stomp, 1997). In *Lathyrus sativus* studies callus proliferation was strictly dependent on the genotype, explant type and combinations of growth regulators in the medium. Barik and Kar (2005) noted the best rate of grass pea callus formation on the MS medium supplemented in 3.0 mg/l NAA and 0.3 mg/l BAP. Such combination of growth regulators was also successfully applied in other callus culture of *L. sativus* (Barik and Kar, 2005; Barik *et al*., 2006; Roy *et al*., 1993; Roy *et al*., 1991; Sinha *et al*., 1982). However, in our studies callus proliferation was the most intense on the medium containing 0.5 mg/l NAA and 1.0 mg/l TDZ. Thidizuron was also used in callus *Lathyrus* research of Zambre *et al*. (2002). In combination with IAA it stimulated formation of green, morphogenic tissue. Contrary, in Polish cultivars this set of regulators was not effective (Piwowarczyk, 2009). In presented experiments the best type of explant was fragments of root or internode what was genotype dependent. Barik and Kar (2005) found that most suitable for callus induction was stem explant followed by leaf, hypocotyl and epicotyl.

In our study, the only form of morphogenesis was rhizogenesis. Interestingly, the formation of roots were observed in both varieties, all types of explant, on all media as well as in the dark and on the light. That confirms statement of Orinos and Mitrakos (1991) that rhizogenesis is a labile morphogenic reaction in the sense that it is influenced by various factors tested. In our experiment during the whole cultivation period rhizogenesis was the strongest on the medium with 2iP. In contrast this cytokinin can inhibit root formation in higher concentration (Orinos and Mitrakos, 1991). The frequent and spontaneous rhizogenesis on media with different combinations of growth regulators and in different grass pea genotypes was also mentioned by Sinha *et al*. (1983). These researchers observed also that the tissue producing roots differentiated shoots very rarely. On the other hand, in some plant species shoots regenerated only from callus with the high level of rhizogenesis (Hsia and Korban, 1996). Because auxin is gradually degraded on the light (Stasinopoulos and Hangarter, 1990), hence may arise a much higher degree of the rhizogenesis noted for the explants cultivated in the dark.

CONCLUSION

The aim of the presented study was the evaluation of different factors influence on induction and proliferation of callus tissue as well as any morphogenic changes in grass pea callus. 'Derek' and 'Krab' – two grass pea Polish cultivars were used as plant material. Callus culture were initiated from different *in vitro* seedling fragments. Explants were cultivated on the media with NAA and different cytokinins both on the light and in the dark.

The rate of tissue proliferation was significantly increased in both tested cultivars by light. The most intensive callus mass increase was noted from 'Krab' internode fragments (380 mg/one explant) and 'Derek' root fragments (361 mg/one explant). On media with the addition of thidiazuron callus tissue grew better (852 mg/one explants) than on media with zeatin and kinetin (56–164 mg/one explant). The only organogenesis observed during study was formation of roots. Most frequently (38%), most numerous (1.6) and the longest roots (12.5 mm) were regenerated in 'Derek' callus on LP medium.

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