# Influence of PEG Generated Osmotic Stress on Shoot Regeneration and Some Biochemical Parameters in *Lathyrus* Culture

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## Abstract

Piwowarczyk B., Kamińska I., Rybiński W. (2014): **Influence of PEG generated osmotic stress on shoot regeneration and some biochemical parameters in** *Lathyrus* **culture**. Czech J. Genet. Plant Breed., **50**: 77–83.

Several *Lathyrus* species and in particular *Lathyrus sativus* (grass pea) display much tolerance to drought and have great agronomic potential as grain and forage legumes. Studies on these crop species could improve our understanding of the mechanisms of plant resistance to water stress. This investigation was aimed at examining the effect of low water potential generated by polyethylene glycol (PEG) 6000 on seed germination, shoot regeneration and some biochemical parameters related to drought stress. Seeds of eight *Lathyrus* accessions (six of *L. sativus*, one of *L. cicera*, one of *L. tingitanus*) were cultured on MB (MS macro and microelements with B5 vitamins) medium with addition of 5 mg/l BAP (6-benzyloaminopurine). Different levels of water-stress were induced by using three concentrations of PEG 6000 (50, 100 and 150 g/l) in MB medium. Osmotic stress had no influence on seed germination of any of the accessions tested. However, an increased concentration of PEG in the media caused a decrease in the multiplication rate and vigour of newly regenerated shoots. The percentage content of dry matter and proline content in *Lathyrus* shoots increased significantly under osmotic stress. In turn, water stress had a little effect on photosynthetic pigments.

**Keywords**: drought; grass pea; *in vitro* screening; multiplication rate; proline

In plant production, the most important factors limiting crop productivity are environmental stresses. Disruption of plant water management caused by drought, salinity or low temperature is a major yield-decreasing factor (Jaleel *et al.* 2009).

According to available sources, the world's land area affected by doubled from the 1970's to the beginning of the 21st century (Shabir *et al.* 2010). A growing human population and global climate changes mean that water deficit is becoming more significant. According to the World Water Council, approximately 66% of consumed water is used for irrigation (in arid regions up to 90%). Such a large consumption of water poses a serious problem for crop production in the future. The cultivation of species able to withstand abiotic stresses while maintaining high productivity, could be a solution to this problem. Several *Lathyrus* species and in particular *Lathyrus sativus* (grass pea) show a great deal of tolerance to drought and have a great agronomic potential as grain and forage legumes. Furthermore, studies on these crops could improve our understanding of plant resistance mechanisms to this stress factor.

Water shortage causes fundamental problems for metabolic processes in plant cells, induces growth inhibition and reduces yield. The main effects of water deficit in cells are: reduction of water potential, increasing concentrations of compounds in the cell sap, decreasing turgor pressure, and changes in structure and conformation of macromolecules (Smirnoff 1993). Physiological and biochemical processes, such as photosynthesis, respiration, translocation of assimilates, ion uptake, carbohydrate metabolism, nutrient metabolism are disrupted (Jaleel *et al.* 2009). Jaleel *et al.* (2009) also reported that reactions of plants to water stress differ significantly at various organizational levels depending upon the intensity and duration of stress as well as plant species and its stage of growth. Research on the tolerance of plants to stress is based mainly on defining genetic factors. Furthermore, the interests of scientists are focused on determination of regulating and controlling factors that affect the cell metabolism under stress conditions, and unravelling the mechanisms of signal transduction and adaptation of plants to stress conditions. Understanding plant responses to drought is fundamental for making crops stress-tolerant.

The available literature reports that plants of the genus *Lathyrus* are less sensitive to adverse water conditions and salinity than other members of the family *Fabaceae* (Vaz Patto *et al.* 2006), but there are few studies on this topic. Generally, the genus *Lathyrus* is neglected, especially in Europe. The influence of water stress on the content of selected chemical constituents in *Lathyrus* tissues has been the subject of limited research (Tyagi *et al.* 1995; Xiong *et al.* 2006; Vaz Patto *et al.* 2011). The experiments presented here will be helpful in understanding mechanisms of water stress tolerance in *Lathyrus* and providing a basis for selecting the lines/objects for further breeding work.

The aim of this study was to determine the effect of water stress on germination and regeneration capabilities as well as on some morphophysiological and biochemical parameters of eight *Lathyrus* accessions.

## MATERIAL AND METHODS

**Plant material**. The experimental material comprised four samples from the seed collection at the Institute of Plant Genetics of the Polish Academy of Sciences (Poznań, Poland) and represented three species of *Lathyrus*: *L. sativus*, *L. cicera* and *L. tingitanus*. Seeds of *L. sativus* originated from Germany (LAT 444) and France (LAT B 1705), *L. cicera* from Greece (LAT 209/84) and *L. tingitanus* were of unknown origin (LAT 109/74). Polish gene resources were represented by two cultivars of *L. sativus*: Derek and Krab and two mutants, D4 and K12, derived from original cultivars after chemomutagenesis (Rybiński 2003).

**Explants, growth conditions and treatments**. Intact seeds were used as explants, after being 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 the seeds were put on a basal medium composed of MS macro and microelements (Murashige & Skoog 1962) and Gamborg $B_5$ vitamins (Gamborg *et al.* 1968) with 30 g/l sucrose and 5 mg/l 6-benzylaminopurine (BAP) and solidified with 5 g/l Phytagel (Sigma-Aldrich, St.Louis, USA). Osmotic stress was induced by adding polyethylene glycol (PEG-6000) at a concentration of 50, 100 and 150 g/l to the basal medium. For each combination four disinfected seeds were placed in each of three vessels, and experiments were repeated twice. All plant materials were cultured at 25 ± 1°C under a 16/8 h light photoperiod of 50 μmol per $m^2$/s photosynthetic photon flux density.

**Estimation of the influence of selective agent**. After 4 weeks the multiplication rate (all newly formed shoots and buds per explant) was assessed. The shoot vigour rate was evaluated by using a rating scale: 1 = weak growth (0 shoots 10 mm in length or more per explant), 2 = below average (1–3 shoots), 3 = average (4–6 shoots) and 4 = above average (7 and more shoots). Shoots were oven-dried at 120°C for 24 h to assess their dry weight.

The content of photosynthetic pigments as well as proline was assessed in regenerated shoots after 4 weeks. Total chlorophylls ($Chl_{a+b}$), chlorophyll *a* ($Chl_a$), chlorophyll *b* ($Chl_b$), and carotenoids (Car) were determined spectrophotometrically using 80% acetone as a solvent (Lichtenthaler 1987). Proline content was measured by the method of Bates *et al.* (1973). Three replicates per object and treatment were obtained from newly regenerated shoots after 4 weeks of culture.

**Statistical analyses**. Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA) was used to carry out statistical analyses. Significant differences between means were determined on the basis of SE (standard error).

## RESULTS

**Multiplication and shoot vigour rate**. The comparison of shoot regeneration efficiency on the media with and without selective agent revealed that multiplication rate was adversely affected by PEG (Figure 1). Multiplication rates compared to media without the addition of PEG showed substantial differences between the genotypes studied (Table 1). It is interesting that in the medium supplemented with various concentrations of PEG these differences disappeared. However, multiplication rates of tested plants were significantly lower on the medium with selective agent than those of their respective controls (Table 1), although with the lowest level of PEG (50 g/l), the number of shoots per explant was significantly reduced only in *L. cicera* and *L. sativus* Derek (Table 1). Conversely, higher concentrations of selective agent (100 and 150 g/l PEG) had an inhibitory effect on the regeneration capacity of all tested objects with the exception of Krab on the medium with 100 g/l

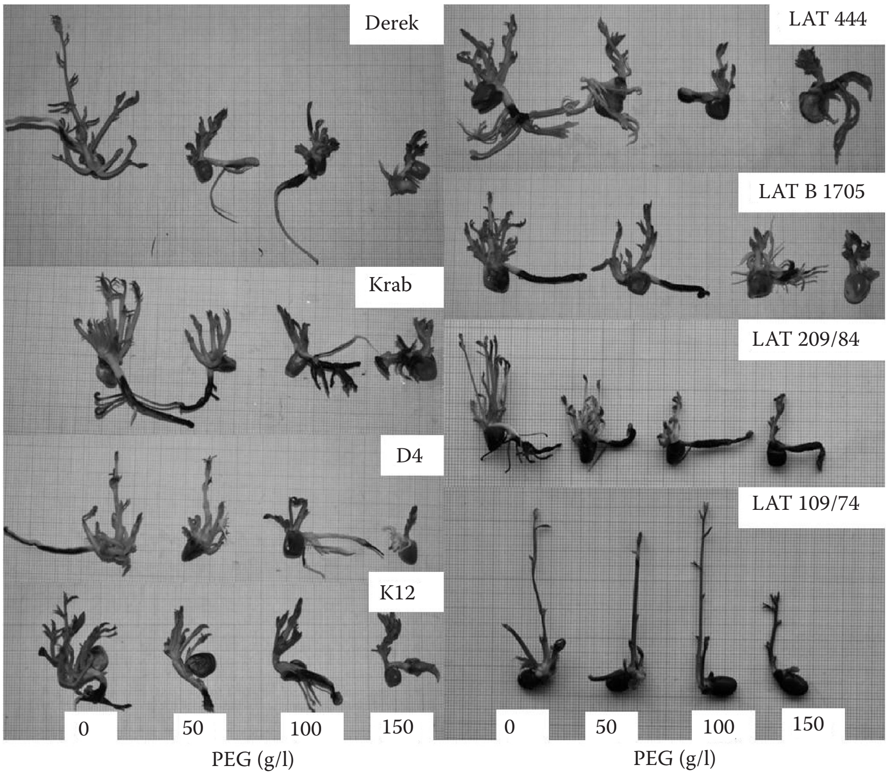

Figure 1. Shoot regeneration of eight *Lathyrus* genotypes depending on different concentrations of polyethylene glycol (PEG)

PEG. Increasing doses of PEG from 50 to 150 g/l in the media caused a substantial reduction in multiplication rate responses among the tested accessions. Generally, drought conditions generated by PEG significantly influenced the vigour of regenerated shoots. However, the lowest dose of selective agent caused a significant decrease in shoot vigour rate only for Derek (Table 1). In turn, on the media with the addition of 100 g/l PEG we noticed a considerable reduction of shoot vigour with the exception of Krab, K12 and LAT 444. Nevertheless, when the level of PEG in the medium was increased to 150 g/l, shoot vigour was noticeably reduced for all

Table 1. Multiplication rate, shoot vigour rate and dry weight percentage of shoots in eight *Lathyrus* genotypes regenerating on media with different concentrations of polyethylene glycol (PEG in g/l) (mean ± standard error)

| Genotype | Multiplication rate | | | | Shoot vigour rate | | | | Dry weight percentage of shoots | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PEG | | | | PEG | | | | PEG | | | |
| | 0 | 50 | 100 | 150 | 0 | 50 | 100 | 150 | 0 | 50 | 100 | 150 |
| Derek | 17.5 ± 1.5 | 10.3 ± 0.7 | 8.5 ± 0.9 | 7.4 ± 0.7 | 3.5 ± 0.1 | 2.6 ± 0.2 | 2.4 ± 0.1 | 2.3 ± 0.1 | 11.6 ± 0.7 | 15.4 ± 0.5 | 18.6 ± 1.1 | 18.7 ± 0.9 |
| D4 | 13.2 ± 2.0 | 10.4 ± 1.0 | 8.4 ± 1.0 | 6.3 ± 0.8 | 3.0 ± 0.2 | 2.6 ± 0.2 | 2.3 ± 0.1 | 2.2 ± 0.1 | 10.1 ± 0.5 | 11.7 ± 0.7 | 12.9 ± 1.0 | 18.5 ± 0.5 |
| Krab | 9.5 ± 1.2 | 9.6 ± 0.9 | 7.8 ± 0.9 | 4.6 ± 0.6 | 3.1 ± 0.2 | 2.7 ± 0.2 | 2.8 ± 0.2 | 1.9 ± 0.2 | 10.4 ± 0.1 | 12.7 ± 0.7 | 17.5 ± 0.7 | 13.6 ± 0.1 |
| K12 | 11.4 ± 0.9 | 12.3 ± 1.1 | 7.9 ± 0.8 | 4.2 ± 0.6 | 2.9 ± 0.2 | 2.8 ± 0.2 | 2.6 ± 0.2 | 2.2 ± 0.1 | 10.5 ± 0.3 | 13.5 ± 0.8 | 16.3 ± 0.4 | 18.3 ± 0.9 |
| LAT 444 | 12.6 ± 0.9 | 10.0 ± 0.9 | 8.4 ± 0.5 | 4.5 ± 0.6 | 2.9 ± 0.2 | 2.6 ± 0.1 | 2.7 ± 0.2 | 2.0 ± 0.1 | 11.4 ± 0.1 | 14.5 ± 1.0 | 18.4 ± 0.8 | 20.7 ± 0.7 |
| LAT B 1705 | 13.9 ± 1.3 | 11.6 ± 0.9 | 8.2 ± 0.4 | 5.4 ± 0.5 | 3.3 ± 0.2 | 2.9 ± 0.1 | 2.2 ± 0.2 | 2.1 ± 0.2 | 13.5 ± 0.1 | 14.5 ± 0.8 | 16.9 ± 0.6 | 17.2 ± 0.3 |
| LAT 209/84 | 16.6 ± 1.4 | 13.0 ± 1.1 | 9.4 ± 0.6 | 6.0 ± 0.5 | 3.0 ± 0.2 | 2.5 ± 0.2 | 2.1 ± 0.1 | 1.9 ± 0.1 | 11.1 ± 0.7 | 16.6 ± 0.5 | 16.4 ± 0.5 | 17.7 ± 0.5 |
| LAT 109/74 | 9.3 ± 0.8 | 9.8 ± 1.0 | 5.0 ± 0.8 | 2.4 ± 0.3 | 2.6 ± 0.2 | 2.5 ± 0.2 | 2.0 ± 0.1 | 2.0 ± 0.1 | 10.4 ± 0.1 | 9.9 ± 0.1 | 11.7 ± 0.7 | 15.6 ± 0.1 |

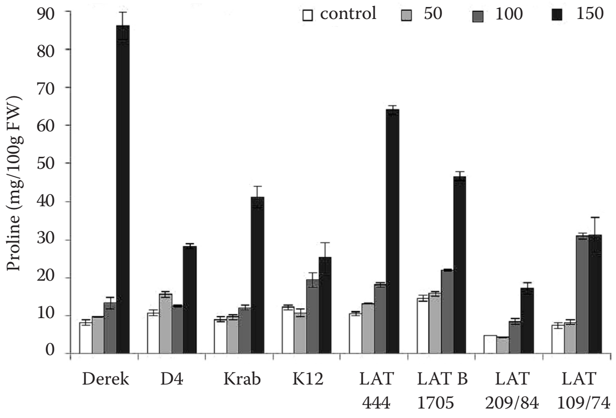

Figure 2. Proline content in shoots of eight *Lathyrus* genotypes regenerating on media with different concentrations of polyethylene glycol (PEG in g/l)
Error bars show the standard error of the mean

studied genotypes compared to the control, but no significant differences were observed between them (Table 1).

**Dry weight of shoots**. stress conditions considerably increased the dry weight of regenerated shoots compared to the control (Table 1). However, the lowest concentration of PEG (50 g/l) did not increase dry weight of shoots regenerated from explants of D4 and LAT 444. Dry weight of LAT 109/74 shoots did not increase on the media with the addition of 100 g/l PEG either (Table 1). Content of dry weight in LAT 444 shoots increased the most and in Krab shoots the least under the influence of severe osmotic stress generated by the highest dose of PEG (150g/l) (Table 1).

**Proline content**. The accumulation of proline was significantly greater under osmotic stress than under unstressed conditions (Figure 2). We noted significant differences between the studied accessions both under control and stress conditions. Without selective agent, newly regenerated shoots of LAT B 1705 had the highest level of proline (14.6 mg/100g) and those of LAT 209/84 the lowest. Proline content in newly regenerated shoots on the media containing PEG ranged from 4.3 mg/100 g FW in LAT 209/84 to 86.3 mg/ 100 g FW in Derek (Figure 2). The highest increase of proline content was seen in Derek and LAT 444 shoots and the lowest in K12 and LAT 209/84 ones under stress generated by 150 g/l PEG (Figure 4).

**Pigments**. Under control conditions, the total chlorophyll content ($Chl_{a+b}$) was significantly higher in newly regenerated shoots of Derek and Krab and K12 than in LAT B 1705 and LAT 109/74 (Figure 3). Carotenoid contents under the same conditions were significantly higher in shoots of Derek and D4 and K12 than in LAT 444, LAT B 1705 and LAT 109/74 (Figure 4). On the other hand, osmotic stress induced by the highest concentration of PEG (150 g/l) caused a significant decrease of total chlorophylls in shoots of Krab and K12 (Figure 3). Under all three levels of PEG treatment used, the content of carotenoids increased

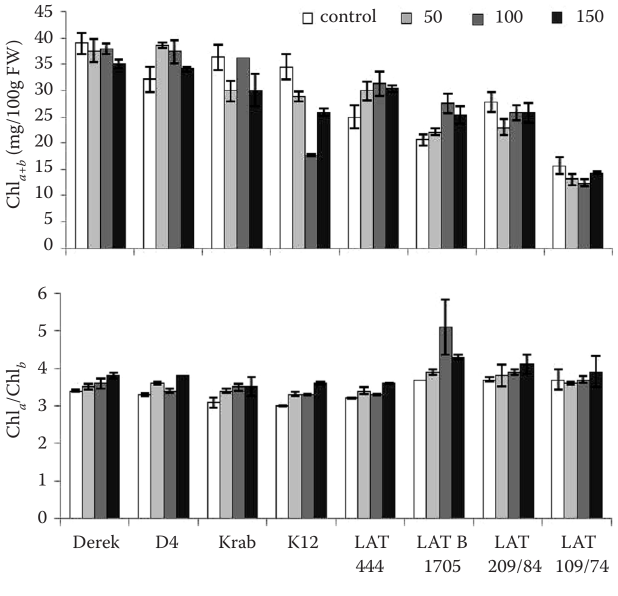

Figure 3. Total chlorophyll ($Chl_{a+b}$) content and the ratio of chlorophyll *a* to chlorophyll *b* ($Chl_a/Chl_b$) in shoots of eight *Lathyrus* genotypes regenerating on media with different concentrations of polyethylene glycol (PEG in g/l)
Error bars show the standard error of the mean

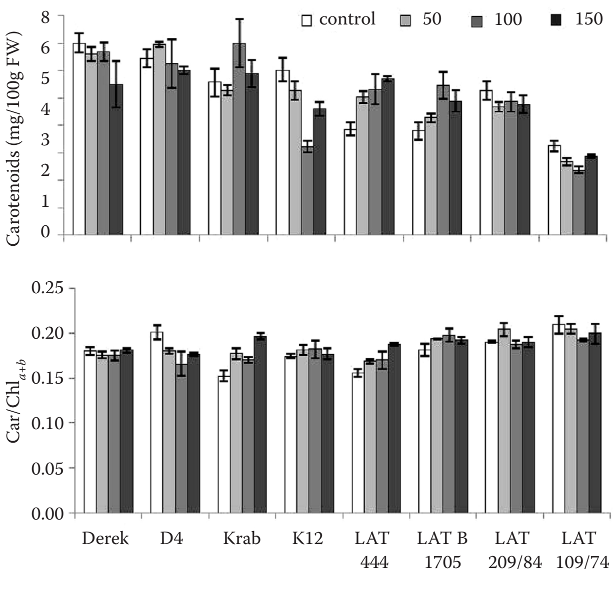

Figure 4. Carotenoids and the ratio of carotenoids to total chlorophylls ($Car/Chl_{a+b}$) in shoots of eight *Lathyrus* genotypes regenerating on media with different concentrations of polyethylene glycol (PEG in g/l)
Error bars show the standard error of the mean

significantly only in the shoots of LAT 444. Osmotic stress induced by 100 g/l of PEG caused an increase in carotenoid pigment content also in the shoots of Krab and LAT B 1705 but the highest dose of PEG (150 g/l) had no such influence. Under severe stress conditions, carotenoids decreased in Derek and K12 (Figure 4). Osmotic stress generated by PEG significantly influenced the ratio of $Chl_a/Chl_b$ in regenerated organs, causing its increase in Krab, D4 and K12 and LAT B 1705 (Figure 3). The ratio of $Car/Chl_{a+b}$ in shoots regenerated on PEG-containing media compared to the control treatment increased in shoots of Krab and LAT 444, decreased in D4 mutant and did not change in the other five genotypes (Figure 4).

## DISCUSSION

Several *Lathyrus* species and especially *Lathyrus sativus* (grass pea) have a great agronomic potential because of their superior drought, flood and salinity tolerance compared to other legume crops (Vaz Patto *et al.* 2006). Research on plant stress responses has been carried out intensively but mostly on model plants such as *Arabidopsis thaliana*. Taking into account physiological processes that differ between plants we can expect that studies on legumes will unravel specific mechanisms involved in abiotic stress resistance/tolerance (Reddy *et al.* 2012). *In vitro* screening techniques are highly desirable in such studies for minimizing the impact of the changing external environment. The creation of drought conditions is not a problem since non-penetrating and inert polyethylene glycol (PEG), with its high molecular weight, induces water stress in plants (Rai *et al.* 2011). Until now, *in vitro* studies of the influence of water stress on the regeneration ability of species from the genus *Lathyrus* have received little attention (Xiong *et al.* 2006; Vaz Patto *et al.* 2011).

In general, the findings that PEG reduced the multiplication and shoot vigour rates of *Lathyrus* accessions are similar to those reported for *in vitro* drought screening of other plant species (Gopal & Iwama 2007; Sakthivelu *et al.* 2008; Bidabadi *et al.* 2012). It is interesting to note that the intensity of multiplication rate and shoot vigour reduction under certain levels of PEG treatment was not genotype dependent, as this contrasts with the results reported by Sakthivelu *et al.* (2008). With increasing concentration of polyethylene glycol, shoot vigour also declined. This may be a consequence of hampered water and nutrient absorption due to a decreasing water potential of the medium or greatly suppressed cell elongation as a result of the low turgor pressure (Jaleel *et al.* 2009).

Higher dry weight accumulation in plants is a desirable feature under water deficit conditions (Jaleel *et al.* 2009) and has been correlated with tolerance to drought (Sakthivelu *et al.* 2008). Moreover, Kage *et al.* (2004) demonstrated that dry matter partitioning and biomass disposition are strongly connected with plant productivity under drought stress conditions. Soni *et al.* (2011) noted an increase of dry matter content in drought-tolerant genotypes of moth bean under water stress. We also recorded higher accumulation of dry matter under PEG-induced osmotic stress in regenerated shoots. Increasing accumulation of dry biomass is related to two processes: dehydration and new material synthesis required for maintenance of higher osmoticum to continue water absorption (Soni *et al.* 2011).

The accumulation of various chemical compounds, including lipoxygenases (Tyagi *et al.* 1995), free polyamines (Xiong *et al.* 2006) and proline (Tyagi *et al.* 1999), that may be involved in the stress adaptation process has been analysed several times in *Lathyrus* plants . Proline is one of the most widespread amino acids accumulated by plants during different abiotic stresses (Wang *et al.* 2003). It is a compatible solute (an osmotically active agent) able to retain water within the cells under conditions of osmotic stress (Janska *et al.* 2010). Moreover, proline protects membranes and proteins in the presence of high osmoticum concentration (Delauney & Verma 1993). Due to these properties the level of proline accumulated could be a basis for selection of water stress-tolerant plants (Rai *et al.* 2011). In many studies, plants exposed to water deficit that enhanced the proline content were identified as tolerant to drought (Fuller *et al.* 2006; Bidabadi *et al.* 2012; Sultan *et al.* 2012). Tyagi *et al.* (1999) recorded a 65-fold increase in proline content in leaves and 56-fold in stems of 7-days-old grass pea seedlings exposed to drought stress. Our results on proline content revealed also its significant increase in shoots of nearly all tested *Lathyrus* species regenerated under osmotic stress induced by higher doses of PEG. Generally, on the medium without selective agent, Polish grass pea mutants and accessions coming from Western Europe were characterized by higher proline content than Polish grass pea varieties and *L. cicera* and *L. tingitanus.* Whereas on the medium with the least PEG added this trend was about the same, on the medium with the highest PEG content, grass pea varieties accumulated much more proline than grass pea mutants. Since a higher proline content is characteristic for more water stress-tolerant or resistant genotypes (Sultan *et al.* 2012), this would suggest that among the tested accessions the most drought-resistant are Derek and LAT 444, and the most sensitive are K12 and LAT 209/89.

Water deficit influences the composition and content of photosynthetic pigments in field-grown plants (Farooq *et al.* 2009; Jaleel *et al.* 2009). Chlorophyll content using the SPAD method was also analysed in different *Lathyrus* genotypes (*L. aphaca*, *L. cicera*, *L. clymenum*, *L latifolius*, *L. ochrus*, *L. odoratus*, *L. sylvestris*, *L. sativus*) both in well-watered conditions and in water deficit stress. Some genotypes of *L. sativus* retained functional photosystems even under severe water stress (Vaz Patto *et al.* 2011). Although plantlets growing under *in vitro* conditions are characterized by lower pigment content that arises from the specificity of *in vitro* conditions (Hazarika 2006), we decided to test the influence of PEG-induced osmotic stress on it. Oxidative stress generated in plant cells as a result of extended drought causes a reduction of chlorophylls and carotenoid content in many species (Smirnoff 1993; Kiani *et al.* 2008). Nevertheless, carotenoids as a part of the plant antioxidant defence system also play additional roles in plants resisting to drought (Jaleel *et al.* 2009). Total chlorophyll ($\mathrm{Chl}_{a+b}$) content was reduced only in two *Lathyrus* genotypes regenerating shoots under osmotic stress (Krab and K12). Moran *et al.* (1994) ruled out any serious oxidative damage to pea plants that showed under drought little or no decrease in chlorophyll content. On the other hand, carotenoid content increased significantly only in LAT 444 shoots at all three levels of osmotic stress. Water stress provokes also variations in the ratio of chlorophyll *a* and chlorophyll *b* and carotenoids (Farooq *et al.* 2009). The highest osmotic stress in the *Lathyrus* culture induced a rise in the $\mathrm{Chl}_a/\mathrm{Chl}_b$ ratio in shoots of four lines studied (Krab, K12, D4 and LAT B1705). Liu *et al.* (2011) explained the increasing $\mathrm{Chl}_a/\mathrm{Chl}_b$ ratio, due to intensifying drought stress, as a decrease of the peripheral light-harvesting complex. We observed no systematic changes in the ratio of $\mathrm{Car}/\mathrm{Chl}_{a+b}$ in various *Lathyrus* accessions under different levels of osmotic stress, which may be explained by the lack of need for photoprotection by Car (Elsheery & Cao 2008).

Our preliminary results on *Lathyrus* drought resistance utilizing *in vitro* screening do not show clearly which *Lathyrus* accession is the most resistant to water stress. Nevertheless, it is evident that responses to stress are connected to an increase in dry weight and proline content but not with carotenoid content.

***Acknowledgements***. This work was supported by the Ministry of Science and Higher Education of the Republic of Poland, Project No. BM4522.

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Received for publication May 31, 2013
Accepted after corrections December 12, 2013

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