


Different acclimatization mechanisms of two grass pea cultivars to osmotic stress in in vitro culture

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Abstract Grass pea (*Lathyrus sativus* L.) is a legume crop known from its tolerance to various abiotic stresses, especially drought. In this study, we investigated: (1) the response of grass pea seedlings to osmotic stress generated in vitro by polyethylene glycol (PEG); (2) potential drought acclimatization mechanisms of two polish grass pea cultivars. Grass pea seeds of two cultivars were sown on media containing different PEG concentrations (0, 5.5, 11.0 mM) and cultivated for 14 days in controlled conditions. Plants' dry matter increased under osmotic stress (regardless of PEG concentration). In turn, the highest dose of PEG caused a reduction in seedling growth in both cultivars. Furthermore, PEG caused the peroxidase activity increase in whole seedlings and catalase (CAT) activity in roots. However, differences between cultivars were noted in: CAT activity in shoots; while phenols and anthocyanin content as well as electrolyte leakage in shoots and roots. In turn, in both tested genotypes, accumulation of proline increased in shoots under osmotic stress. Obtained results indicate that the examined plants, although belonging to the same species, differ in acclimatization processes leading to elevated tolerance to osmotic stress.

Keywords Catalase · Drought · *Lathyrus sativus* · Polyethylene glycol · Peroxidase · Proline

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Introduction

Water scarcity, a worldwide problem currently is expected to elaborate in the near future (Dai 2013). Water shortages negatively influence plant growth and development, consequently heavily limit plant productivity. A search for plant species that are tolerant to drought and elucidating the mechanisms of their responses to osmotic stress is of significant importance. Grass pea (*Lathyrus sativus*, Fabaceae) is a crop highly tolerant to the stress caused by various abiotic factors (Vaz Patto et al. 2006). Even though the exact mechanism of this tolerance is still not known, there are reports indicating that it can rely on the ability to adjust the plants osmotic potential (Jiang et al. 2013; Piwowarczyk et al. 2014), elevated antioxidant enzyme activity (Jiang et al. 2013), accumulation of polyamines and β -*N*-oxalyl-L- α , β -diaminopropionic acid (Xing et al. 2001; Xiong et al. 2006).

In response to water stress, plants adjust their metabolism utilizing different mechanisms. However, considering the complexity of these mechanisms, we are far from gaining a comprehensive understanding of abiotic plant tolerance, despite many years of research (Farooq et al. 2009). Additionally, osmotic stress leads to the formation of reactive oxygen species (ROS). ROS impede the normal functioning of cells by degrading/inactivating proteins, lipids, and DNA (Blokhina et al. 2003), and inter alia severely damage cell membranes (Ashraf and Ali 2008). Plant defense against oxidative stress is based on the antioxidant system (Farooq et al. 2009). Earlier studies showed that the mechanism of salinity tolerance in grass pea plants resulted probably from the elevated activity of antioxidant system in the root cells, manifested by increased accumulation of phenolic compounds and peroxidase activity (Piwowarczyk et al. 2016).

The aim of this study was to investigate the response of grass pea seedlings to osmotic stress generated *in vitro* and identification of potential drought acclimatization mechanisms of this species, in relation to different cultivars.

Materials and methods

Plant material and culture conditions

Plant material comprised of grass pea cultivars: ‘Derek’ and ‘Krab’. Derek and Krab were introduced to Polish Register of Original Varieties in 1997 and are the only two, registered, Polish cultivars (Milczak et al. 2001). Both cultivars yield 3.0 t/ha. Cultivars differ from each other in seed size and morphology (Derek: small, creamy-green; Krab: medium, creamy), thousand seed mass (D 124 g, K 184 g) and seed protein content (D 31%, K 28%) (Cichy and Rybiński 2007). There is a lack of information concerning differences in resistance to biotic and abiotic stresses between the studied cultivars. Seeds were surface sterilized according to Piwowarczyk et al. (2016). Sterilized seeds were placed on phytagel solidified (0.5%) MS macro and microelements’ medium (Murashige and Skoog 1962). To induce osmotic stress, polyethylene glycol (PEG-6000, Duchefa Biochemie) in different concentration (0, 5.5, 11.0 mM) was added to basal medium. Vessels with seeds were placed under controlled conditions (light: 16/8 h photoperiod, 50 $\mu\text{mol}/\text{m}^2/\text{s}^1$ photosynthetic photon flux density, temperature: 25 ± 1 °C).

Determination of biometric and physiological/biochemical parameters

After 14 days of culture a series of biometric measurements (shoots and roots length, shoots and roots dry weight) were performed. Moreover, relative water content (%) was calculated on the basis of fresh and dry weight. Fresh samples of shoots and roots, prepared as described by Piwowarczyk et al. (2016), were used to determine the electrolyte leakage (EL) by ion leaching from tissue to ultrapure water (Millipore, Direct System Q3). Fresh tissue (shoot and root separately) was used to analyze antioxidant enzymes. Homogenization of plant material and preparation of reaction mixture was performed as described by Piwowarczyk et al. (2016). Catalase (CAT) activity was determined spectrophotometrically (Double Beam spectrophotometer U-2900, Hitachi High-Technologies Corp.) according to Bartosz (2006) and peroxidase (POD) activity according to Lück (1962). The content of total phenolic compounds was estimated using the photometric method with Folin’s reagent according to Swain and Hillis (1959) with slight modifications (Piwowarczyk et al. 2016).

Anthocyanin content was measured using the spectrophotometric method according to Fukumoto and Mazza (2000). Proline content was assessed according to Bates et al. (1973) and chlorophyll *a* and *b* content according to Wellburn (1994).

Statistical analyses

Results were subjected to statistical analyses using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA). The significant differences between means were determined using Duncan test at $p < 0.05$.

Result and discussion

One of the most visible drought stress symptoms is impaired growth (Farooq et al. 2009). Reduced water availability causes the loss of cell turgor and subsequently hampers cell elongation and plant growth (Taiz and Zeiger 2010). There is a number of reports indicating that drought reduced legume plants’ growth (Okçu et al. 2005; Zeid and Shedeed 2006; Jiang et al. 2013). In this study, only the highest dose of PEG caused a reduction in shoot growth (by $\sim 40\%$) in both cultivars, and roots by $\sim 30\%$ in ‘Krab’ and $\sim 70\%$ in ‘Derek’ (Table 1).

Osmotic stress negatively affects photosynthesis and carbohydrate production, resulting in growth disturbances (Farooq et al. 2009). Decrease of dry matter was noted in many plant species under drought conditions (Okçu et al. 2005; Zeid and Shedeed 2006). Some authors believe that dry matter increases in plants during water stress may indicate tolerance to drought (Jaleel et al. 2009). Furthermore, osmotic stress conditions enhance translocation of assimilates to the roots, thus increasing their water uptake capacity (Farooq et al. 2009). In the presented experiments, dry matter increased in the roots and shoots with increasing osmotic stress (Table 1). Additionally, our results revealed that the rate of dry matter increase in roots and shoots of both grass pea cultivars was similar and the shoot/root dry matter ratio was closed to 1 (Table 1). This may indicate the other than the improved water uptake mechanism of grass pea tolerance to drought stress.

One of the best known reactions to stress is generation of reactive oxygen species (e.g. H_2O_2 , $\bullet\text{O}_2$), which at high concentrations cause oxidative damage and deterioration of normal cell functioning (Farooq et al. 2009). To cope with oxidative stress, plant cells evolved a complex system composed of different compounds with antioxidative properties (Smirnoff 1993). Among these compounds, peroxidases and catalases are the main enzymes involved in H_2O_2 removal (Noctor et al. 2014). In presented work, a significant increase in POD activity was observed in shoots

Table 1 Different features of shoot and root of 14 day-old grass pea seedlings under osmotic stress

Feature	Organ	Cult.	Concentration of PEG (mM)		
			0.0	5.5	11.0
Length (mm) ± SD	Shoot	D	72.2 (±17.5) ^a	65.4 (±40.3) ^a	42.1 (±24.3) ^b
		K	68.0 (±22.9) ^a	57.4 (±35.3) ^a	43.1 (±21.3) ^b
	Root	D	85.6 (±26.7) ^a	76.3 (±31.6) ^a	24.4 (±24.0) ^b
		K	78.4 (±35.2) ^a	74.1 (±44.3) ^a	53.5 (±22.8) ^b
Dry weight (mg) ± SD	Shoot	D	27.2 (±1.2) ^c	35.0 (±2.2) ^b	39.5 (±1.0) ^a
		K	26.4 (±0.7) ^c	34.2 (±3.0) ^b	40.0 (±2.5) ^a
	Root	D	24.4 (±3.1) ^c	30.5 (±2.7) ^b	37.3 (±3.5) ^a
		K	22.3 (±0.9) ^c	27.8 (±2.2) ^b	36.6 (±3.3) ^a
Relative water content (%) ± SD	Shoot	D	89.1 (±0.5) ^a	86.0 (±0.9) ^b	84.2 (±0.4) ^c
		K	89.4 (±0.3) ^a	86.3 (±1.2) ^b	84.0 (±1.4) ^c
	Root	D	90.3 (±1.2) ^a	87.8 (±1.1) ^b	85.1 (±1.0) ^c
		K	91.1 (±0.4) ^a	88.9 (±0.9) ^b	85.4 (±1.3) ^c
Electrolyte leakage (%) ± SD	Shoot	D	64.4 (±7.5) ^a	61.4 (±8.1) ^a	65.4 (±6.7) ^a
		K	65.2 (±14.4) ^b	68.1 (±9.7) ^b	40.1 (±1.4) ^a
	Root	D	71.6 (±11.1) ^a	70.1 (±4.8) ^a	66.7 (±4.0) ^a
		K	60.9 (±10.4) ^b	65.4 (±6.7) ^b	43.0 (±6.4) ^a
Total phenols (mg/100 g) ± SD	Shoot	D	522.3 (±50.5) ^a	619.7 (±98.6) ^a	559.2 (±16.5) ^a
		K	490.4 (±48.6) ^b	576.9 (±0.0) ^{ab}	714.2 (±115.9) ^a
	Root	D	287.9 (±100.1) ^a	309.7 (±25.6) ^a	410.5 (±34.1) ^a
		K	316.5 (± 28.1) ^c	408.0 (±32.8) ^b	592.0 (±33.9) ^a
Anthocyanins (mg/100 g) ± SD	Shoot	D	23.9 (±7.3) ^a	29.8 (±10.4) ^a	18.9 (±7.6) ^a
		K	27.6 (±2.8) ^b	25.4 (±5.3) ^b	42.2 (±4.7) ^a
	Root	D	38.8 (±2.3) ^a	44.2 (±2.6) ^a	61.6 (±3.2) ^a
		K	52.1 (±2.3) ^a	73.3 (±5.9) ^a	85.5 (±4.6) ^a
Proline (mg/100 g) ± SD	Shoot	D	72.7 (±18.7) ^b	111.4 (±53.1) ^b	195.7 (±29.6) ^a
		K	49.7 (±4.6) ^b	88.7 (±25.0) ^b	301.5 (±42.8) ^a
	Root	D	55.9 (±9.6) ^d	58.8 (±13.6) ^{cd}	125.5 (±71.4) ^{bcd}
		K	50.8 (±3.3) ^a	73.6 (±19.7) ^a	66.3 (±12.5) ^a
Chlorophyll <i>a</i> + <i>b</i> (mg/g) ± SD	Shoot	D	0.63 (±0.33) ^a	0.49 (±0.17) ^a	0.37 (±0.05) ^a
		K	0.37 (±0.10) ^a	0.41 (±0.0) ^a	0.51 (±0.07) ^a

D Derek, K Krab

Different letters—significant difference at $p < 0.05$ within one organ and cultivar

of both cultivars under drought conditions (Fig. 1a). However, increase of POD activity in roots was related to PEG concentration. Lower concentrations significantly increased POD activity in ‘Krab’ seedlings. On the other hand, the effect of PEG on POD activity was more pronounced in ‘Derek’. Our results are in contrary to earlier studies on grass pea reaction to PEG treatment that showed decline of POD activity (Jiang et al. 2013; Gengsheng et al. 2001).

In turn, changes in CAT activity were not that unidirectional (Fig. 1b). In ‘Derek’ roots PEG, in all concentrations stimulated CAT activity, whereas in ‘Krab’ enhanced it only under the strongest osmotic stress applied. Furthermore, only the highest PEG concentration induced

alterations in CAT activity. Surprisingly the activity of CAT in shoots under the highest PEG concentration was downregulated in ‘Derek’ and upregulated in ‘Krab’ (Fig. 1b). A decrease in CAT activity in grass pea plants was also reported by Gengsheng et al. (2001) and an increase by Jiang et al. (2013). These differences may arise from differences in PEG concentrations used and treatment length. Small molecule compounds (including phenols) with ROS scavenging abilities constitute the second branch of the antioxidant (Weidner et al. 2009). In general, differences in phenol content were observed between tested cultivars (Table 1). In ‘Krab’ seedlings, accumulation of phenols increased with increasing levels of osmotic stress (Table 1). In ‘Derek’, phenol content did not change

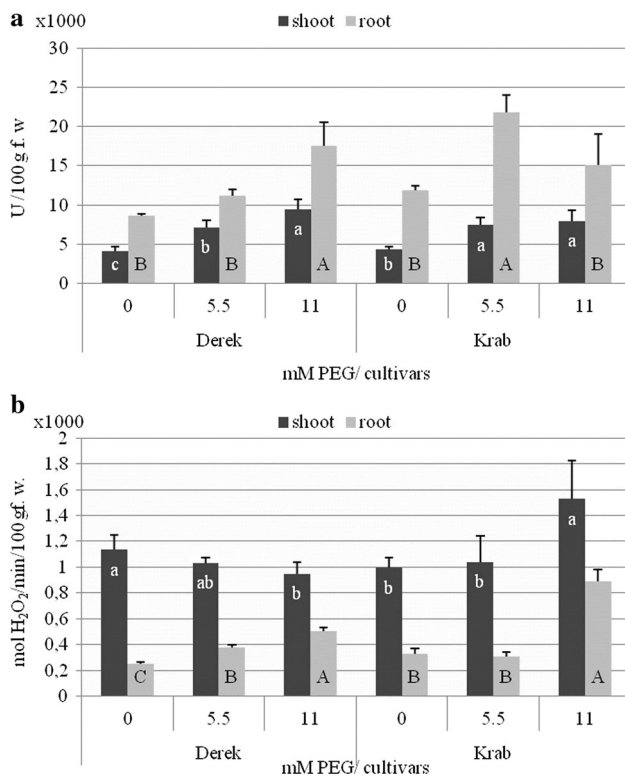


Fig. 1 Activity of antioxidant enzymes of 14 day-old grass pea seedlings under osmotic stress **a** peroxidase activity (U/100 g f.w.); **b** catalase activity ($\mu\text{mol}(\text{H}_2\text{O}_2)/\text{min}/100\text{ g f.w.}$). Different letters significant difference at $p < 0.05$ within one organ and cultivar

significantly in stressed seedlings compared to control ones (Table 1). It is believed that phenolic compounds play a significant role in protection of photosynthetic apparatus during water shortage conditions (Hura et al. 2009), hence their increased accumulation in plants may provide a better adaptation to the emerging stress (Hura et al. 2009; Tattini et al. 2004).

Abiotic stress induces the accumulation of anthocyanins (Kovinich et al. 2014; Chalker-Scott 1999). Here, only 'Krab' seedlings accumulated anthocyanins in elevated concentrations under the highest PEG dose used (Table 1). Although, the exact mechanism of anthocyanins action under drought stress is not known yet, it is believed that, apart from an osmo-regulatory role (Chalker-Scott 1999), they scavenge reactive oxygen species (Kovinich et al. 2014). In previous study on grass pea seedlings, an increase in the phenol concentration was observed under salinity stress (Piwowarczyk et al. 2016). Taking into account obtained results as well as literature data, the accumulation of phenolic compounds may be considered as a significant element of grass pea tolerance mechanism to abiotic stresses. Other compound that can act as a free radical scavenger under stress conditions is proline (Kavi Kishor et al. 2005). Proline is known for its controversial role as

compatible solute (Piwowarczyk et al. 2016); it may also be an osmoprotectant (Hasegawa et al. 2000). Our results showed that only seedling shoots from the medium with 11 mM PEG showed significantly greater accumulation of proline (Table 1).

Reactive oxygen species can cause lipid peroxidation and lead to disturbances in plasma membranes structure and functions. Such disorders are manifested by an increase in the membranes' permeability (Ashraf and Ali 2008). Results presented here show no significant increase in electrolyte leakage from shoot and root cells of both cultivars under osmotic stress (Table 1). On contrary, a significant decrease in ion leakage in 'Krab' cultivar seedlings growing on the medium with the highest PEG concentration was observed (Table 1). The ability to rapidly rebuild plasma membranes to enhance their integrity can be one of the key processes of acclimatisation to the emerging stress. Such a process was observed in 'Krab' seedlings. An upward trend in chlorophyll accumulation in seedlings, on higher osmotic stress treatments, indirectly confirmed this hypothesis (Table 1).

In summary, the results indicate that the examined plants, although belonging to the same species, differ in acclimatization processes leading to elevated tolerance to osmotic stress. In cells of Krab cultivar, in response to the occurring stress are triggered not only the enzymatic antioxidants and osmoprotectants as in the case of Derek cultivar, but also non-enzymatic antioxidants as phenols, including anthocyanins. Moreover, improved biometric parameters of 'Krab' seedlings' root from the severest stress applied suggest that a crucial role in tolerance to osmotic stress may play abilities to rapid plasma membranes rebuilding.

Author contribution statement BP designed and performed experiments, analyzed data and wrote the paper; KT discussed the results and wrote the paper; WM and AŁ gave technical support and performed experiments.

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