Drought-induced Changes in the Antioxidant System in *Pisum sativum* L.

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ABSTRACT

Background: This study was carried out for an understanding of the antioxidant mechanisms of field pea varieties under osmotic stress conditions caused by a lack of water. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx) and glutathione reductase (GR) were examined and analyzed. The gene expression levels of *Cu/Zn SOD*, *cAPx* and *GR* genes were also examined.
Methods: Osmotic stress was stimulated using PEG 6000 with the osmotic potential of -0.1 MPa in 10 days old plant seedlings. The activity of the antioxidant enzymes was measured in the shoots and roots of pea seedlings. The gene expression levels of genes coding antioxidative enzymes were examined by the reverse transcription polymerase chain reaction (RT-PCR) technique. *Arabidopsis* 18S rRNA was used as endogenous control.
Result: Osmotic stress changed the activities of antioxidant enzymes in the shoots and roots of pea seedlings. Varieties more tolerant to osmotic stress showed a significant increase in antioxidant activities in shoots and roots, while sensitive varieties showed a significant decrease. The results of the analysis of the expression of genes, coding for antioxidant enzymes, showed that the reaction of the tested cultivars to ROS was the result of increased expression of the tested genes in tolerant cultivars, *i.e.* decreased expression in sensitive cultivars.

Key words: Antioxidative enzymes, Drought, Field pea, Gene expression, Osmotic stress.

INTRODUCTION

During their growth, crop plants are exposed to different environmental stresses that can limit their growth and productivity (Fleury *et al.*, 2010). Drought is, besides salinity, one of the main abiotic stresses limiting the agricultural production of many important crops. Water stress can be defined as reduced water availability; either by lack of water (drought), osmotic stress (caused by high salt concentrations), or water saturation (too much water). Water stress may change the metabolic and growth patterns in the plant, reduce photosynthesis, respiration and ion uptake and in extreme cases result in plant death. Plants respond to drought stress by closing their stomata and reducing water losses through transpiration (Kausar *et al.*, 2023). When plants suddenly encounter water stress it is important to respond as quickly as possible. A faster drought response means less water loss and an increase in the survival rate of the plants (Seleiman *et al.*, 2021).

Drought and osmotic stress lead to oxidative stress in plants due to a breach in the process of electron transport in some cell organelles and the creation of a large quantity of reactive oxygen species (ROS). They are associated with several forms of cellular damage. Activated oxygen species such as superoxide (O_2·-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH) can seriously disturb normal plant metabolism through oxidative damage of the lipids, protein and nucleic acids. In order to neutralize the damaging effects of ROS plants have developed severe antioxidant defense mechanisms, non-enzymatic and enzymatic. Non-enzymatic

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antioxidants in plants include glutathione, carotenoids, tocopherols and flavonoids (Dumanoviæ *et al.*, 2021). Anti-oxidative enzymes of plants are superoxide dismutase (SOD), glutathione peroxidase (GPx), ascorbate peroxidase (APx), glutathione reductase (GR), glutathione S-transferase (GST), dehydroascorbate reductase (DHAR), peroxide reduction (PRx), mono-hydro ascorbate reductase (MDAR) and catalase (CAT) (Qamer *et al.*, 2021). Both types of antioxidants are crucial for ROS homeostasis (Mahmood *et al.*, 2020). There is a need to understand how plants interact with the environment, particularly in the face of global climate change in order to create more stress-resistant genotypes. Antioxidant

enzymes could be used for screening sensitive and tolerant plant genotypes against abiotic stresses.

The aim of this research was to determine the biochemical and molecular basis of drought stress tolerance of field pea plants, which would significantly improve comprehension of oxidative stress effects on those crops.

MATERIALS AND METHODS

Plant material

Seven genotypes of the genus *Pisum* were selected. Studies were carried out in the period 2019-2020., at the Institute of Field and Vegetable Crops, Novi Sad, Serbia. Seeds were sterilized with 0.5% sodium hypochlorite solution for 1 min and after that, they were washed with distilled water. Seeds of peas were germinated in 15×24 cm plastic boxes fulfilled with sand. For each variety, 4 replicates by 100 seeds for each treatment were sown.

Osmotic stress experiment

Osmotic stress was stimulated by an osmotic potential level of -0.1 MPa using PEG 6000. Solutions of PEG 6000 were prepared according to Michel and Kaufmann (1973).

Plastic boxes were placed in a germination chamber at $20°C$ under conditions of a 12 h light/dark cycle. The sand was moistened daily with distilled water for control and the solution of PEG for treatments. Shoots and roots were taken on the 10th day after sowing, immediately frozen in liquid N_2 and stored at -80°C for further analyses.

Enzyme assays

Frozen shoots were ground in liquid nitrogen using mortar and pestle and the powder was suspended in extraction buffer (50 mM potassium phosphate, pH 7.0 and 0.1 mM EDTA) containing 5% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at $15,000 \times g$ for 20 min and the supernatant was used for the assays. All steps were carried out at 4°C. Protein concentration in the extracts was determined according to Bradford (1976), using Bio-Rad assay kit with bovine serum albumin as standard.

Superoxide dismutase

(EC 1.15.1.1) activity was measured according to Beauchamp and Fridovich (1971). The crude extract (50 µL) was added to the reaction mixture (1.5 mL) containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 13 mM methionine, 2 µM riboflavin and 75 µM nitro blue tetrazolium (NBT). Riboflavin was added last and the tubes were shaken. The reaction was started by exposing the mixture to cool white fluorescent light. After 15 min the light was switched off, the tubes were mixed and the absorbance was measured at 560 nm. SOD activity was expressed as ΔA_{560} min^{-1} (mg of protein)$^{-1}$.

Ascorbate peroxidase

(EC 1.11.1.11) activity was measured as described by Nakano and Asada (1981) in a reaction mixture containing a 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate and 20 µL of crude extract. H_2O_2-dependent oxidation of ascorbate was monitored by reading absorbance decrease at 290 nm. APx activity is expressed as IU mg^{-1} protein.

Glutathione reductase

(EC 1.6.4.2) activity was measured according to Connell and Mullet (1986) in 0.1 M Tris HCl (pH 8.0) containing 0.1 mm EDTA and 0.5 mM GSSG in a final volume of 1 ml. Reactions were initiated by adding NADPH to a final concentration of 0.2 mm and the progress of the reaction was monitored by the decrease in absorbance of NADPH at 340 nm. One unit of enzyme is that amount of protein that will oxidize 1 µmol of NADPH min^{-1} at 250°C.

Gene expression analysis

RNeasy Plant Mini Kit (Qiagen, Germany) was used for RNA extraction, according to the manufacturer's manual. The quality and quantity of the extracted RNA was checked with a UV/VIS spectrophotometer (Evolution 100, Thermo Scientific, USA). Prior to the cDNA synthesis DNA was removed using Ambion 171 DNA-free DNase Treatment and Removal Reagents.

Synthesis of cDNA was done using a reverse transcription kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The cDNA was used as a template in the PCR reaction.

The PCR was carried out using premix of 2x PCR Master Mix, (Fermentas, Lithuania) containing 4 mM MgCl$_2$, 0.4 mM dNTP, 0.05 units/µl Taq DNA Polymerase (recombinant). PCR was performed in a final volume of 25 µl of PCR mix containing 0.2 pmol/µl primers and approx. 50 ng cDNA was used. The list of primers is given in Table 1. Arabidopsis 18S rRNA was used for reference gene (Rivera-Becerril *et al.*, 2005).

Amplifications were carried out in an Eppendorf Mastercycler Gradient (Eppendorf, Germany) under the following programs: denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 53-57°C for 30 sec and 72°C for 1 min and the ûnal extension was carried out at 72°C for 10 min.

The amplification fragments were determined using electrophoresis on 1% agarose gel containing ethidium bromide (0.5 g/mL). The expected size of the ampliûed fragments was estimated by comparison with FastRuler DNA Ladder, Low Range (Fermentas, Lithuania).

The agarose gel was visualized using a UV transilluminator and the images were captured with BioDoc Analyze system (Biometra, Germany).

Statistical analysis

Data given in percentages were subjected to arcsine transformation before statistical analysis. For all investigated parameters, analysis of variance was performed using the Statistical software (Sigmaplot 10.0., Systat Software Inc., San Jose, CA, U.S.A.). Significant differences among the mean values were compared by Student's t-test (p<0.05).

Figures were drawn using Sigmaplot. For the purpose of comparing the genotypes and treatments, two factorial analyses of variance (ANOVA) with a significance level of *P<0.05, **P<0.01 and ***P<0.001 was performed.

RESULTS AND DISCUSSION

Enzyme activities

Exposure to PEG solutions has been effectively used to mimic osmotic stress because PEG binds to water, but due to the size of its molecules, plants cannot absorb it. Practically, PEG causes a state of physiological drought, *i.e.* allows the plant to absorb water (Muscolo *et al.*, 2014). Osmotic stress leads to creation of reactive oxygen species (ROS) in plants. Plants have developed antioxidant defense mechanisms in order to neutralize the effects of ROS. The aim of this research was to determine the molecular basis of osmotic stress tolerance in 7 field pea (*Pisum sativum* L.) varieties at the early seedling stage. A previous study of the effects of osmotic stress on germination and seedling growth of different field pea varieties (Petrović *et al.*, 2021) showed that different concentrations of PEG in germination media significantly affect the seed germination of field pea. An increase in osmotic stress significantly decreased germination percentage (GP) in all the tested varieties. Increased mean germination time (MGT) and decreased total germination time occurred already at the lowest level of stress (-0.1 MPa). Based on those results osmotic stress in this study was stimulated by an osmotic potential level of -0.1 MPa. The activity of antioxidant enzymes was changed in all pea cultivars under stress compared to the control.

However, different enzymes were activated in the tested cultivars, which might indicate a different degree of tolerance.

The effect of osmotic stress on the activity of the enzyme superoxide dismutase

SOD constitutes the first line of defense against highly toxic superoxide radicals by rapidly converting superoxide to hydrogen peroxide (H_2O_2) and molecular oxygen (Konieczna, 2023).

The average values of SOD, in U/mg protein, in shoots and roots of field pea seedlings, in control and in plants under osmotic stress, are presented in Table 2. The results showed that there was an increase in SOD activity in most of the varieties. This increase was statistically significant in the varieties Mraz, Partner, Trezor and Pionir, while it was not statistically significant in the variety Dukat. In Junior and Javor varieties, there was a significant decrease in the average values of SOD activity in shoots after 10 days of treatment. When the effect of osmotic stress (-0.1 MPa PEG) on SOD activity in shoots is shown as a percentage relative to the control (100%) (Fig 1a), it can be noticed that the most prominent increase in the activity of SOD in relation to the control was in variety Mraz and amounted to 184.74% in relation to control, while maximum reduction of activity was in varieties Javor (69.06%) and Junior (58.71%).

In roots of the control group the Javor variety had a significantly higher SOD activity compared to the other varieties, while the activity of this enzyme in treated group was the lowest. Pionir showed the highest SOD activity under stress conditions. Those differences were highly significant. Dukat showed a slight increase in stressed seedlings, but

Table 1: Sequences of oligonucleotide primers.

Gene	Sequence (5′-3′)	Reference
18S rRNK	F: CCAGGTCCAGACATAGTAAG	Zdunek-Zastocka 2008
	R: GTACAAAGGGCAGGGACGTA	
Cu/Zn SOD	F: TTCACAAACTCTCGTCTCACC	Vanacker *et al.* 2006
	R: CACACCACAAGCTAATCTTCC	
GR	F: TCGCAGCACTCTCTTCTTCA	Vanacker *et al.* 2006
	R: CTCCATCCAAAACCATTGCT	
APX	F: TCCTTTCGGAACAATTAAGC	Vanacker *et al.* 2006
	R: TCCTTCTCACCAGTCAACAA	

Table 2: Superoxide dismutase activity (SOD) (U/mg prot) in shoots and roots of field pea varieties.

Varieties	SOD (U/mg prot)			
	Shoot		Root	
	Control	-0.1MPa	Control	-0.1MPa
Mraz	5.90±0.2254	10.90**±1.6879	6.95±0.2254	9.25***±0.5665
Junior	10.90±1.2832	6.40***±0.6705	9.95±0.2254	11.80***±0.3121
Javor	13.90±1.3468	9.60***±0.3642	11.85±0.6590	8.10***±0.9075
Dukat	6.40±1.1561	8.15^{ns}±0.7746	7.20±0.5954	8.40^{ns}±0.8670
Pionir	8.50±0.2832	10.80*±1.1445	7.15±1.2023	12.10**±1.9942
Partner	7.60±0.7514	12.30**±1.9364	6.50±0.4220	9.20***±0.3468
Trezor	7.80±0.8902	11.50***±0.6936	6.65±0.7168	10.80***±0.3757

* - P<0,05, ** - P<0,01, *** - P<0,001, ^{ns} - Not significant.

that increase was not significant. Changes in SOD activity in other varieties were significant. The percentage of SOD activity in roots relative to control (Fig 1b) indicates that Mraz showed the highest increase (133.09%), while Javor showed the highest decrease (68.35%).

SOD activity in both shoots and roots only at the Dukat variety showed an insignificant increase. It is noticed that Javor showed a significant decrease, while Mraz showed a significant increase in shoots and also in roots.

The effect of osmotic stress on the activity of the enzyme ascorbate peroxidase

Ascorbate peroxidase is a hydrogen peroxide binding enzyme that is specific to plants and algae and is crucial for protecting chloroplasts and other cellular components from damage by hydrogen peroxide and the hydroxyl radicals produced from it (Mishra *et al.*, 2023; Murtaza *et al.*, 2010). The average values of APx, in nmol/min/mg protein, in shoots and roots of field pea seedlings, in control and in plants under osmotic stress, are presented in Table 3. The results showed that there was a statistically significant increase in APx activity in most of the varieties. It is especially noticeable in the cultivar Mraz.Junior and Javor varieties, showed a significant decrease in the average values   of APx activity in shoots after 10 days of treatment.

There were similar results in roots of the treated group. Variety Mraz showed the highest increase, while the Javor variety had a significantly higher decrease in APx activity, compared to the other varieties.

Effect of osmotic stress (-0.1 MPa PEG) on APx activity in shoots and roots is shown as a percentage relative to the control (100%) (Fig 2a, Fig 2b). The percentage of APx activity relative to the control indicates that Mraz showed the highest increase in shoots (821.88%) and roots (479.09%), while Javor showed the highest decrease in shoots (74.37%) and roots (15.66%), compared to the control.

The effect of osmotic stress on the activity of the enzyme glutathione reductase

In plants, GR is the GSH-regenerating enzyme of the ascorbate-glutathione cycle, which removes hydrogen peroxide (H_2O_2) (Mishra *et al.*, 2023).

The average values of GR, in nmol/min/mg protein, in shoots and roots of field pea seedlings, in control and in plants under osmotic stress, are presented in Table 4. Osmotic stress in the variety Mraz led to an increase in GR activity in both organs, while decreased GR activity was observed in the variety Javor.

The effect of osmotic stress (-0.1 MPa PEG) on GR activity in shoots and roots is also shown as a percentage relative to the control (100%) (Fig 3a, Fig 3b). Again, variety Mraz showed a significantly higher increase and Javor a significantly higher decrease in shoots and roots of the treated group.

Table 3: Ascorbate peroxidase activity (APx) (nmol/min/mg prot) in shoots and roots of field pea varieties.

Varieties	APx (nmol/min/mg prot)			
	Shoot		Root	
	Control	-0.1 MPa	Control	-0.1 MPa
Mraz	32.00±0.8960	263.00**±4.6243	39.20±1.5029	187.80***±0.751
Junior	231.70±6.3006	28.70**±0.4624	75.00±0.6358	91.80***±2.0809
Javor	159.60±7.4855	118.69**±0.1850	182.00±0.8092	28.50***±0.6358
Dukat	37.49±0.9306	50.00***±1.4451	13.16±1.1792	39.20***±1.5029
Pionir	36.00±1.1676	74.70**±0.2312	48.60±0.8266	35.80***±1.0000
Partner	27.54±0.6359	61.79***±0.9711	35.70±1.3410	31.50*±1.5029
Trezor	27.60±0.7688	42.9**±0.7167	79.80±0.8902	154.30***±1.584

* - P<0,05, ** - P<0,01, *** - P<0,001, ns - Not significant.
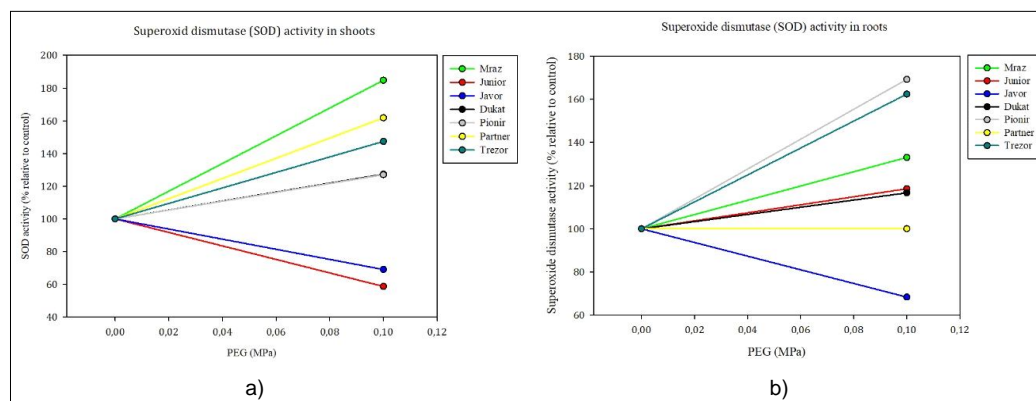
Fig 1: Superoxide dismutase activity in shoots and roots of field pea varieties (% relative to the control).

Osmotic stress changed the activities of SOD, APx and GR in the shoots and roots of pea seedlings (Fig 1, Fig 2, Fig 3). Cultivars more tolerant to osmotic stress showed a significant increase in antioxidant activities, while sensitive cultivars showed a significant decrease (Table 2, Table 3, Table 4). Thus, the mechanism of eliminating ROS in shoots and roots of the tolerant cultivar Mraz is based on the constantly increased activity of all three enzymes. The activity of enzymes was decreased in cultivar Junior, more sensitive to drought stress, as well as in the cultivar Javor in which sensitivity to drought stress is particularly strong.

It is well known that the antioxidant system controls the level of ROS and eliminates their detrimental effects. In this system, antioxidant enzymes, like SOD, POX, CAT and APx, are the most active and efficient protective mechanisms against oxidative stress (Sutulienë *et al.*, 2023; Javadian *et al.* 2010). In the long-term drought stress, SOD, APx and CAT activity in pea leaves increased markedly (Karatas *et al.* 2014). Rodríguez-Serrano *et al.* (2006) obtained that the analysis of the enzymatic activity of antioxidants in pea roots exposed to Cd showed a significant reduction of GR and GPx and, to a lower extent, of CAT, while total SOD activity showed a slight increase by the metal treatment.
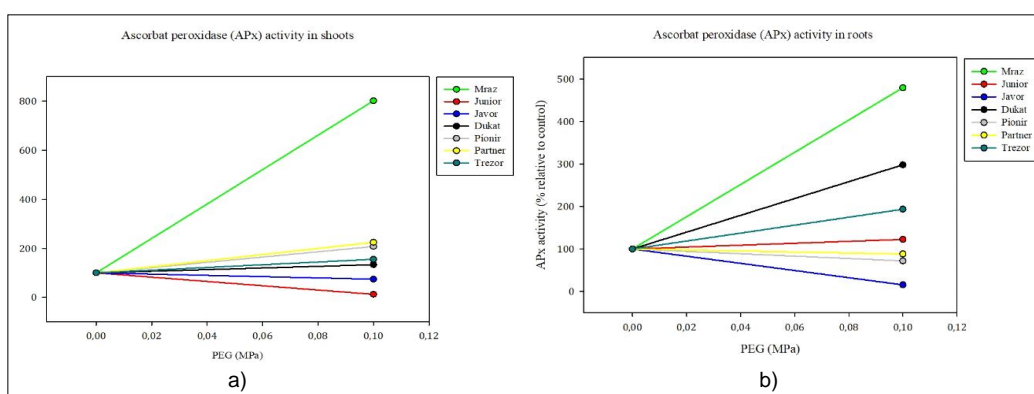

Fig 2: Ascorbate peroxidase activity in shoots and roots of field pea varieties (% relative to the control).
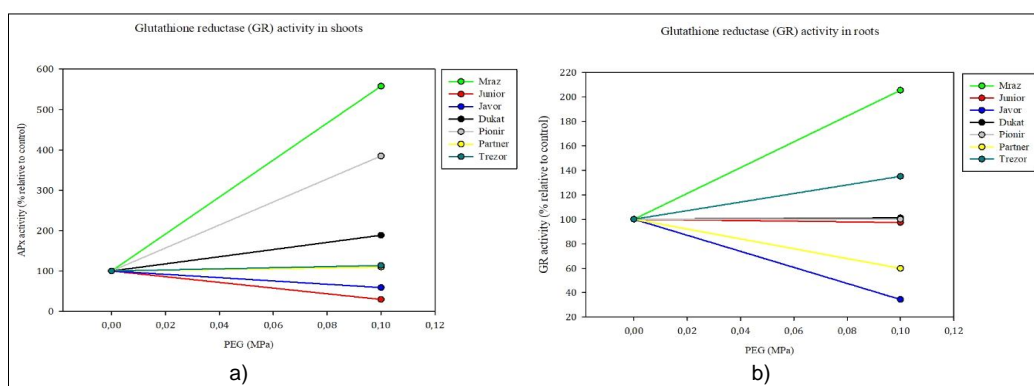

Fig 3: Glutathione reductase activity in shoots and roots of field pea varieties (% relative to the control).

Table 4: Glutathione reductase activity (GR) (nmol/min/mg prot) in shoots and roots of field pea varieties.

Varieties	GR (nmol/min/mg prot)			
	Shoot		Root	
	Control	-0.1MPa	Control	-0.1MPa
Mraz	14.00±0.5144	78.10***±1.0925	45.60±1.5087	93.75***±1.2023
Junior	43.74±2.0173	12.90**±1.9133	23.20±1.4509	44.40***±0.9017
Javor	50.16±1.5491	29.60***±1.0116	94.2±0.8266	32.40**±0.9075
Dukat	22.70±1.7457	42.80***±1.4393	17.40±0.1040	17.60^{ns}±0.3642
Pionir	12.00±0.7746	46.20***±1.1792	17.30±0.9711	18.07^{ns}±1.7284
Partner	20.30±1.9884	22.25^{ns}±1.5607	62.00±0.8439	37.10***±1.4046
Trezor	29.70±1.7572	33.80**±0.5434	49.00±1.6416	66.25***±0.7746

* - P<0,05, ** - P<0,01, *** - P<0,001, ^{ns} - Not significant.

Gene expression analysis in osmotic stress experiment

As part of molecular research on the influence of osmotic stress, the expression of genes encoding 3 antioxidant enzymes (*Cu/Zn SOD*, *cAPx* and *GR*) was monitored. The results are shown for selected most tolerant and most sensitive varieties (Fig 4, Fig 5, Fig 6).

Changes in the expression levels of the *Cu/Zn SOD* transcript in the shoots and roots of seedlings in selected pea varieties, under the influence of osmotic stress, are presented in Fig 4.

The highest increase in SOD activity compared to the control in the, under the action of osmotic stress, measured in the Mraz variety (184.74% compared to the control) (Fig 1a), probably occurred as a result of enhanced gene expression for SOD, primarily *Cu/Zn SOD* (Fig 4a). In the variety Javor, there was a decrease in gene expression (Fig 1), which resulted in a decrease in SOD activity in the shoots (69.06% compared to the control) (Fig 4a).

On the 10th day of treatment, osmotic stress led to an increased expression of the gene for *Cu/Zn SOD* in the root of the Mraz variety (Fig 4b), in which the highest SOD activity was observed compared to the control (Fig 1b). A decrease in enzyme activity, which was 68.35% of the control values, occurred in the Javor variety (Fig 1b) and a reduced expression of the *Cu/Zn SOD* gene was also observed (Fig 4b).

The study of the expression of enzymatic antioxidants showed an osmotic stress-induced down-regulation of APx and GR in shoots and roots of the sensitive variety Javor, which was parallel to the decrease in enzyme activity described earlier (74.37% and 59.01% respectively). On the contrary, an up-regulation of APx and GR was observed (Fig 5, Fig 6), along with an increase in the activity of encoding enzymes in the tolerant variety Mraz (Fig 2, Fig 3).

The molecular analysis can explain changes in antioxidant enzyme activity. The results show that antioxidative enzymes was up-regulated in tolerant varieties (Trezor, Pionir,
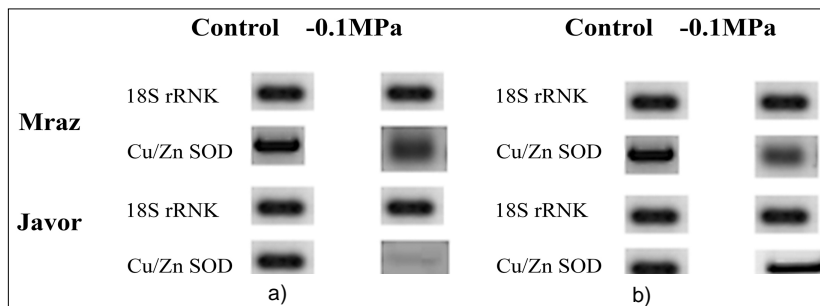
Fig 4: The influence of osmotic stress on the expression of the Cu/Zn SOD gene in the shoots (a) and roots (b) of field pea varieties.
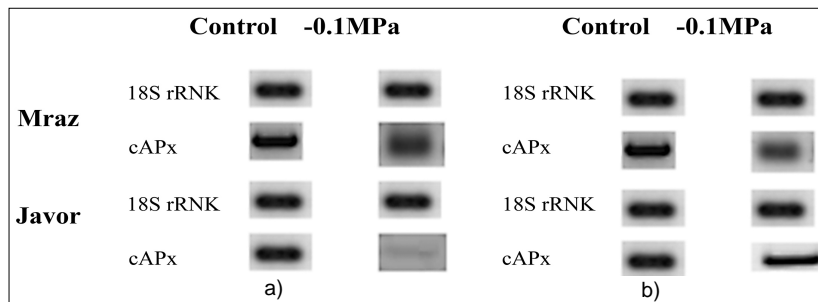
Fig 5: The influence of osmotic stress on the expression of the cAPx gene in the shoots (a) and roots (b) of field pea varieties.
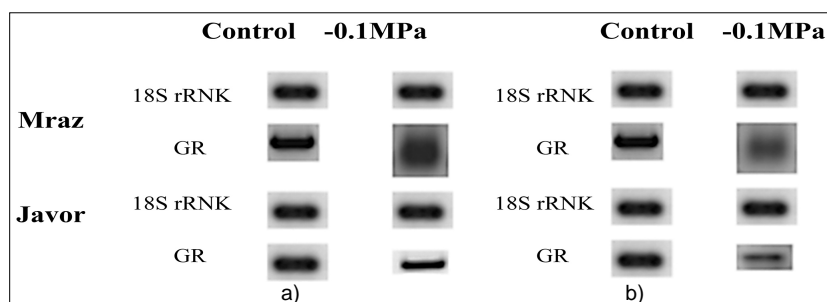
Fig 6: The influence of osmotic stress on the expression of the GR gene in the shoots (a) and roots (b) of field pea varieties.

Mraz) increases the activity of antioxidant enzymes, in both shoot and root, i.e. down-regulated in more sensitive varieties, which proved to be cultivars Junior and Javor.

The results of the analysis of gene expression coding for antioxidant enzymes (*Cu/ZnSOD*, *cAPx*, *GR*) showed that the reaction of the tested varieties to ROS, created under drought stress, was the result of increased expression of the tested genes in tolerant varieties, *i.e.* decreased expression in sensitive varieties.

Earlier studies on heavy metal stress in pea roots showed a significant reduction of GR and GPx and, to a lower extent, of CAT, while total SOD activity showed a slight increase by the metal treatment (Rodríguez-Serrano *et al.*, 2006). Karatas *et al.* (2014) find that drought elevated the activities of total SOD, APx, CAT and POx in the leaves of a pea. The activities of ROS scavenging enzymes (SOD, CAT, POx) gradually increased with the increase in the severity of drought. However, the activity of APx slightly increased in drought-stressed plants compared to control plants. Comparing our results with the results of other researchers (Rodríguez-Serrano *et al.*, 2006, Karatas *et al.*, 2014), it can be concluded that differences in enzyme activity and gene expression are due to different mechanisms involved in the elimination of oxidative stress of different origins. Also, researches were carried out in different stages of growth and development of the plant (seedling, stage of three pairs of leaves).

CONCLUSION

Filed pea is drought sensitive like many other leguminous crops. The activity of antioxidant enzymes was changed in all field pea varieties under stress compared to the control. However, different enzymes were activated in the tested varieties, which might indicate a different degree of tolerance. It turned out that Javor is the most sensitive and Mraz the most tolerant variety to osmotic stress. The results of the analysis of gene expression, coding for antioxidant enzymes, showed that the reaction of the tested varieties to ROS was the result of increased expression of the tested genes in tolerant cultivars, *i.e.* decreased expression in sensitive cultivars. The obtained results show that biochemical and molecular analyses for antioxidant enzymes can be useful for testing tolerance to drought stress in field pea genotypes. Further studies and determinations about antioxidant defense mechanisms might lead to the development of drought-tolerant varieties and cultivation of this crop on dry soils.

ACKNOWLEDGEMENT

Conflict of interest: None.

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