

**Differential response of three contrasting pea (*Pisum arvense*, *P. sativum* and *P. fulvum*) species to salt stress: assessment of variation in antioxidative defence and miRNA expression****Jovanka Miljuš-Djukić<sup>1\*</sup>, Nemanja Stanisavljević<sup>1</sup>, Svetlana Radović<sup>2</sup>, Živko Jovanović<sup>1</sup>, Aleksandar Mikić<sup>3</sup> and Vesna Maksimović<sup>1</sup>**<sup>1</sup>University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, 11000 Belgrade, Serbia<sup>2</sup>University of Belgrade, Faculty of Biology, Studentski Tgr 16, 11000 Belgrade, Serbia<sup>3</sup>Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia\*Corresponding author: [jovanka@imgge.bg.ac.rs](mailto:jovanka@imgge.bg.ac.rs)**Abstract**

Soil salinity is one of the major abiotic stresses leading to crops yield failure. For investigation of salinity response in pea, *in vitro* cultures of three pea species (*Pisum arvense*, *P. sativum* and *P. fulvum*) were induced. Regenerated shoots of these pea species were grown on a medium with moderate and high NaCl concentrations (75, 120, 150 and 200 mM NaCl) to compare the antioxidative capacity in response to salt stress. Growth reduction was observed in all species and it correlated with the increase of NaCl concentration. After four weeks of treatment, the crude tissue extracts were used for measuring the stress response parameters. Level of lipid peroxidation increased in all three species, but in *P. fulvum* the effect of NaCl was less pronounced. Total chlorophyll and phenolic content showed differences among the three species, while the DPPH-scavenging activity was the most evident in *P. fulvum*. The northern blot analysis of the miRNA398 expression showed a similar pattern between *P. sativum* and *P. arvense* but different to that of *P. fulvum*, as in *P. fulvum* miRNA 398 was only expressed at moderate NaCl concentration, with total inhibition at high concentrations. On the basis of these results it can be concluded that *P. fulvum* could represent a gene pool for improving *P. sativum* and *P. arvense* stress defence capacity.

**Keywords:** pea, NaCl, salt stress, FC assay, MDA, DPPH assay, miRNA398a.**Abbreviations:** BAP\_6-benzylaminopurine; DPPH\_2,2-dyphenyl-1-picrylhydrazyl radical; FC\_Folin-Ciocalteu reagent; IAA\_indole-3-acetic acid; miRNA398a\_micro RNA 398a; MDA\_malondialdehyde; NAA\_α-naphthaleneacetic acid; ROS\_reactive oxygen species.**Introduction**

Salinity is considered to be one of the major abiotic factors limiting plant productivity. Plant physiological processes, like seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set, are adversely affected by high salt concentrations, ultimately causing diminished economic yield and also quality of products. According to the FAO Land and Nutrition Management Service (2008), over 6% of the world's land is affected by either salinity or sodicity which accounts for more than 800 million ha of land. Direct consequence of agricultural land salinization at extensive scale is massive economic loss at the global level (Yadav et al., 2011). It is assumed that salt stress would cause an imbalance of the cellular ions resulting in ion toxicity and osmotic stress, thus affecting plant growth, morphology, and survival (Khan, 2010). Metabolic imbalances caused by ionic toxicity, osmotic stress, and nutritional deficiency under salinity may lead to oxidative stress (Zhu, 2002), and to increased production of reactive oxygen species (ROS) for example, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (OH•), and superoxide anion (Simaei, 2011). Salinity reduces photosynthetic activity by destruction of green pigments, lowering leaf area or by decreasing the activity of photosynthetic enzymes in calvin cycle (Misra et al., 1997). It has been shown that salt stress causes membrane damage and lipid peroxidation leading to malondialdehyde (MDA)

accumulation. MDA concentration in plant tissue is being extensively used as efficient criterion to discriminate crop cultivars in respect to salt tolerance (Sairam et al., 2005). Plants have developed two different mechanisms to cope with oxidative stress: enzymatic and nonenzymatic antioxidative system including ascorbic acid, glutathione and thiols (Michalak, 2006; Agarwal and Pandey, 2004). Total antioxidative capacity of low molecular weight antioxidants can be used as summary parameter to detect changes on antioxidative metabolism during oxidative stress caused by various abiotic factors including salt stress (Saleh and Plieth, 2009). DPPH assay can be efficiently used for screening antioxidant capacity of complex samples (Magalhaes et al., 2008). Currently, there are a lot of data suggesting a strong correlation among the antioxidative capacity and NaCl tolerance (Agarwal and Pandey, 2004). Leaf phenolics are also important protective components of plant cells. They have the potential to act as hydrogen donors, reducing agents and quenchers of singlet oxygen (Rice-Evans et al., 1997). FC (Folin-Ciocalteu) reducing capacity of preferentially phenolic compounds can be applied by means of determination of antioxidative activity of plant samples as proposed in Magalhaes et al. (2008). Salinity tolerance may be defined as the ability of a plant to grow and complete its life cycle under stressful salt conditions like NaCl or with

association of other salts (Yadav et al., 2011). The understanding of the molecular basis of salt stress signalling and tolerance mechanism is essential for breeding and engineering salt stress tolerance in crop plants (Chinnusamy et al., 2005). The tissue culture is an efficient method for plant material multiplication which can be further used for different purposes, such as genetic transformation, acclimatization and seed production, and cryopreservation. In vitro culture can also be used for studying the effects of different abiotic stress factors, like drought (Wang et al., 2003) and heavy metals (Stanisavljevic et al., 2012). Also, there are a lot of data on using in vitro technique for salt stress studies in different plant species. For example, in vitro selection was used for selection of salt tolerant cell lines of *Solanum tuberosum* L. (Queiros et al., 2007). Singh et al. (2000) reported that in vitro screening procedure can be used for checking the grape genotypes for salinity tolerance. They also reported reduction in chlorophyll content and phenolic production. Recently, pea callus line (*Pisum sativum* L.) resistant to salinity has been reported (El Sayed and El Sayed, 2011). Authors reported that resistant line was able to grow on 350 mM NaCl in a medium. There was a similar report for in vitro propagated shoots of *Eucalyptus camaldulensis* (Woodward and Bennett, 2005). MicroRNAs (miRNAs) were recognized as important regulators of gene expression at posttranscriptional level, and thus involved in plant stress response (Zhu et al., 2011; Sunkar, 2010; Bouche, 2010). Among stress-responsive miRNAs, miRNA398a is proposed to be directly linked to the plant stress regulatory network and regulates plant responses to oxidative stress, water deficit, salt stress, ultraviolet stress, copper and phosphate deficiency, high sucrose and bacterial infection (Zhu et al., 2011). Predicted targets for mi398 are at least four mRNAs, among them the subunit of the mitochondrial cytochrome c oxidase and the cytosolic CSD1 (copper/zinc superoxide dismutase 1), which is one of the most important enzymes implicated in superoxide anion radical scavenging, and an important factor of ROS scavenging network in plants (Bouche, 2010). Pea is one of the most important legume crops in the world, used both for food and feed. Since legume plants, including beans, soybeans, peanuts and peas are an inexpensive and nutritionally important source of plant proteins, they are cultivated all around the world. However, legume seed yields and protein contents are very variable, mostly because of their high sensitivity to frequent environmental stresses (Bourin et al., 2009). The aim of this study was to compare antioxidant capacities of widely used pea species (*P. sativum* and *P. arvense*), also widely used in the area of Balkans, with the wild pea species of *P. fulvum*. There are no data on salt stress response in *P. fulvum*, which could be used as gene pool to improve the protein pea values. In addition, we decided to analyse the miRNA398a expression during salt stress in species which showed differences in stress response, having in mind there are no data on its expression in peas.

## Results

### Induction of in vitro culture and salt stress treatments

We established in vitro pea culture with the aim to perform salt stress experiments. Regenerated shoots micropropagated on an SM medium (Table 1) were transferred to SM supplemented with NaCl in following concentrations: 75, 120, 150 and 200 mM. After four weeks, shoots were collected and the salt response was measured in shoot tissue

crude extracts. Increasing NaCl concentrations in a medium induced the growth retardation of all three species (Fig. 1).

### Level of lipid peroxidation

Level of lipid peroxidation in shoots was determined by measuring the amount of malondialdehyde. The MDA was produced by the thiobarbituric acid reaction, and results were expressed in nmol per g of dry weight. Salt stress increased the level of lipid peroxidation in treated shoots, but the three pea genotypes responded differently. In *P. arvense* shoots, MDA increased dramatically at 120 mM NaCl (2.7 times higher), and then declined with further NaCl concentration increase. In *Pisum sativum*, the maximum of MDA production was also reached at 120 mM NaCl but it was not that high as in *P. arvense* shoots, and it was also followed by a decline in MDA content. In both genotypes, at 150 and 200 mM, the MDA production decreased even to a lower level than in control plants, suggesting tissue necrosis. In *Pisum fulvum*, the effect of NaCl concentration on MDA production was less pronounced at all levels of NaCl concentrations. 75mM NaCl caused only 31% increase in MDA concentration (not significant). At higher concentrations MDA content kept increasing following a moderate trend. Not even in 200mM NaCl was the decline in MDA caused by tissue necrosis observed (Fig. 2).

### Total chlorophyll content

One of the stress symptoms in salt treated tissue is a decrease in chlorophyll content. On the basis of these results, it is evident that chlorophyll contents decreased with an increasing salinity level and maximum inhibiting effect was recorded at high salt stress (200mM). In all of the three pea genotypes the increase of NaCl in a medium was accompanied with a gradual decrease in total chlorophyll content. However, in *Pisum fulvum* and *P. sativum*, the total chlorophyll content decreased at a lower rate compared to *P. fulvum* (Table 2, Fig. 3).

### Total phenolic content

In *P. arvense*, the phenolic content increased slightly compared to control till it reached 120 mM NaCl, where it was about 25% higher than in control (Fig. 4). At higher concentrations the phenolic content decreased. In *P. sativum*, the phenolic content at 75 mM NaCl was at its maximum (about 40% higher compared to control), and further decreased with the NaCl increase. In *P. fulvum*, the phenolic contents in control and at 75 mM NaCl were almost the same, and they decreased at higher rate during further increase of NaCl concentration (Fig. 4).

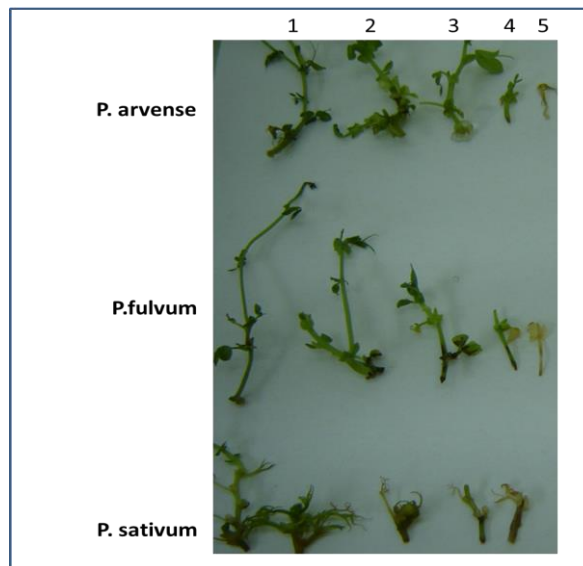
### DPPH-scavenging activity of low molecular antioxidants

In *P. arvense* extracts, we observed statistically significant decline of DPPH scavenging activity caused by NaCl concentrations of 75, 150 and 200 mM. In *P. sativum* 75 mM NaCl provoked slight increase of DPPH scavenging activity. At higher concentrations of NaCl, the scavenging activity decreased compared to control, but it did not seem to be in a concentration dependent manner. The maximum of DPPH-scavenging activity in *P. fulvum* was observed in control extracts, and it slightly decreased at 75, 120 mM and 200 mM NaCl, while the decrease at 150 mM was not significantly different compared to control (Fig. 5).

**Table 1.** Media used for in vitro culture induction, shoot induction, multiplication and salt-treatment experiments of *P. sativum*, *P. arvense* and *P. fulvum* induction.

Species	Germination medium (GM)	Shoot induction medium (SI)	Shoot multiplication medium (SM)	Salt treatment (mM NaCl)
<i>Pisum arvense</i>	½ MS and vitamins	MS +BAP 20 µM, NAA 0.1 µM	MS + BAP 10 µM, IAA 1 µM	SM + 75, 120, 150, 200 mM NaCl, respectively
<i>Pisum sativum</i>	½ MS and vitamins	MS +BAP 20 µM, NAA 0.1 µM	MS + BAP 10 µM, IAA 1 µM	SM + 75, 120, 150, 200 mM NaCl, respectively
<i>Pisum fulvum</i>	½ MS and vitamins	MS +BAP 20 µM, NAA 0.1 µM	MS + BAP 10 µM, IAA 1 µM	SM + 75, 120, 150, 200 mM NaCl, respectively

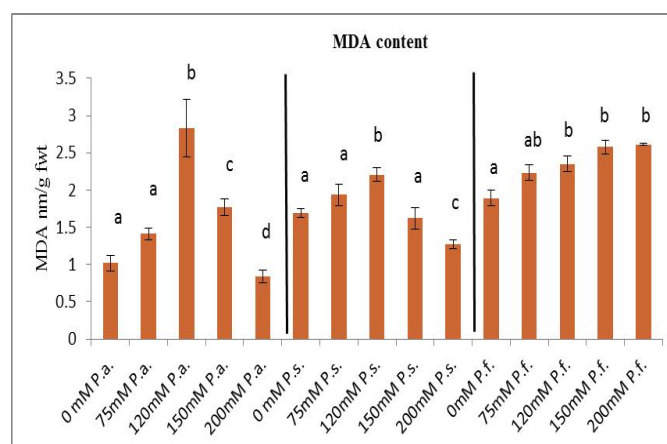
MS- Murashige and Skoog basal mineral medium. GM- germination medium, SI- shoot induction medium, SM- shoot multiplication medium.



**Fig 1.** Micropropagated shoots of *P. arvense*, *P. fulvum* and *P. sativum* treated with different NaCl concentrations. Lanes 1 - controls, 2 - 75 mM NaCl, 3 - 120 mM NaCl, 4 - 150 mM NaCl, 5 - 200 mM NaCl.

**Table 2.** Comparison of chlorophyll slopes in chlorophyll decrease for the three investigated species of pea.

Genotype	Slope of chlorophyll decrease	Statistical significance
<i>Pisum arvense</i>	- 0.397	a
<i>Pisum sativum</i>	- 0.267	bc



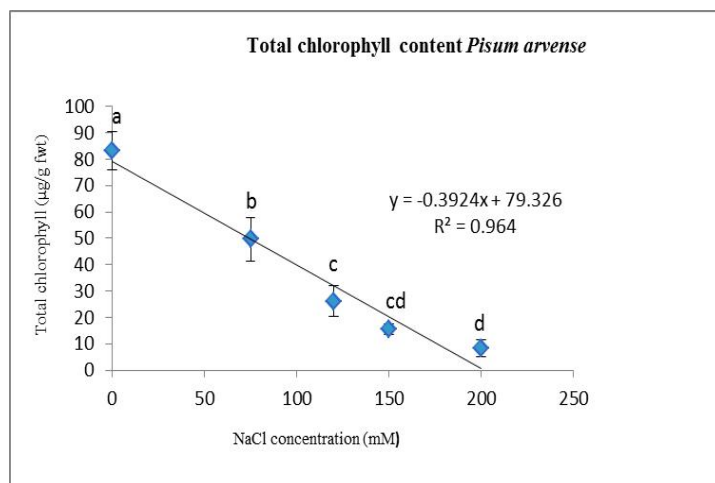
**Fig 2.** Salinity stress induced changes in MDA content. P. a. – *Pisum arvense* (0 mM P.a., 75 mM P.a, 120 mM P.a, 150 mM P.a, 200 mM P.a); P. s.- *Pisum sativum* (0 mM P.s., 75 mM P.s, 120 mM P.s, 150 mM P.s, 200 mM P.s); P.f.- *Pisum fulvum* (0 mM P.f., 75 mM P.f, 120 mM P.f, 150 mM P.f, 200 mM P.f). MDA content is expressed as nmol per g of fresh weight (nmol g<sup>-1</sup>fw). Values are means of the three replications (N=3±SD). Values marked by the same small case letter are not significantly different at p<0.005 (Bonferroni correction).

### miRNA398a expression

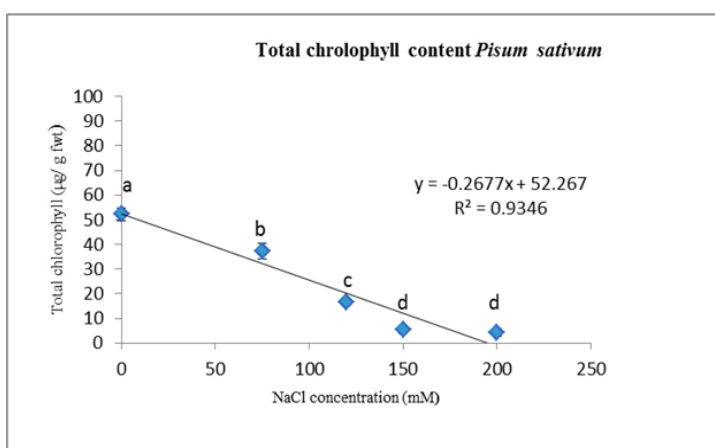
Northern blot analysis was performed with all three species, and results are shown in Fig. 6. and Fig 7. In *P. sativum*, the maximum expression was observed in control sample, while concentration of 75 mM NaCl led to almost complete inhibition of miRNA398a expression. Contrary to this, with the further NaCl increase, miRNA398a expression was reconstructed again. In *P. arvense*, a similar pattern of expression was observed, with the maximum in control, and a very weak signal at 75 mM NaCl, while the expression was restored again at higher NaCl concentrations, but with much weaker intensity than in *P. sativum*. In *P. fulvum*, the maximum expression was observed in control sample, while among NaCl treated samples, miRNA398a was only expressed at a moderate NaCl concentration, with total inhibition at higher concentrations.

### Discussion

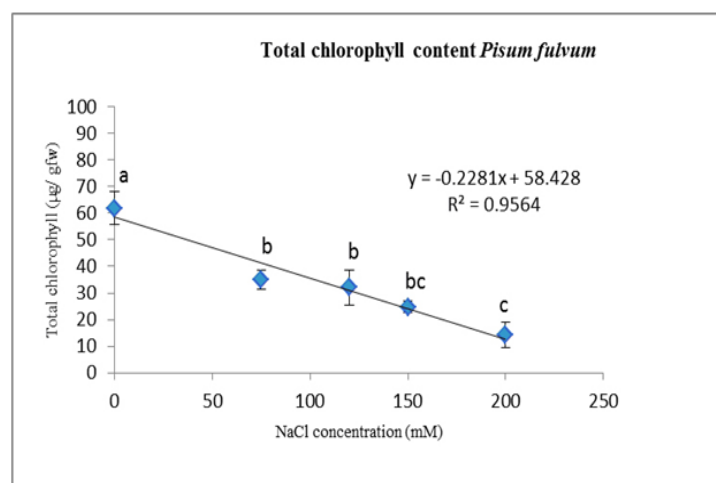
We used in vitro method for pea shoots propagation and salt treatments, as is seemed suitable for this type of experiment and a possible selection of salt tolerant lines. Developing salt-tolerant crops is essential for sustaining food production (Chinnusamy et al., 2005). With the aim of discovering exactly what is being selected through in vitro culture, it would be interesting to ascertain the salt response changes induced by in vitro culture in the long term (Rus et al., 2000). The chlorophyll content gradually decreased in all of the three pea genotypes with the increase of NaCl in a medium, but in *P. fulvum* and *P. sativum*, the total chlorophyll content decreased at a lower rate compared to *P. arvense*, and can be classified as less susceptible to NaCl induced chlorophyll degradation. Yet, comparing the slopes of chlorophyll decline in Table 2 and Fig. 3, the observed decrease of chlorophyll had the lowest rate in *Pisum fulvum*, leading to conclusion that *Pisum fulvum* has a higher capacity to tolerate salt stress, compared to other two genotypes. Other reports examining the effects of salts on tissues in vitro suggest that chlorophyll is an appropriate measure of the plant stress status (Singh et al., 2000; Santos et al., 2001). Salt stress is known to result in extensive lipid peroxidation (Hernandez et al., 2001). The level of lipid peroxidation can be considered an indicator of oxidative status of the plant. An enhanced level of MDA indicates oxidative damage of plants tissues. There are recent studies showing that salt stress and level of lipid peroxidation are in correlation (Davenport, 2003). Authors describe the changes in the leaf apoplast as response to NaCl high concentrations in two pea cultivars, suggesting there are different degrees of sensitivity. Ahmad (Ahmad et al., 2008) reported that the level of lipid peroxidation was higher in salt sensitive cultivar of *Pisum sativum* compared to the tolerant one. In other species like wild chicory, (Sergio et al., 2012) a decrease in MDA was observed, while in salt tolerant wild tomato, the MDA level decreased compared to cultivated tomato (Mittova et al., 2004). Our results showed that values of malondialdehyde content differ among the three pea genotypes. Salt stress significantly enhanced lipid peroxidation in *P. arvense* and *P. sativum*, but at higher concentrations MDA content surprisingly declined even to lower level than in control plants which could be attributed to tissue necrosis. That is in agreement with results of Qui (Queiros et al., 2007) who reported in vitro selection of salt tolerant potato callus line. In *P. fulvum*, level of MDA increased in all genotypes, but elevation compared to control was not so distinct, as it was in other two genotypes, although NaCl concentration dependent trendline in increase was



A

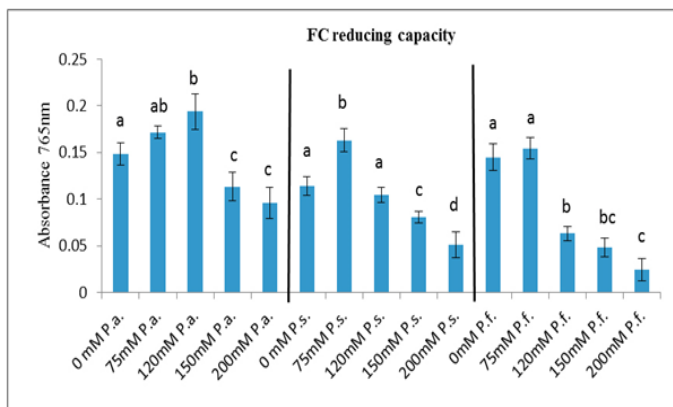


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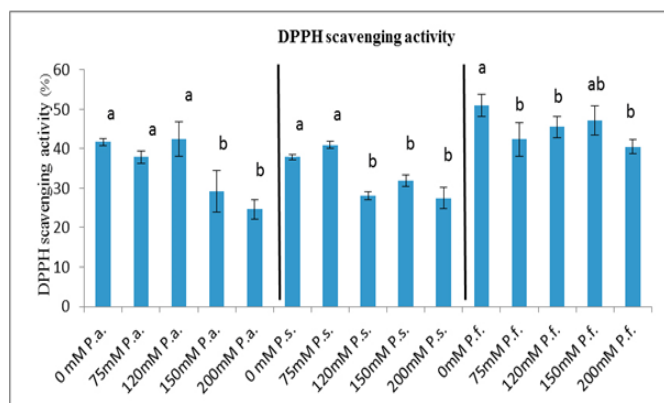


C

**Fig 3.** Total chlorophyll content in pea shoots of *P. arvense* (A), *P. sativum* (B), and *P. fulvum* (C). Pigment contents are expressed as µg per g of fresh weight (µg pigment g<sup>-1</sup> fw). Values are means of the three replications (N=3±SD). Values marked by the same small case letter are not significantly different at p<0.005 (Bonferroni correction).



**Fig 4.** Effects of different concentrations of NaCl (0, 75 mm, 120 mm, 150 mM, 200 mM) in the medium on FC-reducing activity capacity in tissue extracts of *P. arvense* (0 mM P.a, 75 mM P.a, 120 mM P.a, 150 mM P.a, 200 mM p.a); *P. sativum* (0 mM P.s, 75 mM P.s, 120 mM P.s, 150 mM P.s, 200 mM P.s) and *P. fulvum* (0 mM P.f, 75 mM P.f, 120 mM P.f, 150 mM P.f, 200 mM P.f). The values are expressed as percentage relative to the control. Values are means of the three replications (N=3±SD). Values marked by the same small case letter are not significantly different at p<0.005 (Bonferroni correction).

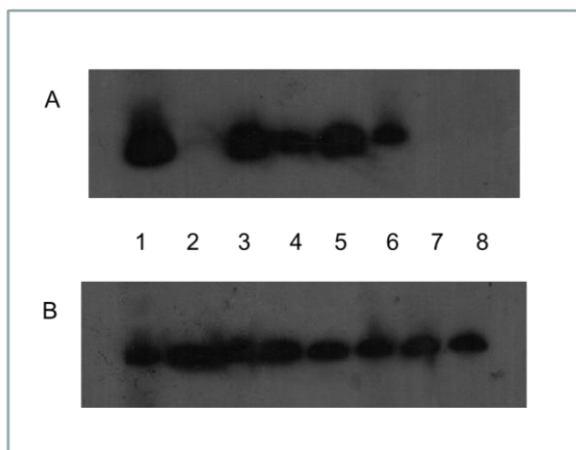


**Fig 5.** Effects of different concentrations of NaCl in the medium on DPPH-scavenging activity in tissue extracts of *P. arvense* (0 mM P.a, 75 mM P.a, 120 mM P.a, 150 mM P.a, 200 mM P.a), *P. sativum* (0 mM P.s, 75 mM P.s, 120 mM P.s, 150 mM P.s, 200 mM P.s), and *P. fulvum* (0 mM P.f, 75 mM P.f, 120 mM P.f, 150 mM P.f, 200 mM P.f). The values are expressed as percentage relative to the control. Values are means of the three replications (N=3±SD). Values marked by the same small case letter are not significantly different at p<0.005 (Bonferroni correction).

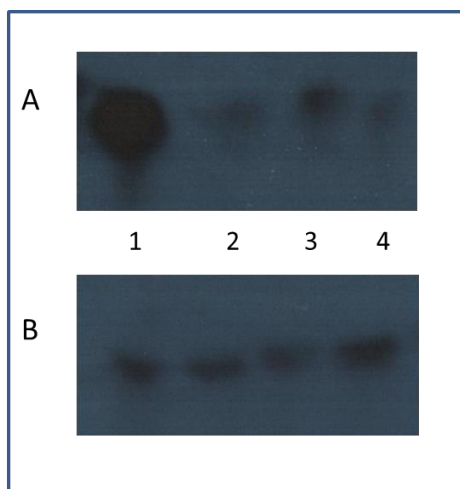
observed. The most prominent feature was the absence of MDA decline at high NaCl concentrations. Considering obtained MDA content in *P. fulvum* shoots, it can be assumed as less sensitive/more tolerant to NaCl in a medium, compared to *P. arvense* and *P. sativum*. The synthesis of phenolics is generally affected in response to different abiotic stresses including salt stress (Ashraf et al., 2010). It has been shown that salt stress induced disturbances in the secondary metabolic pathways lead to an increase in phenolic compounds. Moreover, total phenolic contents are reported to be directly associated with antioxidant activity. However, in

Lopez-Berenguer (2009) results showed a decrease in the phenolic compounds under salinity in leaves at a higher rate when salinity levels increased. In study of Noreen and Ashraf (2009) results showed that response of different pea cultivars in terms of antioxidant defence system, including phenolics, was cultivar specific. At moderate NaCl concentrations the increase of phenolics content was recorded in all genotypes. According to our results, all of the three pea genotypes showed a decrease in phenolics content at higher NaCl concentrations, but it was the most pronounced in *P. fulvum*. It is known from the literature that at high salt concentrations the uptake of phosphorus and potassium, which are principal substances of secondary metabolism such as polyphenols, decline (Waring and Pitman, 1985). Also, due to disturbances of enzymatic activities in high salinity, photosynthesis declines; therefore, growth and production of polyphenols decrease (Wong et al., 2006). This can explain the observed reduction of polyphenol compounds at high salt concentrations in our experiments. Literature data confirm that there are not always phenolic level increases under stress conditions (Szafranska et al., 2012). Among many of phenolic functions, they represent important structural components in the plant cell wall, and lignification represents a mechanical mechanism under biotic and abiotic conditions (Szafranska et al., 2012). Therefore, the decrease in phenolic amount could indicate not a less intense phenolic synthesis, but rather their polymerization which is undetected using standard colorimetric methods (Szafranska et al., 2012). In Baatour et al. (2011) polyphenol content increased with salinity in two analysed *Origanum majorana* genotypes. Authors postulated that in plants, the polyphenol biosynthesis is stimulated in response to abiotic/biotic stress, such as salt, considering that secondary metabolites play a role in stress adaptation. Scavenging of DPPH free radical is the basis of a common antioxidant assay (Sharma and Bhat, 2009) and is used to measure total antioxidant activity of medicinal and aromatic plants (Ogles and Yalcin, 2012; Stanisavljevic et al., 2012; Baatour et al., 2011). DPPH showed a capacity of plants to fight stress. Our results showed that at lower NaCl concentrations pea shoots still have the capacity to cope with salt stress, while at higher NaCl concentrations, tissues suffer from serious damage, and necrosis appears. Generally *P. fulvum* extracts exhibited the highest scavenging activities among studied genotypes. It seems that *P. fulvum* has a greater capacity to withstand salt stress at higher concentrations (150, 200 mM), as changes in DPPH activity did not vary significantly with the increase of NaCl concentrations. It seems that *P. fulvum* has a different stress response profile from that of *P. sativum* and *P. arvense*, and that some other metabolites are included in antioxidant defence and stress tolerance. It is reported that the expression of miRNA could be dependent not only on the type of abiotic stress, but also on its intensity (Wang et al., 2011). Our results, obtained by analysing the miRNA398a expression in *P. sativum*, *P. arvense* and *P. fulvum* shoots, showed that these genotypes had a different pattern of expression. In *P. fulvum* salt stress caused inhibition of miRNA398a expression, but in *P. sativum* and *P. arvense* this effect was observed only at a moderate salt concentration. This could be attributed to activation of a different protective system, having in mind that one of possible target genes for miRNA398 is a superoxide dismutase, and that these genotypes obviously possess different protective mechanisms. These are preliminary results on miRNA398a expression in peas, and





**Fig 6.** Expression pattern of miRNA398a in micropropagated pea shoots of *P. sativum* and *P. fulvum* (A). The accumulation of all miRNAs was quantified according to U6 snRNA loading control (B). Lanes 1-4: 1- *P. sativum* control, 2- 75 mM NaCl, 3- 120 mM NaCl, 4- 150 mM NaCl. Lanes 5-8: 5- *P. fulvum* control, 6- 75 mM NaCl, 7- 120, 8- 150 mM NaCl.



**Fig 7.** Expression pattern of miRNA398a in micropropagated pea shoots of *P. arvense* (A). The accumulation of all miRNAs was quantified according to U6 snRNA loading control (B). Lanes 1-4: 1- *P. arvense* control, 2- 75 mM NaCl, 3- 120 mM NaCl, 4- 150 mM NaCl.

further experiments are necessary to obtain more data to explain possible role of miRNA in pea stress response and to confirm different expression patterns among these three species. The results derived from in vitro culture can be used to predict the responses of plants to environmental conditions and thus reduce the cost of subsequent conventional whole plant experiments. Our results showed that among the three treated genotypes, *P. fulvum* is the least sensitive to high NaCl concentrations in a medium, so *P. fulvum* could represent a gene pool which could be used for improving the characteristics of field pea. As pea is one of the oldest crops in the world, it would be of great benefit for cultivated pea species if some of the environmental plasticity and rusticity found in wild relatives were transferred to the cultivated pea (Ochatt et al., 2004).

Given the amount by which food production will have to increase, it seems reasonable to predict that changing the salt

tolerance of crops will be an important aspect of plant breeding in the future, if global food production is to be maintained (Flowers, 2004).

## Materials and methods

### Plant material and culture in vitro

Seeds of three pea species (*Pisum sativum* var. *arvense*, *P. sativum* and *P. fulvum*) were obtained from The Institute of Field and Vegetable Crops, Novi Sad. Seeds were surface sterilized with 70% ethanol for 1 min, following with 20 min in 1% (w/v) sodium hypochlorite, and then rinsed 3 x 5 min in distilled water. Seeds were germinated on a hormone-free basal medium containing ½ MS salts (Murashige and Skoog, 1962), vitamins, 3 % sucrose, and 0.7 % agar (Torlak, Serbia). The medium pH was adjusted to 5.8 and autoclaved for 25 min at 114 °C. The growth conditions were: temperature 25 ± 2 °C, photoperiod 16 h, and photosynthetic photon flux density of 45 μmol m<sup>-2</sup> s<sup>-1</sup> provided by white fluorescent tubes (Tesla, Pancevo, Serbia, 65 W, 4,500 K). Explants from germinated plantlets (hypocotils, apical shoots and cotyledons) were subcultured to a shoot induction medium MS + 20 μM BAP and 0.1 μM NAA (Smykal et al., 2007). Regenerated shoots were further maintained on a medium supplemented with 10 μM BAP and 1 μM IAA (Srejovic and Neskovic, 1981) (Table 1).

### Salt treatments

For salt treatments, similar size shoots of the three pea genotypes were transferred to an SM medium supplemented with NaCl in following concentrations: 75, 120, 150 and 200 mM (Table 1). The treatments lasted four weeks, and after that treated shoots were stored at -70 °C till needed.

### Malondialdehyde assay

The level of lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction (Heath and Packer, 1986). The crude extract (shoots were ground in liquid nitrogen using mortar and pestle) was mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95 °C for 30 min in the thermo-stated water bath and then quickly cooled in an ice bath. After 10 min centrifugation at 3500 x g the absorbance of the supernatant was monitored at 532 nm. The MDA content was expressed in nmol fw<sup>-1</sup>. The MDA content was calculated according to: MDA equivalents(nmol x ml<sup>-1</sup>)=[(A<sub>532</sub>-A<sub>600</sub>)/155000]10<sup>6</sup> (Heath and Packer, 1986).

### Chlorophyll determination

For pigment analysis, 0.5 g of fresh shoots were ground in liquid nitrogen using mortar and pestle and extracted with 5 ml methanol in the dark for 2 h. The extract was centrifuged at 14 000xg for 5 min, and the absorbance of supernatant was read at 632 nm, 652 nm, 665 nm and 696 nm. Total chlorophyll content was calculated according to Ritchie (Ritchie, 2008). The results were expressed as μg fw<sup>-1</sup>.

### Folin–Ciocalteu-reducing capacity assay

Folin–Ciocalteu-reducing capacity was determined using Folin–Ciocalteu (FC) reagent, according to Julkenen-Titto (1985) with modifications. Shoot samples (50 mg) were

homogenized in 80 % acetone and homogenate was centrifuged at 10000 x g for 10 min. One-hundred microliters of the supernatant were diluted with 2 mL of water and 1 mL of FC reagent, and shaken vigorously. Then, 5 mL of 20 % sodium carbonate solution was added and volume was raised to 10 mL with distilled water. Absorbance was read at 765 nm in ULTROSPEC PRO 3300 spectrophotometer (GE Healthcare, Uppsala, Sweden). The FC reagent is non-specific to phenolic compounds only, as it can be reduced by many non-phenolic compounds (e.g. aromatic amines, sulfur dioxide, ascorbic acid, etc). Therefore, FC reagent has recently been recommended for the sample's total reducing capacity measurements. The correlations between FC and other electron transfer-based assays (TEAC-trolox equivalent antioxidant capacity and DPPH for instance) are usually good, thus this method can be considered as useful and relevant in determination of antioxidant capacity (Magalhaes et al., 2008). The values were expressed as relative absorbance units at 765nm.

#### **DPPH-scavenging activity**

For DPPH (2,2-dyphenyl-1-picrylhydrazyl radical) assay, plant material was ground in liquid nitrogen with a mortar and pestle. Ten volumes of ice cold buffer (50mM Tris/HCl pH 7.4, 1mM Triton X-100) were added to powder and vortexed, filtered through filter paper and passed through membrane filter (MWCO=10kDa, Amicon Ultra). The scavenging activity of DPPH radicals in tissue extracts was measured according to the method reported by Wang et al. (2002). Assays were performed in 3 mL reaction mixtures containing 2.0 mL of 0.1 mM DPPH-ethanol solution, 0.9 mL of 50 mM Tris-HCl buffer (pH 7.4), and 0.1 mL of deionized H<sub>2</sub>O (as control) or test plant extracts. The solution containing 1 mg mL<sup>-1</sup> ascorbate, 1 mg mL<sup>-1</sup> gallic acid, and 1 mg mL<sup>-1</sup> reduced glutathione was used as standard. After 30 min of incubation at room temperature, absorbance of the reaction mixtures was determined at 517 nm. The inhibitory effect of DPPH was calculated according to the following formula: inhibition (%) [(absorbance control - absorbance sample)/ absorbance control] x 100. The major determinant of the reaction is steric accessibility of DPPH radical, since small molecules that have better access to the radical site, have higher antioxidant capacity.

#### **RNA isolation and small RNA hybridization**

Total RNA was isolated according to Van Kan et al. (1992), using GHME buffer (8 M guanidine hydrochloride, 20 mM 2-(N-morpholino) ethanesulfonic acid (MES), 20 mM ethylenediaminetetraacetic acid (EDTA), 2% β-mercaptoethanol, pH7.0) and phenol:chloroform:isoamyl alcohol (25:24:1, by vol). RNA was precipitated on ice for 20 min, with 2 volumes of cold ethanol (100%)+1/20 volumes of 4 M sodium acetate (pH 5.2), and resuspended in 100 µl RNase-free water. 15 µg of total RNA was separated on 15% polyacrylamide gel with 7M urea, in MOPS buffer (pH 7.0), along with the microRNA marker (New England Biolabs, UK). The samples were electro-blotted onto Hybond-NX membrane (GE Heltcare, USA) and chemically crosslinked with EDC and 1-methylimidazole, according to Pall et al. (2007). For detection of small RNA, locked nucleic acid (LNA) probes (Exiqon, Denmark) were used. Probes were end-labelled with γ<sup>32</sup>P-ATP (PerkinElmer, USA) for 1h at 37°C using T4 kinase (ThermoScientific, Lithuania). As a loading control, small nuclear RNA U6 was used. Hybridizations were performed at 42°C overnight using

ULTRAhyb-Oligo buffer (Ambion, USA), membranes were washed at 42°C using 2X SSC + 0.1% SDS solution and visualized after 2 days. The membranes were stripped by incubation in boiled 0.1% SDS solution and hybridized with U6 probe.

#### **Statistical analysis**

All experiments were carried out in triplicate, and data were expressed as means ± standard deviations. The data were subjected to ANOVA and Bonferroni correction using SPSS 19 software to determine significant differences at p<0.005. Values are means of three replications (N=3±SD). Values marked by the same small letter case are not significantly different at p<0.005 (Bonferroni correction).

#### **Conclusions**

*In vitro* approach was successfully applied in the assessment of salinity tolerance of three pea species (*Pisum sativum*, *Pisum arvense* and *Pisum fulvum*). According to changes in levels of lipid peroxidation, chlorophyll content, total phenols and DPPH-scavenging activity, it could be concluded that *P. fulvum* is more tolerant to salinity stress up to 150 mM NaCl than *P. sativum* and *P. arvense*. Profile of miR398a expression in *P. fulvum* is also different compared to *P. sativum* and *P. arvense*. The northern blot analysis of the miRNA398 expression showed a similar pattern between *P. sativum* and *P. arvense* while different to that of *P. fulvum*, as in *P. fulvum* miRNA 398 was only expressed at a moderate NaCl concentration, with total inhibition at high concentrations. This is the first report about miR398 expression in pea species. As a wild species, *P. fulvum* could represent a gene pool which could be used for improving the characteristics of pea.

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