General and Applied Plant Physiology – 2009, Volume 35 (3–4), pp. 162–171

Available online at http://www.bio21.bas.bg/ipp/

Special Issue (Part I) – Proceedings of the XI National Conference on Plant Physiology
18–19 November 2009, Sofia, Bulgaria

IMPROVEMENT OF TOLERANCE TO PARAQUAT IN BARLEY (*HORDEUM VULGARE* L.) BY A SYNTHETIC THIOUREA COMPOUND: EFFECTS ON GROWTH AND BIOCHEMICAL RESPONSES

Yonova P.[1*], S. Gateva[2], N. Mincheva[1], G. Jovchev[2], M. Stergious[2], V. Kapchina-Toteva[3]

[1]Acad. M. Popov Institute of Plant Physiology, BAS, Acad. G.Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria

[2]Central Laboratory of General Ecology, BAS, 2 Gagarin Str., 1113 Sofia, Bulgaria

[3]Department of Plant Physiology, Faculty of Biology, University of Sofia, 8 Dragan Tsankov Str., 1164 Sofia, Bulgaria

Received: *24 February 2010* Accepted: *26 March 2010*

Summary. In pot experiments, the influence of a synthetic compound (FTMP) as a potential protector against paraquat in young barley plants was studied. Seeds with growing root meristems were used as an experimental material. Treatment with FTMP ($5x10^{-6}$, $5x10^{-5}$, $5x10^{-4}$ and 10^{-3}M) preceding paraquat (10^{-5}M) application was performed. Pre-treatment with FTMP reduced the inhibiting effect of PQ on the growth of shoots to 20% and of roots to 7.5% in 9-days-old barley plants. PQ alone did not decline the chlorophyll content that increased in leaves of the PQ/FTMP pretreated plants. Biochemical responses on the bases of activities of antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), ascorbate- and guaiacol-peroxidases (AsPO and GPO) as well as estimations of lipid peroxidation and hydrogen peroxide content in leaves and roots were determined on the 9th day after chemical treatments. The effect of FTMP pretreatment followed by paraquat resulted in a lower level of oxidative stress (H_2O_2 and MDA) in leaves (except for 10^{-3}M) and higher in roots (except for $5x10^{-6}$M) compared to PQ only. A stimulation of AsPO, SOD and GPO in roots of PQ/FTMP pre-treated plants along with an increase in the level of lipid peroxidation was found. At a sublethal paraquat dose (10^{-5}M), pretreatment with our synthetic compound led to a protective effect against paraquat, the effect being dependent on the applied concentration. The best among the four tested concentrations was $5x10^{-6}$M that eliminated completely the paraquat-induced oxidative damages in leaves and roots of barley plants. The protective mechanism of FTMP against paraquat in *Hordeum* is discussed.

Key words: Antioxidant, *Hordeum vulgare,* Paraquat, protector, thiourea.

Abbreviationst: ROS – reactive oxygen species; AsPO – ascorbate peroxidase; CAT – catalase; Chl – chlorophyll; FTMP – 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine; FW – fresh weight; GPO – guaiacol peroxidase; MDA – malondialdehyde; PQ – paraquat; SOD – superoxide dismutase; DMSO – dimethylsulfoxide.

*Corresponding author: pyonova@yahoo.com

INTRODUCTION

Several environmental stress conditions are thought to generate oxidative stress in plants (Foyer and Mullineaux, 1994). Paraquat as a herbicide, introduced in the early 1960s, greatly facilitated weed control in many crops. Paraquat (PQ) is a redox-cycling herbicide that intercepts electrons from various electron transport chains, thereby reducing oxygen to superoxide ($O_2^{\cdot-}$) (Dodge, 1994; Takizawa et al., 2007). PQ belongs to non-translocated herbicides when it is applied foliar, but Paraquat's derived toxic products are able to diffuse within the cell from their site of production in the chloroplast to the tonoplast and plasmalemma where their action stimulates the visible symptoms of wilting and necrosis. A major problem associated with the use of herbicides for a long time is the occurrence of herbicide-resistant weeds (Jasieniuk et al., 1996). As a consequence of this phenomenon, there is a continuous need for the development of new products with a new mode of action. To stop the creating great number of chemicals, there is two alternative ways: 1) increase in the herbicide selectivity, or 2) increase in the herbicide tolerance of sensitive cultural plants. The second way may be realized by using herbicide safeners (antidotes). Up to now, limited evidences in the literature are presented on paraquat antidotes. Studies with leaf slices from the broadleaf weed *Arctotheca calendula* have shown that some polyamines (putrescine, cadaverine, spermidine) when applied concomitantly with PQ can reduce the toxic effects of PQ (Soar et al., 2004). Mascher et al. (2005) have proved the role of 2-aminoethanol (biogenic amine) in the protection against paraquat (0.1 - 1 mM) and water deficit in barley by pre-treatments of barley shoots. Pre-treatment of 12-day-old barley seedlings with 500 μmol/L salicylic acid (SA) before PQ (10 μmol/L) application through the roots stimulated the activities of antioxidant enzymes in both the chloroplasts and the other compartments of the cell (Ananieva et al., 2004). The protective effect of a cytokinin benzyladenine (BA) against paraquat toxicity is investigated in the leaves of maize. Pre-treatment with BA (1, 10 and 100 μM) retarded PQ-induced decreases in chlorophyll, carotenoid and ascorbic acid contents whereas at 10 and 100 μM significantly increased SOD activity after 8 h but not after 12 and 24 h of PQ treatment. The peroxidase activity is significantly increased in 100 μM of BA pre-treated leaves (Durmus and Kadioglu, 2005). Foliar-treatment of young pea plants with 2.5 mM H_2O_2 before PQ (0.2 mM) application increased plant survival through stimulation of cellular antioxidant potential (Moskova, 2009). Our earlier investigation has shown that our synthetic compound FTMP at concentrations of 5-50 μmol/L causes oxidative stress (high H_2O_2 content) in roots of barley plants along with an increase in the activity of SOD and AsPO. We hypothesized that pretreatment with a low concentration of FTMP would act like H_2O_2 and enhance the ability of plants to reduce the PQ damage effects by changing the antioxidant activities.

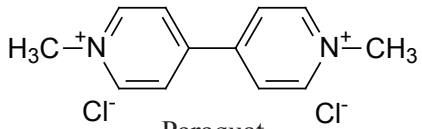
Here, we examined the effectiveness of FTMP pretreatment as a potential protector against PQ during early growth of barley seedlings. To assess the role of FTMP in alleviating PQ toxicity, the growth parameters, quantity of photosynthetic pigments, level of oxidative stress and activities of major antioxidant enzymes

were determined in leaves and roots of PQ/FTMP pre-treated plants on the 9th day after chemical treatments.

MATERIALS AND METHODS

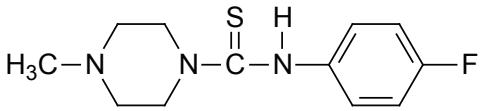
Chemicals

The herbicide paraquat as paraquat dichloride (1,1'-dimethyl-4,4'-bipyridiniumdichloride) was used [Methyl viologen (trade name Gramoxone) from Sigma- Chemie, Deisenhofen, Germany].



Paraquat

The compound tested 1-(4-fluorophenylthiocarbamoyl)-4-methyl-piperazine (FTMP) was synthesized earlier in our lab (Stoilkova and Yonova, 2007).



FTMP

Plant material and chemical treatments

Seeds of *Hordeum vulgare* L., standard karyotype of barley with growing root meristems, ~2mm length [SRM] were used. Water stock solution of the test compound FTMP (10^{-3}M) was prepared by dissolving 12.65 mg in 1 ml DMSO, then diluting with distilled water to 50 ml. A range of aqueous solutions of FTMP at concentrations of $5x10^{-6}$, $5x10^{-5}$, $5x10^{-4}$ and 10^{-3}M was prepared. FTMP solutions were applied each for 1h in the dark. After intertreatment time of 4h, paraquat (10^{-5}M) treatment was applied for 0.5h in the dark. Control seeds [K_1] were treated with distilled water. A second control [K_2] was introduced with seeds being treated with 2% (v/v) aqueous solution of DMSO. The treated material [SRM] was rinsed in running tap water for 10 min and cultivated in Petri dishes (15 cm i.d.) on moist filter paper at 27°C for 48h. Barley seedlings were planted into 10 cm of glass pots containing tap water and grown in a growth chamber (25°C, 12h light (120 $\mu mol.m^{-2}.s^{-1}$ PFD)/12h darkness). Barley plants were harvested on the 9th day after chemical treatments. Leaves and roots were separated. Each sample included 20 uniform plants.

Biochemical analyses

Fresh plant material (leaves and roots) was immediately extracted and assayed. The hydrogen peroxide content was determined spectrophotometrically by monitoring the absorbance (A_{390}) of the KJ-oxidized product using a standard curve (Alexieva et al., 2001). Lipid peroxidation was estimated by measuring the amount of malondialdehyde using the thiobarbituric acid reaction (Dhindsa et al., 1981). Chlorophylls and carotenoids were extracted in acetone (80%, v/v) and estimated according to Arnon (1949).

Enzyme analyses

After homogenized with liquid nitrogen, 0.250 g of leaves and roots were suspended in 5 ml of ice-cold phosphate buffer (0.1 M, pH 7.0) containing 1.0 mM Na_2-EDTA and 1% (w/v) PVPP, and assays were made on the crude extract. The phosphate buffer (0.1 M, pH 7.0)

containing 1.0 mM Na_2-EDTA, 1% (w/v) PVPP and 5 mM ascorbic acid was used for AsPO extraction. Enzyme activities were determined spectrophotometrically at 25°C according to the following protocols: Superoxide dismutase (EC 1.15.1.1) (Beuchamp and Fridovich, 1971); Catalase (EC 1.11.1.6) (Beers and Sizer, 1952); Ascorbate peroxidase (EC 1.11.1.11) (Nakano and Asada, 1981) and Guaiacol peroxidase (EC 1.11.1.7) (Dias and Costa, 1983). Soluble protein content was determined by the method of Bradford (1976) using BSA as a standard.

Statistics

The data presented are means of three independent experiments with three replications each. Differences were analyzed with one-way ANOVA and Least Significant Difference (LSD). *P*-values of <0.05 were considered to be significant.

RESULTS AND DISCUSSION

Effect of FTMP in counteracting paraquat inhibition in barley

PQ has the unusual property of being active only by direct spray onto plants and not by uptake from soil in which strong binding deactivates it. Our experiments have two special features: 1) the model system used - FTMP and PQ solutions were applied to barley seeds with growing root meristems [SRM]; 2) the growth and biochemical parameters were determined after post-treatment period of 9 days. Paraquat applied alone caused a significant inhibition on root growth (65% of the control) and a weaker effect (26.2% of the control) on shoot growth of barley plants. The symptoms of PQ-inhibiting effect on the treated SRM were evident due to either stoppage of germination and growth of seedlings, or poor development of roots. At the end of a 48-h efflux period, the growth of only PQ-treated seedlings was clearly retarded compared to that of controls and PQ/FTMP pre-treated seedlings [data not shown]. Pre-treatment of SRM with four concentrations of FTMP ($5x10^{-6}$, $5x10^{-5}$, $5x10^{-4}$ and 10^{-3}M) reduced the inhibiting effect of PQ on fresh weight of shoots by 11-20% and of roots by 6.4-7.5%. The safening effect of FTMP was more pronounced about shoot growth. From the all four concentrations of FTMP, the most active antidote concentration was $5x10^{-6}$ M, whereas the concentration $5x10^{-4}$ M had the lowest reducing effect to shoot growth only (Fig. 1A, B).

The order of activity in relation to the % of inhibition of shoot growth of the control was:

$$PQ > 5x10^{-4} > 5x10^{-5} > 10^{-3} > 5x10^{-6}$$

The order of activity in relation to the % of inhibition of root growth of the control was:

$$PQ \geq 5x10^{-4} > 5x10^{-5} \approx 10^{-3} > 5x10^{-6}$$

When applied alone, PQ treatment did not decrease chlorophyll content and slightly increased carotenoids content in leaves of the 9-day-old barley plants (Fig. 2). We could suggest that either a limited quantity of active herbicide is translocated to chloroplasts (the green tissues appeared for 3-4 days past PQ treatment), or the enhanced content of natural antioxidants, carotenoids, protects from degradation of the chlorophyll-protein complex. Pretreatment of barley seeds+root tips with three concentrations of FTMP ($5x10^{-6}$, $5x10^{-5}$, 10^{-3}M)

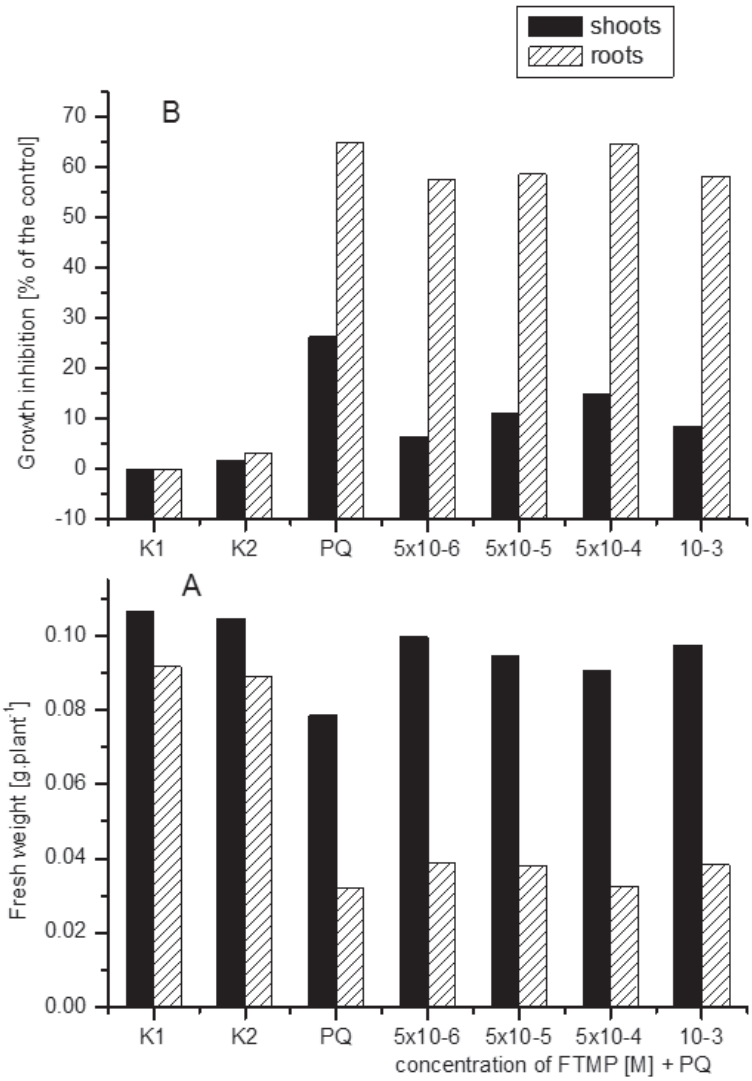


Fig. 1. Effects of potential protector (FTMP) applied at different concentraions on the herbicidal action of paraquat (PQ) on growth (A) and fresh weight (B) of young barley plants, 9 days after chemical treatments [K1 – Control water; K2 – Control 2% DMSO; PQ 10^{-5}M].

resulted in higher chlorophyll and lower carotenoids contents compared to those in leaves of the PQ-treated barley plants. The carotenoids content was limited to that of the control. PQ is a well-known oxidative stress inducer when applied to green plant tissues in the light. In our case, the level of oxidative stress was low in leaves (in respect to H_2O_2 only) and moderate in roots of the PQ-treated barley plants (35% and 39% more H_2O_2 and MDA, respectively, compared to the control). Therefore, the lipid molecules in cell membranes of roots were more sensitive to PQ**.** The application of FTMP reduced differently the paraquat-induced oxidative stress in leaves and roots. The pre-treatment with the lowest concentration of FTMP ($5x10^{-6}$M) decreased the level of oxidative stress (H_2O_2 and MDA content) in both leaves and

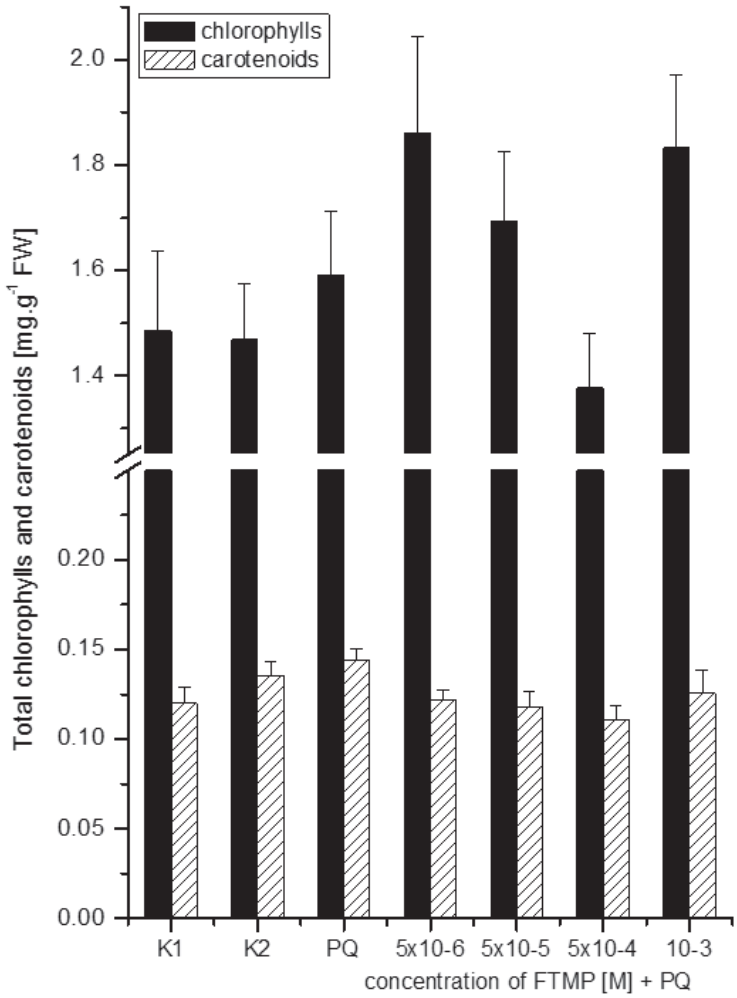


Fig. 2. Changes in the content of total chlorophylls and carotenoides in leaves of PQ and PQ/FTMP pre-treated barley plants 9 days after chemical treatments [K1 – Control water; K2 – Control 2% DMSO; PQ 10^{-5}M].

roots while the other two concentrations ($5x10^{-4}$ and $5x10^{-5}$M) - only in leaves (Fig. 3A, B).

The order of activity in relation to the level of oxidative stress in leaves and roots of the PQ/FTMP pre-treated barley plants was:

Leaves: 10^{-3} > PQ > $5x10^{-4} \approx 5x10^{-5}$ > $5x10^{-6}$ (H_2O_2)

Roots: $5x10^{-4} > 5x10^{-5} > 10^{-3} >$ PQ > $5x10^{-6}$ (MDA)

We found that the determined changes in the activity of antioxidant enzymes CAT, SOD, GPO and AsPO, participating in H_2O_2 metabolism, in leaves and roots of the PQ- and PQ/FTMP pre-treated plants are well correlated with the level of oxidative stress in these tissues, and

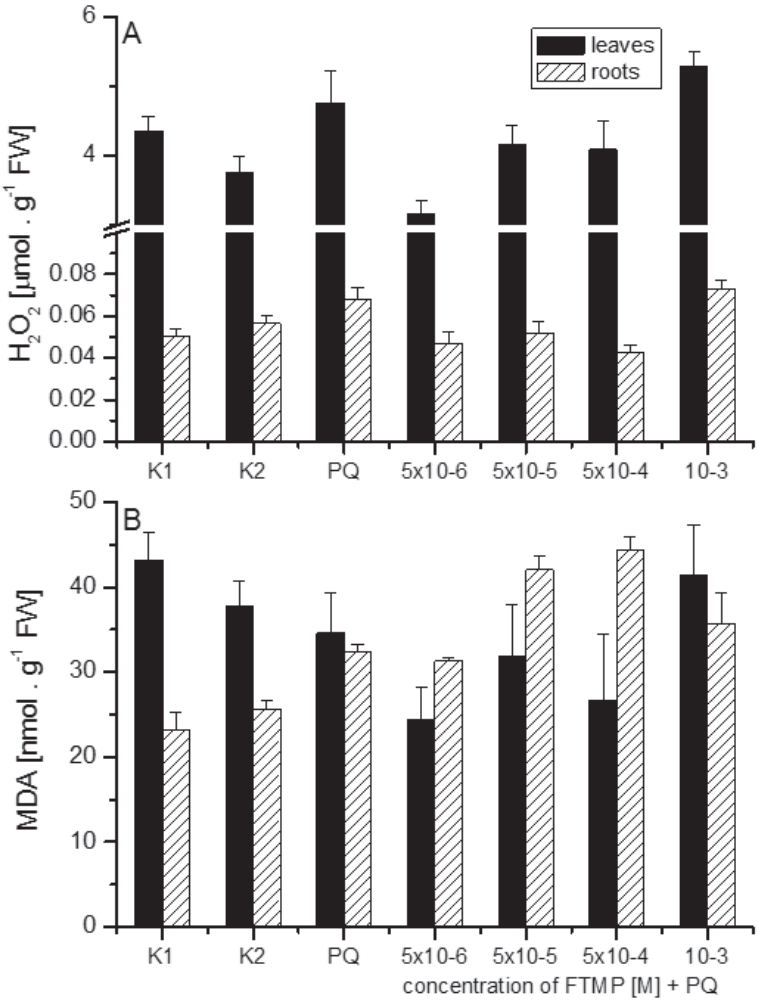


Fig. 3. Changes in the content of H_2O_2 (A) and MDA (B) in leaves and roots of PQ and PQ/FTMP pre-treated barley plants 9 days after chemical treatments [K1 – Control water; K2 – Control 2% DMSO; PQ 10^{-5}M].

further, with the growth parameters.

The photooxidative herbicide PQ alone inhibited SOD and CAT activities in leaves, and GPO and SOD activities in roots. The FTMP pre-treatment did not overcome the enzyme's inhibition in leaves while in roots, it overcame or decreased the enzyme's inhibition. The GPO activity in leaves and CAT activity in roots are only inhibited by the combined treatments. Treatment with PQ alone increased the AsPO activity by 31% in leaves and 16% in roots. The FTMP pre-treatment led to an additional increase in the AsPO activity - more significantly in roots and weakly in leaves (Fig. 4A-D).

The order of AsPO activity in relation to the % of stimulation was:

Leaves: 10^{-3} > PQ > $5x10^{-4}$ > $5x10^{-6}$ = Control ≈ $5x10^{-5}$

Roots: $5x10^{-6}$ > $5x10^{-5}$ ≈ $5x10^{-4}$ > 10^{-3} > PQ

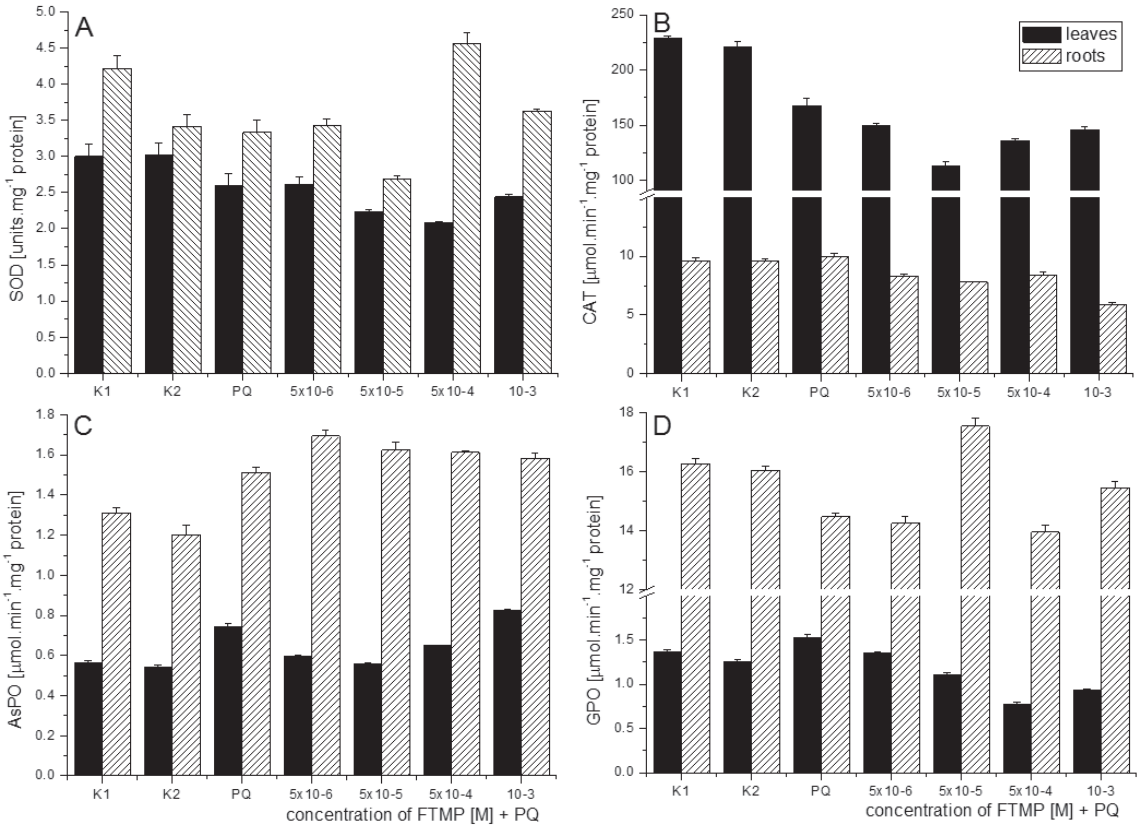


Fig. 4. Changes in the specific activities of SOD (A), CAT (B), AsPO (C) and GPO (D) in leaves and roots of PQ and PQ/FTMP pre-treated barley plants 9 days after chemical treatments [K1 – Control water; K2 – Control 2% DMSO; PQ 10^{-5}M].

We found that the FTMP protective effect could be the result of: 1) FTMP stimulates the activity of four defense antioxidant enzymes, mainly in the roots of PQ / FTMP pre-treated plants. Among the four enzyme activities, AsPO is induced by all four FTMP concentrations, SOD – by three FTMP concentrations (10^{-3}, $5x10^{-4}$, $5x10^{-6}$ M) and GPO – by two FTMP concentrations (10^{-3}, $5x10^{-5}$ M); 2) FTMP ability to reduce the PQ-induced oxidative stress more greatly in leaves ($5x10^{-4}$, $5x10^{-5}$, $5x10^{-6}$M) than in roots ($5x10^{-6}$M); 3) because the tested compound FTMP may possess antioxidant property due to turning thiourea [-N-C(=S)-NH-] group into isothiourea group [-N-C(-SH)=N-]. Thus, we proposed that FTMP as reducing agent contributes to the decrease of oxidative potential in leaves and roots of PQ / FTMP pre-treated plants.

CONCLUSIONS

We have demonstrated here for the first time the protective effect of a synthetic compound 1-(4-fluorophenylthiocarbamoyl)-4-methyl-piperazine (FTMP) applied at four concentrations against herbicide paraquat

in barley. The FTMP pre-treatment of barley seeds with growing root meristems had a higher protective effect on shoots than on roots of 9-day-old barley plants. We suggest that the protective effect of FTMP was mediated by the increased activities of antioxidant enzymes in the roots as an initial defense against PQ. Probably, some paraquat detoxification may occur in the roots as a result of a chain of oxidoreductive processes with the participation of PQ-radical during the first 3-4 days when green leaves had not emerged. Only a limited quantity of the remained active PQ could translocate to green plant tissues.

Acknowledgements: This study was supported by a research project № B-1520 of the National Science Fund at the Bulgarian Ministry of Education and Science.

REFERENCES

Alexieva V, I Sergiev, S Mapelli, E Karanov, 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ, 24: 1337-1344.

Ananieva E, K Christov, L Popova, 2004. Exogenous treatment with salicylic acid leads to increased antioxidant capacity in leaves of barley plants exposed to paraquat. J Plant Physiol, 161: 319-328.

Beauchamp, CH, I Fridovich, 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem, 44: 276-287.

Beers RF, IW Sizer, 1952. A spectrophotometric method for measuring breakdown of hydrogen peroxide by catalase. J Biol Chem, 195: 133-140.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72: 248-254.

Dhindsa RS, P Plumb-Dhindsa, TA Thorpe, 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot, 32: 93-101.

Dias A, M Costa, 1983. Effect of low salt concentration on nitrate reductase and peroxidase of sugar beet leaves. J Exp Bot, 34: 537-543.

Dodge AD, 1994. Herbicide action and effects on detoxification processes. In: Causes of photooxidative stress and amelioration of defense systems in plants (Eds. CH Foyer and PM Mullineaux), CRC Press, Boca Raton, FL, 219-236.

Durmus N, A Kadioglu, 2005. Reduction of paraquat toxicity in maize leaves by benzyladenine. Acta Biol Hung, 56: 97-107.

Foyer CH, PM Mullineaux, 1994. Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL.

Jasieniuk M, AL Brule-Babel, IN Morrison, 1996. The evolution and genetics of herbicide resistance in weeds. Weed Sci, 44: 176-193.

Mascher R, E Nagy, B Lippmann, S Hornlein, S Fischer, W Schneiding, A Neagoe, H Bergmann, 2005. Improvement of tolerance to paraquat and drought in barley (*Hordeum vulgare L.*) by

exogenous 2-aminoethanol: effects on superoxide dismutase activity and chloroplast ultrastructure. Plant Sci 168: 691-698.

Moskova I I, 2009. Protective effect of hydrogen peroxide against herbicide paraquat in pea plants. PhD Thesis, Sofia.

Nakano Y, K Asada, 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol, 22: 867-880.

Soar CJ, C Preston, J Karotam, SB Powles, 2004. Polyamines can inhibit paraquat toxicity and translocation in the broadleaf weed *Arctotheca calendula.* Pest Biochem Physiol 80: 94-105.

Stoilkova G, P Yonova, 2007. Protective effect of two synthetic compounds against chlorsulfuron injury in maize (*Zea mays* L.). Acta Agron Hung, 55: 283-292.

Takizawa M, K Komori, Y Tampo, M Yonaha, 2007. Paraquat-induced oxidative stress and dysfunction of cellular redox systems including antioxidative defense enzymes glutathione peroxidase and thioredoxin reductase. Toxicol. *In Vitro* 21: 355-363.