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The effect of Fe-deficiency on antioxidant enzymes' activity and lipid peroxidation in wheat leaves

Wpływ deficytu Fe na aktywność enzymów antyoksydacyjnych i peroksydację tłuszczu w liściach pszenicy

Summary. Studying the physiological role of nutrient elements has an unraveling capacity in understanding plant's behavioral pattern in order to acquire a high and stable yield. For this purpose, the durum wheat variety, P1252, was selected and planted in hydroponic way. To investigate the effects of Fe deficiency, the element was eliminated from media solutions. Results showed that the lack of Fe affected superoxide dismutase (SOD), guaicol peroxidase (GPX) and catalase (CAT) activities significantly. Meanwhile, ascorbate peroxidase (APX) was the only antioxidant enzyme not shows any significant with control. The SOD/(APX + GPX + CAT) ratio as an index of assessing the balance between hydrogen peroxide (H_2O_2)-producing and H_2O_2-scavenging enzymes increased leading to the accumulation of H_2O_2 in cell. The elevation of SOD/APX + GPX + CAT ratio and H_2O_2 accumulation indicates the occurrence of oxidative stress in the leave cells in the element-deletion conditions. Other oxidative stress indices, cell death as well as malondialdehyde (MDA) did not show any significant change in the absence of Fe. The reason is assumed to be the non-occurrence of Haber-Weiss reaction in Fe absence so that hydroxyl, a very dangerous radical, is produced, leading to increased damage to cell bio-molecules and apoptosis subsequently.

Key words: catalase, Haber-Weiss reaction, superoxide dismutase

Abbreviations. APX – ascorbate peroxidase, CAT – catalase, GPX – guaiacol peroxidase, MDA – malondialdehyde, ROS – reactive oxygen species, SOD – superoxide dismutase, POX – peroxidase, AsA – ascorbate, TBA – thiobarbituric acid, TCA – trichloroacetic acid (TCA), CRD – completely randomized design, LSD – least significant differences, EDTA – Ethylenediaminetetraacetic acid

INTRODUCTION

Durum wheat (*Triticum turgidum* L.) is one of the most important cereal crops in Iran, which is playing a special role in people's nutrition. It is the one of the important food crop in the world population and contributes calories as well as proteins to the world [Shewry 2009]. Iron is one of the important constituent elements in the structure of some carriers in electron-transferring chain. The Fe-deficiency can disturb the process of electron-transference due to the lack of Fe-compounds resulting from the reduction of other carriers of electron-transferring chain [Tewari *et al.* 2005]. Upon carriers' transformation into their reduced form, electrons are transferred onto O_2, producing superoxide radical ($O_2^{\cdot-}$) as well as other forms of reactive oxygen species (ROS). Though plants are equipped with defense mechanisms including antioxidant enzymes which known as SOD, CAT, APX, GPX and etc as well as antioxidants which known as ascorbate, glutathione, tocopherol, carotenoides and etc [Ahmed *et al.* 2009, Esfandiari *et al.* 2010b]. Nevertheless, oxidative stress is likely to occur in the plant when ROS is produced in a rate which is beyond the handling efficiency of defense mechanisms in detoxifying ROS.

Plants which suffering Fe-deficiency are likely to stress a more intensive form of oxidative stress since Fe is a constituent element of those enzymes involved in detoxifying H_2O_2 or CAT [Kono and Fridovich 1983], peroxidase or POX [Gara *et al.* 2003] and dismutating $O_2^{\cdot-}$ or Fe-SOD [Martinez *et al.* 2001]. Some components such as CAT [Iturbe-Ormaetxe *et al.* 1995], POX [Iturbe-Ormaetxe *et al.* 1995] and APX [Ishikawa *et al.* 2003, Zaharieva *et al.* 2004] have been reported to decrease their enzyme activity in decomposing H_2O_2 under Fe-deficiency conditions. According to Ranieri *et al.* [2001], Fe deficiency resulted in oxidative stress in the sunflower due to H_2O_2 accumulation which follows from a drop in the activity of level of POX and APX. Becana *et al.* [1998] found that the lack of catalytic iron can protect the plant against any oxidative stress. A decreased activity of Fe-SOD has been reported in Fe-deficiency conditions for different plants such as citrus [Sevilla *et al.* 1984, pea [Iturbe-Ormaetxe *et al.* 1995] and tobacco [Kurepa *et al.* 1997]. In contrast, Kurepa *et al.* [1997] did not observed any significant effect of Fe deficiency on Cu/Zn-SOD property, while increased Cu/Zn-SOD activity in Fe-deficient pea is observed by Iturbe-Ormaetxe *et al.* [1995] and supported a compensatory increase in the expression of another SOD from when the expression of one SOD form decreased, and is suggestive of increased generation of O_2^-.

Evidently, grasping the physiological roles of nutrient elements in plants seems neceessary in order to promote plant's stability and performance. For this propose and having in mind fact that Fe participates in the structure of carriers of electron transference chain as well as Fe-SOD, CAT, APX and GPX, Fe was omitted from the nutrient solution of the plant in this investigation so that it can provide a chance to scrutinize the effect of Fe-deficiency on the enzyme activity of antioxidants involved in defense mechanisms as well as the intensity of oxidative stress as it occurs.

MATERIALS AND METHODS

Trial protocol

In order to study Fe deficiency effects on the activity of antioxidant enzymes, oxidative stress indexes (MDA and H_2O_2 content) and cell death, a durum wheat variety, called

P1252, was planted in hydroponic way at controlled conditions. The composition of the nutrient solution was: (mmol) 1 calcium nitrate [$Ca(NO_3)_2$ $4H_2O$]; 0.1 monopotassium phosphate (KH_2PO_4); 0.5 potassium sulfate (K_2SO_4); 0.5 magnesium sulfate ($MgSO_4$) and (μmol) 10 boric acid (H_3BO_3); 20 manganese chloride ($MnCl_2$ $4H_2O$); 0.5 zinc sulfate ($ZnSO_4$ $7H_2O$); 1 copper sulfate ($CuSO_4$ $5H_2O$); 0.1 molybdenum trioxide (MoO_3), and 100 iron sulfate ($FeSO_4$ $7H_2O$). These solutions were continuously aerated by electrical pumps (Resun, AC 9904, China) and renewed every three days. $FeSO_4$ was eliminated from media solution in Fe treatment [Esfandiari *et al.* 2010a].

The seedlings were planted on half dose of nutrition solution till 2–3 leaves stage and following on full nutrition solution. To maintain nutrition-elements balance, the solutions were renewed twice a week. The pH of solutions was set around 5.2–5.5. The temperature, day length and light density were 25 ±2°C, 14 h and 200 μM photon m^{-2} s^{-1}, respectively. The seedlings were grown under these conditions for two months. Sampling was made from completely-expanded leaves and was immediately placed in liquid nitrogen. Leaf samples were kept in -20°C till measurement time.

Enzyme extraction

For SOD, CAT and GPX extraction, leaf samples (0.5 g) were homogenized in 10 ml ice cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in Beckman refrigerated centrifuge for 15 min at 15,000g. The supernatant was used for enzyme activity assay.

For APX extraction, leaf samples (0.5 g) were homogenized in ice cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA, 2 mM ascorbate (AsA) and 5% poly vinyl pyrrolidin with pre-chilled pestle and mortar. Other stages were similar to extraction of other enzymes [Esfandiari *et al.* 2007].

Enzyme activity assay

SOD activity was estimated by recording the decrease in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme [Sairam *et al.* 2002]. About 3 ml of reaction mixture, containing 0.1 ml of 200 mM methionine, 0.01 ml of 2.25 mM nitro-blue tetrazolium, 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml distilled water and 0.05 ml of enzyme extraction, were taken in test tubes from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml riboflavin (60 μM) and placing the tubes below a light source of two 15 W florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal color. A non-irradiated complete reaction mixture which did not develop color served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

CAT activity was measured according to Aebi [1984]. APX activity was measured according to Yoshimura *et al.* [2000]. GPX activity was measured according to Panda *et al.* [2003]. The enzyme produced a colorful product by using H_2O_2 and guaiacol as substrates. The absorbance of the product was monitored at 470 nm (E = 26.6 mM^{-1} cm^{-1}),

and peroxidase activity was expressed as units/mg protein. Protein content of samples was determined by the method of Bradford [1976], bovine serum albumin used as a standard.

Peroxidation product estimation

MDA was measured by colorimetric method. 0.5 g of leaf samples were homogenized in 5 ml of distilled water. An equal volume of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) solution was added and the sample incubated at 95°C for 30 min. The reaction stopped by putting the reaction tubes in an ice bath. The samples were then centrifuged at 10000 g for 30 min. The supernatant was removed, absorption read at 532 nm, and the amount of nonspecific absorption at 600 nm read and subtracted from this value. The amount of MDA present was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1} [Stewart and Bewley 1980].

Determination of H_2O_2 content

H_2O_2 levels were determined according to Sergiev *et al.* [1997]. Leaf tissues (0.5 g) were homogenized in ice bath with 5 ml 0.1% (w/v) TCA. The homogenate was centrifuged at 12000×g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M potassium iodide (KI). The absorbancy of supernatant was read at 390 nm. The content of H_2O_2 was given on standard curve which is fitted from original dataset.

Determination of cell death

Aliquots consisting of four leaf discs were removed from treatments and submerged in 1 ml of 0.25% Evans blue in 10 ml disposable plastic beakers and incubated on a platform shaker at 80 rpm for 20 min. The beaker contents were poured into a small Buchner funnel and the discs rinsed well with deionized water until no more blue stain was eluted. The discs were ground with using a pestle and the homogenate diluted with 0.5 ml of deionozed water. The tube was capped, vortexed and centrifuged at 10000×g for 5 min. A 0.8 ml aliquot of the supernatant was removed and the optical density determined spectrophotometrically at 600 nm [Baker and Mock 1994].

Statistical analysis

Enzyme activity, cell death, MDA and H_2O_2 content of samples were recorded with five replications. The data were analyzed in completely randomized design (using MSTATC 1.42 program) and the means were compared through (lease significance differences) LSD method.

RESULTS AND DISCUSSION

The results of normality tests (Kolmogorov-Smirnov) and residual analysis for some of the measured traits indicated data normality and providing assumptions of normal distribution and homogeneity of error in both experiments (data are not shown). The

results of present study indicated that, in Fe-deficiency conditions, the enzyme activity of SOD and CAT significantly decreased compared to control treatment (Fig. 1A and 1B). The SOD is assumed to be an important enzyme in cell's defense mechanisms and $O_2^{\cdot-}$ is turned to H_2O_2 by the activity of SOD [Ahmed *et al.* 2009, Gill and Tuteja 2010]. By removing Fe, in this study, the enzyme activity of SOD was cut down (Fig. 1A). This can somehow contribute to the accumulation of $O_2^{\cdot-}$. Along with the finding of this study, Tewari *et al.* [2005] reported a considerable decrease in SOD activity in Fe-deficiency conditions. They proposed restricted activity of Fe-SOD isozyme as the main explanation for their finding.

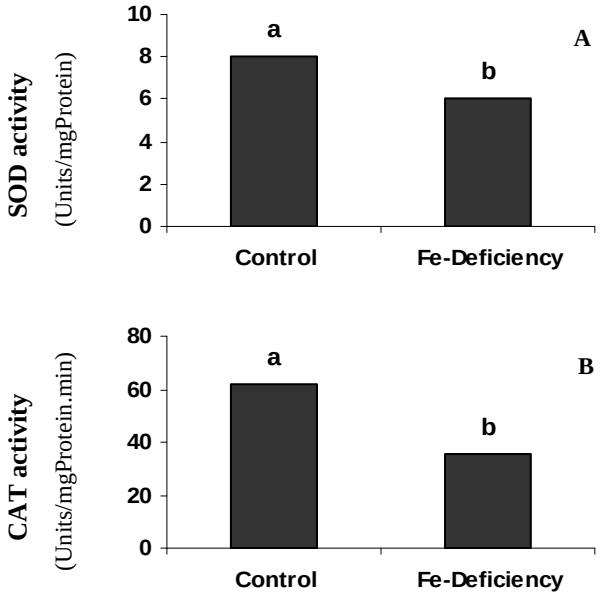

Fig. 1. The effect of Fe-deficiency on (A) SOD and (B) CAT activates in durum wheat seedling
Rys. 1. Wpływ niedoboru Fe na aktywność (A) SOD i (B) CAT w kiełkach pszenicy durum

Also, the enzyme activity of GPX significantly decreased compared to control treatment (Fig. 2A) while APX was the only antioxidant enzyme not to show any significant difference with control treatment (Fig. 2B). According to Ahmed *et al.* [2009] and Gill and Tuteja [2010], APX, GPX and CAT are considered as the most important H_2O_2--scavenging enzymes in plants. In this investigation, the activity of GPX and CAT, were different from APX and decreased with Fe deletion from wheat seedlings since Fe is a constituent element of these enzymes having an effective role in their performance. The observed decreases in the activities of CAT and APX in Fe-starved plants are in consonance with several earlier studies [Agarwala *et al.* 1981; Ranieri *et al.* 2001].

Furthermore, the activity ratio of SOD/APX+GPX+CAT, indicated 176.49% increase in comparison to control treatment in Fe-deficiency conditions (Fig. 3A). Besides, SOD/APX+CAT+GPX ratio is a reliable criterion to examine the balance between generation rate and scavenging rate of H_2O_2 [Halliwell 2006; Esfandiari *et al.* 2010b].

Although generation in plant cells occurs somewhere other than SOD activity, its increased production rate is an indicative of the fact that H_2O_2 production rate by SOD is more than other enzymes involved in its scavenging. The absence of Fe resulted in the significant accumulation of H_2O_2 compared to control (Fig. 3B). The reduced activity of CAT and GPX alongside the increased ratio of SOD/APX+CAT+GPX can justifiably explain H_2O_2 accumulation.

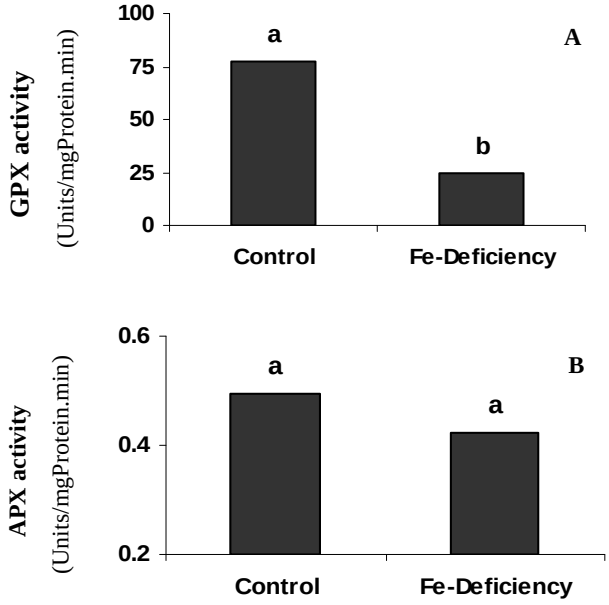

Fig. 2. The effect of Fe-deficiency on (A) GPX and (B) APX activities in durum wheat seedling
Rys. 2. Wpływ niedoboru Fe na aktywność (A) GPX i (B) APX w kiełkach pszenicy durum

Also, the amount of MDA and cell death did not show any significant variation from control (Fig. 4A and 4B). It is well known that $O_2^{\cdot-}$ and H_2O_2 enjoy a very high affinity when reacting with vital bio-molecules [Gill and Tuteja 2010]. These oxidants can target critical metabolistic areas so that the sum of resulting damages would lead to metabolistic disorders including lipid peroxidation and damage to membranes [Ahmed *et al.* 2009, Gill and Tuteja 2010]. Being a critical part of a cell and organelles, the membrane has a special role in regulation of plant cell metabolism. Also, the sum of damages incurred to vital phases of metabolism will lead to cell deaths in plants [Esfandiari *et al.* 2007, 2010c].

The accumulation of $O_2^{\cdot-}$ and H_2O_2 is explicable in view of decreased activity of SOD, CAT and GPX as well as the imbalance between H_2O_2 generation and scavenging. Therefore, lipid peroxidation, as well as the cell death resulting from it, are expected to increase compared to control in the absence of Fe. However findings related to these two parameters are against this expectation, showing no significant difference from control in

Fe absence. In similar findings, Iturbe-Ormaetxe *et al.* [1995] and Tewari *et al.* [2005] reported that the amount of lipid peroxidation, protein denaturation, total glutathione ratio and tocopherol did not show any significant variation from the control in Fe-removal conditions despite a decrease in the enzyme activity of CAT, APX and SOD antioxidants. They proposed non-occurrence of Haber-Weiss reaction and the low generation rate of $HO^{\cdot}$ as the main cause of moderate oxidative stress.

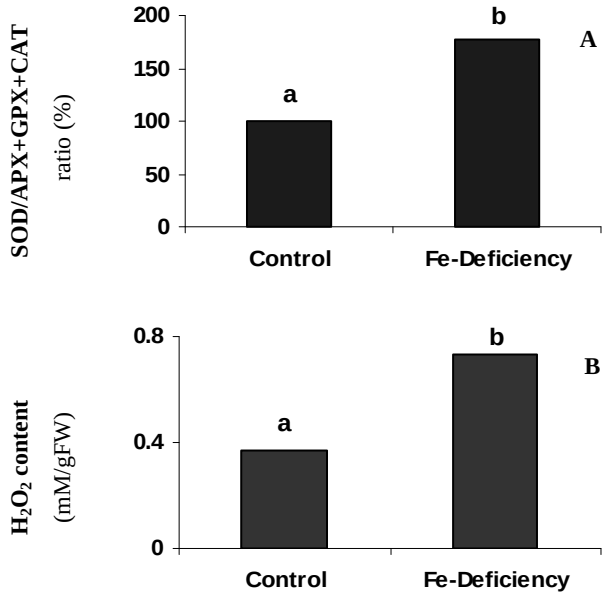

Fig. 3. The effect of Fe-deficiency on (A) SOD/APX+CAT+GPX ratio and (B) H_2O_2 activates in durum wheat seedling

Rys. 3. Wpływ niedoboru Fe na stosunek (A) SOD/APX+CAT+GPX oraz (B) aktywność H_2O_2 w kiełkach pszenicy durum

Many authors consist on Iturbe-Ormaetxe *et al.* [1995], Edreva [2005], Tewari *et al.* [2005], and Halliwell [2006] found that $HO^{\cdot}$ radical is generated from $O_2^{\cdot-}$ and H_2O_2 during Haber-Weiss reaction when Fe is available. By and large, it is concluded that despite reduced activity of antioxidant enzymes and accumulation of $O_2^{\cdot-}$ and H_2O_2 in Fe-deficiency conditions, $HO^{\cdot}$ is not generated due to non-occurrence of Haber-Weiss reaction leading to a slight oxidative stress in plant cells. It can also be concluded that $HO^{\cdot}$ is the most perilous form of active oxygen in damaging cellular bio-molecules. When SOD activity was high, ROS superoxide radical, scavenging was done and damage to membranes and oxidative stress decreased, leading to the increase of tolerance to oxidative stress while if this radical is not scavenged by SOD, it disturbs vital bio-molecules [Mittler 2002]. Also, it inactivates antioxidant enzymes, which are very important for H_2O_2 scavenging such as CAT [Kono and Fraidovich 1983]. This radical attacks

vital biomolecules and damages to membranes happen in wheat and other crops [Martinez *et al.* 2001, Candan and Tarhan 2003, Zhao *et al.* 2006, Esfandiari *et al.* 2007] which found similar results in SOD activity and decrease in oxidative damage.

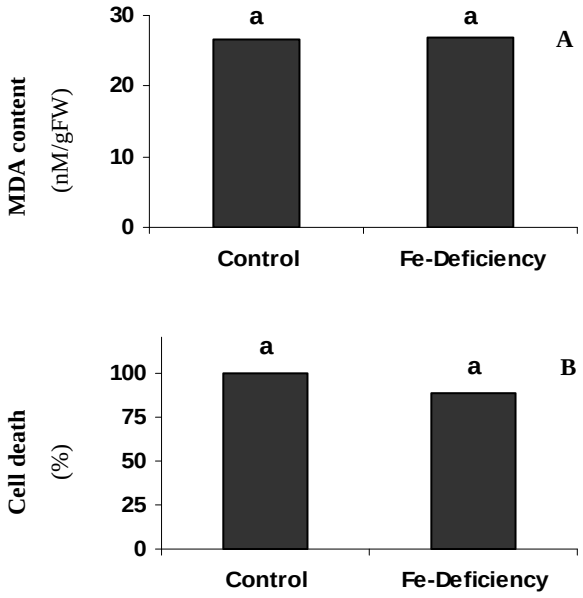

Fig. 4. The effect of Fe-deficiency on (A) MDA and (B) cell death activates in durum wheat seedling
Rys. 4. Wpływ niedoboru Fe na (A) MDA i (B) śmiertelność komórek w kiełkach pszenicy durum

It was shown by that wheat is important dietary sources of iron, particularly for low income people. Information about wheat as a dietary source of iron has been important and conflicting. Iron deficiency is involved in the failure of plants to produce sufficient chlorophyll. Under severe Fe deficiency conditions, the new growth may appear completely devoid of chlorophyll and turn white. Furthermore, iron is essential element for human nutrition [Grusak and Penna 1999] and cereals are a main staple for humans. The nutritional value of grains may be enhanced by increasing accumulation without reducing the availability of the metals or by increasing their bioavailability [Frossard *et al.* 2000].

CONCLUSIONS

However, due to high importance of iron in plants and human nutrition, it is essential to increase iron in plant tissues and especially in grains. It seems that developing new cultivars through various genetically plant improvements procedures using different wheat global germplasms. Attention to iron deficiency from agronomic practices and plant breeding efforts cause to solve this problem in crop production as well as human nutrition.

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Streszczenie. W poznaniu modelu zachowania roślin warunkującego obfite i stabilne plonowanie ogromne znaczenie mają badania nad fizjologiczną rolą składników odżywczych. W tym celu wybrano odmianę pszenicy twardej P1251 uprawianej w kulturze hydroponicznej. Aby określić wpływ niedoboru Fe, pierwiastek ten został wyeliminowany z roztworów pożywki. Wykazano, że brak Fe wywarł znaczący wpływ na dysmutazę ponadtlenkową (SOD) oraz aktywność peroksydazy glutationowej (GPX) i katalazy (CAT). Jednocześnie peroksydaza askorbinianowa (APX) była jedynym enzymem antyoksydacyjnym, który nie wykazał znaczących różnic wobec kontroli. Stosunek SOD/(APX + GPX + CAT) jako wskaźnik oceny równowagi między enzymami produkującymi a enzymami unieczynniającymi nadtlenek wodoru (H_2O_2) zwiększył się, prowadząc do gromadzenia się H_2O_2 w komórce. Wzrost stosunku SOD/APX + GPX + CAT oraz kumulacja H_2O_2 wskazują na występowanie stresu oksydacyjnego w komórkach liści w warunkach usunięcia pierwiastka. Inne wskaźniki stresu oksydacyjnego – śmierć komórek oraz obecność aldehydu dimalonowego – nie wskazały na znaczące zmiany w warunkach braku Fe. Wydaje się, że powodem tego jest niewystępowanie reakcji Habera-Weissa przy braku Fe. Produkowany jest wówczas hydroksyl, bardzo niebezpieczny rodnik, co prowadzi do zwiększonego uszkodzenia biomolekuł, a następnie do apoptozy komórek.

Słowa kluczowe: katalaza, reakcja Habera-Weissa, dysmutaza ponadtlenkowa