Protective Reactions of Seedlings Against Systemic and Local Zearalenone Application to Tolerant and Sensitive Wheat Varieties – Epr Studies

Apolonia Sieprawska (apolonia.sieprawska@up.krakow.pl)
Pedagogical University https://orcid.org/0000-0002-2700-2044
Maria Łabanowska
Jagiellonian University: Uniwersytet Jagiellonski w Krakowie
Magdalena Kurdziel
Jagiellonian University: Uniwersytet Jagiellonski w Krakowie
Maria Filek
Pedagogical University of Cracow of the National Education Commission: Uniwersytet Pedagogiczny im Komisji Edukacji Narodowej w Krakowie
Magdalena Skórka
Pedagogical University of Cracow of the National Education Commission: Uniwersytet Pedagogiczny im Komisji Edukacji Narodowej w Krakowie
Anna Barbasz
Pedagogical University of Cracow of the National Education Commission: Uniwersytet Pedagogiczny im Komisji Edukacji Narodowej w Krakowie

Research article

Keywords: zearalenone, EPR, wheat seedlings, infection, redox homeostasis

Posted Date: March 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-307893/v1

Version of Record: A version of this preprint was published at BMC Plant Biology on August 21st, 2021.
See the published version at https://doi.org/10.1186/s12870-021-03177-1.

Abstract

BACKGROUND

Among the environmental stressors, which oxidative action is currently widely studied, are mycotoxins. The aim of presented studies was to investigate the response of leaves of seedlings to zearalenone (ZEN) applied directly to leaves and to grains (indirectly) of tolerant and sensitive wheat cultivars.

RESULTS

Biochemical analyses of antioxidative activity were performed for chloroplasts and showed the similar decrease of this activity independently of plant sensitivity and the way of ZEN application. On the other hand, higher amounts of superoxide radicals (microscopic observation) were formed in leaves of plants grown from grains incubated in solution of ZEN and in sensitive variety. Electron paramagnetic resonance (EPR) studies showed that upon ZEN treatment larger amount of Mn - aqua complexes was formed in leaves of tolerant wheat cultivar than in those of sensitive one whereas the degradation of Fe-protein complexes occurred independently of the sensitivity of plant varieties.

CONCLUSION

The changes in the quantity of organic stable radicals, which were formed by stabilizing reactive oxygen species on biochemical macromolecules, pointed to the higher ability to their generation in leaf tissues subjected to foliar ZEN treatment indicating the important role of these radical species in protective mechanism mainly against direct toxin action. The way of activation the defense mechanisms depended on the method of applications toxin.

Background

Maintaining redox homeostasis in plant cells during actions of the environmental stressogenic factors is vital for proper plant development and yielding. The imbalance of this homeostasis results from the generation of excess of reactive oxygen species (ROS) in relation to the reduction possibilities of the cell [1]. Inactivation of the surplus ROS occurs through the activation/synthesis of enzymatic and non-enzymatic antioxidants, which effectiveness depends, among others, on plant tolerance/sensitivity to stress [2]. Therefore, recognition of differences in the mechanisms of stimulating the stress resistance between varieties belonging to the same species leads to obtaining new genotypes more tolerant toward the changes of the environmental conditions. Since chloroplasts are the organelles in which the ROS production may be one of the steps of photosynthesis process, and that the correct course of photosynthesis is necessary for the proper functioning of plants, hence the changes occurring in these organelles under the influence of stress are of particular importance.

In the reactions occurring in chloroplasts, the disturbance of direct electron transfer from reduced ferredoxin to O_2 evokes changes in the oxidation state of paramagnetic metal ions (mainly Mn, Fe, Cu)

which constitute the redox centres in proteins, involved in electron transfer in photosystems [3]. The study of photosynthesis processes, in which redox reactions occur, including changes in the oxidation state of metal ions as well as the formation of radical species are carried out by different methods. One of them is electron paramagnetic resonance spectroscopy (EPR), which allows recognition of paramagnetic centers, including Fe(III), Mn(II), Cu(II) and various kinds of radicals. High amounts of Fe(III) as well as Fe(II) ions are contained in ferritin, protein molecule, which basic function is iron storage. In conditions of Fe deficiency, ferritin can serve as a donor of this metal ions, however, when Fe is in the excess - for its accumulation [4]. This protective property of ferritin is particularly important because the high concentration of iron ions can act as the oxidative stress stimulator and can lead to oxidative damage of proteins and membrane lipids. In our earlier studies Fe(III) species, the form of Fe ions determined by EPR method in standard conditions, were analyzed based on differences in the character of their signals registered at different temperatures. Variations in the content and character of manganese ions, occurring upon oxidative stress, were also studied, based on EPR spectra [5]. Characteristic hyperfine structure of manganese signals visible in the spectra of inorganic compounds of Mn(II) in oxide environments, for example in the $[Mn(H_2O)_6]^{2+}$ aqua-complexes allowed differentiating these species from organic structures containing interacting manganese ions [6, 7]. Moreover, the signal intensities of these manganese aqua-complexes were different in the spectra of both genotypes which allowed them to be distinguished [5].

In addition to signals derived from paramagnetic metal ions, the EPR technique allows the registration of radical spectra. The measurement of reactive radical species requires the use of a trapping technique, but stable organic radicals, so-called “long-lived radicals” could be recorded without much difficulty. Examination of these species is particularly important in the study of the mechanism of oxidative stress because it has been shown that they are formed on organic molecules, mainly polysaccharides and proteins, as a result of "trapping" electrons generated in excess under stress [8].

Toxinogenic species of the *Fusarium* are cereal pathogens and produce numerous mycotoxins, the most important of which is zearalenone [9]. Their accumulation in plant cells initiates oxidative stress, which disturbs redox homeostasis and therefore is responsible for quantitative and qualitative crop losses. Additionally, mycotoxins taken with plant foods stimulate disease reactions in human and animal cells. In our earlier studies it was found that zearalenone (ZEN) may be localized in chloroplasts and interact with these organelles [10, 11]. The accumulation of ZEN in chloroplasts caused a decrease in the total content of chlorophyll in wheat cells, especially in sensitive variety, indicating semi-destruction of the photosystem [12]. In these studies, the observed effects on leaf chloroplasts resulted from the long-distance translocation of this toxin taken from infected seeds. Another, equally possible way of infection by ZEN is foliar absorption, after colonization by *Fusarium*, leading to significant crop losses of agronomically important plants. The modifications in the structure of chloroplasts in cereal leaves after direct treatment by ZEN were observed by Kornaś et al. [13]. These changes could have been a consequence of ZEN damage of chloroplast membranes resulting from ROS interactions with lipids and/or changes in membranes hydrophilic/hydrophobic structure associated with location of this toxin

[14]. The specific organization of chloroplast membranes containing significant amounts of galactolipid fractions (MGDG and DGDG) can provide an environment in which ROS and free electrons may be trapped at sugar groups of these lipids creating long-lived radicals [5].

The aim of presented studies was to find whether the way of ZEN application determined the oxidative changes in chloroplasts and to what extent these potential changes depended on the resistance of plants to stress conditions. Oxidative potential was examined by biochemical methods in isolated chloroplasts and verified using EPR technique to study changes of paramagnetic centres in leaves of the tolerant and sensitive wheat cultivars subjected to intra- and extracellular ZEN treatment. EPR studies were performed directly on leaves to prevent the influence of additional stressors during sample preparation which could obscure mechanism of ZEN action. Wheat varieties have been chosen for the research, because wheat is the most popular cereal in whole Europe; however, the mechanism of its protection against mycotoxins is still not fully recognized.

Results

The biochemical analysis

Antioxidative properties of chloroplasts described by the percentage of DPPH radical scavenging activity indicated that in control conditions antioxidative activity was higher in Parabola chloroplasts than in Raweta ones (Fig. 1A). Both types of ZEN application resulted in the decrease of this parameter, however, slightly more visible in chloroplasts obtained from plants grown from grains infected by ZEN than in chloroplasts isolated from directly infected leaves. The changes were more noticeable for Raweta cultivar.

Antioxidative activity, expressed as ascorbic acid concentration, was in control plants greater for chloroplasts of Parabola than that of Raweta (Fig. 1B). After ZEN treatment, chloroplasts of Parabola exhibited similar decrease of the antioxidative activity, independently on the way of infection. For Raweta, lower values were obtained when grains were infected by this mycotoxin than when the toxin was absorbed directly by leaves.

In the calculations of reducing power based on the capability of system to deactivate electrons (Fe(III)/Fe(II) changes) Parabola chloroplasts were characterized by higher content of Fe(III) in control conditions, versus Raweta. This meant that Parabola cells contained less free electrons (more efficiently neutralized by antioxidant systems) capable of reducing the Fe(III) ions. ZEN application generally decreased the values of Fe(III) levels, more significantly in chloroplasts of plants directly treated with this mycotoxin (Fig. 1C).

Visualization of ROS

Microscopic observation showed the presence of superoxide radicals in tested chloroplasts, better visible in control samples of Parabola, than that of Raweta, suggesting higher concentrations of these radicals

in tolerant variety (Fig. 2). After ZEN treatment an increase of radical amounts was noted in the chloroplasts and it was greater for these obtained from plants grown from infected grains, compared to chloroplasts originating from foliar treated leaves. The changes were more pronounced in Raweta cultivar.

Electron Paramagnetic Resonance analysis

EPR measurements revealed that regardless of the registration temperature, the characteristic six lines of hyperfine structure (HFS), overlapped on a broader signal at g = 2.00, were observed in the spectra of control samples of leaves originating from both wheat genotypes (Fig. 3A", D). In the spectra recorded at room temperature these signals were not disturbed and slightly more intensive for Raweta. Between the third and the fourth line of hyperfine structure, a narrow signal R at g = 2.00, with similar intensity for both genotypes, was observed. At room temperature the line at g = 2.25, more distinguished in the spectrum of Parabola, was visible (Fig. 3A, A'). Decrease of registration temperature to 77 K led to the appearing of broad lines at g = 2.6 and g = 2.4 which became the main signals of the spectra. Additionally, low intensive lines between the main ones of HFS became visible (Fig. 3D, D') and not very intense signal at g = 4.26 appeared at spectra of both genotypes. The character of signal R changed, the line broadened and increased its intensity (signal R') (Fig. 3D, 4D).

In the spectra of leaves of wheat plants grown from grains subjected to ZEN treatment more significant changes were observed for Raweta. In the range of g values 2.2–2.5 new signals appeared in the spectrum recorded at room temperature (Fig. 3B). At 77 K, signals in this range disappeared and lines at g = 2.4 and 2.6 became visible, similarly as it was detected in control samples (Fig. 3E). Simultaneously, signal R changed to R', as it was observed in control samples.

Spectra of leaves directly treated with ZEN were different than those of plants grown from grains infected with this mycotoxin (Fig. 3C, F). The main change in the spectrum of Raweta recorded at room temperature was connected with the appearance of a strong line at g = 2.06 and four lines around g = 2.4, with small, equal intensities, whereas in Parabola spectrum the vanishing of the signal at g = 2.25 with simultaneous appearing of signal at g = 2.5 was observed (Fig. 3C'). In Raweta spectrum recorded at 77 K the signal at g = 2.6 decreased and mainly line at g = 2.4 was visible (Fig. 3F), whereas in Parabola spectrum the broad signal at g = 2.6 only slightly decreased (Fig. 3F'). In order to scrutinize the narrow line R, the spectra were recorded in the range of 5 mT.

The simulation of line R at g = 2.00 in the spectrum of control plant showed that it consisted of two components: signals A and B (Fig. 4A). Signal A with a contribution to the spectrum about 70 % exhibited small anisotropy and parameters: g_1 = 2.0064, g_2 = 2.0042, g_3 = 2.0037, g_{av} = 2.0048, A_{av} = 0.2 mT, whereas signal B was isotropic, with g = 2.0050, A = 0.7 mT. Spectra of leaves originating from plants grown from ZEN treated grains (Fig. 4B) were composed of signals A and B with increased g values to g_{av} = 2.0054 and g = 2.0058, respectively. The anisotropy of signal A diminished, whereas the hyperfine constant for center B increased to 1.1 mT. Simultaneously, a weak signal (signal C) at g = 2.0035 split to

four almost equidistant lines (A_1 = 0.8 mT, A_2 = 1.6 mT) with similar intensities had to be added to the simulated spectrum for its good fitting to the experimental line. Foliar ZEN treatment resulted in the spectrum (Fig. 4C) containing signal A, signal B with increasing value of HFS constant (A_1 = 1.6 mT) and signal C with higher contribution than that found in spectrum of leaves of plants grown from ZEN treated grains. The temperature decrease led to the change of signal R into a new one, significantly broader than R, marked as R'. Its parameters for control samples: g_1 = 2.0081, g_2 = 2.0045, g_3 = 2.0013, g_{av} = 2.0045, A_2^{β} = 1.7 mT (Fig. 4D) changed slightly for signals observed in spectra of leaves directly treated with ZEN (Figs. 4E, F).

Discussion

Biochemical analysis performed on chloroplasts isolated from studied plants seedlings allowed detecting the differences in ability of these organelles to protect plants against oxidative stress resulted from ZEN accumulation, depending on stress tolerance of wheat varieties and the way of application of this mycotoxin. Higher amounts of superoxide radicals observed in chloroplasts of the sensitive variety versus tolerant might indicate less efficient action of antioxidant system in this variety. It was interesting, that this phenomena was observed in samples regardless of the method of application and subsequent transport of ZEN to chloroplasts. Better potential antioxidant properties of chloroplasts of the tolerant cultivar compared to sensitive one were confirmed by higher values (under control conditions) of all tested indicators (DPPH, AA, Fe(III)/Fe(II)).

DPPH appears in the presence of hydrogen donating antioxidants in the non-radical form [15]. The decrease of scavenging activity upon ZEN treatment could result from involvement of antioxidants in removal of ROS generated under stress. The greater decrease associated with long-term ZEN-stress (application to grains) could be caused by more effective synthesis of antioxidants than after short-term interaction of ZEN (foliar treatment), similarly as it was observed in microscopic visualization of superoxides.

Ascorbic acid (AA) takes part as a factor in redox reactions in which one reactive substance is reduced at the expense of the oxidation of another [16]. Also the values of "reducing power" expressed as the ability of the reduction Fe(III) ions to Fe(II) indicated that redox reactions in chloroplasts under ZEN application played an important role in defense processes. A higher content of Fe(III) ions in control samples of tolerant plants was connected with the lower level of free electrons that might reduce these ions to Fe(II) and pointed to more effective action of the antioxidative system of this variety. The decrease of Fe(III) amounts, caused by ZEN application (greater in case of foliar treatment than when grains were ZEN infected) indicated the increase of free electrons amounts, generated in stress conditions. The decrease of reducing power was bigger in samples originating from sensitive wheat genotypes, confirming that antioxidant system was less efficient in this genotype.

The studies of EPR signals, despite the fact that the measurements were not performed directly on chloroplasts, allowed characterization of paramagnetic metal ions whose main locations and functions

were associated with these organelles. Moreover, changes in redox status of metal ions upon ZEN treatment could be monitored. The analysis of parameters of EPR signals appearing in spectra of studied plant material combined with literature data allowed attribution of observed signals to particular paramagnetic species. The signal of six hyperfine lines overlapping a broad one was ascribed to Mn species. Well resolved hyperfine structure observed at room temperature originated from freely rotating aqua - complex of Mn(II) and was often found in EPR spectra of various plants [6, 10], while the broad line was ascribed to dipole - dipole interacting Mn(II) ions situated mainly in protein matrix [17]. Signals observed in g range between 2.2–2.5 were attributed to inorganic antiferromagnetically coupled paramagnetic Fe(III) ions forming Fe-O-Fe clusters, ferric oxides, oxyhydroxides and/or phosphates which were accumulated in the “iron-core” of ferritin [18, 19], whereas broad signals at g = 2.4 and 2.6, appearing at 77 K, were ascribed to Fe(III) ions bonded to protein matrix in the ferritin protein shell, containing ferric and ferrous ions [20, 21]. The small line with g = 4.26, observed in all spectra recorded at 77 K, was attributed to non-hem high spin Fe(III) with rhombic symetry [6].

Calculation of intensity of particular signals, recorded at room temperature, indicated that at control conditions, signal of Mn(II) ions had a significant contribution to the spectrum, especially for Parabola, in which its intensity was about 4 times bigger than that of Fe(III), while for Raweta only about 2.5 times (Fig. 5A’, A”). Upon ZEN treatment the observed increase of integral spectrum intensity of Parabola leaves (Fig. 5A), measured at room temperature, was caused mainly by changes of manganese signal, whereas for Raweta genotype it resulted from the growth of Fe(III) signals at g in the range 2.2–2.5 (Fig. 3B, 5A”). The stronger increase of signal intensity of manganese aqua complexes for Parabola than for Raweta, treated with ZEN independently of application way (Fig. 5A’, A”), could suggest better accumulation of water in tissues of tolerant genotype. The binding of water molecules in plant tissues may be considered as one of factors in the cell stress protection ensuring the appropriate quaternary structure of proteins participating in photosynthesis processes. In sensitive genotype growing intensity of Fe(III) signal could result from oxidation of Fe(II) species by ROS, generated during ZEN action (Fig. 3B, 5A’, A”). At applied EPR measurement conditions Fe(II) ions are invisible in spectra. The strong line at g = 2.06 and four lines around g = 2.4, situated in the place of Fe(III) signals, observed in the spectrum of leaves of Raweta directly treated with ZEN, were ascribed to Cu(II) ions in square planar complexes of proteins [6] (Fig. 3C). Hence, it could be suggested that Cu(I) species, silent in EPR, were probably present in untreated plant material and underwent oxidation to Cu(II) upon ROS, similarly as it was observed for Fe species.

In the spectra of control plants measured at 77 K, contrary to room temperature, the signals of Fe(III) ions with g equal to 2.4 and 2.6 were dominant (Fig. 5B”) and two times higher in spectra of Parabola genotype. Upon ZEN treatment the intensity of these signals decreased, whereas that of Mn(II) signals increased for both varieties. The changes of Fe(III) signals were more visible for Parabola when grains were ZEN-infected, whereas for Raweta these changes were smaller. In the latter, Fe(III) signal (g = 2.4) in the spectrum of ZEN treated leaves overlapped Cu(II) signal (Fig. 3F, 5B”), hence the intensity of the line was a sum of those originating from Fe and Cu species.

Decrease upon ZEN of amount of Fe(III) species in plant tissues (Fig. 5B', B") resulted probably from the degradation of Fe(III) - protein complexes and was observed for all plants treated with ZEN, regardless of the way of its introduction (directly or indirectly) confirming that ZEN exerted negative influence on plants. The amount of Fe(III) ions present in plants subjected to ZEN treatment was the result of two processes: degradation of ferric protein complexes (this process was visible in the spectra recorded at 77 K) and oxidation of ferrous ions probably by ROS generated upon ZEN stress (observed in the spectra measured at 293 K). The observed phenomenon indicated that the way of ZEN treatment influenced the mechanism of damage of plant structures.

The changes of signals originating from radical species can provide further data on the influence of ZEN on wheat plants. Both overlapping signals, A and B, giving line R recorded at room temperature in spectra of control samples, were characteristic for carbon centered radicals located in carbohydrate molecules [5, 21]. The changes in intensities of these signals upon ZEN treatment were connected with modification of the signal R by the appearance of additional lines situated symetrically around line R (Fig. 4B, C), which were more intensive in spectra of leaves directly treated with ZEN. The lower value of g factor of signal C could indicate that when ZEN interacted directly with leaves, radicals were created at carbohydrate molecules with lower molecular weight, whereas the species created in plants grown from infected grains, giving signals with higher g factor, were situated at carbohydrates with higher molecular weight, for example in starch.

Intensity of signal R measured at room temperature was slightly lower for Raweta control plants than Parabola ones. ZEN treatment of grains decreased it, whereas application to leaves increased its value, more visibly in Raweta (Fig. 6A). The creation of stable carbohydrate radicals occurred as a result of transfer of free electrons from ROS, formed upon ZEN action, to carbohydrate molecules. Such stabilization of electrons, more effective in Raweta, was one of the defense mechanisms of plant against stress, as it was postulated by our earlier hypothesis, assuming that short direct stress led to the increase of the amount of stable carbohydrate radicals mainly in sensitive genotypes, whereas in tolerant ones other mechanisms were activated [5].

A new signal R', recorded at 77 K (Fig. 4D-F), observed in many plant systems was ascribed to the stable tyrosyl radical [22], acting as an electron transfer in biochemical redox processes [23]. Its amount increased strongly in leaves being in the direct contact with ZEN, simultaneously with increasing g_1 parameter to 2.0091, suggesting that upon ZEN treatment the disturbance of tyrosyl radical geometry occurred. It was probably caused by removal of the hydrogen atom from the hydroxyl group of the tyrosine molecule [19, 22] by reactive oxygen species formed upon ZEN stress. It pointed to destruction of organic matrices, as it was indicated by disorder in biochemical surroundings of metal species, more noticeable in sensitive genotype. The lack of significant alterations in tyrosyl radical concentration in plants grown from grains treated with ZEN was in line with observed small changes in amounts of carbohydrate radicals and could indicate that if more time elapsed from the moment of contact with ZEN, the redox equilibrium in plant tissues was re-established, whereas after direct stress processes of radical transformation continually occurred.

Conclusion

EPR studies pointed that infection of plants with ZEN led to damage of iron - protein complexes and to changes of the redox status of metal ions being important components of antioxidative system. Moreover, it was found that mechanism of ZEN interaction with biochemical structures depended on the way of ZEN application. In the case of directly infected leaves the defense mechanism based on stable organic radicals generation was started, mainly in sensitive genotypes, whereas plants growing from grains contaminated by ZEN adapted to disturbed equilibrium in their tissues probably by increasing accumulation of water and starch.

These observations could suggest that the mode of defense mechanisms activated in plants depended on the method of ZEN application.

Methods

Chemicals

All reagents (including zearalenone) were purchased from Sigma-Aldrich Company (Germany, Munich).

Plant material

Spring wheat of two cultivars: Parabola and Raweta were obtained from the Polish Plant Breeding Stations (Radzików and Strzelce, Poland). The spring wheat varieties with varied resistance to oxidative stress (confirmed in our earlier experiments [10] i.e.: Parabola (tolerant) and Raweta (sensitive) were used. The grains of both cultivars were germinated during 2 days in distilled water and in ZEN solution (30 $\mu mol.dm^{-3}$) at 20°C (dark). Next, seedlings were grown in perlite to obtain the 3-leaves plants (with 2nd well developed leaf) in greenhouse conditions at 20/17°C (day/night) with 16 h photoperiod and 800 $\mu mol.dm^{-3}$ (photon) $m^{-2}.s^{-1}$ light. Then, pool of plants not treated with ZEN was divided into two groups. Plants of one group were used as controls (0), whereas these of the other one were the objects of experiment, in which the second leaves of seedlings were covered with ZEN solution (10 $\mu mol.dm^{-3}$) by brushing. Measurements were performed 24 hours after treatment. The lower ZEN concentration, in comparison to this used in grain treatment, was chosen on the basis of Kornaś et al. [13] studies, which showed that there were no significant differences in the structure of chloroplasts obtained from samples of intra- (grains) and extracellular (leaves) ZEN treated. For each treatment 10 pots with 12 plants in three independent replication were used. All experiments were performed on the 2nd leaves of seedlings.

Chloroplast isolation

Fresh leaves, collected from plants were immediately homogenized in buffer solution containing 50 $mmol.dm^{-3}$ Tris-HCl, 5 $mmol.dm^{-3}$ EDTA, 0.33 $mol.dm^{-3}$ sorbitol (CIB), pre-filtered on a nylon mesh with a mesh size of 1000 μm and centrifuged (400 x g). The supernatant was than centrifuged at 1000 x g. The chloroplast containing sludge was suspended in CIB and purified in Percol (40%/80%) gradient to obtain

the pure organelles, according to procedure described earlier [24]. The whole experiment was carried at 4°C. Visualization of superoxide radicals were performed on freshly prepared chloroplasts. For biochemical analysis chloroplasts were frozen in liquid nitrogen and stored at − 80°C in dark.

Determination of antioxidant activity

Chloroplasts were homogenized in methanol and centrifuged for 10 minutes at 1000 x g. The supernatant was used for further research. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) deactivation was analyzed spectrophotometrically (Thermo Scientific Evolution 200) at λ = 517 nm after 30 minutes incubation of extract with a mixture of 3 ml of 0.004% DPPH solution, in dark [25].

The antioxidant activity described by Prieto et al. [26] based on the reduction of Mo(VI) to Mo(V) and creation of a green phosphate/Mo(V) complex at acid pH. The plants extract was incubated (95°C for 90 min) with the solution containing 300 $mol.dm^{-3}$ sulfuric acid, 28 $mmol.dm^{-3}$ sodium phosphate and 4 $mmol.dm^{-3}$ ammonium molybdate and measured at λ = 695 nm spectrophotometrically. The antioxidant activity was calculated as the mg of ascorbic acid (AA) per 1 g of FW.

Analysis of the reducing power with Fe(III)

This parameter was determined in samples containing 100 µl of the extract mixed with 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferrocyanide solution, incubated at 50°C for 20 min and centrifuged at 1000 x g for 10 min. Then 0.1% $FeCl_3$ solution was added to supernatant, and centrifuged again. The samples were measured spectrophotometrically at λ = 700 nm.

Cellular superoxide radical assay

Superoxide radicals were detected using a Cellular Superoxide Detection Assay kit (Abcam, Cambridge, UK). The isolated chloroplasts were incubated with superoxide detection mix for 30 min at 37°C. The positive control was incubated with 0.2 ml/l pyocyanine. After washing, superoxide-positive chloroplasts (red) were observed using a Canon EOS 60D camera (Tokyo, Japan) and Delta IB-10 optical microscope set (Poland) with filter fluorescence set (x 400 magnification).

Electron Paramagnetic Resonance (EPR) spectroscopy

Measurements of EPR signals of the freshly collected leaves (samples weight: 0.02–0.03g) were recorded at X-band Bruker Elexsys 500 spectrometer (Karlsruhe, Germany) with 100 kHz field modulation. Analyses were made at 293 K and 77 K, with the microwave power of 3 mW and 10 mW in the range of 5 mT and 500 mT, respectively. DPPH (1,1-diphenyl-2-picrylhydrazyl) was used as a g-factor standard. The parameter g and constants of hyperfine splitting of particular radical signals were found by the simulation procedures with program SIM 32 [27]. Integral intensities of whole spectra and radical ones, as well as intensities of signals of particular paramagnetic metal ions were determined. The accuracy of g-factor was ± 0.0005 and ± 0.1 mT of A parameter for radical signals and of ± 0.05 and ± 0.5 mT for g and A parameters respectively, for signals of transition metals ions.

Statistical analysis

All data were presented as means ± SE and significance of differences (at $P < 0.05$) were calculated from Duncan's multiple range test and Student's t-test using SAS, version 10.0 (SAS/STAT software).

Abbreviations

AA- Ascorbic acid; HFS -hyperfine structure; CIB- chloroplast isolation buffer; Cu- cooper; DPPH-2,2-diphenyl-1-picryl-hydrazyl-hydrate; EPR- electron paramagnetic resonance; Fe- iron; MGDG – monogalactosyldiacylglycerol; DGDG- digalactosyldiacylglycerol; Mn- manganese; SOD – superoxide dismutases; ROS- reactive oxygen species; ZEN- zearalenone.

Declarations

Authors information

[1]Institute of Biology, Pedagogical University, Podchorążych 2, 30-084 Krakow, Poland

Apolonia Sieprawska, Maria Filek, Magdalena Skórka, Anna Barbasz

[2]Faculty of Chemistry, Jagiellonian University, Gronostajowa 2, 30-387 Krakow, Poland

Maria Łabanowska, Magdalena Kurdziel

Correspondence to: apolonia.sieprawska@up.krakow.pl

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding The research was financially supported by National Center of Science (NCN) Poland No. 2014/15/B/NZ9/02192. The funding organization provided the financial support to the research, but

were not involved in the design of the study, data collection, analysis of the data, or the writing of the manuscript.

Authors' contributions

SA and FM conceived and designed the experiments; SA, ŁM, KM, SM and BA ran the experiments; SA, ŁA, KM and FM analysed the data and wrote the main manuscript. All authors read and approved the manuscript.

Acknowledgements

Not applicable.

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Figures

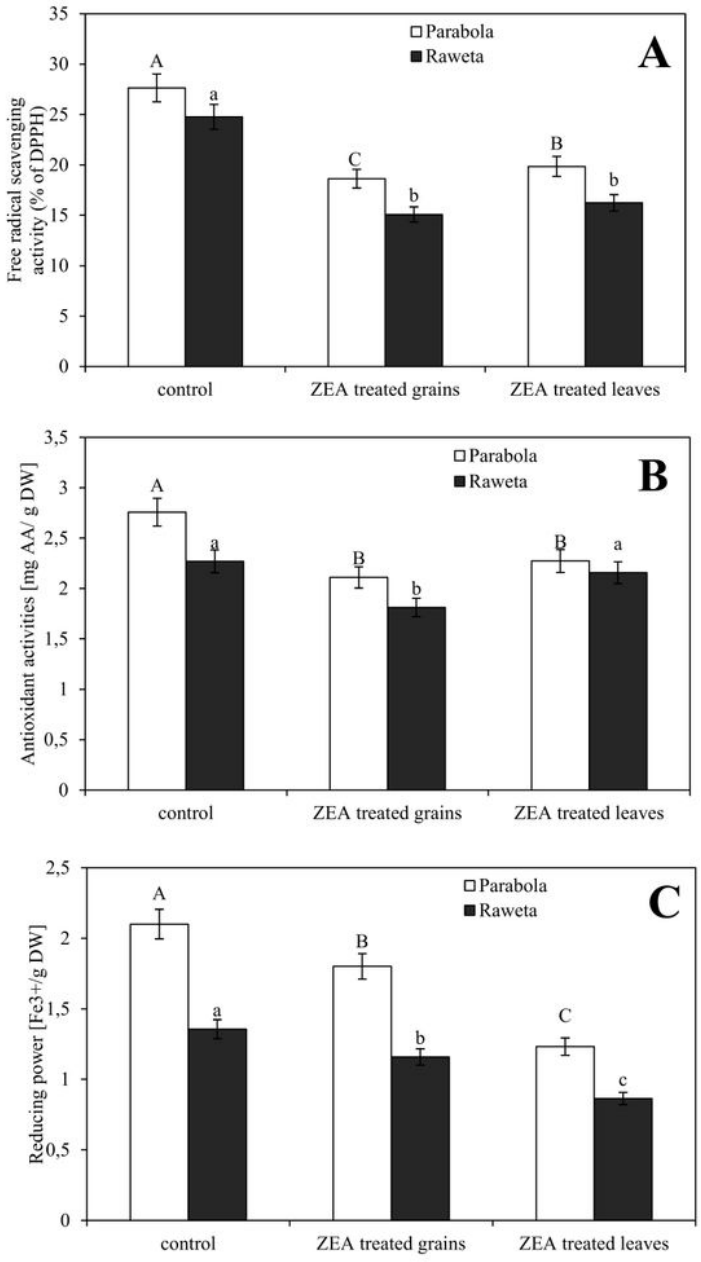

Figure 1

Antioxidant activities (expressed as percentage of reduced DPPH, the amount of ascorbic acid, AA and Fe3+ ions) in the chloroplasts of Parabola and Raweta wheat obtained from control, ZEN treated grains and foliar ZEN treatment. Data represent the mean from three independent experiments ± standard error (SE). Different letters indicate significant inter-group differences, $p \leq 0.05$.

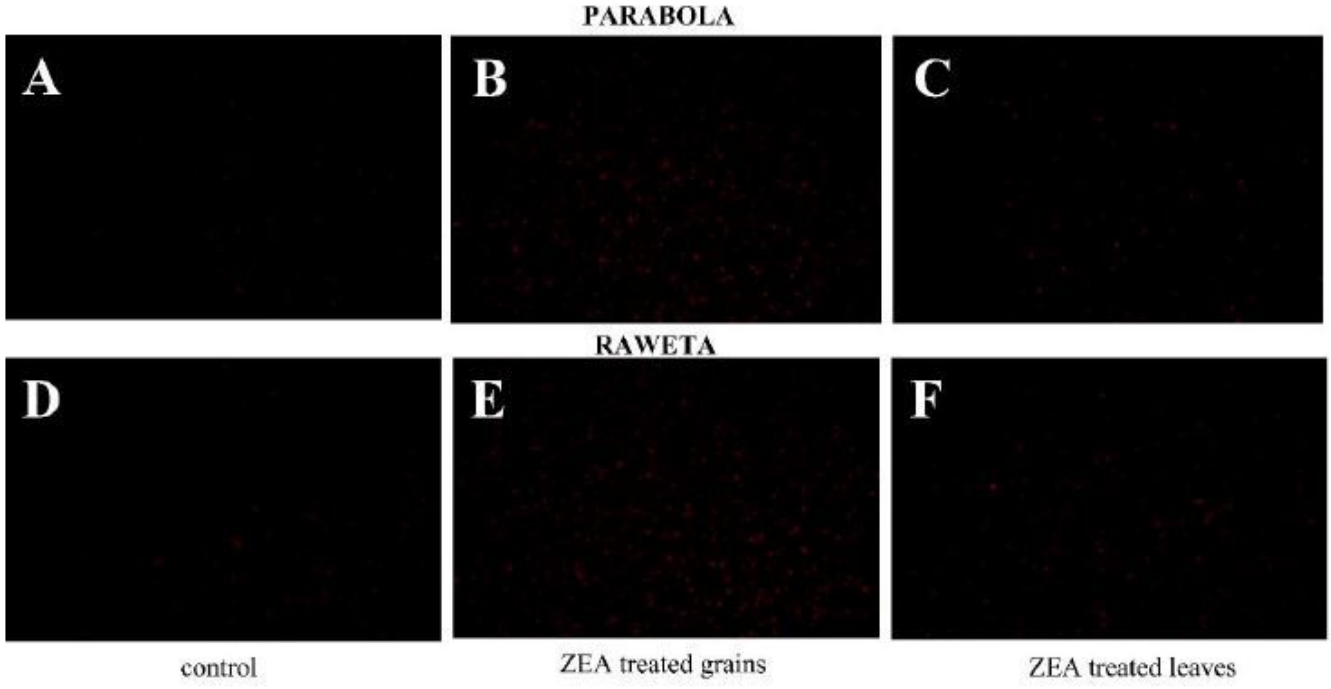

Figure 2

Superoxide radicals activity in the chloroplasts of Parabola and Raweta wheat obtained from control, ZEN treated grains and foliar ZEN treatment. Data represent the mean from three independent experiments ± standard error (SE). Different letters indicate significant inter-group differences, $p \leq 0.05$.

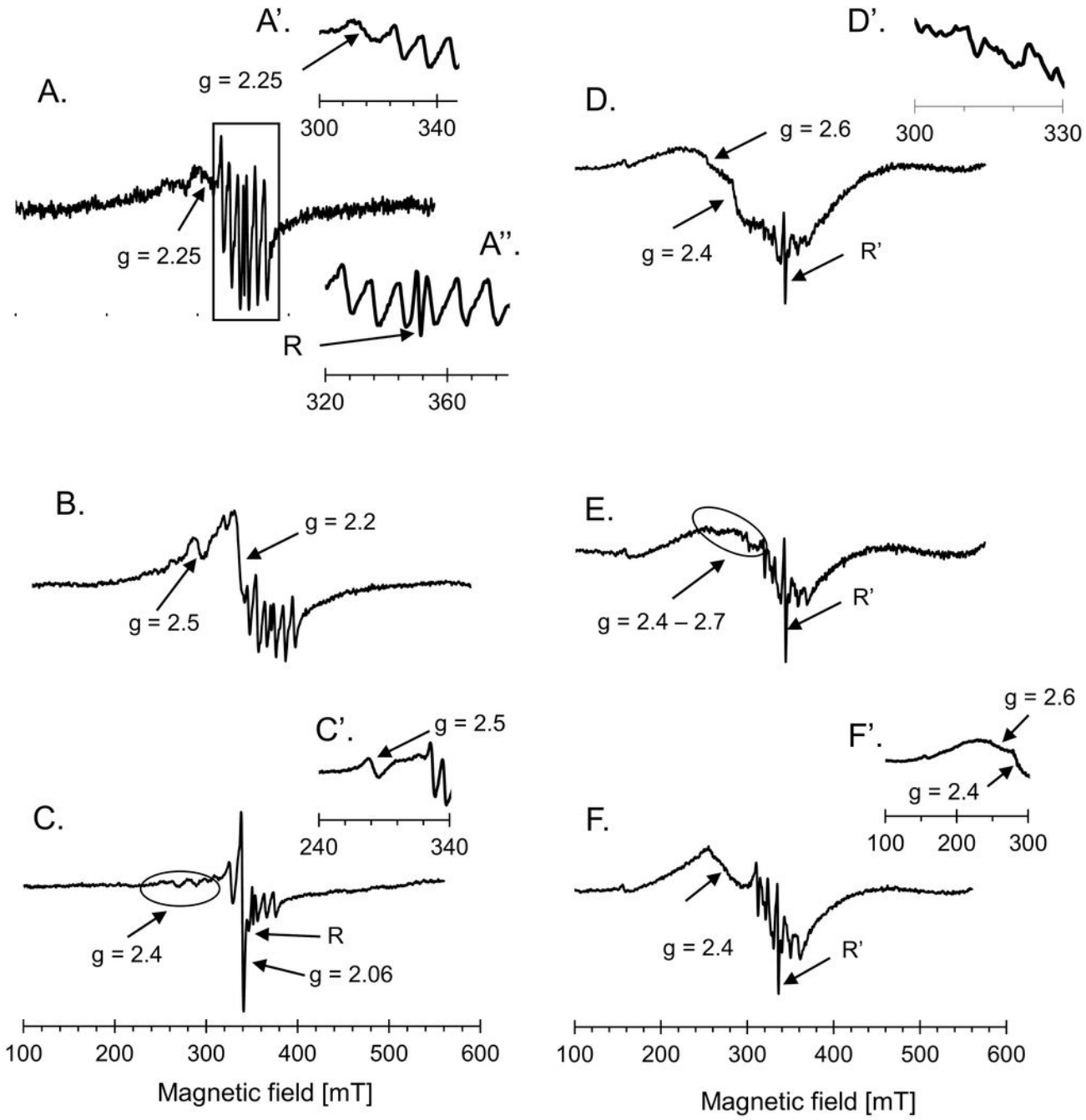

Figure 3

Examples of EPR spectra of Raweta leaves recorded at the range of 500 mT, measured at 293 K: A – control, B – obtained from ZEN treated grains, C – foliar ZEN treatment; A' – Parabola control (part of spectrum), A" – enlarged part of spectrum of Raweta control with HFS and R signals, C' – infected leaves of Parabola (part of spectrum): measured at 77 K: D – control, E – obtained from ZEN – treated grains, F

– foliar ZEN treatment; D’ – hfs structure of Raweta control, F’ – infected leaves of Parabola (part of spectrum).

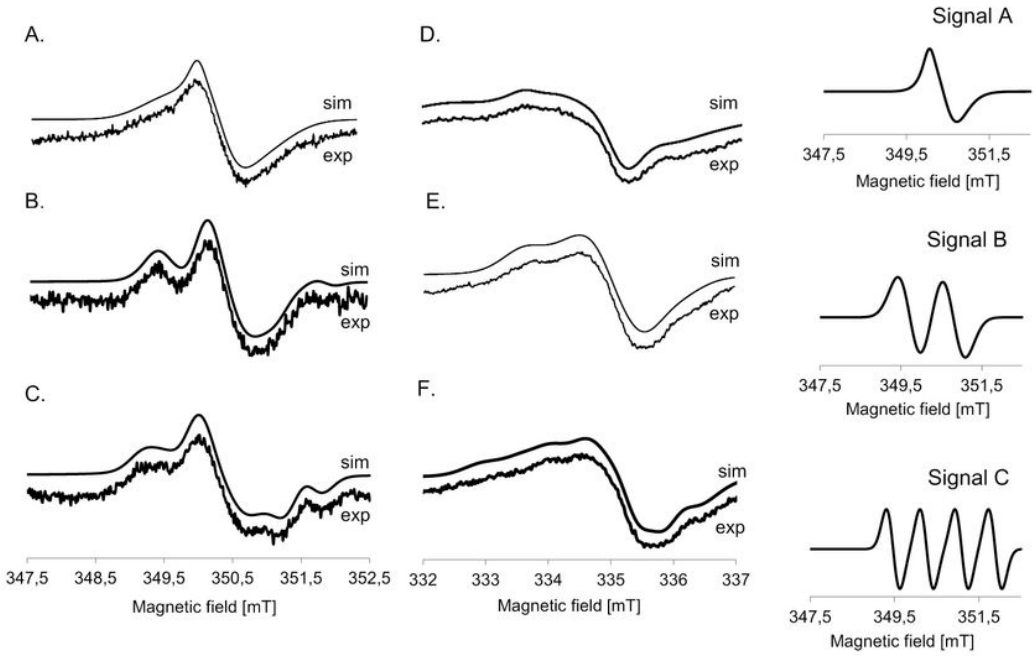

Figure 4

Examples of EPR spectra of organic radicals in Raweta leaves recorded at the range of 5 mT, measured at 293 K: A – control, B – obtained from ZEN – treated grains, C – foliar ZEN treatment; measured at 77 K: D – control, E – obtained from ZEN – treated grains, F – foliar ZEN treatment. Signals A, B and C were used to simulation of spectra recorded at 293 K.

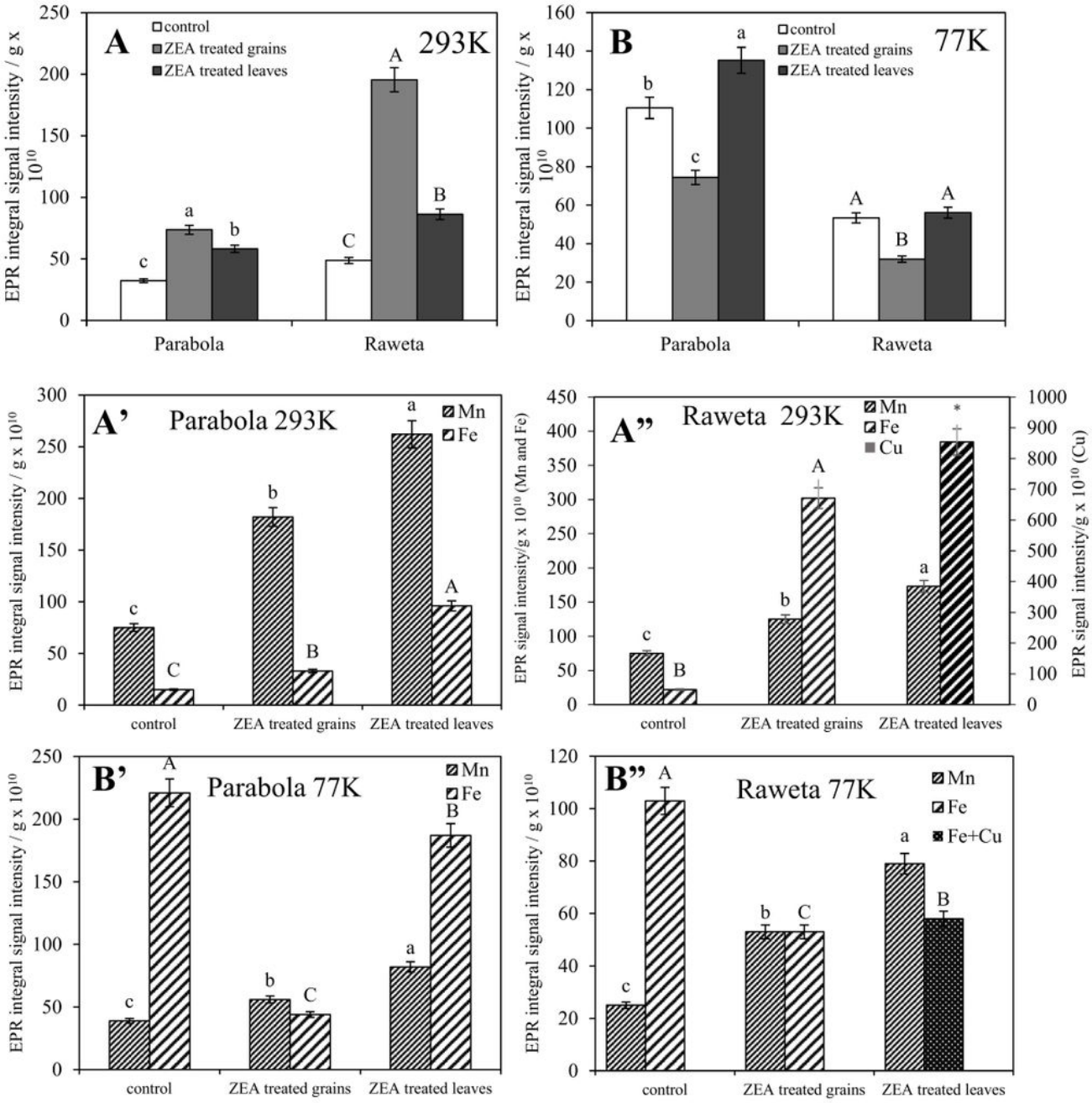

Figure 5

EPR Integral signal intensity of spectra recorded in the range of 500 mT at temperature 293 K (A) and 77 K (B) for Parabola and Raweta leaves. The results are the means (n=5) ± SE; significant differences (P<0.05) between treatments separately for each genotype are marked by different letters. Intensity of Fe(III), Cu(II) and Mn(II) signals recorded in the range of 500 mT at temperature 293 K (A', A") and 77 K (B',

B”). The results are the means (n=5) ± SE; significant differences (P<0.05) between treatments separately for each element are marked by different letters.

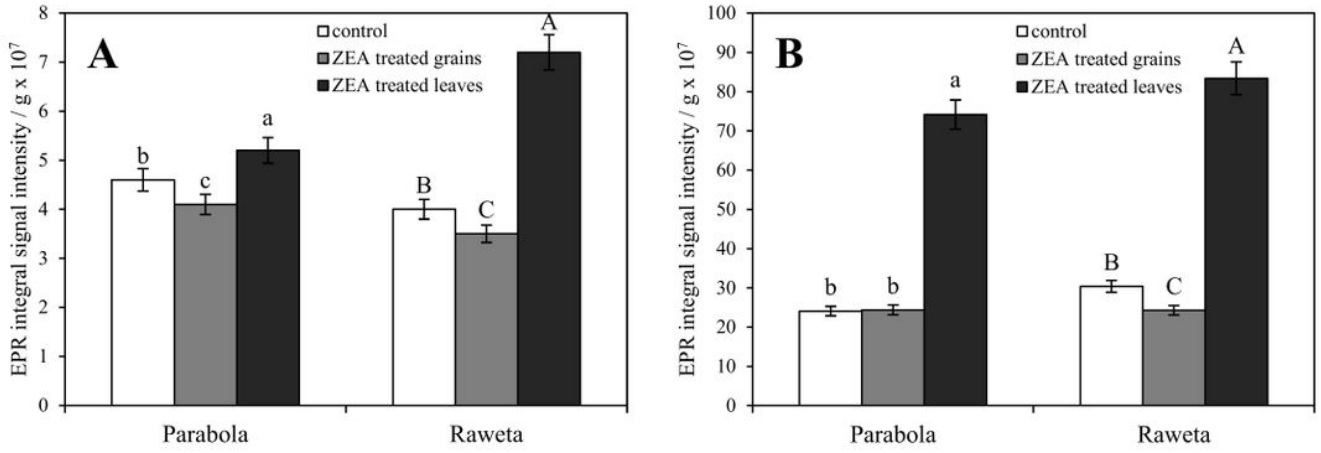

Figure 6

Integral signal intensity of organic radicals recorded in the range of 5 mT at temperature 293 K (A) and 77 K (B). The results are the means (n=5) ± SE; significant differences (P<0.05) between treatments separately for each genotype are marked by different letters.