



Research article

The role of chloroplasts in the oxidative stress that is induced by zearalenone in wheat plants – The functions of 24-epibrassinolide and selenium in the protective mechanisms

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ABSTRACT

This study focused on the idea that the toxic effect of zearalenone (ZEA) and the protective actions of the brassinosteroid - 24-epibrassinolide (EBR) as well as selenium are dependent on its accumulation in chloroplasts to a high degree. These organelles were isolated from the leaves of oxidative stress-sensitive and stress-tolerant wheat cultivars that had been grown from grains that had been incubated in a solution of ZEA (30 μM), Na_2SeO_4 (Se, 10 μM), EBR (0.1 μM) or in a mixture of ZEA with Se or EBR. Ultra-high performance liquid chromatography techniques indicated that ZEA was adsorbed in higher amounts in the chloroplasts in the sensitive rather than tolerant cultivar. Although the brassinosteroids and Se were also accumulated in the chloroplasts, higher levels were only found in the tolerant cultivar. The application of EBR increased the homocastasterone content, especially in the chloroplasts of the tolerant plant and after the addition of ZEA. The presence of both protectants caused a decrease in the ZEA content in studied organelles and resulted in diminishing of the oxidative stress (i.e. changes in the activity of the antioxidative enzymes). Moreover, a recovery of photosystem II and decrease in the negative impact of ZEN on *Hsp90* transcript accumulation was observed in plants.

1. Introduction

Zearalenone (ZEA), a mycotoxin that is produced by *Fusarium*, is a known growth regulator and its occurrence in plants at low concentrations may stimulate the generative development in wheat (Biesaga-Kościelniak and Filek, 2010). However, when it is accumulated at a higher level than the hormonal content, it is responsible for losses in yield and the infection of the seeds of plants that are important in the food industry (Beccari et al., 2017). In recent years, the complex research that is being undertaken by many scientific institutions has been aimed at attempting to understand the mechanisms of the stressogenic effects of mycotoxin activity in order to find the best protection methods against this substance. The finding that ZEA inhibits H^+ extrusion via changes in the ATPase activity and therefore affects the depolarization of transmembrane potentials (Pietsch, 2017) suggests that it affects the membrane properties. Interactions between ZEA and

membrane components can not only modify the activity of the proton pump, but also other ion pumps and channels, thereby leading to changes in the permeability and disturbances of the homeostasis of the elements that are important for proper cell functioning. The impact of zearalenone on the disruption of the photosynthesis process has primarily been studied in terms of the hormonal effects of this substance (Kościelniak et al., 2009). It was found that even at low concentrations of ZEA, its impact on photosystems was dependent on the plant species and usually reduced the parameters of photosynthesis (i.e. the energy flux per cross section (CS) for absorption (ABS/CS), trapping (TRo/CS) and electron transport (ETo/CS)). On the other hand, in some cases, for example, in soybeans, ZEA treatment induced the opposite reaction and the values of those parameters increased (Kościelniak et al., 2011). The changes in photosynthetic activity were mainly affected by disturbances in the functioning of the photosystems that are located in the chloroplasts and it is obvious that ZEA might interact with these

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organelles. However, until now, it has not been shown whether ZEA can be accumulated in these organelles, especially in the case of its long-distance translocation from infected roots (or grains) to the developing uninfected leaves.

In our earlier studies (Filek et al., 2017), it was found that 24-epibrassinolide (EBR) and/or Se ions when applied at low, non-stress-inducing amounts into wheat grains diminished the ZEA uptake and its translocation into the germinating hypocotyls. EBR and Se were selected as alternatives to the many protection agents that can produce toxic effects in plant cells themselves. Brassinosteroids (BRs) are plant steroid hormones and are commonly found in various plants. At a hormonal concentration, they modify the development and defense processes under many stressful conditions (Janeczko et al. 2010, 2016). In spite of studies that have demonstrated the significance of the presence BRs in plant cells, the mechanism of their action has not yet been explained in detail. Furthermore, EBR, which is usually used for exogenous applications, can be metabolized into other BRs, and therefore, it is not known which forms of BRs might be really responsible for modifying the BRs-stimulated processes. In studies of Janeczko et al. (2010), it was indicated that treating wheat with EBR affected the synthesis of brassinolide, castasterone and 24-epicastasterone. In our earlier experiments in which *in vitro* wheat cells were directly treated with EBR (Filek et al., 2018), homocastasterone, except for castasterone, was synthesized in significant amounts. Because these cultures were grown in the dark, the difference in the EBR metabolism might have been an effect of the lack of light radiation and the presence of only pro-plastids (the precursors of chloroplasts) (Filek et al., 2010a). The effect of improving the photosynthesis parameters that was observed after EBR application (Xia et al., 2009) seems to confirm the significance of well-developed chloroplasts in the BR-stimulated physiological processes.

The other protectant that was used, selenium, which is an important element in the human diet, has been proposed to be used for fertilizing cereals in countries in which the soils are poor in this nutrient. The beneficial effect of Se ions in improving photosynthesis effectivity in oxidative stress conditions was well documented in Sieprawska et al. (2015). Recently, Jiang et al. (2017) indicated that Se ions visibly reversed the destructive effects of salinity on the photochemical efficiency of photosystem II (PSII) in maize. The of Se was particularly analyzed in heavy metal-stressed plants (Qing et al., 2015). It was indicated that the activation of the photosynthesis process by an accumulation of Se under Cd-stress conditions was connected with an improvement in the chloroplast membrane structure that had been damaged by Cd (Filek et al., 2010b). The participation of Se in the direct protection of the stabilization of the bio-membranes under stress conditions was confirmed by Gzyl-Malcher et al. (2017).

The aim of the presented studies was to analyze the effect of ZEA that was applied at a toxic concentration and EBR and Se that was used in protective amounts in wheat seedlings in order to determine the significance of chloroplasts as the organelles that participate in the induction of both the stress and defense mechanisms. The experiments focused on studies of (i) the accumulation of ZEA, EBR and Se in the chloroplasts; ii) the impact of these substances on the parameters of photosynthesis and the chlorophyll content and (iii) the stimulation of an antioxidative response. Changes in the antioxidative enzyme activity have commonly been used as indicators of the intensity of stress action as the indirect factor of ROS generation. We analyzed the level of the antioxidative enzymes (superoxide dismutase SOD and ascorbic peroxidase APX) and non-enzymatic antioxidants – carotenoids. The isoforms of the SODs that participate in the overall catalysis of the reduction of reactive superoxide (O_2^-) to hydrogen peroxide and peroxidases (mainly APX), which are involved in the decomposition of H_2O_2 to water, were found in the chloroplasts. Since carotenoids are usually considered to be substances that are involved in protecting membranes (Gruszecki and Strzałka, 2005), their content was determined. Moreover, (iv) changes in the *Hsp90* expression were also

examined. Recently, heat shock protein 90 (*Hsp90*) has been intensively studied as a mediator of stress signal transduction (Xu et al., 2012a, b). Thus, the accumulation of the transcript of *Hsp90* was also analyzed in order to enrich the information about the potential initiation of ZEA stress and the protective role of EBR and Se in ZEA stress.

A comparison of the observed biochemical effects between stress sensitive and stress tolerant cultivars may be helpful in understanding the mechanisms that counteract environmental stresses in which one of the first physiological symptoms is a disturbance in the functioning of the photosystems that are located in the chloroplasts.

2. Material and methods

2.1. Plant material

Two cultivars of spring wheat with varying resistances to oxidative stress (confirmed in earlier experiments (Filek et al., 2017)), i.e. Parabola (tolerant) and Raweta (sensitive) were used in the study. After sterilization, the grains were germinated for two days in solutions containing in addition to distilled water (control, 0) ZEA (30 μ M), Se (10 μ M), EBR (0.1 μ M) and mixtures of ZEA + Se (30 μ M + 10 μ M) and ZEA + EBR (30 μ M + 0.1 μ M) at 20 °C (dark). Then, the germinated seeds were placed into pots with perlite and cultivated (for about 14 days) to obtain the seedling phase (a second well-developed leaf) in the controlled conditions of a greenhouse at 20/17 °C (day/night) with a 16 h photoperiod and 1000 μ mol (photon) $m^{-2} s^{-1}$ light. For each treatment, ten pots with 12 plants were used in three independent replications. All of the experiments were performed on the second leaves. For the biochemical experiments, leaf samples were frozen in liquid nitrogen and stored at -80 °C.

2.2. Chloroplast isolation

For chloroplast isolation, fresh leaves that had been directly collected from the plants were carefully homogenized in a buffer containing 50 mM Tris-HCl, 5 mM EDTA, 0.33 M sorbitol (CIB) in a porcelain mortar and centrifuged (1000 g). The supernatant was purified in a Percol (40%/80%) gradient as was described earlier (Filek et al., 2010a). All procedures were carried out at 4 °C. The chloroplasts were stored at -80 °C in the dark until further analysis.

2.3. Determining the zeaxalenone concentration

An ultra-high performance liquid chromatograph (UPHPLC; Infinity 1260, Agilent Technologies) coupled with a quadruple mass spectrometry detector (QQQ 6410) was used to detect the ZEA content in the leaves and chloroplasts of the studied plants according to a previously described procedure (Gromadzka et al., 2015). The samples were initially homogenized with acetone:water (95:5; v/v) and purified on a Bond Elut Mycotoxin column (45 mm/1000 mg, Agilent Technologies). Then, the extract was introduced into an analytical column (LN B10006, Poroshell 120 Phenyl-Hexyl) at 3.0×100 mm and 2.7 μ m. A mixture of acetonitrile, water and methanol (46:46:8, v/v/v) was used as the mobile phase. The ion mode was monitored in a quadruple mass analyzer with a positive ratio of atomic mass (m) to charge (z) at $m/z = 317.2$ to identify the amounts of ZEA. A stable isotope labeled internal standard was for the calibration.

2.4. Determining the selenium concentration

The selenium content in the chloroplasts was detected according to the method described in detail by Tobiasz et al. (2014) using inductively coupled plasma mass (ICP MS) spectrometry (Elan DRC-e, Perkin Elmer, Shelton, CT, USA) with Se(82) as a standard.

2.5. Analyzing the brassinosteroid contents

Quantitative and qualitative analyses of the content of BRs in the leaves and chloroplasts were performed according to (Oklestkova et al., 2017). After extraction in ice-cold 80% methanol (5 mL per 0.3 g plant material) and centrifugation (2000 g), the samples were enriched with deuterium labeled BRs. Next, they were placed on Discovery columns (Supelco, Bellefonte, PA, USA) and the eluent was evaporated (under a vacuum) to 3 mL (Rotavapor R-215; BUCHI, Flawil, Switzerland) and to dryness under N₂. Then, after re-suspension in a 7.5% methanol buffer (50 mM NaH₂PO₄, 15 mM NaCl, pH 7.2), they were passed through an immunoaffinity column (Laboratory of Growth Regulation, Olomouc, Czech Republic). Next, the samples were evaporated and dissolved in methanol for the analysis of the BRs using a UHPLC (ACQUITY UPLC-Class System; Waters, Milford, MA, USA) coupled with a Xevo™ TQ-S MS triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK). BRs were analyzed in the positive ion mode as [M + H]⁺. The capillary voltage, cone voltage, collision cell energy and ion source temperatures were optimized for each individual compound using the same setup. The MS settings were as follows: capillary voltage, 3.0 kV; cone voltage, 20 V; source temperature, 120 °C; desolvation gas temperature, 550 °C; cone gas flow, 70 L h⁻¹ and desolvation gas flow, 600 L h⁻¹. The dwell time of each MRM channel was calculated to provide 16 scan points per peak with an inter-channel delay of 0.1. The MS data were recorded in the multiple reaction monitoring mode (MRM). All of the data were processed using MassLynx™ software (ver. 4.1, Waters). The following brassinosteroids were measured: EBR, brassinolide, castasterone and 28-homocastasterone (homocastasterone).

2.6. Analyzing the antioxidative enzymes

The chloroplasts were homogenized in a phosphate buffer (pH 7.0) and the activity of the superoxide dismutases (SOD) and ascorbic peroxidase (APX) were assayed spectrophotometrically according to the method described in detail by Filek et al. (2018).

2.7. Chlorophyll *a* fluorescence – estimating the PSII efficiency

Photochemical efficiency was estimated by means of the chlorophyll *a* (Chl) fluorescence measurements. The measurements were taken in the middle part of the second leaf using a FMS2 modulated fluorescence system and a Handy PEA fast chlorophyll fluorescence induction kinetics fluorimeter (Hansatech, Kings Lynn, UK) as was previously described in detail (Wójcik-Jagla et al., 2013).

After the leaves were adapted light (about 5–8 min at 400 μmol (quanta) m⁻² s⁻¹) when the fluorescence signal (F_s) became constant, the FMS2 system was used to calculate the following parameters: (1) the PSII antenna trapping efficiency (F'_v/F'_m) where F'_v = F'_o–F'_m (F'_o is the chlorophyll fluorescence yield when all of the PSII reaction centers and electron acceptor molecules are fully oxidized in a light-adapted leaf and F'_m is the maximum fluorescence yield in a light-adapted leaf); (2) the photochemical light energy quenching coefficient (qP) is qP = (F'_m – F_s)/(F'_m – F'_o) and (3) the quantum yield of electron transport at PSII is ΦPSII = (F'_m – F_s)/F'_m (described in Kościelniak et al., 2011).

The fast kinetic of chlorophyll *a* fluorescence was measured after 45 min of dark adaptation of the leaves in clips (PEA, Hansatech, Kings Lynn, UK). The parameters were calculated based on the theory of

energy flow in PSII and using the JIP-test. The energy that was absorbed in the PSII antennas (ABS/C_{Sm}) and that was dissipated from PSII (DIO/C_{Sm}) as well as the maximum number of active reaction centers (RC/C_{Sm}) were calculated per excited leaf cross section (C_{Sm}) together with the overall performance index of the PSII photochemistry (PI_(ABS)). Based on this, the following indexes were calculated: φ_{po} (maximal quantum yield of primary photochemistry), Ψ_o (efficiency with which a trapped exciton could move an electron into the electron transport chain further than Q_A); and φ_{eo} (maximum yield of electron transport; the relation between the energy transferred to electron transport chain and the absorbed energy). Additionally, the following parameters of the dissipation of energy per the absorption (DIO/ABS_{Sm}) and reaction centers per absorption (RC/ABS) were calculated. The detailed equations for all calculations are given and explained in Strasser et al. (2000). The chlorophyll fluorescence induction curves are attached as a Fig. 1 - supplementary materials.

2.8. Determining the chlorophyll content

The chlorophyll content in the chloroplasts was determined spectrophotometrically as was described by Lichtenthaler et al. (2013) after the extraction of these organelles in acetone (1.5 mL per 0.1 g of fresh weight) and centrifugation (4000 g) at λ = 645 nm (chlorophyll *b*) and λ = 662 nm (chlorophyll *a*). These analyses were performed in three replications.

2.9. Raman spectroscopy

Identifying and determining the concentration of carotenoids in the leaves were performed using a Thermo Electrons Nicolet NXR FT-Raman Module with a Micro Stage Microscope apparatus that was equipped with an InGaAs detector and a 1064 nm 1 W Nd: YAG laser. The power of the laser was 100 and 300 mW and the samples were moved on micro states at a fixed, 2-dimensional characterization within marked areas that had been selected based on the images of the sample surface.

2.10. Analyzing the Hsp90 transcript accumulation

The quantitative PCR amplification and analyses were performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). After collection the samples (approximately 0.05 g of the central part of the second leaf) were frozen in liquid nitrogen. RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The concentration and quality of the RNA was determined using a Q5000 UV-vis Spectrophotometer (Quawell, San Jose, CA, USA). Two μL of RNA (containing approximately 400 ng of RNA), 1 μL of Quantiscript Reverse Transcriptase, 4 μL of a Quantiscript RT Buffer and 1 μL of RT Primer Mix were used in each reverse transcription reaction, which was combined with the elimination of genomic DNA (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany). The PCR amplifications of the Hsp90 genes were run in triplicate. The primers and probe sequences were designed using Primer Express Software v. 3.0.1 (Applied Biosystems, Foster City, CA, USA) and are given in Table 1 together with the sequence origins. The PCR reactions were conducted as was described by Jurczyk et al. (2012). Each reaction contained 900 nM of the primers and 250 nM of the probes. The amplification was started with an incubation (95 °C for 10 min) after which 40 PCR cycles

Table 1

Sequences of the origins and primers and the sequences of the probes that were used in the study.

Gene name	GenBank ID	Forward primer	Reverse primer	TaqMan MGB Probe
<i>Hsp90</i>	JN052206.1	TGTCCTGTTTGTGCCCAAGAG	GTTGTTGGCCCTTCTTCTGTGTGTC	FAM-CCCTTCGACCTCTTC-MGB
<i>Actin</i>	AB181991.1	AGATCCTGACAGAAAGAGGGTACTC	GCCACGTAAGCGAGCTTCT	FAM-TCTCTGACAATTTCCC-MGB

were performed (95 °C for 15 s, 60 °C for 1 min). The PCR data was analysed using the 7500 real-time PCR Sequence Detection Software v 1.3. The relative standard curve method (Applied Biosystems) was used for calculating the gene expression. The results are presented relative to Actin as the reference (endogenous control) gene (An et al., 1996) (Table 1).

3. Statistical analysis

All of the data, which are presented as the means \pm SE and significance of differences (at $p < 0.05$), were calculated using Duncan's multiple range test and the Student's *t*-test using SAS, version 10.0 (SAS/STAT software).

4. Results

The physiological observations indicated that the seedlings that were obtained from the grains that had been treated with ZEA were characterized by a decrease in fresh weight of about 0.030 ± 0.008 g and 0.052 ± 0.009 g per one plant compared to the control (grains that had not been treated with ZEA) for Parabola and Raweta, respectively (Fig. 1A). The presence of Se and EBR in the mixture with ZEA reduced this effect and the values of the seedling weight were close to those that were observed for the plants that had not been treated with ZEA, especially for Parabola. When selenium and EBR were applied individually, they did not result in any significant changes of this parameter compared to the control plants.

The measurements of the ZEA content in the tested samples showed that this mycotoxin was accumulated in the leaves as well as in the chloroplasts of the studied cultivars, but in smaller amounts in the tolerant Parabola than in the sensitive Raweta (Fig. 1B). The level of ZEA was reduced when Se and EBR were co-administrated and the decrease in ZEA level was more visible in the leaves and chloroplasts of Parabola.

Analyses of the chloroplasts in terms of the Se concentration showed no measurable amounts of this element in the control samples of both of the studied cultivars. After Se-treatment, it was absorbed to a greater degree in Raweta than in the Parabola (Fig. 1C). The presence of ZEA (ZEA + Se) additionally increased the amount of Se in the chloroplasts of the tested cultivars, but more effectively in Parabola.

The quantitative and qualitative detections of the BRs revealed differences in the content and ratio of the identified steroids between Parabola and Raweta (Table 2). Moreover, it was shown that BRs had also accumulated in the chloroplasts. The following BRs were found in the chloroplasts of Parabola and Raweta – EBR, castasterone and homocastasterone. Additionally, brassinolide was found but only in the

leaves of plants treated with EBR and ZEA + EBR. Interestingly content of brassinolide was significantly lower in ZEA + EBR treated plants than in EBR treated plants of both cultivars. The amount of homocastasterone was significantly (even few times) higher than the other BRs. Generally, in the tissues and chloroplasts of Parabola, there were more BRs than in Raweta. Larger disproportions, especially in the homocastasterone content between the tested cultivars, occurred in the chloroplasts. When EBR was applied to the grains, the content of homocastasterone and the homocastasterone/castasterone ratio increased in leaves and chloroplasts. When EBR was applied together with ZEA, the increase in this ratio was even more evident.

The studies of the antioxidative enzymes (Table 3) that were present in the chloroplasts revealed that the activity of SOD in the control plants of Parabola was only slightly (statistically insignificantly) higher, whereas APX was lower than in Raweta (Table 3). The presence of ZEA in the chloroplasts generally decreased the activity of SOD (more significantly in Raweta) and increased the activity of APX in both cultivars. The addition of Se and EBR to the mixture with ZEA did not affect the changes in SOD (compared to the control) in the Parabola chloroplasts, but increased the activity of these enzymes in Raweta. The APX activity decreased when both protectants were applied together with ZEA compared to the action of ZEA in both cultivars. The effect was statistically significant only in the Parabola chloroplasts.

Considering the concentration of the pigments in the chloroplasts of the control samples as well as after the addition of Se and EBR, no statistically significant differences were observed between the cultivars (Table 3). The application of ZEA decreased the content of chlorophyll *a* and *b*. When ZEA was added to the mixture with Se and EBR, the level of chlorophylls reached values that were close to those of the control.

The carotenoid content that was measured in the leaves as the average intensity (of I_{1529} spectra) per 1 mm^2 of the surface did not visibly differ in the Parabola seedlings after the application of various treatments (Fig. 2). In Raweta, a rise in the accumulation of carotenoid in the leaf surface (1 mm^2) was observed in the ZEA-treated plants.

Measurements using the FMS2 system enabled the parameters of the photochemical efficiency to be calculated. In the Parabola samples, all of the treatments resulted in a small increase (although in some cases statistically insignificant) in the efficiency of the antennae (F_v/F_m), photochemical quenching (qP) and the quantum of the efficiency of the PSII (Φ PSII) parameters (Table 4). In Raweta, ZEA treatment resulted in a decrease in the values of these parameters. The addition of ZEA + Se/EBR, partly reversed the changes in the direction of the values of the factors that were calculated for control. For the individual treatments with EBR and Se, the data for the photochemical efficiency were close to those that were obtained for the control (for Parabola and Raweta).

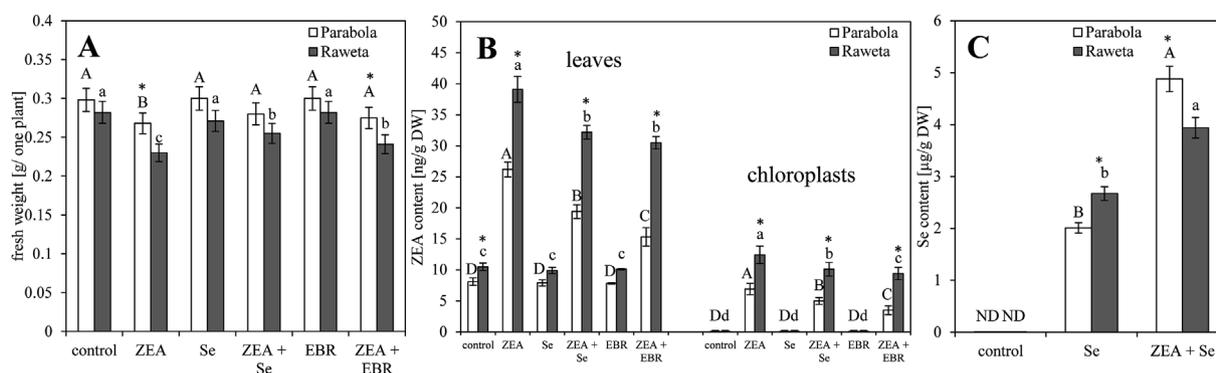


Fig. 1. Fresh weight (g/plant) (A) of wheat seedlings of Parabola and Raweta and B – Zearalenone content in the leaves and chloroplasts of wheat seedlings that had been obtained from the control, ZEA, Se, ZEA + Se, EBR and ZEA + EBR treated grains. C – Selenium content in the chloroplasts of wheat seedlings that had been obtained from the control, Se and ZEA + Se treated grains. Data are the means of ten replications \pm SE. B. Significant variances between the treatments (separately for each cultivar, for $P \leq 0.05$) are indicated by different letters (uppercase for Parabola and lowercase for Raweta). The differences between the cultivars are indicated by “*”. ND – not detectable.

Table 2
Brassinosteroid content in the leaves and chloroplasts of Parabola and Raweta seedlings that had been grown from the control grains and grains that had been treated with EBR and the mixture of ZEA + EBR. Data are the means of five replications ± SE. Means followed by the same letters for each cultivar are not significantly different ($P < 0.05$). The differences between the cultivars are indicated by “*”.

Treatment	Chloroplasts									
	Leaves		Parabola		Raweta		Parabola		Raweta	
	24-Epibrassinolide [pM/g FW]	Brassinolide [pM/g FW]	Castasterone [pM/g FW]	Homocastasterone [pM/g FW]	Homocastasterone/Castasterone ratio	24-Epibrassinolide [pM/g FW]	Castasterone [pM/g FW]	Homocastasterone [pM/g FW]	Homocastasterone/Castasterone ratio	Homocastasterone/Castasterone ratio
Parabola										
Control	0.45 ± 0.02 ^{ab**}	–	0.57 ± 0.04 ^{**}	2.02 ± 0.06 ^b	3.54 ± 0.11 ^c	1.28 ± 0.03 ^{c*}	1.07 ± 0.02 ^a	6.93 ± 0.08 ^{c*}	6.48 ± 0.09 ^{**}	
EBR	0.51 ± 0.04 ^{a†}	0.099 ± 0.011 ^{ab*}	0.57 ± 0.03 ^{ab*}	3.00 ± 0.05 ^a	5.26 ± 0.13 ^b	1.79 ± 0.03 ^{bc*}	1.09 ± 0.04 ^a	7.30 ± 0.11 ^{bc*}	6.70 ± 0.10 ^{bc*}	
ZEA + EBR	0.49 ± 0.03 ^{ab*}	0.066 ± 0.018 ^{ab*}	0.50 ± 0.03 ^{ab*}	3.04 ± 0.04 ^a	6.08 ± 0.21 ^a	2.40 ± 0.05 ^{cd**}	1.08 ± 0.04 ^a	9.46 ± 0.23 ^{ab*}	8.76 ± 0.25 ^{ab*}	
Raweta										
Control	0.40 ± 0.01 ^b	–	0.44 ± 0.02 ^a	2.00 ± 0.05 ^b	4.54 ± 0.15 ^{c*}	0.99 ± 0.02 ^a	1.24 ± 0.03 ^{bc*}	4.39 ± 0.07 ^c	3.54 ± 0.06 ^c	
EBR	0.46 ± 0.03 ^{a†}	0.053 ± 0.009 ^a	0.41 ± 0.02 ^a	3.20 ± 0.06 ^{ab*}	7.38 ± 0.09 ^{bc*}	1.02 ± 0.04 ^a	1.28 ± 0.05 ^{bc*}	4.90 ± 0.06 ^b	3.83 ± 0.06 ^b	
ZEA + EBR	0.42 ± 0.03 ^{ab†}	0.010 ± 0.025 ^b	0.40 ± 0.03 ^{ab}	3.15 ± 0.02 ^{ab*}	7.88 ± 0.10 ^{ab*}	0.91 ± 0.01 ^b	1.58 ± 0.02 ^{bc*}	6.35 ± 0.09 ^a	4.02 ± 0.10 ^a	

Generally, the parameters that were designed on the basis of the fast kinetics of chlorophyll *a* fluorescence were only modulated to a small extent in the presence of studied substances (Table 4). In the Parabola leaves (control), they had similar values for the ϕ_{po} , DIO/ABS (CSm) and $PI_{(ABS)}$ and a lower value for 10RC/ABS compared to the Raweta control plants. Other parameters, i.e. Ψ_o and ϕ_{eo} , were higher in Parabola than in Raweta. Although the application of ZEA resulted in changes in these parameters, it was in a similar manner for both cultivars, i.e. the parameters 10RC/ABS and $PI_{(ABS)}$ decreased whereas DIO/ABS(CSm) increased. The Ψ_o and ϕ_{eo} remained at the same level as in the control. When ZEA was applied in the mixture with EBR and Se, the values of almost all of the studied parameters increased or remained at the same level as those when ZEA was applied alone. When added separately, the EBR and Se did not significantly change the parameters that were calculated based on the chlorophyll *a* fluorescence compared to the controls of both cultivars.

The transcript accumulation of *Hsp90* in the control Parabola plants was close to those of Raweta (Fig. 3). However, when ZEA was added, it changed the reaction that induced the transcript level of *Hsp90* of the studied cultivars. Whilst in the Parabola leaves the values of *Hsp90* were not significantly changed (compared to the control), in the Raweta plants a decrease of about 20% was observed. The individual treatment with Se and EBR generally increased the *Hsp90* transcript level (compared to the control) similarly as in the combination with ZEA (both ZEA + Se and ZEA + EBR). For Raweta, these changes (compared to only the action of ZEA) were greater than for Parabola.

5. Discussion

The observation of the passage of ZEA from infected grains to germinating seeds (Filek et al., 2017) prompted the next studies the aim of which was to check the possibility of its transport into the developing leaves of seedlings and further – its location in the cells, especially in the chloroplasts. The presence of small amounts of ZEA in the leaves of the control plants was expected as this mycotoxin is known to be a hormone that is involved in stimulating the generative development of plants (Biesaga-Kościelniak and Filek, 2010). Interestingly, the increase in the level of this toxin (about three-fold compared to the control) that was observed in the presented studies acted as a stress factor that caused a decrease in the mass of plants, mainly in the sensitive cultivar.

The possibility of ZEA transport between the tissues in plants that have been infected by this toxin had been suggested earlier in some articles (Snighda et al. 2015; Filek et al., 2017). However, although the ZEA-stimulated changes in the activity in photosystems has been confirmed (Kościelniak et al., 2009, 2011), it has not yet been determined in what proportion this substance might be directly accumulated in the chloroplasts. In this study, we found that depending on the stress tolerance of a cultivar, the chloroplasts were able to absorb ZEA at about 26–44% of the amounts that were uptaken to the leaves. A higher ZEA content was registered in the chloroplasts of the sensitive (Raweta) cultivar. The relationship between ZEA accumulation and the tolerance to stress conditions that was observed for the tested wheat cultivars allowed us to speculate (in more detail) about the mechanism of the incorporation of mycotoxin molecules into plant cells. The physico-chemical properties of zearalenone [6-(10-hydroxy-6-oxo-trans-1-undecenyl)b-resorcylic-acid-lactone] are similar to those of the steroids that occur naturally in the biomembranes (Gromadzka et al., 2008; Filek et al., 2018). Thus, the possibility of the straightforward incorporation of ZEA into the membrane structure of a chloroplast is proposed on the basis of the presented experiments. This interference of ZEA molecules may stimulate functional changes not only in lipids but also in the protein complexes (enzymes and transporters) that are located in the biomembranes, which would lead to metabolic changes in the cell homeostasis and as a consequence to oxidative stress.

The ability of EBR to partly remove ZEA from the cell membranes was postulated in earlier studies (Filek et al., 2017, 2018). However,

Table 3

The SOD and APX activity and pigment (chlorophyll *a*, *b*) content in the chloroplasts from the leaves of Parabola and Raweta seedlings that had been grown from the grains of the control and grains that had been treated with ZEA, Se, ZEA + Se, EBR and ZEA + EBR. Data are the means of five replications (for pigments) and ten replications (for enzymes) with \pm SE. Means followed by the same letters for each cultivar are not significantly different ($P < 0.05$). The differences between the cultivars are indicated by “*”.

Treatment	SOD [U/mg protein]	APX [U/mg protein]	Chlorophyll <i>a</i> [mg/g FW]	Chlorophyll <i>b</i> [mg/g FW]
<i>Parabola</i>				
Control	0.090 \pm 0.006 ^a	0.0011 \pm 0.0002 ^b	0.15 \pm 0.01 ^a	0.054 \pm 0.003 ^b
ZEA	0.068 \pm 0.003 ^{b*}	0.0016 \pm 0.0002 ^a	0.12 \pm 0.01 ^b	0.048 \pm 0.002 ^{c*}
Se	0.089 \pm 0.004 ^{a*}	0.0010 \pm 0.0002 ^b	0.16 \pm 0.02 ^a	0.055 \pm 0.002 ^{b*}
ZEA + Se	0.079 \pm 0.005 ^a	0.0011 \pm 0.0001 ^b	0.15 \pm 0.01 ^a	0.060 \pm 0.002 ^{a*}
EBR	0.090 \pm 0.005 ^a	0.0011 \pm 0.0002 ^b	0.16 \pm 0.01 ^a	0.055 \pm 0.002 ^b
ZEA + EBR	0.079 \pm 0.005 ^a	0.0011 \pm 0.0001 ^b	0.16 \pm 0.02 ^a	0.062 \pm 0.004 ^{a*}
<i>Raweta</i>				
Control	0.081 \pm 0.004 ^b	0.0013 \pm 0.0001 ^b	0.14 \pm 0.02 ^a	0.050 \pm 0.002 ^a
ZEA	0.042 \pm 0.004 ^c	0.0016 \pm 0.0001 ^a	0.09 \pm 0.01 ^b	0.040 \pm 0.001 ^b
Se	0.080 \pm 0.003 ^b	0.0012 \pm 0.0002 ^b	0.13 \pm 0.02 ^a	0.050 \pm 0.002 ^a
ZEA + Se	0.090 \pm 0.004 ^{a*}	0.0015 \pm 0.0002 ^{a*}	0.11 \pm 0.01 ^{ab}	0.049 \pm 0.002 ^a
EBR	0.082 \pm 0.003 ^b	0.0013 \pm 0.0001 ^b	0.14 \pm 0.01 ^a	0.051 \pm 0.002 ^a
ZEA + EBR	0.092 \pm 0.003 ^{a*}	0.0015 \pm 0.0001 ^{a*}	0.11 \pm 0.01 ^{ab}	0.050 \pm 0.002 ^a

the possibility of BRs conducting long-distance transport from the roots to the upper parts of plants and their location in the cell organelles, especially after the grains have been treated, have not been unambiguously demonstrated. The distribution of bioactive BRs differs greatly in various parts of a plant (the highest is in the reproductive organs, lower in the shoots and the lowest in the roots) which may suggest their transport between organs (Ross et al., 2006). Our observations of BRs in the leaves and chloroplasts when only the grains had been treated with EBR suggest that these substances might be exported to other organs during plant development and can also be accumulated in the organelles. This does not eliminate the possibility that the EBR itself was transported to cells where it influenced on the synthesis of other BRs. Interestingly, both the EBR that was applied separately and in the mixture with ZEA stimulated especially the synthesis of hydrophobic form of BRs, i.e. homocastasterone. A similar way of activating the synthesis of BRs was observed during the direct treatment of wheat cells with EBR and ZEA in *in vitro* cultures (Filek et al., 2018). In both experiments (cells and seedlings), the application of ZEA resulted in a higher content of homocastasterone. This suggests that even if it is assumed that the synthesis of BRs was activated independently in various tissues, the presence of ZEA together with EBR might better stimulate the translocation of these hormones between organs and thus increase their accumulation in the chloroplasts. However, the action of ZEA + EBR leads to a decrease in the uptake of the toxin in both the leaves and chloroplasts as is shown in the presented

studies. Presumably, as has been suggested in other works (Filek et al., 2018), ZEA, which has a steroid-like structure that is physico-chemically similar to BRs, could compete to occupy the same membrane locations. Confirmation of this assumption was the observation that in the chloroplasts, which absorbed the most hydrophobic BRs (homocastasterone), there was a decrease in the ZEA accumulation.

Finding that the Se ions were accumulated in the chloroplasts and that their absorption resulted in a decrease in ZEA uptake led to the conclusion that the primary action of the protectors that were used reduced the levels of toxins in the cells and chloroplasts. In earlier studies (Filek et al., 2010b), it was shown that Se caused a partial reverse of the changes in the chloroplast properties that had been initiated by Cd-stress of *Brassica* plants, especially in the hydrophilic part of an envelope. Furthermore, the experiments in model systems indicated the participation of Se in protecting membranes against ZEA toxicity (Gzyl-Malcher et al., 2017). Thus, blocking the incorporation of ZEA into cells *via* the membranes and next a translocation into the chloroplasts was presumably the main effect of both the EBR and Se protective actions. On the other hand, observing that both of the protectants that were studied are accumulated in the chloroplasts, and especially to a higher extent in the tolerant cultivar, led to an assumption about their specific intervention in the mechanism of the oxidative stress. The studies of Barbasz et al. (2018) on the cell lines (U-937) indicated that the Se protection was related to blocking the excess of all kinds of ROS, while the EBR action was mainly responsible for

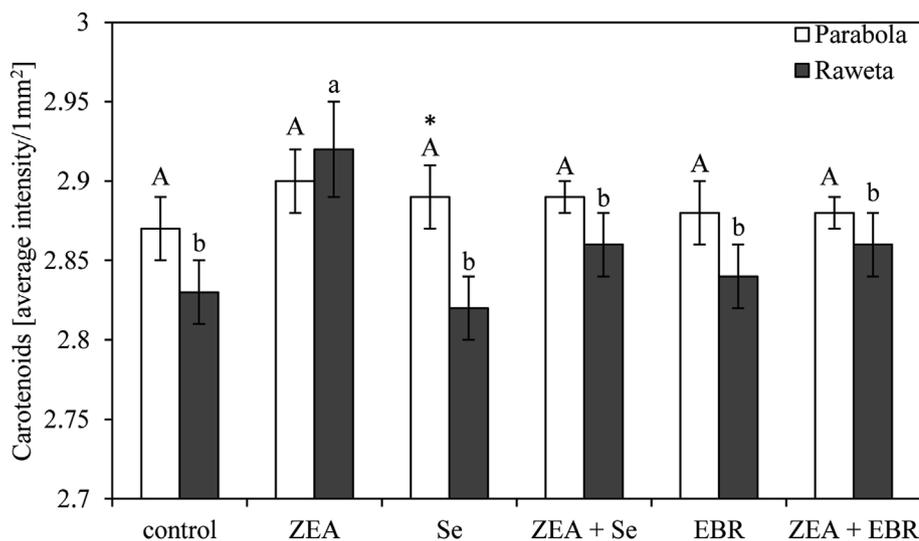


Fig. 2. Carotenoid content in the Parabola and Raweta leaves of the seedlings of control and the plants that had been cultured with ZEA, Se, EBR and mixtures of ZEA + Se and ZEA + EBR. Data are the means of five replications with \pm SE. Means are indicated by different letters (uppercase for Parabola and lowercase for Raweta) between the treatments separately for each cultivar, for $P \leq 0.05$. The differences between the cultivars are indicated by “*”.

Table 4
The PSII efficiency of the leaves of Parabola and Raweta seedlings that had been grown from the grains of the control and grains that had been treated with ZEA, Se, EBR, ZEA + Se and ZEA + EBR. The results are the means of five biological replicates each in three technical replicates \pm SE. Means followed by the same letters (separately for each cultivar) are not significantly different ($P < 0.05$). The differences between the cultivars are indicated by “*”.

Treatment	Fv/Fm	qP	Φ PSII	Φ_{po}	Ψ_o	Φ_{so}	IORC/ABS	PI(ABS)	Dio/ABS(CSm)
Parabola									
Control	0.819 \pm 0.004 ^{ba}	0.779 \pm 0.004 ^d	0.527 \pm 0.003 ^c	0.804 \pm 0.003 ^a	0.538 \pm 0.002 ^{ba}	0.434 \pm 0.002 ^{ba}	3.99 \pm 0.02 ^a	1.94 \pm 0.03 ^a	0.194 \pm 0.002 ^b
ZEA	0.835 \pm 0.005 ^{aa}	0.797 \pm 0.002 ^{ca}	0.562 \pm 0.004 ^{ab}	0.796 \pm 0.004 ^b	0.543 \pm 0.003 ^{ba}	0.432 \pm 0.002 ^{ba}	3.86 \pm 0.01 ^c	1.78 \pm 0.04 ^b	0.204 \pm 0.003 ^a
Se	0.820 \pm 0.004 ^{ba}	0.780 \pm 0.004 ^d	0.528 \pm 0.004 ^c	0.804 \pm 0.004 ^a	0.539 \pm 0.004 ^b	0.434 \pm 0.002 ^{ba}	3.99 \pm 0.03 ^a	1.93 \pm 0.03 ^a	0.193 \pm 0.003 ^b
ZEA + Se	0.838 \pm 0.006 ^{aa}	0.813 \pm 0.004 ^{ba}	0.557 \pm 0.003 ^{ba}	0.801 \pm 0.004 ^a	0.547 \pm 0.003 ^{ba}	0.438 \pm 0.003 ^a	3.91 \pm 0.03 ^b	1.90 \pm 0.02 ^{aa}	0.202 \pm 0.002 ^a
EBR	0.821 \pm 0.005 ^{ba}	0.782 \pm 0.003 ^d	0.529 \pm 0.004 ^c	0.805 \pm 0.003 ^a	0.538 \pm 0.003 ^{ba}	0.433 \pm 0.002 ^{ba}	3.98 \pm 0.03 ^a	1.95 \pm 0.04 ^a	0.193 \pm 0.002 ^a
ZEA + EBR	0.834 \pm 0.004 ^{aa}	0.827 \pm 0.004 ^{ba}	0.569 \pm 0.004 ^{ab}	0.802 \pm 0.003 ^a	0.549 \pm 0.002 ^{aa}	0.439 \pm 0.002 ^{aa}	3.90 \pm 0.02 ^{ab}	1.91 \pm 0.02 ^{aa}	0.201 \pm 0.002 ^a
Raweta									
Control	0.808 \pm 0.004 ^a	0.794 \pm 0.004 ^{ab}	0.530 \pm 0.003 ^c	0.804 \pm 0.003 ^a	0.526 \pm 0.003 ^b	0.423 \pm 0.002 ^b	4.23 \pm 0.02 ^{aa}	1.93 \pm 0.03 ^a	0.197 \pm 0.002 ^b
ZEA	0.788 \pm 0.005 ^b	0.775 \pm 0.005 ^b	0.522 \pm 0.003 ^d	0.798 \pm 0.003 ^a	0.528 \pm 0.004 ^b	0.423 \pm 0.003 ^b	3.92 \pm 0.01 ^{ba}	1.76 \pm 0.01 ^c	0.203 \pm 0.002 ^a
Se	0.810 \pm 0.004 ^a	0.793 \pm 0.003 ^{ab}	0.529 \pm 0.003 ^c	0.803 \pm 0.004 ^a	0.527 \pm 0.003 ^b	0.422 \pm 0.002 ^b	4.23 \pm 0.02 ^{ab}	1.91 \pm 0.02 ^a	0.199 \pm 0.002 ^b
ZEA + Se	0.811 \pm 0.005 ^a	0.789 \pm 0.004 ^{ab}	0.539 \pm 0.004 ^b	0.799 \pm 0.002 ^a	0.540 \pm 0.003 ^a	0.432 \pm 0.003 ^a	3.89 \pm 0.03 ^b	1.82 \pm 0.02 ^b	0.197 \pm 0.002 ^a
EBR	0.811 \pm 0.005 ^a	0.795 \pm 0.005 ^{ab}	0.530 \pm 0.003 ^c	0.803 \pm 0.003 ^a	0.526 \pm 0.003 ^b	0.423 \pm 0.003 ^b	4.22 \pm 0.02 ^{ab}	1.92 \pm 0.03 ^a	0.196 \pm 0.003 ^b
ZEA + EBR	0.811 \pm 0.004 ^a	0.788 \pm 0.003 ^{ab}	0.548 \pm 0.004 ^a	0.800 \pm 0.003 ^a	0.543 \pm 0.002 ^a	0.432 \pm 0.002 ^a	3.88 \pm 0.02 ^c	1.80 \pm 0.03 ^b	0.200 \pm 0.003 ^a

removing of other than the superoxide radical itself. The dependence between presence of BRs and the generation of H₂O₂ was observed by Zhu et al. (2016).

The strong inhibition of the SOD activity that was observed in the sensitive ZEA treated plants may indicate that the high ROS production was responsible for the damage of the protein structure of these enzymes. Such an explanation was described by Casano et al. (1997) and this effect has been shown in many studies. The reversal of the direction of changes in SOD activity in the presence of Se and EBR (increase in relation to ZEA) may indicate a protective effect of studied substances in order to reduce the concentration of ROS. An increase in APX activity is usually perceived as an increase in the generation of H₂O₂. Moreover, this ROS molecule has been described as a signaling substance (Niu and Liao, 2016). Thus, the observed increase in APX in the presence of ZEA indicates that, independent of the application of exogenous Se and EBR, the generation of signaling molecules (as H₂O₂) may be included in the mechanisms that is responsible for intracellular defense. The increase in the content of carotenoids after ZEA treatment also proves the involvement of non-enzymatic antioxidants in the defense mechanisms. The presence of carotenoids may improve the stability of the chloroplast membranes (especially in the sensitive cultivar) under oxidative stress conditions (Gruszecki and Strzałka, 2005).

Although chloroplasts are the organelles in which the generation of ROS is a natural physiological process that occurs in photosynthesis reactions and the activation of antioxidative substances eliminate the excess production of ROS, our finding is that ZEA treatment can reduce the sum of chlorophylls in the tested plants, which indicates that even relative small amounts of this toxin (which is uptaken by chloroplasts) may modify the process of photosynthesis. Changes in the chlorophyll content, which was greater in the ZEA-stressed sensitive cultivar, suggest that there are also modifications in the chlorophyll fluorescence parameters, which should be more visible in the Raweta than in the Parabola plants. In fact, these changes were not spectacular, probably because of the semi-adaptation of the plants to the action of this mycotoxin, since the ZEA was only applied during grain germination. However, even after a relatively long time from the application of the stress factor, the photochemical activity of PS II was differentiated, to some extent, in both the tolerant and sensitive (to oxidative stress) cultivars, especially in the early stages of photosynthesis. An increase in the Fv/Fm factor, which characterizes the potential efficiency of the antenna system in terms of the ability to use the energy that is absorbed by chlorophyll for the photochemical reaction and qP (photochemical quenching). The participation of the pull of Qa⁻ in the total pull of Qa, as well as the quantum yield of electron transport (Φ PSII) that was calculated for the tolerant Parabola after ZEA-treatment was consistent with the observation of Kościelniak et al. (2009, 2011). They used this mycotoxin as a hormonal substance to influence the generative development of plants and suggested that stimulating the photochemical efficiency may be one of the factors that accompany the initiation of flowering of plants. Rather small changes in the photosynthesis parameters were also observed by Kalaji et al. (2011) after the application of short-term (24 h) salt-stress in barley, in which, during this period, only minor differences were observed between the tolerant and sensitive cultivars.

The inhibition of the activity of photosynthesis in the presence of ZEA, which is associated with disturbances in chlorophyll synthesis (similar to what was observed for sensitive Raweta) was found by Von Willert et al. (1995) and Maxwell and Johnson (2000). A decrease in the photochemical efficiency that was correlated with the degree of infection in wheat plants was described by Kuckenberget al. (2007). Murchie and Lawson (2013) suggested that these parameters were suitable for detecting fungal diseases. A noticeable decrease in the content of chlorophyll a in the chloroplasts of the Raweta cultivar, which was also visible as a decrease in the total chlorophyll pool (a + b) indicated the participation of ZEA in the semi-destruction of the photosystems in the non-tolerant plants. However, even in these plants,

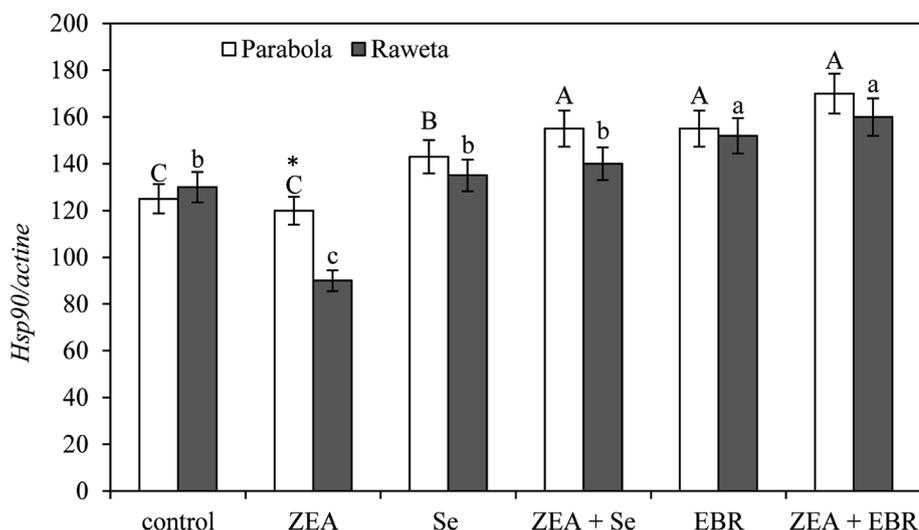


Fig. 3. Relative transcript level of *Hsp90* in the Parabola and Raweta leaves of the seedlings of control and the plants that had been cultured with ZEA, Se, EBR and mixtures of ZEA + Se and ZEA + EBR. The results are the means of five biological replicates, each in three PCR technical replicates \pm SE. Means are indicated by different letters (uppercase for Parabola and lowercase for Raweta) between the treatments separately for each cultivar, for $P \leq 0.05$. The differences between the cultivars are indicated by “*”.

both of the protectants that were used were able to reduce the effects of ZEA stress.

Measurements of the fast kinetics of fluorescence indicated that ZEA strongly decreased the energy flow through the photosynthetic centers, which was expressed more in the sensitive cultivar. A small increase in these parameters after the application of Se and EBR together with ZEA may be considered to be an element of the mechanism that is involved in improving the photochemical reaction that is modified when only ZEA is applied. The activity of the reactive centers, especially the density of the reactive centers (the parameter $10RC/ABS$), changed about 8% for Raweta, whereas for Parabola this drop was smaller (only about 3%). This effect was reversed by EBR and Se in Parabola, which could be connected to its better stress tolerance. The decrease in the values of the performance index ($PI_{(ABS)}$), confirmed that the presence of ZEA decreased the efficiency of the light reactions of photosynthesis within PSII while EBR or Se treatment together with ZEA neutralized the effect. Moreover, the action of ZEA (in both cultivars) was also accompanied by a significant increase in energy dissipation (loss) in the form of heat release in relation to the energy that was absorbed by the antennas (DIo/ABS), but EBR and Se applied together with ZEA did not alleviate this effect.

In the studies of the ZEA-initiated reaction in plants, an analysis of the *Hsp90* transcript level may be helpful in inferring its stressful properties as these proteins indirectly participate in signal transduction by interacting with specific proteins that involve the pivotal stress-related genes (Xu et al., 2012a, b). HSP - heat shock proteins were discovered as protectants in plants exposed to high temperature but later there was confirmed their role also in other stresses. These proteins have chaperone function, they stabilize new proteins to ensure their correct folding. They also help to refold proteins that were damaged as a result of stress. The finding that the level of *Hsp90* transcript was diminished by ZEA and that this process occurred only in the sensitive cultivar, confirms that the action of ZEA is the stress agent for Raweta plants. The decrease of the *Hsp90* transcript accumulation in the presence of ZEA could be (among other) the effect of the oxidative stress and the excess generation of ROS in this cultivar. On the other hand, the increase in the *Hsp90* transcript abundance as a response to the presence of the protectants (especially EBR) in the mixture with ZEA may confirm the importance of these substances in the initiation of the protective mechanisms under the stress that is caused by a fungal infection and by mycotoxins.

6. Conclusion

The results confirmed the participation of both BRs and Se as

protectors in mycotoxin (ZEA) stress conditions and indicated the significance of their translocation into the chloroplasts in the defence mechanisms. An analysis of the content of the tested substances indicates the possibility of the transport and accumulation of Se and BRs into the chloroplasts, which had not yet been clearly demonstrated. Higher accumulation of homocastasterone in the presence of EBR may suggest that the more hydrophobic derivatives of BRs were better transferred *via* membranes. The chloroplast membranes, which are rich in unsaturated fatty acids, may be a convenient hydrophobic environment for the location of homocastasterone and its accumulation in these organelles. Selenium translocation into the chloroplasts seemed to be more dependent on the interaction with the polar part of the membrane, i.e. the carbohydrates that form galactolipids, which are the main lipids of chloroplasts. The stress tolerance of the tested wheat cultivars may be connected with differences in their ability to accumulate protectors; the higher level of the studied protectors was characteristic for the tolerant cultivar and was accompanied by a lower content of the toxin in these organelles. As both tested BRs and Se were engaged in stimulating the effectivity of photosynthesis, their presence in the chloroplasts reduced the disturbances during the early stages of electron transport in PSII. Moreover, in the protective reactions, the accumulation of the *Hsp90* transcript, the activity of the enzymatic antioxidants and the presence of non-enzymatic antioxidants in the chloroplasts were involved.

Author contributions

M.F. participated during the entire project. M.F. and A.S. ran the experiments. J.K. investigated the parameters of photosynthesis and the content of pigments. A.J. made extraction and purification of BRs, J.O. analysed the brassinosteroids on UHPLC/MS-MS. B.J. analysed the accumulation of the *Hsp90* transcript. A.T. analysed the selenium content. All of the authors helped to design the experiments and write the article.

Conflicts of interest

The authors declare no conflict of interest.

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brassinosteroids.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.01.030>.

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