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Silicon-induced alterations in the expression of aquaporins and antioxidant system activity in well-watered and drought-stressed oilseed rape

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ABSTRACT

Progressing climate change necessitates the search for solutions of plant protection against the effects of water deficit. One of these solutions could be silicon supplementation. The aim of the study was to verify the hypothesis that silicon changes aquaporin expression and antioxidant system activity in a direction which may alleviate the effects of drought stress in oilseed rape. The accumulation of BnPIP1, BnPIP2-1-7 and BnTIP1;1 aquaporins and the expression of their genes, the level of catalase, superoxide dismutase activities and hydrogen peroxide content as well as total non-enzymatic antioxidant activity were analyzed in leaf tissue from control and silicon-treated oilseed rape plants growing under well-watered and drought conditions. Silicon was applied in two forms – pure silicon and a silicon complex. It was shown that under drought conditions, both pure silicon and the silicon complex (with Fe) significantly increased the accumulation of aquaporins and improved the activity of enzymatic and non-enzymatic components of the antioxidant system, while under well-watered conditions, these effects were observed only in the case of the silicon complex. The presented study proves that silicon supplementation in oilseed rape improves the regulation of water management and contributes to the protection against oxidative stress caused by drought.

1. Introduction

The uptake of water by plants and the regulation of their water balance depends on the availability of water in the environment. More and more frequently, an important role in these processes and in the protection against the effects of water deficit in plants is attributed to silicon (Si) supplementation (Helaly et al., 2017). Silicon regulates root hydraulic conductivity, alleviates the deficiency of potassium ions responsible for proper cell turgor, reduces the transpiration rate, and increases water uptake and the capacity for osmotic adjustment (Zhu and Gong, 2014). In the plant kingdom, the accumulation of silicon varies widely between species. Silicon accumulation in *Brassica* is relatively low compared to e.g. *Graminae*. Nevertheless, in our earlier studies (Saja-Garbarz et al., 2021) we showed that Si supplementation, also in *Brassica napus* var. *napus*, affects the accumulation of this element in plant shoots. Thus far, the beneficial effect of silicon on plant water management has been studied in many species of monocotyledons (Gong and Chen, 2012) and dicotyledons under both artificially induced and naturally occurring (Gong and Chen, 2012) drought. However, the exact mechanism of action of silicon has not been clearly defined so far. According to Chen et al. (2018), silicon can contribute to initiating processes allowing the plant to overcome barriers in water uptake or eliminate excessive water loss. Undoubtedly, in the case of the first pathway, a key role is played by aquaporins (AQPs) – proteins found in membranes and responsible for the transport of water as well as many plant metabolites (Afzal et al., 2016; Deshmukh and Belanger, 2016). Aquaporins in plants occur in many isoforms, which increases the possibility of transporting various substances in various tissues and improves the selectivity of this process (Deshmukh et al., 2013), both to and from cells. The studies of changes in the expression of AQPs are very useful for the description of water transport regulation under, among others, drought stress conditions, but the effectiveness of AQPs varies greatly depending on the plant's growth conditions, its stage of

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Abbreviations

AP	alkaline phosphatase
AQP	aquaporin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CAT	catalase
DPPH	1,1-diphenyl-2-picrylhydrazyl
H_2O_2	hydrogen peroxide
KCN	potassium cyanide
NBT	p-nitroblue tetrazolium chloride
NIP	nodulin 26-like intrinsic protein
PAGE	polyacrylamide gel electrophoresis
PIP	plasma membrane intrinsic protein
PVPP	poly(vinylpolypyrrolidone)
RH	relative humidity
ROS	reactive oxygen species
Si	silicon
SIP	small basic intrinsic protein
SOD	superoxide dismutase
TBS-T	Tris buffered saline with Tween 20
TIP	tonoplast intrinsic protein
XIP	X intrinsic protein

development, type of tissue, duration and intensity of stress (Alexandersson et al., 2005; Galmes et al., 2007), as well as the type of aquaporin. AQPs include plasma membrane intrinsic proteins (PIP), nodulin 26-like intrinsic proteins (NIP) and unclassified X intrinsic proteins (XIP), which are located along the entire length of the cell membrane. Moreover, the occurrence of small basic intrinsic proteins (SIP) and some NIPs has been confirmed in the endoplasmic reticulum (Deshmukh and Belanger, 2016). Another group of AQPs – tonoplast intrinsic proteins (TIP) – can be found in the vacuole membrane. Furthermore, it is hypothesized that some PIPs and TIPs may be located in the inner membrane and thylakoids of chloroplasts (Deshmukh and Belanger, 2016). There are 120 homologs of AQPs identified in *Brassica napus* so far (Sonah et al., 2017). Plasma membrane intrinsic proteins (PIP) – molecular weight of ca. 30 kDa – are the most important water channels regulating the uptake and loss of water by cells. Based on a sequence similarity analysis, the occurrence of two subgroups – PIP1 and PIP2 – was determined (Kapilan et al., 2018), with PIP1 aquaporins being less efficient as water channels than PIP2. Moreover, isoforms from the PIP2 subgroup probably provide water communication between cells. Plants also use tonoplast intrinsic proteins (TIP) for water transport. They weigh ca. 25–28 kDa, occur around the tonoplast, provide constant cell turgor pressure and regulate osmotic balance of the cytoplasm (Kapilan et al., 2018). During abiotic stress, some AQP isoforms can be activated in selected plant tissues, others in the whole plant. It has also been shown on the basis of AQP gene expression analysis that changes in plant water relations due to stress cause complex transcriptional and post-translational AQP responses (Kapilan et al., 2018), often contradictory for different isoforms. Many studies have reported that PIP channels are present as monomers and/or dimers after separation by SDS/PAGE (Bienert et al., 2012) and that the monomer/dimer ratio depends on the presence of redox-active substances, such as dithiothreitol (DDT), suggesting that PIP dimers are covalently linked by disulfide bonds.

Environmental stress factors including drought cause disruption in redox homeostasis in plant cells, leading to overproduction of reactive oxygen species (ROS) and induction of oxidative stress causing serious damage to cells (Kapilan et al., 2018). ROS such as H_2O_2 (hydrogen peroxide), O_2• (superoxide anion) and •OH (hydroxyl) radicals are generated as a by-product of plant's metabolic activity. However, when plants are facing stress factors such as drought, ROS are overproduced, leading to damage of photosynthetic pigments, membrane lipids, proteins and nucleic acids, and as a consequence, to cell death (Hasanuzzaman et al., 2018). As H_2O_2 is the most resilient of ROS, it is possible to evaluate the level of oxidative stress based on the measurement of its amount. To keep the level of ROS under control, plants have enzymatic and non-enzymatic antioxidant systems, which protect cells from oxidative damage (Mittler, 2002). Si has been shown to increase the activity of key antioxidant scavenging enzymes (Zhu and Gong, 2014), including superoxide dismutase (SOD) and catalase (CAT), thus ameliorating the effects of oxidative stress. An improvement in physiological functioning of the plant and strengthening of antioxidant defense systems due to silicon application have been reported in many plant species (Hasanuzzaman et al., 2017). In the studies of Maghsoudi et al. (2016) conducted on wheat, exogenous application of silicon was shown to alleviate the damage caused by oxidative stress in plants subjected to drought, among others through increasing relative water content, cell membrane stability and photosynthesis rate. In oilseed rape a decrease in H_2O_2 level was confirmed after Si supplementation accompanying drought treatment (Haddad and Kamangar, 2015). Also Hasanuzzaman et al. (2018) observed an increase in the activity of antioxidant enzymes and a decrease in ROS production after the application of silicon in oilseed rape growing under temporary water deficit in the soil. Si-mediated drought tolerance mechanism and the signaling pathways involved therein are still unknown.

The purpose of our study was to examine the changes in the accumulation of BnPIP1, BnPIP2-1-7 and BnTIP1;1 proteins, as well as transcript accumulation of genes encoding BnPIP2-1-7 and BnTIP1;1 proteins in silicon-treated oilseed rape, both optimally watered and cultivated under drought. Further, we analyzed also silicon effects on the plant antioxidant system, which is important from the point of view of oxidative stress induced by drought. Silicon was supplemented as orthosilicic acid and as commercial preparation Optysil containing silicon and iron. This approach allowed additionally to verify the hypothesis that interaction of silicon and iron ions allows for more effective plant protection against stress, and the use of a commercial preparation in the study was also important from the point of view of agricultural practice.

2. Materials and methods

2.1. Plant material, experimental design and sampling

Oilseed rape (*Brassica napus* var. *napus* L.) cv. Markus was chosen for the experiments. Seeds were obtained from the Institute of Plant Protection – National Research Institute, Poznań, Poland. The experimental design included two experiments. In the first one, preliminary analyses were carried out to check whether silicon (at selected concentrations) had an effect on the accumulation of aquaporins and enzymatic activity in the leaves of oilseed rape. Plants were growing in well-watered conditions. In the second experiment, there were two groups of plants tested – well-watered and also drought stressed. The aim was to verify the influence of silicon on plants under drought conditions vs well-watered plants (Fig. 1).

In the first experiment (Experiment 1) seeds were sown on Petri plates with filter paper, and kept in growth chambers at the temperature 25 ± 2 °C, RH 65–70%, in the dark for 48 h. Plastic pots (1.5 l in volume) were filled with garden soil, "black soil" (*chernozem*) and sand (1:2:1; v/v). The pH of the homogeneous mixture was ca. 7.0–7.3. Five seedlings were planted into each pot. Over the next week the plants grew in growth chambers (25 ± 2 °C, 14h photoperiod), and afterwards the pots were moved to a growth tunnel for 2 more weeks (air temperature 25/16 ± 4 °C day/night, relative air humidity 37–80%). Plants were watered with tap water.

23-day-old plants with 2 leaves unfolded [growth stage: leaf development, code 12 on the BBCH-scale (canola) (Meier, 2001)] were separated into three groups. The control group was watered with 60 mL

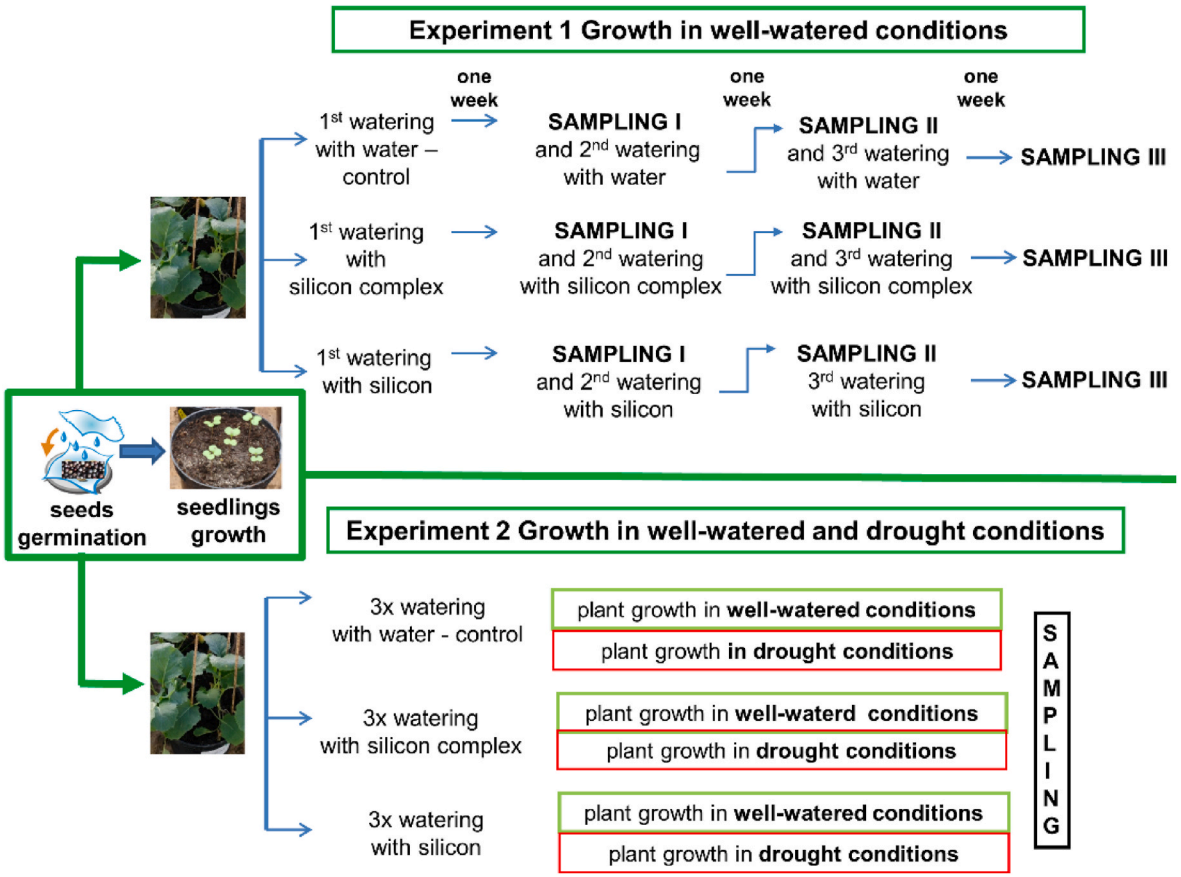

Fig. 1. Experimental design for Experiment 1 and 2.

tap water per pot, the silicon complex group with 60 mL of commercial growth stimulator Optysil (Intermag, Olkusz, Poland) per pot and the silicon group with 60 mL of a solution of silicon (orthosilicic acid tetraethyl ester, Sigma-Aldrich, St. Louis, Missouri, USA) per pot. Watering with water (control) and the aforementioned solutions of silicon was performed at three time points – 30-day-old plants, 37-day-old plants and 44-day-old plants (Fig. 1). The silicon content in the silicon complex (Optysil) and silicon (orthosilicic acid tetraethyl ester) was the same at 3.4 mM Si. Additionally, the silicon complex (Optysil) also includes iron (in ethylenediaminetetraacetic acid (EDTA) chelate form) in the concentration of 0.00027 mM Fe at the second oxidation state. This experiment investigated the effect of silicon on the accumulation of BnPIP1 and BnPIP2-1-7 aquaporins and on the activity of superoxide dismutase (SOD) and catalase (CAT) in the leaves of oilseed rape plants. Samples of control and two silicon-treated groups of plants were collected at three time points: I – one week after the first watering with water (control) and solutions of silicon, II – one week after the second watering with water (control) and solutions of silicon, and III – one week after the third watering with water (control) and solutions of silicon (Fig. 1). In summary, the first sampling was performed on 30-day-old plants one week after watering with water (control) and solutions of silicon, and the second and third sampling after two and three weeks, respectively (Fig. 1).

In Experiment 2, similar to Experiment 1, seeds were germinated for 48h, seedlings were planted in pots and cultured for 3 weeks. The pots were divided into three groups – control (plants watered with water), the silicon complex group (plants watered with Optysil preparation) and the silicon group (plants watered with orthosilicic acid tetraethyl ester). Pots with 44-day-old plants were further split into two subgroups. In the first subgroup, plants grew for 10 days in well-watered conditions – 65–70% of field water capacity (FWC) of the soil. In the second subgroup, plants grew for 10 days in drought conditions – 30% of FWC of the soil. Air temperature was 27/18 ± 2 °C day/night, and relative air humidity was 35–40%. In earlier studies of Saja-Garbarz et al. (2021), it has been confirmed that plants exposed to water deficit in the same model of experiment suffered growth limitation, in comparison to well-watered plants. Leaves of 54-day-old plants were sampled and the following measurements were performed: the accumulation of BnPIP2-1-7 and BnTIP1 aquaporin, the accumulation of *BnPIP2-1-7* and *BnTIP1* transcripts, hydrogen peroxide (H_2O_2) concentration, activity of antioxidant enzymes: superoxide dismutase (SOD) and catalase (CAT), as well as total non-enzymatic antioxidant capacity. Soil moisture conditions at the moment of sampling were as follows: (1) plants growing under well-watered conditions: soil moisture in pots 65% of FWC on the day of sampling, (2) drought-stressed plants: soil moisture in pots 30% of FWC on the day of sampling.

2.2. Measurements

2.2.1. Measurement of protein concentration in crude leaf extract

The leaves of oilseed rape plants were collected, cut into fragments, and divided into 1 g samples. The samples were homogenized at 4 °C in 2.5 mL of a Tricine buffer containing 100 mM Tricine, 3 mM $MgSO_4$, 1 mM DTT, 3 mM EGTA, adjusted to pH 8.0 with 1 M Tris, according to the procedure described by Sadura et al. (2020). After homogenization and centrifugation for 5 min at 14,000 RPM (Hettich, Tuttingen, Germany), the supernatant was collected. Protein concentration in the obtained crude extract was measured according to Bradford (1976). For each of the three independently collected pool samples from one treatment (3 biological replicates), consisting of three to four leaves from ten plants, at least two measurements (2 technical replicates) were performed, giving in total six measurements for each treatment.

2.2.2. Analysis of the accumulation of BnPIP1, BnPIP2-1-7, and BnTIP1 aquaporins using immunoblotting

For the analysis of BnPIP1, BnPIP2-1-7, and BnTIP1 aquaporins, 20 μg of total proteins (PIP analysis) or 10 μg of total proteins (TIP analysis) extracted from the studied plant material were loaded on 12% polyacrylamide gels and electrophoretically separated for 1 h and 30 min at 38 mA in upper gel and 46 mA in lower gel, as described by Laemmli (1970). After electrophoretic separation, proteins were blotted onto a nitrocellulose membrane. Transfer of proteins took place for 1 h and 15 min at 45.5 mA (7–9 V) using the Trans-Blot SD semi-dry transfer cell. Next, the membranes were blocked for 1 h with low-fat milk powder which had been dissolved in a Tris buffered saline with Tween 20 (TBS-T) (containing 0.9% NaCl and 10 mM Tris). After blocking, the membranes were washed with the TBS-T buffer three times for 5 min and then incubated in the appropriate primary antibody (Agrisera) (anti-PIP1, anti-PIP2-1-7, 1:2000, for 1 h 30 min; anti-TIP1, 1:1000, for 1 h 30 min). Then the membranes were washed again with the TBS-T buffer (three times for 5 min) and incubated in secondary, alkaline phosphatase (AP) conjugated anti-rabbit antibody (Sigma-Aldrich) (1:2000, for 1 h 20 min). Next the membranes were washed again with the TBS-T buffer (twice for 5 min) and then with AP buffer (three times for 5 min). Visualization of protein bands corresponding to PIP1, PIP2-1-7 and TIP1 was performed using Nitro blue Tetrazolium Chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (NBT/BCIP) solution kit for alkaline phosphatase staining. In order to quantify the differences in the level of the analyzed aquaporins between the studied samples, densitometric analyses were performed. Staining intensity of the bands corresponding to PIP1, PIP2-1-7 and TIP1 was studied with the use of ImageJ software (National Institutes of Health, USA). For each of the three independently collected pool samples from one treatment (3 biological replicates), consisting of three to four leaves from ten plants, at least one measurement (1 technical replicate) was performed, giving in total three measurements for each treatment.

2.2.3. Accumulation of the transcripts of BnPIP1, BnPIP2-1-7, and BnTIP1: RNA isolation, cDNA synthesis and real-time PCR

The accumulation of the BnPIP1, BnPIP2-1-7 and BnTIP1 transcripts was measured using real-time quantitative PCR amplification and the analyses were carried out using a CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Inc., CA, USA). Samples – leaf blades of oilseed rape (approximately 140 mg of mixed third and fourth leaves) – were collected, frozen in liquid nitrogen and ground with a mortar and pestle to a fine powder under liquid nitrogen. Isolation of total RNA from the leaves, removal of residual DNA and inactivation of DNaseI were performed using methods according to Balarynová and Fellner (Balarynova and Fellner, 2019). Then first-strand DNA mixture was prepared from 1 μg of total RNA using PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Inc., Tokyo, Japan). Synthesis of cDNA and PCR reaction were conducted using methods described previously (Balarynova and Fellner, 2019). Gene expression was analyzed using Sensi Fast SYBR Lo-Rox kit (Bioline - Meridian Bioscience, Memphis, TN, USA) and a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc., CA, USA). For each of the three independently collected pool samples from one treatment (3 biological replicates), consisting of three to four leaves from ten plants, at least one measurement (1 technical replicate) was performed, giving in total three measurements for each treatment.

2.2.4. Determination of endogenous hydrogen peroxide content

Endogenous concentration of hydrogen peroxide was measured using Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes, Eugene, Oregon, USA). Oilseed rape leaves (0.1 g fresh weight) were ground in 0.5 mL of the reaction buffer (provided in the kit). The achieved homogenate was centrifuged for 5 min at 14,000 *g* and then 50 μL of the supernatant was incubated with 50 μL of the working solution containing 100 mM Amplex Red reagent and 0.2 units mL^{-1} horseradish peroxidase for 30 min at room temperature under dark conditions. H_2O_2 concentration was measured using a spectrofluorometer equipped with a 96-well microplate reader Synergy 2 (BioTek Instruments, Inc., Winooski, VT, USA) under the excitation wavelength of 530 nm and fluorescence detection at 590 nm. The standard curve in the range 0–1 μM H_2O_2 concentration was prepared using H_2O_2 provided in the kit. For each of the three independently collected pool samples from one treatment (3 biological replicates), consisting of three to four leaves from ten plants, at least one measurement (1 technical replicate) was performed, giving in total three measurements for each treatment.

2.2.5. Protein isolation, native polyacrylamide gel electrophoresis (PAGE) and visualization of superoxide dismutase (SOD) and catalase (CAT) activity on gels

Plant material (1 g fresh weight) was homogenized in a mortar at 4 °C in 1.5 mL of 50 mM phosphate buffer, pH 7.8 containing 1 mM DTT, 1% poly(vinylpolypyrrolidone) (PVPP) and protease inhibitor cocktail (cOmplete, Roche Molecular Systems, Inc., Basel, Switzerland). The homogenates were centrifuged for 5 min at 14,000 RPM. The supernatants were collected and stored at −80 °C. Total protein concentration was determined in the supernatant according to Bradford dye-binding method (Bradford, 1976) using Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

The activity of SOD or CAT was visualized on 12% or 10% polyacrylamide gels, respectively, after electrophoretic separation of protein fractions using the Laemmli (1970) buffer system without sodium dodecyl sulfate (SDS) at 4 °C and 180 V. Each well was loaded with the same amount of protein (12 μg for SOD and 7 μg for CAT). The bands corresponding with SOD activity were visualized using the activity staining procedure described by Beauchamp and Fridovich (1971) – the gels were incubated in a staining buffer (50 mM potassium phosphate buffer, pH 7.8, containing 0.372 g l^{-1} EDTA, 31% (v/v) Temed, 7.5 mg l^{-1} riboflavin and 0.2 g l^{-1} NBT) for 30 min in the dark at room temperature and then exposed to white light until the SOD activity bands became visible. For the identification of SOD isoforms, selective inhibitory staining was performed. H_2O_2 in the concentration of 5 mM was added into the staining solution in order to inhibit copper/zinc superoxide dismutase (Cu/ZnSOD) and iron superoxide dismutase (FeSOD), while 3 mM potassium cyanide (KCN) was used to inhibit Cu/ZnSOD. For CAT inhibition, 10 mM 3-amino-1,2,4-triazole (AT) was added into the staining solution. The bands corresponding to CAT activity were visualized using the method described by Woodbury et al. (1971). Gels were scanned using the Epson Perfection V700 Photo scanner (Epson America, Inc., Long Beach, CA, USA). Densitometry analyses using ImageJ software were used to determine the differences in the intensities of visualized bands corresponding to the activities of SOD isoforms or CAT. The results were presented in arbitrary units which correspond to the area under the densitometric curve. For each of the three independently collected pool samples from one treatment (3 biological replicates), consisting of three to four leaves from ten plants, at least one measurement (1 technical replicate) was performed, giving in total three measurements for each treatment.

2.2.6. Estimation of total antioxidant activity in leaf tissue

Three independent pool samples, each comprising four leaves from two plants, were collected and after FW estimation promptly frozen. Samples were lyophilized and ground with ball mill MM400 (Retsch, Haan, Germany) in Eppendorf vials and, after the addition of 1 mL of 50% ethanol, shaken for 2 h in the dark at room temperature so as to produce extracts. The extracts were then centrifuged for 20 min in a centrifuge at 18,000 *g* (MPW-350R, Warsaw, Poland) and the supernatant was utilized for the measurements. Total antioxidant activity (of non-enzymatic antioxidants) in the tissues was estimated using 1,1-diphenyl-2-picrylhydrazyl method according to Brand-Williams et al. (1995) with some alterations adjusting it to 96-well microtitre plates and to the assessment of absorbance by microtitre plate reader (Laskoś et al., 2021). The method utilized 0.5 mM stable free radical 1,

1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, Inc., Merck KGaA, Darmstadt, Germany) in methanol. Absorbance was assessed after 30 min of the reaction at 37 °C at 515 nm with reader Model 680 (Bio-Rad Laboratories, Hercules, CA, USA). The results are presented as μmoles of Trolox equivalents. For each of the three samples from one treatment (3 biological replicates) at least three DPPH measurements (3 technical replicates) were carried out, giving in total nine measurements for each treatment.

2.2.7. Primers design

All primers were designed using Primer Express Software v3.0.1 (Applied Biosystems, Foster City, CA, USA) and their sequences are listed in Table 1.

The levels of BnPIP2-1-7 and BnTIP1 transcripts were determined relative to Actin as reference gene.

2.3. Statistical analysis

For each measured parameter three independent biological samples, consisting of three to four leaves from ten plants (biological replicates) were collected. For each sample at least one (accumulation of aquaporins and their transcripts, SOD and CAT activity, H_2O_2 content), two (protein concentration in crude leaf extract) or three (total antioxidant activity) measurements (technical replicates) were performed, giving in total three, six and, nine replicates for one treatment, respectively.

Statistica v. 13.1 (StatSoft, Inc., Tulsa, OK, USA) was employed for the statistical analysis and graphic presentation of the results. One-way analysis of variance (ANOVA) was utilized to ascertain the key effects of the treatments on the physiological parameters. Duncan's multiple range test at 0.05 probability level was used to estimate the significance of differences among the treatment means. Correlations between the measured parameters were tested at the probability of $p \leq 0.05$. The figures include mean values ± standard deviation (SD).

3. Results

3.1. Effect of silicon on the accumulation of BnPIP1 and BnPIP2-1-7 aquaporins in oilseed rape leaves growing under well-watered conditions (Experiment 1)

Both the identification of BnPIP1 and BnPIP2-1-7 (see Supplementary Materials Figs. SM1 and SM2) on the nitrocellulose membrane with the use of specific antibodies, as well as the results of densitometric measurements of the intensity of the obtained bands (Figs. 2 and 3) revealed the presence of both aquaporins in the analyzed material and indicated differences in the level of their accumulation.

In the case of BnPIP1, control plants and plants treated with silicon complex exhibited a significantly higher amount of this aquaporin in the leaves of the oldest plants compared to younger ones in a given treatment group. This effect was not observed in plants watered only with silicon (Fig. 2). Moreover, the highest accumulation of BnPIP1 was detected in the leaves of plants watered three times with silicon complex. However, no clear correlation was found between the accumulation of the studied protein and the presence of silicon in the environment.

In the case of BnPIP2-1-7, the presence of both monomers and dimers of this protein was detected (see Supplementary Materials Fig. SM2). The total (performed for the sum of monomers and dimers) densitometric analysis of band intensity revealed the same tendency as in the case of the accumulation of BnPIP1 aquaporin, indicating a significantly higher level of BnPIP2-1-7 accumulation in the leaves of the oldest plants watered with water (control) and silicon complex, in which case it was the highest (Fig. 3). In the leaves of plants treated only with silicon, this tendency was opposite and the highest level of BnPIP2-1-7 accumulation was observed in the youngest plants. Similar to BnPIP1, the highest level of BnPIP2-1-7 accumulation was detected after watering plants three times with silicon complex, and this relationship was visible in the case of both monomers and dimers.

3.2. Effect of silicon on superoxide dismutase (SOD) and catalase (CAT) activity in the leaves of oilseed rape plants growing under well-watered conditions (Experiment 1)

The analysis of superoxide dismutase (SOD) activity in the leaves of oilseed rape plants growing under well-watered conditions showed particularly high total SOD activity in plants treated three times with silicon complex (Fig. 4). The visualized bands corresponding with the activity of SOD isoforms - see Supplementary Materials (Fig. SM3). All plants exhibited the presence of three SOD isoforms, which were identified as MnSOD, FeSOD and Cu/ZnSOD (Fig. 4). The activity of MnSOD isoform was particularly high in plants watered with silicon complex after the third watering (Fig. 4a). A similar tendency was observed in the case of Cu/ZnSOD (Fig. 4b). FeSOD activity was lower in the oldest plants treated with silicon solutions compared to both younger plants in the same treatments and control plants (Fig. 4c).

In the case of catalase (CAT), the highest activity of this enzyme was observed in plants treated only with silicon (Fig. 5). In plants treated with silicon complex this tendency was reversed, with the highest CAT activity observed after the first application of the preparation. Control plants, on the other hand, maintained a relatively high level of CAT activity throughout the entire period of plant growth, with no differences related to their age.

3.3. Effect of silicon on the accumulation of BnPIP2-1-7 aquaporin (protein and transcript) in the leaves of oilseed rape plants growing under drought (Experiment 2)

BnPIP2-1-7 aquaporin is considered to be the most efficient water transporter. The accumulation of protein BnPIP2-1-7 was analyzed in the leaves of oilseed rape plants growing under drought and well-watered (control and silicon treated).

Similar to plants growing under well-watered conditions, the presence of both monomers and dimers of this protein was detected in drought-stressed plants – see Supplementary Materials (Fig. SM5). Band intensity analysis reflecting the amount of BnPIP2-1-7 aquaporin revealed that plant supplementation with Si significantly reduced the accumulation of this protein compared to control plants, regardless of the source of silicon (Fig. 6a). However, it did not correlate with the transcriptional activity of the *BnPIP2-1-7* gene, where an increase in gene expression was observed after supplementation with silicon complex compared to control (Fig. 6b).

Table 1
Sequence origins and primers used in the study.

Gene name	GenBank ID for forward primer	Forward primer	Reverse primer
BnPIP2-1-7	NM_001316255.1	AAGGGTTTCAGACAAGAGACTATCAAG	GCTCCGCCGGGTCAA
BnTIP1	NM_001316109.1	CAATGGAGCCACCACTCCTT	CGAAGAGACCGAAAGCATGAG
Actin	GQ339782.1	CGTCCTCAGTGGTGGTTCAA	TGCCGTGATCTCTTTGCTCAT

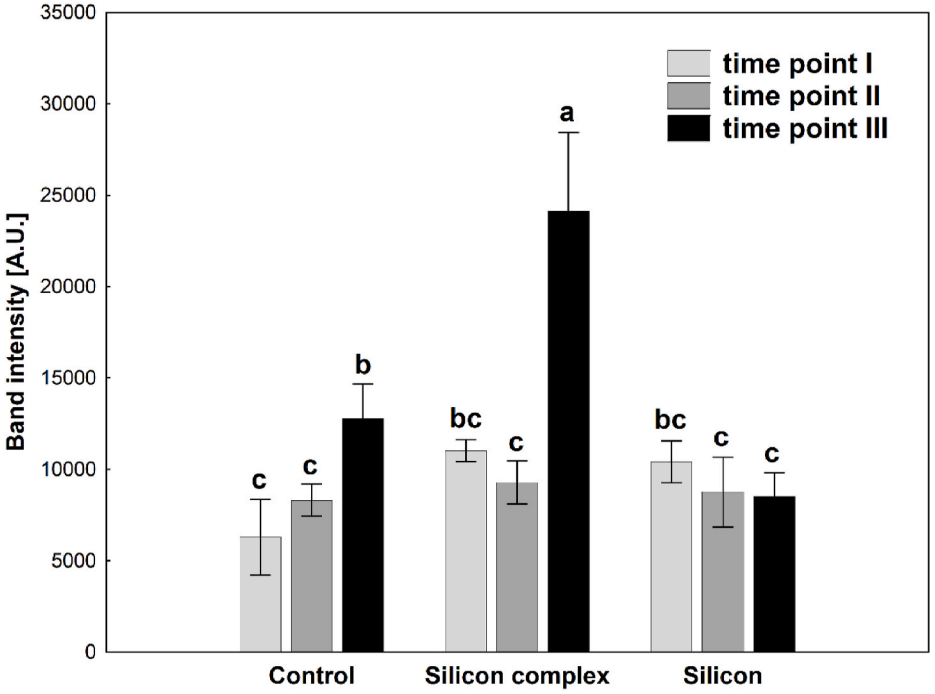

Fig. 2. The accumulation of protein BnPIP1 in leaves of well-watered oilseed rape measured weekly, at three time points I-III - the effect of silicon. Control – plants untreated with silicon. The amount of accumulated BnPIP1 was estimated based on a densitometric analysis of the band intensity staining. The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

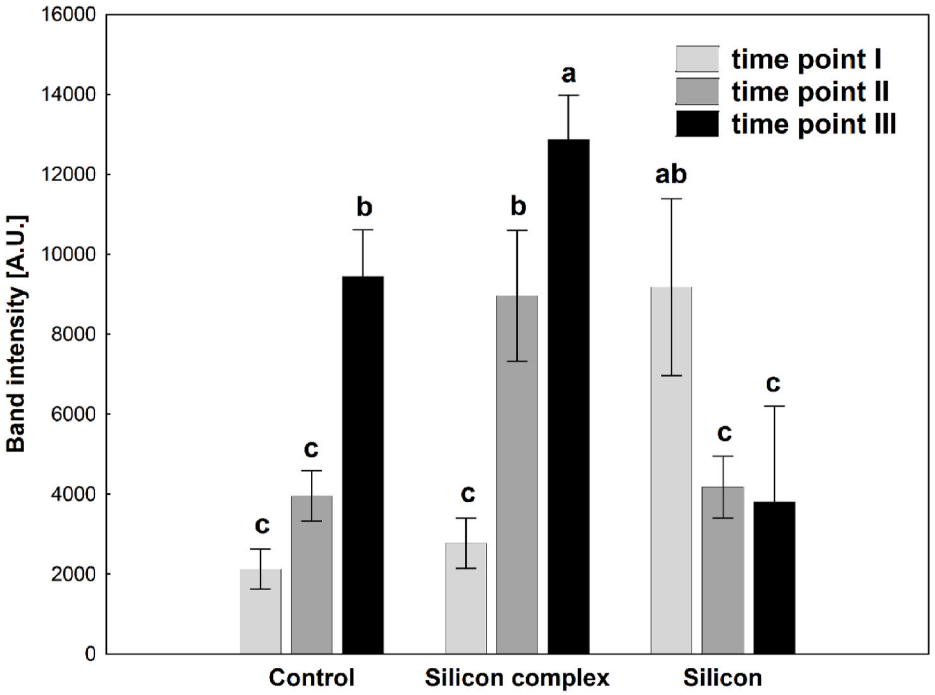

Fig. 3. The accumulation of protein BnPIP2-1-7 in leaves of well-watered oilseed rape measured weekly, at three time points I-III - the effect of silicon. Control – plants untreated with silicon. The amount of accumulated BnPIP2-1-7 was estimated based on a densitometric analysis of the band intensity staining. The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

3.4. Effect of silicon on the accumulation of BnTIP1 aquaporin and its transcript in the leaves of oilseed rape plants growing under well-watered conditions and drought (Experiment 2)

As tonoplast aquaporins are considered to be markers under the conditions of temporary soil water shortage, the accumulation of BnTIP1 aquaporin was compared in the leaves of oilseed rape plants growing under well-watered conditions and under drought. Moreover, an analysis was performed of the regulation of changes induced by silicon. It was revealed that both under well-watered growth conditions and under drought the presence of both monomers and dimers of BnTIP1 protein became clearly marked – see Supplementary Materials (Fig. SM6).

The densitometric analysis showed that under well-watered growth conditions the accumulation of this protein was significantly higher in plants treated with silicon complex compared to control plants and plants watered only with silicon (Fig. 7a). Under drought, on the other hand, a clear effect of silicon supplementation was observed, regardless of their source, through significantly higher BnTIP1 accumulation compared to control plants. The analysis of *BnTIP1;1* transcriptional activity revealed the same tendency in transcript as in protein accumulation in plants growing under well-watered conditions, though this tendency was reversed in plants supplemented with Si under drought

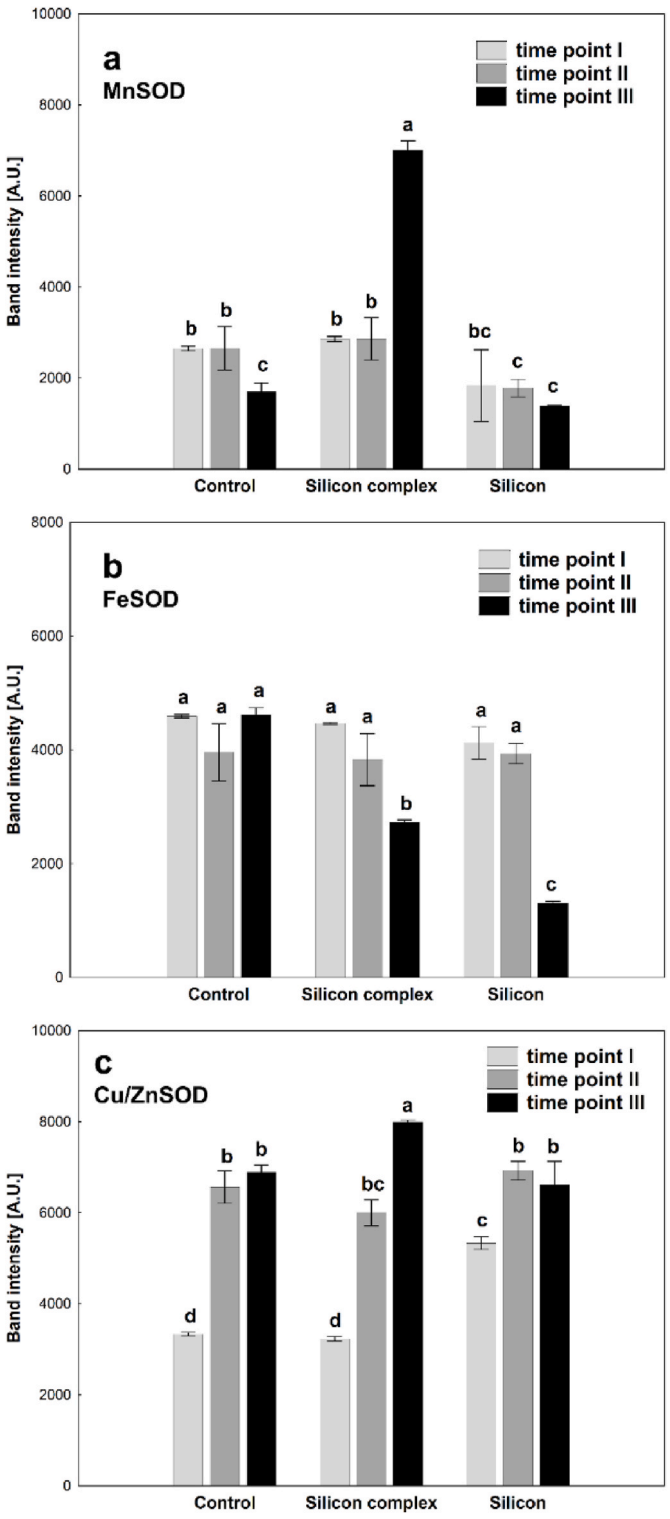

Fig. 4. Densitometric analysis of the total bands intensity (shown on Fig. 4) corresponding with different SOD isoforms – MnSOD (a), FeSOD (b), Cu/ZnSOD (c) in leaves of well-watered oilseed rape measured weekly, at three time points I-III - the effect of silicon. Control – plants untreated with silicon. The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

(Fig. 7b). Control plants exhibited the highest *BnTIP1;1* transcript accumulation compared to plants watered with solutions containing silicon, though statistically significant differences were also observed depending on the source of silicon. Higher transcript accumulation was detected in plants treated with silicon compared to silicon complex.

3.5. Effect of silicon on hydrogen peroxide (H_2O_2) concentration in the leaves of oilseed rape plants growing under well-watered conditions and drought (Experiment 2)

Under well-watered conditions, Si supplementation had a statistically insignificant effect on the increase in H_2O_2 level in plants treated with silicon compared to control plants, while a significant increase in H_2O_2 level was observed in the leaves of plants treated with silicon complex (Fig. 8). Under drought there were no significant differences in H_2O_2 level among plants grown under different silicon treatments. However, in control plants drought caused a sharp increase in H_2O_2 level, while in plants supplemented with silicon this change was milder and not statistically significant (Fig. 8).

3.6. Effect of silicon on antioxidant system activity and total antioxidant capacity of extracts from the leaves of oilseed rape plants growing under well-watered conditions and drought (Experiment 2)

3.6.1. Superoxide dismutase (SOD) enzyme activity

The analysis of the activity of superoxide dismutase (SOD) isolated from the leaves of oilseed rape plants growing under drought revealed an increase in total SOD activity resulting from an increase in all identified isoforms, with the highest activity observed in the case of Cu/ZnSOD – see Supplementary Materials (Fig. SM7). The densitometric analysis of band intensity confirmed that under drought total SOD activity was high and that SOD activity increased significantly in plants supplemented with silicon, while no evidence was found for this change to be affected by the source of silicon (Fig. 9).

3.6.2. Catalase (CAT) enzyme activity and concentration

The measurement of catalase (CAT) activity performed on a polyacrylamide gel revealed higher activity of this enzyme in control plants compared to plants supplemented with silicon, both under well-watered conditions and under drought – see Supplementary Materials (Fig. SM8). The densitometric measurement showed that CAT activity in the leaves of plants growing under well-watered conditions was significantly higher compared to plants growing under drought (Fig. 10) – the exact opposite effect compared to SOD. On the other hand, similar to SOD, the source of silicon (silicon or silicon complex) was not observed to affect CAT activity. An additional densitometric analysis of CAT on the basis of band intensity on the gel confirmed that under well-watered growth conditions its activity is higher than under drought. Moreover, the highest activity of this enzyme under well-watered conditions was detected in control plants (Fig. 10). Under drought, on the other hand, no significant differences in CAT activity were observed between the different silicon treatments.

3.6.3. Total non-enzymatic antioxidant capacity

In order to augment the assessment of antioxidant activity in oilseed rape leaves, an analysis of the activity of non-enzymatic, low-molecular weight antioxidants was performed. It revealed that under well-watered conditions, the highest activity of these antioxidants was exhibited by leaves of control plants and plants treated with silicon complex compared to plants treated only with silicon (Fig. 11).

On the other hand, under drought the lowest activity of these antioxidants was exhibited by plants from the control treatment in comparison to those treated with silicon. Moreover, the source of Si was shown to have a significant effect on the activity of the low molecular weight antioxidants, as the highest activity of these antioxidants was detected in plants supplemented with silicon complex, compared not

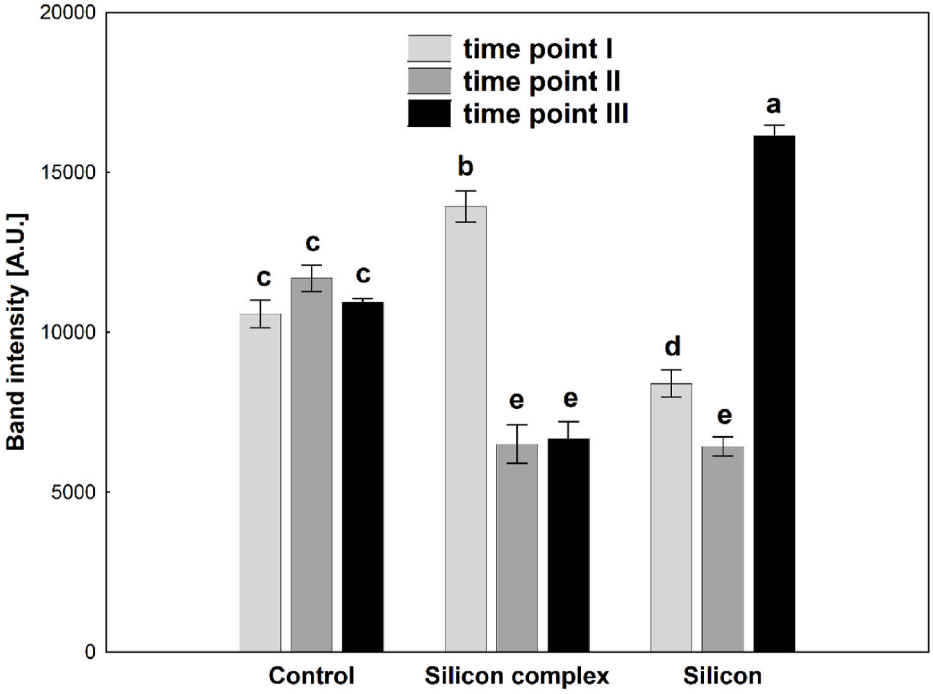

Fig. 5. CAT activity in protein fraction isolated in leaves of well-watered oilseed rape measured weekly, at three time points I-III - the effect of silicon. Control – plants untreated with silicon. The activity of CAT was estimated based on a densitometric analysis of the band intensity. The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

only to control plants but also to plants watered with silicon.

4. Discussion

Maintaining water balance in the face of changing environmental conditions is a crucial survival strategy for plants (Mahdieh et al., 2008). It is well known that silicon protects plants against the adverse effects of periodic water shortage in the soil, improving their hydration level and water use efficiency (Zhu and Gong, 2014). As shown thus far in our research, supplementation of oilseed rape plants with Si does not significantly affect the morphological structure of the shoots (height and dry weight), and its effects on the efficiency of metabolic processes are varied in the studied physiological aspects (Saja-Garbarz et al., 2021). In addition to physiological adjustments, a significant role in regulating water management is played by aquaporins (AQPs), which exhibit different levels of accumulation and activity (Ranganathan et al., 2017).

It was shown that under temporary soil water shortage, supplementation with silicon – silicon and silicon complex – caused a significant reduction in the amount of BnPIP2-1-7 aquaporin (Fig. 6a), which may indicate minimization of water flow through cell membranes in order to maintain turgor in leaf cells (Alexandersson et al., 2005). This is especially important as all PIP2 proteins have a high water transport capacity (Chaumont et al., 2000). In plants treated with the silicon complex, the decrease in this protein's content was associated with a significant increase in the amount of its transcript (Fig. 6b), as previously demonstrated by Rios et al. (2017) in the roots of plants treated with silicon under salt stress. However, as the level of transcript accumulation of a given gene can be significantly differentiated depending on the plant organ, it is difficult to unambiguously compare this effect. Moreover, not all AQP transcripts are converted into functional proteins due to protein post-translational modifications (PTMs), during which they achieve their final form and are able to perform their functions (Yu et al., 2005). According to a recent review by Santoni (2017), over 70 phosphorylation sites, especially in serine residues, have been identified in PIP, TIP and NIP subgroups of AQPs in different plant species. Moreover, AQPs are also widely regulated by N-terminal protein PTMs, which rely on deamidation, glycosylation, methylation and ubiquitination. Particularly the last type of posttranslational protein modification might be of special importance since, as already mentioned, ubiquitination under water stress leads to aquaporin degradation and long-term downregulation of plasma membrane water permeability (Santoni, 2017). As reported by Alexandersson et al. (2005), the decrease in relative PIP2 expression may also result from a general decrease in the transcription level of genes non-essential for the plant under drought stress. In contrast, the studies by Kapilan et al. (2018) showed that overexpression of the PIP2 gene may indicate a significant increase in water permeability. However, this explanation is not entirely clear due to the highly differentiated expression of AQPs depending on the stress factors (Afzal et al., 2016). Galmes et al. (2007) proved that severe stress can significantly reduce the expression of AQPs in leaves in the initial phase of its action, but under prolonged stress, the expression levels of AQPs return to normal. As described by Jang et al. (2007), in Arabidopsis the overexpression of PIP1;4 and PIP2;5 caused rapid loss of water from plants and increased plant susceptibility to water stress. According to a recent study by Sonah et al. (2017), reduced expression of AQPs prevents the loss of metabolic energy in situations of severe stress and/or prevents the loss of water from the root to the surrounding hypertonic environment. Therefore, these results indicate that it was the use of silicon in our studies that more significantly affected AQP-induced regulation of water management in oilseed rape, which exhibited the significantly lowest relative BnPIP2-1-7 expression under drought conditions (Fig. 6b).

In addition to PIP aquaporins, plant water balance is regulated by tonoplast aquaporin (TIP) gene expression and protein accumulation (Rios et al., 2017). Both in well-watered conditions and under drought, plant supplementation with the silicon complex was found to increase the accumulation of BnTIP1;1. This is consistent with results previously described by Vera-Estrella et al. (2004), according to which the disturbance of water balance caused by drought stress increases the amount of proteins in tonoplast fractions. Moreover, a marked increase in relative BnTIP1;1 expression was observed under drought compared to well-watered conditions (Fig. 7b). In well-watered conditions Si had no substantial effect on the expression of *BnTIP1;1* gene or BnTIP1;1 protein. The results suggest that in unstressed conditions the silicon complex promotes stability and synthesis of BnTIP1;1 protein, thus increasing plant fitness (e.g. hydration level). It can be hypothesized that

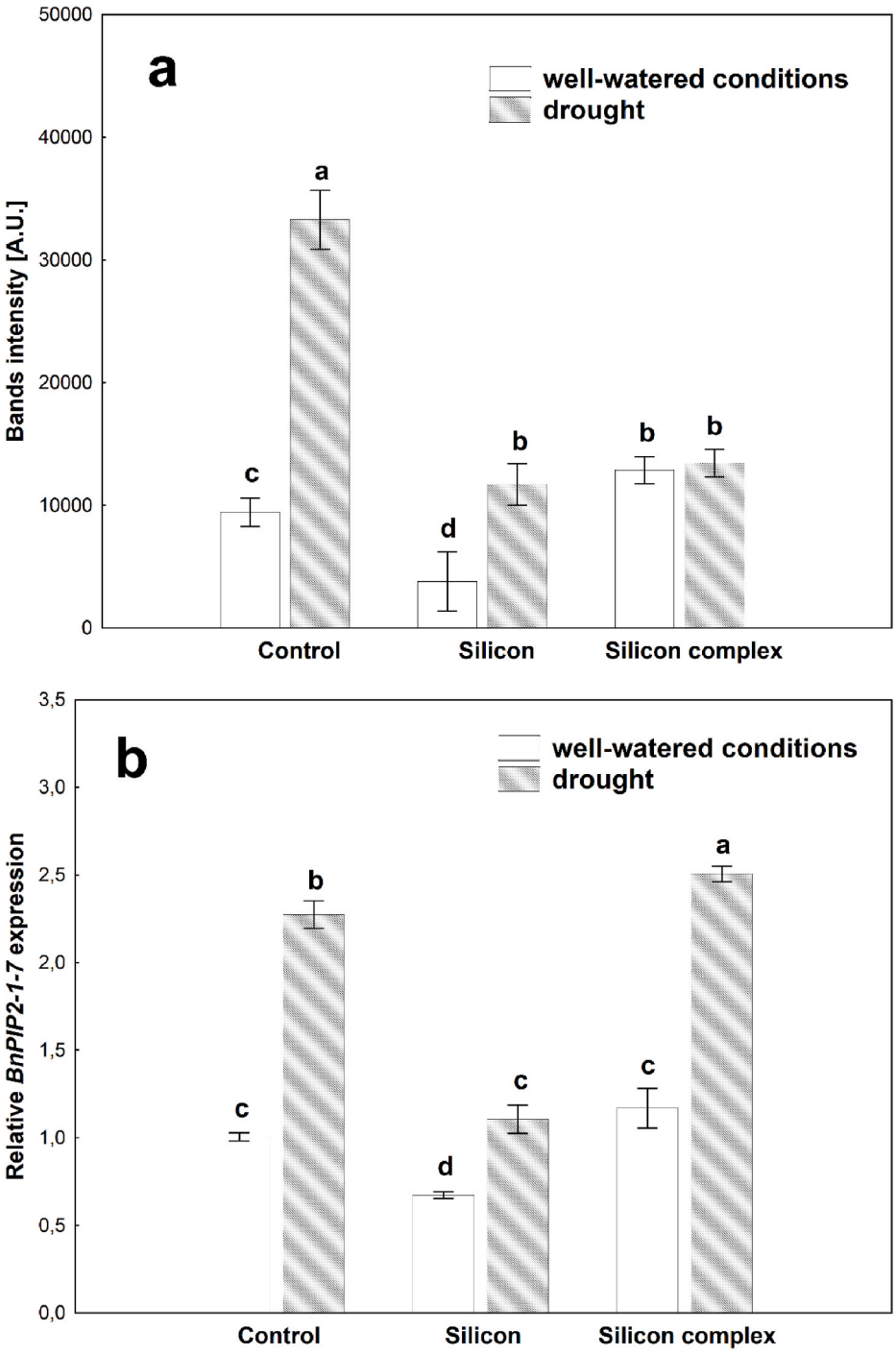

Fig. 6. The effect of drought on accumulation of protein BnPIP2-1-7 (a) and transcript *BnPIP2-1-7* (b) in leaves of silicon untreated control and silicon treated plants of oilseed rape.
The amount of accumulated BnPIP2-1-7 was estimated based on a densitometric analysis of the band intensity staining (a). *BnPIP2-1-7* transcript accumulation is rendered as the fold change in the expression of a specific gene in a particular sample in contrast to the endogenous reference gene – Actin (b). The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

under drought stress conditions, the silicon complex increases stability and translatability of BnTIP1;1. Though the amount of transcript decreased significantly after plant supplementation with silicon from both sources, ultimately the amount of functional protein would indicate that it is necessary for maintaining a water balance allowing plants to survive under drought conditions. Aquaporins participate in the transport of different molecules – not only water, and thus explaining the exact nature of these processes is not easy. On the basis of expression patterns it can be supposed that an increase or decrease in water transport under stress conditions depends on the activity of aquaporins (Javot and Maurel, 2002).

Both biotic and abiotic stress factors cause a number of interrelated processes in plants, triggering the initiation of defense mechanisms. In addition to responses induced in order to alleviate the negative effects of a particular factor, there are also processes leading to the mitigation of the associated oxidative stress caused by reactive oxygen species (ROS) overproduction due to metabolic disturbances. SOD is considered the first line of defense enzyme, which converts the toxic superoxide anion ($O_2\bullet^-$) into the less toxic hydrogen peroxide (H_2O_2), further decomposed into water among others by CAT (Gratao et al., 2005). The ability to maintain a balance between ROS production and the activity of antioxidant systems mitigating the effects of ROS may correlate with the plant's drought tolerance (Tsugane et al., 1999). A number of studies have been conducted on the activity level of selected elements of the antioxidant system under drought stress in *Brassica napus* (Abedi and Pakniyat, 2010). Drought stress preferentially increased the activity of

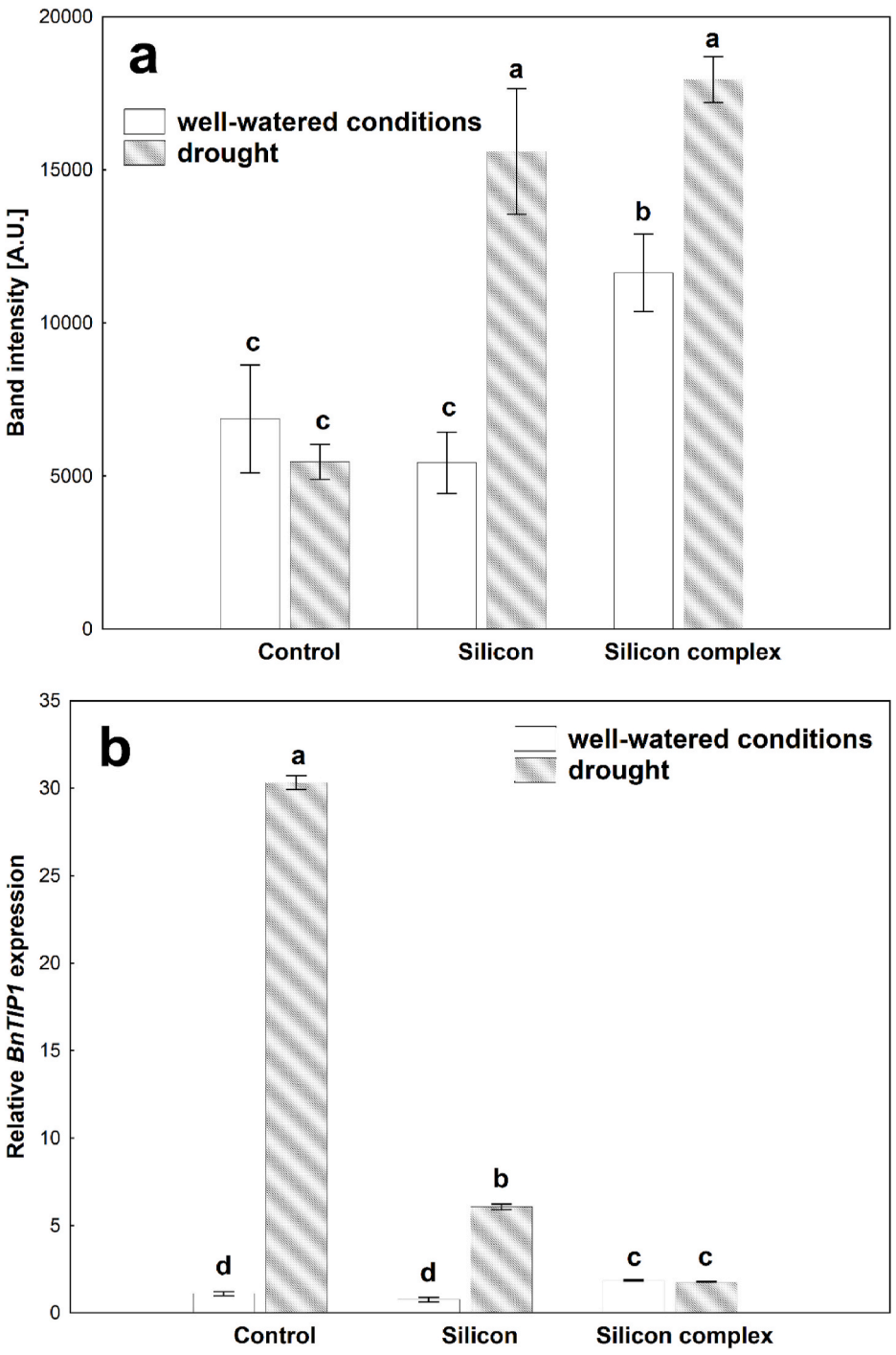

Fig. 7. The effect of drought on accumulation of protein BnTIP1;1 (a) and transcript *BnTIP1;1* (b) in leaves of control and silicon treated plants of oilseed rape. The amount of accumulated BnTIP1; 1 was estimated based on a densitometric analysis of the band intensity staining (a). *BnTIP1;1* transcript accumulation is rendered as the fold change in the expression of a specific gene in a particular sample in contrast to the endogenous reference gene – Actin (b). The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

superoxide dismutase (SOD) and peroxidase (POX), while it reduced the activity of catalase (CAT) (Abedi and Pakniyat, 2010). Qin and Tian (2005) suggested that silicon is beneficial in the processes of plant protection against all stresses through activating antioxidant systems and generating phenolic compounds which act as antioxidants. According to our results, SOD activity in plants supplemented with Si was found to be indirectly dependent on the source of supplemented silicon. Under well-watered growth conditions, an increase in the activity of two SOD isoforms – MnSOD (Fig. 4a) and Cu/ZnSOD (Fig. 4c) – was observed in plants treated with the silicon complex. The obtained results are in agreement with the data published by Zhang et al. (2018), who found an increase in SOD activity under the influence of silicon in plants. In contrast to the increase in the above-mentioned SOD isoforms, a decrease in FeSOD was observed in plants growing in well-watered conditions under silicon complex supplementation. FeSOD is an isoform localized in chloroplasts, while Cu/ZnSOD can be detected in both chloroplasts and cytoplasm of plant cell (Alscher et al., 2002). It has already been stated that FeSOD and Cu/ZnSOD expression is reciprocal in response to a large number of stress treatments and their functions might be interchangeable (Pilon et al., 2011). Thus we cannot exclude the possibility that the pattern of activity of SOD isoforms described in our study is due to the fact that the decrease in FeSOD activity was compensated by a strong increase in the activity of Cu/ZnSOD isoforms. Therefore, it can be concluded that Si supplementation activates the antioxidant system in plants, but the involvement of the additional iron element present in the *Optysil* preparation can also be of importance.

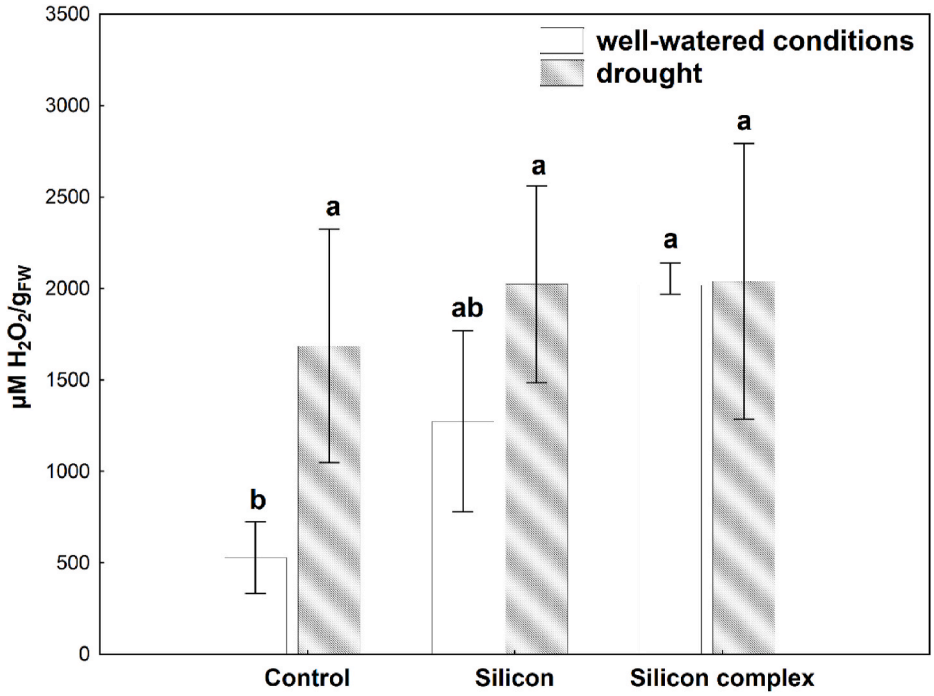

Fig. 8. Changes in the endogenous hydrogen peroxide content in leaves of oilseed rape plants growing under well-watered conditions or drought – the effect of silicon. Control – plants untreated with silicon. The same letter marks mean values (n = 8) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

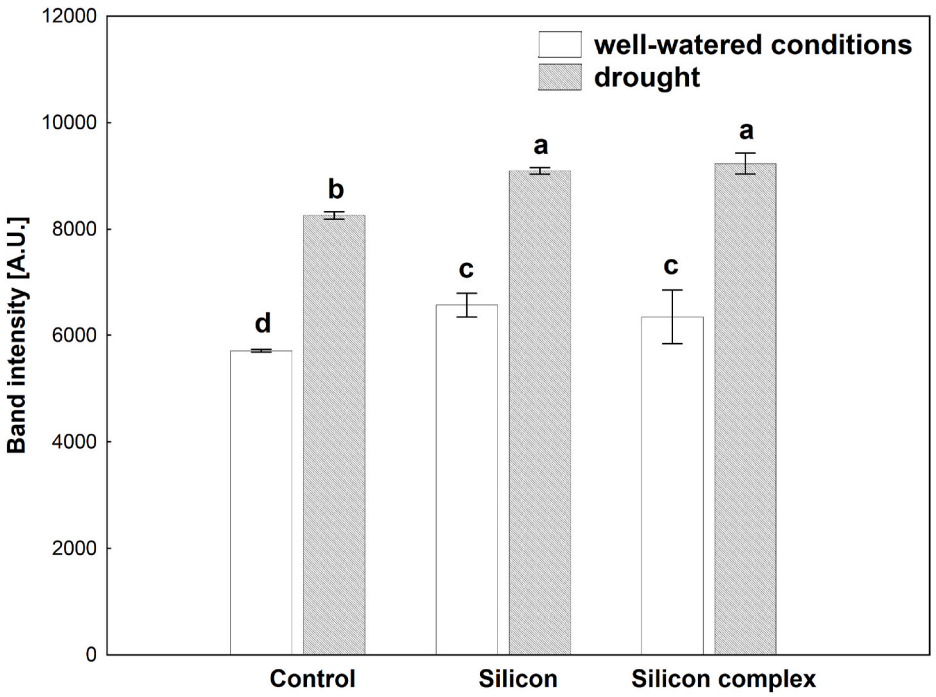

Fig. 9. SOD activity in protein fraction isolated from leaves of oilseed rape plants growing under well-watered conditions and drought – effect of silicon. Control – plants untreated with silicon. The activity of SOD was estimated based on densitometric analysis of the total bands intensity corresponding with different SOD isoforms. The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

SOD activity under drought was higher in plants supplemented with silicon compared to control (Fig. 9). Increased activity of SOD, in combination with enhanced activity of H_2O_2 scavenging enzymes such as CAT and POX, is considered an important anti-drought mechanism in crops suffering from oxidative stress under drought (McKersie et al., 1999).

In their study performed on *Brassica napus*, Hasanuzzaman et al. (2018) proved the activation of the antioxidant system and the protective role of silicon at moderate drought intensity. The results of spectrophotometric measurements of endogenous H_2O_2 level obtained in our studies indicated that it was significantly higher in plants subjected to drought stress than in plants growing under well-watered conditions (Fig. 8). Moreover, the amount of H_2O_2 content increased in plants growing under well-watered conditions, which may suggest that supplementation with the silicon complex leads to disturbances in cell metabolism, inducing an increase in ROS level, which was not

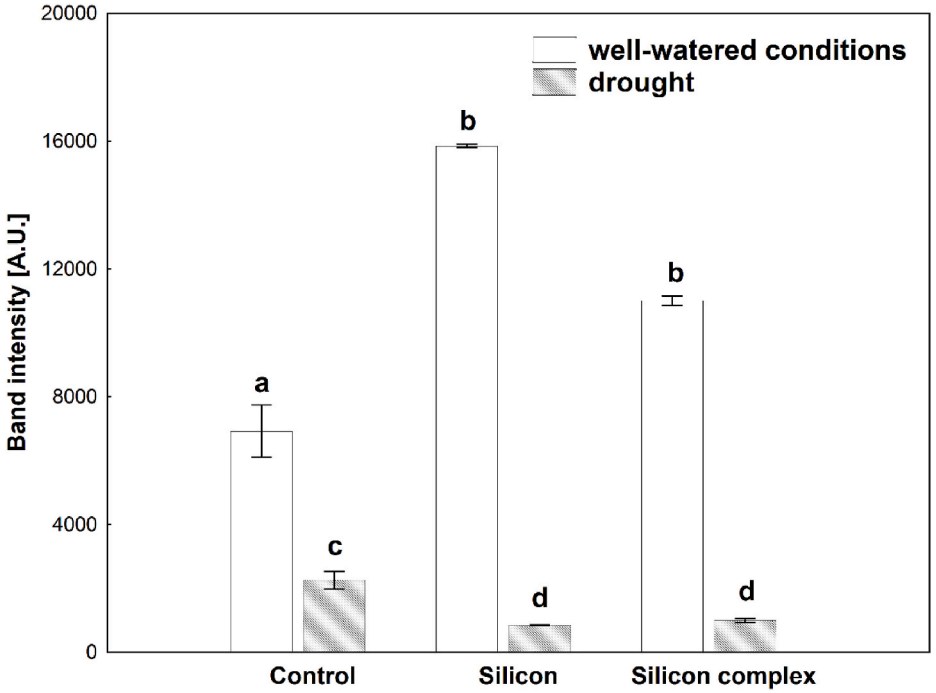

Fig. 10. CAT activity in protein fraction isolated from leaves of oilseed rape plants growing in well-watered conditions and drought – effect of silicon. Control – plants untreated with silicon. The activity of CAT was estimated based on a densitometric analysis of the band intensity. The values are shown as arbitrary units (A.U.). They correspond to the area under the densitometric curves. The same letter marks mean values (n = 5) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

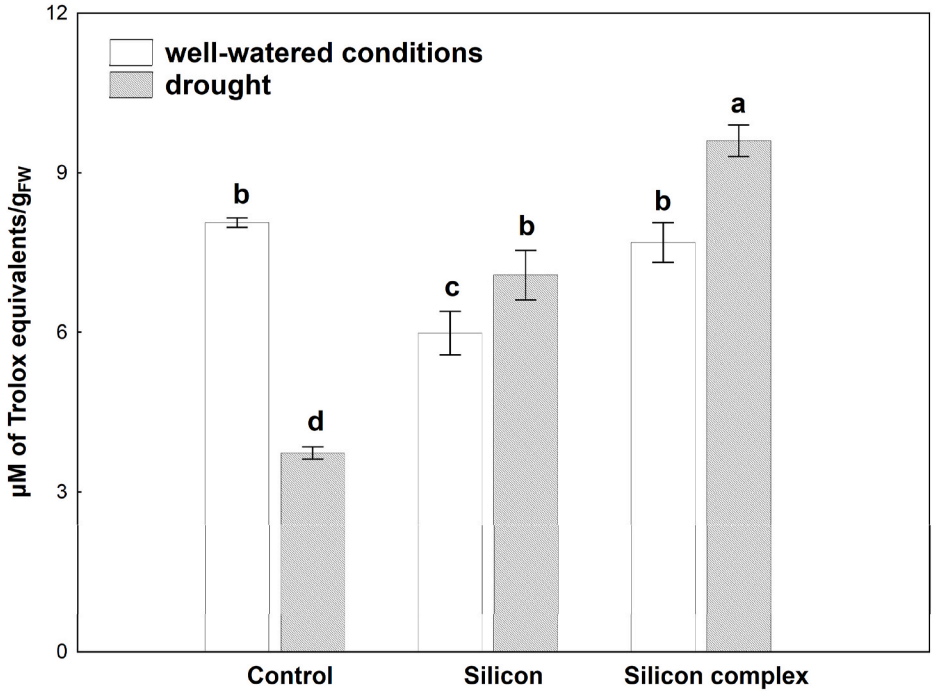

Fig. 11. Changes in total non-enzymatic antioxidant activity in leaves of oilseed rape plants growing under well-watered conditions and drought - effect of silicon. Control – plants untreated with silicon. The same letter marks mean values (n = 9) ± SD which do not differ significantly (Duncan's multiple range test, $p < 0.05$).

observed under drought. CAT is a well-known H_2O_2 scavenger in both plant and animal cells (Comba et al., 1998). An increase in the concentration of silicon in the medium was accompanied by a decrease in the activity of CAT, both in plants growing under well-watered conditions (Fig. 6a) and under drought (Fig. 10). We can hypothesize that the increase in CAT activity might be due to the increase in photorespiration. CAT is the main enzyme destroying hydrogen peroxide produced in the photorespiratory pathway. Photorespiration could serve as an energy container preventing the over-reduction of the photosynthetic electron transport chain and photoinhibition, which is especially important under stress conditions, leading to reduced rates of photosynthetic CO_2 assimilation. Increased CAT activity after the third watering with silicon might indicate that this treatment with Si alone induces stress. Taking into account the results of enzymatic antioxidant activity as well as total non-enzymatic antioxidant capacity (Fig. 11), it can be concluded that the ameliorating effect of silicon on the reduction of oxidative stress caused by drought could rely to a large extent on the activation of low molecular weight antioxidants. This undeniably

confirms their alleviative role in combating the effects of oxidative stress accompanying drought.

The obtained results confirmed the hypothesis about the important role of silicon supplemented during the growth and development of *Brassica napus* var. *napus* L. in the mitigation of the effects of drought stress. Although oilseed rape is not widely recognized as a silicon accumulating plant species, supplementation with silicon solutions was proven to affect its accumulation in the plant and to be important in the regulation of its water management and in the protection against oxidative stress caused by drought. It was shown that in well-watered conditions, the silicon complex significantly increases the accumulation of selected PIPs (BnPIP1- Fig. 2; BnPIP2-1-7 - Figs. 3 and 6a) and TIP (BnTIP1;1 - Fig. 7a) aquaporins. Furthermore silicon and silicon complex improve the performance of enzymatic elements (SOD - Fig. 9; CAT - Fig. 10) of the antioxidant system. It may indicate that during oilseed rape growth under well-watered conditions, the presence of silicon alone in the environment is of little importance. Enrichment of supplementation solutions with additional compounds, as in the case of the commercial *Optysil* preparation (silicon complex), significantly improves the accumulation of aquaporins and increases the activity of antioxidative enzymes. Under drought stress, on the other hand, regardless of the source of silicon, Si supplementation improves the accumulation of BnTIP1;1 aquaporin (Fig. 7a) and increases the activity of antioxidative enzymes (SOD - Fig. 9) and non-enzymatic elements of the antioxidant system - (Fig. 11), which proves that silicon plays an essential role in the plant's effective coping with water deficit. In the previous work (Saja-Garbarz et al., 2021) it was shown that the silicon complex can improve the effectiveness of drought protection. In this paper, we explain that the mechanism of this effect may lie in changes in the accumulation of aquaporins induced by silicon and consequently in an improvement in water management.

Based on the research carried out so far, both in this and the authors' previous study (Saja-Garbarz et al., 2021), it can be assumed that the regulation of water balance in drought conditions under the influence of silicon may be related to facilitating the penetration of water to the roots of the plant. Chen et al. (2018) suggested that the mechanism of such a strategy in the root may depend on, among others, aquaporin activity through up-regulating the expression of PIP aquaporin genes and alleviating ROS. Although, based on the results obtained from the leaf experiments, no such relationship was observed in the case of PIP aquaporins, the effect was visible in the case of TIP aquaporins, where the accumulation of this protein under drought after the application of silicon was higher than in the control. Moreover, despite the fact that the results of the endogenous hydrogen peroxide content analysis were comparable between treatments under drought, the activity level of low molecular weight antioxidants was higher after the use of silicon compared to the control, which would confirm its mitigating character. As the mechanism of silicon action described by Chen et al. (2018) concerns roots, further experiments are planned in the future to investigate also these organs in oilseed rape plants growing under drought stress and treated with silicon.

Summarizing, it was shown that in case of well-watered plants, silicon complex with iron (but not silicon alone) significantly increases the accumulation of selected PIP and TIP aquaporins and improves the performance of enzymatic and non-enzymatic elements of the antioxidant system. Under drought stress, on the other hand, these effects were observed regardless of the source of silicon. It suggests that silicon may play an essential role especially in the plant's effective coping with water deficit.

Author contributions

D.S-G. conceived, designed and conducted all experiments, performed the biochemical and molecular analyses, processed the data, prepared figures and tables, and wrote the manuscript (conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; supervision; validation; visualization; writing – original draft; writing – review & editing). M. L-K. participated in standardization of biochemical experiments and contributed to the final version of the manuscript (data curation; formal analysis; investigation; methodology; resources; validation; writing – review & editing). M.F. helped analyze the data for aquaporins transcript, and contributed to the final version of the manuscript (investigation; methodology; resources; validation; writing – review & editing). B.J. designed starters for aquaporins transcript (methodology; validation; writing – review & editing). F.J. performed the measurements, described the methods and analyzed the data for the estimation of total antioxidant activity, and contributed to the final version of the manuscript (data curation; formal analysis; investigation; methodology; resources; validation; visualization; writing – review & editing).

The corresponding author certifies that all authors read and approved the final version of the manuscript and warrant that the article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.01.033.

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