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ORIGINAL PAPER

ROLE OF AN ANION IN THE EFFECT OF TETRABUTYLAMMONIUM SALTS ON COMMON RADISH SEEDLINGS: GROWTH INHIBITION AND OXIDATIVE STRESS*

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Abstract

Quaternary ammonium salts (QAS) are a group of popular chemical compounds often used in various industries. Consequently, their use is widespread, giving rise to increasing concerns regarding the environmental contamination due to excessive use of these compounds. In this study, the aim was to study the influence of QAS on the growth and development of common radish by using tetrabutylammonium chloride ([TBA][Cl]) and tetrabutylammonium bromide ([TBA][Br]). Both compounds were added to the soil at various concentrations: 0, 1, 10, 100, 400, 700 and 1000 mg compound per 1 kg of soil dry weight (DW). For each concentration of TBA salts, 3 independent samples were prepared. Both test compounds inhibited the growth of aerial parts of the plants and led to an increase in the content of dry matter in radish leaves. Furthermore, the presence of test compounds in the soil adversely affected the content of all assimilation pigments in radish leaves. Moreover, the test compounds induced oxidative stress, as evidenced by an increase in the content of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). In response to the oxidative stress, the common radish plants activated the enzymatic antioxidant system. An increase in the activity of enzymes, such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), was observed. All changes observed in the plants were strongly related to the concentration of test compounds in the soil. However, there were no major differences in the impact of [TBA][Cl] and [TBA][Br] on common radish plants.

Keywords: quaternary ammonium salts, phytotoxicity, dry matter, assimilation pigments, antioxidant enzymes, hydrogen peroxide, malondialdehyde.

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INTRODUCTION

Quaternary ammonium salts (QAS), including ionic liquids (ILs), belong to chemical substances that can cause soil contamination. ILs are chemical compounds which enjoy a tremendous interest from scientists around the world. Some of the desirable properties of these compounds include non-flammability, thermal and electrochemical stability, and low vapour pressure. Owing to these excellent and diverse properties, ILs are used in various industries, for example in the processes of catalysis, separation and extraction, chemical analysis, and in the electrochemical, pharmaceutical, nanotechnological and biotechnological industries; and they are also used as plant protection agents (Cvjetko Bubalo et al. 2017). However, such a large interest in ILs poses a real threat that these compounds can permeate into the natural environment and contaminate it. Studies conducted by various researchers show that ILs exhibit toxic properties in relation to microorganisms, fungi, algae, invertebrates and vertebrates (Peric et al. 2013, Liu et al. 2017, Zhang et al. 2017). They can also get into the soil and can be absorbed by the plants.

Common radish (*Raphanus sativus* L. subvar. *radicula* Pers.) is a popular vegetable known in the world since ancient times. Due to its low calorific value (18 kcal 100 g^{-1}) with a concurrently high content of vitamins such as vitamins C, A, D, E, and B (thiamine, riboflavin, niacin, cobalamin, and piridoxine), macroelements such as phosphorus, calcium, potassium, magnesium, and sodium as well as micronutrients such as iodine, iron, copper, zinc, and manganese, this vegetable has become a permanent ingredient of many diets (Banihani 2017).

Therefore, in this study, the aim was to evaluate the effect of two ILs, namely, tetrabutylammonium chloride ([TBA][Cl]) and tetrabutylammonium bromide ([TBA][Br]), on the growth and development of common radish. To the best of our knowledge, there are no reports regarding the effect of [TBA][Cl] and [TBA][Br] on terrestrial plants, including common radish. Basic phytotoxicity indices, such as yield of fresh weight and dry matter content, were analyzed to determine the effect of the test compounds on common radish. There are some reports in the literature regarding the toxic effects of ILs on plants (Egorova et al. 2017). Having this in mind, we evaluated the level of oxidative stress in the plants. To achieve this, we estimated the content of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and assimilation pigments in addition to the measurement of changes in the activity of enzymes such as peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD).

MATERIAL AND METHODS

Evaluation of TBA salt phytotoxicity

Determination of the phytotoxicity of TBA salts was performed according to the OECD/OCDE 208, (2006) guidelines. Following the recommendations of this guideline for phytotoxicity study, the following concentrations of TBA salts were used: 0, 1, 10, 100, 400, 700 and 1000 mg compound per 1 kg of soil dry weight (DW). For each concentration of TBA salts, 3 independent samples were prepared. The granulometric analysis showed that the soil used in this study was loam with about 10% of a fraction of grains <0.002 mm in diameter, containing 9.5 g kg^{-1} of organic carbon and having pH 5.9. Plastic pots were filled with this soil and which were then seeded with 20 seeds of common radish (*Raphanus sativus* L. *radicula* Pers.) derived from the same source. Seed germination and plant growth (14 days) were carried out under strictly controlled conditions: soil moisture at 70% water holding capacity, temp. of 20 ± 2°C and constant illumination equal 170 μmol m^{-2} s^{-1} in a 16 h day/8 h night system. The phytotoxicity of TBA salts towards common radish was estimated from the amount of dry weight content. The fresh weight inhibition factor was calculated according to the study published by Wang et al. (2009).

Furthermore, MDA and H_2O_2 content, photosynthetic pigments content and antioxidant enzymes activities were measured in plant leaves and compared.

Determination of oxidative stress parameters

Oxidative stress was measured based on the content of MDA and H_2O_2, and antioxidant enzyme activities. MDA and H_2O_2 were assayed according to the procedures described by Hodges et al. (1999) and Singh et al. (2007), respectively. Superoxide dismutase (SOD) [EC 1.15.1.1] activity was determined spectrophotometrically by measuring the degree of reduction of nitroblue-tetrazolium (NBT) by a superoxide anion formed by the photochemical reduction of riboflavin in the presence of light (Giannopolitis, Ries 1977). Catalase activity (CAT) [EC 1.11.1.6] was determined by decomposition of H_2O_2 by this enzyme for 15 min, and H_2O_2 remaining in the reaction mixture was titrated with 0.01 N $KMnO_4$ (Kar, Mishra 1976). The activity of peroxidase (POD) [EC 1.11.1.7] was determined spectrophotometrically from the rate of guaiacol oxidation in the presence of H_2O_2 (Abassi et al. 1998).

Determination of the content of photosynthetic pigment and dry matter

Photosynthetic pigment content was determined according to the method reported by Oren et al. (1993). The content of chlorophyll *a*, chlorophyll *b* and carotenoids was tested by measuring the absorbance at 470 nm, 647 nm and 664 nm. The content of photosynthetic pigments was expressed in mg g^{-1} DW. The dry weight level was determined using an oven-dry method, by drying about 1 g fresh weight of the plant at a temp. of 105°C to constant weight.

Statistical analysis

All experimental results were statistically analysed using Statistica 13.1. (Dell Inc. 2016). The data from three measurements (n = 3) were analysed using two-way ANOVA test followed by the post hoc Tukey's test. Differences were considered significant at the $p < 0.05$ level. The results were expressed as mean ± standard deviation. Moreover, the mean standard deviations were determined, which were plotted as vertical lines in the diagrams presented in the paper.

RESULTS AND DISCUSSION

Soil phytotoxicity test

The results of this test revealed that [TBA][Cl] and [TBA][Br] applied into soil showed toxic effects on common radish plants. The most visible toxic effect was found to be the inhibition of plant growth, which resulted in a reduction in the yield of fresh weight. These changes were linearly correlated with the content of TRA salts in soil. An application of salts at a concentration of 400 mg kg^{-1} soil DW resulted in an over 50% inhibition of yield, whereas at the highest applied concentration (1000 mg kg^{-1} soil DW), the inhibition of yield exceeded 80% for both test compounds (Figure 1).

The decrease in the yield of fresh weight of common radish was found to be correlated with an increase in the dry matter of plants. At the highest tested concentration of [TBA][Br], the dry matter content more than doubled compared to control plants. In the case of [TBA][Cl], this increase was found to be even higher, i.e. about 200% more at a concentration of 700 mg kg^{-1} soil DW and over 300% at a concentration of 1000 mg kg^{-1} soil DW (Figure 2).

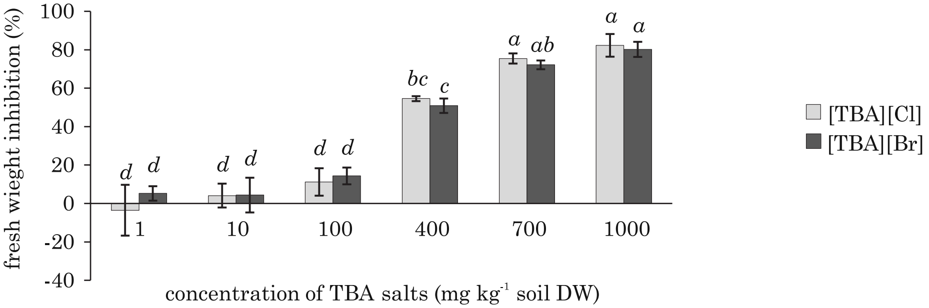

Fig. 1. The inhibition rate for fresh weight of common radish after exposure to TBA salts. Values denoted by the same letters do not differ statistically at $p < 0.05$

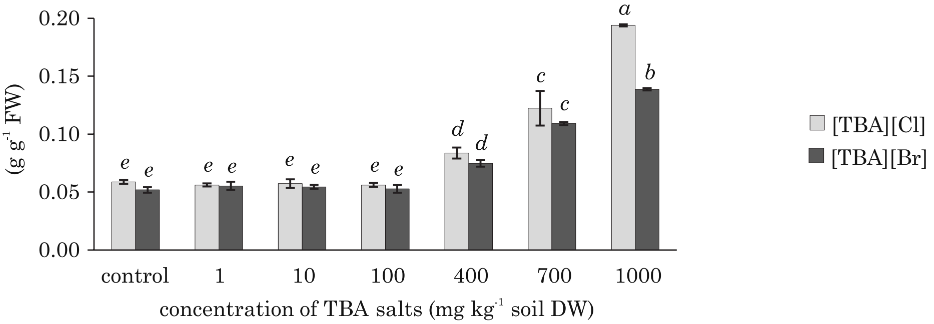

Fig. 2. Effect of TBA salts on the dry matter in common radish leaves. Values denoted by the same letters do not differ statistically at $p < 0.05$

The results obtained in this study agree with the data available in the literature (Liu et al. 2014, 2015*a*,*b*, Biczak, Pawłowska 2016, Biczak et al. 2017*a*, Pawłowska et al. 2017). The authors conclusively demonstrate that different QAS, including ILs, may have an unfavourable effect on the growth and development of plants, and the magnitude of the impact of these compounds was found to be linearly correlated with the tested concentrations. Moreover, it is often possible to observe an opposite effect when using QAS and ILs at low concentrations, which is when the examined substances act as stimulants for plant growth, like plant hormones do (Egorova et al. 2017).

An important indicator of the effect of test compounds on plant productivity is the dry matter content. Previous studies (Biczak et al. 2015, 2016, 2017*b*, 2018) have reported an increase in the dry matter content along with a concomitant increase in the concentration of ILs in the soil. An increase in the dry matter content was correlated with a decrease in the yield of fresh plant mass.

Effect of TBA salts on the content of photosynthetic pigments

Chlorophylls and carotenoids are pigments responsible, *inter alia*, for light absorption, which facilitates the process of photosynthesis in plants. They also protect the plants against photooxidation by dissipating excess energy. Owing to such important functions of pigments in relation to the survival and functioning of plant organisms, changes in the content of chlorophylls are considered one of the most important indicators of oxidative stress in plants caused by various abiotic factors such as salinity, drought, inappropriate temperature or presence of harmful chemical substances in the environment (Tounekti et al 2011, Rachoski et al. 2015). Therefore, we analyzed changes in the content of chlorophylls and carotenoids.

According to our results, the inhibitory effect of [TBA][Cl] and [TBA][Br] on the content of assimilation pigments in common radish leaves revealed a significant decrease in chlorophyll *a*, chlorophyll *b*, carotenoids and total chlorophyll (Table 1). This decrease in the content of assimilation pigments

Table 1

Changes in the content of assimilation pigments in common radish leaves exposed to [TBA][Cl] and [TBA][Br] in soil (mean ± SD, $n = 3$)

Concentration of TBA salts (mg kg^{-1} soil DW)		Assimilation pigments (mg g^{-1} DW)					
		Chl$a$	Chl$b$	Car	Chl$a$+Chl$b$	Chl$a$/Chl$b$	Chl$a$+b/Car
[TBA][Cl]	0	13.907±0.132^{a}	4.239±0.030^{a}	3.562±0.064^{a}	18.146±0.120^{a}	3.281±0.047^{ef}	5.096±0.058^{efg}
	1	13.181±0.161^{b}	3.793±0.085^{b}	3.397±0.032^{b}	16.974±0.241^{b}	3.476±0.044^{cd}	4.997±0.029^{g}
	10	12.717±0.026^{c}	3.745±0.019^{bc}	3.152±0.005^{c}	16.462±0.041^{c}	3.396±0.014^{de}	5.223±0.007^{bcde}
	100	11.208±0.040^{e}	3.607±0.055^{bcd}	2.915±0.024^{e}	14.815±0.082^{e}	3.108±0.043^{f}	5.083±0.049^{fg}
	400	11.243±0.048^{e}	3.564±0.024^{cd}	2.895±0.012^{e}	14.807±0.072^{e}	3.155±0.008^{f}	5.116±0.018^{efg}
	700	8.465±0.023^{f}	2.402±0.025^{g}	2.103±0.002^{g}	10.867±0.025^{g}	3.524±0.042^{bcd}	5.168±0.008^{cdef}
	1000	4.632±0.025^{j}	1.302±0.011^{i}	1.127±0.007^{i}	5.934±0.033^{i}	3.559±0.023^{bcd}	5.264±0.004^{bc}
[TBA][Br]	0	12.789±0.091^{c}	3.570±0.109^{cd}	3.115±0.046^{cd}	16.359±0.187^{c}	3.584±0.092^{bc}	5.253±0.045^{bcd}
	1	12.907±0.096^{c}	3.757±0.040^{bc}	3.112±0.027^{cd}	16.665±0.134^{bc}	3.435±0.014^{cde}	5.356±0.047^{ab}
	10	12.048±0.150^{d}	3.412±0.169^{de}	3.046±0.051^{d}	15.459±0.318^{de}	3.536±0.135^{bcd}	5.075±0.086^{fg}
	100	11.252±0.044^{e}	3.292±0.054^{e}	2.840±0.039^{e}	14.545±0.041^{e}	3.418±0.066^{cde}	5.122±0.070^{defg}
	400	9.847±0.041^{f}	2.771±0.088^{f}	2.515±0.031^{f}	12.618±0.123^{f}	3.556±0.099^{bcd}	5.018±0.036^{g}
	700	8.820±0.014^{g}	2.319±0.014^{g}	2.032±0.008^{g}	11.139±0.014^{g}	3.803±0.027^{a}	5.481±0.025^{a}
	1000	6.792±0.103^{i}	1.835±0.022^{h}	1.630±0.031^{h}	8.627±0.125^{h}	3.701±0.020^{ab}	5.294±0.053^{bc}

Values in the columns denoted by the same letters do not differ statistically at $p < 0.05$; Chl$a$ – chlorophyll a, Chl$b$ – chlorophyll b, Car – carotenoids was found to be higher with an increased concentration of the tested TBA salts. When the highest salt concentration was tested (1000 mg kg^{-1} soil DW), we observed a three-fold and two-fold decrease in the content of chlorophylls for [TBA][Cl] and [TBA][Br], respectively.

The observed changes in the content of chlorophylls demonstrate conclusively that the presence of QAS in the soil causes oxidative stress in plants. Many authors (WANG et al. 2009, MA et al. 2010, LIU et al. 2015*a*, BICZAK 2016) reported a linear decrease in the content of assimilation pigments in plants together with an increase in the concentration of QAS and ILs in soil. Important parameters indicating the proper development of a plant are the ratios of chlorophyll *a* to chlorophyll *b* (chl*a*/chl*b*) and total chlorophyll to carotenoids (chl*a*+*b*/car). A slight increase in the chl*a*/chl*b* ratio when using the highest concentrations of QAS indicates oxidative stress in common radish. However, an increase in the content of carotenoids in relation to total chlorophyll under the same conditions indicates some activation of the plants' defense system against oxidative stress. A slight increase in the ratio of total chlorophylls to carotenoids indicates damage to the photosystems and disturbances in their functioning, which resulted in the reduction of primary plant production in this study, as previously reported (GENGMAO et al. 2015, BICZAK 2017, CHEN et al. 2018).

Effect of TBA salts on MDA and H_2O_2 content

Oxidative stress in plants induces an increased production of reactive oxygen species (ROS). ROS are highly reactive chemical compounds, and they are involved in the peroxidation of nucleic acids, proteins, lipids in cell membranes, and other cellular structures. MDA, resulting from the decomposition of polyunsaturated fatty acids – building elements of cellular membranes, is an excellent indicator of the degree of lipid peroxidation. Therefore, the level of MDA is often determined to evaluate the level of oxidative stress in plants (LIU et al. 2013, 2014, RACHOSKI et al. 2015, ROSALIE et al. 2015).

According to our results, addition of [TBA][Cl] and [TBA][Br] to the soil resulted in an increase in the MDA production in radish plants. This increase was observed when QAS was added to the soil at a concentration of 400 mg kg^{-1} soil DW, and then it increased with an increasing concentration of the test compounds (Figure 3).

Other researchers (TOUNEKTI et al. 2011, LIU et al. 2013, CVJETKO BUBALO et al. 2017) reported that the presence of QAS and ILs in the soil results in an increased level of MDA in plants. According to them, this increase was found to be correlated with the concentration of the tested compounds. The researchers attribute the changes observed to the fact that high concentrations of QAS and ILs cause such a very high level of oxidative stress in the plants that the plant defense mechanisms are unable to counteract with it. BICZAK (2016) suggests that the level of accumulation of MDA is strongly dependent on the plant species.

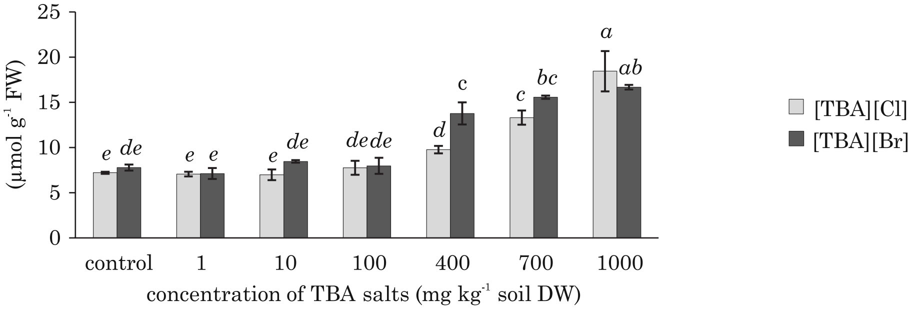

Fig. 3. Effect of [TBA][Cl] and [TBA][Br] on MDA content in common radish leaves. Values denoted by the same letters do not differ statistically at $p < 0.05$

Furthermore, H_2O_2 content is an important biomarker of oxidative stress in plants. It is one of the most stable ROS molecules, and under stress conditions plants produce and accumulate it in large quantities (Liu et al. 2015*b*, Cvjetko Bubalo et al. 2017). Moreover, some authors (Zhang et al. 2013, Liu et al. 2014, Biczak 2016) relate the accumulation of H_2O_2 in duckweed cells, wheat, spring barley and radish seedlings to the tested QAS and ILs concentration in the soil. In this study, we found an increase in the concentration of H_2O_2 in leaves of common radish, which was found to be increasing with an increasing concentration of [TBA][Cl] and [TBA][Br] – Figure 4.

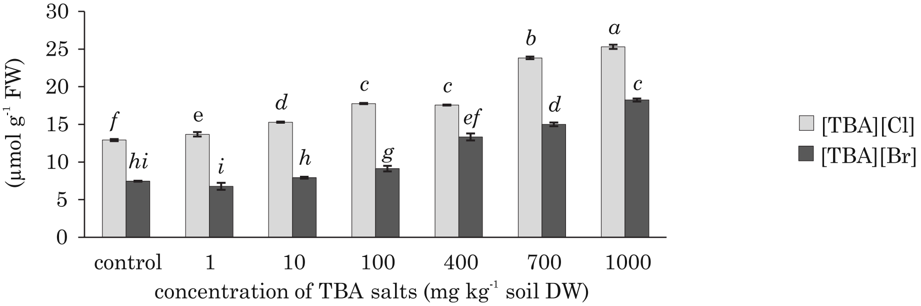

Fig. 4. Effect of [TBA][Cl] and [TBA][Br] on H_2O_2 content in common radish leaves. Values denoted by the same letters do not differ statistically at $p < 0.05$

Meanwhile we must not forget that the plant can never eliminate H_2O_2 from cells, as this compound has functions related to cell signaling (Chen et al. 2014).

Effect of TBA salts on antioxidant enzyme activities

In order to effectively remove ROS, in addition to low-molecular weight antioxidants, the plants develop antioxidant enzymes, such as superoxide dismutases (SOD), catalase (CAT), peroxidases (POD), and glutathione reductase (GR). Antioxidant enzyme activity is a highly specialized and a highly coordinated mechanism of a plant's defensive system, as the product of one enzyme activity is the substrate for the other. These enzymes start to convert the chemical compounds, and this process continues until the toxic compound is converted to one which is nontoxic for plant (Sánchez-Rodríguez et al. 2010, Gengmao et al. 2015).

SOD, an enzyme that is responsible for the decomposition of superoxide radical to H_2O_2 and oxygen, thus preventing lipid peroxidation, is the first line of defense against ROS (Liu et al. 2013, Asensio et al. 2014, Chen et al. 2014, Pawłowska et al. 2017). According to our results, there was a decrease in the activity of SOD when lower amounts of [TBA][Cl] and [TBA][Br] were tested and an increase in the activity of SOD at the highest tested concentrations of the test compounds (Table 2). Similar to our results, the activity of SOD due to the presence of QAS and ILs in wheat, barley and broad bean plants was reported by Liu et al. to increase (2013, 2015*a*) and Cvjetko Bubalo et al. (2017).

The SOD enzyme activity results in the formation of H_2O_2, which is less toxic to the plants than the superoxide radical. CAT, mainly located in peroxisomes and glyoxysomes, is largely responsible for the decomposition of H_2O_2 resulting from SOD activity. Gengmao et al. (2015) observed an increase

Table 2

Activity of superoxide dismutase, catalase and peroxidase in common radish leaves exposed to [TBA][Cl] and [TBA][Br] in soil (mean ± SD, $n = 3$)

Concentration of QAS (mg kg^{-1} soil DW)		The activity of enzymes		
		SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein min^{-1})	POD (U mg^{-1} protein min^{-1})
[TBA][Cl]	0	14.443 ± 0.279^{c}	0.0135 ± 0.0007^{c}	0.700 ± 0.005^{i}
	1	12.411 ± 0.242^{cde}	0.0107 ± 0.0002^{c}	0.636 ± 0.046^{i}
	10	12.819 ± 0.278^{cd}	0.0121 ± 0.0024^{c}	0.649 ± 0.015^{i}
	100	12.463 ± 0.123^{cde}	0.0121 ± 0.0001^{c}	3.538 ± 0.066^{d}
	400	21.240 ± 0.298^{a}	0.0175 ± 0.0001^{b}	6.188 ± 0.053^{b}
	700	18.530 ± 0.531^{b}	0.0213 ± 0.0001^{a}	6.307 ± 0.097^{b}
	1000	17.809 ± 0.169^{b}	0.0195 ± 0.0010^{ab}	6.544 ± 0.121^{a}
[TBA][Br]	0	10.120 ± 0.785^{ef}	0.0181 ± 0.0013^{ab}	1.419 ± 0.019^{h}
	1	10.988 ± 0.601^{def}	0.0177 ± 0.0027^{ab}	1.335 ± 0.030^{h}
	10	9.406 ± 0.784^{f}	0.0193 ± 0.0015^{ab}	1.443 ± 0.054^{h}
	100	8.728 ± 0.618^{f}	0.0185 ± 0.0001^{ab}	1.680 ± 0.011^{g}
	400	19.783 ± 0.507^{ab}	0.0191 ± 0.0013^{ab}	2.390 ± 0.040^{f}
	700	18.745 ± 0.525^{b}	0.0193 ± 0.0005^{ab}	2.763 ± 0.055^{e}
	1000	20.241 ± 0.586^{ab}	0.0173 ± 0.0010^{b}	4.370 ± 0.058^{c}

Values in the columns denoted by the same letters do not differ statistically at $p < 0.05$;
SOD – superoxide dismutase, CAT – catalase, POD – peroxidase

in the activity of antioxidant enzymes, including CAT, in response to various stress factors. In this study, an increase in the activity of CAT was noted (Table 2); however, the changes were small. Not all scientific studies present such an univocal direction of changes in the activity of CAT, which in consequence does not allow to draw conclusions about the direction of changes in this enzyme activity in response to stress factors. Liu et al. (2013, 2015*a*,*b*) reported an increase in the activity of CAT when lower concentrations of ILs were tested, whereas higher concentrations resulted in the decrease in the activity of CAT. They also relate the changes in the activity of CAT to the duration of oxidative stress, the concentration of tested compounds, and the species of the plant on which it was tested. In turn, Pinto et al. (2011) explain that small changes the activity of CAT result from the specific structure of this enzyme, which makes it resistant, *inter alia*, to ILs.

Excess of H_2O_2 in plant cells causes the activation of another enzyme, that is POD. However, Anjaneyulu et al. (2014) argue that POD is responsible only for the regulation of H_2O_2 levels in cells and that the removal of H_2O_2 is primarily due to the activity of CAT. Jbir-Koubaa et al. (2015) have contradictory opinion. According to these authors, POD has a much greater affinity for H_2O_2 than CAT does. Other researches (Wang et al. 2009, Pawłowska, Biczak 2016, Biczak et al. 2017*a*) unambiguously suggest that the activity of POD increases in plants that are exposed to stress and as long as they are able to lead vegetation in these conditions. In this study, we also observed a significant increase in the activity of POD, which linearly correlated with the concentration of TBA salts in the soil. An increase in the activity of POD was observed when [TBA][Cl] and [TBA][Br] were used at a concentration of 100 mg kg^{-1} soil DW (Table 2).

Thus, we suggest that a greater increase in the activity of antioxidant enzymes may be harmful to plants. For instance, high POD activity may lead to an increase in the peroxidation of lipids contained in chloroplast membranes, resulting in the degradation of chlorophyll (Wang et al. 2009).

CONCLUSIONS

According to the results of this study, the tested salts i.e., [TBA][Cl] and [TBA][Br], exhibited phytotoxic properties in common radish plants. The tested compounds decreased the fresh weight of radish plants with a concomitant increase in the dry matter content. A decrease in the content of all assimilation pigments was also observed. In addition, the tested compounds caused oxidative stress in common radish plants as evidenced by an increase in the content of MDA and H_2O_2, increase in the activities of POD and CAT, and the changes in the activity of SOD. All observed changes were found to be increasing with an increasing concentration of QAS. However, according to our results, there were no major differences in the effects of

[TBA][Cl] and [TBA][Br] on the growth of common radish. The changes in the activity POD and decrease in the content of assimilation pigments were found to be notable biomarkers of indicated oxidative stress in common radish plants grown on soil containing TBA salts. These results will enrich the current knowledge on the effect of QAS on plants.

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