Phytotoxicity and Effect of Ionic Liquids on Antioxidant Parameters in Spring Barley Seedlings: The Impact of Exposure Time

Robert Biczak 1,* , Barbara Pawłowska 1, Wiesław Pilis 2, Jan Szczegielniak 3, Jacek Wróbel 4 and Arkadiusz Telesiński 4

1 The Faculty of Science and Technology, Jan Długosz University in Częstochowa, 13/15 Armii Krajowej Av., 42-200 Częstochowa, Poland; b.pawlowska@ujd.edu.pl
2 The Faculty of Health Science, Jan Długosz University in Częstochowa, 13/15 Armii Krajowej Av., 42-200 Częstochowa, Poland; w.pilis@ujd.edu.pl
3 The Faculty of Physical education and Physiotherapy, Opole University of Technology, 76 Prószkowska Street, 45-788 Opole, Poland; j.szczegielniak@po.edu.pl
4 Department of Bioengineering, West Pomeranian University of Technology in Szczecin, 17 Słowackiego Street, 71-434 Szczecin, Poland; jacek.wrob@zut.edu.pl (J.W.); arkadiusz.telesinski@zut.edu.pl (A.T.)

* Correspondence: r.biczak@ujd.edu.pl; Tel.: +48-343614918; Fax: +48-3665322

Received: 9 August 2020; Accepted: 15 September 2020; Published: 17 September 2020

Abstract: The influence of the ionic liquids (ILs) tetrabutylammonium bromide [TBA][Br], 1-butyl-3-methylimidazole bromide [BMIM][Br], and tetrabutylphosphonium bromide [TBP][Br] added at different concentrations to the soil were studied for the growth and development of spring barley seedlings. Samples were harvested at three different time points: day 7, 14, and 21 after addition of ILs. The results show that [TBP][Br] was the most toxic. The introduction of this IL at the dose of 100 mg kg\(^{-1}\) of soil DM decreased the growth of seedlings at all test dates. The addition of the studied ILs to the soil in higher doses resulted in an increase in peroxidase and catalase activity, which may indicate the occurrence of oxidative stress in plants. An increase in the content of plant dry matter weight, contents of \(\text{H}_2\text{O}_2\) and proline and a decrease in the content of photosynthetic pigments in barley seedlings were also observed. The malondialdehyde content and superoxide dismutase activity fluctuated randomly during the experiment. As a result, it was found that the phytotoxicity of ILs and the magnitude of oxidative stress in seedlings depended more on the added doses of these compounds than on the measurement date.

Keywords: ionic liquids; phytotoxicity; oxidative stress; antioxidant enzyme activity; photosynthetic pigments

1. Introduction

Ionic liquids (ILs) are a large group of compounds that have attracted widespread interest. These are ionic compounds and are made of an organic cation and an organic or inorganic anion. These compounds exhibit a number of desirable physicochemical properties such as low vapor pressure, non-flammability, nonvolatility, thermal and electrochemical stability, and good conductivity. Moreover, by the appropriate selection of cations and anions, compounds with the desired properties can be obtained. As a result, ILs have become known as “designer solvents”. The possibility of different combinations of cations and anions to obtain ILs is estimated at \(10^{18}\) [1,2].

Thanks to the possibility of designing the ILs to obtain compounds with interesting properties, these compounds are constantly being studied to apply them in various fields, including analytical chemistry, electrochemistry, organic synthesis, nanotechnology, biotechnology, pharmacology, food,
and agriculture [2,3]. According to Pernak et al. [4], herbicidal ionic liquids deserve special attention, including naturally occurring compounds such as geranium acid or choline. In addition, these compounds are less toxic and therefore more environmentally friendly than traditional herbicides.

There are currently no reports of the presence of ILs in the environment. However, as these compounds are widely used in industry, there is a high risk that they may leak into the water and soil in the near future. Therefore, in 2003, the Royal Society of Chemistry pointed out the need to precisely determine the impact of ILs on various elements of the environment before using them in industry on a larger scale. After the initial admiration of the ILs and their designation as “green solvents”, there have been many reports in the scientific literature on the toxic effects of ILs on different organisms [5–10]. ILs can also cause cytotoxicity, genotoxicity, or lead to DNA damage [7].

The toxic effects of ILs on plants are increasingly explained by the oxidative stress they cause. As a result of ILs, there is an overproduction of reactive oxygen species (ROS) in the organism. In order to defend themselves against ROS, the organisms developed an antioxidant defense system consisting of non-enzymatic antioxidants, i.e., low-molecular chemical compounds, i.e., flavonoids, carotenoids, tocopherol, glutathione and ascorbic acid as well as a specialized enzyme system comprising supernatant dismutase, catalase, guaiacol peroxidase, and the enzymes involved in the Haliwell–Asada cycle [11–13].

Here, we assessed the effect of three IL bromides with different cations—[TBA][Br], [TMIM][Br], and [TBP][Br]—added to soil at different doses. The outcomes were growth, development and some parameters of oxidative stress in spring barley (Hordeum vulgare L.) seedlings. Such studies are relatively rare and described in the available literature and most often concern hydroponic crops [6].

The most important groups of plants in the world are cereals. They form the basis of human and animal nutrition. One of the most important development stages of most plants is their germination and early stages of development, so the presence of any stress factors at these stages of plant growth is one of the most important problems of modern agriculture because it can lead to a significant decrease in the volume and quality of yield. Spring barley is one of the oldest and most important cereals grown in the world. In terms of area of cultivation and production, it is the fourth most popular cereal in the world. In Poland, barley is the most commonly grown spring cereal. This species has a poorly developed root system and short vegetation period, which makes it relatively demanding in terms of soil. It is also sensitive to the pollution that occurs in the environment. The conditions under which the grain is grown can have a major impact on the chemical composition of the grain and its nutritional value [14].

All bromides used in these studies are compounds that can be used in synthesis and catalysis. Furthermore, [TBA][Br] could be used to dehydrochlorinate poly(vinyl chloride) over used with phosphorus pentoxide for greener deoxybromination. [BMIM][Br] can be used in chemo-resistant gas sensors. According to the available knowledge, there is currently no work attempting to assess and compare the effect of [TBA][Br], [TMIM][Br], and [TBP][Br] on spring barley seedlings depending on exposure time. This research will contribute to the broadening of knowledge on the influence of ILs on crops and will bring us closer to understanding the mechanism of ILs’ influence on plant growth and development.

2. Materials and Methods

2.1. Chemicals

Tetrabutylammonium bromide [TBA][Br] (≥98% purity), 1-butyl-3-methylimidazolium bromide [BMIM][Br] (>97.0%), and tetrabutylphosphonium bromide [TBP][Br] (98%) were purchased from Sigma-Aldrich Chemical Co, Poznań, Poland.
2.2. Experimental Design

Phytotoxicity studies of [TBA][Br], [BMIM][Br] and [TBP][Br] were carried out as a pot experiment in the vegetation hall according to guidelines of the OECD/OCDE 208/2006 [15] and PN-EN ISO 11269-2 [16]. The experiment was carried out on soil samples of granulometric composition loamy sand with organic carbon content of 8.5 g kg\(^{-1}\) and pH in 1 M KCl equal to 5.9. The compounds were added to the soil as water solutions at doses of 1, 10, 100, 400, 700 and 1000 mg kg\(^{-1}\) of soil dry matter (DM). The reference was soil without any addition of ILs. The soil samples thus prepared were used to fill plastic pots. Each pot then received 20 identical seeds of spring barley (\textit{Hordeum vulgare} L.) from the same source. The soil humidity was maintained at 70% of the maximum water holding capacity. During the experiment, the plants were illuminated with a radiation intensity at the soil level in the pots of 170 \(\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}\). The photoperiod was established as 16 h of daylight and 8 h of night. On days 7, 14, and 21 after seeding, plant samples were taken for measurements. Averaged samples from all the plants were grown in a given pot. All analyses were performed in three replicates.

2.3. Determination of Basic Phytotoxicity Parameters

The toxicity assessment of [TBA][Br], [BMIM][Br], and [TBP][Br] for spring barley was based on germination potential (GP) and seed germination rate (GR) according to by Li et al. [6] procedure. The growth inhibition of plant’s green parts and roots was determined according to Wang et al. [17]. The yield of fresh plant mass was also determined. The inhibition factor was calculated using the equation:

\[
I = \frac{A_C - A_{ILs}}{A_C} \cdot 100\% \tag{1}
\]

where \(I\) is a inhibition factor in percent, \(A_C\) is a length or weight in the control group, and \(A_{ILs}\) is a length or weight in treatment group ILs. The inhibition factor was calculated for fresh weight yield, length of roots and length of plant green parts.

The dry matter content by the weight-drying method [18] was also determined. The dry matter content was expressed in g g\(^{-1}\) fresh weight (FW).

2.4. Determination of Assimilation Pigments Content

The assimilation pigments (chlorophyll \(a\), chlorophyll \(b\), and carotenoids) content was determined according to Oren et al. [19]. The weight of plant green parts (0.5 g) was homogenized with addition of 80% acetone. After centrifuging the samples, the absorbance of supernatants was measured at wavelengths of 470, 647, and 664 nm. The photosynthetic pigment contents were expressed in mg g\(^{-1}\) DM.

2.5. Determination of Non-Enzymatic Markers of Oxidative Stress

The free proline content was determined colorimetrically according to Bates et al. [20]. This method is based on reaction of proline with ninhydrin. The chromophore was extracted using toluene, and absorbance was measured at 520 nm. Free proline content was given in mg g\(^{-1}\) FW.

Malondialdehyde (MDA) is an important oxidation products and is the main marker in lipid peroxidation. The MDA reaction with thiobarbituric acid (TBA) resulted in a colored compound that was determined spectrophotometrically at wavelengths of 532 and 600 nm [21]. The MDA content was given in \(\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{FW}\).

Hydrogen peroxide (H\(_2\)O\(_2\)) is a reactive oxygen species and the H\(_2\)O\(_2\) content was determined via a spectrophotometric method described by Singh et al. [22]. This method is based on KI oxidation by H\(_2\)O\(_2\) in acidic medium. H\(_2\)O\(_2\) determination relies on absorbance at 390 nm and calculation of the content of that compound using an extinction factor of 155 nm\(^{-1}\) cm\(^{-1}\). The H\(_2\)O\(_2\) was expressed in \(\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{FW}\).
2.6. Determination of Antioxidant Enzyme Activity

The antioxidant enzymes: superoxide oxidase (SOD) [EC 1.15.1.1], catalase (CAT) [EC 1.11.1.6], and peroxidase (POD) [EC 1.11.1.7] were extracted from the green parts of plants with a phosphate buffer of pH 7.4 upon addition of 1 mM EDTA solution and 0.1% polyvinylpyrrolidone (PVP) solution. After centrifugation, the supernatant was used to determine the activity of the enzymes.

The SOD activity was determined spectrophotometrically according to Giannopolitis and Ries [23]. This method is based on the reduction of nitrotetrazolium blue (NBT). Absorbance of the reaction mixture was measured at 560 nm. SOD activity was expressed in units of activity (amount of enzyme causing a 50% decrease in NBT reduction, i.e., U per mg of protein.

CAT was determined by procedure described by Kar and Mishra [24]. This method consists of titration hydrogen peroxide with 0.01 N solution of KMnO₄. Manganate (VII) is reduced and becomes discolored. After oxidation, the excess KMnO₄ stains the titrated solution pink, which indicates that the end point of the titration is reached. CAT activity was expressed as U mg⁻¹ protein min⁻¹.

POD activity was determined spectrophotometrically according to Abassi et al. [25]. The concentration oxidized by POD guaiacol in the presence of H₂O₂ was measured spectrophotometrically at a wavelength of 470 nm. For this purpose, the oxidation rate of guaiacol in the presence of H₂O₂ in 1 min at 470 nm was determined. POD activity was expressed as U mg⁻¹ protein min⁻¹.

Moreover, the total protein content was determined by the Bradford [26] method using Coomasine Blue.

2.7. Data Analysis

Non-linear regression analysis was used to estimate effective concentrations (EC₅₀) for fresh weight, root length, and shoot length using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

All treatments were repeated three times and the results were analyzed statistically. The experimental data were statistically analyzed using STATISTICA 12.5. The Shapiro–Wilk test was used to verify the normal distribution of all obtained data in the analyzed parameters. The test results proved to be statistically insignificant (p > 0.05), which indicates that the distribution of the variable is consistent with normal distribution. The homogeneity of variance was determined using Lavene’s test. In this test, a statistically insignificant result was obtained (p > 0.05). Therefore, we made an assumption about the homogeneity of variance. The results were analyzed using a two-factor analysis of variance (ANOVA) and compared with the post hoc HSD Tukey test at the significance level p < 0.05. The contribution of independent variables to dependent variables was determined by calculating coefficient η² in ANOVA. Moreover, Pearson linear correlation coefficients were calculated between the determined traits at significance levels of p < 0.05 and p < 0.01.

3. Results and Discussion

3.1. Phytotoxicity Assay

The GP and GR of seeds, shoots and root growth inhibition of seedlings, fresh matter yield decrease, dry weight content and EC₅₀ values were determined to assess the phytotoxicity of the examined ILs.

The first stage of growth of many plants is germination. Currently, one of the main problems in agriculture is the presence of various stress factors in the environment. They may negatively influence processes occurring in the early stages of plant development, including germination. Research showed that the examined bromides do not show any influence on the GR. Only [BMIM][Br] using concentrations of 400–1000 mg kg⁻¹ of soil DM inhibits the GP (Table S1).

The GP and GR results suggest that the influence of ILs on these factors depends on the compound used and its dose. A similar lack of influence of ILs on GP and GR was observed by the authors in
their earlier studies on the toxicity of phosphonium ionic liquid for spring barley and radish [27]. However, there are reports on the visible effect of ILs on the GP and GR for different species of higher plants [5,6,28].

Despite little or no influence on the seed germination stage, the ILs studied here inhibited the root and shoot lengths of spring barley seedlings and inhibited the yield of fresh plant mass. Research showed that the roots were most sensitive to the tested compounds. A decrease in the growth of spring barley roots was observed with all studied ILs. The roots keep the plant in the soil, but also provide water and mineral salts to the plant. Damage to the root system causes disorders throughout plant development. These changes include inhibition of fresh plant mass yield. The smallest influence of the examined ILs was on the shoot length of spring barley seedlings. The application of [BMIM][Br] resulted in a clear decrease in the root and shoot lengths of spring barley. A clear inhibition of fresh mass yield on day 7 from seeding to soil was seen with [BMIM][Br] at 400–1000 mg kg\(^{-1}\) of soil DM.

At the second and third research time points (14 and 21 days from seeding to soil), inhibition of the tested parameters was already observed with the use of [BMIM][Br] at 100 mg kg\(^{-1}\) of soil DM. Regardless of the date of analysis, the application of [TBA][Br] led to an inhibition of root length and fresh weight yield of plants already at a dose of 100 mg kg\(^{-1}\) of soil DM. There was a clear decrease in shoot length observed after the application of 700 and 1000 mg kg\(^{-1}\) DM at the first date of analysis and 400–1000 mg kg\(^{-1}\) of soil DM at subsequent dates of analysis. The greatest influence on the length of plants and roots and yield was shown by [TBP][Br]. The application of this compound resulted in inhibition of root length, seedlings, and fresh weight yield inhibition of plants already at 100 mg kg\(^{-1}\) of soil DM, regardless of the test date. Higher inhibition values were noted after addition of higher doses of ILs. Similar results were reported in previous studies [5,8,28,29]. Therefore, we concluded that decrease in the length of the root and shoot is the first symptom of phytotoxicity in plants. Moreover, low doses of ILs do not affect the growth and development of plants; they can sometimes even stimulate plant growth, whereas higher doses of these compounds inhibit the plant growth of plants, shoots, and fresh weight yield. This phenomenon is called hormesis. The inhibition values are very much correlated with an increase in the concentration of the tested compounds in the soil [5,6,28].

To compare the obtained values with the data obtained in other studies, EC\(^{50}\) values were calculated on the basis of root length inhibition, green plant parts, and yield inhibition (Table 1).

A comparison of the obtained EC\(^{50}\) values confirms the observations described above. The root was most sensitive to the examined ILs. The compound with the greatest influence on spring barley seedlings was [TBP][Br], while that with the smallest influence was [TBA][Br]. As mentioned earlier, the root is the body that first comes into contact with all soil contaminants and is therefore the first to react to such contaminants. Roots are also constantly exposed to all harmful substances found in the soil. Constant contact with harmful substances can damage the root cell membrane, resulting in the penetration of toxins into the roots with further transport to other organs [30]. Moreover, in most cases, an increase in EC\(^{50}\) values was observed for individual analyzed parameters in subsequent test dates. This may be because the barley seedlings activate mechanisms to protect the plant against toxic effects of the tested ILs or it may be related to the progressive absorption of these salts on soil colloids. The possibility of ILs absorption on soil colloids was reported by Stepnowski et al. [31] and Studzińska et al. [32].

The dry weight content of plants is another important biomarker for determining the toxicity of chemical compounds. We noted an increase in the dry weight content of plants with ILs. The highest increase in plant dry matter content was recorded for [TBP][Br]. Regardless of the date of analysis, this compound caused an increase in dry matter content at 100 mg kg\(^{-1}\) of soil DM. At higher salt doses, there was a higher increase in dry weight content. The [TBA][Br] and [BMIM][Br] showed an increase in dry matter content only on day 7 from sowing to soil. At subsequent study dates, a clear increase in dry weight content for both compounds was observed only at doses of 400–1000 mg kg\(^{-1}\) of soil DM. All compounds had an increase in dry matter content for subsequent test dates (Table S2).
Table 1. The EC50 (mg kg\(^{-1}\) of soil DM) values and 95% confidence intervals for spring barley seedlings following exposure to ionic liquids (ILs).

| Parameter               | Day 7   | Day 14  | Day 21   |
|-------------------------|---------|---------|----------|
| **[BMIM][Br]**          |         |         |          |
| Inhibition for fresh weight | 189.3 ± 11.2 | 258.8 ± 3.4 | 270.1 ± 1.9 |
|                         | (37.36–959.2) | (150.5–444.9) | (154.5–472.5) |
| Inhibition for root length | 214.1 ± 1.8 | 257.8 ± 3.3 | 218.7 ± 3.6 |
|                         | (172.90–266.1) | (193.1–344.2) | (160.1–298.8) |
| Inhibition for shoot length | 263.5 ± 6.8 | 427.4 ± 17.9 | 882.0 ± 36.0 |
|                         | (93.15–745.4) | (71.38–2559) | (476.5–1446) |
| **[TBA][Br]**           |         |         |          |
| Inhibition for fresh weight | 295.0 ± 31.5 | 470.0 ± 34.8 | 766.0 ± 8.4 |
|                         | (7.712–11,200) | (14.83–14,898) | (375.5–1563) |
| Inhibition for root length | 265.2 ± 12.5 | 434.8 ± 39.5 | 341.7 ± 19.4 |
|                         | (81.03–868.1) | (223.3–2847) | (78.58–1486) |
| Inhibition for shoot length | 1319 ± 107 | 1632 ± 184 | 1748 ± 166 |
|                         | (13.62–127,783) | (10.03–528,100) | (6.68–457,200) |
| **[TBP][Br]**           |         |         |          |
| Inhibition for fresh weight | 78.72 ± 4.49 | 301.6 ± 22.6 | 482.0 ± 48.2 |
|                         | (38.69–159.3) | (22.76–3997) | (8.760–26,527) |
| Inhibition for root length | 82.45 ± 8.28 | 78.70 ± 4.38 | 92.81 ± 3.49 |
|                         | (2.770–245.4) | (14.70–422.3) | (69.43–124.1) |
| Inhibition for shoot length | 145.6 ± 6.9 | 382.0 ± 14.9 | 617.3 ± 44.1 |
|                         | (44.94–472.0) | (76.96–1896) | (23.72–16,062) |

The results are confirmed by the available literature [28,33]. Such authors noted a linear increase in the dry weight content of plants along with an increase in the doses of the studied compounds added to soil. This is most likely due to damage to the root system, which cannot provide the plant with enough water and minerals. The turgidity of the plant cells decreases, which leads to accumulation of dry matter. In contrast, Liu et al. [34] and Tot et al. [8] showed that the dry weight content of beans, wheat, and barley decreased under the influence of contact with ILs. The dry weight content likely depends on the conditions under which the research was conducted, i.e., whether it was a hydroponic crop or an experiment was conducted in the soil (natural conditions), as well as the way the compound was applied.

### 3.2. Effect of ILs on Pigments Content

Photosynthesis is a key process in plants. Assimilation pigments, i.e., chlorophylls and carotenoids (carotenes and xanthophylls) are essential for this process. Chlorophyll \(a\) molecules placed in PSI and PSII photo-systems are the only molecules among the assimilating pigments that can perform photochemical reactions. The remaining pigments are responsible for capturing energy and transferring it to the reaction center. Carotenoids also have a protective function by dissipating excess energy in the form of heat supplied to the photocenter. The malfunction of this system leads to the production of excessive amounts of reactive oxygen species (ROS). ROS oxidize and damage the proteins of the PSII photosystem, resulting in disturbances in the photosynthesis process and consequently reduced plant growth and development. Therefore, it is extremely important to determine the content of photosynthetic pigments in plants in works on the influence of stress factors on plants [9,11].

The results obtained here indicate an inhibitory effect of ILs on the content of assimilation pigments in spring barley seedlings. A decrease in the content of chlorophyll \(a\), chlorophyll \(b\), total chlorophyll \((a + b)\) and carotenoids in spring barley seedlings was observed. No major changes in assimilation pigments were observed with the use of lower concentrations of compounds, i.e., 1–10 mg kg\(^{-1}\) of soil DM. At the highest ILs concentrations, the chlorophyll content decreased by more than 50% compared
to the control. Starting from the concentration of 100 mg kg\(^{-1}\) of soil DM, a linear decrease in the content of assimilative pigments was observed along with an increase in the concentration of the studied ILs in the soil. Moreover, a decrease in chlorophyll content in individual samples was observed over time (Figure 1, Tables S3–S5).

No major changes in chlorophyll \(a/b\) and chlorophyll \(a + b/\text{car}\) ratios were observed. A slight increase in the value of total chlorophyll to carotenoids ratio was observed on day 7 for [TBA][Br] and [TBP][Br] and on days 7 and 14 for [BMIM][Br] (Tables S2–Table S4), but only when the highest doses of the compounds tested were used. The lack of major changes for these two relevant indicators may be because of a comparable percentage decrease in the level of all assimilation pigments. The results are confirmed by numerous literature reports \([6,35,36]\). The authors point to the inhibitory effect of ILs on chlorophyll content in plants. However, there are studies that indicated that ILs do not affect the
chlorophyll content of plants [28]. It follows that the effect of ILs on chlorophyll content depends on the type of compound used, its concentration and the plant species on which it acts.

3.3. Effect of ILs on MDA and H$_2$O$_2$ Content

Malondialdehyde (MDA) is a product of the decomposition of polyunsaturated fatty acids, mainly linoleic acid, present in protein–lipid membranes. The MDA content allows one to determine the degree of lipid peroxidation in plants, and its growth indirectly determines the degree of cell damage and is indisputable evidence of oxidative stress in plants [11,37,38].

Here, there was no clear direction of changes in MDA levels in spring barley seedlings affected by the examined ILs. However, as a rule, a decrease in MDA content can be observed in subsequent test dates, which may indicate an active antioxidant system in spring barley cells (Table S6). The analysis of available literature reports on the influence of ILs on MDA content in plants also does not provide clear conclusions. Moieni-Korbekandi et al. [39] showed that a decrease in MDA content in plants may indicate an increase in plant tolerance to stress factors, whereas Liu et al. [6] and Cvjetko Bubalo et al. [5] observed an increase in MDA content in plants in contact with ILs. Rombel-Bryzek and Pisarek [40] showed that not all stress factors cause an increase in MDA content in plants. These authors investigated the impact of drought on the growth and development of sugar beet and did not observe any changes in MDA content in plants. However, if sugar beet shoots growing under drought conditions were also sprayed with humic acids, then they had an increase in MDA content. Biczak et al. [30,41] further indicated that the effect of xenobiotics on the MDA content is dependent on the plant species on which these substances act. These authors observed that the same ionic liquids and quaternary ammonium salts cause changes in MDA content in spring barley seedlings, but they did not cause MDA accumulation in common radish leaves.

Other important biomarkers of oxidative stress include H$_2$O$_2$ accumulation in cells. H$_2$O$_2$ is one of the most stable ROS molecules. It can diffuse through biological membranes thanks to its electrical inertia. This particle is the result of many natural processes in the organism. At low concentrations, it is a signal molecule that is designed to protect the organism. An increase in the level of H$_2$O$_2$ in the plant always occurs when the cells are subject to increased detoxification of superoxide anion radical (O$_2^-$) and when the enzymatic mechanisms of H$_2$O$_2$ detoxification fail [13,34].

As a result of these tests, an increase in H$_2$O$_2$ content in spring barley seedlings was observed at all analysis dates but only when [TBP][Br] was applied; this increase was positively correlated with the applied compound dose. However, [TBA][Br] and [BMIM][Br] caused slight changes in the content of H$_2$O$_2$ in the plants at the first date of the study; a clear increase in the content of this ROS was seen on days 14 and 21. This increase ranged between 85 and 133% compared to the control, depending on the dose used and the compound under investigation. All the changes were practically linearly dependent on the applied tested compound doses (Table 2).

The changes indicate that the contact of spring barley seedlings with [TBA][Br] and [BMIM][Br] during the first days from seeding to soil causes increased antioxidant activity. This results in a decrease in hydrogen peroxide content. Excessively long contact or very high stress factors disturb the antioxidant system or its insufficient capacity, which results in a significant increase in H$_2$O$_2$ levels in plants.

The results were confirmed in the literature: Zhang et al. [35] for duckweed, Cvjetko Bubalo et al. [5] for barley and Biczak et al. [30] for radish. These groups all observed an increase in hydrogen peroxide content in plants in contact with ILs. These changes were linearly correlated with the IL doses. At the same time, there are also works on the decrease in H$_2$O$_2$ content in plants as a result of contact with ionic liquids [28,41]. These studies showed that changes in H$_2$O$_2$ content as a result of the occurrence of oxidative stress in plants always occur, whereas the direction of their changes may depend on many factors, i.e., the applied ILs concentration, the type of compound used, or the plant species on which the compound acts.
Table 2. Hydrogen peroxide (H$_2$O$_2$) (µg g$^{-1}$ fresh weight (FW)) content in spring barley seedlings exposed to ILs.

| Doses of ILs (mg kg$^{-1}$ of Soil DM) | [TBA][Br] | [BMIM][Br] | [TBP][Br] |
|--------------------------------------|-----------|------------|-----------|
| **Day 7**                            |           |            |           |
| 0                                    | 39.004 ± 1.989 $^{bcd}$ | 32.558 ± 0.358 $^{de}$ | 25.520 ± 0.204 $^i$ |
| 1                                    | 38.587 ± 0.318 $^{cd}$ | 27.925 ± 0.269 $^g$ | 22.868 ± 0.066 $^j$ |
| 10                                   | 41.328 ± 1.272 $^b$ | 27.000 ± 0.247 $^h$ | 22.884 ± 0.235 $^j$ |
| 100                                  | 45.752 ± 0.413 $^a$ | 27.103 ± 0.534 $^h$ | 33.648 ± 0.716 $^e$ |
| 400                                  | 37.890 ± 0.827 $^d$ | 32.039 ± 0.314 $^{ef}$ | 34.842 ± 0.656 $^d$ |
| 700                                  | 38.530 ± 0.223 $^{cd}$ | 33.846 ± 0.294 $^c$ | 40.260 ± 0.065 $^b$ |
| 1000                                 | 37.905 ± 0.694 $^d$ | 34.125 ± 0.158 $^c$ | 47.831 ± 0.297 $^a$ |
| **Day 14**                           |           |            |           |
| 0                                    | 17.431 ± 1.072 $^j$ | 19.162 ± 0.973 $^k$ | 22.557 ± 0.277 $^j$ |
| 1                                    | 15.666 ± 0.146 $^{ik}$ | 17.834 ± 0.134 $^{m}$ | 23.307 ± 0.118 $^j$ |
| 10                                   | 15.607 ± 0.146 $^{ik}$ | 18.978 ± 0.173 $^{lm}$ | 24.589 ± 0.068 $^j$ |
| 100                                  | 25.745 ± 0.819 $^g$ | 24.088 ± 0.414 $^f$ | 33.124 ± 0.268 $^{ef}$ |
| 400                                  | 34.082 ± 1.483 $^{ef}$ | 27.143 ± 0.781 $^{gh}$ | 33.124 ± 0.268 $^{ef}$ |
| 700                                  | 35.296 ± 0.517 $^c$ | 33.568 ± 0.563 $^{cd}$ | 36.743 ± 0.316 $^{c}$ |
| 1000                                 | 40.608 ± 1.034 $^{bc}$ | 37.715 ± 0.257 $^{a}$ | 39.834 ± 0.139 $^{b}$ |
| **Day 21**                           |           |            |           |
| 0                                    | 13.847 ± 0.223 $^b$ | 19.215 ± 0.150 $^{kl}$ | 18.361 ± 0.118 $^k$ |
| 1                                    | 18.717 ± 0.234 $^i$ | 17.595 ± 0.147 $^{pn}$ | 16.930 ± 0.136 $^l$ |
| 10                                   | 16.981 ± 0.064 $^{hi}$ | 19.169 ± 0.117 $^{kl}$ | 17.257 ± 0.199 $^l$ |
| 100                                  | 22.666 ± 0.478 $^b$ | 25.006 ± 0.236 $^{fl}$ | 27.708 ± 0.499 $^h$ |
| 400                                  | 26.751 ± 0.338 $^{gh}$ | 26.149 ± 0.512 $^{hi}$ | 32.570 ± 0.547 $^f$ |
| 700                                  | 27.334 ± 0.319 $^{g}$ | 30.965 ± 0.284 $^{l}$ | 34.729 ± 0.181 $^d$ |
| 1000                                 | 32.073 ± 0.113 $^{f}$ | 35.576 ± 0.066 $^b$ | 35.024 ± 0.401 $^d$ |

Data are means ± SD ($n = 3$); values denoted with the same letters form homogeneous groups at the level of $p < 0.05$ (post hoc Tukey’s HSD test).

3.4. Effect of ILs on Free Proline Content

Our results show a significant increase in the free proline content in spring barley seedlings for all tested ILs at 100 mg kg$^{-1}$ of soil DM. The increase in proline content was simultaneously correlated with the applied doses. When the dose of 100 mg kg$^{-1}$ of soil DM was used, the increase was 32–55%. When the highest IL doses (1000 mg kg$^{-1}$ of soil DM) were used, the increase was already 103–166%, compared to the control (Table 3).

A similar increase in proline content in different plant species exposed to ILs was noted by Pawłowska et al. [28] and Liu et al. [6,42]. This increase was positively correlated with an increase in the dose of the studied compounds in the soil. Some researchers reported that this amino acid is a very important indicator of oxidative stress in plants because it regulates the osmotic cell potential of cells [6,11]. However, Sánchez-Rodríguez et al. [13] believed that the higher concentration of proline in plants is a symptom of oxidative stress in plants, but this amino acid has no antioxidative properties.
The analysis results show that the use of all ILs led to only a slight change in SOD activity in spring barley seedlings was observed after application of the tested compounds. On days 14 and 21, a significant increase in CAT activity in spring barley seedlings was observed due to the significance of this enzyme. However, Herman et al. [45] showed that an increase in CAT activity occurs only up to a certain concentration of the applied ILs, after which there is a decrease. Zhang et al. [35], Liu et al. [37], Cvjetko Bubalo et al. [5] and Chen et al. [44] observed an increase in the activity of IL-treated plant cells. However, Herman et al. [45] showed that an increase in CAT activity occurs only up to a certain concentration of the applied ILs, after which there is a decrease.

In order to protect against reactive oxygen species, the plants developed an antioxidant system that includes enzymes in addition to low-molecular chemicals. The operation of all elements of this system is very closely linked to each other. The enzyme that constitutes the first line of defense against ROS is SOD. This is responsible for the breakdown of superoxide radicals. Changes in SOD activity in barley seedlings grown in soil with ILs were evaluated due to the significance of this enzyme.

### 3.5. Effects of ILs on Antioxidant Enzymes Activities

In order to protect against reactive oxygen species, the plants developed an antioxidant system that includes enzymes in addition to low-molecular chemicals. The operation of all elements of this system is very closely linked to each other. The enzyme that constitutes the first line of defense against ROS is SOD. This is responsible for the breakdown of superoxide radicals. Changes in SOD activity in barley seedlings grown in soil with ILs were evaluated due to the significance of this enzyme. The analysis results show that the use of all ILs led to only a slight change in SOD activity in spring barley. There was no clear direction of change or correlation with dose (Table S7).

Some papers [1,5,34] indicated that there is no correlation between the dose of tested ILs and SOD activity. Stimulation effect of ILs on SOD activity was reported by Liu et al. [34] for rice seedlings, Liu et al. [6] for wheat seedlings, Cvjetko Bubalo et al. [5] for barley seedlings and Liu et al. [34] for bean plants. Fan et al. [1] and Liu et al. [43] noted an initial increase in SOD activity using low doses of ILs followed by a decrease in this enzyme activity, observed at the highest doses of the studied ILs. Chen et al. [44] also reported a decrease in SOD activity in wheat seedlings with an increase in IL doses.

Hydrogen peroxide is formed by the dismutation of superoxide anion radicals and is removed by two groups of enzymes: peroxidases and catalases. CAT decomposes H$_2$O$_2$ into H$_2$O and O$_2$. The literature provides different information about the trend of changes in SOD as well as CAT due to ILs. Liu et al. [6,34] proved that the contact of plants with ILs decreases CAT activity. In contrast, Zhang et al. [35], Liu et al. [37], Cvjetko Bubalo et al. [5] and Chen et al. [44] observed an increase in the CAT activity in IL-treated plant cells. However, Herman et al. [45] showed that an increase in CAT activity occurs only up to a certain concentration of the applied ILs, after which there is a decrease.

We noted a slight inhibition of CAT activity on day 7 after sowing the seeds to the soil containing higher doses of all ILs. On days 14 and 21, a significant increase in CAT activity in spring barley seedlings was observed after application of the tested compounds > 400 mg kg$^{-1}$ of soil DW (Figure 2).

| Doses of ILs (mg kg$^{-1}$ of Soil DM) | [TBA][Br] | [BMIM][Br] | [TBP][Br] |
|-------------------------------------|-----------|------------|----------|
| Day 7                              |           |            |          |
| 0                                  | 12.392 ± 0.582 m | 10.321 ± 0.557 jk | 11.334 ± 0.352 m |
| 1                                  | 11.606 ± 0.607 n | 11.181 ± 0.785 jk | 11.014 ± 0.549 m |
| 10                                 | 13.558 ± 0.392 bm | 10.137 ± 0.114 k | 11.872 ± 0.554 bm |
| 100                                | 18.295 ± 0.516 f | 16.010 ± 0.696 f | 17.530 ± 0.468 h |
| 400                                | 20.246 ± 0.335 ph | 16.051 ± 0.577 f | 21.867 ± 0.985 f |
| 700                                | 24.902 ± 0.857 h | 24.465 ± 0.624 d | 23.309 ± 0.309 e |
| 1000                               | 29.110 ± 0.453 c | 28.375 ± 0.604 c | 25.576 ± 0.446 c |
| Day 14                             |           |            |          |
| 0                                  | 13.956 ± 0.180 hl | 12.326 ± 0.375 hi | 13.255 ± 0.513 hl |
| 1                                  | 13.694 ± 0.432 klin | 12.093 ± 0.295 hj | 14.040 ± 0.441 h |
| 10                                 | 14.237 ± 0.334 kli | 12.141 ± 0.122 hj | 14.624 ± 0.401 lk |
| 100                                | 19.330 ± 0.372 h | 17.826 ± 0.650 e | 19.264 ± 0.288 h |
| 400                                | 21.634 ± 0.602 f | 17.982 ± 0.343 e | 24.125 ± 0.297 de |
| 700                                | 26.743 ± 0.458 d | 25.217 ± 0.492 d | 25.402 ± 0.375 cd |
| 1000                               | 31.143 ± 0.588 b | 30.992 ± 0.693 b | 28.888 ± 0.202 b |
| Day 21                             |           |            |          |
| 0                                  | 15.730 ± 0.454 l | 14.224 ± 0.456 g | 15.823 ± 0.422 g |
| 1                                  | 15.136 ± 0.692 l | 13.674 ± 0.063 g | 16.536 ± 0.447 hi |
| 10                                 | 15.753 ± 0.476 l | 13.952 ± 0.611 g | 17.022 ± 0.313 hi |
| 100                                | 21.051 ± 0.491 h | 18.791 ± 0.356 e | 21.831 ± 0.361 f |
| 400                                | 23.118 ± 0.435 f | 19.226 ± 0.380 e | 26.222 ± 0.359 f |
| 700                                | 28.831 ± 0.365 c | 28.774 ± 0.634 c | 30.074 ± 0.502 b |
| 1000                               | 34.872 ± 0.339 a | 35.538 ± 0.609 a | 32.156 ± 0.293 a |

Data are means ± SD (n = 3); values denoted with the same letters form homogeneous groups at the level of p < 0.05 (post hoc Tukey’s HSD test).
Figure 2. Changes in enzymatic activities of peroxidase (POD) and catalase (CAT) in seedlings of spring barley treated with ILs; data are means ± SD (n = 3); values denoted with the same letters form homogeneous groups at the level of \( p < 0.05 \) (post hoc Tukey’s HSD test).

POD, unlike CAT, requires an electron donor, i.e., guaiacol, benzidine, pyrogallol, and ascorbic acid, to remove \( \text{H}_2\text{O}_2 \). Here, stimulation of POD activity was observed in spring barley seedlings due to application of tested ILs to soil. A significant increase in POD activity after application of the tested compounds at concentrations > 100 mg kg\(^{-1}\) of soil DW was observed. This increase was higher for higher concentrations of tested ILs. The highest stimulation of POD activity was reported after application of the highest dose of [BMIM][Br] and [TBP][Br]. An increase in POD activity was also observed over time (Figure 2). The results were confirmed in the literature [30,34,37,41]. There was an increase in POD activity in plants in contact with ILs. Herman et al. [45] also proved that an increase in POD activity with a simultaneous decrease in chlorophyll content may be indicative of premature plant aging. However, Chen et al. [44] reported a linear inhibition of POD activity in wheat seedlings due to imidazolium ILs.
3.6. Interactions between Tested Parameters

Statistical analysis determined the significance of the effect of particular parameters on the phytotoxicity indices and biomarkers of oxidative stress in barley seedlings. The analysis showed that most of the parameters were influenced by the applied dose of ILs. However, the exposure time of plants to the tested salts (term) had the greatest effect on the content of MDA (for all ILs used) and the level of $\text{H}_2\text{O}_2$ for [TBA][Br]. Similar conclusions cannot be drawn in the case of changes in SOD activity and values of $\text{chl}_a$/chl$b$ and (chl$a$ + chl$b$)/car ratios due to high error values (Table 4).

Table 4. Participation of variable factors in the formation of determined parameters in spring barley seedlings exposed to ILs.

| Parameter          | Term (A) | Dose of ILs (B) | A × B  | Error |
|--------------------|----------|----------------|--------|-------|
| [TBA][Br]          |          |                |        |       |
| POD                | 27.391   | 56.641         | 15.173 | 0.796 |
| CAT                | 16.809   | 28.407         | 50.308 | 4.476 |
| SOD                | 37.809   | 17.043         | 30.705 | 14.443|
| MDA                | 79.658   | 10.416         | 8.608  | 1.318 |
| $\text{H}_2\text{O}_2$ | 54.828   | 24.647         | 20.176 | 0.349 |
| Proline            | 4.574    | 94.573         | 0.486  | 0.366 |
| Chl a              | 6.603    | 88.618         | 4.011  | 0.768 |
| Chl b              | 6.931    | 87.203         | 4.868  | 0.997 |
| Chl a + Chl b      | 6.296    | 88.804         | 4.269  | 0.632 |
| Chl a/Chl b        | 15.817   | 18.550         | 41.609 | 24.024|
| Car                | 1.509    | 93.767         | 4.068  | 0.656 |
| (Chl a + Chl b)/Car| 46.411   | 14.335         | 26.983 | 12.241|
| Dry weight         | 0.554    | 94.551         | 3.331  | 1.364 |
| Yield              | 23.219   | 72.536         | 3.665  | 0.579 |
| [BMIM][Br]         |          |                |        |       |
| POD                | 25.888   | 67.404         | 4.293  | 2.414 |
| CAT                | 51.902   | 27.154         | 15.204 | 5.739 |
| SOD                | 82.565   | 3.686          | 4.493* | 9.256 |
| MDA                | 71.395   | 20.018         | 6.939  | 1.648 |
| $\text{H}_2\text{O}_2$ | 17.090   | 68.209         | 14.437 | 0.263 |
| Proline            | 4.820    | 94.019         | 0.837  | 0.324 |
| Chl a              | 3.557    | 89.629         | 6.664  | 0.150 |
| Chl b              | 0.652    | 92.854         | 6.222  | 0.272 |
| Chl a + Chl b      | 2.602    | 90.933         | 6.288  | 0.178 |
| Chl a/Chl b        | 17.628   | 30.442         | 39.883 | 12.047|
| Car                | 3.751    | 92.367         | 3.759  | 0.124 |
| (Chl a + Chl b)/Car| 7.291    | 24.571         | 21.511 | 46.627|
| Dry weight         | 0.035 *  | 98.502         | 0.680  | 0.783 |
| Yield              | 39.772   | 55.747         | 3.953  | 0.528 |
| [TBP][Br]          |          |                |        |       |
| POD                | 33.124   | 55.730         | 10.089 | 1.056 |
| CAT                | 0.082 *  | 66.465         | 25.675 | 7.796 |
| SOD                | 47.434   | 28.123         | 11.401 | 13.042|
| MDA                | 60.049   | 29.547         | 9.577  | 0.826 |
| $\text{H}_2\text{O}_2$ | 10.571   | 85.526         | 3.809  | 0.094 |
| Proline            | 11.037   | 83.761         | 9.330  | 0.148 |
| Chl a              | 6.161    | 83.285         | 12.795 | 0.194 |
| Chl a + Chl b      | 5.577    | 83.798         | 10.472 | 0.153 |
| Chl a/Chl b        | 9.158    | 37.116         | 51.695 | 2.031 |
| Car                | 2.547    | 92.400         | 4.912  | 0.140 |
| (Chl a + Chl b)/Car| 25.412   | 22.529         | 51.815 | 0.244 |
| Dry weight         | 1.661    | 93.352         | 4.599  | 0.388 |
| Yield              | 23.198   | 71.511         | 4.862  | 0.379 |

* statistically insignificant at $p < 0.05$. 
The calculated Pearson linear correlation coefficients showed a significant relationship between the majority of the parameters determined. The highest positive correlation was found between the content of assimilation pigments. However, the highest significant negative correlation was found between dry matter content and carotenoid content (Table 5).

Table 5. Pearson linear correlation coefficients between the determined traits in spring barley exposed to [TBA][Br], [BMIM][Br], and [TBP][Br].

| Traits | POD | SOD | MDA | H₂O₂ | Pro | Chlα | Chlβ | Car  | DW  | Yield |
|--------|-----|-----|-----|------|-----|------|------|------|-----|-------|
| POD    | 0.639 ** | 0.012 | -0.164 * | 0.293 ** | 0.807 ** | -0.801 ** | -0.784 ** | -0.735 ** | 0.793 ** | -0.361 ** |
| SOD    | -0.224 ** | 0.194 * | 0.466 ** | 0.527 ** | -0.604 ** | -0.596 ** | -0.588 ** | 0.679 ** | -0.615 ** |
| MDA    | -0.193 * | 0.189 * | -0.086 | 0.055 | 0.049 | 0.025 | 0.020 | -0.135 |
| H₂O₂   | 0.636 ** | 0.056 | -0.093 | -0.130 | -0.203 * | 0.262 ** | -0.636 ** |
| Pro    | -0.556 ** | 0.086 | -0.055 | -0.531 ** | -0.534 ** | -0.626 ** | 0.656 ** | -0.802 ** |
| Chlα   | -0.908 ** | -0.891 | -0.909 ** | 0.907 ** | -0.586 ** |
| Chlβ   | 0.987 ** | 0.975 ** | 0.975 ** | 0.975 ** | 0.975 ** |
| Car    | -0.913 ** | 0.699 ** |
| DW     | -0.945 ** | 0.731 ** |

* significant at level of p < 0.05, ** significant at level of p < 0.01, POD, peroxidase, CAT, catalase, SOD, superoxide dismutase, MDA, malondialdehyde, Pro, proline, Chlα, chlorophyll a, Chlβ, chlorophyll b, Car, carotenoids, DW, dry weight.

4. Conclusions

The results show that the tested ionic liquids with bromide anion ([TBA][Br], [BMIM][Br], and [TBP][Br]) had phytotoxicity to spring barley seedlings. The magnitude of the toxic effect was correlated with the concentration. However, the exposure time of plants to ILs also influenced some of the parameters. The strongest effect on plant growth and development was from [TBP][Br], whereas the weakest influence on the growth and development of spring barley seedlings was from [TBA][Br].

Inhibition of the growth of aboveground parts of plants and roots was observed under the influence of contact of spring barley with the examined ILs; there was also inhibition of fresh plant mass yield. A decrease in the content of assimilative dyes and an increase in dry matter, free proline, and hydrogen peroxide were also observed. An increase in catalase and peroxidase activity was also observed. All changes were observed only with higher concentrations of the studied compounds. Lower concentrations of the compounds (1–10 mg kg⁻¹ of soil DM) had no significant effect on plant growth and development. The changes observed after the application of higher concentrations of the compounds clearly prove that oxidative stress occurred in plants under the influence of these compounds.

The results can protect the environment from liquid pollution. The results may also be useful in the reuse of areas not contaminated with such compounds by introducing plants that are resistant to these chemicals. In addition, these results can be used to design new compounds with selective or total herbicidal properties as an alternative to conventional plant protection products. At the same time, these results are another step closer to developing a mechanism of the influence of chemical compounds including ILs on plants.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/9/1175/s1, Table S1: Effect of ILs on the germination potential (GP) and germination rate (GR) of spring barley. Table S2: Dry weight (g g⁻¹ FW) content in spring barley seedlings exposed to ILs. Table S3: Photosynthetic pigment (mg g⁻¹ DW) contents in spring barley seedlings exposed to [BMIM][Br]. Table S4: Photosynthetic pigment (mg g⁻¹ DW) contents in spring barley seedlings exposed to [TBA][Br]. Table S5: Photosynthetic pigment (mg g⁻¹ DW) contents in spring barley seedlings exposed to [TBP][Br]. Table S6: Malondialdehyde (MDA) (µg g⁻¹ FW) content in spring barley seedlings exposed to ILs. Table S7: Superoxide dismutase (SOD) (U mg⁻¹ protein) activity in spring barley seedlings exposed to ILs.
Author Contributions: R.B.—Conceptualization, Validation, Project administration, Methodology, Resources and Funding acquisition. R.B. and B.P.—Investigation, Data Curation, Writing—Original Draft, and Supervision. A.T.—Formal analysis. W.P., J.W. and J.S.—Resources. R.B., B.P., A.T., W.P., J.W., J.S.—Writing—Review & Editing. All authors have read and agreed to the published version of the manuscript.

Funding: The work was financed by a statutory activity subsidy from the Polish Ministry of Science and Higher Education for The Faculty of Science and Technology of Jan Długosz University in Częstochowa (SBR/WNŚPiT/KBBE/16/2019) and West Pomeranian University of Technology in Szczecin (503-07-083-08/04).

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Fan, H.; Jin, M.; Wang, H.; Xu, Q.; Xu, L.; Wang, C.; Du, S.; Liu, H. Effect of differently methyl-substituted ionic liquids on *Scenedesmus obliquus* growth, photosynthesis, respiration, and ultrastructure. *Environ. Pollut.* 2019, 250, 155–165. [CrossRef]
2. Cvjetko Bubalo, M.; Radošević, K.; Radojičić Redovniković, I.; Halambek, J.; Srček, V.G. A brief overview of the potential environmental hazards of ionic liquids. *Ecotoxicol. Environ. Saf.* 2014, 99, 1–12. [CrossRef] [PubMed]
3. Egorova, K.S.; Ananikov, V.P. Fundamental importance of ionic interactions in the liquid phase: A review of recent studies of ionic liquids in biomedical and pharmaceutical applications. *J. Mol. Liq.* 2018, 272, 271–300. [CrossRef]
4. Pernak, J.; Czerniak, K.; Niemczak, M.; Ławniczak, Ł.; Kaczmarek, D.K.; Borkowski, A.; Praczyk, T. Bioherbicidal Ionic Liquids. *ACS Sustain. Chem. Eng.* 2018, 6, 2741–2750. [CrossRef]
5. Cvjetko Bubalo, M.; Hanousek, K.; Radošević, K.; Srček, V.G.; Jakovljević, T.; Radojičić Redovniković, I. Imidazolium based ionic liquids: Effect of different anions and alkyl chains lengths on the barley seedlings. *Ecotoxicol. Environ. Saf.* 2014, 101, 116–123. [CrossRef]
6. Liu, T.; Zhu, L.; Xie, H.; Wang, J.; Wang, J.; Sun, F.; Wang, F. Effects of the ionic liquid 1-octyl-3-methylimidazolium hexafluorophosphate on the growth of wheat seedlings. *Environ. Sci. Pollut. Res.* 2014, 21, 3936–3945. [CrossRef] [PubMed]
7. Xu, Y.; Wang, J.; Du, Z.; Li, B.; Juhasz, A.; Tan, M.; Zhu, L.; Wang, J. Toxicity Evaluation of Three Imidazolium-based ionic liquids ([C₆mim]R) on *Vicia faba* Seedlings Using an integrated biomarker response (IBR) index. *Chemosphere* 2020, 240, 124919. [CrossRef] [PubMed]
8. Tot, A.; Vraneša, M.; Maksimović, I.; Putnik-Delić, M.; Daničić, M.; Belič, S.; Gadžurić, S. The effect of imidazolium based ionic liquids on wheat and barley germination and growth: Influence of length and oxygen functionalization of alkyl side chain. *Ecotoxicol. Environ. Saf.* 2018, 147, 401–406. [CrossRef]
9. Jin, M.; Wang, H.; Li, Z.; Fu, L.; Chu, L.; Wu, J.; Du, S.; Liu, H. Physiological responses of *Chlorella pyrenoidosa* to 1-hexyl-3-methyl chloride ionic liquids with different cations. *Sci. Total Environ.* 2019, 685, 315–323. [CrossRef]
10. Egorova, K.S.; Ananikov, V.A. Toxicity of ionic liquids: Eco (cyto) activity as complicated, but unavoidable parameter for task-specific optimization. *ChemSusChem* 2014, 7, 336–360. [CrossRef]
11. Anjaneyulu, E.; Reddy, P.S.; Sunita, M.S.; Kishor, P.B.K.; Mergia, B. Salt tolerance and activity of antioxidative enzymes of transgenic finger millet overexpressing a vacuolar H+-pyrophosphatase gene (*SbVPPase*) from *Sorghum bicolor*. *J. Plant Physiol.* 2014, 171, 789–798. [PubMed]
12. Rosalie, R.; Joas, J.; Deytieux-Belleau, C.; Vulcaín, E.; Payet, B.; Dufossé, L.; Léchaudel, M. Antioxidant and enzymatic responses to oxidative stress induced by pre-harvest water supply reduction and ripening on mango (*Mangifera indica* L. cv. ‘Cogshall’) in relation to carotenoid content. *J. Plant Physiol.* 2015, 184, 68–78. [CrossRef] [PubMed]
13. Sánchez-Rodríguez, E.; Rubio-Wilhelmi, M.A.M.; Cervilla, L.M.; Blasco, B.; Rios, J.J.; Rosales, M.A.; Romero, L.; Ruiz, J.M. Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant Sci.* 2010, 178, 30–40.
14. Gaśционowski, H. Jeczmienn. *Chemia i Technologia, (Pod Red.); PWRiL: Poznań, Poland, 1997. (In Poland)*
15. OECD/OCDE 208. *Guidelines for the Testing of Chemicals;* Terrestrial Plant: Seedling Test: Seedlings Emergence and Seedling Growth Test; Organisation for Economic Co-operation and Development: Paris, France, 2006.
16. PN-EN ISO 11269-2. Jakość Gleby. Oznaczanie Wpływ Zanieczyszczeń na Flory Glebowe. Część 2: Wpływ Zanieczyszczeń Gleby na Wschody i Wczesny Wzrost Roślin Wyższych; Polski Komitet Normalizacyjny: Warsaw, Poland, 2013.

17. Wang, L.-S.; Wang, L.; Wang, L.; Wang, G.; Li, Z.-H.; Wang, J.-J. Effect of 1-butyl-3-methylimidazolium tetrafluoroborate on the wheat (Triticum aestivum L.) seedlings. Environ. Toxicol. 2009, 24, 296–303.

18. Kowalska, I. The content of selected components of spinach (Spinacia oleracea L.) grown at varying levels of calcium. Rocz. Akad. Roln. Poznań 2004, CCCLX, 105–110.

19. Oren, R.; Werk, K.S.; Buchmann, N.; Zimmermann, R. Chlorophyll-nutrient relationships identify nutritionally caused decline in Picea abies stands. Can. J. For. Res. 1993, 23, 1187–1195. [CrossRef]

20. Bates, L.S.; Waldren, R.P.; Teare, I.D. Rapid determination of free proline for water-stress studies. Plant Soil 1973, 39, 205–207.

21. Hodges, D.M.; DeLong, J.M.; Forney, C.F.; Prange, R.K. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 1999, 207, 604–611.

22. Singh, H.P.; Batish, D.R.; Kohli, R.K.; Arora, K. Arsenic-induced root growth inhibition in mung bean (Phaseolus aureus Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. Plant Growth Regul. 2007, 53, 65–73.

23. Giannopolitis, C.N.; Ries, S.K. Superoxide dismutase. I. Occurrence in higher plants. Plant Physiol. 1977, 59, 309–314.

24. Kar, M.; Mishra, D. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiol. 1976, 57, 315–319. [CrossRef] [PubMed]

25. Abassi, N.A.; Kushad, M.M.; Endress, A.G. Active oxygen-scavenging enzymes in developing apple flowers and fruits. Sci. Hortic. 1998, 74, 183–194. [CrossRef]

26. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72, 248–254. [CrossRef]

27. Biczak, R.; Pawłowska, B.; Feder-Kubis, J. The effect of ionic liquids with (−)-menthol derivative containing a chloride anion to weed. Ecol. Chem. Eng. S 2017, 24, 637–651. [CrossRef]

28. Pawłowska, B.; Feder-Kubis, J.; Telesiński, A.; Biczak, R. Biochemical Responses of Wheat Seedlings on the Introduction of Selected Chiral Ionic Liquids to the Soils. J. Agric. Food Chem. 2019, 67, 3086–3095. [CrossRef] [PubMed]

29. Tot, A.; Vraneš, M.; Maksimović, I.; Putnik-Delić, M.; Gađžurić, S. Evaluation of the impact of different alkyl length and type of substituent in imidazolium ionic liquids on cucumber germination, growth and oxidative stress. Environ. Sci. Pollut. Res. 2018, 25, 35594–35601. [CrossRef]

30. Biczak, R.; Śniószek, M.; Telesiński, A.; Pawłowska, B. Growth inhibition and efficiency of the antioxidant system in spring barley and common radish grown on soil polluted ionic liquids with iodide anions. Ecotoxicol. Environ. Saf. 2017, 139, 463–471. [CrossRef]

31. Stepnowski, P.; Mrozik, W.; Nithnhauser, J. Adsorption of alkylimidazolium and alkylpyridinium ionic liquids onto natural soils. Environ. Sci. Technol. 2007, 41, 511–516. [CrossRef]

32. Studzińska, S.; Kowalkowski, T.; Buszewski, B. Study of ionic liquid cations transport in soil. J. Hazard. Mater. 2009, 168, 1542–1547. [CrossRef]

33. Biczak, R.; Bachowska, B.; Balczewski, P. Study of phytotoxicity of ionic liquid 1-(methylthiomethylene)-3-butylimidazolium chloride. Proc. ECOpole 2010, 4, 105–114. (In Poland)

34. Liu, H.; Zhang, S.; Zhang, X.; Chen, C. Growth inhibition and effect on photosystem by three imidazolium ionic liquids in rice seedlings. J. Hazard. Mater. 2015, 286, 440–448. [CrossRef] [PubMed]

35. Zhang, B.; Li, X.; Chen, D.; Wang, J. Effects of 1-octyl-3-methylimidazolium bromide on the antioxidant system of Lemma minor. Prototaxa 2013, 250, 103–110. [CrossRef] [PubMed]

36. Li, Y.; Yang, M.; Liu, L.; Zhang, R.; Cui, Y.; Dang, P.; Ge, X.; Chen, X. Effects of 1-butyl-3-methylimidazolium chloride on the photosynthetic system and metabolism of maize (Zea mays L.) seedlings. Ecotoxicol. Environ. Saf. 2018, 161, 648–654. [CrossRef]

37. Liu, H.; Zhang, S.; Hu, X.; Chen, C. Phytotoxicity and oxidative stress effect of 1-octyl-3-methylimidazolium chloride ionic liquid on rice. Environ. Pollut. 2013, 181, 242–249. [CrossRef]
38. Wang, X.; Dinler, B.S.; Vignjevic, M.; Jacobsen, S.; Wollenweber, B. Physiological and proteome studies of responses to heat stress during grain filling in contrasting wheat cultivars. *Plant Sci.* **2015**, *230*, 33–50. [CrossRef] [PubMed]

39. Moieni-Korbekandi, Z.; Karimzadeh, G.; Sharifi, M. Cold-induced Changes of Proline, Malondialdehyde and Chlorophyll in Spring Canola Cultivars. *J. Plant Physiol. Breed.* **2014**, *4*, 1–11.

40. Rombel-Bryzek, A.; Pisarek, I. Wpływ kwasów huminowych na aktywność metaboliczną buraka cukrowego w warunkach suszy. *Proc. ECOpole* **2017**, *11*, 279–286. (In Poland)

41. Biczak, R.; Pawłowska, B.; Telesiński, A.; Kapuśniak, J. Role of cation structure in the phytotoxicity of ionic liquids: Growth inhibition and oxidative stress in spring barley and common radish. *Environ. Sci. Pollut. Res.* **2017**, *24*, 18444–18457. [CrossRef]

42. Liu, T.; Wang, J.; Wang, J.; Zhu, L. Assessing the influence of 1-dodecyl-3-methyl-imidazolium chloride on soil characteristics and *Vicia faba* seedlings. *Ecotoxicol. Environ. Saf.* **2018**, *152*, 114–120. [CrossRef]

43. Liu, D.; Liu, H.; Wang, S.; Chen, J.; Xia, Y. The toxicity of ionic liquid 1-decylpyridinium bromide to the algae *Scenedesmus obliquus*: Growth inhibition, phototoxicity, and oxidative stress. *Sci. Total Environ.* **2018**, *622–623*, 1572–1580. [CrossRef]

44. Chen, Z.; Zhou, Q.; Guan, W.; Wang, J.; Li, Y.; Yu, N.; Wei, J. Effects of imidazolium-based ionic liquids with different anions on wheat seedlings. *Chemosphere* **2018**, *194*, 20–27. [CrossRef] [PubMed]

45. Herman, B.; Biczak, R.; Gurgul, E. Effect of 1,10-phenanthroline on peroxidase and catalase activity and chlorophyll, sugar, and ascorbic acid contents. *Biol. Plant.* **1998**, *41*, 607–611. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).