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Biochemical Responses of Wheat Seedlings on the Introduction of Selected Chiral Ionic Liquids to the Soils

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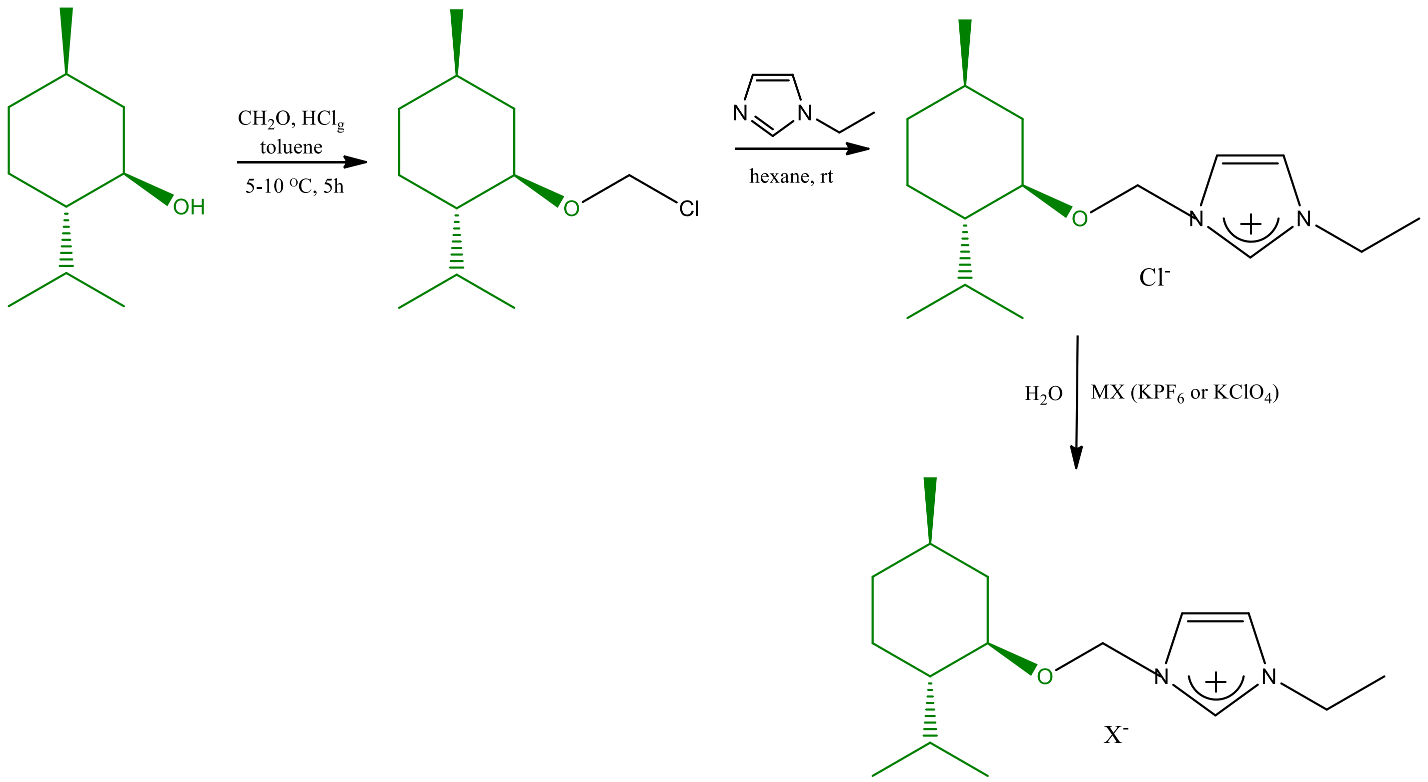
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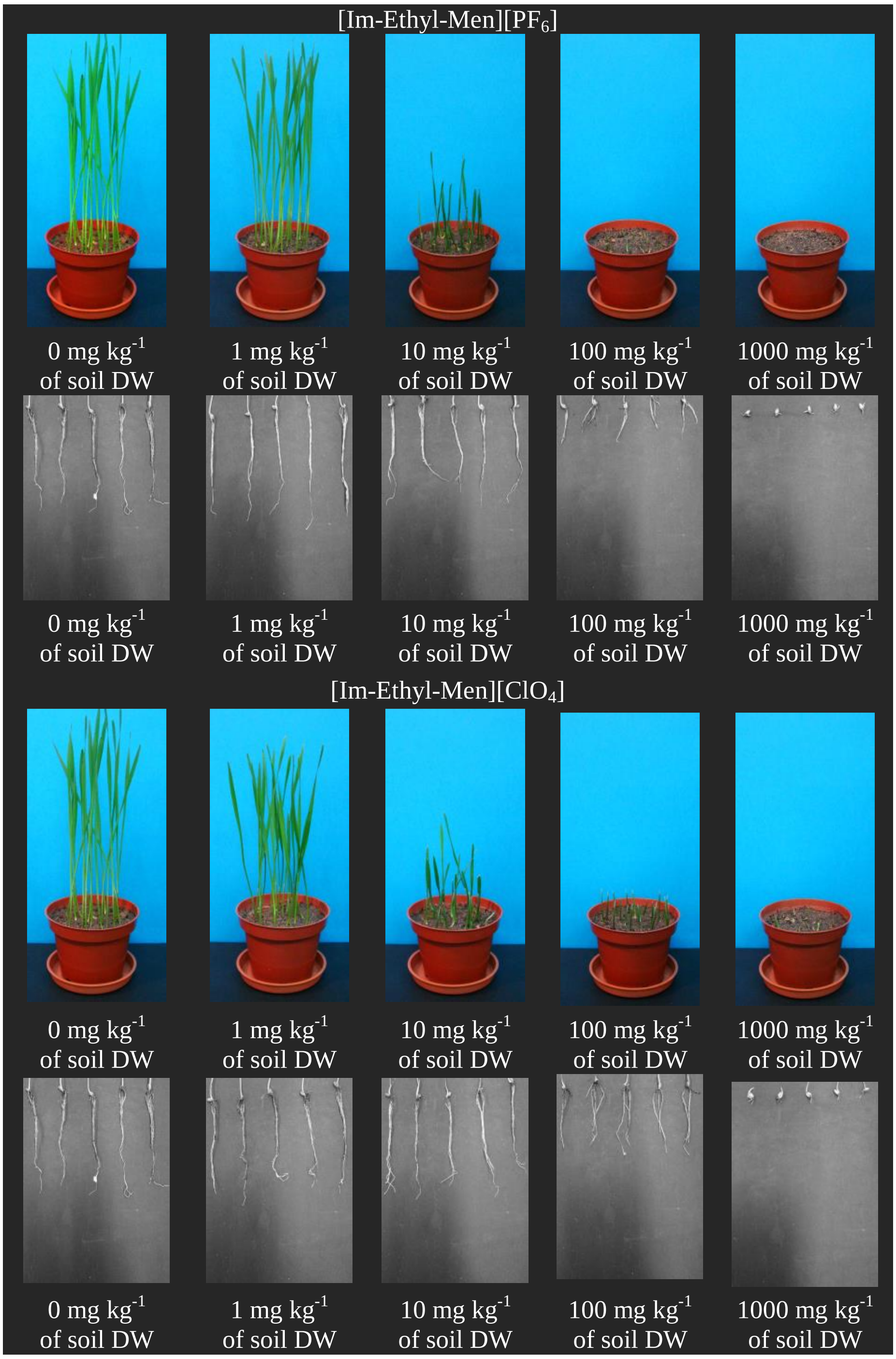
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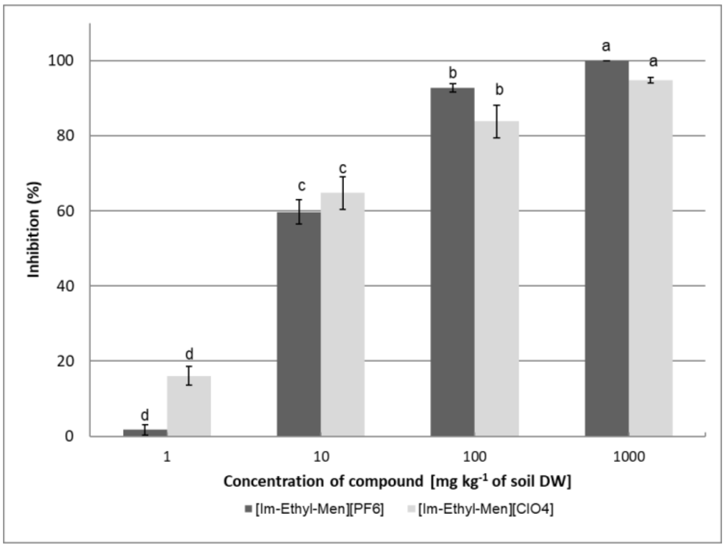
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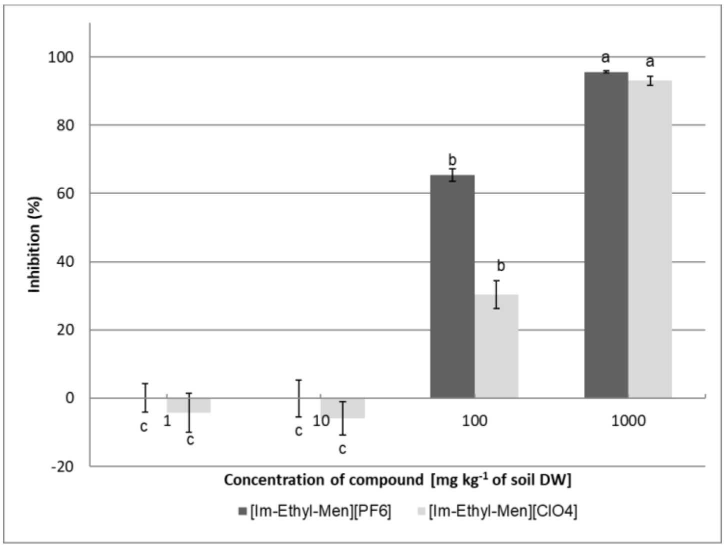
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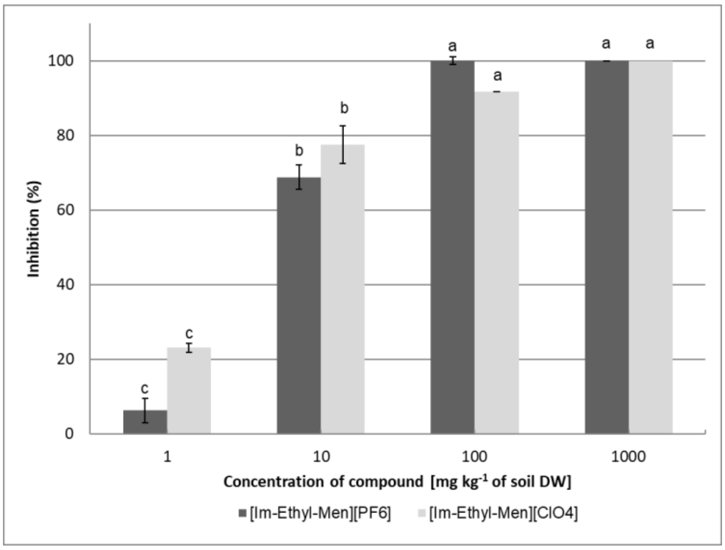
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Concentration of compound [mg kg^{-1} of soil DW]
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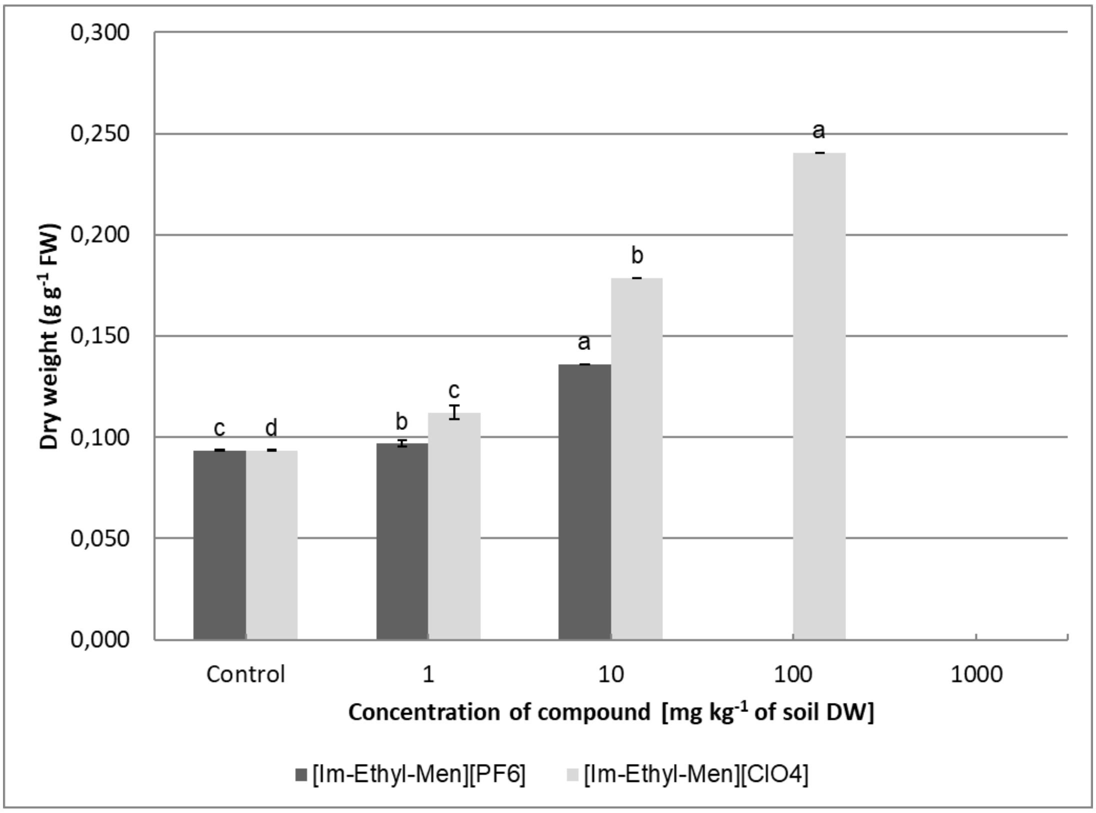
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Dry weight (g g-1 FW)
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c d
b
Control
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10
100
1000
Concentration of compound [mg kg-1 of soil DW]
[Im-Ethyl-Men][PF6]
[Im-Ethyl-Men][ClO4]

Biochemical Responses of Wheat Seedlings on the Introduction of Selected Chiral Ionic Liquids to the Soils

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ABSTRACT

In this study, new chiral ionic liquids (CILs) were obtained from the natural-origin material (1*R*,2*S*,5*R*)-(–)-menthol. The physicochemical characteristics of the studied imidazolium salts were investigated. The obtained 3-ethyl-1-[(1*R*,2*S*,5*R*)-(–)-menthoxy-methyl]imidazolium salts are non-volatile, non-flammable, and stable in air, in contact with water, and in commonly used organic solvents. The influences of the obtained chiral salts on physiological and biochemical parameters were determined for wheat (*Triticum aestivum* L.) seedlings. Both salts led to changes in plant metabolism, which resulted in decreased assimilation pigments, decreased fresh weight, and increased dry weight and proline in wheat seedlings. Moreover, the growth of the above-ground parts and roots was inhibited. Additionally, there was a drop in the potential and germination capacity of wheat seeds after using the highest concentrations of the ionic liquids. The salts caused oxidative stress in wheat seedlings, which was demonstrated by increased malondialdehyde content. In response, the plants engaged their defensive system against free oxygen radicals, which resulted in increased catalase and peroxidase activity and decreased H_2O_2 levels in the plants. There was no changes in the activity of superoxide dismutase. All of the changes observed in the levels of determined biomarkers of oxidative stress in the plants were linearly correlated with the increase in the concentrations of the chiral ionic liquids in the soil. The salt with hexafluorphosphate anion exhibited slightly higher toxicity towards wheat seedlings than the other salt. The CILs led to premature aging of plants, which was demonstrated by the increase in peroxidase activity and a decrease of chlorophyll in the seedlings. The experiment also showed good correlation between the increase in peroxidase activity and the decrease in chlorophyll level, which proves that the decrease in chlorophyll content resulted from not only the increase in CILs concentration in the soil, but also the increased POD activity, which leads to the damage of chlorophyll particles.

Keywords: raw material of natural origin, oxidative stress, growth inhibition, antioxidant enzyme activity

INTRODUCTION

Ionic liquids (ILs) are a group of chemical compounds that have sparked great interest among numerous researchers around the world. These compounds have a wealth of desirable properties, such as non-flammability, non-volatility, high ionic conductivity, good chemical and thermal stability, and good catalytic activity. Thus, they have been used in catalysis, chemical preparation, extraction, the pharmaceutical industry, electrochemistry in capacitors, and plant protection products.[1-3] A very interesting group of ILs are herbicidal ionic liquids (HILs), which have stronger herbicidal properties than previously used herbicides. Like the traditional ILs, these compounds are made of ions with one having confirmed herbicidal properties, such as 2,4-D, MCPA, MCPP, MCPB, dicamba, TBA, glyphosate, clopyraid, and MSM. The latest trends in the synthesis of HILs are directing the attention of researchers towards the synthesis of HILs from naturally occurring substances such as *Pelargonium roseum*, rapeseed oil, choline, and D-glucose. he resulting compounds are called Bio-HILs (bioherbicide ionic liquids).[4,5]

However, due to the wide possibilities of application of ILs, there is high risk that these compounds may penetrate into the natural environment and contaminate it. Due to the good solubility in water and high stability, ILs can easily enter the soil from waste water and sewage sludge, as well as from failures of production systems or disasters during transport. These salts may also be washed from post-production piles and enter the soils in the form of leachate or post-production discharge. The soil is not only a natural store of plant nutrients but also a “natural dustbin” in which all contaminants accumulate.[6]

Ionic liquids that enter the soil may be retained by soil colloids through sorption. The strength of soil sorption depends on both the structure of the ILs and the physicochemical properties of different sorbents. Furthermore, the cation exchange capacity, soil organic matter and clay minerals content are significant factors. The most important soil colloid is organic matter, which consists of substances with high molecular weight, highly complex structure and specific properties. Soil humus is a colloid with a negatively charged core that can successfully bind the cations of ILs. The anions of ionic liquids are not adsorbed in such conditions. Soil ILs can be retrieved by higher plants and affect their growth, development, and the size and quality of yield.[6-8]

Herein the influences of two alkyl imidazolium ILs were evaluated for wheat, one of the world's most commonly cultivated cereals. The level of oxidative stress caused by contact with the examined salts, was evaluated in wheat seedlings. The fresh and dry weight (DW) content and the growth inhibition of above-ground parts of the plants and their roots were also analysed. Oxidative stress was evaluated based on the content of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), proline, and photosynthetic pigments, as well as changes in the activity of the enzymes peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD).

New ILs were selected, which contain the natural component (1*R*,2*S*,5*R*)-(−)-menthol in the cationic part and hexafluorophosphate or chlorate(VII) in the counterion. It is very important to synthesize compounds that are safe for the environment using the safest components. Therefore, the interest in synthesizing ILs has also turned towards naturally occurring source materials, such as pelargonic acid from the flowers of *Pelargonium roseum*, coconut oil, rapeseed oil or just (–)-menthol.[5,9,10]

Considering the beneficial properties of (–)-menthol, such as a ~~non-toxicity,~~ relatively low cost, and environmentally friendliness, it is interesting to study the phytotoxicity of IL containing this natural terpene component. We would like to emphasize, that the Food and Drug Administration (FDA) consider menthol as an safe and effective substance, which help in common cold symptoms and labeled with low toxicity profile.[11,12] This compound is widely used in the food and pharmaceutical industries. Menthol has been indicated to have cooling, analgesic, but also muscle relaxing activities.[13] This well-known representative of the monoterpene class also enhances smooth muscle relaxation, reduces lower esophageal sphincter tone, and the skin barrier by vasodilation, etc.[14,15] The safety profile of menthol has been demonstrated by in vitro and in vivo studies and most investigations reveal a low potential for toxicity in humans.[11] It has been proven that menthol also has antioxidant and antibacterial properties. Mahdavikia and Saharkhiz[16] demonstrated that peppermint extracts containing menthol in their composition may influence the germination of growth and development of higher plants, including weeds. It should be stress that the natural origin of presented alcohol means more safety to the people and the environment.

The imidazolium salts examined are chiral ionic liquids (CILs) where the chirality is located in the cation and associated with the presence of the optically active monoterpene substituent. Previous works have presented various quaternary salts that contain (−)-menthol derivatives and exhibit a number of interesting properties. Some of the most encouraging properties are their biological functions, which include antifungal activity,[~~11~~17] antibacterial activity,[~~12~~18] and stabilizing lacccase in enzymatic systems.[~~13~~19] Compounds with a similar structure have also been tested for their herbicidal properties.[~~14~~20] Other properties of ILs with a menthol derivative include catalytic activity,[~~15~~21] antielectrostatic activity,[~~16~~22] and usefulness in HPLC techniques.[~~17~~23] There are many technological areas where these salts might be successfully applied, such as enzymology, microbiology, and wood protection systems. Hence, the study of ILs with this naturally occurring terpene derivative seems to be very advisable.

Furthermore, the type of anion involved in the ILs structure, is greatly important. One of the projects, in our lab is looking for good or excellent solvents in separation science studies. We have already shown very good results in this area, and terpene-based ILs were used as chiral selectors in HPLC analysis.[~~17,18~~23,24] In those studies only chlorides chiral salts were tested, and we want to go further in testing other possible anions that are good for separation.

The unique properties of ILs combined with their potential uses have already increased the interest of researchers in the field of separation science.[~~19-21~~25-27] Ionic liquids with [ClO$_4$] and [PF$_6$] anions are well known as good additives in chromatographic analysis.[~~22,23~~28,29]

Despite the potential benefits, it is necessary to examine the toxicity of these compounds for important cereals such as wheat. The results will enable risk assessment of environmental contamination with these compounds and the evaluation of their influence on wheat cultivation.

MATERIALS AND METHODS

Chemicals, Materials and General Techniques

The materials used were sourced as follows: (1*R*,2*S*,5*R*)-(–)-menthol (≥99%), paraformaldehyde (powder, 95%), hydrochloric acid (35-38%), sulphuric acid (≥96%), imidazole (≥99,5%), sodium (cubes, contains mineral oil, 99.9% trace metals basis), bromoethane (98%), potassium hexafluorophosphate (≥99%), and potassium perchlorate (≥99.99%) were purchased from Sigma-Aldrich. The drying agents sodium sulphate (anhydrous, pure) and phosphorus pentoxide (powder, anhydrous, ≥98%) were provided by P.O.Ch (Gliwice, Poland). All reagents and solvents were dried beforehand using standard techniques. For NMR analysis, deuterated chloroform was purchased from Merck.

The structure and purity of all of the synthesized substances were confirmed by spectral analysis. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX instrument with tetramethylsilane as the standard (at 400 and 150 MHz, respectively). Elemental analyses were carried out for all of the synthesized substances using a VARIO EL-III. Melting points were determined using an electrothermal digital-melting-point apparatus (model JA 9100, temperature resolution ± 0.1 °C; accuracy ± 1%; ramp rate 1.0 °C/min). The cationic active content was assayed by direct two-phase titration according the EN ISO 2871-2 procedure.[~~24~~30]

General Methods for Synthesis

1-Ethylimidazole was prepared according to a published method[~~25~~31] and purified by vacuum distillation. Chloromethyl (1*R*,2*S*,5*R*)-(–)-menthyl ether was obtained by passing HCl through a mixture of paraformaldehyde and (1*R*,2*S*,5*R*)-(–)-menthol, similar to the method by Pernak et al.[~~26~~32] The precursor of the ILs, 3-ethyl-1-[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]imidazolium chloride, was synthesized according to previously described procedures.[~~25~~31]

Synthesis of new chiral ionic liquids containing (1*R*,2*S*,5*R*)-(–)-menthol derivative

Next, 0.031 mol of the solid inorganic salts potassium hexafluorphosphate, and potassium perchlorate were dissolved in water at room temperature. Then, the prepared solution was added to saturated water containing 3-ethyl-1-[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]imidazolium chloride (0.03 mol). The reaction mixture was stirred at room temperature and the product precipitated from it. After 24h, the crude product was separated and washed with distilled water until chloride ions were no longer detected using $AgNO_3$ solution. Prior to any measurements, 1-alkyl-3-[(1*R*,2*S*,5*R*-(–)-menthoxymethyl]imidazolium salt samples were dried at 40 °C for 48h under vacuum.

3-Ethyl-1-[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]imidazolium hexafluorophosphate, [Im-Ethyl-Men][PF_6]. ^{1}H NMR ($CDCl_3$, 400 MHz, 25°C) δ [ppm] = 0.48 (d, $J = 7.1$ Hz, 3H); 0.88 (m, 9H); 1.22 (m, 1H); 1.40 (m, 1H); 1.58 (m, 5H); 1.92 (sept d, $J^{1,3} = 6.9$ Hz, $J^{1,2} = 2.5$ Hz, 1H); 2.10 (d, $J = 12.0$ Hz, 1H); 3.38 (td, $J^{1,3} = 10.6$ Hz, $J^{1,2} = 4.4$ Hz, 1H); 4.45 (kw, $J = 7.1$ Hz, 2H); 5.48 and 5.72 (d, $J = 10.4$ Hz, 2H, AB system); 7.31 (t, $J = 1.9$ Hz, 1H); 7.20 (t, $J^{1,2} = 1.9$ Hz, $J^{2,1'} = 1.6$ Hz, 1H); 9.01 (t, $J = 1.1$ Hz, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz, 25°C) δ [ppm] = 15.4; 15.5; 20.7; 22.0; 22.6; 25.1; 31.0; 33.9; 40.2; 45.1; 47.5; 76.6; 79.4; 120.6; 121.5; 136.2. Elemental analysis calc (%) for $C_{16}H_{29}ON_2PF_6$ (410.38): C 46.83, H 7.12, N 6.83; found: C 46.90, H 7.23, N 6.72

3-Ethyl-1-[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]imidazolium chlorate(VII), [Im-Ethyl-Men][ClO_4]. ^1H NMR ($CDCl_3$, 400 MHz, 25°C) δ [ppm] = 0.48 (d, J = 7.1 Hz, 3H); 0.87 (m, 9H); 1.22 (m, 1H); 1.40 (m, 1H); 1.58 (m, 5H); 1.90 (sept d, $J^{1,3}$ = 6.9 Hz, $J^{1,2}$ = 2.5 Hz, 1H); 2.11 (d, J = 12.0 Hz, 1H); 3.37 (td, $J^{1,3}$ = 10.6 Hz, $J^{1,2}$ = 4.4 Hz, 1H); 4.45 (kw, J = 7.1 Hz, 2H); 5.52 and 5.79 (d, J = 10.4 Hz, 2H, AB system); 7.41 (t, J = 1.9 Hz, 1H); 7.25 (t, $J^{1,2}$ = 1.9 Hz, $J^{2,1'}$ = 1.6 Hz, 1H); 9.37 (t, J = 1.1 Hz, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz, 25°C)δ [ppm] = 15.2; 15.4; 20.7; 22.1; 22.6; 25.0; 31.0; 33.9; 40.0; 45.1; 47.5; 76.6; 79.3; 120.6; 121.5; 136.5. Elemental analysis calc (%) for $C_{16}H_{29}O_5ClN_2$ (364.86): C 52.67, H 8.01, N 7.68; found: C 52.73, H 8.11, N 7.61

Phytotoxicity Study

Growth Conditions and Treatment

The phytotoxicity of the ILs was determined according to the OECD/OCDE 208 Guidelines for the testing of chemical. Terrestrial plant: seedling test: seedlings emergence and seedling growth test.[~~27~~33] Based on these guidelines, the following concentrations of CILs were used: 0, 1, 10, 100, and 1000 mg of compound per 1 kg of DW of soil. When preparing these concentrations, appropriate amounts of CILs were dissolved in acetone, followed by mixing with quartz sand. After the overhead evaporation of acetone, quartz sand containing imidazolium salts was mixed with the soil. Three independent samples were prepared for each concentration of CILs. Control samples were prepared using the same procedure by adding acetone to the sand but not CILs.

Granulometric analysis showed that the loam used in this study as a soil contained about 11% content with a diameter of <0.02 mm, 9.5 g kg^{-1} of organic carbon, and pH 6.1. Plastic pots were filled with the prepared medium and then seeded with 20 seeds of wheat (*Triticum aestivum* L.), which were derived from the same source. Seed germination and plant growth were carried out for 14 days under strictly controlled conditions: soil moisture at 70% ppw, temperature of 20 ± 2 °C, and constant illumination equal to 170 μmol m^{-2} s^{-1} within a system of 16 h day/8 h night. Stable growth and development conditions were assured by a vegetation hall that is part of the Department of Biotechnology, Biochemistry and Ecotoxicology at Jan Dlugosz University in Czestochowa.

The phytotoxicity of the CILs for wheat was estimated based on the fresh weight yield of the plants, dry weight content, and the length of the aerial parts of the plants and their roots. The inhibition factor of fresh weight, plant length (shoot length), and root lengths were calculated according to a study by Wang et al.[~~28~~34] Based on the calculated inhibition, non-linear regression analysis was used to estimate EC_{50} with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The germination potential (GP) and germination rate (GR) of the wheat seeds were also determined.

$$GP = \frac{Number\ of\ germinated\ seeds\ on\ the\ 3^{rd}\ day}{Seeds\ number\ for\ the\ test} \times 100\%$$

$$GR = \frac{Number\ of\ germinated\ seeds\ on\ the\ 7^{rd}\ day}{Seeds\ number\ for\ the\ test} \times 100\%$$

Seeds with a germ more than 2 mm long were considered germinated.[~~29~~35]

The content of all assimilation pigments, proline, malondialdehyde, H_2O_2, and enzyme activity were determined in fresh plant material. Samples treated with high concentrations of CILs were not included in some analyses. For some of the samples investigated, the inhibition of growth of the aerial parts of wheat was so large that we failed to collect plant material for research purposes.

Determination of Pigments Content

The content of assimilation pigments was determined by spectrophotometry according to the methodology presented by Oren et al.[~~30~~36] A weighed portion (200 mg) of fresh leaves was homogenized with the addition of 80% acetone solution that had been cooled to 4 °C. The resulting extract was transferred into centrifuge tubes and left in the dark for 24 h. The extract was then centrifuged, and in the filtrate obtained, the content of assimilation pigments was determined by measuring the absorbance at 470, 647, and 664 nm. The content of chlorophylls and carotenoids was expressed in mg g^{-1} of plant DW.

Determination of Lipid Peroxidation and Hydrogen Peroxide

A weighed 500-mg portion of fresh leaves was homogenized with the addition of 0.1% trichloroacetic acid solution that had been cooled to 4 °C. After centrifugation, MDA and H_2O_2 content was determined in the supernatant according to procedures described by Hodges et al.[~~31~~37] and Singh et al.[~~32~~38] respectively. Thiobarbituric acid was used as a substrate to determine MDA. The MDA content was determined by measuring the absorbance at 532 nm and 600 nm. To determine the content of H_2O_2, the absorbance was measured at a wavelength of 390 nm for a reaction mixture consisting of supernatant, phosphate buffer pH 7.0, and potassium iodide. The content of MDA and H_2O_2 was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed in μmol g^{-1} fresh weight (FW).

Determination of Free Proline

The free proline content was determined according to the method reported by Bates.[~~33~~39] A fresh sample (0.5 g) was homogenized with 5 ml of 3% sulphosalicylic acid. Next, 2 ml of the supernatant was added to a mixture of 2 ml of glacial acetic and 2 ml of 2.5% (w/v) acidic ninhydrin before boiling at 100 °C for 1 hour. The reaction was terminated in an ice bath. After the solution was cooled, 5 ml of toluene was added for extraction prior to reading the absorbance of the upper red layer (toluene) at 520 nm and calculating the content of proline (mg g^{-1} FW).

Antioxidant Enzyme Activity Determination

Enzymatic extracts for the determination of antioxidant enzymes activity were obtained by homogenizing a weighed portion (500 mg) of fresh leaves with the addition of cooled (4 °C) extraction mixture. The mixture contained phosphate buffer pH 7.4, 1 mM EDTA solution, and 0.1% polyvinylpyrrolidone solution. After centrifugation, the obtained supernatant was also used to determine the protein content.

The activity of superoxide dismutase (SOD) [EC 1.15.1.1] was determined spectrophotometrically by measuring the degree of reduction of nitroblue-tetrazolium (NBT) by superoxide anions that formed as a result of the photochemical reduction of riboflavin in the presence of light.[~~34~~40] NBT reduction is inhibited by SOD. To determine the activity of SOD, the absorbance of the reaction mixture was measured at a wavelength of 560 nm. Superoxide dismutase activity was expressed as U mg^{-1} protein, where 1[U] is the amount of enzyme that induces 50% inhibition of NBT reduction.

Catalase (CAT) activity [EC 1.11.1.6] was determined by the decomposition of H_2O_2 by this enzyme for 15 min, and the H_2O_2 remaining in the reaction mixture was titrated with 0.01 N $KMnO_4$.[~~35~~41] Catalase activity was expressed as U mg^{-1} protein min^{-1}.

The activity of peroxidase (POD) [1.11.1.7] was determined spectrophotometrically by the rate of guaiacol oxidation in the presence of H_2O_2 via enzymes occurring in a given volume of extract for 1 min.[~~36~~42] To measure the activity of POD, the absorbance of the reaction mixture was measured at a wavelength of 470 nm. POD activity was expressed as U mg^{-1} protein min^{-1}.

The total protein content necessary to calculate the activity of SOD, CAT, and POD was determined using the Bradford assay.[~~37~~43]

Statistical Analysis

The results obtained from three independent measurements were subjected to statistical analysis using one-way analysis of variance (ANOVA). Tukey's test with *p*<0.05

(STATISTICA 12.5) was used to determine the significance of reported differences. The data presented in tables and figures are expressed as the mean ± standard error (SE) obtained from 3 measurement replicates.

RESULTS AND DISCUSSION

Synthesis and ~~the~~ Characterization of New Chiral Ionic Liquids Containing (1R,2S,5R)-(–)-Menthol Derivative

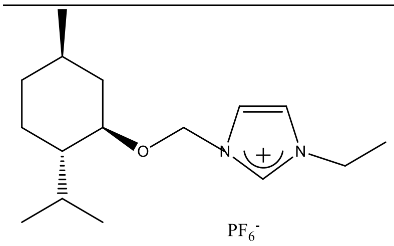
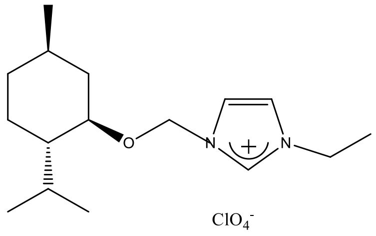
Two CILs were prepared with a natural-origin material in the cation, (1*R*,2*S*,5*R*)-(–)-menthol, and hexafluorophosphate and chlorate(VII) as the counterion (Scheme 1) using previously described methods.[~~25,26~~31,32] In the first step, chloromethyl (1*R*,2*S*,5*R*)-(–)-menthyl ether was obtained by chloromethylation of (1*R*,2*S*,5*R*)-(–)-menthol under isothermal conditions at 10 °C. Then, 3-ethyl-1-[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]imidazolium chloride was prepared using a published method.[~~25~~31] Chiral imidazolium chloride containing a monoterpene moiety was prepared by the Menschutkin reaction. Quaternization was achieved using distilled 1-ethylimidazole and freshly distilled chloromethyl (1*R*,2*S*,5*R*)-(–)-menthyl ether under strictly anhydrous conditions. The reaction was carried out in anhydrous hexane at room temperature, and the product precipitated from the solution. Quaternization takes place immediately and proceeds readily at room temperature. The chiral chloride with (1*R*,2*S*,5*R*)-(–)-menthol derivative was used as a precursor for the new CILs, which were obtained via metathesis reaction. The anion exchange processes progressed smoothly at room temperature, and the final products were obtained in very good yields (98.6% and 98.2%). The total three-step synthesis process is energy efficient and very effective.[~~25,26~~31,32]

Scheme 1. The synthesis of ionic liquids using (1*R*,2*S*,5*R*)-(–)-menthol as a substrate.

Table 1 shows a list of the 3-ethyl-1-[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]imidazolium salts, which have not been described previously in the literature. The are hydrophobic and stable in both air and aqueous solutions. The purities of the hexafluorophosphate and chlorate(VII) salts were determined by a direct two-phase titration technique (EN ISO 2871-2: 2010)[~~24~~30] with a range of 99.9% for the hexafluorphosphate salt and 99.8% for the perchlorate salt. The rest consists of water. The determination could be performed due to the structure of the cation, and the obtained salts were titrated using this method.

Table 1. Structures and physicochemical characteristics of ionic liquids.

Structure and name	**Formula**	***M***			

Structure and name	**Formula**	M_r (g·mol^{-1})	Yield$^{a;b}$ (%)	Melting point^{c} (°C)	Surfactant content^{d} (%)
 3-ethyl-1-[(1*R*,2*S*,5*R*)-(−)-menthoxymethyl]imidazolium hexafluorophosphate [Im-Ethyl-Men][PF_6]	$C_{16}H_{29}N_2OPF_6$	410.44	98.6	99.2–99.5	99.9
 3-ethyl-1-[(1*R*,2*S*,5*R*)-(−)-menthoxymethyl]imidazolium chlorate(VII) [Im-Ethyl-Men][ClO_4]	$C_{16}H_{29}N_2O_5Cl$	364.92	98.2	96.3–96.8	99.8

[a] Isolated yield after purification and drying; [b] Accuracy ± 0.1%; [c] Accuracy ± 0.1 °C; [d] Accuracy ± 0.1%.

The obtained salts are solid, and their melting point does not exceed 100 °C.-Therefore, they should be considered as ILs according to the definition.[~~38~~44]

Phytotoxicity Assay of CILs

The chiral ILs exhibited a phytotoxic effect towards wheat seedlings. After sowing the seeds in soil with the addition of [Im-Ethyl-Men][PF_6] and [Im-Ethyl-Men][ClO_4], there was a drop in the potential and germination capacity of the seeds in comparison to the controls. These changes were higher with increasing CIL concentrations (Table 2).

Table 2. Effects of CILs on the germination potential (GP) and germination rate (GR) of wheat. Data are expressed as a mean ± SE of three replicates for each concentration. Values denoted by the same letters in the columns do not differ statistically at *$p<0.05$*.

CILs concentration (mg kg^{-1} soil DW)	**[Im-Ethyl-Men][PF_6]**		**[Im-Ethyl-Men][ClO_4]**	
	GP (%)	**GR (%)**	**GP (%)**	**GR (%)**
0	91.67 ± 1.67^a	96.67 ± 1.67^a	91.67 ± 1.67^a	96.67 ± 1.67^a
1	86.67 ± 4.41^a	96.67 ± 1.67^a	96.67 ± 3.33^a	81.67 ± 7.39^a
10	81.67 ± 4.41^a	95.00 ± 2.89^a	96.67 ± 1.67^a	73.33 ± 11.61^a
100	6.67 ± 4.41^b	81.67 ± 4.41^a	93.33 ± 1.67^a	70.00 ± 5.00^{ab}
1000	0^b	3.33 ± 1.67^b	66.67 ± 1.67^a	5.00 ± 2.89^b

GP – germination potential; GR – germination rate.

Figure 1. Digital photographs of wheat seedlings and roots on the 14th day after introduction to the soil [Im-Ethyl-Men][PF_6] and [Im-Ethyl-Men][ClO_4] (in mg kg^{-1} of soil DW).

The growth of the plants and their roots was inhibited as the compounds concentrations increased (Figure 1). Inhibition of the root pattern is considered by some authors to be one of the most significant phytotoxic biomarkers.~~[39]~~[45] This is connected with the functions of these bodies, which include keeping the plant in the substrate, as well as the collection and transport of water and nutrients. Therefore, proper development and condition of the roots determine the optimal growth and development of the plant, which in turn translates into the size and quality of the crop. The wheat seedling growth inhibition was over 90% in comparison to the control when using [Im-Ethyl-Men][PF_6] at concentrations of 100 and 1000 mg/kg of soil DW and [Im-

Ethyl-Men][ClO_4] at 1000 mg kg^{-1} of soil DW. The direct effect of the plant growth and their roots consisted of a drop in fresh weight yield of the wheat seedlings in comparison to the controls, which is positively correlated with the increase of the CILs in soil (Figure 2). The EC_{50} values were estimated from the inhibition levels in reference to the root length, above-ground part length, and fresh weight yield. The confidence intervals for the individual EC_{50} values are given in parentheses. In the case of hexafluorphosphate, the values were 75 (41.7-138), 8 (4.75-12.8), and 6 (5.24-6.82) mg kg^{-1} of soil DW, respectively. The EC_{50} values after the use of perchlorate were 171 (70.3-426), 10 (8.33-12.2), and 7 (5.60-8.13) mg kg^{-1} of soil DW.

Figure 2. The inhibition rate for shoot length (a); root length (b); and fresh weight (c) of wheat after exposure to CILs. Data are expressed as a mean ± SE of three replicates for each concentration.

A significant element that determines the influence of the compounds on the plants is the changes in dry weight of the plants. A significant increase in dry weight of the wheat seedlings was observed with the increase of the concentration of CILs in soil (Figure 3). The CIL concentration was a very important biomarker, that had a decisive impact on the growth and development of the wheat seedlings.

Figure 3. Variations in the dry weight (g g^{-1} FW) for wheat seedlings following the introduction of specific amounts of the compound (in mg kg^{-1} of soil DW) to the soil (mean ± SE; n = 3). Values denoted by the same letters in the concentrations do not differ statistically at $p<0.05$.

The results are confirmed by the literature. Studzińska and Buszewki[8] studied the influence of increasing doses of imidazolium ILs on the growth and development of the watercress. They observed a drop in the germination potential of watercress seeds and clear discoloration on the leaves under the influence of these compounds. In previous studies on the determination of phytotoxicity of ILs with iodide anions, we also observed a small effect of these salts on seed germination, which was only visible when compounds were used at the highest concentrations.[~~40~~46] However, other studies show a lack of influence of some imidazolium ILs on the germination potential of seeds of common radish and spring barley.[~~41~~47] These results are corroborated by Liu et al.[~~29~~35] Cvjetko Bubalo et al.[1] and Wang et al.[~~28~~34] These authors also stated that the level of phytotoxicity of chemical compounds introduced to a substrate depends on the concentration. Low IL concentrations may even stimulate plant growth, acting similarly to growth hormones, whereas high concentrations lead to an almost linear drop in yield, sprout length, and root length. [Im-Ethyl-Men][PF_6] had a slightly stronger phytotoxic effect, which may be related to the fact that it contains fluorine. This element has a negative effect on assimilation and photosynthesis, which results in plant growth inhibition.[~~42-44~~48-50] Similar conclusions were also drawn by Matzke et al.[~~45~~51] when studying the effect of ILs with a $CF_3SO_3^-$ anion on the growth and development of wheat.

A very important indicator of phytotoxicity is the changes in the dry matter level of the plants. The growth of dry matter content in both mono- and dicotyledonous plants that have had contact with chemical compounds in the substrate has also been reported by Biczak et al.[7] and Biczak et al.[~~40~~46] A higher increase in dry matter usually correlated with a greater the decrease in the fresh weight of plants.

Effect of CILs on Pigment Content

The yield size is inseparably linked to the efficiency of photosynthesis, in which the content of photosynthetic pigments (i.e. chlorophylls and carotenoids) plays a very important role.[~~46,47~~51,52] Numerous researchers report an almost linear correlation between the concentration of the chemical substance in the environment and the drop of photosynthetic pigment content in plants.[~~29,48-51~~35,54-57] The results of our study demonstrate a drop in Chl*a*, Chl*b*, Chl(*a*+*b*), and carotenoid content, which was positively correlated with an increase of the content of the examined CILs in the substrate. However, no significant changes were observed in the ratio of chlorophyll *a* to chlorophyll *b* and the ratio of total chlorophyll to carotenoids (Table 3).

Table 3. Effects of [Im-Ethyl-Men][PF_6] and [Im-Ethyl-Men][ClO_4] on photosynthetic pigment in wheat seedlings. Data are expressed as a mean ± SE of three replicates for each concentration. Values denoted by the same letters in the columns do not differ statistically at *$p<0.05$.*

	Concentration of CILs (mg kg^{-1} soil DW)	**Pigments (mg g^{-1} DW)**					
		Chl*a*	**Chl*b***	**Car**	**Chl*a*+Chl*b***	**Chl*a*/Chl*b***	**Chl(a+b)/Car**
[Im-Ethyl-Men][PF_6]	0	13.684±0.014^{a}	3.752±0.010^{a}	2.958±0.004^{a}	17.436±0.013^{a}	3.647±0.012^{a}	5.894±0.007^{a}
	1	12.489±0.015^{b}	3.461±0.008^{b}	2.689±0.006^{b}	15.950±0.014^{b}	3.608±0.010^{a}	5.912±0.007^{a}
	10	11.889±0.255^{b}	3.345±0.006^{c}	2.602±0.003^{c}	15.234±0.270^{c}	3.554±0.073^{a}	5.854±0.101^{a}
	100	-	-	-	-	-	-
	1000	-	-	-	-	-	-
[Im-Ethyl-Men][ClO_4]	0	13.684±0.014^{a}	3.752±0.010^{a}	2.958±0.004^{a}	17.436±0.013^{a}	3.647±0.012^{a}	5.894±0.007^{c}
	1	11.475±0.064^{b}	3.227±0.025^{b}	2.253±0.009^{b}	14.702±0.088^{b}	3.556±0.011^{b}	6.525±0.031^{a}
	10	9.814±0.009^{c}	2.666±0.017^{c}	1.950±0.006^{c}	12.481±0.024^{c}	3.681±0.022^{b}	6.399±0.02^{b}
	100	6.692±0.053^{d}	1.723±0.040^{d}	1.287±0.021^{d}	8.415±0.090^{d}	3.886±0.065^{a}	6.541±0.040^{a}
	1000	-	-	-	-	-	-

Chl *a* – chlorophyll *a*, Chl *b* – chlorophyll *b*, chl *a*+*b* – chlorophyll *a* + chlorophyll *b*, car – carotenoides, a/b – chlorophyll *a*/chlorophyll *b*, chl/car –(chlorophyll *a*+ chlorophyl *b*)/carotenoides

Chloroplasts are very susceptible to the production of ROS. The decrease of the level of photosynthetic pigments in plants is related to the fact that the ROS oxidize and damaged proteins contained in the PSII photosystem. This leads to a decrease in photosynthesis efficiency, thus restricting the appropriate growth and development of plants. The harmful influence of the CILs on wheat can also be demonstrated by the drop of the carotenoid content, which constitutes a basic line of defense against ROS.~~[50,51]~~[56,57]

Effects of CILs on Free Proline Content

Changes in proline content constitute an important biomarker of the influence of chemical compounds on a plant, as it regulates the osmotic potential of plant cells, which decreases during oxidative stress. Proline protects the subcellular structure and enzymes and increase cellular osmolarity, which provides the elasticity necessary to multiply cells under stress conditions.[~~29,46~~35,51] Various studies observed higher levels of proline accumulation under salt-stress conditions for wheat, Sorghum bicolor, and green gram.[~~46,52-54~~51,58-60] However, Sánchez-Rodríguez et al.[~~55~~61] believe that the increase of the level of proline is not important for antioxidants and only serves as a marker of stress in plants. In the present study, an increase in the content of this amino acid was observed in wheat seedlings and was positively correlated with the increase of CILs in the soil (Table 4). The increase of proline content in wheat seedlings subjected to imidazolium IL was also observed by Liu et al.[~~29~~35]

Table 4. Effects of a [Im-Ethyl-Men]$[PF_6]$ and [Im-Ethyl-Men]$[ClO_4]$ on the content of proline (mg g^{-1} FW); malondialdehyde (μmol g^{-1} FW) and hydrogen peroxide (μmol g^{-1} FW) in wheat seedlings. Data are expressed as a mean ± SE of three replicates for each concentration. Values denoted by the same letters in the columns do not differ statistically at $p<0.05$.

Concentration of ILs (mg kg^{-1} soil DW)		**Contents**		
		Proline [mg g^{-1} FW]	**MDA [μmol g^{-1} FW]**	**H_2O_2 [μmol g^{-1} FW]**
[Im-Ethyl-Men]$[PF_6]$	0	22.549 ± 1.628^b	35.757 ± 0.395^b	23.661 ± 0.214^a
	1	27.308 ± 0.030^b	34.922 ± 0.329^b	22.532 ± 0.069^b
	10	40.549 ± 1.166^a	40.272 ± 0.426^a	16.611 ± 0.293^c
	100	-	-	-
	1000	-	-	-
[Im-Ethyl-Men]$[ClO_4]$	0	22.549 ± 1.628^b	35.757 ± 0.395^c	23.661 ± 0.214^a
	1	28.643 ± 1.170^b	40.407 ± 0.290^b	21.677 ± 0.353^b
	10	75.958 ± 1.842^a	45.706 ± 0.074^a	19.344 ± 0.037^c
	100	81.242 ± 1.479^a	46.402 ± 0.291^a	17.525 ± 0.128^d
	1000	-	-	-

Effects of CILs on the Generation Rate of H_2O_2 and Lipid Peroxidation

To determine the level of oxidative stress in plants exposed to CILs, the content of MDA was also determined. MDA is a decomposition product of polyunsaturated fatty acids present in the protein-lipid membranes (mainly linoleic acid). The increase in MDA content in plant cells is an indisputable indicator of prevailing oxidative stress and also indirectly reflects the degree of cell damage. An increase of MDA in wheat seedlings was observed with the increase of the content of CILs (Table 4). This increase is explained by the ILs causing strong oxidative stress that prevents the plants from removing ROS.[~~1,29,56~~][1,35,62] This results in increased peroxidation of the protein-lipid membranes that surround different cellular organelles.

The amount of H_2O_2 in plant cells is another very important marker of oxidative stress in plants. The accumulation of H_2O_2 in plants is always observed when an intensive detoxication of the superoxide anion radical ($O^{\bullet -}{}_2$) is catalyzed by superoxide dismutase and when the plant's defensive mechanisms against H_2O_2 fail.[~~49,51,57~~][55,57,63] H_2O_2 is also the most stable chemical molecule of all ROS, capable of rapidly penetrating all cell membranes.[~~58~~][64] A decrease in H_2O_2 content was observed with the increase of CIL concentration (Table 4), which may be related to the increased effect of hydrogen peroxide decomposing the enzymes CAT and POD. However, it appears that oxidative stress in plants is not always manifested by a decrease in H_2O_2 content according to the literature. Zhang et al.[~~49~~][55] and Biczak[~~41~~][47] studied duckweed and radish and indicated an increase in H_2O_2 content in these plants under the influence of 1-octyl-3-methylimidazolium bromide and selected quaternary ammonium salts with hexafluorphosphate anion, respectively. The situation may be related to the various activities of H_2O_2 detoxifying enzymes in various plants.

Effects of CILs on antioxidant enzymes activities

To overcome oxidative stress, plants have developed an enzymatic defense system that includes SOD, CAT, POD, and GR. The first line of defense against ROS is SOD, which decomposes the superoxide anion radical into H_2O_2 and O_2. The elimination of $O_2^{\bullet -}$ by SOD prevents lipid peroxidation, which is why the activity of this enzyme is one of the first to be determined in studies on the determination of the impact of oxidative stress on biochemical and physiological changes in plants.[~~46~~52] However, the literature does not allow for an unambiguous determination of the direction of changes of SOD activity in plants exposed to oxidative stress.

Increases in SOD activity under the influence of ILs in the substrate were observed by Liu et al.[~~56~~62] for wheat seedlings, Cvjetko Bubalo et al.[1] for barley, and Biczak[~~41~~47] for common radish. The present study did not reveal any significant changes in SOD activity in wheat seedlings under the influence of the CILs (Table 5). A similar lack of SOD activity was also observed by Rozpądek et al.[~~59~~65] in cabbage subjected to oxidative stress caused by ozone fumigation.

Table 5. Enzymatic activities of SOD; CAT and POD in wheat seedlings treated with [Im-Ethyl-Men][PF_6] and [Im-Ethyl-Men][ClO_4]. Data are expressed as a mean ± SE of three replicates for each concentration. Values denoted by the same letters in the columns do not differ statistically at *$p<0.05$*.

	Concentration of CILs (mg kg^{-1} soil DW)	**The activity of enzymes**		
		Superoxide dismutase [U mg^{-1} protein^{-1}]	Catalase [U mg^{-1} protein^{-1} min^{-1}]	Peroxidase [U mg^{-1} protein^{-1} min^{-1}]
[Im-Ethyl-Men][PF_6]	0	11.723 ± 0.293^{a}	0.0520 ± 0.0020^{b}	10.928 ± 0.196^{c}
	1	11.504 ± 0.303^{a}	0.0528 ± 0.0016^{b}	13.968 ± 0.136^{b}
	10	11.796 ± 0.124^{a}	0.0778 ± 0.0000^{a}	16.480 ± 0.323^{a}
	100	-	-	-
	1000	-	-	-
[Im-Ethyl-Men][ClO_4]	0	11.723 ± 0.293^{a}	0.0520 ± 0.0020^{c}	10.928 ± 0.16^{d}
	1	10.165 ± 0.177^{b}	0.0536 ± 0.0008^{c}	12.604 ± 0.526^{c}
	10	10.020 ± 0.064^{b}	0.0712 ± 0.0014^{b}	14.538 ± 0.183^{b}
	100	9.377 ± 0.208^{b}	0.0807 ± 0.0012^{a}	16.737 ± 0,180^{a}
	1000	-	-	-

As a result of $O^{\bullet -}_2$ dismutation, a very strong H_2O_2 particle is formed, which has the ability to penetrate all cellular membranes. To protect their cells, plants have developed two oxidative enzymes, CAT and POD, which carry out H_2O_2 detoxification. Catalase is mainly located in peroxisomes and glyoxysomes and breaks down H_2O_2 directly into H_2O and O_2. Peroxidase occurs in many cell organelles and is responsible for the degradation of H_2O_2 with the participation of phenolic compounds (pyrogallol, benzidine and guaiacol) or some antioxidants (ascorbic acid).[~~46~~52] CAT and POD activity increased with the increase of CILs in soil (Table 5). This is corroborated by previous studies that observed an increase of the activity of these antioxidant enzymes in plants subjected to stress factors.[~~46,50~~52,56]

A largely typical marker of oxidative stress is the increase of POD activity, which always increases when the plant is subjected to stress factors and as long as the plant is able to vegetate in such conditions.[~~28~~34] It is also believed that an increase in peroxidase activity and simultaneous decrease in chlorophyll content is a clear indication of premature aging of a plant.[~~41~~47] Furthermore, according to Wang et al.[~~28~~34] Liu et al.[~~29~~35] and Biczak[~~41~~47] peroxidase activity is the most reliable biomarker of the presence of oxidative stress in plants.

In summary, new quaternary imidazolium salts containing a natural-origin material, (1*R*,2*S*,5*R*)-(–)-menthol, were successfully prepared very high efficiency. The synthesized compounds are solid and stable in air, water, and commonly used organic solvents. The salts are ILs since their melting points do not exceed 100 °C, and they are also CILs since they possess three chiral centres in the cation counterpart. The anion exchange processes proceeded smoothly with efficiency exceeding 98% in each case. The general properties such as the melting point, solubility, and surfactant content of the chiral ILs were studied.

The biological results clearly demonstrate that [Im-Ethyl-Men][PF_6] and [Im-Ethyl-Men][ClO_4] exhibit phytotoxicity toward wheat seedlings. The level of this influence primarily depended on the compound concentration. The CILs with hexafluorphosphate anion exhibited slightly stronger phytotoxic properties. The decrease in the level of fresh weight of the plants resulted from the decrease of the content of photosynthetic pigments, which are responsible for the primary production of green plants.

The CILs used in the pot experiment produced oxidative stress in the plants, which was demonstrated by the increased proline content, MDA content, and activity of CAT and POD. This led to a decrease in the level of H_2O_2 in the wheat seedlings. The data obtained could be used to evaluate the risk that these chemical compounds pose to the natural environment and could be applied for their industrial use.

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Conflicts of Interest:

The authors declare no conflict of interest.

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Figures captions:

Scheme 1. The synthesis of ionic liquids using (1*R*,2*S*,5*R*)-(–)-menthol as a substrate.

Figure 1. Digital photographs of wheat seedlings and roots on the 14th day after introduction to the soil [Im-Ethyl-Men][PF_6] and [Im-Ethyl-Men][ClO_4] (in mg kg^{-1} of soil DW).

Figure 2. The inhibition rate for shoot length (a); root length (b); and fresh weight (c) of wheat after exposure to CILs. Data are expressed as a mean ± SE of three replicates for each concentration.

Figure 3. Variations in the dry weight (g g^{-1} FW) for wheat seedlings following the introduction of specific amounts of the compound (in mg kg^{-1} of soil DW) to the soil (mean ± SE; n = 3). Values denoted by the same letters in the concentrations do not differ statistically at $p<0.05$.