Inhibition of germination and early growth of rape seed (*Brassica napus* L.) by MCPA in anionic and ester form

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Abstract MCPA (4-chloro-2-methylphenoxy) acetic acid is a common synthetic auxin used as a herbicide. The purpose of this study was to determine the effects of four new forms of MCPA being the herbicidal ionic liquids (HILs) with MCPA as an anion and two previously known formulations (potassium–sodium salt and 2-ethylhexyl ester) on seed germination and seedling development of winter oilseed rape (*Brassica napus*). Rape plants are susceptible to MCPA and volunteers can be a big problem in crop rotation. Seedling fresh weight and root length were quantified, mitotic activity, as well as lipid, starch, hydrogen peroxide and polyphenol contents were assessed by light and fluorescence microscopy and the computer-aided cytophotometer. In primary roots mitotic activity was almost completely inhibited under the influence of herbicides, cell elongation zones and root hair zones were significantly reduced, and a characteristic bolded root segment formed

just above a meristem. In contrast to the traditional salt formulation the new HILs were weak inducers of hydrogen peroxide synthesis, but were potent stimulators of the synthesis of phenolic compounds and storage as well as emergency substances such as lipids and starch. All tested forms of MCPA caused strong phytotoxic effect on winter rape seedlings, but the tested HILs were more effective.

Keywords Autofluorescence of phenols · Auxin herbicides · Hydrogen peroxide · Ionic liquids · Root meristems · Seed germination

Introduction

Synthetic auxins such as phenoxy-carboxylic acids (MCPA, mecoprop, 2,4-D), benzoic acids (dicamba) and pyridine-carboxylic acids (picloram, clopyralid, fluroxypyr) are widely used in agriculture and horticulture as selective herbicides for mostly postemergence control of annual and perennial broad-leaved weeds (Praczyk et al. 2012). Synthetic auxins act similar to indole-3-acetic acid (IAA), but in contrast to natural phytohormones their degradation in plants occurs slowly (Grossmann 2000). They are absorbed by the leaves or roots, translocated via plant conducting system (xylem and phloem) to the meristematic regions and cause stem twisting, leaf epinasty, inhibition of shoot and root growth and finally cells death. However, the specific site of herbicide action is not well known. It is thought that they are active at multiple levels, among others, they disrupt hormone balance, nucleic acid metabolism and protein synthesis (Monaco et al. 2002). The primary action of synthetic auxins includes also cell wall loosening by increasing the activity of the proton pump that causes the reduction of apoplasmic pH (Senseman 2007). The recent studies showed that there was a

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specific interaction between auxin, ethylene and the abscisic acid biosynthesis (for review see Grossmann 2010).

The purpose of this study was to determine the effects of MCPA on seed germination and seedling development of dicotyledonous plants on the example of winter oilseed rape (*Brassica napus* L. ssp. *napus*). The choice of rape as a model plant was motivated by the fact that rape plants are susceptible to MCPA and volunteers can be a big problem in crop rotation. In our study, we used four new formulations of MCPA being the herbicidal ionic liquids with MCPA as an anion and two known formulations, namely the sodium–potassium salt and 2-ethylhexyl ester.

Ionic liquids (ILs) are defined as organic salts composed entirely of ions that are liquid below 100 °C (Rogers and Seddon 2003a, b; Wasserscheid and Welton 2008; Kirchner 2009; Olivier-Bourbigou et al. 2010). They possess unique properties like low vapour pressure, reasonably good conductivity, wide liquid range, as well as high thermal and electrochemical stability and are excellent solvents for a wide range of compounds. ILs have been receiving considerable global attention in recent years due to their potential application (Plechková and Seddon 2008). Their negligible vapour pressure and non-inflammability reduce or eliminate the risk of polluting emissions to air. The evaluation of ILs proceeds very quickly from the first generation (ILs with unique tuneable physical properties) to the second generation (ILs with targeted chemical properties combined with selected physical properties), and to the third generation (ILs with targeted biological properties combined with selected physical and chemical properties; Hough and Rogers 2007; Hough et al. 2007). The newest third generation ILs was enriched with phytopharmaceuticals called herbicidal ionic liquids (HILs) in which MCPA (Pernak et al. 2011) and 2,4-D are anions (Praczyk et al. 2012). They exhibit higher biological activity than currently used salts of MCPA and 2,4-D. Herbicidal ionic liquids have chemical and thermal stability and show substantially lower water solubility thus reducing soil and groundwater mobility. Acute toxicity of HILs could be controlled by appropriate selection of cation type (Pernak et al. 2011). These novel forms of phenoxy acids possess reduced volatility and drift potential. Ionic liquids are still fairly innovative solvents.

Materials and methods

Plant material

The certificated seeds of winter rape (*Brassica napus* L.) cv. Libomir with germination capacity of 98 % have been used both in laboratory and glasshouse experiments.

Fig. 1 Structures of chemicals used

Chemicals

Two commercial formulations of MCPA: MCPA-salt (Chwastox Extra 300 SL[®] containing MCPA as sodium–potassium salt) and MCPA-ester (Chwastox AS 600 EC[®] containing MCPA as ester 2-ethylhexyl) as well as four new HILs with MCPA as an anion were used (Fig. 1). HILs were obtained according to the method recently described (Pernak et al. 2011). All prepared HILs were characterized by ¹H NMR, ¹³C NMR. ¹H NMR spectra were recorded on a Mercury Gemini 300 spectrometer operating at 300 MHz with tetramethylsilane as the internal standard. ¹³C NMR spectra were obtained with the same instrument at 75 MHz.

Didecyldimethylammonium (4-chloro-2-methylphenoxy)acetate—HIL-1

¹H NMR (CDCl₃) δ ppm = 0.88 (t, *J* = 6.7 Hz, 6H), 1.25 (m, 28H), 1.57 (q, *J* = 6.9 Hz, 4H), 2.24 (s, 3H), 3.12 (s, 6H), 3.21 (t, *J* = 8.5 Hz, 4H), 4.41 (s, 2H), 6.75 (d, *J* = 8.8 Hz, 1H), 7.00 (dd, *J*^{1,2} = 0.6 Hz, *J*^{2,3} = 2.2 Hz, 1H), 7.04 (d, *J* = 0.6 Hz, 1H); ¹³C NMR δ ppm = 14.0, 16.3, 22.5, 22.52, 26.1, 29.06, 29.12, 29.26, 29.30, 31.7, 51.1, 63.3, 68.4, 112.7, 124.1, 126.0, 128.4, 129.9, 156.1, 173.1,

Dicocodimethylammonium (4-chloro-2-methylphenoxy)acetate—HIL-2

¹H NMR (CDCl₃) δ ppm = 0.88 (t, *J* = 6.7 Hz, 6H), 1.25 (m, 44H), 1.57 (q, *J* = 6.9 Hz, 4H), 2.24 (s, 3H), 3.13 (s, 6H), 3.18 (t, *J* = 4.4 Hz, 4H); 4.39 (s, 2H), 6.75 (d, *J* = 8.5 Hz, 1H), 7.02 (d, *J* = 2.8 Hz, 1H), 7.05 (s, 1H),

^{13}C NMR δ ppm = 14.1, 16.4, 22.6, 26.2, 29.1, 29.26, 29.29, 29.34, 29.4, 29.54, 29.59, 29.63, 31.8, 51.2, 63.3, 68.5, 112.7, 123.9, 125.9, 128.3, 129.8, 156.0, 172.9,

Dicocotrimethylammonium (4-chloro-2-methylphenoxy)acetate—HIL-3

^1H NMR (CDCl_3) δ ppm = 0.88 (t, J = 6.7 Hz, 3H), 1.23 (m, 22H), 1.55 (q, J = 3.8 Hz, 2H), 2.21 (s, 3H), 3.08 (s, 9H), 3.15 (t, J = 4.3 Hz, 2H), 4.36 (s, 2H), 6.72 (d, J = 8.2 Hz, 1H), 7.02 (d, J = 3.3 Hz, 1H), 7.04 (s, 1H), ^{13}C NMR δ ppm = 14.1, 16.4, 22.7, 23.0, 26.3, 29.25, 29.30, 29.4, 29.5, 29.6, 29.7, 31.9, 52.9, 66.6, 68.4, 112.7, 124.1, 126.0, 128.3, 129.9, 155.9, 173.1,

Cocodi(2-hydroxyethyl)methylammonium (4-chloro-2-methylphenoxy)acetate—HIL-4

^1H NMR (CDCl_3) δ ppm = 0.88 (t, J = 6.7 Hz, 3H), 1.24 (m, 22H), 1.58 (q, J = 3.8 Hz, 2H), 2.23 (s, 3H), 3.07 (s, 3H), 3.29 (t, J = 5.8 Hz, 2H), 3.45 (t, J = 5.6 Hz, 4H), 3.92 (t, J = 4.4 Hz, 4H), 4.39 (s, 2H), 5.32 (s, 1H), 6.70 (d, J = 8.5 Hz, 1H), 7.04 (d, J = 2.8 Hz, 1H), 7.06 (s, 1H), ^{13}C NMR δ ppm = 14.1, 16.4, 22.5, 22.7, 26.4, 29.2, 29.3, 29.48, 29.51, 29.59, 29.61, 29.7, 31.8, 31.9, 49.9, 55.5, 63.6, 64.1, 68.2, 112.5, 124.4, 126.0, 128.6, 130.0, 155.7, 174.0.

Application of herbicides, seed germination and culture

In laboratory and glasshouse experiments the influence of MCPA forms on the germination and early growth of winter rape seedlings has been evaluated. HIL-3 and HIL-4 were dissolved in water; however, HIL-1 and HIL-2 were dissolved in a water and ethanol solution (3:1). These new forms of MCPA were compared with the commercial herbicides described above. They were applied on the surface of seeds using a spray chamber with flat fan nozzle type F 110/0—6/3 110° LD-015 and an operating pressure of 0.2 MPa. The concentration of all tested substances was established on 0.2 % (v/v), which was similar to 400 g of MCPA per 1 ha with the water volume carrier of 200 L/ha. The experimental design was a randomized complete block with four replications. All experiments were repeated.

In experiments carried out in Petri dishes the seeds of winter rape were sown on wet filter paper (25 seeds per dish), and after application of herbicides they were incubated at 20 °C in darkness for 5 days. After that the seedlings in Petri dishes were grown with 16-h photoperiod (16 h of light—13,000 lx and 8 h of darkness). In experiments carried out in greenhouse the seeds of winter rape were placed on the surface of a sandy loam soil in 0.5-L plastic pots (25 seeds per pot). After application of

herbicides all pots were placed in a greenhouse at temperature 20 °C (± 3 °C) and a 16-h photoperiod. Fresh weight of seedlings was measured 2 weeks (for Petri dishes culture) or 3 weeks (for greenhouse culture) after the herbicide application. The results were expressed as the percent of fresh weight reduction compared with untreated plants.

Feulgen staining

Seedlings were fixed in cold Carnoy's mixture of absolute ethanol and glacial acetic acid (3:1; v/v) for 1 h, washed several times with ethanol, rehydrated, hydrolysed in 4 M HCl for 1 h and stained with Schiff's reagent (pararosaniline; Sigma-Aldrich) according to the standard methods (Maszewski and Kaźmierczak 1998). After rinsing in SO_2 -water (three times) and distilled water, 1-mm-long apical segments of roots were cut off, placed in a drop of 45 % acetic acid and squashed onto Super-Frost (Menzel-Gläser, Braunschweig, Germany) microscope slides. Following freezing with dry ice, coverslips were removed, and the dehydrated dry slides were embedded in Canada balsam.

H_2O_2 detection by the DAB-uptake method

The experimental procedure was performed according to Thordal-Christensen et al. (1997) with some modifications. Three-day-old seedlings of *B. napus* were incubated in a solution containing 1 mg mL^{-1} DAB (3,3'-diaminobenzidine) dissolved in Tris buffer (10 mM Tris, 10 mM EDTA-2Na, 100 mM NaCl, pH 7.6) for 12 h. Then roots were fixed for 40 min (4 °C) in PBS-buffered 4 % paraformaldehyde solution, washed three times with PBS and placed in a citric acid-buffered digestion solution (pH 5.0) containing 2.5 % pectinase, 2.5 % cellulose and 2.5 % pectolyase, and incubated at 37 °C for 25 min. After that the roots were washed as before, rinsed with distilled water and squashed onto glass slides.

Cytochemical localization of lipids and starch

Cytochemical detection of lipids and starch content in root cells was performed using standard procedure of staining with the mixture of Sudan III and IV or Lugol's iodine solution, respectively.

Polyphenols autofluorescence

Several classes of phenolics are autofluorescent under UV light (Schoenwaelder 2008). Seeds were fixed for 1 h in 4 % (v/v) buffered formaldehyde with PBS (pH 7.0) at

Fig. 2 Effect of different forms of MCPA on early growth of *B. napus* seedlings (5 days after application). **a** Control, **b** MCPA-ester, **c** HIL-3. Bar 10 mm

4 °C. Then they were rinsed in PBS three times, dehydrated in a graded ethanol, embedded in paraplast and ultrathin sections (5–7 µm) were prepared using Mikrom microtome. Deparaffinisation was performed by heating in 60 °C and washing in xylene, and then sections were hydrated. Staining was performed using 0.1 % toluidine blue in sodium chloride. Slides were washed three times in distilled water, dehydrated in ethanol gradient 75–100 % and cleared in xylene.

Observations, analyses and statistics

Observations of whole seedlings as well as analyses of mitotic activity, lipid, starch and hydrogen peroxide contents in the cells were performed using binocular microscope SMZ-2T (Nikon) and Optiphot-2 microscope (Nikon) containing phase-contrast optics, respectively, equipped with DXM 1200 CCD camera (Nikon).

Observations of polyphenol autofluorescence were performed using Eclipse E-600 epifluorescence microscope (Nikon) equipped with UV filter ($\lambda = 340\text{--}380$) and DS-Fi1 CCD camera (Nikon).

To evaluate differences in lipid, polyphenol and hydrogen peroxide levels, all images were recorded at exactly the same time of integration. Semiquantitative measurements of the above-mentioned substances staining were made using the computer-aided Cytrophotometr V1.2 (Forel, Poland) after converting colour images into grey scale and expressed in arbitrary units as mean pixel value (pv) spanning the range from 0 (white) to 255 (dark). Each mean value ($\pm$ SD) was based on the analysis of five root meristems (100 cells/meristems). Statistical Student's *t* test was used to establish the significance of the differences between the values. The most typical effects were recorded and are presented as a photo.

Fig. 3 Average length (mm) $\pm$ SD of the embryo axis of *B. napus* seedlings (root + hypocotyls without cotyledons) after 24, 48, 72 and 96 h of culture in distilled water. Before sowing the seeds were either soaked in distilled water (Control) or in the solutions of various herbicides: MCPA-salt (Chwastox Extra 300 SL[®]) and MCPA in anion (HIL-1, HIL-3). Stars show the results where the differences are not statistically significant ($P = 0.05$)

Results

Morphological analysis of seedlings

Initially, the tested forms of MCPA applied on the surface of winter rape seeds similarly disturbed growth and development of seedlings (Fig. 2). Morphological analyses revealed that MCPA-salt, HIL-1 and HIL-3, regardless of their chemical form caused a significant (almost 14-fold after 96 h of culture) inhibition of seedling growth in comparison with the control. The difference in the size of seedlings was mainly due to a significant reduction of root growth (Figs. 3, 4a–d). In the roots under the influence of herbicides cell elongation zone and root hair zone were significantly reduced (Fig. 4a–d). Moreover, after 96 h of

Fig. 4 Morphological differences of *B. napus* seedlings after 96 h of culture in distilled water (**a–d**) as well as differences in the production of hydrogen peroxide released in the reaction with DAB in the roots (**h–k**) and root epidermal cells (**l–o**) of 3-day-old seedlings. Before sowing the seeds were either soaked in distilled water (Control; **a, h, l**) or in the solutions of various herbicides: MCPA-salt (Chwastox

Extra 300 SL[®]; **b, i, m**), MCPA in anion HIL-1 (**c, j, n**) and HIL-3 (**d, k, o**). Differences in the structure of root apex of the seedlings grown from the seeds treated with HIL-2 (**e, f, g**); **black arrows** indicate the zone of root hairs, and **white arrows** indicate bolded zone in the immediate vicinity of meristems. **Black bar** 1 mm, **white bar** 100 μ m

culture, a characteristic protruding section of the root formed just above the meristems. This protruding fragment was the largest in the roots previously treated with HIL-3 (Fig. 4d). During its formation roots often cracked which in consequence led to desiccation and meristem death (Fig. 4e–g).

After 2 weeks of culture in Petri dishes reduction of fresh weight of the seedling whose seeds were treated with HILs varied from 51 to 63 %. Reduction of fresh weight of the seedling whose seeds were treated with commercial forms of MCPA was similar and varied from 55 to 61 % in comparison with the untreated control plants (Table 1).

Table 1 The influence of different forms of MCPA on fresh weight reduction of winter rape seedlings (Petri dishes experiment—as compared to untreated control)

Treatments	Mean (%)	Range (%)
HIL-1	51	50–51
HIL-2	51	47–54
HIL-3	63	61–64
HIL-4	56	51–60
MCPA-salt	61	56–66
MCPA-ester	55	54–55

Table 2 The influence of different forms of MCPA on fresh weight reduction of winter rape seedlings (greenhouse experiment)

Treatments	Mean (%)	Range (%)
HIL-1	83	81–86
HIL-2	86	82–95
HIL-3	78	59–98
HIL-4	75	51–89
MCPA-salt	66	61–72
MCPA-ester	68	63–77

Moreover, the treatments with HILs additionally caused the chlorosis of cotyledons.

Long-term studies conducted in the greenhouse revealed that all tested HILs were more phytotoxic than MCPA-salt or MCPA-ester (Table 2). They caused reduction of seedling fresh weight from 75 to 86 %, while the commercial forms of MCPA from 66 to 68 %. HIL-2 exhibited the highest biological activity.

Hydrogen peroxide content

In the control plants brown polymers of DAB (indicating the presence of hydrogen peroxide) were seen in the root epidermis of cell differentiation and root hair zones (Fig. 4h). Elongated epidermal cells were clearly and homogeneously stained indicating a relatively low level of H_2O_2 in these cells (Figs. 4l, 5). DAB polymers were mainly localized in the wall and, in smaller amounts, in the cytoplasm of root hair cells. MCPA-salt was the potent inducer of H_2O_2 formation. It triggered intense production of H_2O_2 in the root hair zone and cell elongation zone (Figs. 4i, 5). Epidermal cells with limited length were all filled with dark-brown DAB polymers (Fig. 4m). Slightly weaker accumulation of H_2O_2 was observed in the root hair zone and cell elongation zone under the influence of HIL-1 (Figs. 4j, 5) much shorter epidermal cells of these roots contained DAB polymers mainly in the root hair formation sites (Fig. 4n). The lowest content of H_2O_2 expressed as the quantity of polymerised DAB was observed in the roots

Fig. 5 Semiquantitative measurements of hydrogen peroxide staining at the area of cytoplasm, cell wall and organelles in root meristem cells of *B. napus* seedlings after 72 h of culture, expressed as mean pixel value (pv) spanning the range from 0 (white) to 255 (dark) $\pm$ SD. Before sowing the seeds were either soaked in distilled water (Control) or in the solutions of various herbicides: MCPA-salt (Chwastox Extra 300SL®) and MCPA in anion (HIL-1, HIL-3). Stars show the results where the differences are not statistically significant ($P = 0.05$)

under HIL-3 influence (Fig. 5). Clearly the shortest epidermal cells of these roots contained DAB polymers mainly within the root hair zone (Fig. 4k, o).

Phenol content

Autofluorescence of the cross-sections of roots revealed differences in the localization and content of phenolic compounds (Figs. 6, 7). In the control cells, the blue autofluorescence induced by UV light was detected mainly in small vesicles or in a tubular network of endoplasmic reticulum (Fig. 6a–d). After the interference of the herbicides phenols accumulated mainly in relatively numerous large vacuoles. The content of phenols in the vacuoles was much higher in the cells coming from the HIL-treated seeds than in those treated with the traditional MCPA-salt (Figs. 6, 7e–h).

Cytological analysis of cells

Cytological staining of the control root meristems with Feulgen method revealed the presence of cells at each stage of cell cycle (Fig. 8a–g). The cytoplasm of these cells contained small high-density areas, which were visible in the phase-contrast microscope as opalising points (Fig. 8a'–g'). In root meristems under the influence of herbicides mitotic activity was almost completely inhibited (Fig. 9) and almost only interphase nuclei were observed (not shown). However, phase-contrast microscopy showed the presence of post-telophase cells joined by a common cell wall in the meristems coming from the seeds treated with HIL-3 (Fig. 8h, h'). Both the above-mentioned cells and others (not shown) contained significantly larger areas

Fig. 6 Root meristem cells of *B. napus* stained with toluidine blue in the light microscope (**a**, **c**, **e**, **g**) as well as autofluorescence of polyphenols in these same cells under UV light in the fluorescence microscope (**b**, **d**, **f**, **h**). Cells derived from the seedlings grown from

the seeds previously soaked in distilled water (Control; **a–d**) or the solutions of herbicides: MCPA-salt (Chwastox Extra 300 SL®; **e**, **f**) and ionic liquid HIL-3 (**g**, **h**). Bar 10µm; bar in (**h**) also applies to (**a–g**)

of increased cytoplasm density in comparison with the control cells. Furthermore, in the protruding zone of roots large cells filled with numerous Feulgen-positive structures (Fig. 8i) as well as iridescent Feulgen-negative structures were observed (Fig. 8i').

Staining of the meristem cells and of those coming from the protruding zone with the mixture of Sudan III and IV revealed the presence of lipids, while staining with Lugol's revealed also starch grains (Fig. 10). Areas occupied by lipids coincided with those previously seen opalising in the

contrast-phase microscope, while, starch grains in plastids corresponded to Feulgen-positive areas. In the control meristem cells lipid substances were relatively less abundant than in the meristem cells affected by herbicides (Figs. 10a–d, 11). The most abundant lipids were accumulated in the meristem cells under the influence of HIL-3 (compare Fig. 10b–d). Similarly, the cells derived from the protruded zone were rich in lipids (Fig. 10f–h). Significant accumulation of emergency substances characterized also post-telophase cells which already ended the division of

Fig. 7 Phenol content [arbitrary units, (a.u.)] $\pm$ SD in root meristem cells of *B. napus* seedlings after 72 h of culture in distilled water. Before sowing the seeds were either soaked in distilled water (Control) or in the solutions of various herbicides: MCPA-salt (Chwastox Extra 300 SL[®]) and ionic liquid HIL-3

their nuclei but did not complete division of cytoplasm and were connected to a common cell wall (Fig. 10i–k).

Discussion

The current study aimed at comparing the impact of a traditional auxin herbicide, MCPA-salt and new MCPA-ionic liquids on the germination and growth of winter rape seedlings. It was shown that both formulations of MCPA caused similar morphological changes expressed in reduced growth and development of the roots and in consequence of whole seedlings, but it should be noted that MCPA-ionic liquid influence was stronger than that of MCPA-salt. The reason of this difference was looked for at the cellular level by assessing the contents of H_2O_2 and phenols and by analysing the activity of cell division, i.e. the parameters changing under the influence of different stress factors.

H_2O_2 serves as a signalling molecule activates defence responses to various stresses and is one of the constitutive attributes of plant root physiology. On the other hand, H_2O_2 , like other highly reactive oxygen species (ROS), can disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Dat et al. 2000; Neill et al. 2002; Vranová et al. 2002; Mittler et al. 2004; Gechev and Hille 2005; Cheng and Song 2006; Gapper and Dolan 2006; Kwak et al. 2006). Therefore, different antioxidative enzymes (e.g. peroxidases) play a key role in the ROS scavenging system. Peroxidases, depending on their chemical environment, can be considered as bifunctional in relation to H_2O_2 . In the peroxidative cycle they catalyse the reduction of H_2O_2 (thus, converting H_2O_2 into water) by taking electrons from various donors: phenolic compounds, lignin precursors, auxin or secondary metabolites (thus oxidizing them; Passardi et al. 2004). Whereas in the

Fig. 8 Feulgen-stained root meristem cells (a–h) and cells derived from the bolded zone directly adjacent to the meristem (i) in the seedlings of *B. napus* after 72 h of culture in distilled water. Light (a–g, h, i) and phase-contrast (a'–g', h', i') microscopy. Before sowing the seeds were either soaked in distilled water (Control; a nucleus before mitosis, b prophase, c metaphase, d anaphase, e telophase, f post-telophase, g nucleus after mitosis) or in the solutions of ionic liquid HIL-3 (h–i), stars show structures with higher optical density, probably lipids in the cytoplasm, arrows indicate the cell wall between the post-telophase nuclei; n nucleus, p Feulgen-positive areas probably polysaccharides in plastids. Bar 10 μ m; bar in (g') also applies (a–g) and (a'–f')

hydroxylic cycle they can also promote the formation of H_2O_2 , $OH^\cdot$ or $O_2^{\cdot-}$, provided that an appropriately strong reductant is present (Dunand et al. 2007). In our work H_2O_2 content was detected indirectly by the DAB-uptake method. DAB polymerizes giving brown staining of cells as soon as it comes into contact with H_2O_2 in the presence of endogenous peroxidase (Thordal-Christensen et al. 1997).

In the 3-day-old control seedlings of *B. napus* the presence of H_2O_2 was observed mainly in root hairs and also in the walls of root epidermal cells from the differentiation zone (where cell elongation ceases), but not in the root meristem and cell elongation zone. This is in line with the report showing that H_2O_2 is involved in root hair formation and in cell elongation restriction. H_2O_2 reduces root elongation by cross-linking cell wall constituents and diversification of cell wall composition (e.g. by lignin deposition; Foreman et al. 2003; Dunand et al. 2007). Under the influence of traditional MCPA a significant increase in H_2O_2 content (both in the cell differentiation and elongation zones) with simultaneous inhibition of cell and consequently root elongation were observed. The obtained results are consistent with the studies of Šimonovičová et al. (2004) showing that auxin herbicides caused oxidative stress and strong inhibition of barley root growth as a consequence of elevated H_2O_2 production through the root peroxidase system. Analogously, studies carried out by Grossmann et al. (2001) showed that auxin herbicides from different chemical classes caused reduction of internode elongation and stimulated H_2O_2 generation in *Galium aparine* L. shoot tissue. Moreover, results of Jin et al. (2010) indicated that herbicide ZJ0273 (a derivative of 2-pyrimidinyloxy-*N*-aryl benzoate) could deteriorate the enzymatic antioxidant system of rape plants, thereby exposing them to oxidative stress, by accumulating ROS, and ultimately affecting the plant growth. On the other hand, foliar application of 5-aminolevulinic acid (ALA; one of those plant growth regulators being used for stress amelioration) improved relative growth rate (root and shoot) of *B. napus* and also triggered the further accumulation of antioxidants activity, whereas decreased ROS (H_2O_2 and O_2) production (Naeem et al. 2011; Zhang et al. 2008).

Fig. 9 Mitotic index (%) $\pm$ SD in root meristems of *B. napus* seedlings after 24, 48, 72 and 96 h of culture in distilled water. Before sowing the seeds were either soaked in distilled water (Control) or in the solutions of various herbicides: MCPA-salt (Chwastox Extra 300 SL®) and MCPA in anion (HIL-1, HIL-3). Stars show the results where the differences are not statistically significant ($P = 0.05$)

MCPA-ionic liquids (in particular HIL-3) used in these studies, caused a significantly weaker increase in the content of H_2O_2 in cells. Despite this they caused marked reduction of cell growth and inhibited elongation of roots. Research of Dunand and co-workers (2007) using H_2O_2 scavengers revealed that the decrease in the level of H_2O_2 was associated with intensification of cell and root elongation. In our study, lower levels of H_2O_2 , generated by HILs did not correlate with cell and root elongation. On the contrary, cells were significantly shorter than those treated with conventional MCPA. These observations may indicate that mechanisms of HILs action differ from that of the traditional MCPA. On the other hand, in the HIL-treated roots significant (greater than under the influence of traditional MCPA) increase in the content of autofluorescent phenols was observed which may explain the low levels of H_2O_2 in cells. Phenols are effective scavengers of H_2O_2 because

Fig. 10 Lipids (red, orange) and starch (dark) detected using the mixture of Sudan III and IV or Lugol's iodine solution, respectively, in *B. napus* root meristem cells (a–e) and in the cells from the bolded zone directly adjacent to the meristem (f–k) after 72 h of culture in distilled water. Before sowing the seeds were either soaked in distilled

water (Control; a) or in the solutions of various herbicides: Chwastox Extra 300 SL® (b, f), HIL-1 (c, g), HIL-3 (d, h); cw cell wall, l lipids, n nucleus, no nucleolus, p plastids with starch grains. Bar 10 μ m; bar in a also applies to b–e and bar in f also applies to g–k (colour figure online)

Fig. 11 The cell surface occupied by lipids (%) $\pm$ SD in root meristems of *B. napus* seedlings after 72 h of culture in distilled water. Before sowing the seeds were either soaked in distilled water (Control) or in the solutions of various herbicides: MCPA-salt (Chwastox Extra 300 SL[®]) and MCPA in anion (HIL-1, HIL-3)

peroxidases use H_2O_2 for their oxidation (Passardi et al. 2004; Michalak 2006; Dunand et al. 2007). Increased oxidation of phenols with peroxidases might be the reason why peroxidase is not involved in the production of O_2^- required for root elongation in the elongation zone and hence we saw no root elongation (Dunand et al. 2007). Such a hypothesis seems possible but requires further confirmation. The precise role of phenolic substances in the processes of differentiation and morphogenesis is still not clear, but it is known that they play an important role in plant stress resistance. In the current study cells dealt with the excess of exogenous auxin, and it is known that phenols modulate the action of auxin. High levels of phenols, through lowering the endogenous IAA content, might maintain appropriate hormonal balance required for root induction (Volpert et al. 1995; Passardi et al. 2004; Michalak 2006; Dunand et al. 2007). Therefore, it seems possible, that in our study cells triggered the production of phenols in order to restore disturbed physiological balance. Studies of Lee (1977) showed that phenolic compounds such as chlorogenic, caffeic, and ferulic acid could interact with IAA oxidase to reduce the rate of auxin oxidation and hence their degradation. While Faivre-Rampant et al. (2002) suggested that high concentration of chlorogenic acid exceeding a certain threshold concentration could lead to the opposite effect and cause the inhibition of root development. Similarly, the study of Schnablová et al. (2006) showed that monophenolic acid stimulated IAA oxidase activity and thereby contributed to the degradation of auxin.

In the current studies, the lack of root growth under the influence of all applied auxin herbicides was also the consequence of almost complete inhibition of meristematic cell division. In the root meristems there were mainly interphase cells probably blocked in the principal control

points of the cell cycle, i.e. in the G1 and G2 phase (Polit et al. 2003). No abnormal mitotic figures and chromosome aberrations such as: matted chromosomes, chromosome fragmentation and chromosome bridges were observed. They were reported earlier in the study of Sikka and Sharma (1976) and Kumar et al. (2010) who treated *Allium cepa* and *Triticum aestivum* plants with hormonal herbicides. On the contrary, in our results the interphase cells began to accumulate significant amounts of lipids and starch grains. As reported in contemporary literature auxins affect cell division and cell elongation differentially (Campanoni and Nick 2005; Tsugeki et al. 2009; Mithila et al. 2011). Auxin influence on cell division is mediated by a heterotrimeric G-protein whereas that on cell elongation is independent of this protein. Involvement of different receptors may be associated with binding of different ligands. Various auxins should therefore affect cell division and cell elongation with varying dose–response relations through different signal transduction pathways activated by different receptors (Campanoni and Nick 2005). Cytological examination showed that particularly cells treated with HIL-3 that had just passed telophase began rapid growth and accumulation of large amounts of lipids and starch becoming similar to parenchyma cells. Inhibition of cell division and accumulation of large cells in the absence of elongation of epidermal cells result in reduction of meristem and formation of characteristic protruding zone of root. It is also the reason for cracking and detachment root tips. These protruding root segments could also hinder normal development of a vascular tissue as did the abnormal structures formed in roots and shoots under the influence of aminocyclopyrachlor (Flessner et al. 2011). In the present study, it seems that the mechanism or way of standard and ionic MCPA transport-induced changes of varying intensity within the observed cells. Before herbicides reach their site of action they must overcome a number of barriers, both at the tissue level and within membranes of a single cell (Riechers et al. 1994; DiTomasso 1999). Charged molecules definitely influence the efficiency of transport (Zsoldos and Haunold 1979).

Our findings are significant for plant protection based on conjugated phenoxy herbicides because they can lead to long periods of stable and wider performance. Additionally low pricing, worldwide availability, high volume potential, safety for people/environment, cross between green solvents and surfactants are advantageous. The potential of ionic liquids to conceptually fulfil the requirements of environmental sustainability is remarkable.

Author contribution T. Praczyk, J. Pernak and E. Jakubiak prepared the new formulations of herbicides; Ł. Sobiech carried out, analysed and illustrated greenhouse and Petri dishes experiments; J.T. Polit planned, carried

out, analysed, illustrated and described cytological part of the study; G. Skrzypczak was the initiator of research, prepared a theoretical introduction, modified the manuscript and supervised work.

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