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Allelopathic Effect of Aqueous Extracts from the Leaves of Peppermint (*Mentha* × *piperita* L.) on Selected Physiological Processes of Common Sunflower (*Helianthus annuus* L.)

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Abstract

In plants cultivation, some species influencing each other in a favorable manner, and others adversely affect the result of the release of physiologically active substances. These substances, called allelopathic compounds are excreted primarily by underground and aboveground plants' organs or formed during the decomposition of their remains. Allelopathins show the inhibitory or stimulating effects on the processes of seed germination, growth and physiological activity of plants. The aim of the study was to determine the allelopathic effects of aqueous extracts from the peppermint (*Mentha* × *piperita* L.) leaves at various concentrations (1, 3, 5, 10, 15%) on seeds germination and the selected physiological processes of common sunflower (*Helianthus annuus* L.) seedlings. Seeds were germinated and plants were grown under greenhouse conditions for 30 days. Germination of sunflower seeds was reduced and electrolyte leakage from seedlings increased with increasing concentrations of aqueous extracts of the peppermint leaves. Increasing concentrations of aqueous extracts of peppermint also caused decrease of chlorophyll *a* and an increase of chlorophyll *b* content. The highest increase of the maximum photochemical efficiency of photosystem II was observed in *H. annuus* L. treated with 15% peppermint extract in comparison to the lower concentration of extracts and to the control. Non-photochemical and photochemical quenching and vitality index of photosystem II decreased with increasing concentrations of allelopathic substances in peppermint extracts.

Keywords: chlorophyll, electrolyte leakage, fluorescence, photosynthetic activity, seed germination.

Abbreviations: Chl – chlorophyll; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; F_v/F_m – maximum photochemical efficiency of PSII; NPQ – non-photochemical quenching; Rfd – fluorescence decrease ratio; qP – photochemical quenching; PSII – photosystem II.

Introduction

Allelopathic interactions between plants are widely studied issue due to the practical usability of that knowledge (Hao *et al*., 2010; Skoczowski *et al*., 2011; Troć *et al*., 2011; Fabbro *et al*., 2014). Allelopathic substances are produced by plants to counteract competition and to facilitate survival in changing environment (Brown *et al*., 1991; Inderjit and Weiner, 2001; Inderjit and Callaway, 2003; Weston and Duke, 2003; Stokłosa, 2006; Ortega *et al*., 2007; Aziz and Shaukat, 2014). The most commonly observed effects of allelochemicals are morphogenetic changes that eventually reduce the size and quality of the crop yields. A growing interest in the use of allelopathic plants is observed, both in the direct use as plants controlling weeds as well as attempts to isolate specific compounds of high biological activity as natural herbicides (Duke *et al*., 2002; Singh *et al*., 2003; Golisz *et al*., 2004). To the group of plants with medicinal and allelopathic activities, due to the high content of active substances belongs peppermint *Mentha* × *piperita* L. (syn. *M.* × *citrata* Ehrh., *M.* × *piperata* (L.) Hudson). This is a hybrid bred from

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crossing *M. spicata* L. and *M. aquatica* L. It synthesizes monoterpenoids that have been recognized as main allelochemicals in higher plants. The high content of menthol and its isomers, esters of menthol, ketones, and terpene oxides in leaves (Gershenzon *et al*., 2000; Iscan *et al*., 2002; Lawrence, 2007; Eteghad *et al*., 2009; Derwich *et al*., 2010; Kizil *et al*., 2010) makes the peppermint important source of these substances used in food, pharmaceutical and cosmetic industries (Kohlmünzer, 2003). Menthol is known as antifungal and antimicrobial agent (Freire *et al*., 2012; Jha *et al*., 2014) and larvae repellent. Furthemore, menthone is a growth inhibitor (Cavalieri and Caporali, 2010; Tomescu *et al*., 2015), pulegone and other oil components interfere with respiration function of plants (Mucciarelli *et al*., 2001).

Being a hybrid, peppermint does not reproduce by the seeds but only vegetatively, by runners or leaf cuttings. It produces underground stolons, about 10-15 cm long segments, which are seedling material. Establishment of plantations is also carried out using leaves cuttings or vertices of leafy shoots. Due to simple methods of vegetative reproduction, peppermint easily migrates from the plantation to the cultivation of other plants. Therefore, it appears in the excess shading other plants and becoming a weed for the other species of cultivated plant (Burnie *et al*., 2008). In this reason, in addition to its medicinal properties, it is also worth to know its allelopathic effects on other plant species, which can affect not only the seeds germination but also the growth of seedlings by disturbance of some physiological processes. To date there is no scientific information about allelopathic influence on physiological processes of common sunflower. This species is widely cultivated in temperate regions as food crops and ornamental plant. Sunflower seeds contain fatty oil, of economic importance, which is used, among others, as a solvent for lipophilic substances or therapeutically in the diets of artherosclerotic lesions and liver (Kohlmünzer, 2003). Both species, sunflower and peppermint, have similar habitat requirements: they are easy to grow in the temperate climate zone, best in full sunlight on fertile soils and they are characterized by resistance to ground frosts (Burnie *et al*., 2008). Potentially, it is possible the occurrence of both species in cultivation.

The aim of the study was to determine the allelopathic influence of aqueous extracts from leaves of peppermint (*Mentha x piperita* L.) on seeds germination (1), the electrolyte leakage (2), content of chlorophyll *a* and *b* (3) and chlorophyll *a* fluorescence (4) of common sunflower (*Helianthus annuus* L.).

Materials and Methods

Plant material

Dry peppermint leaves (*Folium Menthae piperitae*) from Flos company (Morsko, Poland) and common sunflower seeds (*Helianthus annuus* L.) from PlantiCo company (Zielonki, Poland) were used.

Prepared extract

Aqueous extracts from the leaves of peppermint in percentage concentrations of 1, 3, 5, 10, and 15%, were prepared by flooding in distilled water respectively - 1, 3, 5, 10, and 15 g of dry leaves and leaving them for 24 h, then filtering through paper filters (*Whatman,* USA) using a vacuum pump (*Aga Labor PL2/1*, Warsaw, Poland). Then, the prepared extracts were stored at 4 °C until the end of the experiment.

Seed germination

Germination energy and strength of sunflower seedlings cultured on aqueous extracts from peppermint leaves and on the distilled water (control) were determined. Sunflower seeds washed in distilled water were placed on sterile Petri dishes, 25 seeds on each. At the time of germination, the Petri dishes with seeds were placed in the dark, at a constant temperature conditions (25 °C). Number of germinated seeds was counted every 24 h over 7 days. As germinated seeds were considered ones with sprouts of length equal to half length of the seeds.

Plants

Embraced plants of sunflower, which were germinated on distilled water, and then after 72 h were planted in sand and watered with aqueous extracts from leaves of peppermint for 30 days in greenhouse in natural light and temperature conditions at the turn of May and June 2014. The control plants were watered with distilled water only. Each treatment (1, 3, 5, 10 and 15% of peppermint extract and control) consisted of 5 Petri dishes.

The electrolyte leakage

In order to check an electrolyte leakage, part of sunflower organs (seedlings, roots, shoots and second leaves) were used. Plant material was transferred to polypropylene falcon containing 30 ml of deionized water with a conductivity of 0.05 $\mu S\ cm^{-1}$. Then the tubes were placed on a shaker (*Labnet Rocker International*, New York, USA) for 3 h and for 5 min on Vortex (*Biomix BVX-10*, Blizne Jasiński, Poland). After this time, the measurement of electrolytes flow across cell membranes was made, with the help of MFP (*CX-701 Elmetron*, Zabrze, Poland). After measuring, the plant material was frozen at -80 °C in order to kill the cells. Subsequently the material was defrosted and subjected to the same shaking procedure as before, and conductivity of the total electrolytes content of the tissue was measured. The percentage outflow of electrolytes from cell membranes was calculated (Sutinen *et al*., 1992).

Chlorophyll concentration and fluorescence

The content of chlorophyll *a* and *b* (Chl *a* and Chl *b*) in sunflower leaves was determined by the method of Barnes *et al*. (1992). Measurements of chlorophyll fluorescence were performed using a closed fluorometer FluorCam (*Photon Systems Instruments*, Brno, Czech Republic), according to the method of Lichtenthaler *et al*. (2004). In order to quench the reaction of light phase in photosynthesis, the second leaf of sunflower was cut and placed in the measuring chamber on filter paper lightly dampened with water into the darkness for 20 min. Then the parameters: maximum photochemical efficiency of PSII (F_v/F_m), non-photochemical quenching (NPQ), fluorescence decrease ratio (Rfd), photochemical quenching (qP) were analyzed using FluorCam6 software (http://www.psi.cz/downloads/). The colour scale applied here, shows the absolute values of the studied parameters of sunflower leaves treated with the aqueous extracts from peppermint and control one.

Statistical analysis

The physiological measurements were repeated five times. In the case of chlorophyll the significance of differences was

Table 1. The influence of the aqueous extracts from leaves of *Mentha x piperita* L. on the cell membrane permeability: root, shoot and leaf of *Helianthus annuus* L. watered with *M. x piperita* L. extracts expressed as a percentage of the total electrolytes content in the tissue; data represent the mean value ± SE of 5 replicates

Organs of *Helianthus annuus*	Control	Concentration of aqueous extracts of the *Mentha* × *piperita* leaves L. [%]				
		1	3	5	10	15
Root	20.81 ± 2.11b	19.51 ± 1.72b	19.54 ± 0.74b	19.72 ± 3.10b	20.59 ± 3.01b	33.34 ± 2.09a
Shoot	13.35 ± 0.69d	10.44 ± 0.75e	13.60 ± 0.77cd	16.31 ± 0.29bc	17.00 ± 0.56b	20.69 ± 0.41a
Leaf	19.30 ± 1.44b	8.48 ± 0.76d	9.25 ± 1.11cd	14.30 ± 1.44bc	16.79 ± 0.55b	43.87 ± 1.45a

Note: Different letters between aqueous extracts denote significant differences (Tukey test, $p \leq 0.05$).

Table 2. The influence of the aqueous extracts of *Mentha* × *piperita* L. leaves on the chlorophyll *a* and *b* content [mg g^{-1} FM] in leaves of *Helianthus annuus* L.; data represent the mean value ± SE of 5 replicates

Chlorophyll	Control	Concentration of aqueous extracts of the *Mentha* × *piperita* L. leaves [%]				
		1	3	5	10	15
a	1.47 ± 0.19 a	1.37 ± 0.21 ab	1.24 ± 0.31 b	1.10 ± 0.10 bc	0.85 ± 0.25 c	0.83 ± 0.14 c
b	0.32 ± 0.05 b	0.35 ± 0.04 b	0.32 ± 0.06 b	0.30 ± 0.05 b	0.43 ± 0.19 b	0.66 ± 0.10 a
a + *b*	1.80 ± 0.23 a	1.69 ± 0.25 ab	1.30 ± 0.37 c	1.63 ± 0.14 b	1.58 ± 0.21 b	1.53 ± 0.14 b

Note: Different letters between aqueous extracts denote significant differences (Tukey test, $p \leq 0.05$).

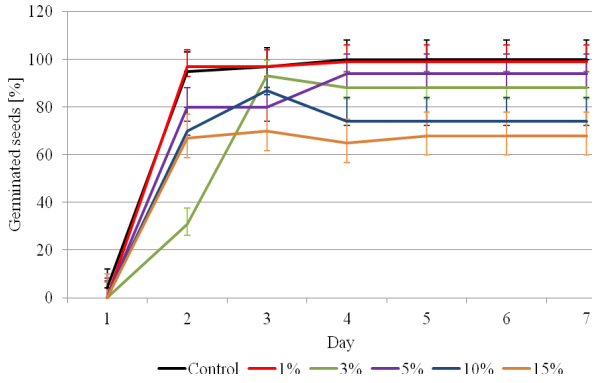


Fig. 1. The influence of the aqueous extracts of the *Mentha* × *piperita* L. leaves on the germination of *Helianthus annuus* L. seeds. Points on lines represent mean value ± SE of 5 replicates

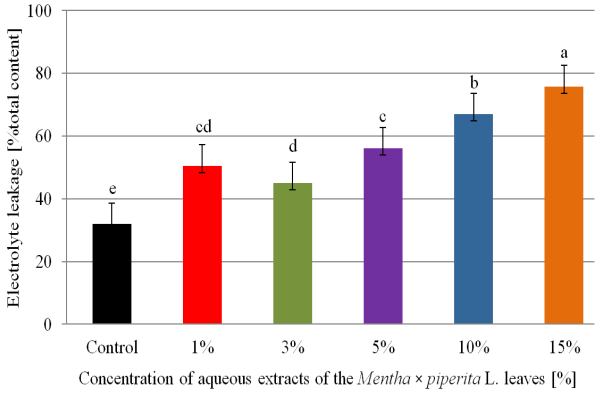


Fig. 2. The influence of aqueous extracts from *Mentha* × *piperita* L. leaves on electrolyte leakage from *Helianthus annuus* L. seedlings, expressed as a percentage of the total electrolyte content in the tissue.
Bars represent mean value ± SE of 5 replicates; different letters between aqueous extracts denote significant differences (Tukey test, $p \leq 0.05$)

examined with inter-facility parametric statistical test - ANOVA, the mean with standard error (SE) using Tukey's test at the level of $p \leq 0.05$. The calculations were performed using Statistica for Windows 10.0 software.

Results

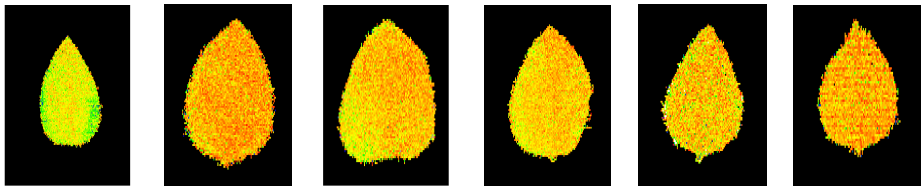
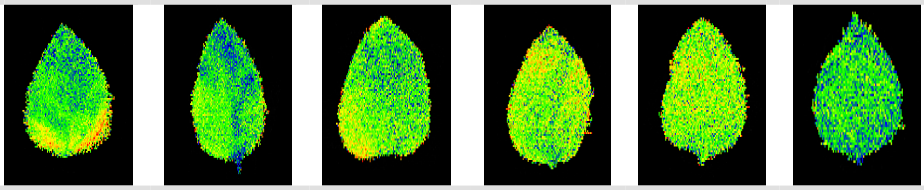
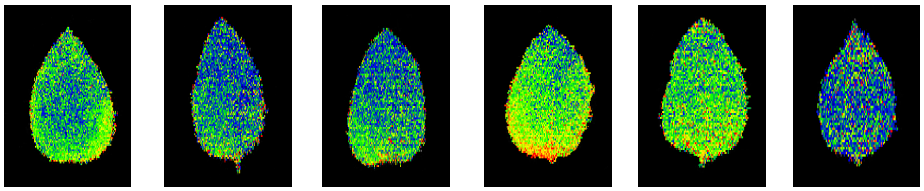
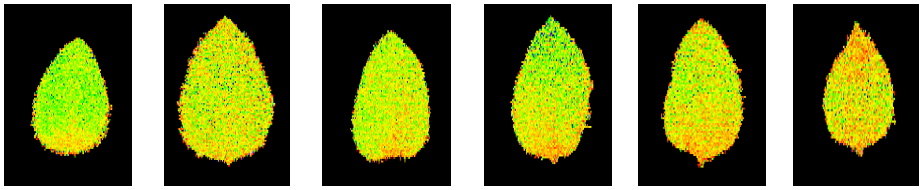
Seed germination

Seeds of *H. annuus* L. placed on substrates with allelopathy extracts were germinating more slowly than the seeds plated on Petri dishes with distilled water (control) (Fig. 1). The fastest rate of germination was observed on the second day of the experiment. The most numerous germinating seeds were observed in control conditions and in 1% of peppermint extract. With increasing concentrations of peppermint extracts the energy of germination decreased and in the dishes with 15% extracts was about 60-70%. On the third day, the highest number of germinated seeds was found on 10% extracts, for which the seeds germinated, up to 90%. On the fourth day, the seeds germination was stable and slow. The curves obtained for the control and at the concentration of 1% were most similar to the curves of control, in contrast to the curves for the germination on 15% extract of peppermint. On the seventh day of the experiment, the percentage of the seeds germinated on the extracts was high at the lower concentrations. The exception was the extract of 15%, which most strongly inhibited the germination of sunflower seeds. In this case, the percentage of the germinated seeds ranged from 60 to 70 (Fig. 1).

The electrolyte leakage

Aqueous extracts from the peppermint leaves significantly increase the permeability of the cell membranes of *H. annuus* L. seedlings compared with the control seedlings (Fig. 2). The least impact on the permeability of the cell membrane had extracts at concentrations of 1 and 3%. The largest increase in the flow of electrolytes was caused by the extract of 15%. The values of the flow of electrolytes in sunflower seedlings fluctuated in the range of 60 to 90%. The flow of electrolytes across cell membranes in roots significantly differs only at the highest concentration of 15%. In shoots, all the extracts from peppermint have caused ionic disturbances, in the leaves the greatest damage was caused by using the extract having a concentration of 15% (Table 1). Generally in all organs of plants of sunflower which were watered with the extracts from peppermint leaves, the flow of electrolytes across membranes have increased without extracts concentration 1 and 3%.

Table 3. Imaging of chlorophyll fluorescence in *Helianthus annuus* L. leaves treated with aqueous extracts of the *Mentha* × *piperita* L. leaves; images obtained from 5 replicates. Selected parameters of chlorophyll fluorescence: F_v/F_m – maximum photochemical efficiency of PSII, NPQ – non-photochemical quenching, Rfd – fluorescence decrease ratio, qP – photochemical quenching

Chlorophyll fluorescence parameters (leaves)	Control	Concentration of aqueous extracts of the *Mentha* × *piperita* L. leaves [%]					Scale
		1	3	5	10	15	
F_v / F_m							
NPQ							
Rfd							
qP							

Chlorophyll content and fluorescence

The content of chlorophyll in plants changed according to the peppermint concentration. There was a significant decrease of Chl *a* and an increase of Chl *b*, especially among the specimens of sunflower watered with the extract of 15% (Table 2).

Imaging chlorophyll fluorescence by FluorCam as opposed to the other methods of measurements, allowed to highlight leaves areas susceptible to the aqueous extracts of peppermint leaves. In the case of maximum fluorescence efficiency for PSII - F_v/F_m, it was different in the leaves of control plants relative to the leaves of plants watered with the extracts in growth. The greatest increase in the value of the parameter in the entire leaf surface was observed among the plants watered with the extracts of 1% (Table 3). Non-photochemical quenching (NPQ) values in leaves of control plants were higher compared to leaves of the plants watered with extracts from peppermint, but only in the lower concentrations. By contrast, with increasing concentrations of extracts, a decrease of NPQ, was observed compared with the control. General heat loss imaged by NPQ exhibited values in the range from 0.5 to 1.5. The chlorophyll fluorescence imaging parameter (Rfd) in leaves was different between the control samples and the plants watered with the extracts. The largest differences were observed among the plants treated with the highest concentrations of extracts and control (Table 3). The values of Rfd parameter ranged from 1.0 to 2.5. In the case of photochemical quenching (qP), visible differences between plants from control group and plants watered with the extracts occurred at the highest concentrations. Changes in qP parameter ranged from 0.0 to 1.5. Changes in chlorophyll fluorescence were observed especially around the petioles and in the upper part of the leaf blade (Table 3).

Discussion

A phenomenon of allelopathy is most often described based on visual changes in plants. There is a delay or inhibition of seed germination (Azizi and Fuji, 2006; Rassaeifar *et al*., 2013), growth inhibition or stimulation of the underground and overground parts of plants (Uddin *et al*., 2014). These actions depend on the concentration of

the active substances contained in the substrate, temperature, light intensity, humidity and other environmental factors (Oleszek, 1996).

Seed germination is a complex cycle of changes, in which the transition from the state of seed dormancy to the phase of vegetative development takes place (Krupa, 1970). During germination, metabolic and structural changes are occurring to ensure harmonious growth and development of seedlings. The first of these are characterized by the intense metabolism, predominantly anabolic processes. The subsequent are characterized by a decrease in the intensity of metabolism and respiration and a decrease of water content in the seed. Simple and easily soluble compounds are rebuilt into hardly soluble complex. In the final stage, the seeds undergo resting phase. With decreasing dormancy, under suitable conditions: moisture, temperature and access of oxygen, in the seeds levels of biochemical processes increase - the result is a germination (Grzesiuk and Kulka, 1981). The aqueous extracts from peppermint leaves used in the experiments have significantly influenced the germination of sunflower seeds. It has been shown that with increasing concentration of the extract, a reduction in the percent of germinated sunflower seeds, relative to the control (distilled water) followed. This effect was most pronounced in the highest concentrations of allelopathic substances (Fig. 1). The differences in the dynamics of seed germination between the seeds, which were treated with aqueous extracts of peppermint and with distilled water (control), prove allelopathic interactions of compounds contained in the peppermint leaves. In our study we chose concentrations in range 1-15% in order to verify the sunflower reaction to varying concentrations of allelopathic compounds in aqueous extracts of the peppermint leaves. The allelopathic influence of the same substance may vary depending on its concentration as well as on the type of seed, plant, the vicinity of other plants and environmental factors (Willis, 2000; Amini *et al*., 2014; Sangeetha and Baskar, 2015).

According to Kohlmünzer (2003), the main active ingredient is peppermint essential oil (*Oleum Menthae piperitae*) comprising in 50% menthol, menthol esters, moreover, ethyl valerate, felandren, pinene, cineol, piperitone, menthofuran, methyl (-) - menthone, phenolic acids. In total, there are about 30 components having different quantitative and qualitative chemical properties. In experiments conducted on extracts from a variety of weed species, it was demonstrated that their toxic properties delay the germination of seeds and growth of seedlings. In addition, they cause anatomical-morphological distortions in root apices to some of them (Halsall *et al*., 1995; Qasem, 1995; Aliotta *et al*., 1996; An *et al*., 1996; Rawat *et al*., 2012). According to Kupidłowska *et al*. (2006) the inhibition of the germination of mustard (*Sinapis arvensis* L.) by extract from the leaves of sunflower is the result of the noise of inherent metabolic processes in cells but not damaged cell organelles. This is because there is an allelochemical impact on seed germination which occurs by interrupting the normal cellular metabolism (Gniazdowska and Bogatek, 2005). On the other hand, Liu and Lovett (1993) said that allelopathic compounds result in the reorganization of the cell structures. The allelopathic substances causes damage to the cell walls, disintegration of organelles, appearing of the lipid globules and increase of the vacuoles. These in turn lead to a slowdown of cell metabolism and dysfunctions of the enzyme systems (Levitt *et al*., 1984). Inhibitory effects of allelopathic compounds were revealed by the inhibition of division and cell elongation, which may take place during periods of swelling, thus delaying the germination and ultimately lead to dying of the embryonic parts of root. The aqueous extracts from peppermint leaves on which the sunflower seedlings grew, caused an increase in cell membrane permeability, as like as measured value of outflow of electrolytes. The most significant increase in outflow of electrolytes from the cell membranes was observed for seedlings and sunflower roots, shoots and leaves in the highest, 15% extract concentration. These indicate that the peppermint extracts contain chemical compounds that are damaging the functioning of the cell membranes, which activity causes interference of the water and mineral economy. We used aquatic extracts of peppermint leaves despite menthol and other alkaloids are slightly soluble in water. Peppermint leaves contain beside menthol essential oil (approx. 2.5%), tannins (6-12%), gentian, phenolic acids, flavonoids (apigenin, and luteolin, diosmetin), carotene, coffeic acid (0.5-2%), chlorogenic acid (0.7%), ursolic acid (0.3%), oleanolic acid (0.12%), betaine, arginine, phytosterol, fats, glucose, rhamnose, mineral salts etc. So, not only menthol and other alkaloids are soluble in water. We dissolved the leaves in water not in e.g. organic solvents because such conditions are the closest to the field conditions. Maffei *et al*. (2001) suggest that the water decreases the solubility of monoterpenes, and increases the activity of terpenoids that interact and interfere with the integrity of cell membranes.

Examples of chemical compounds interfering in the process of photosynthesis, influencing on the lowest chlorophyll content (Huang *et al*., 2010; Ismail and Siddique, 2011; Han *et al*., 2012) are given. Plants subjected to allelopathic stress varied in colour intensity from the healthy plants. Low concentrations of allelopathic substances increase chlorophyll content, while the higher exhibit the opposite effect - lowering its content (Dadkhah, 2012). Disturbances caused by allelochemical compounds can generally reduce the content of plant pigments. In the presented study, a decrease of the chlorophyll *a* and increase of chlorophyll *b* content in sunflower leaves was shown (Table 2). Increase of its amount after application of peppermint extract in comparison to control plant means that photosynthetic apparatus has been damaged. It is probable that some compounds from peppermint extract disturbed synthesis of chlorophylls. In plants, the light-harvesting antennas around photosystem II contain the majority of chlorophyll *b*. Hence, in plants which did not receive enough light (as an effect of field conditions or damages of photosynthetic apparatus), they have an increased ratio of photosystem II to photosystem I, there is a lower ratio of chlorophyll *a* to chlorophyll *b*. This can be adaptive, as increasing chlorophyll *b* increases the range of wavelengths absorbed by the chloroplasts (Kitajima and Hogan, 2003).

In the stress conditions, energy absorbed by the photosynthetic pigments is not used to full effect in the process of photosynthesis. The result is a damage of the PSII complex but the plants are trying to fight against stress in different ways. They block, among others, oxidation processes in photosystem PSII or activate the mechanisms to adapt to new environmental conditions (Havaux, 1993). As the result of allelopathic compounds, the content of secondary metabolites in plants increases (Tang *et al*., 1995). On the other hand, the allelopathic compounds obtained by plants do not always have to disclose their toxic effects. The plants have defence mechanisms allowing them detoxification. Imaging chlorophyll fluorescence by FluorCam allows registering very early changes in the anatomical structures of plants and especially the structure of the PSII, which

is considered extremely sensitive to stress (Kalaji *et al*., 2014; Synowiec *et al*., 2015). In the case of the parameter F_v/F_m growth on the surface of the whole leaf was intense at the highest concentrations of the extracts (Table 3). This proves the high efficiency of electron transport of photosystem PSII induced by the extracts. Generally, heat losses imaged by (NPQ) showed values ranging from 0.5 to 1.5. In particular, areas of the leaves the values of this parameter were lowered relative to the control. For parameter Rfd, especially at the highest concentrations, values were below 1.0, which would indicate a disorder in the process of assimilation of CO_2 during photosynthesis (Kalaji and Łoboda, 2010). In addition, the leaves of sunflower were of high photosynthetic activity and the Rfd reached approximately 2.0-2.5. For the parameter qP, changes in the amount of closed reaction centres were observed under the influence of the saturation of photosynthesis by actinic radiation, with increasing concentrations of the extracts. Changes in fluorescence appeared especially around the petioles and in the upper part of the leaf blade (Table 3). Inhibiting electron transport for energy production and disrupting the proton motive force, protein translocation and synthesis of cellular components are all physiological changes that can result in cell lysis and death (Turina *et al*., 2006).

Conclusion

Aqueous extracts from the *Mentha x piperita* L. leaves at increasing concentrations appear to have the inhibitory effect on physiological processes of *Helianthus annuus* L. Increasing concentration of aqueous extracts negatively influences the germination of seeds (1) and higher leakage of electrolytes from cell membrane of *H. annuus* L. seedlings (2). Depending on the concentration of extracts from peppermint leaves, they have an inhibitory or stimulating influence on the chlorophyll content (3) and functioning of photosystem II (4).

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