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Plant Functioning Under Environmental Stress

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PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS

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PREFACE

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Investigations in the plant stress biology are the multidisciplinary ones, covering aspects of plant physiology, ecology, biochemistry, biophysics, genetics and breeding. They provide information on physiological processes that are disturbed as a result of the influence of the imposed stresses factor. Higher plants, as sessile organisms, during evolution developed defence mechanisms in order to cope with and function under environmental stresses. Physiological basis for plant reaction to environmental stress factors, despite of this, are still the object of researchers interest. Nevertheless, we are far from complete understanding the mechanisms of plant defence against stresses as yet. The modern methodological approach and the applied techniques allow on research at various levels of biological organization beginning with field, through single plants, organs, cells and organelles down to the molecular level. The progress in analytical methods allows the investigation of the successive stages of signal transductions or the role of secondary metabolites taking part both in plant defence reactions against stresses and of those responsible for generating stress at the cellular level. The results of these complementary investigations, important from the academic point of view, also provide much valuable applicable information, which may be used in agricultural and environmental biotechnology including genetic engineering, selection, and breeding as well as in agronomy.

On global scale, 90% of all agriculturally used land is influenced by abiotic or biotic stress and also within the areas that are said to be stress-free, are observed short and of various intensity deviations from the optimum. However, we are still far away from the full and comprehensive knowledge of mechanisms underlying plant acclimatization and adaptation to stress factors. This is due to the complexity of both the reception and transduction of signals, and reactions of plant on various levels of biological organisation, magnified by a multi-gene control of plant responses to stressors, together with the fact, that in many cases there are two or more stress factors appearing together or sequentially. The greater challenge, however, seems to be the proper utilization of the gained knowledge in practice. Further substantial progress in yielding, both quantitatively and qualitatively, obtained through selection, classical breeding, transgenesis in case of genetically modified organisms or through specially designed agro-technologies, may be effective only if the scientific

achievements of physiological, biochemical and molecular bases of plant reactions and tolerance to environmental stress factors are utilized.

Plants, similar to all other living organisms, exist under the influence of the surrounding environment, which in many cases provides conditions far or extremely far from optimal for their growth, development and yielding. Higher plants, as sessile, cannot change their place of existence. Therefore, through evolution, they have developed combinations of molecular, biochemical, physiological, anatomical, morphological, and behavioural features and processes enabling them to adapt to and survive in unfavourable environmental conditions. The processes of acclimatization and adaptation to the changing over time, types and intensity of stressful factors are affected accordingly to the emergence of new threats. The problems of famine in many countries, the losses in food production resulting from drought, floods, too low or too high air temperatures, environmental pollution connected with the industrialization, urbanization, chemization of agriculture and plant diseases, have become problems of world-wide importance, and they will recur on a global scale because the climate is more and more changeable. The tendency of the climate to warm-up, which has been confirmed by meteorologists, as a result of the "greenhouse effect" does not necessarily imply that there will be no more severe winters.

In the popularization of knowledge on plant stress physiology, the important role play scientific conferences aiming at presenting the current state of the research, exchange of opinions and provide the possibly to initiate new scientific projects. Since 1995 the Institute of Plant Physiology, Polish Academy of Sciences, has been organizing international conferences entitles *Plant Functioning under Environmental Stress* in cooperation with the University of Alberta in Edmonton, Slovak University of Agriculture in Nitra, Plant Protection Institute HAS in Budapest, Warsaw University of Life Sciences, Pedagogical University of Cracow, and Agricultural University of Cracow. This years conference is the tenth in the series, *i.e.* a jubilee conference, and on this occasion we would like to avail ourselves of the opportunity to look back on the past two decades of our meetings. The total number of people who participated in the conferences has amounted to 1800, encompassing scientists and students from the majority of Polish universities, Institutes of Polish Academy of Sciences, and National Research Institutes. Around 35% comprised of scientists from other countries – mostly from the EU and other European countries such as Switzerland, Turkey, Russia, Belarus, and Ukraine. Among the participants were also scientists from Canada, USA, Brazil, Uruguay, Egypt, Australia, Japan, India, Pakistan, China, Iran, and Taiwan. The conferences have become a permanent fixture in plant stress physiology meeting calendar, the credit for which is due not only to the organisers but also to the many people who gave us support as well as valuable advice and suggestions throughout these years.

We would like to avail ourselves of this opportunity to express our heartfelt gratitude to Professor Alina Kacperska from the University of Warsaw, to whom – on the occasion of her 80^{th} birthday – we dedicate this year's conference. Professor Kacperska participated in all our previous conferences, giving plenary lectures and actively participating in discussions. The organisers benefited immensely from Professor Kacperska's advice and suggestions arising from her experience as well as scientific and organisational activity in Poland and other countries. Many currently active scientists, including a number of the participants of our conferences, have benefited from Professor Kacperska's scientific output and warm-

ly remember her kindness and support. We wish Professor Kacperska good health and all the best on her birthday and we hope to continue our cooperation.

We would also like to express our sincere gratitude and extend greetings to Professor Michael B. Jackson from the University of Bristol and Professor Karl Dörffling from the Institute of General Botany in Hamburg, who could not come to Cracow this year for personal reasons. We would like to thank Professors Zofia Starck, Angelika Filova, Marian Saniewski, Janusz Związek, and Edward Gwóźdź for their participation in the conference – they also actively participated in our past meetings, chaired sessions and gave plenary lectures. We also welcome Professor Helena Gawrońska, who has used her international contacts to enrich the conference programme with lectures by distinguished scientists from other countries.

We would also like to express our profound gratitude to His Magnificence, Rector Michał Śliwa and the authorities of the Pedagogical University of Cracow for their support in organising the conference and for making the university's venue available for the sessions.

On the occasion of the jubilee we would like to remember those participants of our conferences who are no longer among us, i.e. Professors; Mirosław Zima, Emil Nalborczyk, Adam Markowski, Tadeusz Baszyński, Agnieszka de Barbaro, Bronisław Gej, Włodzimierz Starzecki, and Marian Czarnowski. We are grateful to them for their participation in our conferences as well as for their scientific and organisational support.

Organizers would like to thank for the financial support and for providing some souvenirs to the Polish Botanical Society, Committee of Physiology, Genetics and Breeding Polish Academy of Sciences, Kraków Municipality, Polish Academy of Arts and Sciences, Ministry of Science and Higher Education, Ministry of Agriculture and Rural Development, Ministry of the Environment, Ministry of Culture and National Heritage, National Museum in Kraków and publishers: Springer „Biały Kruk", Social Publishing Institute "ZNAK", Scientific Papers Authors and Publishers Society UNIVERSITAS and sponsors: Enbio Technology Ltd, GEOMOR-TECHNIK Ltd. and PP Systems.

ALINA KACPERSKA – NESTOR OF RESEARCH ON PLANT ADAPTATION TO ABIOTIC STRESS CONDITIONS

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Alina Kacperska-Lewak, formerly Kacperska-Palacz, was born in Warsaw in 1934. She graduated from the Faculty of Biology and Earth Sciences, later Faculty of Biology, University of Warsaw; she earned the Master of Science degree in 1956, and went on to obtain the PhD degree at the Faculty of Biology in 1961. She became a habilitated doctor in plant physiology in 1971, then an associate professor in 1983, and a full professor in 1989, all the while working at the University of Warsaw. Only in the years 1956-1964 did she work at the Institute of Melioration and Grassland Improvement.

Prof. Alina Kacperska-Lewak fulfilled a number of manager functions at the University of Warsaw. Between 1983 and 2003 – almost until her retirement – Prof. Kacperska was the leader of the Department of Plant Resistance, which changed its name several times during that period. She retired in 2004 but did not cease active scientific work. Throughout her research and didactic work, she held a number of administrative positions at the Faculty of Biology, University of Warsaw, and at other institutions. Between 1981 and 1984 she was the deputy-dean, and between 1971 and 1993 – a member of the Scientific Council at the Institute of Botany, Faculty of Biology. During 1991-1994 she held a remarkably responsible position as a member of the commission assessing scientific research projects at the State Committee for Scientific Research (KBN), and from 2012 till now – as an Expert at the National Science Centre (NCN).

Equally significant tasks were entrusted to Prof. Kacperska in scientific societies. In the years 1981-1992 she held the position of vice-chairman and then chairman of the Section of Plant Physiology and Biochemistry in the Polish Botanical Society (PTB). Her innovative undertakings, which invigorated the activity of the Section, included the organization of seminars each month during the period of Martial Law in Poland. At that time, the access to information on current research developments and achievements was very limited. The seminars devoted to the question *What's new in plant physiology?* enjoyed a great popularity, assembling numerous participants from all over Poland. In recognition of her vital contribution to the Polish Botanical Society, Prof. Kacperska was given honorary membership of the Society in 2004.

Prof. Kacperska is also a member of Warsaw Scientific Society and an honorary member of the Polish Society of Experimental Plant Biology (PTBER), as well as a member of the Federation of European Societies of Plant Biology (FESPB), formerly the Federation of European Societies of Plant Physiology (FESPP). As a General Secretary she participated in organising the 3rd FESPP Congress to be held in Warsaw in 1981. The Congress was called off because of the Martial Law in Poland. The detailed programme and invitations for scientists to give plenary lectures were already prepared.

Until 1996 Prof. Kacperska was also a board member of the International Association for Plant Physiology (IAPP). Moreover, she was a member of several committees at the Polish Academy of Sciences and of editorial boards in such journals as *Physiologia Plantarum*, *Acta Physiologiae Plantarum*, *Acta Societatis Botanicorum Poloniae* and *Botanical News.* She also served as a referee for numerous scientific papers submitted for publication. Many authors of these papers know her as a very critical reviewer, who pointed out not only ambiguities or mistakes in the text, but also made suggestions for their correction and introduced very useful suggestions and new lines of thought into the discussion. I personally experienced such a kind and constructive review as author of the monograph *Physiological responses of plants to unfavourable environmental conditions* (Z. Starck et al., 1995), for which I am thankful till now. Her conscientiously crafted reviews have also been highly appreciated by various international journals.

Because of her great scientific personality, Prof. Kacperska was invited as a visiting professor and lecturer to countries ranging from France to China. She has also been frequently invited as a lecturer or Scientific Session Organizer to different Congresses and Conferences in European and other countries, such as USA or India. Her scientific output encompasses over 100 publications, written almost exclusively in English for journals listed in Thomson Reuters Master Journal List, with the number of citations amounting to 730 according to Research Gate. Moreover, Prof. Kacperska is the author of chapters in several acclaimed academic textbooks on plant physiology.

Prof. Kacperska has been the supervisor of nine PhD theses as well as the reviewer in seventeen habilitation procedures and in eleven professorship ones. In recognition of her invaluable scientific and organization work, Prof. Kacperska has been honoured with the Polonia Restituta Knight's and Officer's Crosses, as well as numerous awards granted by the Ministry of Education and university chancellors.

The most notable scientific achievements of Prof. Kacperska with her team can be summarized as follows. From the beginning of her scientific work, Prof. Kacperska's interests focused on the problems of plant adaptation to variable environmental conditions, especially to abiotic stresses, affecting plant growth and development. Her investigations particularly concerned effects of unfavourable temperature (chilling and frost) and water conditions (drought and flooding). The stress-induced plant metabolic responses were of special interest to her. She has also taken into consideration the roles of phytohormones (abscisic acid and ethylene) in plant stress responses. In later years she proposed the stress intensity-dependent mechanisms involved in environmental signals perception.

It should be mentioned that in the research conducted already in 1970s, Prof. Kacperska with her team connected the problem of plant resistance to chilling and frost with the role of proteins, which was far from the common knowledge at that time. Important changes in the electrophoretic pattern of proteins from the cold-treated plants were observed. Later on,

she showed that plant adjustment to variable temperature conditions, in particular to a drop in temperature, modifies energy metabolism in leaves. Modification in ATP level and an increase in the so-called reduction power of a tissue (an increase in the reduced pyridine nucleotide levels) were observed. The later observation corresponds well with the current research data showing an important role of the stress-induced changes in the redox potential of cells. Such a change may activate the alternative metabolic pathways, which was actually observed in the studies on carbon photosynthetic metabolism where a switch from the C_3 to the C_4 path was observed (studies in cooperation with prof. St. Maleszewski) and in the studies on the alternative respiratory pathway (conducted jointly with Prof. Anna Rychter). Prof. Kacperska also proposed that the stress-induced changes in tissue hydration may constitute signals triggering processes resulting in plant acclimation or in plant death. That issue was not fully understood at that time. The identification of the role of the cell wall in plant acclimation to low temperature is another achievement of Prof. Kacperska and her team: cold-induced changes in chemical and physical cell wall characteristics and structure seem to be important factors in plant acclimation to stress conditions. It is worth mentioning that for many years the cell wall had been treated as the "dead part of the cell". Prof. Kacperska and her team also indicated an important role of anthocyanins and phenylpropanoids in plant acclimation to low temperature.

In conclusion to this brief overview of Prof. Kacperska's scientific and organization achievements, greatest appreciation must be expressed for her rich and invaluable scientific achievements as a plant physiologist involved in research and didactic work for over 50 years. She is one of the first Polish scientists who started investigations on plant stress responses as early as the 70s of the 20th century.

Particular mention must be made of her cooperation, resulting in joint publications, with numerous scientists from various universities and institutes both from Poland and from other countries. Despite such an extensive and oftentimes burdensome duties, Prof. Kacperska always finds the time to help or to give advice in solving scientific problems and for consultations with students and co-workers about new and important problems in plant biology.

THE RESPONSES OF *HELIANTHUS ANNUUS* L. OF 28-HOMOBRASSINOLIDE DURING DROUGHT

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Abstract. Experiments studying an impact of brassinosteroids on the reaction of plants stressed e.g. by water deficit are different in various parameters. The influence of application of 28-homobrassinolide (HBR) was studied in young plants of sunflowers (*Helianthus annuus* L.) cv. BELINDA,cv. MAS 97 and cv. SPIROV grown in the greenhouse under optimum and water deficit conditions. The leaves of 32-d-old plants sunflowers were sprayed with (28-HBR) onto the leaves at 0.01 and 1 μM concentrations for 3 days with a 1-day interval. Three levels of drought stress (0, 3, and 5 days withholding water) were applied. Thereafter, the effects of brassinosteroids and water stress were investigated on some biochemical and antioxidative parameters of sunflower plants. Lipid peroxidation, and proline content increased in plants subjected to drought stress. Based on our results it seems that brassinosteroids considerably alleviated oxidative damage that occurred under drought stress. The differences between drought-stressed and well-watered plants, brassinosteroid-treated and -nontreated plants were analyzed. According to the obtained results, it seems that in young sunflowers plants treated with lower concentrations (0.01 and 1μM) of 28-homo, drought had less negative impact on the monitored parameters compared to plants normally watered - growth rate did not change, differences in photosynthetic parameters were smaller. However, the observed differences between HBR-treated and non-treated plants were usually not statistically significant. Proline is accumulated in many plant species in response to osmotic stress, which is stimulated by drought. Results have been shown in sunflowers plants under drought stress, in which a reduction of root weight was correlated to stress severity. Treatment with BR fully compensated for the reduction in biomass caused by mild drought stress. On the other hand, in creases in biomass was correlated with increases in acid inverters activity in young leaves, which likely provided more assimilates to the plant due to their larger sizes. Furthermore, osmotic stress resulted in considerable reduction in the protein contents in all the three varieties of sunflowers. However, BRs not only restored but also stimulated the level of protein and free proline. In leaves of experimental plants cultivars MAS 97 and SPIROV after water stress treatment the level of malondialdehyde (MDA) content has been increased on 11% and 30% respectively. The higher MDA content has been observed in leaves of cultivar BELINDA. The combination of drought and HBR has been shown MDA content in leaves of all experimental plants on control level which can evidence about protection effect of BRs under water deficit treatment on the leaves of experimental cultivars of sunflower plants. BR-regulated stress response as a result of a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction of protein synthesis, and the production of various

chemical defence compounds. BRs open up new approaches for plant resistance against hazardous environmental conditions.

Key words: 28-brassinolidesteroids; Drought stress; Chlorophylls; Helianthus annuus; MDA; Proline

Introduction

Drought stress is defined as a condition in which water available to plants is so low that it is unfavourable for the growth of a plant species (Zhu 2001; Egert and Tevini 2002). Plants will respond to conditions of drought stress through a number of physiological and developmental changes (Inze and Montagu 2000). Under stressful conditions, the stress factors, or the toxic molecules derived from the stress, attack the most sensitive molecules (primary targets) in cells to impair their function (Ingram and Bartels 1996). The damaged targets recover either by repair or replacement via de novo biosynthesis.

When the stress is too intense and severely damages target molecules, catastrophic cascades of events set in, leading to cell death. Cells could be protected either by the endogenous molecular systems or exogenous applied compounds that mitigate the stress (Ingram and Bartels 1996). Capacity of plants to detoxify reactive oxygen species has been related to the stress tolerance of plants (Apel and Hirt 2004).

Oxidative stress is a key underlying component of most abiotic stresses (Mittler 2002, Apel and Hirt 2004, Yin *et al*. 2008) and a major limiting factor to plant growth in the field (Scandalios 2002, Blokhina *et al*. 2003, Mittler 2006). Production of reactive oxygen species (ROS) and other radicals increases dramatically during abiotic stress conditions and oxidative stress occurs when the rate of ROS production outstrips the capacity of antioxidant systems to detoxify them (McCord 2000; Mittler 2002). The result is oxidative damage to key biomolecules such as proteins, lipids, and DNA, leading to cellular dysfunction and ultimately cell death (Wagner *et al*. 2004, Halliwell 2006, Baxter *et al*. 2007). To avoid oxidative damage, plants invoke a molecular response that allows them to cope with and adapt to the oxidative stress situation. Many stress-related genes have been identified and the list of antioxidant enzymes, hormones, and metabolites present in plants continues to be extended (Cao *et al*. 2005). One type of these compounds that have antioxidative characteristics is brassinosteroids (BRs) (Haubrick and Assmann 2006).

Brassinosteroids (BRs) are steroidal phytohormones with a wide scale of effects. BRs, which play an essential role in plant growth and development, have been implicated in many physiological response. By exposing plants to the drought stress as well as to the heavy metals their survival is improved and also resistance and yield are increased. The plant response is regulated directly (by the synthesis of metabolites) or indirectly (by induction of antioxidizing compounds and enzymes), often in the interaction with other phytohormones. BRs are common plant-produced compounds that can function as growth regulators (Bishopp *et al.* 2006). In addition, it has been suggested that BRs could be included in the category of phytohormones (Haubrick and Assmann 2006). Exogenous application of BRs may influence a range of diverse processes of growth and development in plants (Cao *et al*. 2005, Yu *et al*. 2004, Montoya *et al*. 2005). In addition, it is becoming clear that BRs interact both negatively and positively with other major signalling pathways including those regulated by auxin and ethylene (Amzallage and Vaiseman 2006, Haubrick *et al*. 2006). It is clear that BRs provide protection against a number of abiotic stresses (Krishna 2003).

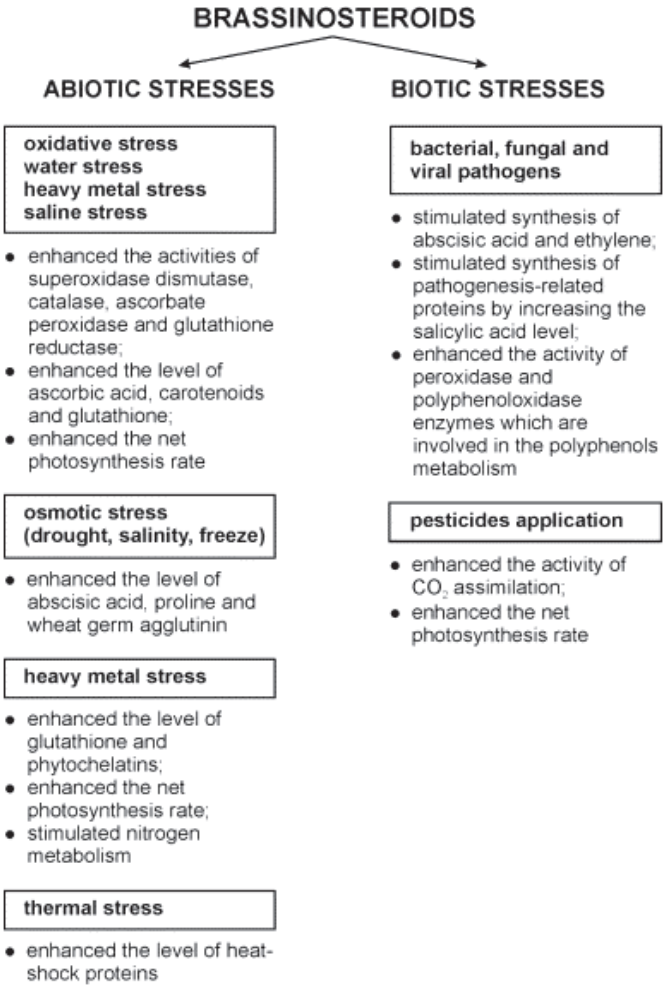

Figure 1: Effects of BRs on plants exposed or subject to different stresses. (Bajguz and Hayat 2009)

Treatment with BRs enhanced the growth of wheat (Sairam 2005), French bean (Upreti and Murti 2004), and sugar beet plants (Schilling 1991) under drought stress. Application of BRs improved tolerance against salt in rice (Anuradha and Rao, 2003; Özdemir *et al*. 2004), groundnut (Vardhini and Rao 2000), wheat (Sairam *et al*. 2005), and chickpea (Ali *et al*. 2007). It was reported that BL induced thermotolerance in tomato (Ogweno *et al*. 2008), rice (Wang and Zang 1993), and brome grass (Wilen *et al*. 1995). Moreover, BRs increased tolerance against high temperatures in *Brassica napus* (Singh and Shono 2005) and brome grass (Wilen *et al*. 1995). One mechanism that may be involved in the resistance to many types of stress is the increased activity of the antioxidant pathway. Several studies have shown that BRs alter the antioxidant capacity of plants under stress conditions

(Yardanova 2004, Yin *et al*. 2008). The present study was an attempt to carry out investigations of the effect of brassinolide on the water stress responses of sunflowers plants, with the aim to elucidate possible mechanisms that might be involved in the BR-promoted antioxidant responses to drought.

Material and methods

The purpose of our experiments was to consider sensitivity of the chosen 3 sunflower cultivars (*Helianthus annuus* L). cv. Belinda (FRA), cv. MAS 97(SVK) and cv. Spirov (BUL) to water stress with the possibility of elimination by means of brassinosteroids on the ground of physiological characteristics (content of dry weight, amount of chlorophylls, proline content, as well as MDA) and to show possible resistance mechanisms of this plant to drought. Sterilized seed were budding for 24 hours in the distilled water and consequently were sprouting on Petri dish (Ø 15 cm) with wetted filter paper in darkness. After 3 – 4 days of sprouting were approximately equally sprouted seeds sown into 15 l plastic bowls with a mixture of peat and perlite (in a ratio of 4:1), poured with distilled water which corresponds to maximum soil sorption capacity (~1000 ml). Plants were left to grow to the beginning phase of butonization – stars (32 day). The leaves of 32-d-old plants sunflowers were sprayed with (28-HBR) onto the leaves at 0.01 and 1 μM concentrations for 3 days with a 1-day interval. Three levels of drought stress (0, 3, and 5 days withholding water) were applied. Thereafter, the effects of brassinosteroids and water stress were investigated on some biochemical and antioxidative parameters of sunflower plants. The experiment was realized in three repetitions. The gained data was analysed by mathematical-statistical methods of programme MS Excel. Meaning of the differences by comparing the sets were determined by the Student test.

Hormone preparation

28-homobrassinolide (HBR) was purchased from Sigma-Aldrich, USA. Stock solution (10^{-4}M) was prepared by dissolving the hormone in 5 ml of ethanol in a 100 ml volumetric flask. Five millilitres of 0.5% surfactant "Tween-20" was added to it, and the final volume was made up to the mark by using double distilled water (DDW). 28-HBR was sprayed onto the leaves at 0.01 and 1 μM concentration for 3 days on alternate days. Three levels of water stress (0, 3, and 5 days withholding water) were applied. After treatment, the third leaves of plants were harvested, and samples were either rapidly dried in an oven at 80 °C to constant weight, which were used for determination of dry weight and further analyses, or were frozen in liquid nitrogen and stored at –80 °C for biochemical analysis.

Biochemical assays

The level of lipid peroxidation in plant tissues was measured by determination of MDA (Heath and Packer 1969) and others aldehydes' (Meirs et al. 1992) breakdown products of lipid peroxidation. MDA content was determined with a thiobarbituric acid (TBA) reaction. Briefly, a 0.2 g tissue sample was homogenised in 5 mL of 0.1% TCA. The homogenate

was centrifuged at 10,000 ×g for 5 min. To a 1 mL aliquot of the supernatant was added 4 mL of 20% TCA containing 0.5% TBA. The mixture was heated at 95 °C for 15 min and cooled immediately. The absorbance was measured at 532 nm. The value for the non-specific absorption at 600 nm was subtracted. The level of lipid peroxidation was expressed as μmol of MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1} and of others aldehydes formed the extinction coefficient was 0.457 × 105 M^{-1} cm^{-1}.

Proline was extracted and its concentration determined as described by Bates et al. (1973). Leaf tissues were homogenised with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 ×g for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined. Proline (Sigma) was used for the standard curve.

Chlorophyll and photosynthesis measurements

The chlorophyll content in fresh leaf samples was measured by using a SPAD chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Japan).

Results and discussion

Tested cultivars of sunflower showed according to the tested parameters, such as length and weight of sprouts and fresh weight of roots, relatively high rate of tolerance. In spite of the fact that no significant visual symptoms of water stress were markedly noticeable, we detected decrease in the content of dry weight of roots (less than 25 – 39 % in comparison with the control of two tested cultivars treated by drought cv. MAS 97 and cv. SPIROV). Cv. Belinda tends to be the most resistant or tolerant to water deficit from the point of view of evaluation of morphological parameters of particular cultivars.

BRs treatment completely compensated reduction of biomass caused by drought. In the comparison with the untreated plants (control and water stress) there was noticed an improvement of root growing in the treated plants. Yuan *et al*. (2010) and Zhang *et al.* (2008) observed that BRs application improved assimilation of carbon and nitrogen by the stabilization of membrane structures in the stressful conditions and also improved general growth and plant photosynthesis. Photosynthetic apparatus cv. MAS 97 and cv. SPIROV reacted the most sensitive to the dose of water deficit what expresses in the content reduction especially of chlorophyll a (by 41%), chlorophyll b (by 22%) and carotenoids (by 29%). Negative impact on the photosynthetic apparatus efficiency is one of the typical signs of the effect of various kinds of abiotic stress such as drought, high temperature, and also heavy metals. Brassinosteroids induced an improvement in photosynthesis efficiency which can be caused by stomatal or non-stomatal factors or by their combination. Influence of BRs on a conductivity of stomas was noticed by Hayat et al. (2010) and Fariduddin et al. (2009). Non-stomatal efficiency limitations of photosynthesis can be related with the photosynthetic pigments, concentration and activity of enzyme Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) and use of assimilative products. Yuan et al. (2010) consider as an acceptable explanation that exogenous application of BRs is increasing the capacity of carbon oxide assimilation in Calvin cycle using increased initial activity of RuBisCo. In the following experiments the BRs treatment reduced the decreasing of chlorophyll content and assimilation speed, increased efficiency of photosystem II and activity of enzymes, such as

RuBisCo, nitroreductase and glutamine synthesis (Li et al. 2010). Carotenoids are pigments protecting chlorophylls from the photo-oxidative damage (Yu et al. 2004). Their content is decreasing with the increasing level of the oxidative stress caused by the water stress in all cultivars of sunflower and is significantly higher in the plants treated by BRs, in the comparison with the controlled ones.

According to the measurement of MDA level it is likely that BRs help the effective catching of ROS by increased activity of the antioxidative enzymes system. Production of MDA and other aldehydes in the plants exposed to the water stress is a dependable indicator of the production of free radicals in the plant tissue and today it serves as the indicator of lipid peroxidation and meanwhile of stress damage, such as water stress at the cell level (Heidari 2010). Considering that drought stress results in increases in reactive oxygen species (Apel and Hirt 2004, Gong *et al*. 2006), peroxidation of lipid membranes is both a reflection and measure of stress-induced damage at the cellular level (Meirs et al. 1992, Borsanio et al. 2001). In the present study the content of MDA, other aldehydes increased significantly as drought progressed in plants (Figures 2). It was previously found that the MDA content increased in 3 genotypes of wheat under drought stress (Sgherri et al. 2000). The cultivars resistant to the drought are able to catch the free radicals and less MDA is produced with the decreasing content of hydrogen peroxide in a comparison with the sensitive cultivars (Divi and Krishna 2009). On the basis of gained data we can claim MAS 97 and SPIROV belong to the sensitive cultivars of sunflower as they produced increased amount of MDA (more than 11 – 30% in a comparison with the control). On the second hand, among tolerant to water stress sunflower cultivars belong BELINDA. Stated cultivar produced lower part of MDA in a comparison with the controlled variant. In the present study, drought stress decreased the peroxidase activity in 5-day drought stressed plants. 28-HBR application positively influenced MDA activity in control and stressed plants. The highest activity was observed in control and 5-day drought stressed plants. The activity of enzyme was 2-fold higher in those plants treated with 1 μM BR when compared with BR-free plants . In 5-day drought stress conditions, the activity of MDA significantly increased. Exogenous application of EBL in drought stressed plants enhanced the activity of this enzyme at both levels of 28-HBR concentration.

With the drought + BRs combination we noticed decreased MDA content in the leaves of all common sunflower cultivars, what indicates possible elimination of water stress in the sunflower plants using the exogenous application of brassinosteroids. Plants for treatment in water stress showed significant reduction in all measured growth parameters (i.e., length, fresh and dry mass of root and shoot, and leaf area) irrespective of varietal difference. However, treatment with HBR to the foliage favored growth and neutralized the negative effects generated by water stress more effectively in all cultivars. Significant reduction in photosynthetic parameters occurred from water deficit, net photosynthetic rate (PN), and relative water capacity (RWC) of three cultivars.

The content of proline progressively increased in plants as the drought levels increased (Figure 2).

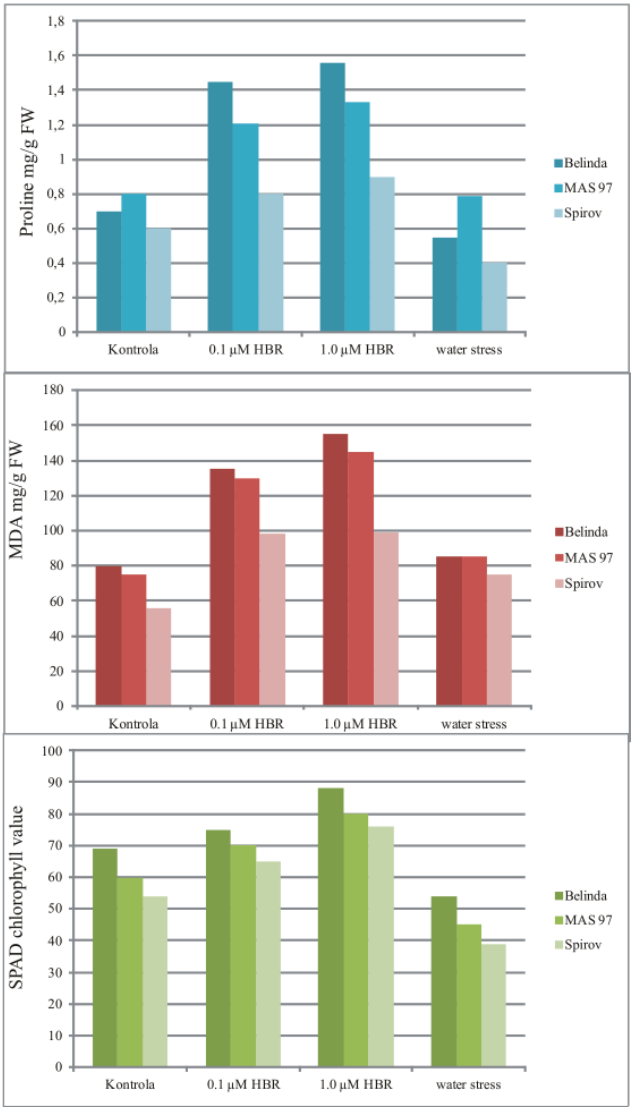

Figure 2: Effects of BR and drought stress on MDA, proline and SPAD chlorophyll value of Helianthus annuus L. cv. BELINDA, cv. MAS 97 and cv. SPIROV. Plants were grown for 1 month under controlled conditions and were divided into 4 groups. One group was control sprayed with distilled water, two groups were pretreted with HBR and other group was exposed to water stress for 3 and 5 days.

In those plants that were either under drought stress or 28-HBR treatments increases in the content of proline were observed. It has been reported that BRtreatments induced expressing of biosynthetic genes of proline (Özdemir et al. 2004).

There are also reports that show that application of BRs (28-HBL and 24-EBL) increased proline content in sorghum plants exposed to osmotic stress (Vardhini and Rao 2003). All varieties showed significantly different responses to the spray 0.1 and 1μM HBR treatments. Enzyme activity increased in response to water stress and hormone treatment in three cultivars, but SPIROV, showed higher enzymatic activity than did BELINDA and MAS 97 in response to the treatments. Our data showed that proline was accumulated in BRtreated plants under stress conditions. Therefore, a role of BR in the accumulation of proline as an important component of protective reactions in sunflower plants in response to drought stress is possible. The foliar spray of either with 28-homoBL significantly enhanced the growth, photosynthesis, antioxidant enzymes and proline content in water deficit stressed sunflower plants.

The exogenous application of plant hormones has been found to counter toxic effects of various abiotic stresses. Brassinosteroids (BRs) are a new type of phytohormones that elicit a wide range of physiological processes in plants (Bajguz and Hayat 2009, Behnamnia 2009, Hayat 2010). At present, 70 analogs have been identified, and among these, three natural brassinosteroids (brassinolide, 24-epibrassinolide (EBL), and 28-homobrassinolide (HBL)) are known to have an economic impact on plant metabolism, growth and productivity, and experience high stability under field conditions (Ali 2007).

Conclusions

Many aspects of the drought efficiency on the plants are clarified, however results of several physiological and biochemical analysis are controversial. At the same time, high variability of plant reaction to the water stress depending up the genotypes complicates unambiguity of the conclusions. Deeper biochemical and molecular-biological analysis can contribute to revealing of other possible mechanisms of sunflower resistance to drought. In conclusion, our results may show that the leaves of sunflower plants subjected to drought stress can endure moderate drought stress because of small changes in enzyme activities. From this result it could be concluded that the of sunflower plants cv. BELINDA is drought tolerant because a did not gradual loss in antioxidant protection in the leaves of plants under drought stress was observed. Pretreatment with BR can ameliorate the adverse effects of drought stress via decreasing the oxidative damage of plant membranes, possibly by the induction of compatible solute for osmotic adjustment and the induction of antioxidant defence system in sunflowers. Finally, the results hint that BR may in future find application for improving plant growth and yield in dry areas. Wide scale of BRs effects on the plants stressed by drought instigates to search for mechanisms and connections which disproves old concepts and motivates development of new methods.

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UP-REGULATION OF PEPPER GENES ENCODING *WRKY* TRANSCRIPTION FACTORS BY SODIUM SALICYLATE TREATMENTS

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Abstract. Plant defense hormones and transcription factor proteins play critical roles in the regulation of defense responses in virus-infected plants. To further explore the complex interactions between plant defense hormones and transcription factors, we studied the effects of exogenously applied sodium salicylate on the expression of four genes encoding WRKY transcription factors in pepper (Capsicum annuum L.) leaves. We identified two novel WRKY genes from the database of a recently completed pepper genome-sequencing project, and these genes were compared with two already known WRKY genes. The induction of the individual pepper WRKY genes by sodium salicylate widely varied and occurred in a dose-dependent manner. The greatest rate of up-regulation was observed for WRKY genes belonging to group III of WRKY transcription factors. Using in silico analysis we identified several hormone-related cis-regulatory elements in the promoter regions of the four WRKY genes. No clear correlation was found between the inducibility of WRKY genes by sodium-salicylate and the occurrence of hormone-related cis-regulatory elements in their promoters.

Key words: pepper, promoter motif, salicylic acid, WRKY transcription factor

List of abbreviations

HR, hypersensitive response; NaSA, sodium salicylate; NCBI, National Center for Biotechnology Information; R-protein, resistance protein; RT-PCR, reverse transcription - polymerase chain reaction; SA, salicylic acid; TMV, *Tobacco mosaic virus*.

Introduction

In plants, resistance to pathogenic virus infection is determined by the timely recognition of the invading virus by intracellular resistance (R) proteins and via rapid deployment of effective defense reactions (Padmanabhan and Dinesh-Kumar 2014). Following the recognition of a viral pathogen, R proteins can activate several downstream signaling cas-

cades in resistant plants. These signals are transmitted to the nucleus and this leads to a rapid and extensive reprogramming of gene expression patterns in affected host plant cells. As a result of these signaling processes, hundreds of plant genes are activated including important defense genes (Whitham et al. 2006). It is well established that transcriptional reprogramming is regulated by a complex, multilayered network where various transcription factors (Li et al. 2004, Gatz 2013) and defense-related plant hormones (Alazem and Lin 2015) play critical roles. In plants, virus resistance is often associated with programmed cell death (hypersensitive response; HR), although the development of necrotic lesions and disease severity do not necessarily correlate (Bendahmane et al. 1999, Király and Király 2006).

The regulation of gene transcription is largely mediated by nuclear transcription factor proteins (DNA binding proteins) that can bind to specific nucleotide motifs located within the promoter region of target genes. In this way, specific transcription factors can up- or down-regulate a large number of genes simultaneously (Li et al. 2004). Transcription factors that are rapidly activated or *de novo* synthesized following a virus infection in resistant plants likely play a critical role in virus resistance. Promising research targets are the plant-specific WRKY transcription factors due to their involvement in plant defense reactions against various pathogenic microorganisms including viruses (Chen and Chen 2000, Eulgem et al. 2000, van Verk et al. 2008, Rushton et al. 2010). WRKY transcription factors are encoded by large multigene families. WRKY protein sequences contain very characteristic and highly conserved sequence motifs: Trp-Arg-Lys-Tyr (WRKY) element followed by Cys_2-His_2 or Cys_2-HisCys zinc finger motifs. WRKY proteins have been categorized into three classes according to the number of WRKY domains and type of zinc finger motifs present (Eulgem et al. 2000). WRKY proteins are most often localized in the cell nucleus (Rushton et al. 2010) and specifically recognize and bind to the W-box nucleotide motif [canonical sequence: (C/T)TGAC(C/T)] within the promoter sequences of target genes (Eulgem et al. 2000). In contrast to bacterial and fungal pathogens, little is known about the role and significance of WRKY transcription factors in virus-infected plants (Chen et al. 2013). In one interesting study, transgenic *Nicotiana benthamiana* overexpressing a *WRKY* gene derived from cotton, exhibited elevated virus resistance (Sun et al. 2012).

The plant hormone salicylic acid (SA; 2-hydroxybenzoic acid) is an important signal in plant defense reactions (Vlot et al. 2009). Substantial biosynthesis and accumulation of endogenous SA was observed in plant foliage following virus inoculation (Malamy et al. 1990, Métraux et al. 1990). Exogenously applied SA effectively induced the expression of several genes that encode a suite of pathogenesis-related (PR) proteins (Ward et al. 1991). SA also up-regulated the expression of various genes encoding antioxidative enzymes, particularly glutathione S-transferases (Fodor et al. 1997, Sappl et al. 2004). In pepper leaves, the expression of various lipoxygenase genes was induced by sodium salicylate (NaSA) treatment (Juhász et al. 2015). SA application strongly activated the expression of numerous *WRKY* genes (Chen and Chen 2000, Li et al. 2004, Park et al. 2006, Zheng et al. 2011). In *Arabidopsis thaliana*, exogenous SA treatment markedly induced the expression of *WRKY* genes that belong to the group III of WRKY transcription factors (Kalde et al. 2003).

To further explore the complex interplay between hormones and transcription factors during anti-virus defense reactions, we investigated the effect of exogenously applied NaSA on the transcription levels of several pepper genes encoding WRKY transcription factors. In addition, we identified the presence of hormone-related *cis*-regulatory nucleotide motifs in the promoter regions of these pepper *WRKY* genes.

Materials and methods

Pepper variety and hormone treatments

Pepper (*Capsicum annuum* L.) cultivar TL 1791 containing the L^3 resistance gene (Tomita et al. 2011) was used for all experiments. The plants were grown under greenhouse conditions as described earlier (Rys et al. 2014) and 55-60 day old plants were used for all experiments. Three middle leaves were treated with 1 or 5 mM aqueous solutions of NaSA by gently brushing the foliage. Application of distilled water was used as the control treatment. Treated and control plants were incubated at 22 °C in a controlled-environment growth chamber, and leaf samples were harvested for total RNA extractions at different time intervals following treatment.

RNA extraction and gene expression analysis by RT-PCR

Reverse transcription - polymerase chain reaction (RT-PCR) was undertaken to determine the expression pattern of pepper *WRKY* genes following NaSA treatment. Total RNA was extracted from 1 or 5 mM NaSA-treated and control leaves ground under liquid nitrogen using the Total RNA Miniprep kit (Viogene, Sunnyvale, CA, USA). Reverse transcription (RT) of 2.5 μg total RNA was carried out with a RevertAid H Minus First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) using an oligo(dT) primer. Semi-quantitative PCR for assaying gene expression levels was conducted with a PTC 200 DNA Engine extended with an ALS-1296 sample holder (Bio-Rad, Hercules, CA, USA). The PCR reaction mixtures contained 4 pmol of each primer, 0.5 U of Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 0.2 mM of each dNTP, 2 mM $MgCl_2$ and 1 μl of template cDNA in a total volume of 20 μl. All oligonucleotide primer pairs used in our studies are shown in Table 1. The PCR amplification began with 2 min denaturation at 94 °C, followed by 22-25 cycles of 30 s at 94 °C, 45 s at specific annealing temperatures (see Table 1 for each primer pair), 45 s at 72 °C and terminated by 10 min extension at 72 °C. Relatively low cycle numbers were used to maintain initial differences in target transcript amounts (semi-quantitative conditions). Expression of a pepper actin (housekeeping) gene served as the constitutive control (Table 1). The amplified PCR products were separated using gel electrophoresis in 1% agarose gels and visualized with GelRed nucleic acid gel stain (Biotium Inc., Hayward, CA, USA).

Bioinformatics

WRKY sequences for this study were obtained from the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Two *WRKY* genes were identified in the database of a recent pepper sequencing project (http://peppersequence.genomics.cn/page/species/index.jsp) (Qin et al. 2014). Database

searches were performed by using the BLAST network service (Altschul et al. 1997). Sequence alignments and comparisons were performed using ClustalW and Muscle programs (http://www.ebi.ac.uk/Tools/msa). The phylogenetic tree was generated by the ClustalW2 Phylogeny software (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and visualized with the MEGA software version 6.06 (Tamura et al. 2013). Promoter sequences of *WRKY* genes were identified in genomic DNA sequences found by conducting nucleotide BLAST searches of the NCBI pepper whole-genome shotgun contig databases (Kim et al. 2014; Qin et al. 2014). For *in silico* promoter analyses, 1500 bp DNA segments upstream of the ATG start codon were selected. Searches for *cis*-acting promoter elements were carried out using the PlantCARE (http://bioinformatics.psb.ugent.be /webtools /plantcare/html/) database (Lescot et al. 2002). The schematic diagram of promoter elements was prepared using the Illustrator for Biological Sequences (IBS) software (Liu et al. 2015).

Results and Discussion

Recently the entire *Capsicum annuum* genome was sequenced by two separate research teams (Kim et al. 2014; Qin et al. 2014) and 73 putative *WRKY* genes were identified (Kim et al. 2014). However, most of these *WRKY* genes have not yet been annotated and characterized. At present, 14 annotated *WRKY* sequences are available in the NCBI GenBank database and these sequences can be classified into three groups according to Eulgem et al. (2000) (Fig. 1). We identified two novel pepper *WRKY* genes in the genomic database published by Qin et al. (2014). These as yet uncharacterized genes are designated as *Capana08g001044* and *Capana10g001548* (Qin et al. 2014). We investigated the up-regulation of these two genes by NaSA and compared their inducibility to the two previously identified pepper *WRKY* genes, *WRKYb* and *WRKY30* (GenBank accession number AY743433 and FJ360844, respectively). Both *WRKYb* and *WRKY30* were up-regulated during the incompatible pepper - *Tobacco mosaic virus* (TMV) interaction or by exogenously applied SA (Lim et al. 2011; Zheng et al. 2011). *WRKYb* is classified into the subgroup II-c of the *WRKY* gene family (Lim et al. 2011) while the other three genes used for our investigations (*WRKY30*, *Capana08g001044* and *Capana10g001548*) belong to group III (Zheng et al. 2011) (Fig. 1).

In this study, we compared the induction of four distinct pepper *WRKY* genes following the exogenous application of NaSA. Treatment of pepper leaves with 1 or 5 mM NaSA did not produce any visible effect on the foliage. The transcript mRNA quantity of *WRKY* genes was monitored via the RT-PCR. The expression of all four *WRKY* genes was up-regulated after NaSA treatment, but the timing and magnitude of gene activation widely varied (Fig. 2). The 5 mM NaSA treatment exhibited slightly stronger *WRKY* gene expression as compared to the 1 mM NaSA treatment. As a constitutive control, expression of a pepper actin gene was determined (Table 1). The expression of actin was not significantly up-regulated by the NaSA treatments (data not shown). Our results confirmed earlier results of the up-regulation of *WRKYb* and *WRKY30* genes by SA (Lim et al. 2011; Zheng et al. 2011).

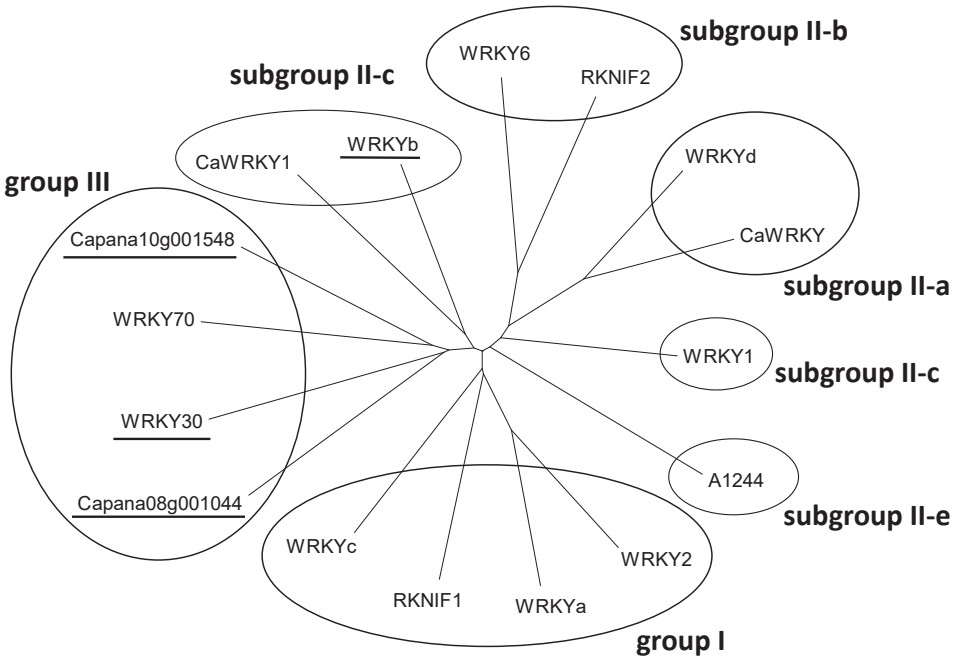

Figure 1. Dendogram of 16 pepper WRKY proteins. Fourteen protein sequences were available in the NCBI GenBank database: CaWRKY1 (GenBank accession number AAO86686), WRKY1 (ABP24358), WRKY2 (ABD65255), WRKY6 (AHD24521), WRKY30 (ACJ04728), WRKY70 (AHA15410), WRKYa (AAR26657), WRKYb (AAW66459), WRKYc (AAW67002), WRKYd (ADD62692), CaWRKY (AAX20040), RKNIF1 (ABA56495), RKNIF2 (ACT80136), and A1244 (AAZ99027). Two novel sequences (Capana08g001044 and Capana10g001548) were identified in the databank of a recent pepper genome project (Qin et al. 2014). The classification of WRKY proteins was made according to Eulgem et al. (2000). The WRKYs studied in the present work are underlined.

In our experiments, the up-regulation of *WRKYb* was very weak, but *WRKY30* showed a marked induction, with transcripts appearing as early as 2 hours following treatment (Fig. 2). The newly identified *Capana08g001044* and *Capana10g001548 WRKY* genes were also strongly induced by NaSA treatment. Among the four *WRKY* genes investigated in this study, *Capana10g001548* was the most substantially activated by NaSA, and the induction occurred 2 hours after treatment (Fig. 2).

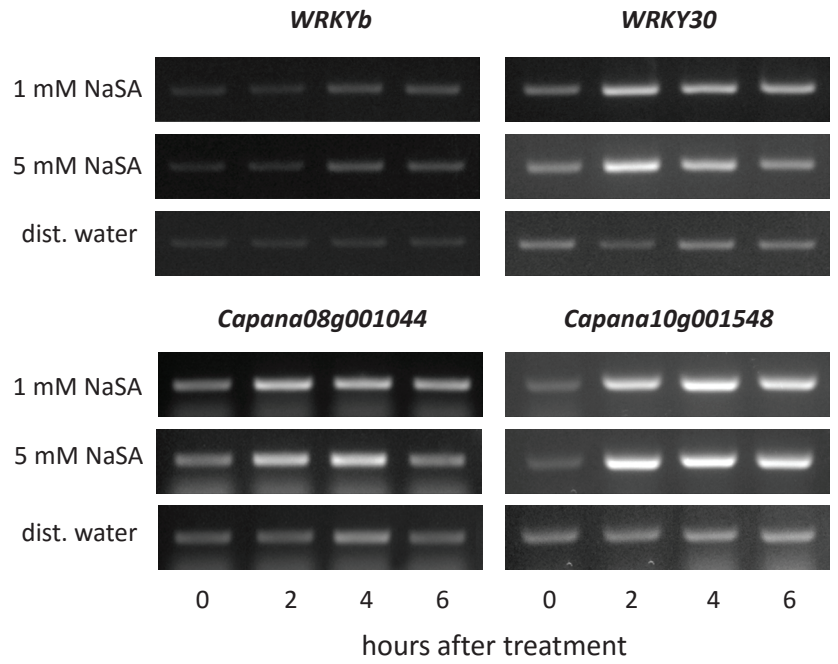

Figure 2. Up-regulation of four WRKY genes in pepper leaves treated with 1 and 5 mM aqueous solutions of sodium salicylate. Semiquantitative RT-PCR was used to detect the expression levels with gene-specific primer pairs (see Table 1). Leaves treated with distilled water were used as controls. Representative results of three independent parallel experiments are shown.

In accordance with the results of Kalde et al. (2003), group III *WRKY* genes (*WRKY30*, *Capana08g001044* and *Capana10g001548*) all exhibited strong inducibility by NaSA treatment (Fig. 2). Although rapid and robust activation of defense responses is necessary for disease resistance, the induction of *WRKY* genes by salicylic acid or by pathogen infection does not necessarily correlate with resistance (Kalde et al. 2003).

To reveal possible connections between the regulation of *WRKY* genes by SA and the occurrence of hormone-related *cis*-regulatory nucleotide elements in their promoters, we conducted *in silico* searches using PlantCARE software (Lescot et al. 2002). This analysis was undertaken to identify regulatory elements that might reside in the 1500 bp nucleotide-segment upstream of the translation start sites in the genomic DNA encoding *WRKY* genes. Promoter sequences were obtained from the published NCBI database of pepper genome data (Kim et al. 2014; Qin et al. 2014). Promoter sequences of *WRKYb*, *WRKY30*, *Capana08g001044* and *Capana10g001548* were identified in genomic DNA sequences with NCBI accession numbers ASJV01094297, ASJU01012274, ASJV01012437 and ASJU01076378, respectively. Upon analysis, the promoter region of all four *WRKY* genes was found to contain several nucleotide segments homologous to well-known *cis*-acting regulatory elements that are involved in the hormonal regulation of defense genes (Fig. 3).

Table 1. Primers used for PCR assays in 5′ to 3′ direction

Target gene (GenBank accession)	Forward primer	Reverse primer	Product length (bp)	Annealing temperature (°C)
WRKY-b (AY743433)	aaggcataaatacactgggt	agcagcgttgtttttagttc	176	59
WRKY-30 (FJ360844)	agtagttcggatgttatcacc	gaaatttcagccgttgacata	151	59
Capana08g001044	tcctcatttgtcggagattgg	caaacatggattcatcgacgtt	239	56
Capana10g001548	ggcaataatcatcaccagttt	ggggtcctaatccaagcaata	198	54
actin (AY572427)	agcaactgggacgatatggagaaga	aagagacaacaccgcctgaatagca	198	55

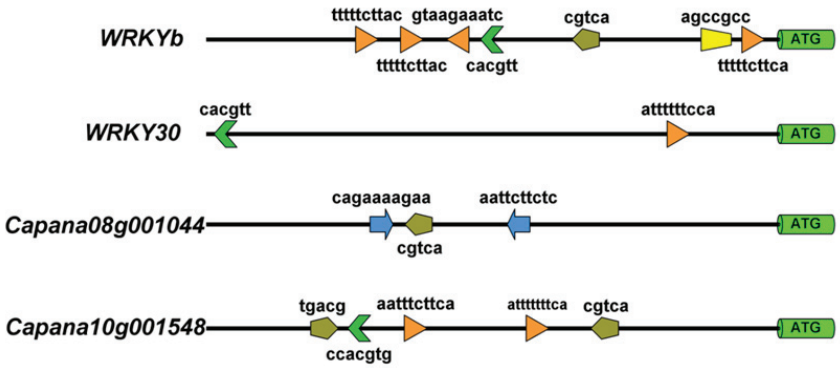

Figure 3. Schematic representation of hormone-related putative cis-acting regulatory elements in promoter sequences of four pepper WRKY genes. For in silico analyses 1500 bp DNA segments upstream of the ATG start codon were selected. The following symbols were used to highlight various promoter elements: yellow trapezoids: ethylene responsive elements (EREs); brown triangles: TC-rich repeats involved in defense and stress responsiveness; olive pentagons: as-1-like elements; green arrowheads: G-box and G-box-like motifs; blue arrows: salicylic acid-responsive TCA-like elements. Promoter motifs were found on both DNA strands, which is represented by the orientation of symbols. The diagram was prepared by the Illustrator for Biological Sequences (IBS) software (Liu et al. 2015).

Two SA-responsive 10 bp TCA-type motifs (CAGAAAAGAA and GAGAAGAATT) (Goldsbrough et al. 1993; Pastuglia et al. 1997) were identified only in the 5' upstream region of *Capana08g001044* (Fig. 3). The presence of these TCA-type motifs in the promoter region may be related to our observation of early and strong induction of *Capana08g001044* by NaSA. Two *as-1*-like elements (TGACG) known to be correlated to salicylic acid (Strompen et al. 1998) and jasmonate-responsiveness (Rouster et al. 1997) were identified in the promoter region of *Capana10g001548* (Fig. 3). The presence of these *as-1*-like motifs in the *Capana10g001548* promoter may contribute to the very strong activation of this gene by NaSA in our experiments. In the promoter region of both *WRKYb* and *Capana08g001044*, one copy of the *as-1*-like element was observed (Fig. 3). One ethylene-responsive element, the GCC-box (AGCCGCC) (Ohme-Takagi et al. 2000), was found in the promoter region of the *WRKYb* gene, but the promoter regions of three remaining pepper *WRKY* genes did not contain any GCC-box (Fig. 3). However, a G-box motif (CACGTGG) (Kim et al. 1992) was identified within the 1500 bp 5' upstream DNA region of the *Capana10g001548* gene. G-box-like segments (AACGTG) were also identified in the promoters of *WRKYb* and *WRKY30* (Fig. 3). G-box type motifs have been associated with jasmonate inducibility (Memelink 2009), but other hormones also appear to activate this region (Menkens et al. 1995). In addition, 1-4 copies of TC-rich repeats that are involved in defense and stress responsiveness (Klotz ad Lagrimini 1996) were found in the promoter regions of *WRKYb*, *WRKY30* and *Capana10g001548* genes (Fig. 3). At this time, no clear correlation was found between the inducibility of *WRKY* genes by NaSA and the occurrence of hormone-related cis-regulatory elements in their promoters. Likely the interaction of multiple cis-elements determines the expression rate of *WRKY* genes. The short nucleotide motifs identified in the present study as putative *cis*-regulatory promoter elements can occur randomly across the entire genome, therefore further experiments are necessary to determine the regulatory role of these sequences.

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INCREASING TOLERANCE TO ABIOTIC STRESSES BY HAPLODIPLOIDIZATION IN *HORDEUM VULGARE* (L.)

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Abstract. The main benefit coming from androgenesis implementation into breeding program is instant production of totally homozygous, doubled haploid (DH) lines. Other beneficial effect is enhanced gene expression for complex traits and increased genotypic and phenotypic variation within offspring population as compared to parental genotypes. In presented study, this technology has been harnessed to improve combined tolerance of winter barley *(Hordeum vulgare* L.) to both freezing and drought stresses. Optimization of the anther culture protocol resulted in effective production of DH offspring. Received progeny was analyzed in respect of freezing and drought tolerance with the use of chlorophyll a fluorescence and an excised leaf water loss assays. Presented results confirmed the assumption that haplodiploidization through androgenesis can increase the genetic variation within the DHs offspring and can be used as a biotechnological tool for breeding progress acceleration. The fact that produced population of DH lines characterizes wide variation for drought and freeze tolerance, makes it an interesting model to study the mechanisms of control and regulation determining tolerance to these stress factors, and a valuable genetic resource for breeding programs.

Key words: androgenesis, barley, drought, freeze, genotypic variation, stress tolerance

Introduction

Plants have evolved various adaptive strategies to deal with abiotic stresses, however during domestication and breeding, in many cases, stress tolerance/resistance has been sacrificed for high yield potential. Now, a big number of economically important crop plants with reduced tolerance against unfavourable environmental conditions has to face a threat posed by changing climate and human impact.

Barley (*Hordeum vulgare* L.) belongs to the most important cereal grains, due to its wide utilization in the malting and brewing industry, for animal feed and human consumption. It has a long history as one of the first domesticated crops originated in Middle East, where it has been cultivated for more than 10,000 years. Today, it is cultivated on 8% of

global grain acreage and its production amounted to approximately 140 million metric tons (*www.statista.com*). However, due to weak winter hardiness (*www.coboru.pl)*, it can be cultivated only at regions with mild winter climate. Cold winter with large temperature fluctuations results in plants damage and eventual plants death. Moreover, although due to high water use efficiency, barley is well adapted to drought at juvenile stages, water deficiency at the generative stage can severely reduce grain yield. That's why the identification of factors that determine drought and freeze tolerance is very important for this crop. Difficulties encountered in research originated from the fact that freezing and drought tolerance are complex and polygenic traits with strong interactions between loci and strong genotype × environment interactions (Busconi et al. 2001, Ceccarelli 2010).

The presented results have been received in the frame of the studies focused on identification of the mechanisms that determine the combined tolerance to both freezing temperatures and drought, which limit winter barley cultivation area and yielding. The first goal was the selection of genotypes extremely different in their tolerance level to both stress factors. It seems highly feasible as physiological background of both stresses is the same, altered water status. Cell water deficit is an obvious cause of all cell damages in drought conditions, whereas in the case of freezing, the formation of ice crystals in the extracellular space dehydrates the cell and disrupts tissue structure (Verslues et al. 2006).

To enhance the actual level of genetic variation existing among genotypes, androgenesis - one of the methods for doubled haploids (DHs) production has been harnessed. It allows for instant production of totally homozygous lines, as DH plants are received by an induction of embryogenesis in immature, haploid cells of male gametophyte (microspores) followed by spontaneous or chemically-induced genome diploidization. Produced population of DH offspring could exploit the whole inherent genetic diversity and potential which is revealed due to the lack of dominance effect, and through initiation of desirable pleiotropic or epistatic interactions (Humphreys et al. 2007). Moreover production of totally homozygous plants in one generation is an additional benefit, speeding up the breeding process and making phenotyping and genotyping more precise (Dwivedi et al. 2015).

Material and methods

Plant material and growth conditions

The plant material consisted of 75 genotypes and F1 crosses of winter barley (*Hordeum vulgare* L.) kindly provided by the Polish (DANKO Hodowla Roślin Sp. z o.o., *Hodowla Roślin Strzelce* Sp. z o. o.) and foreign breeding companies (Secobra Saatzucht GmbH, Saaten-Union GmbH).

Androgenesis induction and anther culture

Anther culture method used in the study was based on the procedures described by Jacquard et al. (2003) and Cistué et al. (2003) with several modifications. Shortly, for androgenesis induction the collected tillers were cold treated for 3-9 days at 4°C. Isolated anthers were pre-cultured on PreMn medium (Cistué et al. 2003) for 3-5 days at 26°C, in the dark. Then, the anthers were transferred to the induction C3 medium (Jacquard at al. 2003) and

kept at the same culture conditions. Androgenic structures (AS) were transferred to M1 regeneration medium (Jacquard et al. 2003) and cultured at 26°C and 16h/8h (day/night) photoperiod. Green regenerants were transferred to the rooting 190-2 medium (Zhuang and Xu 1983). Plantlets with well-developed roots were planted into soil and grown under controlled conditions (at 25°C and 9/15h(day/night) photoperiod) in a greenhouse.

The effectiveness of androgenesis was expressed by the number of green regenerated plants per spike of a donor plant (GR/SP) and the number of albinotic regenerated plants per spike of a donor plant (AR/SP).

Flow cytometry analysis

Ploidy level of regenerants was evaluated by flow cytometry. Leaf samples (10 mg) were placed in modified PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 2.0 g EDTA, 0.5 % BSA, pH 7.0), chopped with a razor blade and filtered through a 30 μm nylon mesh filter (Miltenyi Biotec GmbH, Germany). Suspension of the nuclei was stained with 2 % propidium iodide (PI) solution and analysed with the use of a MACS Quant flow cytometer (Miltenyi Biotec GmbH, Germany) equipped with air-cooled laser (488 nm) and MACSQuantifyTM software. Barley plants derived from the seeds of known diploid DNA content were used as a control.

Drought tolerance assay

Drought tolerance was estimated by the excised leaf water loss assay as proposed by Clarke and McCaig (1982), performed at the 3-4 leaf stage of DH seedlings growth. For analyses, the 100 high-quality DHs were pre-selected. Leaf samples were weighted three times: immediately after sampling (fresh weight; A), then incubated in a hybridization oven at 30°C for 2 h (B), and incubated again at 70°C for 48 h (dry weight; C). The water loss in excised leaves was calculated from the following formula:

$$\%H_2O/h = [((A-B))/((A-C)) \times 100\%]/2$$

Freezing tolerance assay

Pre-selected population of DHs was evaluated in respect of freezing tolerance with the use of procedure described by Rapacz et al. (2011). Leaf samples were collected from DH plants after 20 days of cold hardening at 4/2°C and 9/15h (day/night) photoperiod. The leaves were packed into polyethylene bags and put into the freezing chamber at 2°C. Then, the temperature was progressively reduced to -15°C at a rate of - 2°C/h. After 8 h of freezing and 30 min of dark-adaptation, the parameters of chlorophyll fluorescence were measured using a Handy-PEA fluorometer (Hansatech Ltd. Kings Lynn, UK). Fluorescence measurements were taken in 10 replicates (leaves).

Several parameters of photochemical efficiency were calculated: ABS/CS, ABS/RC, TR_0/CS, TR_0/RC, ET_0/CS, ET_0/RC, DI_0/CS, DI_0/RC, F_v/F_m, ψ_0, φ_{E0}, OEC, PI_{ABS}, PI_{CSm}, PI_{CS0}, RC/CS_0, RC/CS_m where: ABS - absorbed energy flux; CS - leaf cross-section, RC - the single, active PSII reaction centre, TR_0 - trapped energy flux in PSII reaction centres, ET_0 - the energy flux for electron transport, DI_0 - dissipation of energy in PSII reaction

centres, F_v/F_m - the yield of the energy trapping in PSII, ψ_0 - the quantum yield of the electron transport, φ_{E0} - the quantum yield of photochemical reactions, OEC – the yield of oxygen evolving complex, PI – performance indexes of PSII.

Statistical analysis

All data were analysed by one-factor analysis of variance (ANOVA) followed by post-hoc comparison (Duncan's multiple range test , $p \leq 0.05$). with the use of Statistica 10 software (StatSoft. Inc.).

Results and discusion

Combined and optimized anther culture procedures (modified Jacquard et al. 2003, Cistué et al. 2003) allowed for quite effective androgenesis induction and DHs production. Among studied barley genotypes, only seven occurred to be totally nonresponsive to androgenesis-inducing treatment. For the rest, from 2 to 43 DHs has been produced. The efficiency of androgenesis varied significantly between studied genotypes from 0.5 to 10.5 GR/SP and from 0.1 to 5.8 AR/SP, with the mean effectiveness of 2.7 GR/SP and 1.5 AR/SP. In total, 3004 green regenerants and 1787 albinotic regenerants were obtained. Among them almost 57% occurred to be spontaneously diploidized, 30.5% remains haploid, while the rest of plants (12.5%) were mixoploid.

Androgenesis could be used not only for instant production of totally homozygous DH lines which is usually the main purpose of the process implementation into breeding programs. Beneficial side-effect is enhanced, in comparison with parental genotypes, gene expression for complex traits and increased genotypic and phenotypic variation in received offspring population of DHs (Humphreys et al. 2007). One of the examples of such androgenesis exploitation are the studies on *Lulium multiflorum* × *Festuca* ssp hybrids, where produced DHs population enhanced variations in drought resistance (Zare et al. 2002) and freezing tolerance (Zare et al. 1999, Rapacz et al. 2005). Similarly in presented study, high level of variation in tested parameters was detected in the population of DH lines of winter barley produced throughout androgenesis induction with the use of anther culture method.

Rate of water loss from excised leaves estimates cuticular transpiration rate and can be used as the indicator of drought resistance (Clarke and McCaig 1982). High excised-leaf water retention capability was a characteristic of some drought-resistant wheat cultivars (Kirkham et al. 1980, Clarke et al. 1989, McCaig and Romagosa 1991, Lugojan and Ciulca 2011). Moreover, in comparison with relative water content, selection based on this parameter occurred to be less influenced by the stage of plant development (Dhanda and Sethi 1998). In presented experiment, water loss from excised leaves of DH barley plants ranged between 7.3 and 29.4% H_2O/h (Fig. 1). Such high variation among tested genotypes allows for easy selection of drought-susceptible and drought-resistant DH lines. Increased level of genotypic variation could be seen especially clearly for several donor F1 crosses (5213, 5216, 5235, 5237, 5257, 630) for which, among segregating offspring both extremely drought-sensitive and drought-resistant DH lines were selected (Table 1).

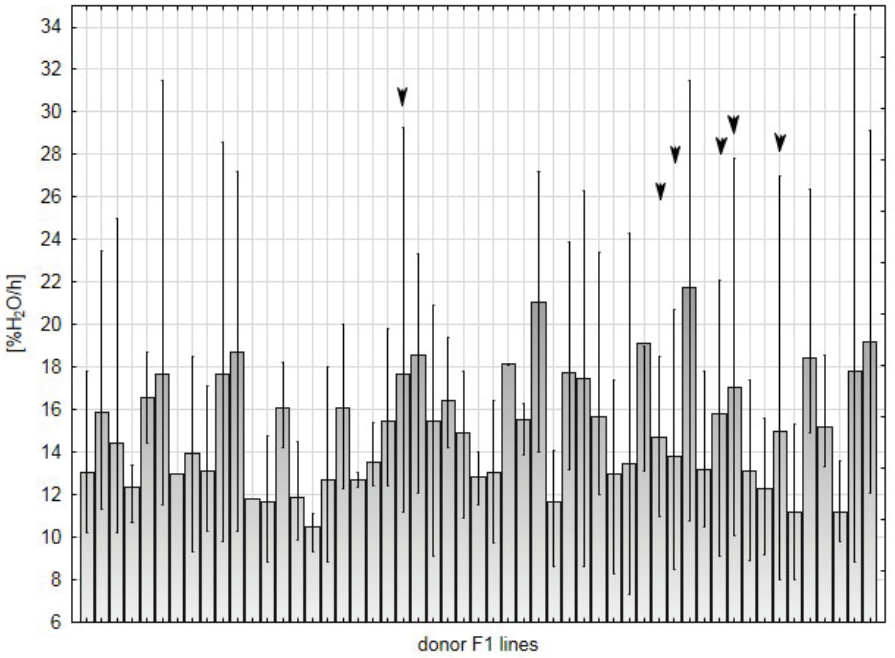

Fig.1. The mean rate of water loss from excised leaves [% H_2O/h] for studied donor F1 cross of winter barley. Whiskers show variation (range between maximum and minimum extremes) among produced DH lines offspring. Asterisks mark donor F1 cross highly heterozygous in respect of screened parameter.

Table.1. Variation among segregating DH progenies of winter barley for tolerance to drought determined by the rate of excised leaf water loss [% H_2O/h].

F1 cross	Drought-resistant genotypes		Drought-sensitive genotypes	
	No. of DH lines	Variation range [% H_2O/h]	No. of DH lines	Variation range [% H_2O/h]
5213	1	11.0	1	18.5
5216	5	8.5-11.0	2	20.4-20.7
5235	1	9.1	3	20.7-22.1
5237	2	10.1-10.2	9	18.8-25.1
5257	8	8.0-11.2	7	18.5-27.0
630	1	11.2	1	27.3

It is well known that in higher plants, photosynthetic system is the most sensitive to various stress factors (Falk et al. 1996). Photosynthetic traits such as chlorophyll fluorescence parameters are standardly used as reliable indicators to evaluate stress effects on growth and yield (Rapacz et al. 2015a, b). In presented work, among parameters describing freezing tolerance of photosynthetic apparatus, especially two: F_v/F_m and RC/CS_m, occurred to be highly diversified throughout the produced DHs population (Fig. 2, Fig. 3). Likewise, as

in the case of drought resistance assay, for some donor F1 crosses (5237, 5257, 630), both highly freeze-tolerant and freeze-susceptible genotypes could be found among their DH offspring (Table 2). Earlier, similar results have been received by Rapacz et al. (2005) and Humphreys et al. (2007) for *Festuca pratensis* × *Lolium multiflorum* amphidiploid cultivars.

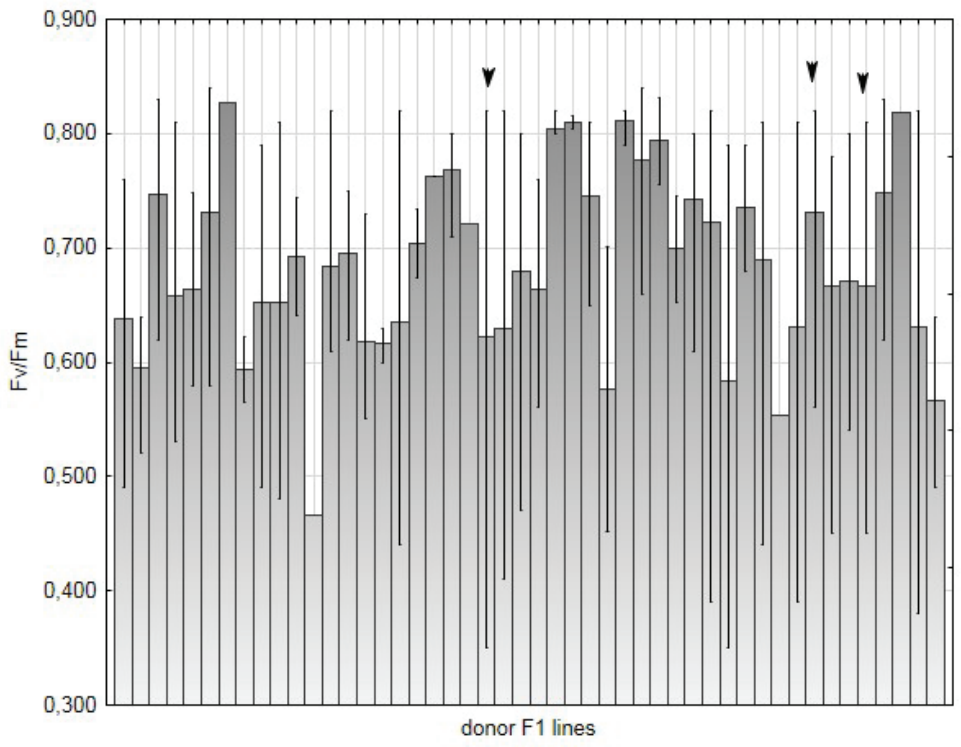

Fig.2. The mean of the yield of the energy trapping in PSII (F_v/F_m) for studied donor F1 crosses of winter barley. Whiskers show variation (range between maximum and minimum extremes) among produced DH lines offspring. Arrows mark donor F1 crosses highly heterozygous in respect of screened parameter.

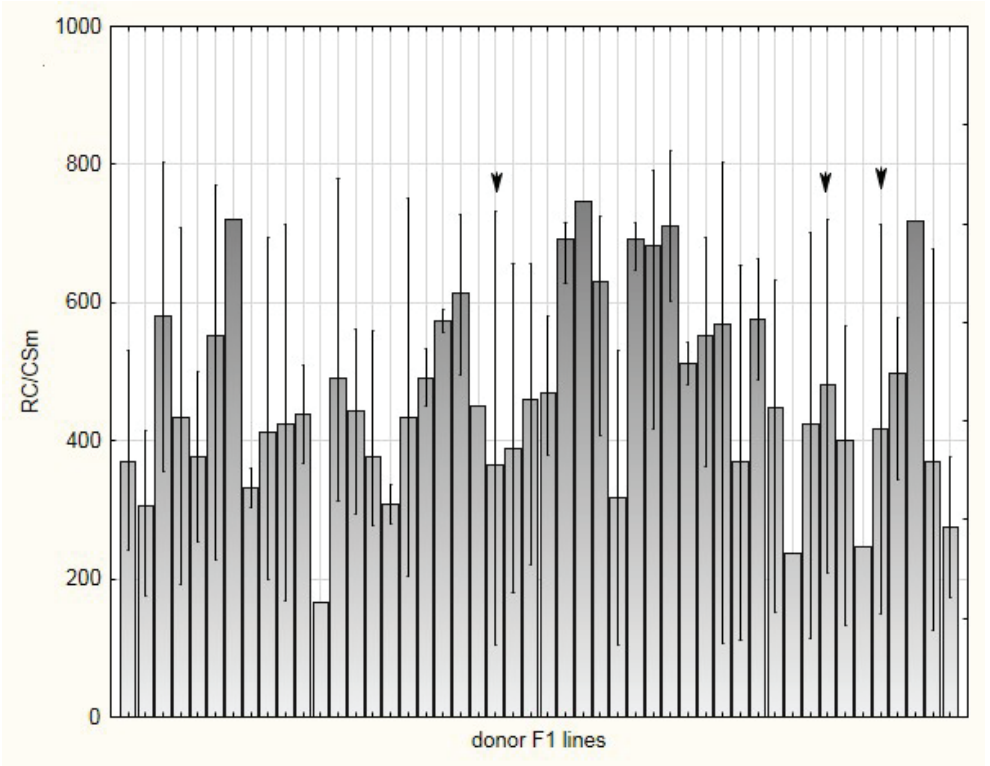

Fig.3. The mean of the number of active reaction centres per excited leaf area (RC/CS_m) for studied donor F1 crosses of winter barley. Whiskers show variation (range between maximum and minimum extremes) among produced DH lines offspring. Arrows mark donor F1 crosses highly heterozygous in respect of screened parameter.

Table 2. Variation among segregating DH progenies of winter barley for tolerance to freeze determined by two parameters of chlorophyll a fluorescence: F_v/F_m (the yield of the energy trapping in PSII) and RC/CS_m – (the number of active reaction centres per excited leaf area)

F1 cross	Freeze-tolerant genotypes			Freeze -sensitive genotypes		
	No. of DH lines	Variation range		No. of DH lines	Variation range	
		F_v/F_m	RC/CS_m		F_v/F_m	RC/CS_m
5257	2	0.796-0.804	661-675.9	2	0.466-0.503	150.2-220.7
5237	1	0.806	678.0	2	0.466-0.503	150.2-220.7
630	1	0.820	731.4	2	0.347-0.544	103.6-233.6

Presented results confirmed the assumption that haplodiploidization through androgenesis can increase the genetic variation within the DHs offspring and can be used as a bio-

technological tool for breeding progress acceleration. The fact that produced population of DH lines of barley were characterized by wide range of variation in drought and freezing tolerance, makes it an interesting model to study the mechanisms of control and regulation determining tolerance to these stress factors, and a valuable genetic resource for breeding programs.

Acknowledments

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MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES IN *SINAPIS ALBA* CV BARKA TREATED WITH AQUEOUS EXTRACTS FROM THE ROOTS OF *HELIANTHUS ANNUUS* L.

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Abstract: Summary of the effects of allelopathic involves secretion by plant into the environment the chemical substances which modify the morphological and physiological processes of other plants. Biological activity of the allelopathic compounds as well as the reaction of the plant to their effects is very different. Sunflower *Helianthus annuus* L. is characterized by high allelopathic potential. The study attempts to demonstrate the effect of aqueous extracts of *H. annuus* roots at concentrations of 5 and 10% on seed germination, plant growth and photosynthetic activity of *Sinapis alba* cv Barka. Allelopathic compounds contained in the roots disrupted the seed germinationprocess with increasing concentration. Regardless of the treatment phase extracts induced reduction in elongation growth of the root and aerial *S. alba*organs and changes in chlorophyll content and photosynthetic activity reduction of the plants.

Keywords: allelopathy, chlorophyll fluorescence, PSII, germination, morphology

Abbreviations:

F_v/F_m – maximum quantum efficiency of PSII,
F_0 – minimum fluorescence,
F_m – maximum fluorescence,
F_v – variable fluorescence,
FW – fresh weight, *H. annuus* – *Helianthus annuus* L., *S. alba* – *Sinapis alba* cv Barka

Inroduction

All plants compete for water, nutrients and light. Growth and development of plants both in the wild and in laboratory cultures can be modified by physical and chemical processes resulting from the presence of other plants. Some plants suppress competition growing out over it, and thus eliminating rivals, sometimes producing a dense dome of leaves

and thereby reducing the amount of light reaching the growing specimens below. The chemical form of limiting the number of competing products is allelopathy (King 2003). Although the impact of allelopathic relationships between plants have been known for a long time, it is still unclear whether production of allelochemicals is a deliberate strategy, developed by the plant to prevent competition or an accidental effect of persisting in subsequent generations, giving the plant an advantage over other individuals (King 2003). All plant organisms, in addition to materials that are products of the so-called basic metabolism, also produce chemical compounds included in the secondary metabolites. They are often referred to as specific, characteristic only for certain species. Many of them are used in plant-plant interactions or as attractants for pollinators (Oleszek et al. 2001).

Some allelopatic compounds are not present in the plant body, but only after the transformation occurring in the soil solution they can be converted into them and undergo permanent or temporary adsorption on soil colloids. The rate of transformation depends largely on the soil type and its oxygenation (Weyman-Kaczmarkowa and Wójcik-Wojtkowiak 1992), and they are produced, among others, during secretion of exudates from the roots. These substances have a simple chemical structure, and with hydrophilic properties can easily penetrate into the soil. So far, they were considered negligible due to the small amount in which they arise in the rhizosphere. In recent years, they have become an important part of the studies on allelopathy (Rasmussen et al. 1992, Kato-Nauchi et al. 2002).

In the era of industrialization and the use of chemicals in agriculture, knowledge of allelopathic interactions is becoming increasingly necessary. In connection with the desire to move away from the use of chemicals in agriculture and popularization of healthy food, experiments on the effect of allelopathic compounds are becoming more popular and are especially important for agriculture. Allelopatins are considered as natural herbicides, and their biggest advantage is rapid biodegradation in the environment and lack of toxic effects on other organisms (Wójcik-Wojtkowiak et al. 1998).

The sunflower is a plant that has considerable allelopathic potential (Siegień et al. 2008). Numerous studies have shown that extracts of various organs such as leaves, stems, roots, flowers or fruits contain compounds exhibiting toxic effects on many other plant species, although in low concentrations can stimulate selected physiological processes in other plants (Batish et al. 2002, Gniazdowska et al. 2007, Siegień et al. 2008). The negative effect of aqueous leaf extracts of *Helianthus annuus* was also observed in the case of germination and growth in *Sinapis alba* (Bogatek et al. 2006). A large scale of impact to other plant organisms is due to the presence of phenolic compounds (Reigosa et al. 1999), the inhibitory effect on plant development of other species have been indicated by a number of authors (Azania et al. 2003). Batish et al. 2002). Terpenoids, also being isolated from sunflower organs, can have a stimulating or inhibiting effect on the growth processes of other plants (Macias et al. 2002).

The aim of this study was to show what impact aqueous extracts from roots of common sunflower (*Helianthus annuus* L.) have on seed germination, plant growth and photosynthetic activity of white mustard (*Sinapis alba*) cv Barka.

Materials and methods

Plant material

For the experiments, dried roots of common sunflower (*Helianthus annuus* L.) and white mustard seeds (*Sinapis alba* cv Barka) were used, obtained from the Cracow Horticultural Breeding and Seed Supply "POLAN". Aqueous solutions of the *H. annuus* root extracts in concentrations of 5 and 10% were prepared by weighing 5 and 10 g of plant material which was poured with 95 ml and 90 ml of distilled water respectively. The whole was left for 24 hours to extract the allelopathic substance contained in the roots. Then extracts were sieved using a vacuum pump on a Buchner funnel with Whatman filter paper and stored in a refrigerator.

Germination ability of *S. alba* seeds on the aqueous extracts from the roots of *H. annuus*

In order to determine germination ability, 100 *S. alba* seeds were placed on sterilized petri dishes lined with filter paper moistened with 6 ml of *H. annuus* root extracts or water (control). The plates were placed in a dark thermostat at 25 °C. The percentage of germinated seeds was counted after 24, 48, 72 and 96 h. Those seeds were regarded as germinated, which had sprout length of not less than the diameter of the seed.

Growth of *S. alba* plants grown from seeds treated with *H. annuus* root extracts during germination and growth phase

In the first part of the experiment seedlings *S. alba*, germinated after 72 hours on media with sunflower root extracts were rinsed with distilled water and planted into sand pots. Grown plants were watered every 48 hours with distilled water and a standard Steiner (1961) medium with macro and micronutrients.

In the second part of the experiment seedlings germinated on distilled water were planted in sand pots and watered every other day with sunflower aqueous root extracts and once a week with the Steiner medium. All pots with seedlings were placed in a thermostat under constant conditions, at 25 °C and light intensity of 300 micromol $m^{-2} \cdot s^{-1}$ and relative humidity (RH) 60-70%.

Morphometric analysis of *S. alba* plants

Biometric analysis was performed by measuring the length of the root, hypocotyl, epicotyl and the rest of the shoot with calipers.

Chlorophyll *a* fluorescence of *S. alba* plants watered with aqueous extracts in germination and growth phase

Measurement of chlorophyll fluorescence was performed using PSM fluorometer (Plant Stress Meter, biographer, Sweden). The source of fluorescence excitation was actinic light in the range of 330-660 nm with a maximum at 500 nm. During the measurements, accli-

matization of plants to the dark for 30 minutes was carried out by means of proprietary clips. The intensity of excitation light was 600 micromol $m^{-2} \cdot s^{-1}$. The effect of stress was evaluated based on the following parameters: F_v/F_m - the maximum quantum efficiency of PSII, F_0 - minimum fluorescence, F_m - maximum fluorescence and variable fluorescence - F_v.

Chlorophyll content in *S. alba* leaves treated with allelopathic root extracts during germination and growth phase

The chlorophyll content was measured according to Barnes et al. (1992). For this purpose, weighed plant material was extracted in 3 ml of DMSO (Sigma-Aldrich) at 65 °C. The absorbance of the extracts was determined at a wavelength of λ = 648 and 665 nm using a Cecil 9500 spectrophotometer (UK). The amount of chlorophyll was calculated as concentration in the fresh weight of the sample [$mg \cdot g^{-1}FW$].

Statistical analysis

The data was subjected to statistical analysis of variance (ANOVA). The significance of differences was determined using the Duncan post-hoc test for homogeneous groups on mean values (n = 5) at $p < 0.05$. The calculations were performed in Statistica v. 10.0.

Results

Germination ability of *S. alba*

After 24 h 17% of seeds germinated on medium containing 5% root extract of *H. annuus*, on the 10% extract - less than 1%, while in the case of control up to 51%. On the second day an increase in the number of germinated seeds on all media was observed. The smallest number was observed again on the medium saturated with the highest concentrations of extract used. In the following hours, highest germination of seeds were found in the control conditions, while it was significantly lower for seeds treated with extract concentrations of 5 and 10% (Tab. 1).

Morphometric analysis of *S. alba* plants

Morphometric analysis showed an inhibition of root growth in each of the concentrations used for aqueous root extracts of *H. annuus*, both during germination and in the growth phase. Compared to control, shorter roots of the plants had grown from seeds watered with extracts. In case of hypocotyl, the length significantly increased in plants watered during germination. The longest epicotyl was present in plants watered during the growth with 5% extract, and the shortest plants had grown from seeds germinated on substrate with 10% extract. The length of the remaining part of the shoot at each concentration of the extract showed lower values in plants treated with during both germination and growth phase (Tab. 2).

Table 1. Percentage of germinated seeds of mustard (Sinapis alba cv Barka) on substrates saturated with distilled water and aqueous root extracts of sunflower (Helianthus annuus L.) at concentrations of 5 and 10%. Mean values ± SD (n = 5) within a row are significantly different according to Duncan test, p <0.05

Time [h]	Control	Concentration of aqueous extracts of root *Helianthus annuus* [%]	
		5	10
	Percentage of germinated seeds		
24	51^{a}±1.41	17^{b}±1.72	0.2^{c}±0.4
48	92^{a}±1.41	79^{b}±2.04	45^{c}±2.69
72	96^{a}±1.33	86^{b}±3.66	75^{c}±2.00
96	98^{a}±1.36	91^{b}±1.02	78^{c}±1.02

Table 2. Organ length of mustard (Sinapis alba cv Barka) plants watered with aqueous root extracts of sunflower (Helianthus annuus L.) at concentrations of 5 and 10% during germination (A) and growth phase (B). Mean values ± SD (n = 5) within a row are significantly different according to Duncan test, p <0.05

Organ	Control	Concentration of aqueous extracts of root *Helianthus annuus* [%]			
		5		10	
		A	B	A	B
	Organ length [cm]				
Root	10.48^{a}±0.32	4.02^{d}±0.04	9.61^{b}±0.08	3.62^{e}±0.12	8.80^{c}±0.11
Hypocotyl	3.04^{a}±0.14	2.17^{b}±0.18	3.18^{a}±0.13	2.09^{b}±0.09	3.01^{a}±0.01
Epicotyl	0.73^{b}±0.03	0.16^{d}±0.05	0.84^{a}±0.04	0.07^{e}±0.01	0.50^{c}±0.01
Remainder of the shoot	0.61^{a}±0.02	0.09^{d}±0.005	0.55^{b}±0.02	0.04^{e}±0.001	0.47^{c}±0.03

Chlorophyll fluorescence

Analysis of the maximum potential PSII efficiency (F_v/F_m) showed the lowest value in plants treated with 10% root extracts of sunflower, both in the germination and during growth phase. For F_m and F_v parameters we observed an increase in plants grown from seeds germinated on substrate with 10% and 5% extracts relative to the control. During the growth phase significant differences were demonstrated between the plants watered with 5% and 10% extract as well as the control. F_0 parameter did not change significantly at any of the concentrations used (Fig. 1a and b).

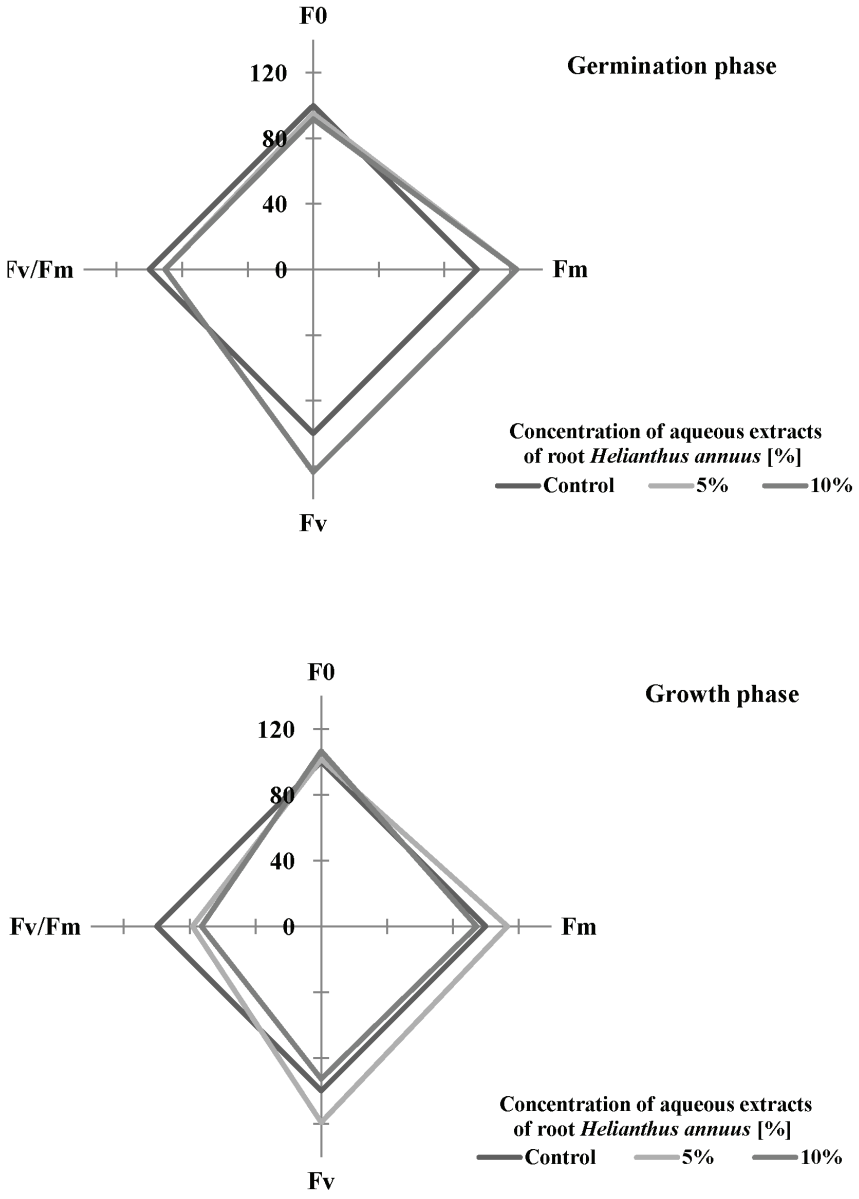

Fig. 1. Chlorophyll fluorescence values (expressed as percent of control) in mustard plants (S. alba cv Barka) grown from seeds treated with aqueous root extracts of sunflower (H. annuus L.) at concentrations of 5 and 10% during germination (A) and growth phase (B). Mean values ± SD (n = 5)

Chlorophyll content

Plants of S. alba watered with aqueous root extracts at concentrations of 5 and 10% both in germination and growth phase showed reduced chlorophyll content as compared to plants grown under control conditions. In plants treated during germination phase, statistically significant differences in chlorophyll b content of compared to the control, it showed only between plants grown from seeds germinated on media with 10% of the extracts. During the growth phase the reduction of chlorophyll b was found at each of the concentrations used. In the case of the total chlorophyll content (a+b) both in the germination as well as during growth phase, a reduction was observed relative to control. The chlorophyll a/b ratio was significantly different between control and plants grown during germination phase and growth phase treated with all root extracts (Tab. 3).

Table 3. Chlorophyll content in first leaf of mustard (Sinapis alba cv Barka) plants watered with aqueous root extracts of sunflower (Helianthus annuus L.) at concentrations of 5 and 10% during germination (A) and growth phase (B). Mean values ± SD (n = 5) within a row are significantly different according to Duncan test, p <0.05

Chlorophyll	Control	Concentration of aqueous extracts of root *Helianthus annuus* [%]			
		5		10	
		A	B	A	B
		Chlorophyll content [mg g^{-1}FW]			
a	1.06^{a}±0.05	0.2^{d}±0.02	0.66^{b}±0.03	0.12^{e}±0.01	0.43^{c}±0.04
b	0.50^{a}±0.01	0.48^{a}±0.04	0.35^{b}±0.03	0.34^{b}±0.02	0.15^{c}±0.03
a+b	1.56^{a}±0.06	0.68^{c}±0.05	1.01^{b}±0.05	0.46^{e}±0.03	0.59^{d}±0.04
a/b	2.14^{b}±0.08	0.42^{c}±0.06	1.90^{c}±0.20	0.37^{b}±0.02	2.97^{a}±0,61

Discussion

Understanding the mechanisms of allelochemicals action poses many difficulties, caused by allelopatics belonging to different classes of chemical compounds, and hence their varied effects on plants (Einhellig 1995). Penetration of allelochemicals can cause metabolic disorders and, consequently, changes in metabolic pathways related to the development and evolution of plant organism (Falińska 1996, Wójcik-Wojtkowiak et al. 1998). Effects of allelopathic interaction can be determined by measuring the rate of seed germination, growth and development of plant organs, photosynthetic activity and other physiological parameters (Ciarka et al. 1996, Parylak 1996, Kupidłowska et al. 2006, Shimabukuro and Haberman 2006, Troć et al. 2009, Możdżeń and Repka 2014, Możdżeń and Oliwa 2015). The inhibition and stimulation of physiological and morphological changes depends on the concentration of the substance. Allelocompounds used in low concentrations, are usually stimulating, while at high concentrations they inhibit plant growth, resulting first of all in primary effects and affecting metabolic and biochemical processes (Oleszek et al. 2001, Skoczowski et al. 2003, Troć et al. 2011, Skoczowski et al. 2011).

Sunflower (*H. annuus*) is an excellent source of terpenoids which have a broad spectrum of activity. Several biochemical studies have shown that it contains monoterpenes, lactones sesquiterpenes, diterpenes, triterpenes, heliannones, heliespirones and phenolic compounds: chlorogenic and isochlorogenic acid, which are classified as active allelopatins. (Macias et al. 1997, Gniazdowska et al. 2004). The inhibitory effect of aqueous root extracts of *H. annuus* had manifested itself already during seed germination of *S. alba*. The higher the concentration of the solution used, the lower the number of germinated seeds (Tab. 1). Germination is a multi-step process of transition from the resting state to the development phase. It includes physical phase - the penetration of water into the seed, hydration and imbibition, biochemical phase - mobilization of storage material and the physiological phase - the growth of the embryo (Grzesiuk and Kulka 1981). Negative allelopathic interactions can be revealed during seed imbibition and lead to anatomical distortion of the seed coat and storage materials. As a result, these deformations delay the germination and contribute to the improper root development leading to the degeneration of the embryo.

During the ontogenetic development of plants, allelocompounds present in the form of alkaloids and phenolic compounds act as an inhibitor of cell division and elongation growth. Allelopatins cause, among others, disturbances of the root by undevelopment of root hairs and lateral roots and decrease horizontal growth and leaf expansion (Baziramakenga et al. 1994, Duer 1996, Wójcik-Wojtkowiak et al. 1998, Oleszek et al. 2001). In this study we demonstrated the inhibitory effect of root extracts from *H. annuus* on root and aboveground organ growth of *S. alba* plants (Tab. 2). Elongation growth inhibition of *S. alba* plants could indicate the presence of alleloinhibitors in aqueous root extracts from *H. annuus*.

Allelocompounds also inhibit protein synthesis, thus changing the photosynthetic activity of plants. Studies on isolated mitochondria revealed that a particularly high activity was shown by monoterpenes and phenol compounds (Politycka and Wójcik-Wojtkowiak 2001). Their effect is primarily a disturbance of oxidative phosphorylation process, thereby reducing the amount of ATP produced (Wójcik-Wojtkowiak et al. 1998). Disturbances in the course of photosynthesis can be caused by lactones and sesquiterpenes that inhibit the functioning of PSII. Phenolic acids cause a decrease in stomatal conductance, increase diffusion leaf resistance and reduce net photosynthesis by 33% (Einhellig 1995). In case of *S. alba* plants watered with root extracts from *H. annuus* demonstrated varying allelopatin impact on the activity of photosystem PSII.

Plants subjected to allelopathic stress such as watering with aqueous root extracts from *H. annuus* during germination and growth phase were of a different color intensity compared to the control plants. Recent research confirms that it is the result of changes in the functioning of chloroplasts, and thus differences in chlorophyll content. Low concentrations of allelopathic substances cause an increase in chlorophyll concentration, while increase of allelopatin concentration produces the opposite effect (Pandey 1994, Stiles et al. 1994). In the case of *S. alba* treated with aqueous root extracts a reduction of the chlorophyll content has been shown in plants treated both during germination and growth phase (Tab. 3).

On the one hand allelopathic substances prove to be useful in combating weeds (Synowiec et al. 2015), bacteria, fungi or other pathogens. On the other hand, however in addition to the favorable effects they may adversely affect the adjacent plant. In many cases,

sowing and planting some plants next to each other should be avoided. Hence the growing popularity of so-called biocommons, the creation of which involves appropriate selection of the neighboring plants. This allows for high yields, while at the same time weed control and prevention of crop pest infestation. In a natural way it allows to avoid losses, yielding a healthy and organic food, and above all protection of the environment, especially in areas contaminated with pesticides and other toxins.

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THE ROLE OF MONOLIGNOLS AND ANATOMICAL STRUCTURE IN CELLULAR MECHANISMS OF LEAF AND ROOTS TOLERANCE TO NATURE FLOODING

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Abstract. The results of comparative anatomical structure and monolignols cytochemical study of *Trapa natans* (water chestnut) submerged organs at the stage of plant flowering were presented. The cytochemical method was used for the study of both distribution monolignols (syringil and guaiacyl) and also relative content of monolignols in cell walls of differ tissues with use of diphenylboric acid-2-aminoethyl ester. The localization of monolignols was analyzed with laser scanning microscope LSM 5 Pascal (Germany) and the content of monolignols was determined with Pascal program. It is established the differences of monolignols content in depend on organs and tissue. The study encourage concept that under plant flood the monolignols of cell walls of submerged organs is involved in cellular and functional mechanisms of tolerance to nature flooding of plants.

Key words: adventitious submerged roots, laser scanning microscopy, monolignols, submerged leaves, *Trapa natans,* flooding

Introduction

The study of morphological and anatomical signs of air-aquatic plants, including of *Trapa natans*, used for systematic of plants, for research of adaptation signs of plants to the flood and also for pharmacological activity of this plants (O'Neill 2006, Prafulla Adkar et al. 2014). It is known that many air-aquatic plants including waterchestnut (*Trapa natans* L.) are heterophyllous. Submerged leaves and roots of such plants differ by the anatomical and structural-functional features from air leaves and roots, including the decreased respiration and weak carbon feed that helps the submerged organs to adapt to the decline of sunlight in water, change of composition of light, content of CO_2 and O_2 in an aquatic environment (Armstrong et al. 1994, Vartapetian and Jackson 1997, Jackson and Colmer 2005).

Lignin, one of the main structures of the polymeric cell wall of higher plants, fulfils a number of functions, among which mention should be made of their remarkable mechanical strength properties, wall impassability for water. Lignin is complex biopolymer of aromatic alcohols, which are synthesized in secondary cell walls. Lignin is highly branched and composed of cross-linked units, three monolignols: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) phenylpropanoid units (Boerjan et al. 2003). Lignin synthesis is de-

pending from tissue type, organ and species (Monties 1998; Fengel and Wegener 1984). This biopolymer can to reduce speed of cell growth and participates in adaptation of plant to the stress**,** changing structure of cellular wall matrix, providing impassability of water and water solutions through walls and forming in an epidermis or other tissues a barrier to the pathogens. It is known that wall lignification intensify plant resistance from pathogens invasion (Moura et al. 2010). It is possible, that such phenomenon is occurs because of lignin is characterized by hydrophobic feature, forming to hydrogen and covalently association between polysaccharides (Boerjan et al. 2003).

It is known that plants which expose to flood or submerged plants (hydrophytes) adapt to constant submerged conditions by the changes of morphology-anatomical structure and change of cell wall structure of epidermal tissue that is the first barrier between plant and water environment. Because the aim of our study was to carry out of the investigation the presence and distribution of individual monolignols in different cell walls and research of anatomical characteristics of submerged organs in *Trapa natans* air-water plant.

Material and methods

Plant material. A research objects were submerged adventitious roots, submerged finely divided feather-like leaves and floating leaves of water chestnut (*Trapa natans* L.) plants that grew on a depth up to 80 cm on the birch of the Rusanivsky channel (left Shore of Dnepr River, in Kiev). Submersed adventitious roots, submerged and floating leaves, were collected at budding-flowering stage (beginning of July). The sun illumination [photosynthetic photon fluency rate (PPFR)] on water surface was 1500-1600 μmol quantum·m^{-2}·sec^{-1}, and on surface of the upper leaves of studied plants (about 8-10 cm below the water surface) was 10-12 μmol quantum·m^{-2}· sec^{-1}. PPFR was measured by the means of Light Meter Li-250 (USA, LI-COR).

Microscopical and cytochemical analyses. The middle part of föoating leaves, submerged adventitious roots, and of needle-shaped particles of submerged dissected leaves were used for research**.** The sections of samples were selected from five plants, which had close identical size and close morphology, and then they were used for the light microscopy and cytochemical study. Immediately after removal from water, samples were fixed on a birch in mixture of 5 % solution of paraformaldehyde and 2 % glutaraldehyde (1: 1, vol) in a 0.5 M phosphate buffer, for 24 h, pH 7.2. Then the fixed material in laboratory terms was washed in buffer, dehydrated in ethanol and acetone, embedded in mixture an epon/araldite resin according to the standard method. Semithin sections (about 12 μm thickness) were stained with Schiff solution and by solution of safranine in accord with standard (Furst, 1979) and were studied on the light microscope (Axioscope, Carl Zeiss).

The cytochemical method of monolignols accordingly to Wuyts et al. (2003) with some modifiation was used for the study of present, distribution syringyl and guaiacyl and the relative content of these monolignols in cell walls. Middle part of floating leaf, needle-like particles of submerged dissected leaf and transversal sections of adventious root (near 1 cm from apex) were hand cut and dipped in the staining solution of saturated (0.25%, w/v) diphenylboric acid-2-aminoethyl ester (DPBA) (Sigma) in deionized H_2O containing 0.02% (v/v) Triton-X-100 for 2-4 min at +25°C; washed in H_2O, put in 0.05% solution of

paraformaldehyde in phosphate buffer at +4°C and then in laboratory samples were washed and investigated on the laser scanning microscope (LSM 5 Pascal, Carl Zeiss, Germany). Our modification is composed of fixation of samples after staining with DPBA. This is helped to preservation of plant material. Complex DPBA–syringyl was excited at 340-380 nm, and fluorescence emission detected at 430 nm. Complex DPBA–guaiacyl was excited at 450-490 nm and fluorescence emission detected at 520 nm using an x 10, x 20 and x 40 objectives. Chlorophyll auto fluorescence was excited at 440 nm and fluorescent emission detected at 662 nm. Fluorescence intensity of monolignols and chlorophyll was measured in the cell walls as a function of emissions wave length using the PASCAL program (LSM 5, Carl Zeiss). The volume tissues in leaflets were measured with using ImageJ programs. Values of results were expressed at the mean and standard errors, using Student's test ($P< 0.05$).

Results and discussion

Morphological and anatomical analysis of leaves and roots. Plants of *Trapa natans* are one-year hydrophyte. The complete cycle of this plant ontogenesis survive about three months (June, July and August) in Ukraine. Plant of *T. natans* is characterized by the presence of developed floating (Fig. 1A), submerged leaves and adventitious submerged roots (Fig. 1B) at the stage of plant flowering. Floating leaves forms a rosette; leaflet is solid and triangular; the middle size of leaflet is 5.2 x 6.4 cm.

Two or four submerged dissected leaves are formed from each internodes of stem. Submerged green dissected leaves have feather-like shape. These leaves are situated oppositely against each other. The middle size of submersed dissected leaf is 53 ± 4.9 cm in long axis and 2.7 ± 0.3 cm −in short axis. Such leaves are consisted of long needle-like particles; the length of that comes up to 25 mm.

An adventitious filaceous roots are formed in base of each stem node near submerged dissected leaves. These roots is very thin and have pinkish color, their length is from 7.5 to 31 cm; root hairs are absent. Away from node stem from 7 to 12 adventitious roots are formed (Fig. 1B). Morphology of floating and submerged leaves at flowering stage was like to that at vegetative stage (Nedukha 2013)

Floating leaf. A dorsoventral structure is characterized for leaflets of floating leaves (Fig. 1C); including of bilamella palisade parenchyma, 6-9 layer of spongy parenchyma, aerenchyma (air cavities) between cells of 6-9 layer of spongy parenchyma and between adaxial epidermis and palisade cells, single-layered adaxial (with stomata) and abaxial epidermis. The middle number of chloroplasts in palisade cell is equaled 13 ± 2.7 on the section. The results of middle area of tissues in leaflets were next: adaxial epidermis – 6.5 %, abaxial epidermis – 6.7 %, palisade parenchyma – 38.5 %, spongy parenchyma – 47.9 %. It is necessary to note, that middle area of aerenchyma cavities was amount 11.9 ± 2.1 %. The size of cells in tissues of floating leaves was like to that in vegetative stage (Nedukha 2013).

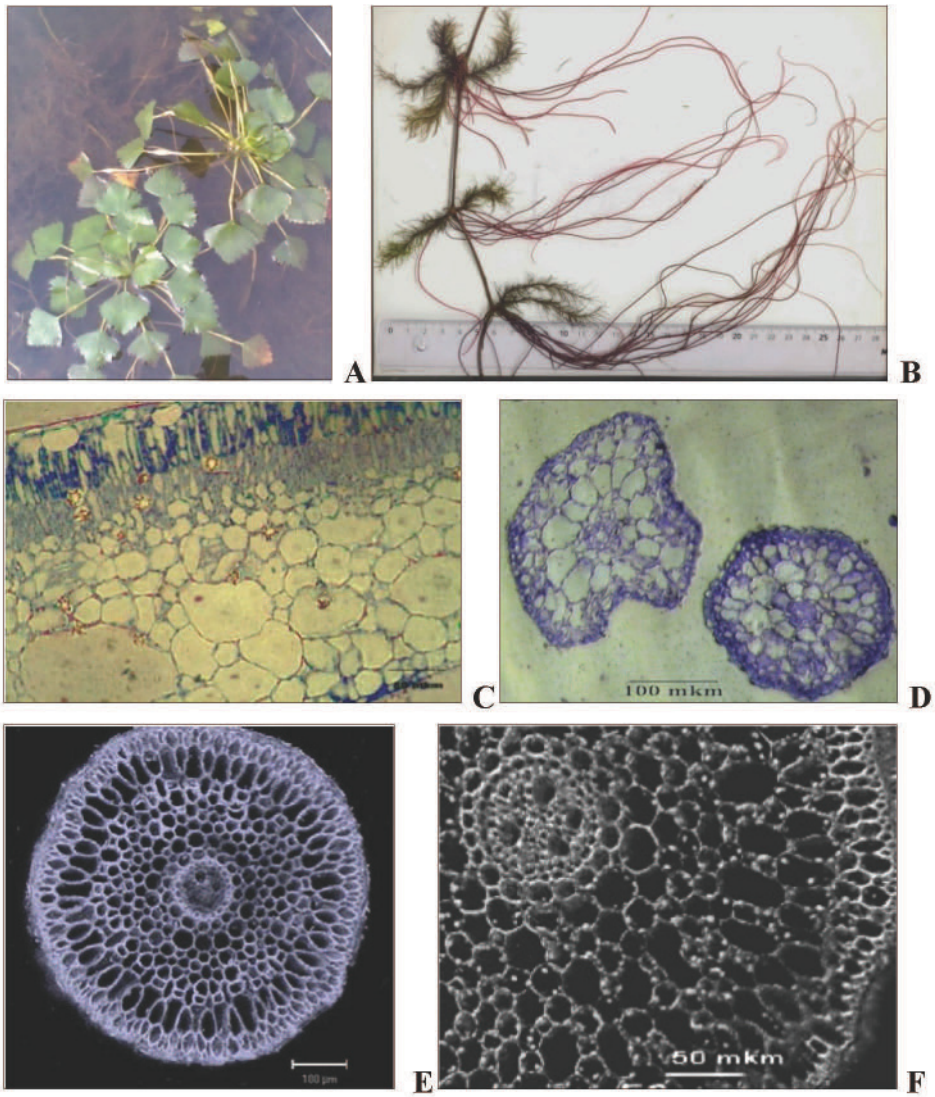

Fig. 1. General view of Trapa natans swimming leaves (a), submerged dissected leaves and adventitious roots (b), and the sections through central plane of swimming leaf (c), submerged leaves (d) and submerged adventitious roots (e, f). Bars: C, F = 50 μm; D, E = 100 μm.

Submerged leaf. Light-microscopic analysis of the transverse sections of needle-shaped particles dissected submerged leaf with rounded or slightly curved lateral shape is exposed unifacial, centric structure leaf (Fig. 1D), including one-layered epidermis, 4-7 layers of radial situated photosynthesizing parenchyma (two outer layers below epidermis contained

a small cells, and inner layers parenchyma – greater cells). The cells of photosynthesizing parenchyma were closely joined. Endodermis and central small vascular bundle with one vessel and small surroundings cells of phloem are situated centripetally below photosynthesizing parenchyma. The middle number of chloroplasts in parenchyma cell is equaled 7.7 ± 0.5 on the section. Very small air volumes were exposed only between cells of inner 3-6 parenchyma layers. Results of middle area tissues in submerged leaflet particles were next: epidermis – 12.9 %, photosynthesizing parenchyma – 83.7 %, conduction bundle – 2.29 % and vessel – 0.13 %. The middle area of small air cavities between inner layers of photosynthesizing parenchyma was amount 0.39 ± 0.03 %.

Adventitious roots. The form of *T. natans* cross-section adventitious submerged roots is round; the middle size of root diameter isequaled 520 ± 23 μm. Roots revealed three distinct regions: outer cell region, cortical region and stele (Fig. 1E, F). Roots are covered by a monolayer epidermis that consists of small cells, by the middle size of 10 ± 2,1 x 7 ± 1,4 μm. Multilayer cortex is founded below the epidermis of root. The cortex was composed of parenchyma cells that are formed exoderm and endoderm. Outer layers of cortex contained large regions of radial aerenchyma (Fig. 1E, F), the amount of layers of that hesitates for different roots (from two to five). A cortex counts 8-10 layers of cells. The form of cortex cells from exoderm to stele is changed from oval to rounded or hexagonal. Chloroplasts were revealed in cells of root cortex (Fig. 2F), the middle number of chloroplasts in cell was 2.9 ± 0.8. The internal layer of cortex is formed thick-walled endoderm. Layer of pericycle is founded below endoderm. Almost rounded cells of pericycle have diameter 15 ± 1.7 μm, and characterizes by thick walls (Fig. 1F). Stele contains the little cells of phloem and four vessels with diameter about 20 ± 2.1 μm. Results of middle area tissues in submerged adventitious root were next: cortex with epidermis – 96.7 %, stele – 2.97 %, four vessels – 0.37 %. The middle area of air cavities (aerenchyma) between cortex cells was 18.9 ± 2.1 %.

Cytochemical analysis of monolignols in *Trapa natans* leaves and roots

Floating leaves. Cytochemical analysis of monolignols in the flooding leaves of *T. natans* are shown as blue fluorescence for syringyl and as green fluorescence for guaiacyl in the walls of epidermis, mesophyll and in xylem vessels (Fig. 2A). The fluorescence intensity of DPKK-syringyl and DPKK-guaiacyl complex was different in the tissues (Fig. 2 B). Periclinal walls of epidermis (Fig. 2B; 3A, 3B) had the greatest fluorescence intensity of DPKK-syringyl complex in comparison with walls of mesophyll and conductive bundle cells, in which guaiacyl predominated. The size of S/G ratio in cells takes place in the next order: walls of epidermis > walls of spongy parenchyma and vessels > aerenchyma > palisade parenchyma. Intensity of monolignols (S + G) fluorescence in different cell walls was next: (in adaxial and abaxial epidermis) periclinal walls – 227 ± 19 and 240 ± 21; anticlinal walls –161±13 and 60 ± 9, acordingly; palisade – 205 ± 19; spongy parenchyma – 148 ± 13; aerenchyma – 176 ± 19 and vessels – 309 ± 22 relative units.

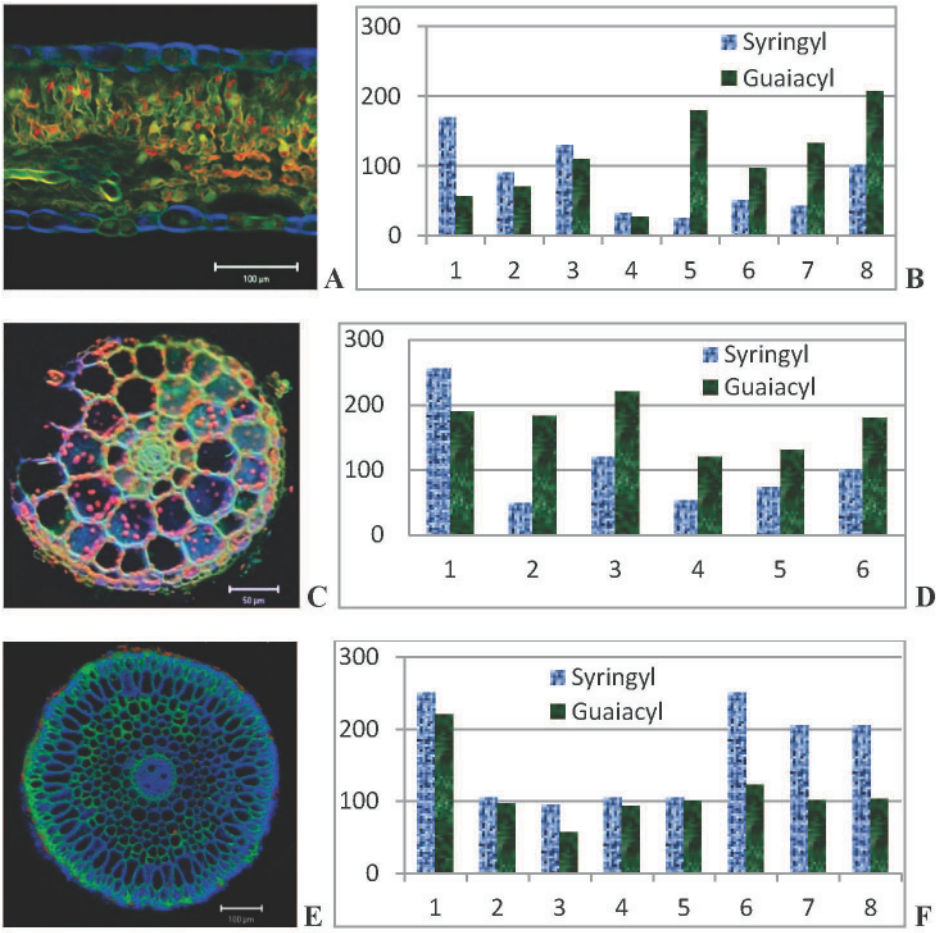

Figure 2. Micrographs of cytochemical fluorescence of monolignols in the floating leaf (A) submerged leaf (C) and adventitious root (E) cells of Trapa natans. Localization of syringyl has blue fluorescence and guaiacyl—green fluorescence; chlorophyll—red auto fluorescence. Changes of the relative content (rel. units) of monolignols in cell walls of floating leaf (B), submerged leaf (D) and root (F). The key on figure B: adaxial epidemis (1, 2) abaxial epidermis (3, 4), 1, 3 – periclinal wall, 2, 4 – anticlinal wall, 5 – palisade parenchyma, 6 – spongy mesophyll, 7 – aerenchyma, 8 –vessels. The key on figure D: 1 – epidermis, periclinal wall, 2 – epidermis, anticlinal wall, 3 – photosynthesizing parenchyma, 4 – endoderm, 5 – phloem cells of conductive bundle, 6 – vessels. The key on figure F: 1- epidermis, 2 – exoderm, 3 – cortex, 4 – aerenchyma in cortex, 5 – endoderm, 6 – pericycle, 7 – stele phloem, 8 - vessels.
Bars: A, E = 100 μm, C = 50 μm.

Submerged leaves. Like fluorescence of monolignols observed in submersed leaves. Cytochemical analysis of the complex DPKK-syringyl and DPKK-guaiacyl in submersed leaves shown blue fluorescence of syringyl and green fluorescence of guaiacyl in cell walls of epidermis, photosynthesizing parenchyma and central vascular bundle (Fig. 2C, 2D). There were some differences in fluorescence intensity of monolignols in cell walls of epidermis, in the photosynthesizing parenchyma and vessels. The level of fluorescence intensity of monolignols is presented in the Figure 2D, 3C and 3D (diagram). It is necessary to note that syringyl is predominated only periclinal wall of epidermis, whereas in walls of inner tissues guaiacyl is prevailed. The size of the relation of S/G cells in submerged leaves are situated in such order: periclinal walls of epidermis > walls of endoderm > vessels and phloem conductive bundle > photosynthesizing parenchyma > anticlinal walls of epidermis. Intensity of monolignols (S + G) fluorescence in different cell walls was next: in epidermis periclinal walls – 444 ± 23; anticlinal walls 232 ± 20; photosynthesizing parenchyma – 340 ± 31; endoderm 173 ± 13; phloem of bundle – 203 ± 22 and vessels – 280 ± 23 relative units.

Adventitious roots. Cytochemical analysis of monolignols in *T. natans* adventitious roots is showed blue fluorescence of syringyl and green fluorescence of guaiacyl in the cell wall of epidermis, exoderm, cortex, aerenchyma in core, endoderm, pericycle, metaxylem, and vessels similar to that in submerged leaves (Fig. 2E). The level of luminescence intensity is presented on the figure 2F, 3E and 3F (diagram). The analysis of monolignols luminescence intensity showed that relative content of syringyl in walls of all tissues was more than that content of guaiacyl (Fig. 2F). The size of S/G ratio in cells takes place in the next order: pericycle and metaxylem > vessels > epidermis and aerenchyma > exoderm and endoderm walls. Intensity of monolignols (S + G) fluorescence in different cell walls of root was next: in epidermis – 470 ± 39; exoderm – 203 ± 24; cortex – 152 ± 13; aerenchyma in cortex – 198 ± 21; endoderm - 205 ± 20; pericycle – 373 ± 29; phloem in stele – 306 ± 31 and vessels – 308 ± 27 relative units.

Thus, anatomic study of *T. natans* submerged organs at the phase of plant flowering is showed forming of adventitious photosynthesizing roots that are formed from stem internodes near submerged dissected leaves, which some authors is named roots (Sculthorpe 1967, Ishimaru et al. 1996). The previous structural investigation of vegetative organs of this species at the stage of vegetative growth did not revealed the presence of adventitious photosynthesizing roots on a submerged stem (Nedukha 2013). We exposed that adventitious photosynthesizing roots began to appear at the beginning of flowering stage and they can to function far nuts formation. Like formation of adventitious photosynthesizing roots was early shown in aquatic plant *Cotula coronopifolia* and *Meionectes brownii.* (Rich et al. 2011, 2012).

Anatomic study of *T. natans* adventitious roots had shown the presence of well-developed aerenchyma that, as known, functions during of plant hypoxia for an accumulation and transport of gases. When hypoxia is arises in submerged organs (root, stem or leaves), an aerenchyma generate in such organs (Vartapetian and Jackson 1997, Jackson and Colmer 2005). Besides, it is known that fundamental value of functioning of chloroplasts in providing of plant both the synthesis of carbohydrates and oxygen (Medvedev 2004). Taking above noted data and the results of our investigation about the presence of chloroplasts in the cells of root cortex, we could to suppose that photosynthesizing cortex

cells of *T. natans* adventitious roots with very developed aerenchyma participate actively in overcoming hypoxia that possible came at flowering stage. it is possible that oxygen, which store into cortex aerenchyma of adventitious roots, transport from aerenchyma cavities in both parenchyma cells and on the surface of root epidermal cells, where, as known, the oxidization of metals ions is occurred (to the reductive forms). Because, metal ions are able to settle on the surface of submerged adventitious roots, these ions block an absorption and transport of nutrients from a water environment into a root (Crawford 1983).

An anatomic structure of particles in dissected submerged leaves of *T. natans* at the stage of flowering is differed greatly from structure of floating leaf. Centric structure, the presence of regular photosynthesizing tissue and chloroplasts in epidermis, the absent stomata and also poorly developed vessel system are promote to adaptation of such leaves to submerged environment by analogical to leaves of real hydrophytes or some air-aquatic plants (Bercu and Fagaras 2002, Bercu 2004, Nedukha 2011).

Besides, it is necessary to note that aquatic nut submerged dissected leaves don't have stele, exoderm and pericycle that is characteristic only for roots (Ezau 1980). On the basis of the received our results and above marked information of literature, we consider the dissected green submerged organs of *T. natans* as leaves, and pink adventitious roots as well as structure of auxiliary photosynthesizing roots, which is formed at the stage of plant flowering.

So, we showed the presence of syringyl and guaiacyl monolignols not only floating leaves, but also in submerged leaves and submerged adventitious roots of *T. natans*. The content of separate monolignols in investigated organs is depended on tissue type and plant organ. There were the general and differentia signs concerning these monolignols in leaves and roots. General signs were: 1) presence of syringyl and guaiacyl at the investigated species regardless of conditions of leaf growth; 2) almost identical (sufficiently great) content of S/G in epidermal walls and vessels cells of all organs, and 3) certain polarity of S/G ratio, that characteristic for every organ. Differentia cytochemical signs were: relative content of syringyl and guaiacyl in tissues of leaves and roots, and also different quantity of S/G tissues of all studied organs. Similar phenomenon was described in *Myriophyllum spicatum, Potamogeton pectinatus* and *P. perfoliatus* submerged leaves (Nedukha 2015).

We revealed that nature submergence environment effected on increase of guaiacyl in photosynthesizing parenchyma of submerged leaf in comparison with that in palisade and spongy mesophyll of floating leaves. Besides we discovered the reliable increase of total amount monolignols (S+G) in epidermis and mesophyll of submerged leaves in comparison with that in floating leaves. Extremely high content of monolignols (S+G) was in both epidermis and in cell walls of root stele.

The revealed high content of syringyl, total amount monolignols (S+G) in cell walls of *T. natans* submerged leaves and adventitious roots can to explain the next manner. According to experimental data the increase of quantity of S/G, as a chemical barrier, is intensificated of cell defense from penetration of water and pathogens (Menden et al. 2007). Besides, the sign (S/G) testifies to the increase of mechanical durability of cells (Christiernin 2006). Take account of these data and the evidence that *T. natans* submerged leaves and submerged roots constantly are situated in a contact with a surrounding water micro flora, we suggest that the increase of monolignols in epidermis (notable in periclinal walls) serve as protection from possible invasion pathogens from submerged environment. Besides, it is

possible, that more content of monolignols in walls of epidermal tissues of submerged organs also is helped opposition to water pressure on surface of submerged organs.

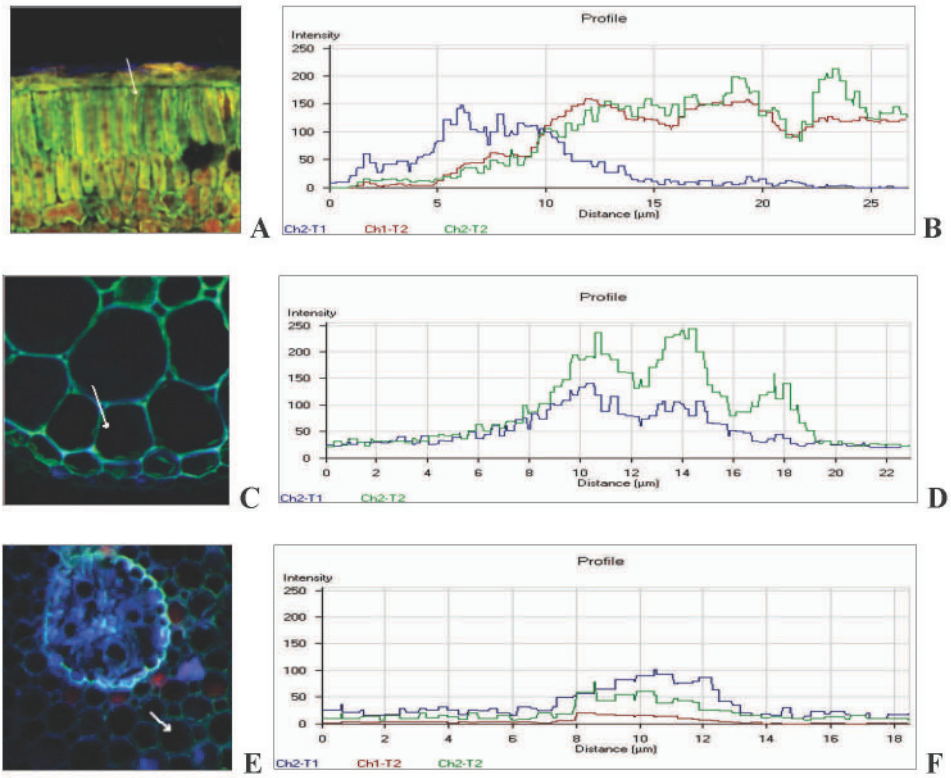

Figure 3. Micrographs of cytochemical fluorescence of monolignols and histogram of fluorescence intensity of syrigyl, guaiacyl and chlorophyll auto fluorescence intensity in the floating leaf (A,B), submerged leaf (C,D) and adventitious root (E, F) cells of Trapa natans. On the histograms (B, D, F) of fluorescence intensity of syrigyl (blue line), guaiacyl (green line) and chlorophyll auto fluorescence intensity (red line) is shown. Ordinate — fluorescence intensity, relative units (pixels). Abscissa — distance (µm), which was scanned on the (A, C, E). This distance is shown as white line on the (A, C, E)

Conclusions

1. The study of an anatomic structure of *Trapa natans* adventitious roots shows that roots are characterized by typical primary structure, well developed cortex, the presence radial aerenchyma and chloroplasts. Stele has a structure, which is typical for roots dicotyledonous.

2. Anatomical structure of *T. natans* submerged leaves differ from floating leaf anatomical structure. Mesophyll of the submerged dissected leaves is not differentiated, its homogeneous with plenty of chloroplasts, stomata are absent.
3. Monolignols (syringyl and guaiacyl) were revealed in cell walls of *T. natans* submerged and floating leaves and also in adventitious roots by the laser scanning microscopy. The location and content of monolignols are depended from organs, tissue and environment conditions. It was found that natural flooding is caused the distribution of monolignols on the tissues. The most content of monolignols was in walls of epidermis and vessels of submerged leaves and also in cell walls of stele and epidermis of submerged adventitious roots.

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PROFILES OF LEAF VOLATILE COMPOUNDS IN HORSE CHESTNUT TREES DIFFERING IN SUSCEPTIBILITY TO HORSE CHESTNUT LEAF MINER (*CAMERARIA OHRIDELLA* DESCHKA & DIMIĆ)

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Abstract. Various species of *Aesculus* have been attacked by the larvae of *Cameraria ohridella* for over 20 years causing the damage to leaves and loss of their aesthetic values. The members of the genus *Aesculus* are characterized by diverse susceptibility to *C. ohridella*. To better understand the biochemical relationships between the pest and its host plants we examined three individuals of the genus *Aesculus* which differ in susceptibility to this leaf miner: *Aesculus* × *neglecta* (resistant) and two individuals of *Aesculus hippocastanum* (more and less susceptible). In these trees we determined during the growing season the profile of volatile compounds emitted by leaves. The results have shown that each horse chestnut tree has different qualitative and quantitative profile of volatiles. Within 165 identified volatile compounds in leaves of all examined trees 104 volatiles were detected in resistant to the pest *Aesculus* × *neglecta* but only 72 and 81 in leaves of *Ae. hippocastanum,* less and more susceptible individual, respectively. Among these compounds 53 were specific (not found in other individuals) for *Aesculus* × *neglecta* tree, 23 were specific for more and 24 for less damaged *Ae. hippocastanum*. However, in more susceptible *Ae. hippocastanum* individual the highest percentage of specific volatile compounds was also shown at the beginning and in the middle of the growing season but amounted to only 10%. In less susceptible *Ae. hippocastanum* the highest percentage of specific volatile compounds was shown in the middle (10%) and at the end (20%) of the growing season. Possible repellent or attractant function of volatile compounds and their impact on differences in resistance of studied horse chestnut trees to *C. ohridella* is discussed.

Key words: horse chestnut leafminer, horse chestnut, plant volatiles, pest susceptibility

Introduction

Aesculus trees are a valuable element of green areas in European towns. For over 20 years, a threat to the esthetic values of horse-chestnut trees has been posed by the inconspicuous moth horse-chestnut leaf miner (*Cameraria ohridella* Deschka & Dimić, Lepidoptera: *Gracillariidae*), first recorded in Macedonia in the 1970's and since 1984 rapidly

spread over central and western Europe (Valade et al. 2009). In Poland *Cameraria ohridella* was observed for the first time in 1998 (Łabanowski and Soika 1998). This pest feeds on different species of *Aesculus* trees causing damages of leaf blades and defoliation. The main host of horse chestnut leafminer is white chestnut (*Aesculus hippocastanum*). A very susceptible to the pest is also Japanese horse chestnut (*Aesculus turbinata*). Other Asian species like *Aesculus assamica, Aesculus chinensis* and *Aesculus indica* are resistant to *C. ohridella*. The resistant species are also: *Aesculus parviflora*, *Aesculus wilsoni*, *Aesculus californica.* Moreover, some species (*Aesculus sylvatica*, *Aesculus pavia*, *Aesculus flava*, *Aesculus glabra*) are colonized by females that deposit eggs on leaves surface but most larvae die during the development (Kenis et al. 2003). The separate group includes interspecific hybrids *Aesculus* × *carnea* and *Aesculus* × *neglecta*, which are resistant or rarely attacked by the pest (Straw and Tilbury 2006, D'costa et al. 2013, 2014). In 2006 red horse chestnut was described as a tree completely resistant to *C. ohridella* (Straw and Tilbury 2006). However, next year other authors have observed that this species was inhabited by horse chestnut leafminer (Dzięgielewska and Kaup 2007). Also the differences in susceptibility to *C. ohridella* between individuals of *Aesculus pavia* have been described (Ferracini et al. 2010). Our several-year observations revealed that individuals of white chestnut are also not equally susceptible to this pest. We have noticed that individuals of *Ae. hippocastanum* growing in the same area, often close to each other, are colonized by horse chestnut leaf miner in varying degree.

The interaction between horse chestnut trees and moth begin from the identification of host plants by imago. It is a very important stage, because the larvae of *C. ohridella* do not have legs and cannot change the place of feeding. An important role in plant-pest interactions is played by volatile compounds, which are emitted by leaves or flowers (Das et al. 2013). Volatile compounds present in leaf blades of horse chestnut trees belong mostly to green leaf volatiles (GLVs) and its profile is characteristic for the species (Schwab and Scheloske 2006). The volatile compounds affect directly the behavior of females which influence the quantity and distribution of eggs deposited on leaves. They can act as attractants or repellents for insects being one of the factors which determines the susceptibility or resistance to this phytophagous insect (Johne et al. 2006 a, b).

The aim of the study was to performe the qualitative and quantitative analysis of volatile compounds emitted by leaves of *Aesculus* trees varied in resistance to horse-chestnut leaf miner. The research was focused on resistant to this pest painted buckeye (*Aesculus* × *neglecta* var. *lanceolata Sargent*) and two individuals of white horse chestnut trees. Our several-year observations revealed that these two individuals of *Ae. hippocastanum* are not equally susceptible to this pest. One of them is colonized by the pest earlier and leaves are damaged to a greater extent than in the case of the other. We presume that leaves of *Aesculus* trees, which are inhabited by *C. ohridella*, may contain attractive volatiles. In contrast, resistant trees probably emit volatile compounds that act as repellents for the moths. The obtained results may allow elucidation of the relationship between preferences of this horse chestnut leaf miner and the chemical composition of plants attacked which could be useful to create effective methods of trees protection.

Material and Methods

The research was conducted on three individuals of genus *Aesculus* which grow in Poznan: two *Ae. hippocastanum* individuals grow at the campus of Poznań University of Life Sciences and one of *Ae.* × *neglecta* located in the Botanical Garden of the Adam Mickiewicz University. Fully developed compound leaves were collected from each tree every 14 days, at the same time of day (between 8 and 9 am) from June to September 2013. Randomly chosen leaves always came from the same and similarly sun exposed place within the tree crown.

In the course of the experiment, simultaneously with the collection of plant material, in every 14 days, observations were carried out on the extent of damage caused by the feeding of *C. ohridella*. The degree of leaf damage was evaluated visually by estimating the size of leaf blade occupied by mines in relation to the total leaf area and it was expressed in percentages. Each time the assessment was performed on randomly chosen 15 compound leaves from each tested individual. The assessment was always carried out by the same person.

Collected material was put into glass containers and transferred to the laboratory within 30-60 min. Plant material (500 mg) was homogenized with 5 cm^3 methanol and filtered through cotton wool. The estimation of volatile compounds in extract was performed by coupled gas chromatography and mass spectrometry (GC-MS) in scan mode (33 – 400 amu) using gas chromatograph Agilent 7890 coupled with mass spectrometer Agilent 5975. The GC-MS method was developed by authors. The GC was equipped with split/splitless injector and a capillary column Supelco SPB -1 (30 m × 0.25 I.D., 0.25 µm). Linear velocity of mobile phase (He 99,9999%) was set at 45 cm/sec. Samples were injected into injector in splitless mode at temperature of 300 °C. The GC oven was programmed from 50 °C for 1 minute, then, 15 °C/min to 230 °C and 25 °C/min to 320 °C. The received mass spectra was compared with the NIST mass spectral library 2011 (National Institute of Standards, and technology, Gaithersburg, MD USA). Only compounds identified with the probability of at least 75 % were taken into consideration. Analyses for each tree and date were assessed in three independent replications. Each replication was a sample of plant material which derived from the middle leaflet of different compound leaves.

Statistical analyses

The repeated measurements for the same experimental units in time suggest applying the profile analysis of variances for longitudinal data. However, the assumptions of normality of distribution and homogeneity of variances were not fulfilled for the obtained data. Thus the Ward's method of cluster analysis (Milligan 1980) for data transformed using Perkal method (Runge 2007), were applied. The Perkal method reduces the obtained results to a common measure without losing the structure of the studied issue. Moreover, the modified Andrews curves were used for the evaluation of the profile of leaf volatile compounds (Khattree and Naik 2002). These curves were designated for the interval $[-\pi;\pi]$. This methods is useful to show differences in leaf chemical composition of particular individuals of *Aesculus* trees, as well as to reveal the difference in leaf chemical composition during the growing season. The statistical calculations were conducted using STATISTICA 10

package but the Andrews curves were prepared using the program written in SCILAB package.

Results

The greatest damage of leaves was found in more susceptible individual of *Ae. hippocastanum* (Fig. 1). Leaf blades area which was covered with mines in this individual increased progresivelly from the beginning of June to the end of the growing season. However, in less susceptible tree of *Ae. hippocastanum* mines area increased not earlier that at the end of July. At the end of the growing season mines area occupied 90% and 60% of leaf blade in more and less susceptible white horse chestnut individual, respectively. The leaf blades of resistant *Ae.* × *neglecta* were inhabited by single females but the symptoms of feeding were never observed.

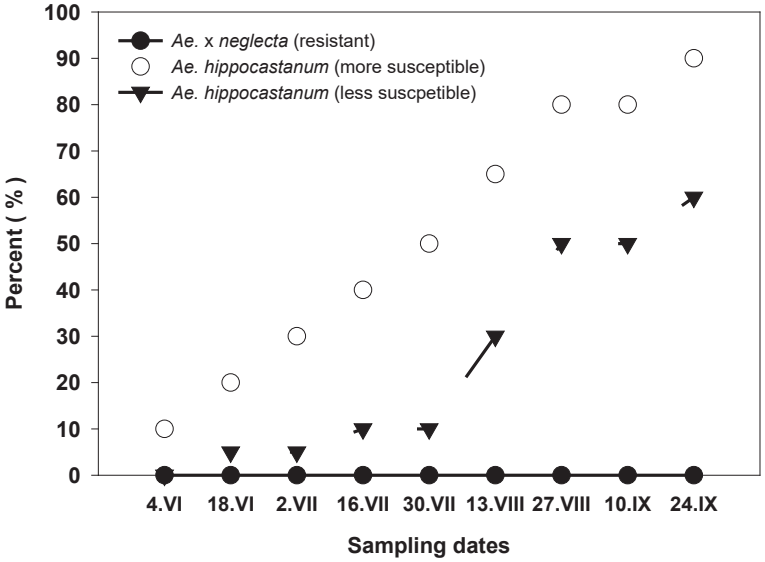

Fig. 1. *The damage of leaf blades in two individuals of white horse chestnut (Aesculus hippocastanum) and painted buckeye (Aesculus* × *neglecta)*

Figure 2 shows results of analysis of profiles, of volatile compounds emitted by examined trees, made on the basis of Andrews curves. Arguments of Andrews functions are different volatile compounds. The curves take into account both qualitative and quantitative composition of volatile compounds in the leaves of chestnut trees throughout the growing season, regardless of the date of sampling. The shape of curve belonging to *Ae.* × *neglecta* is clearly different from the shape of curves for two *Ae. hippocastanum* (more and less susceptible) individuals. Some differences are also visible in the shapes of curves charac-

teristic for *Ae. hippocastanum* individuals. The results of performed analyses revealed that each horse chestnut tree has different qualitative and quantitative profile of volatiles.

A more detailed analysis of differences in the profile of volatile compounds between examined *Aesculus* trees was performed using cluster analysis. The results were shown on two diagrams.

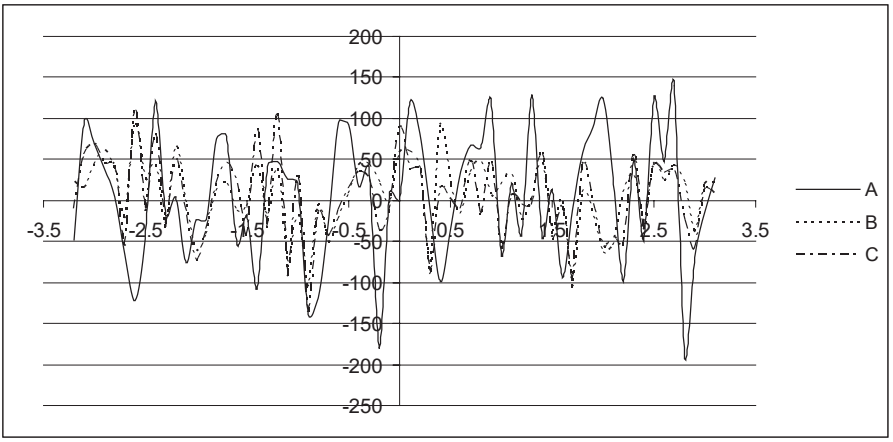

Fig. 2. The curves of the profile of volatile compounds emitted by the Aesculus × neglecta and two individuals of Aesculus hippocastanum, designated using Andrews functions (horizontal axis - radians, vertical axis - values of function); A – Ae. × neglecta (resistant); B – Ae. hippocastanum (more susceptible); C – Ae. hippocastanum (less susceptible)

Figure 3 shows the results of cluster analysis performed for the qualitative and quantitative composition of volatiles compounds emitted by trees in each sampling term. The horizontal axis of the diagram shows the distance between clusters, while the vertical axis grouped trees and sampling terms, taking into account the profile of occurring compounds. The diagram was cut off at a distance of 17 and three clusters can be distinguished. The first cluster, from the bottom, includes both *Ae. hippocastanum* trees (B, C) in terms from 1 (04.VI) to 5 (30.VII). The second cluster includes *Ae. × neglecta* (A) in term 7 (27.VIII) as well as both *Ae. hippocastanum* individuals in term 6 (13.VIII). The third cluster mainly includes terms from 7 (27.VIII) to 9 (24.IX) for both *Ae. hippocastanum* individuals (B, C), terms 3 (02.VII) to 4 (16.VII) for *Ae. × neglecta* (A) and also term 4 (16.VII) for more susceptible *Ae. hippocastanum* (B) tree. Although, *Ae. × neglecta* (A) was not included to any of the clusters in terms 1 (04.VI) and 6 (13.VIII).

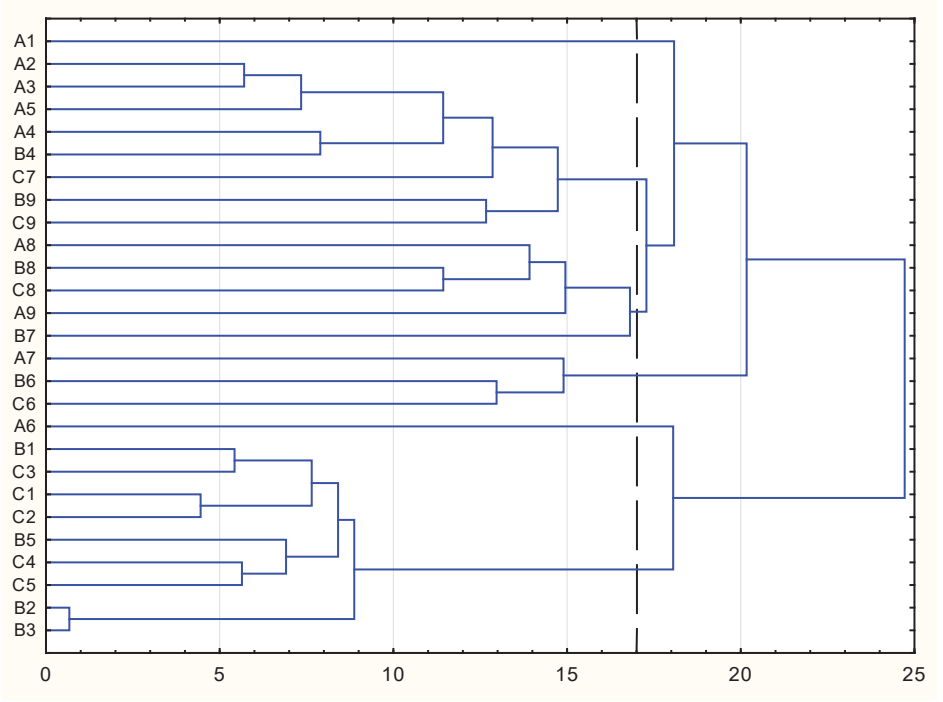

Fig. 3. Cluster analysis taking into account the quantitative and qualitative composition of volatile compounds emitted by leaves of examined chestnut trees depending on the sampling term (numbers 1-8);. A – Ae. × neglecta; B – Ae. hippocastanum (more susceptible); C – Ae. hippocastanum (less susceptible)

Figure 4 shows the results of cluster analysis for the occurrence of 165 identified volatiles compounds in examined horse chestnut trees. Volatile compounds present in the analyzed extracts are marked with consecutive numbers (1, 2, 3, ..., 165). The diagram was cut at the distance of 30, which allows to create four clusters, and part of the compounds outside the clusters. The first cluster, from the bottom, is formed by compounds emitted only by *Ae. × neglecta* (A). The second cluster includes the compounds emitted by all trees, in the third cluster are the compounds emitted mainly by *Ae. × neglecta* (A) and more susceptible *Ae. hippocastanum* (B). However, in the fourth cluster there are mainly volatiles emitted by both *Ae. hippocastanum* trees. The compounds outside the clusters of numbers 2, 3, 18, 54, 97, 100 – 103, 107, 113, 140, 146, 157, 164 are emitted by *Ae. × neglecta* (A). Next, not grouped volatiles are the compounds of numbers 1, 7, 56, 67, 68 emitted by more susceptible *Ae. hippocastanum* (B) and 9, 20, 53 69 emitted by less susceptible *Ae. hippocastanum* (C).

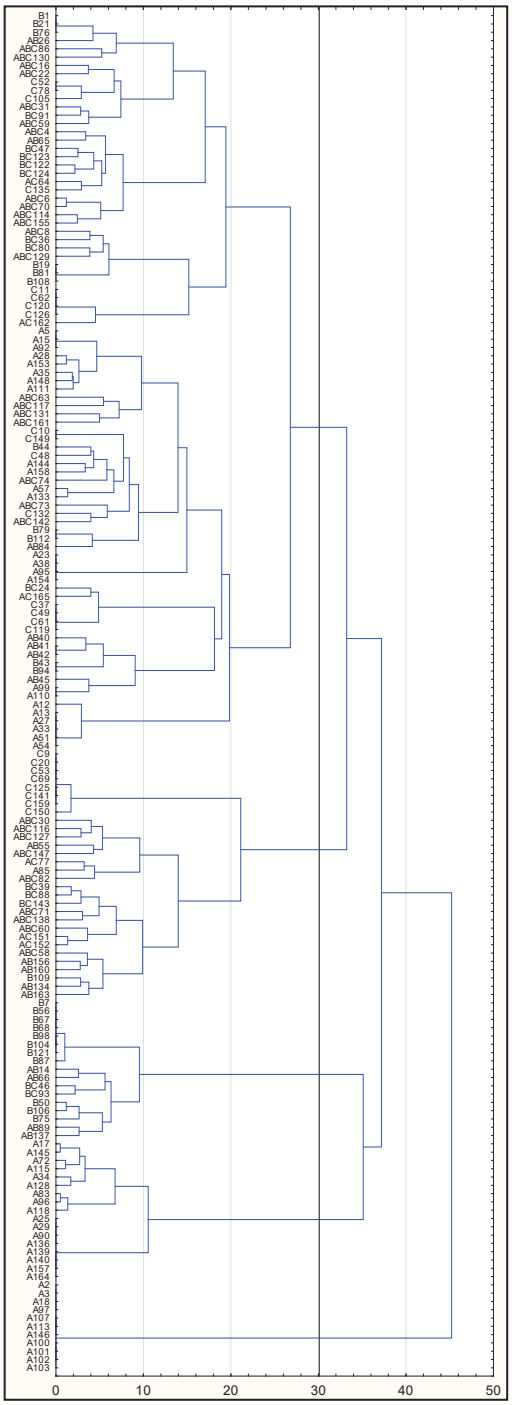

Fig. 4. Cluster analysis of volatile compounds (numbers 1-165) emitted by horse chestnut leaves; A – Ae. × neglecta; B – Ae. hippocastanum (more susceptible); C – Ae. hippocastanum (less susceptible)

Within 165 volatile compounds identified during the growing season in leaves of all examined trees 104 volatiles were detected in resistant to *C. ohridella* painted buckeye but only 72 and 81 in leaves of white horse chestnut less and more inhabited and damaged by the pest individual, respectively (Tab. 1). Among these compounds 53 were specific (not found in other) for painted buckeye horse chestnut tree, 23 were specific for more damaged and 24 for less damaged white horse chestnut.

Table 1. Quantitative composition of volatile compounds present in the investigated trees

Amount of volatile compounds presents in leaf blades of examined *Aesculus* trees			Amount volatile compounds characteristic for particular individuals of *Aesculus* trees		
Ae. × neglecta resistant	*Ae. hippocastanum*		*Ae. × neglecta* resistant	*Ae. hippocastanum*	
	more susceptible	less susceptible		more susceptible	less susceptible
103	81	72	53	23	24

Fig. 5 shows the percentage of characteristic volatile compounds in relation to their entire pool in each sampling date during the growing season in all examined horse chestnut trees. In the entire pool of volatile compounds emitted by leaves of *Aesculus × neglecta,* resistant to horse chestnut leaf miner, characteristic volatiles accounted for a bigger pool than in both individuals of *Ae. hippocastanum*. The percentage of characteristic volatile compounds in *Aesculus × neglecta* was notably high (40%) at the beginning of the growing season, next decreased to 10% in terms 18.VI to 30.VII. It increased in the subsequent date (13.VIII) to 30% than declined gradually to the level of 15% at the end of the growing season. However, in white horse chestnut trees specific volatile compounds accounted only for 3 to 20% of their entire pool. In more susceptible white horse chestnut tree the highest percentage of specific volatile compounds was at the level of 10% at the beginning (04.VI) and in the middle (16.VII and 30.VII) of the growing season. However, in less susceptible white horse chestnut the percentage of specific volatile compounds in the middle (16.VII) and at the end (24.IX) of the growing season, amounted to 10% and 20%, respectively.

The highest abundance of characteristic compounds in the most of terms was in *Aesculus × neglecta* (Tab 2). In leaves of this white horse chestnut tree the highest quantity was detected at the beginning of the growing season and from 13th August to the end of September. Moreover, it is worth noting that the most of volatiles found in this horse chestnut at the beginning of the growing season (04.VI) did not appear in the following terms (Tab. 2). However, in both individuals of white horse chestnut since 04.VI to 30.VII was detected solely single characteristic volatile compounds. Just from 13.VIII in less susceptible and from 27.VIII in more susceptible individual the quantity of characteristic volatile compounds significantly increased.

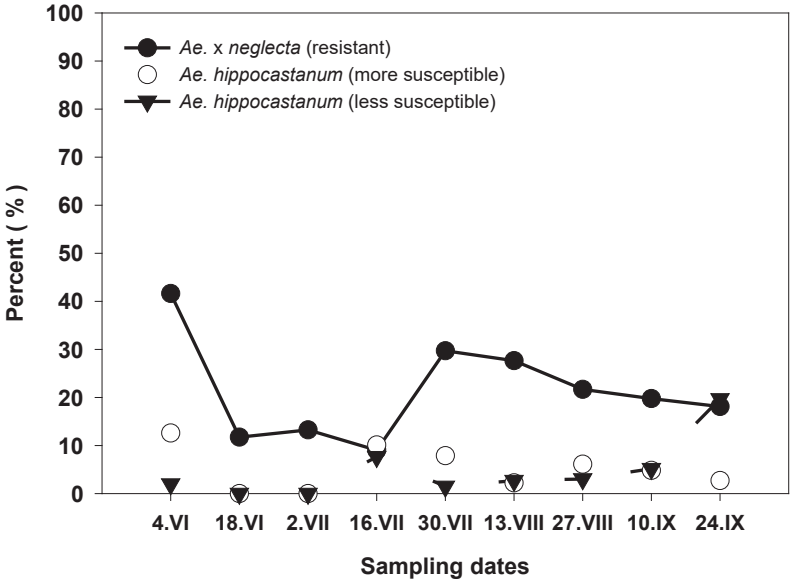

Fig. 5. The percentage of volatile compounds characteristic for examined Aesculus trees in relation to their entire pool during the growing season

Table 2. Volatile compounds characteristic for examined Aesculus trees found in subsequent dates during the growing season

Sampling dates	*Ae.* × *neglecta* resistant	*Ae. hippocastanum* more susceptible	*Ae. hippocastanum* less sussceptible
04.VI.2013	2,3, 18, 97, 100, 101, 102, 103, 107, 113, 118, 133, 146,	44	132
18.VI.2013	57, 118, 133		
02.VII.2013	144, 158,		
16.VII.2013	99, 110,	43, 94,	10, 149
30.VII.2013	85, 144,	79, 112,	48
13.VIII.2013	17, 25, 29, 34, 57, 72, 83, 90, 96, 115,118, 128, 136, 139, 140, 145, 148, 157, 158, 164	50, 75, 106, 109,	9, 20, 53, 69, 125,135, 141, 150, 159,
27.VIII.2013	12, 23, 35,38, 57, 72, 83, 95, 96, 111, 115, 118, 128, 148, 153, 154,	7, 50, 56, 67, 68, 75, 87, 98, 104, 106, 121,	37, 49, 61, 105, 119, 135, 150,
10.IX.2013	12, 13, 17, 27, 28, 33, 34, 35, 51, 54, 72, 115, 128, 144, 145, 148, 153,	1,21, 50, 76, 87, 106,	52, 78, 105, 135,
24.IX.2013	5, 15, 28,34, 35, 92, 111, 128, 133, 144, 148, 153,	19, 81, 108,	11, 62, 120, 126, 135

Discussion and conclusions

The obtained results show that, every *Aesculus* tree included in the experiment had a unique profile of volatile compounds emitted by leaves. Likewise, in research concerning other species of *Aesculus* trees (*Ae. × carnea, Ae. pavia, Ae. flava, Ae. glabra, Ae. parviflora*) it has been shown that volatile compounds profile of leaves was largely dependent on tree species (Schwab 2009). In the presented research significant differences were seen between painted buckeye tree and white horse chestnut trees. However, certain quantitative and qualitative differences in volatile compounds between individuals of white horse chestnut tree divers in susceptibility to pest were detected. Therefore, one can suppose that differences in volatile compounds profile is one of the factors which determine the susceptibility of the analyzed *Aesculus* trees to *C. ohridella.*

In the presented research it has been shown that leaves of painted buckeye contained 53 volatile compounds characteristic only for this tree. Less than half quantity of characteristic compounds was detected in the analyzed white horse chestnut trees. Furthermore, in the first sampling dates *Aesculus × neglecta* emitted markedly more characteristic volatile compounds than white horse chestnut trees, and a lot of these compounds were presented only in this time. Taking into account the fact that volatile compounds play an important role in the localization of the host plant by *C. ohridella* moths (Johne et al. 2006 a), one can assume that painted buckeye was not inhabited by this pest, because it emitted repellent volatile compounds. Repellent volatile compounds produce by plants were observed also in the interaction between tobacco and *Heliothis virescens* moths (De Moraes et al. 2001), and aphids and wheat seedlings (Quiroz et al. 1997). In the first sampling date (4.VI) only one characteristic volatile compound was detected in white horse chestnut more susceptible to the pest. However, the level of this compound comprised 15 % of all volatile compounds emitted by leaves in this term. At this time there was the flight of the first generation of *Cameraria ohridella* moths, which suggests that these molecules are attractants for the pest. Johne et al.(2006 a, b) demonstrated that horse chestnut leaf miner adjust their reaction to the changes in volatile compounds profile and occurrence of only one chemical compound in low concentration may determine the responses of insects. Johne (2006 b) demonstrated that green undamaged leaves of horse chestnut produce volatile compounds acting as attractants. Larvae hatched from eggs deposited on that healthy leaves have an abundance of food (Johne et al. 2006 b, Svatos 2009). In more damaged white horse chestnut was shown an increase in the amount of characteristic volatile compounds in the middle of the growing season (16.VII and 30.VII), but it was other compounds than in the early sampling dates. At this time the percentage of mines covering the leaf blades was more than 40%. Johne et al. (2006 b) show that older and damaged leaves of white horse chestnut emitted volatile compounds which repel *Cameraria ohridella* moths, and therefore impact the degree of plants settlement by horse chestnut leaf miner. The moths detected a signal that this tree is inhabited largely and there may be not enough food for the next generation of larvae. In this paper it has been shown that in white horse chestnut (less susceptible), which was inhabited to a little extent by larvae of *Cameraria ohridella* until the middle of the growing season, was observed increased quantity of characteristic volatile compounds a week earlier – 16th July. From this time the quality and quantity of volatile compounds changed in comparison to their level at the beginning of the growing season. In

each subsequent term new volatile compounds occurred which were not detected earlier in the profile of this individual. These changes were correlated with the increase in the intensity of larvae feeding on this individual. It is suggested that volatile compounds emitted at this time by leaves of this white horse chestnut individual may act as atractants to *Cameraria ohridella*. Similarly Johne et al. (2006 b) and Schwab (2009) revealed that profile of volatile compounds emitted by horse chestnut leaves changes during feeding. However, their observations indicates that that there gradually appear volatile compounds which act as deterrents (furanoid, decanal) on female moths and inhibit the deposition of eggs. Schwab (2009) has shown that leaves of white horse chestnut trees inhabited by the pest were characterized by the decreased level of green leaf volatiles and increased level of total terpenoids.

Summing up the obtained results it can be concluded that the differences in profile of volatile compounds revealed in examined *Aesculus* trees during the growing season may be one of the causes of their varying susceptibility to *C. ohridella*. However, more detailed research is needed to clarify this complex relationships and to find the causes of this varing susceptibility. This is a subject of our current research.

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MKK2-DEFFICIENCY AFFECTS GROWTH BY INCREASED EFFICIENCY OF THE PHOTOSYNTHETIC APPARATUS IN *ARABIDOPSIS THALIANA*

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Abstract. The Mitogen activated protein kinases (MAPK) cascades plays a crucial role in various intracellular transduction pathways in plants. In experiments *mkk2* insertional *T-DNA* mutants (SALK line) of *A. thalina* were used. *Mkk2* mutants exhibited a faster growth rate and increased biomass production in comparison to wild type (WT) plants. These changes were accompanied with increased net CO_2 assimilation rate (P_N), stomatal conductance (g_s), and transpiration (E). Moreover, an improvement in photosystem II (PSII) electron transport rate (ETR(II)) and effective PSII quantum yield (Y(II)) in *mkk2* plants were observed, indicating increased PSII efficiency . The obtained results suggest that MKK2 might play an important role in optimization of growth and developmental responses in *Arabidopsis via* photosynthetic activity.

Key words: *Arabidopsis thaliana*, MAPKs cascade, stress, photosynthesis.

Abbreviations:

ETR - electron transport rate,
mkk2 - mutant plant,
MKK2 - mitogen activated protein kinase kinase 2,
ROS - reactive oxygen species,
WT - wild type

Introduction

The communication between cells, tissues and environment is very important to maintain cellular homeostasis and to regulate the organisms responses to external stimuli. A key role in plant acclimatization to stresses play mechanisms based on perception, integration and intracellular signal transduction of external and internal stimuli. Here, we focused on the kinase cascade, a crucial intracellular transduction pathway for all *Eukaryotes.* The basic mitogen activated protein kinase (MAPK) cascade consists of three levels (Fig. 1). The first component, a MAPK kinase kinase (MAPKKK), activates a MAPK kinase (MAPKK, MPKK or MKK). In turn, phosphorylated MAPKK activates the third component of the pathway, *i.e.,* MAPK. Downstream MAPK activates components of other intracellular pathways, *i.e.* other protein kinases, efector proteins, transcription factors or the components of cytoskeleton etc., orchestrating the plants response to a given stimuli. (Fig. 1, Jonak et al. 2002, Suarez-Rodriguez et al. 2010). In this context MAPKK (MKK) are involved in the transduction of various signals in plant cells *e.g* cold (Teige et al. 2004), pathogens infection (Doczi et al. 2007, Pitzschke et al. 2009b) and osmotic stress (Droillard et al. 2004, Teige et al. 2004).

In *A. thaliana MKK2* encodes a protein, which belongs to the MAPK kinase family (Ichimura et al. 2002 - MAPK Group). The role of MKK2, especially in the context of plant response to various abiotic stresses, has not been fully recognized. It is known that MKK2 integrates various environmental signals *e.g.* salinity, cold (Teige et al. 2004) and pathogen infection (Brader et al. 2007). MKK2 is also a regulator of downstream kinases: MPK4, MPK6, MPK10, MPK11 and MPK13, as it has been shown in yeast two-hybrid assays (Y2H) (Lee et al. 2008) and in some *in vitro* experiments (Qiu et al. 2008).

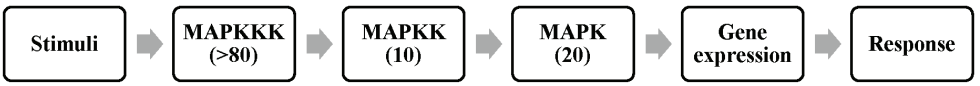

Fig. 1. MAPK-dependent signal transduction pathways in the plant cell (MAPK cascade). Numbers in brackets indicate identified kinases in Arabidopsis genome (www.arabidopsis.org).

MKKs, including MKK2, are very important due to their role in integrating and transduction of signals, earning the well deserved name of the molecular hub of MAPKs' cascade.

The aim of the research was to assess biometric and physiological parameters concerning photosynthetic activity of *A. thaliana mkk2* mutant in comparison to WT plants.

Materials and methods

Seeds of *Arabidopsis thalina mkk2* insertional T-DNA mutants (SALK_127284) in the Col-0 background (NASC -*The European Arabidopsis Stock Centre*) were germinated in COMPO soil in a 12 h photoperiod (100 ± 20 μmol · m^{-2} · s^{-1}). Plants were grown in the

light conditions in a phytotron chamber (21/ 17°C in the light/dark phases) and irrigated with tap water on daily basis.

Fresh weight and rosette area were determined after 6-7 weeks of growth. The surface area of the 6-week-old rosettes was determined using ImageJ (NIH, USA). The changes in chlorophyll *a* (chl *a*) florescence parameters from photosystem II (PSII): effective quantum yield of PSII (Y(II), electron transport rate in PSII (ETR(II)), non-photochemical energy loss in PS II - Y(NPQ) and quantum yield of non-regulated non-photochemical energy loss in PS II - Y(NO) of WT and *mkk2* mutants as a function of photosynthetically active radiation (PAR) were determined. Chlorophyll fluorescence was measured using the Dual-PAM-100 (Walz, Germany) system. Before the measurement plants were dark-adapted *ca.* for 20 min. gas exchange parameters, such as: net CO_2 assimilation rate (P_N), stomatal conductance (g_s), and transpiration (E) were determined with the portable LI-6400 (Li-COR Inc., Lincoln, NE, USA) system on 8-week-old plants.

Statistical analysis

The results were analyzed statistically by Student's *t-test* and analysis of variance (ANOVA). Additionally, Tukey's post-tests for analysis of selected data were used. All calculations were performed using the Sigma Plot 11.0 software.

Results and discussion

According to the obtained results, *mkk2* mutants yielded higher biomass compared to WT plants (Fig. 2A). No changes in rossete surface area were reported. (Fig. 2B). At the end of 9-week vegetation period *mkk2* plants were higher in comparison to WT (Fig. 3).

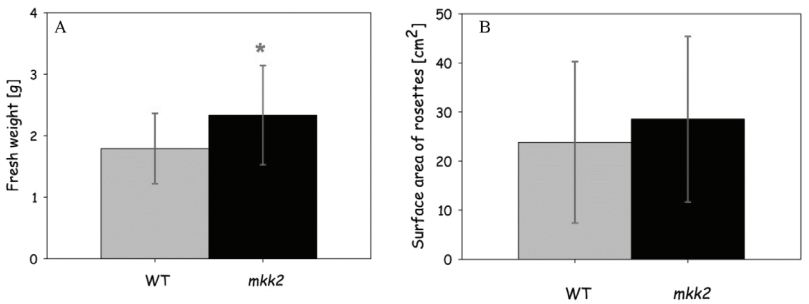

Fig. 2. (A) Fresh weight of 6-7-week-old A. thaliana plants. Data represents mean ± SD (n = 35). (B) Surface area of 6-week rosettes A. thaliana. Data represents mean ± SD (n = 58-59). asterisk above bar indicates statistically significant difference between WT and mkk2 mutants according to Student's t-test: (*) P < 0.05.

MKK2 deficiency resulted in higher plant yield, what may suggest significant alterations in its basic metabolism. On the base of the obtained results (Fig. 2 and 3) we formulated a hypothesis that increased photosynthetic activity is one of the key factors responsible for more efficient biomass production in *mkk2* mutants. To test the hypothesis, gas

exchange and chl *a* fluorescence from PSII were performed. All photosynthetic gas exchange parameters: net CO_2 assimilation (P_N), stomatal conductance (g_s), and transpiration (E) were increased in *mkk2* mutants in comparison to WT plants 27%, 37% and 33% respectively, at 100 µmol · m^{-2} · s^{-1} of PAR (Fig. 4). No differences were reported in higher light intensities. It should be underlined that such light was used during plant cultivation in the growth chamber (see: Materials and methods).

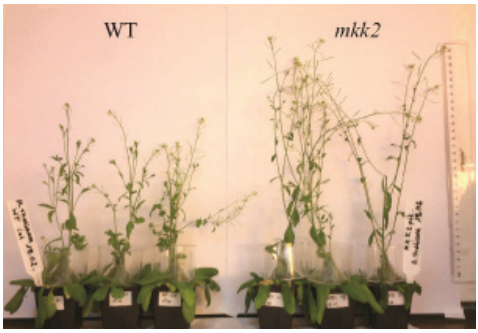

Fig. 3. Comparison of 9-week-old WT and mkk2 A. thaliana plants (phot. P. Zimak-Piekarczyk)

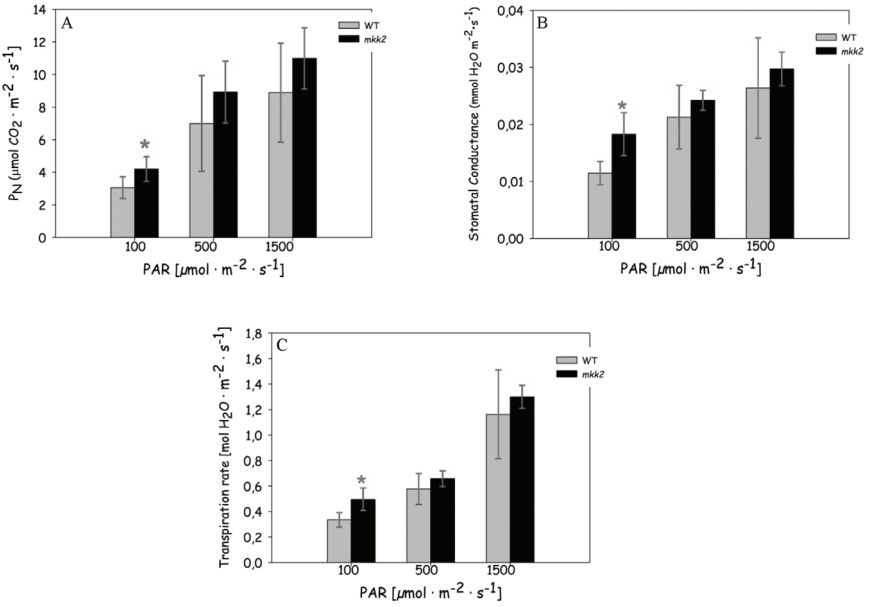

Fig. 4. (A) Net CO_2 assimilation rate (P_N), (B) stomatal conductance (g_s), and (C) transpiration rate (E) of 8-week-old A. thaliana plants. Data represents mean ± SD (n = 5). The asterisk above the bar indicates statistically sigtnificant differences between WT and mkk2 mutants according to Student's t-test:(*) $P < 0.05$.

The activity of the photosynthetic apparatus determined by chllorophyll *a* fluorescence also showed several changes between *mkk2* and WT plants. Increased values of *ca.* 16% ETR(II) and ca. 14% Y(II) indicate effective more efficient: 1) linear electron transport in PSII, and 2) operating quantum yield of PSII, respectively. On the other hand, decreased Y(NPQ) show reduced dissipation of absorbed light energy as heat (Baker, 2008; Murchie and Lawson, 2013). Y(NO) parameter indicated similar values in WT and *mkk2* plants. Higher values of ETR(II), Y(II), and lower values of Y(NPQ) at some light intensities in *mkk2* mutants indicate that *MKK2* somehow increases the efficiency of PSII in comparison to WT. However, significant changes in ETR(II), Y(II), and Y(NPQ) were observed at higher light intensities than *e.g.* the increase of P_N (Fig. 4 and 5), suggesting more sophisticated and subtle role of MKK2 in regulation of photosynthetic activity in *A. thaliana*.

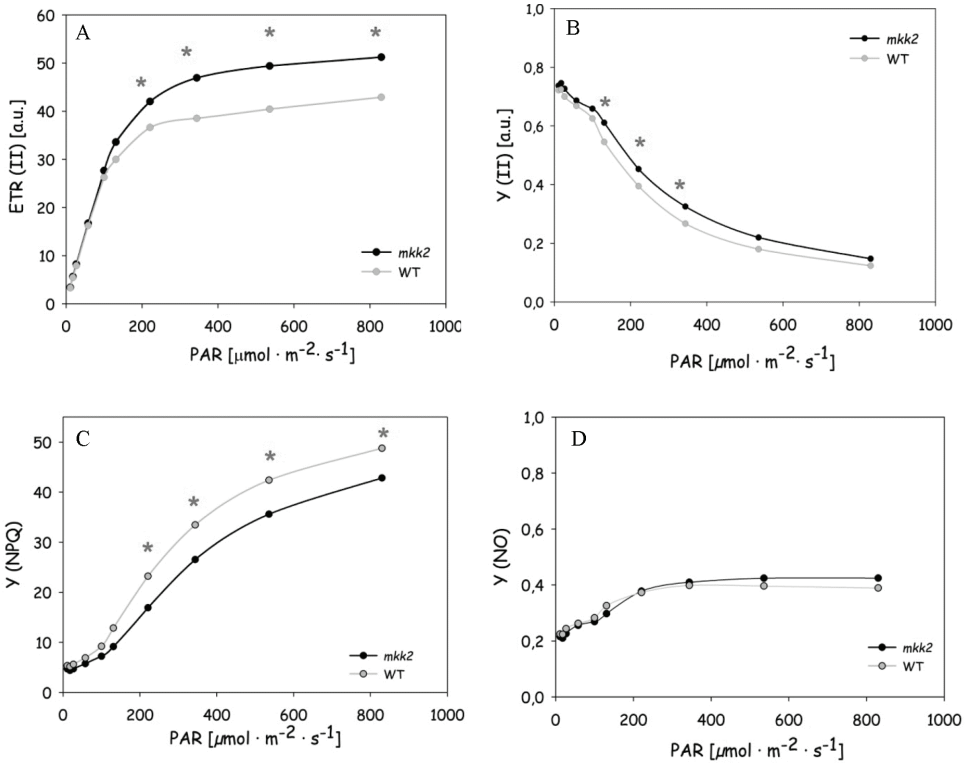

Fig. 5. (A) Photosynthetic parameters: electron transport rate of PSII(ETR(II)), (B) PSII operating efficiency –(Y(II)), (C) Quantum yield of regulated non-photochemical energy loss in PS II - Y(NPQ) and (D) Quantum yield of non-regulated non-photochemical energy loss in PS II - Y(NO) as function of photosynthetically active radiation (PAR) in control conditions. Data represents mean ± SD (n = 5). The asterisk above the bar indicates statistically sigtnificant differences between WT and mkk2 mutants according to one-way ANOVA and Tukey's post-test: (*) P < 0.05.

Conclusion

MKK2 plays a role in optimization of growth and developmental processes in *Arabidopsis via* photosynthetic efficiency. It seems that the *MKK2* is a negative regulator of growth and developmental processes in *A. thaliana*.

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LEAF MODEL OF CHLOROPHYLL A FLUORESCENCE PARAMETERS USED AS PHYSIOLOGICAL MARKERS OF MAIZE HYBRIDS ABILITY TO COPE WITH A SOIL COMPACTION STRESS COMBINED WITH LIMITED OR EXCESS SOIL WATER CONTENT*

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Introduction

Crop plants in natural habitats are subjected to different combinations of soil moisture and soil compaction stresses. Those stresses are multidimensional factors affecting plant growth, development and yield (McKersi and Leshmen 1994; Mittler 2006). It is acknowledged that those stresses have both general and specific impact on physiological processes (Masle 2002). Soil compaction, drought and waterlogging have been studied as a separate stress factors in different plant species and cultivars. High levels of soil compaction are caused mainly by natural processes and by the use of heavy machinery in soil cultivation. In the case of drought, the amount of rainfall does not compensate water loss through transpiration and evaporation, and in the case of waterlogging, the soil is inundated as a result of heavy rainfall or river floods which cause drastic decrease in roots' capability for water uptake as a result of decreased oxygen content in water. The degree of plant restriction by environmental stresses depends mainly on the species, variety and age of the plants. The tolerance of plant species to stress factors is determined by the plants genes, and their expression underlies interactions with actual meteorological conditions. Variations in the degree of tolerance to different stress factors are known to exist within genotypes of plant species and were highlighted in many studies. In numerous papers stress susceptibility indexes are used for comprehension and explanation – on the basis of morphological, physiological and molecular responses – of strategies implemented by plants to alleviate and/or remove an environmental stress influence (Golbashy et al. 2010; Liu 2010; Grzesiak et al. 2016).

The intraspecific variations within genotypes in response to different stresses are significant, though problems related to combined (multistress) influence of soil moisture and physical soil parameters have not been studied thoroughly. Generally, combined and simultaneous exposure to two or more abiotic stresses causes more harmful effect compared to a single stress. However, there are known examples where the effects of exposure to one

factor are alleviated by the other factor. For example, membrane injuries in plants exposed to gas air pollutants might be smaller if the plants are exposed to mild drought.(Ghannoum 2009; Lawlor and Tezara 2009).Both in stress-susceptible and stress-resistant species, changes of morphological and anatomical traits in leaves was observed. In leaves they include changes in their number, area, thickness, specific leaf area (SLA), thickness of epiderma cell and cell wall (Clark et al. 2003; Mommer and Visser 2005; Fageria et al. 2006). Maize is one of the most important cereals in the world and is cultivated under a wide range of climatic conditions. It is also an interesting research model because it has *i.a.* different types of photosynthesis (C4), of bundle sheath structure (Kranz-type syndrome) and of root system structure(Kono et al. 1987;Masle 2002; Mittler 2006; Ashraf 2010).

The fluorescence of chlorophyll *a* is a fast, non-destructive and non-invasive tool to study physiological state of photosynthetic apparatus and it is useful to study the different functional levels of photosynthesis indirectly processes at the pigment level, thylakoid electron transport reactions, primary light reactions, dark enzymatic stroma reactions and slow regulatory processes. At the leaf level, photosynthetic activity is estimated on the basis of chlorophyll fluorescence measurements (Maxwell and Johnson 2000; Medrano et al. 2002). Chlorophyll fluorescence measurements characterize individual elements of the photosynthetic apparatus, on the basis of parameters obtained during a single measurement (Bolhar-Nordenkampf and Öquist 1993). Chlorophyll fluorescence highlights the photochemical efficiency of photosystem II and the effectiveness of utilization of chlorophyll *a* excitation energy in the photosynthesis process. It allows one to estimate the level of openness of reaction centers and the amount of energy reaching the photosynthetic apparatus, which is released as heat (Maxwell and Johnson 2000; Lichtenthaler et al. 2004; Kalaji and Loboda 2010).

In experiments the chlorophyll *a* fluorescence was determined of differences in response to soil compaction stress between maize hybrids. The aim of this study was to compare the effects resistant (Tina) and sensitive (Ankora) in maize genotypes differing in their degree of sensitivity of the deficiency (D) or the excess (W) water in the soil and their simultaneous action with soil compaction stress.

Materials and methods

Plant material

The experiments were carried out using two maize single-cross hybrids (Ankora, Tina) obtained from SEMPOL-Holding Trnava, Slovakia.

Growth and treatment conditions

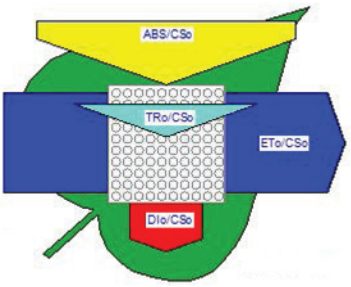
Plants were grown in an air-conditioned greenhouse under the following day/night conditions: temperature 23/18°C (±2.5°C) and relative humidity (RH) 70/60% (±5%), during a 14h photoperiod from 7 am to 9 pm (artificial irradiance from high pressure sodium lamps, Philips SON-T AGRO, 400 W). Photosynthetically active radiation (PAR) was equal to about 350 μmol m^{-2} s^{-1}. Plants were grown in pots (PCV tubes: X cm diameter, X

cm height filled with sand as soil substrate. One pregerminated grain was planted per pot at the depth of 2 cm. Air-dried sand was sieved in a 0.25 cm mesh and mixed with compound fertilizer: N – 28 mg, P – 18 mg, K – 14 mg per 1 kg of soil substrate. Two soil substrate compaction were applied, low (L–1.1 g cm^{-3}) and severe (S – 1.6 g cm^{-3}). Mechanical impedance in soil substrate was measured with penetrometer DIK 5520 (Daiki Rika Kogyo Co. Ltd., Japan).

Field soil capacity (FWC) was determinates according Kopecki method. Air-drained soil samples (110.0 and 160.0 g) were placed inside metal cylinders, with a 1-mm hole at the bottom. For all samples the volume was 100 cm^3. The cylinders with samples were placed inside a container with water for 30 minutes. After 8 h, maximal soil water content in samples was 0.47 and 0.39 g cm^{-3}, and after 48 h it decreased to 0.25 and 0.18 g cm^{-3}, respectively. According to Hillel and van Bavel (1976), the latter values were assumed to be 100% of FWC. During the experiment the PCV tubes were weighed every day, and the amount of water loss through evapotranspiration was refilled to keep the constant mass in each treatment. For control treatments (Lsc+C and Ssc+C) soil water content was maintained at the level of 65-70% FWC from sowing to 28th day. In drought treatment (D) soil water content was kept at the level of 30-35% FWC, and the pots were not watered for 14 days from 14th to 28th day (Lsc+D, Ssc+D). Similarly for waterlogging soil (W) water content was kept at the level of 100% FWC from 14th to 35th day (Lsc+W, Ssc+W) or from 14th to 35th day (L+W, M+W, S+W). In order to obtain waterlogging conditions PCV tubes were submerged in a container in which the water surface was 2 cm above the soil surface.

Measurements

Chlorophyll *a* fluorescence was measured with PEA - Plant Efficiency Analyzer by Hansatech and FMS-2 on a fully expanded leaf. During measurements the actinic light (330–660 nm) was set to 600 µmol (photons) $m^{-2}s^{-1}$ and the induction time was adjusted to 5 s. Prior to measurement leaf were shaded for 15 minutes using special clips. Basic fluorescence parameters were used for a graphic presentation as a leaf model with application of software BIOLYZER (BioenergetigsLaboratory, University of Geneva) (Fig. 1). The measurements was made after 35 days of plant growth under two soil compaction treatment (Lsc-low, Ssc- severe) and after 14 days of drought (D) or waterlogging (W) stress application.



ABS/CSo- absorption flux per CS approximated by Fo

ETo/CSo - trapped energy flux per CS

DIo/CSo - electron transport flux per CS

TRo/CS - dissipated energy flux per CS ○ ● – active or inactive reaction centers, respectively

Fig. 1. Imaging of chlorophyll a fluorescence as a leaf model in time 0 showing phenomenological energy flux per leaf cross section in plants grown under non-stressed condition

Results and Discussion

Drought and waterlogging stresses are the global problems in world agriculture. Drought stress occurs when the water accessible in the soil but its shortage and atmospheric circumstances cause permanent water loss by evaporation and transpiration. Waterloogging stress occurs as a result of heavy rainfall or river flooding. In this experiments determination of the effects of applied stressors (Lsc+D, Lsc+W, Ssc+D, Ssc+W) on the dynamics of chlorophyll fluorescence parameters with particular attention to changes assessed on the basis of the leaf model (JIP) in resistant (Tina) and sensitive (Ankora) maize single-cross hybrids.

In control plants (L_{SC}+C, S_{SC}+C) changes of ABS/CSo, ETo/CSo, DIo/CSo and TRo/CS presented as leaf models shows small differences between both maize hybrids (Fig 2). However significant changes of those parameters were observed in seedlings grown by 35 days under low or severe soil compaction and 21 days under drought (D) or waterlogging (W) conditions. In both maize hybrids and all parameters, differences between drought and waterlogging were observed, and in sensitive to soil compaction maize hybrid Ankora those differences were higher than for Tina. In comparison with control treatments parameters of ABS/CSo, ETo/CSo and DIo/CSo were decreased. Those changes in seedlings grown under severe soil compaction (S_{SC}+D, S_{SC}+W) were higher in comparison with treatment L_{SC} +D, LSC+W and also in seedlings grown under waterlogging condition were higher in comparison with seedlings grown under drought. In comparison to control treatments for parameters TRo/CS - dissipated energy flux per cross section were observed increase its values. Those increase were higher in both hybrids grown under severe soil compaction in comparison with seedlings grown under low soil compaction. Also TRo/CS were higher in seedlings grown under waterlogging conditions in comparison to drought and in sensitive to soil compaction hybrid Ankora than in resistant hybrid Tina.

The applied stresses (soil compaction, drought, waterlogging) caused changes in PSII activity of maize seedlings and the harmful effects of soil compaction stresses combined with drought or waterlogging on PSII activity were apparent in both hybrids. In low soil compaction both genotypes indicates that changes of fluorescence parameters under the waterlogging stress were higher than in soil drought. Whereas the severity of this effect was more visible in sensitive to soil compaction (Ankora) hybrid whereby we considered Tina as resistant hybrid where effects of stresses on PSII activity were less intense (Kalaji and Łoboda 2010)

The most pronounced changes in majority of studied chlorophyll fluorescence parameters were found for non-reducing QA reaction centers (inactive or silent) and electron transport flux per CS. were relatively much more resistant to combined stress factors. This indicates that genotypes resistant to soil compaction (SC) were more resistant to drought (D) or waterlogging (W) stresses and that genotypes resistant to D were also resistant to W. For most of the plants in waterlogged soils the capacity of roots to nutrients and water supply for plant development and growth is inhibited, growth of the shoot and root in waterlogged soil is also inhibited (Lichtenthaler et al. 2006; Kalaji and Łoboda 2010)

The degree of damage in plant is associated with the development and growth stage of plant, soil type, and environmental conditions. In study on water stress tolerance in maize has mainly focused on understanding the structural, morphological, anatomical, biochemi-

cal, and metabolic responses to oxygen deficiency in plant tissues (Subbaiah and Sachs 2003).

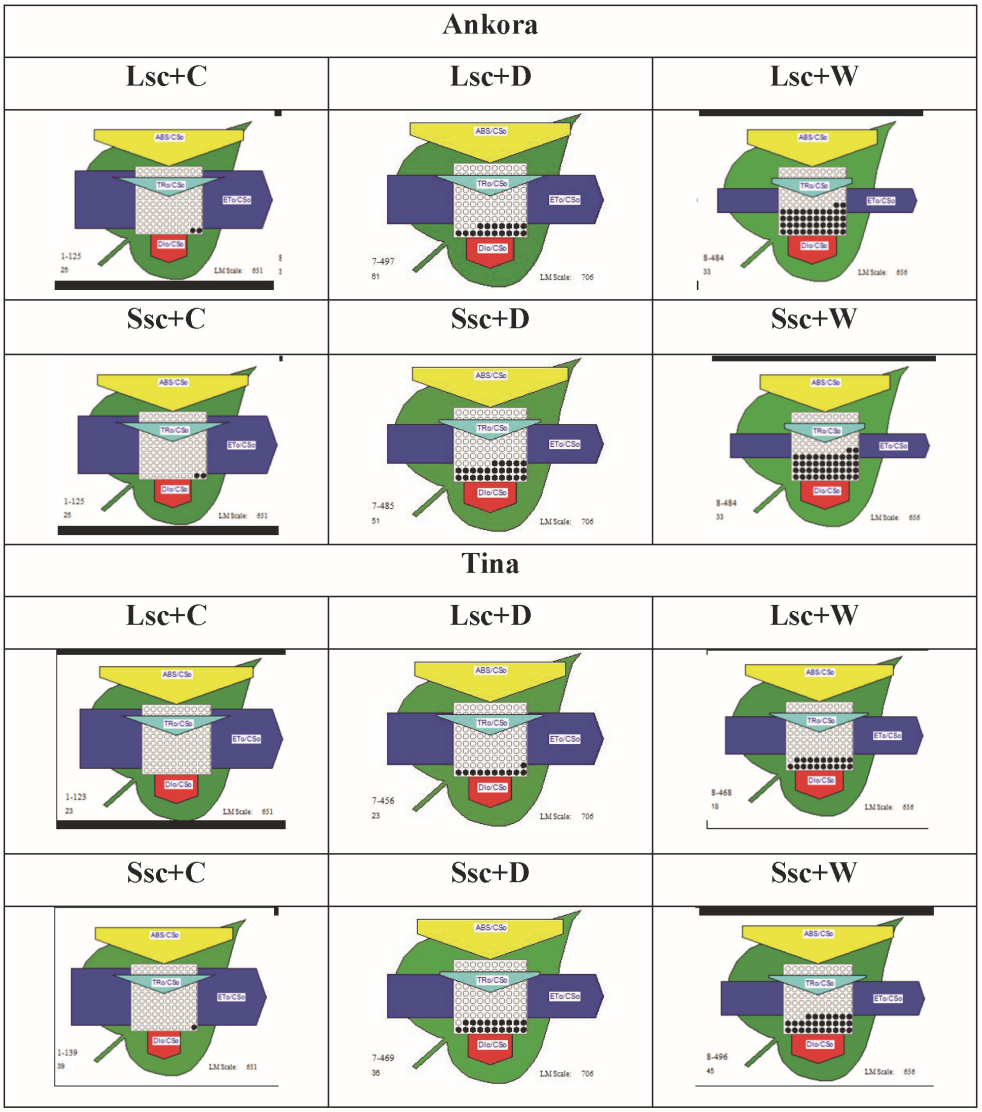


Fig.2. Leaf model for the sensitive (Ankora) and resistant (Tina) maize hybrid grown 35 days under low (Lsc) and severe (Ssc) soil compaction levels combined with drought (D) or waterlogging (W) stresses. For legend see fig 1.

It is known that maize is sensitive to drought and waterlogging at seedling stage, but less known is the fact that leaf stage is the most sensitive to waterlogging stress. Maize is also notably susceptible to soil drought which strongly influence grain yield. maize exhibits a reduction in the photosynthesis rate under the influence of soil drought. Drought stress inhibits plants photosynthesis by closing stomata, reducing the chlorophyll contents and spoil photosynthetic apparatus what disturb the balance between production of reactive oxygen species (ROS) and the antioxidant defense, causing accumulation of ROS (Fagaria et al. 2006:Aschraf 2010;Grzesiak et al.2016)

Conclusions

In maize there exists an intraspecific variation to environmental stresses e.g. soil compaction levels and their combination with drought or waterlogging. Tolerance to a combination of different stress conditions, particularly those that mimic field environment, should be the focus of research programs aimed at developing new crop genotypes with enhanced tolerance.

* *Results were presented as the oral communication during Young scientist Competition and received the second prize in Young Scientists Competition for the best oral presentation at the 10th International Conference "Plant Functioning Under Environmental Stress" Kracow, September 16-19, 2015*

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PHYSIOLOGICAL ACTIVITY OF CUCUMBER (*CUCUMIS SATIVUS* L.) IN CADMIUM STRESS

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Introduction

Abstract. The presence of heavy metals, including cadmium, is an important issue during the cultivation of vegetable plants for food. Even small concentrations of this element can produce physiological responses in both plants and humans consuming them. The aim of the study was to determine the effect of 0.001%, 0.01%, 0.1% cadmium nitrate on germination, growth, chlorophyll content and photosynthetic activity of *Cucumis sativus* L. Plants were grown for 21 days under the following conditions: temperature 25° C (day) and 18° C (night), photoperiod of 12/12 h, light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and relative humidity (RH) 60-70%. Our studies showed that increased concentration of cadmium ions decreased the power and energy of seed germination. Biometric analysis of organs proved the difference in their growth rate. Chlorophyll content and fluorescence activity of *C. sativus* leaves decreased with increasing concentration of cadmium ions.

Key words: cadmium, chlorophyll content, germination, morphology, photosynthetic activity

Introduction

In recent years, much attention is paid to environmental pollution caused by the presence of heavy metals in edible plants. This is related to the threats it is posing for humans as well. Among the heavy metals, cadmium, a highly toxic element, is a serious threat to the environment. Its presence in nature is in large part anthropogenic, and associated with urbanization and industrialization. It is released in large quantities by the metallurgical and mining industries, as well as a byproduct in utilization of mineral fertilizers (Liu *et al.* 2007, Wagner 1993). The problem of heavy metal presence in crops is sometimes caused by too intensive agrotechnical treatments (Szteke 1992). Cadmium, absorbed in the upper soil layers, persists for a long period of time; therefore even in small amounts it can adversely affect the growth of plants. The degree of plant tolerance to heavy metals including

cadmium is typically variable and dependent on the exposure time (Burzyński and Żurek 2007). Some of the crop plants exhibit a high resistance to the adverse effects associated with the accumulation of this metal, even at a concentration of 170 $mg \cdot kg^{-1}$ of dry weight (tomato). Other vegetable species, such as lettuce are characterized by high sensitivity and fast physiological response (Kabata-Pendias and Pendias 1979). Cucumber shows an ability to accumulate cadmium compounds therefore it can also be used as an indicator species for ecotoxicological evaluation of soil (Gratão *et al.* 2005, Tiryakioglu *et al.* 2006). The cadmium content varies in different parts of the plant and symptoms of toxicity in plant cells are non-specific (Kabata-Pendias and Pendias 1999). Cadmium ions penetrate into the plants primarily through the roots and leaves, generating oxidative stress in plant tissues (Schutzendubel *et al.* 2001). Their accumulation in the form of carbonates and phosphates takes place mainly in cell walls of rhizodermis and cortex, in which glucuronic and galacturonic acids function as cation exchangers (Baranowska-Morek 2003, Siedlecka *et al.* 2001). Cadmium and other heavy metals cause considerable disturbance in cell elongation, and also slow down the creation of new cells. This is associated mainly with its effect of decreasing carbon assimilation (Greger and Ögren 1991). Additionally, inhibition of chlorophyll and thylakoid membrane synthesis, results in damage of stress-sensitive photosystem II and a reduction in photosynthesis efficiency. A reduction in water absorption and transport has also been observed, causing disturbances in water photodissociation through the reduction in the number of OEC complexes (Oxygen Evolving Complex) (Janeczko *et al.* 2005, Sanità di Toppi and Gabbrielli 1999). Heavy metals interfere with mineral uptake in plants, disrupting the absorption and distribution of certain nutrients (Krupa *et al.* 2002). Destabilization of key plant metabolic pathways induces visible symptoms such as chlorosis, necrosis, growth inhibition, browning of root tips, and finally death of the entire plant tissues (Kahle 1993, Możdżeń *et al.* 2015b). Despite many studies on the effects of heavy metals on physiological parameters in plants, knowledge of the subject is still insufficient. Several studies take into account the responses of individual species only at relatively high concentrations of cadmium. Therefore, the objective of this study was to determine the effects of (0.001%, 0.01%, 0.1%) cadmium nitrate $Cd(NO_3)_2$ on selected physiological parameters of cucumber (*Cucumis sativus* L.). For this purpose, the chlorophyll content and the photosynthetic activity of the plants grown from seeds germinated on media containing cadmium nitrate solutions was measured. Additional parameters were also determined, including energy and power of germination, the length of individual organs (root, the section from root to the first leaf, first leaf petiole and remainder of the shoot), fresh and dry mass of roots and aboveground parts of *C. sativus*.

Materials and methods

Plant material

The plant material was cucumber seeds (*Cucumis sativus* cv. "Sander F1") acquired from the Cracow Horticultural Breeding and Seed Supply “POLAN”.

Energy and power of plant germination

Seeds of cucumber were first rinsed under running water for 30 minutes and then with distilled/deionized water. After washing they were placed at equal distances from each other on sterilized Petri dishes lined with filter paper. This enabled the uniform conditions for germination. Divided into four experimental groups – a control group and three study groups, seeds were exposed to aqueous solutions of $Cd(NO_3)_2$ at following concentrations: 0.001%, 0.01% and 0.1%. Prepared material was placed in darkness inside a thermostat with air conditioning system that provides constant temperature of 25° C (± 1° C). Number of germinated seeds was counted every 24 hours for seven days.

Plant culture conditions

3-day seedlings sprouted on substrates with different concentrations of cadmium nitrate were planted in containers filled with sand and placed in a thermostat culture (Angelantoni Lifescience, Italy). Photoperiod was set to 12h/12h and the light intensity to 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Temperature was set to 25° C during the day and 18° C at night (± 2° C). During the growth, plants were watered with distilled water and standard Steiner medium containing the necessary macro-and microelements.

Morphometric analysis

After 21 days of culture *C. sativus* plants were subjected to biometric analysis, which included measurements of organ length including root, the section from the root to the cotyledons, first leaf petioles and the remainder of the stem.

Fresh and dry weight of *C. sativus* organs

The fresh weight of underground (roots) and aboveground organs (section from root to the cotyledons, cotyledons, first leaf petioles, first leaf blades and the remainder of the shoot) was determined on the WPS-100-Radwag electronic scale (Poland). In order to determine the dry weight, the plant material was dried for 48 hours at temperature of 105° C in a Wamed SUP - 100 oven (Poland) and then weighed to the nearest of 0.001 g.

Chlorophyll content

Measurements of chlorophyll content was performed according to the method by Barnes *et al.* (1992). Discs cut out from the second leaf of *C. sativus* using a cork borer, were weighed and then transferred to containers with 3 ml of DMSO (Sigma-Aldrich) and extracted for 12 hours at 65° C. The resulting extracts were poured into cuvettes, which were placed in a spectrophotometer CECIL Aquarius 9500 (United Kingdom), and were measured at two wavelengths: $\lambda = 648$ nm and $\lambda = 665$ nm.

Chlorophyll fluorescence

The fluorescence activity of *C. sativus* leaves was measured using a fluorimeter FMS-1 by Hansatech Instruments (United Kingdom). Before the measurement the leaves were adopted to the dark for 30 minutes using corporate clips. Fluorescence excitation source

represented actinic light in the range of 330 – 660 nm with a maximum at 500 nm, the intensity of which was 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Measurement time was one second. Maximum fluorescence (F_m), variable fluorescence (F_v) and maximum photochemical efficiency of PSII (F_v/F_m) were measured.

Statistical analysis

Statistical analysis was performed using one-way ANOVA. The significance of differences was determined via the Duncan test at $p < 0.05$. Calculations were performed using STATISTICA for Windows v.10.0.

Results

The seed germination ability of *C. sativus* on substrates with different concentrations of cadmium ions in cadmium nitrate

During the first day the highest germination energy was observed only in the case of control – about 72%. At the highest concentration of cadmium nitrate (0.1%), the number of germinated seeds was zero. In the second day, the amount of germinated *C. sativus* seeds increased significantly. About 90% of the seeds germinated on media supersaturated with cadmium nitrate solutions in concentrations of 0.001 and 0.01% while in the control - 95%. After 72 h in the highest concentration of $Cd(NO_3)_2$, only about 40% of seeds have germinated while in control and the remaining concentrations more than 90% (Fig. 1).

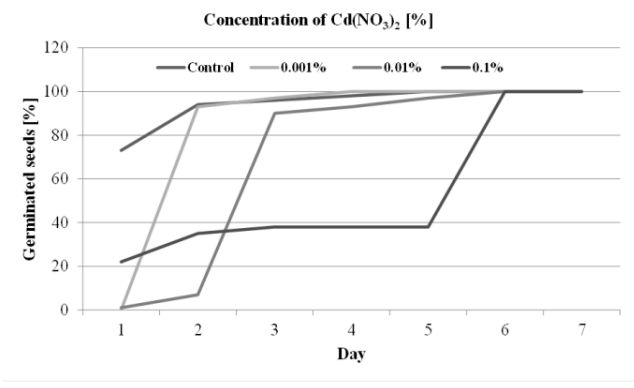

Figure 1. The percentage of Cucumis sativus L. seeds germinated after 7 days on media containing 0.001%, 0.01% and 0.1% of $Cd(NO_3)_2$ and on the medium with distilled water (control), mean values n = 5, differ significantly within the row according to the Duncan test at $p < 0.05$

The effect of cadmium on the growth of underground and aboveground organs of *C. sativus*

Compared to the control, root length of *C. sativus* plant material was inhibited with increasing concentration of cadmium ions. Shortest stem length from the root to the leaves was observed in plants treated with cadmium nitrate solution at a concentration of 0.1%, both relative to the control and to other concentrations. In the case of the first leaf petiole, elongation growth inhibition was found in any of the $Cd(NO_3)_2$ solutions used. The remainder of the shoot was the longest in the control plants and those treated with a 0.001% solution. In turn, statistically significant elongation growth inhibition was observed in the plants treated with 0.01% and 0.1% cadmium nitrate solutions (Tab. 1).

Table 1. The length of Cucumis sativus L. organs grown from seeds germinated on media supersaturated with $Cd(NO_3)_2$ solutions in various concentrations. Mean values n = 5, differ significantly within a row by Duncan test at p <0.05.

Organ	Control	Concentration of $Cd(NO_3)_2$ [%]		
		0.001	0.01	0.1
	Length [cm]			
Root	15.32[a]	12.88[b]	12.16[b]	8.80[b]
Section from the root to the cotyledons	4.06[a]	3.67[ab]	3.44[ab]	3.12[c]
First leaf petioles	2.71[a]	2.26[b]	2.02[b]	1.88[b]
Remainder of the shoot	5.1[a]	4.42[a]	2.30[b]	1.32[b]

Fresh and dry mass of *C. sativus* underground and aboveground organs grown from seeds germinated on substrates with solutions of cadmium nitrate

During the analysis of fresh and dry mass (Tab. 2 and 3) a downward trend was observed for both indicators in all organs of *C. sativus,* which correlated with increasing cadmium nitrate concentrations in the soil. The largest decrease in dry and fresh weight occurred in plants exposed to a concentration of 0.1%. In case of the root, 0.01% concentration already resulted in a significant decrease in organ weight and a further increase in the concentration of $Cd(NO_3)_2$ did not significantly affect the growth of the plant. Similarly blades and petioles of first leaves showed a statistically significant decrease in fresh and dry matter value already at the lowest concentration of cadmium ions (0.001%). These values remained similar during treatment with increasing concentrations of 0.01 and 0.1%. The largest decrease in weight at a concentration of 0.001% in relation to the control of the analyzed parts of the plant was showed by the first leaf blades. On the other hand, the cotyledon decline in both dry and fresh weight was the lowest in comparison with other organs. At the highest content of $Cd(NO_3)_2$ in the medium, decrease in dry weight of each *C. sativus* organ exceed 50% of the control value (Tab. 2 and 3).

Table 2. The fresh weights of Cucumis sativus L. organs treated during germination with aqueous solutions of $Cd(NO_3)_2$. Mean values n = 5, differ significantly within a row by Duncan test, p <0.05.

Organ	Control	Concentration of $Cd(NO_3)_2$ [%]		
		0.001	0.01	0.1
	Fresh mass [g]			
Root	5.48[a]	4.51[b]	2.28[c]	2.26[c]
Section from the root to the cotyledons	1.14[a]	1.03[b]	0.87[c]	0.70[d]
Cotyledons	1.29[a]	1.14[b]	1.00[c]	0.73[d]
First leaf blades	1.14[a]	0.75[b]	0.70[b]	0.69[b]
First leaf petioles	0.42[a]	0.29[b]	0.30[b]	0.25[b]
Remainder of leaves	1.75[a]	1.36[b]	1.00[c]	0.89[d]
Remainder of the shoot	1.12[a]	0.83[b]	0.47[c]	0.29[d]

Table 3. Dry mass of Cucumis sativus L. organs treated during germination with aqueous solutions of $Cd(NO_3)_2$. Mean values n = 5, differ significantly within a row by Duncan test, p <0.05.

Organ	Control	Concentration of $Cd(NO_3)_2$ [%]		
		0.001	0.01	0.1
	Dry mass [g]			
Root	0.57[a]	0.52[b]	0.33[c]	0.28[c]
Section from the root to the leaves	0.08[a]	0.08[a]	0.06[b]	0.04[c]
Cotyledons	0.20[a]	0.17[b]	0.16[b]	0.10[c]
First leaf blades	0.33[a]	0.16[b]	0.10[b]	0.09[b]
First leaf petioles	0.08[a]	0.04[b]	0.02[b]	0.02[b]
Remainder of leaves	0.22[a]	0.12[b]	0.11[b]	0.07[b]
Remainder of shoot	0.08[a]	0.06[b]	0.04[c]	0.02[d]

Chlorophyll content and fluorescence in *C. sativus* leaves

The chlorophyll *a* and *b* content in *C. sativus* leaves grown from seed germinated on media saturated with cadmium nitrate solutions decreased with increasing concentration of the metal ions (Fig. 2). In comparison to the control, the most significant decrease in the amount of both pigments were already visible at 0.001%. At higher concentrations, the differences were slightly higher.

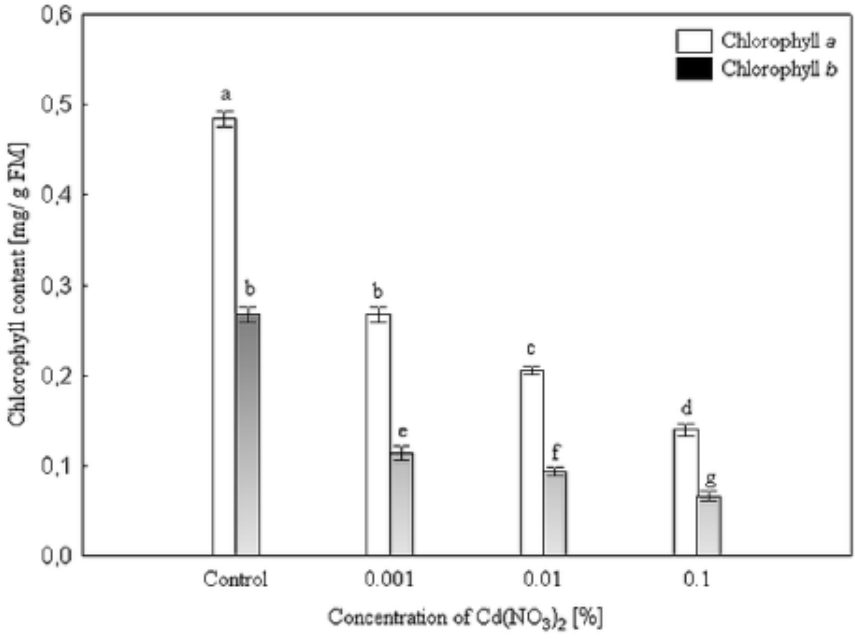

Figure 2. Chlorophyll content a and b in the second leaves of C. sativus grown from seed germinated on media at various concentrations of cadmium nitrate (0.001, 0.01, 0.1%). Mean values ± SD, n = 5, differ significantly by Duncan test, p <0.05

Functioning of PSII in *C. sativus* plants treated with cadmium nitrate in germination phase

Measurement of photosynthetic activity of *C. sativus* leaves based on chlorophyll fluorescence parameters such as: F_m, F_v, F_v/F_m showed a statistically significant effect of cadmium ions on the functioning of PSII (Tab. 4). With the increase of cadmium nitrate concentrations, significant decreases all of the surveyed fluorescence parameters. Compared to the control the lowest fluorescence values were found at the highest concentration of $Cd(NO_3)_2$ - 0.1%. In other cases a slight change in the photosynthetic activity of plants *C. sativus* has also been shown.

Table 4. Chlorophyll fluorescence parameters in the second leaf C. sativus grown from seed germinated on media with different concentrations of cadmium ions in cadmium nitrate (0.001, 0.01, 0.1%). Mean values, n = 5, differ significantly within a row by Duncan test, p <0.05.

Parameters	Control	Concentration of $Cd(NO_3)_2$ [%]		
		0.001	0.01	0.1
F_m	1002.6[a]	1001.4[a]	945.16[b]	821[c]
F_v	786.16[a]	781.6[a]	767[b]	628.2[c]
F_v/F_m	0.818	0.804	0.77	0.67

Discussion

Cadmium compounds induce disturbances in plant growth, water management, and also significantly hinder the germination process (Mishra *et al.* 2006). At the time of germination in newly forming plant organism a number of significant structural and metabolic changes is taking place that are dependent on the external environment. For this reason, the substrate containing anthropogenic contaminations, including heavy metals such as cadmium, significantly influence on the germination process. This element is a subject to geochemical condensation and is easily accumulated in soil the upper layers (Baran *et al.* 2008, Kabata-Pendias and Pendias 1999). Significant delay in cucumber seed germination after treatment with aqueous solutions of cadmium nitrate in various concentrations suggests that the seed coat is not in this case an effective barrier against heavy metals. It might be related to the interaction with other stress factors acting on the developing plant organism.. As a result it can cause lower crop yields (Murkowski 2004, Siwek 2008).Cadmium phytotoxicity cause disturbances in the transport of the necessary micro- and macroelements, which is the cause of poor growth and development of plants (Krupa *et al.* 2002). Reduction of the growth around the root system is due to the fact that cadmium absorption is largely carried out by the underground portion of the plant (Schutzendubel *et al.* 2001). Roots, being directly in the zone of cadmium contamination, are subjected to this detrimental effect in greatest extent (Tiryakioglu *et al.* 2006, Zhang *et al.* 2003). Significant reduction in *C. sativus* root length caused by the accumulation of cadmium was observed also by Gonçalves *et al.* (2007). Root deformities or lack of proper root development significantly affects the water and mineral element uptake in plants (Baran *et al.* 2008). Disorders in the biomass gain can also be caused by a more dense and compact root system, by elongation growth inhibition, lignification and reduction of the cell wall flexibility. Observed in the present study, the linear negative response to a growing concentration of cadmium may be related to deterioration in the functioning of plant antioxidant defense mechanisms and overproduction of reactive oxygen species (Gonçalves *et al.* 2007). This often leads to structural changes in proteins and modification of enzymatic pathways (Cargnelutti *et al.* 2006). Oxidative stress may not only cause irregularities in water and mineral uptake, but also the disruption of chlorophyll synthesis (Burzyński and Żurek 2007). Lowering of the chlorophyll content in cucumber tissues at each of the cadmium nitrate concentrations used, also confirmed by studies of Malinowska *et al.* (2010). However, the mere reduction in pigment content does not provide information about the source of the stress acting on the plant organism because it may be the result of interaction of several environmental factors. In addition to cadmium, germination, growth and development *C. sativus* was also affected by nitrate ions contained in the salt. Antagonistic action between cadmium and other elements sometimes have a significant impact on reducing the effects of toxicity (Kabata-Pendias and Pendias 1999).

Changes in functioning of the photosynthetic apparatus are relatively quick reactions of plants in response to stress factors. Statistically significant, slight reduction in the chlorophyll fluorescence parameters F_m, F_v, F_v/F_m caused by increasing concentrations of cadmium suggests inefficient usage of the light energy delivered to the chloroplasts. Lowering of the maximum fluorescence hints at the incomplete reduction of electron acceptors in PS II, and the low level F_m indicates non-radiant dissipation of energy reaching the leaf blade, which must be absorbed by chlorophyll molecule (Kalaji and Łoboda 2010). This allows

stipulating the existence of disturbances during the light phase of photosynthesis related to PS II operation. Changes in photosynthetic electron transport caused by the accumulation of cadmium, have been confirmed by studies carried out on other plant species (Maksymiec and Baszyński 1996, Myśliwa-Kurdziel *et al.* 2002). Moreover, as evidenced by Burzyński and Żurek (2007), cadmium compounds may affect the dark phase of photosynthesis, even to a greater extent than the light phase. Plant reaction to heavy metals also depends on the exposure time to adverse factors and the stage of plant development. During the growth of the leaf in the presence of cadmium ions, a degradation of the thylakoid internal structure takes place, which then causes defective donor-acceptor transduction (Skórzyńska-Polit *et al.* 1998). This explains why the parameter F_v/F_m in *C. sativus* cotyledons in the short term does not indicate substantial reduction of PS II potential quantum efficiency (Burzyński and Żurek 2007), while the change in fluorescence parameters leaves in this experiment were much more evident. Moreover, in other plant species, measurements of chlorophyll *a* fluorescence carried out on a plant leaf exposed to increased levels of cadmium in the substrate reveal serious disturbances in the photosystem II (Maksymiec and Baszyński 1996, Możdżeń *et al.* 2015a).

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