THE RESPONSES OF *Helianthus annuus* L. TO FOLIAR APLLICATION OF 28-HOMOBRASSINOLIDE

Angelika FILOVÁ
Department of Plant Physiology, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 01 Nitra, Slovak Republic
e-mail: angela.filova@uniag.sk

Abstract: *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. 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 L, 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; INZE and MONTAGU, 2000). 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 (INZE and MONTAGU, 2000). 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 (EGERT and TEVINI, 2002; 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).

BRs are common plant-produced compounds that can function as growth regulators (BISHOP and KONCZ, 2002; 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; NEMHAUSER, 2004). 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.

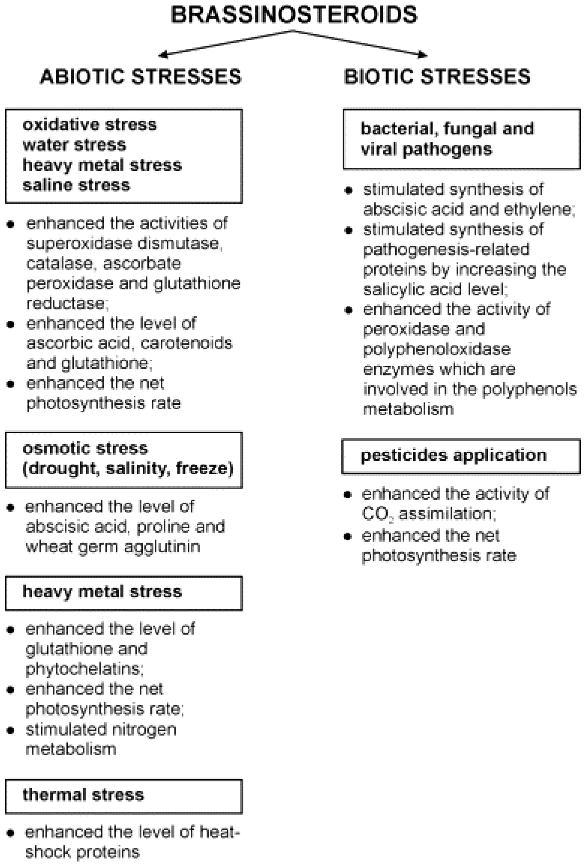

Figure 1: Effects of BRs on plants exposed or subject to different stresses. (BAJGUZ, HAYAT, 2009)

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, 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.

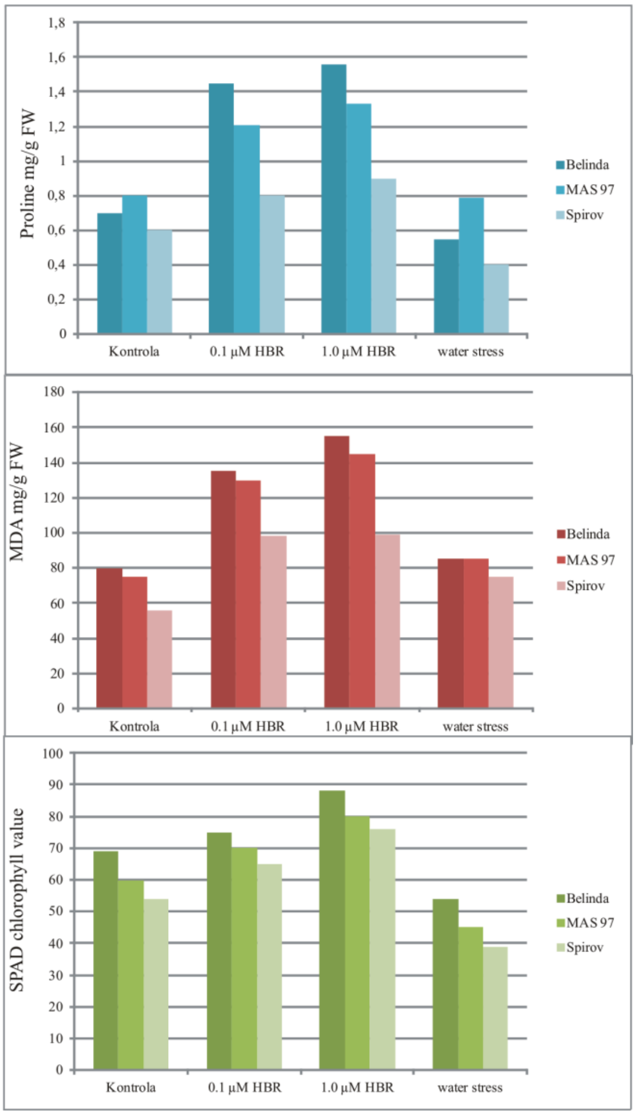

Figure 2: Effects of BR and drought stress on MDA, proline and SPAD chlorophyll value of *Helianthus annuus* L. cv. BELINDA, cv. MAS 97 and cv. SPIROV. Plants were grown for 1 month under controlled conditions and were divided into 4 groups. One group was control sprayed with distilled water, two groups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

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