Iranian Journal of Science & Technology, Transaction A, Vol. 33, No. A1
Printed in the Islamic Republic of Iran, 2009

CHANGE OF SOME OSMOLYTES ACCUMULATION IN WATER-STRESSED COLZA (*BRASSICA NAPUS L.*) AS AFFECTED BY 24-EPIBRASSINOLIDE*

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Abstract – Brassinosteroids are phytohormones possessing a wide spectrum of antistress activity. To see whether this hormone is able to ameliorate the effects of water stress, the effects of 24-epibrassinolise on plant growth, accumulation of osmolytes (proline, sugars and ions), lipid peroxidation and electrolyte leakage were investigated in Colza (*Brassica napus* L. cv. Fusia) plants which were under water stress. The seeds were sown in plastic pots containing sand, clay and peat (in a proportion of 1:1:1). Solution of 24-epi-brassinolid at 10^{-7} M concentration containing 0.01% Tween-20 (polyoxyethylene sorbitan) was sprayed on leaves at intervals of 1, 2 and 3 weeks after sowing. Control plants were sprayed with 0.01% Tween-20, and water treatment was applied 26 days after germination, withholding water for 3 or 4 days. One month after sowing, plants were harvested. Lipid peroxidation and electrolyte leakage significantly increased under water stress, but when plants were pretreated with 24-epibrassinolid and put under water stress these parameters decreased, revealing that less oxidative damage occurred in this group. Proline and reduced sugars content was increased when 24-epibrassinolide were applied. FW and DW were significantly decreased under water stress. Water stress also increased the uptake of K and Ca, but the uptake of Na significantly decreased. 24-epibrassinolide considerably increased ions uptake by plants. Taken together, these results showed that 24-epibrassinolide alleviated the effects of water stress and increased the tolerance of plants to stress by increasing osmolytes accumulation and therefore could be used to improve crops in harsh conditions.

Keywords – Colza (*Brassica napus* L.), 24-epibrassinolide, osmotic adjustment, water stress

1. INTRODUCTION

In nature, plants are frequently exposed to adverse environmental conditions that have a negative effect on plant survival, development and productivity. Water stress is considered the most important abiotic factor, which could be the consequence of many environmental conditions, including drought, salinity, or extremes in temperature, and induces numerous biochemical and physiological responses in plants [1].

Plants can respond to water stress at morphological, anatomical and cellular levels with modifications that allow the plant to avoid stress or to increase its tolerance. The morphological and anatomical adaptations can be of vital importance for some plant species, but they are not a general response of all plant species [2].

Under water stress conditions, plant growth is substantially reduced, partly because the lower turgor pressure in the cells results in a lower cell expansion rate [1].

Some of the observed effects of water stress may be the result of a stress-induced impairment of the biosynthetic machinery required for photosynthetic assimilation of carbon and/or its conversion to metabolically useable forms. In other cases, stress-induced changes may reflect adaptations for stress tolerance. Osmotic adjustment is an important mechanism of a plant's tolerance to a drought environment

*Received by the editor May 20, 2007 and in final revised form December 19, 2009
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[3]. Soluble sugars have been reported to accumulate during drought [4], proline has also been found to increase in response to water stress [5]. These compounds have been generally proposed to act as compatible solutes or osmoprotectants to allow osmotic adjustment of plant cells exposed to water stress. Other proposed roles include, free radical scavenging, protection from photoinhibition, and metabolic detoxification [6], all of which may help a plant to survive various environmental stresses [2].

Because water stress has a negative effect on growth, yield and quality [7], various agronomic and physiological practices are applied to minimize the adverse effect of water stress on plant growth, and use of plant-growth regulators is one that is more practiced [8].

American scientists discovered a new class of plant hormone called brassinosteroid (BR) in 1970. They reported that this hormone has various kinds of regulatory action on the growth and development of plants, such as simulation of cell enlargement and cell division, accretion of biomass, yield and quality of seeds and plant adaptability. Brassinosteroids (BRs) are present in nearly every part of the plant, with the highest concentrations in the reproductive organs (pollen and immature seeds) [9].

Studies with BR biosynthesis mutants and BR insensitive mutants of *Arabidopsis thaliana* have also provided evidence that BRs are essential for plant growth. On other hand, along with their growth promoting effect, they are also reported to have an anti-stress effect on different plants [10].

For instance, it was shown that BRs help to overcome stress exerted by low and high temperatures [11], drought [12], heavy metals [13], infection [14] and salt stress [15]. Although there is valuable information regarding stress alleviation by BRs in many plants, there is little information on the effects of 24-epibrassinolide on Oilseed canola plant (*Brassica napus* L.), which is an important agricultural crop grown in semi arid regions of Iran, primarily for its edible oil. Water stress causes adverse effects on Brassica napus, and limits its growth and productivity, thus tolerance to water stress is a decisive factor in its survival. The increase in drought tolerance in *Brassica napus* by brassinosteroid was associated with the accumulation of osmolytes [12].

Therefore, in this research we studied the effects of 24-epibrassinolide (24-epiBL) on plant growth, accumulation of osmolytes (proline, reduced sugars and ions), lipid peroxidation and electrolyte leakage in plants which were under water stress, to see if this compound has any impact on water stress tolerance.

2. MATERIAL AND METHODS

a) Plant growth and treatments

Colza seeds (*Brassica napus* L. cv. Fusia) were obtained from the Kerman Agriculture Research Institute, sterilized for 10 min with 0.01% (w/v) sodium hypochlorite solution and then thoroughly rinsed with distilled water. They were sown in 12×12 cm pots containing sand, clay and peat (in proportion of 1:1:1). 24-epibrassinolide at 10^{-7} M concentration containing 0.1% tween 20 (polyoxyethylenesorbitan) was used for spray at intervals of 1, 2 and 3 weeks after sowing. The control plants were sprayed with 0.1% tween 20 solutions.

The plants were grown in a growth chamber, (GROUC Company) during the 16 h day and 8 h night cycle. Temperatures were 23±2°C during the day and 16±2°C at night. The intensity of the light above the surface of the plants was 14000 Lux.

Water treatment was applied 26 days after germination by withholding water for 3 (WS_1) and 4 (WS_2) days. At the age of 30 days old, the leaves were used for the experiments. Fresh tissue was sampled from the third fully expanded rosette leaves that have been targeted.

b) Leaf relative water content

Leaf Relative Water Content (LRWC) was calculated based on the methods of Yamasaki and Dillenburg [16]. Leaves were first removed from the stem and then weighed to obtain fresh mass (FM) at the harvest stage. In order to determine the turgid mass (TM), leaves were floated in distilled water inside a closed Petri dish for 6 hours. The leaf samples were weighed after gently wiping the water from the leaf surface with tissue paper, then the leaf samples were placed in an oven at 80º C for 48 h, in order to obtain dry mass (DM). All mass measurements were made using an analytical scale, with a precision of 0.0001 g. Values of FM, TM and DM were used to calculate LRWC using the following equation:

LRWC (%) = [(FM–DM)/(TM–DM)] ×100.

c) Reduced sugar content

Reduced sugar content was measured in fresh leaves according to Somogy [17]. A sample of the leaves was ground in a mortar and pestle, and the tissue was extracted in distilled water at 78º C. The homogenized samples were centrifuged for 10 min at 1000 ×g. A supernatant was used to estimate the sugar content. After keeping for 10 min for color development with coppersulphate and phosphomolibdic acid, solution absorbance was read at 600 nm. The results are expressed in mg sugar g^{-1}DW.

d) Proline

The proline content was estimated by the method of Bates et al. [18]. The plant material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 1000 rpm. Supernatant was used for the estimation of proline content. The reaction mixture consisted of 2 ml supernatant, 2 ml ninhydrin acid and 2 ml of glacial acetic acid, which was boiled at 100° C for 1 h. After termination of the reaction in ice bath, the reaction mixture was extracted with 4 ml of toluene and the absorbance was read at 520 nm. The proline concentration was determined using calibration curve. The results were calculated based on DW.

e) Chemical analysis and weight determination

Three randomly selected leaves per replicate sampled and fresh weights (FW) were recorded. For dry weights determination (DW) and elemental concentration the leaves were washed in a detergent solution to remove any dust on the leaf surfaces, soaked in 0.5 M HCl for 25 S, followed by 3 to 4 rinses in distilled water and then dried at 80° C for 48 h to constant weight.

The dried leaves were ground to powder using a mortar and pestle. Ground samples (0.5 g per replicate) were taken up in 10 ml nitric acid. After 24 h this solution was boiled to remove any acidic gases, then filtered into a 50 ml volumetric flask and filled up to 50 ml with distilled water. Potassium, sodium and calcium were determined in these sample solutions using a Flame photometer (Jenway PFP7, UK). Iron and magnesium concentrations were measured using an Atomic Absorption Spectroscopy (Varian, SpectrAA 220, Australia).

f) Lipid peroxidation

The lipid peroxidation level of the leaves was determined by measuring the content of malondealdehyde (MDA) using the tiobarbitoric acid reaction as described by Heath and Packer, [19]. MDA concentration was calculated by reading the absorbance at 532 nm and the measurements were corrected for nonspecific turbidity by substracting the absorbance at 600 nm. The leaves concentration of MDA was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1}, and the results calculated based on DW.

g) Electrolyte leakage (EL)

EL was measured as described by Lutts et al. [20] using young leaf discs of three plants for each treatment. Samples were washed with deionized water to remove surface adhered electrolytes. Leaf discs were placed in closed vials containing 10 ml of deionized water and incubated at 25° C on a shaker incubator (100 rpm) for 24 h and subsequently, electrical conductivity of the solution (L_1) was determined. Samples were then autoclaved at 120° C for 20 min and the final electrical conductivity (L_2) was obtained after equilibration at 25° C. The EL was defined as follows: $EL(\%)=(L_1/ L_2)\times 100$.

h) Statistical analysis

All analyses were done on a completely randomized design. All data obtained was subjected to two-way analysis variance (ANOVA) and the mean differences were compared by Least Significant Differences (LSD) test. Each data point was the mean of three replicates except for FW of leaves (n=20), and comparisons with P-values<0.05 were considered significantly different. In all the figures, the spread of values is shown as error bars representing standard errors of the means.

3. RESULTS AND DISCUSSION

Water stress induces a reduction in plant tissue water levels and subsequently affects the leaf water potential, leaf elongation, leaf photosynthesis, protein synthesis, N metabolism, and cell membrane properties, leading to a reduction in plant productivity [21].

A positive turgor is required for cell expansion and growth under water-stress conditions [22] and is crucial for the continuation of many biochemical and physiological processes of plants [23].

Osmotic adjustment is a part of water stress avoidance mechanisms to counteract the loss of turgor by increasing and maintaining a higher amount of intracellular compatible solutes in cytosol and vacuole and has been proved to be particularly significant among all the stress adaptation mechanisms [22].

Greater osmotic concentration within the cells with the presence of osmotic adjustment may also contribute some resistance to water stress. Osmotic adjustment helps to maintain a higher water level within the cells and thereby maintain turgor under water-stress conditions [24].

It is well known that as soil water availability is limited, plant growth is usually decreased. This was previously considered due to turgor loss in expanded cells. More recent studies, however, have shown that leaf growth may be inhibited at low water potential despite complete maintenance of turgor in the growing regions as a result of osmotic adjustment. In this study we observed significant reductions in FW, DW and RWC at high water stress compared to the control (Table 1). These results are in agreement with the finding of Kirnak et al. [25] and Smirnoff [26].

In this study, the application of external brassinolide increase FW, DW and RWC content in leaves when compared with those plants exposed to water stress, but external brassinolide was not used (Table 1).

In accordance with our results, the exogenous application of brassinolide showed a protective effect on the growth of leaves, which was decreased due to stress [15].

Proline has been considered as a carbon and nitrogen source for rapid recovery from stress and growth, a stabilizer for membranes and some macromolecules and also, a free radical scavenger [27]. Proline is accumulated in many plants that are exposed to water, salinity or cold stress, and the accumulation of proline was positively correlated with stress tolerance [28].

In maize plants that were exposed to water stress proline content increased significantly [29]. In this study, proline content increased remarkably in the colza plants under water stress and 24-epiBL treatment caused a significant increase in the proline content of plants under water stress; conditions were compared

with those plants which were under water stress alone (Fig. 1). This result is similar to those reported for cucumber by Pustovoitava et al. [30].

Table 1. FW, DW and RWC of colza leaves under water stress (with and without 24-epiBL application). C: control; WS1: 3 days after withholding water; WS2: 4 days after withholding water. Within each column, the same letter indicates no significant difference among treatments (p≤5%), (n=20)

Treatment	FW (mg)	DW (mg)	RWC (%)
Control	685 B	60.5 B	92 A
24-epiBL	755 A	66 A	93 A
WS1	388 D	50.7 C	88 C
WS1+24-epiBL	505 C	59 B	90 B
WS2	287 E	40.7 D	83 E
WS2+24-epiBL	359 D	48.6 E	85 D

It seems possible that 24-epiBL treatment played a protective role for colza plants to prevent them from being severely affected by water stress as reported by Ozdemir et al. [15].

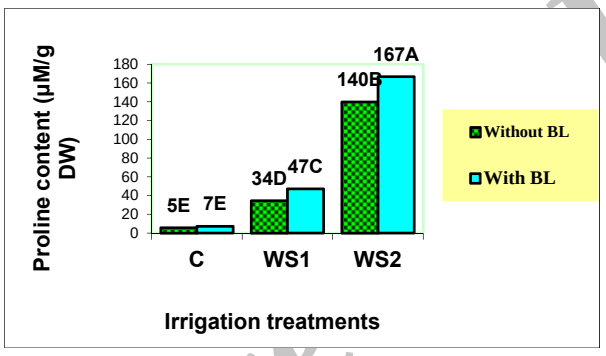

Fig. 1. Proline content (µM/g DW) of colza leaves under water stress (with and without 24-epiBL application) C: control; WS_1: 3 days after withholding water; WS_2: 4 days after withholding water. Values means of three replicates of leaves within each column, the same letter indicates no significant differences among treatments (p≤5%)

Plants may adjust their internal osmotic potential by accumulating some ions from the surrounding solution [13].

In this research we observed a significant increase in the K and Ca content of plants under water stress (Figs. 2 and 3).

Potassium is considered as one of the primary osmotic substances contributing to osmotic adjustment in many plants species [31]. Our results showed that leaf concentrations of K increased with increasing the incidence of water stress (Fig. 2).

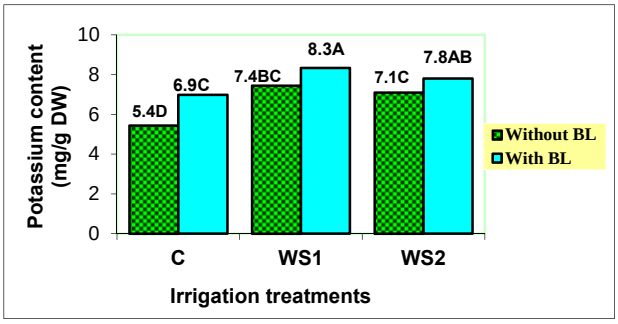

Fig. 2. Potassium content (mg/g DW) of colza leaves under water stress (with and without 24-epiBL application). C: control; WS_1: 3 days after withholding water; WS_2: 4 days after withholding water. Values means of three replicates of leaves within each column, the same letter indicates no significant differences among treatments (p≤5%)

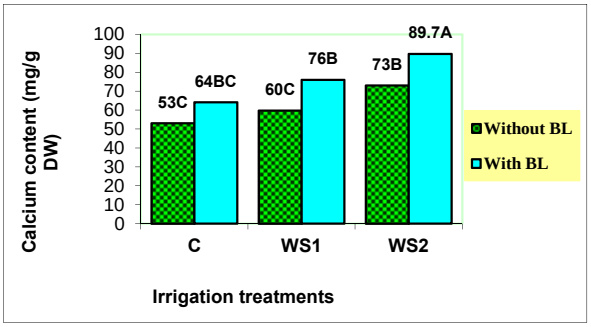

Fig. 3. Calcium content (mg/g DW) of colza leaves under water stress (with and without 24-epiBL application). C: control; WS_1: 3 days after withholding water; WS_2: 4 days after withholding water. Values means of three replicates of leaves within each column, the same letter indicates no significant differences among treatments ($p \leq 5\%$)

In accordance with our result, Kirnak et al. reported that potassium concentration increased in eggplant under water stress [25]. Ashraf et al. also reported that K acts as the primary osmotic contributor in maize under water-stress conditions, and they found significant correlation in cell membrane stability measured by osmotic potential and K concentration in leaf tissues [31].

Ca is involved in the regulation of plant responses to environmental stress conditions. In our results we observed that plants which were exposed to water stress showed a significant increase in calcium content (Fig. 3). Greater Ca concentration in plant tissues may help in achieving better crop survival with improved yields under stress conditions [32 and 33]. Knight et al. indicated that osmotic stress can mediate rapid elevations in cytosolic free calcium in *Arabidopsis* seedlings, and that these changes in Ca^{2+} levels may mediate in increases in the expression of drought-induced genes which have protective functions [34]. In this experiment we showed that 24-epiBL treatment increased Ca content in the colza plants (Fig. 3). Calcium ions increase antioxidant enzyme activities and reduce lipid peroxidation of the cell membrane. It was indicated that increasing Ca^{2+} had the function of preventing cell membrane injury and leakage, as well as stabilizing the cell membrane structure under adverse environmental conditions [35]. In our research lipid peroxidation and EL also decreased with 24-epiBL application. Therefore, Ca plays a vital role in the maintenance of membrane stability and permeability [36].

Also, the increase in stress-induced cytosolic Ca^{2+} has been suggested to up-regulate the biosynthesis, since the induction of transcript for proline biosynthetic enzyme [34].

In this study the sodium content of plants which were exposed to water stress decreased significantly (Fig. 4), which is probably due to less availability of these elements to the plant under water stress condition as reported by Kirnak et al. [25]. In this experiment, it appears that K uptake somehow competes with Na uptake and Ca mitigates the negative effects of Na on the plant tissue.

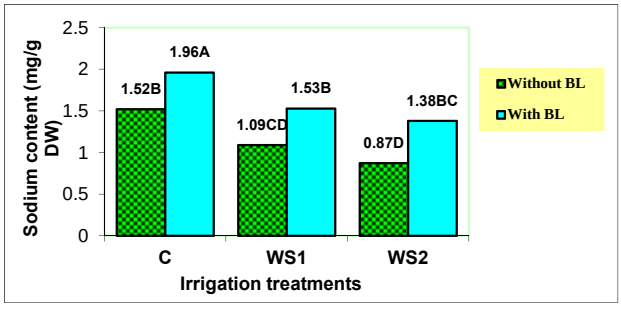

Fig. 4. Sodium content (mg/g DW) of colza leaves under water stress (with and without 24-epiBL application). C: control; WS_1: 3 days after withholding water; WS_2: 4 days after withholding water. Values means of three replicates of leaves within each column, the same letter indicates no significant differences among treatments ($p \leq 5\%$)

In this research we showed that 24-epiBL application increased the ion contents in the colza plants (Figs. 2, 3 and 4). The accumulation of metals (Cd, Zn, Pb, Ca) in 24-epiBL treated plants has been studied for different agricultural plants such as barely, tomatoes, radish and sugar beet [9].

Schulze reported that water loss leads to closure of the stomata, and therefore a decrease in the internal CO_2 and photosynthesis is investigated [37].

As a consequence of the decrease in the rate of CO_2 assimilation caused by drought, the distribution, accumulation and mobilization of sugars could be affected [38].

In spite of the interruption to the supply of photosynthate it has been reported that, absolute amounts of soluble sugars increased in the shoots and roots of stressed plants, and argued that the degradation of starch in the shoots may explain soluble sugar accumulation [1]. This phenomenon might be a physiological response to water stress. Soluble sugars accumulation in plants of many species that are subjected to water stress has been reported [39]. In spinach leaves, water stress stimulated starch breakdown and the accumulation of soluble sugars for the maintenance of turgor [40]. In this research we showed that water stress caused a remarkable increase in reduced sugars content (Fig. 5). Thus, the accumulated sugars might play a role in the osmotic adjustment as the leaf water potential decreases [1].

In this study we observed that the concentration of sugar in leaf sprayed with 24-epiBl increased (Fig. 5).

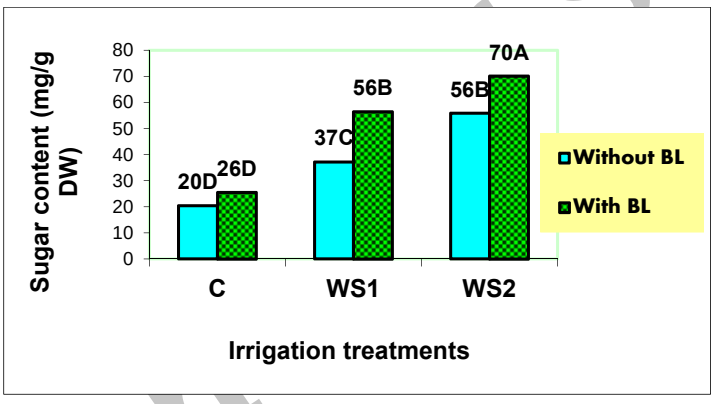

Fig. 5. Sugar content (mg/g DW) of colza leaves under water stress (with and without 24-epiBL application). C: control; WS_1: 3 days after withholding water; WS_2: 4 days after withholding water. Values means of three replicates of leaves within each column, the same letter indicates no significant differences among treatments ($p \leq 5\%$)

Schlüter *et al*. 2002 [41] found that a BR-deficient *Arabidopsis* mutant had an impaired carbohydrate metabolism and reduced biomass in plants such as wheat and groundnut, and reported that the application of BRs increased the sugar content of those plants [42].

In *Cucumis sativus* EBR treatment resulted in large increases in the photosynthetic capacity of leaves, which was accompanied by an increase in Rubisco activity [43].

Khripach et al. suggested that BRs affect the biosynthesis of enzymes via an effect on gene expression and/or the effect of BRs on cell membranes [9]. Increasing photosynthesis, particularly the capacity of CO_2 assimilation in the Calvin cycle, which is mainly attributed to an increase in the initial activity of Rubisco was also discussed by Yu et al. [43]. The accumulation of soluble sugars as a consequence of higher photosynthesis may help plants for osmoregulation and desiccation tolerance contributing to plant survival [44].

A consequence of the drought-induced limitation of photosynthesis is the exposure of plants to excess energy, which, if not safely dissipated, may be harmful to PSII because of over reduction of reaction centers and increased production of reactive oxygen species in the chloroplasts [45].

It is widely accepted that Active Oxygen Species (AOS) are responsible for various stress-induced damages to macromolecules and ultimately to cellular structures [15, 46].

Cell membrane stability was affected by lipid peroxidation resulting from oxidative stress, and its accumulation is considered a manifestation of the detriment of AOS in plants [47, 26]. Lipid peroxidation was evaluated by the determination of MDA concentration in leaf tissues. In this study, water stress increased MDA concentration in leaves, suggesting that active oxygen species accumulated in water-stressed leaves (Fig. 6).

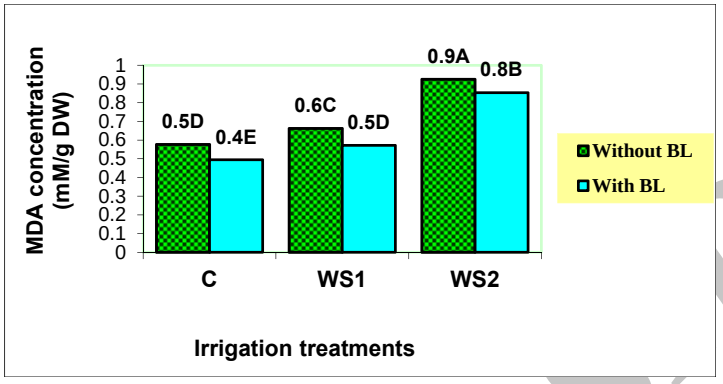

Fig. 6. MDA concentration (mM/g DW) of colza leaves under water stress (with and without 24-epiBL application). C: control; WS_1: 3 days after withholding water; WS_2: 4 days after withholding water. Values means of three replicates of leaves within each column, the same letter indicates no significant differences among treatments ($p \leq 5\%$)

However, MDA concentration decreased with 24-epiBL application under water-stress treatment. Reduction in the accumulation of MDA on 24-epiBL treatment plants indicate reduced lipid peroxidation under water-stress treatment as also mentioned by Sakamoto et al. [2].

Increases in K and Ca concentrations in 24-epiBL treated plants may have prevented membrane damage and also enhanced the capacity to maintain a higher status in plants through osmoregulation.

In this study water stress caused a remarkable increase in EL. Sato et al. reported that phospholipid membranes are destabilized upon water stress by insertion of cellular amphiphiles, phase transition into the gel phase and membrane fusion [47].

El was reduced in 24-epiBL treated plants (Fig. 7), thus indicating BR involvement in membrane stability. Ram Rao et al. discussed how BR was involved in osmoregulation and the stability of the cell membrane [48].

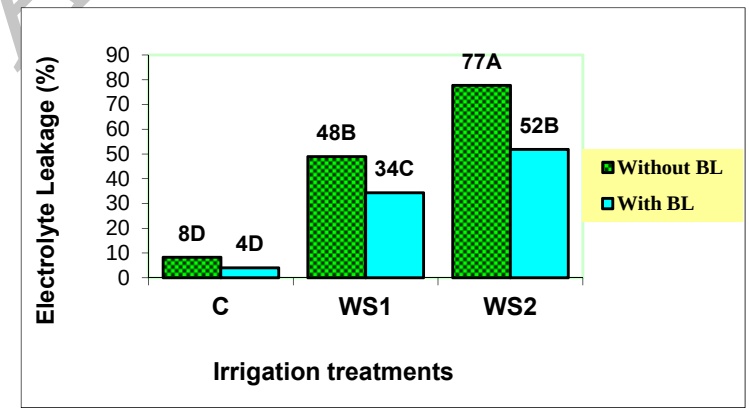

Fig. 7. Electrolyte Leakage (%) of colza leaves under water stress (with and without 24-epiBL application). C: control; WS_1: 3 days after withholding water; WS_2: 4 days after withholding water. Values means of three replicates of leaves within each column, the same letter indicates no significant differences among treatments ($p \leq 5\%$)

Our results indicate that 24-epiBL application influences greater osmotic adjustment during the stress period, and the results suggest that increased ion uptake and accumulation of proline and soluble sugars may have contributed to the promotion of drought tolerance. Increases in K, Ca, and proline concentrations in leaf tissues may have prevented cell membrane damage and also enhanced the capacity to maintain a higher water status in plants.

Based on these properties, the natural phytohormone BR has been approved for agricultural use, and as a result, it may become feasible to grow crops under unfavorable conditions.

Acknowledgments- The authors wish to thank the International Center of Science, High Technology and Environmental Science, Kerman (Iran) for supporting this work. The authors also appreciate Mrs. Soltani for her help in the experimental procedures.

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Iranian Journal of Science & Technology, Transaction A, Vol. 33, No. A1
Printed in the Islamic Republic of Iran, 2009

CHANGE OF SOME OSMOLYTES ACCUMULATION IN WATER-STRESSED COLZA (*BRASSICA NAPUS L.*) AS AFFECTED BY 24-EPIBRASSINOLIDE*

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اثر ۲٤-اپی براسینولید بر تغییر و تجمع بعضی اسمولیتها در گیاهان گلزا (*Brassica napus L.*) تحت تنش کم آبی

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[۱]مرکز بین المللی علوم و تکنولوژی پیشرفته و علوم محیطی(ICST)، کرمان، جمهوری اسلامی ایران
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چکیده: براسینواستروئیدها گروهی از هورمونهای گیاهی با اثرات ضد تنشی قابل توجهی هستند. برای روشن شدن اینکه آیا براسینواستروئیدها موجب بهبود اثرات حاصل از تنش کم آبی در گیاهان می شوند، اثرات ۲٤-اپی براسینولید روی رشد، تجمع اسمولیتها، پراکسیداسیون لیپید و نشت یونی در گیاهان گلزا (*Brassica napus* L. cv. Fusia) تحت تنش کم آبی مورد بررسی قرار گرفت. بذر گیاهان مورد نظر در گلدانهای حاوی شن، رس و خاک برگ کاشته شد و برگهای گیاهان یک، دو و سه هفته بعد از جوانه زنی با محلول 10^{-7} مولار از ۲٤-اپی براسینولید که حاوی توین ۲۰ (۰.۰۱ درصد) بود محلول پاشی شد. گیاهان شاهد با آب ۲ بار تقطیر حاوی توین تیمار گردیدند. ۲۶ روز بعد از جوانه زنی، تیمار کم آبی ۳ و ٤ روز اعمال شد. یک ماه بعد از جوانه زنی برداشت انجام گرفت. افزایش پراکسیداسیون لیپید و نشت یونی که تحت تنش کم آبی مشهود بود در تیمار با ۲٤-اپی براسینولید کاهش معنی داری نشان داد. مقدار پرولین و قندهای احیا کننده در تیمار با ۲٤-اپی براسینولید بطور معنی داری افزایش یافت. FW و DW در طی تنش کم آبی بطور قابل توجهی کاهش یافتند. تنش کم آبی همچنین موجب افزایش Ca و K شد، ولی جذب Na کاهش معنی داری نشان داد. تیمار ۲٤-اپی براسینولید موجب افزایش جذب یونها توسط گیاهان شده است. حاصل آنکه، نتایج نشان داد که ۲٤-اپی براسینولید موجب کاهش اثرات تنش کم آبی شده و با افزایش تجمع اسمولیتها مقاومت گیاهان به تنش را افزایش می دهد، بنابراین می تواند در شرایط سخت برای اصلاح محصولات کشاورزی استفاده شود

*Received by the editor May 20, 2007 and in final revised form December 19, 2009
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