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Effects of 24-epibrassinolide on growth, chlorophyll, electrolyte leakage and proline by pepper plants under NaCl-stress

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Abstract
Brassinosteroids are steroidal phytohormones that have the ability to overcome plant environmental stress. This study was carried out to investigate the role of 24-epibrassinolide in inducing pepper plant salt tolerance as measured by a range of physiological parameters: growth, chlorophyll, electrolyte leakage and proline. *Capsicum annuum* cv. Beldi seedlings were sprayed with 24-epibrassinolide both in the presence or the absence of NaCl and were sampled, 28 days after treatments. As a result of analysing the cultures under salinity stress, it was determined that the biomass and the chlorophyll decreased significantly, while the electrolyte leakage and the proline concentration increased considerably under salinity stress. However, the application of 24-epibrassinolide significantly ameliorated the adverse effects of salinity on the examined parameters, confirming the suppositions of previous authors who have claimed that exogenously applied 24-epibrassinolide can increase growth and protect the integrity of the cellular membrane in stressed plants.

Keywords: Brassinosteroids, chlorophyll, electrolyte leakage, proline, salt tolerance.

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INTRODUCTION

Of various abiotic stresses known in nature, salt stress poses a major threat to crop production. Increasing salinization of arable lands is a problem of paramount importance to crop production in many parts of the world and especially in irrigated fields of arid and semi-arid regions (Grattan and Grieve 1992), where limited rainfall, high evapotranspiration and high temperature associated with poor water and soil management contribute to the salinity problem and become of great importance for agriculture production in these regions (Eraslan et al. 2008). In general, an excess of soluble salts resulting from natural processes or intense human practices causes ion imbalance and hyper osmotic stress which severely depress various physiological and biochemical processes (Munns 2002) leading to a decline in plant growth and yield.

Pepper (*Capsicum annuum* L.) is moderately sensitive to salt stress (Rhoades et al. 1992). In response to high NaCl concentration, pepper plants accumulate toxic ions such as Na^+ and Cl^- (Günes et al. 1996, Chartzoulakis and Klapaki 2000). This leads to an imbalance in mineral elements, resulting in increased membrane permeability (Kaya et al. 2001), reduced of the photosynthetic activity and stomatal conductance (Chartzoulakis and Klapaki 2000, Martinez-Ballesta et al. 2004) and inhibited chlorophyll biosynthesis (Kaya et al. 2001). However, it increases the level of proline (Günes et al. 1996) that acts as an osmoprotectant, membrane stabilizer and reactive oxygen species (ROS) scavenger (Kavi Kishor et al. 2005). Remedial measures, such as the exogenous application of plant growth regulators (Stevens et al. 2006, Tuna et al. 2008) have been tested earlier to

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overcome physiological constraints leading to enhanced production in several crops.

Brassinosteroids (BRs) are common plant-produced compounds that can function as growth regulators (Bishop et al. 2006). They play prominent roles in various physiologic processes, including the induction of a broad spectrum of cellular responses such as stem elongation, pollen tube growth, xylem differentiation, leaf epinasty, root inhibition, induction of ethylene biosynthesis, proton pump activation, regulation of gene expression and photosynthesis (Sasse 2003). BRs have been reported to protect plants from various abiotic/biotic stresses such as drought stress (Behnamnia et al. 2009), temperature stress (Ogweno et al. 2008), pathogen infection (Nakashita et al. 2003), heavy metals (Ali et al. 2008 a, Fariduddin et al. 2009), and salt stress (Hayat et al. 2010).

The objective of the present study was to test the hypothesis that foliar sprays of 24-epibrassinolide, a highly active and stable steroidal hormone (Khripach et al. 2000), could ameliorate the stress in pepper plants caused by NaCl-stress. For this purpose, pepper plants were sprayed with 24-epibrassinolide solution and the effects of salt exposure for 28 days on growth, chlorophyll content, electrolyte leakage and proline accumulation were studied.

MATERIAL AND METHODS

Plant material and experimental conditions

The seeds of pepper (*Capsicum annuum* L.) cv. Beldi were obtained from 'High Institute of Agronomy, Chott Mariem' (Tunisia) and sown directly in peat and germinated in a glasshouse. After the second true leaves appeared, uniforms pepper seedlings were transplanted at a rate of one plant per 17cm plastic pot containing peat. The substrate characteristics were pH= 5.8-6.8, EC 0.40 dS.m-1, dry matter 35% and organic matter 25%.

7 days after transplantation, the seedlings were irrigated with salt water (4 g L^{-1} NaCl) and were sprayed with distilled water (control) or 0.5 mg L^{-1} 24-epibrassinolide (EBR was dissolved in a minimal volume of ethanol and then made up to volume with distilled water as described in our previous study (Houimli et al. 2008). The selection of the concentration was based on the experiment presented in earlier papers (Houimli et al. 2008). At this concentration, 24-epibrasinolide had the greatest effect on the growth of pepper seedling at 4 g L^{-1} NaCl. A Hoagland's solution was added weekly to the plants. Growth took place in glasshouse where the temperature for day/night was 28/20°C; the relative humidity was 60-80% and photoperiod of 14 h/d. The plan of the experiment was simple randomized design. Samples were collected on the 28th day after salt and 24-epibrassinolide treatments application to assess the following parameters.

Plant growth

The plants kept under various treatments were up-rooted carefully, washed with distilled water and then the fresh weight of leaves, stems and roots were recorded. The samples were oven dried at 70°C for 72 h and then the dry weights were recorded.

Chlorophyll content

The chlorophyll from the fresh leaves was extracted in 80% acetone and the absorbance was read spectrophotometrically at 663 and 645 nm. Chlorophyll content was estimated by the method of Arnon (1949).

Electrolyte leakage

Electrolyte leakage was measured using an electrical conductivity meter as described by Lutts et al. (1996). Leaves were excised and washed with deionized water. After drying with filter paper, 1 g fresh weight of leaves were cut into small pieces (about 1 cm^2) and then immersed in 20 mL deionized water and incubated at 25°C. After 24 h, electrical conductivity (EC1) of the bathing solution was recorded. These samples were then autoclaved at 120°C for 20 min to completely kill the tissues and release all electrolytes. Samples were then cooled to 25°C and the final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was expressed following the formula $EL = EC1/EC2 \times 100$.

Proline content

The proline content was determined using the method described by Bates et al. (1973). Proline was extracted from leaf samples of 100 mg FW with 2 mL of 40% methanol. 1 mL of the extract was mixed with 1 mL of a mixture of glacial acetic acid and orthophosphoric acid (6 M) (3: 2, v/v) and 25 mg of ninhydrin. After 1 h incubation at 100°C, the reaction was terminated by putting the tubes in ice bath, 5 mL toluene was added. The absorbance of the upper phase was spectrophotometrically determined at 520 nm. The proline concentration was determined using a standard curve.

Data analysis

The values for the parameters were subjected to one-way analysis of variance (ANOVA) and the mean differences were compared by Duncan Test. Each data point was the mean of six replicates (n= 6) and comparisons with P-values <0.05 were considered significantly different.

RESULTS

By comparison to controls plants (Fig. 1), NaCl-stress induced significant reductions in the fresh weight of plant organs (leaves: 34%, roots: 30% and stems: 28%). However, the spraying of leaf tissues with 24-epibrassinolide to both unstressed as well as stressed plant significantly enhanced the values of this parameter by reducing the loss of fresh weight for the leaves (20% against 34%) and stems (17% against 28%), over the respective controls. The beneficial action of 24-epibrassinolide was not observed for the roots (Fig. 1).

The dry weights of leaves proved to be alleviated by spraying of 24-epibrassinolide upon the leaf tissues of the NaCl-stressed plants. However, the roots and stems dry weights were unaffected by this treatment (Fig. 2).

Table 1 highlight that NaCl-stress caused significant reductions in total water content of leaves (21%), stems (18%) and roots (17%). The 24-epibrassinolide treatment produces an increase in the water content of the various organs (5-10%). When sprayed upon NaCl-stressed plants, EBR reduced the percentage of water loss of leaves (14% against 21%) induced by NaCl but restored the water content of the roots and stems to the value of the control.

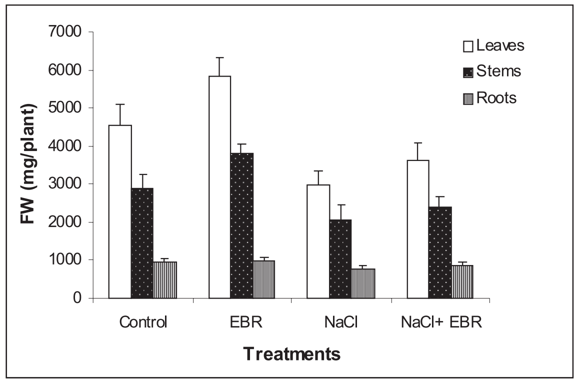

Fig. 1. Effect of 24-epibrassinolide (EBR) (0.5 mg L^{-1}) on leaves, stems and roots fresh weights (mg) in pepper plants exposed to NaCl-stress (4 g L^{-1}).

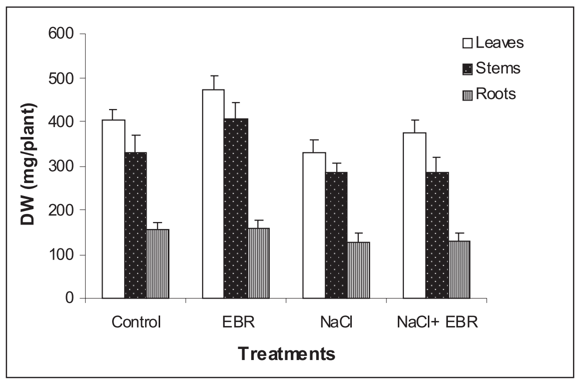

Fig. 2. Effect of 24-epibrassinolide (EBR) (0.5 mg L^{-1}) on leaves, stems and roots dry weights (mg) in pepper plants exposed to NaCl-stress (4 g L^{-1}).

Table 2 show that NaCl-stress caused significant reduction ($P<0.05$) of the leaves contents in chlorophyll a (15.6%) and in chlorophyll b (39.2%), leading to an increase in the Chl a/b ratio. The foliar application of 24-epibrassinolide upon control plants had no significant effect in the pigment contents. But spraying of 24-epibrassinolide upon NaCl-stressed plants resulted in significant rises of Chl a (10.4%) and Chl b (21.7%) contents ($P<0.05$), therefore, the Chl a/b ratio was significantly lowered (Table 2).

The electrolytes leakage (EL) constitutes

Table 1. Effect of 24-epibrassinolide (EBR) (0.5 mg L^{-1}) on leaves, stems and roots water contents in pepper plants exposed to NaCl-stress (4 g L^{-1}).

Treatments	Leaves	Stems	Roots
control	1025 (100%)	784 (100%)	605 (100%)
EBR	1126 (110%)	849 (108%)	636 (105%)
NaCl	806 (79%)	648 (82%)	504 (83%)
NaCl +EBR	878 (86%)	737 (94%)	578 (95%)

Table 2. Effect of 24-epibrassinolide (EBR) (0.5 mg L^{-1}) on the Chl a, Chl b contents (mg kg-1 F.W) and Chl a/b ratio in pepper plants exposed to NaCl-stress (4 g L^{-1}).

Treatments	Chl a (mg Kg^{-1} F.W)	Chl b (mg Kg^{-1} F.W)	Chl a/b
control	1032 a	717 a	1.44 c
EBR	1053 a	741 a	1.42 c
NaCl	869 c	419 c	2.07 a
NaCl +EBR	972 b	556 b	1.75 b

an indicator of the membrane permeability and it was measured in the leaves. The exposure of the plants to NaCl-stress resulted in a significant increase (73%) in electrolytes leakage (Fig. 3). On the other hand, EL was unaffected by spraying of 24-epibrassinolide upon control plants conversely to NaCl-stressed plants where 24-epibrassinolide reduced partially, but significantly the electrolytes leakage (29%).

Concerning the proline content, it was significantly increased (65%) by exposure to NaCl (Fig. 4), but spraying of controls plants with 24-epibrassinolide had no significant effect upon the proline content. On the other hand, its spraying upon NaCl-stressed plants allows a partial reduction (21%) of proline content.

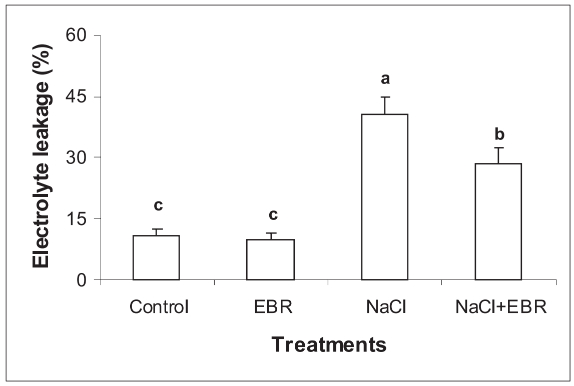

Fig. 3. Effect of 24-epibrassinolide (EBR) (0.5 mg L^{-1}) on the contents of proline (μmol g-1 MF) in pepper plants exposed to NaCl-stress (4 g L^{-1}).

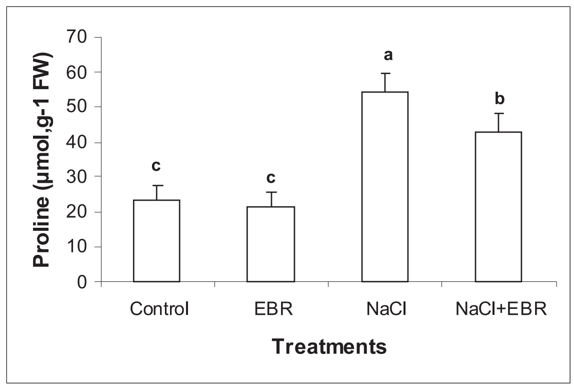

Fig. 4. Effect of 24-epibrassinolide (EBR) (0.5 mg L^{-1}) on the contents of proline (μmol g-1 MF) in pepper plants exposed to NaCl-stress (4 g L^{-1}).

DISCUSSION

The results showed that NaCl-stress caused a decline in overall growth of plants due to reduced masses of fresh and dry materials of the various organs. This is in agreement with previous work on pepper (Chartzoulakis and Klapaki 2000, Lycoskoufis et al. 2005).

The foliar application of EBR upon NaCl-stressed plants reduces the reduction of the fresh and dry weights of leaves produced by NaCl-stress with change in the reduction of the water content percentage. No changes by EBR inhibitory effects of dry masses due to NaCl-stress on the stems and roots were observed. Only the water content of these organs, reduced by NaCl-stress, was restored to the value of the control by the 24-epibrassinolide.

NaCl-stress led to a significant reduction in the contents of chlorophylls a and b. This effect agrees with earlier work (Günes et al. 1996, Kaya et al. 2001, Lycoskoufis et al. 2005) and may results from an inhibition of chlorophyll biosynthesis or from an activation of the chlorophyllase (Günes et al. 1996). The NaCl-stress modifies the chlorophyll b content more than that of chlorophyll a, which appears less sensitive than chlorophyll b to NaCl-stress. These results in an increase in the chl a/b ratio, showing that chl b was being degraded at a higher rate than chl a. This can be explained by the fact that the first step in the degradation of chl b implies its conversion to chl a (Fang et al. 1998).

The exogenous application of 24-epibrassinolide does not modify the contents of pigments. However, when its foliar pulverization was associated with NaCl-stress, it helped to increase the content of chlorophylls a and b. The stimulation of the accumulation of chlorophyll by the brassinosteroids under salt stress was observed in other researchers (Anuradha and Rao 2003, Ali et al. 2007). This result may suggest that 24-epibrassinolide protect photosynthetic apparatus from salt induced oxidative stress. This view is further supported by the fact that chloroplast is a major source of reactive oxygen species production (ROS) in plants (Ormaetxe et al. 1998). Indeed, many works related a protective effect of exogenous BRs against oxidative stress generated by several environmental factors such as salinity (Nunez et al. 2003, Arora et al. 2008), metals (Hayat et al. 2007, Ali et al. 2008a), drought (Behnamnia et al. 2009) and thermal stress (Ogweno et al. 2008). Anuradha and Rao (2003) showed that brassinosteroids remove the inhibitory effects of salt stress on pigment levels and that this could be one of the reasons for the noticed growth stimulation induced by brassinosteroids under saline conditions.

The extent of membrane damage by salinity was assessed by an indirect measurement of solute leakage. NaCl-stress induced significant increases in electrolyte leakage compared to the control as shown in Fig. 3. This phenomenon already observed by several authors and on various crops (Lutts et al. 1996, Kaya et al. 2001, Ghoulam et al. 2002) would be associated to chain reactions initialized by free radicals (Mazliak 1983). Among these reactions, the lipid peroxidation due to the accumulation of the ROS, are the principal causes of membrane damage (Sairam et al. 2005). Maintaining integrity of the cellular membranes under salt stress is considered an integral part of the salinity tolerance mechanism (Stevens et al. 2006). 24-epibrassinolide treatment lowered the electrolyte leakage in NaCl-stressed pepper plants (Fig. 3). These results are concordant with Ali et al. (2008b) for mustard. It seems that BRs may help membrane integrity by enhancing the level of the antioxidant system that protects the plant from the oxidative damage (Arora et al. 2008, Ali et al. 2008b).

Proline accumulation is a common metabolic response of higher plants to salinity stress. In this study, proline was significantly increased in pepper leaves after exposure to NaCl-stress but was unaffected by the foliar application of 24-epibrassinolide. However, when the foliar spraying of 24-epibrassinolide was associated with NaCl-stress, it reduced the proline accumulation induced by salt stress.

An increase in proline under the effect of salinity has been reported for various plants such as sugar beet (Ghoulam et al. 2002), rice (Demiral and Türkan 2005), and maize (Cha-Um and Kirdmanee 2009). Such an increase in the proline was also observed under the effect of other types of stress: water (Zhu et al. 2005), thermal (Taulavuori et al. 2005) and UV radiations (Saradhi et al. 1995). Proline increases in plants may be an adaptive mechanism for reducing (i) the level of accumulated NADH, and (ii) the acidity; $2NADH+2H+$ is used for synthesizing each molecule of proline from glutamic acid (Venekemp et al. 1987). Proline as a cytosolic osmoticum and a scavenger of OH· radical can interact with cellular macromolecules such as DNA, protein and membranes and stabilize the structure and function of such macromolecules (Kavi Kishor et al. 2005).

The foliar spray of 24-epibrassinolide upon NaCl-stressed plants resulted in a decrease in proline. The significant reduction in the levels of this compatible solute is in keeping with the previously observed properties of this group of plant growth regulators (Sasse 1997, González-Olmedo et al. 2005). EBR reduced partially the electrolyte leakage and proline induced by NaCl-stress. This effect is consistent with a protective membrane against the attack of free radicals, agreeing with the results of Alia et al. (1997) who showed that among various compatible solutes, proline is the only molecule that has been shown to be able to protect plants against singlet oxygen and free radical induced damage resulting from stress. In addition, the increased content of chlorophylls could result from protection by proline of thylacoidal membranes against the attack of ROS as reported by Kavi Kishor et al. (2005).

CONCLUSION

From this study, it can be concluded that 24-epibrassinolide overcame the deleterious effects of salinity stress on the parameters investigated by enhancing growth parameters and by improving cell membrane stability. Based on these findings, 24-epibrassinolide treatment may help alleviating the negative effect of salinity on the growth and fruit yield of pepper plant. Further experiments will be carried out to determine the effect of 24-epibrassinolide solution spraying on the plant fruit yield.

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NaCl Stresi Altindaki Biber Bitkilerinde 24-epibrassinolit'in Büyüme, Klorofil, Elektrolit Kaybi ve Prolin Üzerine Etkileri

Özet

Brassinosteroidler, çevresel streslerin üstesinden gelme özelligine sahip steroid yapisinda bitki hormonlaridir. Bu çalisma, 24-epibrassinolit'in biber bitkisinde tuz toleransi tetiklemedeki rolünü incelemek üzere gerçeklestirilmis ve su fizyolojik parametreler ölçülmüstur: Büyüme, klorofil, elektrolit kaybi ve prolin. *Capsicum annuum* cv. Beldi tohumlari üzerine, NaCl varliginda ve yoklugunda 24-epibrassinolit püskürtüldü ve uygulamadan 28 gün sonra örnek alindi. Tuzluluk stresi altindaki kültürlerin analizi sonucu, biyokütle ve klorofilin önemli ölçüde azaldigi, elektrolit kaybi ve prolin yogunlugunun ise tuzluluk stresi altinda önemli miktarda arttigi tespit edildi. Ancak, 24-epibrassinolit uygulamasi, incelenen parametrelerde tuzluluk stresinin olumsuz etkilerini önemli ölçüde hafifletti ve bu durum, harici 24-epibrassinolit uygulamasinin stress altindaki bitkilerde büyümeyi artirabilecegi ve hücre zarinin yapisini koruyabilecegini iddia eden önceki yazarlarin varsayimlarini dogrular niteliktedir.

Anahtar Kelimeler: Brassinosteroidler, elektrolit kaybi, klorofil, prolin, tuz toleransi.

