Ethephon Seed Treatment Impacts on Drought Tolerance of Kentucky Bluegrass Seedlings

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ADDITIONAL INDEX WORDS. drought stress, antioxidant enzyme, lipid peroxidation

SUMMARY. Ethephon [ETH (2-chloroethylphosphonic acid, an ethylene-releasing compound)] has been used as a plant growth regulator in turfgrass management. The aim of the study was to assess the effects of ETH seed treatment on drought tolerance of kentucky bluegrass (*Poa pratensis*) seedlings. Seeds of two kentucky bluegrass cultivars, Midnight and Nuglade, were exposed to ETH treatment or untreated as controls. Seedlings were then exposed to two water regimes: well-watered conditions and polyethylene glycol (PEG)-induced drought conditions. ETH-treated plants exhibited better turf performance relative to the untreated control under PEG-stressed conditions illustrated by higher relative water content (RWC) and lower lipid peroxidation and lower electrolyte leakage (EL). In both cultivars, ETH treatment increased enzyme activity of ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT); proline content; and soluble protein content under PEG-induced drought conditions. The results suggest that ETH seed treatment can improve drought tolerance in kentucky bluegrass seedlings.

Kentucky bluegrass, a perennial grass species, is widely used for both turf and forage. Drought stress is one of the major factors affecting growth, function, and turf performance of kentucky bluegrass in water-limited areas. Extensive studies have shown that application of trinexapac-ethyl could improve water use efficiency in turfgrasses (Burgess and Huang, 2014; Etemadi et al., 2015; Hejl et al., 2015; Roo-hollahi et al., 2010). The potential of using other plant growth regulators to promote plant performance under drought conditions has also been evaluated and reported, including salicylic acid (Ahmed et al., 2014), abscisic acid (ABA) (Khan et al., 2012), and auxin-simulating growth promoters (Bingham and McCabe, 2006; Burgess and Huang, 2014).

Ethephon is an important plant growth regulator which is used in crops and vegetables to induce flowering (Duyvelshoff and Cline, 2013; Garcia et al., 2008) and enhance fruit maturation (Goldschmidt, 1998; Rony

et al., 2013). Ethephon penetrates into plant tissues and is then further translocated, where it decomposes to ethylene, the active substance (Zhang and Wen, 2010). Ethylene affects a variety of processes throughout the plant including stress responses (Wang et al., 2013). Ethylene-mediated growth enhancement can improve plant performance under adverse conditions (Pierik et al., 2007; Yang et al., 2013; Yu et al., 2001). Therefore, ETH might be a candidate for potentially improving plant tolerance to stresses. Morgan et al. (1977) pointed out that ETH treatment might also result in the accumulation of endogenous ABA, an inducer of a wide spectrum of antistress pathways in plants. Many reports confirmed that ETH played a key role in promoting plant tolerance to biotic stress. ETH treatment induced disease resistance in grapevine [*Vitis vinifera* (Belhadj et al., 2008)], mature tomato [*Solanum lycopersicum* (Wang et al., 2011)], and banana [*Musa acuminata* (Tang et al., 2010)] by mediating the expression of

different pathogenesis-related genes. Moreover, ETH treatment stimulated the gene expression of *phospholipase D* (implicated in several responses to stress) following mechanical wounding in cucumber [*Cucumis sativus* (Peng and Mao, 2011)]. Ethephon enhanced the germination of tassel flower [*Amaranthus caudatus* (Bialecka and Kepczynski, 2009, 2010)] and cucumber (Chang et al., 2010) seeds.

There are quite a few studies investigating the effect of ETH application on drought responses in plants, but the results are inconsistent, being dependent on plants' genetics, intensity, and duration of drought treatment and many other unknown factors. For instance, Kasele et al. (1994) revealed that under drought conditions, ETH application increased corn (*Zea mays*) yield at high plant densities, whereas it decreased yield at low plant densities. Shekoofa and Eman (2008) reported that the yield response of corn to ETH varied with plant density and available water. Zhang and Kirkham (1990) showed that ETH treatment increased the turgor potential of drought-sensitive cultivars of sorghum (*Sorghum bicolor*) but not of drought-resistant ones. Kirkham (1983) also found that ETH treatment did not increase resistance to drought in winter wheat (*Triticum aestivum*). Moreover, excessive ETH application induced oxidative stress (Xu et al., 2012), which might have negative effects on plant abiotic stress tolerance.

To minimize the oxidative damage induced by drought and other environmental stresses, plants have evolved various enzymatic and non-enzymatic mechanisms to detoxify harmful oxygen species, including enzymes that combat superoxide (O_2^-) and hydrogen peroxide (H_2O_2), such as superoxide dismutase (SOD), CAT, and POD (Smirnoff, 1993). Ascorbate peroxidase is a H_2O_2 -scavenging

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
0.1	bar	MPa	10
29.5735	fl oz	mL	0.0338
2.54	inch(es)	cm	0.3937
28.3495	oz	g	0.0353
0.001	ppm	mg·g ⁻¹	1000
1	ppm	mg·L ⁻¹	1
1	ppm	μg·g ⁻¹	1
(°F - 32) ÷ 1.8	°F	°C	(°C × 1.8) + 32

enzyme that is unique to plants and is indispensable to protect chloroplasts and other cellular constituents from damage caused by H_2O_2 and hydroxyl radicals ($\cdot\text{HO}$) produced from it (Asada, 1992). Better turf quality in kentucky bluegrass under drought has been related to increases in antioxidant activities (Fu and Huang, 2001; Xu et al., 2011a). Proline, an amino acid, plays a highly beneficial role in plants when they are exposed to various stress conditions (Hayat et al., 2012). Besides acting as an excellent osmolyte, proline plays three major roles during stress; i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat et al., 2012). As osmolytes, soluble proteins also play an important role in maintaining leaf turgor and promoting the root's ability to uptake more water under drought (Wu et al., 2015). Ethephon might affect the drought tolerance of plants by mediating changes in antioxidant enzyme activity, and proline and soluble protein metabolism. Iqbal et al. (2017) conclude that ETH regulated proline and antioxidant metabolism under salinity stress to increase photosynthetic function in mustard (*Brassica juncea*). Ozturk and Demir (2003) showed that under saline conditions, ETH increased CAT activities and proline content but decreased POD activity. Kim et al. (2016) proved that the presence of ETH led to significantly higher levels of proline in a green alga (*Chlorella vulgaris*). Yu et al. (2017) also found that ETH significantly increased the accumulation of proline and the activities of SOD, POD, and CAT which in turn improved corn performance under drought stress. Zhang et al. (2013) demonstrated that ETH application promoted root hair development in trifoliate orange (*Poncirus trifoliata*), which could be beneficial to plants' response to drought.

In the turf industry, foliar application of ETH has been used to suppress seed head numbers and vegetative growth in a few turfgrass species (Fry, 1991). Jiang and Fry (1998) indicated that foliar application of ETH could increase the turf quality of perennial ryegrass under drought. The efficacy of using ETH as a seed treatment to promote drought resistances of turfgrass seedlings has not been widely studied.

For this work, we investigated physiological parameters in seedlings of two kentucky bluegrass cultivars under drought and well-watered conditions to analyze if ETH seed treatment can alter antioxidant enzyme activity, and proline and soluble protein content, and if this affects drought tolerance in kentucky bluegrass seedlings.

Materials and methods

PLANT MATERIALS AND GROWING CONDITIONS. Seeds of kentucky bluegrass cultivars Midnight and Nuglade were surface sterilized in 1% sodium hypochlorite (NaClO) for 5 min and then washed in sterilized water. Seeds of both cultivars were either immersed in 100 mL of 150 $\text{mg}\cdot\text{L}^{-1}$ ETH solution for 24 h or in 100 mL of distilled water for 24 h for ETH treatment and control, respectively. The ETH concentration of 150 $\text{mg}\cdot\text{L}^{-1}$ was used based on our previous study where it had maximum effect (Han, 2014). Afterward, the seeds were put onto sieves in plastic containers (length 45 cm, width 30 cm, and depth 25 cm) filled with half-strength Hoagland solution (Hoagland and Arnon, 1950) and maintained in hydroponic culture in a greenhouse for 1 month. Seedlings were grown at an average temperature of 25 °C under a 16/8 h (light/dark) photoperiod, with a light intensity of 600 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The hydroponic solution was aerated by small aquarium air pumps (Atman EP-9000; Chuangxing Electrical Appliance Co., Zhongshan, China). Hoagland solution was replaced with fresh solution every week.

TREATMENT AND EXPERIMENTAL DESIGN. Seedlings of both ETH-treated, and non-ETH-treated control plants were grown until the fourth leaf was fully expanded and the first leaf was ≈ 13 cm long during the 1-month period of the experiment. All the seedlings were exposed to one of two water regimes: 1) well-watered conditions, in which seedlings were growing in half-strength Hoagland solution and 2) PEG-induced drought conditions, in which seedlings were growing in half-strength Hoagland solution plus PEG 6000 (30%; Beijing Kebaiao, Beijing, China). Water potential of the PEG solution was -1.20 MPa (Steuter et al., 1981). The pH of all solutions was adjusted to 6.0.

The seedlings were maintained under different water regimes for 10 d. Ethephon treatment and water regimes were arranged as a completely randomized block design with four replications. Each replication was many seedlings pooled for sampling.

RWC AND EL. Physiological changes observed in kentucky bluegrass plants were evaluated by measuring leaf RWC and cellular membrane stability expressed as EL. Measurements took place at 0, 5, and 10 d after the initiation of water regime treatments. Leaf RWC was determined based on three weights from collected tissues; fresh weight [FW (≈ 0.15 g)], dry weight (DW), and turgid weight (TW) using the formula $\text{RWC (percent)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$. Leaves were collected from plants and immediately weighed for determination of FW. Samples were then immersed in deionized water and kept in dark for 12 h at 4 °C after which they were removed from deionized water, gently blotted dried, and weighed for TW. The samples were then dried in an oven set to 80 °C for at least 72 h and weighed for DW (Barrs and Weatherley, 1962). To measure EL, fresh leaves (0.1 g) were rinsed with deionized water to remove external solutes, and immersed in 20 mL deionized water. Initial conductivity of the solution (C_{initial}) was measured using a conductivity meter (DDSJ-318; HinoTek Instrument, Shanghai, China) following incubation in deionized water for 24 h on a shaker (HY-5; Ronghua, Jintan, China). Leaf samples were then autoclaved at 121 °C for 15 min and cooled at room temperature for final conductivity (C_{max}) measurement. EL percentage was then calculated as $(C_{\text{initial}} / C_{\text{max}}) \times 100$ (Blum and Ebercon, 1981).

ANTIOXIDANT ENZYME ACTIVITY AND LIPID PEROXIDATION. Fully expanded leaf blades were excised and then wrapped in foil paper, immersed in liquid nitrogen, and stored at -20 °C until measurement was determined. Determination of enzyme activity was carried out using the method previously described by Xu et al. (2011a). Briefly, 0.25 g of fresh leaf tissue was frozen in liquid nitrogen and then ground to a fine powder using a mortar and pestle, and the extraction was performed with 4 mL of extraction buffer (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid, 1% polyvinylpyrrolidone, and 1 mM dithiothreitol; pH

of 7). The extractions were centrifuged at 15,000 g_n for 30 min at 4 °C and the supernatant was collected for

enzyme assays and malondialdehyde (MDA) content measurement. The SOD [Enzyme Commission (EC)

number: 1.15.1.1] activity was measured by recording the rate of p-nitro blue tetrazolium chloride reduction

Fig. 1. Effect of ethephon (ETH) seed treatment on relative water content and electrolyte leakage of Kentucky bluegrass cultivars Midnight and Nuglade under well-watered and polyethylene glycol (PEG)-stressed conditions. Control represents distilled water-treated control plants and ETH represents ETH-treated plants. Bars indicate $\pm$ SD ($n = 4$). Bars which have the same letter indicate no significant difference ($P \leq 0.05$) according to Fisher's protected least significant difference test.

based on absorbance at 560 nm using a spectrophotometer (Biomate 3S; Thermo Fisher Scientific, Waltham, MA). The activity of CAT; EC: 1.11.7.6, POD; EC: 1.11.1.7, and APX; EC: 1.11.1.1 was determined by following the H_2O_2 changes in absorbance at 240, 470, and 290 nm, respectively. An absorbance change of 0.1 in 1 min was defined as one unit of enzyme activity. Lipid peroxidation was measured in terms of MDA content (Dhindsa and Matowe, 1981) with some modifications. A 1-mL aliquot of supernatant was mixed with 1 mL of 0.67% thiobarbituric acid. The mixture was heated at 95 °C for 15 min, quickly cooled on ice, and then centrifuged at 10,000 g_n for 5 min. The absorbance of the supernatant was read at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mm^{-1} \cdot cm^{-1}$ (Heath and Packer, 1968).

PROLINE AND SOLUBLE PROTEIN CONTENT. Randomly selected tillers from each treatment were harvested at 0, 5, and 10 d after the initiation of the water regime treatments. The samples were flash-frozen in liquid nitrogen for at least 1 h and then stored at -70 °C for measurement of proline content and soluble protein content. Proline content was determined as previously described (Bates et al., 1973). Soluble protein content was determined using Bradford's methods (Bradford, 1976).

DATA ANALYSIS. Effects of PEG treatment and ETH seed treatment were determined by analysis of variance according to the general linear model procedure of SAS (version 9.0; SAS Institute, Cary, NC). Differences between means were separated by Fisher's protected least significance difference test at the 0.05 significance level.

Results and discussion

EFFECT OF ETH SEED TREATMENT ON RWC AND EL OF KENTUCKY BLUEGRASS UNDER PEG-STRESSED CONDITIONS. Relative water content has been widely used to determine the physiological water status of plants. The results of this study indicated that PEG reduced RWC to 68% for 'Midnight' and 78% for 'Nuglade' (Fig. 1). Liu et al. (2006) also reported a gradual decrease of RWC in wheat cultivars after

application of PEG. Under PEG-stressed conditions, ETH-treated 'Midnight' maintained a higher RWC of 76.6%, relative to the control (67.8%) (Fig. 1). 'Nuglade' had a higher RWC of 81.8% with ETH treatment compared with the untreated controls 77.7% under PEG-stressed conditions. ETH-treated plants had higher RWC in both cultivars under PEG stress compared with control plants. It is reported that plants with higher RWC under drought stress may have greater drought resistance (Schonfeld et al., 1988; Xu et al., 2011b). Our results suggested that ETH seed treatment might improve drought tolerance in both cultivars through improving plant water status. It has been observed that ethylene can influence stomatal responses via cross talk with ABA (Daszkowska-Golec and Szarejko, 2013; Desikan et al., 2006). ETH seed treatment might regulate stomatal responses which play critical roles in maintaining cell turgor and physiological functions, particularly during plant dehydration. EL has been widely used to estimate cell membrane stability (Blum and Ebercon, 1981). PEG treatment increased EL in both cultivars (Fig. 1). ETH-treated plants had lower EL values than the control plants for both cultivars under PEG-stressed conditions (35% relative to 41%, and 37% relative to 42% for 'Midnight' and 'Nuglade'

respectively) (Fig. 1). Our results indicated that ETH seed treatment alleviated membrane damages caused by PEG stress. Yu et al. (2017) also found that ETH improved membrane stability in corn seedlings. Under nonstressed control conditions, ETH seed treatment reduced EL values for 'Nuglade' but not for 'Midnight', possibly because the cultivar sensitivity to ethylene may be different between the two. Previous studies also revealed that mustard cultivars with different ethylene sensitivities responded to ETH treatment differently (Iqbal et al., 2012).

EFFECT OF ETH SEED TREATMENT ON LIPID PEROXIDATION AND ANTIOXIDANT ENZYME ACTIVITIES OF KENTUCKY BLUEGRASS UNDER PEG-STRESSED CONDITIONS. Drought stress induced by PEG increased lipid peroxidation, measured as MDA content in leaves, compared with well-water conditions (Fig. 2). ETH treatment reduced MDA levels relative to the control under PEG-stressed conditions. This suggests that ETH treatment may improve antioxidant activity in kentucky bluegrass seedlings (Xu et al., 2011a).

Ethephon seed treatment promoted higher levels of SOD activity relative to control under well-watered conditions for 'Midnight' and under PEG-stressed conditions for 'Nuglade' (Fig. 3). Under PEG-stressed conditions, ETH increased SOD activity of

Fig. 2. Effect of ethephon (ETH) seed treatment on malondialdehyde of kentucky bluegrass cultivars Midnight and Nuglade under well-watered and polyethylene glycol (PEG)-stressed conditions. Control represents distilled water-treated control plants and ETH represents ETH-treated plants. Bars indicate $\pm$ SD ($n = 4$). Bars which have the same letter indicate no significant difference ($P \leq 0.05$) according to Fishers protected least significant difference test; $1 \mu g \cdot g^{-1} = 1 \text{ ppm}$.

‘Nuglade’ by 48%, compared with controls. This result suggested that ETH treatment improved the plant’s ability to combat O_2^- under PEG-stressed conditions for ‘Nuglade’ but not for ‘Midnight’. It also confirmed that the ways in which SOD responds to ETH treatment varies between cultivars. CAT activities were increased by ETH treatment under both well-watered and PEG-stressed conditions in both cultivars (Fig. 3) compared with their respective controls. It is worthy to note that under PEG-stressed condition, ETH treatment prompted $\approx 133\%$ and 41% higher CAT activity for ‘Midnight’ and ‘Nuglade’. In agreement with our results, Ozturk and Demir (2003) found that ETH treatment increased CAT activities in salt-stressed spinach leaves. In addition, ETH seed treatment promoted higher enzyme activity of

POD (Fig. 3) and APX (Fig. 3) under PEG-induced drought conditions. Many studies have revealed that ethylene responsive factors (ERFs) play important roles in conferring plant stress tolerance by promoting gene expression of CAT and other defense-related genes (Li et al., 2012; Sewelam et al., 2013; Wu et al., 2008). ETH treatment might trigger expression of ERFs, which in turn enhance the expression of antioxidant genes. In general, higher levels of antioxidant enzyme activity in ETH-treated seedlings indicate that the ability to scavenge for singlet oxygen and H_2O_2 in kentucky bluegrass was enhanced by ETH treatment, which may result in less free radical-mediated damage, including lipid peroxidation (demonstrated by MDA production, Fig. 2) and improve plant drought tolerance.

EFFECT OF ETH SEED TREATMENT ON PROLINE CONTENT IN KENTUCKY BLUEGRASS UNDER PEG-STRESSED CONDITIONS. Polyethylene glycol treatment increased proline content ≈ 3 -fold in comparison with the control under well-watered conditions in both cultivars (Fig. 4). Ethephon treatment increased proline content in both cultivars under PEG-stressed conditions compared with well-watered controls. It is well known that proline accumulates in plants during adaptation to various types of abiotic stresses (e.g., drought). Grzesiak et al. (2013) also reported that proline could be the most important osmoprotectant in osmotic stress induced by PEG. Our data support that proline accumulation in plants has a protective function (Fig. 4). It is suggested that ABA plays a vital role in proline synthesis/accumulation (Hare et al., 1999), and ETH seed

Fig. 3. Effect of ethephon (ETH) seed treatment on activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) in kentucky bluegrass cultivars Midnight and Nuglade under well-watered and polyethylene glycol (PEG)-stressed conditions. Control represents distilled water-treated control plants and ETH represents ETH-treated plants. Bars indicate $\pm$ SD ($n = 4$). Bars which have the same letter indicate no significant difference ($P \leq 0.05$) according to Fishers protected least significant difference test; 1 U/g = 28.3495 U/oz.

Fig. 4. Effect of ethephon (ETH) seed treatment on proline content in kentucky bluegrass cultivars Midnight and Nuglade under well-watered and polyethylene glycol (PEG)-stressed conditions. Control represents distilled water-treated control plants and ETH represents ETH-treated plants. Bars indicate $\pm$ SD ($n = 4$). Bars which have the same letter indicate no significant difference ($P \leq 0.05$) according to Fishers protected least significant difference test; $1 \mu\text{g}\cdot\text{g}^{-1} = 1 \text{ ppm}$.

Fig. 5. Effect of ethephon (ETH) seed treatment on soluble protein content in kentucky bluegrass cultivars Midnight and Nuglade under well-watered and polyethylene glycol (PEG)-stressed conditions. Control represents distilled water-treated control plants and ETH represents ETH-treated plants. Bars indicate $\pm$ SD ($n = 4$). Bars which have the same letter indicate no significant difference ($P \leq 0.05$) according to Fishers protected least significant difference test; $1 \text{ mg}\cdot\text{g}^{-1} = 1000 \text{ ppm}$.

treatment might increase ABA synthesis/accumulation, which activates proline synthesis (Verbruggen and Hermans, 2008) and other stress defense pathways in a coordinated manner.

EFFECT OF ETH SEED TREATMENT ON SOLUBLE PROTEIN CONTENT IN KENTUCKY BLUEGRASS UNDER PEG-STRESSED CONDITIONS. Ethephon-treated seedlings of both kentucky bluegrass cultivars showed

greater soluble protein content than nontreated plants in response to PEG-induced drought stress (Fig. 5). Plants adapt to drought stress via osmotic adjustment through the accumulation of soluble proteins and proline, as well as other compatible solutes (Blum, 2017). In this study, the soluble protein (Fig. 5) and proline contents (Fig. 4) were all increased with ETH seed treatment under PEG stress, which

suggested that ETH seed treatment might contribute to enhancing osmotic adjustment by increasing the contents of soluble proteins and proline (Fig. 4) under PEG-induced drought stress. The increases in soluble protein content in ETH-treated 'Midnight' and 'Nuglade' over their respective control under PEG-stressed conditions were 30% and 22%, respectively.

The generation of reactive oxygen species can cause the oxidation of amino acids and damage protein structure; therefore, oxidative stress might be responsible for the reduction of soluble proteins in kentucky bluegrass plants under PEG stress. Higher antioxidant enzyme activity promoted by ETH seed treatment (Fig. 3) provided better protection against oxidative damage induced by PEG stress which might cause less reduction in soluble protein content in ETH-treated plants.

In conclusion, ETH seed treatment might improve drought tolerance in kentucky bluegrass seedlings through promoting antioxidant enzyme activity and the accumulation of proline and soluble proteins. However, more research is needed to evaluate the efficacy of ETH on the improvement of drought tolerance in established kentucky bluegrass turf as well.

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