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Drought and Biostimulant Impacts on important Attributes of Perennial Ryegrass for Orchard and Vineyard Floor in the Intermountain West Region of the United States

Shahla Mahdavi[1] and Esmaeil Fallahi[2]

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Abstract

Water shortage is a critical issue worldwide, mandating the use of efficient irrigation systems (i.e. drip and micro-jet) in modern orchards and vineyards to irrigate only the target trees and vines. Therefore, this issue may adversely impact orchard floor vegetation, which is needed to prevent tractor traffic compaction. Thus, the impact of two levels of evapotranspiration-based (ETc) water stresses and biostimulants consisting of abscisic acid (s-ABA), Glycine betaine (GB), digoxin, nano silica and some of their combined applications on perennial ryegrass (*Lolium perenne,* L.*)* under climatic and soil conditions of the Intermountain West, USA were studied. Comparing two levels of irrigation, grass clippings with 50% ETc had higher electrolyte leakage and proline but lower visual performance, chlorophyll index (CI) and chlorophyll b than those with 75% ETc. With the exception to the digoxin at 0.5 $mg \cdot l^{-1}$, clippings from all biostimulant-treated plots had significantly better visual performance and higher CI, proline, chlorophyll a and b and potassium (K) concentration than those from the un-treated control. These results underscore the value of these biostimulants for improving the orchard and vineyard floor grass covers under drought stress conditions that prevail in the western United States.

The population increase, meshed with the worldwide water shortages is becoming an increasingly critical issue that mandates minimum use of irrigation water with maximum efficiency and productivity in all agricultural crops. Under such conditions, priority for water use will be given to food crops and as a result landscape plants like turf will be subjected to additional water shortage. At the same time, orchard and vineyard floor cover grasses are needed to reduce soil erosion and compaction from tractors, while maintaining herbicide strips (Rowley et al., 2012).

To conserve water, it is necessary to adopt efficient irrigation systems such as drip and micro-jet in modern orchards (Fallahi et al., 2011), and these irrigation methods may adversely impact vegetation in row middles. Among the possible solutions for maintaining a bio-mulch in these alleyways is the use of drought resistant grasses such as crested wheat grass (*Agropyron cristatum*) (Fallahi et al, 2015) and osmoprotectants or biostimulants (Farooq et al., 2009). Various supplemental chemicals, including plant biostimulants, enhanced survival and recovery of various plants after dehydration. Since the discovery of abscisic acid as a growth retardant by Addicott et al. (1964), a synthetic form was used to induce dormancy (Goggin et al., 2009), flower induction (Greene et al., 2011), fruit set in apples (Greene. 2012), and color enhancement in grapes (Peppi et al., 2006). Plant biostimulants such as abscisic acid play an important role in the regulation of plant tolerance to various environmental stresses, especially drought stress (Quarrie, 1989). Both natural (Lopez-Garbonell et al., 1994) and synthetic forms of plant biostimulants increase tolerance to abiotic stresses (Farooq

[1, 2] PhD graduate and Professor of Pomology, respectively, University of Idaho, Parma Research and Extension Center, 29603 U of I Lane, Parma, ID, USA

et al., 2009). Elevated ABA levels in xylem and root during dehydration can induce closure of stomata as a mechanism to conserve water (Wilkinson and Davis, 2002), inhibit leaf growth or transpiration (Alves and Setter, 2000) and adjust osmotic enhancement (Zhaolong, 2002). Tworkoski et al. (2011) reported that s-ABA was effective in delaying dehydration-induced wilt of shoot tips in apple trees. Recently the role of s-ABA for reducing stress in landscape plants has received particular attention.

Glycine betaine (GB) is an ammonium compound in plants and was discovered in the 19th century. This compound increases in response to dehydration stress (Ashraf and Foolad, 2007). Glycine betaine accumulates under abiotic stress conditions in many plants including barely *(Hurdeum vulgare L.)* (Nomura et al., 1995). In many plants endogenous GB is not sufficient to reduce abiotic stresses dehydration (Subbarao et al., 2000) and exogenous application of GB improves the growth and production of plants under stress (Hussain et al., 2008). Foliar application of GB enhanced the growth of plants under drought conditions by maintaining osmotic pressure and improving stomatal conductance, leading to an enhanced carboxylation efficiency of Rubisco and photosynthesis (Sakamoto and Murata, 2002).

Next to oxygen, silicon is the most abundant element on the surface of the earth. Although not considered as an essential element for higher plants, silicon has major roles in leaf turgidity of monocots and cell wall structure (Gong et al., 2003). Silicon can be effective against both biotic and abiotic stresses (Epstein, 1999). Gong et al. (2003) suggested that application of silicon could improve growth of wheat in arid or semi-arid areas. Hattori et al. (2005) reported that nano silicon enhanced drought tolerance in two cultivars of sorgum (*sorgum bicolour*). Agarie et al. (1998) reported that silicon decreased electrolyte leakage and increased cell walls polysaccharides in rice, suggesting that silicon could be involved in thermal stability of lipids in cell membranes and prevent structural and functional deterioration of cell membranes when rice plants are exposed to abiotic stresses.

Digoxin (Dig) has been exclusively used in human tissues. According to Lelievre and Lechat (2007), digoxin is a cardiac glycoside that binds to and inhibits sarcolemma-bound (Na^+/K^+-) Mg^{2+}-ATPase in human. The inhibition induced by Dig leads to an efflux of K from the cell and, in proportion to the extent of inhibition of the ATPase, an increase in internal sodium ion concentration ($[Na^+]$) at the inner face of the cardiac membranes. This local accumulation of sodium causes an increase in free calcium concentrations via the Na^+–Ca^{2+} exchanger. This free cellular Ca is responsible for the inotropic action of Dig, secondary to the release of Ca^{2+} from the sarcoplasmic reticulum, including the clinical and molecular basis (Lelievre and Lechat, 2007). Edner et al. (1993) studied the influence of Dig on muscular and symphatoadrenergic activity and the serum potassium concentration. The role of this compound is not known in plant tissues.

Grasses are the most common cover crops in apple orchards. Newhouse and Dana (1989) used perennial ryegrass in strawberries. They reported that this living mulch significantly increased strawberry yield and quality and protected strawberry crowns from spring and winter winds and cold temperatures. Granatstein et al. (2010) reported that using living mulch with a mix of perennial ryegrass and tall fescue (*Schedonorus phoenix* (Scop) Holub) in a modern 'Gala' apple orchard system provided good weed control, soil quality benefits and meaningful N contribution. Stefanelli et al. (2009) used a mixture of mammoth red clover (*Trifolium pratense* Var. perenne, L.) and endophytic perennial ryegrass as living mulch for orchard floor management and reported this as a suitable combination in an organic apple production.

Perennial ryegrass is a popular cool season grass, which is commonly used alone and in mixture with other species in modern orchard and vineyard floors. However, information on the use of various levels of water stress and biostimulants on this grass, particularly as living mulch for orchard floors is lacking. Thus, our objective in this experiment was to study the effect of two levels of ET-based drought in combination with exogenous s-ABA, glycine betaine (GB), s-ABA plus GB, two levels of digoxin, two levels of nano silica and digoxin plus nano silica on performance, and other attributes of perennial ryegrass climatic conditions of the southwest Idaho in the Intermountain West Region of United States.

Materials and Methods

Experimental Site and Turf Establishment. The experimental site was located at the Pomology and Viticulture Orchards and Vineyards of the University of Idaho, Parma Research and Extension Center, Parma, Idaho, USA. Perennial ryegrass (*Lolium perenne* L.) was planted in May of 2013. The history of cultural practices and maintenance of this lawn was well recorded and kept at the University of Idaho Research and Extension Center. This was considered a well-established and perfectly suitable lawn, resembling floor cover (alley way) in many commercial orchards and vineyards in the region. The experiment was performed during spring and summer of 2015.

Calculations for ETc, Water Applications. The University of Idaho Parma Research and Extension Centre Weather station (Agri-Met) was located at the experimental site and recorded ET data for all agricultural crops including lawns. There were two irrigation systems to create water deficiency at 50% ETc or 75% ETc of a full ETc. Water requirement was calculated based on ETc where ETc = ETr x Kc (Allen et al., 1998) and the details of irrigation calculations were similar to an earlier reports by Fallahi et al. (2011) and Mahdavi et al. (2016).

Biostimulant Treatments. Biostimulant treatments were prepared and applied twice. The first application was made between 8 a.m. and 1 p.m. on 28 July 2015. On this day, temperatures during application ranged from 21° C to 28° C with a clear sky and wind was calm (less than 2 $km \cdot hr^{-1}$). The second applications were made on 11 Aug. 2015. On this day, temperatures during application ranged from 29° C to 37° C with a clear sky, and the wind was calm (about 1 km/hr). Each solution was uniformly sprayed at the rate of 0.78 $l \cdot m^2$. The non-treated control plots were also sprayed with water at the 0.78 $l \cdot m^2$. The treatment at each application were as follows:

1. ProTone SG® (s-abscisic acid ; s-ABA; 20% soluble granule formulation; Valent BioScience Inc, Libertyville, Illinois, USA) at 100 μM.
2. Glycine betaine (GB; ≈ 99% purity, Sigma Life Science, Sigma-Aldrich Louis, MO, USA) at 100 mM.
3. S-ABA at 100 μM and GB solutions at 100 mM applied separately at each application time.
4. Digoxin (Dig; Powder; DSM Pharmaceuticals Inc. Greenville, NC, USA) at 0.25 $mg \cdot l^{-1}$ (Dig_1) or 0.5 $mg \cdot l^{-1}$ (Dig_2).
5. Nano silica (NanSi; ≈ 99% purity, 20-30 nm, amorphous, US Research Nanomaterials Inc., Houston, TX, USA) at 1 mM ($NanSi_1$) or at 2 mM ($NanSi_2$).
6. Dig at 0.25 $mg \cdot l^{-1}$ and NanSi solutions at 1 mM prepared and applied separately (not in the same solution) at each application time.
7. Un-treated control (water application only).

Measurements. Soil moisture (volumetric water content; VWC) was measured with a fully computerized soil moisture meter, equipped with two 7.5-cm rods, designed for lawn moister (FieldScout Digital Soil Moisture Meter, Model TDR 300, Spectrum Technologies, Aurora, IL, USA) before and after each irrigation and sometimes in between irrigations. At each time, VWC from three different locations within the same plot was measured and averaged. Although ETc was the bases for irrigation scheduling, the soil VWC was measured to monitor and compare the ETc-based water application with that shown with a soil moisture meter. Soil moisture measurements were made between 27 July and 7 Sept.

Performance of the lawn was visually rated based on a combination of several factors, including greenness, growth, density and appearance on a scale of 1 to 9, according to the guideline recommended by Morris (2002); where 1 is poor performance, 7 is acceptable and 9 is outstanding. Visual performance ratings for 7 different dates were averaged and reported.

Chlorophyll indices between 10:00 a.m. to 2:00 p.m. were determined on 12 dates between 29 July and 3 Sept. 2015, using equipment based on a new technology (FieldScout Chlorophyll Meter, Model CM 1000, Spectrum Technologies, Aurora, IL, USA). With this instrument, the ambient and reflected 700 nm and 840 nm wavelengths are used to calculate the relative chlorophyll index. It measures conical viewing areas between, 30 and 180 cm from the lens. The instrument measures index of relative chlorophyll content ranges from 0 to 999. At each time, chlorophyll indices of three different locations per plot were measured and averaged. The average values of chlorophyll indices over these 12 dates are presented in this report.

A composite grass-clipping sample from three locations per plot was taken at a height of approximately 3 to 4 cm from the soil level on 24 Aug., 2015. Clippings were washed in a mild solution, containing 1% Liqui-Nox anionic detergent (AlcoNox Inc., White Plains, NY, USA), rinsed in three different 25-l containers of distilled water, and dried in a forced-air oven at 65°C. Clippings fresh weight (FW) and dry weight (DW) were used to calculate dry weight percentage (DW %). The dried leaves were ground to pass a 40-mesh screen using a Cyclotec Sample Mill (Model 1093; Tecator, Hoganas, Sweden). For mineral analysis, specific guidelines and methods described by Gavlak et al. (2005) were used. Leaf tissue was analysed for potassium (K) by dry ashing at 500°C, nitric/perchloric digestion and the use of Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Perkin-Elmer; Norwalk, CT, USA).

Free proline content was determined according to the method described by Bates et al. (1973). Fully expanded leaves (0.1g) were extracted with 10 mL of 3% (w/v) sulphosalicylic acid. The extract was centrifuged for 10 min. at 3000 RPM. Two mL of supernatant was transferred to fresh test tubes and 2 mL acid ninhydrin and 2 mL glacial acetic acid were added and incubated for 1 hour in a boiling water bath followed by an ice bath. Three mL toluene was added and mixed vigorously until the chromosphere phase separated from the aqueous phase. A calibration curve was prepared from a set of standards consisting of 0, 20, 40, 80 and 100 ppm proline (≈ 99.5% purity, Sigma Life Science, Sigma-Aldrich Louis, MO, USA), using the same procedures as described for samples. The absorbance was read at 520 nm using a spectrophotometer (Bio Mate UV Visible, ThermoScientific, Madison, Wisconsin). This instrument estimated concentrations of proline in unknown samples from a standard curve.

Electrolyte leakage (EL) of leaves was measured according to the method of Valencovic et al. (2006). With this method, 0.5 g of leaves were excised and cut into 2 cm segments after being rinsed three times with distilled deionized water. Leaf segments of each sample were placed in a sterile test tube containing 25 mL of distilled deionized water. Test tubes were shaken on a shaker for 24 hours and the initial conductivity (EC_1) was measured using EC meter (FieldScout Direct Soil EC Meter w/8in Probe, Spectrum Technologies, Aurora, IL, USA). Leaf samples were then autoclaved at 121°C for 1 hour in hot water bath followed by an ice bath and conductivity of killed tissues (EC_2) was measured after tubes cooled to room temperature. The percent electrolyte leakage was calculated as $(EC_1/EC_2)_*100$.

Photosynthetic pigments from turfgrass leaves were extracted as described by Arnon (1949). For this purpose, 0.25 g fresh leaves were ground and completely homogenized in 2 ml of 80% (v/v) acetone using a mortar and pestle, and then centrifuged 10 minutes and 6000 RPM. Absorbance of the resulting extracts was measured at three wavelengths 663, 470 and 646 nm for chlorophyll a (Chl a) and chlorophyll b (Chl b), using Bio Mate Uv Visible, ThermoScientific (Madison, Wisconsin) and converted to mg/g leaf fresh weights.

Layout, Experimental Design, and Statistical Analyses. The experimental design was a randomized-complete-block split-plot design with two levels of irrigations as the main plot (main-effect) and stress-inducing biostimulants, either alone or in some combinations, and an un-treated control as sub-plots, each with four blocks. The size of each sub-plot was 2.0 m x 1.5 m Sufficient buffer zones were kept within each block and between different blocks to prevent cross contamination of irrigation regimes. Analyses of variance and all possible correlation coefficients among all attributes were conducted using SAS Version 9.4 Programme (SAS Institute, Cary, NC, USA), with PROC GLM. Means were compared by Duncan's multiple range test at $P \leq 0.05$.

Results and Discussion

Interaction. There was no significant interaction between water stress levels and biostimulant treatments for any of the parameters measured in this study.

Soil Volumetric Water Content (VWC). Irrigation was scheduled based on daily ETc data. However, an approximately 25% difference in VWC existed between the 50% ETc and 75% ETc treatments during the course of this study (Fig.1), which confirms the validity of the use of ETc for creation of these two levels of stress in this study.

Water Stress Effects. Grass clippings with 50% ETc had significantly higher proline and electrolyte leakage but lower visual performance, chlorophyll index (CI), and chlorophyll b than those with 75% (Table 1) and the positive correlation coefficient between chlorophyll index and visual rating was extremely strong (r = 0.97). Grass clippings with 75% ETc tended to have higher K concentration than those with 50% although differences were not significant (Table 1). Although we did not have a 100% ETc treatment in this study, high visual ratings (as high as a rating of 9 out of 9) in the 75% ETc indicated that

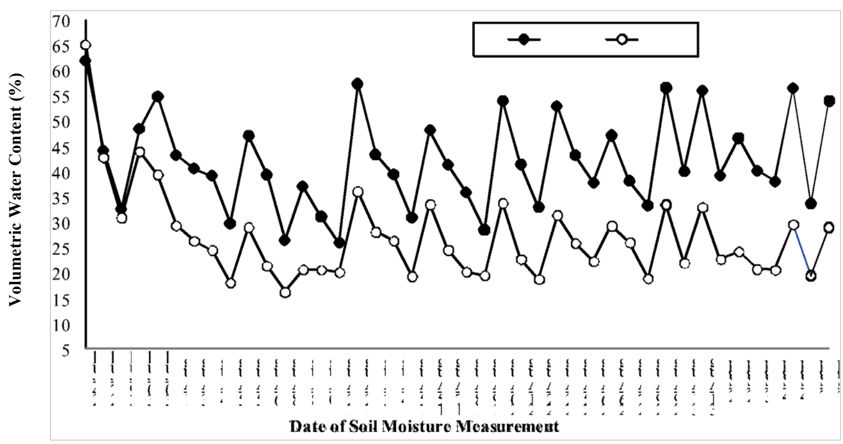

Fig. 1. Soil volumetric water content of 50% ETc and 75% ETc plots during the experiment

Table 1. Effect of water stress and biostimulants on some attributes in orchard and vineyard floor cover perennial rye grass.

Treatment	Visual performance (1-9)*	Chlorophyll Index (1-999)*	Proline (μM/gr FW)	EC (%)	Chlorophyll a (mg/gr FW)	Chlorophyll b (mr/gr FW)	K (% dwt)
Water stress level†							
50% ETc	5.97 b ‡	349 b	13.8 a	28.3 a	0.671 a	0.258 b	1.10 a
75% ETc	8.34 a	510 a	7.7 b	10.4 b	0.703 a	0.340 a	1.41 a
Biostimulant†							
s-ABA	7.33 a	445 a	11.9 ab	17.1 c	0.68 abc	0.273 bc	1.35 a
GB	7.21 a	428 ab	11.8 ab	16.4 c	0.700 ab	0.327 ab	1.24 ab
s-ABA& GB	7.38 a	448 a	13.2 a	17.1 c	0.805 a	0.385 a	1.36 a
Dig_1	7.34 a	440 a	10.1 bc	14.9 c	0.678 abc	0.312 ab	1.32 a
Dig_2	6.68 b	403 bc	8.4 cd	25.2 b	0.615 bc	0.269 bc	1.11 b
$NanSi_1$	7.30 a	436 a	11.7 ab	15.1 c	0.716 ab	0.327 ab	1.27 a
$NanSi_2$	7.15 a	433 ab	11.6 ab	20.2 bc	0.740 ab	0.258 bc	1.27 a
Dig_1NanSi_1	7.31 a	438 a	10.4 abc	16.3 c	0.697 abc	0.318 ab	1.33 a
Control	6.71 b	396 c	7.3 d	32.2 a	0.555 c	0.219 c	1.10 b

* Visual performance rating: 1= poor performance, progressively to 9 = highest (best) performance. Chlorophyll index= Index of relative chlorophyll content= 0 to 999.

† Abbreviations: ETc = Evapotranspiration of lawn; s-ABA= s-abscisic acid; GB= glycine betaine; Dig= Digoxin; NanSi= nano-silica; FW= fresh weight; dwt= dry weight; EC %= Percentage electrolyte leakage; K= Leaf potassium Content.

‡ Mean values within each column of irrigation or biostimulant treatments followed by different letter (s) were significantly different at $P \leq 0.05$ by Duncan's multiple ranges test.

this level of irrigation was sufficient. However, application of water at 50% ETc over the period of this study was unacceptably stressful.

Effects of Biostimulants. With the exception to the Dig_2 treatment, application of each biostimualnt alone or in combination, significantly increased visual performance, chlorophyll index, proline, chlorophyll a and b, and K concentration and decreased percentage electrolyte leakage as compared to control (Table1) and this observation underscores the value of these biostimulants in drought stress reduction. Our study was conducted on a large scale and under field conditions, similar to a realistic commercial orchard and vineyard floor conditions. However, our results with GB was consistent with Hu et al. (2012) who reported a higher chlorophyll content in perennial ryegrass under abiotic stress in greenhouse conditions. They did not include s-ABA in their study. It is essential to conduct an economical analysis to see if the use of these biostimulants, particularly digoxin, to reduce lawn stress is justifiable. Also, further study to understand the mode of action of digoxin spray in plants is necessary.

Higher K in the s-ABA-treated clippings is in agreement with a previous report in Cathamus plants (Gadallah, 1996). Concentrations of K in clippings were negatively correlated with lawn temperatures (r=-0.70) and with clipping percentage electrolytes leakage (r = -0.69), which confirms the positive relationship between K and drought resistance (data not shown). Since application of most biostimulants that resulted in a better orchard floor performance also increased leaf K concentration, it is important to study the physiological and biochemical relationship between K and drought tolerance in more detail. For example, it is essential to see if a simple potassium spray could induce and increase drought tolerance ofperennial ryegrass. If this theory is proven to be correct, a simple K spray can replace application of rather expensive biostmulants, while saving water.

Conclusions

High visual ratings in the 75% ETc is a clear indication that this level of irrigation was sufficient to maintain a reasonable turf. However, 50% ETc treatment was too stressful and long-term application of this level of irrigation may lead to poor turf quality in alleyways of orchards and vineyards. Nevertheless, based on the results of this study, application of biostimulants can slow the process of grass quality decline under extremely severe drought conditions (i.e. 50% ETc). This area deserves further study to see if applications of other stress levels such as 65% ETc with these biostimulants can further reduce stress and maintain turf visual quality.

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