HortScience 60(3):310–316. 2025. https://doi.org/10.21273/HORTSCI18179-24

Effects of Chemical and Biological Inhibitors of Ethylene on Heat Tolerance in Annual Bluegrass

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Keywords. aminoethoxyvinylglycine, *Paraburkholderia aspalathi*, *Poa annua*, plant growth promoting rhizobacteria, root morphology

Abstract. **Heat-stress-induced ethylene accumulation in plants inhibits growth and intensifies damage. Suppressing ethylene production in heat-stressed plants through chemical and biological inhibitors has been effective in promoting heat tolerance in plants. Aminoethoxyvinylglycine (AVG), is a chemical ethylene inhibiter that impedes the ethylene synthesis enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. Biological ethylene inhibitors include bacteria with ACC deaminase (ACCd) enzyme activity, which suppresses ethylene synthesis by breaking down its precursor, ACC. This study tested two ethylene inhibitors, AVG and a novel strain of the ACCd rhizobacteria *Paraburkholderia aspalathi,* 'WSF23', on annual bluegrass (*Poa annua* L.) to see whether they are effective in promoting its heat tolerance. *P. annua* plants were subjected to heat stress conditions for 21 d in controlled-environment growth chambers. Plants were separated into three treatment groups: 1) 25 mL of water (untreated control); 2) 25 mL of 25 μmol AVG; 3) 25 mL of *P. aspalathi* inoculant suspension. Treatments were applied once before imposing temperature treatments and then every 7 d during 21 d of heat stress. *Poa annua* treated with either AVG or the *P. aspalathi* had higher turf quality, green canopy cover, leaf relative water content, and chlorophyll contents during heat stress than the untreated control. Additionally, root characteristics were promoted under heat stress after ethylene inhibitor application, where *P. annua* treated with AVG had greater root depth and dry weight, while the *P. aspalathi* treatment resulted in greater total root length. Both ethylene inhibitors improved *P. annua* performance under heat stress, as characterized by delayed chlorophyll degradation and root maintenance.**

Heat stress is a primary environmental factor limiting the growth of cool-season turfgrass species, and the severity and frequency of heat stress has been intensified due to global warming (Abbas et al. 2024; Beard 1973; Sun et al. 2024; Tate et al. 2023; Xu et al. 2021). Heat damage in cool-season turfgrasses includes decline in turf performance, leaf senescence, inhibition of shoot and root growth, reduction in water and nutrient use, and interruption of hormone metabolism (Abbas et al. 2024; Li Q et al. 2022, 2023; Li Z et al. 2022; Rossi and Huang 2023a, 2023b; Xu and Huang 2000a, 2000b; Zhang et al. 2024). Annual bluegrass (*Poa annua*) is traditionally considered a weed. Despite this, *Poa annua* is the third most abundant cool-season grass species found on golf courses in the United States (Gelernter et al. 2017; Shaddox et al. 2023). This species has been increasingly maintained and cultivated as turfgrass on golf course putting greens due to its adaptation of low mowing and prolific growth in cool climates. However, *P. annua* is highly sensitive to heat stress compared with other cool-season turfgrass species, which has been largely attributed to its shallow root system, making management difficult during the summer (McBride et al. 2024; Petrovic et al. 1985; Wehner and Watschke 1981).

Hormones play a key role in regulating plant growth, and among them, ethylene is considered a stress hormone because it accumulates in plants in response to abiotic stresses, including heat stress (Abeles 2012; Bleecker and Kende 2000; Hays et al. 2007; Morgan and Drew 1997). Ethylene plays an important role in plant development and response to environmental stress (Chen et al. 2021). However, at higher concentrations, stress ethylene can inhibit root and shoot growth, initiate the accumulation of reactive oxygen species (ROS), and cause premature leaf senescence (Hays et al. 2007; Nordström and Eliasson 1984; Qin et al. 2019; Street et al. 2015; Xia et al. 2015). Suppressing ethylene accumulation under heat stress may delay leaf senescence and root growth inhibition (Ma et al. 1998; Zhang et al. 2024). Aminoethoxyvinylglycine (AVG) is a chemical inhibitor of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, a key enzyme involved in ethylene biosynthesis in the conversion of S-adenosylmethionine to ACC, which is ethylene's direct precursor (Even-Chen et al. 1982). Past studies have observed the positive effect of AVG on alleviating damages in plants from abiotic stresses, such as heat stress in soybeans (*Glycine max* L. Merr.), drought stress in wheat (*Triricum aesticum* L. cv. Buck Poncho), and waterlogging conditions in cotton (*Gossypium hirsutum* L.) (Beltrano et al. 1999; Djanaguiraman et al. 2011; Najeeb et al. 2015). For example, AVG was effective in preventing heat-stress-induced leaf senescence in creeping bentgrass (*Agrostis stolonifera*) by regulating chlorophyll (Chl) metabolism, antioxidant metabolism, and amino acid metabolism (Jespersen et al. 2015; Rossi and Huang 2023b; Xu and Huang 2009).

Endophytic bacteria can also be beneficial in improving plant abiotic stress resistance (Chang et al. 2023). Certain plant growth promoting rhizobacteria (PGPR) produce an ACC deaminase (ACCd) enzyme that breaks down ACC, suppressing ethylene production (Singh et al. 2015). Bacteria with ACCd activity have been tested on plants to observe their effect on promoting growth under different abiotic stresses (Cheng et al. 2016; Mahdavi et al. 2020). One PGPR, *Paraburkhulderia aspalathi*, an endophytic bacterium that contain enzymes with strong ACCd activity, was found to improve drought tolerance in creeping bentgrass (Errickson et al. 2023). *Burkholderia phytofirmans* PsJN and *Burkholderia gladioli* RU1 were found to improve salt tolerance in perennial ryegrass when plants were colonized by this PGPR (Cheng et al. 2016).

Although the benefits of AVG-inhibiting ethylene production to improve plant stress tolerance have been well documented across different plant species, large-scale commercial use in turfgrass management may be difficult because of its chemical nature and overall costs. The use of ACCd-PGPR, a biological ethylene inhibitor, is believed to be a promising eco-friendly alternative for alleviating abiotic stress (Singh et al. 2022; Turan et al. 2017). However, how the biological form of ethylene suppressor, PGPR with ACCd activity, affect turf and root growth under heat stress and the relative effectiveness to improve heat tolerance of cool-season turfgrasses compared with the chemical ethylene inhibitor, AVG, are not well documented.

Received for publication 3 Sep 2024. Accepted for publication 11 Nov 2024.
Published online 7 Feb 2025.
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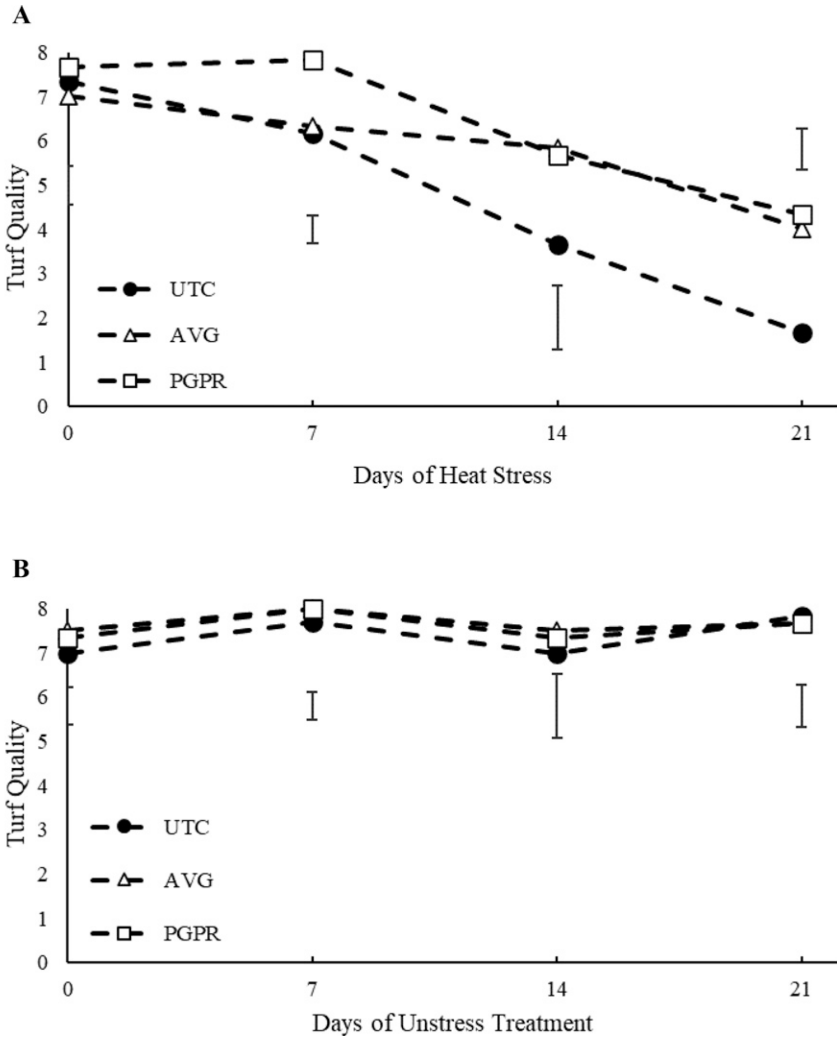


Fig. 1. Effects of ethylene inhibitors [aminoethoxyvinylglycine (AVG) and plant growth promoting rhizobacteria (PGPR) with ACC deaminase activity] on *Paraburkholderia annua* visual turf quality under heat stress (**A**) and unstressed (**B**) conditions. Vertical bars represent least significant difference values denoting significant differences between treatments ($P = 0.05$).

More information is needed to understand the benefits ethylene inhibitors may have on alleviating heat stress in different cool-season plant species. The objectives of this study were to determine the effects of AVG and PGPR with ACCd activity (ACCd-PGPR) on turf performance and root growth for *P. annua* under heat stress. The information from this study provides further insights into physiological functions of ethylene inhibition on heat tolerance in cool-season turfgrass species and can be useful to develop effective management programs to improve summer performance of heat-sensitive *P. annua*.

Materials and Methods

Plant material and growth conditions. Sods of *P. annua* were collected from field plots at the Rutgers Horticulture Research Farm II in North Brunswick, NJ, USA, and transplanted into plastic containers (5 cm in diameter and 17 cm in depth) (Stuewe and Sons Inc., Corvallis, OR, USA) filled with sand. Plants were established in a greenhouse for 30 d before being moved to controlled-environment growth chambers (Environmental Growth Chambers, Chagrin Falls, OH, USA). Chambers were maintained at the following setting: 22/18 °C (day/night), and 750 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux at a 14-h photoperiod. Plants were kept well watered and fertilized using half-strength Hoagland's nutrient solution every 7 d throughout the establishment period (Hoagland and Arnon 1950). Plant shoots were trimmed every 7 d to a height of 2.54 cm.

Experimental treatments and design. After 7 d of acclimation to the growth chamber conditions, plants were foliar sprayed with 25 mL of water (untreated control) or 25 mL of 25 μmol AVG (Sigma-Aldrich Co., St. Louis, MO, USA) or soil drenched with 25 mL of *P. aspalathi* 'WSF23' inoculant suspension (PGPR). The AVG concentration was determined based on previous studies testing its effectiveness on creeping bentgrass (Rossi and Huang 2023b). The bacteria inoculant suspension was prepared from frozen stock tubes kept at −80 °C via agar plate streaking. Single colonies were taken and grown in Luria Broth (Miller) (Sigma-Aldrich Co., St. Louis, MO, USA) where they were incubated at room temperature on an orbital shaker for 4 d. Bacterial suspensions were harvested by centrifuging the solution at 10,000 g_n for 5 min at room temperature and then resuspended in deionized water. Once resuspended, the concentration of the bacteria solution was adjusted so that it had an OD_{600} value of 1.0, which was quantified using an Evolution 201 ultraviolet-visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each treatment was applied 3 d before imposing temperature treatments and then every 7 d during 21 d of heat stress. Each treatment had six replicates (containers).

The untreated control, AVG-treated plants, and PGPR-treated plants were maintained in two growth chambers controlled at the optimal temperature at 22/18 °C (day/night) or heat stress at 30/35 °C (day/night) for 21 d. The experimental treatments were arranged in a split-plot design where the temperature was the main plot and application treatments were the subplots. Plants were randomly arranged and relocated within and across chambers every 7 d to mitigate potential impact of variations in growth chamber environments.

Physiological analysis. Measurements to evaluate treatment effects on the aboveground tissue were taken every 7 d throughout the 21-d experimental period. Parameters included visual turf quality (TQ) rated on a 1 to 9 scale based on uniformity, density, and color where healthy turf = 9, minimum acceptable quality = 6, and nearly dead turf = 1 (Beard 1973; Morris and Shearman 1998). Turf green canopy cover (GC) was measured using images taken on a digital camera (Apple Inc., Cupertino, CA, USA) and analyzed using SigmaScan Pro 5 software (Systat Software Inc., San Jose, CA, USA) following methods described in Karcher and Richardson (2003, 2005).

Leaf relative water content (RWC) was measured according to the procedure of Barrs and Weatherley (1962) based on leaf fresh weight (FW), turgid weight (TW), and dry weight (DW) using the formula (%) = 100 × [(FW – DW)/(TW – DW)]. Immediately after clipping leaves the FW was weighed with a mass balance. Then samples were wrapped in tissue paper and submerged in a 25 mL falcon tube filled with deionized water for 24 h. After the 24-h period was over, samples were removed from the tubes and weighed to determine TW. Lastly, samples were dried for 3 d at 80 °C and weighed to determine DW.

Leaf Chl content was taken weekly by collecting 1.0 g of leaf tissue from each replicate plant, submerging the tissue in 10 mL of dimethyl sulfoxide (DMSO), placing tubes in a dark location for 3 d, and then measuring absorbance at 645 and 663 nm using an Evolution 201 ultraviolet-visible spectrophotometer (Thermo Fisher Scientific Inc.) (Hiscox and Israelstam 1979). Chl content ($mg \cdot g^{-1}$ DW) was calculated using DW of leaf tissues and their respective absorbance values following the formula described by Arnon (1949).

Evaluation of root characteristics. At the completion of the experiment, roots were

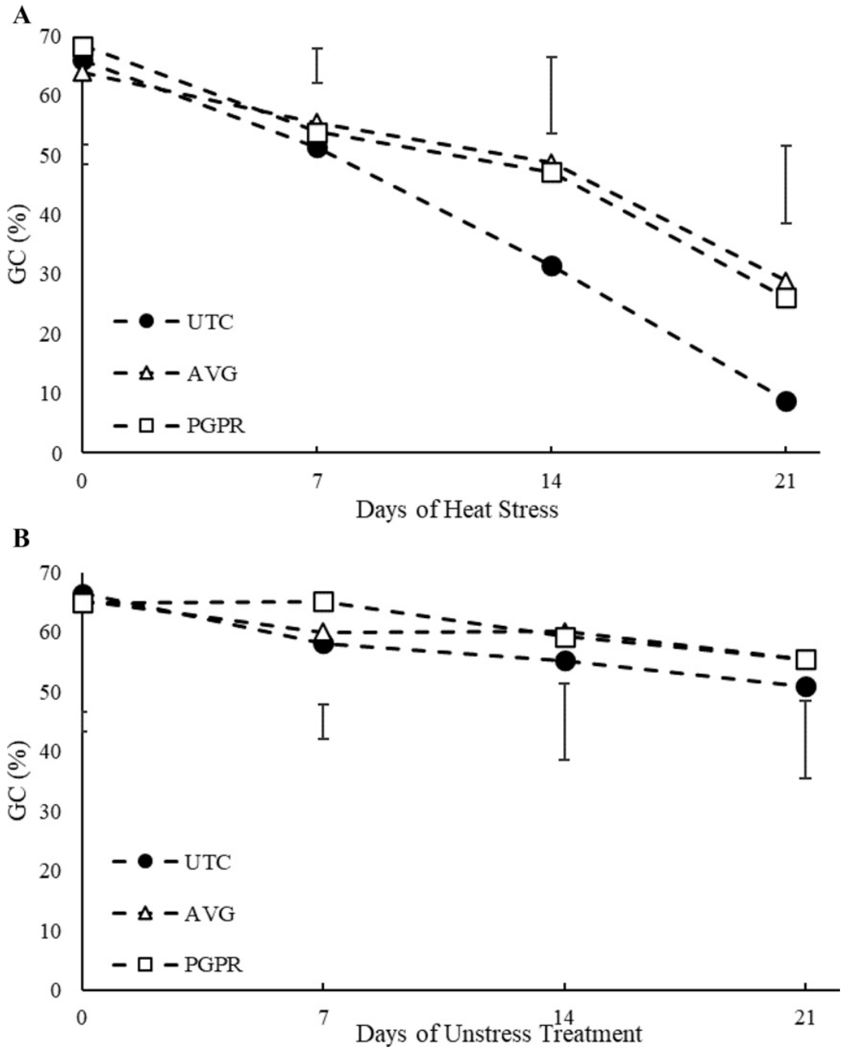


Fig. 2. Effects of ethylene inhibitors [aminoethoxyvinylglycine (AVG) and plant growth promoting rhizobacteria (PGPR) with ACC deaminase activity] on *Paraburkholderia annua* green canopy cover (GC) under heat stress (**A**) and unstressed (**B**) conditions. Vertical bars represent least significant difference values denoting significant differences between treatments ($P = 0.05$).

harvested by washing away any sand. Images were taken in a controlled light box and the maximum rooting depth was recorded for each replicate using a ruler. Crown and shoots were removed, and the harvested roots were stored in 10% ethanol solution at 2 °C before scanning. Total root length (cm), the added length of all the roots in one replicate, was quantified using digital images taken at 400 dpi using an Epson Expression 1680 scanner (US Epson, Inc., Los Alamitos, CA, USA) and then analyzed the image using WinRHIZO Basic V.2002 software (Regent Instruments Inc., QC, Canada). Roots were then dried in an oven set to 80 °C and mass was recorded as g DW.

Statistical analysis. Statistical analysis for aboveground parameters and root characteristics was conducted using analysis of variance (ANOVA) general linear model on SAS (ver 9.2; SAS Institute Inc., Cary, NC, USA). Significant differences were determined based on Fisher's protected least significant difference test at $\alpha = 0.05$.

Results

Effects of ethylene inhibitors on turf performance. The TQ declined over time during heat stress for *P. annua* across all treatment groups, but application of AVG and PGPR with ACCd activity maintained higher quality throughout the study compared with the untreated control (UTC) (Fig. 1A). PGPR-treated plants had significantly higher TQ starting at 7 d of heat stress while AVG-treated plants had significantly higher TQ after 14 d. Application of AVG or PGPR had no significant effect on TQ under unstressed conditions (Fig. 1B).

Green canopy cover (GC) followed similar trends as TQ, with an overall decline occurring across all treatment groups during heat stress, and those treated with AVG or PGPR maintaining higher GC (Fig. 2A). Significant differences were observed starting at 14 d of heat stress for both AVG and PGPR treatments, where AVG had 48.7% GC cover, PGPR had 47.2% and the UTC had 31.4%. AVG or PGPR had no significant effects on GC for unstressed plants (Fig. 2B).

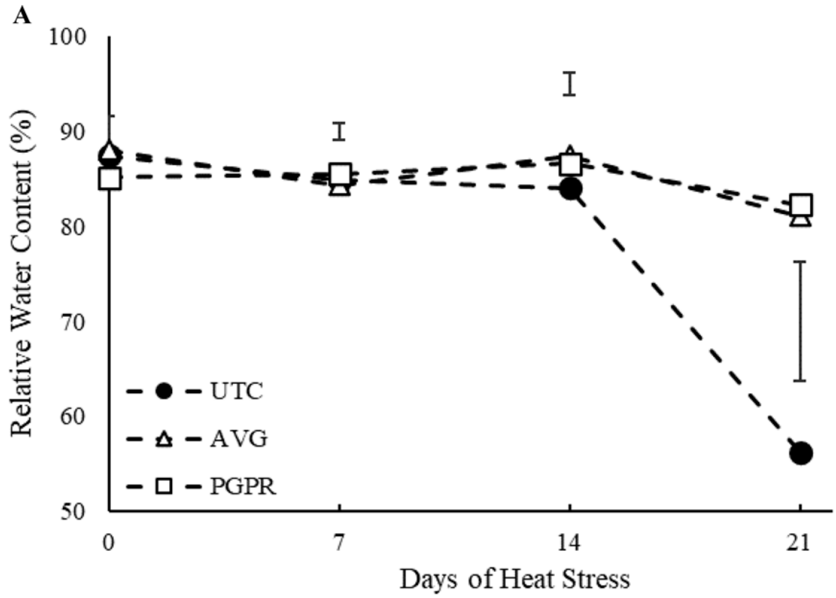
Leaf RWC remained unchanged across all treatments until 21 d of heat stress when the UTC levels dropped. Leaf RWC was maintained higher in plants treated with AVG or PGPR starting at 14 d of heat stress but was most prominent at 21 d when AVG maintained an RWC of 81.1%, PGPR of 82.3%, and the UTC dropped to 56.2% (Fig. 3A).

At 21 d of heat stress, AVG and PGPR treatments had significantly higher Chl contents than the UTC. Chl contents were not significantly affected before 21 d under heat stress. Neither AVG nor PGPR had significant effects on Chl content under unstressed conditions (Fig. 4).

Effects of ethylene inhibitors on root characteristics. The application of AVG or ACCd-PGPR had varied effects on *P. annua* rooting characteristics after 21 d of heat stress, but no significant effects under unstressed conditions (Fig. 5). Root DW was significantly greater in plants treated with AVG than that of UTC under heat stress. Root DW of ACCd-PGPR-treated plants were 2-fold greater than the UTC, although the difference was not statistically significant at 21 d of heat stress (Fig. 5A). The total length of root systems was 71% greater in ACCd-PGPR-treated plants (246 cm) compared with the UTC (144 cm) under heat stress, whereas the effects of AVG on total root length were not significantly different from the UTC under heat stress (Fig. 5B). Length of the longest roots or rooting depth was greater for AVG-treated plants (11.3 cm) than the UTC (7.5 cm), whereas rooting depth of ACCd-PGPR (10.4 cm) depth did not differ significantly from that of the UTC (Fig. 5C). Neither AVG nor ACCd-PGPR had significant effects on root surface area (Fig. 5D) under unstressed or heat stress conditions.

Discussion

Heat-induced ethylene accumulation and the reduction of ethylene by exogenous application of AVG have been reported in creeping bentgrass and perennial ryegrass (*Lolium perenne* L.) (Chen and Huang 2022; Xu and Huang 2007; Zhang et al. 2019). Additionally, AVG-treated creeping bentgrass plants were observed to have delayed leaf senescence and Chl degradation under heat stress compared with untreated control plants (Xu and Huang 2009). Further studies on creeping bentgrass reported the reduction of Chl degradation enzyme activity, in addition to increased Chl synthesis activity, antioxidant activity, and the upregulation of important metabolites involved in osmoregulation and respiration (Jesperson and Huang 2015; Jespersen et al. 2015; Rossi and Huang 2023b). In heat-stressed perennial ryegrass, AVG delayed leaf senescence, reduced ROS production, increased antioxidant enzyme activity, and downregulated both Chl catabolic genes and Chl degradation enzyme activity (Chen and Huang 2022; Zhang et al. 2019). The extent of effects of ethylene inhibitors on alleviating heat stress in plants varies depending on species



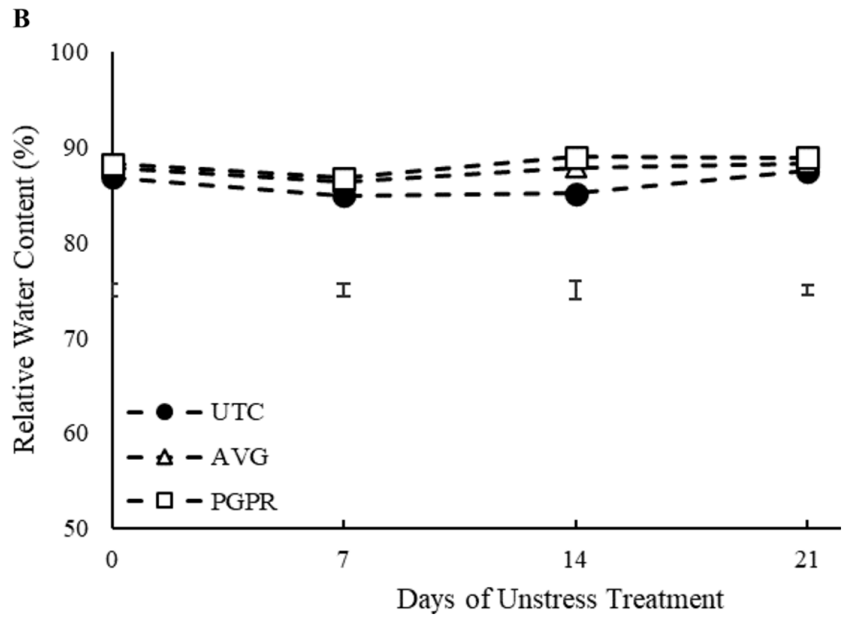


Fig. 3. Effects of ethylene inhibitors [aminoethoxyvinylglycine (AVG) and plant growth promoting rhizobacteria (PGPR) with ACC deaminase activity] on *Paraburkholderia annua* leaf relative water content under heat stress (**A**) and unstressed (**B**) conditions. Vertical bars represent least significant difference values denoting significant differences between treatments ($P = 0.05$).

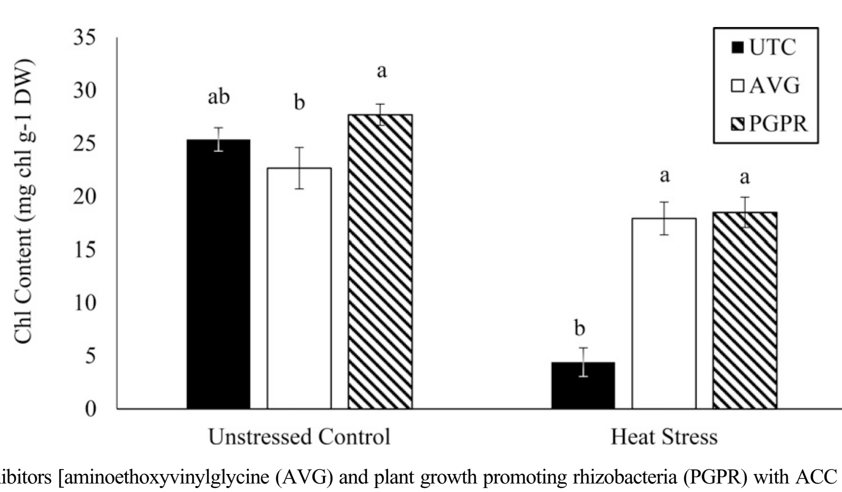


Fig. 4. Effects of ethylene inhibitors [aminoethoxyvinylglycine (AVG) and plant growth promoting rhizobacteria (PGPR) with ACC deaminase activity] on *Paraburkholderia annua* leaf chlorophyll (Chl) content under heat stress and unstressed conditions. Letters denote significant differences between treatments within each temperature group (unstressed control or heat stress) according to Fisher's protected least significant difference test ($P = 0.05$). Error bars represent standard error.

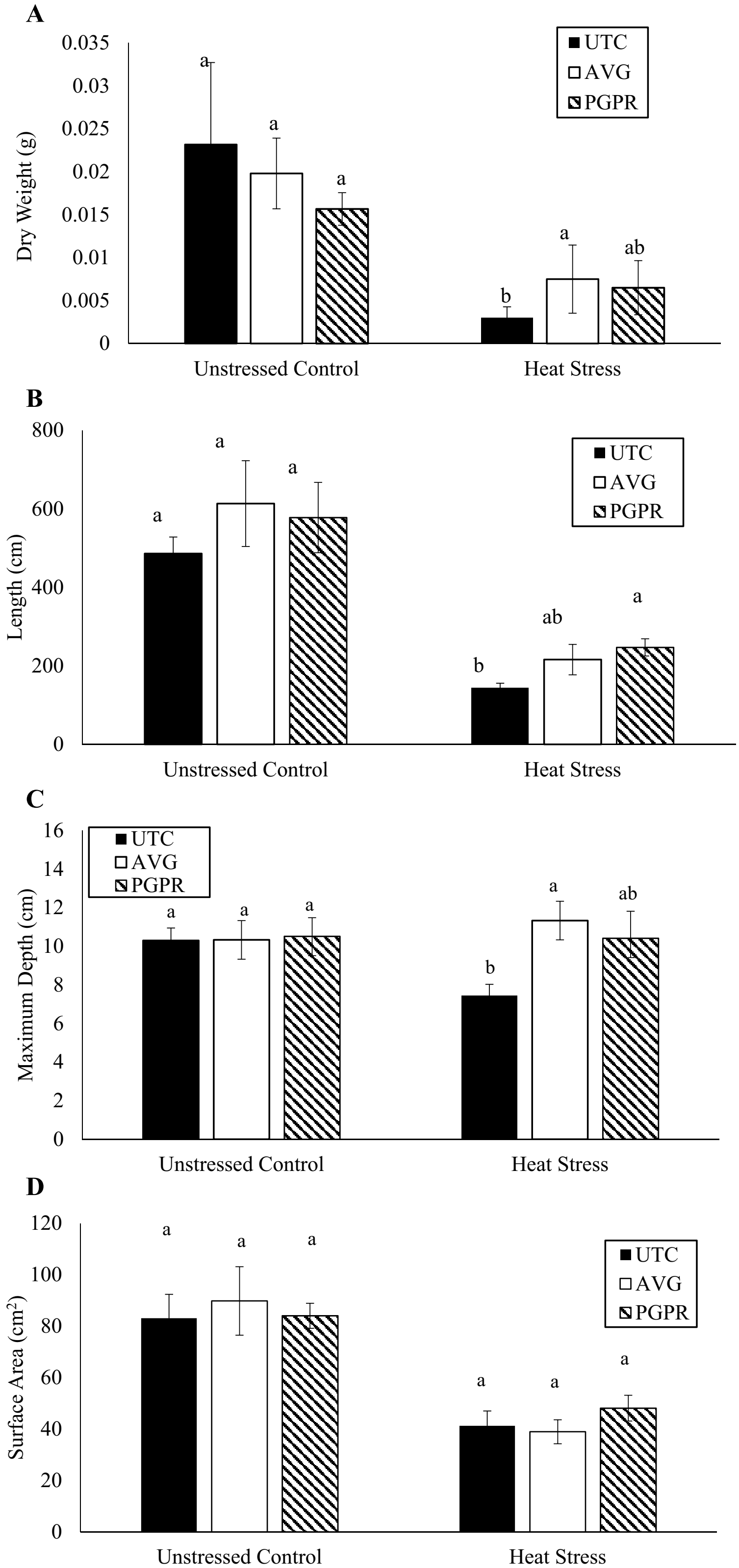


Fig. 5. Effects of ethylene inhibitors [aminoethoxyvinylglycine (AVG) and plant growth promoting rhizobacteria (PGPR) with ACC deaminase activity] on *Paraburkholderia annua* rooting characteristics after 21 d. Characteristics include root DW (**A**), total length (**B**), maximum rooting depth (**C**), and root surface area (**D**). Letters denote significant difference between treatments within each temperature group (unstressed control or heat stress) according to Fisher's protected least significant differences test ($P = 0.05$). Error bars represent standard error.

(Poór et al. 2022). Results of this study demonstrate the effectiveness of the ethylene inhibitors AVG and ACCd-PGPR in promoting physiological and biochemical traits of *P. annua* under heat stress, as manifested by greater TQ, GC, RWC, and Chl content compared with the UTC. Additionally, root characteristics were promoted by both treatments in heat-stressed plants, where AVG-treated *P. annua* had greater root DW and depth, and ACCd-PGPR-inoculated *P. annua* had greater root length compared with the UTC. This is in line with results from previous studies where ethylene inhibitors delayed heat-stress-induced damage on creeping bentgrass, manifested as higher TQ and Chl content (Jespersen et al. 2015; Rossi and Huang 2023b). Higher root number and increased length has been observed to contribute to Kentucky bluegrass (*Poa pratensis* L.) heat tolerance among cultivars (Huang et al. 2012). Furthermore, decreases in root morphological traits such as total root length, volume, DW, and surface area under heat stress have been seen to affect root functionality and is a characteristic of heat sensitivity in wucai (*Brassica campestris* L.) cultivars (Yuan et al. 2016). The promotion of root characteristics, such as DW and total root length, by ethylene inhibitors may play a role in *P. annua* improved heat tolerance compared with the UTC. However, the mechanisms in which AVG and ACCd-PGPR promoted *P. annua* performance under heat stress deserve further investigation.

In this study, *P. aspalathi* 'WSF23' had similar benefits as AVG on heat-stressed *P. annua.* The ACCd-PGPR's effectiveness in promoting heat-stressed *P. annua* performance were comparable to that of AVG, where TQ, GC, RWC, and Chl contents were all improved. Little research has been done on the effects of ACCd-PGPRs on promoting heat tolerance in other cool-season turfgrass species besides what were found in this study with *P. annua*. However, ACCd bacteria strains have been associated with improved heat tolerance in other plant species; including increased biomass, greater RWC, enhanced Chla and Chlb, and enhanced antioxidant enzyme activity in eggplant (*Solanum melongena* L.), rice (*Oryza sativa* L.), and tomato (*Solanum lycopersicum* L.) (Choi et al. 2022; Mukhtar et al. 2020; Sami et al. 2024). Although the mechanisms in which ACCd-PGPRs regulate heat tolerance are not well understood, it is likely that the ACCd activity of these bacteria strains is involved. This assumption is based on previous research discussed earlier in this article where ACCd suppressed ethylene production by converting ACC into alpha-ketobutyric acid and ammonia. Enhanced Chl contents, RWC, and overall plant growth may be direct benefits from ACCd mediated suppression of stress ethylene within plant tissues. This would serve to prevent deleterious effects on plant growth, including oxidative damage from ROS molecules. Additionally, alpha-ketobutyric acid and ammonia may provide an available carbon and nitrogen source for *P. annua* to use under heat stress. Further studies are needed to identify the mechanisms in which *P. aspalathi* functions to promote heat tolerance in *P. annua*. Specifically, how *P. aspalathi* may regulate ethylene and other relevant metabolites in response to heat stress, and how a-ketobutyric acid and ammonia may be used to delay heat-induced TQ decline. ACCd-PGPRs, particularly *P. aspalathi*

'WSF23', have great potential as biostimulants to manage cool-season turfgrasses during heat-stress periods in the summer.

In summary, ethylene inhibitors were effective in alleviating *P. annua* heat stress damage. AVG increased *P. annua* TQ, GC, leaf RWC, Chl content, and root maintenance at 30/35 °C (day/night) temperatures. The biological ethylene inhibitor, *P. aspalathi*, had similar results to AVG, promoting aboveground tissue quality, Chl content, and root maintenance when under heat stress. Comparing each treatment to the control, the only major difference between ethylene inhibitors were their effect on root morphology, where different characteristics were promoted uniquely depending on the treatment, with AVG having significantly greater root DW and depth while *P. aspalathi* had greater total length compared with the UTC. The mechanisms through which these treatments are able to improve *P. annua* heat stress tolerance are not fully understood but are likely a result of suppressing stress ethylene production to prevent inhibition of shoot and root growth, while maintaining higher Chl content. Further research is warranted to investigate the effect AVG and *P. aspalathi* have on ethylene production in *P. annua* under heat stress and the mechanisms by which they can improve *P. annua* heat performance.

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