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Evaluating the Use of Microbial Inoculants in Ameliorating Heat Stress in Creeping Bentgrass (*Agrostis stolonifera*)

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Abstract. **Creeping bentgrass (*Agrostis stolonifera* L.) is a cool-season species sensitive to rising temperature that causes heat stress. The use of microbial inoculants has been advocated as a sustainable approach to enhance plant tolerance to abiotic stress. This study evaluated the effects of two commercial microbial products, BioEnsure® (*Trichoderma harzianum*) and BioTango™ (*Bacillus* spp.), applied individually or in combination on creeping bentgrass under heat-stress or no-stress conditions through foliar spray of mature plants or a germination assay of coated seeds. Turfgrass quality (percent green cover), physiological (electrolyte leakage, pigment content, photosynthetic efficiency), and soil biological health (respiration, urease, and phosphatase activities) parameters were assessed as response variables. Pairwise contrast analysis of each turfgrass parameter between the products and the control did not show any significant difference under stress or no-stress conditions. However, both products significantly affected soil respiration and enzyme activity. An integrated analysis of the data with canonical discriminant analysis revealed clear separation among the treatments, with the control being associated with a more favorable physiological profile than the products under heat stress. Under the no-stress condition, the products were associated with improved turfgrass pigment content and soil enzyme activity. The fungal product resulted in a significantly better germination rate than the control under heat stress, but no significant effect was observed with the bacterial product. These findings emphasize the need for testing microbial products and identifying conditions under which they can be effective.**

More than 16 million hectares are covered by turfgrasses in the United States of America (Fidanza 2023; Simmons et al. 2011). Turfgrasses are vital components of urban and suburban landscapes (golf courses, sports fields, private lawns, and parks) as they provide essential ecosystem services in maintaining and enhancing water, soil and air quality (Chang et al. 2021; Christians et al. 2016; Fan et al. 2020; Stier et al. 2013). Although turfgrasses are crucial to the well-being of the environment and global economy, they face significant challenges due to climate change, which exacerbates abiotic stresses associated with rising temperature, drought, and soil salinity, negatively affecting plant health and productivity (Campos et al. 2023; IPCC 2021). Abiotic stresses can disrupt plant development and physiological processes, leading to metabolic dysfunction, reduced photosynthetic efficiency, and overall growth inhibition (Fan et al. 2020; Mittler 2006; Yadav et al. 2020). Rapid changes in climate affect plant and soil systems, altering soil fertility and plant productivity (Hatfield 2017).

Creeping bentgrass (*Agrostis stolonifera L.*) is a common turfgrass species widely used in temperate regions worldwide, appreciated for its tolerance to low mowing heights and rapid recovery from traffic (Fan and Jespersen 2022). As a cool-season turfgrass species, *A. stolonifera* is adapted to mild temperatures, with optimal range of ~15 to 24 °C for the aboveground parts and 10 to 18 °C for the root zone (Sun et al. 2024). Due to its sensitivity to higher temperatures, creeping bentgrass is particularly susceptible to weather extremes, especially during summer time, which makes it challenging to maintain high turfgrass quality (Dernoeden 2012). In response to these challenges, turfgrass managers often rely on increasing consumption of water resources and other inputs that are unsustainable in the long term (Bosi et al. 2023; Gómez-Armayones et al. 2018; Vishwakarma et al. 2016).

Research in improving abiotic stress tolerance in turfgrasses has mainly focused on breeding efforts that rely on the selection of genes that confer resistance (Casler 2001; Duncan and Carrow 2001; Fan et al. 2020; Huang 2008; Humphreys et al. 2004; Zhang et al. 2006). However, breeding is a time-consuming and labor-intensive endeavor (Casler 2001). Moreover, it suffers from the limited availability of genomic data and insufficient molecular markers for turfgrass species (Huang et al. 2014; Jiuxin and Liebao 2022). The use of biostimulants is often advocated as an additional approach worth exploring (Mall et al. 2017). The word "biostimulant" is commonly used to refer to a collection of products that include microbial inoculants, phytohormones, amino acids, seaweed extracts, and humic and fulvic acids (Rouphael and Colla 2020). Many studies have shown that the use of biostimulants can be beneficial to plants (Ammaturo et al. 2023; Colla et al. 2015; Nardi et al. 2002; Parađiković et al. 2011). There is increasing interest in the use of microbial inoculants in agriculture due to the potential benefits they provide to plants and soil health as sustainable alternatives or supplements to conventional inputs to enhance their efficiency (Alori et al. 2017; Shukla et al. 2022).

Previous studies have focused on the direct impact of microbial inoculants on turfgrass growth and development (Alori et al. 2017; Bolton et al. 2022; Coy et al. 2014; Fidanza 2023; O'Callaghan et al. 2022). However, microbial inoculants can also affect turfgrass indirectly by enhancing soil health parameters that support plant growth. One key mechanism is through the stimulation of microbial activity to enhance nutrient cycling and organic matter decomposition, thereby releasing essential nutrients for turfgrass uptake (Vishwakarma et al. 2016). In the present study, we tested two products: BioEnsure® and BioTango™ (Adaptive Symbiotic Technology, Seattle, WA, USA), which contain endophytes belonging to *Trichoderma harzianum* and *Bacillus spp.* to evaluate their impact on heat stress tolerance and soil health in creeping bentgrass. BioEnsure® is described as containing a dynamic fungal inoculant that imparts abiotic stress tolerance and improves nutrient use efficiency in plants, while BioTango™ contains *Bacillus* spp. that enhance plant nutrient availability. According to the manufacturer, they are best used in tandem to

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enhance their benefits. BioEnsure® has been tested with corn and cotton under heat and water stress, resulting in 26% and 18% increases in yield as compared with untreated controls, respectively (McCauley 2021). To our knowledge, there have not been previous studies on turfgrass.

Materials and Methods

Experimental set-up

The study assessed the effects of two commercial microbial products, BioEnsure® and BioTango™ (Adaptive Symbiotic Technologies), on turfgrass growth and soil biological health under heat stress. The experiment included four treatments: (1) BioEnsure® (BE), (2) BioTango ™ (BT), (3) BioEnsure® + BioTango™ (BB), and (4) control (C). The commercial products were applied at a rate indicated on the product label [BE: 7.3 mL/ha; BT: 2.47 g/ha; BB: 7.3 mL/ha (BE) + 2.47 g/ha (BT)]. The BioEnsure® liquid solution and the BioTango™ powder were diluted in distilled water. The control treatment consisted of the application of deionized water. BioEnsure® is listed as having a minimum of 1×10^6 viable spores mL^{-1}. The product label for BioTango™ includes five *Bacillus* spp. (*B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis*, and *B. amyloliquefaciens*) at 1×10^8 cfu mL^{-1} and dextrose powder as an inactive ingredient (Supplemental Fig. 2). The treatments were applied under heat-stress or no-stress conditions. A split-plot design was used in the study, and the main plots were the stress or no-stress conditions, with product types as subplots. Each treatment was replicated four times. The plants under the stress treatment were kept under a 35/30 °C day/night temperature cycle in a growth chamber (E41L2, Percival, Perry, IA, USA). The no stress plants were kept at 20/15 °C day/night temperature cycle, which is the optimal range for creeping bentgrass (Miller and Brotherton 2020). Both chambers were set with a photoperiod of 12 h light/12 h dark.

To set up the study, turfgrass plugs (*A. stolonifera* L. var. Pure Eclipse) were obtained from research field plots located at the University of Georgia Griffin Campus. The roots were cut at ∼2 cm below the crown. Each plug was set in a 4 cm-diameter × 20 cm-depth pot containing a mixture of sand and organic matter (at a ratio of 9:1) with landscape fabric at the bottom. Plants were established before treatments in the greenhouse under standard (no stress) conditions for about 4 weeks (irrigation was provided to meet the water requirements of plants; temperature was kept at a 20/15 °C day/night temperature cycle, with 50% relative humidity). The trial timeline is shown in Supplemental Fig. 1. Once the establishment period was completed, five consecutive treatment applications (I, II, III, IV, and V) were performed by foliar spray with an interval of 2 weeks between each application. Each condition (stress vs. no stress) was set up in two sets to allow destructive sampling of plant and soil samples after the fourth (T1) and fifth treatment applications (T2).

Turfgrass quality and growth measurements

Visual turfgrass quality (TQ). The visual evaluation of turf quality was done according to the National Turfgrass Evaluation Program criteria (Morris and Shearman 1998). A score from 1 to 9 was assigned to each sample at each collection time (T1 and T2). The value of 1 was assigned to dead turf, while 9 referred to perfectly healthy and dense turf. A score equal to or above 6 is considered acceptable.

Digital image analysis. Turfgrass quality was also expressed in percent green cover that was estimated by taking pictures with a camera (Canon G7X, Canon Inc., Melville, NY, USA) at a resolution of 5472 × 3648 and ISO-400 under a lightbox that ensured the same light quality and height. The pictures obtained were processed using the ImageJ software (Gallagher 2014).

Photosynthetic efficiency (FvFm). After dark adaptation for 30 min, a modulated chlorophyll fluorometer (Opti-Science, OS5p+) was used to measure the minimum fluorescence (F0), in which all reaction centers of photosystem II (PSII) were open. Subsequently, the maximum fluorescence (F_m) was measured using a short saturating pulse of high intensity light. Three readings were taken for each sample. The following ratio represents the maximum potential efficiency of PSII $= \frac{F_v}{F_m}$, where F_v (variable fluorescence) $= F_m - F_0$, and F_m = maximum fluorescence (Baker and Rosenqvist 2004).

Electrolyte leakage (EL). Electroconductivity was measured using a conductivity meter (Cole-Parmer, Vernon Hills, IL, USA) following the protocol described by Bajji et al. (2002). Leaf tissues were rinsed in deionized water and incubated on a shaker for 24 h in deionized water, and an initial conductivity reading was taken (*Eci*). Tissues were then autoclaved and placed back on the shaker, after which a final conductivity reading was taken (*ECf*). The electrolyte leakage was measured as % damage$=(ECi/ECf) \times 100$.

Chlorophyll and carotenoid contents. The chlorophyll and carotenoid contents were measured as described by Wellburn (1994). Briefly, 0.1 g of grass tissue was collected and chopped into 1 cm sections, placed in a 15 mL tube containing 5 mL of dimethyl sulfoxide (DMSO), and tightly closed. The tubes were wrapped in aluminum foil and stored in the dark for one week. The samples were diluted into DMSO at a 1:5 ratio. Using a spectrophotometer (Evolution 300 UV-VIS; Thermo Fisher Scientific, Waltham, MA, USA), the absorbance was measured at three different wavelengths (665, 649, and 480). After 3 d, the dry weight of each sample was recorded, and the parameters were calculated as below.

$$\mathrm{Ca} = 12.19 \times A_{665} - 3.45 \times A_{649} \quad [1]$$

$$\mathrm{Cb} = 21.99 \times A_{649} - 5.32 \times A_{665} \quad [2]$$

$$\mathrm{Total\ C} = [(\mathrm{Ca} + \mathrm{Cb}) \times \mathrm{dilution\ factor}]/\mathrm{DW} \quad [3]$$

$$C_{x+c} = (1000\ A_{480} - 2.14\mathrm{Ca} - 70.16\mathrm{Cb})/220 \quad [4]$$

where Ca indicates chlorophyll a; Cb indicates chlorophyll b; A_{665}, A_{649}, and A_{480} indicate absorbance at wavelengths 665, 649, and 480, respectively; total C indicates total chlorophyll content; the dilution factor is 5; DW indicates dry weight (mg); and C_{x+c} indicates carotenoid content.

Measurements of soil biological health parameters

Soil health parameters were assessed to understand the mechanisms by which microbial application influenced turf growth and quality. In particular, soil parameters such as soil respiration and enzyme activities were monitored to determine changes in soil biological health. Soil samples were destructively collected during each sampling time from both no stress and stress conditions. Before analysis, each soil sample was passed through a 2 mm sieve to remove nonsoil materials. The sieved soil was then stored in sterile plastic bags at 4 °C briefly until analysis. The refrigerated samples were brought to room temperature and then mixed thoroughly to homogenize the samples for analysis. Soil respiration, an indicator of microbial activity, was measured as described in Zibilske (1994) using a CO_2 gas analyzer (EGM-5; PP Systems, Amesbury, MA, USA). Urease and phosphatase activities were measured to evaluate potential changes in the transformations of nitrogen and phosphorus as described by Tabatabai (1994) and Wallenstein and Weintraub (2008).

Isolation and characterization of endophytes

To evaluate whether the inoculants colonized root tissues under heat stress, the protocol described by Hallmann et al. (2006) was used for isolating endophytes from the roots. Roots were initially macerated manually with the addition of sterile water at a rate of 1:10 (root:water) using a sterile mortar and pestle. The suspension obtained from the maceration was then plated on selective media for fungi (glucose rose-bengal agar with streptomycin)

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or bacteria (nutrient agar with nystatin), followed by incubation at 30 °C. Afterward, individual colonies were transferred to selective liquid culture tubes and grown for 4 to 5 d. The genomic DNA of the isolates and the commercial products were then extracted using a fungi/yeast genomic DNA isolation kit (Norgen Biotek Corporation, Thorold, Canada) and a genomic DNA purification kit (Thermo Fisher Scientific).

The fungal DNA extracts were subjected to polymerase chain reaction (PCR) for identification, targeting the 18S rDNA gene with the nu-SSU 0817F and nu-SSU 1196R primer pair as described in Borneman and Hartin (2000). The bacterial DNA was subjected to PCR, targeting the 16S rDNA gene with 968F and 1401R primers (Zhang et al. 2013). All PCRs were conducted in a SimpliAmp™ thermal cycler using the Applied Biosystems Power UP™ Master Mix (Thermo Fisher Scientific). All the primers were synthesized by Eurofins Genomics (Louisville, KY, USA). The PCR amplicons were purified before sequencing with a commercial kit (Wizard PCR Preps DNA Purification System; Promega, Madison, WI, USA) and quantified with a Qubit fluorometer (Thermo Fisher Scientific). The amplicons were sent to Genewiz® (Azenta Life Sciences, South Plainfield, NJ, USA) for sequencing. The sequences were compared against the existing sequences in the GenBank database of the National Center for Biotechnology Information for identification (http://blast.ncbi.nlm.nih.gov/).

Germination assay

Additional trials were also conducted to evaluate the effects of the microbial inoculants on turfgrass germination under heat-stress or no-stress conditions in two substrates. The seeds of creeping bentgrass (*A. stolonifera* L., var. 007) were sterilized as described by Wang and Zhang (2010). Petri dishes were prepared under the following conditions: (1) two layers of filter paper with 5 mL of deionized (DI) water, and (2) 10 g of sand + organic matter mixture (ratio 9:1) with 2 mL of DI water. The sterilized and dried seeds were coated with BE-FP (BioEnsure® FP: powder form of the BioEnsure®), BT, or BB. Seeds were mixed with a product in a paper envelope until all the seeds appeared to be evenly coated on their entire surface. Additionally, control seeds without any coating were included (C). A split-plot design (main plots = stress or no stress, subplots = microbial products) was employed for this study. One hundred seeds were counted and placed into a petri dish (60 mm × 15 mm sterile polystyrene). The plates were then placed into growth chambers with different temperature settings: (1) no stress temperature (20/15 °C day/night) and (2) heat stress (35/30 °C day/night). The chambers were set with a photoperiod of 8 h dark/16 h light cycle. Six replications were used for each treatment. Deionized water was added to the plate as needed to moisturize the plates throughout the trial. The germination rate was measured every 2 d by counting germinated seeds that developed a radicle and shoot with a minimum of 1 mm in length. The product carrier powders were analyzed using a laser diffraction particle size analyzer (model PSA 1190; Anton Paar, Graz, Austria) to determine their particle sizes under the following parameters: sample read time, 3 s; vibrator duty cycle, 40%; vibrator frequency, 30 Hz;

Table 1. Comparison of turfgrass growth and quality parameters of the microbial treatments [BioEnsure® + BioTango™ (BB), BioEnsure® (BE), and BioTango™ (BT)] against the control (*P* values of emmeans – estimated marginal means) at two sampling times (T1 and T2).

Turfgrass growth and quality parameters	Comparison	Sampling time	*P* value	
			Heat stress	No stress
Electrolyte leakage (EL)	BB-C	T1	0.304	0.059
	BE-C	T1	0.568	0.953
	BT-C	T1	0.146	0.499
	BB-C	T2	0.939	0.121
	BE-C	T2	0.606	0.646
	BT-C	T2	0.501	0.845
Turf quality (TQ)	BB-C	T1	1.000	1.000
	BE-C	T1	0.747	1.000
	BT-C	T1	0.629	0.645
	BB-C	T2	0.648	1.000
	BE-C	T2	0.364	1.000
	BT-C	T2	0.364	0.055
Photosynthetic efficiency (Fv/Fm)	BB-C	T1	0.200	0.414
	BE-C	T1	0.793	0.080
	BT-C	T1	0.398	0.333
	BB-C	T2	0.787	0.256
	BE-C	T2	0.849	0.085
	BT-C	T2	0.780	0.126
Percent green cover	BB-C	T1	0.411	0.291
	BE-C	T1	0.645	0.331
	BT-C	T1	0.400	0.051
	BB-C	T2	0.984	0.785
	BE-C	T2	0.364	0.447
	BT-C	T2	0.740	0.181
Chlorophyll content	BB-C	T1	0.998	0.381
	BE-C	T1	0.177	0.596
	BT-C	T1	0.067	0.160
	BB-C	T2	0.945	0.315
	BE-C	T2	0.556	0.853
	BT-C	T2	0.568	0.225
Carotenoid content	BB-C	T1	0.526	0.450
	BE-C	T1	0.197	0.562
	BT-C	T1	0.168	0.075
	BB-C	T2	0.674	0.244
	BE-C	T2	0.578	0.472
	BT-C	T2	0.493	0.129

C = control; Carotenoid = carotenoid content; Chl = chlorophyll content; EL = electrolyte leakage; FvFm = photosynthetic efficiency; Green = green cover percentage; TQ = visual turf quality score.

Table 2. Comparison of soil health parameters (respiration, urease, phosphatase) of the microbial treatments [BioEnsure® + BioTango™ (BB), BioEnsure® (BE), and BioTango™ (BT)] against the control (*P* values of emmeans) at two sampling times (T1 and T2).

Turfgrass growth and quality parameters	Comparison	Sampling time	*P* value	
			Heat stress	No stress
Soil respiration	BB-C	T1	**0.000**	0.159
	BE-C	T1	**0.001**	0.050
	BT-C	T1	**0.012**	0.768
	BB-C	T2	0.811	0.076
	BE-C	T2	0.989	0.059
	BT-C	T2	0.605	**0.044**
Urease activity	BB-C	T1	**0.000**	0.757
	BE-C	T1	**0.034**	0.186
	BT-C	T1	**0.003**	0.067
	BB-C	T2	0.791	0.528
	BE-C	T2	0.261	0.681
	BT-C	T2	0.673	0.113
Phosphatase activity	BB-C	T1	0.873	0.094
	BE-C	T1	0.944	0.201
	BT-C	T1	0.612	**0.050**
	BB-C	T2	0.121	0.884
	BE-C	T2	0.199	0.881
	BT-C	T2	0.895	**0.000**

Significant *P* values (≤0.05) are reported in bold type.

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Table 3. Sequence similarities of the isolates from this study against known bacterial strains based on their partial 16S (bacteria) and 18S (fungi) rDNA sequence.

Isolate ID	Strain with which isolates had the maximum sequence similarity	Similarity (%)
Bacteria		
1B	*Caballeronia mineralivorans* strain NJ-XFW-1-B	90
2B	*Bacillus cereus* strain IARI-A-8	99
3B; 8B	*Pseudomonas* sp. strain B4C38_PSIA_2_14	99; 99
4B	*Paenibacillus alvei* strain LT431	99
5B; 6B	*Janthinobacterium* sp. strain M169	99; 99
7B	*Bacillus cereus* strain MSK	99
9B	*Paenarthrobacter* sp. strain WCUF-2Pae	99
10B	*Pseudomonas fluorescens* strain 14f	83
11B; 12B	*Pseudomonas fluorescens* strain AFS029165	99; 99
Fungi		
1F; 2F; 3F; 4F; 5F; 6F; 9F; 10F; 11F; 14F; 16F	*Microdochium nivale* strain MAFF 236681	99; 99; 99; 99; 99; 99; 99; 99; 99; 99; 99
7F	*Penicillium* sp. strain ELF-4	99
8F; 12F	*Scolecobasidium ramosum* strain UTHSC 12-1082	100; 99
13F	*Discostroma fuscellum*	99
15F	*Mortierella* sp. strain A38	99

and air pressure, 1300 mBar (Cruz-Padilla et al. 2023).

Statistical analysis

Linear models. The data were analyzed using linear model (*lm*) or generalized linear model (*glmmTMB*), depending on the distribution and best fit for each parameter in R (version 4.4.2; RStudio, PBC, Boston, MA, USA). For normally distributed traits, models assumed a Gaussian distribution with identity link (TQ, green cover, soil respiration, urease, and phosphatase). When the distribution was not normally distributed, the data were log-transformed to meet model assumptions of normality and homoscedasticity (EL and Chl). The *beta_family (link= "logit")* distribution and link function were used for FvFm, while *family = Gamma (link= "log")* was used for carotenoid content. The fixed effects for all the models were: treatment (microbial product), time (T1 and T2), and their interaction (treatment × time). The function *contrast* in the *emmeans* package (method = "Dunnett") was used to evaluate the statistical significance of any differences (P value $\leq$ 0.05) between the microbial products and the control for each of the turfgrass and soil health parameters under stress or no-stress conditions separately. The data from the two sampling times (T1 and T2) were also analyzed separately.

Canonical discriminant analysis (CDA). CDA was performed in R (version 4.4.2; RStudio) to analyze the data in an integrated way to discern any patterns among treatments. The *candisc* function in the *candisc* package (Friendly and Fox 2024) was used to define treatment group centroids, 95% confidence ellipses, and vectors (using the standardized canonical coefficients) representing the turfgrass growth/quality and soil biological health response variables to identify which parameters distinguished the treatment groups. Following the CDA, post-hoc pairwise comparisons were assessed using the *pairwise.t.test* function with the Hochberg P value adjustment (Hochberg 1988) to determine the statistical significance of differences.

Germination assay. Analysis of variance was conducted to determine the statistical significance ($P \leq 0.05$) of the effects of the microbial products on germination rate under stress or no-stress conditions separately. When significant differences were observed, Tukey's post hoc test was used for means separation. The statistical analysis for the germination assay was performed using SAS software (SAS, Cary, NC, USA).

Results

Turfgrass and soil health response to treatments

No significant differences were found between each treatment and control under heat-stress or no-stress conditions in any of the turfgrass parameters (Table 1 and Supplemental Table 1). However, there were significant treatment effects on soil health. Soil respiration and urease activity were significantly lower with the microbial products than control under heat stress at T1 (Table 2, Supplemental Figs. 3 and 4 and Supplemental Table 2). Similarly, soil respiration was significantly lower under BT than control under no stress at T2. BT improved phosphatase activity at T2 as compared with the control under no-stress conditions (Table 2, Supplemental Fig. 5 and Supplemental Table 2). Moreover, none of the endophytes isolated from the root samples of the turfgrass were identical to the inoculants in the microbial products (Table 3), indicating the lack of colonization of the inoculants in the root tissues.

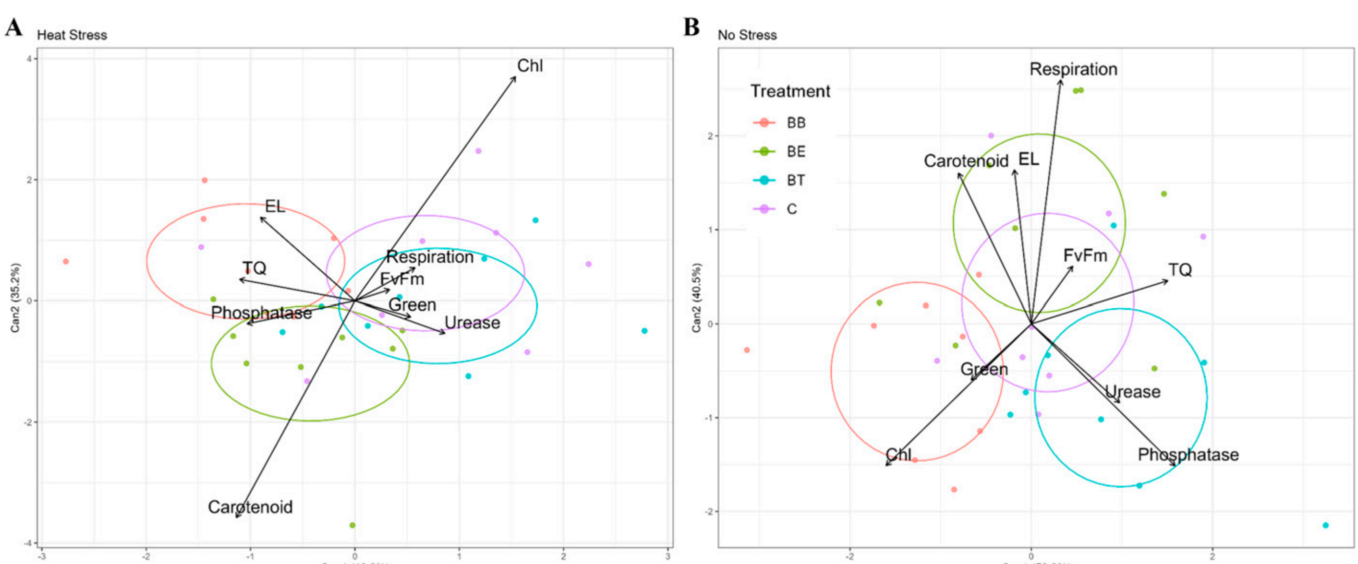

Fig. 1. Canonical discriminant analysis of turfgrass quality/growth and soil biological health (respiration, urease, phosphatase) parameters of creeping bentgrass under (**A**) heat-stress or (**B**) no-stress conditions. The four treatments are represented: BioEnsure® + BioTango™ (BB), BioEnsure® (BE), BioTango™ (BT), and control/nontreated (C). Carotenoid = carotenoid content; Chl = chlorophyll content; EL = electrolyte leakage; FvFm = photosynthetic efficiency; Green = percent green cover; TQ = visual turf quality score.

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Table 4. Canonical standardized coefficient of the independent variables and *P* value of the pairwise comparison of each pair of treatments [control (C), BioEnsure® + BioTango™ (VV), BioEnsure® (BE), and BioTango™ (BT)] on the first two canonical discriminant variates of heat-stress and no-stress conditions.

	Canonical discriminant variate			
	Heat stress		No stress	
Variable	Can 1	Can 2	Can 1	Can 2
EL	−0.823	1.255	0.640	0.194
TQ	−1.005	0.321	−0.079	0.696
FvFm	0.303	0.169	**−0.681**	−0.642
Green	0.486	−0.239	−0.341	0.682
Chl	**1.401**	**3.372**	0.194	0.259
Carotenoid	−1.033	−3.257	−0.282	−0.254
Respiration	0.524	0.497	0.138	**1.104**
Urease	0.784	−0.484	0.414	−0.357
Phosphatase	−0.939	−0.340	0.674	−0.643
Squared canonical correlation	0.403	0.328	0.426	0.372
P value	0.464	0.559	0.43	0.581
Pairwise comparison (*P* value)				
C-BT	0.810	0.575	0.235	0.211
C-BE	0.108	**0.029**	0.853	0.305
C-BB	**0.009**	0.702	**0.038**	0.305
BT-BE	0.084	0.273	0.235	**0.005**
BT-BB	**0.006**	0.457	0.001	0.585
BE-BB	0.449	**0.013**	**0.047**	**0.019**
Accounted variance (%)	48.79	35.19	50.79	40.51

The two variables that primarily drive the separation of the treatment groups per each canonical discriminant variates and the significant values (*P* value ≤ 0.05) of the pairwise comparison are reported in bold type. C = control; Can = canonical axis; Carotenoid = carotenoid content; Chl = chlorophyll content; EL = electrolyte leakage; FvFm = photosynthetic efficiency; Green = green cover percentage; TQ = visual turf quality score.

An integrated examination of the plant and soil data with CDA, however, revealed clear separation among the treatments based on turfgrass physiological and soil health parameters under heat stress (Fig. 1A). The first two canonical axes (Can) explained 84% of the total variation, with Can 1 accounting for 48.8% and Can 2 accounting for 35.2% (Table 4, heat stress). The control group aligned with higher values of chlorophyll content, Fv/Fm, soil respiration, urease activity, and green cover index, traits typically associated with improved physiological functioning. In contrast, the BB treatment aligned strongly with turfgrass quality and electrolyte leakage, suggesting a mixed response: improved visual quality but greater membrane damage. The BT and BE treatments were positioned between control and BB, showing an intermediate response, with more carotenoid production under BE. Table 4 (heat stress) presents the *P* values from the pairwise comparisons of treatments along the two canonical axes. Along Can 1, significant differences were observed between the control and BB treatments, as well as between BB and BT. Along Can 2, significant separation was found between the control and BE and between BE and BB.

Under no-stress condition (Fig. 1B), the CDA revealed distinct yet moderate clustering of plant and soil health parameters associated with the microbial products. The first two canonical axes explained 91.3% of the total variation (Can 1: 50.8%, Can 2: 40.5%) (Table 4, no stress). The BB treatment aligned most closely with chlorophyll content and green cover index, suggesting that this treatment might have improved photosynthesis under no-stress conditions. The BE treatment appeared to be associated with increased respiration, carotenoid accumulation, and electrolyte leakage, possibly indicating a stimulated plant-stress response. The BT treatment, clustered in the lower-right quadrant, is associated with phosphatase and urease activity. In contrast, the control was centrally located and overlapped with all the microbial treatments, aligning with Fv/Fm and turf quality, which reflected stable photochemical efficiency and turf quality in the absence of inoculants. These results suggest that microbial products induced measurable physiological changes in the absence of stress; however, the pairwise comparison of each treatment against the control along Can 1 showed significant separation only when compared with BB (Table 4B). Other significant separations were found on Can 1 between BT and BB and between BE and BB. On Can 2, significant separation was found between BT and BE and between BE and BB but not between the control and any of the microbial products.

Germination assay

The germination of creeping bentgrass seeds on filter paper did not show significant differences between the control and any of the products under stress or no-stress conditions (Fig. 2A). In soil, however, the BE-FP (fungal inoculant) led to a significantly higher germination rate than the control under heat stress (Fig. 2B). The particle size analysis of the carrier powders

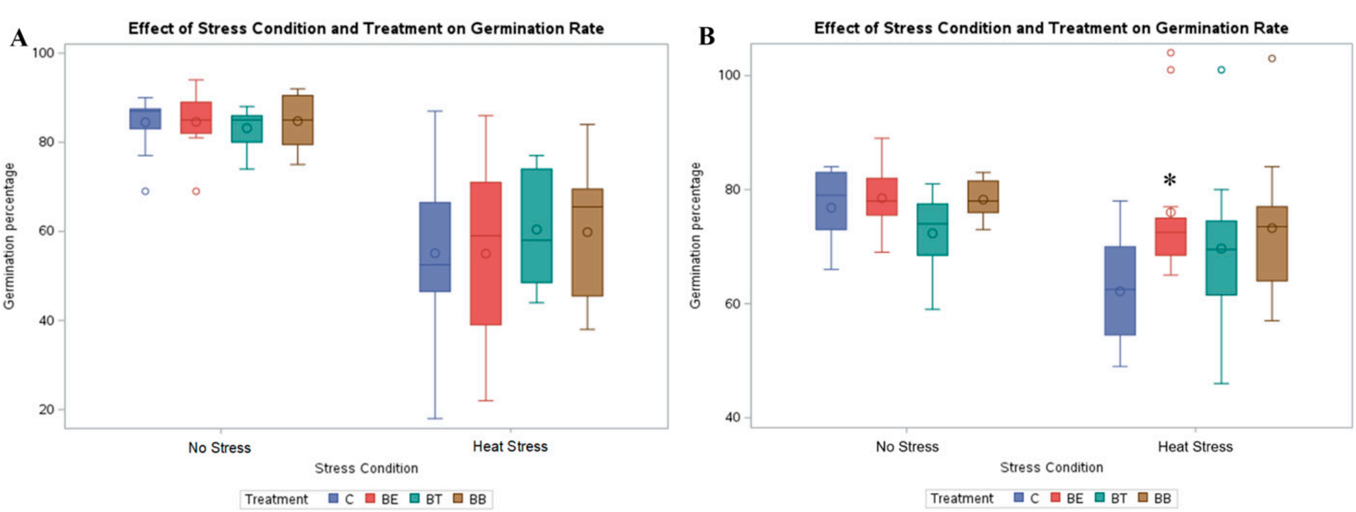

Fig. 2. Germination rate of creeping bentgrass on (**A**) filter paper and (**B**) soil substrate at day 8. The four treatments are represented: BioEnsure® + BioTango™ (BB), BioEnsure® (BE), BioTango™ (BT), and control/nontreated (C). In comparison with the control (C), a statistically significant difference is expressed by an asterisk (*P* value ≤ 0.05).

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Table 5. Particle size analysis on seed coating powders [BioEnsure®FP (BE-FP), BioTango™ (BT), and BioEnsure® + BioTango™ (BB)].

Treatment	D10 (μm)	D50 (μm)	D90 (μm)	Mean size (μm)
BE-FP	1.4 ± 0.2	8.8 ± 1.4	35 ± 0.6	14.5 ± 0.7
BT	7.1 ± 2.0	89.3 ± 2.2	311.8 ± 6.8	137.9 ± 2.9
BB	1.5 ± 0.0	9.8 ± 1.4	37.6 ± 0.4	15.6 ± 0.9

The values are expressed as means ± standard deviation. D10, D50, and D90 identify diameter distributions obtained at 10%, 50%, and 90% cumulative percentile volumes, respectively.

in both products revealed that the BE-FP and BT products had distinct particle size distributions (Table 5 and Figs. 3 and 4).

Discussion

The absence of significant treatment impact on turfgrass growth and quality is most likely due to the failure of the microbial inoculants to colonize the roots. The inability of inoculants to colonize the roots could be due to a reduction of root exudates because of the heat stress or competition from the native microbial communities (Compant et al. 2010; Trabelsi and Mhamdi 2013; Vurukonda et al. 2016). Additionally, microbial inoculants may exhibit functional redundancy or antagonism when combined, potentially triggering mild host stress responses (Finkel et al. 2017). The effect we saw with BB based on CDA, increased electrolyte leakage (EL) that is associated with membrane instability, might be suggestive of such a phenomenon (Blum and Ebercon 1981). The CDA also indicated that BB aligned with higher turf quality, suggesting a discrepancy between visual and physiological stress markers. While turfgrass appearance may have been maintained, the increase in underlying stress indicators (such as EL and reduced Fv/Fm) suggests that cellular integrity was compromised. The observed mismatch between EL and TQ may be partly attributed to the shortcomings of visually scoring turfgrass quality, which is innately subjective and may fail to capture early or subtle physiological stress responses (Habib et al. 2022).

Under no-stress conditions, the physiological shifts induced by microbial inoculants may reflect altered metabolic prioritization rather than a direct benefit to plant performance. The BB treatment appeared to stimulate pigment-related metabolism based on CDA, likely through improved nutrient assimilation or hormone signaling. While this could be interpreted as a positive physiological shift, its relevance is limited in optimal conditions, under which nutrient availability and photosynthetic function are not constrained. The activation of such responses can be related to the "induced resistance" or "priming" caused by the inoculants that, without a corresponding stressor, may represent an unnecessary metabolic cost (Pieterse et al. 2012; Van Hulten et al. 2006).

We expected a boost in soil health because of the products. The expectation was based on the fact that they can be sources of labile carbon and that their colonization of the root would increase exudate production, enhancing microbial activity (Anderson and Domsch 2010; Diera et al. 2020). We observed the opposite effect under heat stress, under which soil respiration and urease activity were negatively affected by the products compared with the control. The heat stress alone does not account for this decrease, as it would have equally affected soil health in the control treatment as well. One possible explanation is that there was an antagonistic interaction between the inoculants and the soil microbial community temporarily, as the effect was mainly apparent at T1 (Trabelsi and Mhamdi 2013). At T2, effects of the products were either neutral or beneficial, as in the positive impact of BT on phosphatase activity. The BT product contains *Bacillus* spp. that are known for mediating processes that improve nitrogen and phosphorus availability in soil (Richardson et al. 2009).

Like the effect we saw with the fungal product in the germination study, previous studies report success in observing the inoculant effect through seed coating. Yildirim et al. (2006) reported improved growth of *Cucurbita pepo* under salinity stress when the seeds were treated with *T. harzianum*. Similarly, Mastouri et al. (2010) reported that *T. harzianum* alleviated biotic and abiotic stresses in germinating seeds and seedlings. A consortium of *Bacillus* spp. had a similar effect in improving *Pisum sativum* growth when introduced via seed coating (Raza et al. 2024). In our case, only the product with the fungal inoculant had a positive impact. A lack of a positive impact from the product containing the bacterial inoculant might partly be explained by the sizes of the particles in the carrier powder (mean size, 140 μm), which were bigger than those of the fungal product (15 μm) (Table 5). To ensure uniform coating of the inoculants on the seeds, carrier powders with particle sizes of less than 75 μm are recommended (Afzal et al. 2020). A significant germination effect from the microbial product under heat stress was observed only with seeds germinated in soil, while no effect was found on filter paper. These results may indicate the interaction between the inoculants and the soil system in which seed coating treatment

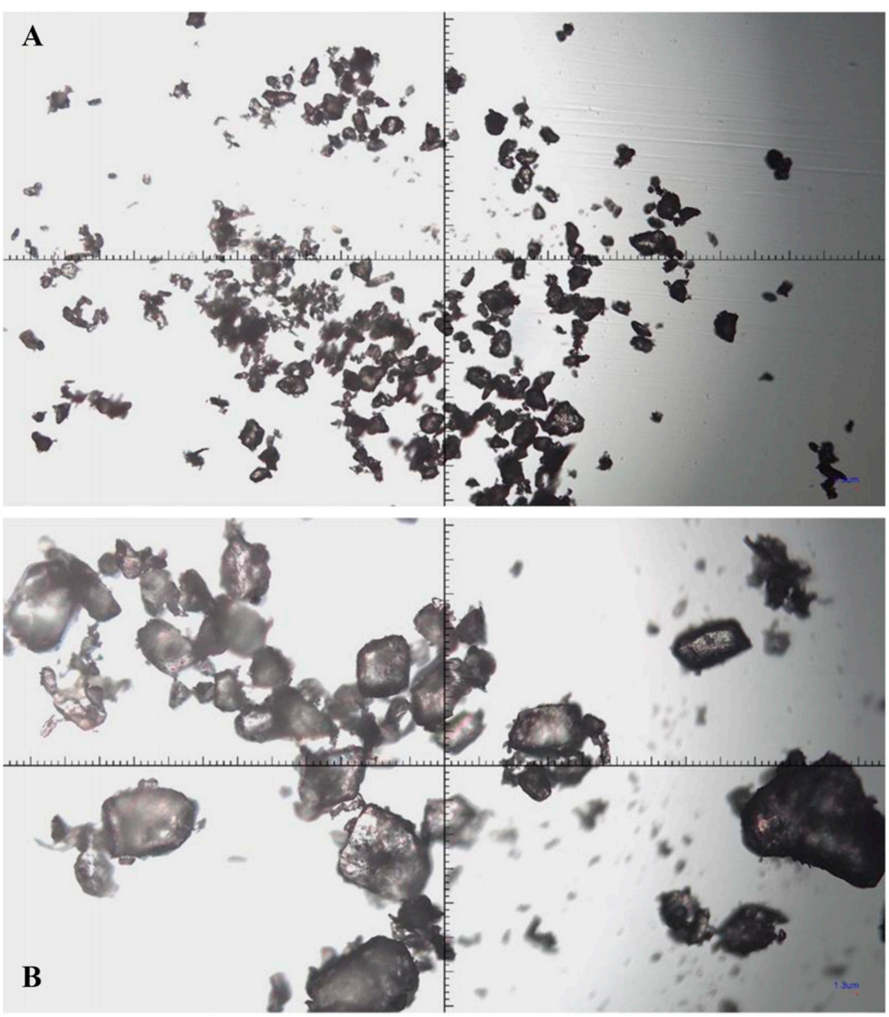

Fig. 3. Images of coating powders BioEnsure®FP (**A**) and BioTango™ (**B**).

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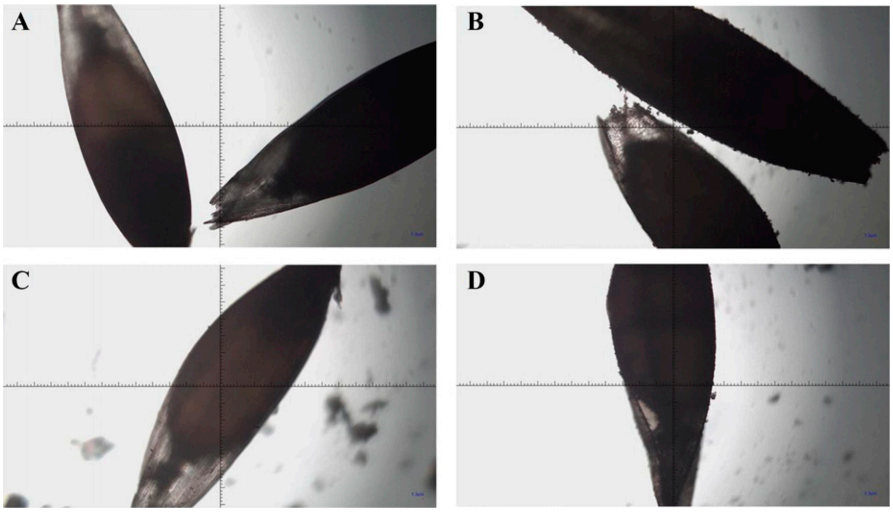

Fig. 4. Images of coated seeds of control (**A**), BioEnsure®FP (**B**), BioTango™ (**C**), and BioEnsure® + BioTango™ (**D**).

enhanced nitrogen uptake by roots (Amirkhani et al. 2016).

In conclusion, the study findings indicate that the performance of microbial products in turfgrass differed under stress and no-stress conditions. Under heat stress, the microbial products did not result in improvements in turfgrass growth and quality. In some cases, they were associated with stress-related indicators such as elevated electrolyte leakage. The absence of significant, positive impact may be linked to the inability of the inoculants to colonize the plant roots, microbial antagonism, or functional mismatch with the host plant's needs under stress. In contrast, under no-stress conditions, the microbial products altered plant metabolic responses, particularly in pigment production and soil enzyme activity, without a consistent impact on plant performance. The germination assay showed that the fungal product improved the germination rate in soil under heat stress, highlighting the potential advantage of introducing the inoculants during germination over application in soil after growth as a delivery method. However, the efficacy of the inoculants might have also been affected by the carrier powders in the products. The study findings highlight the importance of testing microbial products before their use. Future research should prioritize understanding microbial colonization dynamics, improving formulation technologies, and integrating physiological and biochemical metrics to refine the use of microbial products in stress-prone turfgrass systems.

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