

THE USE OF MICROBIAL INOCULANTS TO IMPROVE ABIOTIC STRESS TOLERANCE IN TURFGRASSES

by

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(Under the Direction of Mussie Y. Habteselassie)

ABSTRACT

This study evaluated the impact of microbial inoculants, *Trichoderma harzianum* (BioEnsure®) and *Bacillus* spp. (BioTango™), on the abiotic stress tolerance of three turfgrass species—bermudagrass (*Cynodon* spp.), zoysiagrass (*Zoysia* spp.), and creeping bentgrass (*Agrostis stolonifera*). In a greenhouse trial, the effects of the inoculants, applied individually or in combination, on turfgrass growth and quality and soil biological health were assessed under drought and heat stress conditions. Microbial treatments influenced plant responses, which varied with species and environmental conditions. Combining the treatment did not always result in better outcome over individual applications. Seed coating with *Trichoderma harzianum* significantly improved germination under heat stress, indicating it to be a more effective method than foliar spray. Treatments had limited impact on soil biological health. These findings highlight the potential of microbial inoculants in sustainable turfgrass management and highlighted the need for further research to optimize application strategies.

INDEX WORDS: microbial inoculants, *Trichoderma harzianum*, *Bacillus* spp., turfgrass, abiotic stress, drought tolerance, heat stress, sustainable turf management

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DEDICATION

To the family that has always been my roots and the one that is yet to bloom.

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CHAPTER 1

INTRODUCTION

Turfgrasses are plants that belong to the monocotyledons group (Christians et al., 2016). Many different grass species have been identified, differentiating from each other for their specific botanical characteristics. However, to be suitable for turf, the species must be tolerant to frequent mowing (Kaufmann, 2020). Turfgrasses are defined by Christians et al. (2016) as “a gramineous, root-bearing plant that covers the land surface and tolerates traffic and defoliation”, and by Kaufmann (2020) as “a grass that maintains its growing point close to the soil surface”. They are an essential constituent of urban and suburban landscapes, as they are commonly used in golf courses and sports fields, private lawns, and parks (Fan et al., 2020; Chang et al., 2021; Stier et al., 2013).

In the United States, more than 16 million hectares of the country’s surface are covered by turfgrasses, indicating the societal and economic importance of maintaining high-quality turf (Simmons et al., 2011). Moreover, the turfgrass industry is an integral component of the economy (Chawla et al., 2018). Therefore, the agronomic practices, such as mowing and irrigation, used for turfgrass management must be tailored to meet aesthetic and financial expectations (Stier et al., 2013). However, they require substantial use of agrochemicals and water, which could negatively impact the environment (Bosi et al., 2023; Gómez-Armayones et al., 2018; Vishwakarma et al., 2016). Nowadays, it is crucial to develop strategies to decrease synthetic inputs in agriculture and reduce the detrimental impacts of agrochemicals on ecosystems. Moreover, climate change and associated environmental challenges, such as extreme temperatures and moisture fluctuations, are

affecting the management and productivity of crops worldwide, including turfgrasses (Bhupenchandra et al., 2022; Mall et al., 2017). In light of the evolving climate and societal needs, it is essential to propose innovative approaches to provide sustainable alternatives. These alternatives should enable farmers to maintain productivity and profitability while safeguarding the environment and the communities it supports (Gomiero et al., 2011). The present research on microbial inoculation for turfgrass stress tolerance is a timely and crucial step in this direction.

Microbial inoculants, which are defined by Okon and Baker (1987) as “crop-yield enhancers,” have gained interest as part of a sustainable agricultural management plan due to their potential benefits on several aspects of plant health and soil fertility (Vishwakarma et al., 2016). Microbial inoculants are known for their biostimulant effect, as they can improve plant nutrient uptake, growth, and stress tolerance (Hassen et al., 2016). Moreover, the use of a consortium of microbial inoculants may induce an additive or synergistic effect of the microbial actions, enhancing the beneficial mechanisms that promote plant growth and enzymatic activity (Bosi et al., 2023). Microbial consortia can cooperate, providing a wider range of functions and mechanisms of action that can enable their colonization and persistence in the soil despite its complexity (O’Callaghan et al., 2022). The application of microbial inoculants is also expected to improve microbial activity in the soil system, thereby enhancing the activity of soil enzymes. Such improvement in the dynamics of the soil microbiome can boost plants’ uptake of nutrients, enhance plants’ defensive capacity against diseases (induced systemic resistance), and improve biotic and abiotic stress tolerance (Vijayabharathi et al., 2016).

The present study aims to understand if the use of two commercially available microbial inoculants, BioEnsure® composed of the fungus *Trichoderma harzianum* and the *Bacillus* spp. consortium contained in BioTango™ will impact turfgrass performance and soil microbial activity

under temperature and moisture stress in a controlled environment. The specific objectives of the present study are to: (1) evaluate if two microbial inoculant-based commercial products improve turfgrass growth under temperature and moisture stress in controlled environment; (2) understand if the mechanisms by which these products bring about relief in abiotic stress include changes in the soil microbial activity.

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CHAPTER 2

LITERATURE REVIEW

The importance of turfgrass

Turf areas, covering over 163,000 km² of the United States' surface, are critical for the preservation of the aesthetic value of the country's landscape (Fidanza et al., 2023). The USA is currently among the top countries for surfaces dedicated to turfgrasses; however, this industry is a robust economic sector worldwide; examples are China, Australia, the UK, and the Netherlands (Chawla et al., 2018).

Besides the visual value, managed turfgrasses, especially those in urban and suburban areas that are more prone to pollution, can offer pivotal ecosystem services by improving soil, water, and the atmosphere (Christians et al., 2016). Ecosystem services include the removal of pollutants, carbon dioxide sequestration, erosion control, and improvement in water infiltration, thereby enhancing groundwater recharge (Monteiro, 2017; Chang et al., 2021). Beard and Green (1994) classify the benefits provided by turfgrass to society into three groups according to the nature of the positive action it brings: functional, recreational, and aesthetic benefits. The functional benefits of turfgrass are strongly related to the connection of the turf cover with the protection and relationship it has with the soil system (Christians et al., 2016). In fact, the high density of shoots, a peculiar characteristic of turf species, acts as a shield in the protection of soil from water and wind erosion. In turn, the massive root system growing in the shallow layer of the soil provides stability to the land surface, reducing the risk of runoff. Moreover, turfgrasses reduce sediment loss by about three times compared to bare soils, and they have a crucial role in carbon

sequestration through photosynthesis (Stier et al., 2013; Christians et al., 2016). Recreational and aesthetic benefits are significant to human's mental and physical health. Services such as safe outdoor activity surfaces and the attractiveness of green parks and lawns around the city have a therapeutic effect by improving productivity and social harmony (Beard and Green, 1994; Stier et al., 2013; Christians et al., 2016). All the above-mentioned ecosystem services reported in Beard and Green's review thirty years ago are still accurate to modern societies, as explained by Braun et al. (2024), which confirms those observations, improving them according to the societal and research advances that occurred in the last three decades, however including a new aspect of the turfgrass impact on the ecosystem which refers to the so-called "ecosystem disservices," mentioning, for example, high water consumption, nutrients and pesticide inputs, lack of biodiversity.

Turfgrass benefits also involve the economic sector. The United States turfgrass industry is in fast and continuous development; it was worth \$57.9 billion in 2002 and had an occupational impact of 822,849 jobs (Haydu et al., 2006). More recently, its worth increased to \$150.4 billion in 2023, accounting for 1.2 million employees with an average growth of 5% per year in the last 5 years (Braun et al., 2024).

Challenges in turfgrass management

The turfgrass industry is facing many challenges in ensuring high-quality production, not only in the United States but also in the rest of the world. Such issues are related, for example, to stricter environmental regulations that limit water use, impose specific fertilizers and pesticide use guidelines, and promote the employment of species resistant to the changing climate. Examples are the Clean Water Act (CWA) of the United States (U.S. EPA, 1972); the Water Framework

Directive (WFD 2000/60/E) and the EU Fertilizing Products Regulation (EU 2019/1009) in the European Union (European Union, 2000; European Union, 2019); the Environment Protection Act, 1986 and Prevention and Control of Pollution Act, 1974 in India (FAO, 1986; CPCB, 1974), and many others that regulate the natural resources management all around the world, intending to reduce the environmental footprint of human activities on our planet.

The biggest challenge of the turfgrass industry and the whole agricultural sector nowadays is not only to find management solutions that meet business ambitions and allow adherence to the above-mentioned environmental guidelines but also the compliance of these two goals with the aim of adapting to the changing climate while safeguarding the environment. Climate change intensifies abiotic stress factors, such as drought, extreme temperatures, and soil salinity, which directly impact plant health and productivity (Campos et al., 2023). Rising global temperatures, altered precipitation patterns, and increased frequency of extreme weather events exacerbate these stressors, making it more difficult for turfgrass and other crops to maintain optimal growth and metabolic functions (IPCC, 2021). Abiotic stresses, including water deficiency and heat stress, can disrupt physiological processes, leading to metabolic dysfunction, reduced photosynthetic efficiency, and overall growth inhibition (Fan et al., 2020; Mittler, 2006). These abiotic stresses severely impact turfgrass species development significantly reducing production by disturbing phenological cycles and physiological processes (Yadav et al., 2020). The fast evolution of these weather conditions affects the plant system and the soil, modifying its composition with a significant effect on the organic matter content and fertility and, in turn, nutrient cycling, leading to a reduction in plant productivity (Hatfield, 2017).

Most of the physiological processes that allow plant growth and development are influenced by the temperature. Rising temperatures can interfere with the growth of cool-season

turfgrasses, such as creeping bentgrass (*Agrostis* spp.), which are adapted to mild temperatures (approximately 15-24°C for the aboveground parts and 10-18°C for the root zone) (Sun et al., 2024). Also, heat stress interferes with shoot and root growth, reducing photosynthesis, biomass, and nutrient and water absorption from the soil (Hatfield, 2017; Tan et al., 2021). Photosynthesis depends on enzyme activity and carbon dioxide diffusion, which are influenced by temperature (Di Paola and Beard, 1992). Even though the abovementioned physiological processes depend on the specific species, photosynthesis will generally be less efficient at temperatures above 30°C. Moreover, higher temperatures can have other negative effects on the plant system, such as reduced carbon dioxide fixation and increased respiratory activity, resulting in higher metabolic energy use (Feller and Vaseva, 2014).

Water stress poses a significant threat to turfgrass sustainability, particularly as changing precipitation patterns and prolonged droughts increase pressure on water resources (Gómez-Armayones et al., 2018). Due to their scarcity, water resources must be managed responsibly in agriculture, which is why they have received significant attention over the past few decades (Musie and Gonfa, 2023). Water conservation practices have become part of the action plan proposed to foster agricultural sustainability and adaptation to climate change (Jazi et al., 2019). According to Cabrera et al. (2013), the maintenance of golf course and landscape turfgrass in Texas entails the consumption of 47% of the total water use in the urban environment. Moreover, Fidanza (2023) mentions one of the most common approaches used for turfgrass irrigation, known as “set and forget” scheduling. This irrigation method often provides excessive amounts of water to the turfgrass canopy, especially during spring and fall, leading to over-irrigation and unsustainable use of the water resource. Efficient water management in the turfgrass industry is crucial in the current climate scenario since, by the end of this century, the entire agriculture sector will be strongly

challenged by water shortage, threatening crop productivity worldwide (Chawla et al., 2018). Reducing the amount of water input to turfgrass systems can have a variety of negative effects such as reduced growth, lower photosynthetic activity, and increased susceptibility to diseases and pest infestations (Fan et al., 2020). Furthermore, drought stress leads to physiological changes such as stomatal closure, osmotic adjustment, and reduced leaf water potential, ultimately compromising turfgrass quality and persistence (Gómez-Armayones et al., 2018; Fan et al., 2020). New strategies are needed to allow efficient and low-impact plant cultivation systems compatible with reduced water input by irrigation (Chartzoulakis and Bertaki, 2015). Examples of these strategies to improve water-use efficiency while mitigating the adverse effects of drought include deficit irrigation and drought-tolerant turfgrass species (Gómez-Armayones et al., 2018; Chawla et al., 2018).

In this study, we analysed whether the application of microbial biostimulants is a feasible strategy to improve the resistance of turfgrasses to drought and temperature stress.

Soil and turfgrass: a unique ecosystem

The importance of the soil system to plants and in sustaining all the other living organisms is intrinsic to its nature and role; therefore, preserving its health and promoting its quality is key to fostering sustainability. Doran and Zeiss (2000) underscore this significance by defining soil health as “the capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health.” The reason soil and its health hold such a considerable value within the agricultural systems – and in turn to humans – is not merely given by its function as the primary growth medium for crops. Soils serve as reservoirs of water supplies, ensuring good

quality when well-maintained (Amundson et al., 2015). Soil is a source of ecosystem biodiversity and provides habitat for microorganisms essential for transforming and synthesizing substances that promote plant health growth (Fierer, 2017). Thanks to these microorganisms, all the soil's functions and the processes driven by it can occur (Kibblewhite et al., 2008). The soil microbial communities play a leading role in climate change mitigation by mediating processes that capture carbon dioxide and other greenhouse gases in the soil; they are important in providing nutrients to the plants by transforming complex molecules into simple forms available for their uptake and, by that, they drive the physiological processes that lead to production (Lal, 2004).

Shi et al. (2007) describe *turf* as a unique ecosystem that comprises the turfgrass plant and the soil below it. One of the focal roles of the soil-turfgrass system that helps understand its importance for the whole ecosystem is the accumulation of organic matter, which, among other things, has a significant impact on the availability of organic nitrogen and phosphorus, essentials to plant development (Broadbent, 1986). It is reported that turfgrass soils can accumulate between two to three times more organic carbon than other agricultural soils, especially when well-established in an ecosystem (Shi et al., 2007). However, this applies only to the shallower layers of the turf. Other than soil organic matter, many soil characteristics in turf ecosystems deeply vary according to age and are functional to their efficiency and sustainability. The soil microbial community's abundance and diversity are among these (Shi et al., 2007).

Biostimulants: an overview

One of the most recent and commonly accepted definitions of biostimulant was proposed by du Jardin (2015): "A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits,

regardless of its nutrients content. By extension, plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms.”. Biostimulants are not considered significant sources of nutrients, unlike fertilizers. Phytohormones, amino acids, seaweed extracts, humic and fulvic acids, and microorganisms are examples of biostimulants known worldwide for their beneficial effects on crop production (Rouphael and Colla, 2020). The ways biostimulants provide benefits to plants and soil are diverse. The modes of action and the details of chemical and physiological processes by which they act have not yet been fully understood due to the diversity and complexity of these substances and organisms (Brown and Saa, 2015; Yakhin et al., 2017). Nevertheless, many studies have shown that the use of biostimulants on crops can be effective (Nardi et al., 2002; Parađiković et al., 2011; Colla et al., 2015; Ammaturo et al., 2023). These studies indicated that the impact of biostimulants is through their interaction with plant signaling processes, which enables them to reduce the negative stress response. Moreover, biostimulants can indirectly benefit the plant system by stimulating endophytes and other microorganisms to produce metabolites (Brown and Saa, 2015). Biostimulants improve plant productivity and quality. They positively affect germination and impact root and shoot growth. They are also involved in the response to biotic and abiotic stress and the uptake of essential nutrients (Wozniak et al., 2020). There is increasing interest in using microorganisms as biostimulants in the agricultural field due to the benefits they provide to plants and soil health by forming symbiotic relationships and for being sustainable alternatives to agrochemicals and disruptive agricultural practices (Alori et al., 2017; Shukla et al., 2022).

Microbial inoculation as a sustainable tool to improve abiotic stress tolerance

Bacterial and fungal strains are commonly used as biostimulants in agriculture (Alori et al., 2017; Fidanza, 2023; O'Callaghan et al., 2022). Previous studies have assessed the use of microbial inoculants to stimulate growth and development in turfgrass through the application of diverse types of microorganisms such as *Klebsiella variicola*, *Gluconacetobacter diazotrophicus*, *Azospirillum brasilense* and plant growth-promoting rhizobacteria (PGPR) to assess their potentiality in the establishment and functionality or their effectiveness to reduce nitrogen and water input in turf production (Bolton et al., 2022; Coy et al., 2014).

Li et al. (2022) reviewed a series of 97 studies published between 2010 and 2020 about the use of microbial inoculants in crop production and their effect on nutrient availability and alleviation of stresses. They found that more than half (53.95%) of the investigations reported improved yield, and that *Pseudomonas*, *Enterobacter*, and *Bacillus* are the major contributors to the amelioration of crop yield. In particular, the inoculation of *Bacillus subtilis* has been observed to be a key factor in the improvement of tolerance to abiotic stresses such as drought and salinity. They show that, compared to conventional agriculture practices, microbial inoculant application is responsible for the increase in dry biomass of the crop by 64.73% via alleviation of biotic and abiotic stress, even though plant height only increased by 13.4%. Moreover, the root system of inoculated plants increased in dry weight by 45.35% and length by 72.71%. The benefits brought by the application of microorganisms were found to be dependent on several factors, such as the microbial species and the crop type. However, this sustainable approach effectively increased crop yield and stress tolerance.

Research is increasingly being conducted to develop new formulations and understand the best way of using microbial inoculation to foster sustainability in the agricultural sector and

improve abiotic stress tolerance in plants (Li et al., 2023; Enebe and Babalola, 2018; Phurailatpam and Mishra, 2020). Applying microbial inoculants is currently considered the “most feasible biotechnology to fulfill plant growth requirements in association with crop protection against biotic and abiotic constraints” (Bittencourt et al., 2023). However, using microbial inoculants in a way that is effective and beneficial to turfgrass should consider the following important considerations: the method of delivery and strategic application, the ability of the inoculants to survive and coexist with the indigenous soil microorganisms in a competitive soil environment (O’Callaghan et al., 2022).

Microbial inoculants in agriculture: challenges and limitations

The application of microbial inoculants in agriculture presents several challenges that limit their effectiveness. One major issue is the inconsistency in performance across different soil types and environmental conditions. Many commercial inoculants fail to establish successfully in the soil due to competition with native microbial communities, predation by soil microfauna, and unfavorable abiotic factors such as pH, temperature fluctuations, and moisture levels (O’Callaghan et al., 2022). Additionally, the survival and efficacy of introduced microbes depend on their ability to colonize plant roots and persist in the rhizosphere, which can be inhibited by soil texture, organic matter content, and nutrient availability (Bashan et al., 2014). Even when microbial inoculants are established in the soil system, their long-term persistence remains a challenge, as environmental stresses, antagonistic interactions, and nutrient limitations can reduce their populations over time (Campos et al., 2023).

Another challenge is the lack of standardization in product formulation and storage conditions, which can lead to reduced viability of inoculants before they reach the field (Malusá & Vassilev, 2014).

Beyond biological limitations, other factors restrict the utilization of inoculants in agriculture. Farmers are often skeptical about adopting microbial inoculants due to their uncertain performance compared to synthetic fertilizers and pesticides (Abbott, 2018). Some microbial inoculants are less persistent than synthetic substances, making them less effective when applied in the field with high variability of environmental conditions that can enhance their degradation (Parnell et al., 2016). Additionally, effective application methods, such as co-inoculation with compatible microbial species or the use of protective carriers, require further optimization to enhance inoculant survival and efficacy under field conditions (Campos et al., 2023). Addressing these challenges requires improved techniques, better carrier materials, and strategies to enhance microbial survival and activity under diverse field conditions.

Bacillus spp. and *Trichoderma harzianum*: uses in agriculture

Endophytes, like the two types used in the present study, are microorganisms that colonize plant tissues and build a solid connection capable of regulating the metabolism of the host, enabling the plant to cope with unfavorable environmental conditions that cause abiotic stress (Pandey et al., 2022). Several physiological mechanisms are involved in helping the plant to be resilient to stressful conditions. These include improved nutrient uptake, the production of hormones and secondary metabolites, and enhanced defense mechanisms (Hassen et al., 2016; Pandey et al., 2022; Fidanza, 2023). Moreover, they are fundamental to promoting soil health, particularly its

structure, by affecting soil aggregation, root adherence, and organic matter decomposition (O’Callaghan et al., 2022).

The role of bacteria in agriculture is widely studied for their diverse benefits to crops, soil system, and the environment. *Bacillus spp.* is known for being one of the main genera of microbes used in agriculture with various applications such as biocontrol, plant growth promotion, and phytohormone synthesis (Khan et al., 2022). The *Bacillus spp.* consortia used in the present study comprises five species: *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis*, and *B. amyloliquefaciens*. These bacteria contribute to soil fertility by solubilizing phosphorus, fixing nitrogen, and producing phytohormones that stimulate root development (Vishwakarma et al., 2016; Muras et al., 2021). Additionally, they enhance plant resistance to abiotic stresses such as drought and salinity by inducing systemic tolerance and producing exopolysaccharides that improve soil moisture retention (O’Callaghan et al., 2022; Zhao et al., 2021; Luo et al., 2022; Dobrzyński et al., 2022; Blake et al., 2021). Their ability to form resilient endospores further ensures their long-term viability in soil, making them promising candidates for sustainable agriculture (Hassen et al., 2016). All these benefits derived from the application of a variety of bacterial species, often described as a microbial consortium, develop an effect where the various benefits are amplified, intensifying the efficacy of the application. One of the key factors of the consortia is the interaction between different organisms within this microbial community. Through these connections, microorganisms can cooperate to enhance their beneficial services to the crops (Lahiri et al., 2022).

Fungi belonging to *Trichoderma spp.* are known for their beneficial effects on crops related to phytostimulation and the production of elicitors and other substances able to interact with the plant roots to enhance growth and improve the plant defense mechanisms and abiotic stress

tolerance (Hidangmayum and Dwivedi, 2018; Zin and Badaluddin, 2020). The reason why *Trichoderma spp.* has gained much research interest is that these fungi can survive under stressful conditions. They have a high reproductive capacity and produce secondary metabolites that act as mycoparasites to control pathogenic fungi (Waghunde et al., 2016). The tolerance to abiotic stress in plants colonized by *Trichoderma* is enhanced by the production of compounds, such as phytohormones (auxins, gibberellins, ethylene), antioxidants, enzymes, and phenols. These microbial metabolites improve root growth and their ability to explore soil with extensive branching (Hidangmayum and Dwivedi, 2018). According to Abdullah et al. (2021), improved nutrient availability in plants colonized by *Trichoderma* is due to the increased root biomass and the secretion of organic acids that acidify the rhizosphere, enhancing the solubilization of nutrients. In particular, *Trichoderma harzianum* is shown to enhance yield, increase phosphorous uptake, increase chlorophyll content, and promote plant growth through the production of metabolites such as harzianic acid and harzianolide (Abdullah et al., 2021).

Effect of microbial inoculants application on turfgrass and soil health

Studying dynamics and relationships between plant and soil health is essential for understanding which processes drive changes in quality and biological parameters following the introduction of biological substances into the system. Barrios (2007) defines the soil system as a “regulatory center” of the whole ecosystem, underlying the importance of its role in managing an agricultural context. At the same time, he retraces the processes carried out by the soil microorganisms through which they break down the complex molecules of organic matter into simpler substances to facilitate their decomposition and allow the mineralization of nutrients into forms available for plant uptake. The above-described chain of reactions is the basis of a

sustainable and feasible agriculture management practice to ensure productivity and gains for the producers and the consumers, either when related to crop yields or recreational services such as sports fields, yards, and other turfgrass sites (Guggenberger, 2005).

The origin of the vastity of microbial diversity and abundance in the soil is strongly related to plant needs. Indeed, the plant seeks help through the soil systems and builds direct relations with the microbes by producing a large variety of substances synthesized by the roots and secreted to the rhizosphere, known as root exudates (Dubey et al., 2019). These substances include primary metabolites, such as amino acids, carbohydrates, and organic acids, and other secondary metabolites, such as hormones, sterols, flavonoids, and enzymes. The pathways by which root exudates are secreted vary according to the nature of the exuded substance and other factors, such as integrity and characteristics of the root cells (Bardi and Vivanco, 2009; Vives-Peris et al., 2020).

Bacteria and fungi are among the most important living organisms that inhabit the soil and impact plant physiology. The primary examples of symbiotic relationships between plant and soil microorganisms are the well-known plant-growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi. These microorganisms are fundamental to plants, besides the mineralization of organic phosphorus and nitrogen, also for the mitigation of stresses, production of phytohormones, and synergistic relationships with other bacteria. Moreover, they can induce plant systemic resistance and synthesize volatile compounds that inhibit the development of pathogenic microbes (Vimal et al., 2017).

The essential role of the soil microbiome in the health and quality of plants and the soil itself has been studied and confirmed in depth; however, it is important to discuss what can be the effect of the application of external biological sources in the system, as for the example of the present study.

Microbial inoculants contribute to turfgrass health not only through direct plant growth promotion but also by enhancing soil health parameters that indirectly support plant development. One key mechanism is the stimulation of soil enzymatic activity, which plays a crucial role in nutrient cycling and organic matter decomposition. Enzymes such as phosphatase and urease are produced or stimulated by beneficial microbes, facilitating the breakdown of organic and inorganic compounds to release essential nutrients for turfgrass uptake (Vishwakarma et al., 2016). Additionally, microbial inoculants enhance soil respiration, an indicator of microbial activity and organic matter decomposition, improving soil aeration and structure (Hassen et al., 2016). Increased microbial respiration is often associated with higher microbial biomass and activity, which contributes to the stabilization of soil organic matter and improved soil fertility (Gómez-Armayones et al., 2018). By improving soil structure, microbial inoculants improve roots' penetration and resilience against abiotic stresses such as drought and excessive water retention (Fan et al., 2020). Furthermore, microbial inoculants play a role in disease suppression through competitive exclusion and the production of antimicrobial compounds that inhibit pathogenic fungi and bacteria in the rhizosphere (O'Callaghan et al., 2022). Additionally, microbial inoculants contribute to carbon sequestration by enhancing soil organic matter decomposition and stabilization, reducing carbon loss through CO₂ emissions (Vishwakarma et al., 2016). This process not only benefits turfgrass growth by improving soil nutrient availability and root development (Gaskin et al., 2005) but also aligns with sustainable management practices by promoting carbon storage in soil ecosystems (Liu et al., 2023).

Knowledge gaps and future research directions

Since the beginning of the 21st century, many scientists have offered solutions to overcome the abiotic stress issue in turfgrasses, frequently proposing breeding programs with the employment of existing genes that confer resistance (Casler, 2001; Duncan and Carrow, 2001; Humphreys et al., 2004; Zhang et al., 2006; Huang, 2008; Fan et al., 2020). Even though the use of breeding techniques can be considered an environmentally sustainable alternative to the use of chemicals in building abiotic stress tolerance in plants, it has the drawback of being time-consuming and labor-intensive (Casler, 2001). Moreover, the breeding approach suffers from limited availability of genomic data and insufficient molecular markers for turfgrass species (Huang et al., 2014; Jiuxin and Liebao, 2022). For this reason and considering the imminent need to adapt the agricultural sector to the changing climate, there is a necessity to explore additional methods for growing turfgrass in challenging environments (Anderson et al., 2020; Burke and Emerick, 2016).

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CHAPTER 3

EFFECTS OF MICROBIAL INOCULANTS ON TURFGRASS GROWTH UNDER
DROUGHT AND HEAT STRESS¹

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Abstract

Abiotic stresses such as drought and heat significantly impact turfgrass physiology, reducing growth, photosynthetic efficiency, and overall turf quality. The use of microbial inoculants has been proposed as a sustainable strategy to enhance plant resilience to combat stress. The present study evaluated the effects of two microbial inoculants, *Trichoderma harzianum* (BE) and *Bacillus* spp. (BT), applied individually or in combination (BB) on three turfgrass species—bermudagrass (*Cynodon* spp.), zoysiagrass (*Zoysia* spp.), and creeping bentgrass (*Agrostis stolonifera*)—under drought or heat stress under controlled environment. Turfgrass performance was evaluated by measuring photosynthetic efficiency (Fv/Fm), relative water content (RWC), green percentage, and turf quality. A germination study was also conducted with creeping bentgrass to evaluate seed coating effects on germination rate (GR). The microbial treatments did not positively impact the turfgrasses under stress conditions. In bermudagrass, BE showed a reduction of 22% in Fv/Fm and 19% in RWC after four applications compared to the control (C). Similarly, BT resulted in a reduction of 17% in Fv/Fm and RWC compared to C repeated treatment applications. No significant differences were observed among treatments in zoysiagrass and creeping bentgrass at any collection time under stress condition. The BE treatment improved germination rate by 13% compared to the control when germinated on soil substrate under heat stress. The outcome of this study suggests that microbial inoculants have the potential to improve abiotic stress tolerance in turfgrass; however, more research on application methods and frequency of application is needed to improve knowledge in this field.

Keywords: microbial inoculants, turfgrass, abiotic stress tolerance, BioEnsure®, BioTango™

1. Introduction

Turfgrass plays a crucial role in providing aesthetic and environmental benefits in sports fields, golf courses, and urban green spaces (Christians et al., 2016). However, turfgrass species are frequently exposed to abiotic stresses such as heat and drought, which significantly impact their physiological performance, growth, and overall quality (Huang, 2008). The ability of turfgrass to withstand and recover from stress conditions is essential for maintaining high-performance turf systems. To address this problem, there has been an increasing interest in the use of microbial inoculants as a sustainable strategy to improve plant resilience against abiotic stresses (Bashan et al., 2014).

Plants actively seek support from their surrounding microbial communities, establishing a symbiotic relationship through the secretion of various chemical compounds known as root exudates (Kuzyakov and Razavi, 2019). These exudates, released into the rhizosphere—the narrow region of soil influenced by root activity—comprise a complex mix of primary and secondary metabolites (Vives-Peris et al., 2020). Primary metabolites include amino acids, carbohydrates, and organic acids, which serve as energy sources for microbial populations (Sanchez and Demain, 2008). Secondary metabolites, such as hormones, sterols, flavonoids, and enzymes, further influence microbial interactions and soil health (Dubey et al., 2019; Canarini et al., 2019). The composition and release of root exudates depend on multiple factors, including plant species, environmental conditions, and the plant's physiological state (Bardi and Vivanco, 2009; Vives-Peris et al., 2020).

Microbial inoculants have gained attention due to their ability to enhance plant growth, improve stress tolerance, and optimize water and nutrient use efficiency (Vejan et al., 2016). Among these, BioEnsure® and BioTango™ are two commercially available microbial inoculants

developed to assist plants in coping with abiotic stresses by modulating physiological and biochemical responses. These inoculants contain endophytic microorganisms capable of colonizing plant tissues and influencing plant metabolism to enhance stress resilience (Redman et al., 2011).

Bermudagrass (*Cynodon* spp.), zoysiagrass (*Zoysia* spp.), and creeping bentgrass (*Agrostis stolonifera* L.) widely used in managed turfgrass systems and have differing responses to abiotic stress conditions (Trenholm et al., 2000). Specifically, creeping bentgrass is highly susceptible to heat stress, while bermudagrass and zoysiagrass are more tolerant to high temperatures but are sensitive to drought stress (Huang and Gao, 2000). These attributes make them ideal for stressed related studies.

Treatment response of turfgrass is often evaluated by measuring parameters that reflect changes in growth and physiology of the plant. Photosynthetic efficiency is one such indicator, a decline often signals damage to the photosynthetic apparatus and reduced carbon assimilation (Maxwell and Johnson, 2000). Turf quality indicators (normalized difference vegetation index, % green cover) provide a visual assessment of overall plant vigor and photosynthetically active plant tissues (Christian and Patton, 2016; Huang and Gao, 2000). Relative water content is widely used as it reflects the ability of the turfgrass to maintain hydration under drought conditions (Barrs and Weatherley, 1962). Chlorophyll and carotenoid contents are crucial for assessing pigment stability under heat stress, as chlorophyll degradation is a common response to thermal damage (DaCosta and Huang, 2006). Moreover, electrolyte leakage provides insight into membrane integrity, with increased leakage indicating cell membrane damage and loss of cellular function under extreme environmental conditions (Blum and Ebercon, 1981).

This study provides a unique contribution to the expanding field of microbial inoculants and their role in plant stress tolerance by specifically investigating their effects on turfgrass under heat or drought stress. Differently from previous studies, which often focused on crops for food production or single microbial strains, the present research evaluates both fungal (*Trichoderma harzianum*) and bacterial (*Bacillus* spp.) inoculants, as well as their combined effects in a turfgrass system. By directly comparing these treatments under controlled conditions, this study offers novel insights into the potential synergistic or additive benefits of microbial co-inoculation, highlighting its relevance for sustainable turfgrass management and improved stress resilience.

The primary objective of this study is to evaluate if the use of BioEnsure® and BioTango™ improves turfgrass tolerance to heat stress in creeping bentgrass and drought stress in bermudagrass and zoysiagrass. Given the increasing frequency and severity of climate-related stress, identifying sustainable strategies is essential for maintaining turfgrass quality while reducing dependency on chemical inputs (Zhang et al., 2013).

2. Materials and Methods

2.1 Experimental set-up

This study assessed the effects of two commercial microbial inoculants, BioEnsure® and BioTango™ (Table 3.1), on turf growth under temperature and moisture stress conditions. The experiment included four inoculation treatments: (1) BioEnsure® (BE), (2) BioTango™ (BT), (3) BioEnsure®+BioTango™ (BB), and (4) Control (C). The commercial inoculants were applied at a rate indicated on the product labels [BE: 9.1µl/ml; BT: 1.64mg/l; BB: 16.98µl/ml (BE) + 5.67mg/l (BT)]. The control treatment consisted of the application of deionized water. The treatments were applied under stress or standard conditions. The experiment was performed in the

greenhouse of the University of Georgia, Griffin Campus. A completely randomized experimental design was used for the two warm-season species with drought stress where the temperature was controlled at a range of 18-35°C. The moisture stress was introduced in the form of a reduced irrigation rate, 50% of the rate used under standard moisture condition in two warm-season turfgrass species: Bermudagrass (*Cynodon* spp. var. Tifeagle) and Zoysiagrass (*Zoysia* spp. var. Mayer). A split-plot design was used for the cool-season species. To evaluate heat tolerance of Creeping bentgrass (*Agrostis stolonifera* L. var. Pure Eclipse), it was exposed to 35°C in a growth chamber (VWR, Inc.). The optimal air temperature for Creeping bentgrass ranges between 18 °C and 24°C and between 10°C and 18°C for the root zone (Miller and Brotherton, 2020). The non-stressed plants were located in a greenhouse with a controlled temperature ranging from 10-24°C. The experiment consisted of 4 replicates per treatment and was performed on the three turfgrass species involved in the study (Bermudagrass, Zoysiagrass, and Creeping bentgrass).

The turfgrass plugs were obtained from the experimental fields located at the University of Georgia, Griffin Campus. The sample roots were cut at approximately 2 cm below the crown. Each plug was set in a 4 cm diameter x 20 cm depth pot containing a mixture of sand and organic matter (at a ratio of 9:1) with landscape fabric at the bottom. The prepared pots were then maintained in the greenhouse under standard conditions for about four weeks [irrigation was provided to meet the desired moisture conditions; temperature ranged between 18-32°C and 45% relative humidity for warm-season species; and 10-24°C, 50% relative humidity for cool-season species (Hatfield, 2017)], to allow establishment before the start of the trial. The greenhouse trial timeline is shown in Figure 3.1. Once the establishment period was completed, five consecutive treatment applications (I, II, III, IV, V) were performed by foliar spray with a 2-nozzles boom sprayer with CO₂ backpack tank. The applications were performed at an interval of two weeks. Turfgrass

samples were collected by cutting the turf at the crown. Leaves, roots and soil were separated for downstream analysis. The first (pre-stress) collection (a) was obtained after the third treatment application (III). After that, the stress conditions were imposed and maintained until the end of the trial. The second sample collection (b) was done before the fifth and last treatment application (V). The last collection of samples (c) occurred two weeks after the last treatment application.

2.2 Turf growth measurement in greenhouse

Above-ground growth was evaluated through the assessment of visual turf quality (TQ), green cover percentage with digital image analysis (DIA), and photochemical efficiency (F_v/F_m) via chlorophyll fluorescence. Electrolyte leakage and chlorophyll content were measured for creeping bentgrass. Relative water content (RWC) was measured for the two warm-season species, bermudagrass and zoysiagrass, as an indicator of the leaf hydration of these two species under drought stress (Barrs and Weatherley, 1962).

2.2.1 Visual Turf Quality (TQ)

The visual evaluation of turf quality was done according to the National Turfgrass Evaluation Program (NTEP) criteria (Morris and Shearman, 1998). A score from 1 to 9 was assigned to each sample at each collection time (a, b, c) throughout the trial. 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.

2.2.2 Digital Image Analysis (DIA)

The DIA estimated the percentage of green cover compared to the total leaf area. The measurement was performed using a camera (Canon G7X) at a resolution of 5472 x 3648 and ISO-400 and a lightbox that ensured the same light quality and height of the pictures for all the samples. The samples were located in the lightbox and the pictures obtained were processed using the ImageJ software (Gallagher, 2014). Digital images of turfgrass canopies were converted from RGB to HSB (Hue, Saturation, Brightness) color space, and each channel was processed separately. A color threshold was applied to isolate green pixels based on the following HSB ranges: Hue: 26-125; Saturation: 40-225; Brightness: 40-245. The percentage of green cover was calculated as the proportion of green pixels within a defined area of interest (AOI), standardized across samples using a fixed circular AOI (325 × 325 pixels). Threshold values were visually optimized and consistently applied across all images to ensure comparability.

2.2.3 Photosynthetic efficiency

After a dark adaptation of 30 minutes, using a modulated chlorophyll fluorometer (OptiScience, OS5p+), the minimum fluorescence (F_0) was measured with a modulated light source [660nm (red) and 450nm (blue) LED], which was not strong enough to start the photosynthesis reaction and ensures that all the reaction centers of the photosystem II (PSII) were open. Subsequently, the maximum fluorescence (F_m) was measured using a short saturation pulse of high intensity (white LED with 690nm filter), which allowed the complete photosynthetic reaction to occur (Baker and Rosenqvist, 2004). Three readings were taken for each sample. The following ratio represents the maximum potential efficiency of PSII:

$$\frac{F_v}{F_m}$$

Where:

$F_v = F_m - F_0$ = variable fluorescence

F_m = maximum fluorescence

2.2.4 Electrolyte leakage

Electrolyte leakage was measured using a conductivity meter (Cole-Parmer Instrument Company). A sample of 0.1 g of leaves was collected and chopped into segments of about 1cm, subsequently rinsed with deionized (DI) water, and placed into 50 mL tubes with 35 mL of DI water. The tubes were placed on a rotary shaker (100 rpm) overnight at room temperature. The initial electroconductivity (ECi) was measured at 25°C. The samples were autoclaved (Consolidated Sterilizer Systems, Inc.) at 121.1°C for 20 min. After cooling down, the tubes were placed on a rotary shaker overnight. The final electroconductivity (ECf) was measured at 25°C. The measurement follows the protocol described by Bajji et al. (2002). The electrolyte leakage was measured as follows:

$$\% \text{ of damage} = (ECi/ECf)/100$$

2.2.5 Chlorophyll and carotenoids contents

The chlorophyll and carotenoid contents were measured as described in Wellburn (1994). Briefly, 0.1g of grass tissue was collected and chopped into 1cm sections, then 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 rate of 1:5. Using a spectrophotometer (Evolution 300 UV-VIS), the absorbance was measured at

three different wavelengths (665, 649, 480). After three days, the dry weight of each sample was recorded, and the parameters were calculated as below.

$$Ca = 12.19 \times A_{665} - 3.45 \times A_{649}$$

$$Cb = 21.99 \times A_{649} - 5.32 \times A_{665}$$

$$Total\ C = ((Ca + Cb) \times \text{dilution factor})/DW$$

$$C_{x+c} = (1000 A_{480} - 2.14Ca - 70.16Cb)/220$$

Where:

Ca = chlorophyll a

Cb = chlorophyll b

A_{665} ; A_{649} ; A_{480} = absorbance at wavelengths 665/649/480

Total C = total chlorophyll content

Dilution factor = 5

DW = dry weight (mg)

C_{x+c} = carotenoid content

2.2.6 Relative water content (RWC)

The RWC was measured as described in Mullan and Pietragalla (2012) by collecting 50-100 mg of trimming of leaf tissues from each pot during each sampling time (a, b, c). The fresh weight was measured, and the samples were placed in DI water overnight at room temperature. The turgid weight was measured after 24 h. The tissues were dried at 70°C for three days. The dry weight was measured, and RWC was calculated as follows:

$$RWC = (FW - DW)/(TW - DW) \times 100$$

Where:

FW = fresh weight

DW = dry weight

TW = turgid weight

2.3 Endophyte colonization of root tissues

Root tissues were collected to determine if the endophytic inoculants applied by foliar spray colonized the root tissues. The protocol described by Hallmann et al. (2006) was used for isolating endophytes. After rinsing the roots with water to remove any soil residue, the root surface was sterilized by dipping it in 2% sodium hypochlorite for two minutes. To validate the sterilization, the root surface was imprinted on culture media in Petri dishes, and after 24 hours, if no growth appeared, the endophyte recovery process would be continued. The roots were manually macerated with the addition of sterile water at a rate of 1:10 with the use of mortar and pestle previously autoclaved. The suspension obtained from the maceration was then cultured on nutrient agar plates selective for fungal growth (glucose-peptone Rose-Bengal agar with streptomycin) and bacterial growth (nutrient agar with nystatin). The plates were incubated at 30°C until growth appeared.

Once the fungal and bacterial colonies were grown, single isolates were transferred into culture tubes with selective liquid media and grown for 4-5 days. The DNA of each isolate was then extracted. The microbial genomic DNA was also extracted from the two commercial products used as treatments. The fungal DNA extraction was performed with the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation, Thorold, Ontario, Canada). The genomic DNA from bacterial cells was extracted using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). The fungal DNA extracts were then subjected to polymerase

chain reaction (PCR) to identify the fungal isolates targeting the 18S rDNA gene with nu-SSU 0817F and nu-SSU 1196R primers (Borneman and Hartin, 2000). The PCR conditions were: initial denaturation of 10 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, 2 min at 72°C. The bacterial DNA was subjected to PCR targeting the 16S rDNA gene with 968 F and 1401R primers (Zhang et al., 2013) with the following conditions: initial denaturation of 2 min at 94 °C followed by 35 cycles of 1 min of 94 °C, 1 min of 58.5 °C, and 2 min of 72 °C. There was a final extension for 10 min at 72 °C. All PCR reactions were conducted with a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). All PCR reactions were performed using the Applied Biosystems Power UP™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). All the primers used in this study were synthesized by Eurofins Genomics, Louisville, KY, USA. The PCR amplicons were purified with a PCR purification kit (Wizard PCR Preps DNA Purification System, Promega, Madison, WI, USA) and quantified with Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were send for Sanger sequencing to Genewiz® (from Azenta Life Sciences, South Plainfield, NJ, USA). The results from DNA sequencing were then compared against the existing sequences in the GenBank database of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/>) to investigate the presence of the applied microorganisms in the analyzed root samples.

2.4 Germination assay

After the completion of the greenhouse study, a germination trial was conducted to evaluate whether a different application method (seed coating) would affect the interaction of the microbial inoculants with the turfgrass under stress condition. The germination assay was performed on creeping bentgrass seeds (*Agrostis stolonifera* L., var. 007) under heat stress. The seeds were

soaked in 95% ethanol for 1 minute and then in 2% (v/v) sodium hypochlorite for 15 minutes. The seeds were rinsed three times with deionized water and let dry overnight (Wang and Zhang, 2010). Petri dishes were prepared with two different substrates: (1) two layers of filter paper and (2) 10g of sand + organic matter mixture (ratio 9:1). Before adding the seeds, 5 ml and 2 ml of deionized water were added to the plates with filter paper and soil, respectively. The sterilized and dried seeds were coated with BioEnsure®FP (BE-FP), BioTango™ (BT), and BioEnsure®FP + BioTango™ (BB) powder. The seeds were not treated for the control (C) (Figure 3.8). A paper envelope was used to mix the seeds with the coating powder. Hundred seeds were counted and placed into a Petri dish (60mm x 15mm sterile polystyrene; Fisherbrand). The plates were then placed into two growth chambers with different temperature settings: (1) standard temperature (20/15 °C day/night) and (2) heat stress (35/30 °C day/night). The chambers were set with a photoperiod of 8h dark/16h light. Six replications were used for each treatment and each temperature condition. Deionized water was added to the plate as needed to keep the moisture level standardized among all the plates throughout the trial. The germination rate (GR) was measured every 2 days by counting and removing from the plate the germinated seeds that developed a radicle and shoot of a minimum 1 mm length.

2.4.1 Particle size analysis (PSA)

The powders used as coating treatments for the creeping bentgrass seeds were analyzed using a Laser Diffraction Particle Size Analyzer (Model PSA 1190, Anton Paar, Graz, Austria) to estimate their particle size under the following parameters: sample read time: 3 s; vibrator duty cycle: 40%; vibrator frequency: 30 Hz; and air pressure: 1300 mBar (Cruz-Padilla et al., 2023).

2.5 Statistical analysis

The greenhouse trial for the warm-season species—bermudagrass and zoysiagrass—was conducted using a completely randomized experimental design. A split-plot design was employed for the creeping bentgrass greenhouse trial and the germination assay.

Analysis of variance (ANOVA) was carried out to investigate the statistical significance ($p \leq 0.05$) of the effects of the different treatments and the control on turf quality and growth. When significant differences were observed, a post-hoc analysis using the Tukey test was performed to identify which treatment groups were significantly different. The statistical analysis was performed using statistical software (SAS on Demands for Academics, Cary, NC, USA).

3. Results

3.1 Impacts of treatment on turf growth

3.1.1 Bermudagrass

Turf growth parameters did not show significant differences among treatments in any collection times under non-stress conditions (Figure 3.2a, c, e, g). However, the green percentage significantly reduced at time c for all the treatments (Figure 3.2c). Figure 3.2e shows that from time a to time c, the relative water content of bermudagrass treated with the fungal inoculant (BE) significantly decreased, while C, BT and BB did not experience a significant reduction. The visual turf quality score (Figure 3.2g) of C significantly reduces from time a to time c, while no significant reduction was reported for the inoculated treatments.

Under drought stress condition, bermudagrass treated with BE and BT had a significantly lower photosynthetic efficiency than C, at time b (Figure 3.2b). No significant differences were found among treatments in turf quality (green percentage and visual turf quality score) (Figure

3.2d and h). BE and BT led to a significant reduction in relative water content when compared to C at time b; no differences were found at time c (Figure 3.2f).

3.1.2 Zoysiagrass

In zoysiagrass, photosynthetic efficiency and relative water content did not show significant differences among treatments and collection times under no-stress and drought stress conditions (Figure 3.3a, b, e, f). The green percentage significantly declined from time a to time b for all the treatments under no-stress condition (Figure 3.3c). However, under drought stress, the green percentage did not significantly change from time b to time c (Figure 3.3d). The visual turf quality score under no-stress condition decreased over time, with a significant decline from time a to time c with treatments C and BB; BE and BT did not lead to a significant reduction over time (Figure 3.3g). Under drought stress, no significant differences were reported between treatments and collection times (Figure 3.3h).

3.1.3 Creeping bentgrass

For creeping bentgrass, the treatments did not significantly impact growth parameters under no-stress conditions at any time (Figure 3.4a, c, e and g). However, the photosynthetic efficiency decreased significantly from time a to time c with treatments C, BE and BT (Figure 3.4a). Moreover, visual turf quality was significantly lower from time a to time c with treatment BT (Figure 3.4e).

Under heat stress, at time b, the photosynthetic efficiency was significantly lower with BB compared to BT. No significant differences were found between C, BE and BT. Moreover, the number of applications influenced the photosynthetic efficiency, which improved significantly

with BE and BB from time b to time c. C and BT did not result in an increase in photosynthetic efficiency (Figure 3.4b). Turf quality parameters (visual turf quality score and percentage of green) did not show any significant differences among treatments under stress condition (figure 3.4d and f). The percentage of cell membrane damage at time b, under heat stress, was significantly lower with BT than with BB. However, there were no significant differences between BT and C or BE. No significant difference was observed among treatments at time c (Figure 3.4h).

Chlorophyll and carotenoid contents exhibited similar trends of variation across treatments and collection times. (Figure 3.5). Under non-stress condition, no significant differences between treatments were found at any collection time. However, a significant decline was reported with treatment BT from time b to time c (Figure 3.5a). Similarly, the carotenoid content was not significantly different among treatments. However, treatment BT initially led to a significant increase from time a to time b and then declined from time b to time c. Additionally, with treatments BB a significant reduction of carotenoid content from time b to time c was reported (Figure 3.5c).

Under heat stress, chlorophyll content did not show significant differences among treatments at any collection time. However, with C and BT, there was a significant reduction from time b to time c (Figure 3.5b). The carotenoid content brought by BT was significantly higher than BE at time b. No significant differences were found with the other treatments. At time c, no differences among treatments were found; however, the carotenoid content declined for all treatments compared to time b (Figure 3.5d).

3.2 Endophyte colonization

Table 3.2 illustrates the similarities between the bacterial and fungal isolates collected from the roots of creeping bentgrass and zoysiagrass samples with existing microorganisms. Notably, none of the endophyte isolates recovered from the root samples were identical with any microorganisms in the microbial products.

3.3 Germination assay

At day 8, creeping bentgrass seeds' germination on filter paper did not show significant differences between treatments in any temperature condition (Figure 3.6). However, on soil substrate, under heat stress condition, the fungal treatment BE led to an increase of 13.13% of the germination rate compared to the control (Figure 3.7).

From the PSA, BE-FP and BT coating powders had different particle diameters. As shown in Table 3.3 the fungal powder particles (BE-FP) had a mean value of $14.5 \pm 0.7 \mu\text{m}$; while the mean particle size of BT powder was $137.9 \pm 2.9 \mu\text{m}$, showing that the bacterial coating powder was 9.5 times larger than BE-FP (Figure 3.9).

4. Discussion

4.1 Impacts of treatment on turf growth

The effects of microbial inoculants on turfgrass physiology varied depending on the species, treatment, and collection time. While microbial inoculation is often associated with improved stress resilience, the results from this study indicate that its effects was highly species-specific and time dependent. This suggests that the interaction between the plant host and microbial inoculants is crucial in determining stress responses (Kumar and Verma, 2018).

4.1.1 Bermudagrass

Under non-stress conditions, the microbial products did not significantly affect most turf growth parameters at any frequency of product application. This implies that when water availability is not limiting, bermudagrass may already possess sufficient physiological and metabolic capacity to maintain growth and turf quality without microbial assistance. This stability is expected, as well-established turfgrass species typically operate at optimal photosynthetic efficiency under favourable conditions (Haag, 2013). However, after the fifth application, the control resulted in a significant reduction in green percentage and visual turf quality as compared to the microbial treatments, indicating a positive response to the product. This could imply that microbial inoculants delay senescence by modulating enzyme activity and reducing oxidative stress, as previously reported for microbial-plant interactions (Munir et al., 2022; Muhammad et al., 2024). Interestingly, the relative water content (RWC) in plants treated with BE significantly dropped after four applications, even though no significant differences among treatments were found. This might be explained by a potential trade-off between microbial activity and water retention, where increased microbial metabolic activity could have temporarily altered osmotic adjustments or root exudate composition, affecting water uptake efficiency (Kaushal and Wani, 2016).

Under drought stress, photosynthetic efficiency and RWC were significantly lower in BE and BT than in C after the fourth application. This could be due to an initial reallocation of resources by plants toward microbial interactions and root-microbe signaling, temporarily reducing investment in photoprotection mechanisms (Bashan et al., 2014). No significant differences were observed in green percentage and turf quality as drought progressed, which may suggest that the beneficial effects of microbial inoculants on drought tolerance may require longer

time to manifest, as previous studies have suggested that microbial inoculation enhances plant drought responses through cumulative root-associated benefits (Kim et al., 2012).

4.1.2 Zoysiagrass

In zoysiagrass, microbial inoculation did not significantly alter photosynthetic efficiency or RWC under stress or non-stress conditions. Zoysiagrass is known for its intrinsic drought tolerance, which is attributed to deep rooting, efficient stomatal regulation, and a robust antioxidant defense system (Huang, 2008), which may explain why microbial inoculants did not significantly impact stress response parameters. Even though no significant differences were found under drought stress in photosynthetic efficiency, the reduction after five applications was more intense with C, BT, and BB than BE, suggesting that fungal inoculation helped preserve photosystem integrity over time. This may be due to enhanced antioxidant enzyme production induced by fungal endophytes, which has been reported to protect PSII from oxidative damage under drought stress (Miranda et al., 2023; Rehman et al., 2022). Under no-stress conditions, C and BB resulted in a significant decline in turf quality over time, while BE and BT maintained more stable visual quality. This suggests that microbial inoculants may have prevented chlorophyll degradation and maintained turf aesthetics longer (Heidari and Golpayegani, 2012).

4.1.3 Creeping bentgrass

Creeping bentgrass, being a cool-season grass, is more susceptible to heat stress (Pote et al., 2006). Under heat stress, BB significantly reduced photosynthetic efficiency after four product applications compared to BT, which may indicate negative microbial interactions or competition effects. This aligns with findings that combined inoculation could lead to microbial competition,

reducing overall plant benefits (Bashan et al., 2014). BE and BB significantly improved photosynthetic efficiency from the fourth to the fifth application, which could indicate that, over time, fungal inoculation enhanced photoprotection mechanisms such as the synthesis of antioxidants and enzymes (Pinnola and Bassi, 2018). However, the difference was not significant from the control and from BT at any collection time. Electrolyte leakage analysis revealed that BT led to the lowest membrane damage percentage, significantly lower than BB, indicating that bacterial inoculation may enhance membrane stability through antioxidant regulation and osmoprotection. This is consistent with studies reporting that *Bacillus* spp. can upregulate stress-responsive genes involved in membrane stability (Khan et al., 2020). However, BT was not significantly different from C and BE.

Under no-stress condition, the initial boost and following decline of chlorophyll and carotenoids content brought about by BT and BB, might be due to an early stimulation of pigment synthesis followed by degradation as microbial interactions shifted metabolic priorities. These results are in line with the chlorophyll and carotenoid content trend under heat stress condition; suggesting that *Bacillus* spp. might have helped maintain pigment at an initial stability by reducing chlorophyll degradation pathways (Radhakrishnan et al., 2017).

4.2 Endophyte colonization

None of the endophytes isolated from the root matched any of the isolates from the microbial products, suggesting the failure of the microbes in the applied product to colonize the turf root. This might account for absence of significant treatment effects. The foliar application might not be an effective way to achieve root colonization. Microbial colonization of plant roots depends on multiple factors, including the application method, microbial strain compatibility,

environmental conditions, and plant-microbe interactions (Compant et al., 2010). Through foliar application, microorganisms face multiple barriers to achieve root colonization. They have to initially survive on the leaf surface and subsequently migrate to the rhizosphere (Rastogi et al., 2013). With direct soil inoculation or seed coating, the inoculants make direct contact with the seeds or plant roots. Moreover, foliar application can subject the inoculants to competition against the phyllosphere microbial community (Vacheron et al., 2013). The lack of endophytic recovery of the inoculated microbes may indicate that the applied strains did not survive on the leaf surface long enough to colonize root tissues successfully or they were able to colonize only the leaves tissues.

4.3 Germination assay

Seed coating has been shown to be a promising application method for inoculants to improve plant response to abiotic stress. In particular, the application of *Trichoderma harzianum* on seeds grown on soil substrate under heat stress has shown to be effective. Previous studies have reported the effectiveness of *T. harzianum* as a biological seed treatment under stress conditions. Yildirim et al. (2006) reported the amelioration of plant growth parameters under salinity stress when *Cucurbita pepo* plant were treated with the fungi. Moreover, Mastouri et al. (2010) show that the application of *T. harzianum* under different biotic and abiotic stress conditions, positively affected plant response; while no effect was reported with no-stress, suggesting a relationship between the plant and the fungus that helped tolerate the stress. These observations align with the results of the present study. A previous study has shown that *Bacillus spp.* consortium used as seed coating treatment positively affects the plant growth parameters in *Pisum sativum* (Raza et al., 2024). However, the product with bacterial inoculant used in our study did not significantly affect

germination compared to the control. The explanation for this result might lie in the difference in particle sizes of the powders that were used as carriers of the microbial products. The carrier powder in the bacterial product had bigger particle sizes than the fungal product carrier. As such, the bigger sized particles in the bacterial products might not have allowed bacterial cells to adhere to the seeds properly. Raza et al. (2024) used liquid suspensions of the inoculants for the seed coating. The PSA results showed that the BioTango™ powder contained particles of 137.9µm (mean value). To ensure uniform distribution of the microbial cells on the surface of the seeds, Afzal et al. (2020) reported that the particle sizes in the carrier powder have to be less than 75 µm.

Interestingly, significant effects derived by the microbial inoculants under heat stress were observed only in seeds grown on soil substrate, while no significant effect when germinated on paper. These results may indicate a biostimulant interaction between the inoculated microorganism and the soil system, as suggested by Amirkhani et al. (2016), who reported that seed coating enhanced the nitrogen uptake by the roots at a higher level than the initial nitrogen introduced with the coating treatment.

5. Conclusions

The present study evaluated the use of microbial inoculants for improving turfgrass resilience under drought or heat stress while also underscoring the complexity of plant-microbe interactions in different turf species. The results from the greenhouse study indicated that microbial treatments did not provide consistent benefits across all species and conditions. Even though BT contributed to better membrane protection in creeping bentgrass under heat stress compared to the combined application BB, no differences were found compared to C and BE. Moreover, the effects on bermudagrass and zoysiagrass were more variable and time-dependent, suggesting that

microbial inoculants may be most effective when tailored to species-specific stress responses rather than applied as a universal solution. Moreover, the failure of root colonization by the introduced inoculants might explain for the absence of significant impact of the inoculants on turfgrass. The results from the germination assay indicated that the mode of product application might have played a very important role when it came to root colonization by the inoculants.

Further research is needed to explore plant-microbe interactions and optimize application methods and timing in the greenhouse. Moreover, the long-term benefits of microbial treatments in turfgrass management in real-world conditions need to be assessed and examined. Integrating microbial inoculants as a sustainable turfgrass management strategy could reduce reliance on chemical inputs and irrigation while enhancing turfgrass performance in increasingly challenging environmental conditions.

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Table 3.1 Products information

Treatment	Trade name	Manufacturer	Product type	Type of microorganism	Strain(s)	Other ingredients (inactive)	Trial
BE	BioEnsure®	Adaptive Symbiotic Technologies	liquid	fungi	<i>Trichoderma harzianum</i>	Water (H ₂ O)	greenhouse
BT	BioTango™	Adaptive Symbiotic Technologies	powder	bacteria	<i>B. licheniformis</i> <i>B. megaterium</i> <i>B. pumilus</i> <i>B. subtilis</i> <i>B. amyloliquefaciens</i>	Dextrose powder	greenhouse and germination
BB	BioIQ®	Adaptive Symbiotic Technologies	powder	bacteria + fungi	<i>B. licheniformis</i> <i>B. megaterium</i> <i>B. pumilus</i> <i>B. subtilis</i> <i>B. amyloliquefaciens</i> <i>T. harzianum</i>	Dextrose powder Talc powder	germination*
BE-FP	BioEnsure®FP	Adaptive Symbiotic Technologies	flowable powder	fungi	<i>Trichoderma harzianum</i>	Talc powder	germination

*treatment BB for the greenhouse study was obtained by mixing BioEnsure® and BioTango™.

Table 3.2 Sequence similarities of the isolates against known bacterial strains based on their partial 16S (bacteria) and 18S (fungi) rDNA sequence data.

Isolates ID	Bacterial strain with which isolate had the maximum sequence similarity	% similarity	Source
1B	<i>Caballeronia mineralivorans</i> strain NJ-XFW-1-B	90	B ^a
2B	<i>Bacillus cereus</i> strain IARI-A-8	99	B
3B; 8B	<i>Pseudomonas</i> sp. strain B4C38_PSIA_2_14	99; 99	B; B
4B; 16B; 17B; 18B; 22B; 23B; 24B; 25B; 26B	<i>Paenibacillus alvei</i> strain LT431	99; 99; 99; 99; 99; 99; 99; 99; 99	B; Z ^b ; Z; Z; Z; Z; Z; Z; Z
5B; 6B	<i>Janthinobacterium</i> sp. strain M169	99; 99	B; B
7B	<i>Bacillus cereus</i> strain MSK	99	B
9B	<i>Paenarthrobacter</i> sp. strain WCUF-2Pae	99	B
10B	<i>Pseudomonas fluorescens</i> strain 14f	83	B
11B; 12B	<i>Pseudomonas fluorescens</i> strain AFS029165	99; 99	B; B
13B	<i>Bacillus thuringiensis</i> strain p93_F04	99	Z
14B	<i>Pseudomonas</i> sp. strain MB71901	92	Z
15B	<i>Paenibacillus alvei</i> strain MRFV4	93	Z
19B; 20B	<i>Burkholderia</i> sp. strain DIV109	99; 99	Z; Z
21B	<i>Paraburkholderia polaris</i> strain RP-4-7	99	Z
Fungal strain with which isolate had the maximum sequence similarity			
1F; 2F; 3F; 4F; 5F; 6F; 9F; 10F; 11F; 14F; 16F	<i>Microdochium nivale</i> strain MAFF 236681	99; 99; 99; 99; 99; 99; 99; 99; 99; 99; 99	B; B; B; B; B; B; B; B; B; B; B
7F	<i>Penicillium</i> sp. strain ELF-4	99	B
8F; 12F	<i>Scolecobasidium ramosum</i> strain UTHSC 12-1082	100; 99	B; B
13F	<i>Discostroma fuscillum</i>	99	B
15F	<i>Mortierella</i> sp. strain A38	99	B
17F; 18F	<i>Fusarium proliferatum</i> isolate pk3	100; 100	Z; Z
19F; 22F; 24F	<i>Fusarium fujikuroi</i> isolate PK4	100; 99; 100	Z; Z; Z
20F	No significant similarity was found		Z
21F	<i>Fusarium oxysporum</i> isolate NN1	100	Z
23F	<i>Penicillium</i> sp. strain ELF-4	99	Z
25F	No significant similarity was found		Z

^aIsolates from the roots of creeping bentgrass; ^bIsolates from the roots of zoysiagrass

Table 3.3 Mean particle sizes of product powder carriers¹.

Treatment	D₁₀	D₅₀	D₉₀	Mean size
	µm	µm	µm	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

¹Values are expressed in means ± standard deviation. D10, D50, and D90 identify diameter distributions obtained at 10%, 50%, and 90% cumulative percentile volumes, respectively.

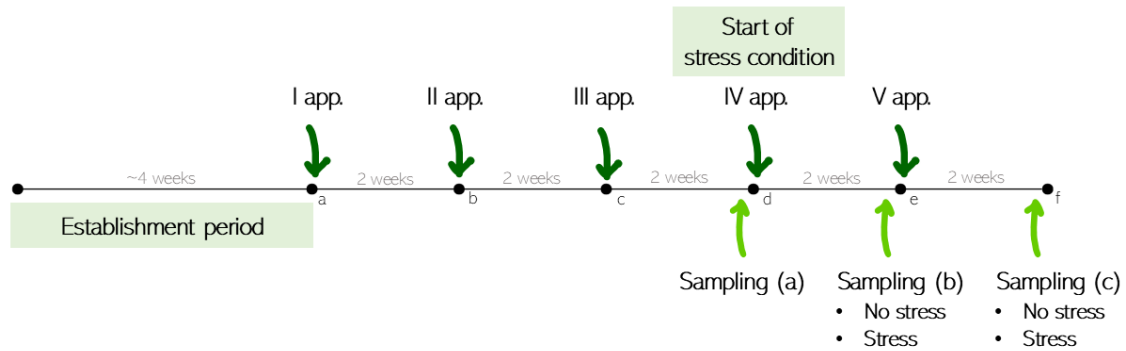


Figure 3.1 Greenhouse trial timeline

^aI application: 4/8/2024 (zoysiagrass); 6/12/2024 (creeping bentgrass); 7/8/2024 (bermudagrass)

^bII application: 4/22/2024 (zoysiagrass); 6/26/2024 (creeping bentgrass); 7/22/2024 (bermudagrass)

^cIII application: 5/6/2024 (zoysiagrass); 7/10/2024 (creeping bentgrass); 8/5/2024 (bermudagrass)

^dIV application and sampling (a): 5/20/2024 (zoysiagrass); 7/24/2024 (creeping bentgrass); 8/19/2024 (bermudagrass)

^eV application and sampling (b): 6/3/2024 (zoysiagrass); 8/7/2024 (creeping bentgrass); 9/2/2024 (bermudagrass)

^fSampling (c): 6/17/2024 (zoysiagrass); 8/21/2024 (creeping bentgrass); 9/16/2024 (bermudagrass)

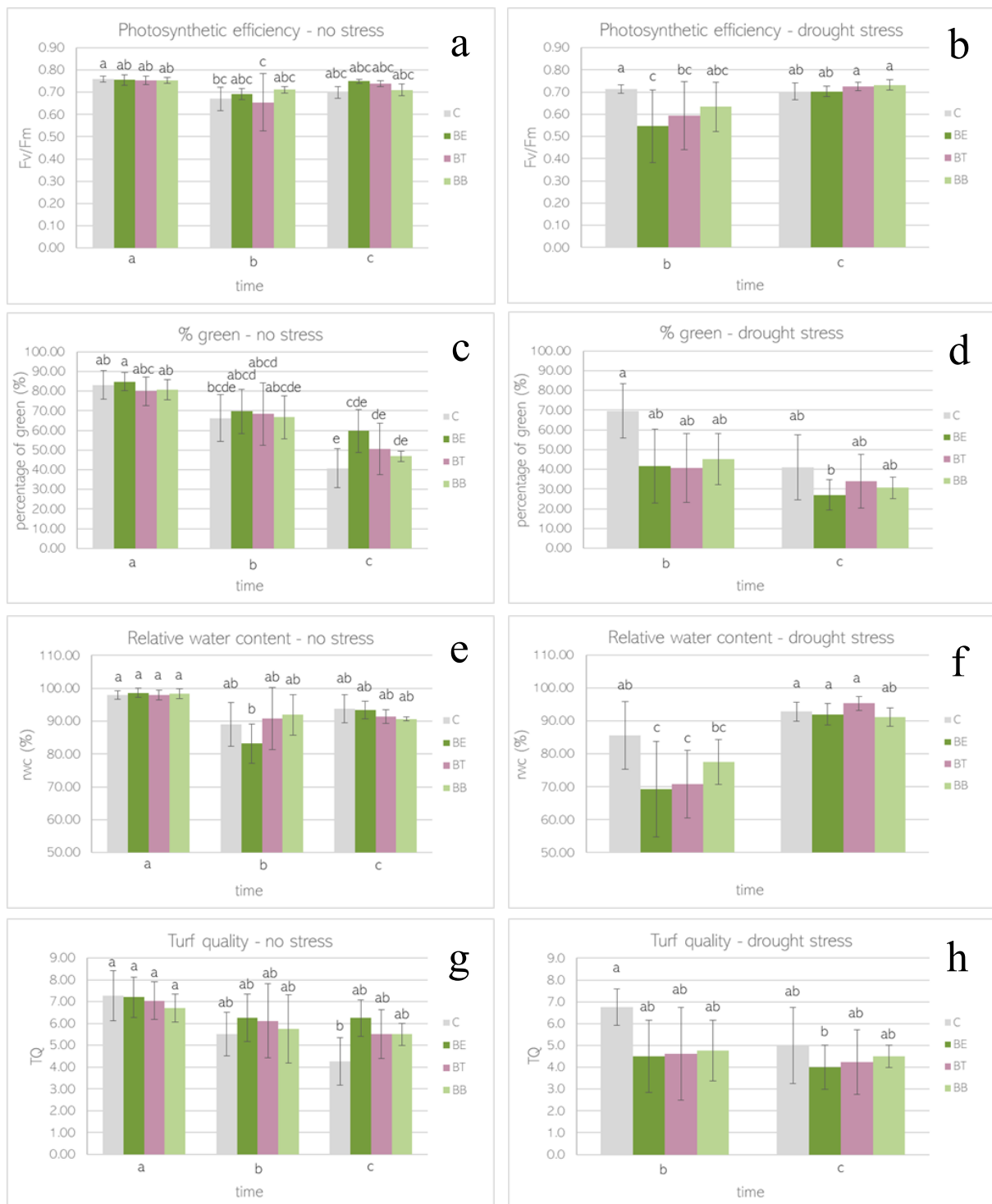


Figure 3.2 Photosynthetic efficiency (a, b), green percentage (c, d), relative water content (e, f) and turf quality (g, h) of bermudagrass under standard condition and drought stress. Statistically significant differences are expressed by different letters (p-value ≤ 0.05). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

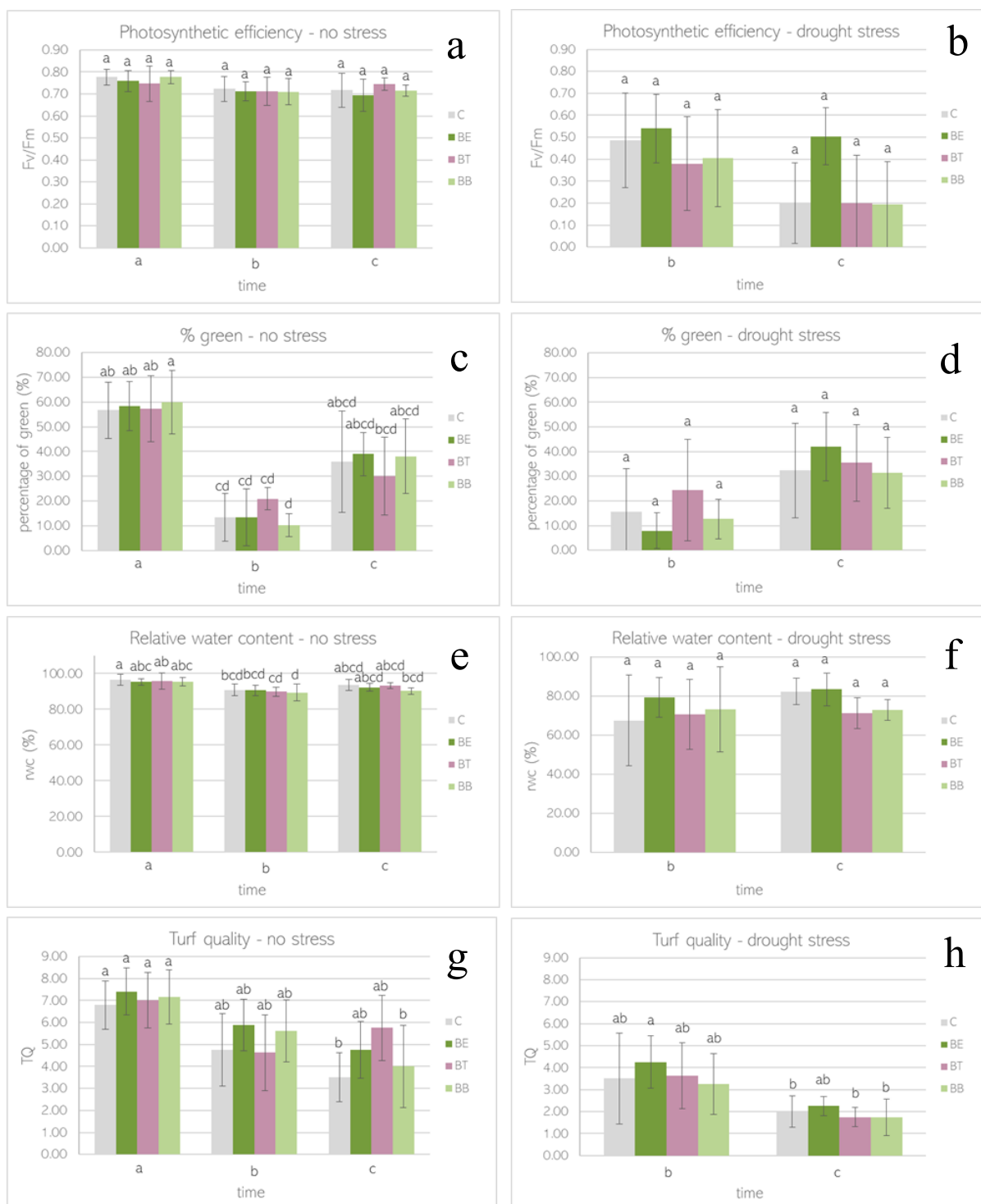


Figure 3.3 Photosynthetic efficiency (a, b), green percentage (c, d), relative water content (e, f) and turf quality (g, h) of zoysiagrass under standard condition and drought stress. Statistically significant differences are expressed by different letters (p -value ≤ 0.05). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

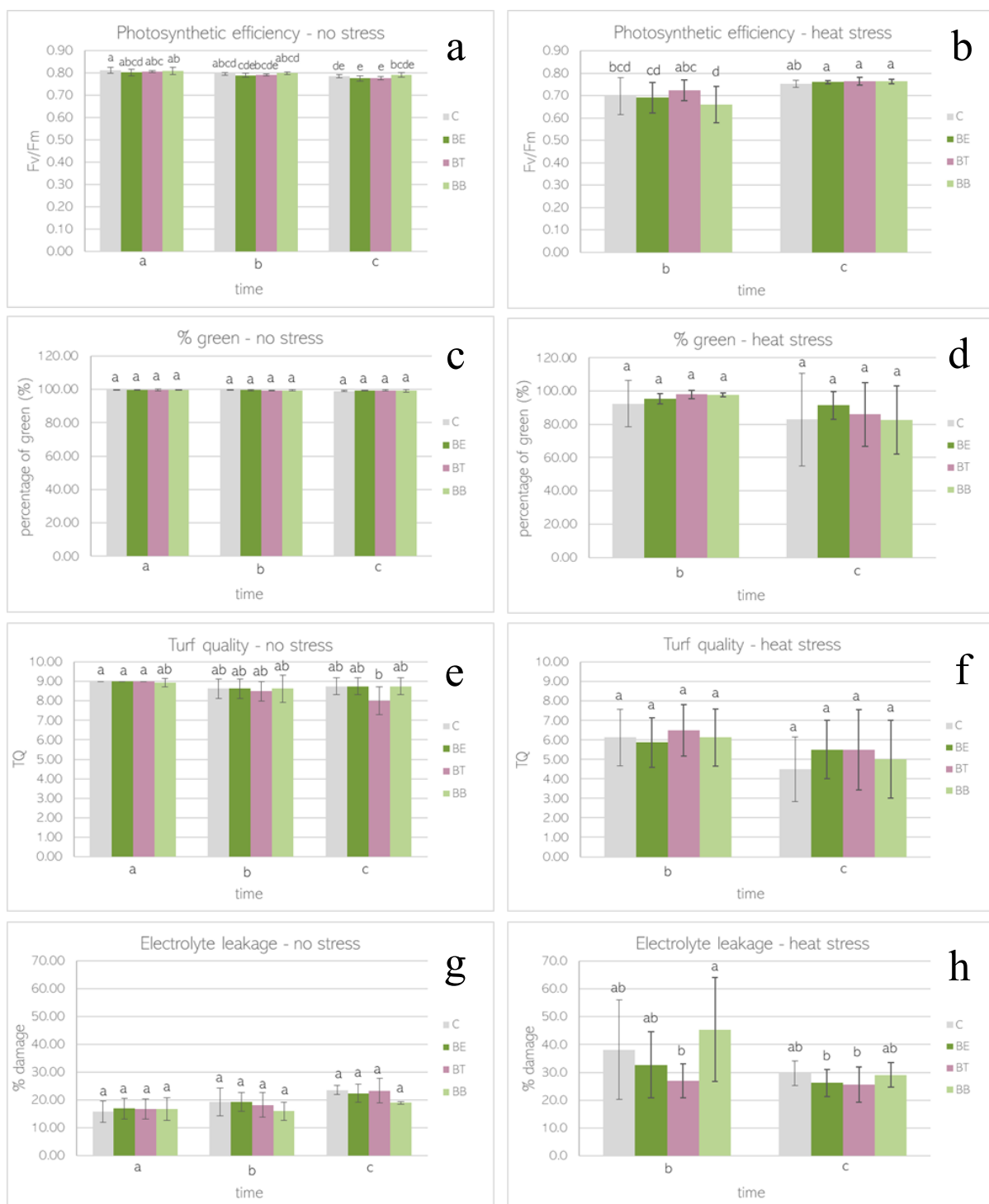


Figure 3.4 Photosynthetic efficiency (a, b), green percentage (c, d), turf quality (e, f) and electrolyte leakage (g, h) of creeping bentgrass under standard condition and heat stress. Statistically significant differences are expressed by different letters (p-value ≤ 0.05). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

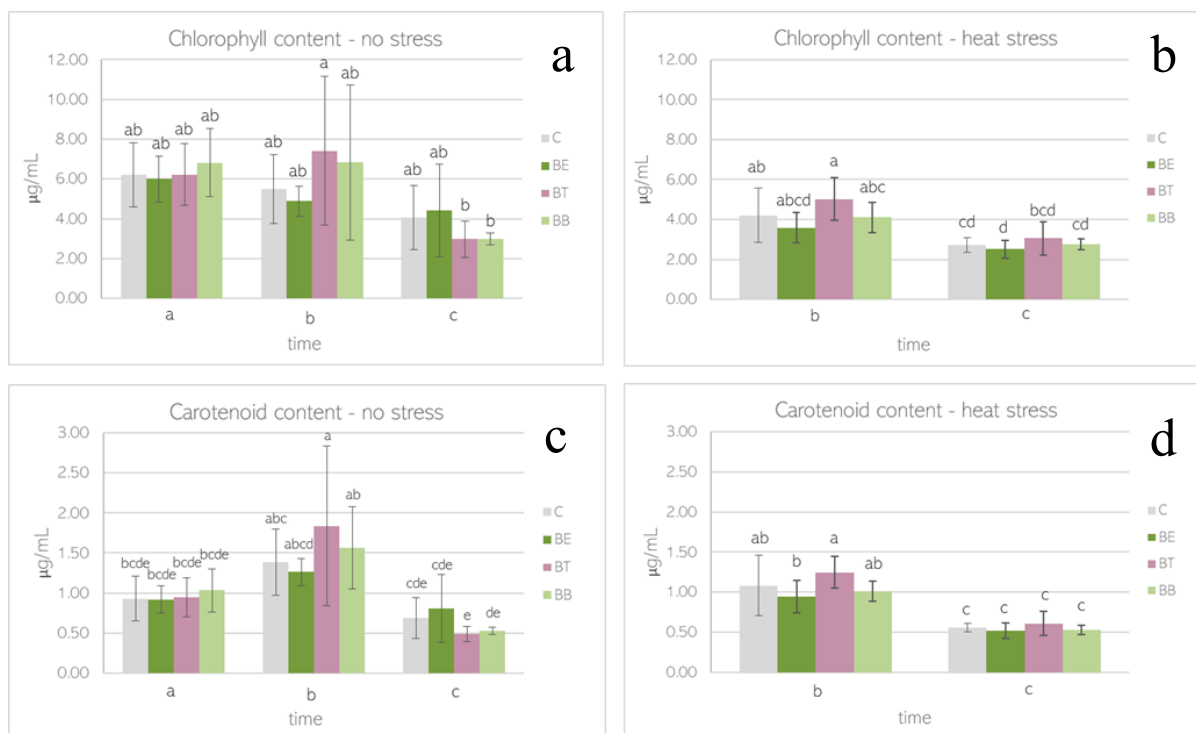


Figure 3.5 Chlorophyll content (a, b) and carotenoid content (c, d) in creeping bentgrass under standard condition and heat stress. Statistically significant differences are expressed by different letters (p -value ≤ 0.05). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

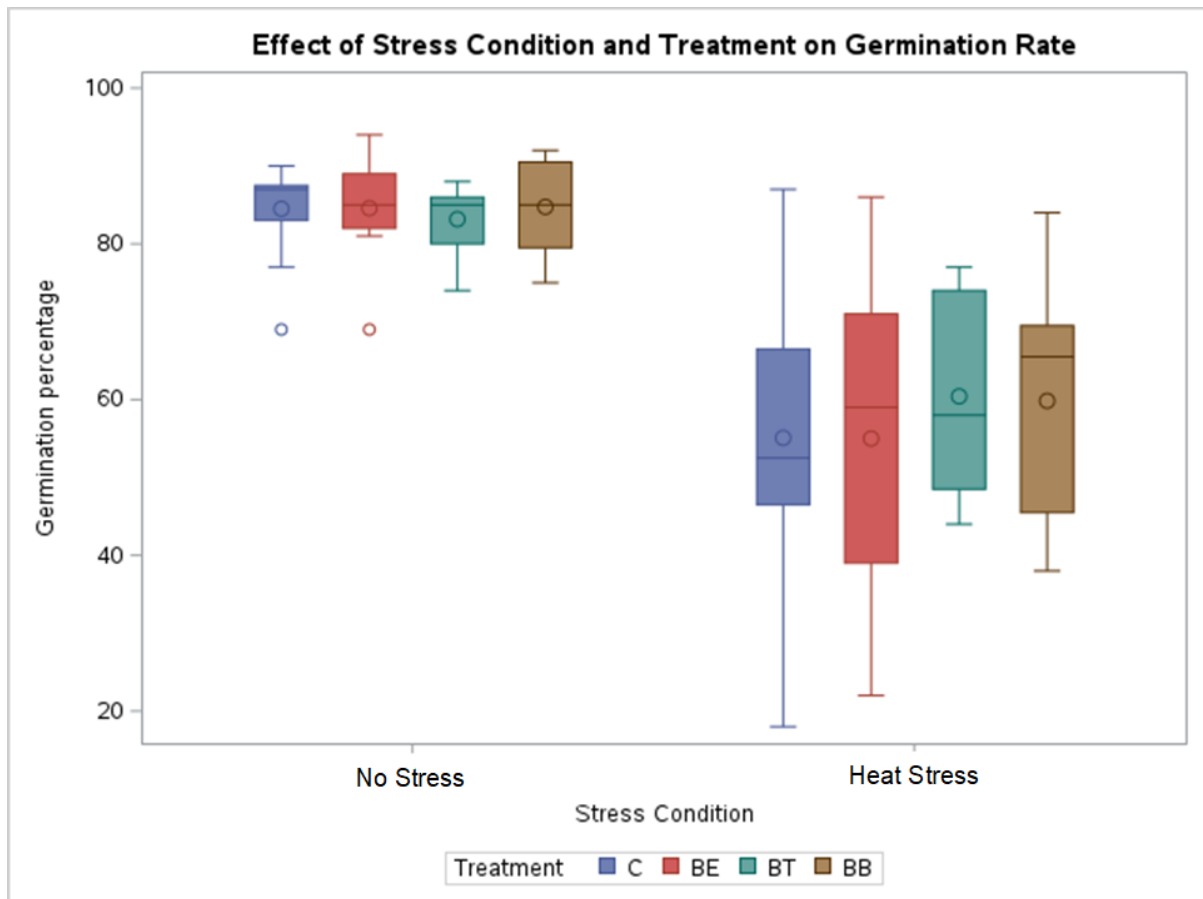


Figure 3.6 Germination rate of creeping bentgrass on filter paper at day 8. Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

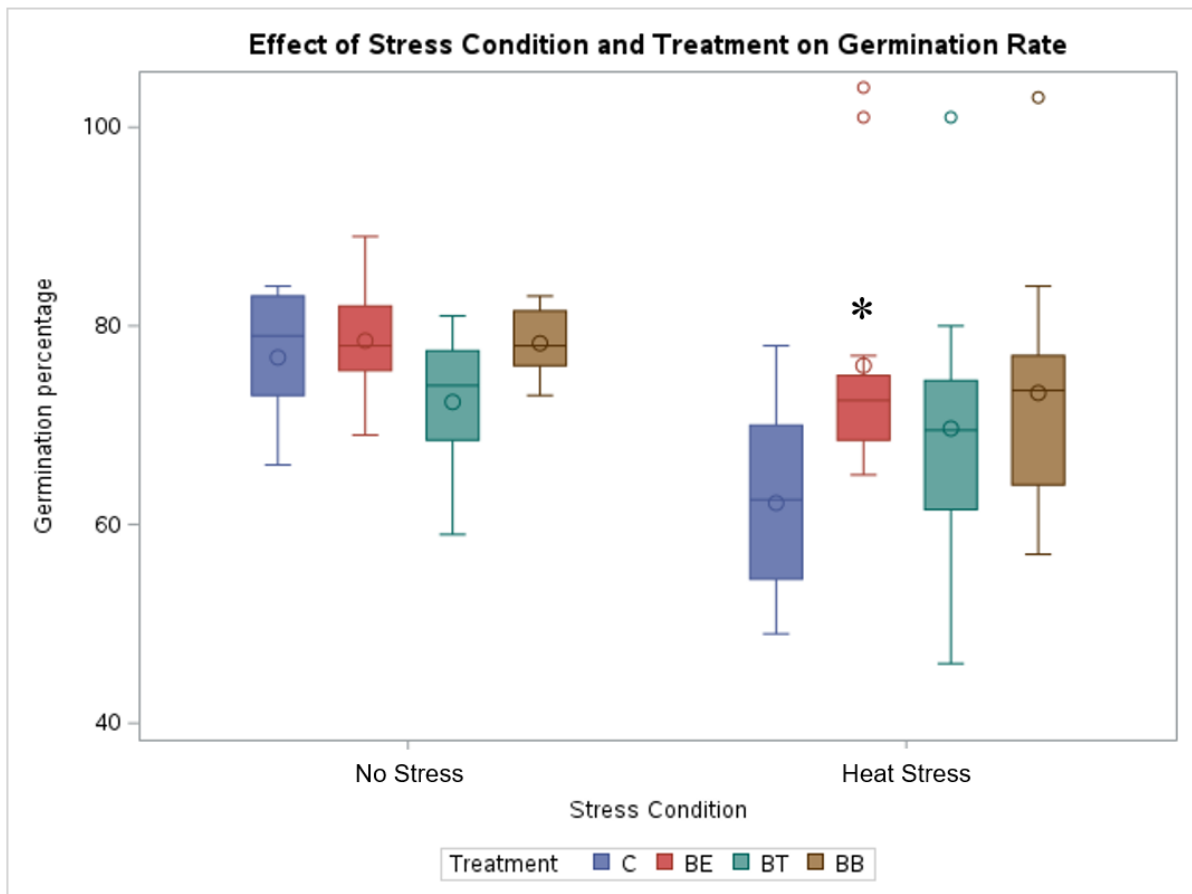


Figure 3.7 Germination rate of creeping bentgrass on soil at day 8. In comparison with the control (C), statistically significant differences are expressed by the symbol * ($p\text{-value} \leq 0.05$). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

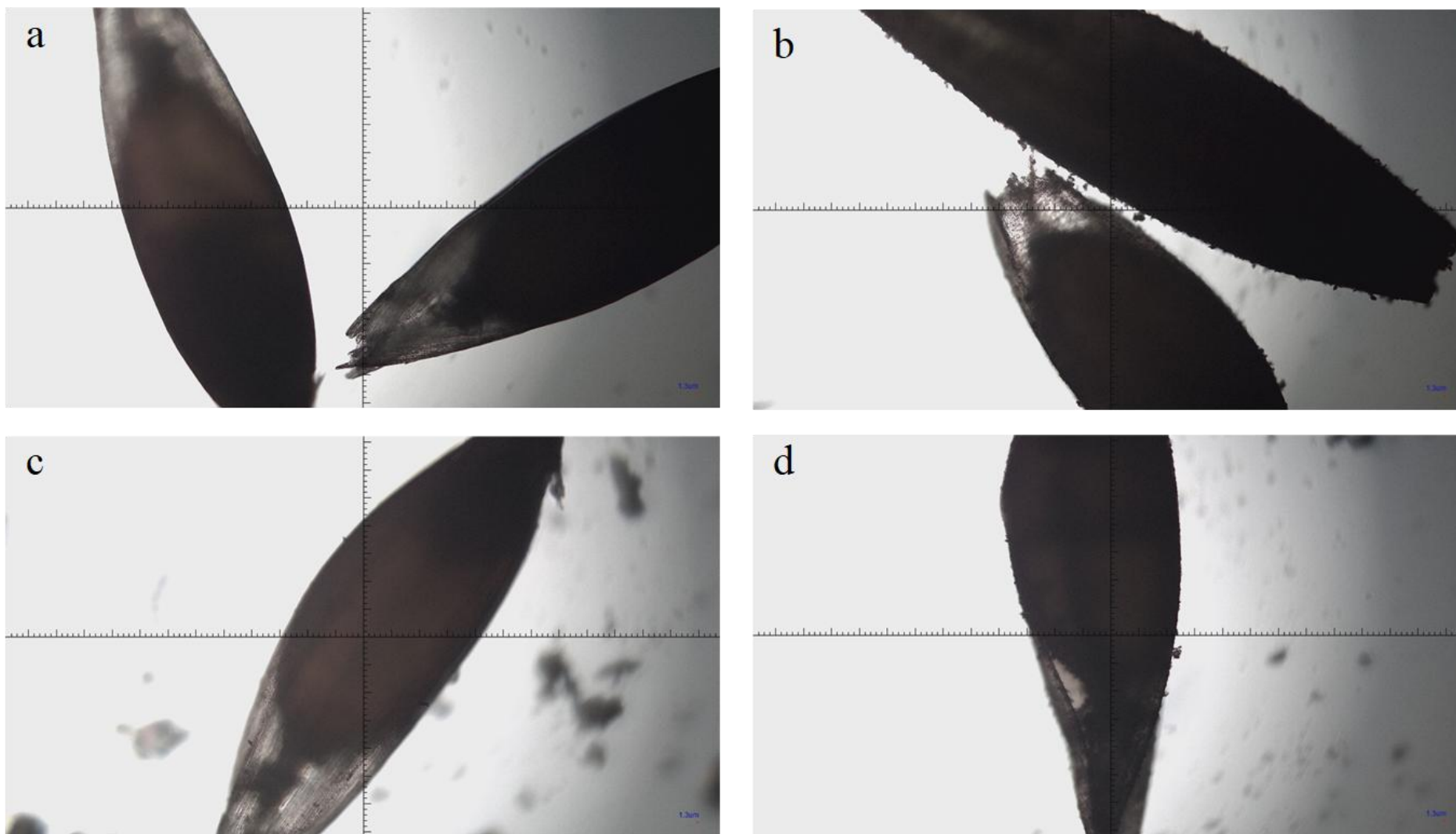


Figure 3.8 Coated seeds. Control (a), BioEnsure®FP (b), BioTango™ (c), BioEnsure®FP + BioTango™ (d). The pictures were taken with a Digital Microscope Camera (M4KHD2/M 4kHD) incorporated in a microscope (Olympus BX51) at 10X.

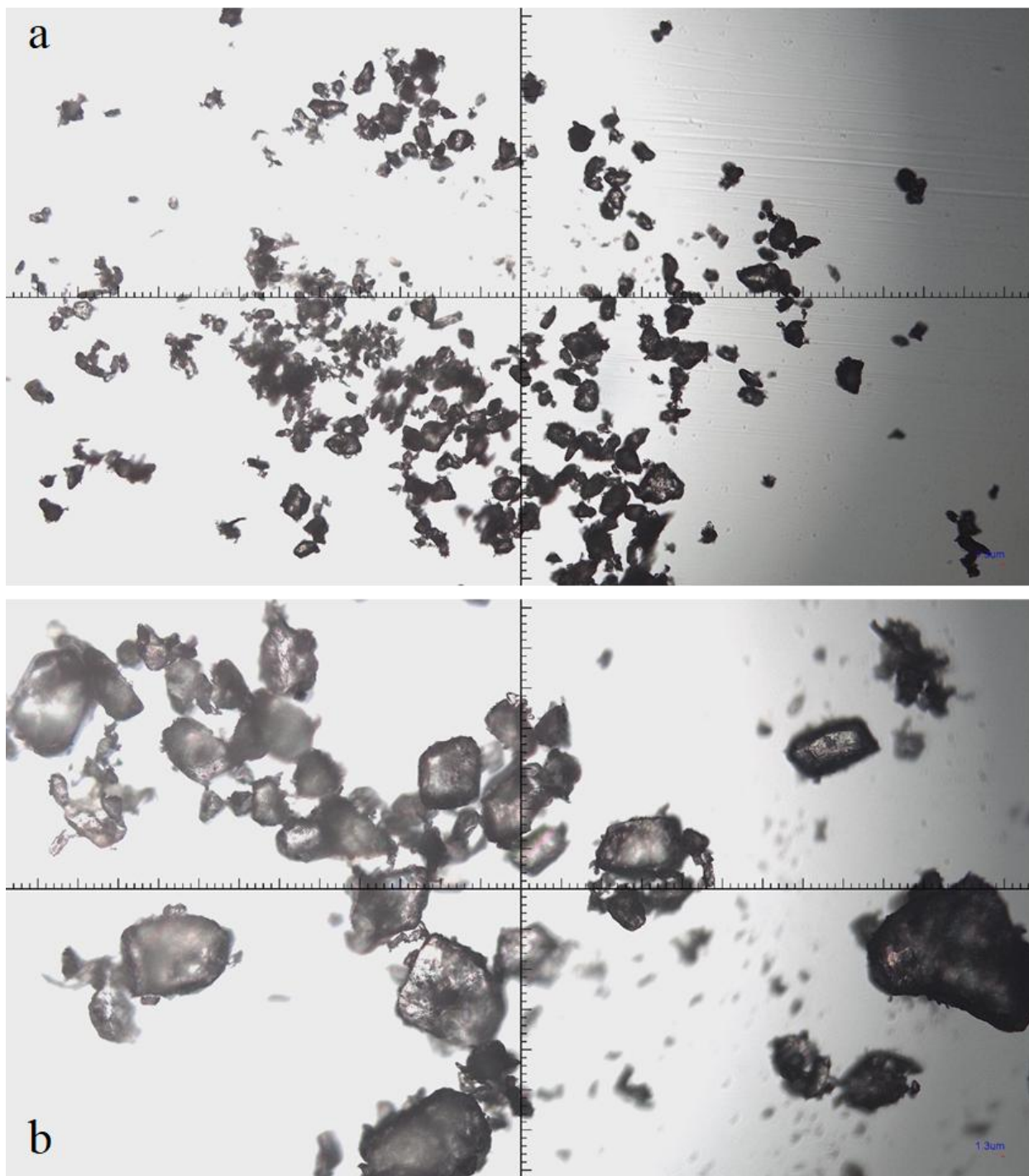


Figure 3.9 Coating powders BioEnsure®FP (a) and BioTango™ (b). The pictures were taken with a Digital Microscope Camera (M4KHD2/M 4kHD) incorporated in a microscope (Olympus BX51) at 10X.

CHAPTER 4

MICROBIAL INOCULANTS: EFFECTS ON SOIL BIOLOGICAL HEALTH²

² Chiara Ammaturo, Mussie Y. Habteselassie and David Jespersen. To be submitted to *Applied Soil Ecology*.

Abstract

Turfgrasses are highly susceptible to water and heat stresses that are being exacerbated by climate change. The use of microbial inoculants is often advocated to improve plant stress because of their potential impact on soil biological health among other factors. The present study evaluated the impact of two microbial products, BioEnsure® (BE) and BioTango™ (BT), applied individually or in combination (BB), on soil biological health under temperature and moisture stress in a greenhouse study. The study was performed on three turfgrass species: two warm-season – Bermudagrass (*Cynodon* spp.) and Zoysiagrass (*Zoysia* spp.) – and one cool-season – Creeping bentgrass (*Agrostis stolonifera*). Measurements of enzyme activities and soil respiration were used as ways of evaluating soil biological health. There was no significant treatment effect on urease activity; however, urease activity showed a tendency to increase after multiple applications of the products under drought stress. On the contrary, it decreased over time in the soil under heat stress. Phosphatase activity was significantly higher with BT compared to the other treatments after multiple applications under no stress conditions. BB showed higher soil respiration than C and BT under drought stress. The lack of significant results might lie in the failed colonization by the inoculants in the roots tissues, probably due to the application method (foliar spray) used in this study. Future studies are needed to determine product application method that would result in microbial colonization of the root system.

Keywords: microbial inoculants, BioEnsure®, BioTango™, soil respiration, urease, phosphatase, *Trichoderma harzianum*, *Bacillus* spp.

1. Introduction

Turfgrass plays a crucial role in providing aesthetic and environmental benefits in sports fields, golf courses, and urban green spaces (Christians et al., 2016). However, turfgrass species are frequently exposed to abiotic stresses such as heat and drought, which significantly impact their physiological performance, growth, and overall quality (Huang, 2008). The ability of turfgrass to withstand and recover from stress conditions is essential for maintaining high-performance turf systems. To address this problem, there has been an increasing interest in the use of microbial inoculants as a sustainable strategy to improve plant resilience against abiotic stresses (Bashan et al., 2014).

Microbial inoculants are often put as one category of plant biostimulants that are defined as inputs with the potential to improve nutrient efficiency, enhance tolerance to environmental stresses, and/or boost crop quality, without being significant sources of nutrients (Du Jardin, 2015). Unlike traditional fertilizers, these products do not serve as direct sources of nutrients but enhance plant performance through several mechanisms (Rouphael & Colla, 2020). One of such mechanisms is through their impact on soil biological health, which encompasses the role of soil microorganisms in nutrient cycling, organic matter decomposition and disease suppression (Doran and Zeiss, 2000). Soil biological health plays a very important role in maintaining key ecosystem services that support plant growth (Guggenberger, 2005; Patle et al., 2023). A well-functioning soil microbial community leads to improved soil structure, increased nutrient availability, and enhanced plant resilience to abiotic and biotic stresses (Diera et al., 2020). However, the effectiveness of microbial based products on soil biological health remains inconsistent, as their impact may depend on factors such as soil conditions, application methods, and their interactions with native microbial communities (Diera et al., 2020).

To assess changes in soil biological health, several key indicators are commonly used. Soil respiration is widely recognized as a measure of microbial activity and organic matter decomposition, providing insight into the overall metabolic activity of soil microorganisms (Zibilske, 1994). Additionally, enzyme activities, such as urease and phosphatase, are used to evaluate microbial contributions to nutrient cycling (Burns et al., 2013). Urease activity is an indicator of nitrogen cycling, as it catalyses the hydrolysis of urea into ammonia, which can be utilized by plants (Diera et al., 2020). Phosphatase activity reflects phosphorus mineralization and availability, playing a critical role in plant nutrient acquisition (Nannipieri et al., 2018). These parameters provide a comprehensive assessment of how microbial inoculants influence soil functions and nutrient dynamics, and, in turn how they affect plant growth.

The objective of this study was to evaluate the impact of two microbial products on soil biological health under temperature or moisture stresses in warm and cold season grasses in a greenhouse study. Soil respiration and two enzyme activities (urease and phosphatase) were used for assessing changes in soil biological health in response to the products.

2. Materials and Methods

2.1 Experimental set-up

For the detailed experimental set-up of the study, see Chapter 3, section 2.1.

2.2 Measurements of Soil Biological Health Parameters

2.2.1 Sample collection and preparation

Soil samples were destructively collected from the pots during the three sample collection times (a, b, and c). For the first sampling (a), the collected plants were not subjected to stress, as

shown in Figure 3.1. Sampling (b) and (c) consisted of collecting samples from the no-stress and stress conditions. In order to determine changes in soil biological health, soil respiration and enzyme activities were monitored. Before the soil analysis, each soil sample was sieved through a 2 mm sieve. The sieved soil was then stored in sterile plastic bags at 4°C for not more than a few weeks before analysis. The refrigerated samples were equilibrated to room temperature and mixed thoroughly to homogenize the sample before analysis. Soil moisture content was measured by oven drying 10 g of fresh soil at 100°C for 24h.

2.2.2 Soil respiration

Soil respiration is the process of organic matter decomposition by microorganisms and is a good indicator of the overall microbial activity (Wang et al., 2003). Soil respiration was measured by incubating 20 g of soil sample in a glass Mason jar (total volume 950 ml), tightly closed with a lid, in an incubator (VWR, Inc.) at 25°C for 24 hours. After incubation, a syringe was inserted into the jar through a rubber septum at the center of the lid. Ten milliliters of air were collected from the headspace of the jar and was injected into a CO₂ Gas Analyzer (EGM-5, PP Systems). The output was in ppm (part per million) and converted to mg of released CO₂/g of soil/d.

2.2.3 Enzyme activity

The two enzyme activities that were measured to gauge changes in microbial activity were urease and phosphatase activities that are involved in the transformations of nitrogen and phosphorus in the soil. Methods described in Tabatabai (1994) and Wallenstein and Weintraub (2008) were followed. Microorganisms are largely responsible for producing these enzymes, and their activity is indirect indicators of microbial activity.

2.2.3.1 Urease activity

Two bi-plate Petri dishes were used for each soil sample; one for control and the other for treatment. 1 g of soil was placed in one of the sections of the treatment bi-plate. In the same section, 3 ml of Tris-maleate buffer ([0.5 M], pH 7.0) was added alongside 1 ml of urea solution [6 M] (or 1 ml of distilled water in the control plate). In the other section, 3 ml of boric acid [2%] indicator was added. The Petri dishes were covered with the lid and left for incubation for 1 h at room temperature. After the incubation time, 0.5 ml of silver sulfate [10 mM] and 1 ml of potassium carbonate [3 M] were added to allow the release of ammonia produced by microorganisms. The plates were closed and put in zip lock bags and left for incubation at room temperature for 24 h. The boric acid-indicator solution was then titrated with HCl [0.02 M], and the volume of hydrochloric acid was noted. The amount of ammonia released was expressed as $\mu\text{mol/g soil/h}$.

2.2.3.2 Phosphatase activity

Two dark centrifuge tubes (15 ml total volume) were used for each sample, one for control and one for treatment. In each tube, 1 g of soil was added alongside 4 ml of Tris-maleate buffer ([0.5 M], pH 7.0). 1 ml of p-nitrophenylphosphate [100 mM] was added to the treatment tubes. The incubation was initiated for all the tubes (treatment and control) on a shaking rack for 1 h at room temperature. After the incubation, 1 ml of p-nitrophenylphosphate [100 mM] was added to the control tubes. Subsequently, the reaction was immediately terminated by adding 1 ml of calcium chloride [0.5 M] and 4 ml of sodium hydroxide [0.5 M] to both tubes. The tubes were centrifuged at 10,000 rpm (12,360 X g) at 4° C for 10 minutes (VWR, Inc.). In a 96-well plate, a series of standard solutions in Tris-malate buffer were prepared at different concentrations (10 μM , 5.0 μM , 3.0 μM , 2.0 μM , 1.0 μM , 0 μM). 300 μL from each centrifuge tube was transferred to

each well. The absorbance was read in a plate reader (Epoch 2, Bio Tek Instrument, Inc.) at 400 nm. After preparing the standard curve, sample absorbance readings were converted to concentration unit and phosphatase activity was expressed in $\mu\text{mol/ g soil/ h}$.

2.3 Statistical analysis

Analysis of variance (ANOVA) was carried out to investigate the statistical significance ($p \leq 0.05$) of the effects of the different microbial inoculants' treatments and the control on soil biological health indicators. When significant differences were observed among treatments, a post-hoc analysis using the Tukey test was done to identify which treatment groups were significantly different. The statistical analysis was performed using SAS (SAS on Demands for Academics, Cary, NC, USA).

3. Results

3.1 Soil biological health parameters

3.1.1 Soil respiration

Soil respiration did not show any significant differences among treatments or sampling times under drought stress and no-stress conditions in bermudagrass (Figures 4.1a and b). In zoysiagrass, under no-stress condition, Soil respiration significantly decreased in C from time a to time c while BE, BT, and BB were stable throughout the trial (Figure 4.1c). Under drought stress, no differences between treatments were found at time b; however, at time c, BB showed a significant improvement in soil respiration compared to C and BT (Figure 4.1d). In creeping bentgrass' soil under no-stress conditions, soil respiration increased significantly from time a to time b, with control (C) and BE treatments. However, from time b to time c, all inoculated

treatments (BE, BT, BB) exhibited a significant decline in soil respiration, whereas the control remained stable (Figure 4.1e). Under heat stress, no significant differences were found among treatments at any time; however, C significantly decreased from time b to time c (Figure 4.1f).

3.1.2 Urease activity

No significant differences were found among the four treatments in soil urease activity with no stress or stress conditions in bermudagrass (Figure 4.2a). In zoysiagrass soil, no significant differences were found among treatments at any times and any moisture conditions. However, under no-stress, when variations of the parameter over time were observed, treatments BB significantly improved urease activity from time a to time b and with BT the activity of this enzyme was significantly enhanced from time a to time c. No differences were found over time for C and BE (Figure 4.2c). Under drought stress, no significant differences were found between treatments; however, C, BT and BB significantly improved urease from time b to time c (Figure 4.2d). In creeping bentgrass soil, no differences were found among treatments under no-stress condition; however, all treatments significantly reduced urease activity at time c (Figure 4.2e). Under heat stress, at time b, BB led to a significant reduction in urease activity compared to C. No differences were reported between BE and BT in comparison to C or BB. Even though all treatments significantly declined, no differences among treatments were reported at time c (Figure 4.2f).

3.1.3 Phosphatase activity

There were no significant differences among treatments in bermudagrass at any collection times or moisture conditions (Figure 4.3a and b). In the zoysiagrass soil, no significant differences were found among treatments at any collection time under both moisture conditions. However, the

phosphatase activity significantly increases with no-stress from time b to time c with BE and BB (Figure 4.3c). Under drought stress, the increase from time b to time c was significant for all treatments (Figure 4.3d).

In creeping bentgrass soil, under no-stress, no differences were found among treatments at time a and time b; however, at time c, BT resulted in a significantly higher phosphatase activity than the rest of the treatments. Additionally, C significantly declined from time b to time c (Figure 4.3e). Under heat stress conditions, no significant differences were found between treatments and collection times (Figure 4.3f).

4. Discussion

The results of the present study provide critical insights into the mechanisms underlying the turfgrass growth responses observed in the previous chapter. The interaction between soil microbial activity and turfgrass stress tolerance highly depends on microbial establishment, plant species, and environmental conditions (Mendes et al., 2013; Berg et al., 2016). Soil respiration and enzyme activities are key indicators of microbial activity, nutrient cycling, and organic matter decomposition, which directly influence plant growth and stress resilience (van der Heijden et al., 2008; Singh et al., 2018). The findings suggest that microbial inoculants impact turfgrass physiology by modifying soil microbial processes, affecting nutrient availability and stress responses.

4.1 Soil respiration

Soil respiration reflects microbial metabolic activity and carbon turnover, influencing nutrient cycling and root exudate dynamics (Paterson et al., 2007). Sanaullah et al. (2011) analysed the effects of drought stress on microbial biomass carbon (MBC) and enzyme activity in the

rhizosphere of the two grasses, *Lolium perenne* and *Festuca arundinacea*. While the drought stress had no effects on MBC in the case of *L. perenne*, an increase of 36% was registered in MBC in soils with *F. arundinacea*. Other studies have reported that the changes in soil microbial activity are closely related to the turf growth parameters such as photosynthesis and relative water content (Hartman & Tringe, 2019). In zoysiagrass, BB treatment improved soil respiration under drought stress after five applications compared to C and BT, suggesting that the combination of fungal (*Trichoderma harzianum*) and bacterial (*Bacillus* spp.) inoculants had a synergistic effect leading to higher microbial respiration. This enhancement in soil respiration is likely due to increased microbial activity, as both fungal and bacterial inoculants contribute to organic matter decomposition, root exudation, and nutrient cycling (Nannipieri et al., 2018). *Trichoderma harzianum* is known to promote plant growth by enhancing root development and increasing the release of root exudates, which serve as a carbon source for soil microbes (Shoresh et al., 2010). Similarly, *Bacillus* spp. are recognized for their role in nitrogen fixation and phosphorus solubilization, further supporting microbial communities involved in respiration (Compant et al., 2010). However, this increase in soil respiration was not reflected in any of the turf growth parameters discussed in the previous chapter.

A study conducted by Dell et al. (2012) on creeping bentgrass, reported that, under high temperatures, the microbial biomass was reduced due to cell death and higher energy requirements that might have led to an increase in cellular storage material degradation. Figure 4.1f shows a similar situation for the creeping bentgrass soil community under heat stress. In fact, the microbial inoculation reduced soil respiration after the fourth application compared to the no-stress condition, which was not seen in the control treatment. This trend might be due to a competitive behavior of the inoculated microorganisms with the indigenous community already present in the

soil leading to a general reduction of the microbial abundance and, in turn, to a lower soil respiration. Moreover, this result is also in accordance with the results from the electrolyte leakage analysis discussed in the previous chapter, which showed that the percentage of cell membrane damage was higher with the combined application (BB).

4.2 Soil enzymatic activity

Soil enzyme activity serves as a proxy for microbial activity, with urease and phosphatase mediating N and P transformations (Burns et al., 2013). Enzyme activities correlate well with soil respiration (Steinweg et al., 2012) and complement each other as measures of soil biological health. Enzyme activities are responsive to soil moisture stress as ability of microbes to access substrates is limited because of lack of water for transport (Dijkstra and Cheng, 2007; Claassen and Steingrobe, 2024; Marschner and Rengel, 2023). Temperature is another important driver of soil enzyme activity in soils (Moyo et al., 1989; Steinweg et al., 2012). The results of the present study contrast the literature discussed above for drought stress conditions. In fact, even though the combined application of BioEnsure® and BioTango™ (BB) in zoysiagrass soil under drought stress was shown to improve soil respiration after multiple applications, the abundance of microbes did not significantly improve the enzymatic activity under drought stress and did not positively affect the turf growth parameters discussed in the previous chapter.

In bermudagrass, where microbial inoculants had a variable effect on RWC and turf quality, enzyme activity fluctuations may explain why microbial benefits were not consistently observed. The lack of significant increases in enzyme activity could indicate that microbial inoculants did not affect soil microbial activity enough to influence plant physiology consistently (Lemanceau et al., 2017; Mendes et al., 2013). Alternatively, native microbial communities in

bermudagrass soils may have competed with the introduced inoculants, limiting their effectiveness.

In creeping bentgrass, urease activity did not significantly differ between microbial treatments and the control, suggesting that inoculants did not directly influence nitrogen cycling in this study. The lack of increased urease activity could indicate that soil nitrogen availability was not a limiting factor, reducing the potential for microbial inoculants to induce significant changes (Paterson et al., 2009; Hartmann et al., 2009).

Riah-Anglet et al. (2015) state that under heat stress condition, the enzymatic activity in the soil is inhibited after prolonged incubation of the soil to 50°C. In contrast, the experiment conducted by Dell et al. (2012) on creeping bentgrass reported that the enzymatic activity significantly increased when temperatures changed from 12 to 22°C and from 22 to 34°C. In the present study, the phosphatase activity was lower under heat stress than no-stress condition in treatment C after four applications, while treatments BE, BT, BB helped keep the phosphatase activity stable under both conditions (Figure 4.3e and f). The improved chlorophyll retention and electrolyte leakage reduction observed in creeping bentgrass might explain the trends in phosphatase activity (Goswami et al., 2016; Ojuederie et al., 2019).

The observed increase in urease activity in the creeping bentgrass soil under temperature stress is consistent with findings by Koçak (2020), who reported that prolonged temperature exposure enhanced enzyme activity. However, in this study, microbial inoculants did not significantly amplify this effect, suggesting that native microbial populations may have already adapted to thermal stress, limiting additional benefits from external inoculation. A study by Bai et al. (2018) shows that soil warming increased urease activity by 29% and 9% in soils according to different warming times, respectively, less than two years and more than two years, respectively.

These statements are mirrored in the urease activity analysis of the creeping bentgrass soil under control treatment where, under temperature stress, the enzymatic activity increased by 30% compared to the no-stress condition. However, when microbial inoculants were applied, this increase was not observed (Figure 4.2e and f). Bai et al. (2018) also suggest that the increase in urease activity due to higher temperatures declines over time. Accordingly, Figures 4.2e and f show that at time c, the urease activity drops in both temperature conditions.

5. Summary and Conclusions

The present study evaluated the potential of microbial inoculants to enhance soil biological health. The bacterial BioTango™ resulted in significantly improved phosphatase activity compared to the other microbial treatments and the control under standard temperature conditions after multiple applications of the foliar spray treatment. Moreover, soil respiration was improved by the combined application (BB) in zoysiagrass under drought stress. These results reflect the possibility that microbial inoculants can improve soil health. The results from this soil study suggest that the effectiveness of microbial inoculants in turfgrass might be closely related to their ability to enhance soil biological health. Treatments that increased soil respiration and enzyme activity were more likely to show physiological improvements in turfgrass, particularly in creeping bentgrass under heat stress. Further research on the application of microbial inoculants to improve soil health should focus on understanding whether different methods of application of the product, frequency of application, and product dosage would affect the soil microbial community differently in order to maximize the efficacy of the application. In addition, exploring long-term effects and experimenting in a field environment would broaden the understanding of microbial inoculants' stability when interacting with a more complex system under real-world conditions.

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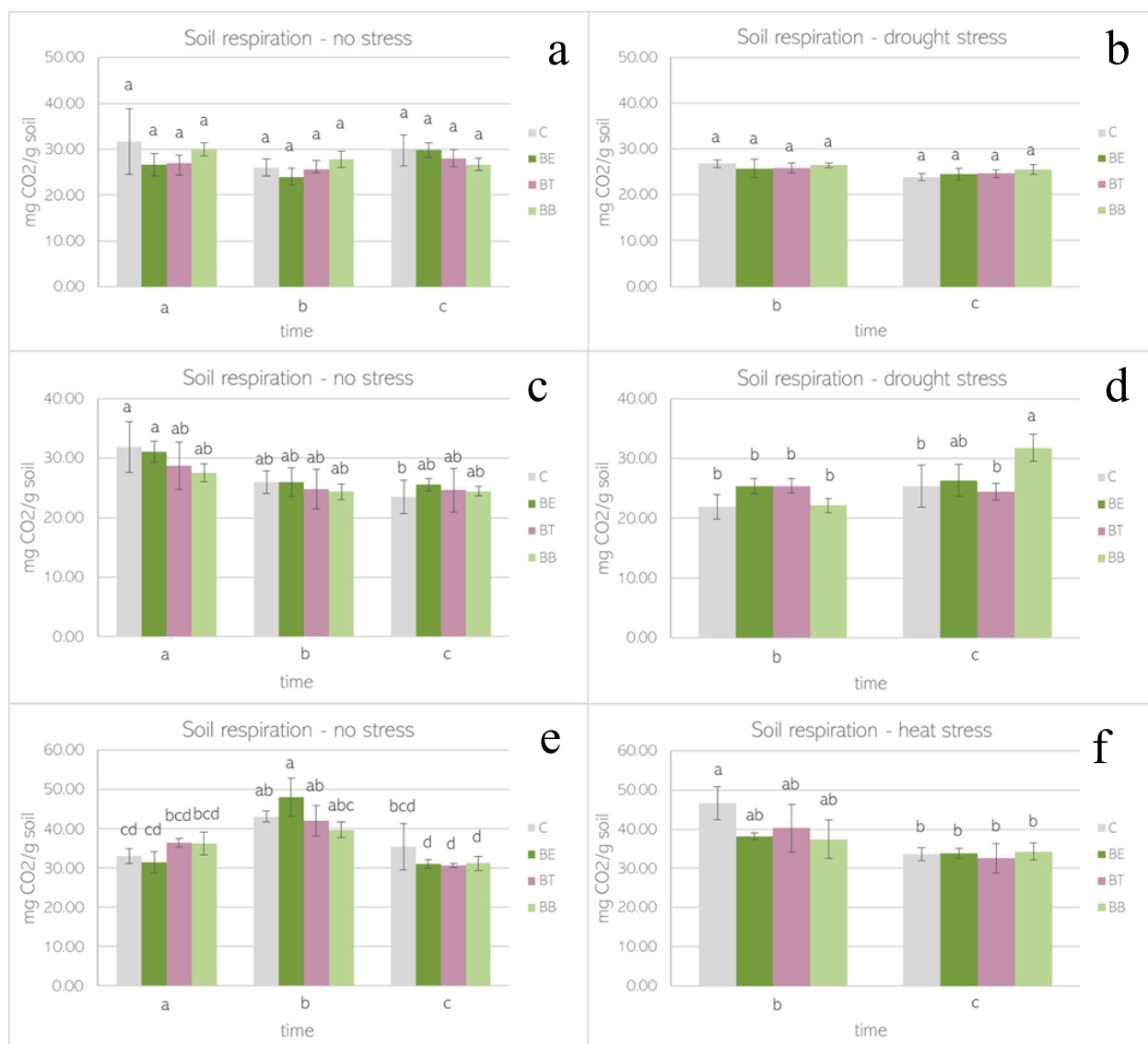


Figure 4.1 Soil respiration of bermudagrass greenhouse soil (a, b), zoysiagrass greenhouse soil (c, d) and creeping bentgrass greenhouse soil (e, f) under no stress and drought/heat stress condition. Statistically significant differences are expressed by different letters (p-value ≤ 0.05). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

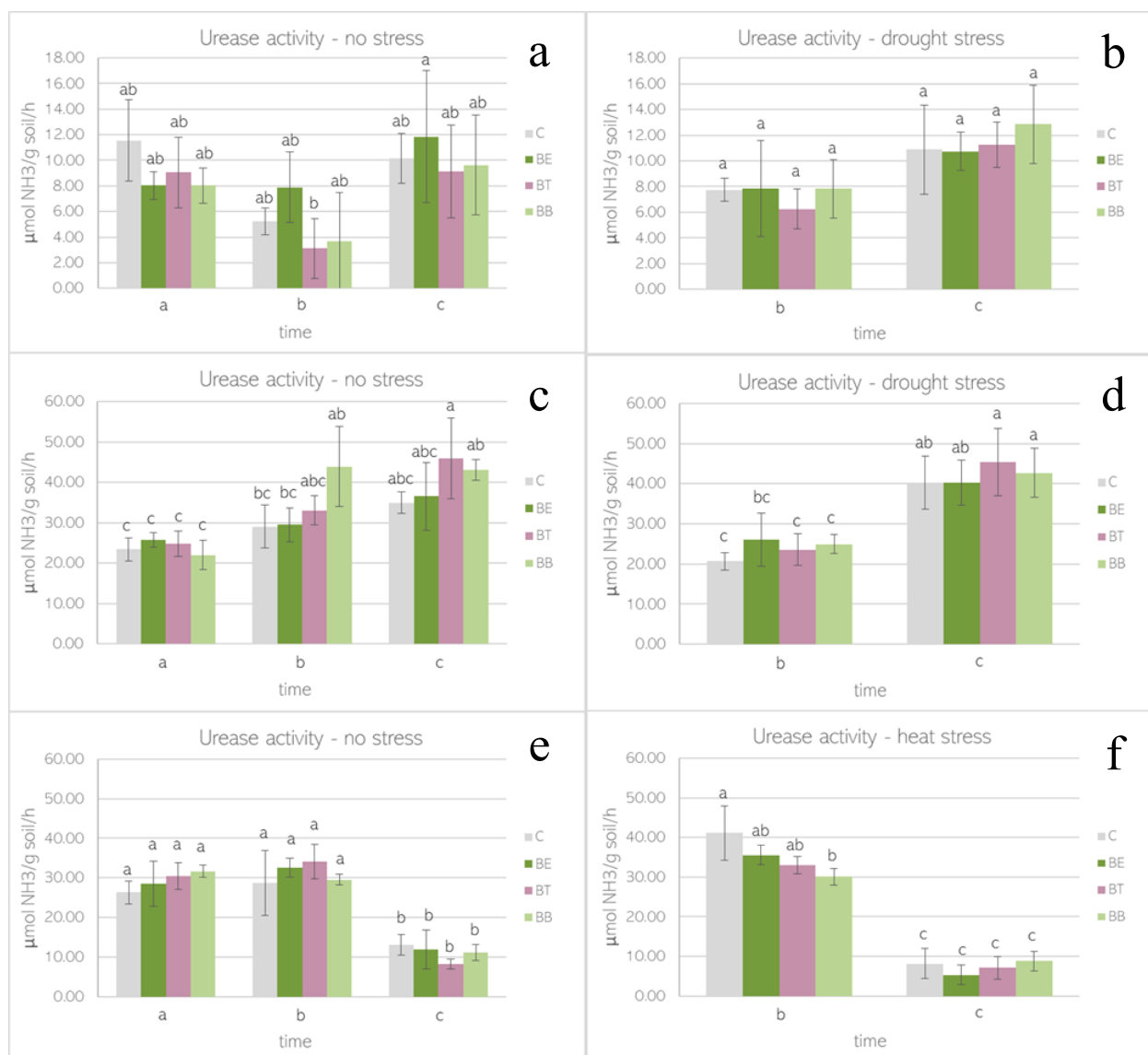


Figure 4.2 Urease activity of bermudagrass greenhouse soil (a, b), zoysiagrass greenhouse soil (c, d) and creeping bentgrass greenhouse soil (e, f) under no stress and drought/heat stress condition. Statistically significant differences are expressed by different letters (p-value ≤ 0.05). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

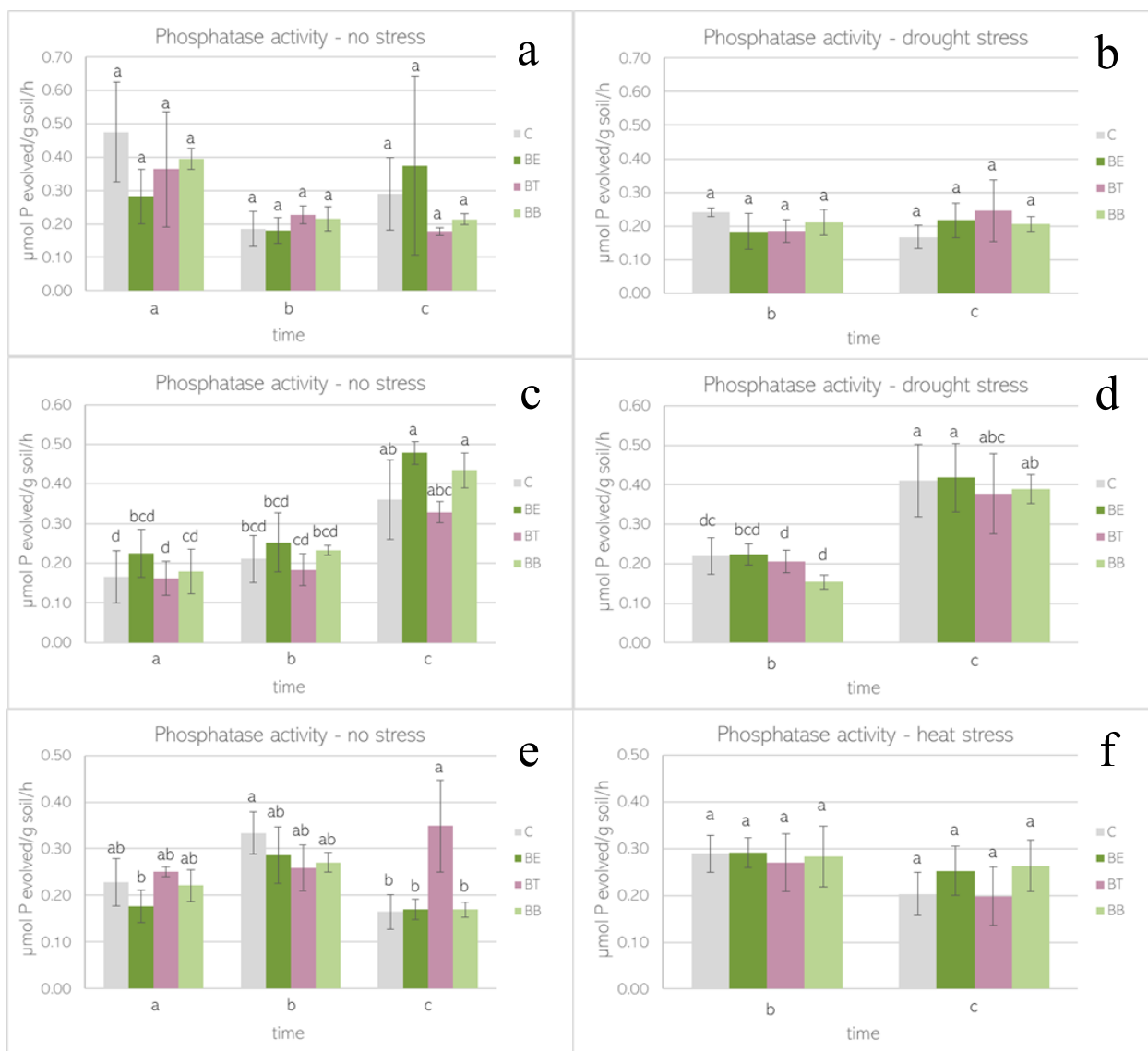


Figure 4.3 Phosphatase activity of bermudagrass greenhouse soil (a, b), zoysiagrass greenhouse soil (c, d) and creeping bentgrass greenhouse soil (e, f) under no stress and drought/heat stress condition. Statistically significant differences are expressed by different letters (p-value ≤ 0.05). Treatments abbreviations: C-control; BE-BioEnsure®; BT-BioTango™; BB-BioEnsure®+BioTango™.

CHAPTER 5

SUMMARY AND CONCLUSIONS

This study investigated the use of microbial inoculants, specifically *Trichoderma harzianum* (BioEnsure®) and *Bacillus* spp. (BioTango™), to improve the abiotic stress tolerance of three turfgrass species—bermudagrass (*Cynodon* spp.), zoysiagrass (*Zoysia* spp.), and creeping bentgrass (*Agrostis stolonifera*)—under drought and heat stress conditions. The research aimed to assess the effectiveness of these microbial treatments in enhancing turfgrass physiological responses, growth parameters, and soil biological health. The experimental approach consisted of greenhouse and germination trials where microbial inoculants were applied individually (BE and BT) or in combination (BB), and their effects were measured. The analyzed parameters included photosynthetic efficiency, relative water content, green percentage, turf quality, and germination rate under stress conditions. Additionally, soil health indicators, such as enzymatic activity and microbial respiration, were measured to evaluate the broader impact of inoculants on soil microbial activity.

The application of microbial inoculants had variable effects, depending on turfgrass species and stress conditions. No significant differences were observed in turf growth parameters for the two warm-season turfgrass species; however, under drought stress, the combined application of fungal and bacterial inoculants improved the soil respiration in zoysiagrass soil. Moreover, BT significantly improved phosphatase activity under standard condition in creeping bentgrass. Seed coating with microbial inoculants showed positive effects on creeping bentgrass germination, especially under heat stress, with BE treatment significantly improving germination rates in soil-

grown samples compared to the control. The microbial treatments enhanced soil enzymatic activity and microbial respiration, indicating potential benefits in improving soil health. However, the combination treatment (BB) did not consistently outperform individual applications, suggesting that the interaction between *Trichoderma harzianum* and *Bacillus* spp. may not always result in a synergistic effect. The findings suggest that microbial inoculants have the potential to enhance turfgrass resilience to abiotic stress, but their effectiveness is highly context-dependent. The variability in responses across species and environmental conditions underscores the need for further optimization of application methods, frequency, and dosage. While microbial inoculants present a promising sustainable alternative to traditional turf management practices, additional field trials under diverse environmental conditions are necessary to validate their practical applicability. Future research should explore the long-term effects of microbial inoculation on turfgrass ecosystems, including potential interactions with native soil microbiomes and nutrient cycling processes. In conclusion, this study provides valuable insights into the role of microbial inoculants in turfgrass stress management. Although some benefits were observed, further refinement is needed to maximize their effectiveness and integration into sustainable turfgrass maintenance programs.