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Using bacterial endophytes to treat *Fusarium*-induced seedling wilt in *Sorghum bicolor* under abiotic stress

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Abstract
Sorghum (*Sorghum bicolor*) is the fifth-most grown crop in Africa and plays a prominent role in the culture and diet of African countries including Ethiopia, South Africa, Kenya, and Egypt. While well-adapted to the African climate, sorghum is highly susceptible to the fatal fusarium wilt, caused by the fungal pathogen *Fusarium oxysporum*, which can lead to crop losses of up to 30%. Treatments have historically centered around practices such as hybridization and fungicides. Due to recent biotechnological advances, scientists have become interested in endophytes—microscopic organisms that form symbiotic relationships with the host plant—as a more sustainable alternative. However, current research has investigated the endophytic biocontrol behavior of *Fusarium* under only ideal moisture, temperature, and nutrient levels *in vitro.* A major gap therefore exists with regard to endophytes' effectiveness under suboptimal field conditions. Therefore, in this study, the two bacterial endophytes *Bacillus subtilis* and *Pseudomonas fluorescens* were inoculated with *Fusarium oxysporum* in Petri dishes and in sorghum plants under simulated abiotic conditions to determine if endophytes could still be successfully applied under suboptimal conditions for possible field implementation. Furthermore, because *Fusarium oxysporum* can be transmitted to sorghum seedlings *via* contaminated soil and insect feeding, this study assessed the efficacy of endophytes in controlling *Fusarium* present in the soil and injected into plant stems. *In vitro*, both endophytes significantly limited *Fusarium* growth; in planta, only *Bacillus subtilis* was found to be effective in limiting fusarium wilt.

Keywords
Fusarium wilt, *Fusarium oxysporum*, Endophyte, Sorghum, Biocontrol, Abiotic stress, Agricultural control, *Bacillus subtilis*, *Pseudomonas fluorescens*, Facultative saprophyte

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Introduction

In the next 30 years, the global human population is projected to increase by two billion to 9.7 billion in 2050 (1). As the global population continues to increase, the primary objective of many researchers is to improve agricultural productivity. This will help mitigate food scarcity and insecurity by increasing plant crop yield and controlling insect and disease outbreaks (2-5).

Solutions such as chemical fertilizers, fungicides, and insecticides have proved unsustainable, inducing widespread resistance to pathogens and polluting the soil microbiome (5). Excessive use of nonbiodegradable chemical fertilizers and pesticides may have contributed to reduced soil fertility, the bioaccumulation of heavy metals, the loss of soil microbial diversity, and mineral groundwater leaching, ultimately leading to decreased food safety and an increase in the risk of cancer in humans (3, 5). Moreover, the usage of such chemicals has proved ineffective in itself, plant pathogens still account for more than 15% of losses in the global harvest despite the implementation of pesticides, hybrids, and other advanced agricultural techniques (6). Traditional management practices such as exclusion, avoidance, and eradication of the disease vectors – while effective – are suboptimal; especially in the face of long-lasting outbreaks, where they can only slow the pathogen's development at best (7).

Of the 15% of crop losses due to pathogens, 30% are caused by fungal pathogens, totaling about $2.4 billion per year of produce loss (6). One of the most significant plant diseases is fusarium wilt, a fatal fungal infection caused by the fungus *F. oxysporum* (*FO*). *FO* is one of the major diseases affecting economically significant crops in southern Africa, causing rot, blight, and canker on crops like corn, wheat, and soybeans (5). In Kenya, *FO* primarily infects cereal grains such as sorghum. Sorghum plays a prominent role in traditional Kenyan culture, and while the seed itself is cheap and drought-resistant, it is highly susceptible to fusarium wilt (8).

Scientists have increasingly turned toward biological control options as a more sustainable and effective solution. Although the ideal strategy would be to implement resistant crop cultivars, resistance to insects and diseases within a cultivar can vary to a great extent and is highly dependent on growing condition variables (7). Furthermore, pests might become resistant to the secondary metabolites produced by resistant cultivars, limiting crop yield potential. Another more promising solution is the usage of endophytes - bacteria or fungi that live in plant tissues and form symbiotic relationships with the plant - to prevent diseases.

Endophytes, especially those of genus *Pseudomonas*, *Bacillus*, and *Serratia*, have shown previous potential in limiting the proliferation of *FO* in crops by producing antifungal metabolites and altering the expression of plant proteins (9-10). The *Bacillus* and *Pseudomonas* species investigated in this study are harmless to healthy, non-immunocompromised humans. Hence, ingesting these plants that contain these bacterial species is unlikely to cause bacterial disease (11-12). However, endophytic biocontrol behavior has only been studied

under relatively favorable conditions *in vitro* (e.g., ideal moisture, temperature, and nutrient levels); not under real-world sub-optimal abiotic conditions, lending to a gap in the current knowledge (2, 13).

Hence, the objective of this study is to investigate the effects of endophytes on sorghum growth under field conditions with simulated biotic and abiotic stresses. More specifically, this study will seek to answer the research question "Can *Bacillus subtilis* and *Pseudomonas fluorescens* control fusarium wilt in sorghum plants by decreasing fungal infested plant area, decreasing symptoms of wilt in plants, and increasing seed germination rates under the suboptimal abiotic conditions of low pH, heat stress, and excess soil moisture as effectively as the endophytes do under the optimal abiotic conditions of neutral pH, normal temperatures and optimum soil moisture?" If *Bacillus subtilis* (*BS*) and *Pseudomonas fluorescens* (*PF*) show evidence of limiting *FO* growth in stressful field conditions, then they can be cultivated and employed to prevent this disease in sorghum-dependent areas of the world such as Kenya under stressful conditions, helping to maximize sorghum yield.

Literature review

Grain sorghum (*Sorghum bicolor*) is one of the most important cereals in the world, cultivated in over 100 countries on five continents and totaling 62 x 10^6 tons annually (14-15). Kenya is the major sorghum production area, where sorghum is ranked as the second- to fifth-most important cereal crop given its many human health benefits, accessibility and affordability as livestock feed, in addition to its drought-resistant vascular system (15-16). However, grain yields in the field are relatively low compared to those in experimental plots (14). Previous studies have shown that this discrepancy is primarily due to fungal contamination, which is often complex and additionally involves disease and insect pests (14, 17). Several notable fungal sorghum pathogens include species from the genera *Fusarium*, such as *F. thapsinum*, causing stalk rot and leaf sheath blight; *F. culmorum*, causing head blight and crown rot; and *FO*, causing fusarium wilt (5, 16, 17). Of these *FO* is the greatest cause for concern and will be the focus of this study.

FO specifically has been documented to cause severe symptoms of fusarium wilt in up to 80% of cases and can lead to sorghum crop losses of up to 30% in the form of germination failures, seedling fusarium wilt, or foliar diseases (15). As a facultative saprophyte, *FO* can also persist in soils for up to 20 years, surviving off of agricultural detritus, and spreading rapidly *via* contaminated soil, machinery, and vegetative propagative material (18). To understand the dynamics of, and ways to maximize the endophytic biocontrol of *FO* and this study's applicability, the effect of *FO* in Sub-Saharan Africa on sorghum crops and the mechanism of endophytes and fungal pathogens to prevent and cause disease respectively, was examined.

Fusarium species; mechanisms of disease causation

The specific incidence and severity of fusarium wilt can vary considerably among agricultural zones and subzones within Kenya given the differences in soil pH, soil nutrient content, soil

temperature, soil moisture, humidity, and altitude (7, 15, 17, 18, 19).

FO disease development is favored by low dewpoints and humidity – caused, in part, by later plantings during the wet season in East African countries - that is conducive for *FO* species to germinate, colonize substrates, and produce mycotoxins (7, 16).

Many studies have concluded that the pH of the soil has an inverse correlation with the severity of *FO* disease (18, 20). Wrather et al. found that a low soil pH of 5 enhanced *FO* growth and encouraged fusarium wilt in bananas (17). However, others questioned the results of pH experiments, citing studies which reported that manipulating a single variable cannot measure the effect of individual variables given the soil's complexity (18, 19). For example, while more severe cases of *FO* were found in soils with low pH, these observations could not be reproduced experimentally (20). Indeed, manipulating soil pH altered the bioavailability of other vital nutrients such as iron, manganese, zinc, copper, calcium, zinc, silicon, potassium, and phosphorus (18). Instead of predicting changes in *FO* severity solely on the pH of the soil, it is better to frame severity in terms of the availability of soil nutrients. Iron and manganese were directly correlated with disease severity, while the nitrate to ammonium ratio, organic matter content, levels of calcium, zinc, silicon, potassium, phosphorus, and boron were inversely correlated (18, 21).

There are additional contradictions within the literature. With regard to the effect of soil temperatures on *FO* disease severity, Wrather et al. found that cooler soil temperatures resulted in greater *FO* growth, while Ferrocino et al. and McLaren et al. found that soil temperature was instead positively correlated with disease incidence. Acknowledging this discrepancy, Orr et al. suggested that because temperature was a difficult variable to control practically, given the vast tracts of land (as opposed to greenhouses) crops are grown on, it was impractical to study soil temperature in isolation.

Depending on the agricultural region's climatic conditions, plants may be infected with varying degrees of fusarium wilt, exhibiting signs of wilting, yellowing, necrosis, and brown leaf tips (17). Plants may also remain asymptomatic (16). *FO* produces millions of spores, and once spores of *FO* germinate and penetrate seedling tissues *via* root wounds, the fungus grows systematically and colonizes the tissues of the apical meristem (5, 7, 18). During plant tissue colonization, *Fusarium* produces a variety of mycotoxins - a group of secondary toxic metabolites such as fumonisins, moniliformin, trichothecene, deoxynivalenol, fumonisin-B1, moniliformin, and beauvericin - which are responsible for fusarium wilt in sorghum, as well as plant unrelated diseases such as esophageal cancer and neural tube defects in humans, and feed refusal, vomiting, and suppressed immune functions in livestock (5, 14, 16).

Although many practices are employed to protect sorghum crops from *FO* infection, it is difficult to eliminate *Fusarium* from fields. Chemical pesticides can almost decimate the soil microbiome and hence affect the entire ecosystem (9). Furthermore, *FO* is a vascular pathogen, it may escape contact with

fungicides. While the selection of a *FO*-resistant variety may be a productive alternative, a widely accessible and affordable one has eluded discovery (18). Recent interest in microorganisms has shown that specific bacterial or fungal strains known as endophytes can help suppress soil-borne diseases such as *FO* by inhibiting the production of mycotoxins and strengthening the plant's chemical defenses (18).

Endophytes; mechanisms of disease prevention and control

First coined by the German botanist Anton de Bary in 1866, the term *endophyte* refers to the organisms that reside in the endosphere tissues of leaves and stems, and the term was later refined in 1989 to describe those that have evolved to form mutualistic relationships with their hosts by assisting with nutrient availability, stress tolerance, biocontrol abilities against pathogens and receiving nutrients in return (2, 4, 6). In their natural habitats, endophytes function as both plant biocontrol agents and detritivores; however, researchers have begun to investigate the role of endophytic metabolites in being effective crop insecticides and biofertilizers, in addition to their properties as commercial antibiotics, anticancer, antidiabetic, immunosuppressant, and antiviral drugs (20, 22). Indeed, endophytes have a considerable biotechnological potential that is yet to be explored.

The diversity of endophytes that colonize plants in a variety of climates and ecological niches is significant (2). Endophytes can be classified based on ecology: clavicipitaceous or non-clavicipitaceous; sexual or asexual reproducers; vertical or horizontal host transmitters; biotrophic or necrotrophic nutrient obtainers; symptomatic or asymptomatic infection expressors; and foliar or root host entry facilitators (4). Endophytes can be additionally characterized based on their functionalities as either plant-growth-promoters, biocontrol agents, or plant-stress homeo-regulating microbes, as well as by their distribution in the ecosystem as either obligate, facultative, or passive (2, 6). Obligate endophytes depend solely on the host to survive with either pathogenic or beneficial relationships. Facultative endophytes are host-optional and develop in rhizospheric soil. Passive endophytes do not intentionally colonize the host, but they may enter the host through wounds on root hairs or through insect feeding. Moreover, facultative endophytes can be classified by their colonizing aptitude, the specificity of the host to the endophyte, and the allocation of resources within the host (2).

Each endophyte has evolved to be specific to its host, and the endophyte and the host's metabolic pathways have become so attuned that the beneficial endophyte can be pathogenic to other plants, such as in the case of *Pseudomonas fluorescens;* which is pathogenic in the leatherleaf plant (2, 4). However, endophytes can be applied to a variety of hosts and still confer various advantages. Each endophytic strain can display a wide spectrum of biocontrol abilities. Only through this specificity can endophytes successfully defend the host from select pathogens.

The allocation of endophyte-friendly nutrients is most heavily concentrated in the root hairs and rhizomes (23). Roots are also the most

easily damaged part of the plant and often secrete exudate and substrate metabolism that facilitates the establishment of microbial hotspots (4). Taken together, the host's roots are the first channel through which an endophyte penetrates the plant (4, 23).

The ability of an endophyte to colonize the host's root endospheric regions is additionally dependent on a variety of abiotic factors such as soil salinity, moisture, temperature, pH, and nutrient content (2, 4, 10, 24, 25). For example, Lin et al. suggest that a high level of nitrogen, potassium, and phosphorus (NPK) content in soils induced a much higher endophytic population compared to an NPK-deficient treatment (25). Kracmarova et al. present an alternate view, however: they argue that soil endophytic distributions have more to do with the ratio of nutrients (26). In their study, they found that a moderate but balanced level of NPK fertilizer led to an increased abundance and diversity of endophytes such as *BS* compared to insufficient or excessive fertilizer levels.

In addition to the roots, endophytes may also enter the host's system *via* the seed coat or direct insect feeding. After an endophyte enters the system, the endophyte increases in number and colonization capability as the plant develops, and some species such as *BS* produce reproductive spores that can endure adverse environmental conditions, assisting with endophyte dissemination and survival (4, 20). Endophytes can secrete a variety of extracellular lytic enzymes such as pectinase, amylase, cellulase, and catalase that enable them to easily penetrate and spread throughout plant tissues (4, 6).

Once fully active, endophytes contribute to the growth and defense of the host in a variety of ways, including stress tolerance, microbial and insectile biocontrol, and plant growth promotion (2, 5).

Stress tolerance

In both aerobic and anaerobic conditions, endophytes such as *P. microspora* have been shown to degrade the plastic polymer polyester polyurethane (PUR) and toxic metals by secreting enzymes such as oxidoreductases and serine hydrolase (2). Additionally, bacterial genera such as *Bacillus*, *Pseudomonas*, and *Serratia* produce the lytic enzymes chitinases and proteases, which break down fungal chitin, hemicellulose, cellulose, and lignin, further contributing to endophytic bioremediation ability (5).

Besides degrading toxic compounds and organic detritus and making nutrients more accessible to the host in times of ecological stress, endophytes can also play a more active role in mediating stress tolerance. Once an endophyte detects stress in the host's physiological or environmental conditions, it triggers a response through systemic gene expression, resulting in changes in metabolism and protein synthesis (4, 13). For example, *Trichoderma* ssp., fungal endophytes, significantly increased seed germination rates and promoted strong root growth and deep root penetration (13). Another example is *B. phytofirmans*, which regulated cellular homeostasis, transcription, and ROS detoxification to improve the fitness of potatoes in drought (2). Although endophytic species have been well-noted for their abilities to enhance plant growth, it is worth noting that

in the absence of such stress, endophytes do not necessarily increase plant growth (5).

Plant growth promotion

To promote plant growth, endophytes can utilize nitrogen fixation, phosphate solubilization, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, iron chelation, and metabolite production. Metabolic compounds known to aid in cell division, shoot elongation, and phosphorus acquisition such as indole-2-acetic acid (IAA), gibberellic acid, phytohormones, cytokinins, phytohormones, and auxins are especially emphasized as the primary growth-promoters (4-5). *B. vietnamiensis* inoculation in wild cottonwood induced IAA production, which in turn led to an increase in dry biomass, root length, and nitrogen content (2). *G. diazotrophicus* from sugarcane can be used to enhance the root growth of rice plants, while *H. seropedicae* from sorghum can also colonize corn and assist with nitrogen fixation (2).

Microbial and insectile biocontrol

Endophytic bioproducts such as terpenoids, steroids, flavonoids, alkaloids, and peptides have been extracted, and they all have been reported to have antimicrobial properties (4). Endophytes can also control insect herbivory. For example, fungi from the order *Hypocreales* release peramine, a compound that prevents nematodes, insects, and parasites from feeding on the plant. These fungi have been reported to control borer insects that attack sorghum seedlings (2).

Endophytic biocontrol of pathogens is dependent on the endophytes producing anti pathogenic compounds, as well as related to the endophytes' ability to alter plant gene expression. Endophyte-produced volatile organic compounds (VOCs) and terpenoids, steroids, flavonoids, alkaloids, and peptides are recognized by plant receptors, which then trigger an effective immune response with multiple hormonal resistance patterns (4, 20). Two types of plant resistance pathways exist. Induced systemic resistance (ISR) is resistance triggered by a pathogen and produces ethylene and jasmonic acid, compounds involved in regulating plant defense and stress response. The systemically-acquired resistance is resistance induced by endophytes and produces salicylic acid, the primary hormone in fighting pathogen infection. Endophytes simultaneously activating both resistance pathways strengthen plant defenses to successfully fend off diseases, maximizing the production of pathogen repellant enzymes and proteins (2).

Suppression of disease vectors can either be specific with direct antagonism between organisms and lipopeptide production, or it can be general with non-targeted competition between organisms for space and nutrients, siderophore production, and antibiotic production (5, 6, 18).

In specific suppression, endophytes secrete lipopeptides (LPs), a diverse group of metabolites produced by genera such as *Bacillus* and *Pseudomonas* that were previously shown to possess antimicrobial, antitumor, immunosuppressant, and surfactant properties (10). Depending on the number and configuration of the amino acids in the peptide chains, LPs of *Bacillus* can be classified into surfactin (ex. heptapeptide variants of esperin, lichenysin, pumilacidin, surfactin groups),

iturin (ex. iturin A and C, bacillomycin D, F L, mycosubtilin), or fengycin (ex. fengycin A and B) (10, 24). LPs of *Pseudomonas* can be classified into viscosin, amphisin, tolaasin, or syringomycin; structurally outlier LPs include arthrofactin, putisolvins I and II, orfamide, and pseudo desmins A and B. Such a wide variety of LPs suggests great diversity in natural function (10).

Indeed, LPs assist with antagonism toward viruses, mycoplasma, fungi, bacteria, and oomycetes, in addition to organism motility and attachment to surfaces. For instance, minimal doses (∼1 μM) of surfactin inhibited fungal aerial hyphae growth in the rice pathogen *M. grisea*, effectively preventing the fungus from releasing spores and disseminating (10). The primary method in which LPs achieve pathogen inhibition is by creating membranous pores in fungi that lead to toxic imbalances in calcium and hydronium ion concentrations (10).

In general suppression, endophytes such as *BS* B6 produce siderophores, which limit the available soil iron content, a major growth factor for host pathogens (5). Additionally, bacteria of the genus *Bacillus* produce 167 antibiotics, 66 of which are from *BS* and 23 of which are from *Bacillus brevis* (24). These antibiotics are mainly active against plant-disease-causing gram-positive bacterial genera such as *Rathybacter*, *Leifsonia*, and *Clavibacter*.

However, just as abiotic factors can influence the distribution of endophytes in a plant's rhizosphere, a variety of interrelated abiotic factors including nutrient content and pH may influence endophytic biocontrol abilities. For example, the application of NPK fertilizers can modify the pH of the soil (21). By decreasing the intracellular pH and hygroscopicity of the host, endophytic inhibition of fungal growth decreased (10). Katz & Demain found that a decline in bacitracin production by *Bacillus licheniformis* was "due to the low pH caused by the organism's metabolism" (24). Furthermore, hydroxamate siderophores—low-molecular-weight iron-chelating agents—are known to undergo pH-dependent transformations that significantly affect their iron-binding capacity (2, 27). This, in turn, promotes plant growth by aiding in DNA synthesis and reproduction and limits pathogenic fungal growth. While pH and other abiotic conditions can be a contributing factor to the attenuation of antifungal effects using endophytes such as *Pseudomonas fluorescens*, abiotic conditions may not have as large of an impact on species that produce resting spores, such as *BS*, which allow for the strain to withstand environmental stressors. It is thus advisable to formulate biocontrol agents using endophytes that produce resting spores such that the impact of unfavorable abiotic conditions on antifungal potency is minimized (28).

Research gap

The current literature addresses endophytic biocontrol under stable and ideal conditions optimal to endophytic biocontrol. Fadiji et al. identified the need for more focused work in adapting endophyte application to agricultural conditions for biotic/abiotic stress tolerance (2).

For example, in their *in vitro* trials, Orr et al. (18) identified a combination of abiotic factors that decreased *Fusarium* severity. However, they did not test *Fusarium* in field conditions, and they recommended that their experimental setup would need to be manipulated into field settings and combinations of region, soil types, and crop cultivars would need revision. Another example can be seen with seed germination rates boosted by endophytic symbiosis. Sorghum seeds that exhibited a 90% viability using standard germination tests nevertheless exhibited significantly reduced emergence rates in the field (7). Finally, although fungicides such as phosphate, ambuic acid, organotin mandelates, carbendazim, carboxin, propiconazole, benomyl, and difenoconazole have proven to be successful in inhibiting *Fusarium* growth *in vitro*, in planta, only carbendazim and indoleacetic acid were successful, and even then, these results have not yet been validated under field conditions (20). Tewari et al. (6) attributed these differences to the unpredictable and dynamic variations in the soil such as the pathogenicity and competition from soil-borne fungi; among others.

The two endophytic species to be tested against *FO* are *BS* and *PF*. *BS* originates from *B. campestris*, and there is an abundance of research attributing significant biocontrol abilities to the endophyte (6, 28, 29). More specifically, under field conditions, fusarium wilt of banana was controlled by *BS* with a 55% efficacy (20). *PF*, from *O. europaea*, was reported to control fusarium wilt of banana with a 79% efficacy as well as the fusarium wilt of chickpea (20).

Furthermore, because this research is geared toward investigating if endophytes can be applied in simulated Kenyan agricultural conditions to successfully treat fusarium wilt, this study will only focus on the relevant pH, temperature, and moisture level conditions. In East Africa, soil pH generally ranges from strongly acidic (< 5) to moderately alkaline (7.4-8.4). In eastern regions of Kenya, between approximately 5^{o}N-5^{o}S and 32^{o}E-40^{o}E, the pH is generally moderately acidic (5.2-6.0), with parts slightly acidic (6.1-6.5) (30). During the planting/wet seasons, the soils absorb significant amounts of water, supporting crop growth along with soil waterlogging. With heavy rainfall, the top layer of organic content and nutrients is eroded, and the soil is compacted, reducing aeration and drainage capacity. Nutrients such as nitrogen, potassium, phosphorus, iron, and calcium are leached into subsurface levels of soil, reducing their bioavailability to plants.

Another large gap in the current literature is the paucity of information reporting the effect of individual abiotic factors on *Fusarium*'s antagonistic or endophytic biocontrol abilities. Studies that try manipulating a single variable cannot measure the effect of individual variables given the soil's complexity (e.g., manipulating the bioavailability of soil nutrients influences soil pH; soil temperature influences soil moisture content, aeration, and nutrient content) (18, 19, 31). Therefore, multiple abiotic factors such as low pH, heat stress, and excess soil moisture will be studied in tandem in this study. This will most effectively simulate Kenyan field conditions as well.

If successful, such research will provide information to Kenyan farmers with an inexpensive and accessible form of fusarium wilt biocontrol. The application of endophytes is simple and does not require multiple field applications or booster doses (6). Once endophytic metabolites are extracted and manufactured, commercial formulation abilities will become more stable than pure endophyte biocontrol (20) Given the ability of endophytes to enter the host system *via* rhizosphere roots or through the phyllosphere stomata, field application methods can include seed coating, soil drenching, or foliar feeding (32).

Materials

The school science laboratory provided potato dextrose agar and nutrient agar, in addition to incubators, ovens, sterile Petri plates, distilled water, grow lights, bleach, pH test strips, sealable containers, plastic wrap, inoculating loops, and corn syrup. The microvave oven was rated at 1.2 kW (General Electric, model no. JES737WM01). Sargent Welch (Rochester, NY, USA) supplied one slant each of *P. fluorescens* and *B. subtilis* (strain designation unknown due to years of subculturing). Carolina Biological (Burlington, NC, USA) supplied a tube of *F. oxysporum*, strain designation IMI 141140. Two hundred Dale Sugar Sorghum seeds (ASIN ID: B07FVLZTS3) were purchased from Amazon. MiracleGro® clay loam soil and MiracleGro® sphagnum peat moss was obtained from the local Lowe's Home Improvement store.

Methods

Design

In the field, fusarium wilt is transmitted to sorghum plants through two vectors: *via* uptake or nematode feeding from soil that has been previously inoculated with *FO* or *via* piercing by infected feeding insects. These vectors were simulated by germinating seeds in *FO*-infected soil and by piercing seedlings with needles brushed with *FO* to assess if the endophytic bacteria *BS* and *PF* could inhibit Fusarium growth under these conditions. In addition to a control group that evaluated the strains' health, these two transmission vectors also constituted the experimental groups. The independent variables were the presence of endophytes (which consisted of three levels: *BS, PF,* or no endophyte), abiotic stress conditions, and vector of *Fusarium* transmission. The dependent variables were fungal areas on the plant and visual symptoms of fusarium wilt, identified as either low seed germination success rates or seedling wilt (33). Symptoms of fusarium wilt were defined as the plant being entirely wilted or exhibiting brown and yellow leaf tips.

FO also thrives in warmer and wetter weather (e.g., 29^{o} C and high humidity) and is more severe in acidic soils (e.g., 5-6) (34). Therefore, the suboptimal conditions for *FO*, *BS*, and *PF* included heat stress/higher temperatures, pH stress/acidic soil, and moisture stress/high moisture levels to simulate those prevailing in Kenya during the wet planting season.

Experimental

Multiple batches of 12.00 grams of nutrient agar and 20.00 grams of potato dextrose agar (PDA) were weighed on an electronic balance (d=0.01g) and then transferred to one-liter beakers. Then, 500 mL of distilled water was measured with a graduated cylinder and poured into each one-liter beaker. The agar was stirred

until fully dissolved. The resulting solution was sterilized by microwaving the beaker until the colloid solution appeared transparent. After letting it cool, the solution was then poured into sterilized 90 x15 mm plastic Petri dishes. The agar dishes were incubated for 24 hours at 27°C and checked for contamination. Fresh plates were selected for use in experiments. To create *BS*-, *PF*-, and *FO*-inoculated agar, a sterilized inoculation loop was used to streak samples of *BS*, *PF*, and *FO* from their original slants onto separate plates of fresh agar plates. Then, each plate was inoculated at 27°C for five days to establish significant culture inoculum samples.

A sterilized innoculation loop was used to streak *FO* along the diameter of two plates. Then, *BS* was streaked on ends perpendicular to the *FO* streak in each plate. The same procedure was repeated with two separate plates using *FO* and *PF*. The inoculating loop was sterilized between streaks. Each plate was sealed with Parafilm^{R}. Then, the plates were incubated according to experimental design. For testing the effects of endophytic bacteria under optimal conditions without seeds, they were incubated at 27^{o}C for five days, taking pictures and recording fungal areas daily. For testing the effects of endophytic bacteria under suboptimal conditions without seeds, the plates were incubated at 32^{o}C for four days, taking pictures and recording fungal areas daily. For *FO*-only-dishes (i.e. the negative control groups), fungal areas were approximately ellipses and calculated as such. For dishes with endophytes and *FO*, fungal areas were predominantly bean-shaped. To calculate these areas, the width of the wider end was added to the width of the smaller end, multiplied by 0.45 and multiplied by the length from end to end ((A+B) x 0.45 x Length).

15 mL of corn syrup was mixed with 300 mL of distilled water to prepare a 4.76% sugar solution. A square-inch-sized sample of *FO* inoculum was added to the solution and mixed vigorously. A microwavable container was filled with twenty-four cups of Miracle Gro® cactus mix soil (sandy loam and acidic) and microwaved for 90 seconds to sterilize the soil (35). Although there is no guarantee that colonization would have occurred in non-sterilized soil, the soil in this study was sterilized to eliminate any confounding variables from the study (35). Sterilized soil was rehydrated with distilled water. Fifteen sterilized Petri dishes were filled with sterilized cactus soil.

Similarly, A microwavable container was filled with twenty-four cups of Miracle Gro® potting (regular) soil (loamy and with a pH of 7) and microwaved for 90 seconds. Sterilized soil was rehydrated with distilled water. Fifteen sterilized Petri dishes were filled with sterilized regular soil. The liquid culture was poured into thirty sterilized soil plates, thus forming 15 *FO*-inoculated cactus soil and 15 regular soil Petri dishes. Each plate was sealed with Parafilm^{R}. The plates was incubated at 27^{o}C for five days to establish significant fungal growth in the different soils. 216 sorghum seeds were sterilized using a 50% bleach solution with agitation and then rinsed three times with water. Sixty seeds were brushed with *BS* and divided among five *FO*-inoculated cactus soil and five *FO*-inoculated regular soil Petri dishes. 60 seeds were brushed with *PF* and divided between five *FO*-inoculated cactus soil and five *FO*-inoculated regular soil Petri

dishes. The remaining sixty seeds were divided among five *FO*-inoculated cactus soil and five *FO*-inoculated regular soil Petri dishes. Each plate was sealed with Parafilm^{R}. They were incubated and hydrated according to experimental design: for testing the effects of endophytic bacteria under optimal conditions, five dishes (regular soil) of *FO* only, five of *BS* and *FO*, and five of *PF* and *FO* were incubated at 25^{o} C for ten days (and no additional moisture was added). For testing the effects of endophytic bacteria under suboptimal conditions, five dishes (cactus mix soil) of *FO* only, five of *BS* and *FO*, and five of *PF* and *FO* were incubated at 32^{o}C for ten days. 15 mL of distilled water was added to each dish.

Four sterilized cups were filled with sterilized cactus soil (sandy and pH of 6) and another four with sterilized regular soil. Six seeds were germinated with a square-inch-sized chunk of *BS*-inoculated agar in each of the two soil types at 27^{o}C. This procedure was repeated with a square-inch-sized chunk of *PF*-inoculated agar in each of the two soil types and without any bacteria-inoculated agar in the remaining soil cups. Each cup was sealed completely with Parafilm^{R}. After germination, the Parafilm^{R} cover was modified to form a 6-inch dome to allow for vertical growth. 8 days after germination, a fine needle was brushed against the *FO* culture and then pierced into each stem in each of the six cups. A sterilized fine needle was used to pierce the stems in the remaining two cups to act as a control. Each cup was sealed with Parafilm^{R}. The three cactus soil cups were incubated at 32^{o}C for five days under a wavelength of 225 nm and 17600 lux grow light using a 16-hour on-off cycle. The three control soil cups were incubated at 25^{o} C for five days under the grow light.

Although MiracleGro® regular potting soil and cactus soil contained trace amounts of slow-release nitrogen (0.06%), phosphorus (0.02%), and potassium (0.04%), sterilization significantly decreased all bioavailable nutrient concentrations (36). Additionally, MiracleGro® slow-release nutrients are designed to be released over two months, and those released over the experimental period were assumed to be negligible.

Safety

All experimentation involving bacteria *PF* and *BS* and fungus *FO*, species all identified as BSL 1, were conducted under a biosafety containment hood with appropriate BSL 1 precautions.

Statistical analysis

Stapplet.com was used to analyze statistical data. One way ANOVA tests, one sided student t tests and Chi-square tests were performed on appropriate data with an $\alpha = 0.05$.

If the endophytic bacteria yielded smaller fungal areas, greater number of seeds germinated, and fewer symptoms of fusarium wilt, then it could be concluded that the endophytic bacteria *BS* and *PF* limited the growth of fusarium wilt in sorghum plants under abiotic stress. The null hypothesis was that the endophytic bacteria *BS* and *PF* do not affect the growth of fusarium wilt in sorghum plants under abiotic stress.

Results

The changes in fungal areas in optimal (defined as incubated at 27ºC) and suboptimal (defined as incubated at 32ºC) conditions are shown in Table 1 and Table 2, respectively.

Table 1. *FO* Fungal Areas (cm^2) Under Optimal Conditions in the Presence of Endophytes

Condition	Day 2	Day 3	Day 4	Day 5
No endophyte	18.85	22.95	22.62	26.41
BS	15.93	12.25	18.43	16.96
PF	15.08	16.49	18.16	12.32

Note: Data on the first day was not collected because of special circumstances.

Table 2. *FO* Fungal Areas (cm^2) Under Suboptimal Conditions in the Presence of Endophytes

Condition	Day 2	Day 3	Day 4	Day 5
No endophyte	15.08	21.99	20.89	21.36
BS	10.21	12.72	13.19	E
PF	7.54	4.71	11.31	8.80

Note: Data on the first day was not collected (NC) because of special circumstances. The plate with *BS* exhibited signs of contamination on the fifth day and was excluded (E) from analysis.

This data was analyzed using a one-way ANOVA test. The calculated p-values for the data in Tables 1 and 2 were .008 and < 0.001 respectively. Tables 1 and 2 show the fungal areas of the plants with endophytes *BS* and *PF* were hence significantly less compared to fungal areas in plants grown without the endophytes.

The number of germinated seeds of five groups in optimal (defined as incubated at 27ºC) and suboptimal (defined as incubated at 32ºC) conditions are shown in Table 3 and Table 4, respectively.

Table 3. Number of Seeds Germinated Under Optimal Conditions in the Presence of *FO* and Endophytes

Condition	No endophyte	BS	PF
Mean ± SD	5.2 ± 0.44	5.6 ± 0.54	5.8 ± 0.44

Note: n=6. Seed germination was defined as the radicle or stem reaching greater than two inches.

Table 4. Number of Seeds Germinated Under Suboptimal Conditions in the Presence of *FO* and Endophytes

Condition	No endophyte	BS	PF
Mean ± SD	2.60 ± 1.94	5.00 ± 0.70	3.60 ± 0.89

Note: n=6. Seed germination was defined as the radicle or stem reaching greater than two inches.

This data was analyzed with a one-tailed independent samples t-test. The calculated p-values of Table 3's *BS vs.* no endophyte and *PF vs.* no endophyte group were 0.29 and 0.121, respectively. The calculated p-values of Table 4's *BS vs.* no endophyte and *PF vs.* no endophyte group were 0.024 and 0.170, respectively. The null hypothesis could hence be rejected for Table 4 for the case of *BS* only. Table 4 shows that endophyte *BS* significantly improved sorghum seed germination numbers compared to the germination numbers of seeds without endophyte treatment. Figures 1 and 2 show examples of sorghum seedlings with and without evidence of fusarium wilt respectively.

Figure 1. Wilted

Figure 2. Not wilted

Table 5. Number of Plants with Symptoms of Fusarium Wilt in the Presence of Endophytes Under Optimal Conditions

Fusarium wilt	(Mean ± SD)			
	No fusarium (control)	No endophyte	BS	PF
Wilted	0.00 ± 0.00	0.83 ± 0.40	0.20 ± 0.44	0.00 ± 0.00

Note: n=6. Symptoms of fusarium wilt were defined as the plant being entirely wilted or exhibiting brown and yellow leaf tips.

Table 6. Number of Plants with Symptoms of Fusarium Wilt in the Presence of Endophytes Under Suboptimal Conditions

Fusarium wilt	(Mean ± SD)			
	No fusarium (control)	No endophyte	BS	PF
Wilted	0.00 ± 0.00	0.66 ± 0.51	0.00 ± 0.00	0.16 ± 0.40

Note: n=6. Symptoms of fusarium wilt were defined as the plant being entirely wilted or exhibiting brown and yellow leaf tips.

The symptoms of wilt for six sorghum plants under optimal (defined as incubated at 27^{O}C, no excess soil moisture, soil pH of 7) and suboptimal (defined as incubated at 32^{O}C, excess soil moisture, soil pH of 6) conditions are shown in Table 5 and Table 6, respectively.

This data was analyzed with a Chi-squared test. The calculated p-values of Table 5's *BS vs.* no endophyte and *PF vs.* no endophyte group were 0.036 and 0.003, respectively. The calculated p-values of Table 6's *BS vs.* no endophyte and *PF vs.* no endophyte group were 0.014 and 0.079, respectively. The null hypothesis was rejected for Table 5 and for the Table 6's *BS vs.* no endophyte group. Table 5 shows that the presence of endophytes *BS* and *PF* in cups decreased the instances of fusarium wilt (only 1 plant (raw data) displayed symptoms of wilt) compared to cups without endophytes, which had 5 wilted plants (raw data). Table 6 reflected a similar pattern.

Discussion

This study investigated the efficacy of the bacterial endophytes *BS* and *PF* in controlling fusarium wilt in sorghum plants under environmental stresses. The observed decrease in fungal areas between plant cups and seeds Petri dishes with endophytes and plant cups and seeds Petri dishes without endophytes was statistically significant in both optimal and suboptimal conditions. This experiment served as the control group and provided evidence that the endophytic *BS* and *PF* strains used were healthy and effective *in vitro*. Since the t-test results found that the number of seeds germinated in *FO* soil was statistically significantly lesser than the number of seeds germinated in the presence of *BS*, it could be concluded that *BS* is effective in controlling fusarium wilt in sorghum plants under suboptimal conditions. This conclusion was reinforced by the ANOVA test results for symptoms of fusarium wilt in seedlings. Therefore, the endophytic bacteria *BS* was found effective in controlling fusarium wilt in sorghum plants under abiotic stressors such as low pH, heat stress, and excess humidity.

In regions with similar environmental conditions such as those in East and West Africa, *BS* can be applied to sorghum plants at the seed-sowing stage to prevent infection by *FO*, whether it be from *Fusarium*-infected soil or *via* insect transmission. However, given the study's small sample size, conclusions should be made cautiously; replicating this study with greater sample sizes is recommended. Another limitation of this study was that it only considered the effects of abiotic factors in affecting endophytic biocontrol ability. Samples of the microbiome of the target regions could not be obtained and studied due to a lack of available information and rules against culturing unknown microorganisms. The specific strain designation of *BS* and *PF* is unknown: the supplier has subcultured the original strains for many years, and the slants could no longer be identified as belonging to a specific strain. Although this substrain may have shown endophytic biocontrol abilities under abiotic conditions, others may not. Future research should focus on evaluating the effects of biotic factors on the bacterial inhibition of *FO*, identifying effective *BS* and *PF* strains, and investigating the effects of *BS* on the location's microbiome to identify any adverse effects the endophytes would have on regional ecosystems. Future studies should

involve more cups to reduce bias; in Tables 1 and 2, only one fungal area was measured for each group per day; these results could have been skewed due to confounding variables. Additionally, because the soil in this study was sterile and there is no guarantee that colonization will occur in non-sterile soil, future studies should test the efficacy of *BS* and *PF* using non-sterilized samples of Kenyan soil (37).

While future research is required to evaluate if endophytic applications are truly sustainable under biotic field stresses in addition to abiotic stresses, based on this study's current findings, it is recommended that farmers apply *Bacillus subtilis* to both sorghum seeds and sorghum soil in areas with severe abiotic stresses to control the spread and growth of fusarium wilt by controlling physical symptoms and preventing the fungus from overwintering and affecting other crops. Because endophytes have also shown evidence of increasing crop mass, *B subtilis* can also be used to simultaneously increase production. This way, by implementing *B subtilis*, farmers can maximize sorghum and general crop yield, increasing food accessibility in the face of a rising population.

Conclusion

The endophyte bacteria *Bacillus subtilis* was found to significantly decrease the incidence of fusarium wilt in sorghum seeds and plants as evidenced by an increase in the number of seeds germinated, a decrease in the fungal infested plant area and a decrease in the symptoms of wilt under the suboptimal abiotic conditions of low pH (cactus mix soil), heat stress, and excess soil moisture; as exist in sorghum growing countries of South Africa; especially Kenya.

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