

Peer review

Yang, Derek, and Sonya Loughran. 2024. "Using Bacterial Endophytes to Treat Fusarium-Induced Seedling Wilt in Sorghum Bicolor under Abiotic Stress." *Journal of High School Science* 8 (3): 321–40.

I have several comments that need to be addressed.

How is excess soil moisture related to sub-saharan Africa; defined as the belt between south Africa and the Sahara desert?

Please define in the manuscript and the Tables, what "optimal" and 'sub-optimal' conditions are. What was the pH of the soil? Does Miracle Gro potting soil contain plant usable N, P or S ? If yes, what effect - if any - do the levels of these elements have on Fusarium? Please perform a literature search.

Different strains of bacteria exhibit different anti-fungal potencies, see:<https://doi.org/10.3390/pathogens12020254> . Either state the strain of the bacteria used or state this as a limitation in the manuscript (since the strain you used may be effective under adverse abiotic conditions, but other strains may not).

The manuscript needs to mention that abiotic conditions are not as important a factor for endophytes that produce resting spores, such as *B. subtilis*. However, they can be a contributory factor to the attenuation of antifungal effect using endophytes such as *P. fluorescens*; which do not produce resting spores. You will need references. Please search the literature. The manuscript should state that it is advisable to formulate biocontrol agents that produce resting spores such that the impact of unfavorable abiotic conditions on antifungal potency is minimized. see: <https://academicjournals.org/journal/AJAR/article-full-text-pdf/0C9E6A243974> for one reference.

Fusarium oxysporum is most effective at colonization at alkaline pH since this is where the maximum amount of fusaric acid is produced and maximum iron chelation is achieved.

See: <https://doi.org/10.3390/toxins15010050>, and <https://doi.org/10.1016/j.apsoil.2016.05.010> therefore, by decreasing soil pH, you are effectively inhibiting the fungal colonization of the seeds. Please explain why alkaline pH was not studied.

Biocontrol bacteria inhibit pathogenic fungi by producing indole acetic acid (IAA), siderophores, and proteases and chitinases. Is there any evidence in the literature to show that the activity/expression of any of these enzymes, substances, chelating agents is either pH or humidity dependent ? If not, then please explain the rationale of the objective of this study. Explain and discuss in the manuscript.

Why was temperature not tested as an independent variable?

See:<https://doi.org/10.1016/j.apsoil.2013.05.015>, why were decreased pH and increased moisture content tested together, rather than separately as two independent variables? This is not a scientific method of experimental design since now you have no quantitative evidence of how

much each independent factor contributed to the severity of disease or to the inhibition of the bacterial species. Please explain and discuss in the manuscript.

There are various other factors that are equally - if not more - important in wilting severity, such as the NO_3^- to NH_4^+ ratio, soil iron content, organic matter content etc.

See: <https://doi.org/10.1016/j.apsoil.2018.06.019>, why were these not studied?

see reference: <https://doi.org/10.1080/26395940.2024.2323123> and include in the manuscript in the proper context if not already done so.

In those experiments where the stem was punctured and Fusarium introduced using a needle; was a control performed where the stem was punctured but no fusarium was introduced?

I don't understand Tables 5 and 6. BS and PF had 4 and 6 plants with wilt while no endophyte had 1 plant with wilt? Please check the tables and/or the explanation that goes along with the tables.

See text highlighted in yellow as revisions responding to the reviewer's comments + wrote a full-length and comprehensive literature review.

Reviewer's Comments:

1. How is excess soil moisture related to sub-saharan Africa; defined as the belt between south Africa and the Sahara desert?
 - After doing more research into the literature, I found Kenya has major issues with Fusarium wilt during the wet sorghum-sowing seasons. Specified Sub-Saharan Africa as regions of Kenya throughout the paper.
2. Please define in the manuscript and the Tables, what "optimal" and "sub-optimal" conditions are.
 - Done. See text.
3. What was the pH of the soil? Does Miracle Gro potting soil contain plant usable N, P or S? If yes, what effect - if any - do the levels of these elements have on Fusarium? Please perform a literature search.
 - Regular soil: 7; cactus soil: 6. Does not contain. See text.
4. Different strains of bacteria exhibit different anti-fungal potencies, see: <https://doi.org/10.3390/pathogens12020254>. Either state the strain of the bacteria used or state this as a limitation in the manuscript (since the strain you used may be effective under adverse abiotic conditions, but other strains may not).
 - Done. See text.

5. The manuscript needs to mention that abiotic conditions are not as important a factor for endophytes that produce resting spores, such as *B. subtilis*. However, they can be a contributory factor to the attenuation of antifungal effect using endophytes such as *P. fluorescens*; which do not produce resting spores. You will need references. Please search the literature. The manuscript should state that it is advisable to formulate biocontrol agents that produce resting spores such that the impact of unfavorable abiotic conditions on antifungal potency is minimized. see: <https://academicjournals.org/journal/AJAR/article-full-text-pdf/0C9E6A243974> for one reference.
 - Done. However, abiotic conditions do affect endophytes such as *B. subtilis* to some extent.
6. *Fusarium oxysporum* is most effective at colonization at alkaline pH since this is where the maximum amount of fusaric acid is produced and maximum iron chelation is achieved. See: <https://doi.org/10.3390/toxins15010050>, and <https://doi.org/10.1016/j.apsoil.2016.05.010> therefore, by decreasing soil pH, you are effectively inhibiting the fungal colonization of the seeds. Please explain why alkaline pH was not studied.
 - Alkaline pH was not studied because the study's goal is to validate the efficacy of the endophytes in controlling fusarium wilt under Kenyan field conditions, and these conditions do not include alkaline pH. See text.
7. Biocontrol bacteria inhibit pathogenic fungi by producing indole acetic acid (IAA), siderophores, and proteases and chitinases. Is there any evidence in the literature to show that the activity/expression of any of these enzymes, substances, chelating agents is either pH or humidity dependent? If not, then please explain the rationale of the objective of this study. Explain and discuss in the manuscript.
 - Yes; pH-dependent. See text.
8. Why was temperature not tested as an independent variable? See: <https://doi.org/10.1016/j.apsoil.2013.05.015>, why were decreased pH and increased moisture content tested together, rather than separately as two independent variables? This is not a scientific method of experimental design since now you have no quantitative evidence of how much each independent factor contributed to the severity of disease or to the inhibition of the bacterial species. Please explain and discuss in the manuscript.
 - An abundance of previous studies have stated that it is unhelpful to test variables individually because of the complexity and dynamic nature of the soil biome. See text.
9. There are various other factors that are equally - if not more - important in wilting severity, such as the NO_3^- to NH_4^+ ratio, soil iron content, organic matter content etc. See: <https://doi.org/10.1016/j.apsoil.2018.06.019>, why were these not studied?
 - See text.

10. see reference: <https://doi.org/10.1080/26395940.2024.2323123> and include in the manuscript in the proper context if not already done so.
- Done.
11. In those experiments where the stem was punctured and *Fusarium* introduced using a needle; was a control performed where the stem was punctured but no *Fusarium* was introduced?
- Yes; see text.
12. I don't understand Tables 5 and 6. BS and PF had 4 and 6 plants with wilt while no endophyte had 1 plant with wilt? Please check the tables and/or the explanation that goes along with the tables.
- Your understanding is incorrect; BS and PF had 4 and 6 plants with **no** wilt while no endophyte had 1 plant with **no** wilt. See text for improved explanations.

Using Bacterial Endophytes to Treat *Fusarium*-Induced Seedling Wilt in *Sorghum bicolor* Under Abiotic Stresses

In the next 30 years, the global human population will increase by two billion to 9.7 billion in 2050 ("Population," n.d.). 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 individual plant crop yield and controlling insect and disease outbreaks (Fadiji & Babalola, 2020; Khalil et al., 2021; Adeleke et al., 2022; Baard et al., 2023).

Solutions such as chemical fertilizers, fungicides, and insecticides have proved unsustainable, inducing widespread resistance to pathogens and polluting the soil microbiome (Baard et al.). Excessive use of nonbiodegradable chemical fertilizers and pesticides has caused issues such as reduced soil fertility, bioaccumulation of heavy metals, enormous 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 (Baard et al.; Khalil et al.). Moreover, the usage of such chemicals has proved ineffective in itself: according to Tewari et al., 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 (2019). Traditional management practices such as exclusion, avoidance, and eradication of the disease vectors have proven to be ineffective as well, especially in the face of long-lasting outbreaks, and they can only slow the pathogen's development at best (McLaren & Rothmann, 2019).

Of the 15% of crop losses due to pathogens, 30% are caused by fungal pathogens, totaling about 200 billion rupees per annum lost in the global market (Tewari et al.). One of the most significant plant diseases is Fusarium wilt, a deadly fungal infection caused by the fungus *F. oxysporum* (*FO*). *FO* is one of the main diseases affecting economically significant crops in Southern Africa, causing rots, blights, and cankers on crops like corn, wheat, and soybeans (Baard et al.). 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 (Leslie et al., 2005).

Because of recent developments in biotechnology, scientists have increasingly turned toward biological control options as a more novel 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 (McLaren & Rothmann). Furthermore, pests might become resistant to the toxins 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*, however, have shown previous potential in limiting the proliferation of *FO* in crops by producing antifungal metabolites and changing the expression of plant proteins (Verma et al., 2018; Raaijmakers et al., 2010). However, research has only studied endophytic biocontrol behavior under relatively favorable conditions in vitro (e.g., ideal moisture, temperature, and nutrient levels) and has not successfully considered endophyte success under more realistic conditions, lending to a gap in the current knowledge (Mastouri & Bergstrom, 2010; Fadji & Babalola).

The objective of this study, then, 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 areas, 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 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 (responding to comment #1) that have these 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 62×10^6 tons annually (Ruben et al., 2022; Corallo et al., 2023). 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 and accessibility and affordability as livestock feed, in addition to its drought-resistant vascular system (Ngugi et al., 2001; Ruben et al.). However, grain yields in the field are relatively low compared to those in experimental plots (Corallo et al.). Previous studies have shown that this discrepancy is primarily due to fungal contamination, which is often complex and involves disease and insect pests (Corallo et al.; Wrather & Sweets, 2022). 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 (Wrather & Sweets; Ruben et al.; Baard et al.). 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 (Ngugi et al.). 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 (Orr & Nelson, 2018). To understand the dynamics of and ways to maximize the endophytic biocontrol of *FO* and this study's applicability, the impacts of *FO* in Sub-Saharan Africa on sorghum crops and the mechanisms of endophytes and fungal pathogens must be examined.

Mechanisms of Fusarium

The specific incidence and severity of fusarium wilt can vary considerably between agricultural zones and subzones within Kenya given differences in soil pH, soil nutrient content, soil temperature, soil moisture, humidity, and altitude (Ngugi et al.; Wrather & Sweets; Orr & Nelson; Ferrocino et al., 2013; McLaren & Rothmann).

FO disease development is favored by high dews and humidity--caused by later plantings during the wet season in East African countries--that help *FO* species germinate, colonize substrates, and produce mycotoxins (McLaren & Rothmann; Ruben et al.).

Many studies have concluded that the pH of the soil has an inverse correlation with the severity of *FO* disease (Orr & Nelson; Bubici et al., 2019). In Wrather & Sweet's study of the relationship between abiotic conditions and fusarium wilt in bananas, a low soil pH of 5 enhances *FO* growth. However, other studies have cast doubt on the veracity of pH experiments, citing that studies that try manipulating a single variable cannot measure the effect of individual variables given the soil's complexity (Orr & Nelson; Ferrocino et al.; Peng et al., 1999 and Cao et al., 2016, as cited in Orr & Nelson). (responding to comment #8) For example, while more severe cases of *FO* have been found in soils with low pH, such an observation has not been reproduced experimentally (da Silva Junior et al., 2000, as cited in Bubici et al.). Indeed,

manipulating soil pH alters the bioavailability of other vital nutrients such as iron, manganese, zinc, copper, calcium, zinc, silicon, potassium, and phosphorus (Orr & Nelson) (responding to comment #8 as well). Perhaps 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 are directly correlated with disease severity, while nitrate to ammonium ratio, organic matter content, calcium, zinc, silicon, potassium, phosphorus, and boron are inversely correlated (Orr & Nelson; Bonanomi et al., 2007). Responding to comment #3.

There are additional contradictions within the literature. With regards to the effect of soil temperatures on *FO* disease severity, Wrather & Sweet's study has found that cooler soil temperatures result in greater *FO* growth, while Ferrocino et al. and McLaren & Rothmann have found that soil temperature is instead positively correlated with disease incidence. Acknowledging this discrepancy, Orr & Nelson suggest that because temperature is a difficult variable to control practically, given the vast tracts of land (as opposed to greenhouses) crops are grown on, it is impractical to study soil temperature in isolation. Responding to comment #8

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 (Wrather & Sweets). Plants may also remain asymptomatic (Ruben et al.). *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 (Baard et al., Orr & Nelson; McLaren & Rothmann). During plant tissue colonization, *Fusarium* will produce a variety of mycotoxins--a group of secondary toxic metabolites such as fumonisins, moniliformin, trichothecene, deoxynivalenol, fumonisin-B1, moniliformin, and beauvericin--that are responsible for fusarium wilt in sorghum, as well as esophageal cancer and neural tube defects in humans, and feed refusal, vomiting, and suppressed immune functions in livestock (Ruben et al.; Corallo et al.; Baard et al.).

Although many practices are being employed to protect sorghum crops from *FO* infection, it is difficult to eliminate *Fusarium* from fields. Chemical pesticides can almost eliminate the soil microbiome and can cause issues for the entire ecosystem (Verma et al.). Furthermore, *FO* is a vascular pathogen, it escapes contact with fungicides. While the selection of a *FO*-resistant variety may be a productive alternative, a widely accessible and affordable one has not been found yet (Orr & Nelson). 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 (Orr & Nelson).

Mechanisms of Endophytes

First termed by German botanist Anton de Bary in 1866, *endophyte* referred 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 (Adeleke et al.; Tewari et al.; Fadji & Babalola). 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 commercial antibiotic, anticancer,

antidiabetic, immunosuppressant, and antiviral drugs (Bubici et al.; Cui et al., 2024 responding to comment #10). Indeed, they have a considerable biotechnological potential that is yet to be explored.

There has evolved to be an incredible diversity of endophytes that colonize plants in a variety of climates and ecological niches (Fadiji & Babalola). Endophytes can be classified based on ecology: clavicipitaceous or non-clavicipitaceous; sexual or asexual reproduction; vertical or horizontal host transmission; biotrophic or necrotrophic nutrient obtainment; symptomatic or asymptomatic infection expression; and foliar or root host entry (Adeleke et al.). 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 (Tewari et al.; Fadiji & Babalola). Obligate endophytes depend solely on the host to survive with pathogenic or beneficial relationships. Facultative endophytes are host-optional and develop in rhizospheric soil. Passive endophytes do not intentionally colonize the host, but they rather 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 (Fadiji & Babalola).

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* in the leatherleaf plant (Fadiji & Babalola; Adeleke et al.). However, endophytes can be applied in 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 (Johnston-Monje & Raizada, 2011). 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 (Adeleke et al.). Taken together, both Johnston-Monje & Raizada and Adeleke et al. suggest that the host's roots are the first channel through which an endophyte penetrates the plant.

The ability of an endophyte to colonize the host's root endosphere regions is additionally dependent on a variety of abiotic factors such as soil salinity, moisture, temperature, pH, and nutrient content (Adeleke et al.; Fadiji & Babalola; Raaijmakers et al.; Katz & Demain, 1977; Lin et al., 2024). For example, studies from 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. Kracmarova et al. present an alternate view, however: they argue that soil endophytic distributions have more to do with the ratio of nutrients (2020). 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 interaction 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 (Fadiji & Babalola; Bubici et al.). 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 (Adeleke et al.; Tewari et al.).

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 (Fadiji & Babalola; Baard et al.).

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 (Fadiji & Babalola). 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 (Baard et al.).

Besides degrading toxic compounds and organic detritus 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 (Adeleke et al.; Mastouri & Bergstrom). For example, in Mastouri & Bergstrom's 2010 dissertation on the effects of *Trichoderma* ssp. in promoting tomato plant growth under abiotic stress, *Trichoderma* ssp., fungal endophytes, significantly increased seed germination rates and promote strong root growth and deep root penetration (Mastouri & Bergstrom). A second example can be seen with *B. phytofirmans*, which have been shown to regulate cellular homeostasis, transcription, and ROS detoxification to improve the fitness of potatoes in drought (Fadiji & Babalola). 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, plant growth may not be enhanced (Baard et al.).

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 (Adeleke et al.; Baard et al.). *B. vietnamiensis* inoculation in wild cottonwood induced IAA production, which in turn led to an increase in dry biomass, root length, and nitrogen content (Fadiji & Babalola). *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 (Fadiji & Babalola).

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.

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; they have been reported to control borer insects that attack sorghum seedlings (Fadiji & Babalola).

Though Orr & Nelson, Tewari et al., and Baard et al. initially asserted that endophytic biocontrol of pathogens is dependent on endophytes-producing compounds, Mastouri & Bergstrom, Verma et al., and Adeleke et al. assert that biocontrol is most related to the endophytes' abilities to alter plant gene expression. Endophyte-produced volatile organic compounds (VOCs) and terpenoids, steroids, flavonoids, alkaloids, and peptides are recognized by plant receptors, which only then trigger an effective immune response with multiple hormonal resistance patterns (Adeleke et al.; Bubici et al.). Two types of plant resistance pathways exist. Induced systemic resistance (ISR) is resistance triggered by a pathogen and produces ethylene and jasmonic acid, compounds heavily 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 mediating 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 (Fadiji & Babalola).

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 (Orr & Nelson; Tewari et al.; Baard et al.).

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 be antimicrobial, antitumor, immunosuppressant, and surfactant (Raaijmakers et al.). Depending on the number and configuration of the amino acids in the peptide chains, LPs of *Bacillus* can be classified including 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) (Raaijmakers et al.; Katz & Demain). LPs of *Pseudomonas* can be classified as 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 (Raaijmakers et al.).

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 ($\sim 1 \mu\text{M}$) of surfactin inhibited fungal aerial hyphae growth in the rice pathogen *M. grisea*, effectively preventing the fungus from releasing spores and disseminating (Raaijmakers et al.). 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 (Raaijmakers et al.).

In general suppression, endophytes such as *BS B6* produce siderophores, which limit the available soil iron content, a major limiting factor for host pathogens (Baard et al.). Additionally, bacteria of the genus *Bacillus* will produce 167 antibiotics, 66 of which are from *BS* and 23 of which are from *Bacillus brevis* (Katz & Demain). 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 (see "Mechanisms of *Fusarium*"; Bonanomi et al.). By decreasing intracellular pH and hygroscopicity of the host, endophytic inhibition of fungal growth decreased (Raaijmakers et al.). Katz & Demain found that a decline in bacitracin production by *Bacillus licheniformis* was "due to the low pH caused by the organism's metabolism" (p. 451). Furthermore, hydroxamate siderophores—low-molecular-weight iron-chelating agents—are known to undergo pH-dependent transformations that significantly affect their iron-binding capacity (Neilands, 1981; Fadiji & Babalola). This, in turn, promotes plant growth by aiding in DNA synthesis and reproduction and limits pathogenic fungal growth (responding to comment #7). 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 (responding to comment #5) (Sivasakthi et al., 2014).

Conclusion

As extensive as the research that has been conducted is, there remains a significant gap in the research knowledge. The current literature has only studied endophytic biocontrol under stable and ideal conditions optimal to endophytic biocontrol. Fadiji & Babalola and Verma et al. identified the need for more focused work in adapting endophyte application to agricultural conditions for biotic/abiotic stress tolerance.

For example, in their in vitro pot trials, Orr & Nelson identified a combination of abiotic factors that decreased *Fusarium* severity. However, they have yet to test *Fusarium* in field conditions, and they speculate that their experimental setup would need to be manipulated into field settings and combinations of region, soil types, and crop cultivars revised. Another example can be seen with seed germination rates boosted by endophytic symbiosis. Sorghum seeds that exhibit a 90% viability using standard germination tests have drastically reduced emergence rates in the field (Mclaren & Rothmann). 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 have proven successful, and even then, these results have not yet been validated under field conditions (Bubici et al.). Tewari et al. attribute these differences to the unpredictable and dynamic variations in soil attributes such as the pathogenicity and competition from soil-borne fungi.

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 (Woo et al., 2020; Sivasakthi et al.; Tewari et al.). More specifically, under field conditions, fusarium wilt of banana was been controlled by *BS* with a 55% efficacy (Bubici et

al.). *PF*, from *O. europaea*, was reported to control fusarium wilt of banana with a 79% efficacy and fusarium wilt of chickpea (Bubici et al.).

Without studies to validate the efficacy of endophytic biocontrol in field conditions, such research will have no basis by which field application can be supported. Therefore, this research's primary goal is to validate the in vitro efficacy of *BS* and *PF* in controlling fusarium wilt in sorghum crops but under field conditions by simulating the abiotic stressors present in Kenyan regions where *Fusarium* sorghum wilt is pervasive. Furthermore, because this research is geared toward investigating if endophytes can be applied in 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°N-5°S and 32°E-40°E, the pH is generally moderately acidic (5.2-6.0), with parts slightly acidic (6.1-6.5) (Agegnehu et al., 2021) (responding to comment #6). During the planting/wet seasons, the soils absorb significant amounts of water, supporting crop growth and soil waterlogging (responding to comment #1). 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. Responding to comment #9; organic matter content and nutrients are insignificant in field conditions due to heavy leaching and topsoil erosion. Testing the potential of endophytes by testing if endophytes are effective at the minimum degree of fertilization.

Another large gap in the current literature is the inability of researchers to accurately isolate the effect of individual abiotic factors on *Fusarium*'s antagonistic or endophytic biocontrol abilities. As described in "Mechanisms of *Fusarium*", studies that try manipulating a single variable cannot measure the effect of individual variables given the soil's complexity (e.g., manipulating bioavailability of soil nutrients influence soil pH or soil temperature influencing soil moisture content, aeration, and nutrient content) (Ngunjiri, 2016; Orr & Nelson; Ferrocino et al.; Peng et al., 1999 and Cao et al., 2016, as cited in Orr & Nelson). Therefore, multiple abiotic factors such as low pH, heat stress, and excess soil moisture will be studied in tandem. Responding to comment #8 and comment #9. This will most effectively simulate Kenyan field conditions as well.

If successful, such research will provide impoverished 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 (Tewari et al.). Once endophytic metabolites are extracted and manufactured, commercial formulation abilities will become more stable than pure endophyte biocontrol (Bubici et al.) Given the ability for endophytes to enter the host system via rhizosphere roots or the phyllosphere stomata, field application methods can include seed coating, soil drenching, or foliar feeding (Kim et al., 2023).

Methods

Design

Fusarium wilt is transmitted to sorghum plants through two vectors: via uptake or nematode feeding from soil that was 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 truly inhibit *Fusarium* growth. 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 and symptoms of *Fusarium* wilt, identified as either low seed germination success rates or seedling wilt.

FO also thrives in warmer and wetter weather (e.g., 29° C and humid) and is more severe in acidic soils (e.g., 5-6) (Schuh, 2021). Therefore, the suboptimal conditions that *FO*, *BS*, and *PF* were placed in included heat stress/higher temperatures, pH stress/acidic soil, and moisture stress/high moisture levels to match those of Kenya during the wet planting seasons (responding to comment #1).

Apparatus and Materials

The school science laboratory provided potato dextrose agar and nutrient agar, in addition to incubators, ovens, microwaves, sterile Petri plates, distilled water, grow lights, bleach, pH test strips, sealable containers, plastic wrap, inoculating loops, and corn syrup. Sargent Welch supplied one slant each of *P. fluorescens* and *B. subtilis* (strain designation unknown due to years of subculturing). Carolina Biological supplied a tube of *F. oxysporum*, strain designation IMI 141140 (responding to comment #4). Two hundred Dale Sugar Sorghum seeds were purchased from Amazon. Clay loam soil and sphagnum peat moss were obtained from Lowe's Home Improvement. *Stapplet.com* was used to analyze statistical data.

Procedures

First, twelve grams of nutrient agar and twenty grams of potato dextrose agar (PDA) were weighed with a weighing dish and electronic balance 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. Next, the solution was sterilized by microwaving the beaker until the colloid solution was completely clear. After letting it cool, the solution was poured into sterilized Petri dishes. The agar dishes were incubated for 24 hours at 27° and checked for contamination. Fresh plates were selected to be used in experimentation. Next, a sterilized inoculation 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. Then, *FO* was streaked along the diameter of two plates and *PF* was streaked on ends perpendicular to the *FO* streak in each plate. The inoculating loop was sterilized between streaks. Each plate was sealed with Parafilm. The plates were then incubated according to experimental design: for testing the effects of endophytic bacteria under optimal conditions without seeds, they were incubated at 27° C for five days (responding to comment #2), 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° C for four days (responding to comment #2), taking pictures and recording fungal areas daily.

15 mL of corn syrup was mixed with 300 mL of distilled water to create a five-percent sugar solution. A large sample of *FO* inoculum was added to the solution and mixed vigorously.

A microwavable container was filled with twenty-four cups of Miracle Gro's cactus mix soil (sandy loam and acidic) and microwaved for 90 seconds. Sterilized soil was rehydrated with distilled water. Fifteen sterilized Petri dishes were filled with sterilized cactus soil. A microwavable container was filled with twenty-four cups of Miracle Gro's potting soil (loamy and with a pH of 7, responding to comment #3) 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. Each plate was sealed with parafilm. The liquid culture was incubated at 27° C for five days to establish significant fungal growth. 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. They were incubated and hydrated according to experimental design: for testing the effects of endophytic bacteria under optimal conditions, five dishes of *FO* only, five of *BS* and *FO*, and five of *PF* and *FO* were incubated at 25° C for ten days (and no additional moisture was added); for testing effects of endophytic bacteria under suboptimal conditions, five dishes of *FO* only, five of *BS* and *FO*, and five of *PF* and *FO* were incubated at 32° 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, responding to comment #3) 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 under 27° C. This 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 with Parafilm. A fine needle was brushed against the *FO* culture and then each stem was pierced in six of the cups. A sterilized fine needle was used to pierce stems in the remaining two cups to act as a control. Responding to comment #11. Each cup was sealed with Parafilm. The three cactus soil cups were incubated at 32° C for five days under a grow light. The three control soil cups were incubated at 25° C for five days under a 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 nutrient concentrations (Hu et al., 2019). Additionally, MiracleGro slow-release nutrients are designed to be released over two months, and those released over the experimental period are negligible. Responding to comment #3.

Safety

All experimentation involving bacteria *PF* and *BS* and fungus *FO*, species all identified as BSL 1, was conducted under a biosafety containment hood. Chemical disinfectants were used to clean the workspace area and all equipment every day. A lab apron, goggles, and gloves were worn. Standard lab safety precautions, such as not eating/drinking in the work area, washing hands after managing all materials, and being familiar with written instructions regarding handling the species, were followed. General caution was exercised when working with a Bunsen burner for inoculation loop sterilization.

Results

If the endophytic bacteria yielded smaller fungal areas, higher seed germination success rates, and fewer symptoms of fusarium wilt, then it can be concluded that the endophytic bacteria *BS* and *PF* have a limiting effect on the growth of fusarium wilt in sorghum plants under abiotic stress. The null hypothesis is that the endophytic bacteria *BS* and *PF* do not affect the growth of fusarium wilt in sorghum plants under abiotic stress.

The changes in fungal areas in optimal (defined as incubated at 27°C) and suboptimal (defined as incubated at 32°C) conditions are shown in Table 1 and Table 2, respectively. Responding to comment #2

Table 1

FO Fungal Areas (cm²) Under Optimal Conditions in the Presence of Endophytes

<u>Endophyte</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
No endophyte	18.85	22.95	22.62	26.41
<i>BS</i>	15.93	12.25	18.43	16.96
<i>PF</i>	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 (cm²) Under Suboptimal Conditions in the Presence of Endophytes

<u>Endophyte</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
No endophyte	15.08	21.99	20.89	21.36
<i>BS</i>	10.21	12.72	13.19	
<i>PF</i>	7.54	4.71	11.31	8.80

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

This data was analyzed using a one-way ANOVA test with a set .05 alpha level. The calculated p-value of Table 1 was .008, meaning the null hypothesis can be rejected, and the p-value of Table 2 was <.001, meaning the null hypothesis can also be rejected. Tables 1 and 2 show the fungal areas with endophytes *BS* and *PF* being significantly less compared to those without endophytes. Responding to comment #12.

The number of germinated seeds of five groups in optimal (defined as incubated at 27°C) and suboptimal (defined as incubated at 32°C) conditions are shown in Table 3 and Table 4, respectively. Responding to comment #2.

Table 3

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

<u>Endophyte</u>	<u>Group 1</u>	<u>Group 2</u>	<u>Group 3</u>	<u>Group 4</u>	<u>Group 5</u>
No endophyte	5	5	5	5	6
<i>BS</i>	6	6	5	6	5
<i>PF</i>	6	6	6	6	5

Note: Each group had six seeds. 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

<u>Endophyte</u>	<u>Group 1</u>	<u>Group 2</u>	<u>Group 3</u>	<u>Group 4</u>	<u>Group 5</u>
No endophyte	0	2	2	4	5
<i>BS</i>	4	5	5	6	5
<i>PF</i>	3	5	4	3	3

Note: Each group had six seeds. 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 with an alpha level set at .05. 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 can be rejected for Table 4 only. Table 4 shows the endophyte *BS* and *PF* significantly improving sorghum germination rates compared to no endophyte. Responding to comment #12.

The symptoms of wilt for six sorghum plants under optimal (defined as incubated at 27°C, no excess soil moisture, soil pH of 7) and suboptimal (defined as incubated at 32°C, excess soil moisture, soil pH of 6) conditions are shown in Table 5 and Table 6, respectively. Responding to comment #2.

Table 5

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

<u>Fusarium wilt</u>	<u>No Fusarium (control, responding to comment #11)</u>	<u>No endophyte</u>	<u><i>BS</i></u>	<u><i>PF</i></u>
No wilt	6	1	4	6

<i>Wilt</i>	0	5	1	0
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Note: n=6 seedlings. Symptoms of Fusarium wilt was 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

<u>Fusarium wilt</u>	<u>No Fusarium (control, responding to comment #11)</u>	<u>No endophyte</u>	<u><i>BS</i></u>	<u><i>PF</i></u>
No wilt	6	2	6	5
<i>Wilt</i>	0	4	0	1

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

This data was analyzed with a Chi-squared test with an alpha level set at .05. 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 can be rejected for Table 5 and Table 6's *BS* vs. no endophyte group. Table 5 shows the presence of endophytes *BS* and *PF* in cups helping to decrease instances of fusarium wilt (only 1 plant displayed symptoms of wilt) compared to cups without endophytes, which had 5 wilted plants. Table 6 reflects a similar pattern. Responding to comment #12.

Discussions

This study investigated the efficacy of the bacterial endophytes *BS* and *PF* in controlling Fusarium wilt in sorghum plants under environmental stresses, and the hypothesis was that the endophytes would control the fungal disease. This hypothesis is supported by the results. First, the observed decrease in fungal areas between units with endophytes and units without endophytes is statistically significant in both optimal and suboptimal conditions. This experiment serves as the control group and proves that the endophytic *BS* and *PF* strains used are healthy and effective in vitro, just as previous studies have proved. Because the t-test declared that the number of seeds germinated in *FO* soil is statistically significantly less than the number of seeds germinated in the presence of *BS*, it can be concluded that *BS* is effective in controlling Fusarium wilt's effects in sorghum plants under suboptimal conditions. This conclusion is again reflected by the ANOVA test results for symptoms of Fusarium wilt in seedlings. Therefore, the endophytic bacteria *BS* can control 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 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 insect transmission. However, given the study's small sample size, conclusions should be made cautiously; replicating this study with greater sample sizes

could potentially prove the efficacy of *PF* in controlling *Fusarium* as well. 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 tied 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 (responding to comment #4), and investigating the effects of *BS* on the location's microbiome to identify any adverse effects the endophytes would have on ecosystems.

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* 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, *Bacillus* can also be used to simultaneously increase production. This way, by implementing *Bacillus*, farmers can maximize sorghum and general crop yield, increasing food accessibility in the face of a rising population.

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Thank you for addressing my comments. However, the manuscript requires more revisions. I have marked the locations with highlight and bold text in the manuscript that is attached to this review.

Using Bacterial Endophytes to Treat *Fusarium*-Induced Seedling Wilt in *Sorghum bicolor* Under Abiotic Stresses

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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, but 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 and has identified a major gap in the knowledge: 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 plates and in sorghum plants under simulated abiotic conditions to discover if endophytes can still be successfully applied under suboptimal conditions for possible agricultural 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 successfully limited *Fusarium* growth; in planta, only *Bacillus subtilis* was found to be successful in limiting fusarium wilt.

Keywords

Fusarium wilt, endophyte, biocontrol, abiotic stress, agricultural control

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Using Bacterial Endophytes to Treat *Fusarium*-Induced Seedling Wilt in *Sorghum bicolor* Under Abiotic Stresses

In the next 30 years, the global human population will 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 individual 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 has caused issues such as reduced soil fertility, bioaccumulation of heavy metals, enormous 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: according to Tewari et al., 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 have proven to be ineffective as well, especially in the face of long-lasting outbreaks, and 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 USD per year lost in the global market (6). One of the most significant plant diseases is Fusarium wilt, a deadly fungal infection caused by the fungus *F. oxysporum*

(*FO*). *FO* is one of the main diseases affecting economically significant crops in Southern Africa, causing rots, blights, and cankers 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).

Because of recent developments in biotechnology, scientists have increasingly turned toward biological control options as a more novel 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 toxins 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*, however, have shown previous potential in limiting the proliferation of *FO* in crops by producing antifungal metabolites and changing the expression of plant proteins (9-10). The *Bacillus* and *Pseudomonas* species investigated in this study are harmless to healthy humans (i.e., not immuno-compromised); hence, ingesting these plants that contain these bacterial species is unlikely to cause bacterial disease (11-12). However, research has only studied endophytic biocontrol behavior under relatively favorable conditions in vitro (e.g., ideal moisture, temperature, and nutrient levels) and has not successfully considered endophyte success under more realistic conditions, leading to a gap in the current knowledge (2, 13).

The objective of this study, then, 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 areas, 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 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 that have these 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 62×10^6 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 and 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 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 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 impacts of *FO* in Sub-Saharan Africa on sorghum crops and the mechanisms of endophytes and fungal pathogens must be examined.

Mechanisms of Fusarium

The specific incidence and severity of fusarium wilt can vary considerably between agricultural zones and subzones within Kenya given 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 high dews and humidity--caused by later plantings during the wet season in East African countries--that help *FO* species 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). In Wrather & Sweet's study of the relationship between abiotic conditions and fusarium wilt in bananas, a low soil pH of 5 enhances *FO* growth (17). However, other studies have cast doubt on the veracity of pH experiments, citing that studies that try 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* have been found in soils with low pH, such an observation has not been reproduced experimentally (20). Indeed, manipulating soil pH alters 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 are directly correlated with disease severity, while nitrate to ammonium ratio, organic matter content, calcium, zinc, silicon, potassium, phosphorus, and boron are inversely correlated (18, 21).

There are additional contradictions within the literature. With regards to the effect of soil temperatures on *FO* disease severity, Wrather & Sweet's study has found that cooler soil temperatures result in greater *FO* growth, while Ferrocino et al. and McLaren & Rothmann have found that soil temperature is instead positively correlated with disease incidence. Acknowledging this discrepancy, Orr & Nelson suggest that because temperature is a difficult variable to control practically, given the vast tracts of land (as opposed to greenhouses) crops are grown on, it is 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* will produce a variety of mycotoxins--a group of secondary toxic metabolites such as fumonisins, moniliformin, trichothecene, deoxynivalenol, fumonisin-B1, moniliformin, and beauvericin--that are responsible for fusarium wilt in sorghum, as well 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 being employed to protect sorghum crops from *FO* infection, it is difficult to eliminate *Fusarium* from fields. Chemical pesticides can almost eliminate the soil microbiome and can cause issues for the entire ecosystem (9). Furthermore, *FO* is a vascular pathogen, it escapes contact with fungicides. While the selection of a *FO*-resistant variety may be a productive alternative, a widely accessible and affordable one has not been found yet (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).

Mechanisms of Endophytes

First termed by German botanist Anton de Bary in 1866, *endophyte* referred 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 commercial antibiotic, anticancer, antidiabetic, immunosuppressant, and antiviral drugs (20, 22). Indeed, they have a considerable biotechnological potential that is yet to be explored.

There has evolved to be an incredible diversity of endophytes that colonize plants in a variety of climates and ecological niches (2). Endophytes can be classified based on ecology: clavicipitaceous or non-clavicipitaceous; sexual or asexual reproduction; vertical or horizontal host transmission; biotrophic or necrotrophic nutrient obtainment; symptomatic or asymptomatic infection expression; and foliar or root host entry (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 pathogenic or beneficial relationships. Facultative endophytes are host-optional and develop in rhizospheric soil. Passive endophytes do not intentionally colonize the host, but they rather 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* in the leatherleaf plant (2, 4). However, endophytes can be applied in 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, both Johnston-Monje & Raizada and Adeleke et al. suggest that the host's roots are the first channel through which an endophyte penetrates the plant.

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, studies from 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. 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 interaction 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 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, in Mastouri & Bergstrom's 2010 dissertation on the effects of *Trichoderma* ssp. in promoting tomato plant growth under abiotic stress, *Trichoderma* ssp., fungal endophytes, significantly increased seed germination rates and promote strong root growth and deep root penetration (13). A second example can be seen with *B. phytofirmans*, which have been shown to regulate 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, plant growth may not be enhanced (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; they have been reported to control borer insects that attack sorghum seedlings (2).

Though Orr & Nelson, Tewari et al., and Baard et al. initially asserted that endophytic biocontrol of pathogens is dependent on endophytes-producing compounds, Mastouri & Bergstrom, Verma et al., and Adeleke et al. assert that biocontrol is most related to the endophytes' abilities to alter plant gene expression. Endophyte-produced volatile organic compounds (VOCs) and terpenoids, steroids, flavonoids, alkaloids, and peptides are recognized by plant receptors, which only 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 heavily 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 mediating 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 be antimicrobial, antitumor, immunosuppressant, and surfactant (10). Depending on the number and configuration of the amino acids in the peptide chains, LPs of *Bacillus* can be classified including 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 as 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 ($\sim 1 \mu\text{M}$) 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 limiting factor for host pathogens (5). Additionally, bacteria of the genus *Bacillus* will 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 (see "Mechanisms of *Fusarium*"; 21). By decreasing 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" (p. 451). 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

As extensive as the research that has been conducted is, there remains a significant gap in the research knowledge. The current literature has only studied endophytic biocontrol under stable and ideal conditions optimal to endophytic biocontrol. Fadiji & Babalola and Verma et al. identified the need for more focused work in adapting endophyte application to agricultural conditions for biotic/abiotic stress tolerance.

For example, in their in vitro pot trials, Orr & Nelson identified a combination of abiotic factors that decreased *Fusarium* severity. However, they have yet to test *Fusarium* in field conditions, and they speculate that their experimental setup would need to be manipulated into field settings and combinations of region, soil types, and crop cultivars revised. Another example can be seen with seed germination rates boosted by endophytic symbiosis. Sorghum seeds that exhibit a 90% viability using standard germination tests have drastically 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 have proven successful, and even then, these results have not yet been validated under field conditions (20). Tewari et al. attribute these differences to the unpredictable and

dynamic variations in soil attributes such as the pathogenicity and competition from soil-borne fungi.

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 been controlled by *BS* with a 55% efficacy (20). *PF*, from *O. europaea*, was reported to control fusarium wilt of banana with a 79% efficacy and fusarium wilt of chickpea (20).

Purged repetition. Furthermore, because this research is geared toward investigating if endophytes can be applied in 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°N-5°S and 32°E-40°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 and 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 inability of researchers to accurately isolate the effect of individual abiotic factors on *Fusarium*'s antagonistic or endophytic biocontrol abilities. As described in "Mechanisms of Fusarium", studies that try manipulating a single variable cannot measure the effect of individual variables given the soil's complexity (e.g., manipulating bioavailability of soil nutrients influence soil pH or soil temperature influencing 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. This will most effectively simulate Kenyan field conditions as well.

If successful, such research will provide impoverished 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 for endophytes to enter the host system via rhizosphere roots or the phyllosphere stomata, field application methods can include seed coating, soil drenching, or foliar feeding (32).

Methods

Design

In the field, fusarium wilt is **(present tense because *FO* thrives in warmer and wetter weather in normal field conditions, so it is not part of the procedure)** transmitted to sorghum plants through two vectors: via uptake or nematode feeding from soil that had 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 and symptoms of fusarium wilt, identified as either low seed germination success rates or seedling wilt (33).

FO also thrives in warmer and wetter weather (e.g., 29° C and humid) and is more severe in acidic soils (e.g., 5-6) (34). Therefore, the suboptimal conditions that *FO*, *BS*, and *PF* had been placed in included heat stress/higher temperatures, pH stress/acidic soil, and moisture stress/high moisture levels to simulate those of Kenya during the wet planting seasons.

Apparatus and Materials

The school science laboratory provided potato dextrose agar and nutrient agar, in addition to incubators, ovens, microwaves, sterile Petri plates, distilled water, grow lights, bleach, pH test strips, sealable containers, plastic wrap, inoculating loops, and corn syrup. Sargent Welch (based in Rochester, NY, USA) supplied one slant each of *P. fluorescens* and *B. subtilis* (strain designation unknown due to years of subculturing). Carolina Biological (based in Burlington, NC, USA) supplied a tube of *F. oxysporum*, strain designation IMI 141140. Two hundred Dale Sugar Sorghum seeds (ASIN ID: B07FVLZTS3) had been purchased from Amazon. MiracleGro clay loam soil and MiracleGro sphagnum peat moss had been obtained from Lowe's Home Improvement. *Stapplet.com* had been used to analyze statistical data.

Procedures

First, 12.00 grams of nutrient agar and twenty grams of potato dextrose agar (PDA) had been weighed with a weighing dish and electronic balance ($d=0.01g$) and then transferred to one-liter beakers. Then, 500 mL of distilled water had been measured with a graduated cylinder and poured into each one-liter beaker. The agar had been stirred until fully dissolved. Next, the solution had been sterilized by microwaving (General Electric household microwave oven model no. JES737WM01, 1.2 kW) the beaker until the colloid solution was completely clear. After letting it cool, the solution had been poured into sterilized 90x15mm plastic Petri dishes. The agar dishes had been incubated for 24 hours at 27°C and checked for contamination. Fresh plates had been selected to be used in experimentation. To create *BS*-, *PF*-, and *FO*-inoculated agar, a sterilized inoculation loop had been used to streak samples of *BS*, *PF*, and *FO* from the 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.

Next, a sterilized inoculation loop had been used to streak *FO* along the diameter of two plates. Then, *BS* had been streaked on ends perpendicular to the *FO* streak in each plate. Then, *FO* had been streaked along the diameter of two plates, and *PF* had been streaked on ends perpendicular to the *FO* streak in each plate. The inoculating loop had been sterilized between streaks. Each plate had been sealed with Parafilm^R. Then, the plates had been incubated according to experimental design: for testing the effects of endophytic bacteria under optimal conditions without seeds, they had been incubated at 27°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 had been incubated at 32°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 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 the length from end to end $((A+B) \times 0.45 \times \text{Length})$.

15 mL of corn syrup had been mixed with 300 mL of distilled water to create a 4.76% sugar solution. A square-inch-sized sample of *FO* inoculum had been added to the solution and mixed vigorously. A microwavable container had been filled with twenty-four cups of Miracle Gro's cactus mix soil (sandy loam and acidic) and microwaved for 90 seconds to sterilize the soil ("Sterilizing Potting Soil", 2021, 2:50). Although there is no guarantee that colonization would have occurred in non-sterilized soil, the soil in this study had been sterilized to eliminate any confounding variables from the study (35). Sterilized soil had been rehydrated with distilled water. Fifteen sterilized Petri dishes had been filled with sterilized cactus soil. A microwavable container had been filled with twenty-four cups of Miracle Gro's potting soil (loamy and with a pH of 7) and microwaved for 90 seconds. Sterilized soil had been rehydrated with distilled water. Fifteen sterilized Petri dishes had been filled with sterilized regular soil. The liquid culture had been poured into thirty sterilized soil plates, thus forming *FO*-inoculated cactus soil and regular soil Petri dishes. Each plate had been sealed with Parafilm^R. The liquid culture had been incubated at 27°C for five days to establish significant fungal growth. 216 sorghum seeds had been sterilized using a 50% bleach solution with agitation and then rinsed three times with water. Sixty seeds had been brushed with *BS* and divided among five *FO*-inoculated cactus soil and five *FO*-inoculated regular soil Petri dishes. 60 seeds had been brushed with *PF* and divided between five *FO*-inoculated cactus soil and five *FO*-inoculated regular soil Petri dishes. The remaining sixty seeds had been divided among five *FO*-inoculated cactus soil and five *FO*-inoculated regular soil Petri dishes. Each plate had been sealed with Parafilm^R. They had been incubated and hydrated according to experimental design: for testing the effects of endophytic bacteria under optimal conditions, five dishes of *FO* only, five of *BS* and *FO*, and five of *PF* and *FO* had been incubated at 25° C for ten days (and no additional moisture was added); for testing effects of endophytic bacteria under suboptimal conditions, five dishes of *FO* only, five of *BS* and *FO*, and five of *PF* and *FO* had been incubated at 32°C for ten days. 15 mL of distilled water had been added to each dish.

Four sterilized cups had been filled with sterilized cactus soil (sandy and pH of 6) and another four with sterilized regular soil. Six seeds had been germinated with a square-inch-sized chunk of *BS*-inoculated agar in each of the two soil types under 27°C. This had been 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 had been sealed completely with Parafilm^R. After germination, the Parafilm^R cover had been modified to form a 6-inch dome to allow for vertical growth. 8 days after germination, a fine needle had been brushed against the *FO* culture and then each stem had been pierced in each of the six cups. A sterilized fine needle had been used to pierce stems in the remaining two cups to act as a control. Each cup had been sealed with Parafilm^R. The three cactus soil cups had been incubated at 32°C for five days under a wavelength of 225.00 nm and 17600.00 lux grow light for a 16-hour on-off cycle. The three control soil cups had been incubated at 25° C for five days under a 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 (agree with you, found another source that specified that sterilization of soil decreased the amount of available nutrients, found significance too)

nutrient concentrations (36). Additionally, MiracleGro slow-release nutrients are designed to be released over two months, and those released over the experimental period are negligible.

Safety

All experimentation involving bacteria *PF* and *BS* and fungus *FO*, species all identified as BSL 1, had been conducted under a biosafety containment hood and appropriate BSL 1 precautions were taken. + deleted “chemical disinfectants...loop sterilization”

Results

If the endophytic bacteria yielded smaller fungal areas, higher seed germination success rates, and fewer symptoms of fusarium wilt, then it can be concluded that the endophytic bacteria *BS* and *PF* have a limiting effect on the growth of fusarium wilt in sorghum plants under abiotic stress. The null hypothesis is that the endophytic bacteria *BS* and *PF* do not affect the growth of fusarium wilt in sorghum plants under abiotic stress.

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

Table 1

FO Fungal Areas (cm²) Under Optimal Conditions in the Presence of Endophytes

<u>Endophyte</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
No Endophyte	18.85	22.95	22.62	26.41
<i>BS</i>	15.93	12.25	18.43	16.96
<i>PF</i>	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 (cm²) Under Suboptimal Conditions in the Presence of Endophytes

<u>Endophyte</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
No Endophyte	15.08	21.99	20.89	21.36
<i>BS</i>	10.21	12.72	13.19	
<i>PF</i>	7.54	4.71	11.31	8.80

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

This data was analyzed using a one-way ANOVA test with a set .05 alpha level. The calculated p-value of Table 1 was .008, meaning the null hypothesis can be rejected, and the p-value of Table 2 was <.001, meaning the null hypothesis can also be rejected. Tables 1 and 2 show the fungal areas with endophytes *BS* and *PF* being significantly less compared to those without endophytes.

The number of germinated seeds of five groups in optimal (defined as incubated at 27°C) and suboptimal (defined as incubated at 32°C) 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

<u>Endophyte</u>	No Endophyte	BS	PF
<u>Mean ± SD</u>	5.2 ± 0.44	5.6 ± 0.54	5.8 ± 0.44

Note: Each group had six seeds. 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

<u>Endophyte</u>	No Endophyte	BS	PF
<u>Mean ± SD</u>	2.60 ± 1.94	5.00 ± 0.70	3.60 ± 0.89

Note: Each group had six seeds. 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 with an alpha level set at .05. 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 can be rejected for Table 4 for the case of *BS* only. Table 4 shows the endophyte *BS* significantly improving sorghum germination rates compared to no endophyte.

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

Figure 1 shows an example of sorghum seedlings exhibiting evidence of fusarium wilt. Figure 2 shows an example of sorghum seedlings not exhibiting evidence of fusarium wilt.

Figure 1

Seedlings With Evidence of Fusarium Wilt



Figure 2

Seedlings Without Evidence of Fusarium Wilt



Table 5

Number of Plants with Symptoms of Fusarium Wilt in the Presence of Endophytes Under Optimal Conditions **added photo representations of wilt vs no wilt**

<u>Fusarium wilt</u>	<u>(Mean ± SD)</u>			
	<u>No Fusarium (control)</u>	<u>No endophyte</u>	<u>BS</u>	<u>PF</u>
Wilt	0.00 ± 0.00	0.83 ± 0.40	0.20 ± 0.44	0.00 ± 0.00

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

Table 6 (the means and SDs don't mean as much here i think because nature of data is binary; that's why I used a chi square test to analyze tables 5 and 6)

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

<u>Fusarium wilt</u>	<u>(Mean ± SD)</u>			
	<u>No Fusarium (control)</u>	<u>No endophyte</u>	<u>BS</u>	<u>PF</u>
Wilt	0.00 ± 0.00	0.66 ± 0.51	0.00 ± 0.00	0.16 ± 0.40

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

This data was analyzed with a **Chi-squared test (to fit the categorical data of having wilt or no wilt) (Used a Chi-squares test of independence for the second experimental group because I wanted to measure whether or not the plant experienced wilt, which was only categorical data. I used t-tests for the first experimental group because I wanted to do pairwise comparisons between groups to control for type 1 errors and avoid false positives)** with an alpha level set at .05. 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 can be rejected for Table 5 and Table 6's *BS* vs. no endophyte group. Table 5 shows the presence of endophytes *BS* and *PF* in cups helping to decrease instances of fusarium wilt (only 1 plant displayed symptoms of wilt) compared to cups without endophytes, which had 5 wilted plants. Table 6 reflects a similar pattern.

Discussions

This study investigated the efficacy of the bacterial endophytes *BS* and *PF* in controlling Fusarium wilt in sorghum plants under environmental stresses, and the hypothesis was that the endophytes would control the fungal disease. This hypothesis is supported by the results. First, the observed decrease in fungal areas between **plant cups and seeds Petri dishes** with endophytes and **plant cups and seeds Petri dishes** without endophytes is statistically significant in both optimal and suboptimal conditions. This experiment serves as the control group and proves that the endophytic *BS* and *PF* strains used are healthy and effective in vitro, just as previous studies have proved. Because the t-test declared that the number of seeds germinated in *FO* soil is statistically significantly less than the number of seeds germinated in the presence of *BS*, it can be concluded that *BS* is effective in controlling Fusarium wilt's effects in sorghum plants under suboptimal conditions. This conclusion is again reflected by the ANOVA test **results (You can find them underneath table 2!)** for symptoms of Fusarium wilt in seedlings. Therefore, the endophytic bacteria *BS* can control 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 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 insect transmission. However, given the study's small sample size, conclusions should be made cautiously; replicating this study with greater sample sizes could potentially prove the efficacy of *PF* in controlling *Fusarium* as well. 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 tied 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 ecosystems. Future studies should involve more cups to reduce bias. For example, 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. **Added means and SDs for Tables 3, 4, 5, and 6.** Additionally, because the soil in this study was sterile and no guaranteed colonization will occur in non-sterile soil, future studies should test the efficacy of *BS* and *PF* using direct 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* 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, *Bacillus* can also be used to simultaneously increase production. This way, by implementing *Bacillus*, farmers can maximize sorghum and general crop yield, increasing food accessibility in the face of a rising population.

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38. _____
39. accepted, will require extensive cleanup with regard to tense, grammar, language, sentence structure... and inclusion of a conclusion section.