THE ROLE OF RHIZOSPHERE BACTERIA VOCS ON *FUSARIUM OXYSPORUM* F.SP. *VASINFECTUM*

A Dissertation

by

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ABSTRACT

Cotton (*Gossypium spp*), a globally traded commodity, encounters numerous obstacles during the cultivation of both Upland (*G. hirsutum*) and Pima (*G. barbadense*) varieties. *Fusarium oxysporum* f.sp. *vasinfectum* race 4 (Fov4), is a virulent pathogen towards Pima cotton with a unique soil inhabiting lifestyle which makes managing disease difficult. Current disease management predominately utilizes fungicides for transporting disease-free seed, while biological controls are largely under-utilized. The introduction focuses on current research utilizing volatile organic compounds (VOC) from various antagonistic bacteria as biological controls against phytopathogens, including *Fusarium oxysporum* species. Two research objectives were aimed at identifying unique combinations of antagonistic bacteria capable of improving Fov4 inhibition with only VOCs. Fov4 inhibition by VOCs was investigated using bacteria isolated from cotton rhizosphere and bulk soil. Using partitioned petri dishes to physically separate all interested organisms, bacteria, Fov4, and Pima cotton, effects of bacterial VOCs were investigated. Combinations of *Bacillus* Rz141 with *Streptomyces* HC658, and *Paenibacillus* ELP529 with *Brevibacillus* Rz160, increased VOC inhibition to 26% and 27%, respectively. Suppression by VOCs extended to Fov4-infected Pima cotton resulting in symptom reduction. The third research objective was to identify differences in Fov4 genome compared to *Fusarium oxysporum* f.sp. *vasinfectum* race 1, which can help understand host specificity and virulence in Fov races. A putatively identified multi-facilitator superfamily transporter showed differential expression in Fov4 during infection of Pima cotton. A null mutant was generated and further characterized. Growth comparisons of wild type and mutant showed significant difference when grown on NaCl ($p<0.05$). Additional differences were seen in conidiation and pigment production, but no change in pathogenicity to Pima cotton when

compared to wild type. Based on characterization results, this putative MFS transporter was named *Fusarium oxysporum sodium related transporter*-1 (*FoSRT1*). In summary, research showed that soil bacteria VOCs have the potential as biocontrols for their ability to suppress Fov4 growth and Pima cotton infection. The influence of NaCl on Fov4 growth was shown, but unknown is why this gene was differentially expressed during infection. This body of work shows the importance of abiotic and biotic soil characteristics which contribute to Fov4 growth and virulence.

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Contributors

This work was supervised by a dissertation committee consisting of Professor Won-Bo Shim [advisor] and Associate Professor Young-Ki Jo, Assistant Professor Susie Dai of the Department of Plant Pathology and Microbiology and Professor Terry Gentry of the Department of Crop and Soil Sciences.

The gene knockouts in Chapter IV were completed by Zehua Zhou, visiting scholar from China. Preliminary data was conducted by undergraduate students Quinton Reneau and Cymone Campos. All other work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

Fov4	*Fusarium oxysporum* f.sp. *vasinfectum* race 4
Fov1	*Fusarium oxysporum* f.sp. *vasinfectum* race 1
BCA	Biocontrol agent
MVOC	Microbial volatile organic compound

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CHAPTER I

INTRODUCTION

Cotton is a natural plant fiber primarily used in the textile and clothing industry and an important globally traded commodity (NASS, 2018, OECD/FAO, 2016), with India, China, and the US as the major producing countries. Although cotton production acreage in the US has shown a recent decline, cotton is still recognized as a major cash crop in the southern US, and the socio-economic importance needs little introduction. Cotton (*Gossypium* spp) is cultivated in 17 states, with Texas as the top cotton producing state with more than 40% of the total US production (NASS, 2018). Upland cotton (*G. hirsutum*) is the predominant cotton species cultivated in the US. About 3% of the US production is Pima cotton (*G. barbadense*), a finer and higher value fiber, mostly grown in California and Arizona.

US cotton growers encounter numerous diseases caused by a variety of pathogens and pests, including bacteria, fungi, nematodes, and insects, which can also be exacerbated by inhospitable abiotic factors. In this review, we focus on a recent emergence of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 (Fov4) in California (Kim et al., 2005), New Mexico (Zhu et al., 2020), and Texas (Halpern et al., 2018). Eight nominal races of *F. oxysporum* f. sp. *vasinfectum* (Fov) are recognized as the causal agent of Fusarium wilt in cotton worldwide (Armstrong & Armstrong, 1960). Until 2004, race 1 (Fov1) was known to be the predominant Fov present in US cotton producing fields. Fov1, however, requires root-knot nematodes (RKN) to infect cotton, and therefore growers have been able to minimize the disease outbreaks with the use of nematicides and RKN tolerant cotton varieties. In 2004, Fov4 was first identified in the California San Joaquin Valley (Kim et al., 2005). This highly virulent race, formally found in India (Armstrong

& Armstrong, 1960), has since spread and now been confirmed in two west Texas counties during the 2016 crop season, causing field symptoms of dead seedlings and black streaks inside tap roots of wilting plants (Halpern et al., 2018, Yang et al., 2018a).

The recent discovery of Fov4 in fields in west Texas (Fig. 1A) county of El Paso has caused justifiable alarm in the cotton industry. Similar to Fov1, Fov4 colonizes roots and vascular system resulting in discoloration (Fig. 1B-C), wilting (Fig. 1D-E), and death. However, Fov4 does not require association with RKN for infection, thus limiting the effectiveness of Fov1-tolerant varieties and nematicides (Kim et al., 2005, Davis et al., 2006). In addition, due to its seed-borne and soil-borne characteristics, Fov4 can be carried via seeds and on equipment, raising concerns over its containment. Pima cultivars are particularly susceptible to Fov4, and this disease devastated the California Pima cotton industry (Kim et al., 2005). Fov4 is now considered an endemic pathogen in California. Geographically, Texas is also unique as the location where western Pima production transitions to Upland cotton production to the east. Though Upland cotton was once thought to be less susceptible to Fov4, we are now learning that Fov4 may be an inoculum density-dependent disease (Fig. 1F) and can pose serious threat to Upland cotton production as well (Ortiz et al., 2017). Currently there are no known Fov4-resistant cotton varieties documented. Thus, there is an urgent need to prevent further spread of the disease into the major cotton-producing region of the southeastern US. The need for alternative control measures of plant pathogens has led to a rise in research regarding biocontrol agents and even the use of microbial volatile organic compounds (MVOC). Some research has been dedicated to focusing on the MVOC effect on *Fusarium* species.

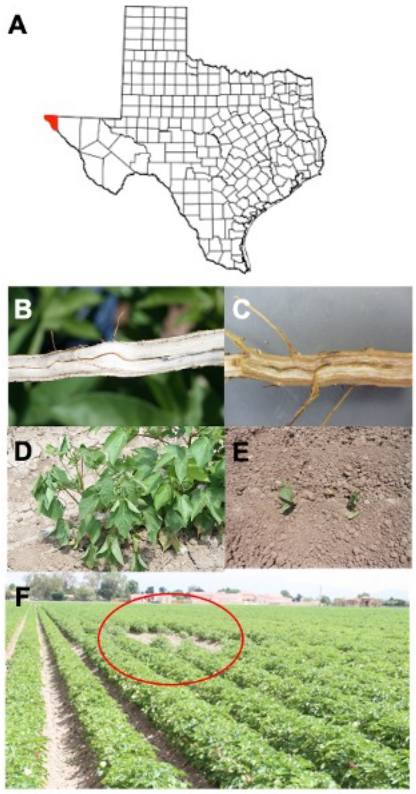

Figure 1. Common symptoms observed in Pima cotton fields infested with Fov4. (A) Location of Fov4 in El Paso County, Texas; (B-C) representative samples showing vascular darkening of Fov4 infected Pima cotton; (D) wilting symptoms of older Pima cotton plants, (E) wilting symptoms of young Pima cotton seedlings; (F) observations of dead spots indicative of Fov4 infested fields. *Photos B - F courtesy of Dr. Tom Isakeit, Texas A&M AgriLife Extension.*

F. oxysporum f. sp. vasinfectum biology and pathology

F. oxysporum species are often referred to as *Fusarium oxysporum* species complex (FOSC), with species grouped into *formae speciales* (f. sp.) primarily based on host specificity. FOSC host specificity and lineages (Ma et al., 2010, van Dam et al., 2016) are well studied, but race differentiation (*i.e.* the ability to infect specific crop cultivars) complicates *Fov* research. The first complete FOSC genome belongs to the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (*Fol*), which showed a large portion (~25%) of the genome harboring lineage-specific (LS)

genomic regions not observed in other sequenced *Fusarium* species (Ma et al., 2010). This comparative genomics study showed that LS regions are rich in transposons and genes that could explain *Fol* host specificity and pathogenicity, including a large set of secreted proteins that can serve as effectors to promote the infection process. Since this publication, other FOSC genomes have been completed and are publicly available, including multiple *Fov* races (Seo et al., 2020, van Dam et al., 2016).

Scientists now have a deeper understanding of the role of effector proteins and other virulence factors play in FOSC (Dai et al., 2016, Niu et al., 2016, van Dam et al., 2016). However, a recent comparative study revealed a very stringent host range in FOSC that may not be simply explained by the presence and absence of effectors and virulence factors (van Dam *et al* 2016). For instance, it is fascinating to discover that *F. oxysporum* f.sp. *niveum* (*Fon*) races are distinct from other FOSC species in that well-defined SIX effectors are not found in *Fon* race 2 genomes (Niu et al., 2016, van Dam et al., 2016) and are not key factors in *Fon* pathogenicity on watermelons. Even more puzzling is that four *Fon* race 2 isolates were segregated into two different subclades when cucurbit-infecting *F. oxysporum* species were phylogenetically analyzed (van Dam et al., 2016). While the presence and absence of putative effectors and virulence factors can help predict host specificity in these species, these data cannot fully explain the complex race structure and virulence mechanism in *Fon* race 2 against vulnerable watermelon varieties.

Current management strategies for cotton Fusarium wilt

As with many soil-borne diseases, the ability to develop and implement control strategies to reduce plant infection and curtail economic loss is challenging. A published report suggests

that long-distance movement of Fov4 is likely due to the contamination of seeds (Doan & Davis, 2015). Furthermore, Fov4 presents a set of lifestyle features, such as the production of chlamydospores that serve as hardy survival structures, that allows the pathogen to establish long-term presence in soil (Hao et al., 2009). Due to chlamydospores and the buildup of Fov4 in soil, approaches to decrease the level of inoculum have been attempted in soil and seed treatments have been important for identifying contaminated seed (Cianchetta & Davis, 2015, Liu et al., 2011). Reducing the spread of Fov4 can be accomplished through early detection using robust diagnostic testing (Ortiz et al., 2017, Crutcher et al., 2016).

Seed treatments through the use of thermotherapy (dry heat or hot water) and chemical fungicides do not entirely eliminate seed-borne Fov4, but effective treatments are those which do not decrease the vigor and germination of treated seed (Doan & Davis, 2015, Bennett & Colyer, 2010). Targeting soil-borne Fov4 has also shown effective reduction in Fov4 through the use of soil solarization, thermotherapy, or solarization in combination with chemical treatments (Cianchetta & Davis, 2015, Bennett et al., 2011). Unfortunately, since Fov4-resistant cotton cultivars are not currently available, fungicides such as carbendazim, thiophanate methyl, thiovit, and dithane M-45 have been frequently used to control Fov4 in Pima cotton (Asif et al., 2020). It is also important to note that since Fov4 does not require RKN for infection, the use of nematicides have not been useful against this race. Management of Fov4 and Fov1 would be most effective through host resistance, however it is unlikely that a Fov4-resistant Pima cotton cultivar will soon become available for growers. Fov4-resistance cultivars started with the Upland cultivar, which is more tolerant, but few Upland cultivars are resistant. Host resistance mechanisms to Fov1 and Fov4 have been identified in Pima and Upland cottons, but not

combined in one cultivar and continues to be the focus of research (Ulloa et al., 2013, Ulloa et al., 2016, Wang et al., 2018, Abdelraheem et al., 2020, Pei et al., 2020).

Biological Control of Soil-borne Fusarium Diseases

Biological controls have gained traction as an alternative to traditional chemical control measures against phytopathogens. Common antagonistic microorganisms studied for biocontrol potential are bacteria, such as *Bacillus* (Yuan et al., 2012), *Streptomyces* (Cordovez et al., 2015, Wu et al., 2015), *Paenibacillus* (Seo et al., 2016, Kim et al., 2004, Naing et al., 2015b), and *Brevibacillus* (Johnson et al., 2020, Yang & Yousef, 2018, Jiang et al., 2015) species. A large portion of biological control of *Fusarium* species has focused on the consortia of beneficial microbes that make up suppressive or conducive soils (Contreras-Cornejo et al., 2014, Mazzola & Freilich, 2017, Syed-Ab-Rahman et al., 2019, Zhou et al., 2019). For an introduced biocontrol agent(s) to persist and function in soil, abiotic and biotic environment must be adequately favorable for practical implementation, requiring comprehensive understanding of the targeted rhizosphere. Unfortunately, our understanding of interactions between the host, pathogen(s), and biocontrol agent(s) is ongoing. This limitation, particularly in soil environments, has been recognized in various earlier studies (Tilocca et al., 2020). Disease suppressive soils are recognized but not completely understood as to how they suppress disease. *Actinobacteria* are described by multiple sources as meeting the criteria for a biocontrol agent in suppressive soils because of slow growing and heat tolerant characteristics(Cha et al., 2016). While *F. oxysporum* species have been identified in both suppressive and conducive soils, the abundance is what determines infection of crops (Yuan et al., 2017, Cha et al., 2016). Based on the suppressive

phenotype, this reaffirms disease incidence dependence on the amount of inoculum as seen in *F. oxysporum* f.sp. *fragariae (Cha et al., 2016)* and *F. oxysporum* f.sp. *cubense* (Zhou et al., 2019).

Crop rotations have long been cited as a control measure against plant diseases by causing a less conducive environment for disease incidence. However, Cha *et al*. (2016) showed a field which had continuously grown strawberries for 15 years was better at suppressing *F. oxysporum* f.sp. *fragariae* than a different field growing monoculture for only 3 years (Cha et al., 2016). Based on the results from Cha *et al*. (2016), it would be interesting to determine how long it takes for suppressive soils to build up tolerance to a specific disease. If a soil can build up tolerance based on microbial communities in a long period of time, *e.g.* greater than ten years, should those fields not be subjected to crop rotation for fear of more detrimental loss because of reduced tolerance to other phytopathogens? Theoretically, microbial community structure could be tested as often as soil chemistry is tested to determine the ideal crop to grow.

Mechanisms of Biological Controls

Mechanisms associated with biological controls can vary as phytopathogens are also constantly evolving themselves. The key to an effective biocontrol agent is determining requirements of different host-pathogen interactions. Biocontrol agents can work from two different perspectives: direct or indirect antagonism. Direct pathogen antagonism can be accomplished through physical contact between biocontrol agents. Indirect biocontrol agents typically work through stimulating induced systemic resistance (ISR) to promote defense and plant growth that would otherwise not occur.

Direct antagonism between biocontrol agent and pathogen can be accomplished through antibiotics, toxins, and production of extracellular cell wall degrading enzymes (Yang et al.,

2008). Four major direct antagonistic bacteria *Paenibacillus* (Aktuganov et al., 2008a, Aktuganov et al., 2008b, Aktuganov et al., 2014, Singh & Chhatpar, 2011, Seo et al., 2016), *Bacillus* (Gu et al., 2017, Kim et al., 2017, Luo et al., 2019, Mihalache et al., 2018, Zouari et al., 2020), *Brevibacillus* (Johnson et al., 2020, Yang et al., 2018b, Yang & Yousef, 2018), and *Streptomyces* (Encheva-Malinova et al., 2014, Essarioui et al., 2017, Danial et al., 2020, Merrouche et al., 2020) have been studied extensively for using at least one of the mentioned antifungal mechanisms (Baindara et al., 2016, Aktuganov et al., 2014, Huang et al., 2013, Naing et al., 2015a, Tseng et al., 2019). For instance, specific secreted metabolites are able to target ergosterol synthesis (Jiang et al., 2015) or inhibit spore germination and mycelial growth of fungal pathogens (Danial et al., 2020). Indirect biocontrol agents can act upon other species within the soil environment. For example, soil bacteria producing insecticides to control nematode populations would be an indirect method of biocontrol (Li et al., 2005, Cheng et al., 2017, Prasanna et al., 2013).

Suppressive soils can putatively be classified as an indirect biocontrol agent. In general suppressive soil provides a basal defense against a broad range of pathogens (Cha et al., 2016) but we do not have a comprehensive understanding of how this pathogen suppression occurs. Manipulating soils to alter conducive soils into suppressive soils has been investigated as a means of biological control. Deng and colleagues (2021) proposed using combined biocontrol strategies to alter soil microbiomes for direct and indirect control of soil-borne pathogens. The authors showed a pre-plant fumigation followed by application of a bioorganic fertilizer, amended with *Bacillus amyloliquefaciens*, helped lower *F. oxysporum* abundance which was also sustained until harvest (Deng et al., 2021). In disease-conducive soils, microbial activity may actually be higher than disease-free soil (Zhou et al., 2019) leading to interesting questions

regarding the interactions between microorganisms in disease conducive soils. One key characteristic of soil microbial communities gaining interest is the role of microbial volatile organic compounds (VOCs) in disease suppressive functions and mediating communication between bacteria, fungi, and plants.

Microbial VOCs as a potential biological control agent

Microbial VOCs (Chaves-Lopez et al., 2015) have been studied for their roles as plant growth promoting organisms through the induction of ISR in host plants. While the use of microbial VOCs to trigger ISR can be considered as an indirect biocontrol method, it is reasonable to recognize that a direct inhibition of phytopathogens does occur upon application. Microbial VOCs may have a regulatory function in soil and other natural habitats, and the combined effort from several different organisms may result in a favorable or an inhospitable rhizosphere environment. The effects of rhizosphere microbial VOCs are also likely dependent upon concentration and relative abundance of key microbial species (Chaves-Lopez et al., 2015). While the study of biocontrol agents for the control of soil borne phytopathogens is vast and all-encompassing fungi and bacteria, we will focus on the impact of bacterial VOCs on *Fusarium* species in this review.

Bacterial VOCs have been shown to inhibit conidia germination, damage fungal cell walls, alter hyphae morphology and even kill fungal pathogens (Wu et al., 2015, Chaves-Lopez et al., 2015, Cordovez et al., 2015). However, fungal cells not completely killed have been shown to regain normal fungal growth after being removed from VOC sources (Wu et al., 2015). Whether these VOC-exposed fungi are still able to infect its host and cause disease is not clear. Additionally, how long the growth inhibition is sustained has not been defined. It is important to

note that bacterial species producing antifungal VOCs also produce compounds that stimulate ISR which can trigger indirect biocontrol mechanism.

A recent study suggests that suppressive soils inhibit *F. culmorum* infection on wheat cultivars due to its rhizobacterial communities and notably recognizes that a certain level of suppression is the result of microbial VOCs (Ossowicki A, 2020). The VOC mediated inhibition showed that emitted volatiles did reduce fungal growth and showed disease reduction in some plants exposed to suppressive soils. However, microbial VOC profiles did not show correlation between suppressive and conducive soils, thus Ossowicki and colleagues (2020) argued that the plant is an important determinant in labeling a suppressive or conducive soil. This can also be said about the VOC profiles of plants and soil microbial communities. VOCs can be either plant growth promoting, ISR stimulants, direct pathogen inhibitors, or nematicides (Syed-Ab-Rahman et al., 2019, Cheng et al., 2017, Lee et al., 2012). Interestingly, VOCs produced by *Paenibacillus polymyxa* and *P. ehmensis* have been studied for indirect mechanism of biocontrol through the control of root knot nematode (RKN) *Meliodogyne incognita* (Hong et al., 2013, Cheng et al., 2017).

Effects of bacterial VOCs on fungal pathogens

To be used as a biocontrol agent (BCA), bacterial VOCs must elicit specific detrimental effect on fungal pathogens. Knowing the specific effect is vital for its use in managing crop diseases. Application of VOCs has largely shown similar results across fungal species including inhibiting conidia germination and reduced mycelial growth (Chaves-Lopez et al., 2015). Reduced mycelial growth from *Bacillus* VOCs has been documented in multiple fungal pathogens, specifically *F. oxysporum* f.sp. *lactucae* (Chaves-Lopez et al., 2015), *Alternaria*

solani (Zhang et al., 2020), and *F. juroshium* (Guevara-Avendano et al., 2020). Similarly, *F. moniliforme* and *Rhizoctonia solani* exposed to *Streptomyces* VOCs also showed reduced mycelial growth (Cordovez et al., 2015).

More specific effects on fungal pathogens from bacterial VOCs have been documented and appear to be species specific. *Bacillus subtilis* VOCs applied to *Alternaria solani* resulted in denser mycelia with reduced pigment, decreased mycelia penetration ability, structural damage, and significant inhibition of spore germination (Zhang et al., 2020). Another study testing multiple fungal pathogens against *Bacillus* VOCs showed fungi with inhibited mycelial growth, supercoiled hyphae, swollen hyphae, and reduced pigment. In another study, *F. oxysporum* f.sp. *lactucae* only showed reduced conidiation during *Bacillus* VOC exposure (Chaves-Lopez et al., 2015). Additionally, two more studies showed that fungal growth, including *F. oxysporum*, was inhibited by bacterial VOCs likely through inhibiting conidia germination (Asari et al., 2016, Wu et al., 2015). After investigating literature in bacterial-fungal VOC communications, it is reasonable to suggest that *Fusarium* species exposed to bacterial VOCs show the common characteristic of reduced conidia germination after exposure to bacterial VOCs. While some fungi like *Alternaria solani* showed more structural damage, it is again evident that the effects of VOCs depend on the microbial community. Fungi can regain growth after removal from bacterial VOC suggesting that VOCs does not pose lethal threat to fungi. If VOCs do not completely kill fungal pathogens, the question of how VOCs can be used as effective BCAs still remains.

Effects of bacterial VOCs on improving plant health

Compounds which serve as plant growth promoters (PGP) have been explored as biocontrol agents to help plants fight off pathogen infection and colonization. Root colonizing bacteria, such as *Bacillus* species, typically emit VOCs that mimic plant hormones, thus promoting plant growth. Microbial VOCs help maintain a symbiotic relationship in the rhizosphere. Microbial VOCs 2,3-butanediol and 3-hydroxy-2-butanone (acetoin) are well known PGP biocontrol agents and have been used as exogenous applications for the stimulation of plant growth (Cordovez et al., 2015, Ryu et al., 2003, Asari et al., 2016, Farag et al., 2013). These two compounds have been found in both *Bacillus* (Asari et al., 2016) and *Streptomyces* (Cordovez et al., 2015) species. Both compounds grow on simple sucrose media but are not produced on more complex media composed of peptides and proteins (Delaplace et al., 2015, Farag et al., 2013, Asari et al., 2016). Interestingly, PGP microbial VOCs appear to be beneficial during early plant growth but as the plant[s] age there is a greater chance of plant death (Delaplace et al., 2015).

While VOC effects on plant growth and disease tolerance through ISR stimulation look promising, there are also many publications describing negative effects on plants exposed to VOCs from bacteria grown in different nutrient component media. While *Bacillus amyloliquefaciens* is an excellent root colonizer, sugar-based media showed improved above-ground biomass, on peptide/protein-based media there was no improvement on growth (Asari et al., 2016). Similarly, *Bacillus* VOCs on *Phytophthora capsici* showed varying effects with regards to plant health. *Arabidopsis thaliana* plants showed greater primary root length and total biomass. However, the same *Bacillus* isolate's PGP properties were only observed when host, bacteria, and pathogen were all present (Syed-Ab-Rahman et al., 2019). PGP VOCs also appear

to diminish as the plants age. Root exudate concentrations decrease, as well as microbial activity, as the root cells mature (Marschner, 2012). The VOCs produced on peptide/protein-based media are also credited with antifungal properties. This may suggest that as concentrations of simple carbon root exudates decrease the root colonizing bacteria is forced to switch carbon sources which changes the VOC profile to more antagonistic VOCs. Whether or not these effects are due to changing root cell structure and components is unclear. If the bacteria are forced to switch to an alternative metabolic process for metabolizing nutrients and if such switch causes a shift in VOC production, this is poses new questions yet to be answered.

Effects of Bacterial VOCs on rhizosphere microbiome

Bacterial VOCs have a significant impact on the rhizosphere structure depending on which organisms can perceive VOC signals and those that secrete VOC signals. Bacterial VOCs include a wide array of acids, alcohols, ketones, aldehydes, esters, terpenoids, aromatic nitrogenous and sulphureous compounds and ethylene. Appendix A provides a detailed list of specific compounds, sources, and antagonism targets. As mentioned, not all VOCs are created equal.

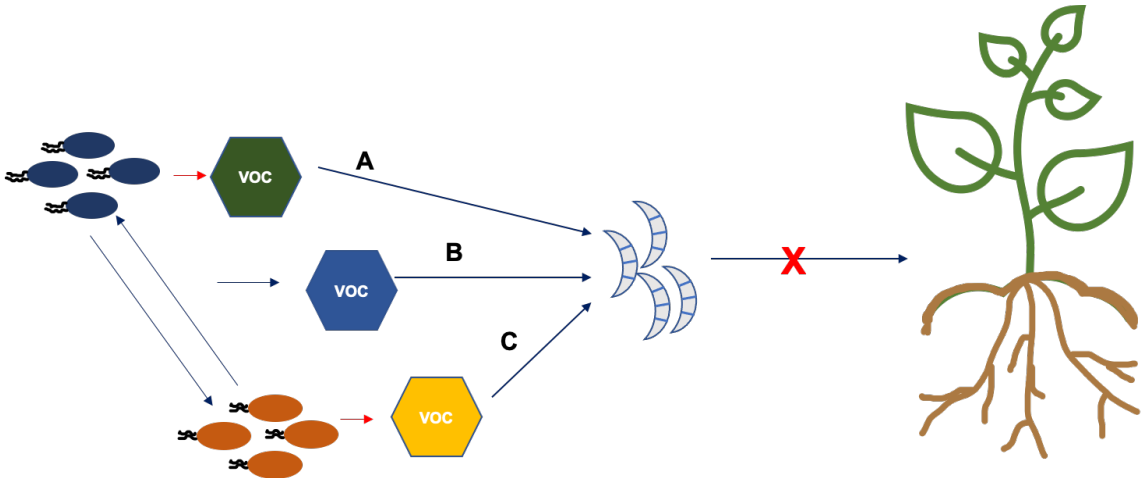

Figure 2. Impact of bacterial VOCs on pathogenic fungi. A simplified diagram of the potential impacts of bacterial species communications effecting the growth and stability of pathogenic fungi like Fov4. Through the communication of two bacterial species, the interaction may produce a unique VOC profile or specific compound which is antifungal with possibly more efficacy against the pathogen, thus preventing or suppressing infection of host plant. Pathways A and C represent how without communication, they each produce VOCs which may not be as effective or ineffective at inhibiting pathogen growth. Pathway B represents the resulting VOC from communication between the two bacteria with the potential to produce a more efficacious VOC inhibiting profile or specific compounds. **Not drawn to scale.*

The over production of some VOCs based on relative abundance of certain species can create a negative feedback loop where plant health can improve for a short time before plant health declines (Delaplace et al., 2015). This can be alleviated with the addition of a cooperative species which improves growth based on the VOC metabolites from another organism (Fig. 2). Positive and short-term growth promotion of *Arabidopsis thaliana* exposed to *Bacillus subtilis* VOCs resulted in modulation of defense signaling pathways, higher photosynthetic capacity and nutrient uptake, reduced disease symptoms and sensitivity to reactive oxygen species while increasing disease resistance (Delaplace et al., 2015). Negative effects on *A. thaliana* plants exposed to bacterial VOCs have been attributed to HCN produced by *Pseudomonas* and *Chromobacterium* species as well as dimethyl disulfide, β-phenyl-ethanol and inorganic volatile NH_3 from *Serratia* species (Delaplace et al., 2015). Literature suggests that having the optimal

bacterial species within the rhizosphere to control the VOC concentrations is vital to establishing a stable and prolonged PGP environment.

Microbial VOCs perceived by bacteria from fungi can also be used to adapt their own behavior. Schmidt *et al*. (2017) performed an interesting investigation into differentially expressed genes in bacteria *Serratia plymuthica* exposed to VOCs from *Fusarium culmorum*. After prolonged exposure to fungal VOCs there were elevated expression in multiple genes, including those related to chemotaxis, motility, energy metabolism, and protein synthesis (Schmidt et al., 2017). One of the effects was *Serratia plymuthica* also producing an 'unusual' terpene compound after fungal VOC exposure. If VOCs from fungi induced higher metabolic activity in bacteria, it is reasonable to anticipate that there is a feedback mechanism sending a VOC signal back to fungi eliciting a second, or perhaps multiple, responses. The feedback loops could also influence the chemotaxis and motility change in the bacterial response to fungal VOCs.

Possibilities and limitations to using microbial VOCs for Fusarium wilt management.

The prospect of microbial VOCs as biocontrol agents is appealing because of their chemical properties, *e.g.* low molecular weight, high vapor pressure, and low boiling points, which make them ideal signals between microorganisms in rhizosphere (Schmidt et al., 2017, Schulz-Bohm et al., 2017, Wu et al., 2015). Microbial VOCs are able to diffuse over long distances, affect below and above ground parts, and increase area of activity through aqueous or air diffusion (Wu et al., 2015, Choudoir et al., 2019, Syed-Ab-Rahman et al., 2019). Microbial VOCs do not often require specialized receptors or transporters to make their way into the cell reducing energy expenditure of receiving organism (Weisskopf et al 2021). High vapor pressure

and low boiling points help promote diffusion under atmospheric pressure and require minimal exact environmental parameters (Schmidt et al., 2017, Sharifi & Ryu, 2018, Schulz-Bohm et al., 2017, Wu et al., 2015). However, the advantages of VOCs as molecular signals can also serve as disadvantages (Fig. 3). Complex interactions within the rhizosphere likely produce the desired antifungal and PGP VOCs. Thus, the desired VOC-mediated biocontrol efficacy is dependent upon a community of microbes and not an individual (Schenkel et al., 2015). The soil properties and microbial population distribution are also important considerations when developing microbial VOC as biocontrol agents (Fig. 3D).

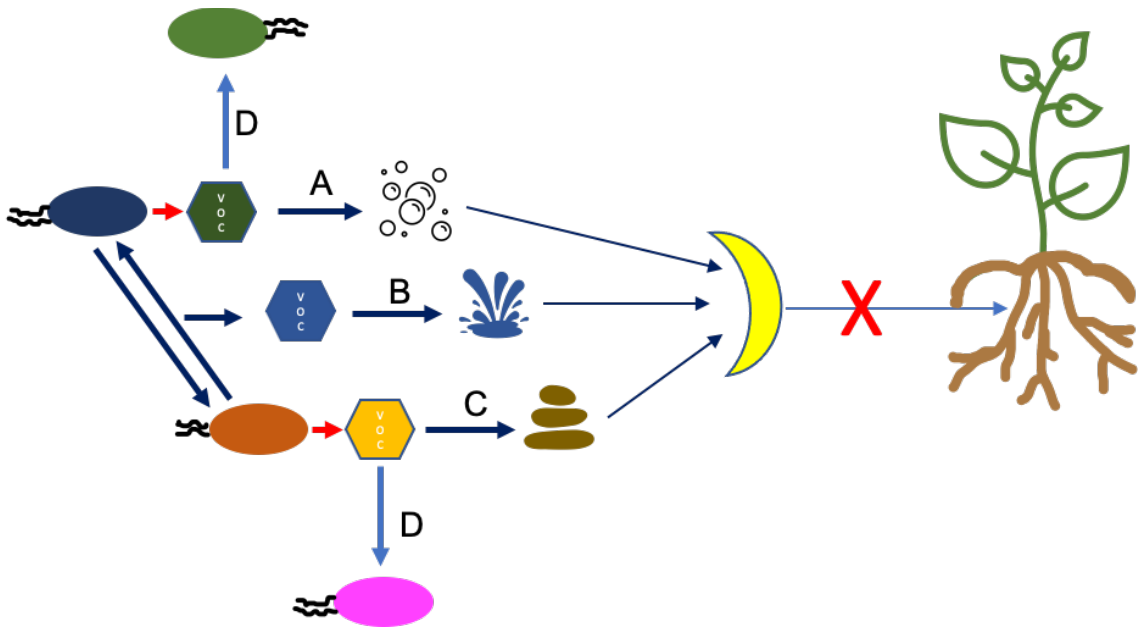

Figure 3. A simplified diagram of the limitations to using MVOCs as biocontrols. (A) VOCs are very prone to evaporation, especially in high temperatures, (B) water can impeded the flow of VOCs and present diffusion as well as altering microbial communities, (C) Soil type and structure is also important in the success of VOCs ability to reach the fungal pathogen, (D) lastly, since VOCs are not very specific in any one pathway, VOCs could be absorbed by other unintended organisms leading to the question of how much is actually needed to cause a suppression of fungal pathogens. **Not drawn to scale.*

Physical and chemical properties of soil can have positive and negative impact on microbial VOCs as biocontrol agents. Water levels have the potential to impede microbial VOC (Fig. 3B) flow in addition to altering microbial populations, *i.e.* favoring anerobic or aerobic microorganisms (Kirchman, 2018). Water can also be used to distribute or dilute micronutrients as well as disturb existing microbial communities. Not only can nutrients be diluted and removed from soil environments, but beneficial antimicrobial peptides can wash away after rain (Yang & Yousef, 2018). Soil pore size should be a concern when utilizing microbial VOCs as biocontrol agents (Fig. 3C). The pore size could impede the flow of volatile signaling molecules depending on composition and water content within the pores. Impediments to air and water flow also risk a microbial VOC derived negative feedback loop altering microbial species abundance. In large macropores, where air and water can move freely by diffusion also means VOC signals can reach their full potential. Conversely, micropores retain more water and prevent the free flow of oxygen and VOC signals (Kirchman, 2018).

In environments limited by carbon, chitonolytic bacteria such as *Paenibacillus* species and facultative mycoparasites, which degrade fungal hyphae, can be found (Aktuganov et al., 2008b, Aktuganov et al., 2008a). The scarcity of carbon sources and extreme temperatures can cause unique adaptations of microorganisms that would normally not be seen elsewhere, such as thermostable enzymes in *Paenibacillus* species. Adaptations to extreme environments cause special diversity in soils due to the ability of organisms to resist changes in temperature, pH, and salt tolerance all which effect enzyme activity (Aw et al., 2016, Sahay et al., 2017, Prasanna et al., 2013, Jiang et al., 2015). The adaptations to extreme environments may cause unique VOC production which could create a positive or negative change to microbial populations and

abundance. In extreme temperatures, VOCs may be evaporated to quickly and not elicit any beneficial effects and the communication between organisms in soil could be impeded (Fig. 3A). Laboratory experiments have documented variation of microbial VOC production dependent on different growth media (Asari et al., 2016). Variations in soil type and microbial VOC production would be an interesting area of research when developing effective biocontrol based on soil type and environmental conditions.

Field application of PGP VOCs, 2,3-butanediol and acetoin, remains challenging due to the lack of effective delivery methods. Naturally, these VOCs are released slowly and at low concentrations raising the problem that a high dose might stress the plant and cause adverse reactions such as stunting growth. Additionally, the risk of compound loss upon field application is significant which means delivery methods for bacterial VOCs are important as are the BCAs themselves (Farag et al., 2013). Delivery methods for bacterial VOCs could involve controlled release mechanisms. In above-soil field applications of PGPRs, rapid evaporation of the beneficial VOCs is a valid concern. Drench application of bacterial VOCs has been tested to pepper roots and seeds, also cucumber seeds, leading to ISR and ISR priming (Farag et al., 2013). Since VOCs can travel from the ground to the above ground parts, defining whether the plant perceives these signals at the roots or the above ground parts is also an important factor to consider.

Summary

Microbial VOCs can serve as a unique biocontrol agent against soil-borne plant pathogens. Years of research has investigated the types and usage of microbial VOCs when the host is in the vicinity of pathogen and beneficial bacteria. As discussed in this review, these

biocontrol agents, when forced to switch nutrient sources from sugars to more complex proteins, can simultaneously change the profile of VOCs produced. And in turn, these altered microbial VOCs can often harm the host plants. There are extensive published reports demonstrating that bacterial VOCs inhibit fungal pathogen growth *in vitro*, but the efficacies of these VOCs and the effective delivery strategies for VOCs as biocontrol agents is still need further characterization. Even with these limitations, the prospect of microbial VOCs as biocontrol agents is appealing and offers a wide array of research and application possibilities.

CHAPTER II

STREPTOMYCES AND *BACILLUS* SPECIES UTILIZE VOLATILE ORGANIC COMPOUNDS TO IMPACT *FUSARIUM OXYSPORUM* F.SP. *VASINFECTUM* RACE 4 VIRULENCE AND SUPPRESS FUSARIUM WILT IN PIMA COTTON[1][2]

Introduction

Cotton is an important globally traded commodity worldwide with primary use in the textile industry. In the US, cotton (*Gossypium* spp) is cultivated in many southern states, with Texas as the top cotton producing state for 2019 with about 32% of cotton and cottonseed, respectively, of the total US production (USDA, 2020). Upland cotton varieties (*G. hirsutum*), with about 3% of the US production being Pima cotton (*G. barbadense*). Pima cotton, a finer and higher value fiber, is grown mostly in California, Arizona, and west Texas (Witt, 2020). One of the key early season diseases of cotton is Fusarium wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) (Skovgaard et al., 2001, Watkins, 1981). In the US, the predominant pathogen of Upland cotton production is Fov race 1 (Fov1). It is important to note that Fov1 requires root-knot nematodes (RKN) to infect cotton, while a highly virulent Fov race 4 (Fov4) does not need RKN assistance. Thus, minimizing disease outbreaks of Fov1 with nematicides or RKN resistant cotton varieties has been successful in past growing seasons (Bell et al., 2017, Watkins, 1981).

Fov4 originated in India, but soon made its way to the United States where it was

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[1] The methods and results in this chapter are printed in an abstract submitted to the American Phytopathological Society 2021 (Reproduced by permission—(c) APS).

[2] This chapter is available on bioRxiv.org preprint server for biology (https://www.biorxiv.org/content/10.1101/2021.10.27.466178v1)

identified in the California San Joaquin Valley in 2004 (Kim et al., 2005), west Texas in 2017 (Bell et al., 2019), and New Mexico in 2020 (Zhu et al., 2020). Fov4 was determined as the pathogen responsible for dead seedlings and black streaks inside tap roots of wilting Pima cotton plants (Watkins, 1981). The detection of Fov4 in the southwestern US has justifiably caused alarm in the US cotton industry. Like Fov1, Fov4 colonizes roots and vascular system resulting in discoloration, wilting and death. Due to its seed-borne and soil-borne characteristics, Fov4 can be transmitted via seeds and on equipment, raising concerns over containment (Liu et al., 2011). Fov4 is now considered an endemic pathogen in California while the presence in Texas is recent and remains spatially isolated. Though Upland cotton was once thought to be less susceptible to Fov4, we are now learning that Fov4 may be an inoculum density-dependent disease and can pose serious threat to Upland cotton production as well (Bell et al., 2019).

The ability to develop and implement control strategies for reducing plant diseases and economic loss from soil borne diseases is challenging, especially for a disease like Fov4. Fov4 presents a set of lifestyle features, such as the production of chlamydospores that serve as hardy survival structures (Watkins, 1981) allowing for the long-distance dissemination through infected seed (Doan & Davis, 2015), which may hinder cotton production. These unique characteristics demand the development of novel control and management strategies. In recent years, an environmentally sustainable alternative to chemicals has utilized a consortia of microbes, or biological control agents, as a means of disease control (Contreras-Cornejo et al., 2014, Mazzola & Freilich, 2017, Syed-Ab-Rahman et al., 2019). Potential microbial biocontrol agents are species with antagonistic properties against other microbes or those stimulating systemic resistance in relevant hosts. Amongst many beneficial microbes, two genera often studied for their antimicrobial properties are *Bacillus* (Asari et al., 2016, Chaves-Lopez et al.,

2015) and *Streptomyces* (Cordovez et al., 2015, Wu et al., 2015). The potential for using beneficial organisms to enhance productivity in agricultural production systems is not new. Many bacterial and fungal agents have been tested as a single strain for their ability to control soil-borne pathogens. However, shaping crop rhizospheres with a beneficial species-rich microbial community remains a major challenge.

Microbes with broad antifungal properties have been attributed to their abilities to produce soluble and volatile secondary metabolites that contribute to direct inhibition of pathogens in soil. A recent review by Tilocca, et. al. (Tilocca et al., 2020) provides a summary of microbial VOC diversity, disease suppressive functions, and mediating communication between bacteria, fungi and plants. The complex relationships between plant growth promoting rhizobacteria (PGPR) and other antagonistic bacteria can produce a wide array of VOCs with antifungal and plant growth promoting properties (Choudoir et al., 2019, Dias, 2017, Stahl & Parkin, 1996). Inhibition of Fov4 through VOCs could be caused by a multitude of factors, such as communication between two or more bacteria genera, making the mainstream utilization of biological controls difficult.

The aim of this study was to investigate the properties of interkingdom signaling, specifically the role of bacterial VOCs in cotton rhizosphere between Fov4, Pima cotton, and select bacterial species. We learned that bacterial VOCs influence Fov4 physiology in our preliminary experiments. Here, we hypothesized that combinations of antagonistic bacterial species and resulting VOC profiles can provide more effective and realistic suppression of Fov4 growth and virulence during Pima cotton infection. To test this hypothesis, we characterized the interaction between Fov4 versus single and two bacterial isolate co-cultures. The types of VOCs produced have been shown to be different when plants are added into the communication

network, thus testing combinations of bacteria in the presence of Fov4 and cotton plants was vital (Syed-Ab-Rahman et al., 2019). We tested the efficacy of the bacterial VOCs by measuring Pima cotton health infected with Fov4.

Materials and methods

Pathogen and antagonistic isolates

Fusarium oxysporum f. sp. *vasinfectum* race 4 (Fov4) strain used in this study was isolated from diseased Pima cotton plants acquired from El Paso, Texas (Courtesy of Dr. Tom Isakeit, Texas A&M AgriLife Extension). Identification of this Fov4 isolate was confirmed using the method described in Doan, et. al. 2014 and AmplifyRP® Acceler8® for Fov4 rapid DNA kit (Product No. ACS 19700/0008) (Doan, 2014). Fov4 culture was maintained on ISP2 agar at 4°C and conidia suspensions used in subsequent studies were made by flooding 7-14 day agar cultures with sterile water and filtered through double layered sterile miracloth before concentration was determined using a hemocytometer. Concentration was adjusted as needed with sterile deionized water.

Antagonistic bacteria were isolated from topsoil samples from both a Pima cotton-producing field (El Paso County, Texas, USA) as well as a non-cotton-producing field as an outgroup (Harris County, Texas, USA) to isolate a diverse collection of bacteria. Soil was air dried and sifted soil prior to using common isolation techniques for species in *Streptomyces* species (Qi et al., 2019, Singh, 2019, Hagn et al., 2008, Yao & Wu, 2010). Subculturing until single colony isolation was achieved used ISP2 media for its ability to support growth of a broad range of bacteria and fungi as well as VOC production (Choudoir et al., 2019). Seed-borne bacteria were also utilized as a source of antagonistic bacteria. Seed associated bacteria were

isolated from 7-week-old Pima cotton plants (*n*=6) showing no sign of disease (Cianchetta & Davis, 2015, Watkins, 1981, Isakeit, 2019). Antagonistic isolates were maintained at 22°C on ISP2 media and stored at 4°C for routine use. Liquid cultures (7 day) of antagonistic bacterial isolates were prepared in separate containers of ISP2 broth and grown at 22°C for 2-5 days on an orbital shaker at 100rpm.

Screening and identification of isolates with antifungal properties against Fov4

To select bacterial isolates showing Fov4 inhibitory properties, we developed a screening method where Fov4 was spot inoculated at the center of an ISP2 agar dish, along with four different bacteria, each 2.5 cm from the center and equal distant from other bacterial isolates (Fig. 4A). Isolates which produced an inhibition zone where Fov4 growth was hindered around the bacterial colony were selected. Examples of observed inhibitions zones shown in Fig. 4 C-G.

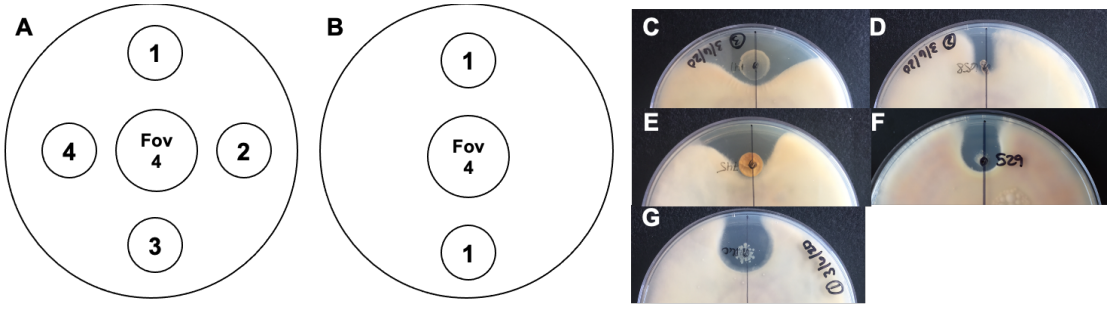

Figure 4. Screening for antifungal bacteria. (A) screening set up of four isolates with Fov4 to determine any antifungal properties by producing inhibition zones, (B) experimental set up confirming Fov4 inhibition on ISP2 agar petri dishes, (C-F) examples of observed inhibition zones during screening.

For identification of bacterial species showing anti-Fov4 properties, we used 16s rRNA gene sequencing. The first set of primers, 16Sf (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 16Sr (5′-GGT TAC CTT GTT ACG ACT T-3′), was used to amplify 16s rRNA gene for all isolates. Primers StrepF (5'-ACG TGT GCA GCC CAA GAC A -3') and StrepB (5'-ACA AGC CCT GGA AAC GGG GT-3'), were used to further identify *Streptomyces* species (Aslam et al., 2013). Template DNA was extracted using Genomic DNA Purification Kit (Thermo Scientific), except for isolates displaying *Streptomyces* colony characteristics. Predicted *Streptomyces* species DNA was extracted by placing colonies into TE buffer and microwaving for 30 seconds followed by centrifugation for 10 minutes at 14,000rpm (Cordovez et al., 2015) and supernatant, containing genomic DNA, was removed and used as PCR template. PCR with Taq polymerase was performed in a 25-µl volume tube following the manufacture's guidelines (New England Biolabs, Ipswich, MA, www.neb.com). PCR amplicons were visualized by agarose gel electrophoresis, subsequently purified using GeneJet Purification Kit (Thermo Fisher Scientific, Waltham, MA) and sequenced (Eton Biosciences, Inc., San Diego, CA). Retrieved sequences were subjected to BLASTn analysis against the NCBI non-redundant database to assign identities to bacterial isolates at the deepest possible taxonomic resolution (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were aligned using Multiple Sequence Alignment (MSA) in Clustal-Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). MSA produced a Neighbour-joining tree which was used to confirm bacterial isolates were all uniquely different.

Here, we further confirmed antifungal properties of each isolated bacteria by measuring inhibition of Fov4 on ISP2 agar. For Fov4, 10 µl conidia suspension ($1x10^4ml^{-1}$) was added to the center of the ISP2 agar petri dish. Single isolate inhibition assays consisted of two opposing

10 µl spots (Fig. 4B). Fov4 growth area was measured by ImageJ software (https://imagej.nih.gov/ij/index.html) at 10-12 days post inoculation (dpi), when Fov4 growth on control samples (devoid of bacteria) covered the entire agar plate. Percent inhibition of Fov4 was calculated from measured Fov4 area using the equation (Positive Control Fov4 Area – Treatment Fov4 Area)/(Positive Control Fov4 Area) x 100%. Significance in growth inhibition was determined by comparing measured Fov4 area of treatment groups to negative control using a one-way ANOVA followed by Tukey's multiple comparisons post-test using a 95% confidence interval on GraphPad prism software (San Diego, CA). Experiment was performed with three biological replicates.

Impact of bacterial VOC on Fov4 growth

To measure the impact of bacterial VOCs on Fov4, we screened multiple bacterial combinations against Fov4 in separate wells of a 24 well plate (Fig. 5). Fov4 growth after exposure to bacterial VOCs was assessed with spectrophotometry (absorbance reading at 350 nm). In a 24 well plate, 10 µl of liquid culture of bacteria in ISP2 broth was added to 1 ml of ISP2 broth of row a and/or row c. To row b, between bacteria wells, 10 µl of Fov4 conidia suspension ($1x10^4$ ml^{-1}) was added to 1 ml of ISP2 broth (n=6). Row d of every plate only contained ISP2 broth which served as a negative control and normalization for the absorbance readings. Toothpicks were added to the left and right side of the plate to slightly prop the 24 well plate lid up and then the entire plate was sealed with parafilm. The plates were shaken on an orbital shaker at 100rpm for 3 days. Absorbance readings were taken on 3 days post inoculation (dpi) at 350 nm on a Spectral Max ID5 plate reader. This wavelength was determined through software optimization. Data were collected for 6 wells of Fov4 per plate and one plate per

treatment group. Plates with Fov4 and only one bacterial isolate served as controls to compare against the combinations of bacteria. Measurements were subject to a one-way ANOVA followed by a Tukey's post-test using GraphPad Prism (San Diego, CA) to determine significant differences with a 95% confidence interval, $p<0.05$. Data was additionally examined by the percent coefficient of variation (%CV) as well as the percent difference between Fov4 only to the Fov4 exposed to bacterial VOCs.

	1	2	3	4	5	6
A	Isolate 1	Isolate 1	Isolate 1	Isolate 1	Isolate 1	Isolate 1
B	Fov4	Fov4	Fov4	Fov4	Fov4	Fov4
C	Isolate 2	Isolate 2	Isolate 2	Isolate 2	Isolate 2	Isolate 2
D	ISP2	ISP2	ISP2	ISP2	ISP2	ISP2

Figure 5. Absorbance VOC inhibition experimental set up for screening bacterial VOC suppression properties. No physical interaction between Fov4 and bacteria. Row D of ISP2 was a plate blank used to normalize absorbance readings as well as a control to show that no exchange of microbes during the shaking and analysis.

To determine the impact of selected bacterial combination VOCs on inhibition of Fov4, we used compartmentalized petri dishes in which bacterial isolate was physically separated from Fov4 with only interaction possible by VOCs and not agar-soluble metabolites. Single isolates were tested in 2-compartment petri dish (Fig. 6A, I-plate, $n=3$) and combinations were tested in 4-compartment petri dish (Fig. 6B, Q-Plate, $n=3$) to ensure physical separation of the isolates. To each compartment of bacteria, 120 µl of liquid culture was streaked over the entire area of the compartment. As for Fov4, 10 µl conidia suspension ($1x10^4$ ml^{-1}) was added to the center of the

remaining compartment. Radial diameter measurements were taken at 4 dpi and used to calculate fungal growth area. Negative controls for both I-plates and Q-plates were measured to ensure the radius was not statistically different between the VOC treatment groups. Measurements were subject to a one-way ANOVA followed by a Tukey's post test to determine significant differences with a 95% confidence interval using GraphPad Prism (San Diego, CA).

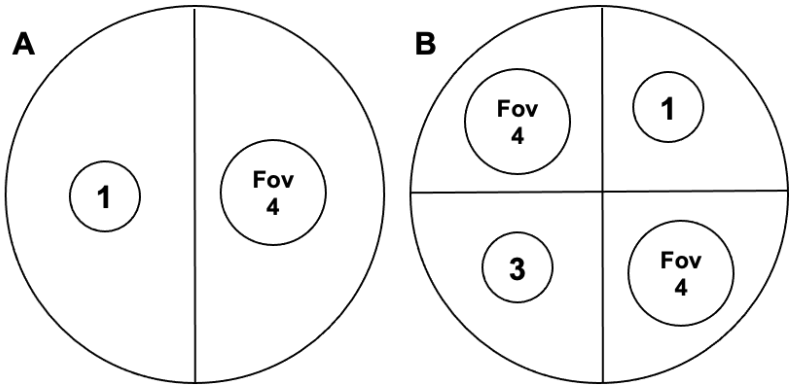

Figure 6. Experimental set up to determine inhibition of Fov4 by only bacterial VOCs. (A) Design with partitioned petri dish with two compartments for determining VOC inhibition by one single isolate, (B) design with four separated compartments to determine the VOC inhibition by two bacterial isolates.

Soil-free Fov4 virulence assay development and VOC impact on Fov4 infection

To further study the effects of bacterial VOCs on Fov4 virulence, we designed a soil-free assay that allowed us to test fungal virulence while minimizing variability due to biotic and abiotic factors, such as seed health and soil characteristics. First, Pima cotton seeds (PhytoGen, No. PHY841RF) were initially surface sterilized in 70% ethanol for 5 minutes, rinsed one time with sterile double deionized water, then shaken in 10% bleach solution, and lastly rinsed in sterile water three times. Seeds were then placed onto sterile cotton circles and covered with a second cotton circle. Sterile double deionized water (5 ml) was applied to cotton circles, placed

inside of plastic sandwich bags, sealed, and incubated under natural sunlight for approximately 5-7 days until cotyledon leaves emerged (Fig. 7A). Once seedlings had reached between 5-7 cm in shoot and primary root length, they were placed on a fresh sterile cotton experiment (Fig. 7C-E).

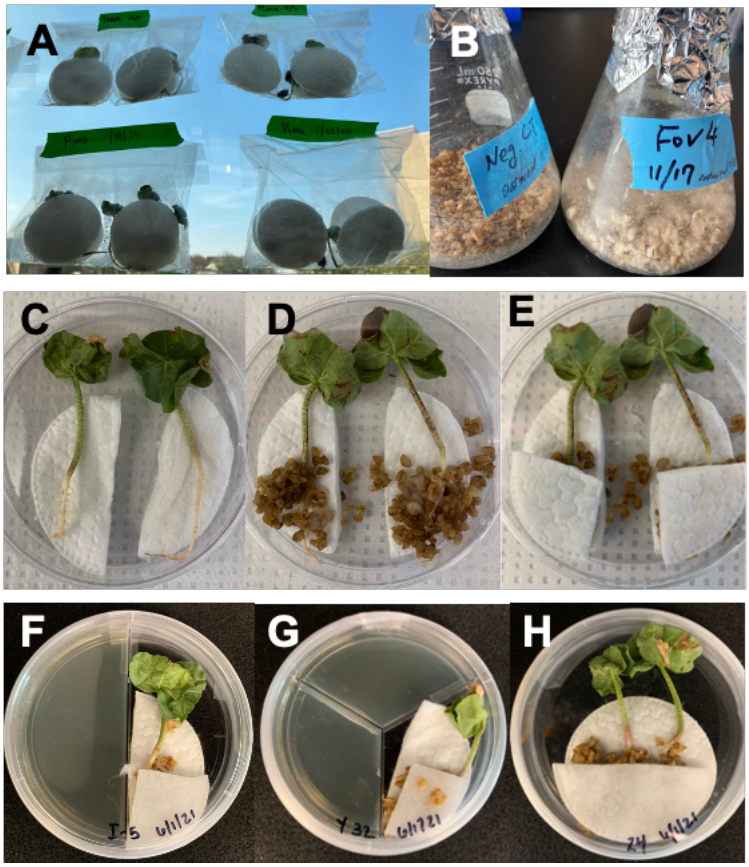

Figure 7. Soil-free pathogenicity assay using Pima cotton seedlings. (A) Cotton seeds were sandwiched between moist cotton circles and placed in plastic sandwich bags for germination. (B) Fov4 inoculum was prepared in twice autoclaved steel cut oatmeal medium. (C) Healthy pima seedlings used in plant assays, (D) pima seedlings with roots covered with twice sterile steel cut oats, (E) final set up when the SCO is covered by a second cotton circle which is then watered with 4ml of sterile water, (F) Experimental set up for I-plate VOC inhibition with Fov4 infected seedlings; (G) Y-plate VOC inhibition of seedlings; (H) sealed petri dish with steel cut oat inoculum for validation of experimental design.

To prepare pathogen inoculum for Fusarium wilt virulence assay on Pima seedlings, twice sterilized steel cut oats (SCO: 20 g organic steel cut oatmeal and 20ml water) in 100 ml

Erlenmeyer flasks were inoculated with 1 ml Fov4 conidia suspension (1 x10^8 ml^{-1}). The flasks were incubated at 28°C for 5-7 days with shaking once a day to maximize fungal growth (Fig. 7B). When cotton seedlings and Fov4 inoculum were ready, we placed Fov4 SCO (1.5 g) on Pima seedings so that SCO covered the primary root (Fig. 7D). A half circle or quarter circle covered the roots and SCO leaving the root shoot junction, shoot, and leaves visible. Before sealing the plates, sterile water (4 ml) was added to the top half or quartered cotton circle (Fig. 7E). These petri dish plates were placed under a 12 hr light/dark cycle at 22°C, and after 6 days the seedlings were removed for examination of Fusarium wilt symptoms. Autoclaved SCO not inoculated with Fov4 served as the negative control. The experiment was completed twice with 8 replicates each containing 2 subsamples for a total of 32 seedling observations. An example of one replicate is shown in Fig. 7H. External tissue was evaluated for color differences between negative (Fov4-) and positive (Fov4+) at the root shoot junction (RSJ), shoot, and leaf wilt. Healthy uninfected seedlings were those which displayed characteristics of the natural seedling variation, such as green or pink RSJ, green shoots, and no wilting or severe discoloration in the leaves. Those which differed were considered related to Fov4 infection. Natural seedling variation using this method was optimized by monitoring cotton seedlings in plates with or without SCO or a parafilm seal around the petri dish. The natural variation was assessed without Fov4 or bacteria.

To test the impact of bacterial VOCs on Fov4 virulence, we adjusted our method so that one seedling was placed in an empty compartment of either an I-plate (2 compartments) to expose the seedling to one bacterial isolate VOCs or a Y-plate (3 compartments) to expose the infected seedling to VOCs from two physically separated bacterial isolates (Fig. 7F-G). In an I-plate petri dish, one side was filled with 10 ml of ISP2 agar and the other was used for cotton

seedling. In a Y-plate, two compartments were filled with 10 ml of ISP2 agar and the remaining compartment was used for cotton seedling. To the ISP2 compartments, 120 µl of liquid ISP2 bacterial culture was streaked over the entire area 2-3 days prior to adding Fov4 SCO and seedlings to establish colony growth and VOC production. Plates were sealed with seedlings once all components were set up as described. The experiment was completed twice, with four replicates and a second experiment with 8 replicates for a total of 12 observations for disease assessment.

Fusarium wilt assessment with VOC exposure

To comprehensively evaluate disease progression under the exposure to bacterial VOCs, we examined the treatment groups based on the external tissue color gradient to determine treatment group with the least significant difference from the negative control (-Fov4, $p>0.05$). We previously determined that terminating experiments 5-6 dpi was ideal because the roots were still intact but showing clear symptoms of Fov4 infection which allowed for suitable evaluation of treatment groups. Incubating longer than 6 days resulted in complete rotting of the plants which made assessing disease difficult when comparing treatment groups against the controls. Additionally, the longitudinal section of the RSJ was observed under 40x magnification of internal tissue for symptoms of infection (Dowd et al., 2004). Disease severity was assessed using a color gradient of the RSJ. Established ratings were given a numerical value and subjected to a non-parametric ANOVA Kruskal-Wallis Test followed by a Dunn's multiple comparison test using Graphpad Prism (San Diego, CA).

Additional disease severity was assessed using a color gradient of the RSJ and shoot color in addition to the number of seedlings with wilted leaves; disease was associated with RSJ

and shoot colors of brown, black, and dark purple. Non-diseased seedlings showed RSJ of green-white, green-pink, and solid pink. The shoot color assessment was based on the same color gradients. The number of observations for each treatment group were analyzed on a contingency table setting in Graphpad Prism (San Diego, CA) and reported as percentage of column total, the observed colors on the RSJ, shoot, and incidence of leaf wilt. For example, a column containing number of observations for pink coloring of the RSJ shows 25 total observed pink RSJ, and 95% of the observations were associated with the negative control treatment group.

Results

Bacillus, Streptomyces, Brevibacillus and Paenibacillus sp. isolated from soil and rhizosphere.

Based on preliminary screening results, a total of seven isolates, four from El Paso County soil samples (ELP524, 528, 529 and 745), one from Harris County soil samples (HC658), and two from Pima cotton root rhizosphere samples (RZ141 and 160), were selected for further investigation. RZ141 (Fig. 8A) exhibited white rippled appearance with fast and lateral growth. RZ160 colonies were light cream colored with smooth surface and turned dark brown as the colonies matured (Fig. 8B). ELP524, 528 and 529 all shared similar colony morphology as shown in Fig. 8C, but with varying colony pigment of cream colored to brown. These strains produced rippled and harder form as the colonies matured. HC658 showed small white spore-forming colonies which resembled typical *Streptomyces* colony phenotypes (Fig. 8E). ELP745 also showed common *Streptomyces* morphology but produced a noticeable brown pigment in ISP2 agar (Fig. 8F). Subsequently, identification of genus using 16s rRNA gene sequences, which were subjected to BLASTn searches against the NCBI non-redundant database. Top five hits (with highest percent identity, query cover, and lowest E-value) were

used to assign taxonomic identities to bacterial isolates: ELP524 (*Paenibacillus sp.*, 531 bp, 99.14% identity), ELP528 (*Paenibacillus sp.,* 600 bp, 97.54% identity), ELP529 (*Paenibacillus sp.,* 1237 bp, 99% identity), ELP745 (*Streptomyces sp.,* 604 bp, 94.56%, identity), HC658 (*Streptomyces sp.,* 1057 bp, 99.5% identity), RZ141 (*Bacillus sp.,* 1297bp, 98% identity), and RZ160 (*Brevibacillus sp.*, 1314 bp, 97% identity). Phylogenetic trees were constructed for the *Firmicute*s species (Fig. 8D) and *Streptomyces* species (Fig. 8G) separately using Clustal Omega alignment and Neighbour-joining tree generation to show distinct species were isolated and related to other species of the same genus.

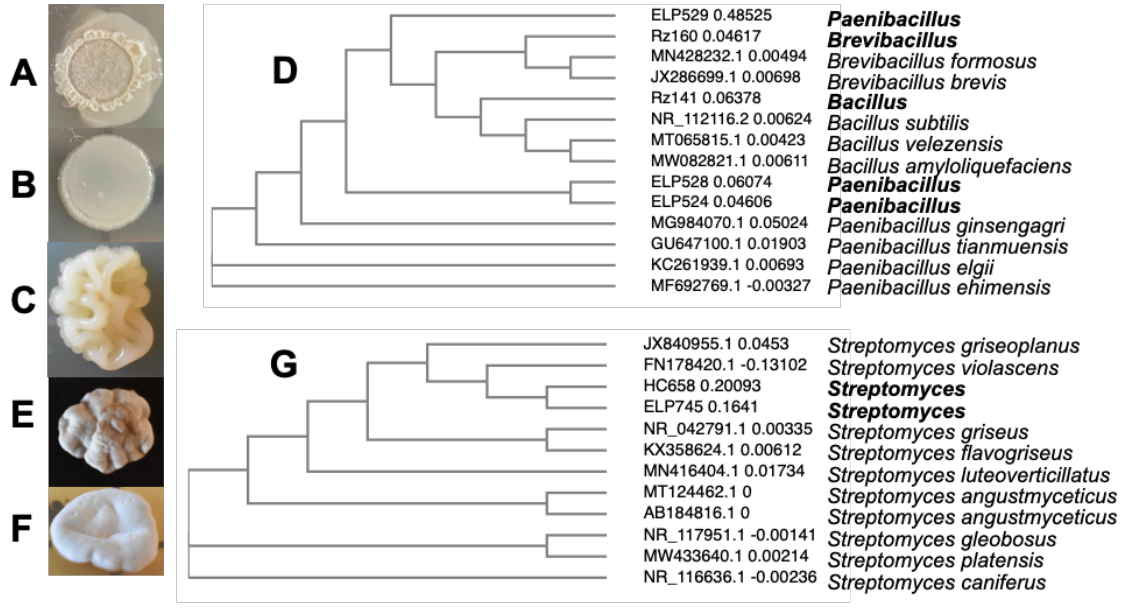

Figure 8. Antagonistic isolates identified by screening and 16s rRNA sequencing. Isolates were from cotton producing field in El Paso County (C and F), non-cotton producing field in Harris County (E) and from healthy cotton rhizosphere (A and B); (A) *Bacillus* Rz 141, (B) *Brevibacillus* Rz 160, (C) *Paenibacillus* ELP 529, (D) Phylogenetic tree of isolated *Firmicutes* species, (E) *Streptomyces* HC658, (F) *Streptomyces* ELP 745, (G) Phylogenetic tree of isolated *Streptomyces* species.

Inhibition of each isolate's antifungal properties against Fov4 was confirmed on ISP2 agar. Bacteria inoculated with Fov4 at the center of the ISP2 agar dish was completed with three replicates. The experiment was terminated after growth of Fov4 without bacteria exposure had covered the entire petri dish. Area measured by ImgJ was used to calculate inhibition (Fig. 9). Area measurements were also subjected to a one-way ANOVA and Tukey post-test which showed $p<0.0001$ for all bacterial isolates compared to negative control.

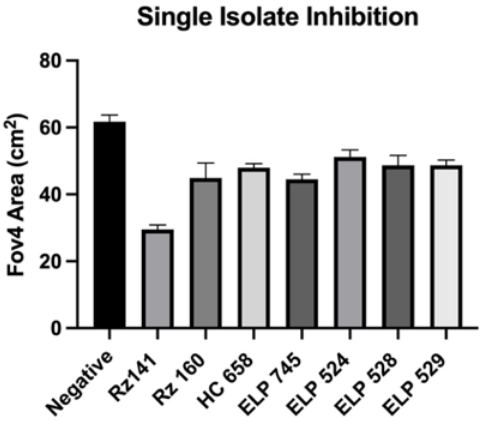

Figure 9. Single isolate Fov4 inhibition on ISP2 agar. Measured Fov4 area exposed to isolated bacterial isolates on ISP2 agar plates. Measured area used to calculate percent inhibition. All isolates resulted in Fov4 area significantly different from negative control ($p<0.0001$). Error bars represent standard deviation based on three replicates.

Table 1 shows the calculated inhibition of each isolate. *Bacillus* Rz141 showed the highest inhibition capability of 52%. *Brevibacillus* Rz160, *Streptomyces* HC658, and *Streptomyces* ELP745 showed inhibition of 27, 22, and 28% inhibition, respectively. *Paenibacillus* ELP524, 528, and 529 showed 17, 21, and 21% inhibition, respectively. Upon

closer examination of *Paenibacillus* species physical colony formation was inconsistent and thus we removed *Paenibacillus* species from further experiments.

Table 1. Single isolate inhibition on ISP2 media.

	%Inhibition	*p* value
Bacillus Rz141	52%	<0.0001
Brevibacillus Rz 160	27%	<0.0001
Streptomyces HC 658	22%	<0.0001
Streptomyces ELP 745	28%	<0.0001
Paenibacillus ELP 524	17%	0.0011
Paenibacillus ELP 528	21%	0.0001
Paenibacillus ELP 529	21%	0.0001

Rz141 and HC658 VOC inhibition of Fov4

In addition to secreted antifungal metabolites produced by bacteria, VOCs are recognized as another important form of compounds that mediate inter-species and inter-kingdom signaling. Based on initial absorbance screening, using 24 well plates, of various VOC inhibition from multiple bacteria combinations, calculated differences between the Fov4 not exposed to bacterial VOCs and various combinations of bacteria was used to determine which combinations warranted further investigation. Two combinations, Rz141 with Rz160 and Rz141 with HC658 showed 58 and 42% difference from Fov4 control. However, Rz141 with HC658 (Fig. 10F) combination had the lower %CV (15%) among the well replicates and was chosen for further investigations.

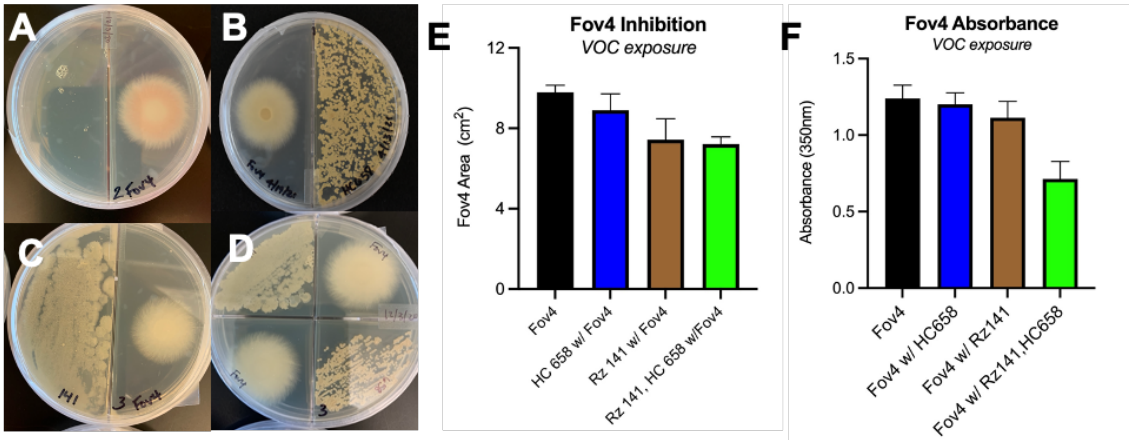

Figure 10. VOC inhibition from the single isolate and combination of Rz 141 and HC 658. (A) Negative sample without Rz141 or HC658, (B) Fov4 exposed to HC658 VOCs grown in a physically separated plate, (C) Fov4 exposed to Rz141 VOCs, (D) Fov4 exposed to physically separated Rz 141 and HC 658 with only VOC exposure possible between Fov4 and bacteria, (E) Measured Fov4 area after exposure to bacterial VOCs, (F) Fov4 absorbance at 350nm taken after 3 days of growth in liquid culture simultaneously exposed to VOCs from HC658 and/or Rz141.

We investigated the capability of Rz141 with HC658 to inhibit Fov4 growth through only VOC interactions and with physical barriers. The use of compartmentalized petri dishes provided a reliable method for investigating VOC inhibition capabilities towards Fov4. After terminating the VOC experiment at 4 dpi, the measurements of Fov4 area from each treatment group were collected and mean areas were calculated (Fig. 10E). Measurements for all replicates (n=3) showed standard deviation less than 1 and coefficient of variation less than 14%. Inhibition by VOC alone was 9, 24, and 26% for HC658, Rz141, and combination, respectively. Measurements showed $p<0.01$ for Fov4 inhibition only when exposed to Rz141 (Fig. 10A) and the combination (Fig. 10D). The VOC inhibition of HC658 alone was not significant (Fig. 10B).

Soil free virulence assay

Based on the Fov4 inhibition caused by a combination of bacterial VOCs, we continued to add more participants to the interkingdom communication network, specifically the host plant Pima cotton. We tested the hypothesis that bacterial VOCs can provide Fov4 suppression during the infection of Pima cotton seedlings. Utilizing a soil-free virulence assay with compartmentalized petri dishes, we were able to investigate the impact of Rz141 and HC658 VOCs on pathogen progression during infection of Pima cotton seedlings. However, we first ensured our plant virulence assay could distinguish between Fov4 infected (Fov+) and uninfected (Fov4-) seedlings. Initial studies, without bacteria or Fov4, tested seedling response to various experimental set ups with and without SCO or parafilm seal around the petri dish. A second study introducing Fov4 was used to establish disease severity criteria.

The first experiment aimed to establish root shoot junction (RSJ) and shoot colors associated with natural variation of Pima seedlings depending on various experimental set up and low disease severity. Groups of 8 replicates, with 2 subsamples, were examined over six days and natural variation was associated with green, green with pink shading (Fig. 11A), and solid pink coloring in the root shoot junction. Moderate rating was associated with a darker and more solid coloring of pink (Fig. 11C). Moderate rating was not seen consistently across all groups like low severity. Fig. 11D shows microscopic examination of internal tissue of the RSJ which is vibrant green. Fig. 11E shows a representative example of what was considered non-wilted leaves. Table 2 shows the number of observations evaluated in a contingency table model bwhere the data set was arranged in columns and percentages were calculated based on the

number of observations (Low, moderate, high, no wilt, wilt) divided by the column total for each experiment.

Utilizing a petri dish with SCO covering seedling primary roots and a parafilm seal around the petri dish was chosen due to the greatest incidence of low severity ratings when compared to other set ups. No seal with SCO was eliminated due to the high percentage of wilted leaves observed (48%). There was no difference between experimental set up as long as the petri dish was sealed. The experimental set up without a parafilm seal and with SCO showed all seedlings with severe leaf wilt.

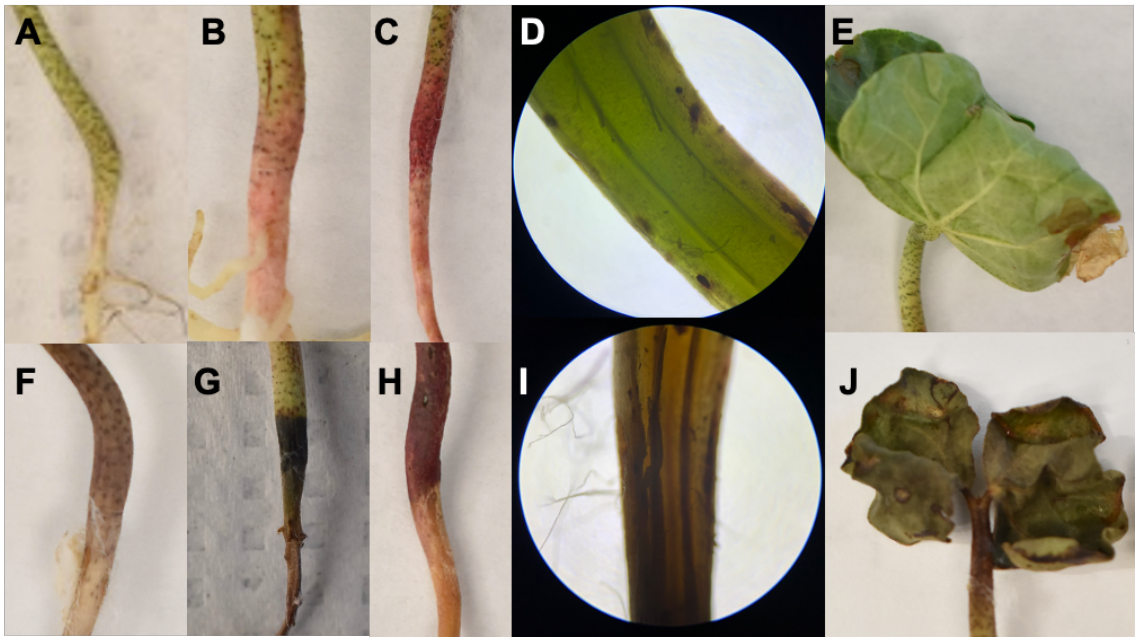

Figure 11. Pima cotton symptoms associated with Fov4 infection. Root shoot junction and leaf appearances associated with uninfected and Fov4 infected seedlings. (A-B) represent root shoot junction colors associated with healthy and uninfected seedlings and these colors were given a disease rating of low, (C) represents a moderate disease rating of pink root shoot junction, (D) microscope image of low moderate disease rating at the root shoot junction, (E) leaf appearance of healthy seedlings associated with no wilt, (F-H) represent examples of root shoot junction colors associated with severe disease rating, (I) microscope image of severe disease rating showing internal tissue darkening, (J) leaf appearance of wilt associated with Fov4 infection.

Table 2. Natural Pima-cotton seedling variation associated with experimental design.

	Root Shoot Junction			Shoot			Wilt	
	Low	*Moderate*	*High*	*Low*	*Moderate*	*High*	*No Wilt*	*Wilt*
No Seal, No SCO	16%	33%	17%	13%	13%	80%	21%	9%
No Seal, SCO	2%	0%	42%	12%	35%	0%	0%	48%
Seal, No SCO	19%	0%	14%	18%	17%	0%	17%	15%
Seal, SCO	21%	33%	8%	21%	9%	0%	19%	12%

Using Fov4 SCO inoculum on Pima cotton seedlings, we were able to establish the "high disease severity" criteria at 6 dpi in a petri dish sealed with parafilm. RSJ and shoot colors associated with infection were dark brown, black, or dark purple coloring (Fig. 11F-H). Internal tissue of RSJ (Fig. 11I) also showed brown coloring. Leaves from the Fov4+ group showed wilting and darkening (Fig. 11J). Table 3 shows positive control group accounted for 100% of all high severity ratings of both RSJ and shoots. Wilt was associated more with the positive group, 91%. Moderate ratings were still associated with the Fov4- groups.

Table 3. Disease severity of Fov4 infected Pima-cotton seedlings.

	Root Shoot Junction			Shoot			Wilt	
Treatment[ab]	*Low*	*Moderate*	*High*	*Low*	*Moderate*	*High*	*No Wilt*	*Wilt*
Fov4-	100%	100%	0%	100%	100%	0%	71%	9%
Fov4+	0%	0%	100%	0%	0%	100%	29%	91%

[a] *n=8, 2 subsamples per replicate, repeated once, total of 32 observations per group.*
[b] *Experiments done with twice sterile steel cut oat inoculum and a parafilm seal around petri dish.*

Bacterial VOCs impacted Fov4 virulence on Pima cotton infection

After determining that our soil-free pathogenicity assay was able to distinguish between healthy and Fov4-infected Pima cotton seedlings, we investigated the impact of bacterial VOCs from Rz141 and HC658 on Fov4 virulence. These assays were independently performed twice, once with 4 replicates and another with 8 replicates for a total of 12 seedling observations and each experiment was terminated 6 dpi. Negative (Fov4-) and positive (Fov4+) controls showed visible symptoms indicating low and high disease severity.

Observations of Fov4- internal RSJ tissue showed low severity, vibrant green, while Fov4+ (Fig. 12A) had the darkest tissue. The treatment groups Rz141 (Fig. 12B), HC658 (Fig. 12C), and Rz141 and HC658 combination (Fig. 12D) showed less tissue darkening than the positive control. Additionally, the observations of external RSJ tissue coloring showed noticeable differences among groups (Fig. 12E).

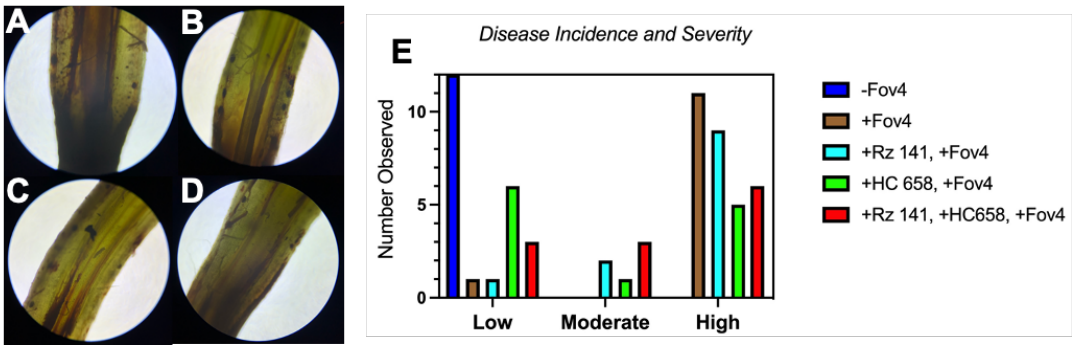

Figure 12. Results of Fov4 infected seedlings response to bacterial VOCs from *Streptomyces* HC 658 and *Bacillus* Rz 141. (A) Internal RSJ tissue from Fov4 infected seedlings; (B) observation of internal RSJ coloring after exposure to Rz 141 VOCs during infection; (C) internal RSJ HC 658 internal tissue; (D) internal RSJ tissue from a combination of Rz 141 and HC 658 VOC exposure; (E) Graphical representation of the incidence of different colors of external tissue based on treatment group. Of the treatment groups, HC 658 had the highest number of seedlings which resembled the negative control group.

Table 4 shows in more detail the graph shown in Fig. 12E. The treatment of HC658 VOCs showed the second highest percentage of low rated RSJ (26%), while the negative control accounting for 52% of all low rated RSJs. The positive control still accounted for the largest percentage of high rated RSJ (35%), shoot (33%), and wilt (33%) among the treatment groups. HC658 treatment group also accounted for the least amount of high rated shoot color (17%) aside from the negative control (0%).

Table 4. Disease severity of Fov4 infected Pima cotton seedlings exposed to Rz141 and HC658 VOCs.

	Root Shoot Junction			Shoot			Wilt	
Treatment [ab]	*Low*	*Moderate*	*High*	*Low*	*Moderate*	*High*	*No Wilt*	*Wilt*
Fov4-	52%	0%	0%	38%	19%	0%	24%	11%
Fov4+	4%	0%	35%	14%	14%	33%	14%	33%
Rz141+, Fov4+,	4%	33%	29%	14%	19%	28%	19%	22%
HC658+, Fov4+	26%	17%	16%	14%	29%	17%	21%	17%
Rz141+, HC658+, Fov4+	13%	50%	19%	19%	19%	22%	21%	17%

[a]*n=4, 6, repeated once, total of 12 observations per group.*
[b]*Experiments done with twice sterile steel cut oat inoculum and a parafilm seal around petri dish.*

In addition to percent incidence analysis, a disease severity rating was established with coloring associated with RSJ. Non-diseased seedlings with RSJ of green-white (1), green-pink (1), and solid pink (2). Green and green-pink were the predominate colors seen in the natural seedling variation experiment. Diseased colors were dark purple (4), brown (5), and black (6) with purple indicating the least amount of severity. A light brown coloring was rarely seen and given a rating of 3. The non-parametric Kruskal-Wallis ANOVA and Dunn's post test comparing treatment groups to negative showed the least variation between negative and HC658 and combination group ($p>0.05$). Rz141 symptoms were the significantly different from the negative, $p=0.0352$.

Discussion

The bacterial isolates collected and investigated in this research, including *Bacillus* (Yuan et al., 2012, Guevara-Avendano et al., 2020), *Streptomyces* (Cordovez et al., 2015, Wu et al.,

2015), *Paenibacillus* (Seo et al., 2016, Kim et al., 2004, Naing et al., 2015b), and *Brevibacillus* (Johnson et al., 2020, Yang & Yousef, 2018, Jiang et al., 2015) species, are widely acknowledged with their antifungal properties and some are commercially used as biocontrol organisms. Notably, *Bacillus* species have shown incredible potential as biocontrol agents because of their antimicrobial metabolites and enzymes (Guevara-Avendano et al., 2020, Chaves-Lopez et al., 2015, Farag et al., 2013, Asari et al., 2016, Syed-Ab-Rahman et al., 2019). In addition to producing soluble metabolites with antifungal capacities, *Bacillus* species have been shown to synthesize diffusible VOC compounds that are capable of inhibiting *F. oxysporum* f. sp. *radicis-lycopersici*, *F. verticilliodes* (Guevara-Avendano et al., 2020) and *F.oxysporum* f. sp. *cubense* (Yuan et al., 2012) and *F. oxysporum* f. sp. *lactucae* (Chaves-Lopez et al., 2015). Carbon disulfide even prevented the growth of *F.o* f.sp. *lactucae* completely (Chaves-Lopez et al., 2015).

Streptomyces species are prominently recognized for producing antibiotics (Danial et al., 2020, Encheva-Malinova et al., 2014, Saadouli et al., 2020), but the species volatile metabolites are gaining much notoriety for their biocontrol applications (Wu et al., 2015, Choudoir et al., 2019, Scholler et al., 2002, Cordovez et al., 2015). While both *Streptomyces* and *Bacillus* species produce some of the same VOCs, such as alcohols and ketones (Cordovez et al., 2015, Wu et al., 2015, Choudoir et al., 2019, Scholler et al., 2002). It is noteworthy that researchers are reporting *Streptomyces* VOCs showing effective inhibition against *Pyrenochaeta lycopersici*, *Sclerotiorum rolfsii*, *F. oxysporum* (Wu et al., 2015) and *Rhizoctonia solani* (Cordovez et al., 2015). A common theme among reviewed literature was the VOC inhibition of fungal pathogens was species specific among bacterial and fungal species (Zhao et al., 2011). As we continued with

our experiments in this study, we were intrigued by the possibility that co-exposure of *Bacillus* and *Streptomyces* species can impact Fov4 physiology and virulence through VOCs.

In this study, we observed the reduction in Fov4 mycelial growth on agar media and suppression of Fov4 infection in cotton through the exposure to *Streptomyces* and *Bacillus* VOCs. Published reports demonstrated the inhibitory effects of VOCs from *Streptomyces* and *Bacillus* species on fungal growth, including *Fusarium* species, *Botrytis cinera, Alternaria brassicola, A. brassicae*, and *Sclerotinia sclerotiorum*. These effects are mostly inhibition of mycelial growth (Cordovez et al., 2015, He et al., 2020, Guevara-Avendano et al., 2020) and conidia germination (Asari et al., 2016, Chaves-Lopez et al., 2015). *Streptomyces* strains have been shown to produce numerous VOCs, and a combination of VOCs is responsible for antifungal properties rather than a single compound (Wu et al., 2015). Effects of bacterial VOCs on fungal growth does not completely kill fungal cells because once removed from VOC source, fungal pathogens have regained growth although at different rates relative to controls (Cordovez et al., 2015, Wu et al., 2015). Our data support these findings as exposure to VOCs did not completely prevent infection but slowed down Fusarium wilt pathogenesis and produced different visual symptom progression than positive controls.

As described by Bell et. al. (Bell et al., 2017), Fov4 does not require root-knot nematode for cotton infection, which raises some intriguing questions regarding the mode of infection when compared to Fov1. It is also confounding to note that wounding and direct injection of Fov4 inoculum into cotton seedlings does not provide reliable symptom development in laboratory assays (Liu et al., 2011). Therefore, current Fov4 virulence assays rely on indirect fungal inoculum injection into soil where cotton seedlings are grown, and this practice can lead to inconsistent and sometimes non-reproducible assay results (Bell et al., 2019, Bell et al., 2017).

With our soil-free assay system, we were able to achieve high reproducibility among treatment groups. In other soil virulence assays, the assessment of disease severity was mostly done on above ground parts by measuring shoot growth suppression, leaf yellowing and overall plant wilting (Bell et al., 2019, Bell et al., 2017, Liu et al., 2011). In this study, we were able to monitor above-ground disease symptom development but also examine root system when experiment was terminated. Symptoms in conventional Fov4 virulence assays in soil were evaluated at 38 dpi (Bell et al., 2019), while symptoms in our study were observable at 6 dpi. As described earlier, some of the recent modified Fusarium wilt assays required large amount of pathogen inoculum, transplanting of cotton seedlings, and inefficient inoculation strategies (Cianchetta & Davis, 2015, Ortiz et al., 2017, Wang et al., 2018). Additionally, our method was easily adaptable to exposing Pima seedlings to bacterial VOCs by placing seedlings with SCO inoculum in an empty well of a divided petri dish. However, a limitation was that while the amount of liquid culture of bacteria added to both I and Y plates was the same, the surface area where the bacteria was allowed to grow was different. A dose dependent analysis with uniform surface area for bacterial growth would help clarify if the combination is truly better because the lack of differences could be due to a reduction in the amount of VOCs exposed to Fov4 infected seedlings in the Y-plate set up. This problem may also be evident when looking at the p values from the absorbance VOC screening compared to the agar plates. Volume was uniform in the absorbance assay which utilized 24 well plates while the agar plate assay was not. This issue further exemplifies the difficulties faced when studying complex biological systems. We acknowledge that this is an artificial assay system that does not truly reflect cotton production field conditions. But for our VOC assays, with sufficient replicates and control samples, this

approach allowed us to determine the difference between VOC treated and non-VOC treated Fov4 infected Pima seedlings.

Our study showed that bacterial VOCs from *Bacillus* and *Streptomyces* isolates are capable of suppressing Fov4 infection, and this outcome has practical and fundamental research implications. First is the prospect of developing VOCs as commercially available biocontrol agents. Currently, many biocontrol agents are sold as whole organisms intended for agricultural use, such as bioorganic fertilizers (Deng et al., 2021). In 2019, for Upland cotton production in Texas, only 5% of suppression methods used biological pesticides while plowing down crop residue with conventional tillage accounted for 68% of prevention methods used on Upland cotton cultivation (USDA, 2020). There are possibilities for biological pesticides in cotton production. More research into prevention and suppression methods in response to Fov4 will be vital as Pima cotton production is predicted to increase in Texas (Witt, 2020). The second research implication is the prospect of recognizing VOCs as a communication tool in soil microbial community and gaining a deeper understanding on how VOCs mediate plant infection at molecular levels. Further research will offer a new opportunity to understand the fundamental mechanisms involved in microbial community interactions via VOCs that lead to plant pathogenesis in root rhizosphere.

CHAPTER III

PAENIBACILLUS ELP529 IN COMBINATION WITH *BREVIBACILLUS* RZ160 SHOWS A SYNERGISTIC RELATIONSHIP CAPABLE OF IMPROVED INHIBITION OF FOV4 THROUGH ONLY VOC EXPOSURE

Introduction

The *Paenibacillus* genus was first described by Ash, et. al. in 1993, most notably as a genus of species that are facultatively anaerobic, or strictly aerobic, endospore forming, gram variable, and prolific producers of antimicrobial compounds (Lee et al., 2004, Aktuganov et al., 2014, Aw et al., 2016, Kim et al., 2004, Haruna et al., 2017). This genus is also characterized as species found in extreme environments (Baindara et al., 2016). In 2004, *Paenibacillus ehimensis* and *Paenibacillus chitinolyticus* became the newest members of the *Paenibacillus* genus after extensive rRNA analysis (Lee et al., 2004). In 2016, *Paenibacillus* genus was comprised of 165 species (Aw et al., 2016), by 2017 it increased to 395 known species (Haruna et al., 2017). As bioprospecting is now a key aspect of sustainable agriculture the number of species is likely to keep increasing.

Paenibacillus species are found in poor soil habitats with limited carbon sources (Aktuganov et al., 2008a). This genus can exhibit broad antagonism against fungal plant pathogens including *Bipolaris sorokiniana, Fusarium oxysporum, Rhizoctonia solani, and Pythium aphanidermatum* (Aktuganov et al., 2008a, Aktuganov et al., 2008b, Kim et al., 2004). While *Paenibacillus* species are capable of producing powerful, water-soluble antimicrobials (Chen et al., 2019, de Araujo et al., 2013, Huang et al., 2013, Baindara et al., 2016, Naing et al., 2015a), they are also known to produce volatile organic compounds (VOC) with antimicrobial

properties of their own. The volatile organic compound (VOC) production of *Paenibacillus ehimensis*, along with other species in the genus, such as *P. polymyxa* (Cheng et al., 2017, Lee et al., 2012), have shown promise for uses as biocontrol agents against fungal pathogens and nematodes.

Biocontrol agents could capitalize on volatile organic compound (VOC) metabolites from *Paenibacillus* species for the control of plant pathogens (Verginer et al., 2010). Along with other rhizosphere bacteria, *Paenibacillus* species are capable of producing unique VOC profile causing differentially expressed genes in other rhizosphere bacteria with functional roles in various metabolic processes (Garbeva et al., 2014). Bacterial VOCs can also increase expression of genes involved in the protection of cells against ROS damage (Garbeva et al., 2014). If *Paenibacillus* species are capable of significant changes in gene expression of co-habituating bacteria, then the symbiotic relationship of *Paenibacillus* with other rhizosphere and soil bacteria is a unique source of biocontrol methods.

A strain of *Paenibacillus* ELP529 was previously isolated from El Paso County, Texas soil, infected with *Fusarium oxysporum* f.sp.*vasinfectum* race 4 (Fov4). Fov4 is a devastating cotton pathogen specifically towards Pima cotton (*Gossypium barbadens*e) which was first identified in the United States in California in 2004 (Kim et al., 2005) and has since been identified in El Paso county, Texas (Crutcher et al., 2016) and New Mexico (Zhu et al., 2020). El Paso County, an arid climate, possesses soils predominated by sandy loams and high salinity where Fov4 thrives. The climate where Fov4 is found is similar to the lifestyle of *Paenibacillus* species which is also salt tolerant at 0-2% (w/v) NaCl (Aw et al., 2016, Kim et al., 2004) but has no growth at 4% (w/v) NaCl (Cho et al., 2017). We noticed *Paenibacillus* ELP529 exhibited unpredictive growth of physical colonies but still possessed unique Fov4 inhibition properties.

Paenibacillus species appear to tell an interesting story with respect to interactions with other soil microbes. They selectively inhibit prokaryotes and eukaryotes depending on environmental stimuli as well as produce unique VOC profiles altering gene expression in surrounding bacteria (Baindara et al., 2016, Aktuganov et al., 2008b, Garbeva et al., 2014). *Paenibacillus* species have also been shown to degrade fungal cell walls and alter hyphae physiology (Aktuganov et al., 2008a).

With the unique characteristics of *Paenibacillus* in mind, this study aimed to investigate microbial relationships which could enhance fungistatic properties of *Paenibacillus* ELP529, which is already present in Fov4 infected soils. The first objective in this study was to characterize microbial relationships between previously identified *Paenibacillus* ELP 529 isolate and other rhizosphere bacterial species: *Bacillus* Rz 141, *Brevibacillus* Rz 160, and *Streptomyces* HC658 and ELP 745. The second objective was to determine the impact of a selected *Paenibacillus* ELP529 combination on VOC suppression of Fov4 growth. Lastly, we determined if a selected *Paenibacillus* combination of VOCs suppressed *Fusarium oxysporum* f.sp. *vasinfectum* race 4 (Fov4) during Pima cotton (*Gossypium barbadense*) interactions.

Materials & Methods

Bacterial Isolates and Fusarium oxysporum f.sp. vasinfectum race 4

Paenibacillus ELP 529 isolate was previously isolated from a El Paso, Texas cotton field (Chapter II). Cultures of ELP 529 were maintained in parafilm sealed petri dishes containing ISP2 agar. Liquid cultures were prepared by placing an agar colony into liquid ISP2 broth in a culture tube filled with only 1ml of air space and shaken for minimum 5 days. Additional bacterial isolates were previously isolated from cotton rhizosphere (*Bacillus* Rz141,

Brevibacillus Rz160), non-cotton producing topsoil (*Streptomyces* HC658), and Fov4 infected cotton field (*Streptomyces* ELP745). These isolates were also maintained in ISP2 agar and liquid cultures were prepared by placing an agar colony into ISP2 broth and allowing to grow for 2-5 days before use.

Fusarium oxysporum f. sp. *vasinfectum* race 4 (Fov4) strain used in this study was isolated from diseased Pima cotton plants acquired from El Paso, Texas (Courtesy of Dr. Tom Isakeit, Texas A&M AgriLife Extension). Identification of this Fov4 isolate was confirmed using the method described in Doan, et. al. 2014 and AmplifyRP® Acceler8® for Fov4 rapid DNA kit (Product No. ACS 19700/0008) (Doan & Davis, 2015, Doan, 2014) . Fov4 culture was maintained on ISP2 agar amended with wheat bran (10g/1L). Fov4 conidia suspensions were made by flooding 7-14 day agar cultures with sterile water and plastic spreader to suspend conidia into solution, then solution was filtered through double layered sterile miracloth.

Growth Promoting Bacterial Relationships

To determine the ecological relationships that *Paenibacillus* ELP 529 has with surrounding soil microbes, we used an ISP2 agar petri dish assay to determine if there were any other antagonistic bacteria which may form a symbiotic relationship under aerobic conditions from co-culture with *Paenibacillus* ELP 529. To provide a comparison of symbiotic relationships, 10µl of liquid 7 day bacterial culture of ELP529 was placed at twelve spots equal distance from each other (Fig. 14A). We added a second set of 12 inoculation spots for a second bacterial isolate (RZ141, RZ160, ELP745, HC658) to be next to *Paenibacillus* ELP529 (Fig. 13B). Treatments were monitored every other day for physical colony growth, at day 10 the experiment was terminated. To determine relationships between the bacteria, a percent formation

was calculated based on number of physical colonies formed, 100% formation meaning 12 physical colonies formed. The experiment was completed with three replicates, each replicate containing 12 colonies of each isolate.

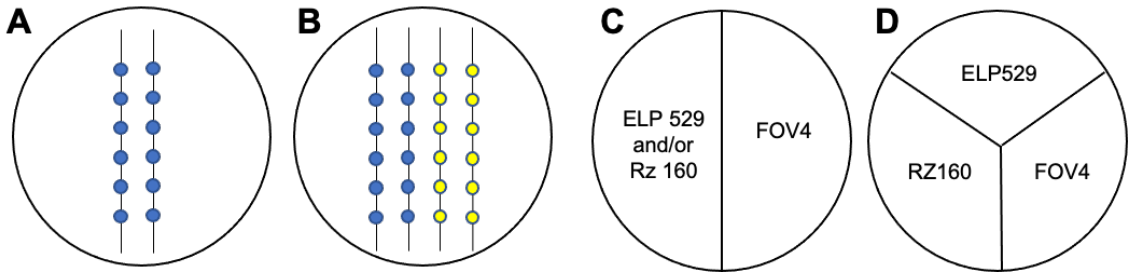

Figure 13. Experimental set up for *Paenibacillus* ELP529 relationships and VOC inhibition. (A) shows how a single bacteria isolate was compared to the combination, (B) the placement of two isolates close to each other on a single ISP2 agar petri dish, (C) shows physical separation to determine an individual isolate or a mixture of the two isolate's VOC inhibition, (D) shows the physical separation of the two isolates to determine VOC inhibition of Fov4.

VOC Antagonism of Fov4

To further investigate the symbiotic bacterial relationships previously tested, we aimed to characterize the VOC impact of the symbionts on the inhibition of Fov4. To a petri dish with a physical barrier creating two equal volume compartments (I-plate) with ISP2 agar, 120µl of liquid ISP2 culture for each isolate was streaked over the entire area on one side of the I-plate (Fig. 13C). Petri dishes containing isolates were sealed with parafilm to allow for colony formation and after 10 days parafilm was removed and 0.5cm diameter ISP2 agar plug of Fov4 was added to the opposite section of the I-plate containing no bacteria (Fig. 13C). Additionally, Y-plates with three equal volume compartments in a petri dish, were utilized to physically

separate bacterial combinations to determine VOC impact on Fov4 when the two isolates were not co-cultured (Fig. 13D). Plates were re-sealed and incubated at room temperature. Experiment was done with three replicates and growth was monitored until the diameter of the positive control, Fov4 only, reached the edge of the plate. Radial Fov4 growth was measured 4 days post inoculation (dpi). Diameter measurements were analyzed using an ordinary one-way ANOVA and Tukey's multiple comparisons posttest using at 95% confidence interval on GraphPad Prism (San Diego, CA).

Virulence of Fov4 exposed to Paenibacillus VOCs.

To test the ability of bacterial VOCs to influence pima cotton growth during Fov4 infection, seedlings were exposed to only bacterial VOCs while infected with Fov4. Methods used in this section are the same used in Chapter II. Pima cotton seeds were initially surface sterilized by rinsing with 70% ethanol for 5 minutes, 1 minute with sterile water, 10 minutes with 10% bleach, followed by three rinses with sterile water. Sterile seeds were placed onto sterile cotton circles and covered with a second cotton circle. The cotton was moistened with 2ml of sterile double deionized water, placed inside of plastic bags and exposed to natural sunlight for approximately 14 days until cotyledon leaves had emerged.

Before the start of the seedling assay, I-plate petri dishes with equal compartments physically separated, one side was filled with 10ml of ISP2 agar and the other side was left empty. Similarly, a Y-plate petri dish with three equal compartments was used to physically separate isolates. To the ISP2 side 120µl of liquid bacterial culture was streaked over the entire area of the well. One set of I-plates contained an equal mixture of isolates. Bacteria was streaked

10 days prior and sealed with parafilm before adding Fov4 steel cut oat (SCO) inoculum and Pima cotton seedling

Fov4 inoculum was prepared by pipetting a 1ml of Fov4 conidia ($1x10^8$) suspension into twice autoclaved steel cut oats and incubated at 28ºC for 5-7 days with periodic shaking between. In I-plates and Y-plates containing bacteria on over ISP2 in one compartment, Pima cotton seedlings with root and shoot lengths between 5-7cm were placed in the neighboring compartment (devoid of ISP2 agar). Seedlings were placed in between one-half cotton circle on bottom and a quarter cotton circle over the steel cut oats inoculum with approximately 1.5g of Fov4 steel cut inoculum.

The experiment was conducted twice for negative (*n*=14), positive (*n*=14), ELP529 (*n*=11), RZ160 (*n*=10), co-culture I-plate (*n*=10), and once for Y-plate culture (*n*=4). Additionally, a separate experiment was conducted with only negative SCO to determine effects of bacterial VOCs on Pima cotton seedlings (*n*=3).

Disease Assessment

Diseased seedlings were rated on a color scale of root shoot junction external coloring. The root shoot junction is an indicator of disease progression and was rated on a scale of low, moderate, or high disease severity. Low rating (1) consisted of coloring of green or green with pink shading. Moderate (2) rating indicated a solid pink coloring while high rating (3) light brown, solid purple (4), brown (5), or blackening (6). Numerical ratings of each disease level were analyzed using a one-way ANOVA non-parametric Kruskal-Wallis analysis with a Dunn's multiple comparisons posttest, with a 95% confidence interval using Graphpad Prism (San Diego, CA).

Results

Paenibacillus sp. and Brevibacillus sp. show symbiotic relationship in co-culture

Of all the combinations tested, ELP529 grown next to Rz160 showed 100% colony formation for both isolates without any phenotypic changes (Fig. 14A). The relationships between ELP529 and HC658 showed 100% formation for each isolate (Fig. 14B). However, ELP529 colonies closest to HC658 showed a change in pigment. Co-culture with ELP745 resulted in the line of ELP529 closest to ELP745 formed half colonies (Fig. 14D). Similar results were seen in the co-culture with Rz141 (Fig. 14C).

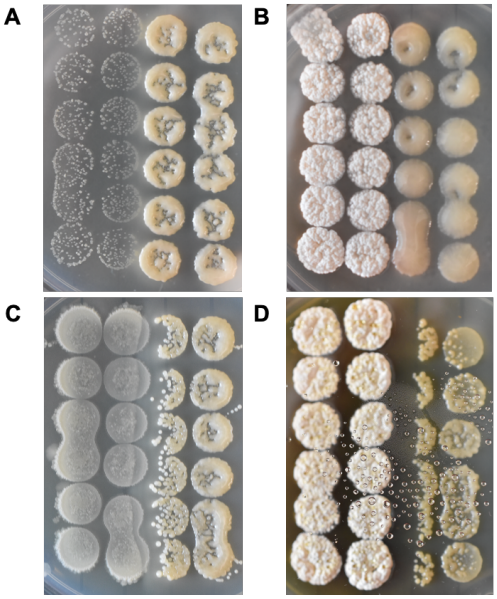

Figure 14. Examined *Paenibacillus* ELP529 bacterial relationships. *Paenibacillus* ELP529 inoculations are shown on the two right columns in each picture (A-D). The two left columns are as follows: (A) *Brevibacillus* Rz160, (B) *Streptomyces* HC658, (C) *Bacillus* Rz141, (D) *Streptomyces* ELP745.

The number of ELP529 colonies formed did not change when it was co-cultured with RZ160 or HC658. However, the pigment change of the six colonies closest to HC658 led us to rule out any further investigation into utilizing ELP529 and HC658 combinations. The combination with Rz160 showed uniform pigment and colony formation for both isolates. We were further interested if this symbiotic relationship improved VOC inhibition of Fov4.

Paenibacillus and Brevibacillus cooperation increased VOC inhibition of Fov4 growth

After determining the mutualistic relationship between ELP529 and RZ160, we aimed to determine if this mutualistic relationship could affect VOC profiles and subsequent impact on Fov4 inhibition through VOC exposure only. We tested the VOC inhibition from only single exposure to ELP529 (Fig. 15A) or RZ160 (Fig. 15B) in I-plates which resulted in 11% inhibition from ELP529 and 2% inhibition from Rz160. However, when the two isolates were co-cultured in an I-plate together (Fig. 15C), this resulted in 10% inhibition. The inhibition was further increased when ELP529 and RZ160 were kept separate on a Y-plate during exposure to Fov4 resulting in 27% inhibition (Fig. 15D). It should also be noted that in Fig. 15A, there was no physical colony formation of ELP529 which is a behavior we had previously noted which led to this investigation.

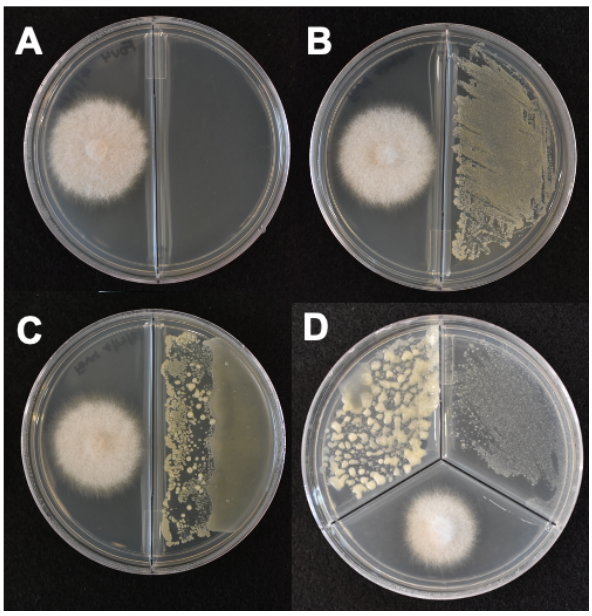

Figure 15. *Paenibacillus* ELP529 and *Brevibacillus* Rz160 VOC inhibition of Fov4. (A) right side inoculated with ELP529, (B) right side inoculated with Rz160, (C) mixture of ELP529 and Rz160 in an I-plate, (D) ELP529 and Rz160 separated in a Y-plate for VOC inhibition.

VOC Impact on Fov4 Pathogenicity in Cotton

With the knowledge that a synergistic relationship with Rz160 and ELP529 existed, we aimed to determine if the cooperative relationship extended to suppressing Fov4 infection of Pima cotton. At 5 dpi a nonparametric Kruskal Wallis and Dunn's posttest showed there was significant difference between the negative and positive controls ($p=0.0030$). There was significant difference between the positive control and I-plate cultures ELP529 and Rz160 ($p<0.05$). Additionally, positive control compared to single culture of ELP529 showed no statistical significance.

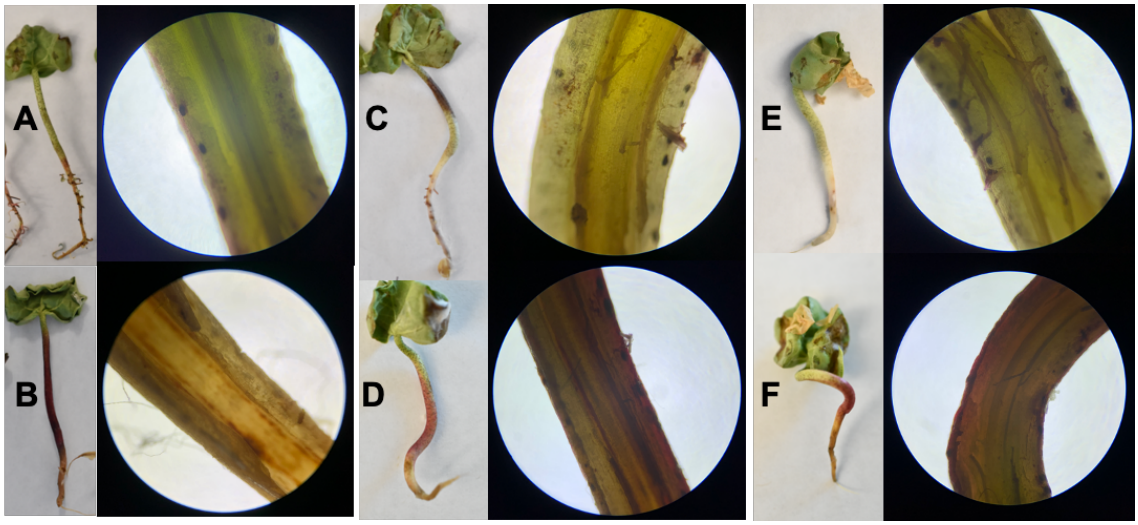

Figure 16. Samples of Fov4 infected Pima cotton also exposed to ELP529 and Rz160 VOCs. (a) Negative control without Fov4 or VOCs, (b) positive control without VOCs, (c) Fov4 infected pima cotton exposed to ELP529 VOCs, (d) exposed to Rz160, (d) exposed to a mix of ELP529 and Rz160 culture, (e) exposed to VOCs from physically separated ELP529 and Rz160.

Discussion

Paenibacillus has been cited for capabilities of altering certain gene expression of neighboring microbes without impacting physical growth of another (Garbeva et al., 2014). The synergistic relationship shown in this study suggests *Paenibacillus* ELP529 has a similar attribute valuable to *Brevibacillus* RZ160 and vice versa. These two species have been isolated together from hot springs (Sahay et al., 2017) and in the same study showed different enzyme production as well as stability. Both species grow in a pH range of 3-8 (Sahay et al., 2017, Singh & Chhatpar, 2011, Prasanna et al., 2013), but *Paenibacillus* appears to produce the most heat stable enzymes, up to 80°C (Singh & Chhatpar, 2011) while stability was 70°C for *Brevibacillus laterosporus* (Prasanna et al., 2013). The other relationships tested between *Paenibacillus* ELP529, *Bacillus* RZ141, *Streptomyces* HC658, and *Streptomyces* ELP745 showed negative impact on growth of ELP529. It should also be noted that when there was no Pima cotton used in

the experiment, the co-culture Y-plate experiment showed the best at inhibiting Fov4 growth. Conversely, once Pima cotton was added to the VOC assay, the I-plate (co-culture) showed the best at suppressing Fov4 infection. There is a more complex interaction occurring depending on participants in the communication network and growth environment.

Paenibacillus species are those which inhabit harsh soil environments while maintaining antimicrobial properties. The cooperative relationship between *Paenibacillus* and *Brevibacillus* has been investigated for promotion of root nodules not just for nitrogen fixation, but also for high concentrations of antimicrobial compounds (Hansen et al., 2020). Soil that Fov4 inhabits in arid, west Texas sandy loam soils with high salinity are uniquely harsh environments showing that effective biocontrols will be those which can endure the same environment as Fov4. Soil type, fertility, and microbial interactions all work together to make a conducive environment for Fov4 growth and survival (Davis 2006). Whether or not these two species would be combined into one biocontrol product is yet to be determined.

The VOC inhibition is of great interest for controlling Fov4 as it does not require proximity to another organisms. The VOC inhibition of the co-culture of ELP529 and RZ160 still had VOC inhibition capabilities, but the Y-plate culture of physically separated ELP529 and RZ160 had an even greater VOC inhibition. Once ELP529 and RZ160 were separated, they reached their full VOC inhibition potential against Fov4. This behavior from the interaction between ELP529 and RZ160 leads these authors to question whether these two organisms should be administered together or should only RZ160 be added to an environment where *Paenibacillus* is already abundant to enhance native fungistatic properties. However, as stated previously in this section, the co-culture in I-plates showed better suppression properties when VOCs were exposed to Fov4 infected Pima cotton. This raises the question on how best to utilize the

combination of *Paenibacillus* ELP529 and *Brevibacillus* Rz160 as biocontrol agents as they had better VOC inhibition before the Pima cotton was added to the experiment. Interestingly, Deng et. al. 2021 showed that using a pre-plant fumigant and a biofertilizer amended with *Bacillus amyloliquefaciens* was able to suppress *Fusarium oxysporum* abundance before planting and sustained until harvest (Deng et al., 2021).

Paenibacillus chitinases have been suggested to use as additives to commercially used fungicide formulations to reduce amount of fungicide used. However, biocontrols have largely been effective *in vitro* but not in field conditions with highly variable results (Shibulal et al., 2017). Singh et. al. 2011 also suggested that to overcome the ineffectiveness of biocontrol agents that we should be thinking about utilizing mixtures of biocontrol components (Singh & Chhatpar, 2011). Using a soil drench has been explored with *Brevibacillus* where it also inhibited *F.o.* f.sp. *cucumerinum* and also showed reduction in nematode populations (Li et al., 2005).

The control of Fov4 has exposed the lack of control measures available to deal with an aggressive emerging pathogen. Previously, Fov race 1 was controlled through the use of nematicides but this is not the case for Fov4 (Bell et al., 2017). Chemical controls for Fov4 infection of cotton consist primarily of carbendazim, thiophanate methyl, thiovit, and dithane M-45 (Asif et al., 2020). Cultural practices rely on the transport of clean seed, sanitation, and hot water treatments with or without fungicides (Cianchetta & Davis, 2015, Bennett & Colyer, 2010). Solarization of soils with double polyethylene film treatment and wet heat treatments were shown to reduced Fov4 chlamydospore viability (Cianchetta & Davis, 2015). Controlling pH of soils can also provide some control as soils that are more basic do not support *Fusarium oxysporum* and disease production. Lastly, biological controls of Fov4 are not currently well documented, but numerous *Fusarium oxysporum* species have gone through investigation to

match the best biological control agent and mechanism of pathogen suppression (Naing et al., 2015b).

This study offers a unique combination which could be an alternative to controlling a disease like Fov4 in harsh soil environments where some biological controls may not thrive. We were able to show that *Paenibacillus* ELP529 and *Brevibacillus* RZ160 together showed a synergistic relationship that was growth promoting for each bacterium as well as a synergistic effect of suppressing Fov4 through VOC exchange only. The VOC inhibition capability also extended to the ability to suppress Fov4 infection in Pima cotton.

CHAPTER IV

CHARACTERIZATION OF *FUSARIUM OXYSPORUM* SODIUM RELATED TRANSPORTER GENE IN *FUSARIUM OXYSPORUM* F.SP. *VASINFECTUM* RACE 4

Introduction

Fusarium oxysporum species complex (FOSC) are a group of common soil borne pathogens with a wide host range, including cotton. *Fusarium oxysporum* f.sp. *vasinfectum* (Fov) is a cotton pathogen leading to root rot, wilt, and eventual death of cotton varieties. Fov is capable of long-term survival through formed chlamydospores which ensure its overwintering between growing seasons (Cianchetta & Davis, 2015). The pathogen is not unique in that multiple races exist and have been characterized based on soil types and requirements of root knot nematodes to complete the infection cycle and host specificity. Fov race 4 (Fov4) is unique in its ability to infect without the assistance of root knot nematodes, while Fov race 1 (Fov1) has long been controlled using nematicides (Bell et al., 2017, Halpern et al., 2020). Fov4 is uniquely different from Fov1 in its host specificity, soil habitat, and no requirement of root knot nematodes for infection. Fov4 has been shown to inhabit different environments than Fov1, which predominately infects Upland cotton.

Upland and Pima cotton are grown in separate regions of Texas. El Paso County is the one small region in Texas where Pima cotton is grown while the production of Upland cotton production dominates the Texas cotton industry (USDA, 2020). However, the cultivation of Pima cotton in Texas is expected to increase (Witt, 2020). Fov4 growth favors neutral to alkaline soils (Liu et al., 2011). Whether or not these soil properties contribute to pathogenicity or long-term survival, or both, has yet to be clearly shown.

When considering infection mechanisms of Fov4, the hypothesis is that the pathogen utilizes effectors when infecting cotton. The first *Fusarium oxysporum* (Fo) effectors were identified in *F.o.* f.sp. *lycopersici* (Fol) in the xylem, subsequently named secreted in xylem (SIX) effector proteins. There are 14 identified, species specific, SIX effectors localized on a few chromosomes (Li et al., 2020). Since the initial identification of SIX effectors in Fol, homologs have also been found in *F.o.* f.sp. *cubense* (Foc) (An et al., 2019) and *F.o.* f.sp. *cepae* (Armitage et al., 2018). A relevant consideration is effector pairs might also be required for compatible infections (Cao et al., 2018). Locations of SIX effector genes has been on chromosome 11-13 in *F.o. cepae* (Armitage et al., 2018) and chromosome 14 for Fol (Li et al., 2020).

The effectors used by Fov4 have yet to be completely identified but with the recent publication of the Fov4 genome, comparative genomics is possible when comparing the Fov4 genome to Fov1 genome. The Fov4 genome is much larger (63Mbp) than Fov1 (50Mbp) and encodes approximately 20,222 genes (Seo et al., 2020). The difference in genome size and number of genes, compared to Fov1, led us to the hypothesis there was added, removed, or mutated genetic material in Fov4 which contributed to differences in pathogenicity and lifestyle.

This study aimed to identify putative genes in Fov4 different from the Fov1 genome which were differentially expressed during Pima cotton infection. A multifacilitator superfamily (MFS) transporter was one of the genes expressed during infection. Through homologous recombination, we were able to generate a gene knockout mutant which was further characterized and found to be related to sodium tolerance.

Materials and Methods

Identification of putative Fov4 genes

The genomes of both races, Fov4 and Fov1, are available in NCBI database organized into scaffolds. Each scaffold was initially aligned using NCBI nucleotide Blast function. Scaffolds which showed 90% or greater identify were further aligned using Vista Align open-source software (http://genome.lbl.gov/vista/index.shtml). Sequences of interest were subjected to NCBI ORF finder, nucleotide Blast, and translated protein Blast queries. Fov4 sequences which did not show homology to Fov1 or showed less than 98% identity to Fov1 sequences were subjected to individual nucleotide and protein sequence analysis. Putative genes were further investigated for relative gene expression during Fov4 infection.

Fov4 Strain used in this study

Fusarium oxysporum f. sp. *vasinfectum* race 4 (Fov4) strain used in this study was isolated from diseased Pima cotton plants acquired from El, Paso, Texas (Courtesy of Dr. Tom Isakeit, Texas A&M AgriLife Extension). Identification of this Fov4 isolate was confirmed using the method described in Doan, et. al. 2014 and AmplifyRP® Acceler8® for Fov4 rapid DNA kit (Product No. ACS 19700/0008) (Doan, 2014). Fov4 inoculum was prepared by growing on ISP2 agar medium (HIMEDIA, 2019) and flooding 7-14 day cultures with sterile water and plastic spreader to suspend conidia into solution, then solution was filtered through double layered sterile miracloth (Bell et al., 2019). Conidia concentration was determined by hemocytometer.

Relative gene expression

To examine relative gene expression of the putative Fov4 genes identified we used a soil-free assay that allowed us to extract RNA from an active infection while minimizing variability due to biotic and abiotic factors, such as seed health and soil characteristics. The soil-free pathogenicity assay described in Chapter II was used for the relative gene expression study. First, Pima cotton seeds (PhytoGen, No. PHY841RF) were initially surface sterilized in 10% bleach and 70% ethanol and rinsed in sterile water three times. Seeds were then placed onto sterile cotton circles and covered with a second cotton circle. Sterile double deionized water (5 ml) was applied to cotton circles, placed inside of plastic sandwich bags, and exposed to natural sunlight for approximately 5-7 days when cotyledon leaves had emerged. Once seedlings had reached between 5-7 cm in shoot and primary root length, they were placed on a fresh sterile cotton circle in a new petri dish for the experiment.

To prepare pathogen inoculum for Fusarium wilt virulence assay on Pima seedlings, twice sterilized steel cut oats (SCO: 20 g organic steel cut oatmeal and 20ml water) in 100 ml Erlenmeyer flasks were inoculated with 1 ml Fov4 conidia suspension (1 $x10^8$ conidia/ml). The flasks were incubated at 28°C for 5-7 days with periodic shaking to maximize fungal growth. When cotton seedlings and Fov4 inoculum were ready, we placed Fov4 SCO (1.5 g) on Pima seedings so that SCO covered the primary root. A half circle or quarter circle covered the roots and SCO leaving the root shoot junction, shoot, and leaves visible. Before sealing the plates, sterile water (4 ml) was added to the top half or quartered cotton circle. These petri dish plates were placed under a 12 hr light and dark cycle at 22°C, and after 6 days the seedlings were removed for examination of Fusarium wilt symptoms. Autoclaved SCO not inoculated with Fov4

served as the negative control. The experiment was completed once with three replicates and terminated at 6 dpi for RNA extraction and qPCR.

To extract RNA, the RNA Plant Mini kit (Thermo Fisher Scientific, Waltham, MA) was used according to manufacturer's guidelines. qPCR analyses were conducted by Step One plus realtime PCR system using the DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA). Primers used are shown in Appendix B. Relative expression levels of each gene were calculated using 2-delta-delta-CT method and normalized with Fov ß- tubulin gene. All qPCR assays were performed three times (Yan & Shim, 2020).

Gene deletion and complementation

Gene deletion and complementation was accomplished using the methods described in Yan, et. al. 2020. Briefly, null mutations were generated in Fov4 through homologous recombination (Yan & Shim, 2020). Gene specific primers used in this study are shown in Appendix B. Template DNA extracted for further studies used an extraction buffer of comprised of 1M Tris-HCl, 0.5M EDTA, 20% SDS, 5M NaCl (0.05:0.1:0.1:0.1 *v:v*). Lysis of Fov4 mycelia was accomplished using warmed extraction buffer and vortex mixing with glass beads. To the lysed solution, chloroform (200µl) was added vortexed and centrifuged before the aqueous layer (200µl) was transferred to a clean tube and chilled 200 proof ethanol (400µl) was added then inverted 5x. The mixture was cooled at -20°C for 30 minutes followed by centrifugation before the supernatant was removed, and pellet resuspended in 70% ethanol, vortex mixed, and centrifuged again to remove ethanol layer. The pellet was allowed to dry in a laminar hood room temperature. The DNA template was resuspended in double deionized sterile water and stored at -20°C until use.

Partial hygromycin B phosphotransferase gene (HYG), designated as HY and YG, were used to fuse with left and right flanking regions of the targeted gene with join-PCR approach. For complementation, we amplified the target gene with its native promoter and terminator, then co-transformed with pB15 plasmid to the mutant protoplast. All gene knockout constructs and complementation fragments in this study were amplified using Phusion Flash High-Fidelity PCR master mix (Thermo Scientific, Waltham, MA) following the manufacturer's instructions. To identify putative null mutation strains, all transformants were screened by PCR using Phire Plant Direct PCR Kit (Thermo Scientific, Waltham, MA) and Taq DNA polymerase (New England Biolabs, Ipswich, MA). Putative mutant strains were further confirmed with qPCR.

Fungal growth study

WT and null mutant growth were characterized by growing from a 0.5cm 0.2xPDA agar plug at the center of plates containing: ISP2 (HIMEDIA, 2019), ISP2 amended with 2% wheat bran, 0.2xPDA, and YEPD. After 6 days of growth, diameter was measured for comparisons. Three biological replicates were used for each treatment.

Carbon utilization was accomplished using Czapek – Dox media amended with maltose (10g/L), sucrose (30g/L), dextrose (10g/L) or fructose (10g/L). After 6-8 days of growth at room temperature (25°C), when WT diameter had reached the edge of the petri dish, diameter of radial growth was measured for comparisons. Experiment was done with three biological replicates for each strain and media. Experiment was repeated two more times.

Stress medium was used to assess phenotype of mutants against the wild type adapted from Yan 2020 (Yan & Shim, 2020). Briefly, stress phenotypes were observed on 0.2xPDA amended with 0.01% SDS, 2mM H_2O_2, 0.75M NaCl, 0.75M KCl, 0.75M $CaCl_2$, or 0.75M

$MgCl_2$. When negative control (0.2xPDA) radial growth had reached 7-8cm diameter, experiments were terminated, and final radial diameter measurements taken. Experiment was done with three biological replicates and entire experiment was completed two more times.

Diameter measurements for growth, carbon utilization, and stress media were compared using one way ANOVA followed by a Tukey post hoc test with 95% confidence interval.

NaCl impact characterization

We next aimed to determine if the difference in radial growth under external NaCl stress extended beyond the 0.75M concentration which had previously been tested. Two additional concentrations of NaCl, 0.5M and 0.25M NaCl were amended to 0.2x PDA. Agar plugs (0.5cm, 0.2xPDA) were placed in the center of the NaCl containing petri dish and grown for 10-12 days at room temperature (25°C). When the negative control (0.2xPDA without NaCl) had reached a diameter of approximately 7-8cm, experiment was terminated, and final diameter measurements were taken from each treatment group. Diameters of WT and null mutant growth were measured for statistical analysis using a one-way ANOVA with Tukey post-test with a 95% confidence interval. Experiment was done with 3 replicates and the experiment was repeated two more times.

The impact of NaCl on conidiation, and pigmentation for WT and null mutant were also of interest as these two properties had been observed early on. To further investigate the impact of NaCl, WT and null mutant were grown in 0.2x potato dextrose broth (0.2xPDB) and 0.2xPDB/0.75M NaCl. To a flask containing 100ml of 0.2xPDB or 0.2xPDB/0.75M NaCl, 200µl of $1x10^9$ conidia was added and mixed on an orbital shaker (100rpm) for three days at room temperature (25°C). Conidia were counted after three days of growth using a hemocytometer and

counts were normalized by calculating log base 10. To observe pigment change under NaCl stress, 1ml of liquid culture was added to each well in three rows (n=18) of a 24 well plate with an additional row only containing growth media (devoid of WT or mutant). The row with only 0.2xPDB was used to normalize absorbance data. The 24 well plates were grown at room temperature (25C) on an orbital shaker (100rpm) for 72 hours before analysis using a Spectral Max iD5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) at 350nm. The absorbance readings from 350nm and conidia concentrations were analyzed by a one-way ANOVA followed by a Tukey's multiple comparisons test using GraphPad Prism (San Diego, CA) with a 95% confidence interval.

Characterization of Fov4 infection

To determine if the null mutant was involved in Fov4 infection in Pima cotton, we used the same pathogenicity assay from relative gene expression method previously described. In addition to preparing WT inoculum as previously mentioned, null mutant inoculum was prepared in the same manner. Pima seedlings inoculated with Fov4 WT or null mutant were grown as previously described in relative gene expression. Experiment was terminated at 4 days post inoculation (dpi). Experiment was done twice with three replicates, each containing two subsamples.

To characterize Fov4 WT and null mutant pathogenicity, we rated external tissue coloring to compare each fungal strain's virulence. Percent incidence was assessed based on external tissue coloring at the root shoot junction. Total numbers were recorded for green-pink, pink, or purple-black coloring. Observations were analyzed in a contingency plot and fraction of column totals were used to determine distribution among treatment groups. We also observed wilting of the

leaves in each treatment group in the same manner. Additionally, the longitudinal section of the RSJ was observed under 0.4x magnification of internal tissue for symptoms of infection (Dowd et al., 2004).

Results

Gene expression and putative identification of major facilitator superfamily transporter

Comparative genomics analysis identified 8 genes different between Fov4 and Fov1, either not present in Fov1 or showed specific nucleotide, and/or amino acid differences. These 8 genes showed homology to three PKS-NRPS genes, one ankyrin protein gene, one aspercryptin biosynthesis cluster, a cargo transport protein, a xylanase β-glucanse, and a major facilitator superfamily (MFS) transporter (Table 5). Four of these genes were differentially expressed. However, what stood out was the expression of two transporter genes (Fig. 17A). The putative 13-MFS gene of interest was in Fov4 scaffold 4 (NCBI Accession Number VINP01000004.1) and showed 100% identity to the MSF transporter in *Fusarium oxysporum* Fo5176, accession number EGU80867.1.

Table 5. Putative Fov4 gene identification and relative expression.

Fov4 Sequence ID	Blast Result	Differentially expressed during Fov4 infection?
1	PKS-NRPS	
2	PKS-NRPS	+
3	Ankryin gene	
5	Aspercryptin biosynthesis cluster	
8	PKS-NRPS	+
11	Cargo transport protein	+
12	Xylanase β-glucanase	
13	MFS transporter	+

Upon closer examination of 13-MFS nucleotide sequences, the reverse complement of Fov1 nucleotide sequence showed a 98% identity to the 13-MFS sequence in Fov1. Clustal omega alignment of Fov4 and Fov1, identified a single nucleotide difference of guanine (G) in Fov4 instead of adenine (A) in Fov1 (Figure 17B). Additionally, the 13-MFS transporter sequence only showed one copy on both Fov4 and Fov1 genome scaffolds. The single point nucleotide difference correlated to a translated protein sequence difference of alanine in Fov4 and valine in Fov1 (Fig. 17C).

We became interested in the expression of the two transporter genes differentially expressed during infection. Through homologous recombination we were able to generate two null mutants of the putative 13-MFS transporter gene in Fov4, further referred to as Δ*13_2* and Δ*13_5*. Deletion of 13-MFS gene was further confirmed by qPCR showing expression of gene 13-MFS in WT and not in the two null mutants.

Figure 17. Relative gene expression and sequence 13-MFS transporter alignment Fov4 scaffold 4 and Fov1 scaffold 4. (A) shows the relative gene expression of transporters and other metabolic associated genes, (B) nucleotide alignment between Fov4 and the reverse complement of Fov1 showing the single nucleotide difference, (C) translated protein sequence of Fov4 and Fov1 13-MFS gene with the resulting amino acid difference.

Growth studies

The MFS mutants *(Δ13_2, Δ13_5*) showed growth variation on different media tested (0.2xPDA, ISP2, ISP2 with 2% wheat bran, and YEPD) shown in Figure 18. Wild type (WT, Fov4) showed statistically significant differences between both mutants when grown on ISP2, YEPD, 2% wheat bran ISP2 and 0.2xPDA ($p<0.05$). When compared to each other, mutants showed no significant differences between each other on all medias. Strains grown on 0.2xPDA showed more violet pigmentation than other growth media. ISP2 amended with wheat bran appeared to show the most vegetative growth and white biomass while 0.2xPDA showed no excess of biomass but showed noticeable violet pigmentation. ISP2 and YEPD appeared to show similar white biomass production. Carbon utilization showed no difference for WT grown on all CD media and Δ*13_5* compared to WT showed $p<0.05$ for dextrose and fructose utilization (Fig. 18). Because of no variation between the two mutants, Δ*13_5* was used in subsequent studies.

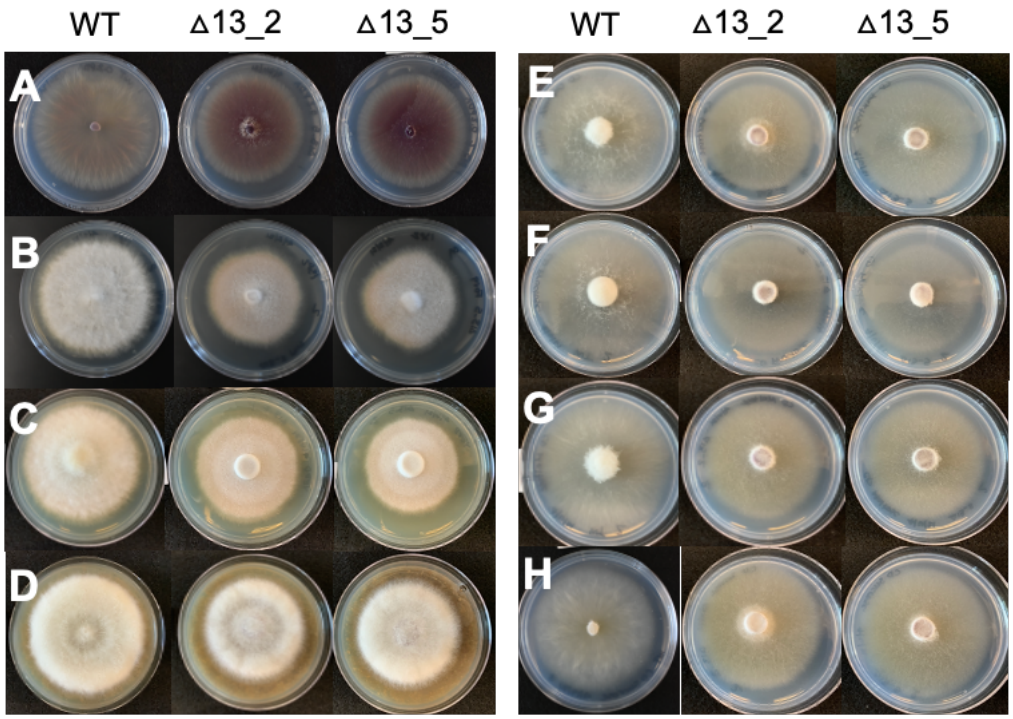

Figure 18. Growth and carbon utilization of 13-MFS transporter null mutant. Growth (A-D) and carbon utilization (E-H) studies to characterize both null mutants. (A) 0.2PDA, (B) ISP2, (C)YEPD, (D) ISP2 amended with 2% wheat bran, (E) Czapek-Dox (CD) media amended dextrose, (F) fructose amended CD, (G) maltose amended CD, (H) sucrose amended CD.

Of the stress media tested, no growth of WT or Δ*13_5* was seen on 0.01% SDS and 2mM H_2O_2. Growth on various 0.75M external concentration of salt compounds, $CaCl_2$, NaCl, KCl, and $MgCl_2$, amended to 0.2xPDA helped further characterize Δ*13_5*. When comparing growth of WT to growth of Δ*13_5*, there was no significant difference in radial diameters on $CaCl_2$, KCl, and $MgCl_2$ amended 0.2xPDA. Interestingly, 0.75M NaCl produced significantly different diameters of growth for WT and Δ*13_5* resulting in $p<0.0001$ (Fig. 19). The influence of NaCl on growth was investigated and characterized further. From this point further, the null mutant is referred to as *Fusarium oxysporum* sodium related transporter -1 *(ΔFoSRT1*).

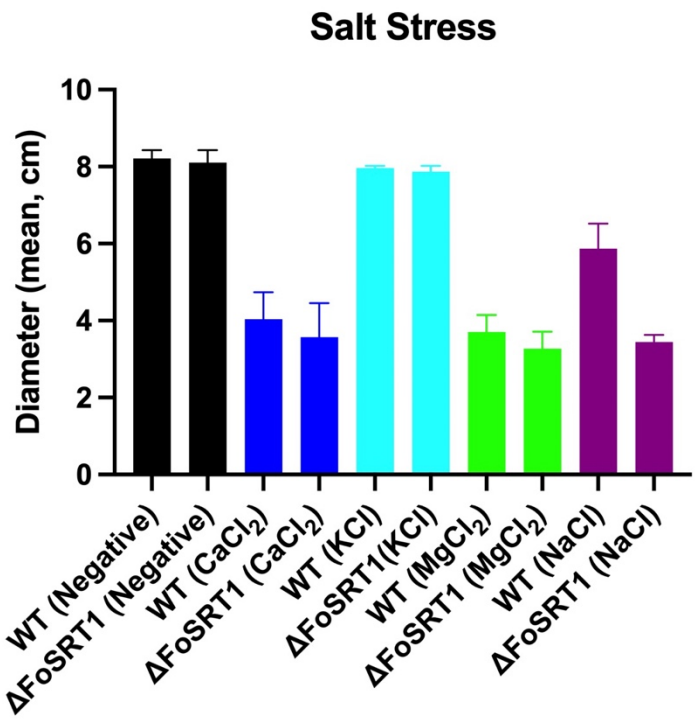

Figure 19. Diameter measurements on various salt containing media. Fov4 WT and Δ*FoSRT1* grown on external concentration of 0.75M of $CaCl_2$, KCl, $MgCl_2$, or NaCl amended to 0.2X PDA.

NaCl Growth Characterization

As we noticed the significant difference of growth between WT and Δ*FoSRT1* with external NaCl concentration of 0.75M, we aimed to determine if a linear relationship existed between radial growth and external NaCl concentration in 0.2xPDA. In addition to 0.75M NaCl concentration in 0.2xPDA, we tested growth on 0.5M and 0.25M NaCl concentrations (Fig. 20A). There were statistically significant differences ($p<0.05$) between WT and Δ*FoSRT1* at both concentrations. A linear regression performed of diameter measurements showed R^2 values of 0.8222 and 0.9225 for WT and Δ*FoSRT1*, respectively (Fig. 20B).

Pigment change of WT and Δ*FoSRT1* was analyzed after three days of growth in 0.2xPDB and 0.2xPDB/0.75M NaCl. Among the groups, a percent coefficient of variation

(%CV) was calculated from all 18 absorbance readings to determine homogeneity of the liquid culture and thus pigmentation. Representative, examples of two wells from each plate are shown in Fig. 20C with noticeable difference in the two wells taken from WT grown in 0.2xPDB/0.75M NaCl. WT grown in 0.2xPDB/0.75M NaCl showed the most variation, highest %CV, among the wells (72%) and lowest absorbance measurements (0.1159 AU) (Fig. 20D). The other treatment groups showed %CV less than 14% among the absorbance data. This correlated to the most significant differences in absorbance ($p<0.0001$) for WT vs WT (with NaCl) and WT (with NaCl) vs Δ*FoSRT1* (with NaCl).

Conidiation change of WT and Δ*FoSRT1* grown in 0.2xPDB/0.75M NaCl showed drastic differences (Fig. 20E). Hemocytometer generated concentrations were evaluated after calculating the $\log_{10}$ because of the extremely high counts from Δ*FoSRT1*. Further analysis showed a statistically significant difference between WT and Δ*FoSRT1* ($p<0.05$) in both 0.2xPDB and 0.2xPDB/0.75MNaCl. There was also significant difference between WT grown in NaCl and WT grown in only 0.2xPDB. However, NaCl did not have a significant effect on conidiation in Δ*FoSRT1* ($p=0.2917$).

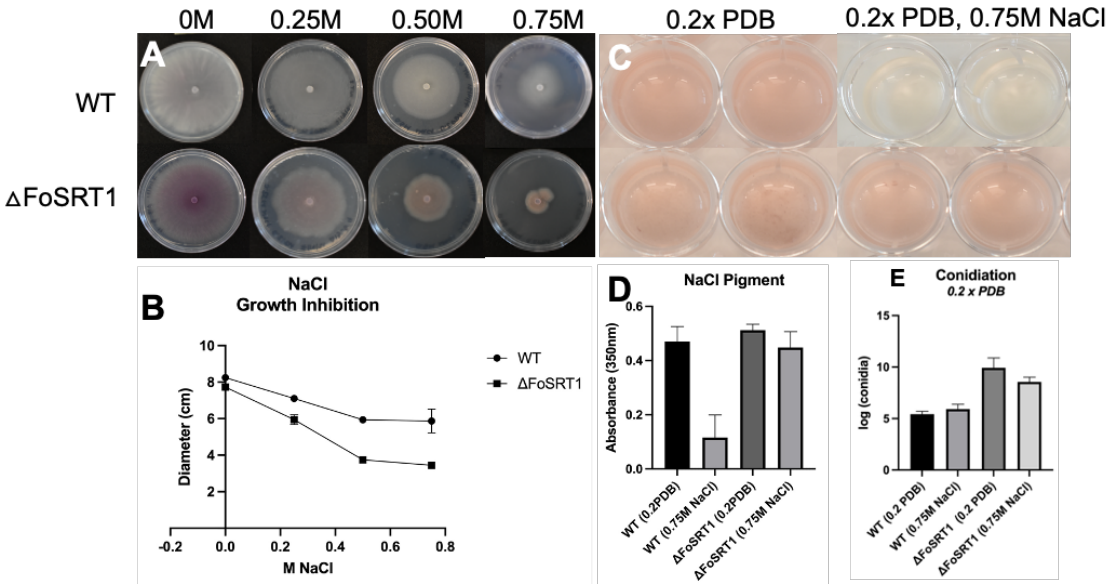

Figure 20. NaCl impact on growth of Fov4 WT and Δ*FoSRT1*. NaCl impact on growth characterization, (A) shows the variation on three concentrations of NaCl on WT and Δ*FoSRT1* growth, (B) linear representation of mean diameter graphed against external NaCl concentration, (C) representative examples of 2 of the 18 wells containing liquid culture of WT and Δ*FoSRT1* grown in 0.2xPDB or 0.75M NaCl amended 0.2xPDB, (D) absorbance measured at 350nm to show variation in pigment change when WT and Δ*FoSRT1* were grown with or without NaCl, (E) variation of conidia concentration resulting from growth with or without NaCl.

WT and ΔFoSRT1 showed no difference in pathogenicity

When observing the external tissues and leaf wilt among the treatment groups, WT and Δ*FoSRT1* did not show any significant differences in ability to cause disease. The root shoot junction is a location which we previously observed as being a reproducible location to observe disease as this is the transition point between below ground and above ground growth which is the earliest indication of disease. Coloring of green or light pink was associated with non-Fov4 infection (Fig. 21A,B). The Δ*FoSRT1* group appeared to show the similar number of observations of root shoot junction coloring to WT (Fig. 21). The negative group accounted for 100% of the observed green-pink root shoot junctions. WT and Δ*FoSRT1* accounted for 56 and 44%, respectively, of purple-black observations. After assessing leaf wilt, WT accounted for

63% of the observations for wilt and Δ*FoSRT1* accounted for 38%. Negative control showed 0% leaf wilt. Microscope observations of the root shoot junction internal tissues showed that WT (Fig. 21C) showed similar browning seen in Δ*FoSRT1* (Fig. 21E). Results indicated no significant impact on pathogen virulence.

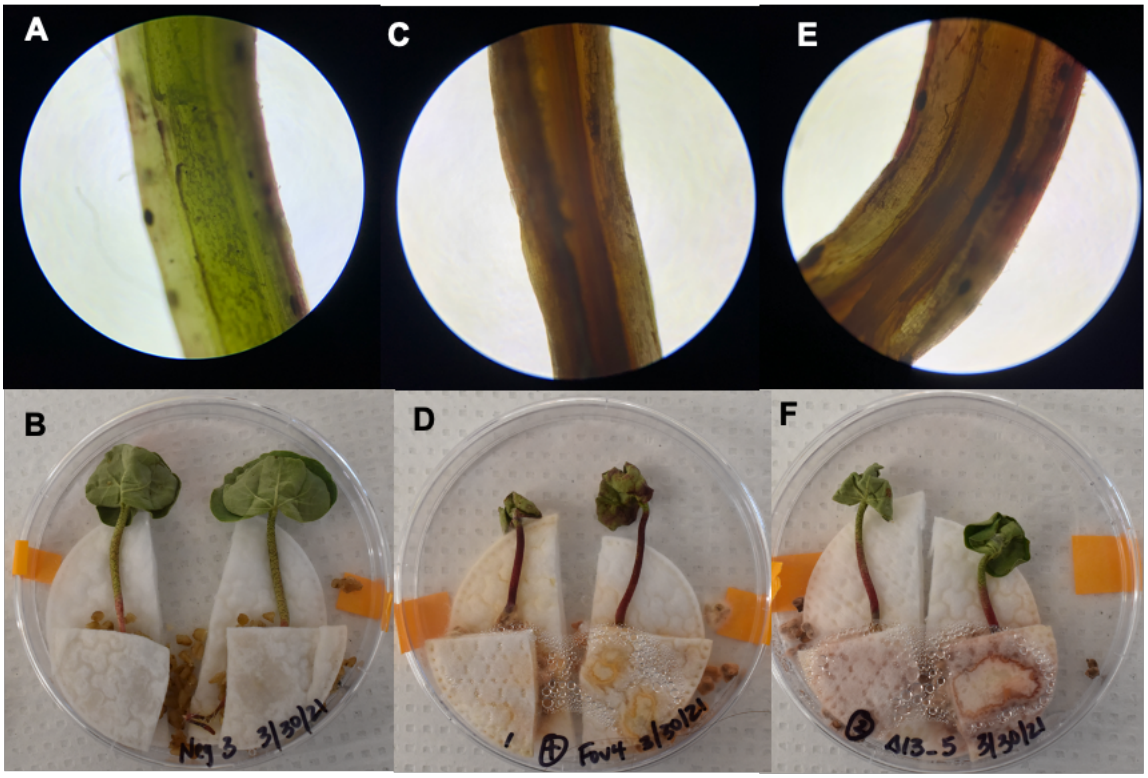

Figure 21. Pathogenicity characterization of WT and Δ*FoSRT1*. (A) internal RSJ tissue of negative controls showing no tissue darkening associated with Fov4 infection, (B) examples of negative controls showing no Fov4 infection, (C) internal RSJ tissue darkening of WT infected Pima cotton, (D) example of WT infected Pima cotton, (E) internal RSJ tissue of Δ*FoSRT1* infected pima seedlings, (F) shows examples of Δ*FoSRT1* infected Pima seedlings.

Discussion

The nucleotide and amino acid differences identified with *FoSRT1* are associated with protein function changes investigated in previous structure-function studies. Alanine to valine substitutions switch a good helix forming residue, alanine, for a poor helix forming residue, valine. The changes in amino acids alter the side chains which in turn, affect hydrogen bonding and overall protein structure (Gregoret & Sauer, 1998). Fov4 contains a good helix forming residue, alanine, while the same location in Fov1 is populated with valine, a poor helix forming residue. The study in Gregoret, 1998 showed that alanine and valine substitutions caused decreases in thermal stability of single and multiple mutants (Gregoret & Sauer, 1998). The role of *FoSRT1* in protein function changes would be an interesting avenue of research given that in addition to NaCl differences between WT and Δ*FoSRT1*, the null mutant showed differences in conidiation and no loss of pigment under NaCl stress indicating more pathways involved.

Multi-facilitator transporters (MFS) are not specific to one function and can serve a variety of functions based on loci. Additionally, *FoSRT1* did not show multiple copies within the Fov4 of Fov1 genome. For example, the MFS transporter that shared the most similarity to the gene deleted in this study came from an investigation into sphingolipid metabolism inhibitors. However, this was not a functional genetics study and did not characterize the gene through gene knockout techniques (Kim et al., 2020). Additional literature investigation led to Crutcher et. al. 2015 which disrupted a MFS transporter gene important to fusaric acid transport (FUB1). However, this study noted that FUB1 gene disruption did not affect conidiation or conidial germination (Crutcher et al., 2015). This is opposite of our findings in that our disruption of the *FoSRT1* gene resulted in increased conidiation. Additionally, the results from our pathogenicity assay showed no difference in infection between the wild type and Δ*FoSRT1* at 4dpi.

Similar to our results, a study into *Fusarium oxysporum* salt stress tolerance showed a loss of function of the pH response transcription factor, PacC, resulted in an increased sensitivity to Li^+ and Na^+ (Caracuel et al., 2003). Interestingly, the mutation did not affect growth at high concentrations of K^+, our results also showed no difference between WT and Δ*FoSRT1* grown on 0.2xPDA/0.75M KCl (Caracuel et al., 2003). Study authors also noted possibilities of two ion transporters, one working at high pH and one working at acidic pH ranges (Caracuel et al., 2003). Interestingly, Fov4 is known to not grow well in acidic soils (Bell et al., 2019, Bell et al., 2017, Cianchetta & Davis, 2015). The *FoSRT1* gene in our study did not show any homology with the genes mentioned in Caracuel, et. al. 2003. A more in-depth investigation into variation of growth in response to multiple pH levels and multiple ion concentrations (Na^+, Li^+, and K^+) may offer a better explanation into the role of *FoSRT1*.

The original intention of this study was to identify genes related to pathogenicity of Fov4. The MFS transporter we identified and subsequently nullified showed no significant difference related to pathogenicity. However, this gene did show different growth characterization from wild type that furthers our knowledge of Fov4 development and growth. The *FoSRT1* shows homology to MFS transporters from multiple *Fusarium* species with literature references. Hypothetical proteins from *Fusarium oxysporum* species, showing homology to *FoSRT1* gene identified in this study, are only annotations in the genome sequence. The *FoSRT1* gene may not offer insight into direct pathogenicity but there are still questions of its role in indirect mechanisms of pathogenicity during host-pathogen interactions

CHAPTER V

CONCLUSIONS

Bacterial VOCs have shown potential as biocontrol agents not only in this body of work, but in numerous peer reviewed journal articles investigating similar bacterial species against multiple phytopathogens, including *Fusarium oxysporum* species. Studies presented in Chapters II and III were the first to investigate the use of VOCs against Fov4 and Pima cotton. Two different combinations of bacterial VOCs were used to determine effects on Fov4-only suppression and the combined effect on an active Fov4 infection in host Pima cotton. Chapter IV investigated the effects of sodium on Fov4 growth. The overall focus in these studies was to characterize the complex interactions between rhizosphere microbes and an abiotic factor NaCl. A change in VOC suppression was expected when comparing a single organism versus a combination of organisms in the bacteria-Fov4 direct assays. Investigating the VOC inhibition of Fov4 infection of Pima cotton showed additional differences in that HC658 and RZ160 showed better disease symptom suppression than bacteria-Fov4 only assays.

The *Bacillus/Streptomyces* VOC combination showed a different response in Fov4-infected Pima than *Paenibacillus/Brevibacillus* VOC combination based on observations of external tissue alone. Interestingly, the two combinations produced similar Fov4 inhibition levels, 26 versus 27% inhibition, from the split plate VOC assays without Pima cotton. In each study from Chapter II and III, it is reasonable to hypothesize that the plant impacted the overall VOC profile of the interaction which affected the Fov4 growth and virulence. If the Pima cotton host were able to change the characteristics of the VOC profile, other factors, such as soil chemistry and structure, should be taken into consideration which may influence the microbial population and subsequently the effectiveness of these organisms as biocontrol agents.

Microbial growth and populations are dependent on multiple limiting factors, including temperature and salt content, in addition to other microbes present in the environment. *Bacillus* species are heat and NaCl tolerant but not in the same manner as *Paenibacillus*, one key difference shown in the characterization of multiple Firmicutes species in Indian Himalayan springs where *Paenibacillus* produced heat stable xylanases at 45, 55, and 65°C while *Bacillus* species did not (Sahay et al., 2017). *Paenibacillus* species are known for their survival in extreme environments. Firmicutes species isolated from a Himalayan hot spring showed *Paenibacillus glycanilyticus* with the most growth (Sahay et al., 2017). *Paenibacillus ehimensis* (Aw et al., 2016, Kim et al., 2004, Wu et al., 2011, Lee et al., 2004), *P. tianmuensis* (Aw et al., 2016) , *P. koreensis* (Wu et al., 2011, Lee et al., 2004, Kim et al., 2004), *P. validus* (Lee et al., 2004), and *P. azoreducens* (Kim et al., 2004) have all been cited for their ability to growth at 50°C. *Bacillus* species have been shown to be very heat tolerance and continue to grow between 40-60°C (Kearl et al., 2019, Ghojavand et al., 2008). *Brevibacillus* species are also capable of tolerating extreme environments and maintain chintinase activity at 20-60°C (Liu et al., 2015). Something that would have helped in this work was conducting experiments at various temperatures. However, heat tolerance and the ability to grow in extreme environments does not necessarily indicate continued antimicrobial activity at high temperatures. For example, *Bacillus cereus* was found to only form spores between 20-45°C (Gonzalez et al., 1999). At what temperature is a bacterium merely trying to survive rather than compete against other microbes and maintain antimicrobial properties? Through the *FoSRT1* null mutant in Chapter IV, Fov4 was shown to be quite sodium tolerant while the null mutant was not. *Paenibacillus* species have shown growth on 0-5% w/v NaCl, *P. koreensis* showing the most impressive growth at 5% NaCl w/v (Cho et al., 2017, Aw et al., 2016, Kim et al., 2004). *Bacillus* species are also very NaCl

tolerant growing between 0-6% (Wu et al., 2020) and 20% in some studies (Ghojavand et al., 2008).

In an environment like El Paso, Texas, where temperatures in July and August can reach 112°F/44°C (NOAA, 2020), the stability of biocontrol agents is a valid concern. Taking the high temperatures of El Paso into consideration, *Paenibacillus* species are those which produce enzymes capable of withstanding temperatures above 44°C. Temperature is an important limiting factor in the survival of microbes which extends to biological controls (Kirchman, 2018). Matching the right biocontrol with the intended environment in which it will be used is important to its success. If the organisms cannot survive, or can survive but lose antimicrobial properties, it renders the application of a biocontrol agent insignificant. Until the mechanism of suppression by the bacterial combinations is understood, it is unknown if specific enzymes impact the overall suppression through VOCs. Utilizing native antagonistic bacteria may be the better method to control Fov4 persistence in soil and virulence. The combination of *Paenibacillus* ELP529 and *Brevibacillus* Rz160 in Chapter III utilized a native bacterial species with a rhizosphere isolated species which helped promote Fov4 inhibition through VOCs. While *Streptomyces* and *Bacillus* species are popular and effective against most pathogens in multiple environments, whether these are good products to use in El Paso has yet to be determined. Chapter IV results suggest that Fov4 is quite tolerant to NaCl and may be a contributing factor in Fov4 to persistence in soil. The application of biocontrol agents should not only focus on hosts, but limiting factors of temperature and NaCl tolerance, as these variables can be used to optimize biocontrol agents.

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APPENDIX A

LITERATURE BACTERIAL VOCS

Appendix A. VOCs from literature sources indicating bacterial source, identified VOC, and target organism.

Bacterial Source	VOC	Antagonism Target	Special Function(s)	Literature Reference
Actinobacteria	butanoic acid methyl ester	Pathogenic *Pseudomonas spp.*	Antibacterial	(Choudoir et al., 2019)
Actinobacteria spp, Bacillus spp.	3-methyl-1-butanol	*Pseudomonas spp., Fusarium oxysporum* f.sp. *lactucae*	Anti-bacterial, antifungal, coiled hyphal growth	(Choudoir et al., 2019, Chaves-Lopez et al., 2015)
Bacillus amyloliquefaciens	2-butanone	*Fusarium oxysporum* f.sp. *lactucae*	Antifungal	(Chaves-Lopez et al., 2015)
	2-propanone			
	2,3-butanedione			
	tetramethyl pyrazine			
Bacillus spp	2-methyl propanoic acid	*Fusarium oxysporum* f.sp. *lactucae*	Antifungal	
Bacillus subtilis	1-butanol	*Fusarium oxysporum* f.sp. *lactucae*	Antifungal, hyphae grew in coiled manner	
	1,3-pentadiene	*Aspergillus flavus*	Antifungal	
	carbon disulphide	*F.o*. f.sp. *lactucae, Aspergillus flavus*	Antifungal, complete inhibition	
	propanone	Fo lactucae	Antifungal	
Bacillus velezensis	2-ethylhexanol	*Sclerotinia sclerotiorum*	Inhibits growth of sclerotia and mycelia and ascospore germination.	(Syed-Ab-Rahman et al., 2019)
Bacillus velezensis, Acinetobacter	benzyl alcohol	*Colletochrium camellia, Phytophthora capsisci*	Inhibition	

Appendix A Continued

Bacterial Source	VOC	Antagonism Target	Special Function(s)	Literature Reference
Acintobacter spp.	3-methylbutanal	*Tuber borchii*	Mycelia inhibition, plant growth promoting	(Syed-Ab-Rahman et al., 2019)
Paenibacillus polymyxa	2-decanol	*Meloidogyne incognita*	Nematicide fumigant, attractant	(Cheng et al., 2017)
	2-decanol acid		Contact nematicide	
	2-decanone		Contact nematicide, fumigant	
	2-nonanol		Contact nematicide, fumigant	
	2-nonanone		Contact nematicide	
	2-undecanol		Contact nematicide, fumigant	
	2-undecanone		Contact nematicide, repellant	
	2-undeundecanone		Nematicide fumigant	
	4-acetylbenzoic acid		Contact nematicide, low concentration repellant	
	acetone		Nematode attractant	
	furfural acetone		Contact nematicide, fumigant, attractant	
Streptomyces albulus	2-pentylfuran	*Sclerotinia sclerotiorum*; *Fusarium oxysporum* f.sp. *cucumerinum*	Antifungal; inhibit fungal germination	(Wu et al., 2015)
	4-methoxystyrene			
	styrene			
	tetradecane			
	toluene			

Appendix A Continued

Bacterial Source	VOC	Antagonism Target	Special Function(s)	Literature Reference
Streptomyces albulus	anisole	*Sclerotinia sclerotiorum*; *Fusarium oxysporum* f.sp. *cucumerinum*		(Wu et al., 2015, Cordovez et al., 2015)
Streptomyces spp.	1,3,5-trichloro-2-methoxybenzene	*Rhizoctonia solani*	Antifungal, melanization	(Cordovez et al., 2015)
	butanone	*Cladosporium cladosporioides*	Inhibit spore germination	
	dimethyl disulfide	*Fusarium moniliforme*	Inhibit mycelial growth	
	methyl 2-methylpentanoate	*Rhizoctonia solani*	antifungal	
Streptomyces spp.; Bacillus amyloliquefaciens	3-hydroxy-2-butanone	*Pseudomonas syringae*	Antibacterial	(Cordovez et al., 2015, Lee et al., 2012, Asari et al., 2016)
Paenibacillus polymyxa Streptomyces spp.; Bacillus amyloliquefaciens	2,3-butanediol	*Fusarium oxysporum, Rhizoctonia solani*	Elicit ISR, direct antifungal, PGPR	(Farag et al., 2013, Cordovez et al., 2015)

APPENDIX B

PCR PRIMERS USED IN CHAPTER IV

Primer ID	Sequence (5'-3')	Function
13_Gene/F	CTTACCAACCTATCAACCCTG	Amplify gene
13_Gene/R	GGACTGCTATCCTTGACCG	Amplify gene
13_F	CGAACTGGTCGCATCAACTA	qPCR of gene
13_R	CCTTGATTTCAGGACTGGAGAG	qPCR of gene
13_LF/F	AGCAACCGAAACCAGAACC	Amplify region upstream of gene
13_LF/R	TAGATGCCGACCGGGAACATTTGCCCATACCCCTGAC	Amplify region upstream of gene
13_RF/F	CCACTAGCTCCAGCCAAGAAACAGTCCAACGAATCCC	Amplify region downstream of gene
13_RF/R	ATAGGTTGGGGTGAAAAGC	Amplify region downstream of gene
HYG/F	TTGGCTGGAGCTAGTGGAGGTCAA	amplify HY fragment
HY/R	GTATTGACCGATTCCTTGCGGTCCGAA	amplify HY fragment
YG/F	GATGTAGGAGGGCGTGGATATGTCCT	amplify YG fragment
HYG/R	GTTCCCGGTCGGCATCTACTCTAT	amplify YG fragment

APPENDIX C

LOCATIONS OF DIFFERENTIALLY EXPRESSED GENES FOUND IN FOV4 GENOME SCAFFOLDS

Gene ID	Fov4 Scaffold Accession Number	Nucleotide Range	Putative Identification
1	VINP01000001.1	6449596-6452532	PKS-NRPS hybrid synthetase
2	VINP01000004.1	3933921-3931483; 1336307-1337845	PKS-NRPS hybrid synthetase
3	VINP01000004.1	4148099-4150097	Ankyrin repeat domain containing protein
5	VINP01000005.1	72975-71383	Aspercryptin biosynthesis cluster transcription regulator atnN
8	VINP01000005.1	843961-845166	Reducing polyketide synthase
11	VINP01000004.1	1137657-1140707	Cargo-transport protein
12	VINP01000004.1	1146228-1147379	Xylanase related
13	VINP01000004.1	1167011-1167826	MFS Transporter