Agricultural Antibiotic Application Alternative: Nisin-A Revealed as Promising Bacteriocin Treatment to Combat Antibiotic Resistant *Pseudomonas syringae,* causative agent of Bacterial Speck of Tomato

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Abstract:

Pseudomonas syringae pv. tomato, the causative agent of tomato bacterial speck, is an agriculturally relevant bacterium that reduces tomato crop yield upon infection. Resistance to broad-spectrum preventative antimicrobial sprays, namely streptomycin and copper sulfate, have been documented since 1990. Current focus lies on identifying alternative prevention methods, one highly attractive option being bacterially produced antimicrobial peptides, bacteriocins, often with narrow killing spectra. Specifically, the bacteriocin-like inhibitory compounds produced by plant-growth-promoting-rhizobacteria (PGPR) are of interest to defend against *P. syringae*. However, of the rhizosphere and intraspecies co-culture screens performed, no single bacterial isolate was more inhibitory than when the bacteriocin, nisin (19.7 μg), was applied directly on each isolate of the pathogenic lawn. This revealed nisin as a potent inhibitor to *P. syringae,* prompting its further study. A series of detached tomato leaf-dipping assays were developed and employed to assess the relative decrease in *P. syringae* colony forming units between the nisin/ streptomycin treatment and control. Both the highly pure nisin (93%) and food-grade nisin (2.5%) were more effective than the 200 PPM streptomycin sulfate after 24 hours on the tomato leaf (91.3%, 96.8%, and 40.0% reduction, respectively). These results reveal that nisin remains highly effective when allowed to dry on a leaf surface. Future directions should continue to assess nisin's activity in-planta in both field and greenhouse settings. This work brings us closer toward implementing nisin as a sustainable agriculture treatment effective at preventing *P. syringae* infection, protecting tomato crop yield, and reducing associated economic loss.

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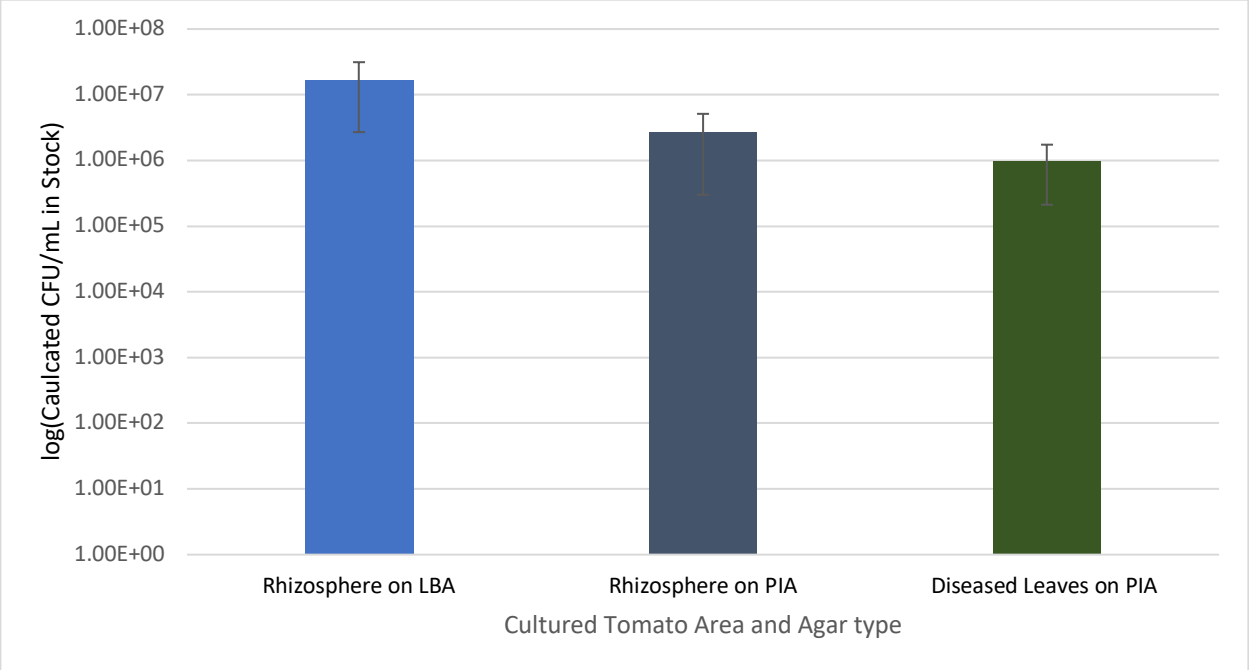

Figure 1. C**alculated concentration of bacterial colony forming units (CFU) per milliliter from rhizosphere and disease leaf microbe isolation stock solution.**
The stock solution concentration was determined by 1:10 serial dilution plating. One single rhizosphere bacterial stock was prepared and cultured on both LBA (left bar) and PIA (middle bar). The isolated bacterial stock solution from diseased tomato leaves was plated on PIA (right bar) in search of obtaining isolates of *P. syringae pv. tomato*. The vertical axis measures the logarithm of the cell concentration in CFU/mL. Bar graph shows the average concentration of three replicates while error bars outline the standard deviation. LBA = Luria Broth Medium, PIA = Pseudomonas Isolation Agar.

Table 1. Isolate profile of *Pseudomonas spp.* cultured on Pseudomonas Isolation Agar (PIA), assessed for fluorescence with UV light on King's B agar, and assessed for cytochrome c oxidase enzyme to identify novel *P. syringae* isolates.

Identification	Number of Isolates
Total Isolates cultured from PIA[a] agar	100
Isolates UV Fluorescent on KB[b] agar	65
Isolates Fluorescent and Oxidase (-)	30

[a] Pseudomonas Isolation Agar.
[b] King's B Medium.

Table 2. Relevant Comparison Criteria from Rhizosphere and Intraspecies Co-Culture Screens used to determine the most effective inhibitor of *P. syringae* for further experimentation.

Analysis Criterion	Rhizosphere Screen	Intraspecies Screen	Nisin-A (19.7 µg)
Percent of Isolates Inhibitory	20%	33%	-
Percent of *P. syringae* inhibited by single best producing isolate	27%	67%	-
Percent of Total *P. syringae* inhibited collectively	40%	97%	100%

Table 3. **Number of leaf replicates used in detached leaf assay to compare 2.5 and 93% pure nisin-A and streptomycin sulfate at *P.* syringae CFU reduction.**

Treatment	sdH_2O	2.5% Pure nisin-A		93% Pure nisin-A		Streptomycin	
Concentration	-	3.93 mg/mL	0.986 mg/mL	3.93 mg/mL	0.986 mg/mL	400 PPM	200 PPM
Drug + *P. syrinage*	6	6	6	6	6	6	6
Drug + No *P. syringae*	6	6	0	6	0	0	6
Total leaves	12	12	6	12	6	6	12

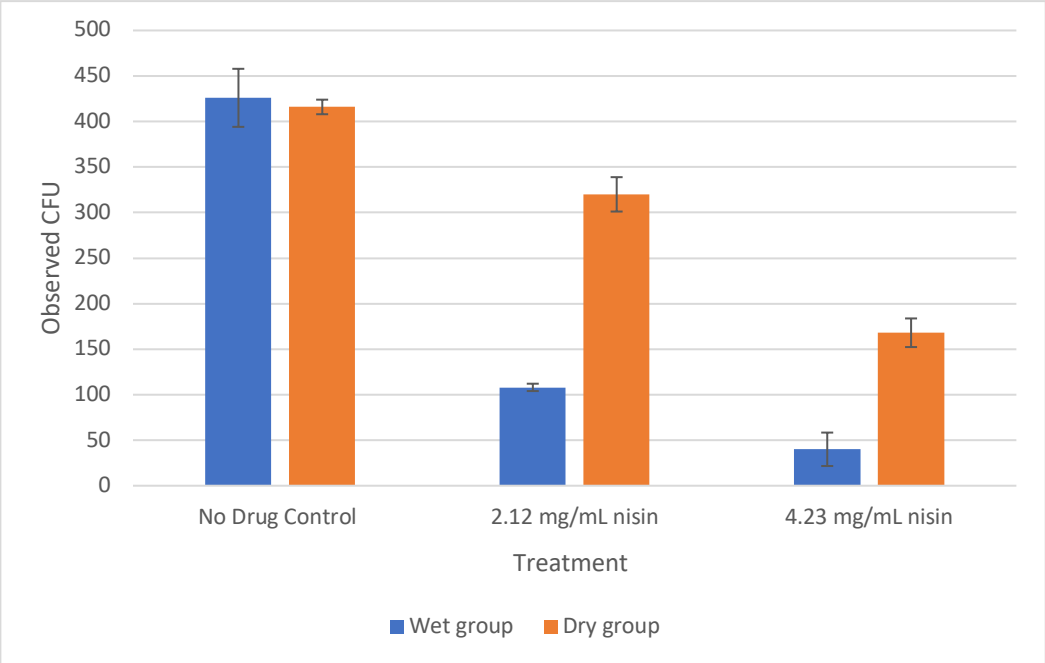

Figure 2. Comparison of nisin-A's efficacy in wet and dry conditions.
Observed *P. syringae* CFU on LBA after treatment with either 2.12 or 4.23 mg of nisin-A in wet (blue) and dry (orange) conditions. Bar graphs show the average of 3 replicates with error bars denoting standard deviation. 1 mL of each nisin solution was added to a petri dish and either allowed to dry for 1 hr or immediately or joined with 1 mL $4.26x10^9$ CFU/mL and 9 mL of 1.7% saline dilution medium (DM). The negative control groups were treated with 1 mL cells, 0 mL nisin, and 9 mL DM in their respective conditions. Relative decrease in observed CFU was calculated by the following formula $((CFU^{Treatment} - CFU^{Control})/ CFU^{Control})$.

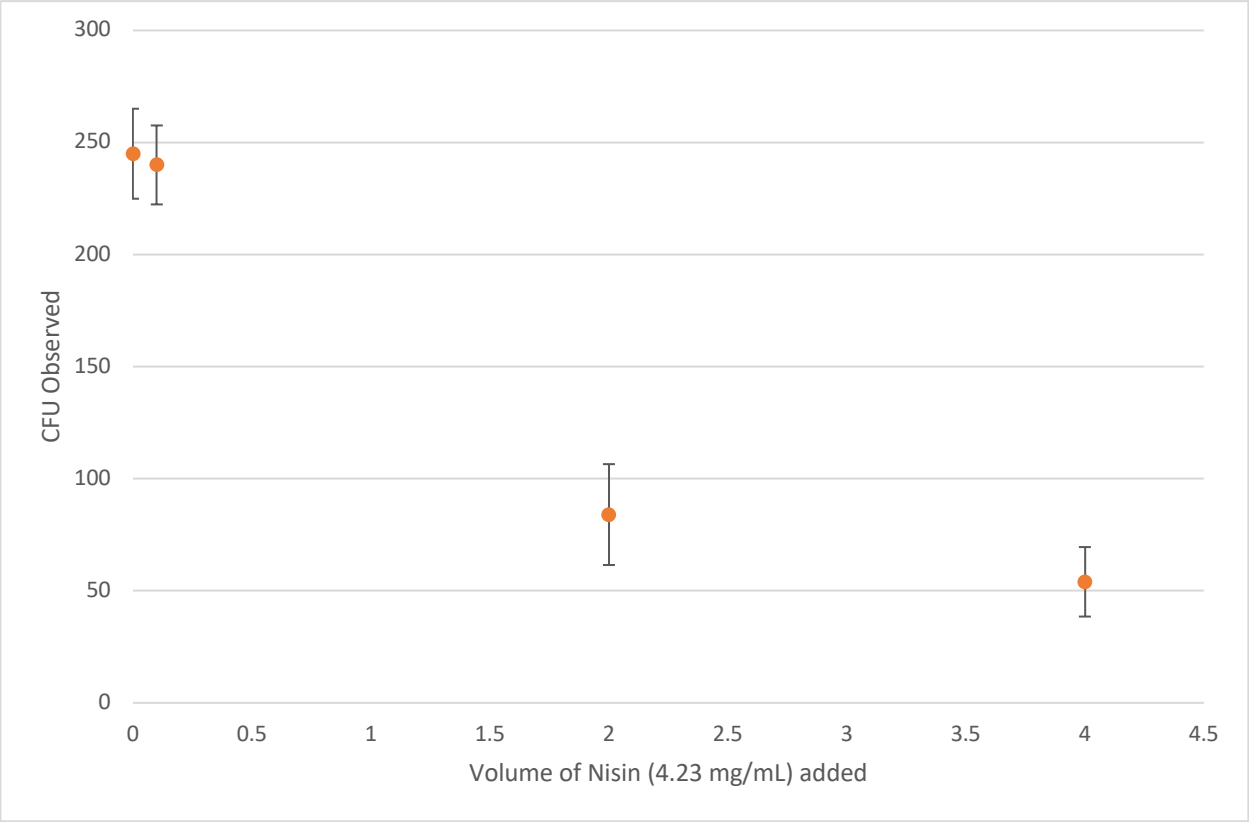

Figure 3. Assessment of CFU viability in dry conditions after nisin treatments.
Scatter plot showing the observed CFU after treatment with either 0, 0.1, 2, or 4 mL of 4.23 mg/mL nisin-A. Dots plot the average of 3 replicates with error bars denoting standard deviation. Orange dots display the results after the application of 1 mL of 10^4 CFU/mL.

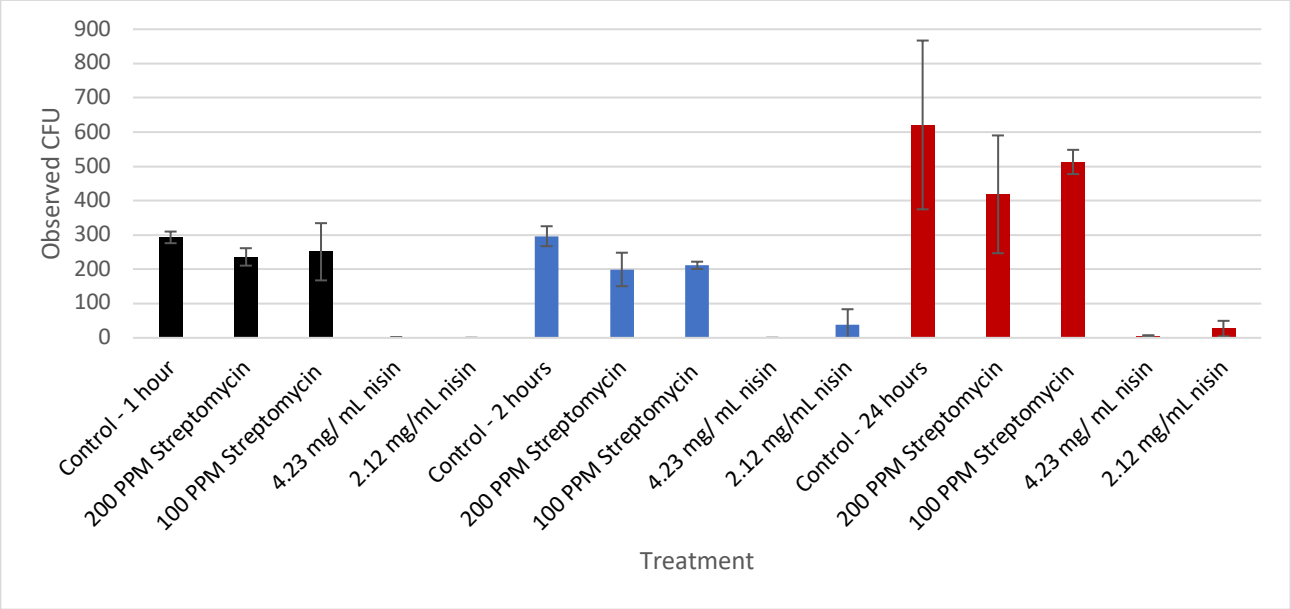

Figure 4. Pilot study with model leaves to observe *P. syringae* colonies following nisin-A or streptomycin sulfate treatment at 3 time points between treatment and 10^4 CFU/mL cell application.

As part of the detached leaf-dipping pilot assay, 16 cm^2 filter paper squares were dipped with either sdH_2O (control), nisin (4.23 or 2.12 mg/mL), streptomycin sulfate (200 or 100 ppm) and allowed to dry for either 1 hour (black), 2 hours (blue), and 24 hours (red) before application with 1 mL $2.4x10^2$ CFU. Bar graphs show the average results of two replicates with error bars displaying the standard deviation. The 24-hour treatment group was dipped in 1 mL of $4.0x10^4$ CFU/mL solution prepared from a different overnight growth flask.

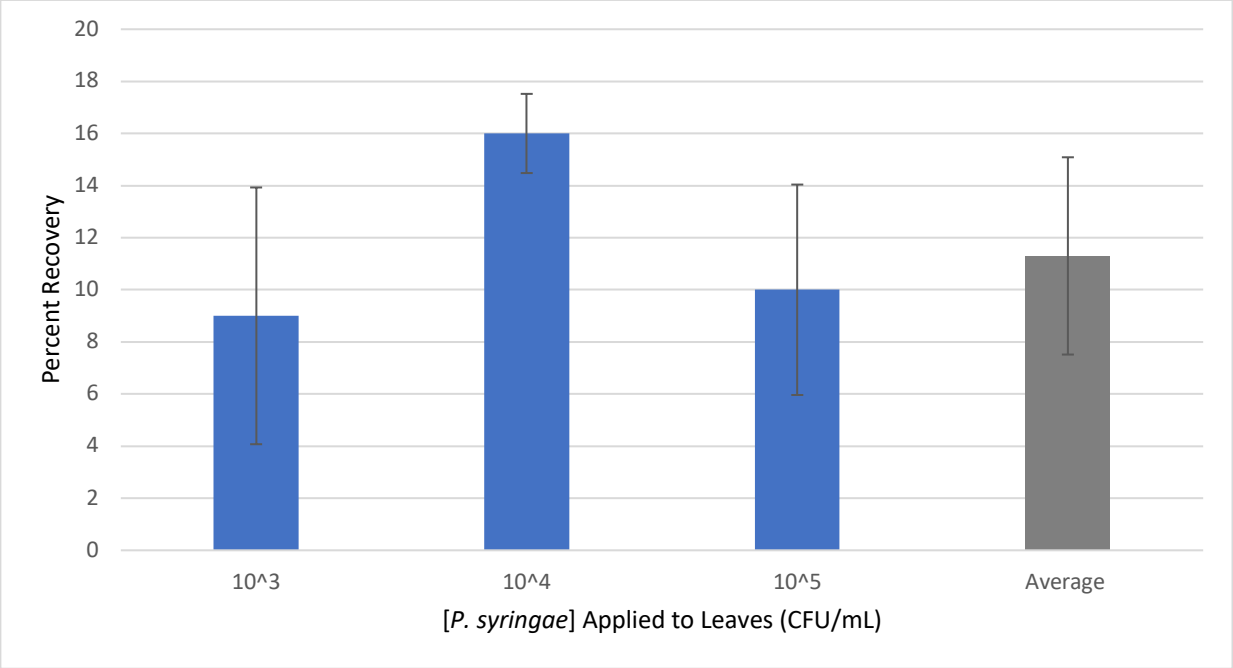

Figure 5. Calculated percent recovery of *P. syringae* CFU after leaf dipping in three distinct cell concentrations.
Percent recovery is defined as the ratio of (observed CFU/ expected CFU) x100. Observed *P. syringae* CFU were found after dipping a leaf in the appropriate solution, immediately transferring to a 10 mL LB test tube, vortexing vigorously, and plating 100 µL on LBA and incubating for 24 hours. Expected CFU were determined through serially diluting the number of CFU/mL in the overnight and plating each dilution to determine how many CFU were present in the originally dipped sample. Bar graphs show the average percent recovery determined from three replicates while error bars display standard deviation. The graph at the far right shows the average of the percent recoveries determined in each cell concentration.

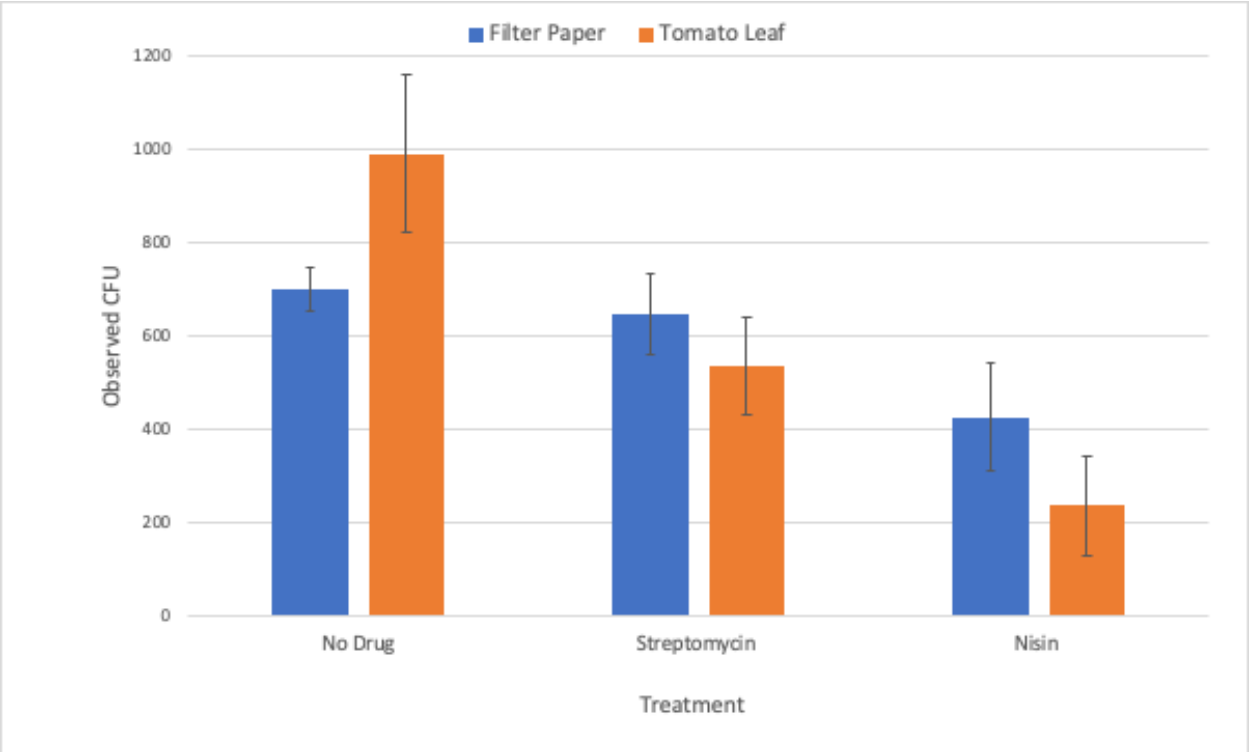

Figure 6. Comparison of Detached Leaf Assay on Filter Paper and Tomato Leaves. Bar graph comparing the observed *P. syringae* CFU for each treatment on either filter paper (blue) or tomato leaf (orange) 24 hours between treatment and $3.73x10^4$ CFU/mL application. Streptomycin sulfate (200 ppm) was compared to nisin (2.12 mg/mL). The control group was treated with sdH_2O only. Bars show the average observed CFU of four replicates with error bars outlining standard deviation.

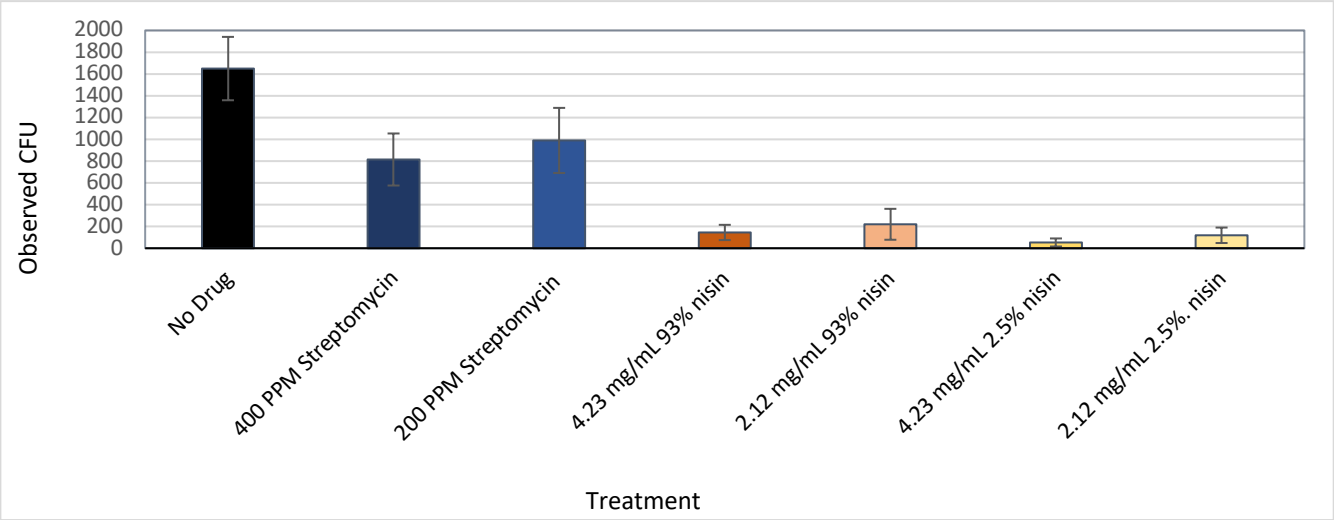

Figure 7. Comparison of highly pure (93%) nisin-A, low purity (2.5%) nisin-A, to Streptomycin sulfate at *P. syringae* CFU reduction on tomato leaves.
Bar graph comparing the observed CFU for each treatment on tomato leaves 24 hours between treatment and 10^4 CFU/mL application. Bars show the average observed CFU of 6 replicates with error bars showing standard deviation. From left to right, each set of leaves were either treated with sdH_2O (black), 400 PPM streptomycin (navy blue), 200 PPM streptomycin (lighter blue), 4.23 mg/mL 93% pure nisin (dark orange), 2.12 mg/mL 93% pure nisin (light orange), 4.23 mg/mL 2.5% nisin (bold yellow), 2.12 mg/mL 2.5% nisin (light yellow).

List of Abbreviations:

LBA - Luria Broth Agar
PIA - Pseudomonas Isolation Agar
KB - King's B Medium
CFU - Colony Forming Unit
DM - Dilution Medium
PGPR - Plant growth promoting rhizobacteria

Introduction

In 2012, *Pseudomonas syringae* was listed as the number one most scientifically and economically relevant phytopathogen (Mansfield et al., 2012) and research investigating effective treatments has continued a decade later. *P. syringae* is a gram-negative, aerobic bacterium which secretes virulence proteins, causing black lesions on the tomato fruit, stem, and leaves surrounded by chlorotic plant tissue (Preston, 2000). These protein toxins, such as coronatine, disrupt the tomato host plant Jasmonate signaling pathway, stunting the growth of the tomato fruit and decreasing tomato yield resulting in significant economic loss (Farmer et al., 1992). The main areas of concern for bacterial speck of tomato are humid, temperate areas such as California, Florida, Oregon, Washington in the United States, and in mid-eastern countries such as Turkey and Israel.

Traditional treatments to prevent *P. syringae* in agriculture are topical preventative sprays of copper sulfate and antibiotics streptomycin and oxytetracycline. However, over several decades of use, these treatments are dwindling in efficacy due to the spread of antibiotic resistance and copper tolerance genes carried and transmitted on bacterial plasmids (Sundin and Bender, 1993). While the use of these antimicrobials is effective against non-resistant *P. syringae* strains, these broad-spectrum bactericide treatments also alter the activity and diversity of the plant microbiota that are vital for plant growth promotion (Cycon et al., 2019**)**. Moreover, these treatments have been linked to the spread of resistance genes in aquatic environments along with heavy metal pollution which pose significant threats to human health (Fagnano et al., 2020)**.**

In the search for alternative bacterial speck preventative treatments, recent research has turned toward investigating alternative treatment methods such as bacteriophage (Svircev et al., 2018), antagonistic plant-growth-promoting rhizobacteria (Liu et al., 2017), and antimicrobial peptides known as bacteriocins (Mirzaee et al., 2021). While the results of these alternatives vary in their efficacy of preventing bacterial speck, the spotlight is only beginning to be shown on bacteriocins for sustainable biocontrol of phytopathogens in agriculture.

Bacteriocins are antimicrobial peptides produced by bacteria that often have a narrow range of inhibitory activity and often target closely related bacteria, such as those within the same genus or species (Riley and Wertz, 2002). Few studies have investigated bacteriocin expression *in planta* for prevention against *P. syringae*, such as putidacin L1 (Rooney et al., 2020). *P. syringae* has shown resistance to this bacteriocin which suggests that more research is needed to identify a bacteriocin with a mechanism of action that targets highly conserved cell components needed for bacterial cell survival.

The focus on bacteriocins in agriculture has largely been on genetic modification of plants, such as in the model organism Arabidopsis, for constitutive expression of the bacteriocin protein rather than investigation of a bacteriocin topical treatment (Mirzaee et al., 2021). Other studies have investigated the inhibitory activity of plant-growth-promoting rhizobacteria (PGPR) which show varying levels of pathogen inhibition (Bashan and de-Bashan, 2002; Liu et al., 2017).

Rhizobacteria found at the intersection of plant roots and soil have also been investigated for their bacteriocin-producing abilities. Plant-growth-promoting rhizobacteria (PGPR) can promote plant growth either directly through the production of plant hormones or nutrient acquisition, or indirectly through the production of antagonistic molecules which inhibit phytopathogens (Beneduzi et al., 2012). These antagonistic molecules include antibiotics, siderophores, lytic enzymes, and bacteriocins (Tariq et al., 2017). While about 500 bacteriocins produced by rhizobacteria have been identified (Mojgani, 2017), the investigation of the tomato rhizosphere has not received significant attention to identify a bacteriocin highly targeted against *P. syringae pv tomato*.

In Yildiz et al.'s 2018 paper, they investigated 524 tomato rhizobacteria for antagonistic activity against *P. syringae pv tomato* and found that 48 showed abilities to inhibit *P. syringae* growth in a traditional co-culture assay, with 39 showing only siderophore production effects (Yildiz et al., 2018). This suggests that bacteriocin production within the tomato rhizosphere may be less frequent, although the isolates tested may not be closely related enough to *P. syringae* to show narrow spectrum antimicrobial activity characteristic of bacteriocins. In their 2019 paper, Dong et al. showed that roughly 36% of the tomato rhizosphere is made up of Pseudomonads (Dong et al., 2019), suggesting that future studies should focus on selection for rhizospheric Pseudomonads for bacteriocin investigation for *P. syringae pv tomato* biocontrol.

In this Honors Thesis, I share the results of a collection of experiments focused on investigating the tomato rhizosphere for bacteria with inhibitory activity characteristic of bacteriocins against *P. syringae* as well as assessing intraspecies bacteriocin production by other *P. syringae* isolates. I conclude that of the co-culture screens performed, no single bacterial isolate was more inhibitory than when the bacteriocin, nisin, was spotted (19.7 ug) directly on each isolate of the pathogenic lawn.

Nisin is a 34 amino acid peptide produced by the gram-positive bacterium *Lactococcus lactis* that has been extensively studied and used as a food preservative since 1988 (Kitagawa et al., 2018). Further, it has been generally recognized as safe by the U.S. Food and Drug Administration. Nisin's high value potential to combat the antibiotic resistance movement has led it to being explored in livestock, specifically against cow mastitis (Kitazaki et al., 2017), synergistically with antibiotics against human pathogens (Jahangeri et al., 2021), and has even shown cytotoxicity against tumor cells (Zainodini et al., 2018). Shockingly, to the best of my knowledge, there are no reports about the use of nisin against strictly plant pathogens nor synergy in combination with conventional antimicrobial treatments such as streptomycin or copper sulfate.

Further testing of nisin was performed against *P. syringae*. In this thesis, I report data about assessing *P. syringae* CFU viability after nisin treatment in solution compared to after drying on a model surface, the development of a detached leaf-dipping assay using filter paper as model tomato leaves, comparing the filter paper assay to actual tomato leaves grown in a greenhouse setting, and then finally comparing the efficacy of 93% and 2.5% pure nisin at reducing observed *P. syringae* CFU compared to the control in a detached-leaf dipping assay. Detached-leaflet assays are highly versatile and have been used as a standard to conduct virulence sensitivity screening and identification of resistant cultivars of tomatoes (Karki and Halterman, 2021).

With relevant data, I conclude that at the concentrations tested, nisin was significantly more effective at inhibiting *P. syringae* CFU on tomato leaves than the streptomycin sulfate at the EPA recommended dose of 200 PPM (EPA Pesticide Fact Sheet No. 186, 1988). A detached leaf-dipping assay was developed and allowed for a consistent way to assess the efficacy of a bacteriocin and antibiotic treatment. This addressed gap in the field of antibiotic alternatives highlights the potential of nisin to prevent bacterial speck of tomato, decrease associated crop loss, and meet global tomato market needs.

Methods

Rhizobacteria Isolation and Concentration determination:

Rhizosphere samples were collected from young tomato plants on Foxcroft Farm in late September 2021. A young, healthy tomato plant was uprooted, and the bulk soil was removed. Tomato roots with adhering soil were excised and stored in a sterile glass bottle. Methods were adapted from Dong et al.'s 2019 paper on bacterial communities in the tomato rhizosphere, phyllosphere, and endosphere.

To isolate bacteria from the tomato rhizosphere, methods were adapted from Barillot et al.'s 2013 standardized method of rhizosphere sampling. Isolated roots were added to a 1 L sterile bottle with 50 mL of 0.9% saline. A bottle with roughly 100g of rhizosphere soil and roots were placed in an orbital shaker set to 150 rpm for 60 minutes at 30 ℃. After 1 hour, the 50 mL sample was placed into two 50 mL centrifuge tubes, each with 25 mL of stock solution. Each tube was centrifuged at 1500 rpm for 10 minutes. The stock solution of each tube was combined into a new 50 mL falcon tube. 1 mL of the stock solution was placed into 9 mL sterile 0.9% saline test tube to dilute the sample 1:10. The 1:10 dilution was repeated until the fifth dilution. This process was repeated three times to achieve 3 replicates of the stock solution serial dilution. To estimate the number of colony forming units isolated from the rhizosphere, 100 μL of each dilution replicate was lawned on Luria Broth Agar (LBA) and incubated 30℃ for 48 hours. The rhizobacteria stock solutions were also plated on Pseudomonas Isolation Agar (PIA, Thermofisher) to select for rhizospheric Pseudomonads. Results shown in Figure 1.

To achieve isolated single colonies of single rhizosphere isolate, standard bacterial streaking procedure was used to achieve single colonies on LBA. Colonies were obtained from PIA plates with countable single colonies, taking careful note of each colony's phenotype and morphology.

Collection and Identification of novel *P. syringae* isolates from diseased tomato leaves:

10 Tomato leaves were collected from Foxcroft Farm in late September 2021 with symptoms consistent with bacterial speck of tomato (black spots with chlorotic tissue). Bacteria were isolated via the same protocol as used for rhizobacteria isolation. Bacteria were serially diluted 1:10 and plated on Pseudomonas Isolation Agar selective for Pseudomonas species.

Colonies achieved from the PIA agar were streaked onto King B media and single colonies were assessed for fluorescence via comparison to known fluorescent (*P. aeruginosa)* and non-fluorescent (*E. coli)* strains.

Diseased leaf isolates that were fluorescent under UV on King B media were assessed for the absence of cytochrome c oxidase. In this test, single colonies of fluorescent strains were spread onto an oxidase test strip (Hardy Diagnostics Z93) via sterile toothpick moistened with sterile distilled water. Because the color change indicative of oxidase enzyme is instantaneous, colonies that did not change to a purple color before 20 seconds were classified as oxidase negative.

Colonies that were selective on Pseudomonas isolation agar, were fluorescent on King B agar, and were oxidase negative were determined to be *Pseudomonas syringae*, and specifically *P. syringae pv tomato* due to being isolated from diseased tomato leaves. Of 100 diseased leaf

isolates tested, 22 *P. syringae* strains were collected. 8 isolates of *P. syringae* were obtained from the Riley Lab freezer collection. Results shown in Table 1.

Rhizosphere Isolate Co-Culture Screen Against *P. syringae:*

Rhizosphere isolates were inoculated into 9.9 mL test tube of sterile LB and were placed in an incubator-shaker at 30 ℃ for 16 hours to achieve a cell concentration of approximately 10^9 CFU/mL. Overnight growth was also prepared for the 30 *P. syringae* isolates. To assess for bacterial antagonism suggestive of bacteriocin production, 60 rhizosphere isolates were spotted in triplicate on each of the 30 *P. syringae* lawns and incubated at 30 ℃ for 24 hours. The plates were then scored using a binary 0 and 1 system to differentiate no inhibition (0) or if a distinct zone of inhibition was present around the spotted rhizosphere isolate (1). Results shown in Table 2.

***P. syringae* sensitivity to nisin-A assay:**

The ability of 4.23 mg/mL of our model bacteriocin, nisin-A, to inhibit *P. syringae* growth was determined via spotting 5 uL of the nisin on each of the 30 *P. syringae* isolates. Pathogenic isolates were prepared from 16 hours overnight growth. Sensitivity was scored with a binary system of 0s and 1s. Results shown in Table 2.

***P. syringae* Intraspecies Co-Culture assay:**

To assess for intra-species bacteriocin production, *P. syringae* isolates were co-cultured against themselves and each of the other 29 *P. syringae* isolates. Overnight growth was prepared for each of the 30 *P. syringae* isolates as stated above. The co-culture assay was employed along with the same scoring system to assess for zones of inhibition. Results shown in Table 2.

Nisin Investigation Assays:

Comparison of Nisin's Efficacy at killing *P. syringae* in Wet and Dry conditions:

Overnight culture of our model *P. syringae* isolate was prepared by inoculating a single colony of *P. syringae* into 10 mL LB flask and shaken at 30 ℃ for 16 hours to achieve a cell density of approximately $4.26x10^9$ CFU/mL. The cell solution was serially diluted to achieve $4.26x10^4$ CFU/mL.

Sterile 16 mm petri dishes were grouped into either wet or dry treatment. 1 mL of 4.23 mg/mL nisin or 1 mL of 2.12 mg/mL nisin was added to each petri dish. In the wet group, 1 mL of 10^4 CFU/mL solution was immediately added to the nisin solution along with 8 mL of 1.7% dilution medium (DM), swirled, and allowed to sit for 1 hour. In the dry conditions, the nisin treatments were allowed to dry for 1 hour at room temperature before adding 1 mL of 10^4 CFU/mL solution and 9 mL of DM to achieve a total volume of 10 mL in the petri dish. The dry group plates were swirled and allowed to sit at room temperature for 1 hour. Each nisin treatment was repeated in triplicate. The negative control plates received a 1 mL treatment with DM instead of nisin.

After 1 hour, 100 µL of each petri dish solution was lawned with sterile glass beads on LBA. The plates were then incubated at 30 ℃ for 24 hours before counting the number of observed CFU. Relative change was calculated by subtracting the average observed CFU from the average CFU observed in the negative control and dividing the difference by the observed CFU in the negative control. Results shown in Figure 2.

Assessment of 4.23mg/mL Nisin Volumes in Dry conditions at reducing *P. syringae* Colony Forming Units:

Overnight culture of *P. syringae* isolate was prepared by inoculating a single colony of *P. syringae* into 10 mL LB flask and shaken at 30 ℃ for 16 hours to achieve a cell density of approximately $3.7x10^9$ CFU/mL. The solution was serially diluted to achieve 10^4 CFU/mL. 0.1, 2, or 4 mL of 4.23 mg/mL nisin was added to a 16 mm petri dish and spread across the dish using sterile glass beads. Nisin treatments were allowed to dry for 1 hour before adding 1 mL of respective cell concentration and 9 mL DM to achieve a total volume of 10 mL in the petri dish. For a negative control, 10 mL of DM were added with no nisin treatment. Dishes were allowed to sit at room temperature for 1 hour before plating 100 µL on LBA and spreading the solution with sterile glass beads. Each treatment was repeated in triplicate. Plates were incubated at 30 ℃ for 24 hours before counting observed CFU. Results shown in Figure 3.

Pilot Study to assess Filter Paper as a model system for a detached tomato leaf assay 1, 2, and 24 hours between treatment and cell application:

Overnight culture of *P. syringae* isolate was prepared by inoculating a single colony of *P. syringae* into 10 mL LB flask and shaken at 30 ℃ for 16 hours to achieve a cell density of approximately $2.4x10^9$ CFU/mL. Cell treatment for the 24-hour group was prepared from a separate overnight growth flask with a calculated density of $4.0x10^9$ CFU/mL The solution was serially diluted to achieve 10^4 and 10^2 orders of magnitude. 4.23 mg/mL nisin was obtained and diluted 1:2 to achieve 2.12 mg/mL nisin. 200 PPM streptomycin sulfate was prepared by dissolving 20 mg in 100 mL of sterile distilled water. 200 PPM streptomycin sulfate was diluted 1:2 to achieve a 100 PPM solution.

Model tomato leaves were prepared by cutting 16 cm^2 squares from filter paper. 1 mL of each respective treatment was added to 3 respective 16 mm petri dishes labeled according to treatment received. Each filter paper was dipped in its respective treatment and allowed to dry at room temperature for 1, 2, or 24 hours. Model leaves in the filter paper group were dipped in DM without nisin nor streptomycin sulfate.

After the respective time interval, each piece of filter paper was fully dipped in either 1 mL of 10^4 CFU/mL or 1 mL 10^2 CFU/mL solution. One petri dish was used per treatment and model leaves were dipped beginning with the lower dilution treatment. In future experiments, each leaf was dipped in its own respective solution with no 'double-dipping.' Two replicates were performed for each drug and cell solution treatment. After cell solution application, the model leaves were allowed to dry for 1 hour before being transferred to LBA. Only one side of the paper was placed on LBA for 10 minutes before being incubated at 30 ℃ for 24 hours. After 24 hours, the number of CFU on each plate was counted. Results shown in Figure 4.

Tomato plant growth conditions:

Ponderosa Pink *Solanum lycoperscium* seeds were obtained from Mountain Valley Seed Co. and sown into 1x1x2" pods in a 7x10 nursery tray. Seedlings were grown in a research greenhouse with 16 hours of artificial light, a day temperature of 70 °F (21 °C) and a night temperature of 65°F (18.3 °C). After 7 weeks, 20 healthy seedlings were transplanted into 6-inch pots and supplemented with Garden Soil potting mix. At 8 weeks, leaves were cut from each tomato plant to achieve a random sample of about the same size leaves. Leaves were stored in sterile petri dishes during transport and used in leaf-dipping assay the same day as collected.

Percent Recovery of *P. syringae* on tomato leaves after CFU application without treatment:

Overnight culture of *P. syringae* isolate was prepared by inoculating a single colony of *P. syringae* into 10 mL LB flask and shaken at 30°C for 16 hours to achieve a cell density of approximately $3.1x10^9$ CFU/mL. The solution was serially diluted to achieve 10^5, 10^4, and 10^3 orders of magnitude. 100 uL of each solution was lawned on LBA to confirm cell density. 1 mL of each cell density was moved into a sterile 15 mm petri dish. Leaves were dipped into respective cell solutions until fully coated. Leaves were then immediately placed in a test tube with 10 mL LB via forceps. Three leaves were dipped per solution in respective 1 mL petri dishes. The test tubes were then thoroughly vortexed for 30 seconds. 100 μL of each leaf solution was plated on LBA, spread with sterile glass beads, and incubated at 30 °C for 24 hours. The number of CFU were counted on each plate after 24 hours. Percent recovery was determined by calculating the ratio of expected CFU in each concentration with the CFU present on the LBA plate. Percent recovery was determined from the averaged percent recovery from each CFU/mL treatment. Results are shown in Figure 5.

Comparison of Detached Leaf-Dipping Assay with Filter Paper and Tomato Leaves:

To compare the success of the leaf dipping assay with filter paper to its efficacy on tomato leaves, four-16 cm^2 model leaves were dipped along with 4 young tomato leaves in either 1 mL of 2.12mg/mL nisin, 200 PPM streptomycin sulfate, or sdH_2O respectively. The leaves were then allowed to rest in thirty-six 90 mm petri dishes for 24 hours before being dipped in 1 mL of $3.73x10^4$ CFU/mL *P. syringae* solution placed in a 16 mm petri dish.

1 hour after cell application, each leaf/model leaf bottom was transferred to a labeled LBA plate and allowed to rest for 10 minutes. After 10 minutes, the top side of the leaf/model leaf was pressed onto another LBA plate. The plates were incubated at 30 °C for 24 hours prior to counting observed CFU on each plate. Results shown in Figure 6.

Efficacy Comparison of nisin purity and streptomycin sulfate on tomato leaves in detached leaf-dipping assay:

66 mature leaves were collected from 8-week-old tomato plants, ensuring leaves were of similar size. Leaves were stored in large petri dishes at 12 °C for about 6 hours before use. 6 leaf replicates were dipped in treatment solutions according to Table 3.

The food grade nisin was obtained from Sigma-Aldrich at 2.5% purity. 314.8 mg of nisin-A (Sigma-Aldrich) were measured and suspended in 2 mL of sdH_2O. To achieve the 1:4

dilution, 250 µL of the stock solution were added to 750 µL of sdH_2O. 4.23 mg/mL ImmuCell Nisin-A was obtained at 93% purity and diluted 1:4 to achieve the final concentrations tested. 400 PPM Streptomycin was achieved by suspending 40 mg in 100 mL of sdH_2O before diluting the sample 1:2 to achieve 200 PPM.

1 mL of each treatment was placed in its respective 16 mm petri dish. Three leaves were dipped in the same 1 mL solution before another 1 mL of treatment was added to the same petri dish and used to treat the remaining 3 leaves in the treatment group. Two leaf replicates were placed in a 90 mm petri dish, covered, and placed in a fume hood with an open water container to increase humidity for 24 hours at room temperature. This was repeated for each of the 66 total leaves.

After 24 hours, the leaves were removed from the petri dishes and dipped in a 10^4 *P. syringae* CFU/mL solution in the same order the treatment was applied. Each leaf replicate was dipped in a respective 1 mL cell solution. The cell-dipped leaves sat for 1 hour at room temperature before the bottom of each leaf was pressed on a 90 mm LBA plate. The leaves were allowed to sit on the LBA plate for 10 minutes before being rotated onto the other side of the plate to capture viable CFU present on the top of the leaf. After 10 more minutes, the leaves were carefully removed and the LBA plates were incubated at 30 ℃ for 20 hours to achieve a countable number of CFU on each leaf. Results shown in Figure 7.

Results

Rhizobacteria Isolation and Concentration determination:

To isolate bacteria from a young tomato plant's rhizosphere, soil samples from the tomato rhizosphere were suspended in 0.9% NaCl to create a stock solution and serially diluted 1:10 before plating on both LBA and PIA. The rhizosphere stock solution was calculated to contain 1.70×10^{7} CFU/mL, approximately 2.71×10^{6} CFU/mL (16%) grew on PIA agar selective for Pseudomonads (Figure 1).

The same bacterial isolation protocol was used to isolate bacteria from tomato leaves with symptoms of bacterial speck. The stock solution prepared from diseased tomato leaves was plated on PIA and determined to have a concentration of 9.75×10^{5} CFU/mL (Figure 1). When plated on PIA, the rhizosphere stock had 3.15x more CFU than the diseased leaf stock.

Collection and Identification of novel *P. syringae* isolates from diseased tomato leaves:

100 colonies grown on PIA were tested for chemical characteristics of *P. syringae*. Of the 100 isolates tested, 65 were fluorescent under UV light when grown on KB agar. Of the 100 isolates tested, 30 were fluorescent on KB and oxidase negative, and thus were identified as *P. syringae* (Table 1). The isolates that were identified as *P. syringae* showed a diverse phenotype on PIA. Many shared a small, round, mucoid, and yellow/tan/beige phenotype. Others were matte, translucent, or cream-colored.

Rhizosphere Isolate and Intraspecies Co-Culture Screen Against *P. syringae:*

To gain a sense of which avenue was best to explore as an antibiotic alternative to prevent against *P. syringae* infection, the inhibitory activity of the tomato rhizobacteria was compared to that of the intraspecies all-by-all assay between each of the 30 identified *P. syringae* isolates. 19.7 μg of nisin-A was spotted on each pathogen isolate to assess its sensitivity to the model bacteriocin.

The intraspecies screen revealed 10 of the 30 isolates (33%) were able to produce a zone of inhibition against at least one *P. syringae* lawned isolate. The rhizosphere screen revealed 12 of 60 isolates (20%) were inhibitory (Table 2). Interestingly, there were at least 9 instances of the spotted isolate being inhibited by the *P. syringae* lawned isolate.

Further, the single isolates that produced the highest number of inhibitory zones were analyzed from their respective screens and compared. The best producing *P. syringae* isolate against other isolates was able to inhibit 20 of the 30 (67%) of the total *P. syringae* lawns. The best producing rhizosphere isolate was only able to inhibit 8 of the 30 (27%) total *P. syringae* lawns (Table 2).

Lastly, each producer's inhibitory activity was considered to determine the percentage of the total 30 *P. syringae* isolates able to be killed. Collectively, the 10 inhibitory rhizosphere isolates were able to inhibit 12 to the 30 *P. syringae* (40%). In the intraspecies screen, the 10 inhibitory isolates were able to collectively inhibit 29 of the 30 (97%) *P. syringae* (Table 2).

However, nisin was the most inhibitory agent assessed against each lawn of *P. syringae*. A 5 uL spot of 4.23 mg/mL (19.7 μg) nisin on a lawn of each *P. syringae* isolate was found to inhibit 30 of the 30 (100%) lawns (Table 2). This result was notable, and the high efficacy led us to proceed with nisin as the most effective potential preventative in future experiments.

Comparison of Nisin's Efficacy at killing *P. syringae* in Wet and Dry conditions:

To assess the efficacy of nisin at reducing *P. syringae* growth after allowing nisin to dry on a model (petri dish) surface, nisin's efficacy was compared in both wet and dry conditions. As expected, nisin was more effective at reducing viable *P. syringae* in the wet conditions versus the dry (Figure 2). Treatment with 1 mL of 4.23 mg/mL nisin resulted in a 90.5% vs. 60.5% relative decrease in *P. syringae* CFU compared to the negative control in the wet and dry conditions, respectively. For the 2.12 mg/mL nisin, the nisin treatment in wet conditions was more effective at reducing viable CFU than in dry conditions (74.6% vs. 24.8% relative decrease).

Assessment of 4.23 mg/mL Nisin Volumes in Dry conditions at reducing *P. syringae* Colony Forming Units:

Having learned that nisin killing efficacy decreased by about one-third compared to the wet conditions, various volumes of applied nisin were tested for a range of efficacy in dry conditions. The reduction in *P. syringae* CFU after 10^4 CFU/mL application was assessed following treatment with 0.1, 2, or 4 mL of 4.23 mg/mL nisin. For the nisin treatments of 0.1, 2, and 4 mL after 10^4 CFU/mL application, there was a 2%, 66%, and 78% relative decrease in the number of *P. syringae* cells compared to the control group, respectively (Figure 3).

Pilot Study to assess Filter Paper as a model system for a detached tomato leaf assay 1, 2, and 24 hours between treatment and cell application:

With an understanding of the activity of nisin in dry conditions, the protocol was refined to a leaf-dipping method. To pilot this new assay, 16 cm^2 filter paper squares were used as model leaves to assess two concentrations of nisin and streptomycin 1, 2, and 24 hours between treatment and either 10^2 or 10^4 *P. syringae* cell application.

Two model leaf replicates were dipped in 10^2 CFU/mL *P. syringae* solution after its respective treatment application and time duration. The number of viable CFU counted after 24 hours incubation (Figure 4). After 1 hour, the 200 PPM streptomycin led to a 22% relative decrease to the number of CFU compared to the negative control whereas the 100 PP streptomycin had a 0% change. Each nisin dilution reduced the number of viable CFU by 100%. After 2 hours, the 200 PPM streptomycin was four times more effective than the 100 PPM streptomycin (-40% vs. -10%). Each nisin dilution reduced the number of viable CFU by 100%. After 24 hours, there was no difference in the percent decrease from the 200 PPM streptomycin treatment compared to the 100 PPM streptomycin treatment (-36%). The 4.23 mg/mL nisin prevented all CFU growth while the 2.12 mg/mL reduced the number of CFU by 93% after 24 hours. While the results show meaningful data, significant conclusions cannot be made from because of the small number of observed CFU even without treatment. Future experiments must apply a higher concentration of *P. syringae* cells.

Two model leaf replicates were dipped in 10^4 CFU/mL *P. syringae* solution after their respective treatment application and time duration. The number of viable CFU were counted following 24 hours incubation (Figure 4). After 1 hour, the 200 PPM streptomycin led to a 19% relative decrease in the number of CFU compared to the negative control whereas the 100 PP streptomycin showed a 14% decrease. Each nisin dilution (4.23 and 2.12 mg/mL) reduced the number of viable CFU by 100%. After 2 hours, the 200 PPM streptomycin was more effective than the 100 PPM streptomycin (-49% vs. -29%). The 4.23 mg/mL nisin dilution reduced the number of viable CFU by 100% whereas the 2.12 mg/mL nisin treatment reduced the number of CFU by 87%. After 24 hours, the 200 PPM streptomycin treatment reduced the number of viable CFU by 33% compared to a 17% decrease in the 100 PPM streptomycin treatment. The 4.23 mg/mL nisin prevented 99% of CFU growth while the 2.12 mg/mL reduced the number of CFU by 96% after 24 hours. There appears to be a narrower standard deviation for observed CFU after 10^4 CFU/mL application compared to the 10^2 CFU/mL solution.

Percent Recovery of *P. syringae* on tomato leaves after CFU application without treatment:

To gain a better understanding of how many *P. syringae* cells are picked up via the tomato leaf dipping assay, leaves were dipped in either 10^5, 10^4, or 10^3 CFU/mL solution and repeated in triplicate before transferring to an LB test tube, vortexing, and plating on LBA. The number of observed CFU divided by the number of expected CFU was defined as the percent CFU recovery. Leaves dipped in the 10^4 CFU/mL solution had the highest average percent recovery (16% ±1.5), followed by the 10^5 CFU/mL solution (10% ±4.0), and finally the 10^3 CFU/mL solution (9% ±4.9) (Figure 5). Despite being dipped in a high range of cell concentrations, there appears to be a consistent percent recovery. The average percent recovery across all three cell concentrations was determined to be 11.3% ±3.8.

Comparison of Detached Leaf-Dipping Assay with Filter Paper and Tomato Leaves:

To ensure the success of the leaf dipping protocol, 16 cm^2 filter paper squares were treated in comparison to 8-week-old tomato leaves. Following the success of the percent recovery data, both the leaves and model filter paper squares were dipped in 10^4 CFU/mL solution. The no drug treatment revealed a higher number of CFU present on the tomato leaf plates compared to the filter paper plates (989 vs. 697 CFU) (Figure 6). Relative change was also used in this assay to assess the efficacy of each treatment. The nisin appeared to be more effective at reducing the number of viable CFU on the tomato leaf than on the filter paper (-76.3% vs. -39.2%). The streptomycin was also observed to be more effective at reducing CFU on the tomato leaves compared to the filter paper (-45.9 vs. -7.4). Interestingly, both tomato leaves -drug,-*P. syringae* and tomato leaves +drug, -*P. syringae* yielded hardly any CFU when plated on LBA.

Efficacy Comparison of nisin purity and streptomycin sulfate on tomato leaves in detached leaf-dipping assay:

Following the previously described data about nisin's efficacy at reducing *P. syringae* both on filter paper and actual tomato leaves, the 93% pure nisin was compared to a 2.5% pure nisin. The 4.23 mg/mL pure nisin reduced the number of viable CFU by 91.3% compared to the lower purity equivalent concentration which reduced the number of CFU by 96.8% (Figure 7). The 2.12 mg/mL pure nisin reduced the number of CFU by 86.7 compared to 92.8% decrease in the lower purity equivalent.

Both the lower and higher purity nisin were significantly more effective than the streptomycin after 24 hours on the tomato leaf. The 200 PPM streptomycin treatment reduced the number of CFU by 40% whereas doubling the EPA recommended application dose to 400 PPM only increased the percent decrease to 50.6%. As previously found, both tomato leaves without drug and *P. syringae* treatment and tomato leaves with drug treatment and no *P. syringae* yielded hardly any CFU when plated on LBA.

Discussion

Rhizosphere Isolate and Intraspecies Co-Culture Screen Against *P. syringae:*

The overarching goal of this set of experiments was to search for an alternative treatment for the tomato bacterial pathogen *P. syringae*. Based on prior investigation, it was reported that bacteria known to be plant-growth-promoting rhizobacteria show potent and targeted antimicrobial activity against plant pathogens (Mitchell et al., 2022). Based on this finding, 60 tomato rhizobacteria were investigated in a co-culture assay for inhibitory activity against 30 isolates of the plant pathogen isolated from tomato leaves with symptoms of bacterial speck. We found that approximately 16% percent of the isolated bacteria from the rhizosphere was made up of Pseudomonads. This result is lower than reported by Dong et. al where they used PCR amplification and sequencing to determine that 36.78% of the rhizosphere were Pseudomonads (Dong et al., 2019). This discrepancy may be due to the methods of sampling. It is known that less than 99% of soil bacteria cannot be cultured by traditional plating methods (Pham and Kim, 2012). If we isolated 1% of the microbes in our 100g soil sample, then we can estimate that we had $1.70x10^9$ CFU/100 g soil which equates to $1.70x10^7$ CFU/ g soil. This estimation is lower than the 10^{10} CFU/g of rhizosphere soil reported by Raynaud and Nunan (2014). Perhaps the soil collected was from a less microbiota-rich rhizosphere than previously expected, or less than 100 grams of soil were collected. Future experiments should be sure to measure how much soil is added and assess colony number and morphology on a range of nutrient agar media to culture as high a diversity as possible.

The hypothesis of the rhizosphere co-culture screen was that it would reveal microbes within the Pseudomonas genus highly effective at inhibiting the growth of a high fraction of the novel *P. syringae* isolates. However, we only found that 12 (20%) of the 60 isolates tested were able to produce at least one zone of inhibition. The best producing isolate was able to inhibit 8 of the 30 *P. syringae*. Collectively, the 12 inhibitory isolates were able to inhibit the growth of 12 of 30 (27%) *P. syringae* isolates. While the abundance of Pseudomonas rhizobacteria producers against *P. syringae* is lower than expected the sample size of rhizobacteria tested against *P. syringae* was small. These results may also be explained because not all rhizobacteria are producers of bacteriocin-like inhibitory substances (Beneduzi et al., 2012), or the co-culture assay may not have been suitable for their production and assessment.

However, the low production of inhibitory compounds is consistent with another study focused on rhizobacteria conducted in 2018. The researchers investigated 524 tomato rhizobacteria for antagonistic activity against *P. syringae pv tomato* and found that 48 (9.16%) inhibited *P. syringae* growth in a traditional co-culture assay, with 39 showing siderophore production (Yildiz et al., 2018). This suggests that bacteriocin production within the tomato rhizosphere may be less abundant. Further, although the isolates tested may not be closely related enough to *P. syringae* to show narrow spectrum antimicrobial activity characteristic of bacteriocins, even the Pseudomonas isolates from PIA media did not show significant production of inhibition. Future screens to investigate novel antimicrobials should rely on bacterial PCR amplification and sequencing to avoid the biases that come with attempting to isolate and screen only a small subset of the total bacterial population. Genomic analysis would first need to be informed by the common sequences and amino acids found within a bacteriocin gene/protein.

The lack of rhizobacteria with significant inhibitory activity across the *P. syringae* isolates led us to turn to assessing for inhibition of *P. syringae* by *P. syringae* itself in a similar intraspecies co-culture assay. We observed that 10 of the 30 (33.3%) *P. syringae* isolates used in the assay showed at least a single zone of inhibition when spotted against another isolate of *P. syringae*. The single best producing isolate was able to inhibit 20 of the 30 (66.7%) isolates, a percentage much higher than observed in the rhizosphere screen. Collectively, the 10 producers were able to inhibit 29 of the 30 isolates at least once, a notable statistic. The higher abundance of inhibition found in this screen is less surprising due to the narrow spectrum of activity many bacteriocins act (Riley and Wertz, 2002). Other work has documented that *P. syringae* can both produce its own bacteriocins, such as Syringacin 4-A and W-1 (Smidt and Vidaver, 1986), and is also sensitive to the protein defense weapons as well (Lavermicocca et al., 2002). To support this claim, in our assay, there were 9 instances of inhibition where the spotted rhizobacterial isolates appeared to be inhibited by the *P. syringae* lawn. This suggests that the *P. syringae* isolates produced some inhibitory compound(s) which inhibited the growth of the spotted rhizobacteria. Further characterization of these isolates and the substance(s) they produce would have carried out if the collected *P. syringae* isolates survived glycerol stock preparation and freezing at -20 ℃ over the 2021-2022 Winter season.

In contrast to the rhizosphere and intraspecies screens that did not reveal a single inhibitory isolate able to inhibit the growth of each *P. syringae* isolate, a 5 uL spot of 4.23 mg/mL nisin produced a distinct zone of inhibition on each *P. syringae* lawn. This result was surprising as the bacteriocin, nisin, produced by gram-positive *Lactococcus lactis* was previously thought to be most effective against gram-positive bacterial species due to nisin's accessibility to the lipid II receptor (Li et al., 2018). However, *P. syringae* is gram-negative and showed high sensitivity to the highly concentrated bacteriocin and warranted further study.

To the best of our knowledge, nisin has not been tested against *P. syringae* in-vitro or in-planta. This finding was also corroborated by a 2020 review on modes of phyllosphere biocontrol (Legein et al., 2020). The lack of report on exploring nisin's efficacy against gram-negative plant pathogens reveals an exciting contribution to the current gap in knowledge. However, nisin's high antimicrobial properties are continuing to be explored. Specifically, nisin has been used as a food preservative via its integration in nanoparticles (Khan and Oh, 2016), but also has high potential for use in food packaging, agriculture, aquaculture, and human health to combat antibiotic resistant pathogens.

Assessment of Nisin on Tomato Leaves:

Comparison of Nisin's Efficacy at killing *P. syringae* in Wet and Dry conditions:

The next set of experiments were focused on developing a protocol to assess the efficacy of nisin at inhibiting *P. syringae* on a tomato leaf compared to the conventional streptomycin antibiotic. To the best of my knowledge, there is no standard way of assessing the efficacy of a bacteriocin or antibiotic on tomato, thus a leaf-dipping assay was developed. Detached-leaflet assays are highly versatile and have been used as a standard to conduct virulence sensitivity screening and identification of resistant cultivars of tomatoes (Karki and Halterman, 2021).

The study began by comparing nisin in solution versus after drying on a petri dish. We found that the efficacy of nisin decreased by an average of 30% when left to dry on the petri dish compared to when in solution (wet conditions). This is a notable drop in efficacy, but nisin is so

effective in solution that it was of high interest to continue exploring its efficacy in dry conditions by adding varying nisin volumes to the petri dish.

Recently, a study was published which added nisin to fungi protein hydrophobin surfaces and found highly activity of nisin against *Staphylococcus aureus* even after being immobilized on the hydrophobic polysystrene surface (Wang et al., 2021). Another study found that nisin's efficacy increased when added to a hydrophilic surface compared to a hydrophobic surface (Karam et al., 2013). These results suggest that even when nisin is adsorbed on a surface, it retains excellent antibacterial activity. How the nisin remains able to kill bacteria after being immobilized is still not well understood as well as the efficacy of nisin on organic hydrophobic plant tissue, such as the tomato leaf.

Assessment of 4.23 mg/mL Nisin Volumes in Dry conditions at reducing *P. syringae* Colony Forming Units:

When 0.1, 2, and 4 mL nisin was allowed to dry before the addition of 1 mL of 10^4 CFU/mL, there was an average of 2%, 66%, and 78% relative decrease in the number of *P. syringae* cells compared to the control groups. One source of error in these data may be the inconsistent amount of sterile glass beads used to the spread the nisin over the surface of the petri dish, thus a differing number of nisin molecules were removed from each petri dish. When there is only a small amount of nisin added to the petri dish, there was a low change in the observed CFU. When a large volume of nisin was added (4 mL), then the number of viable *P. syringae* decreased by approximately 5-fold. This suggests that there needs to be enough nisin molecules present to effectively kill the cells. Future experiments could extrapolate on this work and assess whether this decay function remains consistent at higher volumes of nisin added.

This experiment originally tested two cell conditions (10^2 and 10^4). These concentrations were selected based on the work of Chai et al. who found a range of 10^2-10^4 CFU *P. syringae** g^{-1} infected tomato seeds using PCR detection (Chai et al., 2020). Application with 10^2 CFU/mL *P. syringae* only yielded 2-6 CFU on the LBA plate, rendering the data inconclusive (data not shown). If this is the range of pathogen cells found, then a preventative treatment may need to be effective against that magnitude range of cells. However, it's important to remember that individually painted tomato seeds weigh much less than a gram and thus the number of infected cells may be much smaller on the seed but proliferate upon entry into the soil or tomato plant vasculature. One may infer that the number of cells to enter the tomato's stomata must be large enough to overwhelm the plant's cellular defenses (Freeman and Beattie, 2008). Moreover, nisin's efficacy against a high magnitude of *P. syringae* is believed to also retain high efficacy against a smaller magnitude of cells.

Pilot Study to assess Filter Paper as a model system for a detached tomato leaf assay 1, 2, and 24 hours between treatment and cell application:

After establishing that the application of more nisin molecules resulted in a greater CFU decrease, a leaf-dipping assay was developed using model tomato leaves made from filter paper. In the pilot leaf-dipping assay, model leaves were dipped with 1 dilution of either nisin or streptomycin and allowed to dry for either 1, 2, or 24 hours. After the respective dry time, 1 mL of 10^2 or 10^4 CFU/mL application in respective petri dishes. For the 10^2 CFU/mL treatment

group, each plate had fewer than 10 CFU, making the results of the 10^2 CFU/mL treatment less conclusive. At each time point, both dilutions of nisin prevented all *P. syringae* cell growth, except for the 2.12 mg/mL nisin treatment after 24 hours showed an average of 1 CFU. This is a much greater reduction than observed for the streptomycin sulfate treatment which often did not show a significant relative decrease compared to the control group.

For the 10^4 CFU/mL treatment group, again nisin showed much greater efficacy at reducing *P. syringae* growth compared to either dilution of streptomycin sulfate. For the 1-, 2-, and 24-hour time points, 4.23 mg/mL nisin treatment resulted in 1, 0, and 5.5 observed CFU whereas the 2.12 mg/mL nisin resulted in 0, 38, and 27 CFU respectively. These results suggest that nisin's activity only minimally decreases over a 24-hour period when treated on filter paper. Interestingly, when the concentration of streptomycin sulfate was increased from 100 to 200 PPM, there was not a significant difference in the number of CFU observed. Perhaps there was not enough streptomycin applied to the model leaves, the model leaves somehow trapped the streptomycin molecules within the paper's fabric, or the streptomycin needed more than 1 hour to kill the applied *P. syringae* cells.

This experiment was pivotal in highlighting several potential sources of error. Two different overnight growth solutions of our model *P. syringae* isolate were used in both the 1- and 2-hour treatment, and then a separate solution for the 24-hour time point. Two separate solutions were needed to not reduce cell viability by shaking for too long or storing overnight to risk contamination or cell death. The first 16 hours growth grew to a calculated density of 2.4×10^9 CFU/mL whereas the second overnight was calculated to have a density of approximately 4.0×10^9 CFU/mL. This increase in turbidity may be due to allowing the overnight growth media to shake for slightly longer than 16 hours, or perhaps inoculating the LB flask with a larger colony than used in the first flask. The difference in turbidies of the 16-hour growth solution is reflected in the approximately 2-fold higher observed CFUs in the 24-hours drying period samples. Originally, this was interpreted as the drug treatments losing efficacy over the 24 hours. However, the negative control sample also showed a 2-fold increase in observed CFU compared to the control for 1- and 2- hours. Future experiments must ensure that highly similar cell densities are used during comparison and future experimenters may consider using a spectrophotometer to measure *P. syringae* cell absorbance.

Another source of error may be due to not dipping each model leaf in its own respective petri dish with 1 mL of cell solution. Instead, there were separate petri dishes for each treatment and, starting with the lower concentration of treatment, both dilutions were dipped in the same petri dish with cell solution. If any of the treatment residue entered the cell solution, this would effectively increase the amount of drug on the higher concentration model leaf resulting in a starker decrease in the number of *P. syringae* CFU between the 100 and 200 PPM streptomycin treatment. This is only minimally observed, however. Future experiments must ensure that each leaf replicate is dipped in its own respective dish with cells. Also, both sides of the dipped model leaf should be plated on LBA to observe all CFU present.

Percent Recovery of *P. syringae* on tomato leaves after CFU application without treatment:

Intrigued by the question of whether dipping a tomato leaf in different cell concentrations would influence the percent of CFU that stick to a tomato leaf upon dipping, the percent recovery was determined for three separate *P. syringae* concentrations. It was hypothesized that leaves dipped in a higher concentration of cells may pick up more CFU, leading to a greater

percent recovery. Interestingly, each cell solution resulted in roughly similar percent recovery. This suggests that the number of CFU that stick to the tomato leaf is proportional to the amount of CFU/mL in solution. Rather than a set number of cells contacting the leaf, it was determined that roughly 11.3% of the cells are transferred from the leaves in LB and then onto an LBA plate. This is important data to know because it allows for an estimation of how many CFU stuck to the tomato leaf and encountered the bacteriocin or antibiotic treatment.

Future experiments may wish to explore percent recovery a bit closer; perhaps looking at the connection between leaf surface area and percent recovery. The current protocol makes several assumptions: 1) All of the CFU were released from the leaf upon vortexing the pre-dipped leaf in an LB test tube; 2) All of the CFU observed on the LBA plates were *P. syringae* only, and 3) The expected cell count was 100-fold lower than the magnitude of cells that the leaves were dipped in. Future studies may also wish to compare the percent recovery of *P. syringae* CFU to the surface area of the leaf dipped. Surface area of the leaf may be investigated with digital software such as ImageJ.

Comparison of Detached Leaf-Dipping Assay with Filter Paper and Tomato Leaves:

Now knowing that roughly 10% of the CFUs in the cell solution stick to the tomato leaves, the next step was to compare the efficacy of the dipping assay on filter paper to actual tomato leaves. Several notable results emerged from this experiment. Firstly, there was 1.42x more observed CFU on the tomato leaf with no drug control compared to the filter paper control. Although the tomato leaves showed a greater standard deviation than the filter paper (168 vs. 47), this may be due to the surface area of the tomato leaves being slightly bigger, having a greater percent recovery than the filter paper, or greater transfer of CFU from leaf to LBA plate than the filter paper.

However, the tomato leaves were dipped in 1 mL of a $3.7x10^4$ CFU/mL cell solution and the average number of observed CFU in the no drug, tomato leaf control was 989 CFU and 697 CFU on filter paper. This suggests that the percent recovery of CFU on tomato leaves in this experiment is closer to 2.67% for the tomato leaves and 1.88% for the filter paper. These numbers are less than 10-fold lower than determined in the previous experiment. This suggests that the application of the sdH_2O treatment reduces the ability of *P. syringae* to stick to the leaves, or perhaps leaves that are 24 hours detached from the plant show reduced ability to pick up bacterial CFU.

In addition to more cells sticking to the tomato leaves, each treatment was more effective on the leaves than on filter paper. Specifically, 200 PPM streptomycin sulfate showed an increased CFU reduction on leaves than on the filter paper (-45.9% vs. -7.4%). 2.12mg/mL nisin also showed a greater efficacy on actual tomato leaves (-76.3% vs. -39.2%).

Unexpectedly, 2.12 mg/mL nisin was unable to prevent all CFU growth after only 24 hours on both the filter paper and tomato leaf. While nisin showed high efficacy at killing *P. syringae* on the tomato leaves, this is lower than the near 100% expected found when 1-, 2-, and 24-hour intervals were tested (Figure 4).

Further, in the previous assay 2.12 mg/mL nisin completely prevented all CFU growth on the filter paper, whereas an average of about 400 CFU were observed in this experiment on the treated filter paper. This discrepancy may be due to improvements to the protocol (i.e., ensuring sample is dipped into a separate cell solution sample) or differences in the calculated overnight growth concentration and 10^4 CFU/mL dilution. These discrepancies highlight the importance of

having a solidified protocol that can be replicated to achieve consistent results each time. However, because we are working with living organisms such as *P. syringae* and tomato leaf tissue, there will be a margin of error.

These results suggest that while the model filter paper may have been an appropriate model for development of the protocol, the paper itself seems to diminish the efficacy of the treatments when compared to the actual tomato leaves. This may be due to the paper's high absorbance which could trap some of the small streptomycin molecules within the paper fibers and prevent its access to *P. syringae* CFU. This would underestimate the true efficacy of the treatment. There is a possibility that the tomato leaves do this as well, although to a much smaller extent due to being less absorbent.

Efficacy Comparison of nisin purity and streptomycin sulfate on tomato leaves in detached leaf-dipping assay:

Learning from the previous experiment that nisin was unable to prevent all CFU growth only after 24 hours, the question of how long nisin remains effective on the tomato leaf was foregone to answer another question: Is a lower purity nisin equally effective at killing *P. syringae* CFU on tomato leaves compared to our highly pure nisin solution? The data support this hypothesis that the 2.5% pure nisin-A was equally effective if not more than the 93% pure nisin-A. Both purities were tested at two concentrations. The 4.23 mg/mL concentration equivalent of the 2.5% pure nisin was more effective, on average, at reducing CFU count (96.8% vs. 91.2% reduction). The 2.12 mg/mL equivalent of the 2.5% pure nisin was also more effective at reducing CFU count compared to the 93% pure nisin on average (-92.8% vs. -86.7%).

These results are intriguing and suggest that some of the impurities in the 2.5% pure nisin may be contributing to the fewer observed *P. syringae* CFU on the tomato leaf. The 2.5% pure nisin stock was prepared from powder and the resulting solution was a cloudy, beige, and opaque solution. This was distinct from the 93% pure ImmuCell nisin which was already in solution and had a translucent and slightly yellow-green appearance. The 2.5% purity nisin was 77.5% NaCl which may significantly change the salinity of the tomato leaf and potentially create an inhospitable environment for *P. syringae* growth or survival. Unsurprisingly, most salinity experiments have focused on exploring the effect of salinity plant stress on the plant's susceptibility to pathogens. One team found that salt stress altered the interaction between the Jasmonate and Salicylate signaling pathways in tomatoes but did not significantly impact resistance to *P. syringae* on tomatoes (Thaler and Bostock, 2004).

It is somewhat surprising how effective nisin-A has been when applied on tomato leaves. In addition to nisin-A, there are other forms of nisin with amino acid substitutions that have been naturally produced by *L. lactis* and bioengineered. Nisin-A has been found to have optimal solubility, stability, and activity around a pH of 3.0, wehereas nisin-Z was found to have roughly 2-fold higher solubility at pH 7.0 (Rollema et al., 1995). These data suggest that other forms of nisin may be even more effective on the tomato in agricultural conditions where the pH may be less acidic than the optimal activity for nisin-A.

In contrast to the highly effective 2.5% and 93% pure nisin treatments, both the 400 PPM and 200 PPM showed a much lower relative CFU decrease, -50.6% and -40.0% respectively. This result is shocking. At the current EPA recommendation of 200 PPM, only about 40% of the *P. syringae* cells can be killed. Even when we double the concentration of streptomycin sulfate to twice the EPA recommended dose, we only observe about a 10.6% increase in efficacy. These

results point to a less effective antimicrobial, one that potentially may take significantly longer to kill the bacterial cells or requires a higher treatment concentration.

Although the leaves were allowed to sit for 1 hour after cell application before transfer to LBA and then incubated for 24 hours, these results may be biased toward nisin because nisin kills the pathogen immediately via pore-formation in the membrane or inhibition of cell wall biosynthesis (Prince et al., 2016). Conversely, streptomycin works by inhibiting bacterial protein translation initiation and may need more time to completely kill all cells. To the best of my knowledge, I could not find published data on the time required for aminoglycoside antibiotics to kill their bacteria targets. However, one study modeling the killing of *Pseudomonas aeruginosa* by two aminoglycoside antibiotics and found the time between treatment and death to fall between 0.5 and 6 hours, the time required for synthesis of lethal proteins due to ribosome inhibition (Bulitta et al., 2015).

Lastly, it must be emphasized that the molarities of nisin and streptomycin treatment used are not equal. Nisin was applied in almost 10x the mM quantity than streptomycin sulfate at 200 PPM. Even when the concentration was doubled to 400 PPM, the molarity of streptomycin is 0.137 mM compared to 1.17 mM of the nisin. However, none of these treatments showed negative effects on the leaf tissue appearance, suggesting that nisin can be safely applied at this high concentration without damaging leaf tissue. According to a 2017 nisin safety study that gave mice 225 mg of nisin-A/ kg bodyweight while the ESFA acceptable daily intake (ADI) is listed at 0.13 mg/kg bodyweight (Younes et al., 2017).

Conclusion

This collection of experiments has identified a potential alternative to the conventional antibiotic streptomycin sulfate used to prevent tomato infection by *Pseudomonas syringae pv. tomato*. Not only were the antagonistic activities of tomato rhizobacteria and other *P. syringae* isolates investigated, but nisin-A was revealed to be the most effective growth inhibitor. Through the collection of protocols described, a leaf-dipping assay was developed to provide a measurable way to compare the efficacy of nisin and streptomycin at reducing *P. syringae* cells that survived on treated tomato leaves. These results highlight nisin as a treatment that is highly effective with minimal diminishing effects up to 24 hours after treatment. Further, the equivalent success of a food-grade 2.5% nisin suggests that turning to nisin as a preventative spray may meet economic constraints to ensure growers' profit.

The work is not done, however. Only a single isolate of *P. syringae* was studied in this collection of experiments. Future studies may focus on applying nisin in different conditions, such as humidity, sunlight intensity, or post-rainfall against a large sample size of *P. syringae* isolates. Other studies may experiment with different engineered variants of nisin, such as nisin-Z. The length of time that nisin remains active on the tomato before requiring another treatment is a valid question, along with its comparison to the conventional antibiotic. Further, in-planta assays to confirm the safety of nisin use on tomato plants. Determining the number of *P. syringae* cells to cause symptom development in-planta may prove invaluable in assessing the next wave of antimicrobial treatments using a more realistic concentration of applied cells. This addressed gap in the field of antibiotic alternatives highlights the potential of nisin to prevent bacterial speck of tomato, decrease associated crop loss, and meet global tomato market needs.

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