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Characterization and Interactions of Soil Microbial Communities in Five Physiographic Regions and the Rhizosphere of Common Weeds

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Abstract

This paper explores the symbiotic bacteria and the antimicrobial activity of the common weeds *Plantago major* and *Trifolium repens*, as well as those of North Carolina soils collected from five physiographic regions. The purpose of this study is to characterize the diversity and interactions of symbiotic microbes in the rhizosphere of prolific weeds and different soil types. Cell cultures and isolations were taken from the rhizosphere, phyllosphere, and endosphere of each weed sample as well as bulk soil collected from Blue Ridge Mountains, Piedmont, Sandhills, Lower Coastal Plain, and Upper Coastal Plain physiographic regions. Competition assays between isolates were performed and a final set of disc diffusion assays were conducted to test effectiveness of prolific weed-associated bacteria against *E. coli*. The *T. Repens* associated bacteria produced the highest degree of inhibition against *E. coli* with 33.3% of its symbiotic bacteria displaying inhibition. No microbes from *P. major* were able to inhibit *E. coli*. When competing *T. repens* and *P. major* microbe isolates against one another, 60.38% participated in microbial warfare. For the regional soil types, 35.3% of Mountain, 85% of Piedmont, 71.4% of Upper Coastal, 80% of Lower Coastal, and 33.3% of Sandhills microbes showed zones of inhibition. The Mountain region exhibited the highest degrees of inhibition, while the Lower Coastal region exhibited the lowest degrees. The Mountain and Lower Coastal regions also displayed the largest amount of microbial diversity. In addition to understanding interactions between microbes and their symbiotic effects for plants, these microbes could also serve as the basis for future explorations into natural product discovery.

Keywords: North Carolina, Microbiology, Secondary Metabolism, Microbial Inhibition

1. Introduction

Present-day vegetation has evolved over millennia to recruit environmental microbial communities. Trees, plants, and weeds use these microbes for defense, nutrient acquisition, and systemic processes. For example, some plants can use growth-promoting rhizobacteria in the soil to protect against whiteflies, which typically damage the leaves of plants[1]. Other vegetation, such as legumes, use a combination of mycorrhizal fungi and rhizobacteria in the soil to increase nutrient procurement and seedling recruitment[2]. The plants explored in this study were two weeds: *Plantago major* and *Trifolium repens*.

Weeds are extremely successful at competing in new and unfamiliar environments. Weed species can be found everywhere, from an urban sidewalk to the Arctic tundra. The scientists involved in the current study sought to find whether or not their versatility might be due to their advanced recruitment of the microbes found in such environments. The mechanisms, protection, and nutrients gained from their symbiotic relationship with these microbes could be serving them better than other plant counterparts. The mission of this study is to determine whether microbes in the soil, as well as within the plant, serve to enhance the competitiveness of *P. major* and *T. repens*. Disc diffusion assays, competition assays, and DNA testing were done to identify these microbes and their supportive abilities. Additional

assays were done to compete the isolated symbiotic microbes against *Escherichia coli* in order to test their feasibility in human applications. The scientists went on to sample regional soil samples of North Carolina to determine one that might produce increased competition against other samples.

The antimicrobial properties of plant associated bacteria have long been studied by scientists for medical applications and have helped create numerous antibiotics and antifungals[3 4 5]. The use of chemical properties from symbiotic bacteria has had many utilizations in human pathogenic interactions, as well as agricultural growth aids. The conclusions drawn from this research could one day serve to aid in further antimicrobial development for human and agricultural applications.

2. Methodology

2.1 Native North Carolina Weed Sampling

Isolations were taken from the rhizosphere, endosphere, and phyllosphere of North Carolina native weed species. Three samples, labeled "a", "b", and "c", from two different plants were collected. The first plant was a white clover, or *T. repens*, located within a sidewalk crack. The second plant was a broadleaf plantain, or *P. major*, located on the side of an urban neighborhood road.

Sample methods varied depending on the compartment of the weed being examined. To sample the phyllosphere, the leaf surface was swabbed on both sides using a BD BBL culture swab. To collect samples from the endosphere and rhizosphere, a shovel was first sterilized with alcohol and wiped. The trowel was used to dig out a portion of the weed from the soil including the roots and surrounding soil.

After bringing the weeds back to the laboratory via 50 mL conical Falcon tubes, approximately 2 cm root sections were added to 1 mL of sterile phosphate buffer solution and placed in a sonicator for 5 minutes to break apart the rhizosphere soil from the roots. Roots were then surface sterilized by a two minute soak submerged in 10% bleach. Endophytes were extracted from the surface sterilized roots by macerating the roots with a scalpel.

2.2 North Carolina Soil Sampling

The collection of North Carolina soil was collected in five distinct regions of the state: Blue Ridge Mountains, Piedmont, Sandhills, Lower Coastal Plain, and Upper Coastal Plain physiographic regions. The Sandhills soil came from the Weymouth Forest located in Southern Pines, NC. The Upper Coastal Plain soil came from the Cliffs of the Neuse State Park located in Seven Springs, NC. The Lower Coastal Plain soil came from Croatan National Forest located across the coastal region of eastern North Carolina. The Blue Ridge Mountains soil came from Pisgah National Forest located in Asheville, NC. Finally, the Piedmont soil came from Uwharrie National Forest located in Uwharrie, NC. To collect soil, a shovel and trowel were sterilized using 70% ethanol. Care was taken to move away from the main roads in order to avoid human interaction and traffic that could change the soil environment. The leaf litter and plant debris were removed and a hole that was 10 cm in depth was dug to collect soil from a largely untouched area. The soil at the bottom of the hole was then collected in 50 mL conical Falcon tubes. This process was repeated at five locations within each physiographic region in order to account for within region heterogeneity.

Culturing was performed by mixing 1 mL of sterile PBS with .5 gm of the sample. This was followed by 10 seconds of vortexing, 10 minutes rest, and 60 seconds of centrifuge. 100 µL of the supernatant was then plated onto either nutrient of Rose Bengal agar (see below). After at least 72 hours of growth on the agar plates, phenotypically unique typing was done to "type" different fungi and bacteria isolates from each region. Each isolate was preserved over the course of the study by selecting one colony from each plate using a sterile loop. The colony was then placed in 5 mL of nutrient broth and refrigerated until needed or preserved in 15% glycerol frozen at -80°C.

2.3 Nutrient Agar - Bacterial Isolation

To prepare nutrient agar, 1000 mL of dH_2O was mixed with 15g of nutrient agar preparation mixture (1.5% of total volume) and 25g of broth in a large beaker. Tin foil was placed over the opening of the beaker to cover it. The solution was then placed on a hotplate-stirrer. Autoclave tape was placed over the tin foil before putting it in the autoclave. Solid media was formed by pouring approximately 10 mL molten agar into sterile petri dishes.

2.4 Rose Bengal Agar - Fungal Isolation

To prepare Rose Bengal agar, 500 mL of dH_2O was mixed with 16g of Rose-Bengal agar preparation mixture. The solution was placed in a large Erlenmeyer Flask and placed on a hotplate and stirred until a white film appeared. Tin foil was placed over the opening of the beaker to cover it. Autoclave tape was placed over the tin foil before putting it in the autoclave. Solid media was formed by pouring approximately 10 mL molten agar into sterile petri dishes.

2.5 Nutrient Broth

200 mL of dH2O were combined with 2.5 grams of nutrient broth preparation powder. The broth was mixed on a stirrer. It was then autoclaved with a loose lid and covered in autoclave tape.

2.6 Long-term Storage of each Type Culture

In order to build a collection of bacterial types collected within North Carolina, a representative culture of each type was preserved in glycerol and deep frozen for use if needed at a later time. Two mL cryovials were filled with a solution of 500 µL of 30% glycerol and 500 µL of liquid culture.

2.7 Competition Assays

The isolates were moved from a T-streaked plate into a liquid broth medium. Isolates of each "type" culture were transferred to 5 mL of prepared nutrient broth and grown for up to one week. Competition assays were made by labeling two sides of a nutrient agar dish, and pipetting 10 µL of each bacterial strain 0.5 cm away from each other. All of the bulk soil isolate types from the same region were tested against each other to test the antimicrobial activity. In addition, the bacterial and fungal plant-associated isolates from each of the three sections of the four weed samples were tested against one another. The plates were then incubated overnight and tracked over the following week for zones of inhibition.

2.8 PCR and DNA Sequencing

Total community DNA was extracted from five samples from each of the five physiographic regions using the MoBio PowerSoil DNA Isolation kit according to manufacturer's protocol. PCR was performed to amplify the V4/V5 region of the 16S rRNA gene of the bacterial community of each soil collected from the physiographic regions. Amplification mixture included a 25 µL mix of 5 µL buffer, 2.5 µL Mg, 0.5 µL dNTPs, 0.5 µL 515F (5'-GTGCCAGCMGCCGCGGTAA-3'), 0.5 µL 806R (5'-GGACTACHVGGGTWTCTAAT-3'), 0.25 µL Taq, 1 µL sample, and 14.75 µL dH2O. The 515F/806R[6] primers had adapter overhangs added to allowing for Illumina indexing. The thermocycler program was as follows: 96℃ for 5 min, 25 cycles of 96℃ 30 sec, 58℃ 30 sec, 72℃ 45 sec, and a final extension of 72℃ for 10 min. Electrophoresis was performed in order to measure the success of PCR using a 1% agarose gel at 100 volts for 30 min. Results were transilluminated by UV light to confirm presence of bands. Following the two stage PCR 16S rRNA gene metagenomics protocol outline by Illumina, the samples were barcoded, pooled in an eqimolar ratio, and sequenced on the Illumina MiSeq platform.

2.9 Disc Diffusion Assays

To test the efficacy of microbes deemed active in the competition assay against potential human pathogens, disc diffusion assays were run against *E. coli*. Samples were tested from the two weed species collected, as well as those of the five physiographic regions. Growth plates were prepared by infusing molten dilute nutrient agar with 1e6 cells/mL of either *E. coli*. Cell concentrations were standardized by enumeration of liquid culture concentration using a hemocytometer. 10 mL of infused molten media were poured into a sterile petri dish and allowed to set and cool overnight.

Each type isolate was placed in 5 mL of nutrient broth and left to grow for at least 72 hours. A micropipette was used to take 1 mL of each isolate-broth mixture to place in collection tubes. These isolates were centrifuged for 30 seconds. 20 µL of cell-free supernatant was placed on each sterile filter paper disc. A control disc with 10 µg ampicillin was placed on the same infused nutrient agar plate. Each supernatant disc was placed onto five spots on the growth

plate, separated by 1.5 cm or less.

3. Results

In regards to the North Carolina native weed species, there were three isolates from each part of the plant: endosphere, rhizosphere, and phyllosphere. There were a total of 12 types of bacteria and 12 types of fungi. *T. repens* produced the highest degree of inhibition against *E. coli* with 33.3% of its symbiotic bacteria and fungi displaying inhibition. No microbes from *P. major* were able to inhibit *E. coli*. When competing *T. repens* and *P. major* microbe isolates against one another, approximately 60.38% of all isolates participated in microbial warfare, suggesting that each isolate has withstanding chemical and biological properties rendering them necessary for their respective plant to compete. At the 48-72 hour, 144 hour, and 192 hour windows, the zone of inhibition either grew smaller or was sustained (Figures 1-3).

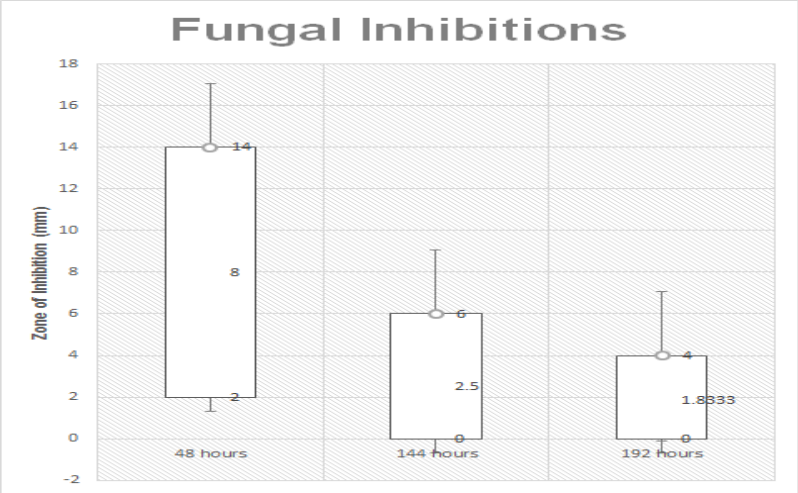

Figure 1. The chart above depicts the minimum, maximum, average, and deviations of each respective time interval of the isolated fungal competitions from North Carolina weeds.

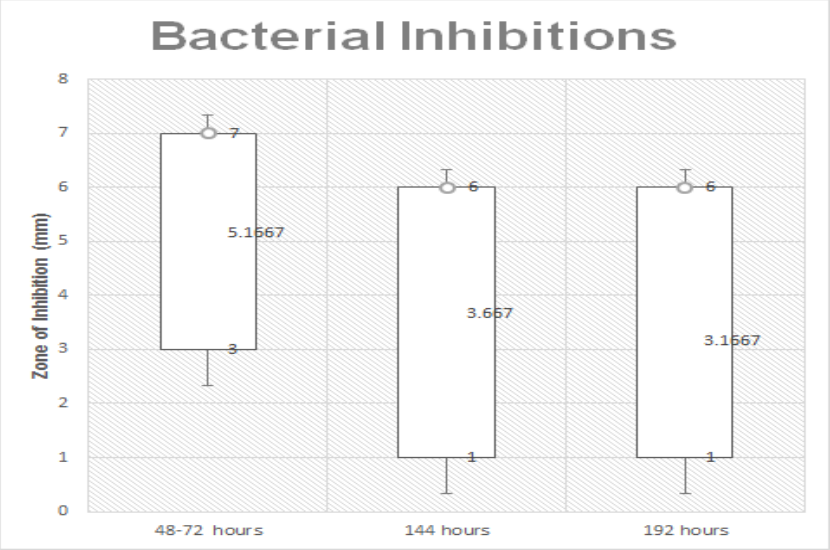

Figure 2. The chart above depicts the minimum, maximum, average, and deviations of each respective time interval of the isolated bacterial competitions from North Carolina weeds..

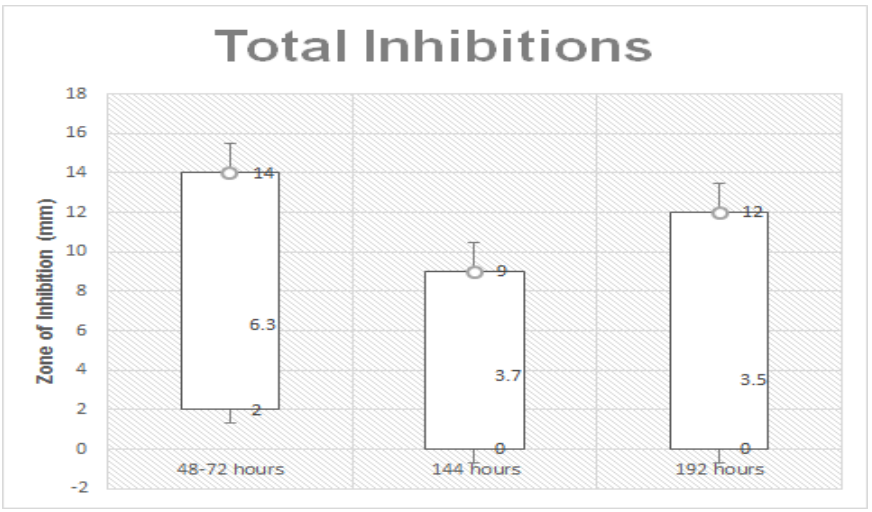

Figure 3. The above chart depicts the minimum, average, maximum, and standard deviations of each respective time interval of all fungal and bacterial competitions of North Carolina weeds.

For the regional soil types, 35.3% of Mountain, 85% of Piedmont, 71.4% of Upper Coastal, 80% of Lower Coastal, and 33.3% of Sandhills microbes showed zones of inhibition. The Mountain region exhibited the highest degrees of inhibition, while the Sandhills region exhibited the lowest degrees (Figure 4). The Mountain and Lower Coastal regions also displayed the largest amount of microbial diversity including both bacterial and fungal isolates. The most types were isolated from the Mountain and Lower Coastal regions, as we were able to see large amounts of variety in the morphology in the colonies after plating the original sample. There were a total of 16 Mountain isolates, 8 Piedmont isolates, 6 Upper Coastal isolates, 9 Lower Coastal isolates, and 8 Sandhill isolates.

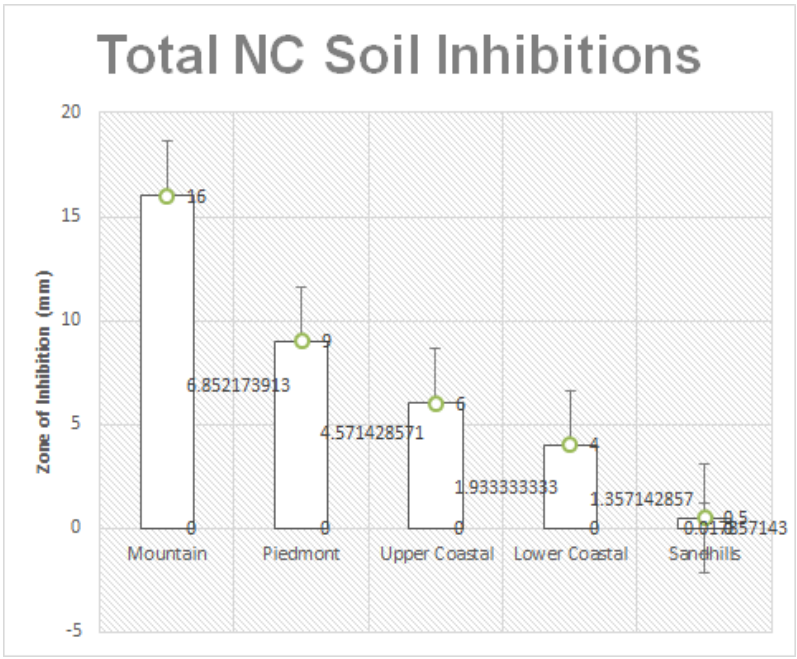

Figure 4. The above graphic shows the minimum, maximum, average, and standard deviations of the inhibitions of four of the five studied NC soil regions.

Bacterial diversity as revealed through 16S rRNA gene sequencing indicated similar diversity patterns to those found

in our isolation type counts for the Mountain region relative to the other four physiographic regions. However unlike isolations, the Upper Coastal region was reported as being higher in bacterial diversity than the Lower Coastal based on next generation sequencing results (Figure 5). This diversity was calculated using the Chao1 diversity estimator, which uses a correction factor for datasets skewed towards low abundance species[7]. The correction is based on the likelihood of resampling members of the same species from within the dataset. Using the Bray-Curtis dissimilarity metric to compare overall bacterial community structure between samples, a principal coordinate analysis represents each sample in an ordination plot (Figure 6). Here, a single dot represents the bacterial diversity of one sample. The closer together two dots, the more similar the bacterial assemblage of those samples are to each other[8]. Overall, the distance between samples within a given region indicates the heterogeneity of soil even within a region. The most intra-region variability is present in the Mountain and Upper Coastal regions, which also have the highest overall diversity.

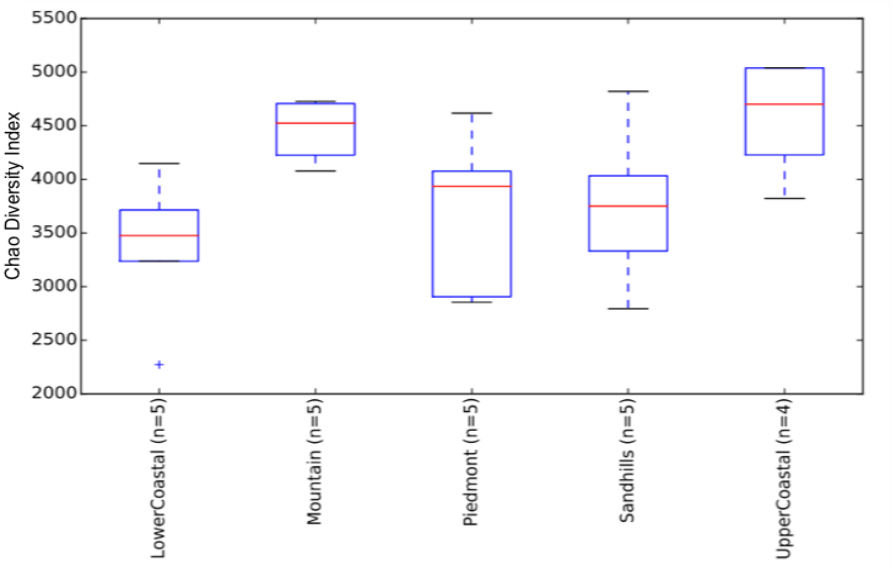

Figure 5. The above plot shows the Chao diversity of each of the North Carolina regional soil communities.

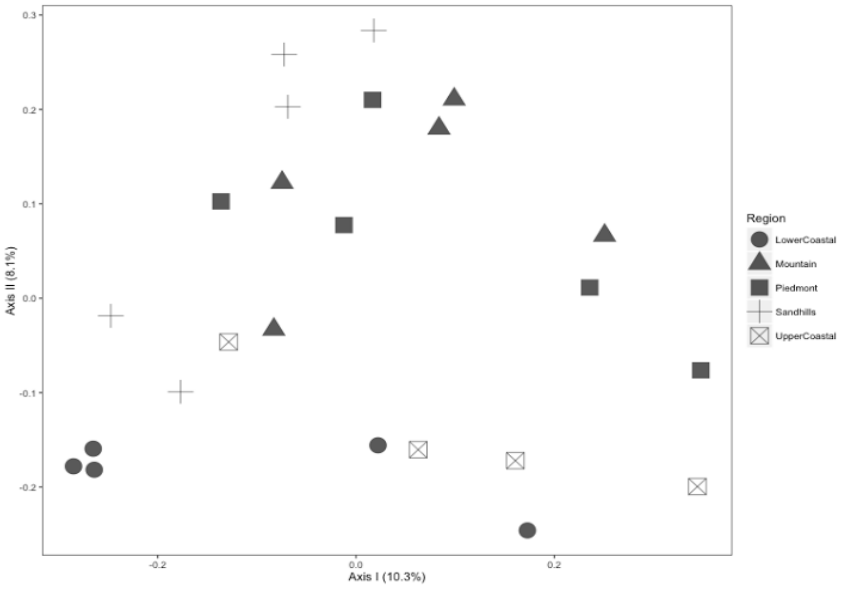

Figure 6. The above image shows the PCoA plot representing the bacterial communities of each North Carolina soil field site.

4. Discussion

The effectiveness of soil microbes in antibiotic production has long been studied. The goal of this experiment came in two parts. First, the scientists involved sought to determine the microbiome of *P. major* and *T. repens* in order to establish if their success depended on symbiotic microbial interactions. Second, the scientists wanted to explore whether differences between soil in several physiographic regions made a strong impact on microbial diversity and competitiveness.

From the results, we show the effectiveness of bacterial and fungal isolates of *P. major*, *T. repens*, and NC regional soils not only against one another, but, for *T. repens*, against *E. coli* as well. Previous studies support these results and the patterns found. In a study done by Veltech Technical University, the researchers described weeds as "disturbance opportunists" for their tendencies to exploit environmental disturbances. Weeds with backgrounds as anti-inflammatories and anti-microbials were examined under various environments and found to be largely successful in contrasting microbial cultures[9]. Other studies have explored communities where farmers and road personnel kill weeds before knowing their antimicrobial properties. These properties include uses as diuretics, antimicrobials, and anti-inflammatories. The missed medical and agricultural usage of these weeds possibly lead to negative impacts[10 11]. These findings suggest more exploration is needed in the microbes of weeds and their respective anti-microbial properties, particularly weeds of varying climates, soils, and populations.

Human errors that might have occurred include mis-labeling of cultures, contamination, incorrect measurements during competition assays, inconsistent data analysis, and misread data.

As for the NC regional soil isolations, there is a correlation between the soil region and bacterial assemblage. It seems there is a link between the amount of sand in the soil and changes in edaphic factors. Bacteria are more diverse in the Blue Ridge Mountain region and Upper Coastal Plain regions, which had the most similar pH (4.9 and 4.8) and these bacteria showed higher zones of inhibition. The pH in these soils were higher than that of the other regions. These results support the idea that pH has the greatest effect on soil bacterial community diversity[12]. Further, the similarity of diversity comparisons between regions using both culture-dependent and culture-independent methods shows that while next generation sequencing is useful in identifying unculturable bacteria, this technique is not necessary when targeting relative diversity comparisons.

The scientists involved in the study intend to produce a plant probiotic based on these findings that could aid in the growth of common North Carolina crops, such as tobacco and corn. The microbes will be isolated from plants growing in extreme environments, such as sidewalk cracks and sand dunes, in order to cultivate a more potent microbial selection. These plans could lead to an increase in stem and leaf growth, produce production, and stress tolerance[13].

These findings have both medical and agricultural implications, as these microbes could be used for both antibiotic and fertilizer development. The same properties, chemicals, and metabolites that make weeds such as *P. major* and *T. repens* competitive in new environments could prove to be beneficial in the fight against both human and plant pathogens. With bacteria becoming increasingly resistant to modern types of antibiotics, finding new antimicrobial metabolites is of paramount importance[14].The results from this study could one day contribute to explorations into various forms of antibiotics based on the virulence mechanisms used by common weeds.

5. Acknowledgements

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