Exploring the Rhizosphere Microbiome of Hydroponically Grown Leafy Greens

Madeline Taylor[a], Amber Newman[a], Relena Ribbons[a]

a. Geosciences, Lawrence University, Appleton, WI

Abstract

Microgreens are immature leafy vegetables that are popular for their short growing times and versatile growing conditions. Classifying the microbial communities for different microgreens can help us understand pathways to prevent diseases and promote plant growth; however, the rhizosphere microbiome of leafy greens is not well classified. We aimed to: 1) optimize hydroponic manifold assembly to support the growth of microgreen monocultures 2) design and optimize the process for harvesting rhizospheric film for DNA analysis and 3) determine the rhizosphere microbiome composition of four microgreens – Swiss chard, lettuce, kale, and basil. We used *16S* rRNA and *ITS* genetic markers to quantify bacterial and fungal abundance for our four microgreens. We engineered a single-level manifold holding 36 seedlings with its own growth light and water reservoir as an ideal hydroponics set-up. We developed, refined, and optimized a root scraping procedure to maximize the amount of rhizospheric film obtained from plant roots to consistently extract viable DNA. Future work should classify and explore the mechanistic role of rhizosphere microbiome in promoting plant growth.

Introduction

The rhizosphere, or the area of substrate adjacent to plant roots, is home to a diverse collection of microbiota that can increase a plant's nutrient uptake and pest resistance (Copant et al., 2010; Berendsen et al., 2012) (Fig. 1). Microbiota include microorganisms like bacteria and fungi that live on or around the plant roots. Plant Growth Promoting Microorganisms (PGPMs) are a subset of the microbiome: bacteria and fungi that benefit the plant in some way. PGPMs are a growing research field because they offer an environmentally friendly alternative to chemical fertilizer when identified and added to a plant's substrate. Through root exudates (i.e sugars, gases, and other rhizodeposits), water, and nutrient uptake, plants shape their own unique rhizosphere community compositions (Hinsinger et al., 2009).

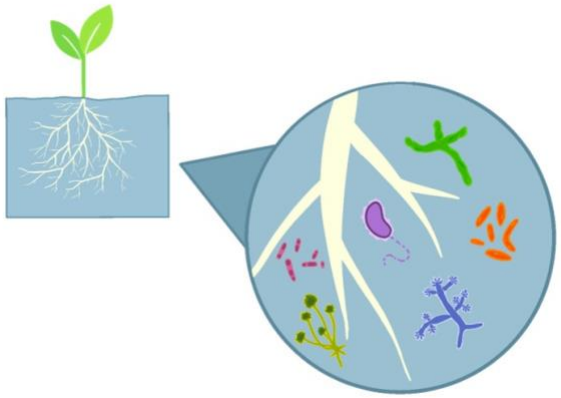

Figure 1. Schematic drawing of buttercrunch lettuce rhizosphere in water containing bacteria and fungi.

Funding and resources provided by the Wisconsin Space Grant Consortium and Lawrence University

Despite the significance of the rhizosphere to plant health, many plant species lack classification of their rhizosphere microbiome compositions. Microgreens – among the unclassified groups – are nutritious and fast-growing leafy greens that have become more desirable agricultural crops. Microgreens grow for one to four weeks or until the first set of true leaves develop, and they reach about two to three inches tall. Microgreens are commonly grown in hydroponic systems.

Hydroponic systems are water-based (soilless) systems where plants grow only in water and a nutrient addition that circulates and recycles through a reservoir system. Growing microgreens in hydroponic systems can increase space efficiency, plant yield, and biomass of crops, and perhaps decrease rates of disease and pest infestations, eliminate the need for soil, allow for year-round production, and be cultivated in a microclimate with controlled conditions (Sharma et al., 2018). Hydroponic systems range in size from tabletop versions, like the popular household AeroGarden® brand, to large-scale commercial operations like VivoSun® or CropKing® and can grow a multitude of harvestable produce.

In this study, we have chosen to use a hydroponic drip system to grow four species of microgreens: 'Buttercrunch' lettuce (*Lactuca sativa* var. *capitata*), 'Red Russian' kale (*Brassica oleracea* var. *sabellica*), Swiss chard (*Beta vulgaris* L. var. *cicla*), and 'Genovese' basil (*Ocimum basilicum* L. cv. Genovese). A previous study by Srichamnog et al. (2020) investigating the effectiveness of antibiotics on rhizobia of butterhead lettuce revealed some dominant bacterial phyla: Proteobacteria, Bacteroidetes, Pseudomonadota, Acidobacteria, and Gammaproteobacteria. Another study investigating endophytic fungi diversity of kale revealed the dominant fungal phyla to be Ascomycota (Poveda et al., 2020). Although these studies provide a rough estimate of microbiome compositions, quantities and general microbiome classifications are not provided—microbiota were identified for those specific contexts, rather than in an effort to classify and characterize rhizobiomes as a whole.

Study Objectives. The first objective for our study is to investigate the complete rhizosphere microbiome for each of our four microgreens. The standard procedure for isolating rhizospheric DNA from hydroponically grown plants includes harvesting root material and substrate growing medium like rockwool (Vargas et al., 2021). In an effort to reduce complexity and cost of this procedure, our second objective is to determine the effectiveness of scraping the roots of microgreens in isolating rhizospheric DNA. Our third objective is to determine the most effective hydroponic assembly for microgreen growth within the confines of using both a drip system and artificial lighting.

Materials and Methods

Focal species of microgreens. We chose to study Swiss chard (*Beta vulgaris subsp. cicla*), 'Buttercrunch' lettuce (*Lactuca sativa*), 'Red Russian' kale (*Brassica oleracea*), and 'Genovese' basil (*Basilicum ocimum*) (Fig. 2). These microgreens were selected because they are common edible plants, they germinate and grow quickly, and the seeds are readily available to purchase and ship. The seeds were planted in rockwool trays with 1-3 seeds per rockwool cube and covered with a plastic ventilating lid to increase humidity in the tray (Soligt). The trays were put in the Lawrence University greenhouse and watered every other day for 2 weeks. After germinating in the greenhouse, the seeds were moved into the lab space and transferred into a hydroponic manifold set-up. When transferred to the hydroponic manifold, one teaspoon of

liquid FloraGro nutrient mixture was added to each monoculture reservoir, and two teaspoons were added to the Mixed Greens reservoir. We monitored the pH levels of the reservoirs using a BlueLab pH pen. Once the plants were large enough to obtain 0.250 grams of root scraping material, the plants were harvested and used for DNA analysis. All the plants were measured for aboveground plant height by using a standard ruler and measuring from the base of the plant at the top of the rockwool cube to the longest leaf. The plants were measured for aboveground fresh weight by clipping the base of the plant above the rockwool and placing the stem and leaves on a balance scale. Between plant rounds, the manifolds were washed and bleached to ensure that microbial DNA would not be carried over to a different species.

Figure 2. The four microgreen species studied in the hydroponic system in the lab at Lawrence University.

Hydroponic set-up and engineering. To start our project, we needed to build a hydroponic system that could be used in our indoor, windowless lab space. We wanted a system to grow both monocultures (one species of plant isolated in the set-up) and mixed greens (multiple species of plants sharing the same water) to compare the microbiome compositions. From previous experiments, Lawrence University had four VivoSun® 3-layer Hydroponic Kits in storage (Fig. 3A). We connected two of the full-sized VivoSun® manifolds so that they shared the same water reservoir with a VivoSun® 24-watt Submersible Water Pump for our Mixed Greens Manifold. Finding an ideal set-up for monocultures was a trial-and-error process that involved moving the light stands, buying new lights, buying new pumps, and buying new PVC parts. We landed on a 1-layer Mini Manifold disassembled from the VivoSun® 3-layer Hydroponic Kit connected to a Pulaco 5-watt Submersible Water Pump as the best assembly for growing monocultures (Fig. 3B).

From our remaining two VivoSun® kits, we created four Mini Manifolds for growing monocultures. We used a rockwool growth medium (Soligt) which is an inert material spun out of basaltic rock into a wool-like texture, and comes in compacted cube shape to hold our seeds in place in the PVC pipe system. We fitted hanging LED Veg grow lights (Exlenvce) onto standard garment racks (Amazon) 2-2.5 feet above each of the four Mini-manifolds and one above the Mixed Greens Manifold. The lights run 24 hours/day on a blue-red-white light combination. Each Mini Manifold holds 36 plants and the Mixed Greens Manifold holds 216 plants.

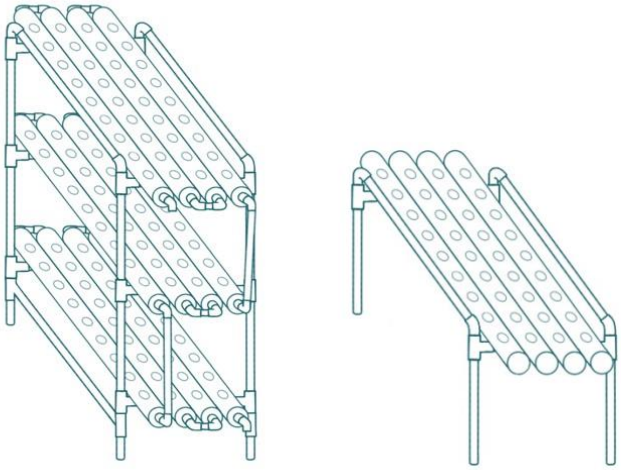

Figure 3. A (left). Schematic drawing of the 3-tier VivoSun® hydroponic manifold and B (right). Schematic drawing of the 1-tier hydroponic Mini Manifold.

Root scraping procedure. We aimed to develop a root scraping procedure that would allow us to maximize the amount of bacterial and fungal DNA in our samples while minimizing the amount of plant DNA. The methods from existing literature on plant rhizospheres included putting the roots straight into DNA isolation tubes or putting the rockwool medium into DNA isolation tubes (Vargas et al., 2021). We observed extremely low DNA concentrations with both of these methods. We wanted to target the thin rhizospheric film coating the plant roots where the number of microorganisms might be most concentrated. We started by cutting the roots with sterilized scissors directly below the rockwool cube and spreading the roots on a sterilized flat petri dish (Fig. 5). We used a sterilized scalpel to gently scrape the roots and collect the rhizospheric film near the edge of the petri dish. We weighed the film to get 0.250g for DNA isolations and disposed of the dry root material in a waste bucket.

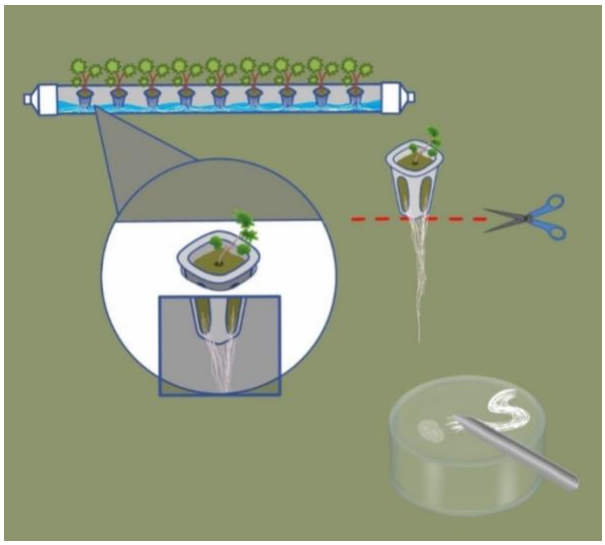

Figure 5. Schematic drawing of the root scraping procedure.

DNA isolation and PCR. We used a Zymo Research Quick-DNA™ Fungal/Bacterial Miniprep Kit (California, USA) and followed the instructions as written with minor modifications as described below to isolated DNA from the rhizospheres of hydroponic crops. Before bead-beating, 0.250g of fresh root scraping material was added to the bead-beating tubes. We used the BeadBlaster ™ 24 at 4000rpm for 3x 1:30 min cycles. DNA extracts were quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE), and stored at -20°C until qPCR amplifications. We checked for Nucleic Acid Concentrations within the range of 5-100 ng/µl to assess viable DNA samples for downstream applications. For both the monocultures and the Mixed Greens setting, three plants from each hydroponic set-up were sampled for DNA isolation (n=3). If a DNA sample yielded unviable results, another plant was sampled to ensure an even sample size for each treatment. DNA isolations took place on the same day as plant harvesting, so root material was fresh when extracted. If there was additional root scraping material left after 0.250g were used for DNA isolation, the material was frozen for later use, and stored at -20°C.

DNA was subsequently used for quantitative polymerase chain reactions (qPCRs) to quantify gene copy numbers of two focal gene markers: bacterial *16S* and fungal *ITS*. All qPCRs were run in duplicate on a BioRad CFX 96 Opus machine (BioRad, USA) machine with 20 µl reactions consisting of: 10.0 µl of SYBRGreen (2X) PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA), 0.25 µl each of forward and reverse primers, 1 µl of DNA template, and 8.5 µl of nuclease-free water. *16S* rRNA genes, which indicate bacterial and archaeal abundances, were amplified using Eub338 and Eub518 primers diluted to a working stock of 1:10 (Table 1). qPCR conditions for the *16S marker* were 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 53 °C for 30 seconds, and 72 °C for 20 seconds, with a standard curve comprised of ten-fold serial dilutions which ranged from 10^2 to 10^7 gene copies. Fungal:bacterial ratios were calculated using these ratios of log gene copies. Fungal *ITS* gene abundances were amplified using BITS and B58S3 primers (Table 1) diluted to a working stock of 1:10. qPCR conditions for fungal *ITS* were 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 20 seconds. Standard curves for fungal *ITS* were constructed using ten-fold serial dilutions which ranged from 10^3 to 10^9 gene copies.

Table 1: Primers, primer sequences, and references for qPCR reactions.

Primer target	*Primers*	*Primer Sequence (5'-3')*	*Reference*
All Bacteria (*16S* rRNA)	Eub338 Eub518	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	Fierer et al. (2005)
All Fungi (*ITS*)	BITS B58S3	ACCTGCGGARGGATCA GAGATCCRTTGYTRAAAGTT	Bokulich and Mills (2013)

Results

Plant growth. Over the course of the study, we grew three rounds of Swiss chard, two rounds of lettuce, two rounds of basil, and one round of kale. Swiss chard Round 1 grew in the Mixed Greens manifold with lettuce; Round 2 grew in Mini Manifold 3 as a monoculture; Round

3 grew in Mini Manifold 2 as a monoculture. Lettuce Round 1 grew in Mini Manifold 1 as a monoculture, and Round 2 grew in the Mixed Greens manifold with Swiss chard. Basil Round 1 grew in Mini Manifold 2 as a monoculture, and Round 2 grew in Mini Manifold 4 as a monoculture. Kale Round 1 grew in Mini Manifold 4 as a monoculture. Each round for the different species of microgreens lasted for a different number of growing days because the plants could not be harvested until 0.250g of root scraping material could be collected off their roots. For the monocultures: Lettuce Round 1 - harvested on Day 36; Kale Round 1– harvested on Day 38; Swiss Chard Round 2 - harvested on Day 42; and Basil Round 1 – harvested on Day 58. Between the lettuce and the basil was a 22-day difference in growing time. The basil plants had a large mass of roots early in their growing period, but the roots were noticeably drier than those of other plants and had less rhizospheric film, so they had to grow longer to achieve the target 0.250g amount of material for root scraping. The Mixed Greens manifold containing Swiss Chard Round 1 and Lettuce Round 2 was harvested on Day 24.

New methods. Our developed root scraping procedure was successful. After switching to our new root scraping method, our DNA nucleic acid concentrations increased to viable levels between 10 and 100 ng/µl where they had been <10 ng/µl when using the rockwool material. We also saw success with our manifold set-ups. The three main concerns when experimenting with our manifolds set-ups were 1) finding a set-up to provide equal, strong light coverage to all plants 2) isolating plants by species so that the rhizosphere microbial communities would be unique and 3) creating a set-up that could be easily assembled, dismantled, and moved around the lab. We had initial struggles in all three areas: plants on the top tier of the Mixed Greens set-up received more light than lower tiers, we did not have a submersible water pump with a wattage strength that matched the height of our manifolds, and the PVC pipes on the manifold structure would come apart and dump the water in the system. The unequal light distribution manifested in plants with yellow leaves, leggy stems, and small leaf area. By creating the Mini-Manifold set-ups we were able to hang lights above a single tier and get adequate and equal light coverage to all plants. The Mini-Manifold also allowed for monoculture growth where the rhizosphere microbial communities were separated by plant species.

Aboveground biomass. Each of the 36 plants harvested from a monoculture were weighed to quantify total aboveground biomass (n=36). Basil had the largest average mass (1.642g), followed by lettuce (0.902g), Swiss chard (0.582g), and kale (0.397g) (Fig. 6A). A sample of the plants in the Mixed Greens manifold containing Swiss Chard Round 1 and Lettuce Round 2 were also weighed for aboveground biomass. The Mixed Greens manifold contained 35 plant units – 3 plant units were exclusively Swiss chard, 3 plants units were exclusively lettuce, and 29 plant units were Swiss chard and lettuce growing together in the same rockwool cube. The Swiss chard plants (n=3) had an average mass of 0.462g, the lettuce plants (n=3) had an average mass of 0.346g, and the Swiss chard/lettuce plant units (n=29) had an average mass of 0.725g (Fig. 6B).

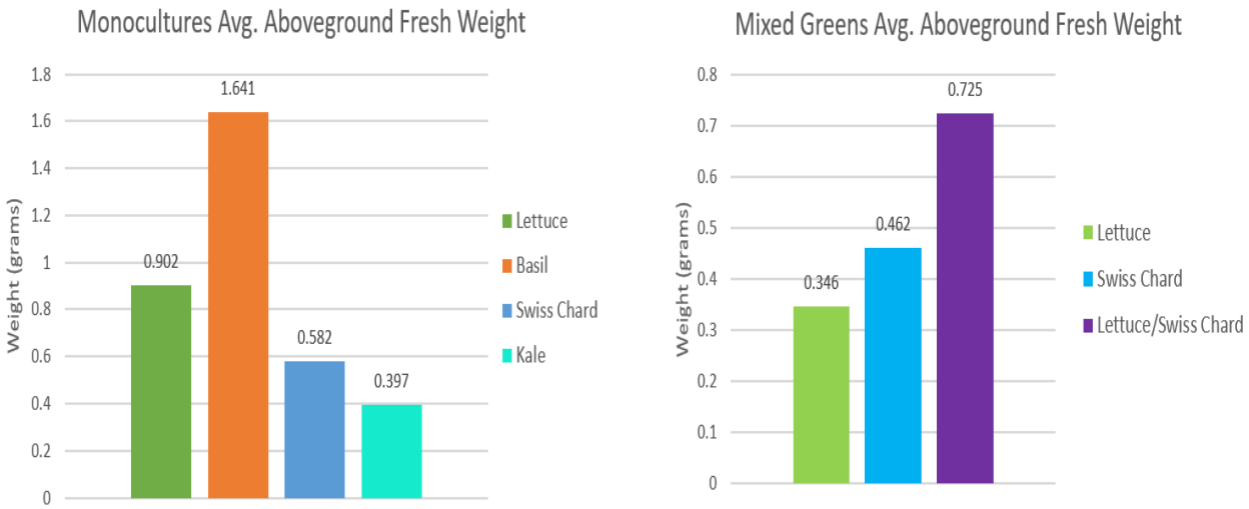

Figure 6: Mean Aboveground Fresh Weight of Lettuce, Basil, Swiss Chard, and Kale grown in monocultures (A, left). Mean Aboveground Fresh Weight of Lettuce, Swiss Chard, and Lettuce/Swiss Chard grown in the Mixed Greens manifold B (right).

Preliminary qPCR findings: archaeal and bacterial *16S* and fungal *ITS* abundance. Lettuce and chard had the greatest log gene copies of bacterial and archaeal *16S*, with 9.14 and 9.09 respectively, followed by kale with 7.42 and basil with 6.08 (Figure 7 A). Kale had the greatest abundance of log fungal *ITS* gene copies with 7.61, followed by chard with 6.92, basil with 6.75, and lettuce with 6.12 (Figure 7 B). We found a great deal of variability in both the lettuce and chard *16S* results, with less variability in kale, and the least variability for basil rhizospheres. For fungal *ITS*, we observed a more equal spread of variation about mean values for all crops, with chard encompassing the most, followed by kale, basil, and lettuce. Final values for qPCR runs will only include monoculture crops, and might reduce the variability expressed in these figures.

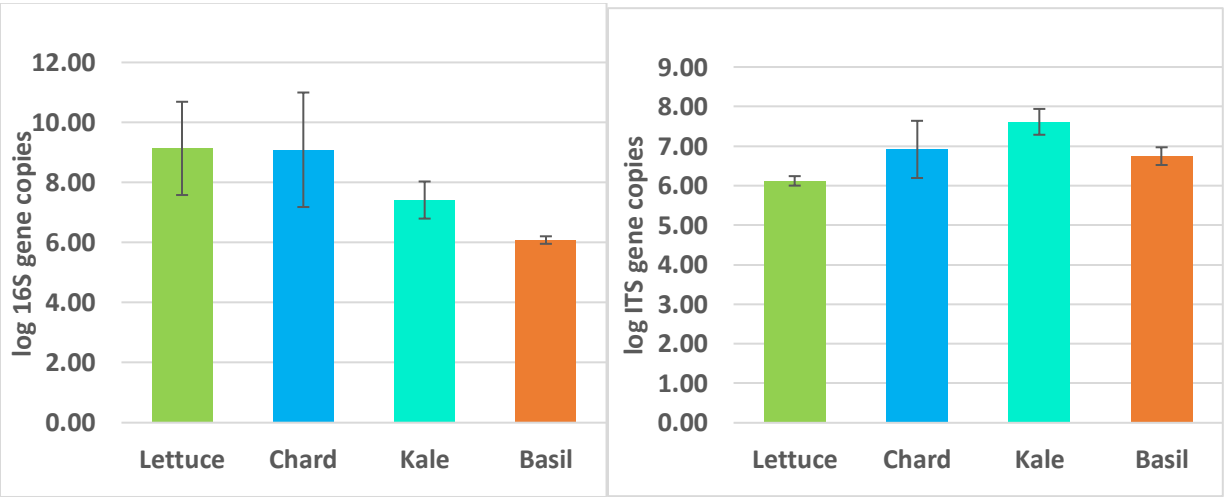

Figure 7: Mean log gene copies for archaeal and bacterial rhizospheric 16S (A, left) and fungal ITS (B, right).

Conclusions

Our research is just scratching the surface of microgreen microbiome research. There has been relatively little information published on microgreen rhizospheres and only a few lettuce varieties have taxonomic information on their bacterial and fungal abundances and community compositions. Engineering an ideal manifold set-up and developing a root scraping procedure enabled us to gain insight into how microgreen research should be conducted. The root scraping procedure, while simple, provided better results than any of the existing methods in published literature that we tried. We isolated DNA from four microgreen monocultures that, once sequenced, will provide novel bacterial and fungal taxonomic information. The success of our initial engineering solutions in this stage of the project will allow us to continue hydroponic microgreen work at a more efficient pace in the future.

Commercial hydroponic systems sold for individual household consumption of microgreens are becoming more popular. Investigating the rhizosphere microbial communities of plants grown in specific commercial systems is a future step in hydroponic and microgreen research. Our lab is continuing microgreen microbiome work with the Lyko™ Aerogarden, a small 9 plant set-up targeted at the at-home gardener. We have preliminary microbiome PCR data from 'Buttercrunch' lettuce grown as a monoculture in the Lyko™ Aerogarden. Our lab is also continuing microgreen microbiome work with the larger ForkFarms™ hydroponic system – a 266 plant vertical growing tower marketed for both at-home and industry use. Knowledge of the microbiomes of plants in specific commercial set-ups can help create PGPMs and other nutrient add-in solutions that benefit plant growth and offer a safer alternative to chemical fertilizers.

Acknowledgements

We would like to thank the National Aeronautics and Space Administration, Wisconsin Space Grant Consortium, and Lawrence University for providing us with funding for our project. This material is based upon work supported by NASA under Award No. RIP22_3-0 issued through Wisconsin Space Grant Consortium. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Aeronautics and Space Administration. Thank you to Ariela Rosa for grant support. Thank you to Eric Lewellyn, Daniel Martin, Meghan Raebel for technical assistance and support.

References

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. Doi: 10.1016/j.tplants.2012.04.001

Bokulich, N. A., & Mills, D. A. (2013). Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Applied and environmental microbiology*, *79*(8), 2519-2526.

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for

utilization. *Soil Biol. Biochem.* 42, 669–678. Doi: 10.1016/j.soilbio.2009.11.024

Fierer, N., Jackson, J. A., Vilgalys, R., & Jackson, R. B. (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Applied and environmental microbiology*, *71*(7), 4117-4120.

Hinsinger, P., Bengough, A. G., Vetterlein, D., and Young, I. (2009). Rhizosphere: biophysics, biogeochemistry and ecological relevance. *Plant Soil* 321, 117–152. Doi: 10.1007/s11104-008 9885-9

Sharma, N., Acharya, S., Kumar, K., Singh, N, and Chaurasia, O. P. (2018). Hydroponics as an advanced technique for vegetable production: An overview. *Journal of Soil and Water Conservation.* 17(4), 364-371. Doi: 10.5958/2455-7145.2018.00056.5

Srichamnog, W., Kalambaheti, N., Woskie, S., Kongtip, P., Sirivarasai, J., Matthews, K. R. (2020). Occurrence of antibiotic-resistant bacteria on hydroponically grown butterhead lettuce (*Lactuca sativa car. Capitata*). *Food Science & Nutrition* 9, 1460-1470. Doi: 10.1002/fsn3.2116.

Vargas, P., Bosmans, L., Van Calenberge, B., Van Kerckhove, S., Lievens, B., Rediers, H. (2021). Bacterial community dynamics of tomato hydroponic greenhouses infested with hairy root disease. *FEMS Microbiology Ecology*, 97(12), 1-10.

Poveda, J., Zabalgogeazcoa, I., Soengas, P., Rodríguez, V. M., Cartea, M. E., Abilleira, R., & Velasco, P. (2020). Brassica oleracea var. acephala (kale) improvement by biological activity of root endophytic fungi. *Scientific Reports*, *10*(1), 1-12.