# THE EFFECTS OF MICROBIOMES ON FOOD CROP YIELD AND QUALITY IN AQUAPONIC SYSTEMS

by

**Yi-Ju Wang**

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# THE PURDUE UNIVERSITY GRADUATE SCHOOL
# STATEMENT OF COMMITTEE APPROVAL

**Dr. Hye-Ji Kim, Chair**

Department of Horticulture and Landscape Architecture

**Dr. Lori A Hoagland**

Department of Horticulture and Landscape Architecture

**Dr. Amanda J Deering**

Department of Food Sciences

**Dr. Zhi (George) Zhou**

Department of Civil Engineering and Environmental and Ecological Engineering

**Approved by:**

Dr. Linda S; Prokopy

*Dedicated to my beloved family and all my friends*

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# ABSTRACT

Facing challenges for increasing demands for agricultural land, water, and energy, aquaponics has emerged as a sustainable solution that can contribute to global food production while minimizing environmental impacts. In a recirculating aquaponic system, the waste produced by aquatic animals is processed through microbes and breaks down into compounds for plant uptake. By recycling nutrients and water between hydroponics and aquaculture systems, aquaponics can reduce the waste of fish feeds and the use of chemical fertilizers and use 90-99% less water than conventional aquaculture. However, a few studies reported that nutrient use efficiency is still low in aquaponics, and only 10-37% and 20-30% of nitrogen (N) is typically assimilated by plants and fish, respectively. Yield reduction is commonly reported for plants in aquaponics. Due to the unique water physical and chemical environment, the microbiomes are more diverse in aquaponics than in hydroponics. While the most important microbial group is considered nitrifying bacteria, *Nitrosomonas* spp. and *Nitrobacter* spp. mediating the N conversion process from ammonia into nitrate, some plant growth-promoting bacteria (PGPB) in soils were found in aquaponics indicating their important function in the system. Meanwhile, the use of aquaculture wastewater can introduce and promote the growth of harmful microbial pathogens, posing a food safety concern.

The goal of this research is to investigate the effects of microbiomes in aquaponic systems. A series of studies were conducted to examine the effects of different bacterial groups on food crop yield and quality and investigate the potential risk of contamination with enteric pathogens in aquaponic systems. The specific objectives are: to 1) examine whether enteric pathogens present in aquaponics and hydroponics; 2) investigate the effects of plant age and root damage on internalization of STEC *E. coli* in leafy vegetables and herbs. 3) examine the effects of pH on the plant yield in aquaponics; and 4) investigate the effects of PGPB on lettuce in aquaponics and hydroponics3. The data obtained from this research will fill the knowledge gap and provide new management strategies for cultivating crops in aquaponics, which will greatly promote the application of aquaponics to provide a solution for the increasing food demands in the future.

# CHAPTER 1. INTRODUCTION

Facing the challenge of food demand and sustainable agricultural practices, aquaponics has emerged as an important concept of global food production. Therefore, it is necessary to adjust a suitable environment for three organisms to increase yield and quality in aquaponics. In aquaponics, most fish species only absorb 20-30% of nitrogen in the diet, but release the rest 70-80% as waste into water, which could be potential nutrient for crop growth (Yang et al., 2018; Wongkiew et al., 2017a). Within these systems, the waste produced by aquatic organisms is filtered through tanks of microbes, which convert ammonia ($NH_4^+$) to nitrite ($NO_3^-$) for plants uptake (Wongkiew et al., 2017a, 2018). Plants can absorb nitrogen either in the nitrate or ammonium form, but high concentration of $NH_4^+$ will be toxic and reduce plant yields (Britto and Kronzucker, 2002). Beside of nitrogen, Phosphorus (P), Potassium (K), and other micronutrient nutrient, also affect plant yields.

The major source of P in aquaponics is from fish feeds. Fish only use 15% of the phosphorus in fish feeds, and plants have different availability to absorb phosphorous from recycling aquaculture wastewater based on different aquaponics designs (Schmautz et al.; Rafiee and Saad, 2005). The available phosphorus for plants depends on the pH (Cerozi and Fitzsimmons, 2017). Phosphorus turn into insoluble complexes under high pH environment and 30–65% of the phosphorus are remained in solid fish sludge, which is unavailable to plants. Fish feeds also contain K and other micronutrients, but the amount of K and other micronutrients is limited since fish have minimal requirements of many metal ions (Fe, Mn, Mg, Cu), and lower requirements for K (Rakocy, 2012; Villarroel et al., 2011; Seawright et al., 1998). Therefore, some aquaponic systems apply synthetic salts into the system water or apply a foliar spray to prevent the deficiency in K, Cu, Ca, Mg, Mn and Fe (Rakocy, 2012; Roosta, 2014).

## 1.1 Microflora

The major nitrogen source in aquaponics is derived from the fish feed. Nitrifying bacteria breakdown fish wastes to nitrite ($NO_3^-$) via a two-step process known as nitrification. The main nutrient conversion process is the transformation of ammonia to nitrate by two different groups of nitrifying bacteria, *Nitrosomonas* spp. and *Nitrobacter* spp. (Wongkiew et al., 2017a, 2018).

Ammonia oxidizing bacteria (AOB) is one kind of *Nitrosomonas europaea* which was well studied in nitrification (Arp and Stein, 2003). Besides nitrifying bacteria, Schmautz et al. (2017) found that the largest phylum in aquaponic systems is *Proteobacteria*, to which many plant growth-promoting bacteria belong (Schmautz et al., 2017).

Schmautz et al (2017) investigated the deviation of microbiomes in aquaponics, and they found most nitrifying bacteria grew in the biofilter and nitrifying bacteria were not the largest group of bacteria in the aquaponic system (Schmautz et al., 2017). The largest phylum in aquaponic systems is *Proteobacteria*, which has many plant growth-promoting bacteria, such as *Pseudomonas* spp., *Acidovorax*, and *Sphingobium* (Schmautz et al., 2017). Also, *Firmicutes*, which contain *Bacillus* spp., were found in fish feces. *Bacillus subtilis*, which is used as PGPB in soil culture systems can be used as probiotics in aquaculture systems (Arkhipova et al., 2005; García-López et al., 2018; Mills et al., 2011; Fu et al., 2014; Idris et al., 2007). *B. subtilis* can increase the weight gain, survival rate, and nutrition usage in shrimp culture and also enhance tilapia immunity to against fish pathogens, such as *Streptococcus iniae*, *P. fluorescens*, and *Aeromonas hydrophila* (Zokaeifar et al., 2012; Aly et al., 2008). Bartelme et al. (2018) outlined that PGPB may play a major role in the plant's ability to uptake iron in an aquaponic system, because many different PGPB, like *P. fluorescens* Pf-5 and *Chryseobacterium* C138, can increase the bioavailability of iron through siderophore production in iron deficient plants(Radzki et al., 2013; Loper and Henkels, 1997; Bartelme et al., 2018)

Many studies show that PGPB or microflora can enhance plant performance in soil-cultured environment (Bartelme et al., 2018; Goddek et al., 2015), but there are only a few studies published on PGPB in aquaponic systems. Therefore, there is a great opportunity to investigate the effects of PGPB in aquaponic systems. For example, *Pseudomonas fluorescens* Pf-5, which is found in soil, can increase siderophore production in roots, and the siderophores can bind and facilitate iron to transport into plant roots. Therefore, *P. fluorescens* Pf-5 may be a potential PGPB in aquaponics to solve the issue of iron deficiency (Bartelme et al., 2018; Goddek et al., 2015).

## 1.2 Plant Performance

Although fruiting crops have higher value than leafy vegetables, fruiting crops have longer production periods and less marketable yield in aquaponics (Bailey and Ferrarezi, 2017). Flowering crops are more difficult to grow in aquaponic systems due to their heavy nutrient requirements of phosphorous and potassium (Rakocy, 2012). Thus, leafy vegetables have been the preferred crop to grow in aquaponics since they can grow well in nitrogen concentrated water, have a short production period, do not have high nutrient requirements, and are in high demand (Bailey and Ferrarezi, 2017). Commercial aquaponic growers most commonly grew basil (81%), salad greens (76%), non-basil herbs (73%), *Solanum lycopersicum* (tomatoes) (68%), *Lactuca sativa* (head lettuce) (68%), *Brassica oleracea* (kale) (56*%), Beta vulgaris subspecies cicla* (chard) (55%), pak choi (51%), *Capsicum annuum* (pepper) (48%), and *Cucumis sativus* (cucumbers) (45%) (Love et al., 2015).

Different plants have different requirements for growth. For example, Yang et al (2018) found that water use efficiency of cherry tomato, basil, and lettuce were related to thermal requirement of different plants (Yang et al., 2018). However, plant performance also showed the same trend in different aquaponics design (Yang and Kim, 2019a, 2020c). For example, Yang grew cabbage (*Brassica rapa*), lettuce (*Lactuca sativa*), mustard (*Brassica juncea*), chia (*Salvia hispanica*), basil (*Ocimum basilicum*) and Swiss chard (*Beta vulgaris*) with different flow rate in tilapia-based aquaponics, and she found that all of six different plant species had better performance in high loading rates (3.3 $m^3$ /$m^2$ /day) (Yang and Kim, 2020c).

## 1.3 Fish Species in Aquaponics

Many different aquatic organisms can be applied in aquaponics system. Love et al. (2015) investigated 257 commercial aquaponic farms and found that 69% used tilapia (*Oreochromis niloticus*), 43% used ornamental fish, and 25% used catfish (*Siluriformes*) (Love et al., 2015). Rakocy et al. (2006) also reported rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), barramundi (*Lates calcarifer*), Largemouth bass (*Micropterus salmoides*), pacu (*Piaractus mesopotamicus*), crappies (*Pomoxis*) and murray cod (*Maccullochella peelii*) were used in aquaponic systems (James E. Rakocy et al., 2006). Other species of aquatic organisms (i.e white leg shrimp (*Litopenaeus vannamei*), Amazon River shrimp (*Macrobrachium amazonicum*)) have

been suggested to work well in aquaponic systems (Lima et al., 2019; Castilho-Barros et al., 2018). The most commonly used and most successful fish species used in aquaponics is Nile tilapia, followed by Carp and African Catfish (Love et al., 2015). Additionally, Tilapia is ideal for reaching the nutrient demands of plants in aquaponics since tilapia can live in an environment with a wide range of pH, temperature, and dissolve oxygen (El-Sayed, A. F. M., 2006). Different combinations of plants and aquatic organisms can result in different yields of plants and fish. For example, Knaus and Palm (2017) compared the effect of different plant species, cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), and lettuce (*Lactuca sativa*), and fish, Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*), combinations. They found that the use of common carp effluent resulted in higher yields of cucumber over tilapia effluent, while tilapia effluent resulted in higher yields of tomatoes (Knaus and Palm, 2017). However, it is not clear why tomatoes grew better with tilapia effluent over carp effluent since very limited scientific research conducted to directly compare the different fish and plant species combinations, so the physiological interactions between them in an aquaponic system is still undiscovered.

## 1.4 Food Safety in Aquaponics

Fish feces can be a potential contamination source of human pathogens even though pathogens are not permanent flora and correlated with the level of environmental contamination because foodborne pathogens can be carried in fish intestines up to 7 days (Geldreich and Clarke, 1966; WHO, 1989; Pillay, 2004). Nutrient solutions are collected and reused in recirculating hydroponic systems (Kim et al., 2018a; Gómez et al., 2019a), which may be a source of contamination if not properly treated and managed. Additionally, contaminated seeds and/or irrigation water could potentially be the source of contamination (Deering et al., 2012a).

Food safety concerns related to aquaponics have emphasized the need for more research in food safety interventions such as UV-treatment (Pantanella et al., 2015), ozonation (Kim et al., 2003), and organic acids (Sirsat and Neal, 2013). However, careful handling of aquaponic systems can limit most of these potential concerns (Wang et al., 2020b). Indeed, Shiga toxin-producing E. coli was found in fish feces, the recirculating water, and on the plant root surfaces but was not found in lettuce leaves, basil leaves, and tomato fruits in greenhouse-based aquaponic systems

(Wang et al., 2020b). However, there is still lack of scientific reports on the risks of enteric pathogen transfer to plants in aquaponics and management strategies on aquaponic crop production.

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# CHAPTER 2. OCCURRENCE OF SHIGA TOXIN-PRODUCING *E. COLI* IN AQUAPONIC AND HYDROPONIC SYSTEMS

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**Yi-Ju Wang[1], Amanda J. Deering[2], and Hye-Ji Kim[1,3]**
[1]Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, United States
[2]Department of Food Science, Purdue University, West Lafayette, IN 47907, United States
[3]Corresponding author: hjikim@purdue.edu
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## 2.1 Abstract

Food safety concerns have been raised over vegetables and herbs grown in aquaponics and hydroponics due to the reuse of wastewater and spent nutrient solutions. This study was conducted to determine the occurrence of foodborne pathogens in greenhouse-based aquaponic and hydroponic systems. Fish feces, recirculating water, roots, and the edible portions of lettuce, basil, and tomato were collected at harvest and microbiological analyses were conducted for bacterial pathogens shiga toxin-producing *E. coli* (STEC), *Listeria monocytogenes*, and *Salmonella* spp. Enrichments and selective media were used for the isolation and the presumptive positive colonies were confirmed by PCR. STEC was found in fish feces, in the water of both systems, and on the surface of the roots of lettuce, basil, and tomato regardless of the system. However, the contaminated water did not lead to the internalization of STEC into the roots, leaves, and/or fruit of the plants. Meanwhile, *L. monocytogenes* and *Salmonella* spp. were not present in any samples examined. Our results demonstrate that there are potential food safety hazards for fresh produce grown in aquaponic and hydroponic production systems.

**Key words:** food safety, *E. coli*, agricultural water, PCR detection, indoor farming

## 2.2 Introduction

Food safety has become an important issue in fresh produce production worldwide due to many factors such as importation of fresh produce from various countries, potential sources of bacterial pathogens from the growing environment, and inappropriate domestic food preparation

(Redmond and Griffith, 2004; Tirado et al., 2010). Nearly 48% of foodborne outbreaks are linked to the consumption of fresh fruits and vegetables (Hoagland et al., 2018). The number of outbreaks associated with *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. is increasing in recent years (Deering et al., 2012b; Strawn et al., 2013) largely due to improved detection methods and traceback procedures following an outbreak (Abadias et al., 2012; Brashears and Durre, 1999; Walker et al., 1990). In the United States, the Center for Disease Control and Prevention (CDC) estimates that 1 in 6 people experience a foodborne illness every year (Painter et al., 2013). From 2009 to 2015, the Foodborne Disease Outbreak Surveillance System (FDOSS) received reports of 344 outbreaks (27%) and 2288 illnesses (9%) associated with aquatic animal consumption and 334 outbreaks (26%) and 9746 illnesses (37%) associated with vegetable consumption in the United States and Puerto Rico (Dewey-Mattia et al., 2018). Outbreaks caused by *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. were responsible for 4241 hospitalizations (82%) and 117 deaths (82%) (Dewey-Mattia et al., 2018). According to a recently released annual summary from FDOSS (Dewey-Mattia et al.), fruit and vegetable crops were in the top 5 pathogen-category pairs resulting in outbreak-associated illnesses in 2017, and 32 outbreaks (14%), 919 illnesses (25%), and 205 hospitalizations (51%) were associated with vegetables. Shiga toxin-producing *E. coli* (STEC) were responsible for 110 illnesses associated with vegetables, while *Salmonella* spp. were responsible for 178 illnesses associated with fruits and vegetable crops (Dewey-Mattia et al.).

Fruit and vegetables grown in the field are typically at a greater risk of contamination because of increased exposure to contamination sources such as through manure application, wildlife activities, and polluted irrigation water (Strawn et al., 2013). Bacterial pathogens can survive for a prolonged period in animal feces and may serve as a potential inoculum onto plants (Hoagland et al., 2018). Once plants are exposed to bacteria, it is often difficult to remove the contamination from the fruit and vegetables (Alegbeleye et al., 2018; Strawn et al., 2013). Meanwhile, vegetables and herbs grown in aquaponics and hydroponics in controlled environment agriculture (CEA) are often thought to be at a lower risk for contamination than its field-grown counterparts due to less exposure to environmental contaminants (Despommier, 2011). Spent nutrient solutions are collected and reused in recirculating hydroponic systems (Kim et al., 2018a; Gómez et al., 2019a), which may be a source of contamination if not properly treated and managed. In addition, the potential for internalization of *E. coli* O157:H7 and *Salmonella* spp. has been

demonstrated in lettuce, spinach, and tomato grown in an inoculated hydroponic system (Guo et al., 2002; Warriner et al., 2003; Franz et al., 2007; Macarisin et al., 2014) where contaminated seeds and/or irrigation water could potentially be the source of contamination (Deering et al., 2012b).

Aquaponics, integrated food production of aquaculture and hydroponics, is a sustainable farming method to address many agricultural challenges such as water scarcity, resource stewardship, and global food security (Quagrainie et al., 2018; Yang and Kim, 2019a). Recirculating aquaponic systems are designed to increase nutrient use efficiency and decrease environmental wastes generated in agricultural food production (Yang and Kim, 2019a; Chalmers, 2004; Rakocy, 2012). Nitrifying bacteria convert ammonia into nitrate from aquaculture wastewater. This process not only provides a nitrogen source and other nutrients for plant growth, but also cleans the water for the fish which allows water recirculation and reduces wastewater disposal issues associated with aquaculture (Enduta et al., 2011). However, the use of waste products derived from fish cultivation raises food safety concerns because foodborne pathogens can be carried in fish intestines up to 7 days (Geldreich and Clarke, 1966). Although some evidence demonstrates that they are not permanent flora and correlated with the level of environmental contamination (WHO, 1989; Pillay, 2004), fish feces can be a potential contamination source of bacterial pathogens in aquaponic systems.

Considering the recent expansion of indoor aquaponics and hydroponics (Gómez et al., 2019a), it is important to determine possible factors that can adversely affect the food safety of fresh produce. The objective of this study was to investigate the occurrence of bacterial pathogens in fish feces, recirculating water, roots and edible portions of the crops grown in aquaponic and hydroponic systems. We targeted shiga toxin-producing *E. coli, L. monocytogenes*, and *Salmonella* spp., the most common foodborne pathogens of concern associated with fresh produce (Painter et al., 2013; Rangel et al., 2005; Silk et al., 2013; Jackson et al., 2013), to assess the presence of bacterial pathogens and the likelihood of contamination of these systems.

## 2.3 Materials and Methods

### 2.3.1 System design

Six experimental units were operated in the greenhouse in West Lafayette, IN (lat. 40°N, long. 86°W) which consisted of three aquaponic and three hydroponic systems. Each unit was equipped with a fish tank or a nutrient reservoir (350 L), a clarifier (20 L), a two-stage biofilter (40 L) (Yang and Kim, 2019a; Wongkiew et al., 2017b) and a deep-water hydroponic grow bed (350 L; 1.0 $m^2$). A month prior to the study, systems were filled with reverse osmosis water. Nile tilapia (*Oreochromis niloticus* L.) fish were obtained from Animal Sciences Research and Education Center at Purdue University which had been cultivated in a conventional aquaculture system for 4-months. Fish fresh weight was measured (an average of 300 g per fish) and evenly distributed to three different fish tanks with a stocking density of 20 $kg/m^3$ in each aquaponics system. The pH of the aquaponic systems was maintained at 6.5 to 7.0 using a combination of KOH and $Ca(OH)_2$. The biofilter was connected to a peristaltic pump (Masterflex, Cole-Parmer, USA) to recirculate nutrient solution within a system unit. In each hydroponic system, the nutrient solution reservoir and hydroponic culture unit were filled with reverse osmosis water blended with nutrient stock solution at 1:100 dilution rate which was used as initial and follow-up daily replenishment used for leafy vegetables and herb crops (CropKing, Lodi, OH, US) and fruity vegetable (CropKing, Lodi, OH, US). The electrical conductivity (EC) was maintained at 2.0 dS/m by adding and replenishing nutrition solution daily and the pH of hydroponics was adjusted at around 6. The total water volume in both aquaponic and hydroponic systems was 700 L with a flow rate of 138 L/h, giving a water retention time [(surface area × water depth) / flow rate] of 5 hours in a hydroponic culture unit of each system (**Figure 1**). Aquaponic solution or nutrient solution flowed through the hydroponic culture unit of each system and back to the fish tank or reservoir. Each aquaponic and hydroponic system had air stones to maintain dissolved oxygen (DO) concentrations at full saturation and aquatic heaters were set in aquaponic systems to maintain the temperature in the ranges of 25 to 28 °C. Water temperature, pH, EC, and DO were measured daily using the HQ40d Portable Water Quality Lab Package (HACH Corp., Loveland, CO, USA). Total ammonia nitrogen, nitrite, and nitrate concentrations were analyzed immediately using HACH reaction kits (Loveland, Co. Ltd., USA), namely Ammonia Reagent Powder Pillows, Nitrite Reagent Powder Pillows, and Nitrate Reagent Powder Pillows, respectively. The same

water samples were used to confirm the concentrations of nitrogen species by ion chromatography (Dionex ICS–5000, Thermo scientific, Co. Ltd., USA).

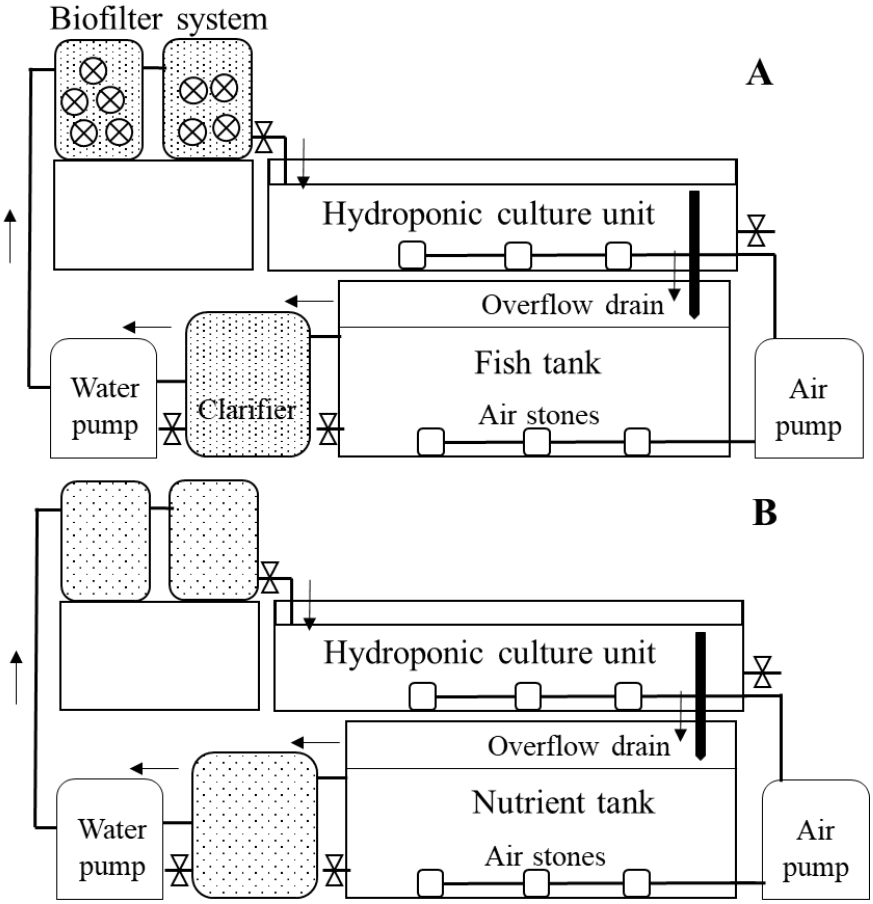


Figure 2.1. Schematic diagram of the experimental units (A) aquaponic system and (B) hydroponic system adapted from Yang and Kim (2019).

### 2.3.2 Plant materials and growing conditions

Three crops were tested: lettuce (*Lactuca sativa*), basil (*Ocimum basilicum*), and tomato (*Solanum lycopersicum*). Seeds were purchased from a commercial source (Johnny's Selected Seeds, Winslow, ME) and sown in Agrifoam soilless plugs (SteadyGROWpro, Syndicate Sales, Kokomo, IN) with few days interval to ensure uniform seedling size at the time of transplanting. Seeds were germinated as described by Kim et al. (2018) and 2 to 3 weeks old seedlings were transplanted to aquaponic and hydroponic systems. Planting densities were 24 plants per $m^2$ for

lettuce and basil and 8 plants per $m^2$ for tomato. During the study period, the fish were fed 60 g with a complete diet (41% protein, 1.1% phosphorus) with 4.8-mm floating pellets (AquaMax Sport Fish 500, Purina Mills, St. Louis, MO) at 9:00 am daily. The experiment was conducted between December 2017 and February 2018. The photoperiod was 14-h (8:00 am to 10:00 pm) consisting of natural daylight with supplemental lighting using high-pressure sodium (HPS) lamps (600-W, P.L. Light Systems Inc., Beamsville, ON, Canada). A supplemental photosynthetic photon flux (PPF) of the greenhouse was measured using a quantum sensor (LI-250A light meter; LI-COR Biosciences, Lincoln, NE) and maximum photosynthetically active radiation in the greenhouse was averaged at 168 µmol/$m^2$/s. Day (8:00 am to 10:00 pm) and night (10:00 am to 8:00 am) temperatures were set at 24 and 18 °C, respectively.

### 2.3.3 Plant sample collection and microbial isolation

Leaf and root samples of lettuce and basil were collected at 30 and 60 days after transplanting, respectively, while fully ripe tomato fruit and root samples were collected at 60 days after transplanting. A total of six biological replicates (each with three technical replicates) were collected from each plant source in both the aquaponic and hydroponic systems. To investigate surface contamination, plant samples were blended with peptone water for 15 secs without any surface-sterilized procedure. To examine if the bacteria of interest were able to internalize within the plant tissue, plant samples were surface-sterilized to remove surface-located bacteria using 0.1% sodium hypochlorite for 10 secs followed by sterile water for 30 secs. This procedure was repeated 3 times for each sample. A total of 5 g of fresh produce was blended with 45 mL of buffered peptone water (PW; Difco, Detroit, MI) in a 50 ml centrifuge tube, allowing the sample to be completely homogenized (Guzel et al., 2017; Andrews et al., 2018; Feng et al., 2002). The homogenized samples were then incubated at 37 °C for 6 hours to allow for recovery of the bacteria and potential enrichment. The samples were then serially diluted and 0.1 mL spread plated in duplicate on MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC) agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), Oxford Listeria-selective agar (Becton, Dickinson and Company), and Xylose Lysine Tetrathionate 4 (XLT4) agar with supplement (Becton, Dickinson and Company) for the selection of *E. coli* O157, *L. monocytogenes*, and *Salmonella* spp., respectively (Andrews et al., 2018; Feng et al., 2002). Plates were incubated for

24 h at 37 °C for the isolations of *E. coli* O157 and *Salmonella* and for 48 h at 30 °C for the isolation of *L. monocytogenes*.(**Figure S1**).

### 2.3.4 Microbial detection in water and fish feces samples

Water samples were collected immediately after crop harvest. Each sample was randomly collected from two duplicate systems with a total of six biological replicates (each with three technical replicates) from each system. Fish feces were collected from the clarifier tank where most solid waste was found and excess water was carefully drained.

A separate fish-growing system was constructed to confirm if the fish feces were the source of contamination in aquaponics. Tilapia (2.5 kg per tank) were placed in a fish tank (125 L) filled with 50 L reverse osmosis water and grown for two weeks. Approximately 20% of the water was replaced daily to maintain water quality. Fish feces were collected from the fish-growing system. The 20 mg fish feces sample from each system was then mixed with 180 µL water and vortexed for 15 secs. Water samples and fish feces samples were plated after collection without enrichment. The water samples and fish feces samples were serially diluted and spread plated as described above in duplicate on CT-SMAC agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), Oxford Listeria-selective agar (Becton, Dickinson and Company), and XLT4 agar with supplement (Becton, Dickinson and Company) for the selection of *E. coli* O157, *L. monocytogenes*, and *Salmonella* spp., respectively.

### 2.3.5 PCR assay for detection of virulence genes

A total of 90 isolated colonies each from presumptive positive STEC and *Salmonella* spp. cells (no presumptive *L. monocytogenes* positive colonies were isolated) were picked into 20 µl of distilled water and then heated to 90 °C using a dry heat bath (Benchmark Scientific Inc., Edison, NJ, USA) for 5 min. PCR was then performed to amplify the *stx1* gene from *E. coli* O157 (Fode-Vaughan et al., 2003) and the *iro*B gene for *Salmonella* spp. (Ziemer and Steadham, 2003) (**Table1**). A total of 5 µl samples of the PCR product was mixed with 1 µl of 6X DNA safe stain loading dye (Bullseye DNA Safe Stain C138, MIDSCI, USA) and loaded into a 1% agarose gel (H26855 Agarose D1-LE , Thermo scientific, Co. Ltd., USA) in 50 ml of 1x TAE buffer (Tris-acetate-EDTA, Thermo scientific, Co. Ltd., USA). The gel was run at 110 V for 35 min. using a

PowerPac$^{TM}$ Basic Power Supply (PowerPac$^{TM}$ Basic Power Supply, Bio-Rad, USA). The gel was then imaged using a Gel Doc EZ Imager (Gel Doc EZ Imager, Bio-Rad, USA).

### 2.3.6 Statistical methods

For the analysis of water conditions in aquaponic and hydroponic systems, a completely randomized design and two-way ANOVA were used. The water quality parameters in the aquaponic and hydroponic systems were tested by using Tukey's Honest Significant Difference. The statistical analysis used post-hoc pairwise comparisons in R 3.6.1 (R, Comprehensive R Archive Network, USA) at a significance level of 0.05.

Table 2.1. PCR primers for **STEC**, *L. monocytogenes*, and *Salmonella* spp.

| | ***Sequence*** | ***PCR program*** | ***Reference*** |
|---|---|---|---|
| Shiga Toxin-Producing *E. coli* (STEC)<br>*stx1*-F<br>*stx1*-R | CAGTTAATGTGGTGGCGAAG<br>CACCAGACAATGTAACCGCTG | 95°C for 3 min, 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, repeat 2-4 for 30 times, 72°C for 10 min | (Fode-Vaughan et al., 2003) |
| *Salmonella* spp.<br>*iro*B-F<br>*iro*B-R | TGCGTATTCTGTTTGTCGGTCC<br>TACGTTCCCACCATTCTTCCC | 95°C for 3 min, 95 °C for 30 sec, 60°C for 30 sec, 72°C for 1 min, repeat 2-4 for 30 times, 72°C for 10 min | (Ziemer and Steadham, 2003) |

## 2.4 Results and Discussion

### 2.4.1 Water conditions for aquaponic and hydroponic systems

The water quality parameters in aquaponic and hydroponic systems are shown in **Table 2**. The water temperatures of aquaponics averaged 27.5 ± 1.4, 25.9 ± 1.2, and 27.5 ± 1.7 °C for lettuce-, basil-, and tomato-based systems, respectively, and those of hydroponics averaged 20.3 ± 1.2 °C. Tilapia is a tropical fish that can grow in a wide range of temperature from 22 to 34 °C; however, the feed conversion ratio, fish weight gain in relation to feed consumption, and daily weight gain of tilapia are known to be better at temperatures between 26 - 30 °C (Azaza et al., 2008). The pH values averaged 6.7 ± 0.5 and 5.7 ± 0.5 in aquaponics and hydroponic systems, respectively (**Table 2**). There were no significant differences between aquaponic and hydroponic systems in EC levels and nitrogen species concentrations, which averaged respectively at 1.2 ± 0.5

and 1.6 ± 0.1 and 72.9 and 76.6 mg/L. The average DO level was significantly lower in aquaponics (6 mg/L) than in hydroponics (10 mg/L) although it was maintained at full saturation in both systems.

Table 2.2**.** Water quality parameters in lettuce-, basil-, and tomato-based aquaponic and hydroponic systems during the experimental period.

| Production system | Vegetable | DO (mg/L) | pH | Temperature (°C) | EC (dS/m) | $NH_4^+$ (mg/L) | $NO_2^-$ (mg/L) | $NO_3^-$ (mg/L) |
|---|---|---|---|---|---|---|---|---|
| Aquaponics | Lettuce | 6.1 ± 0.7 | 6.9 ± 0.6 | 27.5 ± 1.4 | 1.1 ± 0.4 | 1.8 ± 0.4 | 0.8 ± 0.2 | 74.7 ± 10.8 |
| | Basil | 6.2 ± 0.7 | 6.5 ± 0.5 | 25.9 ± 1.2 | 1.3 ± 0.5 | 3.0 ± 1.1 | 0.3 ± 0.1 | 50.8 ± 4.9 |
| | Tomato | 6.1 ± 0.7 | 6.8 ± 0.5 | 27.5 ± 1.7 | 1.2 ± 0.5 | 2.9 ± 0.7 | 1.2 ± 0.5 | 83.1 ± 13.8 |
| Hydroponics | Lettuce | 10.0 ± 0.4 | 5.7 ± 0.5 | 20.3 ± 1.4 | 1.7 ± 0.1 | 0.8 ± 0.3 | nd | 77.5 ± 7.8 |
| | Basil | 10.0 ± 0.4 | 5.7 ± 0.5 | 20.2 ± 1.2 | 1.6 ± 0.1 | 0.9 ± 0.2 | nd | 73.9 ± 7.0 |
| | Tomato | 9.9 ± 0.5 | 5.8 ± 0.6 | 20.5 ± 1.2 | 1.5 ± 0.2 | 1.2 ± 0.4 | nd | 95.5 ± 9.4 |
| Significance | | | | | | | | |
| System | | *** | *** | *** | *** | *** | *** | *** |
| Vegetable | | ns | ns | ns | ns | ns | ns | ns |
| System × Vegetable | | ns | ns | ns | ns | *** | ns | ns |

Each value in the table is the mean of 4 replicates ± SD.
nd means not detected.
ns, *, **, *** mean no significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

It is well documented that environmental factors such as temperature, pH, nutrient availability, and DO affect bacterial populations (Tirado et al., 2010; Hoagland et al., 2018; D'Souza et al., 2004; Shadbolt et al., 2001). Bacterial pathogens survived for a longer time (up to 91 days) at cold (4–8 °C) and frozen (−4 °C) temperatures than warmer (20–30 °C) temperatures (up to 84 days) when they were inoculated in river water and sterilized soil (Guan and Holley, 2003). *E. coli*, *L. monocytogenes,* and *Salmonella* s.v Typhimurium, can survive in animal waste at 28 °C during anaerobic digestion (Kearney et al., 1993) and the population can be affected by the interaction between temperature and pH (Ellajosyula et al., 1998). Environmental factors in our study were similar between aquaponics and hydroponics, and therefore, the differences in temperature and pH between aquaponic and hydroponic systems are not likely to affect the population of bacteria between the systems. For example, the pH values in aquaponic and

hydroponic systems were 6 and 7 which are in optimal ranges of 6 to 9 for enteric pathogens (Matches and Liston, 1972; Lungu et al., 2009; Ukuku et al., 2009) and at pH 6, the population densities of *E. coli* O157:H7 have been reported to be the same at 20 and 30 °C (Clavero and Beuchat, 1996).

EC is a common indicator of soluble salts dissolved in nutrient solution because soluble salts carry an electrical charge and their presence increases the EC in the solution. Availability of nutrients (e.g., nitrogen) and energy sources is a key factor affecting the survival of bacteria in the environment (Hoagland et al., 2018). It has been demonstrated that the viability of *E. coli* O157:H7 is increased in nutrient-rich soils (van Elsas et al., 2011). Since the high level of nutrients in hydroponic solution is also ideal for the growth of bacteria, the irrigation water containing a high concentration of nutrients poses the biggest contamination risk in soilless culture systems (Settanni et al., 2013). In fact, it was reported that the nutrient reservoirs in hydroponic systems can be the contamination sources of bacteria (Allende and Monaghan, 2015; Coleman et al., 2017). Enteric pathogens are facultative anaerobic bacteria (Lungu et al., 2009; Semenov et al., 2011). The average DO levels observed in our study are sufficient to allow the growth of *E. coli* O157:H7, *Listeria* spp., and *Salmonella* spp. The implication of these environmental conditions is that pathogenic bacteria can grow in greenhouse-based aquaponic and hydroponic systems if they are introduced by any means.

### 2.4.2 Occurrence of Shiga Toxin-Producing *E. coli* (STEC)

We tested the presence of STECs in fish feces in aquaponic and aquaculture system and found STECs in fish feces and the water regardless of the systems **(Table 3)**. The colonies were confirmed after incubation at 37 °C for 20 hours and PCR targeting *stx1* gene for the detection **(Figure S2)**. STEC were also detected on the root surfaces, but was not found to internalize into the roots or the edible parts of lettuce, basil, and tomato grown in the aquaponic systems (**Table 3; Figure S2**). These results indicated that the water contaminated by fish feces is likely the primary source of root surface contamination.

Table 2.3**.** Occurrence of *STEC*, *L. monocytogenes*, and *Salmonella* spp. in recirculating water and the roots, leaves, and/or fruit of lettuce, basil, and tomato grown in aquaponics and hydroponics.

| Vegetable | Tissue type | Shiga Toxin-Producing *E. coli* | | *L. monocytogenes* | | *Salmonella* spp. | |
|---|---|---|---|---|---|---|---|
| | | Aquaponics | Hydroponics | Aquaponics | Hydroponics | Aquaponics | Hydroponics |
| Lettuce | Internal leaves | – | – | – | – | – | – |
| | Leaf surfaces | – | – | – | – | – | – |
| | Internal roots | – | – | – | – | – | – |
| | Root surfaces | + | + | – | – | – | – |
| | Water | + | + | – | – | – | – |
| | Fish feces | + | NA | – | NA | – | NA |
| Basil | Internal leaves | – | – | – | – | – | – |
| | Leaf surfaces | – | – | – | – | – | – |
| | Internal roots | – | – | – | – | – | – |
| | Root surfaces | + | + | – | – | – | – |
| | Water | + | + | – | – | – | – |
| | Fish feces | + | NA | – | NA | – | NA |
| Tomato | Fruit | – | – | – | – | – | – |
| | Internal roots | – | – | – | – | – | – |
| | Root surfaces | + | + | – | – | – | – |
| | Water | + | + | – | – | – | – |
| | Fish feces | + | NA | – | NA | – | NA |

The symbols, + and –, indicate presence and absence, respectively. NA means no fish feces in hydroponics.
Each symbol in the table is the results from 18 samples (6 biological replicates x 3 technical replicates). Ten isolates per positive plate were examined for PCR confirmation.

Irrigation water is often found to be the major source of contamination in outbreaks associated with bacterial pathogens and this can be particularly true for the field grown vegetables due to overhead irrigation with contaminated water, damaged roots and shoots by a herbivore, or groundwater contamination by a plume of wastewater (Franz and Bruggen, 2008; Allende and Monaghan, 2015; Pagadala et al., 2015; Kokkinos et al., 2017). However, our study suggests that there is a potential risk associated with aquaponic produce even when the solutions are directly applied from the roots due to water contamination. Our separate aquaculture system confirmed that fish feces was the major source of contamination with STEC in the aquaponic system. These results indicate that introducing contaminated fish can be a source of foodborne pathogens in aquaponics. Previous work has shown that fish reared in the ponds where the concentration of coliforms was low, a small number of *E. coli* O157:H7 cells were recovered from the fish intestines, but not fish muscle (Buras et al., 1987; Apun et al., 1999). The bacterial intestinal flora of fish can survive up to 84 days in water at 20–30 °C (Wang and Doyle, 1998; Youssef et al., 1992).

Therefore, tilapia fish used in this study may have been contaminated prior to the receipt and the STEC could have been carried over by the fish to the tanks, contaminating the water in aquaponic systems. Importantly, the contaminated water did not lead to the internalization of STEC into the roots and edible parts of lettuce, basil, and tomato grown in aquaponic systems suggesting the main source of contamination during production would be from accidental splash of the water to the edible portions of the plant during harvest (**Table 3; Figure S2**).

STEC were also detected in the water of the hydroponic systems. The bacteria may have been introduced accidentally to the hydroponic systems during experimental setup or handling, possibly allowing the formation of biofilms on the surfaces of the hydroponic culture unit, nutrient reservoir, and/or irrigation tubing. A variety of factors, such as water temperature, pH, nutrient availability, solar radiation, the presence of other microorganisms, and the ability to form biofilms, influence both survival and proliferation of STEC in water (Wang and Doyle, 1998; Schuster and Maertens, 2016; Chen et al., 2018). STEC concentrations in irrigation water are also affected by diurnal and seasonal variation (Pachepsky et al., 2011). In fact, the biofilm of *E. coli* O157:H7 can grow rapidly and have a high number of adherent cells even at low nutrient availability and low temperature (Somers et al., 1994; Dewanti and Wong, 1995). Once formed, it is hard to remove them from the system because the biofilm increases the survival rate of *E. coli* O157:H7 even in the exposure to hydrogen peroxide, quaternary ammonium sanitizer, and citric acid (Uhlich et al., 2006). Therefore, we speculate that the hydroponic systems in this study may have been contaminated with STEC due to incomplete sanitation before cultivating plants.

Human activities can also increase the risk of contamination. In a study to determine the source of contamination in hydroponic tomato greenhouses, work shoes and personal shoes were identified as a vehicle for transmission of *E. coli* O157:H7 (Orozco et al., 2008b). In this study, there is a potential that the bacteria may have been introduced from the aquaponic system to the hydroponic systems from visitors during their visit to the greenhouse, during the feeding of fish, during sample collection, and/or by cross-contamination from other human activities (Gómez et al., 2019a; Joyce et al., 2019). Contrary to our results, very low levels of generic *E. coli* and undetectable pathogenic *E. coli* O157:H7 and *Salmonella* spp. were found in water and plant samples of aquaponic produce originated from outdoor aquaponic farms located in a tropical climate (Fox et al., 2012). In their study, solar radiation and heat might have played a key role in controlling the bacterial levels in the system (Joyce et al., 1996; Crane and Moore, 1986). However,

the results cannot be translated into the indoor aquaponic and hydroponic systems located in a temperate climate since the solar radiation is often limited and the environmental conditions are very different from the situation in a tropical climate.

Our results indicate that contamination with bacterial pathogens could likely be reduced in aquaponic and hydroponic systems if the entire systems are thoroughly sanitized before each use and pathogen-free fish are used for the operation. One of the major routes of the enteric pathogen internalization is through the sites of biological or physical damage, and/or through natural openings on the plant surface such as stomata, lenticels, and sites of lateral root emergence (Deering et al., 2012b). The contaminated irrigation water in our study did not have direct contact with the aerial edible parts of the plants as there was a foam board between the water and the plants and the plants were not disturbed until harvest. It should be noted that damaged roots during handling allow entry points for the bacterial pathogens, and therefore, the risk of contamination can be avoided if the plant tissues, particularly the roots, are carefully handled during production and harvest. Similarly, previous reports found that foodborne illness was well controlled in aquaponics when the following practices were carried out: cleaning and sanitation of reusable plastic containers, environmental controls, handwashing, and the use of clean irrigation water (Barnhart et al., 2015; Turkon, 2018).

### 2.4.3 Occurrence of Listeria monocytogenes

While the optimal water temperature and the pH poses a potential contamination concern of *L. monocytogenes* in aquaponics and hydroponics (**Table 1),** our selective growth medium and colony PCR assay did not detect the occurrence of *L. monocytogenes* in fish feces, recirculating water, and plants grown in aquaponic and hydroponic systems. These results suggest that there was no contamination source of *L. monocytogenes* in both systems (**Table 3; Figure S2**). One source of *L. monocytogenes* is from the soil, and there was no soil in the greenhouse during this experiment. Other potential sources of *L. monocytogenes* are human activities and contaminated seeds. In the event of pathogen introduction to the systems, *L. monocytogenes* can internalize through natural openings on the plant surface or through the sites of biological or physical damage (Deering et al., 2012b). Although the fish feces or human activities were demonstrated as potential sources of contamination for STEC, our results showed that these were not the major sources of

contamination for *L. monocytogenes* and the production practices employed in this study were not associated with the risk of contamination with *L. monocytogenes* in aquaponic and hydroponic systems.

### 2.4.4 Occurrence of *Salmonella* spp.

Various environmental factors such as nutrient, pH, and water temperature affect the population of *Salmonella* spp. (Matches and Liston, 1972). The optimal pH range for the growth of *Salmonella* spp. is between 6.5 and 7.5 and the temperature range is between 20 and 30 °C in the soil (Guan and Holley, 2003). Our results showed that there was no *Salmonella* spp. in water samples collected from fish tanks (or nutrient reservoir) and hydroponic culture units, although the pH and temperature were within the optimal range for their growth and the nutrient level was sufficient to allow the growth of *Salmonella* spp. in both systems (**Table 1**). Further, *Salmonella* spp. was not present in other samples such as fish feces and the roots and edible portions of plants (**Table 3; Figure S2**).

Outbreaks associated with fruits and vegetables contaminated with *Salmonella* spp. have also been attributed to contaminated irrigation water sources (Gould et al., 2013). It should be noted that we used reverse osmosis water to fill and replenish the systems. It is apparent that the plants in the aquaponic and hydroponic systems did not have contact with contamination sources of *Salmonella* spp. A previous study reported that tomato fruit was not contaminated with Salmonella spp. even when the nutrient solution was inoculated with an avirulent strain of *S.* Typhimurium at a level of $10^5$ colony-forming units (CFU)/mL in a hydroponic system (Coleman et al., 2017) and when the plants were irrigated with 350 ml of $10^7$ CFU/ml of *S.* Montevideo every 2 weeks for 10 weeks (Miles et al., 2009).

## 2.5 Conclusions

This study demonstrated that there is a potential risk of pathogen contamination in fresh produce even when the crops were grown in indoor aquaponic and hydroponic systems. STEC was found in fish feces, the recirculating water, and on the plant root surfaces in aquaponic and hydroponic systems. Importantly, the bacteria did not internalize the roots and the edible parts of plants regardless of the production systems. Fish feces were considered the major source of the

pathogenic bacteria in the aquaponic system possibly due to the introduction of contaminated fish. Therefore, it is essential to follow proper handling, cleaning, and sanitizing practices in order to minimize the risk of contamination of fresh fruit and vegetables grown in an aquaponic system.

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**Author contribution statement**

YW conducted experiment, collected data, undertook data analysis and interpretation, and drafted the manuscript. AD supervised the microbial analysis and participated in production of the manuscript, and made critical revisions. HK coordinated and supervised the research, and completed the final version of the manuscript.

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

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Figure S1. Bacterial isolation using a selective medium for *E. coli* O157 by plating on CT-SMAC agar with supplement. The colorless colonies on the plate are presumptive positive *E. coli* O157. A total of 10 colonies from each sample were confirmed for the presence of *stx1* gene using colony PCR.

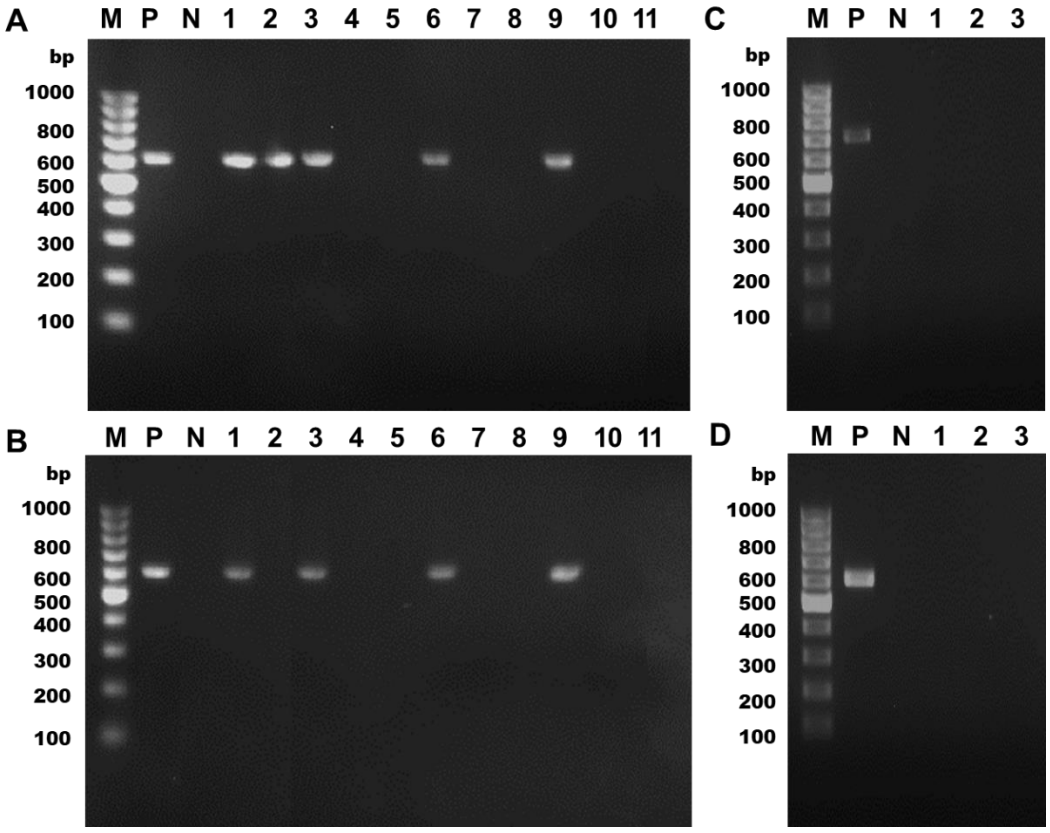


Figure S2. Colony PCR of selected colonies of (A) STEC in aquaponics and (B) STEC in hydroponics. M: 100bp Marker, P: Positive control (*E. coil* O157:H7), N: Negative control ($H_2O$), 1: Recycled water, 2: Fish feces, 3: Lettuce root surface, 4: Lettuce internal roots, 5: Lettuce leaves, 6: Basil root surface, 7: Basil internal roots, 8: Basil leaves, 9: Tomato root surface, 10: Tomato internal roots, and 11: Tomato leaves; Colony PCR of selected colonies of (C) *L. monocytogenes* and (D) *Salmonella* spp. in aquaponics and hydroponics. M: 100bp Marker, P: Positive control (*L. monocytogenes* or *Salmonella*), N: Negative control ($H_2O$), 1: Recycled water in aquaponics, 2: Recycled water in hydroponics, and 3: Fish feces.

# CHAPTER 3. EFFECTS OF PLANT AGE AND ROOT DAMAGE ON INTERNALIZATION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN LEAFY VEGETABLES AND HERBS

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**Yi-Ju Wang [1], Amanda J. Deering [2] and Hye-Ji Kim [1,*]**
[1]Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, USA; wang3707@purdue.edu
[2]Department of Food Science, Purdue University, West Lafayette, IN 47907, USA; adeering@purdue.edu
*****Correspondence: hjikim@purdue.edu
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## 3.1 Abstract

Our previous study reported that fresh produce grown in aquaponic and hydroponic systems can pose potential food safety hazards due to an accidental introduction of contaminated fish and cross-contamination between the systems. In this study, we examined the effects of plant species and age on the likelihood and level of internalization of Shiga toxin-producing *Escherichia coli* (STEC) in aquaponic and hydroponic systems. Four plant species, basil (*Ocimum basilicum* L. cv. Genovese), cilantro (*Coriandrum Sativum* L.), lettuce (*Lactuca sativa* cv. Cherokee), and kale (*Brassica oleracea* var. *sabellica*), received root damage treatment as seedlings before transplanting or mature plants at three weeks after transplanting by cutting off 1-cm tips of one-third of the roots. Enrichments and selective media were used for the isolation, and presumptive positive colonies were confirmed by PCR for the presence of *stx*1 gene in plant tissues, recirculating water, and fish feces collected at four weeks after transplanting. In hydroponic systems, STEC was found neither in the solution nor in the roots and leaves of all four plant species, possibly through improved sanitation and hygiene practices. However, consistent with our previous findings, STEC was found in the water, on the plant roots, and in the fish feces in aquaponic systems, even after thorough sanitation prior to the study. Regardless of plant age, STEC was internalized in the roots of all plant species when the roots were damaged, but there was no difference in the degree of internalization with STEC among plant species. STEC was present in the leaves only when seedlings received root damage treatment and were grown to maturity, indicating that root damage allows STEC to internalize in the roots within a week, but a

longer period is required for STEC to internalize into the leaves. We concluded that root damage on seedlings can cause the internalization of *E. coli* O157:H7 in the edible parts of leafy vegetables and herbs in soilless production systems.

**Keywords:** food safety; *E. coli*; aquaponics; hydroponics; agricultural water; PCR detection; indoor farming

## 3.2 Introduction

The global food demand and security have been increased with the increasing population (Alexandratos, 2012). FAO (Food and Agriculture Organization of the United Nations) estimates that 10.8% of people worldwide suffered from undernourishment in 2018, and Sub-Saharan Africa experienced a sharp increase of undernourishment from 20.6% in 2015 to 22.8% in 2018 (Food and Agriculture Organization of the United Nations, 2019). Facing the challenge of food demand, the urban farming fulfilled 37% of vegetable needs in Kathmandu, Nepal, 45% of local food needs in Hong Kong, and almost 60% of all Cuban vegetable demands (Rees, 1997; Garnett, 1996; Daniel Hoornweg and Paul Munro-Faure, 2008). Therefore, it is a potential way to feed urban residents in the world with fresh produce (Brook and Dávila, 2000).

Meanwhile, nearly 48% of foodborne outbreaks are linked to the consumption of fresh fruits and vegetables due to the bacterial contamination of fresh produce (Luna-Guevara et al., 2019; Hoagland et al., 2018). The Foodborne Disease Outbreak Surveillance System (FDOSS) pointed out that 32 outbreaks (14%), 919 illnesses (25%), and 205 hospitalizations (51%) reported in 2017 were associated with the consumption of raw vegetables, and 110 illnesses (11%) were associated with Shiga toxin-producing *E. coli* (STEC) (Dewey-Mattia et al.). In the United States, leafy greens and other vegetable crops are a major source of STEC infections, and 51 foodborne disease outbreaks linked to leafy greens were reported to the Centers for Disease Control and Prevention (CDC) from 2014 to 2018 (Painter et al., 2013). Moreover, the UK and Germany reported 531 and 3785 cases of illness, respectively, in association with leafy vegetables between 2008 and 2010 (Hoc, 2007; Authority, 2011).

Microbial contamination of produce can occur at any point from farm to fork. In field-grown vegetables, foodborne pathogens can be introduced from polluted irrigation or postharvest-washing water, soil, animal feces, and by handlers during harvest, post-harvest, or packing

(Hoagland et al., 2018; Riggio et al., 2019; Deering et al., 2012b). The multi-state outbreak of *E. coli* O145:H28 infections in southern Arizona was associated with STEC originated from stray dog and coyote feces in a major leafy green production field at the United States-Mexico border (Jay-Russell et al., 2014).

Soilless culture is an applied technology of food production in a controlled environment, which has a higher water and land use efficiency compared with traditional field production (Daniel Hoornweg and Paul Munro-Faure, 2008; Akash Bhargaw and Priyamvada Chauhan, 2020; Gómez et al., 2019a; Chen et al., 2020; Yang and Kim, 2020b, 2019a) and can eliminate the risk of wild animal fecal contamination (Azevedo et al., 2014; Oliver et al., 2018). Hydroponics is one of the production methods in a controlled environment and known to produce safe and clean vegetables, and more than 404.7 ha of vegetables are produced hydroponically in the United States (Quagrainie et al., 2018). Aquaponics is another soilless production method, which contains major three organisms: aquatic animals, microorganisms, and plants (Wirza and Nazir, 2020; Chalmers, 2004; Wongkiew et al., 2017b). While hydroponics uses chemical fertilizers containing high levels of nitrogen and phosphorus (Yang and Kim, 2020a), aquaponics uses waste products generated from aquatic animals' digestion of fish feed. Contaminated seeds, growing media, irrigation water, worker health and hygiene, field and harvest sanitation, and sanitation of packing facilities can be the potential microbial contamination source in both systems (Hoagland et al., 2018; Hoc, 2007; Riggio et al., 2019; Deering et al., 2012b; Orozco et al., 2008a; Hochmuth, 2001); however, aquatic animals can be an additional contamination source in aquaponics if an externally or internally contaminated aquatic animal is accidentally introduced and releases bacteria directly or indirectly via animal feces into the solution (Riggio et al., 2019; Chalmers, 2004; Wang et al., 2020b).

Contamination with enteric pathogens may occur at relatively low levels in aquaponics and hydroponic systems (Weller et al., 2020). Nonetheless, our previous study showed that STEC was present in the water of both aquaponic and hydroponic systems, possibly due to the introduction of contaminated fish and cross-contamination between the systems, but not present in plant tissues (Wang et al., 2020b). Several studies suggested that mechanical or biological damage in the roots is associated with the internalization of enteric pathogens in roots but shoots. Moriarty et al. (2019) found that *E. coli* O157:H7 internalized in the roots of lettuce grown in hydroponic systems regardless of the degree of root damages (Moriarty et al., 2019). Similarly, Hora et al. (2005) found that *E. coli* O157:H7 was present in the roots but not in the leaves of spinach grown in soil

regardless of root damage treatment (mechanical or biological root damages) (Hora et al., 2005). Meanwhile, *E. coli* O157:H7 was found in the leaves at 10 days after inoculation with *E. coli* O157:H7 when lettuce plants were physically damaged from being cracked along the central vein or infected with bacteria (*Xanthomonas campestris* pv. *vitians*) (Aruscavage et al., 2008). Although plant species and age have not been considered in most studies, these factors can affect the degree of internalization of human pathogens possibly due to the different levels of defense mechanisms. This aspect was demonstrated in a study by Jablasone et al. (2005), in which they inoculated seeds with *E. coli* O157:H7 and germinated on the solidified hydroponic medium and found that cress and spinach had a higher population of *E. coli* O157:H7 than did lettuce and radish (Jablasone et al., 2005). A higher percentage of internalization was observed in 30-day-old green ice leaf lettuce (11%) than 12-day-old ones (7%) when they were grown in pots and irrigated with *E. coli* O157:H7-contaminated water (Mootian et al., 2009).

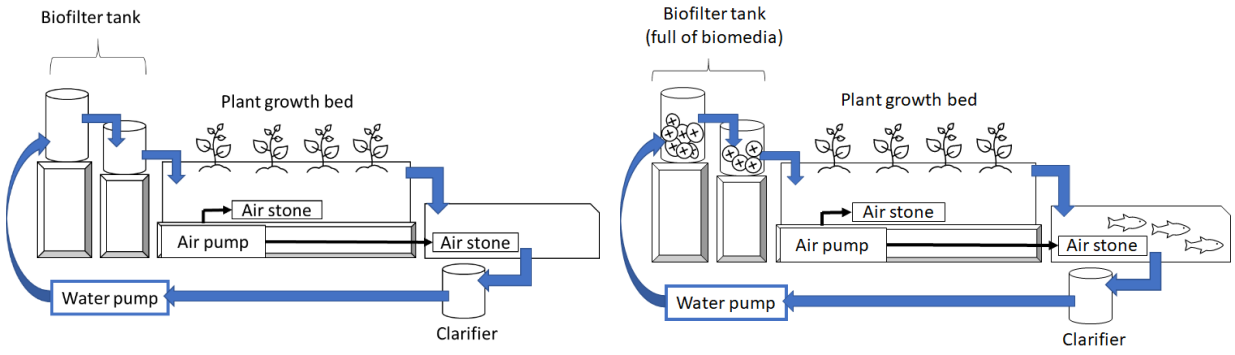
Therefore, the objective of this study was to examine the likelihood and level of internalization of STEC into plant tissues caused by root damage in association with plant species and age. The results will help understand critical factors affecting the internalization of STEC in plants and minimize the risk of contamination in soilless culture systems.

## 3.3 Materials and Methods

### 3.3.1 System Setup

Six experimental units consisting of three aquaponic and three hydroponic systems had been set up and operated in the greenhouse in West Lafayette, IN (lat. 40°N, long. 86°W), for nearly five years. Each unit was equipped with a fish tank or a nutrient reservoir (350 L), a clarifier (20 L), a two-stage biofilter (40 L) (Yang and Kim, 2019a; Wongkiew et al., 2017b), and a deep-water hydroponic grow bed (350 L; 1.0 $m^2$). A month prior to the study, systems were thoroughly sanitized and disinfected except biofilters, and then they filled with reverse osmosis water. Nile tilapia (*Oreochromis niloticus* L.) fish were obtained from the Animal Sciences Research and Education Center at Purdue University, which had been cultivated in a conventional aquaculture system for 4-months. At least a month prior to the study, fish weight was measured (an average of 300 g per fish) and evenly distributed to three different fish tanks at a stocking density of 15 $kg/m^3$. The biofilter was connected to a peristaltic pump (Masterflex, Cole-Parmer, Chicago, IL, USA) to

recirculate nutrient solution within the system. In each hydroponic system, the nutrient solution reservoir and hydroponic culture unit were filled with reverse osmosis water blended with the nutrient stock solution at 1:100 dilution rate which was used as initial and follow-up daily replenishment used for leafy vegetables and herbs (CropKing, Lodi, OH, US). The electrical conductivity (EC) was maintained at 1.5 dS/m by adding and replenishing nutrition solutions daily. The pH of the aquaponic and hydroponic systems was automatically adjusted by a Bluelab pH controller (Walchem, Iwaki America Inc., Holliston, MA, USA) and maintained at around 6.5, using a combination of 1 M KOH and 200 mM $Ca(OH)_2$. The total water volume in both aquaponic and hydroponic systems was 700 L with a flow rate of 138 L/h (3.3 $m^3/m^2$/day), giving a water retention time ((surface area × water depth)/flow rate) of 6 h in a hydroponic culture unit of each system (Figure 1) (Yang and Kim, 2020c). Aquaponic solution or nutrient solution flowed through the hydroponic culture unit of each system and back to the fish tank or reservoir. Each aquaponic and hydroponic system had air stones to maintain dissolved oxygen (DO) concentrations at full saturation. Aquatic heaters were set in aquaponic and hydroponic systems to maintain the temperature in the ranges of 25 to 28 °C. Water temperature, pH, EC, and DO were measured daily using the HQ40d Portable Water Quality Lab Package (HACH Corp., Loveland, CO, USA).




(**A**) (**B**)

Figure 3.1**.** Schematic diagram of the experimental units, (**A**) aquaponic system and (**B**) hydroponic system.

### 3.3.2 Plant Materials and Growing Conditions

Basil (*Ocimum basilicum* L. cv. Genovese), cilantro (*Coriandrum sativum* L.), lettuce (*Lactuca sativa* cv. Cherokee), and kale (*Brassica oleracea var. sabellica*) were cultured in aquaponic and hydroponic systems for 30 days. Seeds were purchased from a commercial source (Johnny's Selected Seeds, Winslow, ME, USA) and sown in Agrifoam soilless plugs (SteadyGROWpro, Syndicate Sales, Kokomo, IN, USA) with a few day intervals to ensure uniform seedling size at the time of transplanting. Seeds were germinated as described by Kim et al. (2018) and 2- to 3-week-old seedlings were transplanted to each system (Kim et al., 2018a). During the study period, the fish were fed daily (9:00 am) with a 4.8-mm floating pellet (AquaMax Sport Fish 500, Purina Mills, St. Louis, MO, USA) containing a complete diet (41% protein, 1.1% phosphorus) at a constant weight of 60 g. The experiment was conducted between December 2018 and February 2019. The photoperiod was 14 h (8:00 a.m. to 10:00 p.m.), consisting of natural daylight with supplemental lighting using high-pressure sodium (HPS) lamps (600-W, P.L. Light Systems Inc., Beamsville, ON, Canada). A supplemental photosynthetic photon flux (PPF) of the greenhouse was measured using a quantum sensor (LI-250A light meter; LI-COR Biosciences, Lincoln, NE, USA), and maximum photosynthetically active radiation in the greenhouse was averaged at 168 μmol/$m^2$/s. Day (8:00 a.m. to 10:00 p.m.) and night (10:00 p.m. to 8:00 a.m.) temperatures were set at 24 and 18 °C, respectively.

### 3.3.3 Root Damage Treatment

After the third true leaf of the seedlings emerged, uniform healthy seedlings were randomly and equally divided into three groups: no root damage (Control), root damage at transplanting (T1), and root damage at preharvest (T2). Control plants were carefully removed from seedling trays, transplanted into mesh pots (diameter: 7.6 cm, height: 6.4 cm) each containing clay pebbles, then transferred to a hydroponic unit of aquaponic and hydroponic systems. The plants were grown for 4 weeks without disturbance. Meanwhile, T1 plants were removed from seedling trays, and one-third of the root system was cut off at 1-cm behind the root tips with alcohol sterilized scissors. After the treatment, T1 plants were transplanted into mesh pots and then transferred to aquaponic and hydroponic systems and grown for 4 weeks. The cut surfaces of the roots were fully submerged and maintained lower than the level of the solution. Likewise, T2 plants were transplanted into

mesh pots and then transferred to and grown in the aquaponic and hydroponic systems. After 3 weeks, the whole plant was carefully removed from the systems, and one-third of the root system was cut off at 1-cm behind the root tips with sterile scissors. The plants were promptly transferred back to the systems and cultivated for another 7 days.

### 3.3.4 Plant Sample Collection and STEC E. coli Isolation

Leaf and root samples were collected at the end of the experiments. Six plant samples were blended with 45 mL buffered peptone water (PW; Difco, Detroit, MI, USA) for 15 s without surface-sterilization. Six plant samples were surface-sterilized to remove surface-located bacteria using 0.1% sodium hypochlorite for 10 s, followed by sterile water for 30 s (Medina et al., 2000). A total of 5 g of plant tissue was blended with buffered peptone water in a 50 mL centrifuge tube, allowing the sample to be completely homogenized (Guzel et al., 2017; Andrews et al., 2018; Feng et al., 2002). The homogenized samples were then incubated at 37 °C for 6 h to allow for the recovery of bacteria and potential enrichment. The samples were then serially diluted, and 0.1 mL was spread plated in duplicate on MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC) agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the plates were incubated for 24 h at 37 °C.

### 3.3.5 Microbial Detection in Water and Fish Feces Samples

Incoming water sources were tested for the bacterial pathogens before, during, and after the study, and the results were negative for STEC. Water samples from aquaponic and hydroponic systems were collected from six different locations immediately after harvest. Fish feces were collected from the clarifier tank where most solid waste was found, and excess water was carefully drained.

A 20 mg fish feces sample from each system was mixed with 180 µL water and vortexed for 15 s. Water samples and fish feces samples were plated after collection and enrichment for 6 h. The water samples and fish feces samples were serially diluted and spread plated as described above in duplicate on CT-SMAC agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for the selection of *E. coli* O157. Samples were cultured on MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC) agar (Becton, Dickinson and Company, Franklin Lakes, NJ,

USA) for 24 h at 37 °C, and the colorless colonies were confirmed after incubation at 37 °C for 20 h and detected by PCR targeting of the *stx1* gene.

### 3.3.6 PCR Assay for Detection of Virulence Genes

A total of 90 isolated colonies each from presumptive positive STEC were picked into 20 µl of distilled water and then inactivated at 90 °C using a dry heat bath (Benchmark Scientific Inc., Edison, NJ, USA) for 5 min. PCR was then performed to amplify the *stx1* gene from *E. coli* O157 (Fode-Vaughan et al., 2003) (Table 1).

Table 3.1**.** PCR primers for Shiga toxin-producing *Escherichia coli* (STEC)**.**

| | ***Sequence*** | ***PCR Program*** | ***Reference*** |
|---|---|---|---|
| Shiga Toxin-Producing *E. coli* (STEC) | | | |
| *stx1*-F | CAGTTAATGTGGTGGCGAAG | 95 °C for 3 min, 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, repeat steps 2–4 30 times, 72 °C for 10 min | (Fode-Vaughan et al., 2003) |
| *stx1*-R | CACCAGACAATGTAACCGCTG | | |

### 3.3.7 Experimental Design and Data Analysis

Treatments consisted of two production systems, four plant species, and three root treatments. Each system had three replicates based on a split-plot design. Plant species and root treatments were arranged in a completely randomized design within the system. Six plants per system were randomly chosen as biological replicates, totaling 18 replicates per system. Three subsamples (technical replicates) per plant were taken for the analysis of bacterial pathogens. The statistical analysis used post-hoc pairwise comparisons in R 3.6.1 (R, Comprehensive R Archive Network, USA, http://cran.us.r-project.org/; Last accessed on Dec. 15, 2020) at a significance level of 0.05.

## 3.4 Results and Discussion

### 3.4.1 Water Conditions for Aquaponic and Hydroponic Systems

The average values of water quality parameters in the aquaponic and hydroponic systems are shown in Table 2. It is well-documented that environmental factors, such as DO, pH, water

temperature, and EC, affect bacterial populations (Tirado et al., 2010; Shadbolt et al., 2001). We controlled and maintained these factors similar between aquaponic and hydroponic systems (Table 2) to minimize environmental variations that may affect the STEC population in the recycling nutrient solution. For example, enteric pathogens can survive for a longer time at cold and freezing temperatures than at warmer (20–30 °C) temperatures (Guan and Holley, 2003), and STEC can survive in water for 12 weeks at 25 °C (Wang and Doyle, 1998). *E. coli* and *Salmonella* sv. Typhimurium can grow better at pH 4.7 than 5.2 (Ellajosyula et al., 1998). EC is a common indicator of soluble salts dissolved in a nutrient solution and a key factor affecting the survival of bacteria in the environment (Hoagland et al., 2018). A high level of nutrients is ideal for bacterial growth; therefore, the viability of *E. coli* O157:H7 increases in nutrient-rich soils and hydroponics (Macarisin et al., 2014; van Elsas et al., 2011; Shaw et al., 2016). The measured DO, pH, water temperature, and EC in our study were sufficient to support the growth of STEC. This implies that pathogenic bacteria can grow in greenhouse-based aquaponic and hydroponic systems if they are accidently introduced.

Table 3.2. Water quality parameters in aquaponic and hydroponic systems during the experimental period.

| **Production System** | **DO [z] (mg/L)** | **pH** | **Temperature (°C)** | **EC (dS/m)** |
|---|---|---|---|---|
| Aquaponics | 7.81 ± 0.03 [y] (7.73–8.09) | 6.5 ± 0.0 [x] | 22.9 ± 0.1 (22.7–23.1) | 1.52 ± 0.04 (1.48–1.58) |
| Hydroponics | 7.78 ± 0.04 (7.70–7.84) | 6.5 ± 0.0 [x] | 23.0 ± 0.1 (22.7–23.3) | 1.54 ± 0.02 (1.50–1.56) |
| Significance | ns | ns | ns | ns |

Abbreviations: [z] dissolved oxygen (DO); electrical conductivity (EC). [y] Each value is the mean of four replicates (2 sample replicates per system) ± SE (standard error). [x] means that pH was automatically adjusted by pH controllers in this study. ns means no significant difference.

### 3.4.2 The Effects of Growing System and Plant Species on the Occurrence of STEC

We assessed the presence of bacterial pathogens and the likelihood of contamination in aquaponic and hydroponic systems. Unlike our previous study, in which STEC was found in recycling water of both systems due to the cross-contamination between adjacent aquaponic and hydroponic systems (Wang et al., 2020b), STEC was absent in recycling water of hydroponic systems in this study but present in recycling water and fish feces of aquaponic systems (Table 3). The discrepancy could be attributed to the differences in sanitation and management practices

between these studies. Recognizing the presence of STEC in these systems, we thoroughly sanitized the systems before this study and avoided cross-contamination through improved management practices. It turned out to be that these practices were effective in controlling STEC for hydroponic systems but not for aquaponic systems. Although plant growth beds and fish tanks were thoroughly sanitized, the fish had not been cleaned or changed since the last experiments (Wang et al., 2020b); therefore, the preexisting condition of the fish should be attributable to the current results. As we discussed previously (Wang et al., 2021), tilapia are considered filter feeders and can efficiently harvest filamentous and planktonic algae (Popma and Masser, 1999). It is likely that a fish ingested STEC from filamentous or planktonic algae, and the fish feces was released into water contaminating the system (M.B. Timmons, personal communication, 9 September 2020). If a fish is grown in polluted water, STEC can survive on fish skin and internal organs (e.g., kidney, liver, and digestive tract) (Suhalim et al., 2008; Wang and Doyle, 1998). When tilapia, catfish, common carp, and silver carp were grown in the wastewater infested with a high level of *E. coli* ($10^6$ $g^{-1}$), the concentration of *E. coli* as high as $10^8$–$10^9$/g was recovered in the digestive tract (Suhalim et al., 2008). Especially if a contaminated fish is introduced to a recirculating aquaponic system like this, it will pollute the water and subsequently contaminate the entire system, making it difficult to eliminate the foodborne pathogens from the system. Therefore, these results indicate that improved sanitation and management practices can ensure producing safe foods in hydroponics, but different strategies may be required in aquaponics to reduce foodborne illness. For example, if the system is contaminated with foodborne pathogens, the existing fish should be removed, the entire system needs to be thoroughly sanitized, and clean fish stock should be introduced. Adding UV-radiation is another efficient way to inactivate STEC in an aquaponic system, as it can decrease 10 to 100 times coliform bacteria in aquaponic solution (Moriarty et al., 2018).

Table 3.3. Occurrence of *STEC* in recirculating water, fish feces, and the roots and leaves of basil, cilantro, lettuce, and kale grown in aquaponic and hydroponic systems.

| Vegetable | Tissue Type | *Control* | | *Root Damage at Transplanting (T1)* | | *Root Damage at Preharvest (T2)* | |
|---|---|---|---|---|---|---|---|
| | | **Aquaponics** | **Hydroponics** | **Aquaponics** | **Hydroponics** | **Aquaponics** | **Hydroponics** |
| Basil | Internal leaf | –[z] | – | +(18/18) | – | – | – |
| | Internal root | – | – | +(18/18) | – | +(18/18) | – |
| | Root surface | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Water | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Fish feces | +(18/18) | NA | +(18/18) | NA | +(18/18) | NA |
| Cilantro | Internal leaf | – | – | +(18/18) | – | – | – |
| | Internal root | – | – | +(18/18) | – | +(18/18) | – |
| | Root surface | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Water | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Fish feces | +(18/18) | NA | +(18/18) | NA | +(18/18) | NA |
| Lettuce | Internal leaf | – | – | +(18/18) | – | – | – |
| | Internal root | – | – | +(18/18) | – | +(18/18) | – |
| | Root surface | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Water | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Fish feces | +(18/18) | NA | +(18/18) | NA | +(18/18) | NA |
| Fort Smith, Arkansas Kale | Internal leaf | – | – | +(18/18) | – | – | – |
| | Internal root | – | – | +(18/18) | – | +(18/18) | – |
| | Root surface | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Water | +(18/18) | – | +(18/18) | – | +(18/18) | – |
| | Fish feces | +(18/18) | NA | +(18/18) | NA | +(18/18) | NA |

[z] The symbols, + and –, indicate presence and absence, respectively. NA means no fish feces in hydroponics. Each symbol in the table is the result of 18 samples (six biological replicates × three technical replicates). The numbers in the parentheses mean the number of STEC-positive samples/the number of total samples. Ten isolates per positive plate were examined for PCR confirmation.

Although STEC was found on the root surface of all four plant species grown in aquaponic systems, plant species had no effect on the degree of internalization (Table 3). There is very little information on the influence of plant species on internalization and persistence of STEC in hydroponic or aquaponic systems. Most studies conducted in the soil-based systems reported that STEC colonization and internalization were varied by plant species (Wright et al., 2013; Merget et al., 2019). Wright et al. (2013) found that *E coli* can colonize on the roots and translocate to the aerial parts in a similar process as endophytic bacteria (Wright et al., 2013). The root exudates, which contain sugars, protein, or other nutrients, can provide a rich environment for the growth of not only plant pathogens but also human pathogens (Hou et al., 2013; Erlacher et al., 2015). Merget et al. (2018) examined four plant species, fenugreek, alfalfa, spinach, and lettuce in soil after inoculation with STEC and found that the internalization of STEC in spinach and lettuce was 10 times higher than that in fenugreek and alfalfa, and that spinach extracts supported a higher level of biofilm formation compared to lettuce extracts (Merget et al., 2019). Wright et al. (2017) demonstrated the variations in the level of internalization with *E. coli* O157:H7 Sakai among plant species and that the bacterial growth was restricted in spinach and lettuce at the internal boundary of the epidermal cell but not in *Nicotiana benthamiana,* in which a 400-fold increase in the number of bacteria was found in their leaves compared to lettuce and spinach after 20 days (Wright et al., 2017). They also reported that the internal population of *E. coli* O157:H7 Sakai was affected only in low dose ($10^3$ CFU/mL; colony forming unit per ml) but not in high dose ($10^7$ CFU/mL).

This study, however, did not find plant species effects on the degree of internalization with STEC (Table 3). The contradictory results may be due to the different concentrations of human pathogens, application methods, and culture systems. It should be noted that the level of STEC in our systems was extremely low to a level that requires enrichment for the detection, unlike the above studies. Moreover, we grew all plant species in soilless systems, which allow exudates from damaged roots to release easily into the recycling water, attracting various microbes including foodborne pathogens to the roots, if present. Besides, unlike soil-based systems, STEC can freely flow through the water-based systems, attach, and form biofilms on the roots, and detect entry points in the roots. In supporting our view, a different level of internalization with *E. coli* O157:H7 was found in spinach, where both the roots and shoots were internalized in hydroponic medium, but only in the roots when grown in soil (Sharma et al., 2009). Therefore, unlike soil-based systems, the impact of root exudates in water-based systems is considered significant for STEC in finding

the point of entry even with low-density contamination, due to no spatial boundaries of root spread and dynamic water movement.

### 3.4.3 The Effects of Plant Age and Root Damage on the Degree of Internalization of STEC in Plants

STEC was present in the roots of both T1 and T2 plants in aquaponics, while it was present in the leaves of T1 plants only (Table 3). The results demonstrate the interaction between plant age and root damage, which together play important roles in determining the degree of internalization of STEC in plants. STEC can colonize and internalize the roots and rhizosphere of plants, and the growth of STEC can be supported by root metabolites (Hou et al., 2013; Erlacher et al., 2015). In our study, four plant species were subjected to root damage treatments either at transplanting or at preharvest. The seedlings were 14-day-old after the seeds were germinated at the time of transplanting for both T1 and T2 plants. In T1 treatment, following the root damage treatment, the seedlings were grown in contaminated water for 30 days. In T2 treatment, plants were 20 days old at the time of root injury and grown for additional 7 days in contaminated water until harvest. Root damage can occur during transplanting or handling; therefore, this approach suggests a potential mechanism and provides practical implications to develop strategies by targeting and minimizing the potential risk of food pathogen internalization in soilless culture systems.

Most research on the internalization of human pathogens focused on the effect of injury on the internalization and reported that damaged roots caused by mechanical or biological damages were associated with the internalization of enteric pathogens (Deering et al., 2012b, 2011; Bernstein et al., 2007a, 2007b). Internalization of *E. coli* to roots was detected in lettuce grown in hydroponics after root cutting and in spinach after exposure to the northern root-knot nematode (Moriarty et al., 2018; Hora et al., 2005). Ward and Mahler (1982), inoculated phage f2 at the midpoint of corn and bean plants in hydroponic systems and found a large number of phage were taken from cut roots within 1 day, but no phage uptake through intact roots (Ward and Mahler, 1982). Bernstein et al (2006) also indicated that the plants with damaged roots had 27.8 times higher number of *Salmonella enterica* serovar Newport in roots than the plants with intact roots at 2 days after inoculation (Bernstein et al., 2007a).

Our study showed that plant age plays a critical role in the internalization of STEC in water-based systems and that infection during the early stage of plant development can increase the

persistence of human pathogens in the plant. STEC was found in the leave of T1 plants but not in the leaves of control or T2 plants (Table 3), suggesting that it takes more than 7 days for enteric pathogens to translocate to edible parts of mature lettuce plants through damaged root tissues. Root damage allows STEC to attach and internalize in the roots within a week, but a longer period is needed for STEC to internalize into the shoots. When plants are young, their root systems are small and more susceptible to plant pathogens or human pathogens (Raftoyannis and Dick, 2002). As plants mature, they can develop additional defense mechanisms against plant or human pathogens (Asraful Islam et al., 2010). For example, Islam et al. (2010) evaluated the effect of plant age on anti-human pathogenic activity of endophytes in balloon flower roots and found the highest anti-human pathogenic activity (70%) in 3-year-old roots (Asraful Islam et al., 2010). Due to the differences in plant maturity at the time of root damage and the growing period afterward, the two-time points may allow attachment, internalization, and persistence of STEC to occur differently. The differences in the internalization in these age groups may be also related to the differences in the level of reactive oxygen species and callose deposition against *E. coli* O157:H7 (Jacob and Melotto, 2020).

However, some studies have demonstrated that the internalization of STEC is negatively associated with the age of plants (Mootian et al., 2009; Bernstein et al., 2007b). Bernstein et al (2007) reported that *Salmonella* was present in 33-day-old lettuce but not in 17-day-old lettuce at 2 days after inoculation of 17- and 33-day-old lettuce with $10^6$–$10^8$ CFU/g *Salmonella* in soil (Bernstein et al., 2007b). Mootian et al. (2009) observed a higher number of *E. coli* O157:H7 internalization in 30-day-old lettuce compared to 12-day-old lettuce in soil culture system after exposure to $10^1$–$10^4$ CFU/g *E. coli* O157:H 7 (Mootian et al., 2009). As discussed earlier, the discrepancy among the studies may be related to the differences in the concentration of human pathogens, application method, and culture systems, and this aspect needs further clarification.

## 3.5 Conclusions

Sanitation and good hygiene intervention is effective in interrupting STEC contamination and internalization in hydroponics. Contamination with STEC can be eliminated in hydroponic systems if the entire system is thoroughly sanitized before each use. However, such intervention may not be possible in aquaponics as the biofilters should not be disturbed for the microbiome and

may not be effective once polluted fish is introduced to the system. Therefore, obtaining a clean fish stock is of prime importance in aquaponic operation. STEC may be present at an undetectable level without enrichment but can colonize roots even at low density.

Enteric pathogens can internalize in the roots by mechanical injury, and the internalization of STEC in the leaves can occur, especially when the roots are damaged at transplanting, allowing sufficient time for STEC to transmit to shoots. Since plant age at the time of root injury is critical for the degree of transmission of STEC in plant tissues, it is essential to prevent root damages during transplanting to minimize the risk of contamination of fresh vegetables and herbs grown in soilless production systems.

## Author Contributions

Y.-J.W. conducted the experiment, collected data, undertook data analysis and interpretation, and drafted the manuscript. A.J.D. supervised the microbial analysis and was involved in manuscript production. H.-J.K. coordinated and supervised the research, made critical revisions, and completed the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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## Institutional Review Board Statement

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Purdue University, West Lafayette, IN 47907, USA (protocol number 1507001272) on September 30, 2015.

**Informed Consent Statement**

Not applicable.

**Data Availability Statement**

Data are available from the corresponding author on a reasonable request.

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**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

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# CHAPTER 4. INTERACTIVE EFFECTS OF PH AND NUTRIENT UPTAKE ON GROWTH OF SIX PLANT SPECIES IN AQUAPONIC AND HYDROPONIC SYSTEMS

## 4.1 Abstract

Aquaponics, a sustainable food production system of growing aquatic organisms and plants symbiotically in a recirculating system, can address agricultural challenges such as water scarcity and global food security. An optimal pH range of 7.0 to 7.2 is suggested to maintain reasonable nitrification rates for nitrification, the critical step in aquaponics, which occurs at pH 8.0; however, the pH is not desirable for plant growth. To determine the effects of pH in an aquaponic system, Swiss chard (*Beta vulgaris* L. var. cicla), kale (*Brassica oleracea var. sabellica*), mustard green (*Brassica juncea* cv. Golden Frill), cilantro (*Coriandrum Sativum* L.), lettuce (*Lactuca sativa* 'Big Boston'), and arugula (*Eruca vesicaria* (L.) Cav.) were cultured with tilapia (*Oreochromis niloticus*) in 5-year-old coupled aquaponics at three pH levels: 6.0, 6.5, and 7.0. Morphological and physiological growth parameters of vegetable and fish crops were measured regularly. At the end of the study, ammonia oxygen bacteria (AOB) in aquaponics were analyzed by qPCR. The results showed that feed conversion ratio (FCR) and fish biomass were not affected by pH, but lower pH positively affected fresh and dry mass of all plant species. Swiss chard, kale, cilantro, and mustard green had higher SPAD value at pH 6.0 than at pH 6.5 and pH 7.0 in both systems. Especially, Swiss chard, kale, and cilantro had significantly a higher Fv/Fm value at pH 6.0 than at pH 7.0 in aquaponics, but the value was not affected by pH in other plant species. Although there was no significant difference in photosynthetic rate among the pH treatment, the biomass of all plant species was greater at pH 6.0 than at pH 6.5 and pH 7.0. Meanwhile, the pH treatment did not affect the copy number of AOB, indicating that pH has little effect on nitrification in mature aquaponic systems used in this study. There was no significant difference of nutrient level in aquaponic and hydroponic solutions, but the nutrient concentrations in plant tissues were significantly affected by pH treatment. Arugula, cilantro, lettuce, and Swiss chard had a higher plant biomass and higher nutrient concentrations in plant tissues at lower pH. Mustard green and kale also had a higher biomass at lower pH, but tissue nutrient concentrations were not affected by pH in both aquaponic and hydroponic systems. This study suggests that pH greatly affects plant

performance and yield in aquaponics and that pH 6 is desirable to improve crop yield in aquaponic systems.

## 4.2 Introduction

Facing the challenge of food demand and sustainable agricultural practices, aquaponics has emerged as an important concept of global food production. Aquaponics, an integration of aquatic organisms and plants symbiotically in a recirculating system, has been suggested as a sustainable food production system, which can address agricultural challenges such as water scarcity and global food security.

The major source of nutrients in aquaponics is fish feed. In aquaponics, most fish species only absorb 20-30% nitrogen (N) in the diet, but release the rest 70-80% as waste into water, which could be used as nutrients for crop growth (Yang and Kim, 2019b). Within these systems, the waste produced by aquatic organisms is filtered through tanks of microbes, which convert ammonia ($NH_4^+$) to nitrite ($NO_3^-$) for plants uptake (Wongkiew et al., 2017a, 2018). Plants can absorb N either in the nitrate or ammonium form, but high concentration of $NH_4^+$ is toxic and reduces plant yields (Britto and Kronzucker, 2002).

Meanwhile, fish use only 15% of the phosphorus in fish feeds, and plants have different availability to absorb phosphorous from recycling aquaculture wastewater based on different aquaponic designs (Schmautz et al.; Rafiee and Saad, 2005; Yang and Kim, 2020b). Fish feed also contains K and other micronutrients, but the amount of K and other micronutrients can be limited for plant growth since the fish feed contains insufficient levels of iron (Fe), manganese (Mn), magnesium (Mg), copper (Cu) other than fish requirements (Rakocy, 2012; Villarroel et al., 2011; Seawright et al., 1998). Therefore, it is common to observe some nutrient deficiency in plants when aquaponics relies on fish feed for plant nutrients (Rakocy, 2012; Villarroel et al., 2011; Seawright et al., 1998). Quagrainie et al (2018) pointed out that aquaponic growers are hard to get profit from vegetables compared to hydroponics growers (Quagrainie et al., 2018). To prevent the deficiency of these nutrients, it is suggested to apply synthetic salts into the system water or apply a foliar spray (Rakocy, 2012; Roosta, 2014).

The nutrient compositions in aquaponic solution are affected by many factors, such as fish feeding rate, hydraulic loading rate, and pH (Wongkiew et al., 2017a; Yang and Kim, 2020c, 2019b). Especially, pH is a critical factor to regulate the nutrient level in aquaponics. The pH should be set to balance among three different organisms. The critical step in aquaponics is nitrification, which converts toxic $NH_3$ to $NO_3^-$ for plants uptake (Wongkiew et al., 2017a, 2018). The growth of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) is restricted at pH 5.8 and 6.5, respectively, and the activity ceases typically below pH 5.5 in liquid pure culture (Gieseke et al., 2006; Jiang and Bakken, 1999; Hankinson and Schmidt, 1988). As a result, it has been suggested that aquaponic systems should be set at pH 7.0 to make the best performance of nitrification in aquaponics. Contrarily, the recommended pH for hydroponics is 5.5–5.8 (Bugbee, 2004). The available phosphorus for plants depends on the pH (Asao, 2012). Such high pH can limit nutrient availability, negatively impacting plant growth. For example, when pH increases above 7.0, most P turns into insoluble complexes, and 30–65% phosphorus remains in solid fish sludge as an unavailable form to plants. Moreover, when pH is higher than 6.5, iron, copper, zinc, boron, and manganese become less available for plant uptake (Asao, 2012).

In this study, we established deep-water coupled aquaponic systems to investigate the interactive effects of pH and nutrient uptake on the growth and yield of six plant species in aquaponic and hydroponic production systems. Our findings will lead to better understanding on how pH affects nitrification and plant yield and find the potential solution for limited nutrients and plant growth in aquaponics.

## 4.3 Materials and Methods

### 4.3.1 System design

Experiments were repeated three times between February and July 2019. Three identical aquaponic systems and three hydroponic systems were assembled in a greenhouse at Purdue University in West Lafayette, USA (40º 25’ 26.4’’ N, 86° 55’ 44.4’’ W) (Fig. 4.1). Each unit equipped with a fish tank or a nutrient reservoir (350 L), a clarifier (20 L), a two-stage biofilter (40 L) (Yang and Kim, 2019; Wongkiew et al., 2017b) and a deep-water hydroponic grow bed (350 L; 1.0 m2) (Fig. 4.1). The biofilter was connected to the peristaltic pumps (MasterflexLive™ Cole-Parmer L/S Digital Drive, Vernon Hills, IL, USA). The biofilter was connected to a

peristaltic pump (Masterflex, Cole-Parmer, USA) to recirculate nutrient solution within a system unit. In aquaponics, plants were cultured in combination with tilapia (Oreochromis niloticus) with fish feed (Purina® AquaMax® Sport Fish MVP, USA), which is the major source of fertilizer for vegetables (Table 4.1). Each plant growth bed was filled with 350L water and was covered with a 1 $m^2$ foam raft. The plant holders allowed for densities of 24 plants per $m^2$. The total water volume in each aquaponics/hydroponics unit was 760 L under a flow rate of 125 L/h, giving a water retention time of 168 min in fish tank/nutrient solution reservoir and in floating system unit. Each of aquaponic and hydroponic system had air stones to maintain dissolved oxygen (DO) concentrations above 5 mg/L and heater to maintain temperature at 23°C. Temperature, pH, electrical conductivity (EC) and DO were measured everyday by using HANNA Instrument HI 9811-5 (Hanna Instruments, Inc., Smithfield, RI, USA). The photoperiod was 14-h (8:00 am to 10:00 pm) consisting of natural daylight with supplemental lighting using high-pressure sodium (HPS) lamps (600-W, P.L. Light Systems Inc., Beamsville, ON, Canada). A supplemental photosynthetic photon flux (PPF) of the greenhouse was measured using a quantum sensor (LI-250A light meter; LI-COR Biosciences, Lincoln, NE) and photosynthetically active radiation in the greenhouse was averaged at 134 µmol/$m^2$/s. Day (8:00 am to 10:00 pm) and night (10:00 pm to 8:00 am) temperatures were set at 24 and 18°C, respectively, with an hour transition between two temperature regimes. Depending on ambient temperature, the greenhouse was cooled as needed using a fan-and-pad evaporative-cooling system, heated using radiant hot-water-pipe heating, and retractable shade curtains regulated by an environmental control system (Maximizer Precision 10, Priva Computers Inc., Vineland Station, ON, Canada).

### 4.3.2 Plant and Fish Materials

Arugula (*Eruca vesicaria* (L.) Cav.), cilantro (*Coriandrum Sativum* L.), kale (*Brassica oleracea var. sabellica*), mustard green (*Brassica juncea* cv. Golden Frill), lettuce (*Lactuca sativa* 'Big Boston') and Swiss chard (*Beta vulgaris L. var. cicla*) were used in this study. Seeds were purchased from a commercial source (Johnny's Selected Seeds, Winslow, ME) and sown in Agrifoam soilless plugs (SteadyGROWpro, Syndicate Sales, Kokomo, IN) with few days interval to ensure uniform seedling size at the time of transplanting. Seeds were germinated as described by Kim et al. (2018) and 2 to 3 weeks old seedlings were transplanted to aquaponic and hydroponic

systems. Planting densities were 24 plants per $m^2$ (Kim et al., 2018b). Seeds were irrigated with tap water, and gradually increase to a half-strength fertilizer solution after germination. Seedlings were irrigated with full fertilizer after true leaves developed (Kim et al., 2018b). The fertilizer was combined with two water soluble fertilizers (3:1 mixture of 15N–2.2P–12.5K Cal-Mag Special and 21N–2.2P–16.6K Multi-Purpose fertilizers, respectively; Everris NA, Dublin, OH) (Table 4.1).

Nile tilapia (*Oreochromis niloticus* L.) fish were obtained from the Animal Sciences Research and Education Center at Purdue University. Fish fresh weight was measured and evenly distributed to three different fish tanks with a stocking density of 20 $kg/m^3$ in each aquaponic system. The fish feed used in this study was a complete diet containing 41% protein and 1.1% phosphorus (AquaMax Sport Fish 500, Purina Mills, St. Louis, MO) with 4.8-mm floating pellets, and the nutrient composition and concentration are shown in Table 4.1. Fish were fed by 1% of fish weight once per day at 10:00 am. Fish were weighed at the beginning, weekly, and at the end of the experiment by removing fish from fish tank to another tank filled with water. The average weight was used to determine the amount of fish feeds. Water temperature was maintained at 26–28 °C for tilapia by aquarium thermostat heaters (Eheim Jager TruTemp, Germany).

### 4.3.3 Measurement of water quality parameters

The electrical conductivity (EC) was at 1.5 dS/m and maintained by adjusting the feeding rate in aquaponics and hydroponics. The pH of the aquaponics and hydroponics was maintained at pH 6.0, 6.5, and 7.0 by 10% $H_2SO_4$ or a combination of base solutions (0.02 mM $Mg(OH)_2$ and 0.02 mM $Ca(OH)_2$. =1:1 (v:v)). Each culture system had air stones to maintain dissolved oxygen (DO) concentrations at full saturation and aquatic heaters were set in aquaponic systems to maintain the temperature in the ranges of 22 to 25 °C. EC, pH, water temperature, and DO were measured daily before feeding by HQ40d portable water quality lab package (HACH Corp., Loveland, CO, USA).

### 4.3.4 Quantitative PCR of ammonia oxidizing bacteria

The abundances of AOB were quantified via SYBR Green chemistry qPCR using specific primers targeting *amo*A. The forward primer used (*amo*A-1F; 5'-GGGG TTTCTACTGGTGGT) targets a stretch corresponding to positions 332 to 349 and the reverse primer used (*amo*A-2R; 5'-

CCCCTCKGSAAAGCCTTCTTC [K =G or T; S= G or C]) targets a stretch corresponding to positions 802 to 822 of the open reading frame published previously for the *amo*A gene sequence of AOB. The standard thermal profile used for the amplification of the amoA target sequence was as follows: 5 min at 94°C; pause at 80°C to add polymerase; then 36 cycles (pure cultures) or 42 cycles (environmental samples) consisting of 90 s at 60°C (annealing), 90 s at 72°C (elongation), and 60 s at 94°C (denaturation); and a final cycle consisting of 90 s at 60°C and 10 min at 72°C. The qPCRs were performed in duplicate on iQ5 real-time PCR thermal cycler and analyzed with iCycler iQTM software (BioRad Laboratories, Hercules, CA, U.S.A.). Standard curves for qPCR were generated via serial decimal dilutions of plasmid DNA and primer specificity.

### 4.3.5 Measurement of fish growth rate and feed-conversion ratio

At the beginning and end of experiments, fish samples were collected from each aquaponics system and weighted to get total fish biomass. The fish stocking density was calculated as the total fish mass in each aquaponic system divided by the volume of the fish tank. The Specific growth rate (SGR) and feed-conversion ratio (FCR) was calculated by the following formula:

SGR = (ln final weight of fish−ln initial weight of fish) × 100/days.

FCR = total weight of fish feed applied/total fish biomass increase (wet weight).

### 4.3.6 Measurement of plant biomass and photosynthetic properties

All plant tissues were harvested at 30 days after transplanting into aquaponics and hydroponics. At harvest, all plant samples were divided into roots and shoot (stems and leaves,), and weighed for fresh weight. Plant samples were placed in oven at 70°C for 72 hr. and weighed for dry biomass. All dried plant samples were filtered through a 10-mesh sieve after grinding with a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and kept in plastic vials for nutrient analysis.

The chlorophyll fluorescence parameter Fv/Fm (maximum photochemical efficiency of PSII) been widely used for the evaluation of plant stress response in various species, especially for the early stress detection in plants (Sharma et al., 2015). Fv/Fm were recorded and calculated by Plant Efficiency Analyzer Fluorimeter (PEA, Hansatech Instruments, England). Five leaves per each plant were taken and averaged, and four replicate plants were taken and averaged from each

treatment every three days after transplanting. Averaged Fv/Fm was recorded by a handy chlorophyll fluorescence meter (handy PEA+, Hansatech Instruments, Norfolk, UK). Fully expanded young leaf was shield by dark adaptation leaf clips (PPEA/LC, Hansatech Instruments, Norfolk, UK) for 20 minutes. The meter was set as 3,000 μmol/m2/s at 650 nm to a 4 mm diameter area within the sensor enclosure. The SPAD value (an index of chlorophyll content per unit leaf area) readings were taken weekly on each young fully expanded leaf using a SPAD-502 Chlorophyll Meter (Minolta Camera Co. Ltd., Japan). Five readings per leaf were taken at the central point of a leaf between the midrib and the leaf margin for leafy vegetable and herb, and the terminal leaflet for tomato and the values were averaged. Leaf temperature was measured at the third week after transplanting using a hand-held infrared radiometer (MI-210, Apogee Instruments, Inc., Logan, UT, USA) at a distance of approximately 4.8 cm from the leaf surface. At harvest, all plant samples were divided into different plant tissues (roots, stems, leaves, and/or flowers and fruits), and weighed for fresh weight. All leaf samples were scanned for leaf area by using a LI-3100 leaf area meter (LICOR, Lincoln, NE, USA) immediately after harvest. Plant samples were oven-dried (over 72 h at 70 °C) and weighed for dry weight. All dried samples were filtered through a 10-mesh sieve after grinding with a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and kept in plastic vials for nutrient analysis.

Gas-exchange measurements were performed using a portable gas exchange system (LI-6400XT; LICOR Biosciences, Lincoln, NE) equipped with a 6-cm2 leaf chamber with built-in LEDs (470 and 665-nm peak wavelengths for blue and red LEDs, respectively) on recently fully expanded leaves at each canopy level. Illumination was supplied at a PPF of 400 μmol/$m^2$/s by red and blue LEDs at a ratio of 9:1 under ambient temperature conditions when supplemental lighting was in use. The reference $CO_2$ concentration and flow rate through the chamber were 400 μmol/mol and 500 μmol/s, respectively. One leaf at each canopy level was selected from each plant for the measurements. The measurements of photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (E), and internal CO2 (Ci) were conducted between 9:00 am and 14:00 pm at a PPF of 400 μmol/$m^2$/s. Readings were taken when the coefficient of variation (i.e., sample CO2, sample H2O, and flow rate) was less than or equal to 0.2%, which typically occurred within 10 min.

### 4.3.7 Mediation analysis

Fig 4.2 illustrates the relationship between pH effects, dependent variable (such as Fv/Fm, and shoot and root ratio), and plant yield (harvest index). The harvest index is the ratio of harvested shoot fresh weight to total plant fresh weight, and this can be used to measure the reproductive efficiency (Hay, 1995). The function is shoot fresh weight / total plant fresh weight × 100% (Hay, 1995). Fig. 4.2 depicted the corresponding structural equations would have the form (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012):

X = F1(e1) z = F2(x, e2) y = F3(x, z, e3) (1)

where X, Y, Z are discrete or continuous random variables, F1, F2, and F3 are arbitrary functions, and e1, e2, e3 represent omitted factors. With above formula, we next determined total effect, T $E_{0,1}$ which measures the change in harvest index produced by a unit change in pH effects, and therefore, we had it for the total effect (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012):

TE0,1(e2, e3) = F3[1, F2(1, e2), e3] − F3[0, F2(0, e2), e3] (2)

Then, we determined the natural effects by the following formula (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012):

$$NDE_{x,x'}(Y) = \sum_z [E(Y|X = x', Z = z) - E(Y|X = x', Z = z)]P(Z = z\,|X = x) \quad (3)$$

$NDE_{x,x'}(Y)$ is defined as the expected change in Y induced by changing X from x to $x_0$ while keeping all mediating factors constant at whatever value they would have obtained under X = x, before the transition from x to $x_0$ (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012).

### 4.3.8 Data analysis

The experiment was conducted by using three aquaponic systems and three hydroponic systems. Each system provided space for 24 plants to grow. pH was set as 6, 6.5 and 7 for three aquaponic systems and three hydroponic systems accordingly. Fig 4.2 illustrates the sequence of the steps used to evaluate statistical models. This study focused on the pH effects on plant yields associated with Fv/Fm in soilless production systems. Mediation analysis is a statistic model to illustrate observed relationship between independent variable and dependent variable with a third hypothetical variable (Imai et al., 2010b; Lee et al., 2018; Zhao et al., 2010). For the analysis of

water conditions, plants and fish performance, and nutrient concentration in aquaponic and hydroponic systems, completely randomized design and two-way ANOVA were used. The water quality parameters in the aquaponic and hydroponic systems were tested by using Tukey's Honest Significant Difference. The statistical analysis used post-hoc pairwise comparisons in R 3.6.1 (R, Comprehensive R Archive Network, USA) at a significance level of 0.05.

## 4.4 Results

### 4.4.1 Water Physical and Chemical Parameters

Solution pH was maintained at an average of 6.0, 6.5, or 7.0 in aquaponic and hydroponic systems based on the pH treatment, which led to a significant difference in the usage of pH correction solutions (Table 4.2). Nutrient input was different in aquaponics and hydroponics due to the different nutrient sources (Tables 4.1 and 4.2); however, the average EC was not affected by either the system or pH treatment (Table 4.2). The DO and water temperature were maintained above 7 mg/L and 22°C, respectively, for both systems, and there was no difference between the systems and among the pH treatments (Table 4.2). In aquaponic solution, there was no significant difference in the concentrations of $NH_4$-N, $NO_3$-N, and $NO_2$-N among the pH treatments. Additionally, AOB was not affected by different pH treatments (Table 4.3).

### 4.4.2 The pH effects on fish production

During 3 month experiments period, the fish biomass gain, SGR, and FCR of Nile tilapia were not significantly affected by different pH treatment in aquaponics (Table 4.4).

### 4.4.3 Plant biomass

The average biomass of most plant species was significantly affected by the production system and pH treatment (Table 4.5). Plants had higher fresh and dry weight in hydroponics than in aquaponics regardless of pH treatment except lettuce. In general, the total fresh weight of arugula, cilantro, kale, mustard green, and Swiss chard significantly increased in hydroponic systems by 142.5%, 50.6%, 52%, 48.7%, and 52.2%, respectively, compared to those in aquaponic systems (Table 4.5). Additionally, shoot fresh and dry weight was affected by different pH in all

plant species. Regardless of the production system, plant species grown at pH 7 had lower total fresh and dry weight and shoot fresh and dry weight compared to those at other pH treatments. For example, the total fresh weight of arugula, cilantro, kale, lettuce, mustard green, and Swiss chard was 45.6%, 51.6%, 28.7%, 14.7%, 34%, and 36.3%, respectively, lower at pH 7 than at pH 6 (Table 4.5). In aquaponics, the shoot fresh weight of arugula was 93.7% and 253.4% higher at pH 6 than those at pH 6.5 and pH 7, respectively (Table 4.5), and there was no significant difference of root biomass of arugula between different pH in aquaponics. Similarly, cilantro had 81.2% and 149.3% higher shoot fresh weight at pH 6 than at pH 6.5 and 7, respectively. On the other hand, the biomass of lettuce, and Swiss chard was less affected ($P < 0.05$ and $P < 0.01$, respectively) by pH treatments compared to other plant species ($P < 0.001$).

### 4.4.4 Leaf chlorophyll and Photosynthetic Parameters

SPAD values of crops grown in aquaponics and hydroponics were not significantly affected by the pH treatment when measured at 7 days after transplanting into aquaponics or hydroponics (Table 4.6). The pH significantly affected SPAD value in most plant species after transplanting (Fig 4.3). When grown in aquaponics, SPAD value of arugula, cilantro, mustard green, and kale gradually, especially pH 6.5 and pH 7 (Fig 4.3). Meanwhile, SPAD value of lettuce and Swiss chard was less affected by pH and remained relatively constant throughout production period.

The Fv/Fm value indicates high PSII maximum light conversion efficiencies, and the value of healthy C3 plants is above 0.8 (Kalaji et al., 2012; da Silva Branco et al., 2017; Zhou et al., 2019). The Fv/Fm values of most of plant species were lower than the threshold vale of 0.8 when measured at 7 days after transplanting except when they were grown at either pH 6 or pH 6.5 (Table 4.6). None of the plant species grown at pH 7 reached the threshold value. Both the system and the pH did not affect Fv/Fm value in most plant species.

The Fv/Fm value of six plant species decreased after transplanting into aquaponics and hydroponics, and most of them recovered at 14 days after transplanting (Fig 4.4). There was a tendency that plants grown in aquaponics with pH 6.5 or pH 7 demonstrated relatively a rapid drop of Fv/Fm during the first 7 days after transplanting, which was gradually recovered during the rest of production period (Fig. 4.4). During the later production period, chlorophyll fluorescence of

most plant species were somewhat negatively affected by the aquaponic system, showing lower values than the counterpart (Fig 4.4). In general, there was no dramatic effect of production system nor pH treatment on photosynthetic parameters, such as photosynthetic rate (*Pn*), stomatal conductance (*gs*), transpiration rate (*Tr*), and intercellular $CO_2$ concentration (*Ci*) when measured at 28 days after transplanting (Fig 4.5)

### 4.4.5 The nutrient concentrations in plants

In general, the nutrient concentration in most plant species was significantly affected by the system and pH except mustard green, although there were variations among plant species and the type of nutrients (Table 4.7). There were no interactions between the system and pH in the accumulation of most of the nutrients in plants.

In arugula, the accumulation of N and Ca were the highest at pH 6, but the concentrations of P, K, and Mg reduced by the pH. Similarly, the concentrations of micronutrients (Fe, Mn, B, Cu, and Zn) were also reduced by the low pH.

In cilantro, the concentrations K, Mg, Ca and S were not significantly different between aquaponics and hydroponics (Table 4.7). Cilantro had higher concentrations of N, P, and Fe when grown in hydroponics. A lower pH increased the concentration of these nutrients in plant tissues. Whereas, the concentrations of K and Mg in plant tissues were significantly higher at pH 6.5 and pH 7 than at pH 6. In mustard green, the concentrations of most nutrients were not significantly affected by the system and/or pH except S and Mn (Table 4.7). The concentrations of K, S, Fe, Mn, B, and Zn in kale were increased by aquaponic systems (Table 4.7); additionally, nutrient concentrations in kale were significantly affected by the pH (Table 4.7). The concentration of N, P, Mg, Ca, S, Mn, and B in Kake were significantly higher at pH 6 (Table 4.7). Meanwhile, the production system significantly affected the concentration of P, K, Mn, and Zn in lettuce and concentration of N, P, Ca, Fe, Mn, Cu, and Zn in lettuce were significantly affected by the pH (Table 4.7). Lettuce had a lower level of N, Ca, Fe, and Cu and higher a level of P, Mn, and Zn at pH 6 (Table 4.7). In Swiss chard, the production system and the pH had a significant effect on the concentration of most of the nutrients in plant tissues (Table 4.7). The concentration of P, K, Ca, Fe, Mn, Zn in Swiss chard were significant higher in aquaponics than hydroponics (Table 4.7). Moreover, the concentrations of most of the nutrients in Swiss chard were reduced by lower pH

(Table 4.7). Swiss chard at pH 7 had higher concentrations of P, K, Mg, Mn, B, and Zn compared to the one at pH 6 and pH 6.5 (Table 4.7).

### 4.4.6 The mediation analysis of pH effects on nutrient concentration in plant tissues and total fresh weight

The mediation model is shown in Fig 4.2. We used casual mediation analysis to examine the mediation effect of nutrients, and the data should fit the following functions to fit the mediation model.

Total fresh weight =b0+b1* pH effect +e (4)

Dependent variable =b0+b2* pH effect +e (5)

Total fresh weight =b0+b4* pH effect +b3* dependent variable +e (6)

The *P*-value of average casual mediation effect (ACME) of most plant nutrients was not significant, which indicated those variables were no mediation effects between pH treatment and plant yields (Table 4.8). Meanwhile, the nutrient concentrations in plant tissues had direct effects in pH treatment and plant yields, since the *P*-value of average direct effect (ADE) of all nutrients was lower than 0.05 (Table 4.8). The *P*-value of both ACME and ADE of Mn were lower than 0.05, which indicated the partial mediation effect of pH treatment on Mn and plant yield in aquaponics and hydroponics.

## 4.5 Discussion

### 4.5.1 Physical and Chemical Parameters of Aquaponic Solution

Water physical parameters, such as EC, DO, and water temperature, have direct impacts on plant yield in soilless culture systems. In this study, EC level was maintained above 1.5 mS/cm for plant growth in aquaponics and hydroponics (Oliveira et al., 2011; Yang and Kim, 2020a; Kappel et al., 2021). The EC level in aquaponics increased with time (data not shown), which may due to the accumulated mineral nutrients in aquaponic solution (Yang and Kim, 2020a). The DO was maintained above 6 mg/L to support nitrification and fish growth in aquaponics (Graber and Junge, 2009; Tyson et al., 2004). The water temperature also can affect plant crop yield in soilless culture systems. Thompson et al. (1988) indicated the root temperature can have better market quality and production of lettuce at around 24 °C (Thompson et al., 1998). In this study, water

temperature ranged between 22 and 25°C in all treatments, which is considered optimal for better plant yield and fish feed conversion ratio (Beamish, 1970; El-Sayed, 2019; Thompson et al., 1998).

### 4.5.2 The pH effects on nitrification activity

In the process of nitrification, both AOB and NOB had great influences on the $NH_4$–N removal, $NO_2$−N, and $NO_3$−N production (Xu et al., 2012). The growth rate of nitrifiers are affected by different temperatures and pH values due to the energy requirement of cell maintenance (Chen et al., 2006; De Boer et al., 1991). The pH ranging from 7.0 to 9.0 were reported to be the most efficient pH for nitrification activity in aquaculture biofilters (Wheaton, 1998; Masser et al., 1999). Tyson et al. (2004) examined the effects of pH on nitrogen transformations in the biofilters in aquaponics (Tyson et al., 2004). They found that no nitrification occurred at pH 5.5, and it took 12, 20, and 20 to 24 days at pH 8.5, 7.5, and 6.5, respectively, to decrease total ammonia nitrogen from 5 to 0 mg/L, respectively (Tyson et al., 2004). Additionally, Wongkiew et al. (2018) found that there was higher total ammonia nitrogen and lower nitrate concentrations at lower pH levels (6.0 and 5.2) in aquaponic solution than at pH 7 (Wongkiew et al., 2018). However, the recommended pH for growing plants in hydroponics is 5.5–5.8, and high pH can limit nutrient availability, negatively impacting plant growth (Bugbee, 2004). We hypothesized plant can have higher yield at low pH aquaponics due to the higher ability of nutrient uptake, even though lower pH decreases nitrification activity in aquaponics biofilters. In previous study, Zou et al (2016) found that the maximum nitrogen utilization efficiency reached 50.9% at pH 6.0, followed by 47.3% at pH 7.5 and 44.7% at pH 9.0 in media-based aquaponics (Zou et al., 2016). Moreover, nitrogen level of plant tissues was 34.8%, 30.3%, and 28.5% at pH 6.0, 7.5, and 9.0, respectively. The pH 5 led to significantly higher plant yields (Zou et al., 2016). They also found that the nitrogen level in water was not significantly different among the pH treatments (Zou et al., 2016). Additionally, Day et al. (2021) compared the effects of different concentration of nitrifier bacteria in lettuce-based aquaponics (Day et al., 2021). They did not find nitrifying bacteria in aquaponics in both treatments, even though treating commercial nitrifying bacteria led to a higher lettuce yield (Day et al., 2021). Our results were similar to their finding such that there was no significant difference of $NH_4$–N, $NO_2$−N, and $NO_3$−N among the pH treatment in aquaponics, but plant growth was significantly better at pH 6.0. Additionally, there was no significant difference in AOB

population between different pH treatment in our study. These results indicated that the AOB population and the nitrification activity were not significantly decreased by lower pH, but lower pH increased plant yields due to higher nutrients uptake ability in aquaponics.

### 4.5.3 The pH effects on fish and plant yield

There are numerous studies which compared plant yields in aquaponics and hydroponics (Ayipio et al., 2019). Due to the discrepancy in aquaponics designs, fish feeding rate, water flow rate, and different plant and fish material, the studies showed inconsistent results on yield. For example, some studies showed that aquaponics had higher or similar yields compared to hydroponics, but other studies showed that aquaponics had lower plant yields (Yang and Kim, 2020a; Anderson et al., 2017; Alcarraz et al., 2018; Roosta and Afsharipoor, 2012). Roosta and Afsharipoor (2012) found that growing strawberry with 100% of perlite in aquaponics had higher fruit number and yields than hydroponics although strawberry accumulated less N, P, K in aquaponics (Roosta and Afsharipoor, 2012). Meanwhile, Yang and Kim (2020) compared nitrogen and phosphorous use efficiency for lettuce-, basil-, and tomato-based aquaponics and hydroponics, and the nitrogen and phosphorous use efficiency were significant higher in aquaponics than hydroponics even though plant yields were low in aquaponics (Yang and Kim, 2020a). Various environmental factors can affect plant yields in aquaponics, such as fish feeding rate, water flow rate and pH, since those factors can affect nitrogen transformation in aquaponics (Wongkiew et al., 2017a). Yang and Kim (2019) found that nitrogen use efficiency and crop production were increased in aquaponics with uniform fish feeding rate (Yang and Kim, 2019b). Moreover, Yang and Kim (2020) compared the effect of hydraulic loading rate in aquaponic systems, and they found not only plant performances were improved by higher flow rate (3.3 m/day) but also fish growth rate and biomass (Yang and Kim, 2020c).

Optimizing pH can prevent metabolic stress and avoid mortality of stocked fish in ponds. The optimal pH range of water pH is between 5 to 8 for Nile tilapia, and 5.5–5.8 for plants in hydroponics (Nobre et al., 2014; Bugbee, 2004). The higher pH in aquaponics may result in lower plant yields with lower nutrient uptake (Yang and Kim, 2019b; Anderson et al., 2017). In this study, we grew six different plant species in aquaponics and hydroponics with different pH treatment. There were no significant differences of fish biomass gain, SGR, and FCR between

different pH treatment in aquaponics, and plants had lowest total fresh weight at pH 7 regardless of the production system. This result is similar to previous finding. Anderson et al (2017) grew butterhead lettuce at different pH in hydroponics, and the hydroponic system at pH 7 produced 26% less shoot fresh weight than at pH 5.8 (Anderson et al., 2017). Our results showed that it increased 84.0% in arugula, 106.8% in cilantro, 51.6% in mustard green,40.3% in kale, 17.3% in lettuce, and 56.9% in Swiss chard when pH was adjusted from 7 to 6. These results indicated that plants had higher yield at pH 6 than pH 7 in aquaponics and hydroponics due to higher nutrient uptake ability. In our study, the K level in most plant tissues was higher at higher pH in aquaponics than hydroponics, since the pH was adjusted by KOH and $Ca(OH)_2$. Meanwhile, the concentration of N in arugula, cilantro, kale was higher at pH 6 than pH 7, it indicated that the ability of nitrogen uptake in arugula, cilantro, kale were better at lower pH which resulted in higher plant yields. The higher concentration of P in cilantro, kale, and lettuce at lower pH was associated with higher plant yields in aquaponics and hydroponics. The higher plant yields in lower pH due to higher nutrient uptake ability of plants. The availability of phosphate, Fe, Mn, B, and Zn decreased at alkaline pH (Neina, 2019). Bress and Weston (1992) cultured lettuce in hydroponic systems with different pH level (5.0, 5.5, 6.0, and 6.5) and found the significant influence of solution pH on P, Ca, and Mg (Bres and Weston, 1992). Moreover, Anderson et al. (2017) investigated that the level of N, Cu, and Mo was low at pH 5.8 in hydroponic lettuce which led to 26% and 18% reduction in lower shoot fresh and dry weight, respectively (Anderson et al., 2017). In this study, lower pH may be associated with unstress environment and lead to higher nutrient level, which led to higher plant yields. Arugula, cilantro, lettuce, and Swiss chard had higher nutrient level which resulted higher plant biomass at lower pH. However, Mustard green and kale didn't have higher nutrient level at lower pH but have higher plant biomass at lower pH in aquaponics and hydroponics. It needs to have further study to understand the tolerance of pH for aquaponic plants.

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Table 4.1. Nutrient composition and concentration contained in hydroponic nutrient solutions and aquaponics fish feed.

| Parameter | Hydroponics fertilizer [a] | Aquaponics fish feed [b] |
|---|---|---|
| Macronutrient (%) | | |
| Total nitrogen (N) | 0.043 | > 6.88 |
| $P_2O_5$ -P | 0.093 | > 1.10 |
| $K_2O$-K | 0.035 | 0.99 |
| $SO_4$-S | – | 0.43 |
| Ca | 0.075 | 2.25-2.75 |
| Mg | 0.039 | 0.23 |
| Micronutrient (mg/kg) | | |
| B | 2 | – |
| Cu | 1.05 | 10 |
| Fe | 21 | 40 |
| Mn | 1.9 | 80 |
| Mo | 0.42 | – |
| Zn | 2.1 | 153 |

Data obtained from product description.
"–" means not included or no related information.
[a] Nutrient compositions of fertilizer used in hydroponics were calculated based on 1:100 dilution of commercial fertilizer.
[b] Nutrient compositions of fish feed used in aquaponics were calculated based on g feed per day.

Table 4.2. Average values of physical and chemical water-quality parameters in plant growth bed for 4 weeks

| System | pH | pH correction solution (mL/day) | EC (mS/cm) | DO (mg/L) | Temperature (°C) |
|---|---|---|---|---|---|
| AQU | 6.0±0.0 (5.7-6.3) | 39.5±4.5 (0-170) | 1.57±0.01 (1.45-1.70) | 7.51±0.05 (6.72-8.12) | 22.8±0.0 (22.4-24.0) |
| | 6.5±0.0 (6.4-6.6) | 80.6±7.3 (0-300) | 1.61±0.01 (1.47-1.81) | 7.32±0.05 (6.52-8.12) | 23.3±0.0 (22.6-24.4) |
| | 7.0±0.0 (6.8-7.2) | 91.3±8.5 (0-300) | 1.59±0.01 (1.42-1.73) | 7.25±0.08 (6.52-8.05) | 23.1±0.0 ((22.1-24.0) |
| HYD | 6.0±0.0 (5.8-6.2) | 2.0±1.0 (0-60) | 1.54±0.01 (1.46-1.70) | 7.75±0.03 (6.78-8.12) | 23.2±0.0 (22.5-24.1) |
| | 6.5±0.0 (5.9-6.6) | 0.8±0.6 (0-40) | 1.50±0.00 (1.45-1.65) | 7.80±0.03 (6.65-8.12) | 23.0±0.0 (22.4-23.5) |
| | 7.0±0.0 (6.9-7.1) | 00±0.0 (0-0) | 1.51±0.01 (1.44-1.70) | 7.83±0.03 (6.72-8.05) | 23.6±0.0 (22.5-24.8) |
| ANOVA | | | | | |
| System | | *** | ns | ns | ns |
| pH | | *** | ns | ns | ns |
| System x pH | | *** | ns | ns | ns |

Abbreviations: DO, dissolved oxygen; EC, electrical conductivity, AOB, ammonia oxidizing bacteria. Each value in the table is the mean ± SE (range: min.-max.). nd is not detectable.
ns, *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.
Significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

Table 4.3. Average concentrations of ammonia oxidizing bacteria (AOB), ammonia ($NH_4$–N), nitrite ($NO_2$-N), nitrate ($NO_3$-N) in plant growth bed for 4 weeks.

| System | pH | AOB (Copy numbers/g biomedia) | $NH_4$–N(mg/L) | $NO_2$-N (mg/L) | $NO_3$-N (mg/L) |
|---|---|---|---|---|---|
| AQU | 6.0±0.0 (5.7-6.3) | $5.3\pm0.4x10^5$ | 1.62±0.09 | 0.12±0.08 | 29±1.2 |
| | 6.5±0.0 (6.4-6.6) | $1.1\pm0.1x10^6$ | 1.45±0.11 | 0.09±0.05 | 30.4±0.6 |
| | 7.0±0.0 (6.8-7.2) | $3.2\pm0.6 \times 10^6$ | 1.13±0.08 | 0.12±0.06 | 30.2±0.9 |
| *p* | | ns | ns | ns | ns |

Abbreviations: AOB, ammonia oxidizing bacteria. Each value in the table is the mean ± SE (range: min.-max.).
ns mean no significant.
Significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

Table 4.4. Fish production in aquaponics

| Treatment | Fish feed applied | Initial stocking density ($kg/m^3$) | Final stocking density | Fish biomass gain ($kg/m^3$) | SGR | FCR |
|---|---|---|---|---|---|---|
| pH 6 | 2100 | 19.9 | 27.0 | 2.5 | 8.3 | 0.84 |
| pH 6.5 | 2100 | 20.3 | 27.5 | 2.5 | 8.4 | 0.83 |
| pH7 | 2100 | 20.4 | 27.9 | 2.6 | 8.7 | 0.80 |
| *p* | | ns | ns | ns | ns | ns |

Abbreviations: SGR, Specific growth rate. FCR, Feed conversion ratio.
ns mean no significant.
Significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).
Specific growth rate calculated as SGR = (ln final weight of fish−ln initial weight of fish) × 100/days.
Feed conversion ratio calculated as FCR = total weight of fish feed applied/total fish biomass increase (wet weight).

Table 4.5. Average biomass of arugula, cilantro, and kale in aquaponics and hydroponics.

| Treatments | Fresh weight (g/plant) | | | Dry weight (g/plant) | | |
|---|---|---|---|---|---|---|
| | Total | Shoots | Roots | Total | Shoots | Roots |
| | Arugula | | | | | |
| System | | | | | | |
| Aquaponics | 111.5b | 96.2b | 15.4b | 8.5b | 7.8b | 1.0b |
| Hydroponics | 270.4a | 270.8a | 22.8a | 19.5a | 20.2a | 1.9a |
| pH | | | | | | |
| 6 | 253.7a | 238.0a | 25.6a | 18.2a | 18.0a | 1.9a |
| 6.5 | 181.2b | 188.2b | 20.2ab | 14.1b | 14.0b | 1.7a |
| 7 | 137.9c | 124.4c | 11.5b | 9.6c | 9.9c | 0.8b |
| Significance | | | | | | |
| System | * * * | * * * | * | * * * | * * * | * * |
| pH | * * * | * * * | * * | * * * | * * * | * * |
| System × pH | ns | ns | ns | ns | ns | ns |
| | Cilantro | | | | | |
| System | | | | | | |
| Aquaponics | 72.5b | 55.0b | 23.0b | 6.6b | 4.8b | 2.0 |
| Hydroponics | 109.2a | 80.8a | 27.9a | 9.7a | 7.3a | 2.2 |
| pH | | | | | | |
| 6 | 124.7a | 87.7a | 37.0a | 11.3a | 8.1a | 3.2a |
| 6.5 | 87.6b | 65.0b | 22.6b | 7.7b | 5.8b | 1.9b |
| 7 | 60.3c | 51.0b | 16.9c | 5.4c | 4.4b | 1.1b |
| Significance | | | | | | |
| System | *** | *** | ns | ** | *** | ns |
| pH | *** | *** | *** | *** | *** | ** |
| System × pH | * | ns | * | ns | ns | ns |
| | Mustard green | | | | | |
| System | | | | | | |
| Aquaponics | 218.0b | 195.6b | 17.5 | 17.9b | 16.4b | 1.1b |
| Hydroponics | 324.2a | 300.1a | 19.4 | 26.7a | 25.1a | 1.5a |
| pH | | | | | | |
| 6 | 323.9a | 296.2a | 23.2a | 27.8a | 25.9a | 2.0a |
| 6.5 | 275.6b | 249.3ab | 16.6b | 22.3ab | 20.9b | 1.4b |
| 7 | 213.7c | 198.1b | 15.5b | 16.9b | 15.6c | 0.7c |
| Significance | | | | | | |
| System | *** | *** | ns | *** | *** | * |
| pH | *** | *** | * | *** | *** | *** |
| System × pH | *** | *** | ns | *** | ** | * |

Means within column followed by the same letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$ =0.05). Each value in the table is the mean of 9 replicates of system and 6 replicates of pH treatment. ns, *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 4.5(Cont.) Average biomass of lettuce, mustard green, and Swiss chard in aquaponics and hydroponics.

| Treatments | Fresh weight (g/plant) | | | Dry weight (g/plant) | | |
|---|---|---|---|---|---|---|
| | Total | Shoots | Roots | Total | Shoots | Roots |
| | Kale | | | | | |
| System | | | | | | |
| Aquaponics | 177.3b | 160.3b | 19.1b | 16.0b | 14.5b | 1.6b |
| Hydroponics | 269.5a | 244.4a | 27.9a | 22.8a | 21.4a | 2.0a |
| pH | | | | | | |
| 6 | 247.1a | 223.4a | 22.6 | 22.4a | 20.3a | 1.9 |
| 6.5 | 246.9a | 228.2a | 25.6 | 20.9b | 19.7a | 1.9 |
| 7 | 176.1b | 155.4b | 22.3 | 15.0c | 14.0b | 1.6 |
| Significance | | | | | | |
| System | *** | *** | *** | *** | *** | * |
| pH | *** | ** | ns | *** | *** | ns |
| System × pH | ** | ** | ns | * | ** | ns |
| | Lettuce | | | | | |
| System | | | | | | |
| Aquaponics | 303.9 | 286.5 | 17.2 | 10.4a | 8.9 | 1.3 |
| Hydroponics | 290.0 | 289.6 | 14.4 | 9.1b | 8.4 | 1.0 |
| pH | | | | | | |
| 6 | 334.8a | 315.4a | 20.4a | 11.6a | 9.7a | 1.5a |
| 6.5 | 270.4b | 277.9b | 13.6b | 8.8b | 8.3b | 1.1ab |
| 7 | 285.5ab | 270.9b | 13.5b | 8.8b | 7.9c | 0.9b |
| Significance | | | | | | |
| System | ns | ns | ns | * | ns | ns |
| pH | * | * | ** | ** | ** | * |
| System × pH | ns | ns | ns | ns | * | ns |
| | Swiss chard | | | | | |
| System | | | | | | |
| Aquaponics | 342.9b | 311.7b | 30.3b | 28.4 | 26.7 | 2.5 |
| Hydroponics | 521.9a | 419.2a | 50.0a | 33.5 | 30.4 | 3.0 |
| pH | | | | | | |
| 6 | 526.6a | 393.9a | 53.8a | 38.8a | 35.4a | 3.3 |
| 6.5 | 435.0b | 402.9a | 33.7b | 32.7a | 31.0a | 2.6 |
| 7 | 335.7c | 299.6b | 33.1b | 21.3b | 19.3b | 2.3 |
| Significance | | | | | | |
| System | *** | * | * | ns | ns | ns |
| pH | ** | ns | ns | *** | *** | ns |
| System × pH | ** | ns | ns | ** | ** | ns |

Means within column followed by the same letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$ =0.05). Each value in the table is the mean of 9 replicates of system and 6 replicates of pH treatment. ns, *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 4.6. SPAD value and Fv/Fm of crops grown in aquaponics and hydroponics with different pH. The values were measured at day 7 after transplanting.

| | | SPAD | | | | | | Fv/Fm | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Systems | pH | Arugula | Cilantro | Mustard green | Kale | Lettuce | Swiss chard | Arugula | Cilantro | Mustard green | Kale | Lettuce | Swiss chard |
| AQU | 6.0 | 38.1 | 36.9 | 30.6 | 37.2 | 28.4 | 34.0 | 0.76 | 0.70 | 0.80 | 0.75 | 0.79 | 0.75 |
| | 6.5 | 31.8 | 29.5 | 31.7 | 32.7 | 26.5 | 33.2 | 0.71 | 0.62 | 0.74 | 0.70 | 0.75 | 0.73 |
| | 7.0 | 43.6 | 34.9 | 27.8 | 35.8 | 28.9 | 33.9 | 0.75 | 0.71 | 0.76 | 0.73 | 0.73 | 0.76 |
| | | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| HYD | 6.0 | 45.9 | 35.4 | 32.7 | 37.9 | 27.2 | 31.4 | 0.80 | 0.76 | 0.80 | 0.75 | 0.77 | 0.76 |
| | 6.5 | 38.4 | 38.7 | 30.1 | 43.0 | 30.1 | 31.0 | 0.78 | 0.81 | 0.81 | 0.74 | 0.77 | 0.78 |
| | 7.0 | 39.7 | 34.3 | 30.4 | 32.4 | 26.2 | 32.8 | 0.76 | 0.69 | 0.75 | 0.69 | 0.71 | 0.77 |
| | | ns | ns | ns | ns | ns | ns | ns | ns | * | ns | ns | ns |
| Systems | | ns | ns | ns | ns | ns | ns | * | * | ns | ns | ns | ns |
| pH | | ns | ns | ns | ns | ns | ns | ns | ns | * | ns | ns | ns |
| Systems x pH | | * | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |

Means within systems and pH, and column followed by the same letter are not significantly different based on Turkey's honestly significant difference (HSD) test ($\alpha = 0.05$). ns, *, **,*** mean bi significant or significant at $p \leq 0.05$, 0.01, 0.001, respectively.

Table 4.7. Average mineral nutrient concentrations of arugula, cilantro, and mustard green in aquaponics and hydroponics.

| Treatments | Nutrient concentration (%) | | | | | | Nutrient concentration (ppm) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | P | K | Mg | Ca | S | Fe | Mn | B | Cu | Zn |
| | Arugula | | | | | | | | | | |
| System | | | | | | | | | | | |
| Aquaponics | 5.1 | 0.9a | 9.2a | 0.7a | 2.2b | 0.9b | 48.4a | 135.6a | 42.4b | 12.4a | 275.6a |
| Hydroponics | 5.7 | 0.8b | 8.0b | 0.6b | 2.5a | 1.1a | 42.1b | 62.1b | 44.5a | 7.8b | 164.8b |
| pH | | | | | | | | | | | |
| 6 | 5.7a | 0.8b | 7.8c | 0.5b | 2.8a | 1.3a | 42.5b | 53.8c | 43.1 | 6.8 | 133.5c |
| 6.5 | 5.3b | 0.8b | 9.8a | 0.8a | 2.2b | 1.0b | 51.3a | 112.9b | 46.5 | 10.7 | 187.6b |
| 7 | 5.3b | 1.0a | 8.5b | 0.7a | 1.9c | 0.9c | 41.9b | 129.8a | 40.8 | 13.0 | 339.5a |
| Significance | | | | | | | | | | | |
| System | ns | ** | *** | *** | ** | *** | *** | *** | * | * | *** |
| pH | * | * | ** | ** | ** | *** | ** | *** | ns | ns | *** |
| System × pH | * | ns | ** | ** | ** | *** | ns | *** | * | ** | ** |
| | Cilantro | | | | | | | | | | |
| System | | | | | | | | | | | |
| Aquaponics | 4.0b | 0.6b | 10.1 | 0.4 | 2.1 | 0.4 | 43.7b | 751.1a | 78.5a | 15.4 | 284.8a |
| Hydroponics | 4.7a | 0.8a | 9.1 | 0.5 | 2.2 | 0.4 | 80.3a | 192.3b | 61.1b | 14.2 | 171.3b |
| pH | | | | | | | | | | | |
| 6 | 4.9a | 0.8a | 8.7b | 0.4b | 1.8 | 0.4 | 95.3a | 533.0b | 70.4b | 14.5 | 214.7 |
| 6.5 | 4.2b | 0.7b | 10.1a | 0.5a | 2.3 | 0.4 | 56.5b | 619.8a | 58.5c | 15.3 | 263.7 |
| 7 | 3.9b | 0.6c | 10.0a | 0.5a | 2.3 | 0.4 | 34.2c | 262.3c | 80.4a | 14.7 | 205.8 |
| Significance | | | | | | | | | | | |
| System | ** | ** | ns | ns | ns | ns | *** | *** | ** | ns | *** |
| pH | ** | ** | ** | * | ns | ns | *** | *** | ** | ns | ns |
| System × pH | * | ** | *** | ns | ns | ns | ** | *** | ns | ns | ns |

Table 4.7 continued

| | Mustard green | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| System | | | | | | | | | | | |
| Aquaponics | 4.7 | 0.7 | 7.9 | 0.5 | 2.8 | 0.8b | 40.9 | 83.4a | 36.3 | 5.4 | 165.8 |
| Hydroponics | 4.8 | 0.7 | 8.3 | 0.5 | 3.1 | 1.2a | 35.0 | 62.0b | 40.3 | 6.3 | 156.8 |
| pH | | | | | | | | | | | |
| 6 | 4.3 | 0.7 | 7.8 | 0.4 | 2.9 | 0.9b | 35.2 | 64.3 | 35.7 | 5.0 | 152.8 |
| 6.5 | 4.9 | 0.8 | 8.6 | 0.5 | 3.2 | 1.2a | 37.5 | 59.8 | 39.0 | 6.5 | 142.5 |
| 7 | 5.1 | 0.7 | 7.7 | 0.6 | 2.8 | 0.9b | 41.2 | 84.2 | 40.3 | 6.2 | 188.7 |
| Significance | | | | | | | | | | | |
| System | ns | ns | ns | ns | ns | * | ns | * | ns | ns | ns |
| pH | ns | ns | ns | ns | ns | *** | ns | ns | ns | ns | ns |
| System × pH | * | ns | ns | * | ns | *** | ns | ** | * | ns | * |

Means within column followed by the same letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$ =0.05). Each value in the table is the mean of 9 replicates of system and 6 replicates of pH treatment. ns, *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 4.7(Cont.). Average mineral nutrient concentrations of kale, lettuce, and Swiss chard in aquaponics and hydroponics.

| Treatments | Nutrient concentration (%) | | | | | | Nutrient concentration (ppm) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | P | K | Mg | Ca | S | Fe | Mn | B | Cu | Zn |
| | Kale | | | | | | | | | | |
| System | | | | | | | | | | | |
| Aquaponics | 5.4 | 0.7 | 7.4a | 0.5 | 2.9 | 1.3a | 66.1a | 105.3a | 41.7a | 7.8 | 247.6a |
| Hydroponics | 5.5 | 0.7 | 7.0b | 0.6 | 3.0 | 1.1b | 51.0b | 71.1b | 38.3b | 6.1 | 116.4b |
| pH | | | | | | | | | | | |
| 6 | 5.5a | 0.7a | 6.8c | 0.7a | 4.2a | 1.6a | 41.5c | 93.7a | 51.5a | 6.2b | 192.7b |
| 6.5 | 5.3b | 0.6b | 7.7a | 0.4b | 2.0b | 0.9b | 81.2a | 89.2b | 31.7b | 7.7a | 241.7a |
| 7 | 5.6a | 0.6b | 7.3b | 0.5b | 2.6b | 1.1b | 53.0b | 81.8c | 36.8b | 7.0b | 111.7c |
| Significance | | | | | | | | | | | |
| System | ** | ns | *** | ns | ** | *** | *** | ns | * | ns | *** |
| pH | ** | *** | *** | *** | *** | *** | *** | *** | *** | * | *** |
| System × pH | *** | ** | *** | *** | *** | *** | *** | ns | *** | * | ** |
| | Lettuce | | | | | | | | | | |
| System | | | | | | | | | | | |
| Aquaponics | 4.5 | 1.2a | 9.9a | 0.5 | 2.0 | 0.4 | 253.1 | 386.9a | 33.8 | 13.6 | 156.9a |
| Hydroponics | 4.5 | 1.0b | 9.0b | 0.5 | 1.5 | 0.3 | 231.1 | 294.1b | 29.8 | 11.4 | 102.2b |
| pH | | | | | | | | | | | |
| 6 | 4.4b | 1.2a | 8.8 | 0.5 | 1.7b | 0.4 | 265.2b | 392.5a | 29.5 | 12.5b | 143.8a |
| 6.5 | 4.2c | 1.1b | 8.5 | 0.5 | 1.5b | 0.4 | 334.2a | 362.3b | 30.7 | 14.0a | 106.2b |
| 7 | 4.9a | 1.0c | 11.1 | 0.5 | 2.0a | 0.4 | 127.0c | 266.7c | 35.2 | 11.0b | 138.7a |

Table 4.7 continued

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Significance | | | | | | | | | | | |
| System | ** | *** | *** | ns | ns | *** | *** | *** | ns | ns | *** |
| pH | *** | *** | ns | * | *** | *** | *** | *** | ns | *** | ** |
| System × pH | * | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| | Swiss chard | | | | | | | | | | |
| System | | | | | | | | | | | |
| Aquaponics | 5.1b | 0.6a | 10.7a | 1.0b | 1.5a | 0.3 | 53.1a | 191.9a | 49.7b | 9.4 | 116.2a |
| Hydroponics | 5.4a | 0.5b | 9.5b | 1.1a | 1.1b | 0.4 | 49.8b | 34.9b | 51.1a | 6.1 | 49.9b |
| pH | | | | | | | | | | | |
| 6 | 5.5 | 0.6a | 9.2b | 0.9b | 1.1 | 0.3 | 52.5 | 105.3b | 45.0b | 7.8b | 67.3c |
| 6.5 | 5.2 | 0.5b | 9.2b | 1.2a | 1.3 | 0.4 | 56.0 | 97.0c | 52.8a | 8.0a | 72.0b |
| 7 | 5.1 | 0.6a | 11.7a | 1.1a | 1.5 | 0.3 | 45.8 | 138.0a | 53.3a | 7.5b | 109.8a |
| Significance | | | | | | | | | | | |
| System | ** | *** | ** | * | *** | ns | ** | *** | ** | ns | *** |
| pH | ns | *** | ** | ** | ns | ns | ns | *** | ** | ** | *** |
| System × pH | ** | *** | *** | *** | *** | ns | ns | *** | * | ns | *** |

Means within column followed by the same letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$ =0.05). Each value in the table is the mean of 9 replicates of system and 6 replicates of pH treatment. ns, *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 4.8. Causal mediation modeling results

| Dependent variable | ACME (p-Value) | ADE (p-Value) | Result |
|---|---|---|---|
| N | 0.79 | <0.05 | Direct effect |
| P | 0.16 | <0.05 | Direct effect |
| K | 0.94 | <0.05 | Direct effect |
| Mg | 0.28 | <0.05 | Direct effect |
| Ca | 0.91 | <0.05 | Direct effect |
| S | 0.48 | <0.05 | Direct effect |
| Na | 0.83 | <0.05 | Direct effect |
| Fe | 0.45 | <0.05 | Direct effect |
| Mn | <0.05 | <0.05 | Partial mediation effect |
| B | 0.67 | <0.05 | Direct effect |
| Cu | 0.14 | <0.05 | Direct effect |
| Zn | 0.38 | <0.05 | Direct effect |

Abbreviations: ACME, average causal mediation effect. ADE, average direct effect.

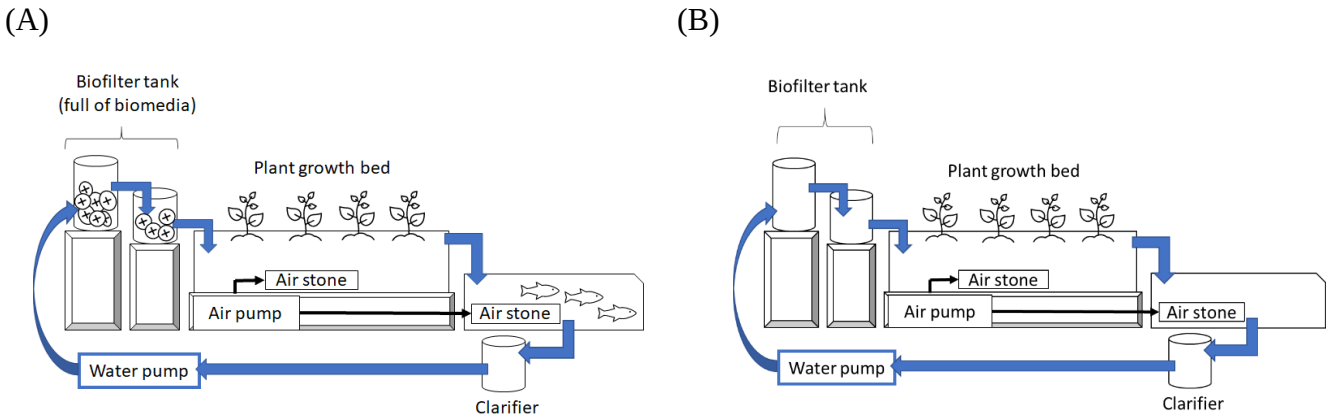


Figure 4.1. Schematic diagram of the experimental units (**A**) aquaponic system and (**B**) hydroponic system.



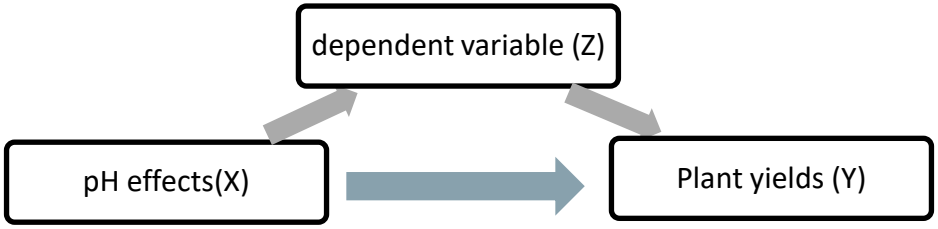


Figure 4.2.A schematic outline of mediation analysis

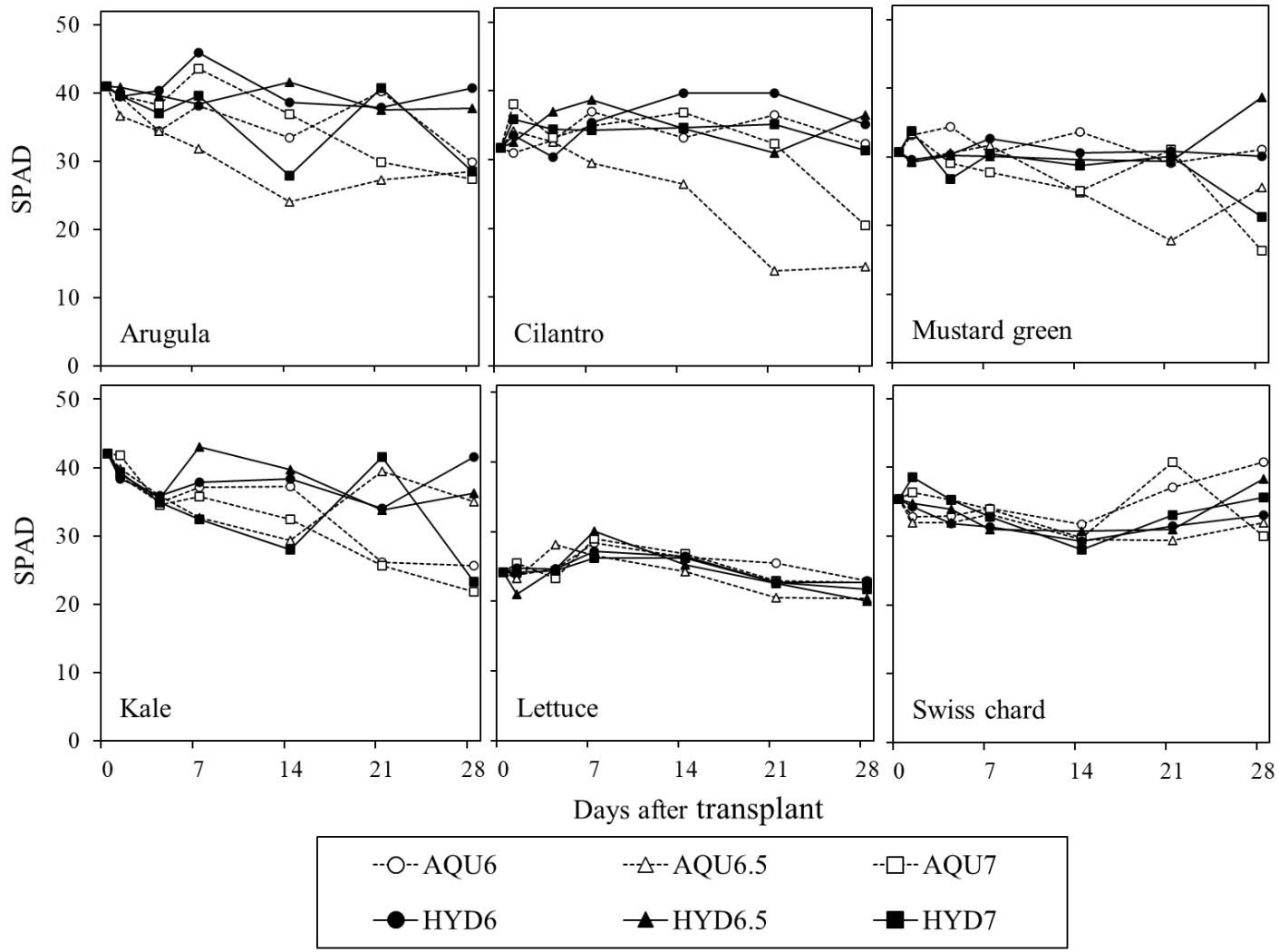


Figure 4.3. Dynamic changes in SPAD value of arugula, cilantro, mustard green, kale, lettuce, and Swiss chard in aquaponics and hydroponics set at pH 6, 6.5, or 7 over 28 days of production period.

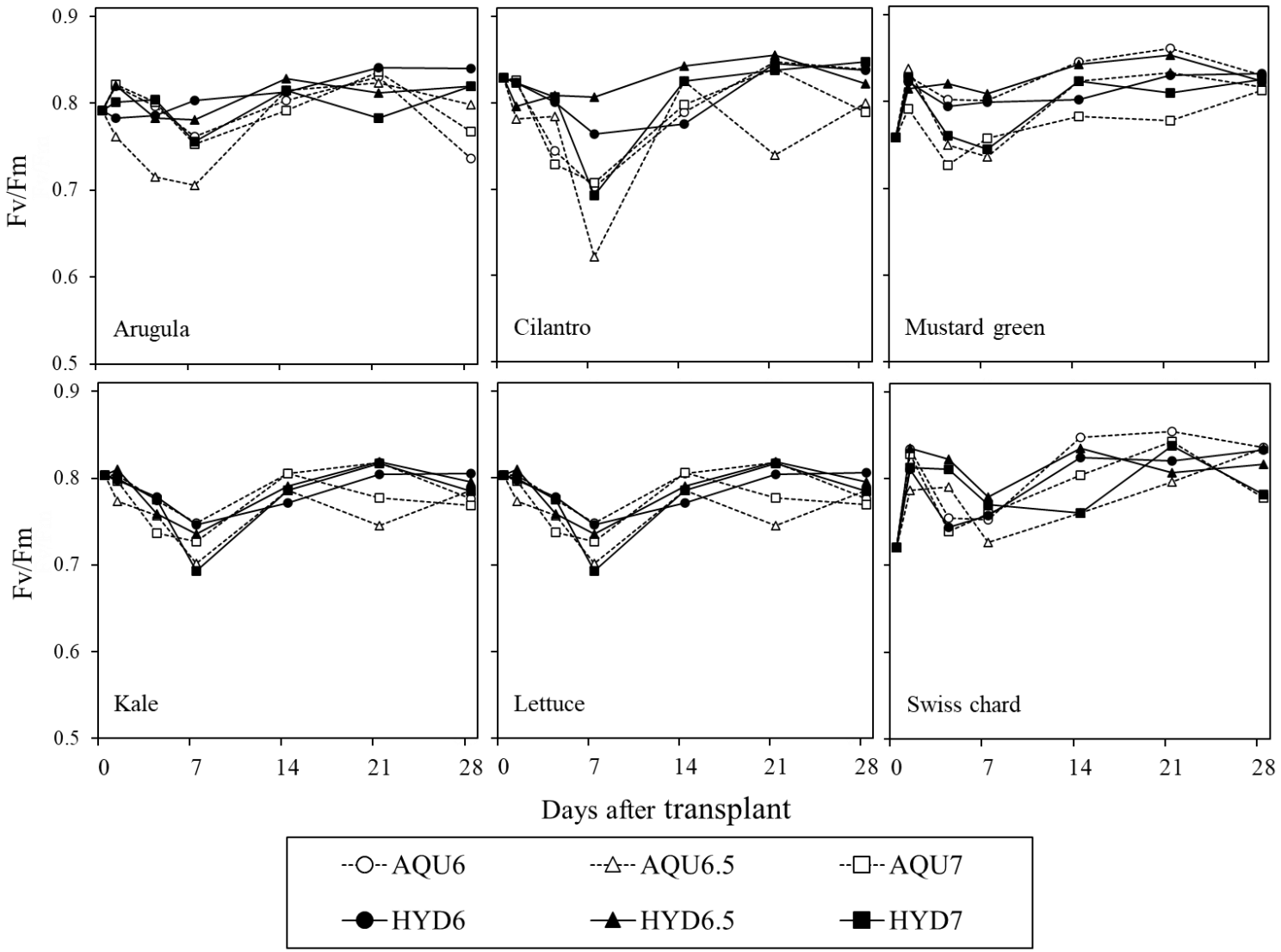


Figure 4.4. Dynamic changes in Fv/Fm value of arugula, cilantro, mustard green, kale, lettuce, and Swiss chard in aquaponics and hydroponics set at pH 6, 6.5, or 7 over 28 days of production period.

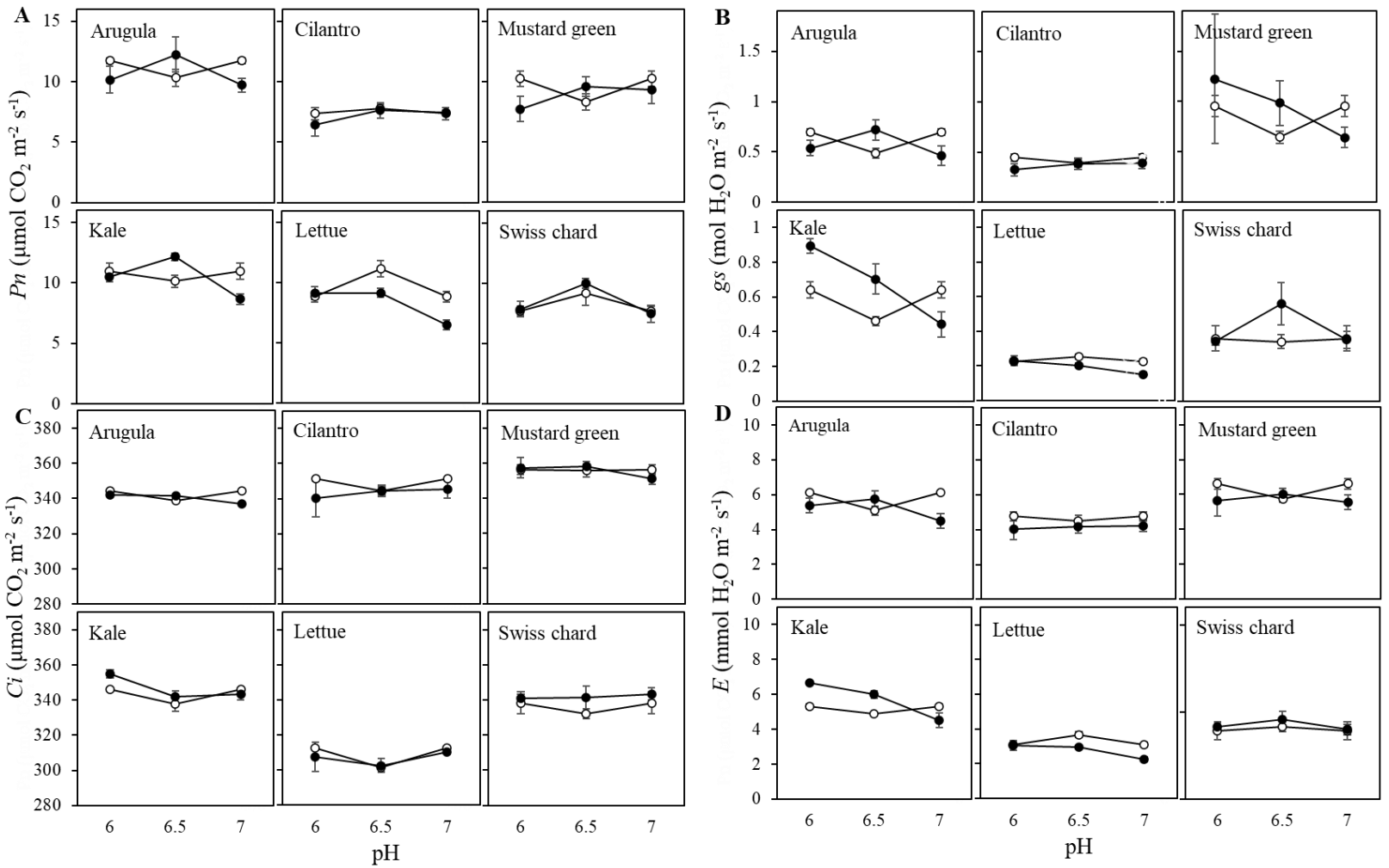


Figure 4.5. Plant photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (E), and intercellular CO2 concentration (Ci)of crops grown in aquaponics with different pH. The values are photosynthetic parameters measured at day 28 after transplanting.

# CHAPTER 5. *PSEUDOMONAS* INOCULATION DIFFERENTIALLY ENHANCES LETTUCE GROWTH AND QUALITY IN AQUAPONIC VERSUS HYDROPONIC PRODUCTION SYSTEMS

## 5.1 Abstract

Inoculating plants with plant growth promoting bacteria (PGPB) in hydroponic and aquaponic production systems has potential to address challenges associated with nutrient deficiencies, while providing additional benefits such as protecting plants against salinity and pathogen stress. In this study, we screened six potential PGPB isolates representing five genera (*Bacillus*, *Pantoea*, *Paenibacillus*, *Pseudomonas*, and *Xanthomonas*) in the laboratory for their capacity to promote root growth in Arabidopsis, since root traits are likely to be critical in obtaining nutrients in these production systems. One of these isolates (O3, *Pseudomonas* spp.), which best enhanced Arabidopsis root growth was then included in a subsequent greenhouse trial evaluating the capacity for this inoculant to enhance key physiological properties such as chlorophyll fluorescence, photosynthetic rate, and stomatal conductance, as well as nutrient uptake and yield in lettuce plants grown in aquaponic and hydroponic production systems. Results of these trials indicate that lettuce plants grown in the aquaponic system had greater a root:shoot ratio, water content, relative growth rate, yield and concentrations of potassium than plants grown in the hydroponic system. Inoculating plants with the PGPB isolate enhanced nutrient uptake in both systems, with higher concentrations of phosphorous, magnesium and zinc in lettuce tissues. However, the effects of the inoculant varied among the production systems, indicating that there could be unique environmental conditions that favor the survival and efficacy of PGPB in these systems. For example, the inoculant only enhanced lettuce yield in the aquaponic system, but increased the uptake of more nutrients in the hydroponic system. Results of this study demonstrate that well managed aquaponic systems can be just as productive, or more productive than hydroponic systems. Inoculating plants with PGPB also has potential to further boost the productivity and nutritional quality of plants grown in these unique production systems.

Key words: aquaponics, hydroponics, plant growth-promoting bacteria, lettuce

## 5.2 Introduction

Growing vegetables in controlled environment agricultural (CEA) systems has many advantages including optimizing plant yield, increasing energy use efficiency, and predicting plant responses to the environment (Gómez et al., 2019b; Engler and Krarti, 2021; Srivani et al., 2019). Many CEA systems employ hydroponic methods to produce crops and there has been a lot of research to optimize these systems (Gómez et al., 2019b). In contrast, far less research has been conducted to optimize aquaponic systems. Aquaponics, a growing trend in food production, integrates aquaculture and hydroponics into one system where plants obtain nutrients from aquaculture wastewater (Rakocy et al., 2004). This form of cultivation is widely considered to be one of the most efficient and sustainable ways to simultaneously obtain animal protein as well as vegetables. Additionally, in terms of water use efficiency, aquaponics is potentially more efficient than current stand-alone conventional recirculating aquaculture (RAS) and hydroponic production systems (Rakocy, 2012). Thus, the number of studies focusing on the advantages and ways to address challenges in aquaponic systems, have increased in recent years (Hao et al., 2020), but there still much work left to be done.

In aquaponic systems, nutrients excreted by fish or left over from fish feeds are transformed into plant available nutrients by microbes before being taken up by hydroponically cultured plants (Nichols and Savidov, 2012; Endut et al., 2009). For example, most fish species only absorb 20-30% of the total nitrogen (N) in their food, and the remaining 70-80% is left in the water providing a valuable source of nutrients for crops (Wongkiew et al., 2018; Hao et al., 2020; Yang and Kim, 2020). Within these systems, these organic waste products are filtered through tanks which are inoculated with a set of specialized microbes that can convert ammonia ($NH_4^+$) to nitrite ($NO_3^-$) in a two-step process called nitrification (Wongkiew et al., 2017a, 2018). This is thought to be a critical step in aquaponic systems since plants can absorb N in both of these forms, but high concentrations of $NH_4^+$ can be toxic and reduce plant yields (Britto and Kronzucker, 2002). Two different groups of nitrifying bacteria, *Nitrosomonas* spp. and *Nitrobacter* spp., are commonly added to aquaponic systems to facilitate this conversion process, and the pH is kept above 7.0 to promote the activity of these microbes (Wongkiew et al., 2017a, 2018).

Besides N, plants also need adequate supplies of phosphorus (P), potassium (K), and several micronutrients for optimal crop growth. Plants grown in aquaponic systems have been

reported to have lower yield and quality than plants grown in hydroponic systems (Cerozi and Fitzsimmons, 2016a). These deficiencies could be caused by the fact that aquaponic systems are generally maintained with a pH above 7, which could negatively affect the availability of other important crop nutrients. For example, lettuce (*Lactuca sativa* 'Cherokee'), basil (*Ocimum basilicum* 'Genovese'), cherry tomato (*Lycopersicon esculentum* 'Washington Cherry'), tomato, and eggplant (*Solanum melongena* L.) grown at high pH in an aquaponic solution had symptoms resembling P deficiency (Resh, 2004; Roosta and Mohsenian, 2012; Roosta, 2014; Yang and Kim, 2020a; Hochmuth, 2001). The availability of P for plant uptake is highly dependent on pH, and P will be held within insoluble complexes under high pH environments (Cerozi and Fitzsimmons, 2017). In addition, 30–65% of P in aquaponic systems is in solid fish sludge, which is unavailable to plants (Yang and Kim, 2019b). Fish feeds contain K and other micronutrients, but the amount of these nutrients is often limiting for plants in aquaponic systems, since fish have minimal requirements of many micronutrients such as iron (Fe), manganese (Mn), magnesium (Mg), and copper (Cu), and lower requirements for K than plants (Rakocy, 2012; Villarroel et al., 2011; Seawright et al., 1998).

One potential way to overcome challenges associated with potential nutrient deficiencies in aquaponic systems, while enhancing the yield and quality of crops in both aquaponic and hydroponic systems, is to inoculate plants with so-called 'plant growth promoting bacteria'. PGPB are a group of beneficial bacteria that colonize plant roots or leaves and enhance plant health through several mechanisms including promoting nutrient uptake, increasing abiotic stress tolerance, and controlling pathogens (Bhattacharyya and Jha, 2012; García-Fraile et al., 2015; Vejan et al., 2016). Some PGPB can enter internal plant tissues and survive within plants as endophytes (Abelrazek et al., 2020a). Endophytic PGPB can be classified as intra- or extra-cellular based on their degree of association with root cells (Gray and Smith, 2005; Martínez-Viveros et al., 2010). The primary mechanism of many intracellular PGPB, such as those belonging to Rhizobia genera, is to fix atmospheric N into plant available forms within specialized root nodules (Prithiviraj et al., 2003; Gray and Smith, 2005). In contrast, extracellular PGPB, such as those belonging to *Bacillus*, *Pseudomonas*, *Erwinia*, *Caulobacter*, *Serratia*, *Arthrobacter*, *Micrococcus*, *Flavobacterium*, *Chromobacterum*, *Agrobacterium*, and *Hyphomycrobium* genera, may increase plant growth by a variety of mechanisms, such as producing phytohormones like indole acetic acid

(IAA) that can stimulate root growth, solubilizing nutrients, or mediating biotic and abiotic stress (Prithiviraj et al., 2003; Gray and Smith, 2005; Abdelrazek et al., 2020a; Abdelrazek et al., 2020b).

Many studies have demonstrated that soil and plant microbiomes are critical in promoting the health and productivity of plants in natural and field-based agricultural systems (Reeve et al., 2016), and inoculants containing PGPB have been shown to enhance performance when plants are grown in CEA settings using soilless potting media (Bartelme et al., 2018; Goddek et al., 2015). However, only a few studies have investigated the role of microbiomes and the application of select PGPB in hydroponic or aquaponic systems. In one recent study, Schmautz et al (2017) characterized microbiomes in an aquaponic system and discovered that while many nitrifying bacteria were present in the biofilter, nitrifying bacteria were not the largest group of bacteria in the rest of the system (Schmautz et al., 2017). The largest phylum in this aquaponic system were *Proteobacteria*, which contain many potential PGPB, including those belonging to *Pseudomonas*, *Acidovorax*, and *Sphingobium* genera (Schmautz et al., 2017). Other bacteria, including those belonging to the *Firmicutes* phylum, which contains *Bacillus* spp., were found in fish feces. Products containing *Bacillus subtilis,* are commonly used to enhance plant growth in soilless potting media-based culture systems, and some have also been used as probiotics to promote fish growth in aquaculture systems (Arkhipova et al., 2005; García-López et al., 2018; Mills et al., 2011; Fu et al., 2014; Idris et al., 2007). For example, *B. subtilis* has been shown to increase weight gain, survival rate, and nutrition in shrimp culture (Zokaeifar et al., 2012), and enhance tilapia immunity against fish pathogens such as *Streptococcus iniae*, *P. fluorescens,* and *Aeromonas hydrophila* (Aly et al., 2008). Bartelme et al. (2018) suggested that some PGPB could also play a major role in the plant's ability to take up micronutrients like Fe in aquaponic systems, since some PGPB, such as *P. fluorescens* Pf-5 and *Chryseobacterium* C138 can increase the bioavailability of Fe and reduce deficiencies in plants by producing siderophores (Radzki et al., 2013; Loper and Henkels, 1997; Bartelme et al., 2018).

In this study, we predicted that inoculating plants with PGPB would enhance nutrient uptake and lead to higher plant yield in both hydroponic and aquaponic production systems. To test our hypothesis, we inoculated lettuce plants with a *Pseudomonas* isolate with PGPB activity and quantified changes in the yield and quality of plants grown in in these two soilless culture systems. The PGPB isolates evaluated in this trial were originally isolated as endophytes from carrot taproot

grown in a field trial, that were subsequently demonstrated to promote carrot growth and suppress disease in laboratory and greenhouse trials (Abdelrazek et al., 2020b). In this study, xix of these isolates (Table 5.1) were first evaluated for their capacity to promote Arabidopsis root growth in order to identify an isolate that could enhance the productivity of multiple crop species. *Pseudomonas* O3 was selected for the subsequent lettuce trials. *Pseudomonas* strains are commonly applied as PGPG to enhance plant growth in soilless potting media based production systems (Loper and Henkels, 1997; Someya et al., 2008; Abd El-Rhman et al., 2009; Cipriano et al., 2016; Adhikari et al., 2021).

## 5.3 Materials and Methods

### 5.3.1 The effect of PGPB on arabidopsis root performance

### 5.3.2 Plant Materials

*Arabidopsis thaliana* seeds (ThermoFisher, Waltham, MA, USA) were placed on 1/2 Murashige and Skoog medium (MS medium) (ThermoFisher, Waltham, MA, USA) supplemented with 2% sucrose (ThermoFisher, Waltham, MA, USA), and allowed to grow for 7days in a growth chamber set at 23 °C, with a 16-h light/8-h dark photoperiod, and 60% relative humidity.

### 5.3.3 Cocultivation conditions

The identity of the six PGPB isolates evaluated in this study were determined Sanger sequencing and the sequences were deposited in the National Institute of Health (NIH) genebank to obtain accession numbers (Abdelrazek et al., 2020b). The PGPB isolates were removed from long-term storage at -80C for this study and revived by culturing at 25 ºC in 1 mL LB broth (ThermoFisher, Waltham, MA, USA) for 20-24 hours. Seven-day-old *A. thaliana* seedlings with uniform growth were transferred to new agar plates containing 1/2 MS agar media supplemented with 2%sucrose, and 50 µL bacterial suspensions ($10^6$ CFU $mL^{-1}$) containing each of the six PGPB isolates (Table 5.1), were streaked 5 cm below the roots using techniques described in Wintermans et al. (2016). Plates streaked with 50 µL of sterile LB were used as the control. Plates containing

the *A. thaliana* and bacteria were placed in a growth chamber for 7 days at 23 °C, with a 16-h light/8-h dark photoperiod, and 60% relative humidity.

### 5.3.4 Measurements of root morphological traits

Differences in Arabidopsis root growth and architecture were quantified using a WinRHIZO root-scanning system and Pro software (WinRhizo Pro v.2005b, Regent Instruments, Québec, Canada). The debris removal filter was set to discount objects less than 1 $cm^2$ in size and a length/width ratio less than 4. Diameter class length (root length within a diameter class) classes were generated from the images of adventitious roots acquired from the system. The roots were divided into 26 diameter classes at 0.25 mm intervals and root length per each root diameter class was calculated. The root diameter class distribution was computed based on the proportion of the root length in each root diameter class compared to the total root length.

### 5.3.5 Statistical analysis

The experiment was repeated in triplicate. Statistical data were computed using the R 3.4.0 (R, Comprehensive R Archive Network, USA) program, with a one-way ANOVA test.

### 5.3.6 The effect of O3 on lettuce yield in soilless culture systems

### 5.3.7 Aquaponic System design

Three identical aquaponic and hydroponic systems were assembled in a greenhouse at Purdue University in West Lafayette, USA (40º 25’ 26.4’’ N, 86° 55’ 44.4’’ W) (Fig. 5.1). Each unit was equipped with a fish tank or a nutrient reservoir (350 L), a clarifier (20 L), a two-stage biofilter (40 L) (Yang and Kim, 2019; Wongkiew et al., 2017b) and a deep-water hydroponic grow bed (350 L; 1.0 m2) (Fig. 5.1). The biofilters in were connected to a peristaltic pump (MasterflexLive™ Cole-Parmer L/S Digital Drive, Vernon Hills, IL, USA) to recirculate nutrient solution within the systems. In the aquaponic systems, plants were cultured in combination with tilapia (Oreochromis niloticus) and fish feed (Purina® AquaMax® Sport Fish MVP, USA), which was the major source of fertilizer for the lettuce (Table 5.2). Each plant growth bed was filled with

350L of water and covered with a 1 $m^2$ foam raft. The plant holders allowed for densities of 24 plants per $m^2$. The total water volume in each aquaponic/hydroponic unit was 760 L under a flow rate of 125 L/h, giving a water retention time of 168 min in each fish tank/nutrient solution reservoir and floating system unit. Each aquaponic and hydroponic system had air stones to maintain dissolved oxygen (DO) concentrations above 5 mg/L, and a heater to maintain temperature at 23°C. Temperature, pH, electrical conductivity (EC) and DO were measured daily using a portable HI9811- 5 pH/EC/TDS/Temperature meter (Hanna Instruments, Inc., Smithfield, RI, USA).

The photoperiod was 14-h (8:00 am to 10:00 pm) consisting of natural daylight with supplemental lighting using high-pressure sodium (HPS) lamps (600-W, P.L. Light Systems Inc., Beamsville, ON, Canada). Supplemental photosynthetic photon flux (PPF) of the greenhouse was measured using a quantum sensor (LI-250A light meter; LI-COR Biosciences, Lincoln, NE) and photosynthetically active radiation in the greenhouse was averaged at 134 µmol $m^{-2}$ $s^{-1}$. Day (8:00 am to 10:00 pm) and night (10:00 pm to 8:00 am) temperatures were set at 24 and 18°C, respectively, with an hour transition between the two temperature regimes. Depending on ambient temperature, the greenhouse was cooled as needed using a fan-and-pad evaporative-cooling system, heated using radiant hot-water-pipe heating, and retractable shade curtains regulated by an environmental control system (Maximizer Precision 10, Priva Computers Inc., Vineland Station, ON, Canada).

### 5.3.8 Plant and Fish materials

Lettuce seeds were purchased from a commercial source (Johnny's Selected Seeds, Winslow, ME), sown in Agrifoam soilless plugs (SteadyGROWpro, Syndicate Sales, Kokomo, IN) and allowed to grow for a few days to ensure uniform seedling size at the time of transplanting. Seeds were germinated as described by Kim et al. (2018) and 2 to 3 weeks old seedlings were transplanted to the aquaponic and hydroponic systems. Planting densities were 24 plants per $m^2$ for lettuce (Kim et al., 2018b). Seeds were irrigated with tap water, and the fertility was gradually increased to a half-strength fertilizer solution after germination. Seedlings were irrigated with full fertilizer after true leaves developed (Kim et al., 2018b). The fertilizer was combined with two water soluble

fertilizers (3:1 mixture of 15N–2.2P–12.5K Cal-Mag Special and 21N–2.2P–16.6K Multi-Purpose fertilizers, respectively; Everris NA, Dublin, OH) (Table 5.2).

Nile tilapia (*Oreochromis niloticus* L.) were cultured in each aquaponic system, and lettuce was cultured in both systems. Fish fresh weight was measured prior to the start of the experiment and evenly distributed to the three fish tanks with a stocking density of 15 kg/m3 in each aquaponic system. The fish feed used in this study was a complete diet containing 41% protein and 1.1% phosphorus (AquaMax Sport Fish 500, Purina Mills, St. Louis, MO) with 4.8-mm floating pellets (Table 5.2). Fish were fed by 1% of fish weight once per day at 10:00 am. Fish were weighed at the beginning, weekly, and at the end of the experiment by removing fish from the fish tank to another tank filled with water. The average weight was used to determine the amount of fish feeds.

### 5.3.9 Bacteria preparation and inoculation

A suspension of bacterial isolate (O3) *(Pseudomonas spp.)* was prepared by inoculating 1 mL of the pre-culture obtained from long-term storage in 15 mL LB broth (ThermoFisher, Waltham, MA, USA). The culture was grown at 125 rpm/min at 26℃ for 24 h. After 24 h, the broth was diluted to OD600=0.6 (approximately $10^6$ CFU/mL). After true leaves of lettuce seedings were fully expanded, the roots were soaked in the O3 broth for 30 second and inoculated lettuce seedlings were placed on new trays for 3 days (Wu et al., 2020). Seedlings soaked in sterilized LB were used as the control. Seedlings were irrigated with a combined fertilizer solution 3:1 mixture of 15N–2.2P–12.5K Cal-Mag Special and 21N–2.2P–16.6K Multi-Purpose fertilizers, for the X and X system, respectively; Everris NA, Dublin, OH) (Table 5.2).

### 5.3.10 Water quality parameter measurements

The EC of the water in the aquaponic system was set at 1.5 dS/m and maintained by adjusting the feeding rate. The pH of the aquaponic and hydroponic systems were maintained at 6.0 using 10% $H_2SO_4$ or a combination of base solutions (1N KOH and 0.05 N $Ca(OH)_2$. =1:1 (v:v)). Each system had air stones to maintain dissolved oxygen (DO) concentrations at full saturation and aquatic heaters were set in aquaponic systems to maintain temperature in the range of 21 to 25 °C. EC, pH, water temperature, and DO were measured daily before feeding using a HQ40d portable water quality lab package (HACH Corp., Loveland, CO, USA).

### 5.3.11 Growth measurements

SPAD readings were taken weekly on each young fully expanded leaf using a SPAD-502 Chlorophyll Meter (Minolta Camera Co. Ltd., Japan). Five readings per leaf were taken at the central point of a leaf between the midrib and leaf margin and the values were averaged. Other plant growth parameters were measured at harvest, and included plant height, leaf number, leaf area, shoot fresh weight and root fresh weight. Plant materials were oven-dried (over 72 h at 70°C) until stable and weighed to obtain dry weight. All dried plant samples were filtered through a 10-mesh sieve after grinding with a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and kept in plastic vials for nutrient analysis. The root: shoot ratio, water content, and relative growth rate were calculated using the following equations:

$$root{:}\,shoot\ ratio\ (\%) = (\frac{root\ fresh\ weight}{shoot\ frehs\ weight} \times 100\%)$$

$$water\ content\ (\%) = \left(\frac{total\ fresh\ weight - total\ dry\ weight}{total\ dry\ weight}\right) \times 100\%$$

$$Relative\ growth\ rate\ (\%) = \frac{(total\ fresh\ weight_{day28} - \ total\ fresh\ weight_{day0})}{days} \times 100\%$$

### 5.3.12 Photosynthetic measurements

During the first and third week after transplanting, and the day before harvest, four representative plants were randomly selected in each treatment to quantify instantaneous photosynthetic rate (μmol/$m^2$/s) on young, fully expanded leaves. Gas-exchange measurements were performed using a portable gas exchange system (LI-6400XT; LICOR Biosciences, Lincoln, NE) equipped with a 6-$cm^2$ leaf chamber with built-in LEDs (470 and 665-nm peak wavelengths for blue and red LEDs, respectively). Illumination was supplied at a PPF of 400 μmol $m^{-2}$ $s^{-1}$ by red and blue LEDs at a ratio of 9:1 under ambient temperature conditions when supplemental lighting was in use. The reference CO2 concentration and flow rate through the chamber were 400 μmol $mol^{-1}$ and 500 μmol $s^{-1}$, respectively. One leaf at each canopy level was selected from each plant for the measurements. The measurements of photosynthetic rate (*Pn*), stomatal conductance (*g*s), transpiration rate (*E*), and internal CO2 (*Ci*) were conducted between 9:00 am

and 14:00 pm at a PPF of 400 μmol $m^{-2}$ $s^{-1}$. Readings were taken when the coefficient of variation (i.e., sample $CO_2$, sample $H_2O$, and flow rate) was less than or equal to 0.2%, which typically occurred within 10 min. The intrinsic water use efficiency (WUE) was calculated by dividing *Pn* by *gs* (Chaves et al., 2004).

### 5.3.13 Chlorophyll fluorescence

Average values of were recorded using a handy chlorophyll fluorescence meter (handy PEA+, Hansatech Instruments, Norfolk, UK). Fully expanded young leaves were shielded using dark adaptation leafclips (PPEA/LC, Hansatech Instruments, Norfolk, UK) for 20 minutes. The meter was set as 3,000 μmol $m^{-2}$ $s^{-1}$ at 650 nm to a 4 mm diameter area within the sensor enclosure. $F_m$ (maximum fluorescence), $F_v$ (variable fluorescence), $F_v/F_m$ were recorded and calculated using a Plant Efficiency Analyzer Fluorimeter (PEA, Hansatech Instruments, England). Measurements were conducted on five leaves per plant and four replicate plants per treatment and averaged every 3 days.

### 5.3.14 Root morphological traits

Whole root samples of lettuce were stored in 50% ethanol solution for root length, diameter, surface area, volume measurement using WinRHIZO root-scanning system (WinRhizo Pro v.2005b, Regent Instruments, Québec, Canada). Root morphological traits were measured as described in 5.3.4.

### 5.3.15 Mediation analysis

Fig 5.2 illustrates the relationship between PGPB effects, dependent variables (such as root:shoot ratio, water content, and relative growth rate), and plant yield (total fresh weight). The nonlinear version of the mediation model, as depicted in Fig. 5.2, was used to develop the corresponding structural equations using previously described equations (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012):

$$X = F1(e1)\ z = F2(x, e2)\ y = F3(x, z, e3) \quad (1)$$

where X, Y, Z are discrete or continuous random variables, F1, F2, and F3 are arbitrary functions, and e1, e2, e3 represent omitted factors. Using this formula, total effects were determined, T $E_{0,1}$ to quantify the change in total fresh weight produced by a unit change due to PGPB effects. The total effect was determined using the following formula (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012):

TE0,1(e2, e3) = F3[1, F2(1, e2), e3] − F3[0, F2(0, e2), e3] (2),

and then natural effects were determined using the following formula (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012):

$$NDE_{x,x'}(Y) = \sum_z [E(Y|X = x', Z = z) - E(Y|X = x', Z = z)]P(Z = z\,|X = x) \quad (3)$$

$NDE_{x,x'}(Y)$ is defined as the expected change in Y induced by changing X from x to $x_0$ while keeping all mediating factors constant at whatever value they would have obtained under X = x, before the transition from x to $x_0$ (VanderWeele, 2008; VanderWeele et al., 2014; Cheong and MacKinnon, 2012).

Hypothesis 1 (h1) was O3-inoculation positively corelated with total fresh weight. Hypothesis 2 (h2) was O3-inoculation negatively corelated with root: shoot ratio. Hypothesis 3 (h3) was O3-inoculation positively corelated with water content. Hypothesis 4 (h4) was O3-inoculation positively corelated with relative growth rate. Hypothesis 5 (h5) was root: shoot ratio negatively corelated with total fresh weight. Hypothesis 6 (h6) was water content positively corelated with water content. Hypothesis 7 (h7) was relative growth rate positively corelated with relative growth rate.

### 5.3.16 Experience design and data analysis

The experiment was conducted using three aquaponic and three hydroponic systems for 28 days. Each system can grow up to 24 plants, and therefore, 24 plants were randomly placed in each system which consisted of 12 control plants and 12 plants treated with O3 (*Pseudomonas spp*.). For the analysis in this study, a completely randomized design and two-way ANOVA were used. The water quality parameters, plant biomass, photosynthesis rate, SPAD value, Fv/Fm, root performance, and nutrient accumulation in the aquaponic and hydroponic systems were tested using Tukey's Honest Significant Difference. The statistical analysis used post-hoc pairwise

comparisons in R 3.6.1 (R, Comprehensive R Archive Network, USA) at a significance level of 0.05.

## 5.4 Results

### 5.4.1 The effect of potential PGPB on Arabidopsis roots

Only O3 and O12 significantly increased the surface area, volume, and average diameter of Arabidopsis roots (Table 5.3). Root length was 39.8% and 27.1% greater, and root surface area was 58.7% and 46% greater than controls plants treated with O3 and O12, respectively (Table 5.3). Plants treated with O3 and O12 also had root volumes that were 96.7% and 53.3% greater than control plants, respectively (Table 5.3). In contrast, roots from plants treated with 11D2, O1, O10 were not significantly different from the control group (Table 5.4). All of the root parameters except average diameter, were lower in plants treated with 11N in comparison with the control (Table 5.3).

### 5.4.2 Physical parameters of aquaponic and hydroponic Solutions

The water quality parameters in the aquaponic and hydroponic systems are shown in Table 5.4. The pH values averaged 6.0 ± 0.0 in both systems, and the pH correction solutions to maintain pH at this level were 34.8 ± 4.7 and 15.0 ± 0.4 in aquaponic and hydroponic systems, respectively (Table 5.4). EC levels were 1.55 ± 0.01 and 1.53 ± 0.01 in aquaponic and hydroponic systems, respectively (Table 4). The average DO were 7.72 ± 0.05 and 7.62 ±0.03 in aquaponic and hydroponic systems, respectively (Table 5.4). The water temperatures of aquaponic systems averaged 23.1 ± 0.1 and the water temperature of hydroponic systems averaged 22.9 ± 0.1 (Table 5.2). Throughout the experiment, there was no significant difference in physical water parameters between the two systems.

### 5.4.3 Lettuce growth and yield

The production systems significantly influenced lettuce growth and plants treated with O3 in the aquaponic system had larger above-ground biomass (Figure 5.3, Table 5.5). Plant fresh

weight was significantly affected by O3 treatment in the aquaponic but not hydroponic systems (Table 5.5). Plants treated with O3 in the aquaponic system had higher shoot fresh weight and dry weight than control plants (Table 5.5). Moreover, O3-inoculated plants had greater leaf area, leaf number, root surface area, root volume, and root average diameter, which led to higher water content and relative growth rate, and a lower root: shoot ratio (Table 5.6-8). In the hydroponic system, treating plants with O3 only affected root dry weight (Table 5.5), which was caused by longer root length, greater surface area, and average diameter of roots (Table 5.8). O3-inoculated plants in the hydroponic systems did not have greater yield than the control plants, but the relative growth rate was slightly higher after treating plants with O3 in this system (Table 5.8).

Root:shoot ratio, water content, and relative growth rate are indicators for the effect of treatments on plants. In our study, we used casual mediation analysis to quantify the relation between O3-inoculation, dependent variables (such as root:shoot ratio, water content, and relative growth rate), and total fresh weight. To do this mediation analysis, we had six different hypotheses, which are illustrated in 2.2.9. We used linear regression to test our hypotheses. The hypotheses (h1, h4, h5, h6, h7) confirmed that O3-inoculation was positively corelated with total fresh weight and relative growth rate; total fresh weight was positively corelated with water content and relative growth rate, and total fresh weight was negatively corelated with root:shoot ratio (Table 5.9). After these hypotheses were confirmed, we used a common method for CMA in R package (Imai et al., 2010b, 2010a). The results indicated that relative growth rate had a partial mediation effect on total fresh weight, and root:shoot ratio and water content were direct factors affecting lettuce total fresh weight (Table 5.10).

### 5.4.4 Leaf chlorophyll and Photosynthetic Parameters

Chlorophyll fluorescence analysis has been used as a non-invasive measurement of photosystem II (PSII) activity for studying plant stress response in a range of species (Gorbe and Calatayud, 2012; Bauriegel et al., 2014). In this study, Fv/Fm dropped after transplanting and was restored to 0.8 after transplanting 5 days (Fig. 5.4). Throughout the experiment, the Fv/Fm was maintained above 0.8, which is considered unstressed in many plant species (Choudhary and Johri, 2009; Bhandari et al., 2018). The SPAD value of lettuce was significantly higher in hydroponics than aquaponics 7 days after transplanting (Table 5.11), but there was no significant differences

between aquaponics and hydroponics at 21 days after transplanting (Table 5.11). Meanwhile, O3-inoculated lettuces had a higher SPAD value than non-inoculated lettuce plants in aquaponics, but not in hydroponics 7 days after transplanting (Table 5.11). The SPAD value was not significantly different between systems and O3 inoculation at any other time point (Fig. 5.4). The SPAD value of commercial green leafy vegetables is often associated with N level (Limantara et al., 2015), but the relationship between SPAD value and N level is this experiment was not strongly correlated (Fig. 5.5). Moreover, the SPAD value was not correlated with total fresh weight, although the SPAD value was correlated with the photosynthesis rate (Fig. 5.4).

At 21 days after transplanting, *gs* and E were significantly lower in O3-inoculated lettuce grown in aquaponics, but there was no significant difference in Fv/Fm, SPAD value, Pn, Ci, and WUE between control and O3 treated plants (Table 5.11). *gs* and E often lead to higher photosynthesis rates and yields. In this study, the *gs* and E of plants at day 7 were correlated with total fresh weight (Fig. 5.6). Additionally, O3-inoculated lettuce plants had 45.7% higher Pn at day 7 and 2% higher total fresh weight than non-inoculated plants in aquaponics. Meanwhile, WUE at day 7 was 47.1% higher in aquaponics than in hydroponics, but it was 46.3% lower in aquaponics at day 21.

### 5.4.5 The nutrient concentrations in lettuce plants

In general, lettuce plants contained higher concentrations of macronutrients (N, P, K, Mg, Ca, S) when grown in aquaponic than hydroponic systems (Fig. 5.7). Additionally, primary macronutrients (N, P, K) were positively correlated with shoot fresh weight, plant height and leaf number (Fig. 5.8A), and negatively correlated with root fresh and dry weight, and average diameter of roots (Fig. 5.7B). Total fresh weight, water content, and relative growth rate were positively correlated with primary macronutrients (N, P, K) (Fig. 5.8). In the aquaponic systems, there was a significant difference in P and magnesium (Mg) between control and O3 treated lettuce plants (Table 5.10). In hydroponic systems, O3-inoculated plants contained significantly higher concentrations of P (11%), K (5%), Mg (16%), Ca (19%), and S (11%) than control plants (Table 5.12). The sodium (Na) level in lettuce tissues was significantly affected by O3-inoculation and production system (Table 5.12). Plants contained more Na in aquaponics than hydroponics, and O3-inoculated lettuce plants contained a higher level of Na regardless of the production system

(Table 5.12). Na level was positively correlated with total fresh weight, N, P, K, *gs*, plant height, and water content (Fig. 5.9). In aquaponics, there was only a significant difference in the micronutrient Zn between O3 treated and control plants (Table 5.12). In contrast, all micronutrients (Fe, Mn, B, Cu, Zn) has significantly greater concentrations in O3 treated plants compared to the control in hydroponics (Table 5.12). Mn, Cu, Zn were positively correlated with total fresh weight, water content, and relative growth rate, but negatively correlated with total dry weight and root:shoot ratio (Fig. 5.10A). B was the only nutrient positively correlated with water content (Fig. 5.10A). Fe and Mn were positively correlated with plant height and leaf number, but negatively correlated with *Pn*, *gs*, *Ci*, *E*, and leaf area (Fig. 5.9B). B was negatively correlated to *Pn*, *gs*, *Ci*, *E*, plant height, leaf number, and leaf area (Fig. 5.9B). Cu and Zn were positively correlated with *Pn*, plant height, and leaf number, and negatively correlated with gs, *Ci*, *E*, and leaf area (Fig. 5.9B).

## 5.5 Discussion

### 5.5.1 Selecting a PGPB isolate with broad adaptability for soilless culture systems

Plants are colonized by an abundant and diverse assortment of microorganisms and some of these can improve the productivity and nutritional quality of crop plants (Reeve et al., 2016; Hoagland et al., 2018; Abdelrazek et al., 2020a). For example, many PGPB have been isolated and demonstrated to enhance plant growth by producing IAA, hydrolyzing organic nutrient sources, and increasing abiotic and/or biotic stress tolerance (Bhattacharyya and Jha, 2012; Meena et al., 2017). Moreover, some PGPB have been reported to increase lateral root length, surface area, root hair number and length in a variety of economically important crops including canola, tomato, lettuce, and wheat, which can have dramatic impacts on nutrient uptake (Bertrand et al., 2000; Khan et al., 2019; Dobbelaere and Okon, 2007; Mantelin, 2004). Consequently, there is a lot of interest in isolating select taxa and applying these as inoculants in agricultural settings to enhance crop performance (Colla et al., 2017).

One of the challenges of developing these products is that microbes may promote the growth of one plant species while providing no benefits, or acting as a pathogen, in other crop

species (Abdelrazek et al., 2020a, 2020b). In addition, the benefits provided by these microbial inoculants can vary given environmental factors associated with different types of production systems (Reeve et al., 2016). In this study, we evaluated six potential PGPB that had originally been isolated from healthy carrot taproots grown in an organic field and demonstrated to stimulate carrot growth and suppress disease by a key pathogen (Abdelrazek et al., 2020b). Subsequent laboratory assays demonstrated that many of these isolates had PGPB activity, including the capacity to produce IAA and siderophores, solubilize P, and fix atmospheric N (Abdelrazek, 2019), indicating that they could be used as microbial inoculants in a range of plant species. For example, others have demonstrated that *Bacillus spp.*, *Pantoea agglomerans*, *Paenibacillus* spp, *Pseudomonas spp.*, and *Xanthomonas spp.* can enhance the growth of carrot, lettuce, and wheat (Araújo et al., 2002; Rosenblueth and Martínez-Romero, 2006; Hashem et al., 2019; Barber and Silberbush, 1984; Kasozi et al., 2021). Results of this study indicate that some of our previously isolated microbes can enhance the growth of Arabidposis and lettuce as well as carrot, indicating that these microbes have broad applicability, and therefore could possibly be commercialized for use in CEA production systems.

In aquaponic production systems, nutrient deficiencies have been reported to be a challenge (Yep and Zheng, 2019). In a recent aquaponic study, Schwartz et al. (2019) observed a positive correlation between lettuce root surface area and fresh weight, and they developed a predictive equation to link these parameters with shoot weight to help overcome challenges in aquaponic systems (Schwartz et al., 2019). Therefore, as a first step in choosing the most promising PGPB isolate for use in CEA systems, we quantified the effect of the six microbial isolates previously isolated from carrot on Arabidopsis root morphology under *in vitro* conditions. By physically separating the microbes from the Arabidopsis seedlings, we were able to demonstrate that most of these isolates are able to stimulate root growth and alter root system architecture via the production of volatile organic compounds (VOCs). Others have demonstrated that VOCs can act as important signaling compounds between plants and microbes, stimulating root growth and in some cases suppressing pathogens and/or inducing plant defense responses (Syed-Ab-Rahman et al., 2019). Therefore, the production of VOCs represents another possible mechanism for how these microbial isolates are able to enhance the health and productivity of plants.

*Pseudomonas* spp. isolate O3 had the most significant enhancement on Arabidopsis root performance, so we chose this isolate for our subsequent aquaponic and hydroponic experiments, since root morphology (root length, surface area, and volume), is often positively corelated with nutrient uptake (Riley and Barber, 1971; Barber and Silberbush, 1984; Macduff et al., 1986). While we did not test the other PGPB isolates originally isolated from carrot taproots, it does not mean that they will not be able to enhance lettuce yield in CEA systems. Many studies have showed that *Bacillus spp*, *Pantoea agglomerans*, *Paenibacillus* spp. and *Xanthomonas* spp. are able to improve plant nutrient uptake and increase the accumulation of nutrients in plant tissues (Cerozi and Fitzsimmons, 2016b; Hussain et al., 2020; Kasozi et al., 2021). For example, *Bacillus* spp. and *Paenibacillus* spp. have been shown to increase Zn (1–15%) and Fe (3–13%) concentrations in the shoots and grains of wheat (*Triticum aestivum* L.) (Hussain et al., 2020). Moreover, when a *Bacillus* spp. was applied to aquaponically grown lettuce, nutrient deficiencies were lower and plant yield was increased (Cerozi and Fitzsimmons, 2016b; Kasozi et al., 2021). Additionally, some PGPB may not show strong PGPB activities under *in vitro* conditions because they promote plant growth using different mechanisms (Ibañez et al., 2014). For example, Kasim et al. (2013) treated wheat plants with two potential PGPB isolates, *Bacillus amyloliquefaciens* 5113 and *Azospirillum brasilense* NO40, and found that they could improve homeostatic mechanisms against drought stress, but this activity was only apparent when plants were under stress (Kasim et al., 2013). Similarly, Hashem et al. (2019) noted that thte primary benefit of *Bacillus subtilis* was in suppressing disease when a pathogen was apparent (Hashem et al., 2019). However, not all of the isolates evaluated in this trial should be considered for use as inoculants. For example, the isolate 11N, which was identified as *P. agglomerans*, reduced Arabidopsis root growth. This microbe has previously been noted to cause disease in some plant species as well as humans (Cruz et al., 2007). This further highlights the challenge in isolating microbial inoculants with broad activity for use in the agricultural industry, and the need to ensure that they will not act as opportunistic pathogens in humans (Hoagland et al., 2018).

### 5.5.2 Effect of production system and O3 inoculation on lettuce growth parameters

Plant growth parameters such as the root:shoot ratio, relative growth rate and water content can be used as indicators to quantify the effect of various treatments on nutrient deficiency, drought, and other stress factors (Trehan and Sharma, 2003; Linker and Johnson-Rutzke, 2005; Robinson and Peterkin, 2019). For example. Neocleous and Savvas (2019) investigated the effect of P limitation on lettuce grown in a recirculating nutrient solution, and found that P limitation increased the root:shoot ratio of plants, and led to a reduction in leaf biomass (Neocleous and Savvas, 2019). In our study, we used casual mediation analysis to investigate relationships between root:shoot ratio, water content, and relative growth rate. We found that all of these parameters affected total lettuce fresh weight. In particular, the root:shoot ratio and water content of lettuce plants directly affected total fresh weight but without any mediation effects, whereas relative growth rate partially mediated total fresh weight. These results indicate that relative growth rate was an important mediating factor for total fresh weight of lettuce in these production systems.

Another important finding in this study was that the root:shoot ratio of lettuce plants was negatively correlated with total fresh weight. Other studies have also observed negative relationships between root:shoot ratios and yield in a variety of economically important crops such as wheat, maize, and peanut (Bonifas et al., 2005; Feng et al., 2013; Ma et al., 2010; Wang et al., 2020a). For example, Ma et al. (2010) pruned roots of winter wheat to lower the root:shoot ratio, and demonstrated that this can increase grain yield (Ma et al., 2010). Similarly, Wang et al. (2020) found that lower root:shoot ratios in cotton were related to higher biomass in reproductive organs, and total boll number under different irrigation methods (Wang et al., 2020a). Thus, results of our study indicate that these relationships are also important in hydroponic and aquaponic production systems.

When comparing production systems, we observed that the lettuce root:shoot ratio was lower in aquaponics than in hydroponics, which likely led to higher total plant fresh weight in the aquaponic system. Lettuce grown in the aquaponic systems also had longer root length and broader root surface area than plants grown in the hydroponic system, which may have increased nutrient uptake and led to greater shoot biomass in the aquaponic system. Consequently, our results indicate that well managed aquaponic systems can be more productive than hydroponic systems and some of this could be due to changes in root architecture rather than total root biomass. Interestingly, while the root:shoot ratio was not affected by O3-inoculation in this study, we did observe greater

shoot biomass and relative growth rates in lettuce plants inoculated with O3 in the aquaponic but not hydroponic system. This could indicate that environmental conditions in the aquaponic system are more conducive to supporting the survival and efficacy of this PGPB.

Leaf water content is a critical component of plant health, and lower water content can result from drought or salinity stress, and negatively affect plant yield (Ingestad, 1982; Karlidag et al., 2010). For example, Tahara et al. (1990) found that higher winter wheat grain yield was associated with higher relative water content (Tahara et al., 1990). Previous studies have demonstrated that inoculating plants with PGPB can influence water content and enhance the productivity of many plant species (Mayak et al., 2004; Marulanda et al., 2009; Karlidag et al., 2010). For example, by producing or inducing production of IAA in plants, PGPB can increase plant water content leading to enhanced drought tolerance production (Marulanda et al., 2009). These relationships can also help mediate salinity stress. For example, Karlidag et al. (2010) demonstrated that inoculating strawberry roots with different PGPB helped plants tolerate salinity stress, and this was correlated with greater chlorophyll content, relative water content, and mineral uptake, and reduced membrane injury (Karlidag et al., 2010).

In our study, the water content of lettuce leaves was affected by both production system and O3-inoculation. Lettuce grown in the aquaponic system had higher water content than plants growing in the hydroponic system, which likely contributed to the higher plant shoot fresh weight observed in the aquaponic system. Interestingly, O3-inoculation increased water content in lettuce grown in the hydroponic, but not aquaponic system, even though total EC and Na concentrations in lettuce leaves were greater in the aquaponic system. However, Na levels in both systems did not appear to be above critical thresholds needed to induce salinity stress. Consequently, the beneficial effect of the O3 in the hydroponic system on water content must have been due to other potential stress factors such as lower nutrient availability.

Finally, plant relative growth rates are often positively correlated with nutrient concentrations and this leads to higher plant yields (Ingestad, 1982; Karlidag et al., 2010). For example, previous studies have demonstrated clear links between relative growth rate and total concentrations of N, P, K, Ca and Mg in plant tissues (Burns, 1991; Kumar et al., 2018). In this study, treating lettuce plants with O3 on lettuce resulted in a 38.6% and 30.7% increase in the relative growth rate of lettuce in aquaponics and hydroponics, respectively. Moreover, lettuce plants in both production systems had greater concentrations of P, Mg, Na and Zn in lettuce tissues

in response to the O3 inoculant, indicating that these parameters are related in these production systems as well with this PGPB.

### 5.5.3 Interactions between production system and O3 inoculation on lettuce nutrient profiles

Many isolates within the *Pseudomonas* genus have been isolated and noted for their PGPB activity with a wide diversity of crop plants using a diverse set of mechanisms including helping plants acquire nutrients and fight pathogens (Andreote et al., 2009; Zhang et al., 2012). Some *Pseudomonas* spp. Have specifically been reported to promote growth and increase nutrient uptake in lettuce, (Someya et al., 2008; Cipriano et al., 2016; Trinh et al., 2018). For example, Trinh et al., (2018) used a qRT-PCR analysis to demonstrate that inoculating lettuce plants with *P. nitroreducens* can enhance nitrate uptake and increase relative chlorophyll content, and changes in these activities were attributed to increased leaf size (Trinh et al., 2018). In our study, treating plants in the aquaponic system with O3 was also correlated with greater leaf area and higher chlorophyll content, which is consistent with the results of Trinh et al. (2018). However, inoculation did not lead to higher N accumulation in the lettuce plants, which may be due to the fact that there were adequate supplies of N in this system. It is unclear why O3 inoculation did not have the same effect in hydroponic systems, but again, could be related to environmental conditions being more conducive for survival of the isolate than the hydroponic system.

Many *Pseudomonas* isolates can increase the availability of P by releasing enzymes that hydrolyze organic P compounds into inorganic P forms that are available for plant uptake (Martínez-Viveros et al., 2010; Jang et al., 2019; Adhikari et al., 2021). For example, Cipriano et al (2016) evaluated 54 *Pseudomonas* strains and found 12 strains could enhance lettuce growth under field conditions via several mechanisms including P solubilization (Cipriano et al., 2016). Others have demonstrated the *Pseudomonas* isolates can help plants obtain Zn as well as P (Esitken et. al., 2010). We previously demonstrated that the *Pseudomonas* isolates obtained from carrots have potential to solubilize P and produce siderophores that aid in Fe scavenging using laboratory assays (Abdelrazek, 2019). The results of this trial verify that O3 can help plants obtain these nutrients when grown in two diverse production systems. Interestingly in our study, lettuce plants

also accumulated more Mg when inoculated with O3 in both production systems. Mg is theorized to be critical in helping *Pseudomonas* spp. form biofilms, and could be an important mechanism in how these bacteria help plants fight pathogens.

A SPAD meter was deployed in this study to quantify relative leaf chlorophyll correlations since this parameter often positively correlated with nutrient levels in many plant species (Bullock and Anderson, 1998; Gáborčík, 2003; Lin et al., 2010). For example, Simko (2020) noted that lettuce fresh weight and SPAD value were significantly lower when N and P levels were low in a nutrient solution. However, SPAD values are not always positively related to nutrient levels. For example, Shams et al., (2019) observed a negative correlation between SPAD and nutrient levels when plants were exposed to excess copper, even though there were adequate supplied of N, P, and K for plant uptake. In our study, lettuce plants treated with O3 had greater SPAD values 7 days after transplanting in both production systems, which was correlated with greater uptake of P, Mg and Z in this study. However, while there were significant differences in SPAD value among O3 treated plants 21 days after transplanting in the aquaponic system, there were no differences in the hydroponic system. Since plants in the hydroponic system had lower biomass than the aquaponic system, this indicates that the plants in the hydroponic system may have been stressed, and the SPAD meter was able to detect this.

While many studies often focus on the critical role of macronutrients, micronutrients such as Fe, Mn, and Zn can also influence essential plant processes such photosynthesis rate, and deficiencies in these micronutrients can lead to lower plant yields (Aravind and Prasad, 2004; Barker and Pilbeam, 2015). For example, Roosta (2018) cultured lettuce under Fe, Mn, and Zn deficiency in a hydroponic system and found that shortages of these micronutrients significantly reduced photosynthetic pigments and chlorophyll fluorescence parameters (Roosta et al., 2018). In our study, Zn and Cu concentrations in lettuce tissues were higher in plants grown in the aquaponic vs. hydroponic system, which could have contributed to higher SPAD rates at 21 days after transplanting and plant growth. In addition, non-inoculated lettuce plants contained lower concentrations of Zn in both production systems, which was correlated with lower photosynthetic rates, stomatal conductance, transpiration rates, and water use efficiency at 7 days after transplanting. These results demonstrate further demonstrate that aquaponic systems can be more

productive than hydroponic systems, and that inoculating plants with PGPB in these systems can aid in the health, productivity and nutritional quality of plants.

Finally, it is interesting to note that many nutrients (K, Ca, S, Fe, Mn, B, Cu) were significantly greater in lettuce plants inoculated with O3 in the hydroponic but not aquaponic system in this study. This indicates that while the *Pseudomonas* isolate appeared to have less capacity to promote plant growth in the hydroponic system, it was still able to colonize lettuce plant roots and provide benefits in this system. Additional studies aimed at determining how environmental conditions in these unique production systems enhance the survival and efficacy of PGPR are recommended to optimize the benefits of these inoculants in the CEA production systems.

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Table 5.1. Potential plant growth-promoting bacteria (PGPB) isolates.

| Isolate[a] | Bacteria | NCBI Accession # of closest hit | NCBI deposit Accession # |
|---|---|---|---|
| 11D2 | *Bacillus spp* | LN878354.1 | TBD[b] |
| 11N | *Pantoea agglomerans* | KY127366.1 | TBD |
| O1 | *Paenibacillus spp* | KX953866.1 | SAMN13006134 |
| O3 | *Pseudomonas spp.* | KX758046.1 | SAMN13006141 |
| O10 | *Xanthomonas spp* | KM252981.1 | SAMN13006129 |
| O12 | *Pseudomonas fluorescens* | FJ225306.1 | SAMN13006133 |

[a] PGPB isolates obtained from carrot taproots (Abdelrazek et al., 2020b; Abdelrazek, 2019)

[b]TBD (to be determined) after manuscript has been accepted for publication

Table 5.2. Nutrient composition and some macro- and micro-nutrient contents in hydroponic nutrient solutions and aquaponics fish feed.

| Parameter | Hydroponics fertilizer [a] | Aquaponics fish feed [b] |
|---|---|---|
| Macronutrient (%) | | |
| Total nitrogen (N) | 0.043 | > 6.88 |
| $P_2O_5$ -P | 0.093 | > 1.10 |
| $K_2O$-K | 0.035 | 0.99 |
| $SO_4$-S | – | 0.43 |
| Ca | 0.075 | 2.25-2.75 |
| Mg | 0.039 | 0.23 |
| Micronutrient (mg/kg) | | |
| B | 2 | – |
| Cu | 1.05 | 10 |
| Fe | 21 | 40 |
| Mn | 1.9 | 80 |
| Mo | 0.42 | – |
| Zn | 2.1 | 153 |

Data obtained from product description.

"–" means not included or no related information.

[a] Nutrient compositions of fertilizer used in hydroponics were calculated based on 1:100 dilution of commercial fertilizer.

[b] Nutrient compositions of fish feed used in aquaponics were calculated based on g feed per day.

Table 5.3. Effects of potential plant growth-promoting bacteria (PGPB) isolates on Arabidopsis root parameters.

| Treatment | Length (cm) | Surface area ($cm^2$) | Root volume ($10^3$ $cm^3$) | Length per volume ($cm/cm^3$) | Average diameter (mm/plant) |
|---|---|---|---|---|---|
| Control | 11.8±3.6 b | 0.63±0.22b | 3±1b | 12.0±4.0b | 0.17±0.01b |
| 11D2 | 12.8±7.7 b | 0.76±0.47a | 3.7±2.1b | 12.8±7.7b | 0.20±0.04a |
| 11N | 7.9±3.5 c | 0.44±0.19c | 2.0±0.7c | 7.9±3.5c | 0.18±0.01b |
| O1 | 12.3±5.7 b | 0.70±0.34b | 3.3±1.8b | 12.3±5.7b | 0.18±0.02b |
| O3 | 16.5±5.3a | 1.00±0.40a | 5.2±2.0a | 16.5±5.3a | 0.20±0.02a |
| O10 | 13.7±4.5 b | 0.81±0.29a | 3.9±1.6b | 13.7±4.5b | 0.18±0.01b |
| O12 | 15.0±3.9 a | 0.92±0.25a | 4.6±1.4a | 15.0±3.9b | 0.20±0.03a |

Each treatment consisted of 36 replicates. Each value in the table is the mean ± SD. Values within columns followed by the same letter are not significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha$ =0.05).

Table 5.4. Physical and chemical water-quality parameters in aquaponic and hydroponic systems averaged for 4 weeks.

| System | pH | pH correction solution (mL/day) | EC (mS/cm) | DO (mg/L) | Temperature (°C) |
|---|---|---|---|---|---|
| Aquaponics | 6.0±0.0 (5.7-6.2) | 34.8±4.7 (0-170) | 1.55±0.01 (1.43-1.66) | 7.72±0.05 (6.78-8.21) | 23.1±0.1 (22.2-24.8) |
| Hydroponics | 6.0±0.0 (5.7-6.2) | 15.0±0.4 (0-15) | 1.53±0.01 (1.45-1.78) | 7.62±0.03 (6.52-8.08) | 22.9±0.1 (21.5-24.0) |
| | ns | *** | * | ns | ns |

Abbreviations: DO, dissolved oxygen; EC, electrical conductivity. Each value in the table is the mean ± SE (range: min.-max.). ns, *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 5.5. Effect of production system and plant growth-promoting bacteria (PGPB) inoculation on fresh and dry weight of lettuce 28 days after transplanting.

| System | Inoculation | Fresh weight (g/plant) | | | Dry weight (g/plant) | | |
|---|---|---|---|---|---|---|---|
| | | Total | Shoots | Roots | Total | Shoots | Roots |
| Aquaponics | Control | 106.4±3.4 a | 104.1±3.6 a | 2.35±0.23 | 4.14±0.14 ab | 3.872±0.136b | 0.271±0.025 |
| | O3 | 108.5±3.6 a | 106.4±3.5 a | 2.16±0.17 | 4.65±0.16 a | 4.413±0.144a | 0.242±0.026 |
| Hydroponics | Control | 82.0±4.0 b | 79.7±4.1 b | 2.37±0.21 | 3.96±0.15 b | 3.691±0.14 b | 0.269±0.032 |
| | O3 | 78.9±3.6 b | 76.7±3.5 b | 2.21±0.17 | 3.61±0.16b | 3.441±0.142b | 0.171±0.03 |
| ANOVA | | | | | | | |
| System | | *** | *** | ns | ns | ns | ns |
| Inoculation | | ns | ns | ns | * | ** | ns |
| System x Inoculation | | ns | ns | ns | ** | ** | ns |

Abbreviations: O3, PGPB (*Pseudomonas* spp.). Each value in the table is the mean ± SD. Values within columns followed by the same letter are not significantly different based on Tukey's honestly significant difference (HSD) test (α =0.05). *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 5.6. Effect of production system and plant growth-promoting bacteria (PGPB) on lettuce growth parameters 28 days after transplant.

| System | Inoculation | Height (cm) | Leaf number | Leaf area($cm^2$) |
|---|---|---|---|---|
| Aquaponics | Control | 8.2±0.2 a | 26±0b | 1381.2±41.1 |
| | O3 | 9.8±0.2 a | 30±1a | 1447.3±45.3 |
| Hydroponics | Control | 9.1±0.3ab | 26±0 c | 1315.2±40.7 |
| | O3 | 9.6±0.3b | 28±0 bc | 1312.8±56.5 |
| ANOVA | | | | |
| System | | ns | ** | ns |
| Inoculation | | ns | ** | * |
| System x Inoculation | | * | ns | ns |

Abbreviations: Each value in the table is the mean ± SD. Values within columns followed by the same letter are not significantly different based on Tukey's honestly significant difference (HSD) test (α =0.05). *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 5.7. Effect of production system and plant growth promoting bacteria (PGPB) on lettuce root:shoot ratio, water content, and relative growth rate 28 days after transplant.

| System | Inoculation | Root:shoot ratio | Water content (%) | Relative growth rate (%) |
|---|---|---|---|---|
| Aquaponics | Control | 2.3±0.51b | 25.1±1.0a | 37.0±1.4b |
| | O3 | 2.1±0.17b | 22.6±0.5ab | 44.2±1.5a |
| Hydroponics | Control | 3.8±0.53a | 19.9±0.9b | 28.3±1.4c |
| | O3 | 3.5±0.15ab | 21.5±0.7b | 31.9±1.3bc |
| ANOVA | | | | |
| System | | ** | *** | *** |
| Inoculation | | ns | * | *** |
| System x Inoculation | | ns | * | ns |

Abbreviations: Each value in the table is the mean ± SD. Values within the same columns followed by the same letter are not significantly different based on Tukey's honestly significant difference (HSD) test (α =0.05). *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 5.8. Effect of production system and plant growth-promoting bacteria (PGPB) on lettuce root parameters 28 days after transplant.

| System | Inoculation | Length (cm) | Surface area ($cm^2$) | Average diameter (mm) | Root volume ($cm^3$) | Length per volume ($cm/m^3$) |
|---|---|---|---|---|---|---|
| Aquaponics | Control | 2397.6±90.2a | 347.4±18.4a | 0.46±0.01 | 4.0±0.2a | 2397.6±90.2a |
| | O3 | 2424.5±125.5a | 329.4±15.7ab | 0.43±0.01 | 3.6±0.2ab | 2397.6±125.5a |
| Hydroponics | Control | 1990.1±96.3b | 282.7±10.7b | 0.46±0.01 | 3.2±0.1b | 1990.1±96.3b |
| | O3 | 2323.3±60.5ab | 314.4±10.9ab | 0.43±0.01 | 3.4±0.2ab | 2323.3±60.5ab |
| ANOVA | | | | | | |
| System | | ** | *** | ns | ** | *** |
| Inoculation | | ns | ns | * | ns | ns |
| System x Inoculation | | ns | ns | ns | ns | * |

Abbreviations: O3, PGPB (*Pseudomonas* spp.). Each value in the table is the mean ± SD. Values within the same columns followed by the same letter are not significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha$ =0.05). *, **, *** mean no significant or significant at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 5.9.Results of the structural equation modeling used to evaluate relationships between plant growth promoting bacteria (PGPB) and lettuce growth parameters

| **Hypothesis** | **Relationship** | **Anticipate Impact** | **p-Value** | **Result** |
|---|---|---|---|---|
| H1 | PGPB-inoculation, TFW[a] | + | < 0.005 | Confirmed |
| H2 | PGPB-inoculation, root:shoot ratio | - | 0.588 | Not-confirmed |
| H3 | PGPB-inoculation, water content | + | 0.571 | Not-confirmed |
| H4 | PGPB-inoculation, relative growth rate | + | < 0.005 | Confirmed |
| H5 | root:shoot ratio, TFW | - | < 0.005 | Confirmed |
| H6 | water content, TFW | + | < 0.005 | Confirmed |
| H7 | relative growth rate, TFW | + | < 0.005 | Confirmed |

[a]Total fresh weight of lettuce



Table 5.10. Results of the causal mediation modeling used to evaluate relationships between plant growth promoting bacteria (PGPB) and lettuce growth parameters

| Relationship | ACME (p-Value) | ADE (p-Value) | Result |
|---|---|---|---|
| PGPB-inoculation, root:shoot ratio, TFW | 0.62 | 0.62 | Not-confirmed |
| PGPB-inoculation, water content, TFW | 0.59 | 0.89 | Not-confirmed |
| PGPB-inoculation, relative growth rate, TFW | < 0.005 | < 0.005 | Confirmed |

Average causal mediation effect (ACME), average direct effect (ADE), total fresh weight (TFW)

Table 5.11. Fv/Fm, SPAD, photosynthetic rate (*Pn*), stomatal conductance (*gs*), intercellular $CO_2$ concentration (*Ci*), transpiration rate (*E*), and water use efficiency (WUE) of lettuce as affected by production system and plant growth-promoting bacteria (PGPB) inoculation at 7 and 21 days after transplanting.

| System | Inoculation | Fv/Fm | SPAD | *Pn* | *gs* | *Ci* | *E* | WUE |
|---|---|---|---|---|---|---|---|---|
| | | *At Day 7* | | | | | | |
| Aquaponics | Control | 0.83±0.00 | 27.5±0.2b | 3.00±0.58 | 0.35±0.05 | 415.8±5.5 | 2.8±0.3 | 11.7±2.9 |
| | O3 | 0.84±0.01 | 29.6±0.5a | 4.37±1.43 | 0.38±0.03 | 392.4±6.5 | 3.3±0.1 | 13.3±4.9 |
| Hydroponics | Control | 0.82±0.02 | 30.9±0.5 | 1.87±0.15 | 0.28±0.03 | 410.2±4.0 | 2.6±0.2 | 9.7±2.6 |
| | O3 | 0.81±0.03 | 31.6±0.8 | 2.16±0.18 | 0.32±0.03 | 407.5±1.1 | 2.8±0.2 | 7.3±0.6 |
| ANOVA | | | | | | | | |
| System | | ns | *** | ns | ns | ns | ns | ns |
| Inoculation | | ns | * | ns | ns | ns | ns | ns |
| System x Inoculation | | ns | ns | ns | ns | ns | ns | ns |
| | | *At Day 21* | | | | | | |
| Aquaponics | Control | 0.81±0.01 | 29.1±0.7 | 2.57±0.23 | 0.45±0.03a | 400.6±1.2 | 6.4±0.2a | 6.2±0.6 |
| | O3 | 0.81±0.01 | 30.6±0.6 | 2.22±0.17 | 0.36±0.03b | 400.7±1.3 | 5.5±0.3b | 6.9±0.8 |
| Hydroponics | Control | 0.82±0.01 | 29.8±0.9 | 2.44±0.17 | 0.37±0.05 | 410.7±7.8 | 5.4±0.5 | 13.2±5.1 |
| | O3 | 0.81±0.01 | 28.9±0.6 | 2.51±0.18 | 0.38±0.06 | 407.8±3.8 | 5.2±0.5 | 11.2±2.6 |
| ANOVA | | | | | | | | |
| System | | ns | ns | ns | ns | ns | ns | ns |
| Inoculation | | ns | ns | ns | ns | ns | ns | ns |
| System x Inoculation | | ns | ns | ns | ns | ns | ns | ns |

Abbreviations: O3, PGPB (*Pseudomonas* spp.). Each value in the table is the mean ± SD following by the same letter are not significantly different based on Tukey's honestly significant difference (HSD) test ($\alpha$ =0.05). ns, *, **, *** mean no significance or significant differences at $p \leq 0.05$, 0.01, or 0.001, respectively.

Table 5.12. Effect of production system and plant growth-promoting bacteria on average mineral nutrient concentrations of lettuce at 28 days after transplant.

| System | Inoculation | N (%) | P (%) | K (%) | Mg (%) | Ca (%) | S (%) | Na (%) |
|---|---|---|---|---|---|---|---|---|
| Aquaponics | Control | 6.13±0.12 | 1.00±0.04 | 8.39±0.09 | 0.40±0.01 | 1.04±0.03 | 0.28±0.01 | 0.24±0.02 |
| | O3 | 6.10±0.14 | 1.04±0.03 | 8.43±0.23 | 0.42±0.01 | 1.08±0.03 | 0.36±0.06 | 0.31±0.02 |
| | | ns | * | ns | * | ns | ns | *** |
| Hydroponics | Control | 5.80±0.17 | 0.82±0.07 | 7.07±0.51 | 0.38±0.02 | 0.94±0.07 | 0.27±0.01 | 0.11±0.01 |
| | O3 | 5.61±0.22 | 0.91±0.09 | 7.42±0.56 | 0.44±0.03 | 1.12±0.10 | 0.30±0.02 | 0.16±0.01 |
| | | ns | *** | *** | * | *** | * | ** |
| ANOVA | | | | | | | | |
| System | | ns | ns | * | ns | ns | ns | *** |
| Inoculation | | ns | ** | ** | * | *** | ** | *** |
| System x Inoculation | | ns | * | ** | ns | *** | ns | ns |

| system | treatment | Fe (ppm) | Mn (ppm) | B (ppm) | Cu (ppm) | Zn (ppm) |
|---|---|---|---|---|---|---|
| Aquaponics | Control | 137.2±5.0 | 142.78±9.13 | 32.78±0.72 | 13.00±0.75 | 81.56±8.71 |
| | O3 | 133.6±2.8 | 149.78±5.51 | 34.33±0.99 | 14.33±0.70 | 83.44±7.64 |
| | | ns | ns | ns | ns | * |
| Hydroponics | Control | 112.4±6.3 | 122.44±14.42 | 38.44±1.47 | 9.33±0.77 | 55.56±6.22 |
| | O3 | 129.4±6.7 | 154.56±17.95 | 43.00±1.63 | 10.89±0.97 | 61.22±7.13 |
| | | * | *** | *** | *** | *** |
| ANOVA | | | | | | |
| System | | ns | ns | *** | *** | * |
| Inoculation | | ns | *** | *** | *** | *** |
| System x Inoculation | | ns | ** | * | * | ns |

Abbreviations: O3, PGPB (*Pseudomonas* spp.). Each value in the table is the mean ± SD. ns, *, **, *** indicate no significance or significant differences at $p \leq 0.05$, 0.01, or 0.001, respectively.

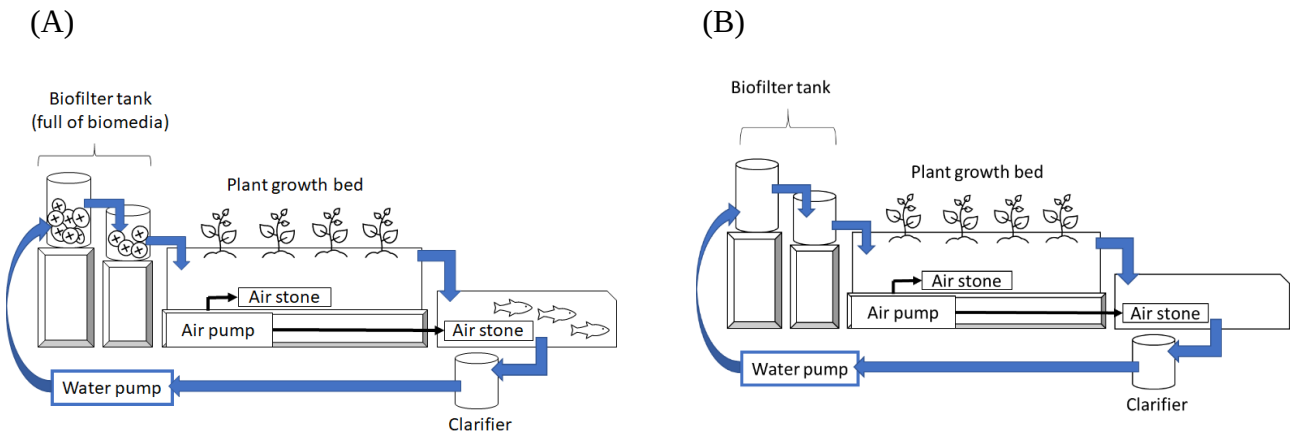



Figure 5.1. Schematic diagram of the experimental units: (**A**) aquaponic system and (**B**) hydroponic system.

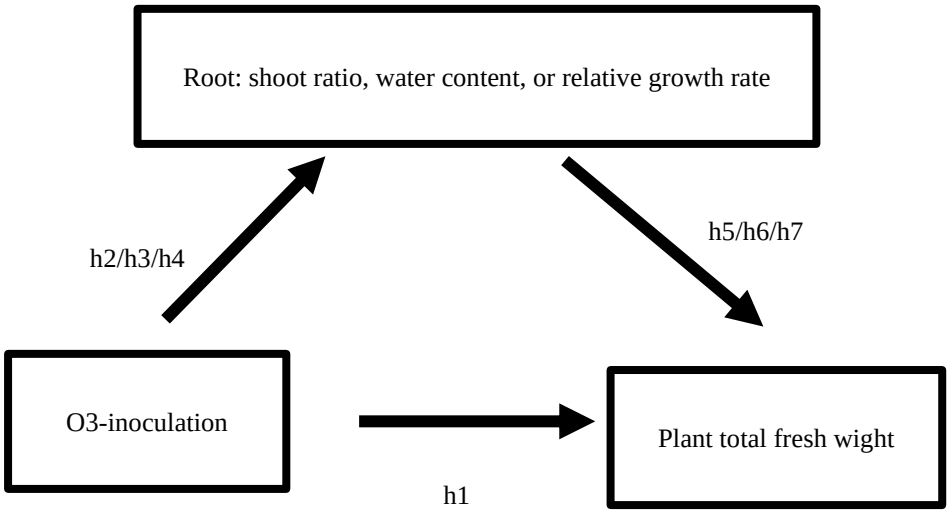
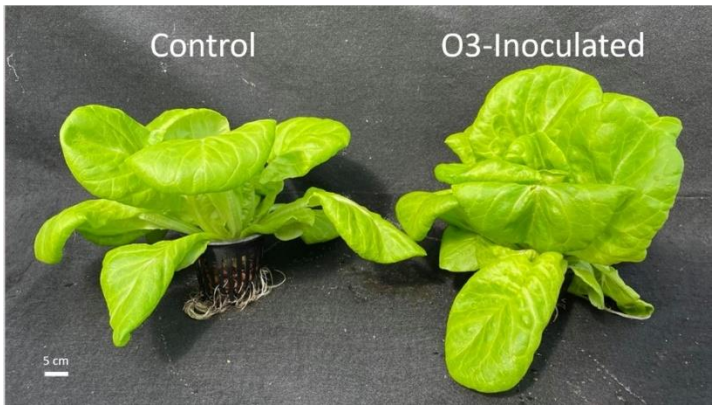



Figure 5.2.Hypotheses associated with the mediation model.

(A)




(B)


Figure 5.3. Whole lettuce plants 28 days after transplanting in aquaponics (A) and hydroponics (B).

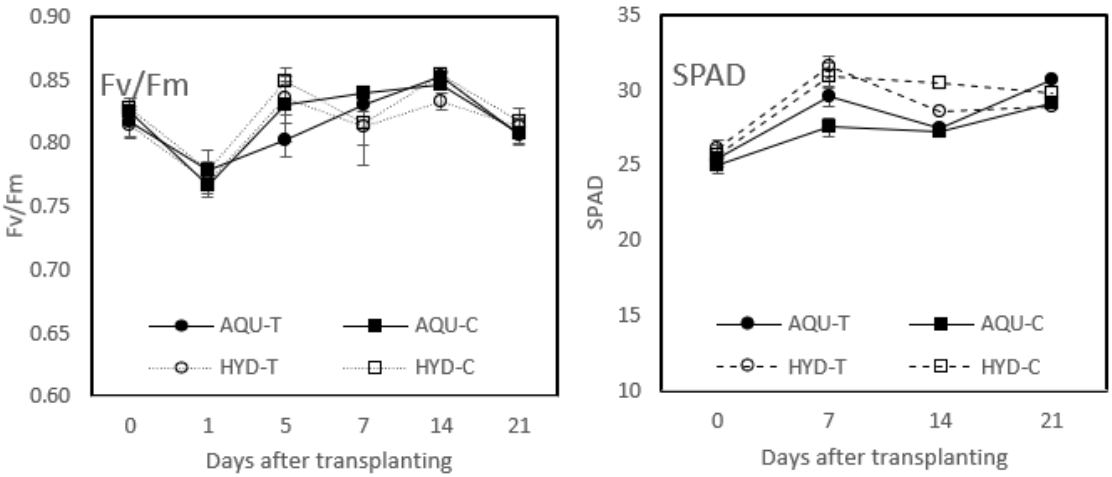



Figure 5.4. Dynamic changes in Fv/Fm and SPAD in lettuce leaves as affected by production system and plant growth-promoting bacteria (PGPB) inoculation. Each data point is the mean of 4 replicates ± SD. AQU-C, control in aquaponics; AQU-I, PGPB inoculation in aquaponics; HYD-C, control in hydroponics; HYD-I, PGPB inoculation in hydroponics.

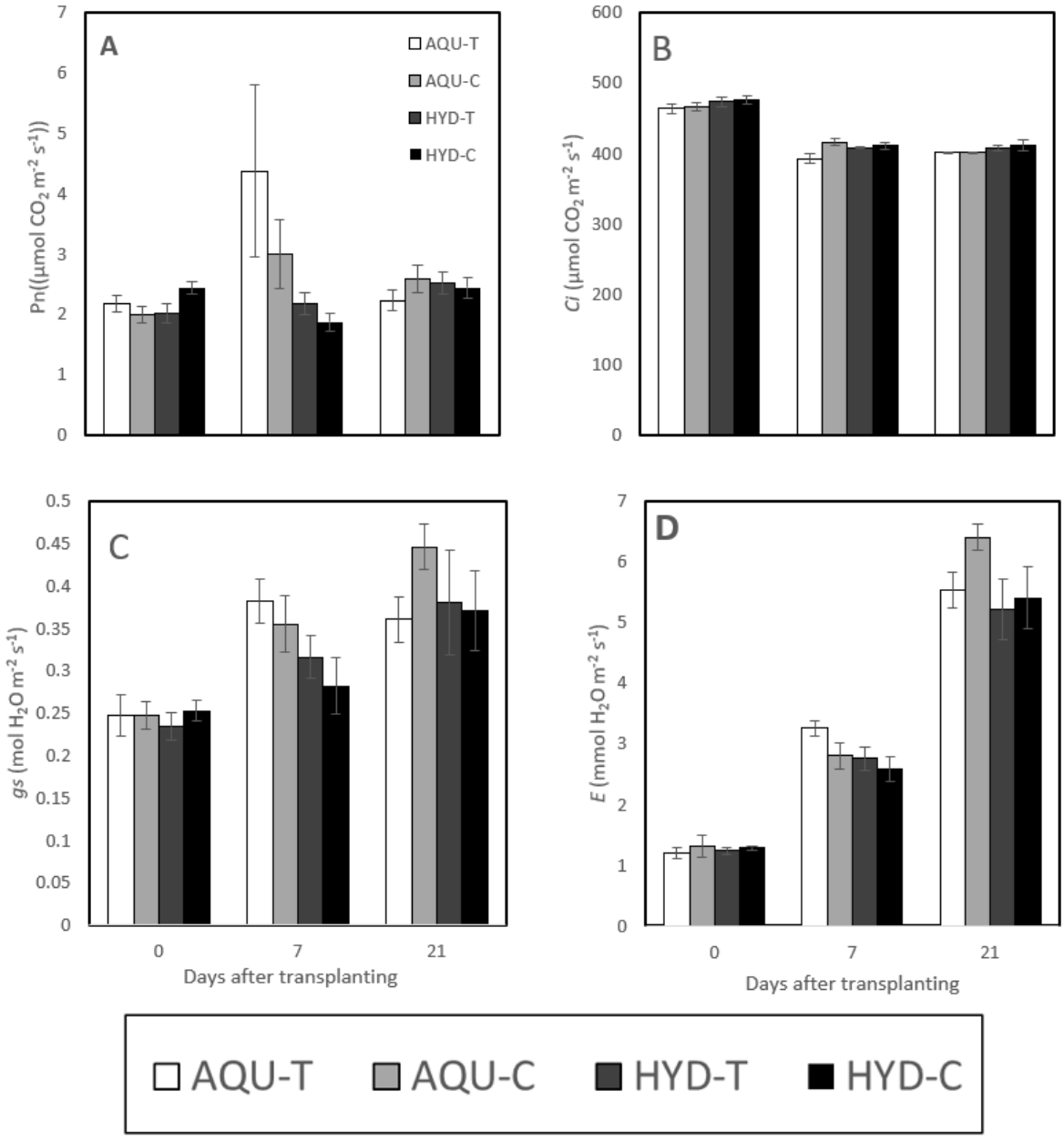



Figure 5.5. Dynamic changes in (A) photosynthetic rate (*Pn*);(B) intercellular CO2 concentration (*Ci*);(C) stomatal conductance (*gs*); and (D) transpiration rate (*E*) of lettuce as affected by production system and plant growth-promoting bacteria (PGPB) inoculation. Each data point is the mean of 5 replicates ± SD. AQU-C, control in aquaponics; AQU-I, PGPB inoculation in aquaponics; HYD-C, control in hydroponics; HYD-I, PGPB inoculation in hydroponics.

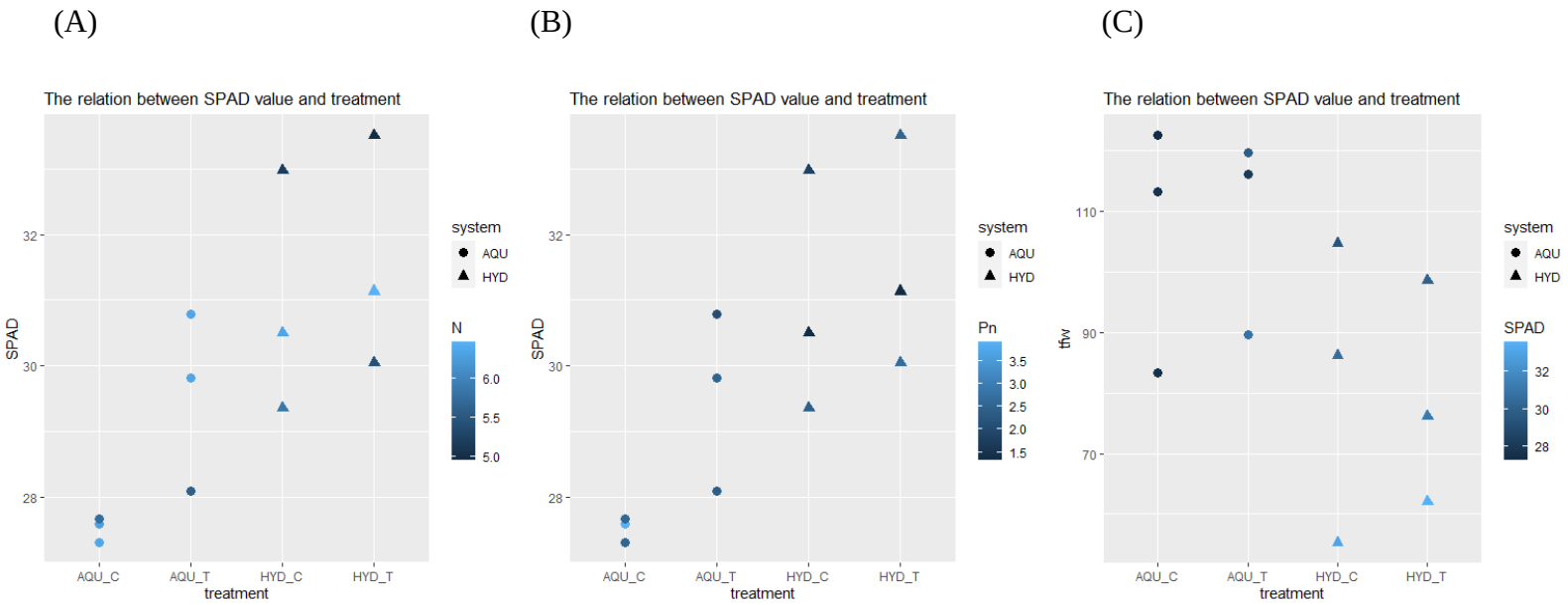



Figure 5.6. Relationships between SPAD value and (A) nitrogen (N) level, (B) photosynthesis rate (Pn), and (C) total fresh weight (tfw) (g). AQU_C, control in aquaponics, AQU_T, treated PGPB in aquaponics, HYD_C, control in hydroponics, HYD_T, treated PGPB in hydroponics.

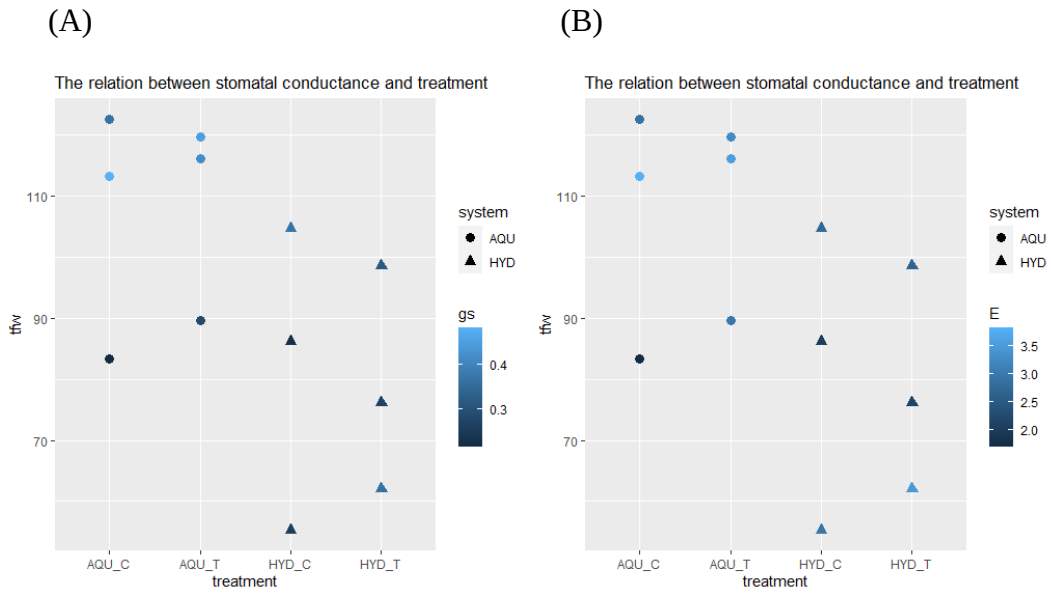



Figure 5.7. Relationships between total fresh weight (tfw) (g) and (A) stomatal conductance (gs) and (B) transpiration rate (E) at day 7 after transplanting in aquaponics and hydroponics. AQU_C, control in aquaponics, AQU_T, treated PGPB in aquaponics, HYD_C, control in hydroponics, HYD_T, treated PGPB in hydroponics.

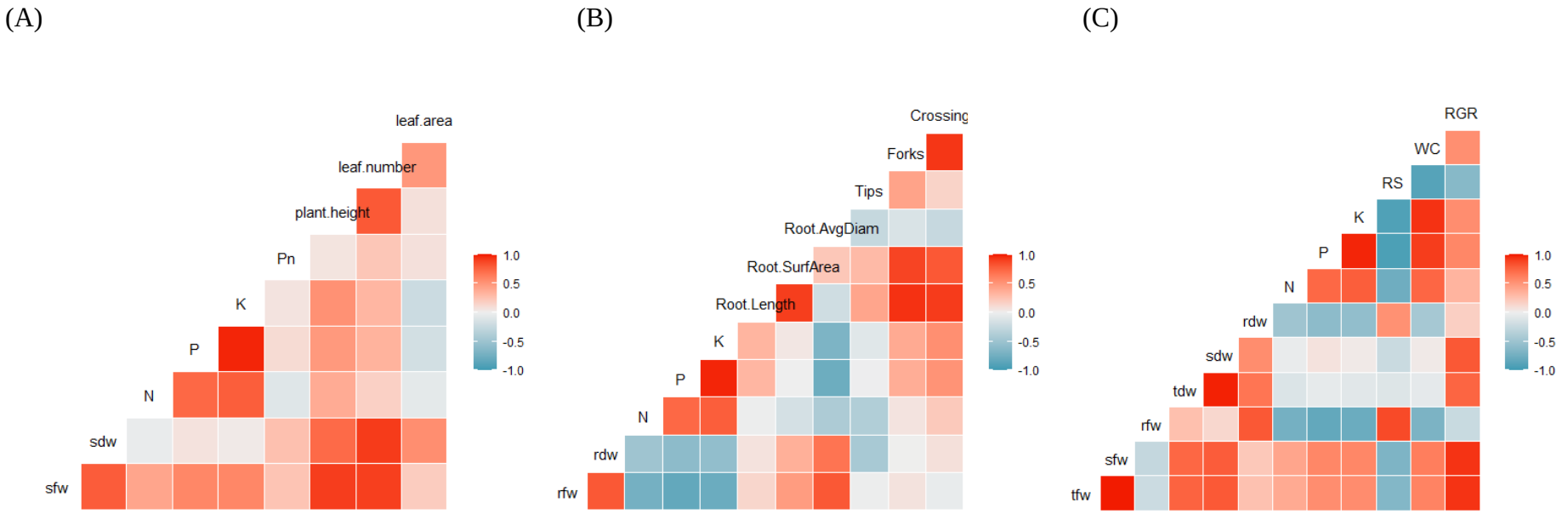



Figure 5.8 Correlations between N, P, K, and (A) shoot fresh weight (sfw), shoot dry weight (sdw) photosynthesis rate (Pn), plant height, leaf number, and leaf area; (B) root fresh weight (rfw), root dry weight (rdw), root length, root surface area, average diameter of roots, root tips, forks, amd crossing; (C) plant biomass, root:shoot ratio (RS), water content (WC), and relative growth rate (RGR).

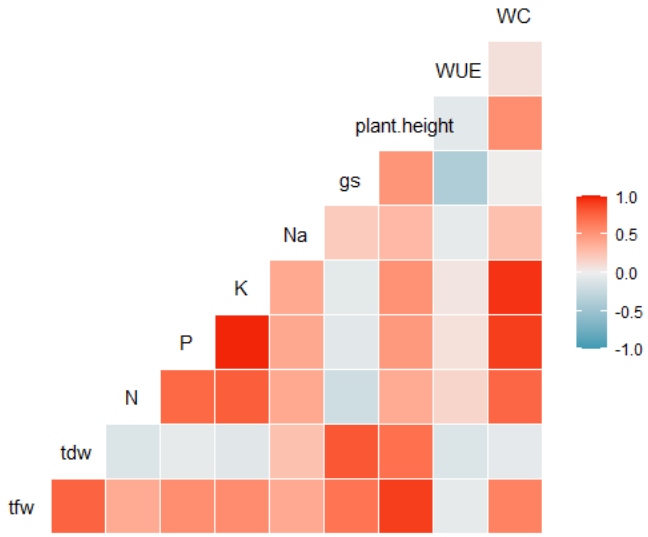
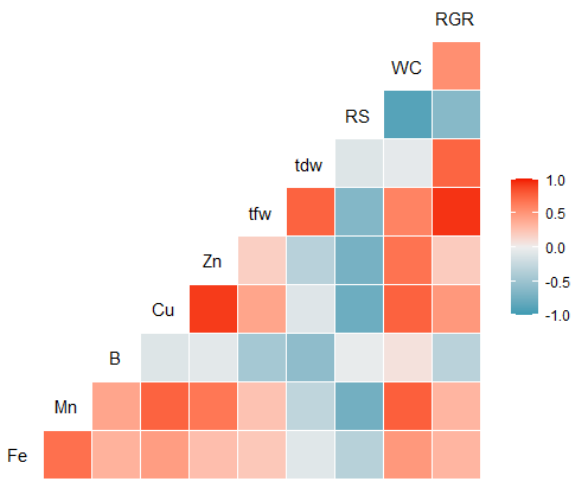



Figure 5.9 Correlations between Na and total fresh weight (tfw), total dry weight (tdw), N, P, K, stomatal conductance (*gs*), plant height, water use efficiency (WUE), and water content (WC).

(A)




(B)

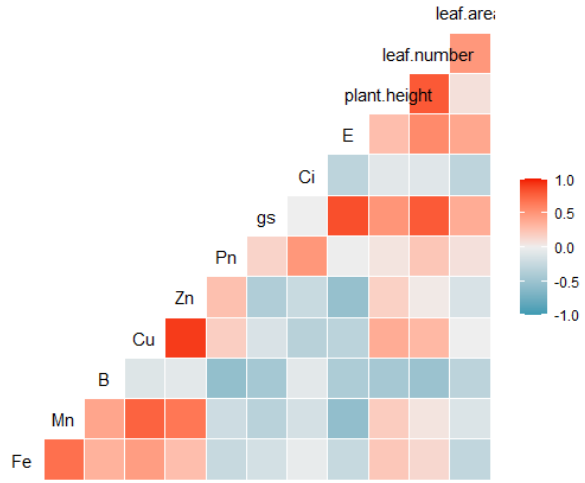



Figure 5.10 Correlations between micronutrients and (A) total fresh weight (tfw), total dry weight (tdw), root:shoot ratio (RS), water content (WC), and relative growth rate (RGR); (B) photosynthetic rate (Pn), stomatal conductance (gs), intercellular CO2 concentration (Ci), transpiration rate (E), plant height, leaf number, and leaf area