ABSTRACT

Title of Dissertation:	INTEGRATING FOOD SAFETY AND SUSTAINABILITY: INVESTIGATING LETTUCE AND KALE INTERACTIONS WITH *SALMONELLA* AS INFLUENCED BY STRESS AND PLANT GROWTH-PROMOTING RHIZOBACTERIA
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As the human population grows and living standards improve, the demand for safe and nutritious food is increasing. Fresh produce provides essential nutrients and lowers the risks of chronic diseases, including cancer. Increased consumption of fresh produce, especially raw leafy greens, is suggested as a restorative practice to maintain and optimize human health. However, leafy greens and raw produce are frequently associated with foodborne pathogens, causing foodborne illnesses and economic loss. Contamination of foodborne pathogens could happen at any stage from the farm to fork continuum, becoming impossible to remove completely once

contaminated. An in-depth understanding of the association between leafy greens and foodborne pathogens could provide insights into this complex association and allow the more efficient restriction of foodborne pathogens on leafy greens. This study investigated the *Salmonella* and human pathogen association from the plant nutrient connection using lettuce and kale as plant material. The impact of plant cultivar, drought, and plant development on *Salmonella* association on leaf surface was evaluated. Our results identified that red loose-leaf lettuce 'Mascara' with high contents of secondary metabolites were less favorable to *Salmonella* compared to green Romaine lettuce. Drought restricted the *Salmonella* association on surfaces of 'Mascara' lettuce and caused a more prolonged lag phase of *Salmonella* in lettuce washes. Leaf and wash phytochemical profiles of 'Mascara' lettuce diverged from Romaine lettuce and shifted in response to drought. The decline of *Salmonella* was positively associated with total flavonoids, phenolics and anthocyanins levels in lettuce tissues. Kale plants from 20 days (baby kale) to 59 days (ready-to-harvest mature kale) post-germination were recruited in this study to identify the impact of drought and plant development on *Salmonella* association. Drought impaired the *Salmonella* association on the baby kale surface and in washes, and shifted the phytochemical profiles, but not on mature kale plants. Baby kale plants supported higher levels of *Salmonella* than mature kale plants. Accumulation of flavonoids, possibly phenolics, but not glucosinolates in kale tissues, are of future interest as *Salmonella* restrictive compounds. To ensure food security under climate change, the impact of two strains of plant growth-promoting rhizobacteria (PGPR) - *Pseudomonas* spp., on plant growth promotion under environmental stresses and epiphytic *Salmonella* restriction were studied. For 26 days old kale plants, root inoculation of *Pseudomonas* spp. showed no growth-promoting effects but limited the *Salmonella* population on kale surface without changing the contents of secondary metabolites under regular watering

condition. Salinity reduced kale plant biomass accumulation and limited *Salmonella* growth on surfaces, with higher antioxidant capacity detected in kale tissues. For both 20 days (baby) and 59 days (mature) kale, the inoculation of *Pseudomonas* spp. protected plants from biomass reduction due to drought and limited the surface survival of *Salmonella* under regular watering condition, with limited changes in overall phytochemical profiles and no changes in secondary metabolites detected. In summary, this study advanced our understanding of the impact of phytochemicals and plant growth-promoting rhizobacteria on the *Salmonella* and leafy green association. Our data suggested that the selection of specific cultivars, regulated abiotic elicitation of secondary metabolites and application of beneficial rhizobacteria are promising strategies to maintain plant growth and enhance food safety and nutritional quality under the great challenge of climate change.

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by

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Chapter 1: Introduction

Salmonella, the leading bacteria that causes the most foodborne illness in the United States, is a primary public health concern and economic burden (Scallan et al., 2011b). Food safety incidents cost over $15.5 billion annually in the USA with non-typhoidal *Salmonella enterica* subsp. *enterica* incurring the highest economic burden compared to all other 14 human foodborne pathogens considered (Hoffman et al., 2015). According to the Centers for Disease Control and Prevention (CDC), 1 in 6 Americans are affected by foodborne illness annually, and 3,000 of those illnesses lead to death (Scallan et al., 2011a; Scallan et al., 2011b). In the USA, from 1998 to 2008, leafy greens were associated with 46% of foodborne diseases (Painter et al., 2013), and from 1973 to 2012, 11% of foodborne illness outbreaks associated with leafy greens were caused by *Salmonella enterica* (Herman et al., 2015). The growth in foodborne disease outbreaks associated with fresh fruit and vegetables (Callejón et al., 2015b; Lynch et al., 2009) may be attributed to the rise in fresh produce consumption, more centralized product processing and distribution, climate change, and pathogen adaptation. The growing consumption of fresh and raw leafy greens requires an in-depth project to study the interaction of leafy greens and human pathogens, thus ensuring the safety of fresh produce.

Under the influence of climate fluctuation, a higher frequency of drought and other abiotic stresses have dramatically reduced crop yield and affected plant physiological traits (Bechtold et al., 2021; Skirycz et al., 2010; Urano et al., 2009; Verslues and Juenger, 2011). Drought is considered the primary threat among abiotic stresses and is predicted to worsen (Cominelli et al., 2009). Moreover, salinity stress is an emerging issue in coastal regions due to sea-level rise and seawater intrusion (White and Kaplan, 2017). Plants display a range of mechanisms

to adapt abiotic stresses such as drought and salinity stress, including stomatal closure to reduce water loss (Azevedo Neto et al., 2004; Flexas and Medrano, 2002; Robinson et al., 1997), accumulation of low-molecular weight osmoprotectants to lower water potential (Singh et al., 1972; Streeter et al., 2001) and antioxidant compounds to cope with oxidative stresses (Eek and Zhang, 2017). Simultaneously, plant growth-promoting rhizobacteria (PGPR), one kind of biotic factor, can colonize plant roots and provide beneficial services to plants (Loper and Henkels, 1999; Upadhyay et al., 2011). PGPR can also alter plant metabolomes (Upadhyay et al., 2012) and the composition of phyllosphere microbial community structures (Ghorbanpour et al., 2013; Massoni et al., 2021), thus impacting the plant-microbe association.

Salmonella is able to colonize plant surfaces under relatively high humidity and survive under dry conditions (Brandl and Mandrell, 2002). The availability of phytonutrients on plant surfaces determined the successful epiphytic growth and population size (Brandl and Amundson, 2008; Goudeau et al., 2013; Hirano and Upper, 1990a; Mercier and Lindow, 2000). Most phytonutrients readily available to *S. enterica* were leached and exuded from plant surfaces (Mercier and Lindow, 2000). Leaf age, plant genotype, and abiotic stresses could impact the composition and abundance of phytonutrients and affect bacterial populations, including enteric pathogens (Han and Micallef, 2016; Hunter et al., 2010; Ryffel et al., 2016). Studies aiming to illustrate plant phytocompound profile changes in response to various conditions and the influence of those changes on enteric pathogen-leafy green associations can provide direction for enhancing food safety from the plant perspective (George and Brandl, 2021; Melotto et al., 2020), albeit little is investigated.

Human pathogens, such as *Salmonella enterica*, could be introduced to produce at any stage of the farm to fork continuum and survive the production, processing, and harvest (Bennett et al., 2015; Harris et al., 2003; Olaimat and Holley, 2012). It is rather complex to reduce and eliminate human pathogens once they establish on the plant surface (Goodburn and Wallace, 2013; Parish et al., 2003). To enhance food safety and reduce its collateral economic losses, Good Agricultural Practices (GAPs) and Produce Safety Rule (PSR) under the Food Safety Modernization Act (FSMA) have been established to offer guidance and recommendations for growers to prevent foodborne outbreaks. Nevertheless, a more thorough understanding of leafy green – foodborne pathogen interaction is required to reduce foodborne outbreaks. The goal of this project is to explore the relationships between the survival of foodborne pathogens on leafy greens and the plant metabolome and surface metabolome that change during development or as affected by abiotic stress (drought and salinity), and in the presence of PGPR, and under the combination of abiotic stress and PGPR. Results from this project can fill current knowledge gaps, then provide solutions to control foodborne illnesses such as salmonellosis caused by *S. enterica.* Furthermore, agricultural practices that can enhance the accumulation of foodborne pathogen growth-limiting compounds or recruit more enteric pathogen growth-limiting microbial organisms will be introduced to growers under the significant challenges caused by climate change.

The central hypotheses of this study were listed below:

Hypothesis 1: The accumulation of antioxidant compounds and phytochemical profile changes in response to plant cultivar, drought stress, or as plants develop restrict the survival of *Salmonella enterica* on the leafy green surface.

Hypothesis 2: Root inoculation of *Pseudomonas* spp. S2 and S4 could alleviate the detrimental effects of drought and salinity stress on kale plants with enhanced resilience against *Salmonella* establishment.

The objectives and specific aims of the study were as follows:

Objective 1: Investigate the epiphytic association of *Salmonella enterica* on leafy greens as influenced by plant cultivar, drought, and plant developmental stage

> Objective 1.1: Determine if drought stress and lettuce cultivar affect the serotype-dependent lettuce- *Salmonella* association.
>
> Objective 1.2: Evaluate the impact of drought stress and plant developmental stage on the survival of *Salmonella* on the kale plant surface and in leaf surface washes.

Objective 2: Integrate metabolomic approaches to understand the association of *Salmonella enterica* on surfaces of leafy greens as influenced by drought and plant developmental stage

> Objective 2.1: Determine if drought stress and lettuce cultivar affect the phytochemical profiles of lettuce leaves and leaf surfaces.
>
> Objective 2.2: Evaluate the impact of drought stress and plant developmental stage on kale leaf and leaf surface phytochemical profiles.

Objective 3: Assess the influence of plant growth-promoting rhizobacteria (PGPR) on the *Salmonella* – kale association under the influence of abiotic stresses and with plant development.

Objective 3.1: Determine the impact of drought stress and plant developmental stage on the *Salmonella*–kale association with PGPR application.

Objective 3.2: Evaluate the influence of salinity stress on the survival of *Salmonella* on kale plants inoculated with PGPR.

Objective 4: Assess physiological impacts of PGPR on kale plants under the influence of abiotic stresses and plant developmental stage

Objective 4.1: Determine growth and phytochemical profile changes of kale plants inoculated by PGPR under drought stress.

Objective 4.2: Evaluate the impact of salinity stress on growth and phytochemical profiles of PGPR-inoculated kale.

Chapter 2: Literature Review

2.1 Salmonella enterica and leafy greens

According to the Produce for Better Health Foundation in 2015, with the advocating of healthy lifestyles, the consumption of fresh vegetables, has vastly increased and expected to grow by 8% over the next 5 years. An epidemiological study reported that the consumption of vegetables is negatively associated with the risk of chronic diseases such as cardiovascular disease (Rosner et al., 2004) and oral cancer (Nobile et al., 2006). However, the incidence of foodborne outbreaks associated with fresh fruits and vegetables increases as they are often eaten raw.

Green leaf lettuce (*Lactuca sativa*) is one of the most popular vegetables among American consumers, with its consumption being mostly fresh and raw. Lettuce is a good source of iron, folate, fiber, vitamin C and bioactive compounds including carotenoids and phenolic compounds, and is low in calories, sodium and fat (Kim et al., 2016). However, lettuce has been highly associated with foodborne disease outbreaks. According to the Centers for Disease Control and Prevention (CDC), 354 outbreaks, 9,105 illnesses, 524 hospitalizations and 5 deaths were caused by consumption of contaminated lettuce from 1998 to 2017. A recent lettuce recall due to pathogenic *E. coil* in 2018 was reported to affect at least seven states in the US, Mexico and Canada, which caused 62 people to fall ill and 25 to be hospitalized in the US (FDA, 2019).

Kale (*Brassica oleracea*) is one of the ten most economically important vegetables in the global agriculture markets (Francisco et al., 2017). It is also one of the most frequently

consumed leafy greens all over the world (Kim Moo et al., 2017). Higher consumption of *Brassica* vegetables is reported to associate with lower rates of cardiovascular disease and cancers due to the abundant bioactive substances in *Brassica* plants such as phenolics and flavonoids. However, conventional cooking remarkably affects the phytonutrient content of *Brassica* plants such as kale (Jones et al., 2006; Lopez-Berenguer et al., 2007). To obtain higher concentrations of bioactive phytonutrients, consuming fresh juvenile kale is recommended (Francisco et al., 2017). However, fresh juvenile kale is also easily contaminated by pathogens and causes foodborne disease. A recent kale recall caused by *Listeria monocytogenes* in 2018 was reported to affect 11 states in the US and five states in Canada (Desk, 2019), which caused a billion dollars loss. Kale, as an increasingly popular leafy vegetable, has caused 4 outbreaks, 19 illnesses and 5 hospitalizations from 1998 to 2017 recorded by CDC.

Salmonella, the bacteria that cause the most foodborne illness in the United States, is a major public concern and economic burden (Scallan et al., 2011b). From 1979 – 2010, *Salmonella* was the most common etiologic agent in multistate outbreak-associated illnesses, with 26% of implicated vehicles being produce commodities, higher than any other commodity classification (Nguyen et al., 2015). This increasing outbreak trend with fresh produce may be due to several reasons, such as the increased consumption of fresh produce with 94% growth in global million tons of production from 1980-2004, more centralized processing and distribution, climate change, and pathogen adaptation (Olaimat and Holley, 2012). Also, a few studies have elucidated details about the mechanisms of the survival of human pathogen *S. enterica* on the surface of leafy greens. The numbers of bacteria on plant surfaces can reach 10^5 to10^7 CFU per gram of leaf under favorable environmental conditions, such as

when high relative humidity or free water is present (Hirano and Upper, 1990b; Obrien and Lindow, 1989). This indicates that nutrients leached from the plant surface could be adequate for bacteria to survive (Han and Micallef, 2016; Hirano and Upper, 1990b; Mercier and Lindow, 2000). Molecules leached from plant leaves include a variety of organic and inorganic compounds, such as sugars, organic acids, amino acids, methanol, and various salts whose abundance can vary with plant species, age of leaves, and growing conditions (Corpe and Rheem, 1989; Purnell and Preece, 1971; Tukey Jr, 1970). Plants capable of supporting high bacterial population sizes were proportionally more depleted of leaf surface nutrients than plants with low epiphytic population (Mercier and Lindow, 2000). In addition, sugar, sugar alcohols and organic acids promote the growth of *Salmonella enterica*, whereas fatty acids inhibit the growth of *S. enterica* (Dev Kumar et al., 2019; Dev Kumar and Micallef, 2017; Han and Micallef, 2016). Plant secondary metabolites might also have a negative impact on *S. enterica* growth and colonization (Han and Micallef, 2016).

2.2 The impact of drought, salinity, and other abiotic stresses due to climate change in Maryland and the USA

According to the National Center for environmental information (NOAA), southern and western parts of the country were influenced by drought in 2018. Moreover, drought in much of the southwest was severe and persistent. Even though drought does not impair human life and kill plants immediately, it is a huge economic burden. NOAA reported that, in the past three decades, drought ranks second regarding phenomena associated with billion-dollar weather disasters. Drought is a serious hazard cost nearing $9 billion per year in the US (NOAA). Drought affects our lives in multiple ways. Farmers spend more money to water

their plants and feed all animals. The decrease in agricultural production then results in a higher price of daily supplies such as dairy products. Soil could turn unfarmable due to severe drought. A long period of drought intensifies the harshness of wildfire, which impacts the environment, society, and economy. Besides drought, increased levels of greenhouse gases are evident to cause the Earth to global warming in response. Reported by National Aeronautics and Space Administration (NASA), the planet's average surface temperature has risen about 0.9 ℃ since the late 19th century. Most of the warming occurred in the past 35 years. In the meantime, the Antarctic and Greenland ice sheets have decreased dramatically along with a 0.2 ℃ increase with the top 700 meters of oceans. Global sea level rose about 8 inches in the last century. However, the rate doubles in the last two decades and is accelerating slightly every year (Nerem et al., 2018). Climate change is increasing the number and severity of winter storms, hurricanes, flood, and other extreme weather events.

Maryland is one of the most vulnerable states to climate change. Climate change has already impacted the state of Maryland in many ways, such as the rising sea level, more frequent and violent thunderstorms, and summer heat waves. Growers in Maryland are facing costly losses due to extreme heat waves and drought. A 0.6 to 1.1℃ rise in temperature and intensive drought stress were observed in the last century in Maryland (Maryland.gov). Long-term temperature data show that the average temperature will continue to rise in the future. Rising temperature increases evaporation, which increases average rainfall, frequency of heavy storms in many places. However, it contributes to a severe drought in others (Maryland.gov). Even though the annual precipitation in Maryland increased by about 5 percent in the last century, precipitation from extremely heavy storms has increased in the eastern United States by more than 25 percent since 1958 (Maryland.gov). Increased precipitation in spring results

in higher evaporation in the soil, which dries the soil during summer and fall. As a result, intensive floods are more likely to occur in winter and spring, and severe droughts take place in summer and fall (Maryland.gov). Sea level rising is a huge challenge in Maryland. Sea levels have risen about one foot during the last 100 years and are expected to continue to rise, then reach another one-foot rise by 2050 (EPA, 2016; Maryland.gov). Furthermore, higher water levels are exacerbating coastal flooding, submerging low lands, eroding beaches and increasing the salinity of estuaries and aquifers. Most importantly, one of Maryland's most important agricultural regions, the Eastern Shore, is experiencing high sea level rise and coupled with subsidence and groundwater extraction, is exacerbating saltwater intrusion into aquifers and soil that is affecting agriculture (Rebecca Epanchin-Niell and Miller, 2018). The ecosystems in Maryland has become more vulnerable, with less grass species surviving in the warmer season and less food for predators (EPA, 2016).

2.3 Plant physiology and metabolome changes due to drought and salinity stresses

Drought, salt and temperature stresses are major environmental factors that affect the geographical distribution of plants in nature, limit plant productivity and threaten food security (Zhu, 2016). The increasing temperature and other extreme weather conditions caused by climate warming have greatly changed the environments of plants. To cope with drought, plants are found to biosynthesize specialized metabolites, which characteristically exhibit antioxidant activity *in vitro* (Nakabayashi and Saito, 2015). As one of the major constraints limiting crop production worldwide, drought impairs plant growth and reduces water use efficiency in plants. Plants display a range of mechanisms to withstand drought, such as reduced water loss by increased diffusive resistance, increased water uptake, and

smaller and succulent leaves to reduce transpiration loss (Farooq et al., 2012). Low-molecular-weight osmolytes, including glycine betaine, proline, and other amino acids, organic acids, and polyols also play vital roles in sustaining cellular functions under drought. Many abiotic stresses directly or indirectly affect the synthesis, concentration, metabolism, transport, and storage of sugars. Soluble sugars act as potential signals interacting with light, nitrogen and abiotic stress to regulate plant growth and development (Kang et al., 2010; Obertello et al., 2010; Osuna et al., 2007). Mutant analysis has revealed that sugar signaling interacts with ethylene (Zhou et al., 1998), ABA (Arenas-Huertero et al., 2000; Finkelstein and Gibson, 2002), cytokinins (Franco-Zorrilla et al., 2005), and light (Mita et al., 1995; Thum et al., 2003). Lettuce has high water content (~95%) (Kim et al., 2016), which makes it susceptible to drought stress. Drought impaired lettuce growth and pushed plants to reallocate resources to achieve maturity quickly (Eriksen et al., 2016). Drought significantly decreased the fresh weight of green leaf lettuce cultivar 'Slobolt', 'Grand Rapids', 'Western Green' and 'Australian' (Eriksen et al., 2016). Drought stress adversely affected the leaf area and fresh and dry weight of kale plants (Issarakraisila et al., 2007). Physiological changes including thickening and whitening of the cuticle and closure of stomata limits the photosynthesis and accumulation of bioactive substances in kale plants under drought stress (Issarakraisila et al., 2007).

Salinity indicates the amount of salt present in soil and water. Saline soil and water is rich in several salts (Na^+, Mg^{2+}, Ca^{2+}, Cl^-, HCO_3^-, and SO_4^{2-}), and excess amount of salt has caused a negative impact on phytochemical and morphological functions of plants. Salinity inhibits germination of seed, plant growth, development, and yield, and it is one of the major abiotic stresses that harm agriculture by decreasing productivity (Zhang and Dai, 2019). Salinity

would impact photosynthetic machinery, transpiration, and gaseous exchange by decreasing chlorophyll and carotenoids content (Pan et al., 2021). Soil salinity lowers could disturb plant water relations by influencing soil water potential and leaf water potential, which may ultimately lead to osmotic stress of plant cells (Navada et al., 2020). Too much salts taken by plants from the saline soil via transporters may also create ion toxicity and disturb mineral uptake of plants. Over-accumulation of ions including Na^+ and Cl^- from saline soil or water would inhibit other ions intake such as K^+ and Ca^{2+} uptake an lead to ionic imbalance (Arif et al., 2020). Salinity also triggers more reactive oxygen species (ROS) and creates oxidative stress in plant cells, which may lead to DNA and protein damage (El Ghazali, 2020). Salinity limited the biomass accumulation of lettuce (*Lactuca sativa* L. cv. 'Paris Islands Cos') (Al-Maskri et al., 2010). Lettuce (*Lactuca sativa* L. cv. 'Tafalla') growth was inhibited under salinity stress even with the help of plant growth-promoting rhizobacterium ad an arbuscular mycorrhizal fungus (Kohler et al., 2009). Higher levels of oxidative stress, decreased plant growth and induced plant death were reported due to salinity (Shannon and Grieve, 1998; Shannon et al., 2000). Reduced fresh and dry weight and leaf area of lettuce (Xu and Mou, 2015) and kale (Shannon et al., 2000) in response to high salinity was identified. Salinity induced the stomatal closure and enhanced antioxidant enzyme activities were observed for lettuce (Eraslan et al., 2007) and kale (Tayebimeigooni et al., 2012).

Stresses such as drought, salt, ultraviolet radiation, and air pollutants can disrupt the cellular homeostasis of cells and enhance the production of reactive oxygen species (ROS) in plants (Mittler, 2002). The production of ROS during stress results from pathways such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration. Studies have shown that the production of ROS due to pathogens, wounding or

environmental stresses was triggered by NADPH oxidases (nicotinamide adenine dinucleotide phosphate-oxidase). The enhanced production of ROS during stress can pose a threat to cells which potentially induce DNA, RNA, protein and membrane oxidation and damage, but it is also thought that ROS act as signals for the activation of stress-response and defense pathways (Desikan et al., 2001; Knight and Knight, 2001). Thus, to mitigate ROS in cells, plants either recruit detoxifying proteins such as superoxide dismutase (SOD), ascorbate peroxidase (APX), or antioxidants such as ascorbic acid and glutathione (GSH), sugars, quinic acid derivatives. Flavonoids that are common in the plant kingdom are responsive to almost all abiotic stresses. Experiments conducted on *Arabidopsis* have proved that the over-accumulation of flavonoids enhanced the plants' oxidative-stress and drought-stress tolerance (Nakabayashi et al., 2014a). Flavonoid accumulation in accordance with abiotic stress exposure was reported as a late response implemented to protect plants (Kusano et al., 2011).

2.4 Drought and salinity stress affects pathogens infection:

Abiotic stresses elicit complex plant responses and can, in turn, impact plant responses to pathogens. Drought stress could make plants more susceptible to pathogens. Drought-treated *Parthenocissus quinquefolia* plants were more susceptible to *Xylella fastidiosa*, the agent of bacterial leaf scorch (McElrone et al., 2001). Cankers caused by *Septoria musiva*, a damaging fungal pathogen, were significantly larger on drought-stressed hybrid poplar trees than those on regularly watered trees (Maxwell et al., 1997). Red pines under moderate drought stress were reported to be more susceptible to various isolates of *Sphaeropsis sapinea* that cause blight (Blodgett et al., 1997). Similarly, common bean under both simultaneous drought

stress and *Macrophomina phaseolina* that cause charcoal rot and seedling blight showed higher leaf temperature and transpiration rate than only drought-treated bean plants (Mayek-PÉrez et al., 2002). It was hypothesized that the protection mechanism is weakened by drought stress intensifying the infection of pathogens and causing further damage to the plants.

In contrast, the application of abiotic stresses activated the plant stress signal transduction network and prepared them for subsequent stresses, including pathogens. Drought stress could help plants to cope with pathogens that prefer humid or wet conditions (Ramegowda and Senthil-Kumar, 2015). Drought stress reduced 50% of the *Botrytis cinerea* infection, suppressed the spread of *Oidium neolycopersici* and increased the endogenous ABA level on tomato cv 'Moneymaker'(Achuo et al., 2006). Compared to regularly watered infected plants, drought-acclimated *Nicotiana benthamiana* plants showed resilience to the necrotrophic fungus *Sclerotinia sclerotiorum* that causes white mold and the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* that causes bacterial speck (Ramegowda et al., 2013). Increased ROS levels and pathogenesis-related protein expression were observed in drought-acclimated *Nicotiana benthamiana* plants, which indicated that accumulated ROS during drought acclimation played a role in tolerance against pathogens (Ramegowda et al., 2013). The accumulation of ABA caused by drought stress proved to increase the plants' resistance to pathogens due to callose deposition (Mauch-Mani and Mauch, 2005). Salinity stress primed wheat plant to wheat blast disease (Cabot et al., 2018).

Less data is available regarding the association of enteropathogens with plants under drought stress. A study reported that when iceberg lettuce plants were under severe drought, levels of

Salmonella Typhimurium retrieved from soil was significantly higher than from regularly watered plants (Zhang et al., 2016). Moreover, the internalization of *Salmonella* Typhimurium through roots in Iceberg lettuce leaves increased significantly when lettuce plants were under severe drought stress (Zhang et al., 2016). Similarly, drought stress was reported to increase the detection frequency and the level of internalization of *Salmonella* Infantis in leafy lettuce cultivar 'Green Salad Bowl' leaves (Ge et al., 2012). However, drought and heat stress did not promote internalization of *Escherichia coil* O157 :H7 in Iceberg lettuce leaves (Zhang et al., 2009). Fresh-cut lettuce exposure to increased salinity showed a decrease in mesophilic bacteria in soilless production system (Scuderi et al., 2011).

2.5 Developmental changes in plant metabolome

Besides abiotic stresses such as drought, plant metabolome and exo-metabolome change as plants develop and mature, and in response to biotic stresses caused by plant pathogens. Leaf development is a complicated process that involves various factors (Van Lijsebettens and Clarke, 1998). The photosynthesis rate of leaf changes as plants age, where it increases until the leaf fully expands, then decreases near abscission (Hardwick et al., 1968; Miller et al., 2000; Noodén, 2012). Leaf death occurs when all the nutrients are removed and recycled (Buchanan-Wollaston et al., 2003). Leaf senescence is a well-regulated process that usually starts from the chloroplast (Cabello et al., 2006). Protein and RNA start degradation along with the chloroplast (Cabello et al., 2006). The degradation of mitochondria and the nucleus happens at the final stages (Gan and Amasino, 1997; Nam, 1997). Nutrients such as sulfur, phosphorus, minerals, and metal ions are also transferred out of the leaves (Himelblau and Amasino, 2001). The structure and function of cellular membranes are impaired due to the

accelerated metabolisms of membrane lipids. The membranes of thylakoids are abundant sources of carbon that can be used as an energy source during senescence (Diaz et al., 2005). Rubisco is the major nitrogen source for mobilization (Diaz et al., 2005). During the development of sunflower (*Helianthus annuus*), the degradation of leaf pigments and decreased photosynthetic activity were observed along with lower content of chlorophyll (Cabello et al., 2006). Soluble protein, starch, and total RNA levels also decreased during aging (Cabello et al., 2006). However, the soluble sugar content increased (Cabello et al., 2006). Nitrogen-rich amino acids were suggested to be accumulated and transported from senescent leaves (Cabello et al., 2006). The photosynthesis rate in the third pair of leaves of *Perilla frutescens* started a gradual decline from full expansion to abscission (Hardwick et al., 1968). Most of the studies, nowadays, focus more on monitoring chlorophyll content and gene expression patterns shifting during leaf senescence. Understanding the nutrient mobilization during leaf senescence is vital, however, analyses of the metabolite profiles during aging still need more in-depth studies.

Higher sugar content is observed during leaf senescence in several plant species, while the amino acid content decreases (Jongebloed et al., 2004; Masclaux et al., 2000). In *Arabidopsis*, the content of glucose and fructose increased, while sucrose stayed stable in senescing leaves (Diaz et al., 2005; Pourtau et al., 2006; Quirino et al., 2001). Ethylene and cytokinin are highly related to the inducing or delaying leaf senescence (Guiboileau et al., 2010). Moreover, cytokinin is reported to have the strongest effect on leaf senescence delaying (Schippers et al., 2007). Gibberellin acid and auxin are capable of promoting plant growth. However, ethylene, salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) are reported to induce leaf senescence (Guiboileau et al., 2010). Plant metabolism changes along

with aging, however, all plant species and genotypes do not senesce at the same time nor in a similar way. Plant senescence can happen naturally or induced by stress. All these factors should be included when considering the interaction between plant aging and the survival of pathogens.

2.6 Plant growth promoting rhizobacteria and abiotic stresses

Bacteria colonizing the rhizosphere that can enhance the growth of plants through various mechanisms are called plant growth promoting rhizobacteria (PGPR). PGPR have been widely recognized as biofertilizers (Khan et al., 2018). Higher yields have been recorded in potatoes, radishes, rice, tomato, and apples as a result of PGPR inoculation(Almaghrabi et al., 2013; Bhattacharyya and Jha, 2012; Han and Lee, 2005; Khalid et al., 2004). According to the interaction of PGPR with plants, they can be classified into two types as extracellular plant growth promoting rhizobacteria (ePGPR), which mainly inhabit between the cells of the root cortex in the rhizosphere and intracellular plant growth promoting rhizobacteria (iPGPR), which exists inside the plant specialized structure such as nodules of plant root cells (Martínez-Viveros et al., 2010). *Pseudomonas, Arthrobacter, Chromobacterium, Micrococcus Serratia, Azospirillum, Bacillus, Caulobacter, Erwinia, Flavobacterium,* and *Burkholderia* are major genera included in the ePGPR group; whereas, *Rhizobium, Bradyrhizobium, Mesorhizobium,* and *Allorhizobium* belong to iPGPR group (Bhattacharyya and Jha, 2012; Hurek and Reinhold-Hurek, 2003; Sevilla et al., 2001; Steenhoudt and Vanderleyden, 2000). PGPR play plant-host dependent growth promoting roles through direct and indirect mechanisms, which includes nutrient fixation, producing volatile organic compounds (VOCs) and enzymes to prevent diseases, and neutralizing biotic and abiotic

stress (García-Fraile et al., 2015). Several compounds such as siderophores and antibiotics produced by antagonistic PGPR showed pathogen control effects and were linked to indirect growth promoting (Beneduzi et al., 2012). PGPR are highly impacted by both biotic and abiotic factors. Plant development stages, plant defense mechanisms, plant genotypes, and plant existing microbial community could affect the role of PGPR as well as climatic conditions, soil composition, and soil management (Vacheron et al., 2013). PGPR can affect the plant metabolome. The inoculation of PGPR strain *Serratia marcescens* NBI1231 increased the content of phenolics including gallic acid, caffeine acid, chlorogenic and ferulic acids in Betel vines (Lavania et al., 2006). Increased activity of polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) was suggested to explain the increased levels of phenolics under the presence of PGPR (Mabrouk et al., 2007).

Abiotic stresses such as drought, UV radiation, extreme temperature, salinity, and floods harm the survival of plants and their development, thus affecting the biomass and productivity. To help plant host to cope with abiotic stresses, mechanisms such as the reduction in oxidative damage, increase water-use efficiency and osmotic adjustment were reported from PGPR studies. PGPR could help plants survive under drought through accumulating exopolysaccharides, producing metabolites associated with stress and biofilm (Sandhya et al., 2010). *Paenibacillus polymyxa* was reported capable of colonizing plant root tips, then forming biofilm-like structure and manipulating the mRNA transcriptions of a drought-response gene *ERD15*, thus protecting the plants from drought stress (Timmusk et al., 2005; Timmusk and Wagner, 1999). *Phyllobacterium brassicacearum* was reported to induce a delay in flowering time, thus increasing biomass accumulation under drought stress (Bresson et al., 2013). The growth and yield of Chinese kale (*Brassica oleracea* var.

alboglabra) were promoted with the inoculation of *Pseudomonas* (Issarakraisila et al., 2007). *P. putida* promoted the shoot and root growth, increased the leaf numbers, leaf relative water content, proline accumulation, and alkaloid content, and induced the activities of antioxidant enzymes when *Hyoscyamus niger* (medicinal plant) were under drought stress (Ghorbanpour et al., 2013).

Chapter 3: Drought-induced responses in loose-leaf lettuce, but not Romaine lettuce, limited *Salmonella enterica* association on leaf surfaces

3.1 Introduction

As the leading enteric bacterial pathogen, *Salmonella enterica* subsp. *enterica* causes the highest monetary cost of foodborne illness among other foodborne pathogens (Morris Jr et al., 2011). Fresh produce accounted for 46% of all reported foodborne illness in the US from 1998 to 2008 (Painter et al., 2013). In 2021, more than 1/3 of multistate outbreaks were associated with fresh produce (CDC) and it was projected to reach a higher proportion of all foodborne outbreaks in the USA (Bennett et al., 2018). The *Salmonella* – Produce pair ranked 8th with regards to the average impacts to cost of illness and quality adjusted life years (Morris Jr et al., 2011). Of these produce, lettuce (*Lactuca sativa*) is one of the most popular vegetables among American consumers, with its consumption being mostly fresh and raw. Simultaneously, lettuce is the most commonly implicated vegetable associated with foodborne outbreaks in the U.S. (Sivapalasingam et al., 2004). Among foodborne pathogens, *Salmonella* Newport was identified the leading serovar in illnesses associated with salads, lettuce and tomatoes in the European Union (Callejón et al., 2015a). Despite the huge economic loss and public health concerns caused by *Salmonella* on fresh produce, the interaction between *Salmonella* and plants remains unclear.

The contamination of human pathogens to produce can happen at any stage from preharvest to postharvest (Bennett et al., 2015; Harris et al., 2003). Once established on the plant

surface, reducing or eliminating human pathogens is complex (Goodburn and Wallace, 2013; Parish et al., 2003). At least 20% of contamination takes place during the preharvest stage and enteric pathogens are capable of associating with plants to persist from farm to postharvest stages, potentially leading to foodborne illness and outbreaks (Brandl and Mandrell, 2002; Yaron, 2014). Excluding introduction of pathogens from field and controlled environment agriculture is the principal way by which contamination is controlled at the pre-harvest stage, since no effective strategies exist to control foodborne pathogens at the production level, once established in the plant niche. *Salmonella enterica* serovars exhibit a differential ability to colonize and grow on fruit and leaf surfaces, which can be partially explained by diversity in traits linked to attachment and biofilm formation (Berger et al., 2009; Beuchat, 2002; Patel and Sharma, 2010). Exploring the traits and drivers that favor *Salmonella* association with leaf vegetables could be helpful in devising solutions to control this human pathogen at the pre-harvest level.

Phytonutrients are crucial for successful bacterial colonization of plants and epiphytes depend on nutrients released to the plant surface (Hirano and Upper, 1990a; Hirano and Upper, 2000; Mercier and Lindow, 2000). Under favorable environments, bacteria could reach up to 10^7 CFU/g of leaf (Hirano and Upper, 1990a; O'brien and Lindow, 1989). Specifically, *Salmonella* can utilize sugars and amino acids as carbon and nitrogen resources to thrive on plant surfaces (de Moraes et al., 2017; Kröger and Fuchs, 2009; Kwan et al., 2015; Megías-Pérez et al., 2020). Plant age, plant anatomical structure and cultivar impact the composition and levels of phytochemicals present and have been shown to mediate *Salmonella* association on produce surfaces (Brandl and Amundson, 2008; Han and Micallef, 2016; Quilliam et al., 2012). Simultaneously, plants deal with adverse conditions by accumulating secondary

metabolites which provide defense against pathogens and herbivores and protect against oxidative damage generated by abiotic stresses. Controlled elicitation of secondary metabolites is a new approach being explored for enhanced medicinal and beneficial value of crops (Thakur et al., 2021). This approach should also be explored to enhance food safety (George and Brandl, 2021; Liu et al., submitted). Drought is one of the most severe stresses plants encounters. Lettuce responds to this stress by accumulating proline (amino acid) and polyphenols (secondary metabolites) (Rajabbeigi et al., 2013). How these phytochemical changes influence the lettuce – *Salmonella* association has not been explored. Thus, in this study, we aimed to investigate the impact of drought stress on two common lettuce cultivars and how drought responses related to the surface survival of two *Salmonella enterica* serovars.

3.2 Material and Methods

3.2.1 Lettuce growth and drought treatment condition

Lactuca sativa L. var. *longifolia* cv. 'Parris Island Cos' (Romaine) and cv. 'Mascara' (loose-leaf, red oak-leaf type) were used in this study. Lettuce seeds (Territorial, OR, USA) were germinated in the potting mix soil (Sunshine LC1, Sungro Horticulture, Canada) at the Research Greenhouse Complex, University of Maryland, and grown under controlled light and temperature conditions (16 h light: 8 h dark photoperiod at 23℃ day and 18℃ night temperature). Four-week-old lettuce plants were subjected to water stress by restricting irrigation for 6 days or watered regularly (control) prior to experimentation. At the end of the watering treatment, lettuce plants were all 34 days old for subsequent analyses.

3.2.2 Lettuce leaf tissue extract and surface wash sample preparation

Whole lettuce leaves from both cultivars were flash frozen using liquid nitrogen and then ground into powders using a pestle and mortar. Plant powder was ready for proline and anthocyanin measurements. For phytochemical analyses, 200 mg of plant powder were accurately weighed and extracted with 1.5 ml 70% methanol (Sigma-Aldrich, MO, USA) with 0.5% formic acid (VWR, PA, USA). Each extract was fully vortexed before it was centrifuged at 3,000 rpm for 10 min. The supernatants were frozen at -20℃ until further analysis. Whole plant washes were collected by placing whole lettuce plants in Whirl-pak bags (Nasco, Wi, USA) immersed in 30 ml 5% methanol/water solution while leaving the root ball out of the bag and shaken at 150 rpm for 24 h at room temperature. Surface compound solutions were filter-sterilized through 0.2 μm cellulose acetate syringe filters (VWR) and then aliquoted to 2 ml as the wash for *Salmonella* growth evaluation and phytochemical profiling.

3.2.3 Salmonella enterica inoculum preparation

Salmonella enterica serovar Newport, an environmental isolate collected from an irrigation pond that matched a recurring tomato outbreak strain (Greene et al., 2008) and *S.* Typhimurium, an isolate recovered from river water in Maryland (Callahan et al., 2019), were used in this study. Both *S.* Newport and *S*. Typhimurium had been adapted to rifampicin (rif) (MilliporeSigma, MA, USA) and were maintained at -80℃ in Brucella broth (BD, MD, USA) containing 15% glycerol (VWR) and 80 μg/ml rifampicin and revived by streaking onto Trypticase Soy Agar (TSA, BD) plates amended with 50 μg/ml rifampicin (TSA-rif) overnight at 35℃. Inocula of *S.* Newport or *S.* Typhimurium were made by suspending fresh

bacterial colonies in 0.1% peptone water (PW, BD) to an OD_{600}=0.5, to obtain a cell density of ~10^9 CFU/ml. The growth of *Salmonella* in plant washes was only evaluated using *Salmonella* Newport. Actual inoculum level for *S.* Newport or *S.* Typhimurium was determined by plating serial dilutions onto TSA-rif plates for direct quantification prior to plant or wash inoculation.

3.2.4 Lettuce surface inoculation with S. enterica

The third or fourth true leaf of either Romaine or 'Mascara' lettuce under both watering conditions were gently marked on the stalk with a black marker. An aliquot of 100 μl of *S.* Newport or Typhimurium were pipetted on the abaxial side of each marked leaf. An estimated total inoculum dose of 10^6 CFU was inoculated per leaf. Inoculated lettuce leaves were harvested 24 hours post-inoculation (hpi). Each leaf was clipped off the stem aseptically with scissors and placed in a sterile Whirl-pak bag. Inoculated leaves were then immersed in 30 ml 0.1% PW, hand massaged for 30 s and sonicated in a Branson Ultrasonic Cleaner (Branson Ultrasonics Corporation, CT, USA) for 1 min at maximum speed to dislodge *Salmonella* cells from the lettuce leaf surfaces, then shaken at 150 rpm for 10 min. Serial dilutions from each leaf rinsate were plated on TSA-rif plates for *S.* Newport or Typhimurium enumeration after 24 h of incubation at 35℃.

3.2.5 Evaluation of S. Newport growth in lettuce surface washes

Twenty microliters of *S.* Newport (~10^6) were added to 2 ml sterile lettuce leaf washes to achieve an initial concentration of *Salmonella* at ~10^4 CFU/ml and incubated at 35℃ at 150 rpm in a shaking incubator. *S.* Newport cell counts were recorded at 0, 2, 4, 6 and 24 hpi. At each time point, serial dilutions were plated on TSA-rif plates for enumeration. Two

milliliters of sterile 5% methanol inoculated with 20 μl of 10^6 CFU/ml *S.* Newport served as negative control.

3.2.6 Phytochemical profiling of lettuce leaf extracts and washes using electrospray ionization-mass spectrometry (ESI-MS)

Phytochemical profiling using lettuce leaf and wash samples were conducted as previously described (Liu et al., submitted). One ml aliquots of lettuce leaf extracts and 30 ml filter-sterilized leaf wash solutions were lyophilized into powder and resuspended in 100 μl 70% methanol and 0.5% formic acid. To ensure all compounds were fully dissolved in the extraction liquid, all tubes were sonicated in a water bath for 2 h at maximum intensity and then transferred into vial inserts for ESI-MS analysis.

A Time-of-Flight mass spectrometer (AccuTOF, JEOL, USA, Inc.) equipped with an ESI ion source for mass spectrometric analysis of the lettuce tissue extracts. Mass spectra were acquired in positive and negative modes at a rate of one spectrum/s (m/z range of 50–800 Da), averaging mass spectra over ~1 min. The AccuTOF MS settings were as follows: needle voltage = 2100 V, orifice 1 temperature = 100_C, orifice 1 = 30 V, orifice 2 = 5 V, ring = 10 V. The desolvating chamber temperature was set at 250_C, and the flow rates of the nebulizing and desolvating gases were 0.6 and 3.0 l/min, respectively. The sample injection volume was 10 µl with a flow rate of 0.25 ml/min. Ten µl methanol were injected prior to and following the analysis of each sample and measurement taken to monitor possible contaminants or carryover. Calibration for exact mass measurements was accomplished using 5 mM cesium iodide as the internal standard. For all samples, measurements were repeated at least twice to ensure reproducibility.

3.2.7 Proline measurement using lettuce leaf powder

Lettuce plant powder (200 mg) was mixed with 1.5 ml of 3% aqueous sulfosalicylic acid (Avantor, PA, USA) in a 2 ml microcentrifuge reaction tube. Samples were fully vortexed and centrifuged at 11,000 rpm for 30 min at 4 ℃. Extract supernatant (300 µl) were mixed with 300 µl ninhydrin (Avantor) and 300 µl glacial acetic acid (VWR Chemicals, BDH) in 2 ml reaction tubes, vortexed thoroughly, incubated at 90℃ on a digital heat block (VWR, PA, USA) for 1 h then placed on ice for 5 min. Toluene (VWR Chemicals, BDH) (900 µl) were added to the reaction tube and thoroughly mixed before going through centrifugation at 10,000 rpm for 5 min and 200 µl of supernatant transferred to a microplate reader (Synergy HTX; Biotek, VT, USA) for absorption measurements at 520 nm. Pure proline (Avantor) was used to make a standard curve and results were expressed as mg proline/g of leaf fresh weight according to the standard curve (Bates et al., 1973).

3.2.8 Phytochemical analyses of lettuce leaf extracts

Antioxidant capacity, and total phenolic and flavonoid content were conducted as previously described (Liu et al., submitted). The methods are described briefly below:

Antioxidant capacity: Modifications were made according to methods previously described by Stratil et al., (Stratil et al., 2006), ABTS solution was prepared by dissolving 31.7 mg 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (TCI, OR, USA) and 8.6 mg $K_2S_2O_8$ (potassium persulfate; (Aldon Corp SE, NY, USA)) in 10 ml water and allowed to stand at room temperature in the dark for 16 h to form a stable radical. The solution was diluted to an absorption of ~1.3 at 734 nm and 100 µl aliquots prepared, to which 20 µl sample extracts were added and allowed to stand for 15 min. Absorption was measured at 734 nm using a

microplate reader. A standard curve from the blank-corrected absorption at 734 nm of Trolox (TCI, OR, USA) was plotted and antioxidant capacity of lettuce leaves was calculated as Trolox equivalent.

Total flavonoids: Ad**a**pted from published method (Zhishen et al., 1999), **a**liquots of 100 µl leaf extracts were added to 20 µl 5% $NaNO_2$ (Avantor, PA, USA). After 5 min, 20 µl 10% $AlCl_3$ (Alfa Aesar, MA, USA) were added, followed by 100 µl 1M NaOH (VWR, PA, USA) 1 min later. Absorption of the mixture was measured at 510 nm on the microplate reader. A standard curve from the blank-corrected absorption at 510 nm of the catechin standard (Enzo Life Sciences, NY, USA) was plotted and total flavonoid content was calculated as catechin equivalent (Ketnawa et al., 2020) using the standard curve.

Total phenolic content: Using a method adapted from previous publications (Kao et al., 2014; Singleton and Rossi, 1965), 80 µl lettuce leaf extracts were mixed in a 96-well plate with 90 µl 10% Folin Ciocalteu reagent (MP Biomedicals, CA, USA), then 40 µl 700 mM Na_2CO_3 (VWR Chemical, PA, USA) added. Samples were vortexed in the microplate reader and allowed to stand for 1 h prior to absorption measurements at 765 nm. A standard curve from the blank-corrected absorption values at 765 nm of the gallic acid (Acros, Organic Fair Lawn, NJ, USA) standard was plotted and total phenolics were calculated as gallic acid equivalents.

3.2.9 Anthocyanin measurement of lettuce leaf powder

This method was modified based on Neff and Chory (Neff and Chory, 1998). Lettuce tissue powder (200 mg) was mixed with 300 µl methanol (VWR Chemicals) with 1% (v/v) HCl (VWR Chemicals) and incubated overnight at 4°C in the dark. To each lettuce sample tube,

200 µl of water and 500 µl of chloroform (VWR Chemicals) were added and tubes centrifuged at 11,000 rpm for 5 min at room temperature. The supernatant was transferred into a new tube, volume adjusted to 800 µl with 60% methanol containing 1% HCL (v/v) and 200 µl transferred to 96-well plates for absorption measurements at 530 nm and 657 nm using 60% methanol-1% HCl as blank. Anthocyanins were calculated based on the following equation:

$$\text{Anthocyanins} = (\text{Ab}_{530} - \text{Ab}_{657}) * 1000 * \text{ powder weight (mg)}^{-1}$$

3.2.10 Statistical analysis

Each experiment was repeated with each treatment tested in triplicate or more replicates and data were pooled for statistical analysis. The retrieval of *S. enterica* Newport and Typhimurium count data on lettuce leaf surface in CFU/plant and *S. enterica* Newport data in lettuce washes in CFU/ml were log transformed prior to statistical analyses. One-way analysis of variance (ANOVA) and Tukey's HSD and Student's *t*-test for pairwise comparisons were conducted using JMP Pro 15.2.0 (SAS Institute Inc., Cary, NC).

To explore relationships among lettuce samples, ordination of the phytochemical profiles acquired from ESI-MS was performed using multidimensional scaling (MDS). Briefly, ESI-MS data consisting of the m/z ratio and relative signal intensity of each compound were imported into RStudio (version 1.3.1093). Distance matrices were generated using the Bray-Curtis dissimilarity coefficient using relative peak with the vegan package (version 2.5-7) and MDS analysis was performed in JMP ver. 15.2.0. For significance testing of sample data, analysis of similarity (ANOSIM) was conducted in PRIMER 6 (Plymouth Routines in

Multivariate Ecological Research version 6.1.15; PRIMER-E-Ltd., Plymouth, United Kingdom) using Bray-Curtis or Jaccard (based on presence/absence of peaks) distance matrices. The *R* statistic in ANOSIM ranges from 0-1, with a value closer to 0 when samples from different treatments are similar and a value closer to 1 when replicates within a treatment are more similar to each other than to samples from other treatments.

3.3 Results

3.3.1 Epiphytic persistence of Salmonella on leaf surfaces of regularly watered lettuce was dependent on cultivar

Decline of *S*. Newport and *S*. Typhimurium populations after 24 h compared to inoculum was measured for both cultivars under regular watering condition. A larger *S*. Newport log reduction of 2.0 log CFU/plant was detected on 'Mascara' compared to 0.7 log CFU/plant reduction on Romaine ($p < 0.003$). The discrepancy was also apparent for *S*. Typhimurium, which decreased by 2.5 log CFU/plant on 'Mascara' compared to 1.4 log CFU/plant on Romaine lettuce ($p = 0.07$, Table 1). On each cultivar under regular watering condition, *S.* Newport exhibited lower decline on both Romaine and loose-leaf than *S.* Typhimurium, however, the differences were not statistically significant.

3.3.2 Salmonella enterica persistence on lettuce leaf surfaces was influenced by drought

Within the same cultivar, the reduction of *Salmonella* Newport and *Salmonella* Typhimurium population after 24 h compared to the inoculum was measured under both watering conditions. The population of *S*. Newport reduced more from drought-subjected Romaine

lettuce leaf surfaces than from regularly watered Romaine, with a 0.7 log CFU/plant difference in log reduction (p=0.06) (Table 1). The reduction of *S.* Newport was also lower on drought-exposed 'Mascara' lettuce than on control plants and a 0.7 log CFU/plant difference in log reduction was measured between the two watering conditions ($p < 0.002$). No statistical difference was found between the log reduction of *S.* Typhimurium on Romaine lettuce grown under different watering conditions (Table 1). By contrast, drought-exposed 'Mascara' lettuce supported lower population of *S.* Typhimurium than regularly watered plants with a discrepancy in log reduction of 0.9 log CFU/plant ($p < 0.02$) (Table 1).

Table 1. Counts of *Salmonella enterica* on Romaine and loose-leaf 'Mascara' lettuce leaf surfaces grown under regular watering (CON) and irrigation withholding (DR) conditions, retrieved after 24 h of bacterial inoculation.

	S. Newport (log CFU/plant)		*S.* Typhimurium (log CFU/plant)	
Watering condition	Inoculum	Decline†	Inoculum	Decline†
Romaine lettuce 'Parris Island Cos'				
CON	5.7–5.9	0.7 ± 0.3 a, A	6.0 – 6.5	1.4 ± 0.5 a, A
DR		1.4 ± 0.4 a*, A		1.9 ± 0.5 a, A
Loose-leaf lettuce 'Mascara'				
CON	5.7–6.0	2.0 ± 0.2 a, B	6.0 – 6.5	2.5 ± 0.4 a, A*
DR		2.7 ± 0.2 b, B, ‡		3.4 ± 0.2 b, B, ‡

Statistically significant differences in *Salmonella* counts are denoted by letters; small letters indicate differences by treatment within the same cultivar (Student's *t*-test, $p < 0.05$), capital letters denote differences by cultivar within the same watering treatment (Student's *t*-test, $p < 0.05$). Values are means of replicates ± standard error (SE), with units of log CFU/ plant. † Data represent the average log CFU/plant reduction between initial inoculum and 24 h retrieval [log (inoculum count/retrieval count)] in at least four independent experiments with at least four replicates. * denotes $p<0.1$ for this comparison. The log decline on loose-leaf 'Mascara' plants under water restriction was higher for *S.* Typhimurium than *S.* Newport (denoted by ‡).

3.3.3 Cultivar-specific and serotype-dependent effects detected on Salmonella persistence on drought-treated lettuce leaf surfaces

Cultivar comparison for plants grown under water restriction showed the same trends as for regularly watered plants in regard to *Salmonella* surface persistence. After 24 h, the population of both serotypes showed less decline on drought-treated Romaine lettuce than on loose-leaf 'Mascara' lettuce, with a 1.3 and 1.5 log CFU/plant discrepancy in log reduction between the two cultivars for *S.* Newport ($p < 0.002$) and Typhimurium ($p < 0.02$), respectively (Table 1).

Comparing serotype behaviour on the same cultivar under drought treatment, no statistical difference in population decline between the 2 serotypes was noted on Romaine lettuce. By contrast, a discrepancy of 0.7 log CFU/plant reduction was measured between *S.* Typhimurium and *S.* Newport on 'Mascara' lettuce under drought, with greater log decline observed for *S.* Typhimurium ($p < 0.02$) (Table 1).

3.3.4 Growth of Salmonella Newport in leaf surface washes was affected by cultivar and drought

Growth data of *Salmonella* Newport in plant surface washes at 2, 4, 6 and 24 h were collected from samples under the same watering condition for each cultivar and pooled for calculation. The growth of *Salmonella* was calculated based on the population level change in relation to the corresponding 0 h samples. Wash samples collected from both 'Mascara' and Romaine lettuce were able to support the growth of *Salmonella* Newport over 24 h under both watering conditions, with lower levels of *Salmonella* reported in 'Mascara' lettuce washes (both $p < 0.05$; Table 2). A cultivar-dependent effect was detected in *Salmonella* Newport growth

curve progression. In washes from control plants, *Salmonella* growth was noted after 2 hours in 'Mascara' washes, but after 4 hours in Romaine washes. At the 24-hour time point, population level change was higher in Romaine than 'Mascara' by 1.4 log CFU ($p < 0.05$; Table 2).

Subjecting plants to drought had an impact on *Salmonella* growth in loose-leaf 'Mascara' leaf washes, but less so on Romaine lettuce. Little growth was detected in washes from drought-subjected 'Mascara' before the 24 h timepoint and *Salmonella* population levels differed from controls at all timepoints ($p<0.05$ for 2, 4 and 6 h and $p=0.09$ at 24 h). This longer lag phase was also apparent in Romaine washes, with a weak difference at 2 h ($p=0.08$), but the lag did not result in a difference in population levels compared to controls at 24 h (Table 2).

Table 2. Counts of *Salmonella enterica* Newport in Romaine and loose-leaf lettuce 'Mascara' leaf surface washes grown under regular (CON) and restricted (DR) watering conditions taken 2-, 4-, 6- and 24-hours post-inoculation and reported as change from 0 h.

	Salmonella Newport log change†			
Timepoint/h	2	4	6	24
Romaine lettuce 'Parris Island Cos'				
Control	-0.01 ± 0.12 a, A	0.20 ± 0.10 a, A	0.55 ± 0.21 a, A	**3.71 ± 0.30** a, A
Drought	**-0.25 ± 0.11** a, A	**0.09 ± 0.11** a, A	**0.30 ± 0.20** a, A	**3.64 ± 0.27** a, A
Loose-leaf lettuce 'Mascara'				
Control	**0.11 ± 0.03** a, A	0.31 ± 0.07 a, A	0.61 ± 0.18 a, A	**2.30 ± 0.22** a, B
Drought	**0.02 ± 0.04** b, B	**0.14 ± 0.04** b, A	**0.02 ± 0.10** b, A	**1.80 ± 0.29** a, B

Statistically significant differences in *Salmonella* Newport counts are denoted by letters. Small letters indicate differences by treatment within same cultivar and timepoint (Student's *t*-test, $p < 0.05$). Capital letters denoted differences by cultivar within same watering treatment (Student's *t*-test, $p < 0.05$). Values are means of replicates ± standard error (SE), with units of log CFU/ ml exudate. † Data represents the average log CFU/ml difference from count at 0 h in at least three independent experiments with at least four replicates. Bold type denotes counts that are significantly different from 0 h count for that treatment.

3.3.5 Proline, phytochemical compounds and antioxidant capacity varied by lettuce cultivar and were induced by drought treatment in loose-leaf lettuce

We measured proline and various phytochemical in leaf tissue extracts to try to explain the differential lettuce-*Salmonella* associations detected. Proline and phytochemical levels varied by cultivar. Proline content was significantly higher in loose-leaf 'Mascara' lettuce than in Romaine lettuce grown under both regular and restricted watering ($p < 0.001$) (Figure 1A). 'Mascara' lettuce under regular watering condition also accumulated more flavonoids ($p < 0.003$) and phenolics ($p = 0.05$) (Figures 1C and 1D) and showed significantly higher antioxidant capacity than Romaine lettuce (Figure 1B; $p < 0.005$). Drought-treated 'Mascara' lettuce showed higher levels of antioxidant capacity, total flavonoids and total phenolics than Romaine lettuce (Figure 1B-1D, all $p < 0.001$).

Water restriction only had an impact on the phytochemical content and proline accumulation of loose-leaf 'Mascara' lettuce. The accumulation of proline increased markedly under restricted watering ($p < 0.05$) (Figure 1A), as did total flavonoid, phenolic and anthocyanin levels (Figures 1C-E; all $p < 0.02$). Antioxidant capacity of 'Mascara' however, showed no difference in response to drought treatment (Figure 1B). Total flavonoid and phenolic levels of Romaine lettuce tissue were unaffected by drought treatment, resulting in a much-enhanced difference in phytochemical levels between Romaine and 'Mascara' lettuce when grown under restricted watering.

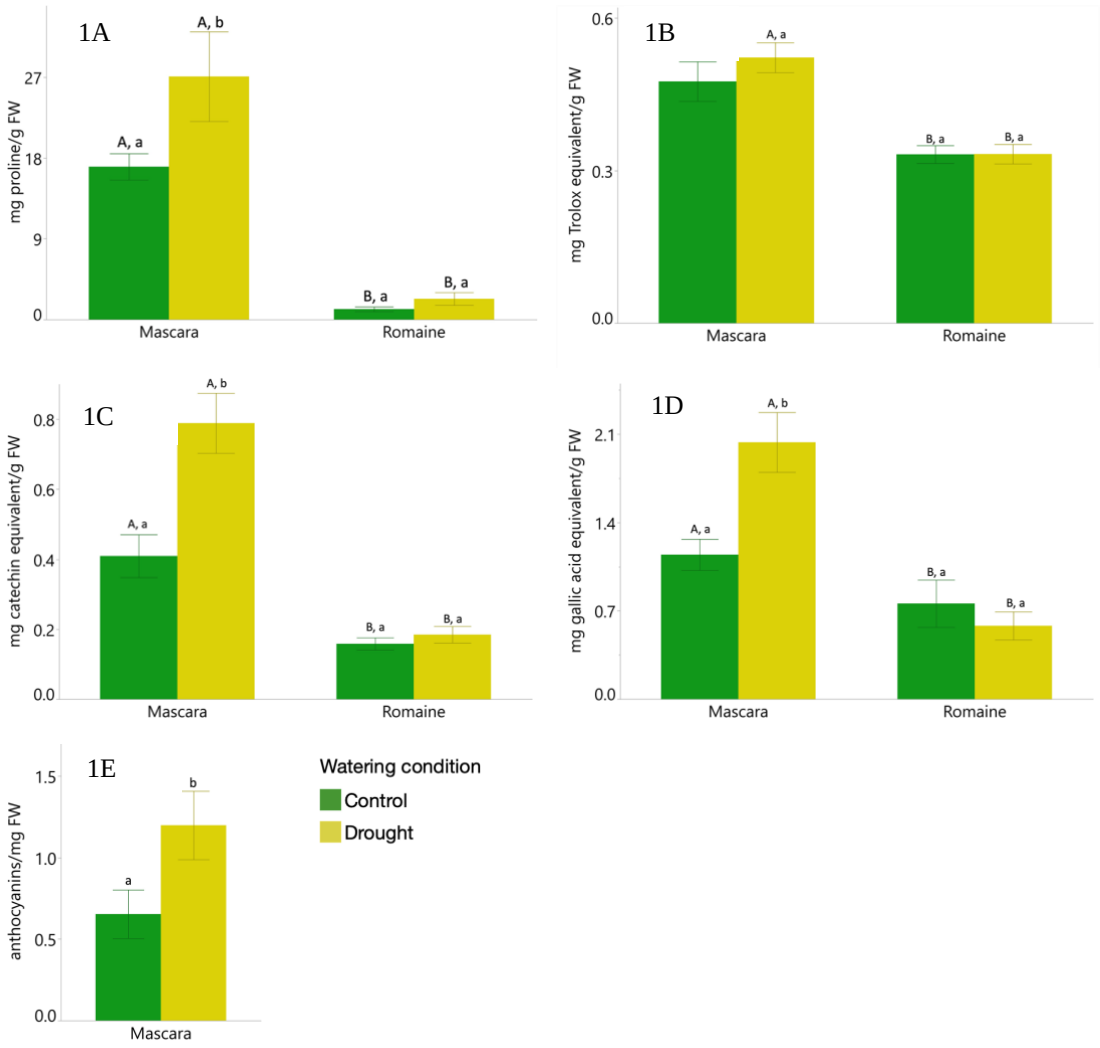


Figure 1. The chemical analyses of (A) proline, (B) antioxidant capacity, (C) total flavonoid, (D) total phenolic, in Romaine and loose-leaf 'Mascara' lettuce leaf tissues and (E) anthocyanins contents in 'Mascara' lettuce, under regular and restricted watering regimes. Different capitalized letters represent significant differences due to watering condition within the same cultivar and different small letters represent significant differences due to cultivar for the same watering condition by Student's t-test for a priori comparisons ($p < 0.05$).

3.3.6 Population decline of Salmonella Newport and Typhimurium was positively associated with phytochemical levels in lettuce leaf samples

Drought and cultivar showed influence on bacterial population decline and plant phytochemical compounds levels. Pearson correlation analyses were conducted between *Salmonella* reduction and phytochemical data. For both serotypes, levels of antioxidant capacity, total flavonoids and phenolics and anthocyanins in lettuce tissues were positively correlated with *Salmonella* population decline (Table 3, all $p < 0.001$), indicating that decline of *Salmonella* population was due to increased contents of photochemical with antioxidant capacity elicited by cultivar or drought.

3.3.7 Phytochemical profiles of lettuce leaf tissue and surface wash were distinct

Both Romaine and loose-leaf lettuce tissue and wash samples were subjected to untargeted phytochemical profiling to reveal any impact of lettuce cultivar and drought stress on leaf tissue and leaf surface metabolomes. Altogether, 1,008 peaks were detected in positive mode and 469 peaks were detected in negative mode (Table 4). Tissue samples yielded 245 and 239 peaks under positive and negative modes, respectively, while wash samples yielded a higher number of peaks under positive mode. In Romaine lettuce, more peaks were found to be in common between tissue and wash samples than for loose-leaf 'Mascara' lettuce (81 versus 53). A much higher number of peaks were shared between the same sample type from plants grown under different watering regimes (Table 4). However, unique peaks by watering treatment (detected only in control or water restricted plants) were also abundant.

Table 3. Correlation analysis between phytochemical measurements (antioxidant capacity, total flavonoids, total phenolics and anthocyanins) in leaf tissues, and against decline of *S.* Newport (SN) or *S.* typhimurium (ST) counts from Romaine and 'Mascara' leaf surfaces under both regular and restricted watering regimes. NS means not significant at α=0.05.

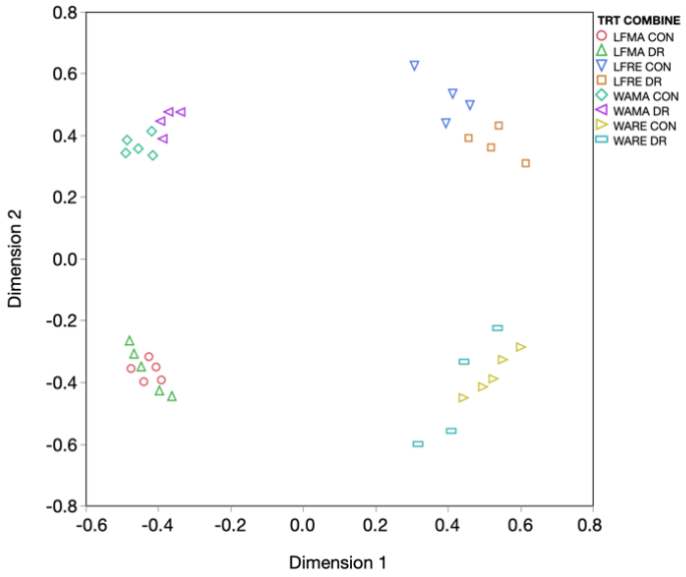
Pearson correlation	Correlation coefficient r	p
Salmonella Newport Decline (log CFU/plant)		
Antioxidant capacity	0.41	<0.001
Total flavonoids	0.46	<0.001
Total phenolics	0.38	<0.001
Anthocyanins	0.44	<0.001
Salmonella Typhimurium Decline (log CFU/plant)		
Antioxidant capacity	0.33	<0.001
Total flavonoids	0.38	<0.001
Total phenolics	0.33	<0.001
Anthocyanins	0.36	<0.001
Pearson correlation	Correlation coefficient r	p
Salmonella Newport Decline (log CFU/plant)		
Antioxidant capacity	0.41	<0.001
Total flavonoids	0.46	<0.001
Total phenolics	0.38	<0.001
Anthocyanins	0.44	<0.001
Salmonella Typhimurium Decline (log CFU/plant)		
Antioxidant capacity	0.33	<0.001
Total flavonoids	0.38	<0.001
Total phenolics	0.33	<0.001
Anthocyanins	0.36	<0.001

The most distinct divergence was detected between tissue and surface phytochemical profiles, regardless of cultivar (Figure 2). The global *R* statistics acquired from ANOSIM for sample type (leaf tissues versus leaf surface) were 0.26 for positive and 0.35 for negative mode (both $p < 0.001$), respectively (Table 5).

Table 4. Number of metabolite peaks acquired from leaf tissue and wash samples from Romaine and loose-leaf 'Mascara' lettuce plants grown under regular (CON) or restricted (DR) watering regimes based on phytochemical profiling with ESI-MS under positive and negative modes.

ID	Sample type	Total peaks detected	Shared peaks		Treatment/ unique peaks	
			Leaf-wash	CON-DR	CON	DR
Peaks detected in positive ionization mode						
Romaine	Leaf	125	15	71	35	19
Romaine	Wash	353		287	55	13
'Mascara'	Leaf	120	46	56	28	36
'Mascara'	Wash	410		220	122	68
Peaks detected in negative ionization mode						
Romaine	Leaf	129	66	122	7	0
Romaine	Wash	147		118	20	9
'Mascara'	Leaf	110	7	55	35	20
'Mascara'	Wash	83		44	24	15

2A



2B

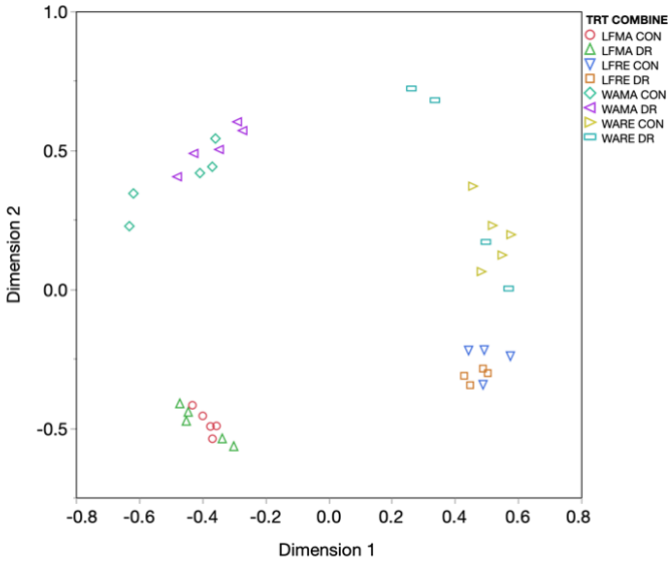


Figure 2. Multidimensional scaling (MDS) plots of phytochemical profiles of lettuce leaf tissue (LF) and leaf surface washes (WA) for both Romaine (RE) and 'Mascara' (MA) based on ESI-TOF-MS under both (A) positive and (B) negative modes. Stress for plots was 0.19 (A) and 0.19 (B).

3.3.8 Leaf and leaf surface metabolomes were affected by lettuce cultivar

For leaf tissue samples, phytochemical profiles of Romaine lettuce differed from loose-leaf 'Mascara' lettuce plants, regardless of watering regime (p <0.05; Table 5, Figure 2). Leaf tissue profiles of Romaine under positive mode were the least related to profiles from 'Mascara' lettuce plants under both regular and restricted watering (both R=1, p < 0.01; Figure 2A), sharing only 12 peaks combined. Under negative mode, the profiles of Romaine lettuce were closer to 'Mascara' lettuce under either watering condition, sharing 39 peaks, but they were still highly divergent overall (both R=1, p < 0.01; Table 5, Figure 2B).

Differences in leaf surface phytochemical profiles obtained from Romaine and 'Mascara' lettuce surface analysis were also detected in positive and negative modes (both R=1, p < 0.01; Table 5; Figure 2). Romaine and 'Mascara' lettuce yielded 353 and 410 peaks in positive mode, respectively, and 20 peaks were shared between the cultivars, while 27 out of 230 peaks were shared between Romaine and 'Mascara' lettuce under negative mode.

Table 5. ANOSIM pairwise comparison of Romaine and loose-leaf 'Mascara' lettuce leaf extracts and leaf surfaces washes based on phytochemical profiling with ESI-MS under positive and negative modes. Plants were 34 days old at time of experimentation and subjected to regular (CON) or restricted (DR) watering. The *R* statistic spans 0-1, with 0 indicating no relationship and 1 indicating highest relatedness between sample profiles. Two distance measures were used to construct the distance matrix for ANOMSIM analysis, Bray-Curtis dissimilarity coefficient which uses relative peak height data and Jaccard Index which considers only the presence or absence of peak.

Lettuce Type	Watering condition	Positive mode		Negative mode	
		R (p value)			
		Bray-Curtis	Jaccard	Bray-Curtis	Jaccard
Leaf tissue extracts					
Romaine	CON vs. DR	0.26 (<0.05)	0.44 (<0.05)	0.42 (<0.05)	0.13 (NS)
'Mascara'	CON vs. DR	0.27 (<0.05)	0.04 (NS)	0.2 (NS)	0.31 (<0.05)
Romaine vs. 'Mascara'	CON	1 (<0.01)	1 (<0.01)	1 (<0.01)	1 (<0.01)
Romaine vs. 'Mascara'	DR	1 (<0.01)	1 (<0.01)	1 (<0.01)	1 (<0.01)
Leaf surface washes					
Romaine	CON vs. DR	0.38 (<0.05)	0.54 (<0.01)	0.24 (<0.1)	0.24 (<0.1)
'Mascara'	CON vs. DR	0.58 (<0.05)	0.41 (<0.05)	0.21 (<0.05)	0.26 (<0.05)
Romaine vs. 'Mascara'	CON	1 (<0.01)	1 (<0.01)	1 (<0.01)	1 (<0.01)
Romaine vs. 'Mascara'	DR	1 (<0.05)	1 (<0.05)	1 (<0.01)	1 (<0.01)

3.3.9 Drought induced shifts in leaf and leaf surface metabolomes

When plants from the same cultivar were subjected to regular or restricted watering, distinct metabolome shifts were detected that could be attributed to watering condition. ANOSIM analysis supported phytochemical profile shifts in Romaine and loose-leaf lettuce tissue in response to water deficit under both positive and negative modes (Figure 2A). Leaf tissues of Romaine lettuce under positive mode yielded 125 peaks and while 71 of these were detected in samples under both regular watering and drought-subjected plants, 19 were detected only in plants under restricted watering (Table 4). No new peaks were detected under negative mode as a result of restricted watering, but 7 peaks were unique to samples under regular watering condition (Table 4). Nonetheless, ANOSIM based on the presence or absence of peaks was not significant for Romaine leaf tissue under negative mode and the difference between the treatments was attributed to variations in levels of shared compounds (Table 5). Similarly, under positive mode detection, the response of 'Mascara' tissue profile to drought was also likely driven by changes in levels of compounds, since no significant difference to control plants was detected using the Jaccard similarity coefficient. By contrast, in negative mode, changes in the presence or absence of peaks between treatments contributed to leaf tissue profile divergence (R=0.31, p<0.05) (Table 5).

Surface metabolite profiles of Romaine lettuce plants under different watering conditions diverged most prominently under positive (R= 0.38, p < 0.05; Figure 2A) compared to negative mode ((R= 0.24, p = 0.07; Table 5, Figure 2B). Under positive mode, 55 and 13 peaks were detected under regular watering or irrigation withholding conditions, compared to 20 and 9 peaks with negative mode ionization (Table 4). Phytochemical profiles of wash

samples from drought-exposed ‘Mascara’ lettuce were markedly different to profiles from regularly watered lettuce under both positive (R= 0.59, $p < 0.05$) and negative (R= 0.21, p <0.01; Table 5) modes, and in both cases, both relative amounts and presence/absence of peaks contributed to the profile shifts detected.

Taken together, results suggest that phytochemical profiles differences between the two lettuce cultivars was greater than the variations induced by drought within the same cultivar. ESI-MS in positive mode was more sensitive in identifying changes in phytochemical profiles in lettuce. Importantly, phytochemical profile shifts were identified in both leaf tissue and on leaf surfaces. Drought had a pronounced impact on phytochemical profiles of lettuce leaf tissues. The surface metabolome (in leaf wash) of ‘Mascara’ was more strongly influenced by drought compared to Romaine lettuce.

3.4 Discussion

In this project, we studied the impact of cultivar and drought effects on the *Salmonella* and lettuce association. Romaine and loose-leaf ‘Mascara’ lettuce and *Salmonella* serotypes Newport and Typhimurium were included. Specific phytochemical compounds and overall phytochemical profiles were detected. Phytochemical data together with *Salmonella* survival collected from both lettuce cultivars were analyzed to test the hypothesis whether alteration of phytochemicals impacted *Salmonella* and lettuce association. Our results showed that Romaine lettuce under regular watering condition accumulated least phytocompounds of antioxidant capacity, with least decline of *Salmonella* population on surface and no lagged growth in washes. On the other hand, restricted *Salmonella* population and higher levels of phytochemicals of ‘Mascara’ lettuce in response to drought was recorded. Taken together,

these results suggested that Romaine lettuce, the most popular green, was at high risk of foodborne outbreaks. ‘Mascara’ lettuce supported less *Salmonella* possibly due to naturally accumulated or drought-induced higher levels of phytochemicals. Results from this research could provide suggestions to farmers and produce industry to cultivate cultivars of low food safety risk and regulate irrigation for enhance food safety.

Population of *Salmonella* Newport and Typhimurium declined after 24 hours of the initial inoculation, indicating reduced fitness of *Salmonella* on lettuce surfaces under regular irrigation and watering withholding conditions. Cultivar-dependent changes of *Salmonella* survival were noted. A larger *S.* Newport population reduction was detected on ‘Mascara’ lettuce under both watering conditions compared to on Romaine. *S.* Typhimurium showed less population decline on drought-treated Romaine lettuce surface than ‘Mascara’. *Salmonella* Newport growth in Mascara washes was limited. Higher levels of total flavonoids, total phenolics, anthocyanins and antioxidant capacity were detected in ‘Mascara’ leaf tissues than Romaine, consistent with divergence in overall phytochemical profiles of leaf tissues and leaf washes between two lettuce cultivars. Drought significantly restricted *Salmonella* Newport but not Typhimurium on Romaine. Drought-treated ‘Mascara’ lettuce showed higher reduction of both *S.* Newport and Typhimurium population compared to ‘Mascara’ under regular irrigation. Growth of *Salmonella* Newport in washes collected from drought-treated lettuce plants showed a prolonged lag phase. Phytochemical compounds were not altered by drought for Romaine lettuce. However, increased accumulation of total flavonoids, total phenolics and anthocyanins were identified in ‘Mascara’ lettuce in response to drought. Drought significantly impacted the phytochemical profiles of leaf tissue and wash profiles of both Romaine and ‘Mascara’ lettuce. Strong positive correlations between

phytochemical measurements and *Salmonella* epiphytic decline were noted, indicating increased phytochemicals in response to cultivar variation and drought stress play a role in epiphytic *Salmonella* restriction on lettuce surface. Overall, as the most popular raw green among American consumers, Romaine lettuce is interestingly more implicated with *Salmonella*. Our results also suggested more restricted actions for Romaine are needed to ensure food safety, especially under well-watered growing condition.

Metabolomic changes due to lettuce cultivar and drought stress were reported in this study. Phytochemical profiles of Romaine and 'Mascara' lettuce diverged for both leaf tissues and wash samples. Drought significantly induced metabolomic changes of 'Mascara' and Romaine lettuce leaf tissue and wash samples. The metabolomic changes were mainly due to presence of compounds unique to fully watered or water deficit situations. Phytochemical analysis of leaf tissue from regularly watered plants revealed that increases in total flavonoid and phenolics contents might contribute to higher antioxidant capacity and differences in metabolomic changes between Romaine and 'Mascara' lettuce. Levels of total flavonoids, phenolics and anthocyanins increased markedly in response to drought treatment, which might associate with the metabolome changes in 'Mascara' lettuce leaf tissues. By contrast, levels of antioxidant capacity, phenolics and flavonoids showed no change after drought for Romaine lettuce. However, the phytochemical profiles of Romaine lettuce leaf tissues were altered, which indicating more compounds might be responsible for the drought-driven metabolome changes. These results could enhance the understanding for lettuce-*Salmonella* in a more systemic way and illuminating innovative solution to ensure food safety.

Decline of *Salmonella* on both regularly watered Romaine and 'Mascara' leaf surface was reported, probably due to poor in available nutrients and the reduced fitness of enterica pathogen on leaf surface (Brandl and Mandrell, 2002; Leveau Johan and Lindow Steven, 2001). Reduction of *S.* Newport and *S.* Typhimurium on regularly watered Romaine and 'Mascara' lettuce showed no difference. Similar results were reported using Romaine leaf pieces, the retrieval of *S.* Newport and Typhimurium showed no differences after 2 hours post-incubation at room temperature (25℃) and 3 days at 30℃ (Kroupitski et al., 2009b). The retrieval of *Salmonella* Newport or Typhimurium from Romaine lettuce surface was higher than from 'Mascara' lettuce, with highest *Salmonella* reported from Romaine under regular watering condition, suggesting for same plant species, the *Salmonella* population varied (Lindow and Brandl, 2003). Taken together, these results suggested that Romaine lettuce might provide more growth-supporting nutrients to *Salmonella* on the surface than 'Mascara' lettuce. In the meantime, carbon- and nitrogen-containing nutrients such as organic acid, amino acids and carbohydrates leached from plant leaves are critical sources for enterica pathogen colonization (Brandl and Amundson, 2008; Leveau Johan and Lindow Steven, 2001; Mercier and Lindow, 2000). More specifically, levels of total sugars on the surface of plants species is positively related to epiphytes population (Mercier and Lindow, 2000). Oak-leaf lettuce in average showed lower sugar content than Cos lettuce (Missio et al., 2018). Fructose and glucose and were the major soluble sugars detected in both Oak leaf and Romaine lettuce (Allende et al., 2006; López et al., 2014). Glucose, a carbon source that can readily utilized by *Salmonella* (Gutnick et al., 1969; Nur et al., 2016). Malic and citric acid, the predominate organic acid found in oak leaf were also found in Romaine lettuce (Allende et al., 2006; López et al., 2014). These organic acids are vital nutrients for epiphyte and

possibly *Salmonella* (Wilson and Lindow, 1994). Moreover, amino acids in leaf tissue could serve as nutrients for epiphytes (Wilson and Lindow, 1994) and are highly involved in *Salmonella* colonization on lettuce surface (Goudeau et al., 2013).

Not only primary metabolites, secondary metabolites, such as phenolics and fatty acids, could impact the surface colonization of *Salmonella* (Dev Kumar and Micallef, 2017; Han and Micallef, 2016). More specifically, fatty acids and phenolics were reported negatively associated with *Salmonella* on tomato surface (Han and Micallef, 2016). Our results seconded with these findings. In our results, under regular watering condition, 'Mascara' lettuce showed higher levels of total phenolics, flavonoids and antioxidant capacity than Romaine and harbored less *Salmonella*. The levels of anthocyanins in Romaine lettuce were lower than the limit of detection, which partially explained the relative low antioxidant capacity of Romaine leaf tissues. Red lettuce cultivars exhibited higher total phenolics and flavonoids, antioxidant capacity and anthocyanins levels than green lettuce (Ozgen and Sekerci, 2011; Tsormpatsidis et al., 2010). Differences in primary and secondary metabolites in leaf tissues play a major role in the divergence in phytochemical profile between two cultivars under regular watering condition.

Phytochemical profile of surface washes diverged between Romaine and 'Mascara' lettuce, indicating that phytocompounds that are directly exposed to epiphytic bacteria for potential utilization altered due to lettuce cultivars under regular watering condition. The growth of *Salmonella* in washes of Romaine was enhanced compared to 'Mascara' over 24 hours under regular irrigation. Combined with the phytochemical measurements for lettuce, we hypothesized that, under regular irrigation, 1) sufficient nutrients were present in Romaine

washes for supporting *Salmonella* growth; 2) *Salmonella* growth-limiting compounds were abundant in 'Mascara' washes.

In addition to cultivar, drought stress restricted the surface survival of *Salmonella* and shifted the metabolomic profiles of both Romaine and 'Mascara' lettuce. A series of mechanisms have been developed by plants to cope with drought, including stomatal closure (Flexas and Medrano, 2002). Stomata is the natural opening for bacteria to enter even invade plants (Mittal and Davis, 1995). Enterica pathogens have been reported to create aggregates and form colonies on leaf site sufficient with nutrients such as stomata and glandular trichomes (Lindow and Brandl, 2003) and reopen stomata on produce surface (Markland et al., 2015). So far, no evidence has been reported regarding the ability *Salmonella,* suggesting that the closed stomata due to drought might negatively affect the nutrients availability to *Salmonella* and its surface survival. In regard to metabolomic changes, drought stimulated the accumulation of osmoprotectants including proline in lettuce plants (Rajabbeigi et al., 2013; Shin et al., 2021), which could support the growth of epiphytic pathogen and possibly utilized by *Salmonella* (Wilson and Lindow, 1994). Simultaneously, lettuce under restricted watering condition showed increased phenolic accumulation, antioxidant capacity and nutritional quality (Oh et al., 2010; Paim et al., 2020). These secondary metabolites could, in turn, negatively affect surface survival of *Salmonella* (Dev Kumar and Micallef, 2017; Han and Micallef, 2016). Our results showed increased accumulation of proline and secondary metabolites in 'Mascara' lettuce in response to drought and an overall phytochemical profile changes due to drought under positive mode. Interestingly, phytochemical measurements for Romaine lettuce showed no difference, but the overall phytochemical profile changes were detected due to drought. Taken together, we may conclude that *Salmonella* declines on lettuce

surface due to drought might be associated with stomata closure and photochemical profile changes including secondary metabolites.

Phytochemical profiles of surface washed showed no convergence after drought treatment for 'Mascara' lettuce, whereas overlaps were reported for Romaine washes samples under negative mode. In addition, these phytochemical profiles changes impacted the growth of *Salmonella* in washes. The growth of *Salmonella* in 'Mascara' washes was lower than in Romaine washes, possibly due to insufficient nutrients or the presence of inhibitory compounds in response to drought.

This research studied the colonization of *Salmonella* on lettuce from both the bacteria and host side. Two *Salmonella* serovars were included in this study to evaluate the fitness of serovars on lettuce surfaces. Green and red lettuce cultivars, as hosts to *Salmonella*, their leaf tissue and surface wash phytochemical profiles were studied to reveal cultivar impact on *Salmonella*. Our results suggested that green Romaine lettuce created a more favorable environment to *Salmonella* with less accumulation of secondary metabolites. Impact of abiotic stress drought on epiphytic *Salmonella* growth and metabolomic changes of lettuce was stated in this study. Based on the findings, drought-treated plants restricted *Salmonella* with altered the phytochemical profiles reported. 'Mascara' was more sensitive to drought treatment in regard to the accumulation of phytochemical of antioxidant capacity. Plant surface washes were able to support the growth of *Salmonella*. Washes from Romaine lettuce supported a larger population of *Salmonella*, possibly due to readily nutrients or the absence of inhibitory compounds. Drought delayed the growth of *Salmonella* in 'Mascara' washes with higher accumulation of secondary compounds of antioxidant capacity. These results

suggested the accumulation of antioxidant secondary compounds might contribute the restriction of *Salmonella* on surface and in washes. Pairwise comparison of phytochemical profiles revealed that differences between lettuce cultivars were not affected by drought and remained in both leaf tissues extract and washes. Significant differences between leaf tissues and washes were stated. Our results expanded insights into the lettuce-*Salmonella* association. Cultivar- and drought-associated *Salmonella* restriction on leaves could be advising in breeding produce and farming practices. Elucidating phytonutrients found both on surfaces and in washes that can be utilized by *Salmonella* and inhibitory compounds due to cultivar or drought is required in the future to enhance produce resilience to human pathogens, thus, enhancing food safety.

3.5 Conclusion

In summary, we focused on the phytochemical profiles of lettuce and their impact on *Salmonella* association. As an epiphyte, successful *Salmonella* colonization on produce surface is highly associated with their availability to nutrients (Mercier and Lindow, 2000; Tukey, 1966). Diversity and contents of these nutrients could possibly impact the overall phytochemical profiles of leaf tissues and washes. Our results demonstrated green Romaine lettuce, the most popular green among Americans, showed less decline to *Salmonella* compared to red 'Mascara' and was of lower nutritional quality. Drought stress further reduced *Salmonella* population on 'Mascara' lettuce surfaces. 'Mascara' lettuce after drought treatment accumulated more phytochemicals and showed higher nutritional quality. Cultivar and drought treatment influenced *Salmonella* growth in lettuce washes. Washes from Romaine lettuce supported higher *Salmonella* growth. Drought significantly delayed the

growing activities of *Salmonella* in ‘Mascara’ washes. Overall, our results further evidenced that phytochemical profiles changes in response to cultivar and drought impacted the epiphytic growth of *Salmonella* on lettuce. It is feasible to manipulate *Salmonella* population through environmental interventions and naturally cultivar variances. Future studies are needed to decipher the metabolomic changes for both leaf tissues and washes in response to cultivar and drought, thus illustrating *Salmonella* growth-limiting compounds or factors and enhancing food safety.

Chapter 4: Kale leaf surface and tissue metabolomes shift with plant development and in response to drought, limiting *Salmonella enterica* leaf association

4.1 Introduction

The upward trend in the incidence of foodborne disease outbreaks associated with fresh fruit and vegetables (Callejón et al., 2015b; Lynch et al., 2009) may be attributed to an increase in fresh produce consumption, more centralized product processing and distribution, climate change and pathogen adaptation (Olaimat and Holley, 2012). Food safety breaches are not only a public health concern, but also precipitate enormous economic losses. It is reported that in the USA, food safety incidents cost over $15.5 billion annually, with non-typhoidal *Salmonella enterica* incurring the highest economic burden compared to all other 14 human foodborne pathogens considered (Hoffman et al., 2015). *Salmonella* accounts for over half of the bacterial foodborne disease outbreaks in both the USA and the European Union, with *Salmonella* serotype Newport emerging as a predominant serotype in illnesses associated with tomatoes, salads and lettuce (Callejón et al., 2015b). In the USA, from 1973 to 2012, 11% of foodborne illness outbreaks associated with leafy greens were caused by *Salmonella* (Herman et al., 2015) and, using multi-year data, leafy greens were estimated to cause over 4% of the foodborne illnesses caused by *Salmonella* (IFSAC, 2021).

Kale (*Brassica oleracea* Acephala group) is one of the ten most economically important vegetables in the global agriculture market (Francisco et al., 2017). It has a diverse phytochemistry and is a highly nutritious leafy green(Sikora and Bodziarczyk, 2012; Šamec

et al., 2019), being a good source of minerals, micronutrients, carbohydrates, amino acids and health-beneficial compounds such as polyphenols and glucosinolates (Sikora and Bodziarczyk, 2012; Velasco et al., 2007). From 1997 to 2019, kale farming grew rapidly by 215% in the USA as this leafy vegetable gained popularity among American consumers (ERS, 2020). In 2016, one kale outbreak caused by *Salmonella* Enteritidis was reported in Minnesota (Larsen, 2016) and several enteric pathogen-related (including *Salmonella*) product recalls containing kale as the major ingredient have been reported in recent years (Vince. and Staff., 2016).

Salmonella interactions with the leaf surface are still not fully deciphered. Bacteria can attain 10^5 to10^7 CFU/g of leaf under favorable environmental conditions, such as under high relative humidity (Hirano and Upper, 1990a; O'brien and Lindow, 1989). Phytonutrients available on plant surfaces, including amino acids, sugars and organic acids can provide the carbon and nitrogen needed for nucleic acid and protein synthesis and bacterial replication (Brandl and Amundson, 2008; Goudeau et al., 2013; Hirano and Upper, 1990a; Mercier and Lindow, 2000). The abundance of these nutrients and other phytochemicals such as phenolics can vary with leaf age, plant genotype, environmental conditions and abiotic stresses, which in turn can affect bacterial populations, including enteric pathogens (Han and Micallef, 2016; Hunter et al., 2010; Ryffel et al., 2016). Elucidating phytocompound profiles in plants under various conditions and investigating how metabolome shifts modulate enteric pathogen-plant associations can provide insights for enhanced food safety (George and Brandl, 2021; Melotto et al., 2020), but research in this area remains scant.

Drought is one of the most taxing abiotic stresses that plants must face and they display a range of mechanisms to withstand it, including stomatal closure (Flexas and Medrano, 2002) , accumulation of osmoprotectants such as amino acids, glycine betaine, sugars and sugar alcohols (Good and Zaplachinski, 1994; Huang et al., 2000; Singh et al., 1972; Streeter et al., 2001), and biosynthesis of antioxidant compounds to overcome oxidative damage (Eek and Zhang, 2017; Oh et al., 2009; Rajabbeigi et al., 2013). In kale, the accumulation of polyphenols, glucosinolates and overall antioxidant capacity has been shown to increase not only in response to stress but also with plant development (Korus, 2011; Velasco et al., 2007). Hence, we sought to use drought stress in kale plants at various stages of development to investigate how leaf tissue and leaf surface metabolomes influence the *Salmonella*-kale interaction. We hypothesized that kale plants under drought or later in development would accumulate higher levels of secondary metabolites that would in turn contribute to limiting *Salmonella* on plants. Data from phytochemical profiling of leaf extracts and leaf surface washes, kale leaf phytochemical analyses and quantification assays of epiphytic *Salmonella* on kale were integrated to gain insight on how plant metabolite profiles may affect *Salmonella*-plant associations.

4.2 Material and Methods

4.2.1 Kale growth and drought treatment condition

Brassica oleracea L. var. *acephala* cv. 'Improved Dwarf Siberian' was used in this study. Kale seeds (Territorial, OR, USA) were germinated in potting mix soil (Sunshine LC1; Sungro Horticulture, Canada) at the Research Greenhouse Complex, University of Maryland, and grown under controlled light and temperature conditions (16 h light:8 h dark photoperiod

at 23℃/18℃ day/night temperatures). Kale plants at different stages (2-8 weeks post-germination) were subjected to water stress by withholding irrigation for 3-6 days (depending on age, adjusted to maximum number of days before leaf wilting occurred) or regularly watered (controls) prior to experimentation. Details regarding water withholding treatment duration and plant age are listed in Table S1. At the end of treatment, kale plants ranged from 20 to 59 days old. Biomass was determined by weighing all above ground tissue after drying in an oven at 70°C for 48 h or until weight no longer changed. Measurements were repeated twice for 2-week-old plants and 3 times for 8-week-old plants with a minimum of 5 plants per treatment in each experiment. For plants of the same age, change in dry shoot weight due to drought treatment was calculated using the following equation and expressed as a percentage.

$$\%\ \text{change in biomass} = \frac{\text{dry weight}_{\text{control}} - \text{dry weight}_{\text{drought}}}{\text{dry weight}_{\text{control}}} \times 100\%$$

S. Newport assays were conducted on plants at all growth stages. Only 20- and 59-day old kale plants were selected for phytochemical analyses and leaf and wash phytochemical profiling. At 20 days old, kale is consumed as a raw salad ingredient (baby kale) and at 59 days old kale is fully mature and marketed for regular consumption.

4.2.2 Preparation of kale leaf extracts and surface washes

Whole kale leaves were flash frozen using liquid nitrogen and then ground into powders using a pestle and mortar and 200 mg of plant powder accurately weighed and extracted with 1.5 ml 70% methanol (Sigma-Aldrich, MO, USA) with 0.5% formic acid (VWR, PA, USA). Each extract was fully vortexed before it was centrifuged at 3,000 rpm for 10 min. The supernatants were frozen at -20°C until further analysis. Whole plant washes were collected

from a subset of plants (Table S1). Leaving the root ball out of the bag, all kale leaves of each plant were immersed in 30 ml 5% methanol/water solution and shaken at 150 rpm for 24 h at room temperature. Surface compound solutions were filter sterilized through 0.2 μm cellulose acetate syringe filters (VWR, PA, USA).

4.2.3 Phytochemical profiling of kale leaf extracts and surface washes using electrospray ionization-mass spectrometry (ESI-MS)

Aliquots (1 ml) of kale leaf extracts and 30 ml filter-sterilized kale wash solutions were lyophilized into powder and resuspended in 100 μl 70% methanol with 0.5% formic acid with at least 4 biological replicates.

To ensure all compounds were fully dissolved in the extraction liquid, all tubes were sonicated in a water bath for 2 h at maximum intensity. The remaining sample extract was then transferred into vial inserts for ESI-MS analysis. A Time-of-Flight mass spectrometer (AccuTOF, JEOL, MA, USA) equipped with an electrospray ionization ion source was used in the mass spectrometric analysis of the extracts. Mass spectra were acquired in the positive and negative modes, respectively, at a rate of one spectrum per second with the m/z range of 50–800 Da. Every mass spectrum was averaged over about one minute. The AccuTOF mass spectrometer settings were as follows: needle voltage = 2100 V, orifice 1 temperature = 100°C, orifice 1 = 30 V, orifice 2 = 5 V, ring = 10 V. The desolvating chamber temperature was set at 250°C and the flow rates of the nebulizing and desolvating gases were 0.6 and 3.0 L/min, respectively. The sample injection volume was 10 μl and the flow rate was set to 0.25 ml/min. Before and after each sample was analyzed, 10 μl methanol were injected and measured to monitor any possible contaminants or carryover. Calibration for exact mass

measurements was accomplished using 5 mM cesium iodide as the internal standard. Measurements were repeated at least twice, and the experimental results showed excellent reproducibility, hence the second set of mass spectral data per sample was used in statistical analysis.

4.2.4 Phytochemical analyses of kale leaf extracts

All absorption readings in the following assays were acquired using a microplate reader (Synergy HTX; Biotek, VT, USA). Each phytochemical analysis was repeated 3 times in separate experiments with at least 3 biological replicates in each treatment. Data were pooled.

Antioxidant capacity: This method was modified from Stratil et al. (Stratil et al., 2006). To prepare the reagent, 31.7 mg 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (TCI, OR, USA) and 8.6 mg potassium persulfate (Aldon Corp Avon, NY, USA) were dissolved in 10 ml water and allowed to stand at room temperature for 16 h in the dark to form a stable radical. The ABTS solution was diluted to an absorption of ~1.3 at 734 nm and 200 μL aliquots prepared, to which 20 μL sample extracts were added and allowed to stand for 15 min. Absorption was measured at 734 nm. A standard curve from the blank-corrected absorption at 734 nm of Trolox (TCI) standards was plotted and antioxidant capacity was calculated as Trolox equivalent using the standard curve.

Total flavonoids: The method was adapted from Zhishen et al. (Zhishen et al., 1999) by mixing 100 μL kale leaf extract to 20 μL 5% $NaNO_2$ (Avantor, PA, USA). After 5 min, 20 μL 10% $AlCl_3$ (Alfa Aesar, MA, USA) were added, followed by 100 μL 1 mol/L NaOH (VWR Chemicals, BDH, Radnor, PA, USA) 1 min later. Absorption of the mixture was measured at

510 nm on the microplate reader. Antioxidant capacity was calculated as catechin equivalent using the standard curve plotted from blank-corrected absorption measurements of the catechin standard at 510 nm (Enzo Life Sciences, NY, USA) (Yoon et al., 2019).

Total phenolic content: Modifications were made to previously published methods (Kao et al., 2014; Singleton and Rossi, 1965) for measuring total phenolic contact in kale tissue. Kale leaf extract (80 μL) was mixed with 90 μL 10% Folin Ciocalteu reagent (MP Biomedicals, CA, USA) and 40 μL 700 mM Na_2CO_3 (VWR Chemicals, BDH) in a 96-well plate. Samples were vortexed and let to stand for 1 h in a microplate reader at 25℃. Absorption measurements were taken at 765 nm. Total phenolics were calculated as gallic acid equivalents using the standard curve prepared from blank-corrected absorption values of gallic acid at 765 nm (Acros Organics Fair Lawn, NJ, USA).

Estimated glucosinolate content: Kale leaf extract (80 μL) was added to 200 μL 2 mM sodium tetrachloropalladate (Acros Organics). The samples were incubated at room temperature for 1 h and absorption measured at 425 nm. Water was then used as a blank. Total glucosinolate content was calculated by putting the OD_{425} of each sample into the predicted formula as follows (Mawlong et al., 2017):

$$Y = 1.40 + 118.86 * A_{425}$$

4.2.5 Salmonella enterica inoculum preparation

Kale plants were inoculated with *Salmonella enterica* serovar Newport, an environmental isolate collected from an irrigation pond that matched a recurring tomato outbreak stain (Greene et al., 2008), to investigate drought treatment and age effects on the ability of kale to

support the enteropathogen. The *S.* Newport strain had been previously adapted to rifampicin (rif) (MilliporeSigma, MA, USA) and was maintained at -80℃ in Brucella broth (BD, MD, USA) containing 15% glycerol (VWR Chemicals, BDH) and 50 μg/ml rifampicin and revived by streaking onto Trypticase Soy Agar (TSA, BD) plates amended with 50 μg/ml rifampicin (TSA-rif) overnight at 35℃. Single strain assays were conducted to control for the high variability in the plant/bacterial system.

Single colonies of *S.* Newport grown overnight were streaked onto fresh TSA-rif plates and incubated overnight at 35℃. *S.* Newport inoculum was made by suspending fresh bacterial colonies in 0.1% peptone water (PW) to an OD_{600}=0.5 to obtain a cell density of ~10^9 CFU/ml. For leaf inoculation, a 100-fold dilution of this suspension in 0.1% PW was used. Prior to each experiment, actual inoculum level was determined by plating serial dilutions onto TSA-rif plates for direct quantification.

4.2.6 Kale leaf surface and leaf wash inoculation with Salmonella Newport

The third or fourth true leaf of each kale plant under different watering regimes were gently marked on the stalk with a black marker pen. The inoculation methods used were optimized in our lab to include direct leaf and leaf surface wash inoculations. An aliquot of 100 µl of *S.* Newport inoculum was pipetted on the adaxial side of each marked leaf distributed in 10 spots. An estimated total inoculum dose of 10^6 CFU was inoculated per leaf. Plants were kept at 18°C and 50% relative humidity and inoculated kale leaves were harvested 24 hours post-inoculation (hpi). Each leaf was aseptically clipped off the stem with scissors and placed in a sterile Whirl-pak bag (Nasco, WI, USA). Inoculated leaves were immersed in 30 ml 0.1% PW, hand-massaged for 30 s and sonicated in a Branson Ultrasonic Cleaner (Branson

Ultrasonics Corporation, CT, USA) for 1 min at maximum speed to dislodge *S.* Newport cells from the kale leaf surfaces. Each leaf was then shaken at 150 rpm for 10 min. Serial dilutions from each leaf rinsate were plated on TSA-rif plates for *S.* Newport enumeration after 24 h of incubation at 35°C.

For leaf washes, 20 µl of *S.* Newport inoculum were added to 2 ml leaf wash solution at an initial concentration of ~10^6 CFU/ml and incubated at 35°C with shaking at 150 rpm. *S.* Newport cell counts were taken at 0, 2, 4, 6 and 24 hpi. At each time point, serial dilutions were plated on TSA-rif plates for enumeration. Sterile 5% methanol in water served as the negative control.

4.2.7 Statistical analysis

Ordination of the phytochemical profiles obtained from ESI-MS was conducted using multidimensional scaling (MDS) to depict relationships among samples. Briefly, data retrieved from ESI-MS containing information regarding the m/z ratio and signal intensity of each compound were pre-processed by binning peaks and aligning relative peak intensity data from different samples. Data were imported into RStudio (v. 1.3.1093). Distance matrices based on the Bray-Curtis dissimilarity coefficient were generated by the vegan package (version 2.5-7) and MDS analysis was performed in JMP Pro v. 15.2.0. (SAS Institute Inc., Cary, NC, USA).

For significance testing of sample data, analysis of similarity (ANOSIM) was conducted in PRIMER 6 (Plymouth Routines in Multivariate Ecological Research version 6.1.15; PRIMER-E-Ltd., Plymouth, United Kingdom). ANOSIM assigns ranks to the similarity matrix built from distances between samples and combines the ranking similarity with Monte

Carlo randomization to assess significance (α=0.5) between groups. In ANOSIM, the test statistic *R* approaches 0 when samples from different treatments are the same, whereas *R* approaches 1 when replicates within one group are more similar to each other than to replicates from another group.

Volcano plots were used to feature differential peaks detected in samples from two age groups under different watering regimes. Pairwise comparison by Student's *t*-test of relative signal intensity of each peak was conducted between samples under different watering regimes (WA 20D CON vs. WA 20D DR, WA59D CON vs. WA59D DR, LF20D CON vs. LF20D DR, LF59D CON vs. LF20D DR) and *p*-values were ranked and corrected for multiple comparisons using the Storey and Tibshirani method (Storey and Tibshirani, 2003). The false discovery rate (FDR) adjusted *p*-values (*q*-value) were transformed [$-\log_{10}$(*q*-value)] before plotting. The fold change (FC) of each peak between drought-subjected and regularly watered plant samples was calculated as $\log_2$ (DR/CON). Volcano plots were generated by ggplot2 R package (Wickham, 2016). Peaks with a $\log_2(\text{FC}) \geq 1$ and a $-\log_{10}(q\text{-value}) \leq 1.3$ ($q \leq 0.05$) indicating up-accumulated peaks after drought treatment and were colored red. Blue symbols denoted down-accumulated peaks after drought treatment with a $\log_2(\text{FC}) \leq -1$ and a $-\log_{10}(q\text{-value}) \leq 1.3$ ($q \leq 0.05$).

For phytochemical and bacterial assays, each experiment was repeated at least three times with each treatment tested in replicates of four to six and data were pooled for statistical analysis. *S.* Newport counts in CFU/plant and CFU/ml, retrieved from kale leaves and wash solutions, respectively, were log transformed prior to analysis. Statistical bootstrapping (Eforn, 1979) was used to resample log transformed data acquired from the survival of

Salmonella on leaf surface after 24 h and the retrieval of *Salmonella* in leaf wash for further Pearson's correlation analysis. *Salmonella* data from either the surface survival or in the leaf wash were resampled 1,000 times for each age group under each watering regime. ANOVA was conducted on *S.* Newport counts and phytochemical measurements in JMP Pro v. 15.2.0.

4.3 Results

4.3.1 Phytochemical profiling of kale leaf tissue extracts and leaf surface compounds using ESI-MS revealed marked differences between leaf tissue and leaf surface metabolomes

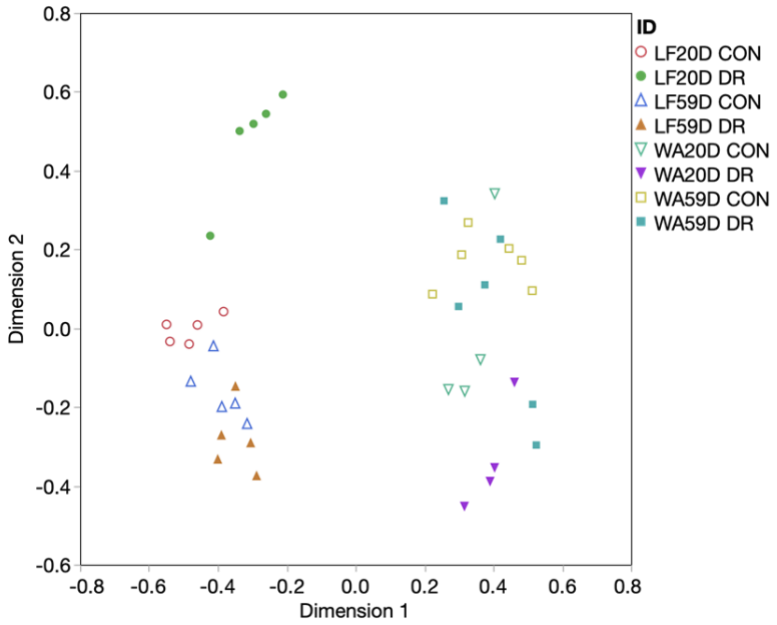
Untargeted phytochemical profiling of kale leaf tissue extracts and leaf surface washes were analyzed with ESI-MS to investigate how the leaf tissue and surface metabolomes changed as plants developed and in response to drought stress. In total, 1,439 peaks were detected in positive mode and 424 peaks in negative mode. Kale leaf tissue extracts yielded 532 peaks in positive mode. Of these, 189 were shared between juvenile and mature samples and 315 peaks were shared between samples under different watering regimes. Under negative mode, 217 peaks were detected in tissue extracts, with 88 shared between both growth stages and 153 peaks were found in samples from both regularly watered and drought-subjected kale. Kale surface wash samples yielded 907 peaks in positive and 207 in negative modes. Of these, 249 and 39 peaks were detected in both growth stages in positive and negative modes, respectively, while 382 peaks in positive and 80 peaks in negative modes were shared between plants under different watering regimes (Table 6). Combined, there were 429 and 313 (in positive mode), and 106 and 85 (in negative mode) unique peaks (detected only in one watering treatment) in juvenile and mature kale, respectively. Under positive mode, 129

and 101 peaks were shared between leaf tissue extracts and leaf surface washes for juvenile and mature kale, respectively. By contrast, in negative mode, fewer shared peaks between tissue and washes were detected for juvenile (9 peaks) and mature kale (18 peaks). Ordination of the data showed no overlap between tissue and wash profiles under either mode, indicating that the differences between leaf tissue and surface metabolite profiles were greater than differences within groups for juvenile and mature plants subjected to regular (Figure 3A) or restricted watering regimes (Figure 3B). ANOSIM analysis based on sample type (tissue versus surface) gave a global *R* statistic of 0.83 (positive mode) and 0.77 (negative mode; both p=0.001).

Table 6. Number of metabolite peaks acquired from leaf tissue and wash samples from 20-day (juvenile) and 59-day old (mature) kale plants under regular watering (CON) or restricted watering (DR) regimes based on phytochemical profiling with ESI-MS under positive and negative modes.

ID	Sample type	Total peaks detected	Peaks in volcano plot	Treatment/ unique peaks↓		Peaks of interest*	
				CON	DR	Up	Down
Peaks detected in positive ionization mode							
LF20	Leaf	324	160	32	132	33	24
LF59	Leaf	208	155	36	17	0	0
WA20	Wash	432	167	183	82	13	7
WA59	Wash	475	215	125	135	0	0
Peaks detected in negative ionization mode							
LF20	Leaf	103	65	17	21	12	9
LF59	Leaf	114	88	17	9	0	0
WA20	Wash	93	25	35	33	8	2
WA59	Wash	114	55	32	27	0	0

3A



3B

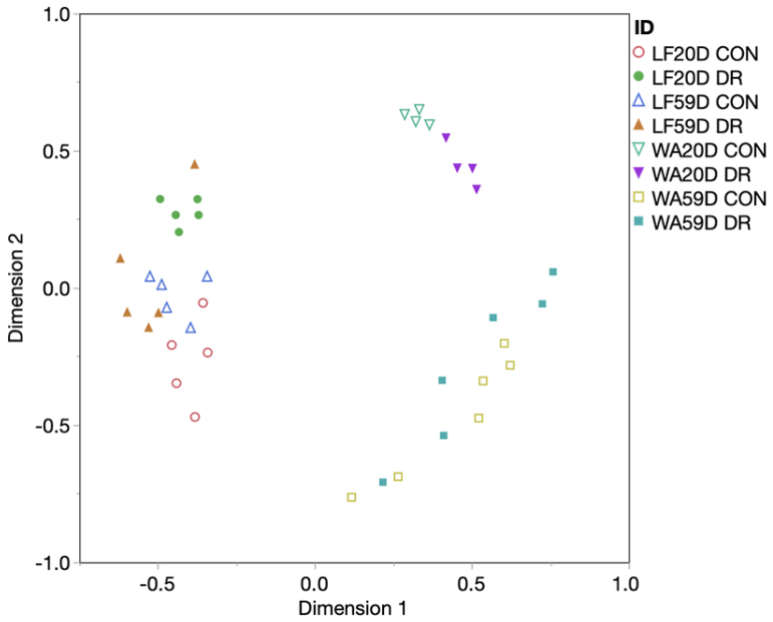


Figure 3. Multidimensional scaling (MDS) plots of phytochemical profiles of kale leaf tissue (LF) and leaf surface washes (WA) at two growth stages of 20-day (20D) and 59-day (59D) for plants under regular (CON) or restricted (DR) watering, based on ESI-TOF-MS under (A) positive and (B) negative modes. Stress for plots was 0.17 (A) and 0.21 (B).

Table 7. ANOSIM pairwise comparison of kale leaf tissue extracts (LF) and kale leaf surfaces washes (WA) based on phytochemical profiling with ESI-MS under positive and negative modes. Juvenile and mature plants were 20 (20D) or 59 (59D) days old at time of experimentation and subjected to regular (CON) or restricted watering (DR). The R statistic spans 0-1, with 0 indicating no relationship and 1 indicating highest relatedness between sample profiles. NS means not significant at α=0.05.

	Positive mode		Negative mode	
Sample comparisons	*R*	*p* value	*R*	*p* value
Leaf tissue extracts				
LF20D CON – LF59D CON	0.496	0.032	0.36	0.008
LF20D DR – LF59D DR	0.956	0.016	0.652	0.016
LF20D CON – LF20D DR	0.948	0.008	0.9	0.008
LF59D CON – LF59D DR	0.116	NS	0.016	NS
LF59D DR – LF20D CON	0.908	0.008	0.388	0.008
LF59D CON – LF20D DR	0.944	0.008	0.892	0.008
Leaf surface washes				
WA20D CON – WA59D CON	0.698	0.01	1	0.005
WA20D DR – WA59D DR	0.345	0.048	0.694	0.005
WA20D CON – WA20D DR	0.792	0.029	0.802	0.029
WA59D CON – WA59D DR	0.091	NS	0	NS
WA59D DR – WA20D CON	0.274	0.076	0.877	0.005
WA59D CON – WA20D DR	0.96	0.005	0.869	0.005

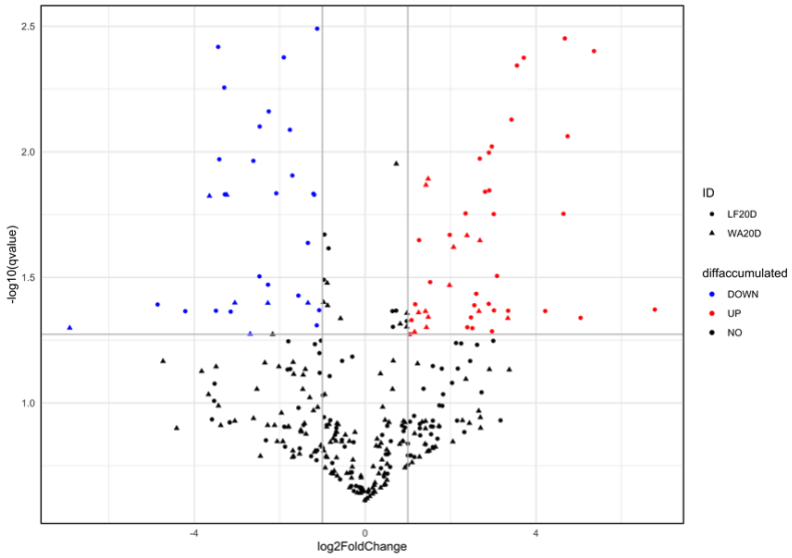
4.3.2 Phytochemical profiling of leaf tissue extracts revealed age-related and water stress-induced shifts

For kale leaf tissue samples, profiles from juvenile plants differed from mature plants, regardless of watering regime ($p<0.05$; Table 7, Figure 3). Profiles of regularly watered juvenile plants were most closely related to profiles from mature plants under the same watering condition, but these were still significantly different (ANOSIM $R=0.50$, $p<0.05$ and $R=0.36$, $p<0.01$, for positive and negative mode separation, respectively; Table 7, Figure 3). For kale plants subjected to water restriction, leaf profiles of juvenile and mature kale diverged more markedly ($R=0.96$ and $R=0.65$, both $p<0.05$), for positive and negative mode separation, respectively). These data indicate a development-dependent shift in leaf metabolites.

A very distinct separation was detected between regularly watered and drought-subjected juvenile plants in both positive ($R=0.95$, $p<0.01$) and negative ($R=0.90$, $p<0.01$) modes (Tables 6 and 7, Figures 3 and 4). Juvenile plants subjected to water stress exhibited the most divergent profiles compared to all other samples, with a total of 153 unique peaks detected in this group (Table 6, Figures 4A and 4B). Among shared peaks in leaf tissue samples, 78 were differentially detected following drought treatment in 20-day old plants (Table 6, Figure 4). Most of these shared peaks were detected under positive mode; 33 and 24 peaks of signal intensity increased and decreased, respectively, after drought treatment (Table 6, Figure 4A), with a less marked metabolome shift revealed under negative mode (Table 6, Figure 4B). By contrast, leaf tissue profiles of regularly watered and water-restricted mature plants converged and no differential peaks (Table 6) or significant difference (Table 7) were

detected between these two groups. Despite several peaks being unique to each group, only 9 (the lowest for any group) were unique to drought-subjected plant profiles (Table 6). All pairwise comparisons of differing age and watering regime where highly significantly different for both positive and negative mode data ($p<0.01$). Taken together, these results suggest that a water stress-dependent shift in the leaf metabolome is related to age, being most pronounced in juvenile plants.

4A



4B

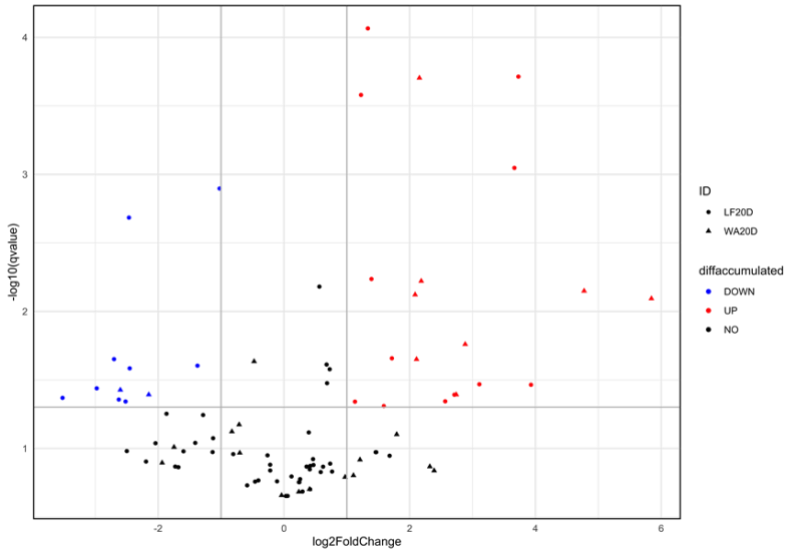


Figure 4. Volcano plots presenting the peaks that were significantly influenced by drought treatment via the non-targeted ESI-TOF-MS analysis under (A) positive and (B) negative modes. In each plot, leaf tissue and wash samples of 20-day old kale under regular watering (CON) and drought-subjected (DR) conditions were included. They were labelled as LF20D (leaf tissues from 20-day old kale; dots) and WA20D (wash samples from 20-day old kale; triangles).

4.3.3 Phytochemical profiling of leaf surface washes revealed age-related and water stress-induced shifts

Metabolome changes were not restricted to leaf tissue but were also apparent for leaf surface compounds. A very distinct age-dependent difference was revealed between regularly watered juvenile and mature plants in negative mode separation (R=1, p<0.01; Table 7). Differences in positive mode were less marked, attributed to some overlap between the two groups (R=0.70, p=0.01; Figure 3A). In water-restricted plants, mature leaf surface profiles diverged most markedly when separated in negative mode (R=0.69, p<0.01; Table 7, Figure 3B). In total, 218 and 115 peaks were detected only in the juvenile control or the water-restricted profiles, respectively. These results provide strong evidence for leaf surface metabolome shifts occurring in a developmentally programmed manner.

Statistically significant differences in leaf surface metabolomes between regularly watered and water-stressed juvenile plants were detected in both positive and negative modes (R=0.79 and 0.80, respectively, both p<0.05; Table 7). On the other hand, surface metabolite profiles of mature plants under different watering conditions converged, indicating similarities between these metabolite profiles and no age or water-stress dependent effects (Table 7, Figure 3A and 4). Despite this convergence, 319 peaks were unique to one or the other group in this comparison, which was higher than for leaf tissue profiles for the same treatment (Table 6). Taken together, these data suggest that water stress induces surface phytochemical shifts in juvenile kale plants, but evidence was not strong for mature plants.

4.3.4 Phytochemical analyses of kale leaves indicated differences due to plant age and water stress

Plant development was a factor in phytochemical traits of leaf tissue extracts. For regularly watered plants, the antioxidant capacity (Figure 5A) and total flavonoid content (Figure 5B) increased with plant age ($p<0.05$), while the rise in total phenolic content (Figure 5C) was not significant and estimated glucosinolates (Figure 5D) were unaffected. Antioxidant capacity correlated strongly with flavonoids and phenolics ($r = 0.74$ and 0.63, respectively, both $p < 0.01$) (Table 8) when data from control plants were grouped by age. Plants under water stress also exhibited some shifts as they matured. The antioxidant capacity of drought-subjected mature plants was higher than for juveniles under the same watering regime ($p<0.05$; Figure 5A). Since juvenile plants under water stress were elicited to produce more secondary metabolites, the age effect on phytochemical levels was less pronounced (Table 8). No change was recorded for total flavonoid content. A marked decrease in estimated glucosinolates ($p<0.05$) was detected as plants matured.

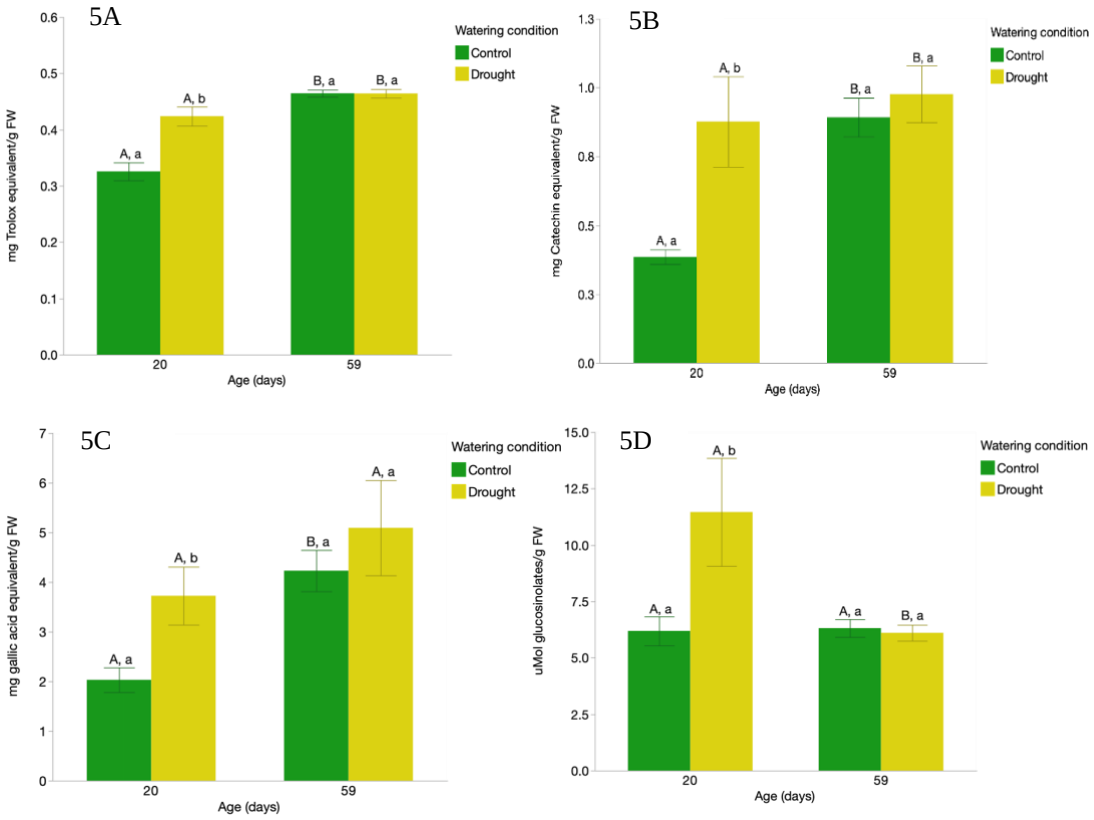


Figure 5. The chemical measurements for (A) antioxidant capacity, (B) total flavonoid, (C) total phenolic, and (D) estimated glucosinolate contents in 20- and 59-day old plant tissues under both watering regimes. Capitalized letters represent significant differences by age under the same watering condition by Student's t- test (p <0.05) for a priori comparisons. Small letters represent significant differences due to treatment within the same age group by Student's t-test (p < 0.05).

Within each growth stage, watering regime affected phytochemical measurements. Increases in antioxidant capacity, total flavonoid and estimated glucosinolate contents were detected in tissues from drought-exposed juvenile kale compared to regularly watered juvenile kale (p<0.05). Total flavonoids, phenolics, glucosinolates and antioxidant capacity all correlated positively with each other (Table 8) in juvenile plants under elicitation by drought. On the

other hand, no further increases in the antioxidant capacity, total flavonoids and phenolics and estimated glucosinolates were detected in tissue extracts from mature plants that were subjected to drought compared to regular watering.

Drought treatment affected plant biomass accumulation ($p<0.05$). Dry weight measurements of juvenile plants subjected to water restriction accumulated 24% less weight compared to controls, while mature plants had an 11% discrepancy.

4.3.5 Plant age negatively affected epiphytic S. Newport association with kale

Regularly watered plants at 7 stages, spanning 20 to 59 days post-germination, were inoculated with *S.* Newport at a level of 4.4 - 4.8 log CFU/plant. *Salmonella* populations declined over a 24 h period. At 24 hpi, the highest *S.* Newport cell count retrieved from plant surfaces was 3.9 log CFU/plant, detected on 20-day old regularly watered kale plants (Figure 6). Within this control group, bacterial cell counts steadily declined with plant age such that counts for 20-day old plants were different from kale at 39-days and onwards ($p<0.05$), with a difference of 1.1 log CFU/plant between day 20 and day 59 (Figure 6). These results indicate that the kale leaf surface becomes less favourable for *S.* Newport association as plants mature.

No consistent age effect on *S.* Newport retrieval was detected in drought-subjected plants. Although counts from 20-day old water stressed plants (2.9 log CFU/plant) were higher than those from 59-day old plants (2.4 log CFU/plant), these differences were not significant. The largest populations of *S.* Newport cells were retrieved from 39-day old (3.2 log CFU/plant) plants compared to 26-, 52- and 59-day old plants ($p<0.05$), with a difference of 0.8, 0.9 and 0.8 log CFU/plant, respectively. The reason for this growth peak at 39 days is not clear. On

the other hand, 59-day-old regularly watered plants yielded *S.* Newport counts that were not significantly different from any other age.

Table 8. Correlation analysis between phytochemical measurements (antioxidant capacity, AOC; total flavonoids, TFLA; total phenolics, TP; estimated glucosinolates, EG) in leaf tissues, and against S. enterica counts retrieved from kale leaf surfaces (EPI) for juvenile (20-day old) and mature (59-day old) plants under both regular (CON) and restricted (DR) watering regimes. NS means not significant at α=0.05.

Pair	Pearson's *r*	*p*	Pearson's *r*	*p*	Pearson's *r*	*p*	Pearson's *r*	*p*
	20D		59D		CON		DR	
AOC vs. TFLA	0.40	<0.001	0.008	NS	0.74	<0.01	0.05	0.034
AOC vs. TP	0.38	<0.001	-0.02	NS	0.63	<0.01	0.12	<0.001
AOC vs. EG	0.32	<0.001	-0.008	NS	0.05	0.019	-0.19	<0.001
TFLA vs. TP	0.32	<0.001	0.02	NS	0.60	<0.01	0.04	0.045
TFLA vs. EG	0.28	<0.001	-0.02	NS	0.05	0.026	-0.03	NS
TP vs. EG	0.26	<0.001	0.02	NS	0.05	0.023	-0.10	<0.001
	20D		59D		CON		DR	
EPI vs. AOC	-0.40	<0.001	-0.02	NS	-0.55	<0.001	-0.18	<0.001
EPI vs. TFLA	-0.32	<0.001	-0.05	0.04	-0.52	<0.001	-0.05	0.015
EPI vs. TP	-0.28	<0.001	-0.06	0.005	-0.45	<0.001	-0.08	<0.001
EPI vs. EG	-0.27	<0.001	0.03	NS	-0.04	0.057	0.167	<0.001

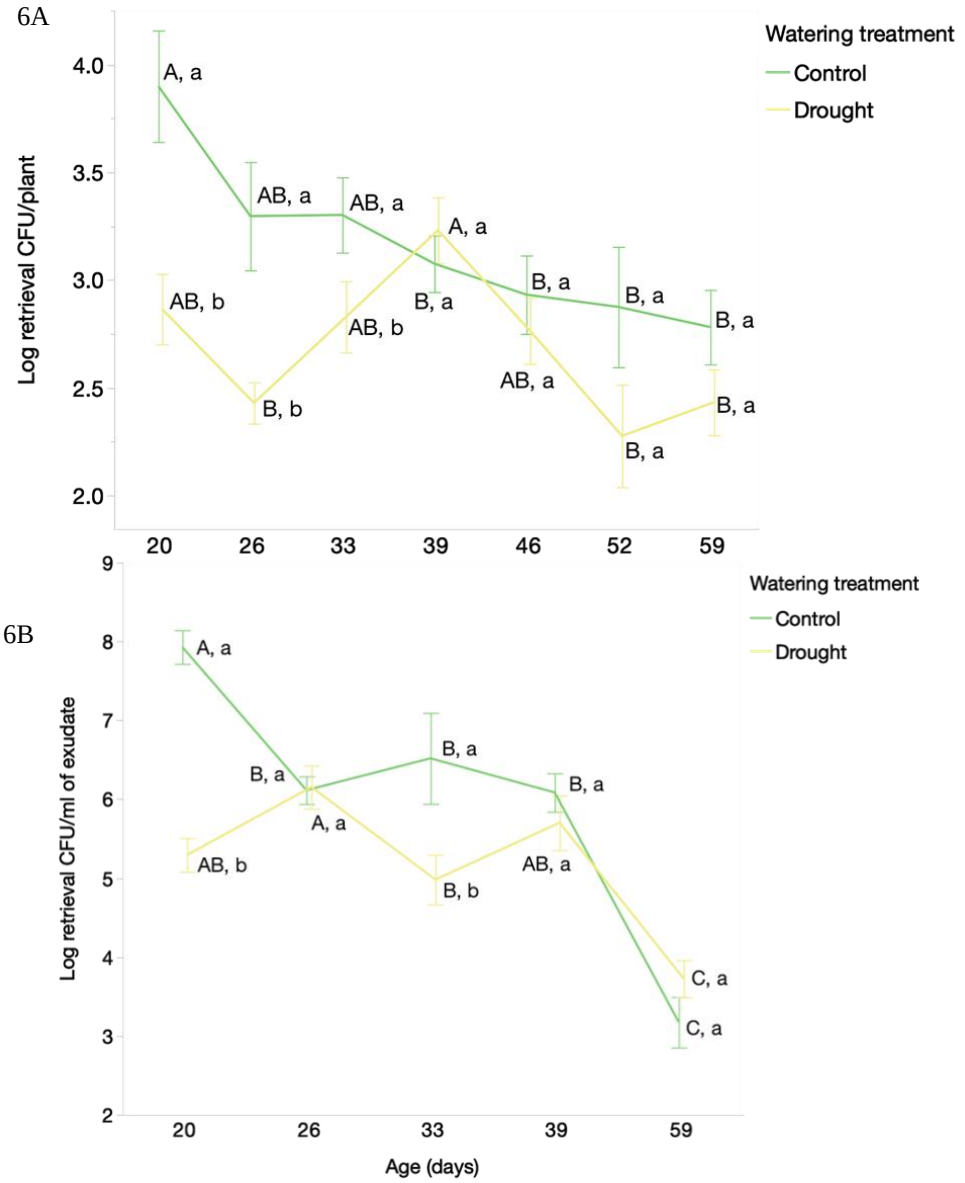


Figure 6. Levels of *Salmonella* Newport on (A) leaf surfaces of regularly watered (green) and drought-subjected (yellow) kale plants and (B) in plant surface washes, at various stages of development between 20 and 59 days old. Capital letters denote differences among age groups under the same watering regime (ANOVA and Tukey HSD; $p<0.05$). Small letters indicated statistical differences for pairwise comparisons between drought-subjected and regularly watered kale plants of the same age ($p<0.05$).

4.3.6 Drought restricted the survival of S. Newport epiphytic on juvenile kale plants

Drought-exposed kale plants supported lower levels of *S.* Newport than regularly watered plants ($p<0.05$; Figure 6). The retrieval of *S.* Newport from control plants ranged from 3.9 to 2.8 log CFU/plant. In contrast, *S.* Newport recovered from water-stressed plants ranged from 3.2 to 2.30 log CFU/plant. The most distinct difference in *S.* Newport counts between the two watering treatment groups was detected on day 20, with a 1.0 log CFU/plant difference ($p< 0.05$) The retrieval of *S.* Newport from regularly watered plants was ~0.7 log CFU/plant lower than the initial inoculum level. By contrast, the retrieval of *S.* Newport on drought-subjected plants decreased ~1.7 log CFU/plant from the inoculum. For 20-, 26- and 33-day regularly watered plants, *S.* Newport counts were higher than for drought-subjected plants ($p<0.05$). However, the difference in *S.* Newport counts due to drought treatment was not sustained over time, indicating that drought did not cause further restriction of *S.* Newport as plants aged. Interestingly, the lower counts on more mature (≥46 days), regularly watered plants approached the lower counts obtained from water-stressed plants of any age. While the retrieval of *S.* Newport from 20-day control plants was higher than both control and drought-subjected 59-day plants, *S.* Newport counts from water-stressed 20-day plants were not significantly different from 59-day kale regardless of watering treatment. The findings suggest that water stress induces changes in the plant that render the leaf surface less favorable for *Salmonella* association at the juvenile stage. No further bacteria-limiting effect could be attributed to drought on mature plants that were inherently less supportive of *Salmonella* association than juvenile plants.

4.3.7 Phytochemical levels in leaf tissue correlated negatively with Salmonella populations on kale leaf surface

We conducted correlation analysis between the phytochemical data and *Salmonella* count data to explore possible effects exerted by phytocompounds on bacterial populations. Strong negative correlations ($p < 0.001$) between epiphytic *Salmonella* counts on kale and all phytochemical measurements were obtained in juvenile, but not mature plants (Table 8), possibly explaining *Salmonella* restriction on juvenile plants subjected to drought. Regularly watered kale plants exhibited the same inverse relationship between *Salmonella* counts and antioxidant capacity, flavonoids and phenolics ($p < 0.001$), and a weaker relationship with glucosinolates ($p = 0.06$), possibly explaining *Salmonella* decline as plants developed. The low *r* values obtained for mature plants (including data from both water treatments) and plants under drought (including juvenile and mature plants) (Table 8) provide further evidence that secondary metabolites elicited by drought stress or induced by age have a limiting effect on *Salmonella* colonization.

4.3.8 Surface washes collected from regularly watered juvenile kale provided a more favorable environment for S. Newport than washes from water-stressed plants

Wash solutions from all age groups except 59-day plants were able to support increases in *S.* Newport counts over 24 h relative to inoculum (Figure 6B). An age-dependent effect was noted with regularly watered kale. The largest difference in *S.* Newport counts between inoculum and wash solution was recovered from 20-day plants (4.3 log CFU/ml). Counts from 20-day surface washes were higher than from 26, 33 and 39-day solutions ($p<0.05$). All were higher than from 59-day solutions ($p<0.05$), which were not supportive of bacterial

growth. By contrast, the growth of *S.* Newport in washes collected from drought-treated plants did not present a marked age-dependent pattern. The difference in *S.* Newport levels between inoculum and solution from 20-day plants was 1.7 log CFU/ml. *S.* Newport levels in solutions from plants 20-39 days of age were similar, though surface washes collected from 26-day drought-subjected plants supported higher *S.* Newport growth than the 33-day plants in the same treatment group ($p<0.05$; Figure 6B). As in the control, 59-day plant washes did not support bacterial growth.

Comparing watering treatments, the retrieval of *S.* Newport was significantly higher from washes collected from control kale than drought-subjected kale at 20 days of age ($p<0.05$). Washes collected from 33-day regularly watered plants also yielded more *S.* Newport than water-stressed plants ($p<0.05$) (Figure 6B). No other differences were noted between watering treatments, especially noteworthy for 59-day samples as both watering treatments showed a marked decline compared to juvenile plants ($p<0.05$). Taken together, these results suggest that surface compounds on juvenile kale leaf surfaces were able to support *S.* Newport growth, which was curbed in more mature plants and when plants were under water stress, regardless of age.

4.3.9 S. Newport counts associating epiphytically correlated positively with growth in leaf surface washes

Epiphytic association of *S.* Newport with kale was age- and drought-dependent. The age-dependent decline in *S.* Newport retrieved from leaves of regularly watered plants between 20 and 59 days was a trend observed also for *S.* Newport inoculated in leaf washes. In drought-subjected plants, *S.* Newport followed the same trend in wash solutions but not on plant

surfaces. Middle-aged, drought-subjected plants also exhibited inconsistent patterns. This prompted us to determine whether a correlation existed between data obtained from these two systems. An overall positive correlation (r=0.35, p<0.001) was in fact detected between *Salmonella* association on plant surface and growth in leaf wash (Table 9). Analyzing watering groups separately showed that a correlation was present in both regularly watered (r=0.38, p<0.01) and, to a lesser degree, drought-subjected (r=0.11, p<0.01) plant groups. Analyzing data by age identified a strong positive correlation in 20-day kale (r=0.53. p<0.001) and in 33-day kale (r= 0.15, p<0.01) but a negative correlation in mature plants (59-day r=-0.152, p<0.01).

Taken together with the inverse correlations obtained between *Salmonella* counts and phytochemical levels in leaf tissue, the data suggest that the age-dependent differential levels of *S.* Newport epiphytic on kale receiving adequate watering may, at least in part, be explained by leaf surface compounds that varied as plants developed. The same may be inferred for juvenile plants responding to drought, exhibiting a diminished favorability to *Salmonella* association.

Table 9. Pearson's correlation coefficient r, between *S. enterica* counts retrieved from kale leaf surfaces (EPI) and leaf surface washes (WA) at five different growth stages under both regular (CON) and restricted (DR) watering regimes. NS means not significant at α=0.05.

Pearson correlation	Correlation coefficient *r*	*p*
Overall EPI vs. WA	0.35	<0.01
CON EPI vs CON WA	0.38	<0.01
DR EPI vs. DR WA	0.11	<0.01
20D EPI vs. 20D WA	0.53	<0.01
26D EPI vs. 26D WA	-0.04	0.06
33D EPI vs. 33D WA	0.15	<0.01
39D EPI VS 39D WA	-0.01	NS
59D EPI vs. 59D WA	-0.15	<0.01

4.4 Discussion

In this study, we investigated the role that plant phytochemicals may play in modulating *Salmonella enterica* Newport association with kale leaf surfaces. *Salmonella* association was most successful with juvenile plants and diminished as plants matured. This finding has implications for food safety, since juvenile plants constitute a product known as 'baby kale', which is frequently consumed raw. The enterobacterial interaction was also impaired on kale suffering water stress. The bacterial dynamics observed were detected in *Salmonella* populations associating epiphytically, as well as in populations inoculated into leaf surface washes containing surface-deposited phytocompounds. Phytocompound measurements were inversely correlated with bacterial levels, supporting the hypothesis that metabolome shifts in response to abiotic stress can modulate plant-bacterial interactions.

We show that both plant development and water stress can induce metabolomic changes, contributing to the complex network of influences that govern the *Salmonella*-kale interaction. Kale phytochemical profiles changed with plant ontogeny under regular watering. Moreover, at the juvenile stage, but not the mature stage, phytochemical profiles shifted markedly in response to drought treatment. Both the leaf tissue and surface metabolomes were altered in this manner. The metabolomic changes were a result of differential up- or down-accumulation of certain compounds, as well as the detection of compounds unique to fully watered or water deficit situations. Phytochemical analysis of leaf tissue from regularly watered plants revealed that increases in total flavonoid contents, but not glucosinolates, partially explained the age-dependent metabolomic shifts, that also resulted in higher antioxidant capacity. On the other hand, higher levels of total flavonoid content and glucosinolates contributed to drought-driven metabolome changes in juvenile, but not mature kale. Kale surface phytochemicals collected in surface washes were able to support the growth of *Salmonella*, although differences were observed based on watering regime. A strong correlation between *Salmonella* population levels associating epiphytically and those growing in surface washes was identified in juvenile plants. The bacterial counts also correlated negatively with flavonoids and phenolics. These findings suggest that the status of the kale leaf tissue and leaf surface metabolomes influence epiphytic *Salmonella* colonization of this crop, especially at the baby kale stage, providing proof of concept that metabolome modulation could lead to innovative solutions for controlling *Salmonella* on vegetable crops and identifying a possible role for secondary metabolites in controlling *Salmonella* on baby kale.

Baby kale, represented by 20- and 26-day old plants in our study, are increasingly popular as a functional raw food. Regularly watered juvenile kale was the most vulnerable to *Salmonella* establishment. Juvenile kale might release more *Salmonella*-supporting chemicals to the leaf surface than mature plants, thus creating a more favorable environment for *Salmonella*. The type of carbon source however could also modulate bacterial growth. While fructose was reported to increase with curly kale development from 28-56 days, glucose (the most abundant sugar measured in that study) and sucrose were highest in young kale (28 days) and lowest in 56-day old kale (Megías-Pérez et al., 2020). In curly kale, sugar levels increased with delayed harvest in mature field grown kale, an effect driven by sucrose but not glucose and fructose (Hagen et al., 2009). *Salmonella* can readily utilize glucose as a carbon source, while sucrose is not preferred and can lead to stasis (Gutnick et al., 1969; Nur et al., 2016). This might explain *Salmonella* preference for juvenile plants. Modulation of sugar levels is complex, however, and environmental factors also play a role in sugar regulation. Decreasing air temperatures have been suggested as a strategy to improve taste for kale grown in controlled environments (Steindal et al., 2015). Low molecular weight carbohydrates such as the sugar alcohol sorbitol and raffinose/fructooligosaccharides increase in certain kale cultivars during water deficits (Pathirana et al., 2017). We did not assess low molecular weight carbohydrates, however, if they increased with drought stress in our system, they had no effect on *Salmonella*. This could be due to the accumulation of non-metabolizable carbon sources, the presence of inhibitory compounds on the leaf, or possibly attributable to leaf thickening. Thickening and whitening of cuticle were reported for 34-day old kale plants under drought for 14 days (Issarakraisila et al., 2007), which may create a barrier for carbon sources to reach the leaf surface and support bacterial growth. A spike in

Salmonella levels was detected in middle-aged plants under water stress. This could be the result of bimodal accumulations of certain compounds. For instance, the sugar alcohol *myo*-inositol, a metabolizable carbon source for *Salmonella* (Kröger and Fuchs, 2009) exhibited two peaks in 28- and 56-day plants (Megías-Pérez et al., 2020). Finally, although data on amino acid level changes with kale development are not readily available, evidence of an essential role for amino acids in the *Salmonella*-plant interaction has been identified (de Moraes et al., 2017; Han et al., 2020; Kwan et al., 2015) and fluctuating availability of amino acids may also play a role in *Salmonella* colonization of kale.

Concurrent to the availability of carbon and nitrogen sources, the levels of specialized compounds potentially having restrictive bacterial effects may be lower in the juvenile stage. Phytochemical profiling of regularly watered kale in our study revealed plant ontogenic shifts in low molecular weight and bioactive compounds present in leaf tissue. Total phenolics, total flavonoids and glucosinolates increased per plant in two kale varieties from the Acephala group measured weekly 14-49 days after transplanting 2-week old seedlings, with antioxidant capacity following the same trend (Yoon et al., 2019). Korus reported a similar maturity-dependent rise in phenolics and antioxidant capacity in three kale cultivars and additionally, an overall increase in vitamin C, although the latter varied by cultivar (Korus, 2011). Kale seedlings had lower antioxidant capacity and flavonoids at seven days than 60 day-old plants (Soengas et al., 2012). More data exist on this age effect in other Brassicas. During the vegetative growth stage, 30 day-old *Brassica oleracea* var. *italica* plants showed higher levels of antioxidant capacity and accumulation of total phenolics compared to 8 day-old plants (Bachiega et al., 2016). Analysis of Chinese cabbage (*Brassica rapa*) plants also revealed higher levels of secondary metabolites with plant development, whereby two-leaved

plants had lower levels of total phenolics, hydroxycinnamic acids and flavonols, and *L*-ascorbic acid than six-leaved plants (Šola et al., 2020). In our study, phytochemical analysis in regularly watered kale indicated, however, that total flavonoid content, but not total phenolic and estimated glucosinolates, increased significantly with plant development and therefore at least in part, flavonoids could have driven the age-related divergence in phytochemical profiles of leaf tissue. Moreover, to our knowledge, this is the first study examining the effects of age-related phytochemical shifts in Brassicas on bacterial associations.

Variation in phytochemical levels brought about by drought stress overrode conditions specific to the stage in plant ontogeny with regards to *Salmonella* association. Drought is a known elicitor of secondary metabolites in Brassicas. Plants under drought stress accumulate osmoprotectants and antioxidant compounds to counteract the induced oxidative stress (Nakabayashi et al., 2014b). Total phenolic acid increased in response to drought in young Chinese cabbage plants (Šola et al., 2021) and broccoli plants showed significantly higher phenolic concentrations in response to drought (Barickman et al., 2020). Timed drought stress has been explored as a strategy to enhance low molecular weight carbohydrates and bioactive compounds in lettuce and kale including phenolics, tocopherols and flavonoids before harvest (Kusano et al., 2011; Nakabayashi et al., 2014a; Oh et al., 2009; Pathirana et al., 2017; Podda et al., 2019). In this study, phytochemical profiles of fully watered and water stressed juvenile kale were among the most divergent comparisons, indicating a strong response to drought at this developmental stage. The enhanced induction of secondary metabolites in younger plants in response to drought may be related to effects on growth since activating the phytochemical pathways needed comes at a cost to the plant. Chinese cabbage that was

subjected to drought starting at 40 days of age had fewer leaves and reduced leaf area and biomass compared to controls (Shawon et al., 2020). In this latter study, phenolic acids and glucosinolates were induced by drought but flavonoids were not. After a seven-day drought treatment, 21 day-old broccoli plants had a lower dry weight than control, while kaempferol accumulation was detected after two weeks of water stress (Khan et al., 2011). We observed a more pronounced effect of biomass reduction in juvenile than mature plants. Secondary metabolites profiles are associated with plant biomass accumulation and chlorogenic acid was negatively associated with biomass accumulation (Turner et al., 2016). Increased accumulation of chlorogenic acid, total phenolics and flavonoids were induced by drought in plants (Gharibi et al., 2019). Adverse effects of drought on biomass accumulation have been reported in Brassicas (Shawon et al., 2020). We therefore hypothesize that the higher levels of secondary metabolites synthesized in juvenile plants in response to drought could have come at the expense of plant growth. The longer period of drought needed to reach the pre-wilting stage would also have had an effect. Despite this biomass reduction, the treatment had a desirable outcome from a food safety perspective. The dissimilarities in phytochemical profiles between fully watered and water stressed juvenile kale were observed in both the leaf tissue and surface metabolomes. We attribute some of this variation to surges in the total amounts of flavonoids and estimated glucosinolates in leaves. As a result of these accumulations, the total antioxidant capacity of drought-subjected juvenile kale was higher compared to regularly watered juvenile kale. Excluding the mature kale plant group, positive relationships between antioxidant capacity and total phenolics and flavonoids in kale tissues were observed. Strong positive correlations between antioxidant capacity and total flavonoids

have been reported in young Chinese cabbage (Šola et al., 2021) and phenolics in 30 plant extracts (Dudonné et al., 2009).

Taken together, these findings suggest that regulated water stress early in development could also be an effective strategy to enhance the nutritional quality of baby kale products. Importantly, we saw a concomitant reduction in the favorability of young water-stressed kale (20-33 days) to *Salmonella*. The mechanism for *Salmonella* restriction is not known. Drought stress results in a surge of reactive oxygen and nitrogen species (ROS/RNS) (Laxa et al., 2019) which has been shown to restrict *Salmonella* on tomato surfaces (Ferelli et al., 2020). The plant's stressed state could itself be playing a role. However, this would not be the case for fully watered mature plants, which were also less favorable for *Salmonella* colonization. Alternatively, therefore, certain phenolics and flavonoids might have a direct antibacterial effect. Nevertheless, the favorability of baby kale to *Salmonella* association is a clear food safety risk. The strong induction of antioxidant compounds in response to drought in baby kale, and the ensuing restrictive effect on *Salmonella*, support secondary metabolite elicitation as a strategy not only for health beneficial purposes but also the added benefit of enhancing food safety of freshly consumed baby kale. Future studies should optimize regulated abiotic stress to minimize the negative impact on plant growth while achieving enteric pathogen control.

We detected no further food safety risk reduction between juvenile plants under water stress (with induced levels of flavonoids and attenuated favorability for *Salmonella* association) and mature plants with augmented levels of these traits even under regular watering. Despite these consistent microbiological outcomes, phytochemical measurements between early and

late ontogenic stages of drought-subjected plants indicated slightly higher antioxidant levels but a sharp reduction in glucosinolates. Phytochemical profiling supported these variations in tissue and surface metabolomes. Although we may partly attribute this divergence in phytochemical profiles to the drop in glucosinolates in plants at a fully grown, harvestable stage, glucosinolate accumulations with development (Yoon et al., 2019) and in response to drought (Podda et al., 2019) have been reported. In fully developed plants that were inherently less favorable for *Salmonella* association, drought treatment did not further enhance *Salmonella* restriction. Congruently, no differences in antioxidant capacity, total phenolic and flavonoid content and estimated glucosinolate levels were detected in mature plants following water stress and only weak negative associations between *Salmonella* and phytocompounds were observed. Moreover, pairwise comparisons of phytochemical profiles from mature kale under different watering regimes were convergent, volcano plots did not reveal any differential compound accumulation, and the association between *Salmonella* growing epiphytically and in leaf wash dissipated, suggesting that drought stress alone did not substantially shift leaf tissue and surface metabolomes in mature plants. Higher levels of specialized compounds, especially flavonoids, in fully watered plants at later stages in plant development might have been sufficient to overcome the abiotic stress brought on by drought treatment and to limit *Salmonella* populations.

In this study we evaluated both the metabolome from leaf tissue extracts and from leaf washes. Although both these components responded similarly to biotic and abiotic factors, they were revealed to be very different in composition. The study of plant surface compounds, how they relate to the plant metabolome, and the role they play in epiphytic bacterial interactions is still scarce. Moreover, the metabolite landscape on the plant surface is

not only governed by the plant's physiological response to development and abiotic factors, but also influenced by microbial interactions. Plant surface compounds are the substrate for epiphytic bacterial colonization. Alterations to carbohydrates on the plant surface reflected microbial activity and metabolism of various constituents present, while signs of changes in phytoalexin biosynthesis suggested a plant response to bacterial epiphytes (Ryffel et al., 2016). In this study, surface washes collected from juvenile kale under regular watering, a stage when plants are actively recruiting constituents to their microbiome, were the most supportive of *Salmonella* growth. On the other hand, regardless of watering regime, leaf wash solutions from mature plants were significantly less favorable, possibly due to insufficient levels of metabolizable substances or the presence of inhibitory compounds. These observations align with negative correlations between bacterial counts from the leaf surface and levels of phenolics and flavonoids. It remains to be determined what phytocompounds are shared between the tissue metabolome and the surface metabolome under any given condition, however the comparable *Salmonella* population outcomes on the plant surface and in surface washes do allude to analogous alterations in composition.

In summary, shifts in plant surface wash phytochemical profiles suggest that a series of phytocompounds in the surface metabolome, the suite of compounds that epiphytic bacteria directly interact with on the plant, changed between 20-day old juvenile plants and 59-day old mature plants. Simultaneously, *Salmonella* counts in wash solutions from regularly watered kale markedly declined between day 20 and day 59, suggesting that a similar shift in the balance of metabolizable carbon sources and restrictive phytocompounds occurs on the leaf surface as internal to the leaf. This was further supported by a strong negative correlation between antioxidant compounds in leaves and epiphytic *Salmonella* counts, and a positive

correlation between epiphytic *Salmonella* quantification data and *Salmonella* growing in leaf surface solutions. In conclusion, we hypothesize that lower flavonoid levels in juvenile kale, and potentially on the surface of kale leaves, facilitate *Salmonella* persistence on baby kale surfaces. Conversely, accumulating levels of specialized compounds with plant ontogeny may hamper *Salmonella* persistence as plants mature. We infer that flavonoids may play an active role in this dynamic. Future work will focus on identifying specific compounds that play a role in hampering *Salmonella* growth, even in the presence of bioavailable nutrients.

4.5 Conclusion

This study provides novel data on kale favorability to *Salmonella* at different harvestable stages and presents an innovative avenue to study complex enteropathogen-plant associations in other systems, as a factor of abiotic stresses. Since a measurable component of these associations are epiphytic, the nutrient connection is vital and this study indicates that plant development and drought impact the metabolomic profiles of kale, and in turn modulate *Salmonella* colonization. Despite the intensifying cultivation and consumption of kale over the past decade, the kale-human pathogen association has received next to no attention (Allard et al., 2019; Shah et al., 2019). We demonstrate that regularly watered juvenile kale, a commonly used baby leaf salad ingredient, provides a more favorable niche for *Salmonella* compared to mature kale, sustaining higher populations. By contrast, mild drought exposure impaired the *Salmonella* association in young kale to the same degree as mature kale plants. As plants matured, drought treatment did not further intensify the constraint on *Salmonella* surface survival. As the frequency of extreme weather events increases, and so the likelihood of periods of drought occurring during a crop cycle, studying the impact of drought on plant

physiology and its effect on plant microbiota, including *Salmonella*, will give growers more guidance for crop management. Simultaneously, since crop contamination can happen at any time along the production and post-harvest processing line, it is beneficial to understand the dynamics of *Salmonella* interaction for various kale and other leafy green products. Decoding plant metabolic responses to development and stress, and determining which compounds serve as *Salmonella* growth-promoting and limiting factors, can serve as the basis for viable strategies to minimize food safety risks. Regulated cold and drought acclimatization have already been suggested as strategies to improve crop nutritional quality. Such interventions could be optimized to have the added benefit of enhancing food safety, especially at the most vulnerable plant growth stages.

Chapter 5: Beneficial *Pseudomonas* spp. protected kale from salinity stress and influenced association with *Salmonella enterica*

5.1 Introduction

Food safety is not only a public health concern, but also an economic burden. An upward trend in produce associated foodborne outbreaks in response to increased produce consumption and climate fluctuation was concerning (Callejón et al., 2015b; Lynch et al., 2009; Olaimat and Holley, 2012). *Salmonella enterica*, the leading agent of foodborne outbreaks in the USA, could be introduced to produce at any stage of the produce production line, survive since then and potentially cause human infections (Bennett et al., 2015; Harris et al., 2003; Olaimat and Holley, 2012). Once established on plant surfaces, it is rather tough to reduce and eliminate human pathogens (Goodburn and Wallace, 2013; Parish et al., 2003). Among all stages during produce production, pre-harvest stage has been reported to be highly associated with foodborne pathogens and outbreaks (Greene et al., 2008; Truitt et al., 2018). To enhance food safety and reduce its collateral economic losses, Good Agricultural Practices (GAPs) and Produce Safety Rule (PSR) under the Food Safety Modernization Act (FSMA) have been established to offer guidance and recommendations for growers to prevent foodborne outbreaks. However, contamination during production lasts (Angelo et al., 2015; CDC, 2008) and effective on-farm interventions are required to ensure produce safety.

Salinity stress not only can deteriorate irrigation land and reduced plant yield for decades, but also impose economic pressures with more than $27 billion annual loss globally (Qadir et al., 2014). Sea level rises caused by climate fluctuation has severely worsened sea water intrusion into freshwater system, which, in turn, altered plant physiology and impaired agricultural production, especially in coastal region (Rebecca Epanchin-Niell and Miller, 2018; Tully et al., 2019; White and Kaplan, 2017). Along with sea water intrusion, biogeochemical changes in soil such as increases in sodium and other cations, ionic strength and alkalinization impose plants under salinity stress (Kaushal et al., 2018; Weston et al., 2010). Stomatal closure is the immediate and short-term strategy for plants to cope with salinity (Hasegawa et al., 2000). Plants regulate ion movement and accumulation of organic osmolytes such as proline and sugars in the long term to adapt to salinity stress (Hasegawa et al., 2000; Lee et al., 2008; Sanada et al., 1995). Higher levels of antioxidant capacity and accumulation of antioxidants including phenolics and flavonoids were noted for salinity-stressed plants (Bistgani et al., 2019; Pérez-López et al., 2013). Nevertheless, interventions are required to develop potential solutions to ensure crop yield under salinity stress.

Plant growth-promoting rhizobacteria (PGPR) are bacteria colonizing on plant surface and mediating promotion of plant growth. PGPR can influence plant phytohormones synthesis and improve nutrients uptake to encourage plant growth in a direct manner (Glick, 1995; Khosravi et al., 2018). *Pseudomonas* spp. is one of the most widely applied rhizobacteria in the field to promote plant development and health and has been shown to promote plant growth directly and indirectly. Root inoculation of *Pseudomonas* was associated with a higher ratio of Na+/K+ and relative water content of plants to alleviate

the influence of salinity (Nadeem et al., 2006). The application of PGPR protects plant from biotic invasion by priming plants through induced systemic resistance (ISR) indirectly (Loon and Glick, 2004; Meziane et al., 2005). Evident results have shown that the application of *Pseudomonas* protects plants from pathogens and fungus via ISR (Duijff et al., 1997; Van Wees et al., 1997). Root inoculation of *Pseudomonas* restricted the epiphytic growth of *Salmonella* on produce (Hsu and Micallef, 2017). However, the mechanism of PGPR inoculation on enteric pathogen associated with produce requires more attention.

This study aims to determine the beneficial effects of PGPR on plant growth and enteric pathogen reduction on produce surface under salinity stress. *Pseudomonas* spp. S4 recruited in this study has proven effects on soybean nodulation enhancement (Dashti et al., 2000; Zhang et al., 1996), growth-promoting effects on spinach, Romaine lettuce and tomato plants and restriction of *Salmonella* on spinach and tomato plants (Hsu and Micallef, 2017). However, plant cultivar and abiotic stresses could impact the effectiveness of *Pseudomonas* (Hsu and Micallef, 2017; Tabassum et al., 2017). Hence, we investigated the growth-promoting effects of *Pseudomonas* spp. S4 on kale and its limiting effects on two serovars of *Salmonella* on kale surface under salinity stress.

5.2 Material and Methods

5.2.1 PGPR preparation and inoculation

Pseudomonas spp. S4 (PS4) were obtained from Dr. Brian Klubek (Southern Illinois University Carbondale) with rifampicin resistance. Frozen stock of PS4 was maintained at -

80℃ in Brucella broth (BD, MD) containing 15% glycerol (VWR Chemicals, BDH) and 50 $\mu g \cdot ml^{-1}$ rifampicin and revived by streaking onto Trypticase Soy Agar (TSA, BD) plates amended with 50 $\mu g \cdot ml^{-1}$ rifampicin (TSA-rif) at 30℃ for 48 hours. Single colony of PS4 was then streaked onto fresh TSA-rif plates and grown for 48h at 30℃. PS4 inocula were made by suspending fresh bacterial colonies in 0.1% peptone water (PW) to an OD_{600}=0.5 to obtain a cell density of ~10^9 CFU per ml. Each PS4-treated kale plant received 2 ml of PS4 inocula suspension and non-PGPR inoculated kale plant received 2 ml of 0.1% PW as negative control. All inocula were applied directly to the base of stems near soil. Two separate root inoculations were conducted when kale plants were 2- and 9-days old (post-germination).

5.2.2 Plant preparation and salinity treatment

Brassica oleracea L. var. *acephala* cv. 'Improved Dwarf Siberian' was used in this study. Kale seeds (Territorial, OR, USA) were germinated in potting mix soil (Sunshine LC1; Sungro Horticulture, Canada) at the Research Greenhouse Complex, University of Maryland, and grown under controlled light and temperature conditions (16 h light:8 h dark photoperiod at 23℃/18℃ day/night temperatures). Kale plants were kept regularly watered after being inoculated with PS4 or 0.1% PW at 2- and 9- days old until they were 21 days old. Kale plant then were subjected to 15 $g \cdot L^{-1}$ NaCl (high salt, HS), 5 $g \cdot L^{-1}$ NaCl (medium salt, MS) or 0 $g \cdot L^{-1}$ NaCl (no salt or regular water, NS) for continuous 5 days. At the end of treatment, kale plants were 26 days old for future analyses.

5.2.3 Biomass measurement of kale plants

PS4-inoculated and non-PGPR inoculated kale plants were harvested after salinity treatment described above. All above-soil biomass of kale was harvested from the base of each plant

and put in a pre-weighted foil tray and baked in an oven at 70℃ for 48 hours for shoot dry weight (SDW) measurements.

5.2.4 Salmonella enterica inoculum preparation

Salmonella Javiana and Newport were recruited in this study. The *S. enterica* Javiana strain is a clinical isolated from a tomato-associated foodborne illness outbreak (Callahan and Micallef, 2019). *S.* Newport strain used is a tomato outbreak strain (Greene et al., 2008). Both Salmonella serovars were adapted for rifampicin resistance. Single colony of each *Salmonella* serovar grown on TSA with 50 $\mu g \cdot mL^{-1}$ rifampin was streaked on fresh TSA-rif plates and grown at 35°C overnight. *Salmonella* inocula were made by suspending bacterial colonies in 0.1% PW to an OD600=0.5, to obtain a cell density of ~10^9 CFU per mL. A 100-fold dilution of each *Salmonella* suspension or 0.1%PW (non-*Salmonella* inoculated control) was used for leaf surface inoculation.

5.2.5 Kale leaf surface inoculation with Salmonella enterica

An aliquot of 100 µl of *Salmonella* Newport or Javiana inoculum was pipetted on the adaxial side of the third or fourth true leaf of kale plants distributed in ~10 spots. An estimated total inoculum dose of 10^6 CFU was inoculated per leaf. Inoculated kale leaves were harvested 24 hours post-inoculation (hpi). Each leaf was aseptically clipped off the stem with scissors and placed in a sterile Whirl-pak bag (Nasco, WI, USA). Inoculated leaves were then immersed in 30 ml 0.1% PW, hand-massaged for 30 s and sonicated in a Branson Ultrasonic Cleaner (Branson Ultrasonics Corporation, CT, USA) for 1 min at maximum speed to dislodge *Salmonella* cells from the kale leaf surfaces. Each leaf was then shaken at 150 rpm for 10

min. Serial dilutions from each leaf rinsate were plated on TSA-rif plates for *Salmonella* enumeration after 24 h of incubation at 35°C.

5.2.6 Phytochemical analyses with kale leaf tissues

After harvesting the inoculated leaf for *Salmonella* survival test, the rest of kale leaves were flash-frozen using liquid nitrogen and ground into powders using a pestle and mortar. Two hundred mg of plant powder was accurately weighed and extracted with 1.5 ml 70% methanol (Sigma-Aldrich, MO, USA) with 0.5% formic acid (VWR, PA, USA). Each extract was fully vortexed before it was centrifuged at 4,226 *g* for 10 min. The supernatants were frozen at -20°C for phytochemical analyses through photo spectrometer. All absorption readings in the following assays were acquired using a microplate reader (Synergy HTX; Biotek, VT, USA). Each phytochemical analysis was repeated at least 2 times in separate experiments with at least 4 biological replicates in each treatment. Data were pooled.

Antioxidant capacity: To prepare the reagent, 31.7 mg 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (TCI, OR, USA) and 8.6 mg potassium persulfate (Aldon Corp Avon, NY, USA) were dissolved in 10 ml water and allowed to stand in darkness at room temperature for 16 h to form a stable radical, then diluted to an absorption of ~1.3 at 734 nm. Twenty μL sample extracts were added to 200 μL ABTS solution and allowed to stand for 15 min, then absorption was measured at 734 nm. A standard curve from the blank-corrected absorption at 734 nm of Trolox (TCI) standards was plotted. Antioxidant capacity was calculated as Trolox equivalent using the standard curve.

Total flavonoids: One hundred μL kale leaf extract were added to 20 μL 5% $NaNO_2$ (Avantor, PA, USA). After 5 min, 20 μL 10% $AlCl_3$ (Alfa Aesar, MA, USA) were added,

followed by 100 μL 1 mol·L^{-1} NaOH (VWR Chemicals, BDH, Radnor, PA, USA) 1 min later. Absorption of the mixture was measured at 510 nm on the microplate reader. A standard curve from the blank-corrected absorption at 510 nm of the catechin standards (Enzo Life Sciences, NY, USA) was plotted. Antioxidant capacity was calculated as catechin equivalent using the standard curve.

Total phenolic content: Eighty μL kale leaf extract were mixed with 90 μL 10% Folin Ciocalteu reagent (MP Biomedicals, CA, USA) and 40 μL 700 mM Na_2CO_3 (VWR Chemicals, BDH) were added in a 96-well plate. All samples were fully vortexed and let to stand for 1 h in a microplate reader at 25℃. Absorption measurements were taken at 765 nm. A standard curve from the blank-corrected absorption values at 765 nm of the gallic acid (Acros Organics Fair Lawn, NJ, USA) standards was plotted. Total phenolics were calculated as gallic acid equivalents using the standard curve.

Estimated glucosinolate content: Kale leaf extract (80 μL) was added to 200 μL 2 mM sodium tetrachloropalladate (Acros Organics). The samples were incubated at room temperature for 1 h and absorption was measured at 425 nm. Water was then used as a blank. Total glucosinolate content was calculated by putting the OD_{425} of each sample into the predicted formula as follows (Mawlong et al., 2017)

$$Y = 1.40 + 118.86 * A_{425}$$

5.2.7 Statistical analysis

For biomass measurement were repeated at least twice with each treatment tested in at least 3 replicates. Pooled data were used for analysis. Bacterial growth experiments for *Salmonella*

Newport and Javiana were repeated twice with 5 replicates included in each PGPR and salinity treatment. Bacteria reduction data were log transformed and pooled for data analysis. Student's *t*-test for *a priori* pairwise comparisons was employed to determine PGPR effects. ANOVA (Tukey HSD) test was included to illustrate salinity effects. Analyses were conducted in JMP Pro 14.0.

5.3 Results

5.3.1 Kale Biomass was affected by salinity level but not the inoculation of Pseudomonas spp. S4

The biomass for each kale plant was measured by drying plant tissue above ground in the oven until no weight loss was measured. For PGPR-negative group, no biomass difference was observed due to the salt treatment. By contrast, salt treatment affected the biomass for PS4-inoculated group. PS4-inoculated plants under medium salt showed higher biomass accumulation than under high salt treatment ($p < 0.05$) and showed no difference with plants from no salt treatment. Biomass accumulation was not affected by PGPR inoculation under the same salt treatment (Table 10).

Table 10. Dry biomass accumulation of 26-day kale plants under high salt (HS), medium salt (MS) and no salt (NS) treated with root inoculation of *Pseudomonas* spp. S4 (PS4) or 0.1% peptone water (PW). Statistically significant differences in dry biomass are denoted by letters. Capital letters denote differences by watering treatment within the same PGPR root inoculation (ANOVA, Tukey HSD test, $p < 0.05$). Small letters indicate differences by root inoculation within same age and watering condition (Student's *t*-test, $p < 0.05$).

Salinity treatment	root inoculation	Dry biomass g/plant
HS	PW	2.9 ± 0.3 A, a
	PS4	2.7 ± 0.2 B, a
MS	PW	3.1 ± 0.3 A, a
	PS4	3.4 ± 0.1 A, a
NS	PW	3.1 ± 0.3 A, a
	PS4	3.2 ± 0.2 AB, a

5.3.2 Decline of Salmonella Newport on kale surface was greater than Salmonella Javiana

After 24 hours, the log change per plant of *Salmonella* Newport and Javiana on kale surface was calculated from subtracting log retrieval per plant from log inoculum per plant. For 0.1% PW inoculated kale group, the decline of both *Salmonella* Newport and Javiana were noted under all salinity treatments. Moreover, a huge reduction of *Salmonella* Newport was reported compared to *Salmonella* Javiana under both medium and high salt conditions with at least a 0.6 log CFU/plant difference detected ($p < 0.05$). No difference was detected between two *Salmonella* serovars under no salt condition for PGPR-negative kale group. Compared to *Salmonella* Javiana, the *Salmonella* Newport population was greatly reduced on surface of kale plants under all salinity treatments, with the most noticeable 1 log CFU/plant difference detected under the medium salinity condition ($p < 0.05$) (Table 11).

5.3.3 Surface survival of Salmonella was affected by salinity treatment and root inoculation of Pseudomonas spp. S4

The change and retrieval of *Salmonella* Javiana was not affected by salinity treatment for non-PGPR inoculate kale plants. However, kale plants with no root inoculation of PS4 showed varied preference for *Salmonella* Newport. *Salmonella* Newport reduced at a level of 1.4 log CFU/plant on the surface of non-PGPR inoculated kale under medium salinity, significantly higher than on surface of kale plants under no salt condition ($p < 0.05$). Inoculation of PS4 showed no impact on the epiphytic change in *Salmonella* Javiana growth under salinity treatment (Table 11). Interestingly, with the root inoculation of PS4, the change of *Salmonella* Newport population was no longer affected by salinity treatment, suggesting that root inoculation of PS4 might alter the surface environment of leaf surface and then affect the *Salmonella* – kale surface association.

Change of *Salmonella* Javiana population 24 hours post-inoculation was not influenced by root inoculation of PGPR under the same salinity treatment, even though a slightly higher reduction was detected on PGPR-inoculated kale surface. Epiphytic growth of *Salmonella* Newport was not affected by PS4 inoculation when kale plants are treated with salt solution. However, under no salt condition, kale plants inoculated with PS4 showed a larger *Salmonella* Newport population decline at a level of 1.4 log CFU/plant than non-PGPR inoculated kale ($p < 0.05$; Table 11).

Table 11. Change and retrieval of *Salmonella* population on surface of 26-day kale plants under high salt (HS), medium salt (MS) and no salt (NS) treated with root inoculation of *Pseudomonas* spp. S4 (PS4) or 0.1% peptone water (PW). Statistically significant differences in Salmonella population are denoted by letters. Capital letters denote differences by watering treatment within the same PGPR root inoculation (ANOVA, Tukey HSD test, $p < 0.05$). Small letters indicate differences by root inoculation within same age and watering condition (Student's t-test, $p < 0.05$). Values are means of replicates ± standard error (SE).

	Salmonella Javiana (log CFU/plant)			*Salmonella* Newport (log CFU/plant)		
Root inoculation	Inoculum	Change↓	Retrieval	Inoculum	Change↓	Retrieval
High salinity (HS)						
PW	4.4 – 4.5	0.3 ± 0.2 A, a	4.1 ± 0.2 A, a	4.4 – 4.8	0.9 ± 0.2* AB, a	3.7 ± 0.2 AB, a
PS4		0.5 ± 0.2 A, a	3.9 ± 0.2 A, a		1.1 ± 0.2* A, a	3.5 ± 0.2 A, a
Medium salinity (MS)						
PW	4.4 – 4.5	0.4 ± 0.2 A, a	4.1 ± 0.2 A, a	4.4 – 4.8	1.4 ± 0.2* A, a	3.2 ± 0.2* B, a
PS4		0.5 ± 0.2 A, a	4.0 ± 0.2 A, a		1.5 ± 0.3* A, a	3.1 ± 0.2* A, a
No salinity (regular watering, NS)						
PW	4.4 – 4.5	0.4 ± 0.3 A, a	4.1 ± 0.2 A, a	4.4 – 4.8	0.6 ± 0.2 B, b	4.0 ± 0.1 A, a
PS4		0.8 ± 0.2 A, a	3.7 ± 0.2 A, a		1.4 ± 0.2* A, a	3.2 ± 0.2 A, b

5.3.4 Phytochemical measurements of kale plants were affected by salinity treatment and Pseudomonas spp. S4 inoculation

Proline, antioxidant capacity, total flavonoids and phenolics and estimated glucosinolates levels were measured using kale leaf tissues left from bacterial growth experiments (Figure 7A- 7E). Proline levels of kale plants under high salinity were significantly higher than under medium or no salt condition for both non-PGPR and PS4 inoculated kale plants ($p < 0.05$). PGPR inoculation did not change proline levels except for medium salt-treated kale plants, where PS4-inocualted kale plants accumulated more proline ($p < 0.05$). Antioxidant capacity was lower on kale plants under no salt condition than non-PGPR inoculated kale plants treated with salt solution ($p < 0.05$). No difference in antioxidant capacity was detected under various salinity treatments when inoculated with PS4. Root inoculation of PS4 lowered the antioxidant capacity of kale plants under medium salt level ($p < 0.05$), but not at the other two salinity treatments. Total flavonoids were not affected by salinity treatment for both 0.1% PW- and PS4-inoculated kale group. Under medium salt condition, PS4-inoculated plants showed a lower accumulation of flavonoids ($p < 0.05$). No differences in flavonoids levels in kale tissues due to PGPR inoculation were detected. Non-PGPR inoculated kale groups showed no change in phenolics levels. However, kale plants under high salt increased the accumulation of phenolics than under no salt condition with the root inoculation of PS4 ($p < 0.05$). Root inoculation of PS4 had no impact on total phenolics levels in kale tissues under the same salinity condition. Salinity treatment played no role in glucosinolate levels in kale tissues with 0.1 % PW inoculation. Kale plants inoculated with PS4 showed significantly lower levels of glucosinolates under no salt condition than under other two salt conditions (*p*

< 0.05). Under high salt treatment, the inoculation of PS4 increased the accumulation of glucosinolates than non-PGPR inoculated group ($p < 0.05$).

5.4 Discussion

Salinity stress reduced the crop yield and has affected plant physiologically in every way including their association with enterica pathogens. This study investigated the impact of three salinity treatment on plant biomass, phytochemical shifts, and epiphytic growth of *Salmonella* with root inoculation of PGPR *Pseudomonas* spp. High salt treatment reduced the biomass of PS4-inoculated plants compared to medium salt treatment. The inoculation of PS4 showed no biomass boosting effects under the same salinity treatment. Decline of *Salmonella* population was found on kale plant surface. *Salmonella* Javiana population decline showed no response to salinity and PGPR treatment and was smaller than the population reduction for *S.* Newport. The change of *Salmonella* Newport on kale surface due to salinity or PGPR root inoculation was identified. Reduced retrieval of *S.* Newport was noted on the surface of PGPR-negative kale under medium salinity treatment compared to under no salt condition, indicating controlled salinity treatment could restrict epiphytic growth of enteric pathogen. Striking decline of *S.* Newport population found on kale surface under no salt condition in response to PS4 inoculation was noted, suggesting application of PS4 can be a promising farming strategy to limit enteric pathogen growth. Salinity treatment and root inoculation of *Pseudomonas* spp. S4 influenced the proline and antioxidants accumulation in plant tissues. Proline accumulation and antioxidant capacity of PGPR-negative plants and phenolics and glucosinolates content in PS4-inoculated kale plants increased after salinity treatment. PGPR inoculation impacted the proline and antioxidants accumulation in kale tissues. Negative

associations between *Salmonella* population change and all phytochemicals were identified, suggesting proline and antioxidants in leaf tissues could play a role in manipulating *Salmonella* population on produce surface. Overall, A huge decline of *S.* Newport population was detected compared to *S.* Javiana under most controlled conditions. For PGPR-negative kale plants, *Salmonella* Newport association was most successful (lowest decline) on regularly watered kale surface with lowest levels of antioxidant capacity and antioxidants (flavonoids, phenolics and glucosinolates) and proline. Salinity treatment showed no impact on *Salmonella* Javiana and Newport decline on PS4-inoculated kale surface but altered phenolics and glucosinolates content. Root inoculation of PS4 restricted the growth of *Salmonella* Newport without changing the levels of antioxidant capacity, antioxidants, and proline. From these results, we conclude that 1) phytochemical profiles matter for non-PGPR kale plants. Phytochemical profile changes due to salinity treatment could impact the enterica pathogen colonization on plant surface; 2) mechanism of *Salmonella* restriction in response to root inoculation of PS4 is not solely associated with phytochemical profile changes under regular watering conditions and induced systemic resistance might be responsible in this setting. Future experiments will be designed based on these conclusions to illustrate the survival mechanism of *Salmonella* on plant surface.

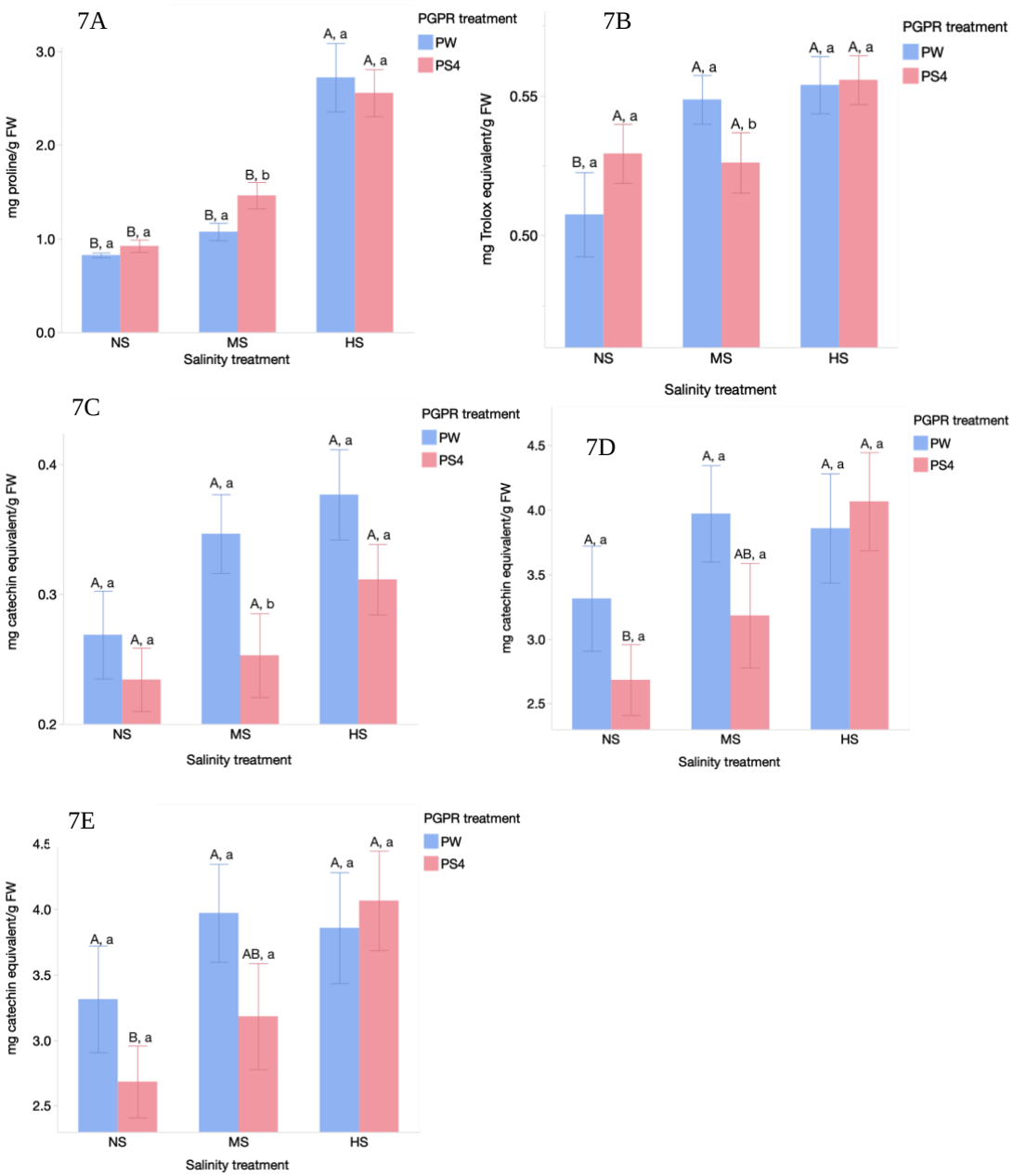


Figure 7. Phytochemical analyses in (A) proline, (B) antioxidant capacity, (C) total flavonoids, (D) total phenolics, and (E) estimated glucosinlates using plant tissues from 26 days old of kale plants under high salt (HS), medium salt (MS) and no salt (regular watering, NS) under the influence of *Pseudomonas spp.* S4 (PS4) or 0.1% PW (negative control, PW).

Salinity treatment (up to 255 mM NaCl) showed no impact on shoot biomass in our study for plants without PGPR inoculation. Most research reported growth restriction effects of salinity on shoot biomass under salinity treatment(Ahmadi et al., 2018). As low as 50 mM NaCl salinity treatment showed growth inhibition effects on *Brassica* plants (Linić et al., 2019). However, no decline in *Brassica napus* dry mass was noted even under 300 mM NaCl condition (Szymańska et al., 2019). With the inoculation of PS4, the biomass reduced under high salt treatment compared to medium salt condition and showed no difference compared to the biomass under no salt condition. The rhizobacteria PS4 we used in this study was isolated from Canadian soil and showed slight ability to improve emergence and nodulation of soybean (Dashti et al., 2000; Zhang et al., 1996) and promote biomass accumulation of spinach, Romaine cv. 'Parris Island Cos' and tomato cv. 'Moneymaker' and 'Nyagous' but not 'BHN602' (Hsu and Micallef, 2017). Based on our research results, root inoculation of *Pseudomonas* spp. S4 showed no growth-promoting effects on kale plants, possibly due to the plant-specific growth-promoting effects of *Pseudomonas* spp. Several studies reported similar results when *Pseudomonas* spp. showed growth-promoting effects to limited cultivar in various farming systems (Agaras et al., 2014; Jiménez et al., 2020; Long et al., 2008). Studies also reported the no growth-promoting effects of the application of PGPR under regular watering condition. However, when salinity stress was present, the biomass of PGPR-inoculated plants showed higher biomass (Vaishnav et al., 2020). Adverse effects of certain PGPR on eggplant growth were also reported (Abd El-Azeem et al., 2012).

Salmonella enterica serovars showed varied fitness on kale plant surface. Decline of *Salmonella* Javiana was slower than *S.* Newport on kale surface under most salinity and PGPR treatments. A slower decline rate of *Salmonella* Javiana compared to *S.* Newport was

reported on cucumber cultivars (Callahan and Micallef, 2019). This *Salmonella* serotype-specific differences have been reported in other produce systems including but not limited to cucumbers, tomatoes and lettuce (Callahan and Micallef, 2019; Han and Micallef, 2014; Kroupitski et al., 2009b; Shi et al., 2007; Trmcic et al., 2018). Several factors might contribute to this serovar-dependent fitness of *Salmonella* on plant surface. From *Salmonella* perspective, the varied capabilities of *Salmonella* serovars to utilize readily available nutrients, tolerate stresses and form biofilms on produce surfaces lead to a serovar-specific *Salmonella* population size (Callahan and Micallef, 2019; Kroupitski et al., 2009b). Cuticle thickness, surface traits, and nutrients composition of different cultivar might favor the growth of particular *Salmonella* serovars (Callahan and Micallef, 2019).

Phytochemical profiles of kale plants responded differently to medium and high salt conditions with no PS4 inoculation. Salinity treatment increased the antioxidant capacity of kale leaf tissues. Proline levels increased solely under high salt condition. Kale seedlings showed relatively high salt tolerance under control short-term salinity environment with the least biomass reduction, increased accumulation of proline to two other Brassica crops (Linić et al., 2019). Salinity treatment (up to 200mM) had no impact on the levels of chlorophylls, total phenolics, and flavonoids in kale seedlings (Linić et al., 2019). This research may partially explain no change in kale biomass, total phenolics and flavonoids under medium salinity treatments in our study. Total phenolic content of radish sprouts decreased after being treated in low salinity (10 mM and 50 mM NaCl) and reported no change after being treated in 100mM NaCl for 7 days compared to regularly watered radish sprouts (Yuan et al., 2010). Total phenolics positively correlate with antioxidant capacity measured by ABTS radicals (Dudonné et al., 2009; Thaipong et al., 2006). Our results found a positive correlation

between total phenolics and antioxidant capacity. However, higher levels of antioxidant capacity due to salinity stress might link to increased accumulation of compounds other than phenolics that can quench ABTS radicals, possibly ascorbic acid (Osman et al., 2021). Glucosinolates content increased with salinity treatment (up to 200 mM) for kale seedlings (Linić et al., 2019). However, lower total glucosinolates contents in kale shoot were identified in high salt (up to 100 mM) (Aghajanzadeh et al., 2018). Total glucosinolates content in radish sprouts declined after low salinity treatment (up to 7 days) at levels of 10 mM and 50 mM NaCl and no change at 100 mM NaCl treatment for 3 days (Yuan et al., 2010). In our study, glucosinolates showed no response under salinity (up to 255 mM) for 5 days. Nevertheless, controlled salinity treatment increased the antioxidant capacity of kale plants and enhanced nutritional quality of kale plants.

Proline and levels of phenolics, flavonoids, glucosinolates and antioxidant capacity showed no difference with root inoculation of *Pseudomonas* spp. S4. Glucosinolates content increased in response to *Pseudomonas* inoculation under high salt condition. *Brassica napus* under the influence of *Pseudonomas* showed no change of proline levels under no salinity and high salinity (300 mM NaCl) treatment, but lower proline levels in medium salinity treament (50 and 150 mM NaCl) (Szymańska et al., 2019). Maze plants inoculated with *Pseudomonas* under salinity treatment (200 mM NaCl) showed higher total phenolics (Kubi et al., 2021). Interestingly, based on our results, application of PS4 increased the proline content and decreased the levels of antioxidant capacity and total flavonoid in kale tissues under medium salt condition (around 85 mM NaCl). Increased proline content in response to *Pseudomonas* spp. root inoculation was reported in radish and wheat with increased gene expression of proline synthesis (Mohamed and Gomaa, 2012; Shavalikohshori et al., 2020).

Increased proline accumulation could provide higher salt tolerance to *Brassica* plants (Linić et al., 2019). Under salinity and other abiotic stresses, proline can not only act as an osmoprotectant but provide antioxidant properties to lower the reactive oxygen species and increase antioxidant enzyme capacity (Kavi Kishor and Sreenivasulu, 2014). Root inoculation of *Pseudomonas putida* reduced levels of antioxidant capacity and phenolic contents but increased flavonoids accumulation in soybean plants (Kang et al., 2014). With these results, in response to *Pseudomonas* spp. S4, under medium salt condition, kale plants increased the proline content, thus increased provided the osmoprotection to kale and increased the antioxidant enzyme capacity, thus boosting the overall tolerance to salinity with less accumulation of flavonoids and levels of non-enzymatic antioxidant capacity.

For PGPR inoculated kale group, proline, phenolics and glucosinolates content increased under salinity treatment in our study. As reported, with root inoculation of *Pseudomonas*, proline levels in *Brassica napus* leaves and rice seedlings increased along with enhanced severity of salinity stress (Jha et al., 2011; Szymańska et al., 2019). Radish plants with *Pseudomonas* inoculation showed increased proline accumulation with enhanced salinity treatment (Mohamed and Gomaa, 2012). Salinity treatment increased the phenolics and glucosinolates content for PS4-inoculated kale plants. An increase of these non-enzymatic antioxidants can potentially help kale to scavenge free radicals due to salinity treatment.

Decline of *Salmonella* was observed and affected by salinity stress on kale plant surface in our study. Under medium salinity condition, a sharp decline of *Salmonella* population on leaf surface was noticed compared to *Salmonella* population under no salt condition. Simultaneously, the antioxidant capacity of medium salt-exposed kale tissues increased

significantly, with slightly high levels of total flavonoids and phenolics and glucosionolates. A previous study in our lab showed the correlation between reduced *Salmonella* population and increased antioxidant capacity on 20-days old kale plant surface. In addition, phenolics, major compounds contributing to antioxidant capacity of plants, on tomato surfaces negatively associated with *Salmonella* colonization (Han and Micallef, 2016). The decline of *Salmonella* might be due to increased accumulation of growth-limiting compounds, but also reduced nutrients present on plant surfaces. Sugars leached from the interior of the plant leaf can serve as carbon-containing nutrients to support the growth of epiphytes, including *Salmonella* (Han and Micallef, 2016; Lindow and Brandl, 2003; Mercier and Lindow, 2000). We did not measure total soluble sugar in kale plants after salinity treatment. Several studies showed varied total sugar contents in plants under salinity treatments. Rapeseed (*Brassica napus)* leaves showed no soluble sugar change after exposure to 110 mM NaCl solution and increased the soluble sugar contents at NaCl level higher than 220 mM (Ahmadi et al., 2018). Soluble sugar contents in soybean plants decreased under 75 mM and 150 mM NaCl condition (Osman et al., 2021). Taken these together, we hypothesize that soluble sugar levels of kale plants under medium salt (~ 80mM NaCl) decreased, thus unable to support the growth of *Salmonella* with antioxidants present. However, under high salt condition (~ 255 mM NaCl), kale plants accumulated more soluble sugar in plant tissue and leached more to plant surface. With more nutrients and the same level antioxidants present on kale surface under high salt condition, *Salmonella* were capable to persist on kale surface. On the other hand, the decline of *Salmonella* population might be due to closed stomata of kale under salinity treatment (Hasegawa et al., 2000). Stomata are one of the most common sites for bacterial colonization (Beattie and Lindow, 1995). Epiphytes including enteric pathogens

could successfully create aggregates and form colonies on stomata and other sites with readily available nutrients present (Lindow and Brandl, 2003). In general, compared to adaxial side, larger population of bacteria were found on abaxial side, possibly due to reduced UV radiation and abundant presence of stomata (Beattie and Lindow, 1995). *Salmonella* showed strong attachment preference to abaxial side compared to adaxial side of the same Romaine lettuce leaf area with no stomata density difference, suggesting that other factors might impact the attachment and following colonization of *Salmonella* (Kroupitski et al., 2011). Moreover, stomata provide an avenue for *Salmonella* internalization (Kroupitski et al., 2009a; Kroupitski et al., 2011), though salinity stress showed no impact on *Salmonella* internalization and persistence in sweet-basil (Bernstein et al., 2017).

Root inoculation of PS4 restricted S. *Newport* population on salinity-free kale surfaces with no measurable non-enzymatic antioxidants and proline contents change, indicating similar levels of *Salmonella* growth-limiting compounds on kale surfaces even with PS4 inoculation. Sugars, vital nutrients for *Salmonella* surface survival, were not measured in this study. Many studies noted sugar level increase in shoot with root application of PGPR. *Bacillus subtilis* SU47 strain increased total soluble sugar contents in wheat (Upadhyay et al., 2012). Root application of *Pseudomonas fluorescence* increased the total soluble sugar contents in beans (*Phaseolus vulgaris* L) (Younesi and Moradi, 2014). Sugars levels increased in Broccolis with root incoulation of *Paraburkholderia* (Jeon et al., 2021). Total soluble sugar content kept the same after the inoculation of *Pseudmonas fluorescenes* and *Bacillus subtillis* for cucumbers (Saberi-Riseh et al., 2020). Phytochemical changes in tissues and washes due to *Pseudomonas* spp. S4 could favor other microbiota with less nutrients available to *Salmonella* (Critzer and Doyle, 2010; Hsu and Micallef, 2017; Mhlongo et al., 2020). Future studies in

microbial communities and phytonutrients in plant tissue and in plant washes in response to PS4 are required to better understand the mechanism of *Salmonella* colonization. Besides metabolites changes, root inoculation of *Pseudomonas* induced priming in plants for enhanced defense responses through various mechanisms (Meziane et al., 2005). *Salmonella*, on the other hand, is considered plant pathogens due to their ability to activate plant immune response and overcome stomatal defense by plant innate defense (Ferelli et al., 2020; Johnson et al., 2020; Shirron and Yaron, 2011). To relieve impact of plant pathogens, *Pseudomonas* spp. activated plant defense pathways and induced transcriptional changes (van de Mortel et al., 2012). *Psedumonas* spp. S4 might help plants to recognize *Salmonella* and lead to *Salmonella* population decline (Hsu and Micallef, 2017). Other PGPR strains reported *Salmonella* restriction effects through ISR as well. Stomatal closure in response to *Bacillus subtilis* inoculation was identified as an effective intervention for *Salmonella* epiphytic growth on lettuce and spinach plants(Johnson et al., 2020; Markland et al., 2015). However, the detailed defense response to *Salmonella* decline is unclear.

Overall, it is evident that salinity and root inoculation of *Pseudomonas* spp. altered plant biomass and phytochemical profiles and impacted the *Salmonella* association in a serovar-dependent manner. Salinity treatment reduced the biomass of kale plants. PS4 failed to promote shoot growth of kale, possibly due to the cultivar-specific growth-promoting effects of *Pseudomonas*(Agaras et al., 2014; Long et al., 2008). More PGPR strains should be included to test growth-promoting effects on kale. A sharp decline of *Salmonella* Newport was detected on kale surface and other produce (Callahan and Micallef, 2019). Salinity treatment impaired the *Salmonella* Newport fitness possibly through two mechanisms, increased non-enzymatic antioxidants and stomatal closure rate. Future studies are required to

identify *Salmonella* growth-limiting antioxidants and their restriction mechanism even in presence of abundant nutrients. ISR-related metabolomic and transcriptional change and microbiota shifts were the potential mechanisms for *Salmonella* restriction due to PS4 inoculation. Revealing mechanisms related to *Salmonella* decline associated with PGPR would further help the application and commercialization of PGPR in real life. Through this research, we provide evidence that the epiphytic growth of *Salmonella* can be manipulated by altering phytochemical profiles of hosts. Medium salinity treatment enhanced plant nutritional quality and reduced *Salmonella* population without cost of biomass. PS4 limited *Salmonella* epiphytic growth, possibly due to enhanced defense response to stresses. Root application of PS4 could be a promising farming strategy to improve plant safety from *Salmonella* and the health of other phytopathogens at the pre-harvest stage.

Chapter 6: Plant Growth-Promoting Rhizobacterium, a new agent to maintain yield under drought and food safety by limiting the surface survival of *Salmonella enterica*

6.1 Introduction

Extreme weather conditions, including drought caused by climate fluctuation, have severely altered plant physiology and impaired agricultural production, which is predicted to worsen with increased frequencies (Li et al., 2009). Drought starts to impact plants when the water potential of the soil drops lower than the water potential of plant root cells. Under this situation, water moves from plant root cells to the soil, and without fully closed stomata, plant cells lose turgor and wilt. The impact of drought on plants depends on the duration of water deficit and the developmental stage of plants (Farooq et al., 2009). During the vegetative stage, water deficiency can increase the level of secondary metabolites and cause morphological changes (Dietz et al., 2021). Drought can impair the size and uniformity of leafy greens, thus lowering their economic values (Dietz et al., 2021). To cope with drought stress, plants can increase the accumulation of soluble molecules such as proline and sugars in the protoplast to lower the water potential of cells and prevent water loss to soil (Pathirana et al., 2017). Urgent attention is needed to develop potential solutions to ensure crop yield under increased drought frequencies.

Food safety is another challenge that is worth further investigation. Among all food commodities related to foodborne illnesses, an upward trend in produce-associated outbreaks was concerning (Callejón et al., 2015b; Lynch et al., 2009). Human pathogens, such as

Salmonella enterica, could be introduced to produce at any stage of the farm to fork continuum and survive the production, processing, and harvest (Bennett et al., 2015; Harris et al., 2003; Olaimat and Holley, 2012). It is rather complex to reduce and eliminate human pathogens once they establish on the plant surface (Goodburn and Wallace, 2013; Parish et al., 2003). To enhance food safety and reduce its collateral economic losses, Good Agricultural Practices (GAPs) and Produce Safety Rule (PSR) under the Food Safety Modernization Act (FSMA) have been established to offer guidance and recommendations for growers to prevent foodborne outbreaks. However, contamination during production lasts (Angelo et al., 2015; CDC, 2008) and effective on-farm interventions are required to produce safety.

Plant growth-promoting rhizobacteria (PGPR) are bacteria colonizing on the plant surface and promoting plant growth directly or indirectly. PGPR can directly influence plant phytohormones synthesis and improve nutrient uptake to promote plant growth (Glick, 1995; Khosravi et al., 2018). *Pseudomonas* can also function as biocontrol agents to protect plants and promote plant growth indirectly via induced systemic resistance (ISR) (Loon and Glick, 2004; Raaijmakers et al., 1995). With the root inoculation of *Pseudomonas,* plants suppress soil-borne plant disease via ISR (Matilla et al., 2010). More interesting, studies report that PGPR-induced ISR might play a role in enteric pathogens survival on produce surfaces. Among all widely used PGPR, *Bacillus* strains have shown limiting effects on foodborne pathogens on produce, including lettuce and spinach, by producing biosurfactants and modulating stomatal apertures (Johnson et al., 2020; Markland et al., 2015; Rani et al., 2020; Zhao et al., 2021). *Pseudomonas* also have been shown to promote produce growth and

restrict *Salmonella* growth, possibly due to ISR (Hsu and Micallef, 2017). However, research regarding the mechanism of PGPR on enteric pathogens on various produce remains scant.

This study aims to study the beneficial effects of PGPR on plant growth and enteric pathogen reduction on produce surface under drought stress long with plant development. *Pseudomonas* spp. S4 used in this study has proven effects on soybean nodulation enhancement (Dashti et al., 2000; Zhang et al., 1996), growth-promoting effects on spinach, Romaine lettuce, and tomato plants (Hsu and Micallef, 2017) and restriction of *Salmonella* on spinach and tomato plants (Hsu and Micallef, 2017). However, drought and plant cultivars could impact the effectiveness of *Pseudomonas* (Hsu and Micallef, 2017; Tabassum et al., 2017). Hence, we investigated the growth-promoting effects of *Pseudomonas* spp. S2 and S4 on kale and its limiting effects on *Salmonella* Newport – kale association under drought stress at two different development stages.

6.2 Material and Methods

6.2.1 PGPR strain preparation and inoculation

Pseudomonas spp. S2 (PS2) and S4 (PS4) were obtained from Dr. Brian Klubek (Southern Illinois University Carbondale) with rifampicin resistance. Frozen stocks of *Pseudomonas* spp. were kept in Brucella broth (BD, MD, USA) containing 15% glycerol (VWR Chemicals, BDH) and 50 μg/ml rifampicin at -80℃. *Pseudomonas* spp. were revived by streaking onto Trypticase Soy Agar (TSA, BD) plates amended with 50 μg/ml rifampicin (TSA-rif) at 30℃ for 48 hours. A single colony of PS2 or PS4 was then streaked onto fresh TSA-rif plates and

grown for 48h at 30℃. PS2 or PS4 inocula were made by suspending new bacterial colonies in 0.1% peptone water (PW) to an OD_{600}=0.5 to obtain a cell density of ~10^9 CFU/ml. Each PS2 or PS4-treated kale plant received 2 ml of PS2 or PS4 inocula suspension and the non-PGPR inoculated kale plant received 2 ml of 0.1% PW as the negative control. All inocula were applied directly to the base of stems near the soil. Two separate root inoculations were conducted when kale plants were 2- and 9-days old (post-germination).

6.2.2 Kale growth and drought treatment

Brassica oleracea L. var. *acephala* cv. 'Improved Dwarf Siberian' was used in this study. Kale seeds (Territorial, OR, USA) were germinated in potting mix soil (Sunshine LC1; Sungro Horticulture, Canada) at the Research Greenhouse Complex, University of Maryland, and grown under controlled light and temperature conditions (16 h light:8 h dark photoperiod at 23℃/18℃ day/night temperatures). This research studied two developmental stages (baby kale and mature kale) of the kale plant.

Baby kale plants: Kale plants were kept regularly watered after being inoculated with *Pseudomonas* spp. S2 or S4 or 0.1% PW at 2- and 9- days old until they were 14 days old. Kale plants were then separated into two groups. One group of plants was subjected to drought stress by withholding irrigation (DR) for six days or regularly watered (CON) prior to experimentation. After treatment, kale plants in this group were 20 days old. Baby kale inoculated with *Pseudomonas* spp. S2 was not involved in biomass measurement and phytochemical profiling, but *Pseudomonas* spp. S4 or 0.1% PW treated kale was used for biomass, bacterial growth evaluation on the surface and in washes, and all phytochemical measurements. Baby kale samples were kept under a relative humidity of 50%.

Mature kale plants: Kale plants were kept regularly watered after being inoculated with *Pseudomonas* spp. S4 or 0.1% PW at 2- and 9- days old until they were 56 days old. Those plants were subjected to drought treatment (DR) or regular irrigation (CON) for three days. Kale plants were 59- days old after treatment. Mature kale inoculated with *Pseudomonas* spp. S4 or 0.1% PW were used for biomass, bacterial growth evaluation on the surface and in surface washes, and all phytochemical measurements. Mature kale plants were grown under 60% relative humidity.

This chapter did not study plant developmental effects since baby kale and mature kale were grown in different environments.

6.2.3 Biomass measurement of kale plants

Kale plants inoculated with PS4 or 0.1% PW were harvested at the baby and mature kale stage under the same growing conditions as described above. All above-soil biomass of kale was harvested from the base of each plant and put in a pre-weighted foil tray, and baked in an oven at 70℃ for 48 hours for dry shoot weight (SDW) measurements.

6.2.4 Salmonella Newport inoculum preparation

The *S. enterica* Newport strain used in this study is a tomato outbreak strain (Greene et al., 2008) adapted for rifampicin resistance. A single colony of *S.* Newport grown on TSA with 50 μg/mL rifampin was streaked on fresh TSA-rif plates and grown at 35°C overnight. *S.* Newport inocula were made by suspending bacterial colonies in 0.1% PW to an OD600=0.5 to obtain a cell density of ~10^9 CFU/mL. A 100-fold dilution of the *S.* Newport suspension or 0.1%PW (non-SN inoculated) was used for leaf surface and surface wash inoculation.

6.2.5 Kale leaf extract and surface wash preparation

As described (Liu et al., submitted), whole kale leaves were flash-frozen using liquid nitrogen and then ground into powders using a pestle and mortar; 200 mg of plant powder were accurately weighed and extracted with 1.5 ml 70% methanol (Sigma-Aldrich, MO, USA) with 0.5% formic acid (VWR, PA, USA). Each extract was fully vortexed before centrifuged at 3,000 rpm for 10 min. The supernatants were frozen at -20°C until further analysis. Whole plants were used to collect surface washes by immersing whole plants in 30 ml 5% methanol/water solution and shaken at 150 rpm for 24 h at room temperature. Surface compound solutions were filtered through 0.2 μm cellulose acetate syringe filters (VWR, PA, USA).

6.2.6 Kale leaf surface and leaf wash inoculation with Salmonella Newport

As described (Liu et al., submitted), an aliquot of 100 μl of *S.* Newport inoculum was pipetted on the adaxial side of the third or fourth true of kale plants distributed in ~10 spots. An estimated total inoculum dose of 10^6 CFU was inoculated per leaf. Inoculated kale leaves were harvested 24 hours post-inoculation (hpi). Each leaf was aseptically clipped off the stem with scissors and placed in a sterile Whirl-pak bag (Nasco, WI, USA). Inoculated leaves were then immersed in 30 ml 0.1% PW, hand-massaged for 30 s, and sonicated in a Branson Ultrasonic Cleaner (Branson Ultrasonics Corporation, CT, USA) for 1 min at maximum speed to dislodge *S.* Newport cells from the kale leaf surfaces. Each leaf was then shaken at 150 rpm for 10 min. Serial dilutions from each leaf rinsate were plated on TSA-rif plates for *S.* Newport enumeration after 24 h of incubation at 35°C.

For leaf washes, 20 µl of *S.* Newport inoculum were added to 2 ml leaf wash solution at an initial concentration of ~10^6 CFU/ml and incubated at 35°C with shaking at 150 rpm. *S.* Newport cell counts were taken at 0, 2, 4, 6, and 24 hpi. At each timepoint, serial dilutions were plated on TSA-rif plates for enumeration. Sterile 5% methanol in water served as the negative control.

6.2.7 Phytochemical analyses using kale leaf tissue extracts

All absorption readings in the following assays were acquired using a microplate reader (Synergy HTX; Biotek, VT, USA) as described (Liu et al., submitted). Each phytochemical analysis was repeated at least three times in separate experiments with at least three biological replicates in each treatment. Data were pooled for analysis.

Antioxidant capacity: Modified based on the previous method (Stratil et al., 2006) was made for ABTS measurement. ABTS preparation: 31.7 mg 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (TCI, OR, USA) and 8.6 mg potassium persulfate (Aldon Corp Avon, NY, USA) was dissolved in 10 ml water and allowed to stand in darkness at room temperature for 16 h to form a stable radical, then diluted to absorption of ~1.3 at 734 nm. Twenty µL sample extracts were added to 200 µL ABTS solution and allowed to stand for 15 min, then absorption was measured at 734 nm. A standard curve from the blank-corrected absorption at 734 nm of Trolox (TCI) standards was plotted. Antioxidant capacity was calculated as Trolox equivalent using the standard curve.

Total flavonoids: Based on methods developed by studies (Zhishen et al., 1999), one hundred µL of kale leaf extract were added to 20 µL 5% $NaNO_2$ (Avantor, PA, USA). After 5 min, 20 µL 10% $AlCl_3$ (Alfa Aesar, MA, USA) were added, followed by 100 µL 1 mol/L NaOH

(VWR Chemicals, BDH, Radnor, PA, USA) 1 min later. The absorption of the mixture was measured at 510 nm on the microplate reader. A standard curve from the blank-corrected absorption at 510 nm of the catechin standards (Enzo Life Sciences, NY, USA) was plotted. Antioxidant capacity was calculated as catechin equivalent using the standard curve (Yoon et al., 2019).

Total phenolics: The method was adapted from previous studies (Kao et al., 2014; Singleton and Rossi, 1965). Eighty μL kale leaf extract were mixed with 90 μL 10% Folin Ciocalteu reagent (MP Biomedicals, CA, USA) and 40 μL 700 mM Na_2CO_3 (VWR Chemicals, BDH) were added to a 96-well plate. All samples were fully vortexed and left to stand for one h in a microplate reader at 25℃. Absorption measurements were taken at 765 nm. The standard curve was plotted from the blank-corrected absorption values at 765 nm of the gallic acid (Acros Organics Fair Lawn, NJ, USA) standards. Total phenolics were calculated as gallic acid equivalents using the standard curve.

Estimated glucosinolate content: Kale leaf extract (80 μL) was added to 200 μL 2 mM sodium tetrachloropalladate (Acros Organics). The samples were incubated at room temperature for one h, and absorption was measured at 425 nm. Water was then used as a blank. Total glucosinolate content was calculated by putting the OD_{425} of each sample into the predicted formula as follows (Mawlong et al., 2017)

$$Y = 1.40 + 118.86 * A_{425}$$

6.2.8 Phytochemical profiling using kale leaf tissue extracts and surface washes by electrospray ionization-time-of-flight mass spectrometry (ESI-TOF-MS)

Phytochemical profiling of kale leaf tissue and wash samples was analyzed (Liu et al., submitted). Aliquots (1 ml) of kale leaf extracts and 30 ml filter-sterilized kale wash solutions were lyophilized into powder and resuspended in 100 µl 70% methanol with 0.5% formic acid with at least four biological replicates.

To ensure all compounds were fully dissolved in the extraction liquid, all tubes were sonicated in a water bath for 2 h at maximum intensity. The remaining sample extract was then transferred into vial inserts for ESI-MS analysis. A Time-of-Flight mass spectrometer (AccuTOF, JEOL, MA, USA) equipped with an electrospray ionization ion source was used in the mass spectrometric analysis of the extracts. Mass spectra were acquired in the positive and negative modes at a rate of one spectrum per second with the m/z range of 50–800 Da. Every mass spectrum was averaged over about one minute. The AccuTOF mass spectrometer settings were as follows: needle voltage = 2100 V, orifice one temperature = 100°C, orifice 1 = 30 V, orifice 2 = 5 V, ring = 10 V. The desolvating chamber temperature was set at 250°C, and the flow rates of the nebulizing and desolvating gases were 0.6 and 3.0 L/min, respectively. The sample injection volume was ten µl, and the flow rate was set to 0.25 ml/min. Before and after each sample was analyzed, ten µl methanol was injected and measured to monitor any possible contaminants or carryover. Calibration for exact mass measurements was accomplished using five mM cesium iodide as the internal standard. The measurements were repeated at least twice for all the samples, and the experimental results showed excellent reproducibility.

6.2.9 Statistical analysis

Data collected from each measurement were pooled for treatment effect analysis. Biomass was repeatedly measured at least three times with each treatment, including at least five replicates. For bacterial epiphytic growth evaluation, each experiment was repeated at least two times with four replicates. Bacterial growth in washes was measured twice with three replicates. *S.* Newport counts retrieved from kale leaves, and washes solutions were log-transformed in CFU/plant and CFU/ml, respectively, prior to analysis. Tukey's HSD test following ANOVA and Student's *t*-test for *a priori* pairwise comparisons were employed to determine PGPR effects and watering regime on *S.* Newport counts. Student's t-test analyzed differences in phytochemical analyses between PGPR effects and watering regimes for *a priori* pairwise comparisons. Data analyses were conducted in JMP Pro 15.2.0 (SAS Institute Inc., Cary, NC). Results of plant developmental stages were not analyzed as the plants grew under different humidity conditions.

Multidimensional scaling (MDS) was involved in analyzing phytochemical profiling data obtained from ESI-TOF-MS to visualize levels of similarity among samples. Data regarding each compound's m/z ratio and signal intensity were retrieved from ESI-TOF-MS and pre-processed by binning peaks and aligning relative peak intensity data from different samples. Distance matrices based on the Bray-Curtis dissimilarity coefficient were generated by the vegan package (version 2.5-7) in RStudion (v. 1.3.1093) and used for MDS analysis performed in JMP Pro v. 15.2.0 (SAS Institute Inc., Cary, NC, USA). Analysis of similarity (ANOSIM) was conducted in PRIMER 6 (Plymouth Routines in Multivariate Ecological Research version 6.1.15; PRIMER-E-Ltd., Plymouth, United Kingdom) based on Bray-Curtis

distance matrices to test similarities among samples. Briefly, ANOSIM tests the null hypothesis that the similarity of samples within the different treatments is not greater than between samples from different treatments and provides test statistic *R*. When all samples from different treatments are the same, the *R* equals 0. By contrast, when samples are more similar within the group than among groups, *R* gets close to 1.

6.3 Results

6.3.1 PGPR inoculation and drought treatment influenced the dry shoot mass of kale plants.

Under both watering conditions, no growth-promoting effects of *Pseudomonas* spp. S4 was reported for both baby and mature kale. Under drought treatment, PS4-inoculated baby kale showed a higher biomass accumulation than PGPR-negative kale plants, but not for mature kale (Table 12). Drought significantly reduced the biomass of PGPR-negative plants at both growing stages ($p < 0.05$, Table 12). Drought-treated baby and mature kale plants inoculated with *Pseudomonas* spp. S4 showed no change in biomass accumulation (Table 12).

Table 12. Dry biomass accumulation of 20-day (baby) and 59-day (mature) kale plants under regular watering (CON) or restricted watering (DR) regimes treated with root inoculation of *Pseudomonas* spp. S4 (PS4) or 0.1% peptone water (PW). Small letters indicate differences by PGPR root inoculation within same age and watering condition (Student's *t*-test, $p < 0.05$). Capital letters denote differences by watering treatment within the same age and root inoculation (Student's *t*-test, $p < 0.05$). * indicated baby kale and mature kale plants were kept under different humidity.

Watering regimes	root inoculation	Dry biomass g/plant
Baby kale (20D)		
CON	PW	0.82 ± 0.06 a, A
	PS4	0.78 ± 0.05 a, A
DR	PW	0.62 ± 0.05 b, B
	PS4	0.81 ± 0.05 a, A
Mature kale (59D) *		
CON	PW	5.66 ± 0.21 a, A
	PS4	5.40 ± 0.23 a, A
DR	PW	5.02 ± 0.22 a, B
	PS4	5.17 ± 0.24 a, A

6.3.2 Salmonella surface reduction was affected by Pseudomonas spp. inoculation and drought treatment

Baby and mature kale were inoculated with *S.* Newport at a 4.4.2 – 4.70 log CFU/plant level and 4.95 – 5.29 log CFU/plant, respectively. The decline of the *Salmonella* population on kale surface was recorded 24 hours post-inoculation, with the highest *Salmonella* Newport decline found on the surface of PS4-inoculated mature kale at a level of 2.93 log CFU/plant under regular watering conditions. By contrast, the lowest reduction of *Salmonella* 1.84 log

CFU/plant was observed on non-PS4 affected baby kale under regular watering conditions (Table 13).

6.3.2.1 Root inoculation of *Pseudomonas* spp. S4 restricted surface survival of *S. Newport* under the regular watering conditions

For baby kale under the normal watering condition, the *Salmonella* population showed the highest reduction levels on the surface of PS4 inoculated kale plants ($p < 0.05$). No difference in *Salmonella* population decline was found on PS2 inoculated kale plants compared to non-PGPR inoculated kale plants. The trend was maintained as the plant aged. Decline in *Salmonella* population for mature kale surfaces in response to *Pseudomonas* spp. S4 inoculation was reported. The *Salmonella* population reduction showed no change due to root inoculation of PS4 on drought-treated plant surfaces for both baby and mature kale plants.

6.3.2.2 Drought restricted the growth of *S.* Newport on non-PGPR inoculated baby kale surface

For the non-PGPR inoculated kale group, *S.* Newport remarkedly declined on the surface of drought-treated baby kale ($p < 0.05$). However, with the root inoculation of *Pseudomonas* spp. S2 or S4, the decline of *S*. Newport on the surface showed no difference under both watering regimes. As the plant developed, the decline of *S*. Newport was not affected by drought within the same PGPR root inoculation condition (Table 12).

Table 13. *Salmonella* Newport retrieval on surface of 20-day (baby) and 59-day (mature) kale plants under regular watering (CON) or restricted watering (DR) regimes treated with root inoculation of *Pseudomonas* spp. S4 (PS4) or 0.1% peptone water (PW). Statistically significant differences in *S.* Newport population are denoted by letters. Capital letters denote differences by watering treatment within the same age and root inoculation (Student's *t*-test, $p \leq 0.05$). Small letters indicate differences by root PGPR inoculation within same age and watering condition (Baby kale: Tukey' HSD test, $p < 0.05$; Mature kale Student's *t*-test, $p < 0.05$). Values are means of replicates ± standard error (SE).

		Surface (log CFU/plant)		Wash (Log CFU/ml of wash)	
Watering regimes	Root inoculation	Log inoculum	Log decline↓	Log inoculum	Log growth‡
Baby kale (20D)					
CON	PW	4.42 – 4.70	1.84 ± 0.23 B, b	3.42 – 3.75	3.99 ± 0.21 A, a
	PS2		2.17 ± 0.13 A, ab		3.87 ± 0.29 A, a
	PS4		2.46 ± 0.10 A, a		4.44 ± 0.10 A, a
DR	PW		2.39 ± 0.11 A, a		1.86 ± 0.75 B, a
	PS2		2.01 ± 0.27 A, a		1.38 ± 0.41 B, a
	PS4		2.48 ± 0.10 A, a		0.72 ± 0.15 B, a
Mature kale (59D) *					
CON	PW	4.95 – 5.29	2.46 ± 0.14 A, b	2.65- 3.35	0.69 ± 0.31 A, a
	PS4		2.93 ± 0.15 A, a		0.96 ± 0.37 A, a
DR	PW		2.61 ± 0.13 A, a		0.54 ± 0.31 A, a
	PS4		2.76 ± 0.15 A, a		0.24 ± 0.22 B, a

6.3.3 Drought impacted the growth of Salmonella in surface washes

Salmonella population was inoculated into the surface wash at a 3.42 – 3.75 log CFU/ml of wash and 2.65 – 3.35 log CFU/ml of wash for baby and mature kale. Exudates collected from baby kale under the regular watering condition with no *Pseudomonas* inoculation supported the highest *Salmonella* growth for 3.99 log CFU/ ml of wash after 24 hours. By contrast,

washes collected from drought-treated mature kale with PS4 support the lowest levels of *Salmonella* population after 24 hours post-inoculation. No differences in *Salmonella* growth were reported due to root inoculation of *Pseudomonas* spp. S2 or S4 at the baby kale stage. Application of *Pseudomonas* spp. S4 showed no impact on *Salmonella* growth after 24 hours for mature kale (Table 13).

For the PGPR-negative kale group, drought significantly restricted the growth of *Salmonella* in baby kale washes. Washes collected from regularly watered baby kale supported a higher population of *Salmonella* than from drought-treated baby kale ($p < 0.05$). The growth of *the Salmonella population in washes was* not influenced by drought for the mature kale group.

The reduced growth of the *Salmonella* population in response to drought for baby kale remained with the root inoculation of *Pseudomonas* spp. For both PS2 and PS4-inoculated baby kale groups, higher populations of *Salmonella* were found in washes collected from regularly watered kale plants. The decline of *Salmonella* in washes of PS4-inoculated mature kale showed a higher *Salmonella* reduction after drought treatment.

6.3.4 Root inoculation of Pseudomonas spp. and drought were responsible for differences detected in phytochemical analyses

Tissues from *Pseudomonas* spp. S2 and S4 or 0.1% PW treated baby kale plants and mature kale plants with PS4 or 0.1% PW were collected for phytochemical measurements, including antioxidant capacity (Figure 8A), total flavonoids (Figure 8B), total phenolics (Figure 8C) and estimated glucosinolates (Figure 8D).

6.3.4.1 *Pseudomonas* inoculation altered the phytochemical measurements

Baby kale showed no changes in phytochemical measurements in response to root inoculation of *Pseudomonas* spp. S2 or S4 under both regular watering and drought treatments. PS4-inoculated mature kale under regular watering conditions showed higher levels of glucosinolates. No changes in phytochemical measurements were reported for drought-exposed mature kale plants in response to *Pseudomonas* spp. S4 root inoculation.

6.3.4.2 Drought affected levels of phytochemicals

Significant changes in overall antioxidant capacity and total phenolics, but not total flavonoids and glucosinolates due to drought, were noted for baby kale plants without *Pseudomonas* spp. Root inoculation. For baby kale plants inoculated with *Pseudomonas* spp. S2 or S4, drought increased the antioxidant capacity ($p < 0.05$), total phenolics ($p \leq 0.06$), and glucosinolates ($p \leq 0.05$) in leaf tissues. By contrast, drought showed no impact on the phytochemical measurements for mature kale no matter the presence of *Pseudomonas* spp. S4.

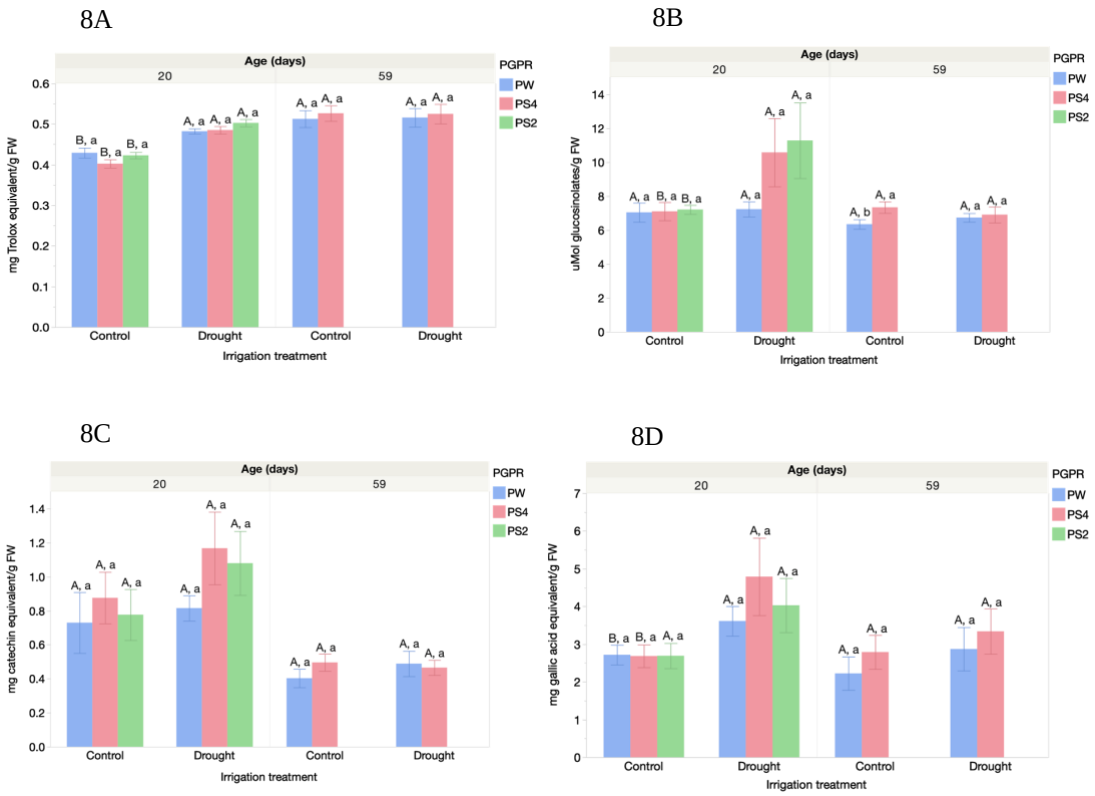


Figure 8. The chemical measurements for (A) antioxidant capacity, (B) total flavonoid, (C) total phenolic, and (D) estimated glucosinolate contents in baby (20-day old) and mature (59-day old) plant tissues under regular and restricted watering regimes impacted by 0.1% PW (blue), *Pseudomonas spp.* S4 (red) and S2 (green). For baby kale plants, capitalized letters represent significant differences by PGPR application under the same watering condition by Tukey's HSD (p<0.05) for *a priori* comparisons. For mature kale plants, PGPR treatment effects were analyzed by Student's *t*-test (p<0.05). Small letters represent significant differences due to treatment within the same age group by Student's *t*-test (p < 0.05).

6.3.5 Differences were identified between phytochemical profiles of kale leaf tissue and wash samples

Leaf tissues and washes from *Pseudomonas* spp. S4 or 0.1% PW inoculated baby and mature kale under both watering regimes were involved for phytochemical profiling using ESI-TOF-MS. Results based on the phytochemical profiling could identify the impact of root inoculation of PGPR and drought on the kale metabolomes (Figures 9 and 10). In total, leaf tissue and wash samples yielded 1,023 and 1,150 peaks under the positive and negative modes, respectively.

6.3.5.1 Phytochemical profiles of leaf and wash samples collected from baby kale diverged

For the baby kale group, 766 and 674 peaks were detected under positive and negative modes. Under positive mode, baby kale leaf and wash samples yield 361 and 986 peaks. More peaks in kale leaf tissues were detected compared to wash samples under negative mode. Distinct differences between kale leaf and wash samples were detected under both positive mode (R= 1, $p < 0.001$) and negative mode (R= 0.97, $p < 0.001$) regardless of the application of *Pseudomonas* spp. S4 and watering treatments (Table 14 and Figure 9). More shared peaks were found in washes from baby plants under the influence of PGPR or 0.1% PW compared to leaf tissues under the positive mode, but fewer shared peaks were found in washes under the negative mode (Table 14).

6.3.5.2 Leaf and wash phytochemical profiles from mature kale showed no overlap

In mature kale leaf and wash samples, 799 and 937 peaks were reported for positive and negative modes using ESI-TOF-MS. More peaks in wash samples were detected than in leaf tissues under positive mode but less reported under negative mode. Phytochemical profiles of

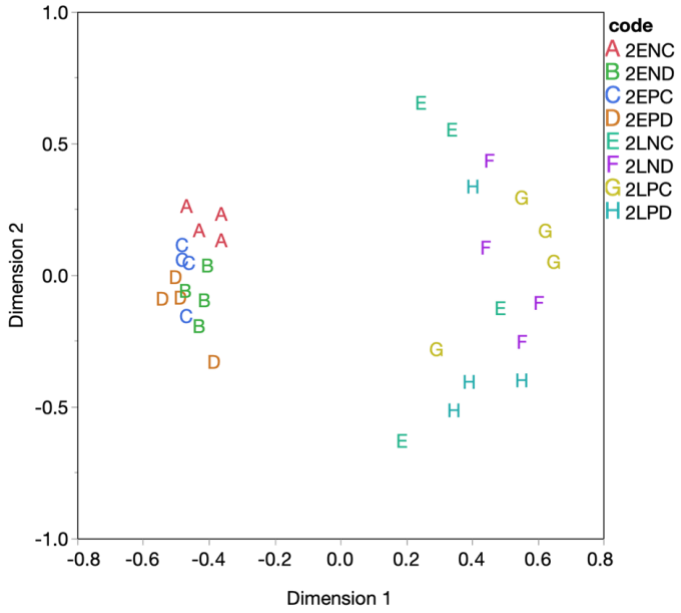
mature kale leaf and wash had no overlap under positive (R= 0.95, p < 0.001) and negative (R= 0.92, p < 0.001) modes (Table 14 and Figure 10). A higher proportion of shared peaks were detected in washes than in leaf tissues for mature kale (77 % versus 38%) under positive mode (Table 14). Leaf samples from mature kale shared more peaks than washes for mature kale under negative mode (Table 14).

6.3.6 Root inoculation of Pseudomonas spp. S4 played a role in phytochemical profiles of baby kale leaf and washes

Under positive mode, no changes in response to the root inoculation of *Pseudomonas* spp. S4 was noted for both baby kale leaf tissues based on the similarity of Bray-Curtis dissimilarity coefficient measurements under both watering conditions. However, under regular watering conditions, *Pseudomonas* spp. S4 impacted baby kale leaf tissues (Jaccard, R= 0.41, p = 0.09). Wash samples of baby kale showed convergent phytochemical profiles based on Jaccard dissimilarity measurements under both watering regimes but differences in washes under regular watering conditions based on Bray-Curtis measurement (R= 0.30, p = 0.06, Table 14).

Divergence in phytochemical profiles of leaf tissue samples under regular watering conditions was found via the negative mode of ESI-TOF-MS for both Bray-Curtis (R= 0.43) and Jaccard (R = 0.37) measurements (both p < 0.03, Table 14). No shifts in phytochemical profiles were identified for wash samples under both watering conditions by ESI-TOF-MS under negative mode.

9A



9B

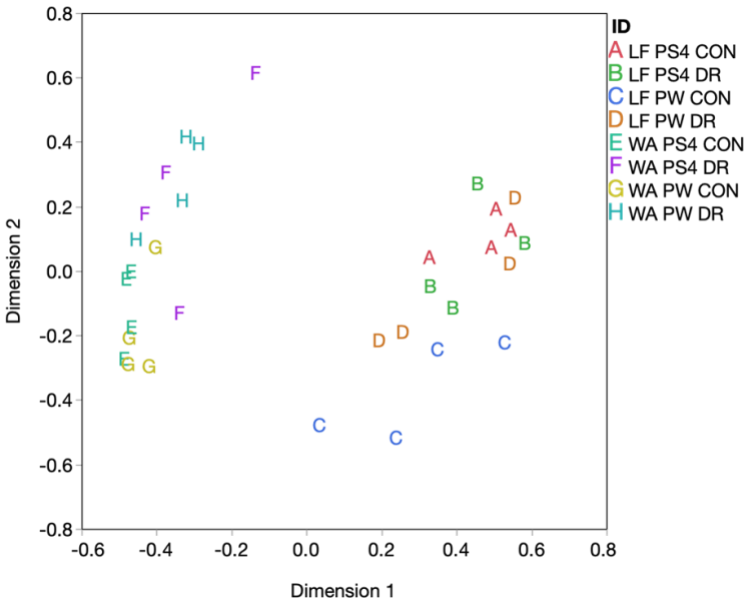


Figure 9. Multidimensional scaling (MDS) plots of phytochemical profiles of leaf tissue (LF) and leaf surface washes (WA) of baby kale with root inoculation of 0.1% PW (PW) or *Pseudomonas spp.* S4 (PS4), based on ESI-TOF-MS under (A) positive and (B) negative modes. Stress for plots was 0.20 (A) and 0.13 (B).

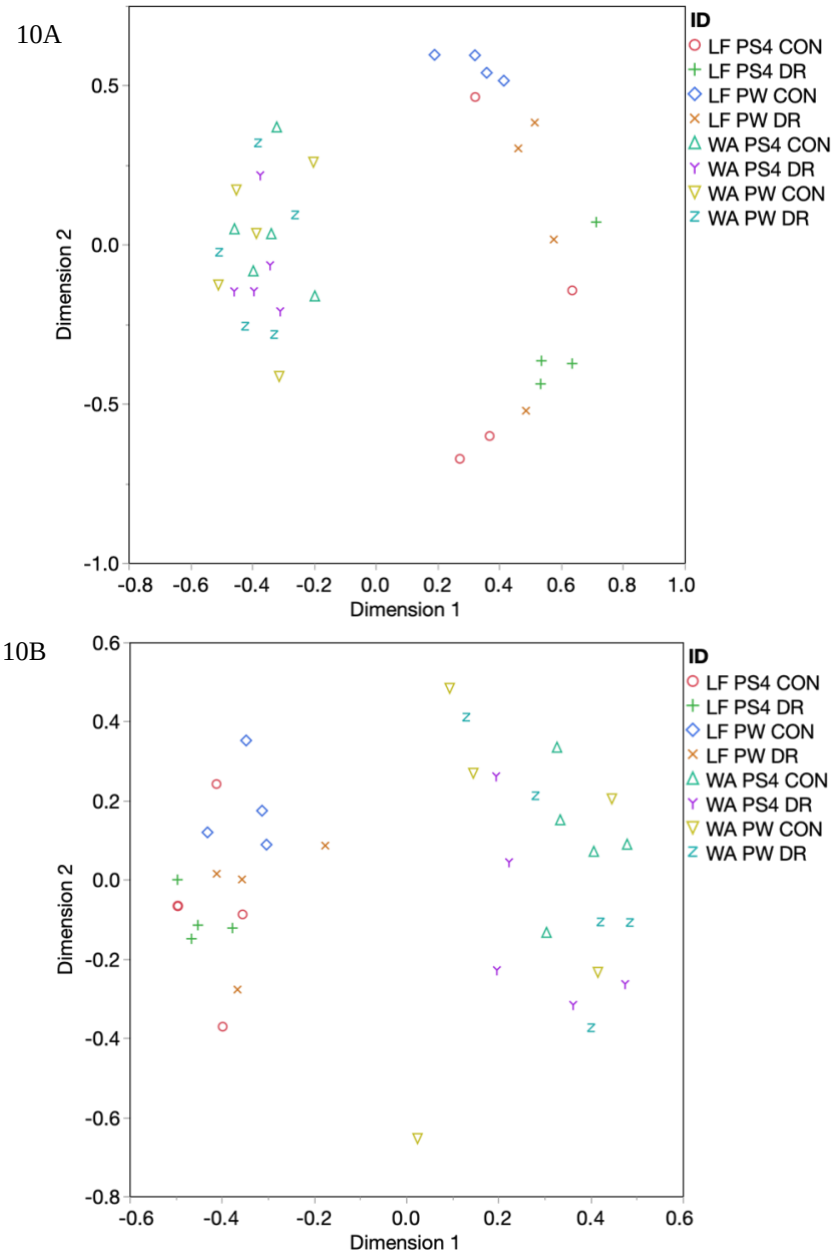


Figure 10. Multidimensional scaling (MDS) plots of phytochemical profiles acquired from ESI-TOF-MS from mature kale leaf tissue (LF) and surface wash (WA) under the absence (PW) or presence (PS4) of *Pseudomonas* spp. S4. Stress for MDS plot under positive mode (A) was 0.20 and 0.16 under negative mode (B).

Table 14. Number of metabolite peaks acquired from leaf tissue and wash samples from 0.1% PW or *Pseudomonas* spp. S4 (PS4) inoculated baby and mature plants under regular (CON) or restricted (DR) watering regimes based on phytochemical profiling with ESI-TOF-MS. † stands for peaks shared for PW or PS4 inoculated samples.

ID	Sample type	Total peaks detected	Shared peaks†	Treatment/ unique peaks	
				PW	PS4
Peaks detected in positive ionization mode					
Baby kale Leaf	CON	191	82	56	53
	DR	170	96	35	39
Baby kale Wash	CON	480	329	80	71
	DR	506	385	64	57
Mature kale Leaf	CON	160	59	28	73
	DR	176	70	63	43
Mature kale Wash	CON	664	509	106	49
	DR	630	490	90	50
Peaks detected in negative ionization mode					
Baby kale Leaf	CON	464	153	216	95
	DR	409	207	125	77
Baby kale Wash	CON	146	99	26	21
	DR	184	121	28	35
Mature kale Leaf	CON	475	245	111	119
	DR	377	205	102	70
Mature kale Wash	CON	329	154	127	48
	DR	300	180	66	54

Table 15. ANOSIM pairwise comparison of kale leaf extracts and kale leaf surfaces washes based on phytochemical profiling with ESI-TOF-MS under positive and negative modes. Leaf and was samples were collected from plant growth-promoting rhizobacterium *Pseudomonas* spp. S4 (PS4) or 0.1% PW (PW) inoculated baby plants (20 days old) under regular watering condition (CON) or irrigation withholding (DR) for 6 days.

Sample comparisons	Positive mode		Negative mode	
	R (*p* value)			
	Bray-Curtis	*Jaccard*	*Bray-Curtis*	*Jaccard*
Leaf tissue extracts				
PW 20D CON – PW 20D DR	0.01 (0.49)	0.09 (0.23)	0.08(0.23)	-0.02(0.60)
PS 20D CON – PS 20D DR	0.03 (0.46)	-0.02 (0.46)	*0.34(0.09)*	0.02(0.40)
PW 20D CON – PS 20D CON	0.25 (0.11)	*0.41 (0.09)*	**0.43(0.03)**	**0.37(0.03)**
PW 20D DR – PS 20D DR	0.18 (0.26)	0.08 (0.26)	0.26(0.11)	0.21(0.20)
Leaf surface washes				
PW 20D CON – PW 20D DR	**0.58 (0.03)**	*0.45 (0.06)*	*0.65(0.06)*	**0.60(0.03)**
PS 20D CON – PS 20D DR	0.17(0.17)	0.06 (0.34)	**0.47(0.03)**	**0.46(0.03)**
PW 20D CON – PS 20D CON	*0.30 (0.06)*	0.14 (0.11)	-0.02(0.29)	-0.10(0.69)
PW 20D DR – PS 20D DR	0.08 (0.29)	0.12 (0.20)	-0.07(0.69)	-0.02(0.60)

Table 16. ANOSIM pairwise comparison of mature kale leaf extracts and surfaces washes based on phytochemical profiling with ESI-TOF-MS under positive and negative modes. Mature kale inoculated with 0.1% PW (PW) or plant growth-promoting rhizobacterium *Pseudomonas* spp. S4 (PS4) were exposure to regular watering condition (CON) or irrigation withholding (DR) for 3 days. Bold and italic numbers in the table indicated *p* value smaller than 0.05 and 0.1, respectively.

Sample comparisons	Positive mode		Negative mode	
	R (*p* value)			
	Bray-Curtis	*Jaccard*	*Bray-Curtis*	*Jaccard*
Leaf tissue extracts				
PW 59D CON – PW 59D DR	**0.39 (0.03)**	**0.52 (0.03)**	0.13 (0.14)	*0.22 (0.06)*
PS 59D CON – PS 59D DR	-0.02 (0.49)	0.00 (0.40)	0.00 (0.49)	0.12 (0.17)
PW 59D CON –PS 59D CON	*0.51 (0.06)*	**0.58 (0.03)**	*0.25 (0.06)*	**0.29 (0.03)**
PW 59D DR – PS 59D DR	0.16 (0.26)	0.41 (0.14)	**0.34 (0.03)**	*0.20 (0.09)*
Leaf surface washes				
PW 59D CON – PW 20D DR	-0.21 (0.97)	-0.23 (0.98)	-0.05 (0.61)	0.00 (0.33)
PS 59D CON – PS 59D DR	-0.14 (0.90)	-0.15 (0.83)	0.22 (0.13)	0.11(0.20)
PW 59D CON –PS 59D CON	-0.10 (0.82)	-0.11 (0.87)	0.08 (0.21)	**0.32 (0.02)**
PW 59D DR – PS 59D DR	-0.06 (0.68)	0.02 (0.36)	-0.12 (0.78)	-0.02 (0.41)

6.3.7 Drought shifted the phytochemical profiles of baby kale leaves and washes

Baby kale leaf samples showed no differences in phytochemical profile under positive detection mode in response to drought treatment. Washes samples from non-PGPR treated baby kale, in contrast, showed phytochemical shifts after drought exposure (Table 15). Under negative detection mode, profiles of leaf tissues from PS4-treated kale samples separated (Bray-Curtis, $R = 0.34$, $p = 0.09$) but not PW-treated baby kale leaf samples. Drought shifts the washes profiles under negative mode for PW- and PS4-treated groups (Table 15).

6.3.8 Application of Pseudomonas spp. S4 had an impact on phytochemical profiles for mature kale samples

Overall phytochemical profiles of regularly watered mature kale leaf samples diverged, especially based on the Jaccard coefficient ($R= 0.58$, $p < 0.05$, Table 15) under positive mode. No differences in washes in response to *Pseudomonas* spp. S4 application were found under the same detection mode. The negative mode of ESI-TOF-MS revealed phytochemical shifts due to *Pseudomonas* spp. S4 for leaf tissues and washes for regularly watered mature kale samples (Table 15). Leaf tissues from drought-treated kale showed separation regarding root inoculation of PS4 under negative detection mode (Table 15).

6.3.9 Drought showed influences on phytochemical profiles of mature kale leaf tissue and washes

Pooling kale leaf and washes samples together, mature kale containing non-PGPR and PGPR-inoculated groups showed no difference in phytochemical profiles to drought under both detection modes. Drought drove the phytochemical profiles differences in leaf tissues but not in washes for mature kale without root inoculation of PGPR under both positive and negative

modes (Table 16). Convergences in PS4-inoculated mature kale leaf tissues and washes were reported under both positive and negative modes (Table 16).

6.4 Discussion

The influence of PGPR root inoculation on kale plant growth, phytochemical profile changes, and epiphytic growth of kale plants at two developing stages under drought treatment were studied in this research. Research results from this study revealed the application of *Pseudomonas* spp. S4 could promote kale plant growth under drought conditions and maintain the regular growth of kale plants under drought at all age stages. Regularly watered baby kale, a common ingredient in a raw salad, is highly implicated with *Salmonella*, which constitutes an extremely high risk to the public. Root inoculation of *Pseudomonas* spp. S4 reduced *Salmonella* population on baby kale surfaces and shifted the overall phytochemical profiles but showed no influence on phytochemical measurements of baby kale. Epiphytic growth of *Salmonella* was restricted for mature kale in response to root application of *Pseudomonas* spp. S4. Phytochemical measurements and overall phytochemical profiles of mature kale shift under the influence of *Pseudomonas* application. Drought treatment showed restrictive effects on *Salmonella* growth on the surface and in washes for both baby and mature kale, with altered phytochemical profiles. Results from this research provided evidence that the application of *Pseudomonas* spp. S4 could be a potential intervention strategy to promote plant growth under abiotic stresses and enhance produce safety during cultivation.

Results from this study showed that root inoculation of *Pseudomonas* spp. S4 showed no growth-promoting effect on kale plants under regular watering conditions. However, it enhanced plant biomass accumulation under drought treatment for baby kale and prevented the plant biomass reduction from drought for baby kale and mature kale. A sharp decline in *Salmonella* population was observed on both baby and mature kale surfaces with root inoculation of *Pseudomonas* spp. S4, but not *Pseudomonas* spp. S2 under regular watering conditions. No phytochemical measurement changes in leaf tissues, but differences in overall phytochemical profiles were reported in response to PS4 treatment for baby kale. These results suggested phytochemical changes other than secondary metabolites induced by *Pseudomonas* spp. S4 might be associated with the *Salmonella* restriction. No differences in *Salmonella* reduction on surface and leaf tissues phytochemical measurements and overall profiles in tissues of baby kale were found under drought treatment due to *Pseudomonas* application. These results suggest that drought overrode the *Salmonella* restriction effects on the surface of *Pseudomonas* spp. S4-treated kale. Age showed no disruption in *Pseudomonas*-induced *Salmonella* restriction. The lower decline of *Salmonella* was reported on the regularly watered mature kale but not under drought treatment in response to the application of *Pseudomonas* spp. S4. Higher accumulation of glucosinolates and altered overall phytochemical profiles were observed in PS4-inoculated mature kale under regular irrigation, suggesting phytochemicals shifts in mature kale are possibly associated with the *Salmonella* decline under regular watering conditions.

Drought reduced the population of *Salmonella* on baby kale surfaces with no presence of *Pseudomonas* spp, with enhanced antioxidant capacity and total phenolics accumulation. Growth of *Salmonella* in baby kale washes was restricted by drought, even with the root inoculation of *Pseudomonas* spp. After drought treatment, a higher antioxidant capacity, total phenolics, and glucosinolate contents were found for both PS2 and PS4 inoculated baby kale. These results inferred that drought treatments were able to alter secondary metabolites accumulations and restrict *Salmonella*. Regulated drought treatment in baby kale could be applied to enhance the health benefits of produce and boost food safety. Limited growth of *Salmonella* was found in PS4-treated mature kale washes due to drought treatment, but not in PW-treated mature kale washes, with no influences on phytochemicals measurements.

Similarity tests based on the overall phytochemical signals acquired from ESI-TOF-MS revealed distinct differences between kale leaf tissue and wash samples. The application of *Pseudomonas* spp. altered the leaf tissue and wash profiles of baby kale under regular watering conditions and mature kale under both irrigation conditions. Drought impacted the leaf tissue profile of PGPR-negative mature kale and the wash profile of baby kale for PGPR positive and negative treatment. These findings further reveal that the kale-*Salmonella* association could be manipulated by altering phytochemicals in leaf tissue due to abiotic (drought) stresses exposure or *Pseudomonas* spp. application.

Drought-induced kale shoot dry mass reduction was reported for both baby kale and mature kale. After keeping lettuce plants in controlled drought (30% of soil water holding capacity)

for 21 days, biomass reduction was reported for 25 days old lettuce plants (Galieni et al., 2015). A higher water depletion rate in soil under drought significantly reduced the biomass of thyme (Ashrafi et al., 2018). Along with plant biomass reduction, drought reduced the plant height and number of bare spikes (Rollins et al., 2013). *Pseudomonas* spp., one of the widely used plant growth-promoting rhizobacterial (PGPR), has long been applied to relieve plants from drought stress (Ma et al., 2011; Macleod et al., 2015). *Pseudomonas* spp. S2 and S4 involved in this study were firstly discovered in 1996 and showed the capacity to improve nodulation and nitrogen fixation of soybean from Canadian soil (Zhang et al., 1996). Growth-promoting effects of these two strains were further reported in soybean, spinach, Romaine lettuce, and selected tomato cultivars (Dashti et al., 2000; Hsu and Micallef, 2017). However, in our study, PGPR showed no enhanced growth on kale plants. This cultivar-specific growth-promoting property of *Pseudomonas* spp. S4 was also reported in other research systems. *Pseudomonas fluorescens* LBUM677 showed different growth and lipid accumulation promoting effects in three oilseed crops, including canola (*Brassica napus*) (Jiménez et al., 2020). Moreover, not all PGPR were able to promote the growth of plants under stress-free conditions (Vaishnav et al., 2020). Decreased dry mass accumulation in response to PGPR inoculation was stated (Abd El-Azeem et al., 2012). In our study, when baby kale plants were exposed to drought, *Pseudomonas* spp. S4 accumulated more dry mass. These growth-promoting effects in response to stresses were stated in several studies. PGPR-inoculated wheat plants showed no growth difference under regular watering conditions but increased biomass under drought compared to non-PGPR inoculated wheat plants (Timmusk et al., 2014).

In 2017, *Pseudomonas* spp. S4 was reported for *Salmonella* epiphytic restriction on spinach and tomato surfaces and *Pseudomonas* spp. S2 showed *Salmonella* growth restriction on spinach surfaces (Hsu and Micallef, 2017). According to our results, a further epiphytic decline of *Salmonella* was reported on regularly watered baby kale surfaces under the influence of PS4, with phytochemical profile changes detected under the negative mode of ESI-TOF-MS. The potential explanations of the reduced *Salmonella* fitness on baby kale were 1) physiological changes due to PGPR inoculation impacted the surface compounds composition and turned it into a less favorable environment for *Salmonella*; 2) altered microbial structure of plant surfaces in response to PS4 inoculation created a more competitive environment for *Salmonella* survival; 3) *Pseudomonas* spp. S4 elicited the induced systemic resistance (ISR) to protect baby kale from *Salmonella*. Firstly, *Pseudomonas fluorescens* were capable of thickening the upper and lower epidermis of tomato leaves (Kolomiiets et al., 2019). Leaf structural changes, including increased cuticle thickness due to PGPR inoculation, were reported in woody plants (Larraburu et al., 2010). This thick cuticle created an enhanced natural barrier from pathogen infection (Jenks et al., 2002) and prevented water loss (Kerstiens, 1996) and leaching of water-soluble compounds to the surface of plants, thus limiting the growth of epiphytes such as *Salmonella* with less readily available nutrients. In addition, the altered composition of surface compounds could impact the microbial communities on the plant surface (Lindow and Brandl, 2003), which might further restrict the growth of *Salmonella.* Microbiota favored from the physiological changes brought by *Pseudomonas* spp. inoculation could dominate the plant phyllosphere and inhibit *Salmonella* colonization (Hsu and Micallef, 2017). Secondly, this restriction might be related to the induced systemic resistance (ISR) brought by *Pseudomonas* spp. S4. ISR has

been widely depicted as an important indirect mechanism for PGPR to protect plants from pathogens. Root application of *Pseudomonas* spp. S4 primed tomato and spinach plants to pathogen challenges (Conrath et al., 2015; Conrath et al., 2002). However, *Salmonella*, usually not considered a plant pathogen, can be recognized by plants and induce plant host defenses. The flagellin of *Salmonella* was recognized as a Pathogen-associated molecular pathogen (PAMP) and resulted in pattern-triggered immune (PTI) of Arabidopsis plants (Garcia et al., 2014). The PTI response can, in turn, limit the growth of *Salmonella* (Meng et al., 2013). However, *Salmonella* has evolved its ability to improve fitness on the plant surface by manipulating the innate perception system of plants (Shirron and Yaron, 2011). *Salmonella* Typhimurium was able to suppress the innate response on the lettuce surface. With the help of ISR brought by PGPR *Bacillus subtilis*, increased stomatal closure rate and less *Salmonella* population were found on lettuce surfaces (Johnson et al., 2020). *Pseudomonas* spp. were reported to affect stomatal closure (Frommel et al., 1991). Thus, it is possible that the *Pseudomonas* spp. S4 included in this study induced the ISR by enforced stomata closure of the kale plant, resulting in more rapid suppression of *Salmonella*. The biological control agent *Bacillus* spray restricted the *Salmonella* population on the lettuce surface (Zhao et al., 2021). Biosurfactant-producing *Bacillus* demonstrated inhibition against *Salmonella* Newport on lettuce surfaces (Rani et al., 2020). *Pseudomonas* strains were also able to produce biosurfactants (Das and Kumar, 2016), which could limit *Salmonella* on produce surfaces. Future studies could stress the above hypotheses to further reveal the mechanism related to *Pseudomonas* spp.-induced *Salmonella* limitation on produce surface.

6.5 Conclusion

In summary, this study showed that *Pseudomonas* application promoted baby kale growth and prevented biomass loss due to drought treatment for both baby and mature kale. Root inoculation of *Pseudomonas* spp. to kale plants under two development stages was capable of enforcing *Salmonella* population reduction on surfaces and shifting phytochemical profiles. The *Pseudomonas-induced Salmonella* restrictive effects on produce surfaces might be associated with altered phytocompounds but not related to secondary metabolites. More studies should be conducted to illustrate phytocompounds and physiological changes in plants related to *Salmonella* inhibition and determine if ISR is involved in this inhibition. The findings of this study provided evidence that the root application of *Pseudomonas* spp. can be an effective farming practice to boost plant growth and limit *Salmonella* contamination during cultivation.

Chapter 7: Conclusion

Safe and nutritious food is an essential component for food security. Fresh produce provides essential nutrients and lowers the risks of chronic diseases, including cancer (Van Duyn and Pivonka, 2000). Increased consumption of fresh produce, particularly raw and leafy greens, is suggested as a restorative practice to maintain and optimize human health (Klerk et al., 1998; Van Duyn and Pivonka, 2000). However, leafy greens and raw produce have been highly associated with foodborne pathogens, causing foodborne illnesses and economic loss. Contamination of foodborne pathogens could happen at any stage from the farm to fork continuum and be impossible to remove once contaminated. An in-depth understanding of the association between leafy green and foodborne pathogens could provide insights into this complicated association and allow the more efficient restriction of foodborne pathogens on leafy greens.

In this study, the impact of plant cultivar and plant development, abiotic stresses, and plant growth-promoting rhizobacteria (*Pseudomonas* spp. S2 and S4) on *Salmonella* growth on plant surface and in washes was evaluated using lettuce and kale plants. Results provided evidence that plant cultivar, plant development, drought and salinity, and *Pseudomonas* spp. S2 and S4 can serve to restrict the *Salmonella* association on produce surfaces. Positive correlations between the *Salmonella* surface survival and growth in washes were identified, providing evidence that the epiphytic growth of *Salmonella* was affected by leafy green surface compounds. Specifically, Romaine lettuce and baby kale, when grown under regular watering condition, provided a more favorable environment for *Salmonella* than 'Mascara' lettuce and mature kale, possibly due to reduced levels of secondary metabolites of

Salmonella growth-limiting properties. Abiotic stresses including drought and salinity led to a more significant *Salmonella* population decline on leafy greens, potentially associated with enhanced accumulation of secondary metabolites and altered physiological traits. Inverse associations between the epiphytic growth of *Salmonella* and the contents of total flavonoids, phenolics in 'Mascara' lettuce and juvenile kale plants and anthocyanins in the lettuce plants. However, as leafy green plants develop during the vegetative stage, drought could not enhance *Salmonella* restriction on mature kale. This might lead to plant physiological changes, such as the advanced development of cuticle, which served as an enhanced physical barrier for *Salmonella* colonization on kale surface. Similarly, *Salmonella* surface restriction in response to root application of *Pseudomonas* spp. S4 was likely due to plant physiological changes and boosted plant immune system but not secondary metabolite accumulation in kale plants. In the future, more studies can be conducted to reveal the mechanisms regarding the Salmonella restrictive effects due to cultivar variability, in response to abiotic stresses including drought and salinity and induced by the root inoculation of *Pseudomonas* spp. Factors, other than the phytochemical profiles of leafy green tissues, could be included to study the *Salmonella*-leafy green association. Looking at the influence of plant surface openings stomata and physical barrier cuticle on the *Salmonella* association are vital for a more inclusive and thorough understanding.

This study provided a novel angle to understand the complex leafy green and *Salmonella* association from plant phytochemical profiles and investigated the potential of plant growth-promoting rhizobacteria (PGPR) in promoting plant growth and restricting *Salmonella*

epiphytic colonization. Results suggested that the *Salmonella* colonization on plant surface can be manipulated by changes in the phytochemical profiles of plant tissues. Accumulation of secondary metabolites, including total flavonoids and total phenolics in leafy greens were associated with lower survival of *Salmonella* on surface. Abiotic elicitation of secondary metabolites in plants is a promising strategy to enhance the safety of leafy greens. Regulated irrigation or other farming practices that could induce the accumulation of secondary metabolites could be applied during produce cultivation to reduce *Salmonella* fitness on produce surface. Future work should focus on identifying specific *Salmonella* growth-limiting compounds and elucidating their metabolic pathways for curbing *Salmonella* persistence during production. Screening of leafy green should be incorporated to identify cultivars that naturally accumulated higher levels of *Salmonella* growth-limiting compounds to minimize *Salmonella* contamination on produce during cultivation. Application of *Pseudomonas* spp. limited the *Salmonella* colonization under regular irrigation conditions and protected plant biomass reduction under drought stress. Though the mechanism for this *Pseudomonas*-induced *Salmonella* restriction remained unclear, this study pointed out the potential of including another tier to impair *Salmonella* association and boost plant growth during cultivation. As we face the pressure of climate fluctuation, our results can provide new directions to enhance plant resistance to *Salmonella* during production while boosting nutritional value and maintaining adequate yield.

Bibliography

Abd El-Azeem, S. A., Elwan, M. W., Sung, J.-K. & Ok, Y. S. (2012) Alleviation of salt stress in eggplant (*Solanum melongena* L.) by plant-growth-promoting rhizobacteria. *Communications in soil science and plant analysis,* 43(9)**,** 1303-1315.

Achuo, E. A., Prinsen, E. & Höfte, M. (2006) Influence of drought, salt stress and abscisic acid on the resistance of tomato to *Botrytis cinerea* and Oidium neolycopersici. *Plant Pathology,* 55(2)**,** 178-186. 10.1111/j.1365-3059.2006.01340.x.

Agaras, B. C., Wall, L. G. & Valverde, C. (2014) Influence of agricultural practices and seasons on the abundance and community structure of culturable pseudomonads in soils under no-till management in Argentina. *Plant and Soil,* 382(1)**,** 117-131. 10.1007/s11104-014-2095-8.

Aghajanzadeh, T., Reich, M., Kopriva, S. & De Kok, L. (2018) Impact of chloride (NaCl, KCl) and sulphate (Na2SO4, K2SO4) salinity on glucosinolate metabolism in *Brassica rapa*. *Journal of Agronomy and Crop Science,* 204(2)**,** 137-146.

Ahmadi, F. I., Karimi, K. & Struik, P. C. (2018) Effect of exogenous application of methyl jasmonate on physiological and biochemical characteristics of *Brassica napus* L. cv. Talaye under salinity stress. *South African Journal of Botany,* 115**,** 5-11. https://doi.org/10.1016/j.sajb.2017.11.018.

Al-Maskri, A., Al-Kharusi, L., Al-Miqbali, H. & Khan, M. M. (2010) Effects of salinity stress on growth of lettuce (*Lactuca sativa*) under closed-recycle nutrient film technique. *International Journal of Agriculture and Biology,* 12(3)**,** 377-380.

Allard, S. M., Callahan, M. T., Bui, A., Ferelli, A. M. C., Chopyk, J., Chattopadhyay, S., Mongodin, E. F., Micallef, S. A. & Sapkota, A. R. (2019) Creek to table: tracking fecal indicator bacteria, bacterial pathogens, and total bacterial communities from irrigation water to kale and radish crops. *Science of The Total Environment,* 666**,** 461-471.

Allende, A., McEvoy, J. L., Luo, Y., Artes, F. & Wang, C. Y. (2006) Effectiveness of two-sided UV-C treatments in inhibiting natural microflora and extending the shelf-life of minimally processed 'Red Oak Leaf' lettuce. *Food Microbiology,* 23(3)**,** 241-249. https://doi.org/10.1016/j.fm.2005.04.009.

Almaghrabi, O. A., Massoud, S. I. & Abdelmoneim, T. S. (2013) Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato

plant growth and nematode reproduction under greenhouse conditions. *Saudi J Biol Sci,* 20(1)**,** 57-61. 10.1016/j.sjbs.2012.10.004.

Angelo, K. M., Chu, A., Anand, M., Nguyen, T.-A., Bottichio, L., Wise, M., Williams, I., Seelman, S., Bell, R. & Fatica, M. (2015) Outbreak of *Salmonella* Newport infections linked to cucumbers—United States, 2014. *MMWR. Morbidity and mortality weekly report,* 64(6)**,** 144.

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J. & Leon, P. (2000) Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. *Genes & Development,* 14(16)**,** 2085-2096.

Arif, Y., Singh, P., Siddiqui, H., Bajguz, A. & Hayat, S. (2020) Salinity induced physiological and biochemical changes in plants: An omic approach towards salt stress tolerance. *Plant Physiology and Biochemistry,* 156**,** 64-77.

Ashrafi, M., Azimi-Moqadam, M.-R., Moradi, P., MohseniFard, E., Shekari, F. & Kompany-Zareh, M. (2018) Effect of drought stress on metabolite adjustments in drought tolerant and sensitive thyme. *Plant Physiology and Biochemistry,* 132**,** 391-399.

Azevedo Neto, A. D. d., Prisco, J. T., Enéas-Filho, J., Lacerda, C. F. d., Silva, J. V., Costa, P. H. A. d. & Gomes-Filho, E. (2004) Effects of salt stress on plant growth, stomatal response and solute accumulation of different maize genotypes. *Brazilian Journal of Plant Physiology,* 16**,** 31-38.

Bachiega, P., Salgado, J. M., de Carvalho, J. E., Ruiz, A. L. T. G., Schwarz, K., Tezotto, T. & Morzelle, M. C. (2016) Antioxidant and antiproliferative activities in different maturation stages of broccoli (*Brassica oleracea* Italica) biofortified with selenium. *Food Chemistry,* 190**,** 771-776. https://doi.org/10.1016/j.foodchem.2015.06.024.

Barickman, T. C., Ku, K.-M. & Sams, C. E. (2020) Differing precision irrigation thresholds for kale (*Brassica oleracea* L. var. *acephala*) induces changes in physiological performance, metabolites, and yield. *Environmental and Experimental Botany,* 180**,** 104253. https://doi.org/10.1016/j.envexpbot.2020.104253.

Bates, L. S., Waldren, R. P. & Teare, I. (1973) Rapid determination of free proline for water-stress studies. *Plant and soil,* 39(1)**,** 205-207.

Beattie, G. A. & Lindow, S. E. (1995) The Secret Life of Foliar Bacterial Pathogens on Leaves. *Annual Review of Phytopathology,* 33(1)**,** 145-172. 10.1146/annurev.py.33.090195.001045.

Bechtold, E. K., Ryan, S., Moughan, S. E., Ranjan, R. & Nüsslein, K. (2021) Phyllosphere Community Assembly and Response to Drought Stress on Common Tropical and Temperate Forage Grasses. *Appl Environ Microbiol,* 87(17)**,** e0089521. 10.1128/aem.00895-21.

Beneduzi, A., Ambrosini, A. & Passaglia, L. M. P. (2012) Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genetics and molecular biology,* 35(4 (suppl))**,** 1044-1051.

Bennett, S. D., Littrell, K. W., Hill, T. A., Mahovic, M. & Behravesh, C. B. (2015) Multistate foodborne disease outbreaks associated with raw tomatoes, United States, 1990–2010: a recurring public health problem. *Epidemiology and Infection,* 143(7)**,** 1352-1359. 10.1017/S0950268814002167.

Bennett, S. D., Sodha, S. V., Ayers, T. L., Lynch, M. F., Gould, L. H. & Tauxe, R. V. (2018) Produce-associated foodborne disease outbreaks, USA, 1998–2013. *Epidemiology and Infection,* 146(11)**,** 1397-1406. 10.1017/S0950268818001620.

Berger, C. N., Shaw, R. K., Brown, D. J., Mather, H., Clare, S., Dougan, G., Pallen, M. J. & Frankel, G. (2009) Interaction of *Salmonella enterica* with basil and other salad leaves. *Isme j,* 3(2)**,** 261-5. 10.1038/ismej.2008.95.

Bernstein, N., Sela , S., Dudai, N. & Gorbatsevich, E. (2017) Salinity Stress Does Not Affect Root Uptake, Dissemination and Persistence of Salmonella in Sweet-basil (*Ocimum basilicum*). *Frontiers in Plant Science,* 8. 10.3389/fpls.2017.00675.

Beuchat, L. R. (2002) Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes and Infection,* 4(4)**,** 413-423. https://doi.org/10.1016/S1286-4579(02)01555-1.

Bhattacharyya, P. N. & Jha, D. K. (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology,* 28(4)**,** 1327-1350.

Bistgani, Z. E., Hashemi, M., DaCosta, M., Craker, L., Maggi, F. & Morshedloo, M. R. (2019) Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of *Thymus vulgaris* L. and *Thymus daenensis* Celak. *Industrial Crops and Products,* 135**,** 311-320. https://doi.org/10.1016/j.indcrop.2019.04.055.

Blodgett, J., Kruger, E. & Stanosz, G. (1997) Sphaeropsis sapinea and water stress in a red pine plantation in central Wisconsin. *Phytopathology,* 87(4)**,** 429-434.

Brandl, M. T. & Amundson, R. (2008) Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *Appl Environ Microbiol,* 74(8)**,** 2298-306. 10.1128/aem.02459-07.

Brandl, M. T. & Mandrell, R. E. (2002) Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Appl Environ Microbiol,* 68(7)**,** 3614-21. 10.1128/aem.68.7.3614-3621.2002.

Bresson, J., Varoquaux, F., Bontpart, T., Touraine, B. & Vile, D. (2013) The PGPR strain *Phyllobacterium brassicacearum* STM196 induces a reproductive delay and physiological changes that result in improved drought tolerance in Arabidopsis. *New Phytol,* 200(2)**,** 558-69. 10.1111/nph.12383.

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page, T. & Pink, D. (2003) The molecular analysis of leaf senescence – a genomics approach. *Plant Biotechnology Journal,* 1(1)**,** 3-22. 10.1046/j.1467-7652.2003.00004.x.

Cabello, P., Agüera, E. & De La Haba, P. (2006) Metabolic changes during natural ageing in sunflower (Helianthus annuus) leaves: expression and activity of glutamine synthetase isoforms are regulated differently during senescence. *Physiologia Plantarum,* 128(1)**,** 175-185. 10.1111/j.1399-3054.2006.00722.x.

Cabot, C., Bosch, R., Martos, S., Poschenrieder, C. & Perelló, A. (2018) Salinity is a prevailing factor for amelioration of wheat blast by biocontrol agents. *Biological Control,* 125**,** 81-89. https://doi.org/10.1016/j.biocontrol.2018.07.003.

Callahan, M. T. & Micallef, S. A. (2019) Waxing and cultivar affect *Salmonella enterica* persistence on cucumber (*Cucumis sativus* L.) fruit. *International Journal of Food Microbiology,* 310**,** 108359. https://doi.org/10.1016/j.ijfoodmicro.2019.108359.

Callejón, R. M., Rodríguez-Naranjo, M. I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. C. & Troncoso, A. M. (2015a) Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathog Dis,* 12(1)**,** 32-8. 10.1089/fpd.2014.1821.

Callejón, R. M., Rodríguez-Naranjo, M. I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. C. & Troncoso, A. M. (2015b) Reported Foodborne Outbreaks Due to Fresh Produce in the United States and European Union: Trends and Causes. *Foodborne Pathogens and Disease,* 12(1)**,** 32-38. 10.1089/fpd.2014.1821.

CDC Centers for Disease Control and Prevention. List of Selected Multistate Foodborne Outbreak Investigations. Available online:

https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html (accessed on 22 April 2022).

CDC (2008) Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items--United States, 2008. *MMWR. Morbidity and mortality weekly report,* 57(34)**,** 929-934.

Cominelli, E., Galbiati, M., Tonelli, C. & Bowler, C. (2009) Water: the invisible problem: access to fresh water is considered to be a universal and free human right, but dwindling resources and a burgeoning population are increasing its economic value. *EMBO reports,* 10(7)**,** 671-676.

Conrath, U., Beckers, G. J. M., Langenbach, C. J. G. & Jaskiewicz, M. R. (2015) Priming for Enhanced Defense. *Annual Review of Phytopathology,* 53(1)**,** 97-119. 10.1146/annurev-phyto-080614-120132.

Conrath, U., Pieterse, C. M. J. & Mauch-Mani, B. (2002) Priming in plant–pathogen interactions. *Trends in Plant Science,* 7(5)**,** 210-216. https://doi.org/10.1016/S1360-1385(02)02244-6.

Corpe, W. A. & Rheem, S. (1989) Ecology of the methylotrophic bacteria on living leaf surfaces. *Fems Microbiology Ecology,* 62(4)**,** 243-249. 10.1016/0378-1097(89)90248-6.

Critzer, F. J. & Doyle, M. P. (2010) Microbial ecology of foodborne pathogens associated with produce. *Current Opinion in Biotechnology,* 21(2)**,** 125-130. https://doi.org/10.1016/j.copbio.2010.01.006.

Das, A. J. & Kumar, R. (2016) Bioremediation of petroleum contaminated soil to combat toxicity on *Withania somnifera* through seed priming with biosurfactant producing plant growth promoting rhizobacteria. *Journal of Environmental Management,* 174**,** 79-86. https://doi.org/10.1016/j.jenvman.2016.01.031.

Dashti, N., Prithiviraj, B., Hynes, R. & Smith, D. (2000) Root and Rhizosphere Colonization of Soybean [*Glycine max* (L.) Merr.] by Plant-Growth-Promoting Rhizobacteria at Low Root Zone Temperatures and under Short-Season Conditions. *Journal of Agronomy and Crop Science,* 185(1)**,** 15-22.

de Moraes, M. H., Desai, P., Porwollik, S., Canals, R., Perez, D. R., Chu, W., McClelland, M. & Teplitski, M. (2017) *Salmonella* persistence in tomatoes requires a distinct set of metabolic functions identified by transposon insertion sequencing. *Applied and environmental microbiology,* 83(5).

Desikan, R., Mackerness, S. A. H., Hancock, J. T. & Neill, S. J. (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. *Plant Physiology,* 127(1)**,** 159-172. 10.1104/pp.127.1.159.

Desk, N. (2019) Government's *Listeria* test causes recall of Eat Smart kale bagged salad kits. *Food safety News*.

Dev Kumar, G., Macarisin, D. & Micallef, S. A. (2019) *Salmonella enterica* Filamentation Induced by Pelargonic Acid Is a Transient Morphotype. *Applied and Environmental Microbiology,* 85(2)**,** e02191-18. 10.1128/AEM.02191-18.

Dev Kumar, G. & Micallef, S. A. (2017) Susceptibility of *Salmonella enterica* Isolates from Tomato Farm Environments to Fatty Acids Naturally Found on Tomato Fruit. *Foodborne Pathogens and Disease,* 14(5)**,** 293-301. 10.1089/fpd.2016.2239.

Diaz, C., Purdy, S., Christ, A., Morot-Gaudry, J.-F., Wingler, A. & Masclaux-Daubresse, C. (2005) Characterization of Markers to Determine the Extent and Variability of Leaf Senescence in Arabidopsis. A Metabolic Profiling Approach. *Plant Physiology,* 138(2)**,** 898. 10.1104/pp.105.060764.

Dietz, K. J., Zörb, C. & Geilfus, C. M. (2021) Drought and crop yield. *Plant Biology,* 23(6)**,** 881-893. https://doi.org/10.1111/plb.13304.

Dudonné, S., Vitrac, X., Coutière, P., Woillez, M. & Mérillon, J.-M. (2009) Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *Journal of Agricultural and Food Chemistry,* 57(5)**,** 1768-1774. 10.1021/jf803011r.

Duijff, B. J., Gianinazzi-Pearson, V. & Lemanceau, P. (1997) Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. *The New Phytologist,* 135(2)**,** 325-334.

Eek, S. J. & Zhang, W. (2017) Optimal Treatment Strategies of Drought and UV-B Stresses before Harvest for Promoting Antioxidants Accumulation in Kale Grown in Plant Factories.

Eforn, B. (1979) Bootstrap methods: another look at the jackknife. *The Annals of Statistics,* 7**,** 1-26.

El Ghazali, G. E. (2020) Suaeda vermiculata Forssk. ex JF Gmel.: Structural Characteristics and Adaptations to Salinity and Drought: A Review. *International Journal of Sciences,* 9(02)**,** 28-33.

EPA (2016) What Climate Change Means for Maryland.

Eraslan, F., Inal, A., Savasturk, O. & Gunes, A. (2007) Changes in antioxidative system and membrane damage of lettuce in response to salinity and boron toxicity. *Scientia Horticulturae,* 114(1)**,** 5-10.

Eriksen, R. L., Knepper, C., Cahn, M. D. & Mou, B. (2016) Screening of Lettuce Germplasm for Agronomic Traits under Low Water Conditions. *HortScience,* 51(6)**,** 669-679.

ERS (2020) Economic Research Service (ERS), U.S. Department of Agriculture (USDA). Food Availability (Per Capita) Data System. Vegetable (fresh). https://www.ers.usda.gov/data-products/food-availability-per-capita-data-system/ Last updated: September 23, 2020.

Farooq, M., Hussain, M., Wahid, A. & Siddique, K. (2012) Drought stress in plants: an overview. In: *Plant responses to drought stress.* Springer.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. & Basra, S. M. A. (2009) Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable Development,* 29(1)**,** 185-212. 10.1051/agro:2008021.

FDA (2019) Investigation Summary: Factors Potentially Contributing to theContamination of Romaine Lettuce Implicated in the Fall 2018 Multi-State Outbreak of E. coli O157:H7.

Ferelli, A. M. C., Bolten, S., Szczesny, B. & Micallef, S. A. (2020) *Salmonella enterica* Elicits and Is Restricted by Nitric Oxide and Reactive Oxygen Species on Tomato. *Frontiers in Microbiology,* 11(391). 10.3389/fmicb.2020.00391.

Finkelstein, R. R. & Gibson, S. I. (2002) ABA and sugar interactions regulating development: cross-talk or voices in a crowd? *Current Opinion in Plant Biology,* 5(1)**,** 26-32. 10.1016/s1369-5266(01)00225-4.

Flexas, J. & Medrano, H. (2002) Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Ann Bot,* 89(2)**,** 183-9. 10.1093/aob/mcf027.

Francisco, M., Tortosa, M., del Carmen Martinez-Ballesta, M., Velasco, P. & Garcia-Viguera, C. (2017) Nutritional and phytochemical value of *Brassica* crops from the agri-food perspective. *Annals of Applied Biology,* 170(2)**,** 273-285.

Franco-Zorrilla, J. M., Martin, A. C., Leyva, A. & Par-Ares, J. P. (2005) Interaction between phosphate-starvation, sugar, and cytokinin signaling in arabidopsis and the roles of cytokinin receptors CRE1/AHK4 and AHK3. *Plant Physiology,* 138(2)**,** 847-857. 10.1104/pp.105.060517.

Frommel, M. I., Nowak, J. & Lazarovits, G. (1991) Growth enhancement and developmental modifications of *in vitro* grown potato (*Solanum tuberosum* spp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. *Plant physiology,* 96(3)**,** 928-936.

Galieni, A., Di Mattia, C., De Gregorio, M., Speca, S., Mastrocola, D., Pisante, M. & Stagnari, F. (2015) Effects of nutrient deficiency and abiotic environmental stresses on yield, phenolic compounds and antiradical activity in lettuce (*Lactuca sativa* L.). *Scientia Horticulturae,* 187**,** 93-101.

Gan, S. & Amasino, R. M. (1997) Making Sense of Senescence (Molecular Genetic Regulation and Manipulation of Leaf Senescence). *Plant Physiol,* 113(2)**,** 313-319.

Garcia, A. V., Charrier, A., Schikora, A., Bigeard, J., Pateyron, S., de Tauzia-Moreau, M.-L., Evrard, A., Mithöfer, A., Martin-Magniette, M. L., Virlogeux-Payant, I. & Hirt, H. (2014) *Salmonella enteric* Flagellin Is Recognized via FLS2 and Activates PAMP-Triggered Immunity in *Arabidopsis thaliana*. *Molecular Plant,* 7(4)**,** 657-674. 10.1093/mp/sst145.

García-Fraile, P., Menéndez, E. & Rivas, R. (2015) Role of bacterial biofertilizers in agriculture and forestry.

Ge, C., Lee, C. & Lee, J. (2012) The impact of extreme weather events on *Salmonella* internalization in lettuce and green onion. *Food Research International,* 45(2)**,** 1118-1122. https://doi.org/10.1016/j.foodres.2011.06.054.

George, A. S. & Brandl, M. T. (2021) Plant Bioactive Compounds as an Intrinsic and Sustainable Tool to Enhance the Microbial Safety of Crops. *Microorganisms,* 9(12). 10.3390/microorganisms9122485.

Gharibi, S., Sayed Tabatabaei, B. E., Saeidi, G., Talebi, M. & Matkowski, A. (2019) The effect of drought stress on polyphenolic compounds and expression of flavonoid biosynthesis related genes in *Achillea pachycephala* Rech.f. *Phytochemistry,* 162**,** 90-98. https://doi.org/10.1016/j.phytochem.2019.03.004.

Ghorbanpour, M., Hatami, M. & Khavazi, K. (2013) Role of plant growth promoting rhizobacteria on antioxidant enzyme activities and tropane alkaloid production of *Hyoscyamus niger* under water deficit stress. *Turkish Journal of Biology,* 37(3)**,** 350-360.

Glick, B. R. (1995) The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology,* 41(2)**,** 109-117. 10.1139/m95-015.

Good, A. G. & Zaplachinski, S. T. (1994) The effects of drought stress on free amino acid accumulation and protein synthesis in *Brassica napus*. *Physiologia plantarum,* 90(1)**,** 9-14.

Goodburn, C. & Wallace, C. A. (2013) The microbiological efficacy of decontamination methodologies for fresh produce: a review. *Food Control,* 32(2)**,** 418-427.

Goudeau, D. M., Parker, C. T., Zhou, Y. G., Sela, S., Kroupitski, Y. & Brandl, M. T. (2013) The *Salmonella* Transcriptome in Lettuce and Cilantro Soft Rot Reveals a Niche Overlap with the Animal Host Intestine. *Applied and Environmental Microbiology,* 79(1)**,** 250-262. 10.1128/aem.02290-12.

Greene, S. K., Daly, E. R., Talbot, E. A., Demma, L. J., Holzbauer, S., Patel, N. J., Hill, T. A., Walderhaug, M. O., Hoekstra, R. M., Lynch, M. F. & Painter, J. A. (2008) Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. *Epidemiol Infect,* 136(2)**,** 157-65. 10.1017/s095026880700859x.

Guiboileau, A., Sormani, R., Meyer, C. & Masclaux-Daubresse, C. (2010) Senescence and death of plant organs: Nutrient recycling and developmental regulation. *Comptes Rendus Biologies,* 333(4)**,** 382-391. https://doi.org/10.1016/j.crvi.2010.01.016.

Gutnick, D., Calvo, J. M., Klopotowski, T. & Ames, B. N. (1969) Compounds which serve as the sole source of carbon or nitrogen for *Salmonella* typhimurium LT-2. *Journal of bacteriology,* 100(1)**,** 215-219.

Hagen, S. F., Borge, G. I. A., Solhaug, K. A. & Bengtsson, G. B. (2009) Effect of cold storage and harvest date on bioactive compounds in curly kale (*Brassica oleracea* L. var. *acephala*). *Postharvest Biology and Technology,* 51(1)**,** 36-42. https://doi.org/10.1016/j.postharvbio.2008.04.001.

Han, H. & Lee, K. (2005) Plant growth promoting rhizobacteria effect on antioxidant status, photosynthesis, mineral uptake and growth of lettuce under soil salinity. *Res J Agric Biol Sci,* 1(3)**,** 210-215.

Han, S., Ferelli, A. M. C., Lin, S.-S. & Micallef, S. A. (2020) Stress response, amino acid biosynthesis and pathogenesis genes expressed in *Salmonella enterica* colonizing tomato shoot and root surfaces. *Heliyon,* 6(9)**,** e04952. https://doi.org/10.1016/j.heliyon.2020.e04952.

Han, S. & Micallef, S. A. (2014) *Salmonella* Newport and Typhimurium Colonization of Fruit Differs from Leaves in Various Tomato Cultivars. *Journal of Food Protection,* 77(11)**,** 1844-1850. 10.4315/0362-028X.JFP-13-562.

Han, S. & Micallef, S. A. (2016) Environmental Metabolomics of the Tomato Plant Surface Provides Insights on *Salmonella enterica* Colonization. *Applied and Environmental Microbiology,* 82(10)**,** 3131-3142. 10.1128/aem.00435-16.

Hardwick, K., Wood, M. & Woolhouse, H. (1968) Photosynthesis and respiration in relation to leaf age in Perilla frutescens (L.) Britt. *New Phytologist,* 67(1)**,** 79-86.

Harris, L., Farber, J., Beuchat, L., Parish, M., Suslow, T., Garrett, E. & Busta, F. (2003) Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh‐cut produce. *Comprehensive reviews in food science and food safety,* 2**,** 78-141.

Hasegawa, P. M., Bressan, R. A., Zhu, J. K. & Bohnert, H. J. (2000) Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology,* 51**,** 463-499. 10.1146/annurev.arplant.51.1.463.

Herman, K. M., Hall, A. J. & Gould, L. H. (2015) Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. *Epidemiology and infection,* 143(14)**,** 3011-3021. 10.1017/S0950268815000047.

Himelblau, E. & Amasino, R. M. (2001) Nutrients mobilized from leaves of Arabidopsis thaliana during leaf senescence. *Journal of Plant Physiology,* 158(10)**,** 1317-1323.

Hirano, S. S. & Upper, C. D. (1990a) Population biology and epidemiology of *Pseudomonas syringae*. *Annual review of phytopathology,* 28(1)**,** 155-177.

Hirano, S. S. & Upper, C. D. (1990b) Population biology and epidemiology of *Pseudomonas-syringe. Annual Review of Phytopathology,* 28**,** 155-177. 10.1146/annurev.py.28.090190.001103.

Hirano, S. S. & Upper, C. D. (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a pathogen, ice nucleus, and epiphyte. *Microbiology and molecular biology reviews,* 64(3)**,** 624-653.

Hoffman, S., Maculloch, B. & Batz, M. (2015) Economic burden of major foodborne illnesses acquired in the United States, EIB-140. *U.S. Department of Agriculture, Economic Research Service*.

Hsu, C. K. & Micallef, S. A. (2017) Plant-mediated restriction of *Salmonella enterica* on tomato and spinach leaves colonized with *Pseudomonas* plant growth-promoting rhizobacteria. *Int J Food Microbiol,* 259**,** 1-6. 10.1016/j.ijfoodmicro.2017.07.012.

Huang, J., Hirji, R., Adam, L., Rozwadowski, K. L., Hammerlindl, J. K., Keller, W. A. & Selvaraj, G. (2000) Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant physiology,* 122(3)**,** 747-756. 10.1104/pp.122.3.747.

Hunter, P. J., Hand, P., Pink, D., Whipps, J. M. & Bending, G. D. (2010) Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca* species) phyllosphere. *Applied and environmental microbiology,* 76(24)**,** 8117-8125.

Hurek, T. & Reinhold-Hurek, B. (2003) *Azoarcus* sp. strain BH72 as a model for nitrogen-fixing grass endophytes. *J Biotechnol,* 106(2-3)**,** 169-78.

IFSAC (2021) Foodborne illness source attribution estimates for 2019 for *Salmonella, Escherichia coli* O157, *Listeria monocytogenes,* and *Campylobacter* using multi-year outbreak surveillance data, United States. *Interagency Food Safety Analytics Collaboration. GA and D.C.: U.S. Department of Health and Human Services' Centers for Disease Control and Prevention and U.S. Food and Drug Administration, U.S. Department of Agriculture's Food Safety and Inspection Service.*

Issarakraisila, M., Ma, Q. & Turner, D. W. (2007) Photosynthetic and growth responses of juvenile Chinese kale (*Brassica oleracea* var. alboglabra) and Caisin (*Brassica rapa* subsp. parachinensis) to waterlogging and water deficit. *Scientia Horticulturae,* 111(2)**,** 107-113. https://doi.org/10.1016/j.scienta.2006.10.017.

Jenks, M. A., Eigenbrode, S. D. & Lemieux, B. (2002) Cuticular waxes of Arabidopsis. *The Arabidopsis Book/American Society of Plant Biologists,* 1.

Jeon, J.-S., Carreno-Quintero, N., van Eekelen, H. D., De Vos, R. C., Raaijmakers, J. M. & Etalo, D. W. (2021) Impact of root-associated strains of three *Paraburkholderia* species on primary and secondary metabolism of *Brassica oleracea*. *Scientific reports,* 11(1)**,** 1-14.

Jha, Y., Subramanian, R. & Patel, S. (2011) Combination of endophytic and rhizospheric plant growth promoting rhizobacteria in *Oryza sativa* shows higher accumulation of osmoprotectant against saline stress. *Acta Physiologiae Plantarum,* 33(3)**,** 797-802.

Jiménez, J. A., Novinscak, A. & Filion, M. (2020) *Pseudomonas fluorescens* LBUM677 differentially increases plant biomass, total oil content and lipid composition in three oilseed crops. *J Appl Microbiol,* 128(4)**,** 1119-1127. 10.1111/jam.14536.

Johnson, N., Litt, P. K., Kniel, K. E. & Bais, H. (2020) Evasion of Plant Innate Defense Response by *Salmonella* on Lettuce. *Frontiers in Microbiology,* 11. 10.3389/fmicb.2020.00500.

Jones, R. B., Faragher, J. D. & Winkler, S. (2006) A review of the influence of postharvest treatments on quality and glucosinolate content in broccoli (*Brassica oleracea* var. *italica*) heads. *Postharvest Biology and Technology,* 41(1)**,** 1-8.

Jongebloed, U., Szederkényi, J., Hartig, K., Schobert, C. & Komor, E. (2004) Sequence of morphological and physiological events during natural ageing and senescence of a castor bean leaf: sieve tube occlusion and carbohydrate

back-up precede chlorophyll degradation. *Physiologia Plantarum,* 120(2)**,** 338-346. 10.1111/j.0031-9317.2004.0245.x.

Kang, S.-M., Radhakrishnan, R., Khan, A. L., Kim, M.-J., Park, J.-M., Kim, B.-R., Shin, D.-H. & Lee, I.-J. (2014) Gibberellin secreting rhizobacterium, *Pseudomonas putida* H-2-3 modulates the hormonal and stress physiology of soybean to improve the plant growth under saline and drought conditions. *Plant Physiology and Biochemistry,* 84**,** 115-124. https://doi.org/10.1016/j.plaphy.2014.09.001.

Kang, S. G., Price, J., Lin, P. C., Hong, J. C. & Jang, J. C. (2010) The Arabidopsis bZIP1 Transcription Factor Is Involved in Sugar Signaling, Protein Networking, and DNA Binding. *Molecular Plant,* 3(2)**,** 361-373. 10.1093/mp/ssp115.

Kao, F.-J., Chiu, Y.-S. & Chiang, W.-D. (2014) Effect of water cooking on antioxidant capacity of carotenoid-rich vegetables in Taiwan. *Journal of Food and Drug Analysis,* 22(2)**,** 202-209. https://doi.org/10.1016/j.jfda.2013.09.010.

Kaushal, S. S., Likens, G. E., Pace, M. L., Utz, R. M., Haq, S., Gorman, J. & Grese, M. (2018) Freshwater salinization syndrome on a continental scale. *Proceedings of the National Academy of Sciences,* 115(4)**,** E574-E583.

Kavi Kishor, P. B. & Sreenivasulu, N. (2014) Is proline accumulation *per se* correlated with stress tolerance or is proline homeostasis a more critical issue? *Plant, Cell & Environment,* 37(2)**,** 300-311. https://doi.org/10.1111/pce.12157.

Kerstiens, G. (1996) Signalling across the divide: a wider perspective of cuticular structure—function relationships. *Trends in plant science,* 1(4)**,** 125-129.

Ketnawa, S., Suwannachot, J. & Ogawa, Y. (2020) *In vitro* gastrointestinal digestion of crisphead lettuce: Changes in bioactive compounds and antioxidant potential. *Food Chemistry,* 311**,** 125885. https://doi.org/10.1016/j.foodchem.2019.125885.

Khalid, A., Arshad, M. & Zahir, Z. A. (2004) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *J Appl Microbiol,* 96(3)**,** 473-80.

Khan, M., Ulrichs, C. & Mewis, I. (2011) Effect of water stress and aphid herbivory on flavonoids in broccoli (*Brassica oleracea* var. *italica* Plenck). *J. Appl. Bot. Food Qual,* 84**,** 178-182.

Khan, N., Bano, A., Shahid, M. A., Nasim, W. & Babar, M. A. (2018) Interaction between PGPR and PGR for water conservation and plant growth attributes under drought condition. *Biologia***,** 1-16.

Khosravi, A., Zarei, M. & Ronaghi, A. (2018) Effect of PGPR, Phosphate sources and vermicompost on growth and nutrients uptake by lettuce in a calcareous soil. *Journal of Plant Nutrition,* 41(1)**,** 80-89. 10.1080/01904167.2017.1381727.

Kim, M. J., Moon, Y., Tou, J. C., Mou, B. & Waterland, N. L. (2016) Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). *Journal of Food Composition and Analysis,* 49**,** 19-34. https://doi.org/10.1016/j.jfca.2016.03.004.

Kim Moo, J., Chiu, Y.-C. & Ku, K.-M. (2017) Glucosinolates, Carotenoids, and Vitamins E and K Variation from Selected Kale and Collard Cultivars. *Journal of Food Quality*.

Klerk, M., Jansen, M., Van't Veer, P. & Kok, F. (1998) Fruits and vegetables in chronic disease prevention.

Knight, H. & Knight, M. R. (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends in Plant Science,* 6(6)**,** 262-267. 10.1016/s1360-1385(01)01946-x.

Kohler, J., Hernández, J. A., Caravaca, F. & Roldán, A. (2009) Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. *Environmental and Experimental Botany,* 65(2)**,** 245-252. https://doi.org/10.1016/j.envexpbot.2008.09.008.

Kolomiiets, Y., Grygoryuk, I., Likhanov, A., Butsenko, L. & Blume, Y. (2019) Induction of bacterial canker resistance in tomato plants using plant growth promoting rhizobacteria. *The Open Agriculture Journal,* 13(1).

Korus, A. (2011) Level of vitamin C, polyphenols, and antioxidant and enzymatic activity in three varieties of kale (*Brassica oleracea* L. var. *Acephala*) at different stages of maturity. *International Journal of Food Properties,* 14(5)**,** 1069-1080.

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D. & Sela, S. (2009a) Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl Environ Microbiol,* 75(19)**,** 6076-86. 10.1128/aem.01084-09.

Kroupitski, Y., Pinto, R., Belausov, E. & Sela, S. (2011) Distribution of *Salmonella* typhimurium in romaine lettuce leaves. *Food microbiology,* 28(5)**,** 990-997.

Kroupitski, Y., Pinto, R., Brandl, M., Belausov, E. & Sela, S. (2009b) Interactions of *Salmonella enterica* with lettuce leaves. *Journal of applied microbiology,* 106(6)**,** 1876-1885.

Kröger, C. & Fuchs, T. M. (2009) Characterization of the *myo*-inositol utilization island of *Salmonella enterica* serovar Typhimurium. *Journal of bacteriology,* 191(2)**,** 545-554.

Kubi, H. A., Khan, M. A., Adhikari, A., Imran, M., Kang, S.-M., Hamayun, M. & Lee, I.-J. (2021) Silicon and Plant Growth-Promoting Rhizobacteria *Pseudomonas psychrotolerans* CS51 Mitigates Salt Stress in *Zea* mays *L*. *Agriculture,* 11(3). 10.3390/agriculture11030272.

Kusano, M., Tohge, T., Fukushima, A., Kobayashi, M., Hayashi, N., Otsuki, H., Kondou, Y., Goto, H., Kawashima, M., Matsuda, F., Niida, R., Matsui, M., Saito, K. & Fernie, A. R. (2011) Metabolomics reveals comprehensive reprogramming involving two independent metabolic responses of Arabidopsis to UV-B light. *Plant Journal,* 67(2)**,** 354-369. 10.1111/j.1365-313X.2011.04599.x.

Kwan, G., Pisithkul, T., Amador-Noguez, D. & Barak, J. (2015) *De novo* amino acid biosynthesis contributes to *Salmonella enterica* growth in alfalfa seedling exudates. *Applied and environmental microbiology,* 81(3)**,** 861-873.

Larraburu, E. E., Apóstolo, N. M. & Llorente, B. E. (2010) Anatomy and morphology of photinia (*Photinia* × *fraseri* Dress) in vitro plants inoculated with rhizobacteria. *Trees,* 24(4)**,** 635-642. 10.1007/s00468-010-0433-x.

Larsen, L. (2016) *Salmonella* Outbreak In Minnesota Associated with Taylor Farms Kale. *Food Poisoning Bulletin*.

Lavania, M., Chauhan, P. S., Chauhan, S. V. S., Singh, H. B. & Nautiyal, C. S. (2006) Induction of plant defense enzymes and phenolics by treatment with plant growth–promoting rhizobacteria Serratia marcescens NBRI1213. *Current Microbiology,* 52(5)**,** 363-368.

Lee, G., Carrow, R. N., Duncan, R. R., Eiteman, M. A. & Rieger, M. W. (2008) Synthesis of organic osmolytes and salt tolerance mechanisms in *Paspalum vaginatum*. *Environmental and Experimental Botany,* 63(1-3)**,** 19-27.

Leveau Johan, H. J. & Lindow Steven, E. (2001) Appetite of an epiphyte: Quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proceedings of the National Academy of Sciences,* 98(6)**,** 3446-3453. 10.1073/pnas.061629598.

Li, Y., Ye, W., Wang, M. & Yan, X. (2009) Climate change and drought: a risk assessment of crop-yield impacts. *Climate research,* 39(1)**,** 31-46.

Lindow, S. E. & Brandl, M. T. (2003) Microbiology of the Phyllosphere. *Applied and Environmental Microbiology,* 69(4)**,** 1875. 10.1128/AEM.69.4.1875-1883.2003.

Linić, I., Šamec, D., Grúz, J., Vujčić Bok, V., Strnad, M. & Salopek-Sondi, B. (2019) Involvement of phenolic acids in short-term adaptation to salinity stress is species-specific among Brassicaceae. *Plants,* 8(6)**,** 155.

Liu, X., Li, Y. & Micallef, S. (submitted) Kale leaf surface and tissue metabolomes shift with plant development and in response to drought, limiting S*almonella enterica* leaf association.

Long, H. H., Schmidt, D. D. & Baldwin, I. T. (2008) Native Bacterial Endophytes Promote Host Growth in a Species-Specific Manner; Phytohormone Manipulations Do Not Result in Common Growth Responses. *PLOS ONE,* 3(7)**,** e2702. 10.1371/journal.pone.0002702.

Loon, L. C. v. & Glick, B. R. (2004) Increased plant fitness by rhizobacteria. In: *Molecular ecotoxicology of plants.* Springer.

Loper, J. E. & Henkels, M. D. (1999) Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Appl Environ Microbiol,* 65(12)**,** 5357-63.

Lopez-Berenguer, C., Carvajal, M., Moreno, D. A. & Garcia-Viguera, C. (2007) Effects of microwave cooking conditions on bioactive compounds present in broccoli inflorescences. *J Agric Food Chem,* 55(24)**,** 10001-7. 10.1021/jf071680t.

Lynch, M. F., Tauxe, R. V. & Hedberg, C. W. (2009) The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology & Infection,* 137(3)**,** 307-315.

López, A., Javier, G.-A., Fenoll, J., Hellín, P. & Flores, P. (2014) Chemical composition and antioxidant capacity of lettuce: Comparative study of regular-sized (Romaine) and baby-sized (Little Gem and Mini Romaine) types. *Journal of Food Composition and Analysis,* 33(1)**,** 39-48. https://doi.org/10.1016/j.jfca.2013.10.001.

Ma, Y., Rajkumar, M., Luo, Y. & Freitas, H. (2011) Inoculation of endophytic bacteria on host and non-host plants—effects on plant growth and Ni uptake. *Journal of Hazardous Materials,* 195**,** 230-237.

Mabrouk, K., Ram, N., Boisseau, S., Strappazzon, F., Rehaim, A., Sadoul, R., Darbon, H., Ronjat, M. & De Waard, M. (2007) Critical amino acid residues of maurocalcine involved in pharmacology, lipid interaction and cell

penetration. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1768(10)**,** 2528-2540.

Macleod, K., Rumbold, K. & Padayachee, K. (2015) A Systems Approach to Uncover the Effects of the PGPR *Pseudomonas koreensis* on the Level of Drought Stress Tolerance in *Helianthus Annuus*. *Procedia Environmental Sciences,* 29**,** 262-263. https://doi.org/10.1016/j.proenv.2015.07.200.

Markland, S. M., Ferelli, A. M., Craighead, S. A., Bais, H. & Kniel, K. E. (2015) Application of *Bacillus subtilis* to the roots of leafy greens, in the presence of *Listeria innocua* and *Salmonella* Newport, induces closure of stomata. *Foodborne pathogens and disease,* 12(10)**,** 828-835.

Martínez-Viveros, O., Jorquera, M., Crowley, D., Gajardo, G. & Mora, M. (2010) Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of soil science and plant nutrition,* 10(3)**,** 293-319.

Maryland.gov, C. C. Science, climate change is real.

Masclaux, C., Valadier, M.-H., Brugière, N., Morot-Gaudry, J.-F. & Hirel, B. (2000) Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. *Planta,* 211(4)**,** 510-518. 10.1007/s004250000310.

Massoni, J., Bortfeld-Miller, M., Widmer, A. & Vorholt, J. A. (2021) Capacity of soil bacteria to reach the phyllosphere and convergence of floral communities despite soil microbiota variation. *Proceedings of the National Academy of Sciences,* 118(41)**,** e2100150118. doi:10.1073/pnas.2100150118.

Matilla, M. A., Ramos, J. L., Bakker, P. A., Doornbos, R., Badri, D. V., Vivanco, J. M. & Ramos‐González, M. I. (2010) *Pseudomonas putida* KT2440 causes induced systemic resistance and changes in Arabidopsis root exudation. *Environmental Microbiology Reports,* 2(3)**,** 381-388.

Mauch-Mani, B. & Mauch, F. (2005) The role of abscisic acid in plant–pathogen interactions. *Current opinion in plant biology,* 8(4)**,** 409-414.

Mawlong, I., Sujith Kumar, M. S., Gurung, B., Singh, K. H. & Singh, D. (2017) A simple spectrophotometric method for estimating total glucosinolates in mustard de-oiled cake. *International Journal of Food Properties,* 20(12)**,** 3274-3281. 10.1080/10942912.2017.1286353.

Maxwell, D. L., Kruger, E. L. & Stanosz, G. R. (1997) Effects of Water Stress on Colonization of Poplar Stems and Excised Leaf Disks by Septoria musiva. *Phytopathology,* 87(4)**,** 381-388. 10.1094/PHYTO.1997.87.4.381.

Mayek-PÉrez, N., GarcÍa-Espinosa, R., LÓpez-CastaÑeda, C., Acosta-Gallegos, J. A. & Simpson, J. (2002) Water relations, histopathology and growth of common

bean (*Phaseolus vulgaris* L.) during pathogenesis of *Macrophomina phaseolina* under drought stress. *Physiological and Molecular Plant Pathology,* 60(4)**,** 185-195.

McElrone, A. J., Sherald, J. L. & Forseth, I. N. (2001) Effects of Water Stress on Symptomatology and Growth of Parthenocissus quinquefolia Infected by Xylella fastidiosa. *Plant Disease,* 85(11)**,** 1160-1164. 10.1094/PDIS.2001.85.11.1160.

Megías-Pérez, R., Hahn, C., Ruiz-Matute, A. I., Behrends, B., Albach, D. C. & Kuhnert, N. (2020) Changes in low molecular weight carbohydrates in kale during development and acclimation to cold temperatures determined by chromatographic techniques coupled to mass spectrometry. *Food Research International,* 127**,** 108727. https://doi.org/10.1016/j.foodres.2019.108727.

Melotto, M., Brandl, M. T., Jacob, C., Jay-Russell, M. T., Micallef, S. A., Warburton, M. L. & Van Deynze, A. (2020) Breeding Crops for Enhanced Food Safety. *Frontiers in Plant Science,* 11. 10.3389/fpls.2020.00428.

Meng, F., Altier, C. & Martin, G. B. (2013) *Salmonella* colonization activates the plant immune system and benefits from association with plant pathogenic bacteria. *Environ Microbiol,* 15(9)**,** 2418-30. 10.1111/1462-2920.12113.

Mercier, J. & Lindow, S. E. (2000) Role of leaf surface sugars in colonization of plants by bacterial epiphytes. *Applied and environmental microbiology,* 66(1)**,** 369-374. 10.1128/aem.66.1.369-374.2000.

Meziane, H., Van Der Sluis, I., Van Loon, L. C., HÖFte, M. & Bakker, P. A. H. M. (2005) Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. *Molecular Plant Pathology,* 6(2)**,** 177-185. https://doi.org/10.1111/j.1364-3703.2005.00276.x.

Mhlongo, M. I., Piater, L. A., Steenkamp, P. A., Labuschagne, N. & Dubery, I. A. (2020) Metabolic profiling of PGPR-treated tomato plants reveal priming-related adaptations of secondary metabolites and aromatic amino acids. *Metabolites,* 10(5)**,** 210.

Miller, A., Schlagnhaufer, C., Spalding, M. & Rodermel, S. (2000) Carbohydrate regulation of leaf development: Prolongation of leaf senescence in Rubisco antisense mutants of tobacco. *Photosynthesis Research,* 63(1)**,** 1-8. 10.1023/A:1006367719639.

Missio, J. C., Rivera, A., Figàs, M. R., Casanova, C., Camí, B., Soler, S. & Simó, J. (2018) A Comparison of Landraces vs. Modern Varieties of Lettuce in Organic Farming During the Winter in the Mediterranean Area: An Approach Considering the Viewpoints of Breeders, Consumers, and Farmers. *Frontiers in Plant Science,* 9. 10.3389/fpls.2018.01491.

Mita, S., Suzukifujii, K. & Nakamura, K. (1995) Sugar-inducible expression of a gene for beta-amylase in *Arabidopsis-thaliana*. *Plant Physiology,* 107(3)**,** 895-904. 10.1104/pp.107.3.895.

Mittal, S. & Davis, K. R. (1995) Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. *tomato*. *Mol Plant Microbe Interact,* 8(1)**,** 165-71. 10.1094/mpmi-8-0165.

Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in plant science,* 7(9)**,** 405-410.

Mohamed, H. I. & Gomaa, E. Z. (2012) Effect of plant growth promoting *Bacillus subtilis* and *Pseudomonas fluorescens* on growth and pigment composition of radish plants (*Raphanus sativus*) under NaCl stress. *Photosynthetica,* 50(2)**,** 263-272. 10.1007/s11099-012-0032-8.

Morris Jr, J. G., Hoffmann, S. & Batz, B. (2011) Ranking the risks: the 10 pathogen-food combinations with the greatest burden on public health.

Nadeem, S. M., Zahir, Z. A., Naveed, M., Arshad, M. & Shahzad, S. (2006) Variation in growth and ion uptake of maize due to inoculation with plant growth promoting rhizobacteria under salt stress. *Soil Environ,* 25(2)**,** 78-84.

Nakabayashi, R., Mori, T. & Saito, K. (2014a) Alternation of flavonoid accumulation under drought stress in *Arabidopsis thaliana. Plant signaling & behavior,* 9(8)**,** e29518.

Nakabayashi, R. & Saito, K. (2015) Integrated metabolomics for abiotic stress responses in plants. *Current Opinion in Plant Biology,* 24**,** 10-16. 10.1016/j.pbi.2015.01.003.

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., Matsuda, F., Kojima, M., Sakakibara, H., Shinozaki, K., Michael, A. J., Tohge, T., Yamazaki, M. & Saito, K. (2014b) Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *Plant Journal,* 77(3)**,** 367-379. 10.1111/tpj.12388.

Nam, H. G. (1997) The molecular genetic analysis of leaf senescence. *Current Opinion in Biotechnology,* 8(2)**,** 200-207. https://doi.org/10.1016/S0958-1669(97)80103-6.

Navada, S., Vadstein, O., Gaumet, F., Tveten, A.-K., Spanu, C., Mikkelsen, Ø. & Kolarevic, J. (2020) Biofilms remember: osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms. *Water Research,* 176**,** 115732.

Neff, M. M. & Chory, J. (1998) Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. *Plant physiology,* 118(1)**,** 27-35.

Nerem, R. S., Beckley, B. D., Fasullo, J. T., Hamlington, B. D., Masters, D. & Mitchum, G. T. (2018) Climate change driven accelerated sea-level rise detected in the altimeter era. *Proceedings of the National Academy of Sciences,* 115(9)**,** 2022. 10.1073/pnas.1717312115.

Nguyen, V. D., Bennett, S. D., Mungai, E., Gieraltowski, L., Hise, K. & Gould, L. H. (2015) Increase in Multistate Foodborne Disease Outbreaks-United States, 1973-2010. *Foodborne Pathogens and Disease,* 12(11)**,** 867-872. 10.1089/fpd.2014.1908.

Nobile, C. G., Pileggi, C., Pavia, M. & Angelillo, I. F. (2006) Association between fruit and vegetable consumption and oral cancer: a meta-analysis of observational studies. *The American Journal of Clinical Nutrition,* 83(5)**,** 1126-1134. 10.1093/ajcn/83.5.1126.

Noodén, L. D. (2012) *Senescence and aging in plants.* Elsevier.

Nur, I. T., Tahera, J., Munna, M. S., Rahman, M. M. & Noor, R. (2016) Impact of Different Carbon Sources on the in vitro Growth and Viability of *Escherichia coli* (SUBE01) and *Salmonella spp.* (SUBS01) Cells. *Bangladesh Journal of Microbiology,* 32(0)**,** 39-44. 10.3329/bjm.v32i0.28476.

O'brien, R. & Lindow, S. (1989) Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. *Phytopathology,* 79(5)**,** 619-627.

Obertello, M., Krouk, G., Katari, M. S., Runko, S. J. & Coruzzi, G. M. (2010) Modeling the global effect of the basic-leucine zipper transcription factor 1 (bZIP1) on nitrogen and light regulation in Arabidopsis. *Bmc Systems Biology,* 4**,** 11. 10.1186/1752-0509-4-111.

Obrien, R. D. & Lindow, S. E. (1989) EFFECT OF PLANT-SPECIES AND ENVIRONMENTAL-CONDITIONS ON EPIPHYTIC POPULATION SIZES OF PSEUDOMONAS-SYRINGAE AND OTHER BACTERIA. *Phytopathology,* 79(5)**,** 619-627. 10.1094/Phyto-79-619.

Oh, M.-M., Carey, E. E. & Rajashekar, C. (2010) Regulated water deficits improve phytochemical concentration in lettuce. *Journal of the American Society for Horticultural Science,* 135(3)**,** 223-229.

Oh, M.-M., Trick, H. N. & Rajashekar, C. (2009) Secondary metabolism and antioxidants are involved in environmental adaptation and stress tolerance in lettuce. *Journal of plant physiology,* 166(2)**,** 180-191.

Olaimat, A. N. & Holley, R. A. (2012) Factors influencing the microbial safety of fresh produce: A review. *Food Microbiology,* 32(1)**,** 1-19. 10.1016/j.fm.2012.04.016.

Osman, M. S., Badawy, A. A., Osman, A. I. & Abdel Latef, A. A. H. (2021) Ameliorative impact of an extract of the halophyte *Arthrocnemum macrostachyum* on growth and biochemical parameters of soybean under salinity stress. *Journal of Plant Growth Regulation,* 40(3)**,** 1245-1256.

Osuna, D., Usadel, B., Morcuende, R., Gibon, Y., Blasing, O. E., Hohne, M., Gunter, M., Kamlage, B., Trethewey, R., Scheible, W. R. & Stitt, M. (2007) Temporal responses of transcripts, enzyme activities and metabolites after adding sucrose to carbon-deprived Arabidopsis seedlings. *Plant Journal,* 49(3)**,** 463-491. 10.1111/j.1365-313X.2006.02979.x.

Ozgen, S. & Sekerci, S. (2011) Effect of leaf position on the distribution of phytochemicals and antioxidant capacity among green and red lettuce cultivars. *Spanish Journal of Agricultural Research,* 9(3)**,** 801-809.

Paim, B. T., Crizel, R. L., Tatiane, S. J., Rodrigues, V. R., Rombaldi, C. V. & Galli, V. (2020) Mild drought stress has potential to improve lettuce yield and quality. *Scientia Horticulturae,* 272**,** 109578.

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J. & Griffin, P. M. (2013) Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging infectious diseases,* 19(3)**,** 407-415. 10.3201/eid1903.111866.

Pan, T., Liu, M., Kreslavski, V. D., Zharmukhamedov, S. K., Nie, C., Yu, M., Kuznetsov, V. V., Allakhverdiev, S. I. & Shabala, S. (2021) Non-stomatal limitation of photosynthesis by soil salinity. *Critical Reviews in Environmental Science and Technology,* 51(8)**,** 791-825.

Parish, M., Beuchat, L., Suslow, T., Harris, L., Garrett, E., Farber, J. & Busta, F. (2003) Methods to reduce/eliminate pathogens from fresh and fresh‐cut produce. *Comprehensive reviews in food science and food safety,* 2**,** 161-173.

Patel, J. & Sharma, M. (2010) Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *International Journal of Food Microbiology,* 139(1)**,** 41-47. https://doi.org/10.1016/j.ijfoodmicro.2010.02.005.

Pathirana, I., Thavarajah, P., Siva, N., Wickramasinghe, A. N. K., Smith, P. & Thavarajah, D. (2017) Moisture deficit effects on kale (*Brassica oleracea* L. var. *acephala*) biomass, mineral, and low molecular weight carbohydrate concentrations. *Scientia Horticulturae,* 226**,** 216-222. https://doi.org/10.1016/j.scienta.2017.08.050.

Podda, A., Pollastri, S., Bartolini, P., Pisuttu, C., Pellegrini, E., Nali, C., Cencetti, G., Michelozzi, M., Frassinetti, S., Giorgetti, L., Fineschi, S., Del Carratore, R. & Maserti, B. (2019) Drought stress modulates secondary metabolites in *Brassica oleracea* L. convar. *acephala* (DC) Alef, var. *sabellica* L. *Journal of the Science of Food and Agriculture,* 99(12)**,** 5533-5540. https://doi.org/10.1002/jsfa.9816.

Pourtau, N., Jennings, R., Pelzer, E., Pallas, J. & Wingler, A. (2006) Effect of sugar-induced senescence on gene expression and implications for the regulation of senescence in Arabidopsis. *Planta,* 224(3)**,** 556-568. 10.1007/s00425-006-0243-y.

Purnell, T. J. & Preece, T. F. (1971) Effects of foliar washing on subsequent infection of leaves of swede (*Brassica napus*) by *Erysiphe-cruciferarum*. *Physiological Plant Pathology,* 1(2)**,** 123-&. 10.1016/0048-4059(71)90021-x.

Pérez-López, U., Miranda-Apodaca, J., Muñoz-Rueda, A. & Mena-Petite, A. (2013) Lettuce production and antioxidant capacity are differentially modified by salt stress and light intensity under ambient and elevated CO2. *Journal of Plant Physiology,* 170(17)**,** 1517-1525. https://doi.org/10.1016/j.jplph.2013.06.004.

Qadir, M., Quillérou, E., Nangia, V., Murtaza, G., Singh, M., Thomas, R. J., Drechsel, P. & Noble, A. D. (2014) Economics of salt-induced land degradation and restoration. *Natural Resources Forum,* 38(4)**,** 282-295. https://doi.org/10.1111/1477-8947.12054.

Quilliam, R. S., Williams, A. P. & Jones, D. L. (2012) Lettuce cultivar mediates both phyllosphere and rhizosphere activity of *Escherichia coli* O157: H7. *PLoS One,* 7(3)**,** e33842.

Quirino, B. F., Reiter, W.-D. & Amasino, R. D. (2001) One of two tandem Arabidopsis genes homologous to monosaccharide transporters is senescence-associated. *Plant Molecular Biology,* 46(4)**,** 447-457. 10.1023/A:1010639015959.

Raaijmakers, J. M., Leeman, M., Van Oorschot, M. M., Van der Sluis, I., Schippers, B. & Bakker, P. (1995) Dose-response relationships in biological control of Fusarium wilt of radish by *Pseudomonas spp*. *Phytopathology,* 85(10)**,** 1075-1080.

Rajabbeigi, E., Eichholz, I., Beesk, N., Ulrichs, C., Kroh, L. W., Rohn, S. & Huyskens-Keil, S. (2013) Interaction of drought stress and UV-B radiation-impact on biomass production and flavonoid metabolism in lettuce (*Lactuca sativa* L.). *Journal of Applied Botany and Food Quality,* 86(1).

Ramegowda, V. & Senthil-Kumar, M. (2015) The interactive effects of simultaneous biotic and abiotic stresses on plants: Mechanistic understanding from drought

and pathogen combination. *Journal of Plant Physiology,* 176**,** 47-54. 10.1016/j.jplph.2014.11.008.

Ramegowda, V., Senthil-Kumar, M., Ishiga, Y., Kaundal, A., Udayakumar, M. & Mysore, K. (2013) Drought stress acclimation imparts tolerance to *Sclerotinia sclerotiorum* and *Pseudomonas syringae* in *Nicotiana benthamiana*. *International journal of molecular sciences,* 14(5)**,** 9497-9513.

Rani, M., Weadge, J. T. & Jabaji, S. (2020) Isolation and Characterization of Biosurfactant-Producing Bacteria From Oil Well Batteries With Antimicrobial Activities Against Food-Borne and Plant Pathogens. *Frontiers in Microbiology,* 11. 10.3389/fmicb.2020.00064.

Rebecca Epanchin-Niell, K. G., Jarrod & Miller, K. T. (2018) Saltwater Intrusion and Coastal Climate Adaptation: Building Community Resilience.

Robinson, M. F., Very, A.-A., Sanders, D. & Mansfield, T. (1997) How can stomata contribute to salt tolerance? *Annals of botany,* 80(4)**,** 387-393.

Rollins, J., Habte, E., Templer, S., Colby, T., Schmidt, J. & Von Korff, M. (2013) Leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (*Hordeum vulgare* L.). *Journal of experimental botany,* 64(11)**,** 3201-3212.

Rosner, B., Hunter, D., Spiegelman, D., Hu, F. B., Colditz, G. A., Hung, H.-C., Joshipura, K. J., Jiang, R., Smith-Warner, S. A. & Willett, W. C. (2004) Fruit and Vegetable Intake and Risk of Major Chronic Disease. *JNCI: Journal of the National Cancer Institute,* 96(21)**,** 1577-1584. 10.1093/jnci/djh296.

Ryffel, F., Helfrich, E. J. N., Kiefer, P., Peyriga, L., Portais, J.-C., Piel, J. & Vorholt, J. A. (2016) Metabolic footprint of epiphytic bacteria on *Arabidopsis thaliana* leaves. *The ISME journal,* 10(3)**,** 632-643.

Saberi-Riseh, R., Fathi, F. & Moradzadeh-Eskandari, M. (2020) Effect of some *Pseudomonas fluorescens* and *Bacillus subtilis* strains on osmolytes and antioxidants of cucumber under salinity stress. *Journal of Crop Protection,* 9(1)**,** 1-16.

Sanada, Y., Ueda, H., Kuribayashi, K., Andoh, T., Hayashi, F., Tamai, N. & Wada, K. (1995) Novel light-dark change of proline levels in halophyte (*Mesembryanthemum crystallinum* L.) and glycophytes (*Hordeum vulgare* L. and *Triticum aestivum* L.) leaves and roots under salt stress. *Plant and cell physiology,* 36(6)**,** 965-970.

Sandhya, V., Ali, S. Z., Grover, M., Reddy, G. & Venkateswarlu, B. (2010) Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes, antioxidant

status and plant growth of maize under drought stress. *Plant Growth Regulation,* 62(1)**,** 21-30.

Scallan, E., Griffin, P. M., Angulo, F. J., Tauxe, R. V. & Hoekstra, R. M. (2011a) Foodborne illness acquired in the United States—unspecified agents. *Emerging infectious diseases,* 17(1)**,** 16.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., Jones, J. L. & Griffin, P. M. (2011b) Foodborne illness acquired in the United States--major pathogens. *Emerging infectious diseases,* 17(1)**,** 7-15. 10.3201/eid1701.P11101.

Schippers, J. H., Jing, H.-C., Hille, J. & Dijkwel, P. P. (2007) Developmental and hormonal control of leaf senescence. *Senescence processes in plants***,** 145-170.

Scuderi, D., Restuccia, C., Chisari, M., Barbagallo, R. N., Caggia, C. & Giuffrida, F. (2011) Salinity of nutrient solution influences the shelf-life of fresh-cut lettuce grown in floating system. *Postharvest Biology and Technology,* 59(2)**,** 132-137. https://doi.org/10.1016/j.postharvbio.2010.08.016.

Sevilla, M., Burris, R. H., Gunapala, N. & Kennedy, C. (2001) Comparison of Benefit to Sugarcane Plant Growth and 15N2 Incorporation Following Inoculation of Sterile Plants with Acetobacter diazotrophicus Wild-Type and Nif¯ Mutant Strains. *Molecular Plant-Microbe Interactions,* 14(3)**,** 358-366. 10.1094/MPMI.2001.14.3.358.

Shah, U., Ranieri, P., Zhou, Y., Schauer, C. L., Miller, V., Fridman, G. & Sekhon, J. K. (2019) Effects of cold plasma treatments on spot-inoculated *Escherichia coli* O157:H7 and quality of baby kale (*Brassica oleracea*) leaves. *Innovative Food Science & Emerging Technologies,* 57**,** 102104. https://doi.org/10.1016/j.ifset.2018.12.010.

Shannon, M. & Grieve, C. (1998) Tolerance of vegetable crops to salinity. *Scientia horticulturae,* 78(1-4)**,** 5-38.

Shannon, M. C., Grieve, C. M., Lesch, S. M. & Draper, J. H. (2000) Analysis of salt tolerance in nine leafy vegetables irrigated with saline drainage water. *Journal of the American Society for Horticultural Science,* 125(5)**,** 658-664.

Shavalikohshori, O., Zalaghi, R., Sorkheh, K. & Enaytizamir, N. (2020) The expression of proline production/degradation genes under salinity and cadmium stresses in *Triticum aestivum* inoculated with *Pseudomonas* sp. *International Journal of Environmental Science and Technology,* 17(4)**,** 2233-2242. 10.1007/s13762-019-02551-9.

Shawon, R. A., Kang, B. S., Lee, S. G., Kim, S. K., Ju Lee, H., Katrich, E., Gorinstein, S. & Ku, Y. G. (2020) Influence of drought stress on bioactive

compounds, antioxidant enzymes and glucosinolate contents of Chinese cabbage (*Brassica rapa*). *Food Chem,* 308**,** 125657. 10.1016/j.foodchem.2019.125657.

Shi, X., Namvar, A., Kostrzynska, M., Hora, R. & Warriner, K. (2007) Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *J Food Prot,* 70(12)**,** 2725-31. 10.4315/0362-028x-70.12.2725.

Shin, Y. K., Bhandari, S. R., Jo, J. S., Song, J. W. & Lee, J. G. (2021) Effect of drought stress on chlorophyll fluorescence parameters, phytochemical contents, and antioxidant activities in lettuce seedlings. *Horticulturae,* 7(8)**,** 238.

Shirron, N. & Yaron, S. (2011) Active Suppression of Early Immune Response in Tobacco by the Human Pathogen *Salmonella* Typhimurium. *PLOS ONE,* 6(4)**,** e18855. 10.1371/journal.pone.0018855.

Sikora, E. & Bodziarczyk, I. (2012) Composition and antioxidant activity of kale (*Brassica oleracea* L. var. *acephala*) raw and cooked. *Acta Scientiarum Polonorum Technologia Alimentaria,* 11(3)**,** 239-248.

Singh, T., Aspinall, D. & Paleg, L. (1972) Proline accumulation and varietal adaptability to drought in barley: a potential metabolic measure of drought resistance. *Nature New Biology,* 236(67)**,** 188-190.

Singleton, V. L. & Rossi, J. A. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture,* 16(3)**,** 144-158.

Sivapalasingam, S., Friedman, C. R., Cohen, L. & Tauxe, R. V. (2004) Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J Food Prot,* 67(10)**,** 2342-53. 10.4315/0362-028x-67.10.2342.

Skirycz, A., De Bodt, S., Obata, T., De Clercq, I., Claeys, H., De Rycke, R., Andriankaja, M., Van Aken, O., Van Breusegem, F. & Fernie, A. R. (2010) Developmental stage specificity and the role of mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress. *Plant physiology,* 152(1)**,** 226-244.

Soengas, P., Cartea, M. E., Francisco, M., Sotelo, T. & Velasco, P. (2012) New insights into antioxidant activity of Brassica crops. *Food Chemistry,* 134(2)**,** 725-733. https://doi.org/10.1016/j.foodchem.2012.02.169.

Steenhoudt, O. & Vanderleyden, J. (2000) Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological

aspects. *FEMS Microbiol Rev*, 24(4)**,** 487-506. 10.1111/j.1574-6976.2000.tb00552.x.

Steindal, A. L. H., Rødven, R., Hansen, E. & Mølmann, J. (2015) Effects of photoperiod, growth temperature and cold acclimatisation on glucosinolates, sugars and fatty acids in kale. *Food Chemistry,* 174**,** 44-51. https://doi.org/10.1016/j.foodchem.2014.10.129.

Storey, J. D. & Tibshirani, R. (2003) Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences,* 100(16)**,** 9440-9445. doi:10.1073/pnas.1530509100.

Stratil, P., Klejdus, B. & Kubáň, V. (2006) Determination of Total Content of Phenolic Compounds and Their Antioxidant Activity in Vegetables Evaluation of Spectrophotometric Methods. *Journal of Agricultural and Food Chemistry,* 54(3)**,** 607-616. 10.1021/jf052334j.

Streeter, J., Lohnes, D. & Fioritto, R. (2001) Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. *Plant, Cell & Environment,* 24(4)**,** 429-438.

Szymańska, S., Dąbrowska, G. B., Tyburski, J., Niedojadło, K., Piernik, A. & Hrynkiewicz, K. (2019) Boosting the *Brassica napus* L. tolerance to salinity by the halotolerant strain *Pseudomonas stutzeri* ISE12. *Environmental and Experimental Botany,* 163**,** 55-68. https://doi.org/10.1016/j.envexpbot.2019.04.007.

Tabassum, B., Khan, A., Tariq, M., Ramzan, M., Iqbal Khan, M. S., Shahid, N. & Aaliya, K. (2017) Bottlenecks in commercialisation and future prospects of PGPR. *Applied Soil Ecology,* 121**,** 102-117. https://doi.org/10.1016/j.apsoil.2017.09.030.

Tayebimeigooni, A., Awang, Y., Mahmood, M., Selamat, A. & Wahab, Z. (2012) Leaf water status, proline content, lipid peroxidation and accumulation of hydrogen peroxide in salinized Chinese kale (Brassica alboglabra). *J Food Agric Environ,* 10**,** 371-374.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L. & Hawkins Byrne, D. (2006) Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis,* 19(6)**,** 669-675. https://doi.org/10.1016/j.jfca.2006.01.003.

Thum, K. E., Shasha, D. E., Lejay, L. V. & Coruzzi, G. M. (2003) Light- and carbon-signaling pathways. Modeling circuits of interactions. *Plant Physiology,* 132(2)**,** 440-452. 10.1104/pp.103.022780.

Timmusk, S., Abd El-Daim, I. A., Copolovici, L., Tanilas, T., Känaste, A., Behers, L., Nevo, E., Seisenbaeva, G., Stenström, E. & Niinemets, Ü. (2014) Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress volatiles. *PloS one,* 9(5)**,** e96086.

Timmusk, S., Grantcharova, N. & Wagner, E. G. H. (2005) *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Applied and environmental microbiology,* 71(11)**,** 7292-7300. 10.1128/AEM.71.11.7292-7300.2005.

Timmusk, S. & Wagner, E. G. H. (1999) The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: A possible connection between biotic and abiotic stress responses. *Molecular Plant-Microbe Interactions,* 12(11)**,** 951-959. 10.1094/mpmi.1999.12.11.951.

Trmcic, A., Chen, H., Trząskowska, M., Tamber, S. & Wang, S. (2018) Biofilm-Forming Capacity of Five *Salmonella* Strains and Their Fate on Postharvest Mini Cucumbers. *J Food Prot,* 81(11)**,** 1871-1879. 10.4315/0362-028x.jfp-18-180.

Truitt, L. N., Vazquez, K. M., Pfuntner, R. C., Rideout, S. L., Havelaar, A. H. & Strawn, L. K. (2018) Microbial Quality of Agricultural Water Used in Produce Preharvest Production on the Eastern Shore of Virginia. *J Food Prot,* 81(10)**,** 1661-1672. 10.4315/0362-028x.jfp-18-185.

Tsormpatsidis, E., Henbest, R., Battey, N. H. & Hadley, P. (2010) The influence of ultraviolet radiation on growth, photosynthesis and phenolic levels of green and red lettuce: potential for exploiting effects of ultraviolet radiation in a production system. *Annals of Applied Biology,* 156(3)**,** 357-366.

Tukey, H. B. (1966) Leaching of metabolites from above-ground plant parts and its implications. *Bulletin of the Torrey Botanical Club,* 93(6)**,** 385-&. 10.2307/2483411.

Tukey Jr, H. (1970) The leaching of substances from plants. *Annual review of plant physiology,* 21(1)**,** 305-324.

Tully, K., Gedan, K., Epanchin-Niell, R., Strong, A., Bernhardt, E. S., BenDor, T., Mitchell, M., Kominoski, J., Jordan, T. E., Neubauer, S. C. & Weston, N. B. (2019) The Invisible Flood: The Chemistry, Ecology, and Social Implications of Coastal Saltwater Intrusion. *BioScience,* 69(5)**,** 368-378. 10.1093/biosci/biz027.

Turner, M. F., Heuberger, A. L., Kirkwood, J. S., Collins, C. C., Wolfrum, E. J., Broeckling, C. D., Prenni, J. E. & Jahn, C. E. (2016) Non-targeted Metabolomics in Diverse Sorghum Breeding Lines Indicates Primary and

Secondary Metabolite Profiles Are Associated with Plant Biomass Accumulation and Photosynthesis. *Frontiers in Plant Science,* 7. 10.3389/fpls.2016.00953.

Upadhyay, S. K., Singh, J. S., Saxena, A. K. & Singh, D. P. (2012) Impact of PGPR inoculation on growth and antioxidant status of wheat under saline conditions. *Plant Biology,* 14(4)**,** 605-611. https://doi.org/10.1111/j.1438-8677.2011.00533.x.

Upadhyay, S. K., Singh, J. S. & Singh, D. P. (2011) Exopolysaccharide-Producing Plant Growth-Promoting Rhizobacteria Under Salinity Condition. *Pedosphere,* 21(2)**,** 214-222. https://doi.org/10.1016/S1002-0160(11)60120-3.

Urano, K., Maruyama, K., Ogata, Y., Morishita, Y., Takeda, M., Sakurai, N., Suzuki, H., Saito, K., Shibata, D., Kobayashi, M., Yamaguchi-Shinozaki, K. & Shinozaki, K. (2009) Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. *Plant Journal,* 57(6)**,** 1065-1078. 10.1111/j.1365-313X.2008.03748.x.

Vacheron, J., Desbrosses, G., Bouffaud, M.-L., Touraine, B., Moënne-Loccoz, Y., Muller, D., Legendre, L., Wisniewski-Dyé, F. & Prigent-Combaret, C. (2013) Plant growth-promoting rhizobacteria and root system functioning. *Frontiers in plant science,* 4**,** 356.

Vaishnav, A., Singh, J., Singh, P., Rajput, R. S., Singh, H. B. & Sarma, B. K. (2020) *Sphingobacterium* sp. BHU-AV3 Induces Salt Tolerance in Tomato by Enhancing Antioxidant Activities and Energy Metabolism. *Front Microbiol,* 11**,** 443. 10.3389/fmicb.2020.00443.

van de Mortel, J. E., de Vos, R. C. H., Dekkers, E., Pineda, A., Guillod, L., Bouwmeester, K., van Loon, J. J. A., Dicke, M. & Raaijmakers, J. M. (2012) Metabolic and Transcriptomic Changes Induced in Arabidopsis by the Rhizobacterium *Pseudomonas fluorescens* SS101. *Plant Physiology,* 160(4)**,** 2173-2188. 10.1104/pp.112.207324.

Van Duyn, M. A. S. & Pivonka, E. (2000) Overview of the Health Benefits of Fruit and Vegetable Consumption for the Dietetics Professional: Selected Literature. *Journal of the American Dietetic Association,* 100(12)**,** 1511-1521. https://doi.org/10.1016/S0002-8223(00)00420-X.

Van Lijsebettens, M. & Clarke, J. (1998) Leaf development in Arabidopsis. *Plant Physiology and Biochemistry,* 36(1)**,** 47-60. https://doi.org/10.1016/S0981-9428(98)80090-9.

Van Wees, S. C. M., Pieterse, C. M. J., Trijssenaar, A., Van 't Westende, Y. A. M., Hartog, F. & Van Loon, L. C. (1997) Differential Induction of Systemic

Resistance in *Arabidopsis* by Biocontrol Bacteria. *Molecular Plant-Microbe Interactions®,* 10(6)**,** 716-724. 10.1094/MPMI.1997.10.6.716.

Velasco, P., Cartea, M. E., González, C., Vilar, M. & Ordás, A. (2007) Factors affecting the glucosinolate content of kale (*Brassica oleracea acephala* group). *Journal of agricultural and food chemistry,* 55(3)**,** 955-962.

Verslues, P. E. & Juenger, T. E. (2011) Drought, metabolites, and Arabidopsis natural variation: a promising combination for understanding adaptation to water-limited environments. *Current Opinion in Plant Biology,* 14(3)**,** 240-245. https://doi.org/10.1016/j.pbi.2011.04.006.

Vince., J. & Staff., P. (2016) The salad's suppliers issued the voluntary recall Tuesday. *Patch*.

Weston, N. B., Giblin, A. E., Banta, G. T., Hopkinson, C. S. & Tucker, J. (2010) The effects of varying salinity on ammonium exchange in estuarine sediments of the Parker River, Massachusetts. *Estuaries and Coasts,* 33(4)**,** 985-1003.

White, E. & Kaplan, D. (2017) Restore or retreat? Saltwater intrusion and water management in coastal wetlands. *Ecosystem Health and Sustainability,* 3(1)**,** e01258.

Wickham, H. (2016) *ggplot2: elegant graphics for data analysis.*: springer.

Wilson, M. & Lindow, S. E. (1994) Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Applied and environmental microbiology,* 60(12)**,** 4468-4477.

Xu, C. & Mou, B. (2015) Evaluation of lettuce genotypes for salinity tolerance. *HortScience,* 50(10)**,** 1441-1446.

Yaron, S. (2014) Chapter 2 - Microbial Attachment and Persistence on Plants. In: Matthews, K. R., Sapers, G. M. & Gerba, C. P. (eds.) *The Produce Contamination Problem (Second Edition).* San Diego: Academic Press.

Yoon, H. I., Kim, J.-S., Kim, D., Kim, C. Y. & Son, J. E. (2019) Harvest strategies to maximize the annual production of bioactive compounds, glucosinolates, and total antioxidant activities of kale in plant factories. *Horticulture, Environment, and Biotechnology,* 60(6)**,** 883-894. 10.1007/s13580-019-00174-0.

Younesi, O. & Moradi, A. (2014) Effects of plant growth-promoting rhizobacterium (PGPR) and arbuscular mycorrhizal fungus (AMF) on antioxidant enzyme activities in salt-stressed bean (*Phaseolus vulgaris* L.). *Agriculture,* 60(1)**,** 10.

Yuan, G., Wang, X., Guo, R. & Wang, Q. (2010) Effect of salt stress on phenolic compounds, glucosinolates, myrosinase and antioxidant activity in radish

sprouts. *Food Chemistry,* 121(4)**,** 1014-1019. https://doi.org/10.1016/j.foodchem.2010.01.040.

Zhang, F., Dashti, N., Hynes, R. & Smith, D. L. (1996) Plant growth promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.] nodulation and nitrogen fixation at suboptimal root zone temperatures. *Annals of Botany,* 77(5)**,** 453-460.

Zhang, G., Ma, L. I., Beuchat, L. R., Erickson, M. C., Phelan, V. H. & Doyle, M. P. (2009) Heat and Drought Stress during Growth of Lettuce (*Lactuca sativa* L.) Does Not Promote Internalization of *Escherichia coli* O157:H7. *Journal of Food Protection,* 72(12)**,** 2471-2475. 10.4315/0362-028X-72.12.2471.

Zhang, Q. & Dai, W. (2019) Plant response to salinity stress. In: *Stress Physiology of Woody Plants.* CRC Press.

Zhang, Y. P., Sallach, J. B., Hodges, L., Snow, D. D., Bartelt-Hunt, S. L., Eskridge, K. M. & Li, X. (2016) Effects of soil texture and drought stress on the uptake of antibiotics and the internalization of *Salmonella* in lettuce following wastewater irrigation. *Environmental Pollution,* 208**,** 523-531. 10.1016/j.envpol.2015.10.025.

Zhao, X., Silva, M. B. R. d., Van der Linden, I., Franco, B. D. G. M. & Uyttendaele, M. (2021) Behavior of the Biological Control Agent *Bacillus thuringiensis* subsp. *aizawai* ABTS-1857 and *Salmonella enterica* on Spinach Plants and Cut Leaves. *Frontiers in Microbiology,* 12. 10.3389/fmicb.2021.626029.

Zhishen, J., Mengcheng, T. & Jianming, W. (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry,* 64(4)**,** 555-559. https://doi.org/10.1016/S0308-8146(98)00102-2.

Zhou, L., Jang, J. C., Jones, T. L. & Sheen, J. (1998) Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. *Proceedings of the National Academy of Sciences of the United States of America,* 95(17)**,** 10294-10299. 10.1073/pnas.95.17.10294.

Zhu, J. K. (2016) Abiotic Stress Signaling and Responses in Plants. *Cell,* 167(2)**,** 313-324. 10.1016/j.cell.2016.08.029.

Šamec, D., Urlić, B. & Salopek-Sondi, B. (2019) Kale (*Brassica oleracea* var. *acephala*) as a superfood: Review of the scientific evidence behind the statement. *Critical reviews in food science and nutrition,* 59(15)**,** 2411-2422.

Šola, I., Stić, P. & Rusak, G. (2021) Effect of flooding and drought on the content of phenolics, sugars, photosynthetic pigments and vitamin C, and antioxidant

potential of young Chinese cabbage. *European Food Research and Technology,* 247(8)**,** 1913-1920. 10.1007/s00217-021-03759-1.