

# MICROBIAL COMMUNITIES OF SPINACH AT VARIOUS STAGES OF PLANT GROWTH FROM SEED TO MATURITY

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partial fulfillment of the requirements for the degree of Master of Science in Life Sciences

In

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**ABSTRACT**

Little is known about how the leaf bacterial community is affected by the seed microbiota at different stages of plant development. The bacterial populations of spinach seed and leaves after germination were compared using DGGE, to assess bacterial community richness, and real-time PCR to compare the abundance of select phyla (total bacteria, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*,  $\alpha$ -*Proteobacteria* and  $\beta$ -*Proteobacteria*). To determine the effect of environment, the plants were grown in the field and growth chambers. Vertical transmission of bacterial community members was evident; the developmental stage of the plant affected the richness and abundance of select bacterial phyla. The bacterial richness of plants grown in the two environments was not affected. However, overall numbers of bacteria increased in field grown samples in comparison to those produced in growth chambers during development. A statistically significant interaction was seen between growth stage and environment with each of the selected phyla. Populations on cotyledons were smaller than mature leaves, but were not significantly different than the 3-4 leaf stage plants. The culturable populations of bacteria on seeds (~5 log CFU/g) were significantly smaller than determined using real time PCR (~7 log copies). Of these bacteria cultured from spinach seeds, isolates belonging to the genera *Pantoea* were found to inhibit growth of *E. coli* O157:H7 *in vitro*. This study highlights the importance of vertical transmission on the bacterial community of plants and suggests the importance of

developing strategies to influence these communities on seed to control human and plant pathogens on the leaf surface.

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**Dr. Monica Ponder**, Department of Food Science and Technology, and **Dr. Greg Welbaum**, Department of Horticulture, were the principal advisors in this research project providing guidance, advice, and resources. Dr. Ponder provided the research facilities and shared her knowledge of microbial ecology (Chapters 1-4). Dr. Welbaum provided expertise in horticulture, research facilities, and helped plant and provide care of the spinach (Chapters 1-4).

**Jon Wooge**, College Farm, helped with the pesticide application and irrigation (Chapter 3).

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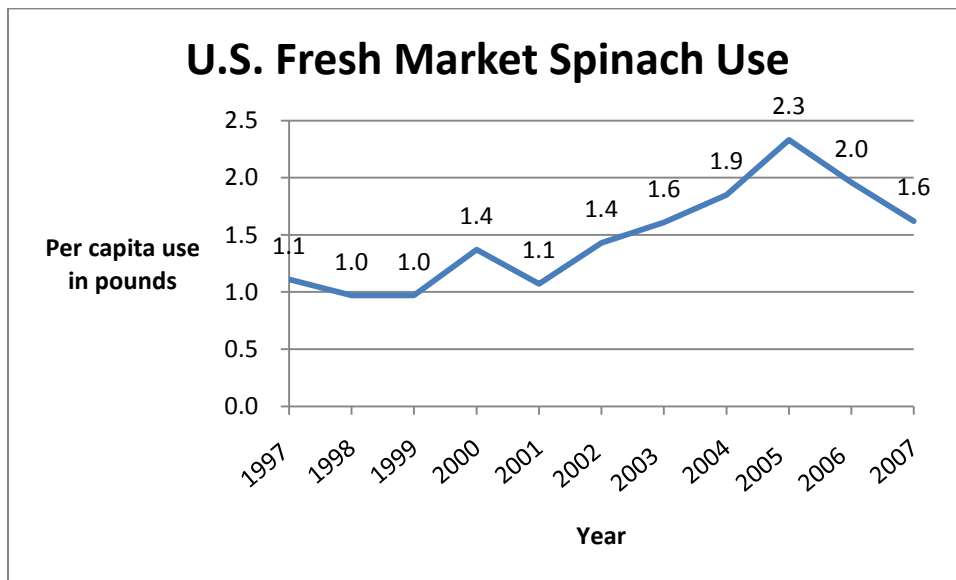
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## CHAPTER 1. INTRODUCTION AND JUSTIFICATION

Microorganisms are found on all plant surfaces including the aerial parts as well as the roots (8, 12). Additionally, microbial communities are known to populate plants at all stages of plant development; from seed to maturity (1). Vegetable seeds harbor large numbers of microorganisms. Some of the microorganisms on seeds are harmless while others are plant pathogens (9). Many of the microorganisms associated with plants are also beneficial. For example, some of the microorganisms found in the soil actually benefit plants by triggering defense mechanisms to protect against diseases (16). Leaves also support a diverse microbial population (17) that is dependent on factors such as leaf age (4), the amount of moisture present (2), the presence of various organic compounds that may be used as nutrients (13), and adverse factors such as UV radiation (10). This research investigates a possible link between bacterial populations on seeds and populations on plants produced from those seeds. To date, the role the seed microbiota plays in the establishment of the microbial communities on the leaves at different stages of plant development is largely unexplored.

In addition to plant pathogens, seeds may also potentially harbor human pathogens. For example, the Center for Disease Control (CDC) recently reported alfalfa sprouts grown from seed were the cause of a *Salmonella* outbreak (7). Human pathogens such as *Salmonella enterica* and *Escherichia coli* O157:H7 can survive on leaf surfaces (4) and their survival is influenced by the number and identity of the microbial communities present on these surfaces (11). Therefore, research exploring the possible connection between seed and leaf microbiota has human health implications.

Recent food-borne illness outbreaks have been traced to the consumption of fresh fruits and vegetables including spinach (12). An outbreak of *E. coli* O157:H7 infections related to fresh baby spinach was associated with 205 illnesses, 3 deaths, and 31 persons developing hemolytic-uremic syndrome (5, 6). In September 2006, the FDA advised consumers to not consume spinach, resulting in a decrease in per capita consumption of fresh spinach in the United States by 0.4 pounds between 2006 and 2007 (Figure 1) (14, 15). In addition, total retail spending on bagged spinach fell by \$201.9 million in the 68 weeks after the FDA’s September 2006 warning to not eat fresh-bagged spinach (3).



Data Source: USDA Economic Research Service 2009 Vegetable and Melons Yearbook (89011) Table 72--U.S. fresh-market spinach: Supply, utilization, and price, farm weight, 1979-2009

Figure 1: Per capita consumption of fresh market spinach in the United States between 1997 and 2007.

These outbreaks and recalls have alarmed consumers, caused economic losses for vegetable producers, and raised questions about how fresh fruits and vegetables can serve as vehicles for human pathogens that cause food-borne illnesses. This study will expand our

understanding of how human pathogens interact with native microbial communities on developing plants from seed to maturity.

Using three different cultivars of spinach (*Spinacia oleracea*) (9), this study compared the bacterial population on the seed with the bacterial population on the leaf surfaces of developing plants after germination. The cultivars were chosen according to leaf type and included savoy, semi-savoy, and flat or smooth leaved cultivars. The plants were grown under controlled conditions in a growth chamber and in a field. The objectives of this research were three-fold. First the richness of the bacterial communities on the seed and leaves of developing plants following germination were compared for all three cultivars. Additionally, the bacterial abundance present on the three different cultivars of spinach seed were compared to the abundance of bacteria on the leaves of the plants at various stages of plant growth. Finally, the bacteria present on the three different cultivars of spinach seed were evaluated as possible antagonists against *E. coli* O157:H7 and *Salmonella enterica*. The data were analyzed by cultivar to determine if there was a significant correlation in richness and abundance of the bacterial community between the seed and the plants they produced. The effect of the environment was assessed for each cultivar to determine if there is a significant correlation in richness and abundance on the leaf bacteria in response to environmental conditions.

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## CHAPTER 2. LITERATURE REVIEW

### Spinach

Spinach (*Spinacia oleracea*) is a vegetable belonging to the Chenopodiaceae family. It produces a rosette of leaves, which are used for food. It is a cool-season annual crop usually grown in the cool coastal valleys of California during late fall, spring, or summer months. In fact, spinach can tolerate temperatures below freezing and can be overwintered in the southern US. However, optimum growth occurs between 50 and 70°F. Spinach is directly sown from seed and is ready for harvest in about 40 to 70 days depending on the cultivar and the temperature. Flower stalk formation, also called bolting, is a result of long days and high temperatures. Once bolting occurs, the leaves are no longer considered suitable for eating. Spinach is dioecious, meaning that male and female flowers are produced on separate plants, although other forms of sex expression may also occur. In the vegetative state it is difficult to distinguish male and female plants. The flowers are wind pollinated (52).

Spinach cultivars are generally categorized according to the leaf blade variations and are described as savoy, semi-savoy, and flat or smooth leaved (52). Savoy cultivars such as ‘Menorca’ have wrinkled leaves. Semi-savoy cultivars such as ‘Melody’ are somewhat wrinkled. Smooth or flat leaved spinach cultivars such as ‘Space’ lack wrinkles and are flat (43). The savoy and semi-savoy cultivars are used for processing while the flat leaf cultivars are generally used for fresh market due to the ease of cleaning (52). The number of stomata and glandular trichomes on spinach leaves differ with cultivar. Stomata are known to be located on the abaxial and adaxial spinach leaf surfaces (26).

Spinach is often infected with several well characterized diseases including 14 fungal, 6 viral, and 3 bacterial diseases (14). Since the focus of this research is primarily bacterial microorganisms, only the bacterial diseases will be discussed. Below is a listing of the 3 bacterial diseases and their causative organisms (Table 1) (2, 46).

Table 1: Bacterial diseases common to spinach and their causative bacteria

Common Name of the Disease	Bacteria Responsible
Bacterial leaf spot	<i>Pseudomonas syringae</i>
Bacterial soft rot	<i>Erwinia carotovora</i>
Witches'-broom	<i>Phytoplasma</i>

Bacterial leaf spot causes necrotic lesions to develop on leaves. The causative organism, *Pseudomonas syringae*, may overwinter on infected plants, may be present on or in the seed, may be found on contaminated tools, or may be found in the soil. These bacteria enter the plant through stomata, hydathodes, or injuries. Overhead irrigation and heavy rains favor invasion of the bacteria into the plant. Control measures include crop rotation, sanitation of equipment, and application of chemicals such as antibiotics (2).

Bacterial soft rot of spinach is caused by *Erwinia carotovora* (46), which can invade spinach growing in the field or harvested spinach. The primary route of infection is entry by way of wounds in the plant tissue. Primarily contaminated tools, soil, and most importantly insects spread the bacteria. The bacteria can live in all stages of the insect life cycle. Once within the plant tissue, the bacteria excrete enzymes in the intercellular spaces that break down pectin and

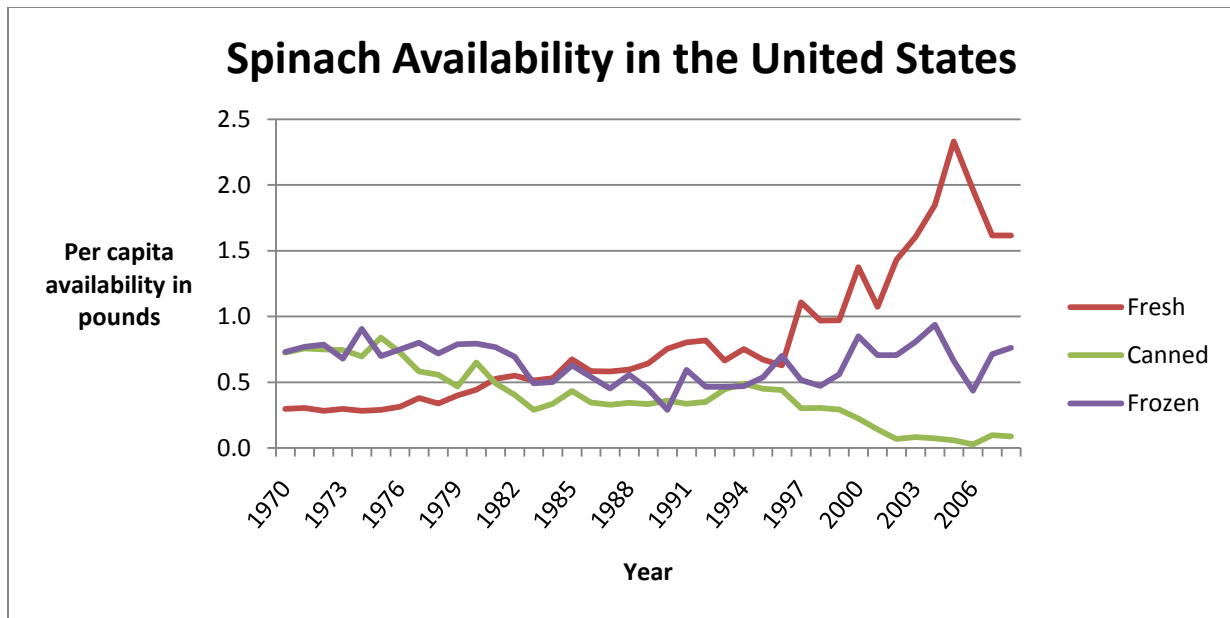
ultimately dissolve the plant's cell walls. Control measures include sanitation of equipment, insect control, crop rotation, the avoidance of plant tissue injury, and storage of harvested spinach in a cool, dry environment. Bacterial soft rot is of minimal importance in the field while the spinach is growing. However, once harvested and packaged in plastic, the bacteria cause rapid decomposition of the leaves (2).

Witches'-broom disease is caused by a group of plant pathogens called phytoplasmas. Phytoplasmas resemble bacteria in that they are prokaryotes except that they contain only a cell membrane and lack a cell wall. Most are not culturable by conventional media (2).

Phytoplasmas are found only in the phloem of plants (34). Phloem is the vascular tissue that transports the products of photosynthesis, minerals, amino acids, and hormones throughout the plant (9). Symptoms include a general yellowing of the plant and stunting of growth (2).

Phytoplasmas infect plants through vectors such as leafhoppers. Reproduction occurs solely in the insect vectors with the phytoplasmas being transferred during feeding. Control measures include insecticides and beneficial insects that feed on leafhoppers (34).

Spinach is an increasingly valuable commodity in the United States. According to the USDA, spinach consumption in the United States is increasing because of new packaging methods. Spinach is now conveniently washed, pre-packaged, and sold as a ready-to-eat fresh market product (49). Responding to market demand, the availability of fresh spinach has steadily increased from 1970 to 2008, while canned spinach has declined (Figure 2) (50).



Data Source: USDA Economic Research Service 2010 Food Availability Data Sets  
<http://www.ers.usda.gov/Data/FoodConsumption/FoodAvailSpreadsheets.htm#vegtot>

Figure 2: Per capita spinach availability in the United States between 1970 and 2008

The US is the second largest spinach producer in the world surpassed only by China (49). California is the top US producer accounting for 73% of the spinach produced in the US during 2004-2006 (27), with California’s Salinas Valley being the major growing area because of its long, cool growing season (52). Arizona is the second largest producer of spinach in the United States accounting for about 12% of total production between 2004 and 2006. New Jersey is also among the top producers of spinach with 3% of the total US production between 2004 and 2006 (27).

Spinach is direct seeded and sprinkler irrigated to germinate the seed and obtain uniform emergence (24). Once the stand is established, the spinach may then be furrow irrigated or sprinkler irrigated. Growers in California and Arizona prefer furrow irrigation (24). Fresh

market spinach is ready for harvest in about 30 to 55 days; whereas, spinach grown for processing typically takes longer depending on the production season and cultivar (24).

There are specific guidelines provided to producers to aide in the production of fresh market spinach that is free of microbes that cause human disease. The FDA and produce industry groups provide microbial food safety guidance for spinach and other leafy greens from farm-to-table (17, 38, 48). The FDA guidelines, “Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Leafy Greens; Draft Guidance”, provide recommendations to reduce microbial contamination during production, harvest, and postharvest including minimal processing, distribution, and end-use guidance (48). Industry recommendations, “Lettuce/Leafy Greens Commodity Specific Guidance Production & Harvest Unit Operations”, focus solely on production and harvest. The industry guidance surpasses the FDA guidance in that it provides not only specific areas of focus, but also includes sampling plans, testing frequencies, levels of acceptance, and decision trees (38). Therefore, the industry has guidelines in place to reduce microbial contamination of spinach during all phases of production, especially production and harvesting.

### **Bacterial Communities on Seeds**

Seeds are known to harbor many microorganisms including fungi and bacteria (13, 18, 19, 28). For instance, both endophytic and epiphytic bacteria and fungi are known to colonize sugar beet seed (13). Seeds can transmit spinach fungal endophytic and epiphytic diseases (19). In a 1986 study, alfalfa, wheat, and mung bean seeds tested positive for the bacterium *Bacillus cereus* spores, which are known to cause food-borne illness in humans (18, 29). A 1995 food borne illness outbreak affecting both the United States and Finland was traced back to alfalfa

seed contaminated with the bacterium *Salmonella enterica* serovar Stanley (28). However, quality vegetable seed intended for commercial use are generally tested for germination and physical purity, vigor, genetic purity, and disease. Also, vegetable seed are usually treated before sale to reduce fungal and/or bacterial numbers. This is accomplished through hot water baths or chemical treatments. Topical chemical treatments may include seed coatings of fungicides, insecticides, and bactericides (53). Overall, little information is available to describe the normal bacterial community present on seed. Studies of bacteria found on seed have primarily focused on seed transmission of plant pathogens and on human food-borne illnesses associated with sprouts.

### **Bacterial Communities on Plants**

Terrestrial plants provide multiple and diverse habitats for microorganisms both on the aerial parts of plants and the roots (3, 11, 41). The above ground region of the plant is known as the phyllosphere (25), and the part of the plant located below soil level is known as the rhizosphere (3). These multiple plant surfaces provide differing environmental conditions. For instance, the phyllosphere presents a harsh environment for bacteria due to the continuously fluctuating climatic conditions including temperature, humidity, wind, solar radiation, and moisture levels (20). Nutrients are less abundant and more spatially distributed on the aerial plant surfaces (3, 25). Several factors encourage the survival of bacterial communities on leaf surfaces. For instance, it is known that bacteria favor the underside of the leaf and leaves that are closer to the ground. These areas provide more nutrients and shelter from environmental stress (3). Additionally, both the upper and lower surfaces of a leaf are not smooth. The leaf's surfaces are made up of sutures, trichomes, and leaf veins, which determine where water tends to

collect on the leaf. Considering the size of a bacterium in relation to the size of a leaf, there are many crevices in the cuticle in which bacteria can survive (25). Leaves are known to produce exudates containing nutrients such as sugars, amino acids, proteins, and salts (4). However, these exudates are not evenly dispersed on the leaf's surface, but tend to be found in areas of injury, near glandular trichomes, and at hydathodes (2, 25). Bacteria are more prevalent near these nutrient rich leaf areas (25). Generally, bacterial colonization is more abundant at the base of trichomes, stomata, the epidermal cell wall junctions, and in the grooves along the veins of the leaf (55). In contrast, the rhizosphere is considered a more stable environment because water availability is more constant, and the area is generally thought to be more nutrient rich because of exudates from the root tips (3).

There are a vast number of microorganisms associated with plants. Some are beneficial while others cause plant diseases. There are many microorganisms, including bacteria and fungi that benefit the plant, especially in the root zone, where some microorganisms encourage root growth. Some bacteria and fungi develop symbiotic relations with plants by increasing the availability of nutrients and by controlling some plant pathogens. Therefore, both the plant and the microbes benefit. Some rhizosphere bacteria produce hormones that stimulate plant growth in response to nutrients leaked by the plant into the root zone. Many times it is the cumulative effect of these different microbes that result in the positive effect on the plant (54). However, some microorganisms adversely affect plant health. Many bacteria, viruses, and fungi, are known to be the cause of plant diseases (2). For example, some spinach diseases caused by bacteria include bacterial leaf spot and bacterial soft rot. Viral diseases of spinach are many and include curly top and spinach blight. There are many fungal diseases of spinach including downy mildew, Fusarium wilt, and Pythium root rot (46).



The leaf blade of the plant is made up of several distinct tissues and cell types. The leaf's outer surface is known as the epidermis and can be thought of as the leaf's skin. It is a network of closely spaced cells. The cuticle is a waxy layer covering the epidermis and acts as a physical barrier (9). The epidermis and cuticle are not smooth. On a microscopic level, these tissues consist of ridges and valleys (25). The topography of savoy spinach is more pronounced than in flat-leaved cultivars. There are openings in the epidermis and cuticle called stomata that allow gas and water vapor exchange between the inner leaf and the surrounding atmosphere. Guard cells control the opening and closing of the stomata to regulate gas exchange. Inside the epidermis, the leaf consists of a network of vascular tissue and mesophyll tissue. The vascular tissue consists of xylem and phloem. The xylem tissues are responsible for carrying water and minerals from the root of the plant to the stem and leaves of the plant. The phloem tissues are responsible for the transport of photosynthetic products and other organic molecules throughout different parts of the plant. The mesophyll tissue just below the upper epidermis is composed of parenchyma cells with chloroplasts, which carry out photosynthesis. The mesophyll tissue in the underside of the leaf is known as spongy mesophyll and consists of irregularly shaped cells interspersed with air spaces. The air spaces are filled with water vapor, nitrogen, CO<sub>2</sub> and O<sub>2</sub> (9).

Bacteria are the most abundant colonizers of leaves. The bacteria that normally colonize leaves are different from the bacteria that normally colonize plant roots, with different metabolic functionalities and tolerance to environmental conditions. Most of the bacteria that colonize the roots, including *Rhizobium* and *Azospirillum*, do not colonize the leaves (25). The leaf may be colonized externally on the cuticle by bacteria and other microorganisms. This is known as an epiphytic or surface colonization (42). The leaf may also be colonized internally by microorganisms that reside inside the stomata, the intercellular spaces, and the mesophyll. This

is known as endophytic or internal colonization of the leaf. Interestingly, it has been found that many microorganisms have a preference to the site that is colonized. For example, both *Pseudomonas syringae* and *Pantoea* are frequently found in the phyllosphere bacterial community on plants (25). *Pseudomonas syringae* is known to colonize both epiphytic and endophytic sites depending on the plant species. In contrast, *Pantoea agglomerans* is known to colonize only epiphytic sites independent of the plant species (42). Strains of *Pseudomonas syringae* and *Pantoea agglomerans* are known plant pathogens, however not all members of the species are pathogenic to all plants (2, 5).

The majority of bacterial pathogens are introduced onto the plant phyllosphere in a number of ways, including water used for crop irrigation or pesticide application (8). Rain may also introduce bacteria onto the leaf surface by splashing contaminated soil onto the leaf or by wind-blown rain droplets carrying bacteria from one plant to another (2). Insects frequently serve as vectors in the transmission of bacteria from contaminated surfaces to leaves (2, 8). Wild animals often deposit bacteria onto the soil through fecal droppings and may transfer bacteria onto leaf surfaces by brushing their fur against the leaf. Agricultural tools such as tractors and workers' boots may disseminate bacteria from contaminated areas to plant surfaces. Flooding also carries bacteria onto leaf surfaces (2). However, it is unknown how the vast majority of non-pathogenic microorganisms are introduced onto the leaf surface.

Seed transmission of bacteria onto plant surfaces has not been fully researched. Contaminated seed are known to transmit fungal plant diseases (19). Also, it is documented that contaminated seed was the cause of a food-borne illness outbreak related to sprouts.

Specifically, *Salmonella* serotype Stanley infected 242 people through alfalfa sprouts from contaminated seeds (27). Possible seed transmission of bacteria needs further research.

Bacteria that normally colonize leaf surfaces also possess the ability to adapt to or change the environment. Many epiphytic bacteria have the capability to secrete substances that change the microenvironment that they occupy on the leaf surface. This enhances their ability to survive the harsh environment on the leaf's surface. For instance, pigmented bacteria are more resistant to UV radiation. Some bacteria, including *P. syringae*, possess genes that aid in the repair of DNA damaged by UV radiation. An increase in UV radiation causes these plasmid genes to begin producing enzymes that repair any UV damaged DNA (25). Some *Pseudomonas* species also have the ability to produce surfactants. The cuticle, since it is a waxy substance, is hydrophobic. It is suggested that the surfactant allows for diffusion of nutrients on the leaf's surface and also helps the bacteria move across the leaf surface in water (25). All *P. syringae* produce the protein syringomycin, which in large quantities causes plant cells to lyse, however smaller amounts act as a surfactant and also cause the formation of ion channels across the plant cell plasma membrane. This in turn causes the release of nutrients from the plant cell. At present, the only epiphytic bacteria known to excrete surfactants and syringomycin are the *Pseudomonas* species (25). In contrast, many bacteria known to colonize the phyllosphere are capable of producing the plant growth hormone auxin. One such bacterium is *Pantoea agglomerans*; although some of the strains are capable of auxin production, while others are not. Once again, the production of this hormone is related to the virulence of the bacterium. However, at very low concentrations, this hormone causes the plant cell wall to loosen, releasing nutrient-rich saccharides (25).

The plant itself also affects the microbial community found on the leaf surface. Most of the current knowledge of a plant's response to the microbial community is based on the study of plant pathogens. However, current thinking is that plants tend to react to both pathogens and non-pathogens in a similar manner. In addition, it is now believed that these reactions are similar for both epiphytic and endophytic microorganisms (3, 25). Plants have the ability to recognize foreign organic molecules such as such as sugars and peptides in very low concentrations (3). A plant's basic responses to microorganisms include self-induced plant cell death to prevent the spread of disease, cell wall thickening in the area of a forming bacterial colony, and the secretion of antimicrobials (1). However, bacteria have developed genes to overcome some of these defenses. The bacterial hrp (hypersensitive response and pathogenicity) genes, collectively known as type III effectors, interfere with the plant's response (25, 45). The type III effectors can suppress or stimulate the plant's immune response (45). These same hrp genes have also been found in non-pathogenic epiphytic bacteria. It has been hypothesized that these non-pathogens use the type III effectors to modify the leaf's surface to enhance the bacteria's survival (25).

Plants are known to produce molecules that deter (detergent-like compounds, salicylic acid, and jasmonic acid, glucosinolates) or encourage microbial growth (carotenoids) (1,41). In one particular study involving the leaves of four different plant species, an increasing  $\beta$ -carotene concentration in the leaves showed a strong correlation with an increase in the number of bacteria present on the plant phyllosphere (41).

Leaf exudates also help to determine the number of bacteria present on a leaf's surface. The concentrations of the exudates are dependent on the amount of water the plant has received.

Exudates are more concentrated if the plant is under moisture stress (4). The concentrations of the exudates are also dependent on leaf age. One particular study examined the growth of bacteria on lettuce leaves with varying concentrations of nitrogen, carbon, and glucose. Nitrogen was determined to be the limiting factor (7). Varying carbon and glucose concentrations did not significantly affect bacterial growth. However, the leaves that were rich in nitrogen had a much higher bacterial population than the leaves that had little nitrogen. This was determined to be a factor of leaf age. Young leaves were found to have much higher nitrogen concentrations than middle leaves, and older leaves had very low nitrogen concentrations (7). Therefore, the amounts of nutrients, especially nitrogen, on the leaf determine to a large extent the size of the bacterial population on the leaf.

To date, there has been very little research to study the bacterial community on the leaves over the life cycle of the plant and to determine if seed bacteria are transferred to the plant phyllosphere. As previously mentioned, the nutrients of the leaf vary in relation to the age of the leaf. Leaf constituents also change over time causing hardening of leaves with age. These factors affect the ability of microorganisms to populate the leaf (7, 16). Several bacterial food-borne illness outbreaks have been associated with sprouts. The source of contamination each time was traced back to bacteria on the seeds used to grow the sprouts (10, 18, 28, 33). *Pantoea agglomerans* tagged with the *gfp* gene and then inoculated onto *Eucalyptus* seeds by soaking in a  $10^8$  CFU/mL suspension for one hour was recovered from seedlings produced from those seeds (15). This research suggests that vertical transmission of bacteria from seed surface to plant phyllosphere is possible.

## **The Study of Microbial Ecology**

Microorganisms are known to live in communities. Microbial communities consist of different types of microorganisms such as bacteria, fungi, and yeasts. The communities also vary in the genera and species of particular microorganisms. Microbial ecology is the study of microorganisms in their habitat. Specifically, it is the study of communities of microorganisms and how they interact with their habitat and other microorganisms within the community (25, 37). Microbial communities are described in terms of diversity, identity, and abundance (21). Microbial ecology of bacterial communities present on seed and plant leaves can be studied using both traditional culture techniques as well as molecular methods.

Traditional culture techniques are based on the ability to grow bacteria on culture media. The success of getting bacteria to grow depends on the make-up of the media and the correct environmental factors. All bacteria require nutrients. For growth, bacteria also require energy and electrons to be present in a useful form. Other growth factors including amino acids for protein synthesis, purines, and pyrimidines for nucleic acid synthesis, and vitamins for enzyme cofactors are also needed by some bacteria. The ability of bacteria to grow is also dependent on environmental factors such as water activity and the concentration of solutes, pH, temperature, the amount of oxygen present, and atmospheric pressure (37). Therefore, the successful growth of bacteria on culture media depends upon a general knowledge of the bacteria thought to be present and proper selection of media and environmental conditions that mimic the microbial habitat. The conditions required for growth are unknown for the majority of microorganisms.

Within limitations, the diversity, identity, and abundance of bacteria present in a particular microbial ecosystem can be determined using traditional culture techniques. However,

this is true only for a few members of the community. Diversity, to a degree, can be studied by varying the media and the environmental factors. Abundance of bacteria present can also be determined based on the number of culturable bacteria present in the original sample, which is determined by plate counts of serial dilutions (37). Identities of some of culturable bacteria can be presumptively determined based on appearance on selective media and biochemical tests. However, due to the fact that many bacteria of different species appear similar on selective media and results of biochemical tests may be similar for bacteria adapted to the same environment.

Culturing of bacteria found in a particular ecosystem can provide valuable information. Foremost, only viable bacterial cells grow when plated. Therefore, no dead bacteria are retrieved from the ecosystem. Culturing also allows for the isolation of one pure bacterial species within a colony. Once isolated, the bacteria can then be studied and categorized according to morphology and the use of metabolic substrates. Biochemical testing on the isolated, pure bacterial colonies can provide information about the ecosystem such as sources of nutrition and electron acceptors that are available for use in respiration (37). This is why culturing is important.

The vast majority of microbes are unculturable, however, necessitating the use of molecular techniques to study diversity, identity, and abundance (21, 31, 56). DNA isolated from complex communities are extracted and the conserved 16s rDNA are amplified using Polymerase Chain Reaction (PCR) (21). Once there are adequate amounts of DNA, the different DNA sequences can be separated by electrophoresis to determine diversity, identity, and abundance within the community.

Denaturing Gradient Gel Electrophoresis (DGGE) is one technique by which the different DNA fragments can be separated. DGGE separates the DNA fragments based on nucleotide composition rather than size (31, 32). DGGE separates fragments through an acrylamide gel containing the denaturants urea and formamide. The gel is poured in such a way that the denaturants increase in concentration from low at the top of the gel to high at the bottom of the gel. As the denaturant becomes more concentrated, the DNA begins to unravel based upon the base pairs. It requires more energy, thus more denaturant, to unravel DNA containing base pairs with higher guanine and cytosine content because these base pairs are held together with three hydrogen bonds; whereas, adenine and thymine base pairs are held together with two hydrogen bonds. The DNA fragments do not completely unravel because one end is held together by a GC clamp. This area of high guanine and cytosine content keeps the DNA from completely unraveling into two single stranded DNA fragments. Once the DNA fragment unravels to the point of the GC clamp, it stops migrating in the acrylamide gel. DNA sequences are therefore separated based on the difference in base pairs, which is different for different bacteria. Once migration has stopped, the DNA fragment is displayed as a band in the acrylamide gel. The number of bands present in the gel represents the richness of microorganisms present in the sample. Each band corresponds to a different genus (21, 31, 32). Although DGGE does not identify the bacteria present, the bands in the acrylamide gel may be excised and the DNA sequence determined. Typically, the conserved 16s rDNA is amplified and the subsequent bacterial community members separated using the DGGE. Excision and sequencing of the 16s rDNA gene product can then be sequenced and compared to a sequence data base to determine the identity of the bacterium at the genus level (22).



Quantitative real-time PCR is another molecular technique that may be used to study the abundance of the members of a microbial community. This technique allows the determination of the starting amount of DNA present in the sample (44). The primers for community analysis are typically designed to amplify members of specific taxonomic levels; typically the phylum (6). Real-time PCR allows quantification of DNA in real time by incorporating a fluorescent molecule, such as SYBR green into the PCR reaction. This molecule binds only to double-stranded DNA and fluoresces only when bound to the DNA, thus fluorescence will only occur when a new copy of DNA is synthesized by PCR. As more DNA is made during the PCR reaction, more fluorescence is detected. A standard curve is made from a standard containing a known amount of beginning DNA. The amount of DNA in an unknown sample is then determined by extrapolation from the standard curve (21, 44). The quantity of each of the different members in the microbial community can be determined using real-time PCR.

Both traditional culture techniques and molecular methods may be used to study microbial ecology. Each has both advantages and disadvantages. Traditional culture techniques only detect living microorganisms. Therefore, transient dead microorganisms are not detected. However, traditional culture techniques only detect a small portion of the actual microorganisms present (22, 47, 56). For example, it is estimated that only 1% of the total bacterial population present in soil samples can be detected using the traditional culture techniques (22, 47). Weakened or dead microorganisms or those that require unknown specific substrates on which to grow will not be detected using general culture techniques (21). Also, microorganisms with longer generation times are often not detected using traditional culture techniques (56). Yang et al. (56) compared leaf bacterial communities of 7 different plant species using both traditional culture techniques and DGGE. DGGE analysis revealed that the bacterial richness of the plant

species were greater than determined using culture based techniques (56). DGGE only requires the presence of DNA from microorganisms. Therefore, it can be used to detect microorganisms that are alive and/or weakened, as well as those that are dead (56). However, DGGE is not without flaws. In an ideal community, each band within a DGGE pattern represents a single group of closely related bacteria. However, it is possible for bands from different microorganisms to co-migrate if the organisms are similar in GC content within the amplified region or the denaturing gradient too broad (35). Another disadvantage of DGGE is the difficulty of reproducibility. The gel characteristics are dependent upon the actual amounts of denaturants put into the gel and the rate at which the gel is poured. It is difficult to pour two gels exactly alike (32). In spite of the disadvantages, molecular methods provide much information on microbial ecology and are the method of choice.

### **Microbial Antagonism**

Bacteria reside within biofilms on the leaf surface, embedded within polysaccharides that form a coating or barrier linking the bacteria to each other and to the surface. Biofilms provide an intricate network allowing the bacteria to share water and nutrients, and are often composed of multiple species (36). These interactions may be beneficial, neutral, or competitive (36, 40). Competition between the microorganisms for limited space and nutrients is termed antagonism. Certain microorganisms out-compete others by being more motile, which allows them to move to a more nutrient rich environment. Other microorganisms are capable of releasing toxins to reduce the numbers of their competitors. In fact, some species are capable of quorum sensing (QS) in that the toxins are only released when the species has reached a certain population size thereby insuring an effective concentration of the toxin. Still other microorganisms feed on their

neighbors (30). Therefore, competition exists in microbial communities for limited nutrients and space.

Antagonists may be useful tools to combat unwanted microorganisms. Microorganisms from the rhizosphere and phyllosphere of sugar beet (*Beta vulgaris*) were determined to antagonize the growth of known sugar beet plant pathogens. The presence of antagonists was determined by the plant developmental stage and that the antagonistic potential was pathogen specific, very few of the antagonists displayed antagonism toward a broad range of microorganisms (57). Therefore, antagonists for plant pathogens are known to exist and are specific. Antagonists also play a role in the ability of food-borne human pathogens to colonize fresh fruits and vegetables. A recent study examined the correlation between the natural bacteria of different lettuce cultivars and the ability of several *Salmonella enterica* cultivars to colonize the lettuce. Some cultivars were more easily colonized than others and that the indigenous microbiota appeared to determine which cultivars were colonized with the human pathogen (23). Brandl (8) provides an overview of the factors affecting the infection of fresh produce with *Salmonella enterica* and *E. coli* O157:H7. Brandl suggests chemical disinfection of produce to reduce microbial counts leaves little competition from the resident microbiota to protect against infection of the produce by human pathogens. On the other hand, indigenous microbiota may indeed help the human pathogens colonize the leaves (8). Clearly, more research is needed to determine the role resident microflora play in the establishment of human enteric pathogens on fresh produce.

One possible use of antagonism is to prevent the infection of plants by both plant and human pathogens. Microorganisms normally present on the surface of seed have the potential to

be antagonistic. For example, rice seed are known to harbor bacteria that are antagonistic toward several plant pathogens (12). Several rhizobacteria, especially those from *Pseudomonas* spp., have the ability to induce plant resistance to plant pathogens with immunity persisting throughout the life of the plant (39). Several strains of *Pseudomonas fluorescens* inhibit the growth of several fungi associated with plant disease (51). The ability of human pathogens to infect the root systems of plants is known to be adversely affected by the presence of natural microflora of bacteria surrounding the roots (23). Several beneficial organisms have been delivered on seeds using bio-priming to test the efficacy of seed treatment with antagonistic bacteria. These studies showed that application of antagonistic bacteria to the seed surface does in fact provide protection against plant pathogens (39, 51). This suggests that human pathogens could be inhibited from colonizing food crops through the use of antagonistic bacterial seed treatments applied prior to planting.

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## CHAPTER 3. MICROBIAL COMMUNITIES OF SPINACH AT VARIOUS STAGES OF PLANT GROWTH FROM SEED TO MATURITY

### Introduction

Microbial communities are known to populate plants at all stages of development from seed to maturity (2). Seeds can harbor significant levels of culturable microorganisms, typically  $10^3$  to  $10^5$  CFU/g for alfalfa (4, 5), mung beans, onion (26), and as many as  $10^7$  CFU/g on rice seeds (24). Some of the microorganisms on seeds are harmless while others are plant pathogens (17). Leaves also support a diverse microbial population (32) that is dependent on factors such as leaf age (7), the amount of moisture present (3), the presence of various organic compounds that may be used as nutrients (27), and adverse factors such as UV radiation (19). Bacteria are the most abundant colonizers of leaves and are often as numerous as  $10^8$  CFU/g of leaf (20). For instance, field-grown tomato leaves have been shown to harbor as many as  $10^4$  to  $10^8$  CFU/g (13). To date, the role the seed microbiota plays in the establishment of microbial communities on the leaves at different stages of plant development is largely unexplored.

In addition to plant pathogens, seeds are carriers of human pathogens. For example, the Center for Disease Control (CDC) recently reported alfalfa sprouts grown from contaminated seed caused a *Salmonella* outbreak (12). Therefore, research exploring the possible connection between seed microbiota and plant leaf microbiota has human health implications. Leafy greens in particular have been implicated in recent food-borne illness outbreaks. According to the CDC, the consumption of fresh spinach caused an *E. coli* O157:H7 outbreak in September 2006 (11). In September 2007, Dole Fresh Fruit Company issued a recall of Dole Hearts Delight Salad Mix due to the detection of *E. coli* O157:H7 by the Canadian Food Inspection Agency (30). These

outbreaks and recalls have alarmed consumers, caused economic losses for vegetable producers, and raised questions about how fresh fruits and vegetables can serve as vehicles for bacterial human pathogens that cause food-borne illnesses.

The bacterial community structure and abundance of spinach (*Spinacia oleracea*) seeds and leaves at different developmental stages from these germinated seeds were compared using molecular techniques. Three cultivars were chosen according to leaf type including savoy 'Menorca', semi-savoy 'Melody', and flat leaved 'Space' to assess differences in microbial community due to leaf topography. Contributions of the environment to the bacterial community on leaves were examined by growing plants under controlled conditions in a growth chamber and under uncontrolled field conditions. The data was analyzed according to cultivar to determine if there is a significant correlation in richness and abundance of the bacterial community between the seed and the plant leaves produced from those seed. It was also determined if there was a significant correlation in richness and abundance of the bacterial community at the different stages of plant development according to cultivar. The effect of the environment was also assessed for each cultivar to determine if there was a significant correlation between richness and abundance of leaf bacteria in response to environmental conditions. Finally, bacteria cultured from the three different cultivars of spinach seed were evaluated as possible antagonists against *E. coli* O157:H7 and *Salmonella enterica* in the interest of developing a natural biological seed coat treatment that could inhibit the establishment of food-borne pathogens in the future.

## Materials and Methods

### Spinach Production and Harvest

Three spinach cultivars (*Spinacia oleracea*) with different leaf variations, ‘Melody’ (semi-savoy) (Lot 714089), ‘Menorca’ (savoy) (Lot 847122), and ‘Space’ (flat) (Lot 34018) from SeedWay® LLC, Hall, NY, USA were seeded under two different growing conditions. Gloves were worn when handling the seed. On 14 September 2009 each of the three cultivars was planted at the Virginia Tech Kentland Research Farm on a 4 x 100 ft plot. The plot was subdivided into 3 subplots approximately 4 x 30 ft each. Four rows of one cultivar were planted per subplot. Prior to planting, the soil was tilled. At the time of planting, an inorganic granular fertilizer (Southern States Cooperative Fertilizer) of 10N-10P-10K was applied at a rate of 574 grams per every 30 ft row and incorporated into the soil before planting. A pre-emergence herbicide (Dual II Magnum®, Syngenta Crop Protection, Inc, Greensboro, NC) was applied at 0.6 pt/ acre two days after planting. Surface water from a creek was filtered and applied by drip irrigation every two days from seeding until final harvest. On September 23, 2009 seeds of each of the three cultivars were planted in 4 inch square plastic pots filled with Sun Gro® Metro-Mix 852 (Sun Gro® Horticulture Canada CM Ltd.) at a rate of five seeds per pot. The pots were placed in Percival growth chambers (Boone, Iowa) and held at 21°F with a 12 hour photoperiod. Pots were watered as needed using tap water, and fertilizer was added to the soil on November 25, 2009 using a water-soluble, inorganic fertilizer providing 250 ppm nitrogen. Care was taken to prevent water or fertilizer from contacting the foliage.

Leaves were harvested at each location when the samples reached the appropriate development stages. The cotyledon, 3-4, and 6-8 leaves were harvested by cutting the base of the

petioles using scissors. Samples were stored in separate sterile plastic bags according to cultivar. Scissors were wiped with ethanol before use and between harvests of different cultivars. Gloves were worn while handling samples. The samples were immediately taken to the laboratory, stored at 4 °C, and processed within 4 hours.

The plants in the growth chamber required more time to reach the 3-4 and 6-8 leaf stages than did the plants grown at Kentland. Therefore, the leaves in the growth chamber were harvested at later dates for these stages (Table 3). Growing Degree Units (GDUs) for Kentland were calculated based on the environmental conditions recorded hourly at the Virginia Tech Kentland Research Farm weather station (<http://www.cals.vt.edu/research/kentland/weather/index.html>) (Table 3). GDUs for the plants in the growth chamber were calculated based on the set temperature of 21°C (Table 3). The GDUs were calculated using 2.2°C as the base temperature and with the following formula (16).

$$GDUs = \sum\{1/2(T_{max} + T_{min}) - T_{base}\}$$

### **Epiphytic Bacterial Isolation and Bacterial DNA Extraction from Seeds and Leaves**

About 10 grams of leaves from each cultivar and growing condition were collected. Experiments were repeated in triplicate. Each sample was assigned a unique number and treated independently as described below. The bacteria present on the surface of the spinach seeds and harvested leaves were removed from the surfaces using 1% (wt/vol) peptone water (Sigma-Aldrich Co., St. Louis, MO) mixed with 10 mM EDTA (ethylenediamine tetraacetic acid disodium salt dihydrate, Fisher Scientific, Pittsburg, PA) and 1% Tween® 80 (Fisher Scientific). For each 10 grams of sample, 90 mL of the peptone water mixture was added into a sterile filter bag (Filtrabag®, Fisher). Similar to the treatment presented by Burke et al. to remove bacterial

DNA from algae surfaces (10), the contents were incubated for 2 hours at room temperature and 80 rpm in an incubator/shaker (innova® 42, New Brunswick Scientific, Edison, NJ) to allow dissolution of the bacteria on the leaf surface into the aqueous solution. After this, the samples were processed in a stomacher (BagMixer® 3500 JumboMix®, Interscience Laboratories, Weymouth, MA) at the lowest speed for 5 minutes. Approximately 15 mL of liquid was decanted from the filtered bag into a sterile centrifuge tube and centrifuged at 4000 rpm at 4°C for 20 minutes. The liquid was discarded and the pellet was suspended in 112 µL sterile water, 44 µL of 3mg/mL lysozyme (Fisher-Scientific), and 44 µL of 1 mg/mL achromopeptidase (Sigma). This mixture was incubated for 30 minutes at 37°C and then transferred into a bead beater tube (VWR™) containing 0.8 grams of 0.1 mm glass beads (Research Products International Corporation, Mt. Prospect, IL). The DNA was then extracted using the ZR Soil DNA Kit™ (Zymo Research Co., Orange, CA) per manufacturer's instructions.

### **DGGE Analysis of PCR Amplified 16s rDNA Gene Fragments**

Microbial community richness was assessed by amplification of the 16s rDNA from the total DNA to generate a 566 bp fragment using the primers 341f (5'-CCT ACG GGA GGC AGC AG-3') and 907r (5'-CCG TCA ATT CMT TTG AGT TT-3') (22). The forward primer was modified to add a 40 nucleotide GC clamp at the 5' end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') (22, 23). Each 50 µL PCR mixture consisted of 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 25 mM Tris-HCl (pH 8.6), 0.2 mM dNTPs, 1% DMSO (dimethylsulfoxide), 0.5 µM of each primer, 0.026 Unit *Taq* DNA polymerase (USB, Cleveland, Ohio), and 100 ng of sample DNA. The size and intensity of PCR products were confirmed using a 0.9% agarose gel (Fisher-Scientific, Atlanta, GA).



The PCR products were run on a 6% acrylamide gel using a 35-60% denaturant gradient of urea and formamide (100% denaturant corresponds to 7 M urea plus 40% (vol/vol) of deionized formamide) using the Bio-Rad DCode™ Universal Detection System (Bio-Rad, Hercules, CA). A 60 µL aliquot of each of the PCR products was separated at a constant 75 volts at 60°C for 16 ½ hours. The DNA bands were visualized by staining with ethidium bromide (2 µg/mL) and photographed using the Molecular Imager ® GelDoc™ XR (Bio-RAD). The bands were analyzed using FP Quest™ Software Version 5.10 (Bio-Rad) and the DGGE profiles were analyzed using the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) using the same software producing dendrograms for the 3 cultivars under the 2 different conditions at each harvest time. Three different runs were made per each sample. Gels were repeated two times.

### **Quantifying Bacteria Belonging to Select Phyla Using Real-time PCR**

Bacterial community abundance was assessed by amplification of phyla specific regions of the 16s rDNA gene as described by Blackwood et al. and Fierer et al. (6, 14). Phylum-specific abundance was determined targeting the following phylogenetic groups: *α-Proteobacteria*, *β-Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Table 2).

Table 2: Phylogenetic specific primer sequences used in real-time PCR including the bacterium used as a positive control

<b>Phylogenetic Target</b>	<b>Isolated Bacterium used as Positive Control</b>	<b>Primer Sequences</b>
Eubacteria	<i>Escherichia coli</i>	Eub 338f ACT CCT ACG GGA GGC AGC AG Eub 518r ATT ACC GCG GCT GCT GG (14)
<i>Actinobacteria</i>	<i>Microbacterium</i> sp.	Actino 235f CGC GGC CTA TCA GCT TGT TG Eub 518r ATT ACC GCG GCT GCT GG (14)
<i>Bacteroidetes</i>	<i>Flavobacterium</i> sp.	Cfb 319f GTA CTG AGA CAC GGA CCA Eub 518r ATT ACC GCG GCT GCT GG (14)
<i>Firmicutes</i>	<i>Exiguobacterium</i> sp.	Lgc 353f GCA GTA GGG AAT CTT CCG Eub 518r ATT ACC GCG GCT GCT GG (14)
<i>α-Proteobacteria</i>	<i>Sphingomonas</i> sp.	Eub 338f ACT CCT ACG GGA GGC AGC AG Alf 685r TCT AGC RAT TTC ACC YCT AC (14)
<i>β-Proteobacteria</i>	<i>Acidovorax</i> sp.	Beta 680f CRC GTG TAG CAG TGA 1392r ACG GGC GGT GTG TRC (6)

Source of primer is cited following the reverse primer

Standard curves for real-time PCR analysis were constructed for each phylum using 16s rDNA universal primers for bacteria previously isolated from spinach leaves and identified at the genus level using 16s rDNA universal primers (Table 2). Frozen stocks of each bacterium were inoculated onto TSA (Trypticase soy agar, BBL™ Becton, Dickinson & Company (BD) Sparks, MD) and incubated for 48 hours at 25°C. Isolated colonies were transferred to 10 mL of TSB (Trypticase soy broth, BBL™ BD) and incubated for 48 hours at 25°C and 100 rpm. DNA from

each phyla except for *Firmicutes* was isolated using the Puregene® DNA purification kit (Gentra systems, Minneapolis, MN) per manufacturer's instructions. DNA from the *Firmicutes* was isolated using a modified CTAB DNA extraction protocol (28). Serial dilutions of the DNA were made to create 10-fold serial dilutions from 100 to 0.001 ng/μL. Standard curves using real-time PCR amplification were prepared for each dilution using phylum-specific primers (Table 2). Each 25 μL reaction contained 12.5 μL of HotStart-IT™ SYBR®Green qPCR Master Mix 2x, which contains 5 and 0.4 mM of MgCl<sub>2</sub>, respectively (USB® catalog # 75770, Cleveland, OH), 10 nM of Fluorescein Passive Reference Dye (USB® catalog # 75767), 1% DMSO (dimethylsulfoxide), 1 mM MgCl<sub>2</sub>, 0.5 μM of forward and reverse primers, and 50 ng of DNA template. PCR conditions were denaturation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, 30 seconds at the annealing temperature, and 30 seconds at 72°C. Annealing temperatures were 60°C for *α-Proteobacteria*, *Actinobacteria*, and *Firmicutes* (14). The annealing temperature for *β-Proteobacteria* was 57 and 65°C for *Bacteroidetes* (6, 14). Each concentration in the standard curve was done in triplicate. Each sample was run in duplicate on separate dates using a standard curve as described above for each run. Amplification was carried out with an iQ5™ Optical System Real-time PCR Detection System (Bio-Rad).

### **Isolation and Identity Determination of Bacterial Antagonists Present on Seeds**

Bacteria on the surface of the three cultivars of spinach seeds were removed from the surfaces as described earlier. An aliquot was serially diluted into peptone water. A 1 mL aliquot of each dilution per cultivar was pipetted into a Petri dish and R2A agar (Difco™ Franklin Lakes, NJ) was poured into each Petri dish. This was done in triplicate for each dilution of each cultivar. The plates were incubated at room temperature and plate counts were done on days 3, 8,

10, and 15. On each day, newly appearing colonies were transferred onto R2A media in square Petri dishes. These bacteria were allowed to grow at room temperature until a sufficient colony size was obtained. The square plate was then refrigerated. At the start of the antagonism assay, the colonies were transferred to new square plates containing R2A agar to maintain growth.

To prepare the lawn and check for antagonism assays one vial of frozen culture for *E. coli* O157:H7 was removed from -80°C storage and thawed on ice. One loop from the vial was streaked onto a SMAC (Sorbitol MacConkey Agar, Difco™ BD) plate and incubated at 37°C for 24 hours. Similarly, one vial of frozen culture for *Salmonella enterica* was removed from -80°C storage and thawed on ice. One loop from the vial was streaked onto a XLT4 (Difco™ BD) plate and incubated at 37°C for 24 hours. One pure colony from each plate was inoculated into separate 100 mL flasks containing ½ strength TSB and incubated at 37°C for 24 hours. The resulting concentration for each organism was determined to be approximately 10<sup>8</sup> cells/mL from plate counts. The broth for each organism was diluted to 10<sup>7</sup> cells/mL using 1x phosphate buffer solution. The lawn was created by spreading 0.1 mL (100 µL) of each culture onto R2A culture plates; thus providing a final concentration of 10<sup>6</sup> cells. Each plate was allowed to dry for 5 minutes and then colonies from the box plate that were isolated from the spinach seed were applied to the lawn in an isolated area of the plate. Each plate was inoculated with 8 different colonies to prevent intersecting zones of inhibition. The plates were incubated for 48 hours at room temperature. The plates were checked at day 2 and thereafter for up to 10 days for a zone of inhibition surrounding the colonies, indicating antagonism.

DNA was extracted from the colonies showing antagonism using the PUREGENE® DNA Purification Kit and the manufacturer's Gram-positive bacteria protocol. The 16s rDNA

was amplified. Each 25  $\mu$ L reaction mixture contained 5x colorless GoTaq® Reaction Buffer (Promega, Madison, Wisconsin), pH 8.5 and MgCl<sub>2</sub> at 1.5 mM per reaction, 0.2 mM dNTPs, 1% DMSO, an additional 4 mM MgCl<sub>2</sub>, 0.3 mM of 27f primer and 0.3mM of 1392r primer, GoTaq® DNA Polymerase (Promega) at 5 units/mL giving a final concentration of 0.026 units per 25  $\mu$ L reaction, and 50 ng of template DNA. The size and intensity of PCR products were confirmed using a 0.9% agarose gel (Fisher-Scientific). The PCR products were then purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, California). The purified DNA was sent to Virginia Bioinformatics Institute (VBI) at Virginia Tech for sequencing. The taxonomic identity of the isolate was determined by comparing the resulting sequence to known 16s rDNA sequences using the NCBI/Blast (Megablast) Nucleotide collection (nr/nt) database at the following website (<http://www.ncbi.nlm.nih.gov/blast/>).

## **Results and Discussion**

This study examined the bacterial richness and abundance of members of select bacterial phyla of three different cultivars of spinach seed and the leaves of the plants grown from those seed at different stages of development (cotyledon, 3-4 leaf, and 6-8 leaf). The three cultivars were chosen to represent different leaf topography; savoy ‘Menorca’, semi-savoy ‘Melody’, and flat leaves ‘Space’. The plants were grown under two conditions; one being growth chamber in which temperature and light were controlled, and the other being outdoors at Kentland Farm where environmental conditions were not controlled. Plants grown in growth chambers may receive contributions to the epiphytic microbial community from the seed coat, soil-less potting mix, and the tap water used for hydration of the plants. The walls of the chambers were not sanitized and may have contributed some microbes as well. The epiphytic microbial community

of spinach plants grown at Kentland Farm may result from the seed coat, soil, filtered stream water used for irrigation and wind. It can be surmised that similar bands in DGGE profiles or bacteria isolated from plants grown in the two environments are of common origin: the seed coat.

### **Environmental Conditions at Time of Harvest**

The cotyledon, 3-4, and 6-8 leaves were harvested at each location when each sample reached the appropriate development stage, which varied between plants grown in growth chambers or in the field (Table 3). Spinach is typically harvested at about 1000 GDUs for immature or baby spinach (21).

Table 3: Dates of harvest and Growing Degree Units (GDUs) of spinach leaves from Kentland Farm<sup>§</sup> and Growth chamber<sup>§</sup> for each leaf stage

<b>Leaf Stage Harvested</b>	<b>Kentland</b>		<b>Growth chamber</b>	
	<b>Date of Harvest</b>	<b>Accumulated GDUs</b>	<b>Date of Harvest</b>	<b>Accumulated GDUs</b>
Cotyledon	10-16-2009	429	10-16-2009	451
3-4 leaf	11-11-2009	629	11-24-2009	1184
6-8 leaf	11-23-2009	711	12-29-2009	1842

<sup>§</sup> Kentland Farm data based on the environmental conditions recorded hourly at the Virginia Tech Kentland Research Farm weather station (<http://www.cals.vt.edu/research/kentland/weather/index.html>). Growth chamber data based on the set temperature of 21°C

The field and growth chamber plants were closest in terms of GDUs for the cotyledon harvest, indicating that both populations accumulated similar thermal time. Thereafter, the GDUs differed widely between the field and growth chambers with GDUs being much higher for the growth chamber plants compared to Kentland Farm. However, upon physical inspection, the

plants at Kentland were larger at comparable stages of development and less spindly and succulent than those grown in the growth chamber. The major difference between the two environments was light intensity. In the growth chamber light intensity from Gro Lite full photosynthetic spectrum fluorescent bulbs was measured as  $74.1 \mu\text{moles m}^{-2} \text{s}^{-1}$  compared to an estimated value of over  $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$  in the field (data not shown). Therefore, photosynthesis was lower in the chamber and development was delayed and stunted compared to the field. Although the plants differed in appearance between the field and chamber the amount (weight) of leaves harvested and processed for each stage was consistent. Since growing environments were different, particularly with regards to light intensity, GDUs were not adequate predictors of maturity after the cotyledon state of develop.

Environmental conditions, which are recorded hourly at the Virginia Tech Kentland Research Farm weather station (<http://www.cals.vt.edu/research/kentland/weather/index.html>) including air temperature, soil temperature, and precipitation, were obtained from the two days prior to and the day of harvest for each time of harvest (Table 4).

Table 4: A summary of the environmental conditions recorded hourly at the weather station at Kentland Farm, Blacksburg, VA from the time of seeding to the last harvest

Leaf Stage Harvested		Air Temperature (°C)	Soil Temperature (°C)	Precipitation (inches)
Cotyledon	Average	7.5 ± 2.1*	13.0 ± 0.7*	0.01 ± 0.03*
	Minimum	4.1	12.3	0.00
	Maximum	12.7	14.5	0.15
3-4 Leaf	Average	8.5 ± 4.4*	9.6 ± 0.5*	0.03 ± 0.03*
	Minimum	-0.4	8.3	0.00
	Maximum	20.0	10.1	0.12
6-8 Leaf	Average	5.1 ± 4.6*	8.6 ± 0.5*	0.00 ± 0.02*
	Minimum	-1.8	7.7	0.00
	Maximum	15.4	9.7	0.11

\* ± standard deviation

The plants in the growth chamber were held under constant temperature at  $21 \pm 1.9^\circ\text{C}$ . When watering, care was taken to prevent the splashing of leaves with soil as much as possible.

The environmental conditions surrounding the three times of harvest at Kentland did not differ drastically in temperature or precipitation between stages of development (Table 4). At no time did the temperature at time of harvest exceed the optimal growth temperature of  $21.1^\circ\text{C}$  ( $70^\circ\text{F}$ ), nor did the temperature at time of harvest fall below the temperature of  $-12.2^\circ\text{C}$  ( $10^\circ\text{F}$ ), which is the temperature spinach can tolerate before frost damage (32). The microbial community is known to differ with temperature and with precipitation (3, 17).

There was not a large air temperature difference around the times of harvest (Table 4). The average air temperature for the three harvests was less than  $10^\circ\text{C}$ . The maximum air



temperature surrounding the times of harvest was between 13 and 20°C, and the minimum air temperature was -1.8 to 4.1°C. The bacteria that were recovered were likely psychrotrophic or bacteria that had adapted to cooler temperatures.

Precipitation was also fairly constant surrounding the three harvest dates at Kentland Farm (Table 4). The bacteria that were present at each harvest were exposed to about the same moisture levels at each harvest.

### Microbial Richness – DGGE Analysis

The richness of the bacterial seed communities varied between cultivars, the similarity between all three cultivars was 23% (Figure 3). This was unexpected since the seeds are all cultivars of spinach and are from the same company. The seeds of ‘Menorca’ and ‘Melody’ were produced in Denmark, while ‘Space’ originated in the Netherlands. Differences in environment during production and harvesting as well as differences in handling may account for some of these differences in seed bacterial community richness.

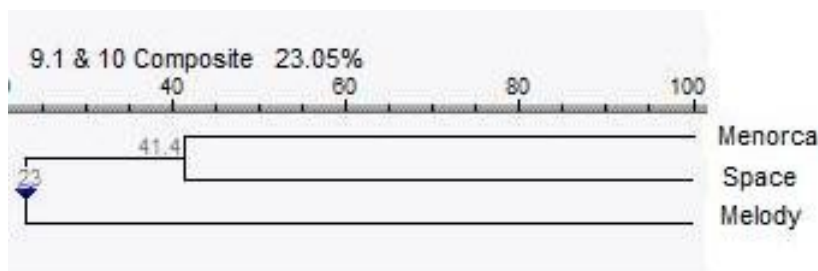


Figure 3: UPGMA dendrogram constructed based on the similarity between DGGE profiles for the three different cultivars of spinach seed. (Percentage of similarity is indicated at each node)

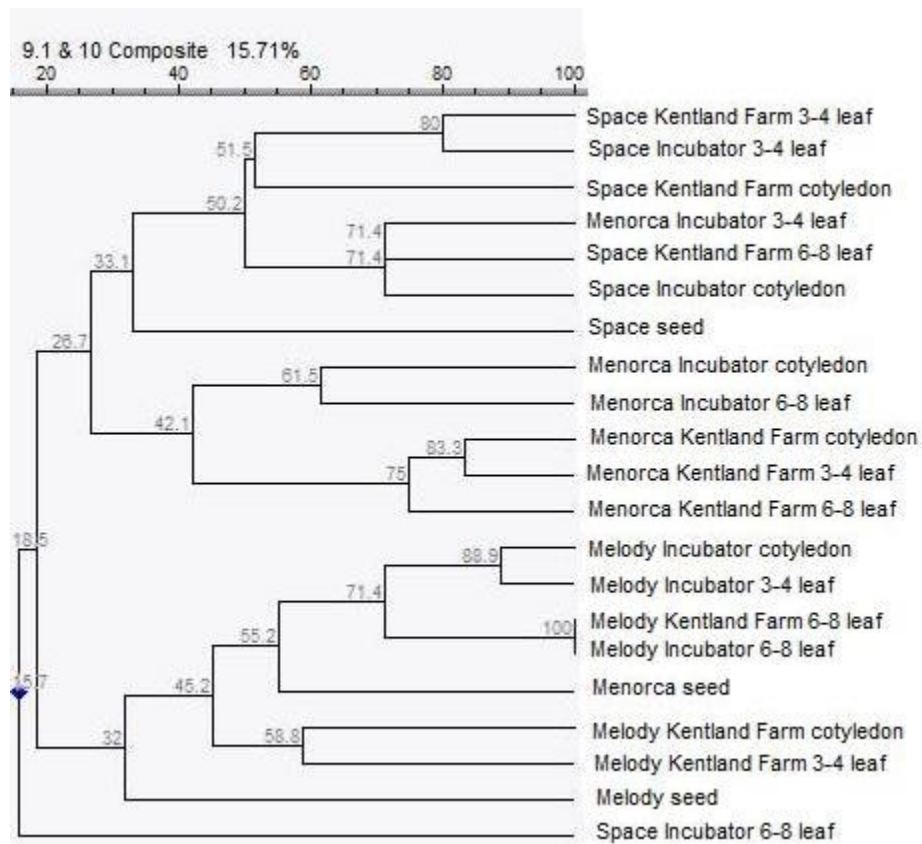


Figure 4: UPGMA dendrogram constructed based on the similarity between DGGE profiles for the three different cultivars of spinach seed and the spinach leaves grown at Kentland Farm and in a growth chamber. (Percentage of similarity is indicated at each node)

The bacterial communities of leaves from the same cultivar were the most similar to each other and to the seed (Figure 4). The seed coat community varied between 19 and 33% similarity amongst leaves of all development stages and the seed (Figure 4). When the DGGE profiles of only the leaves were examined the largest difference in community was due to the cultivar with 12-18% similarity between all cultivars (Figure 5). The cultivar, rather than the environment, accounted for the largest differences in bacterial communities with Melody being only 19%

similar to Menorca and Space and Menorca and Space being 27% similar (Figure 4). The richness of the bacterial communities changed as the plant developed (Figures 5 and 6). The communities of cotyledons and 3-4 leaf stages were most similar for the Menorca and Space cultivars, which also showed the most similarity between leaves of all development stages grown in field (Figure 5). The bacterial communities of the Melody cultivar showed more variation between the different developmental stages than the other cultivars when grown in field (Figure 5).

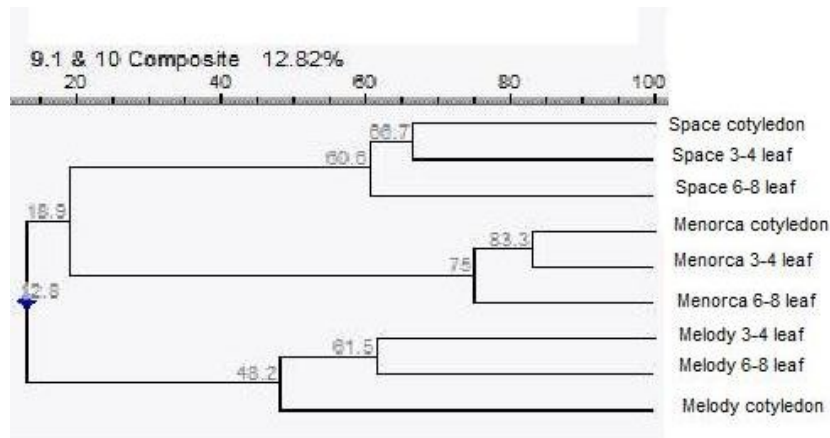


Figure 5: UPGMA dendrogram constructed based on the similarity between DGGE profiles for the three different cultivars of spinach leaves grown at Kentland Farm. (Percentage of similarity is indicated at each node)

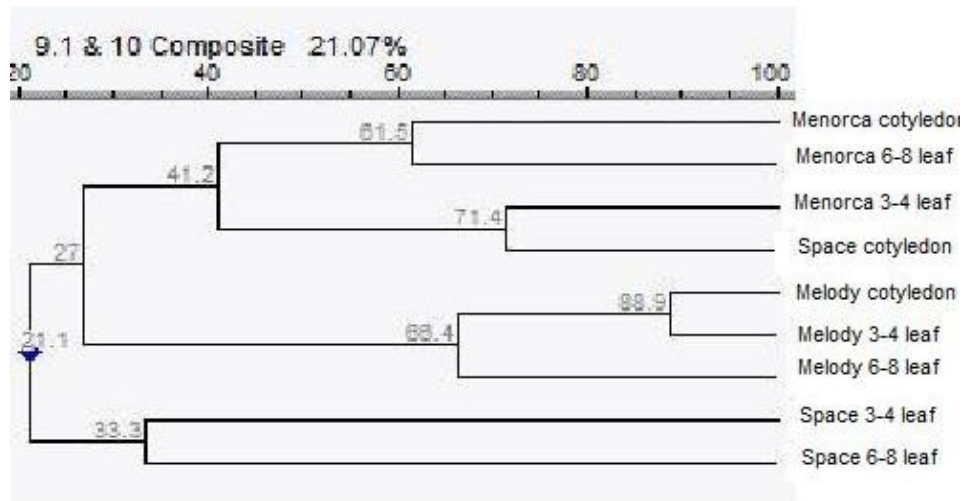


Figure 6: UPGMA dendrogram constructed based on the similarity between DGGE profiles for the three different cultivars of spinach leaves grown in the growth chamber.

(Percentage of similarity is indicated at each node)

In contrast, the most variation in DGGE profiles was observed between the Space cultivar when grown in growth chambers (Figure 6). The leaves clustered by cultivar under both growing conditions with Melody being the most different at Kentland and Space being the most different in the growth chamber (Figures 5, 6).

Age may also play a role in the microbial richness found on the leaves as it was observed that the communities of plants of the same developmental stage but grown in different environments were most similar (Figure 4). Similarly, Melody 6-8 leaf at Kentland matched 100% to Melody 6-8 leaf in the growth chamber (Figure 4). This suggests that vertical transmitted bacteria from the seed are then influenced by conditions on the leaf surface. Previous studies with romaine lettuce have shown that leaves of different age contain different amounts of nutrients (7). The amount of nitrogen in the plant was shown to limit the abundance

of bacteria on the leaf surface, this was associated with the older outer leaves of romaine lettuce. In this study we did not separate the leaves in order of emergence; however, it can be surmised that plants of the 6-8 leaf stage will contain a mix of young and old leaves while the 3-4 leaf stage plants will be mostly younger leaves. The larger differences in bacterial richness of the leaf community of growth chamber samples may reflect the difference in leaf age, as the plants grown in the growth chamber took longer to reach the different plant developmental stages.

### **Microbial Abundance**

The abundance of bacteria on the seeds was determined using culture and culture independent techniques. The numbers of total culturable bacteria on seeds of each cultivar were ‘Melody’ 5.2 log CFU, ‘Menorca’ 2.8 log CFU, ‘Space’ 0.02 log CFU. ‘Melody’ and ‘Menorca’ originated from Denmark, while ‘Space’ originated from the Netherlands. Larger populations of bacteria were determined based on amplification of the universal 16s rDNA.. The total number of bacteria on seeds of each cultivar was not affected; however, the numbers of *Actinobacteria*, *α-Proteobacteria*, *β-Proteobacteria* and *Bacteroidetes* were affected by cultivar (Figure 7). The most difference in abundance was seen between the Melody and Menorca seeds (Figure 7). These differences in abundance may reflect on the differences in bacterial species richness that were observed. Only the *Firmicutes* were constant in numbers across the three different cultivars (Figure 7). Members of these phyla are known to contain a large number of spore forming bacteria that could persist for long periods where other bacteria may die. It is not known how the seed were handled or stored before receipt. Therefore, this could account for the difference in microbial abundance.

The abundance of the bacterial populations of select phyla was quantified for each development stage of the plant grown in the two environments. Overall, the cultivar did not affect the numbers of total bacteria or members of the selected phyla that were amplified (Table 5). This was surprising as we hypothesized that the differences in leaf topography would alter the numbers of bacteria which could be supported within crevices of the savoyed cultivars (33). It is possible that the cultivars suggested do not have differences in amount of nutrients secreted or in numbers of stomata, all factors which have been previously associated with bacterial survival. No measurements of surface area, numbers of stomata or hydathode numbers or nutrient analyses were compared in this study. The abundance of total bacteria, *Bacteroidetes*, *α-Proteobacteria* and *β-proteobacteria* were significantly different between leaves from cotyledon and 6-8 leaf stage plants (Table 5). Numbers of *Bacteroidetes*, *α-Proteobacteria* and *β-proteobacteria* were significantly larger on leaves from 3-4 leaf plants compared to cotyledons (Table 5). The increase in *alpha-Proteobacteria* numbers may be due to increases in *Methylobacterium*, which use methanol emitted from the stomata of the plants as a carbon source (25). The amount of methanol available for use would be relatively low at the cotyledon stage. *Methylobacterium* has been shown to be an important member of the plant community and is associated with carbon cycling (34). The most significant differences ( $p < 0.05$ ) in bacterial abundance were observed for leaves grown in different environment, with Kentland abundance being greater than growth chamber abundance (Table 5). Several factors may explain the increased bacterial abundance on field grown leaves including delivery by soil or wind. The leaf surfaces at Kentland Farm were exposed to soil bacteria due to splashing during rain storms as well as animal and insect vectors; all of which are known to increase the microbial abundance on plant surfaces (1, 8). Precipitation occurred before each of the three harvest dates. This increase

in water on the leaf surfaces could be responsible for the abundance of bacteria on each of the different cultivars. The leaves of plants at the 3-4 leaf and 6-8 leaf stages grown in the growth chamber were smaller than those grown in the field. The size difference was most likely related to differences in light intensity between the field and growth chambers. This difference in size may be associated with less space for bacteria to colonize, resulting in smaller bacterial populations.

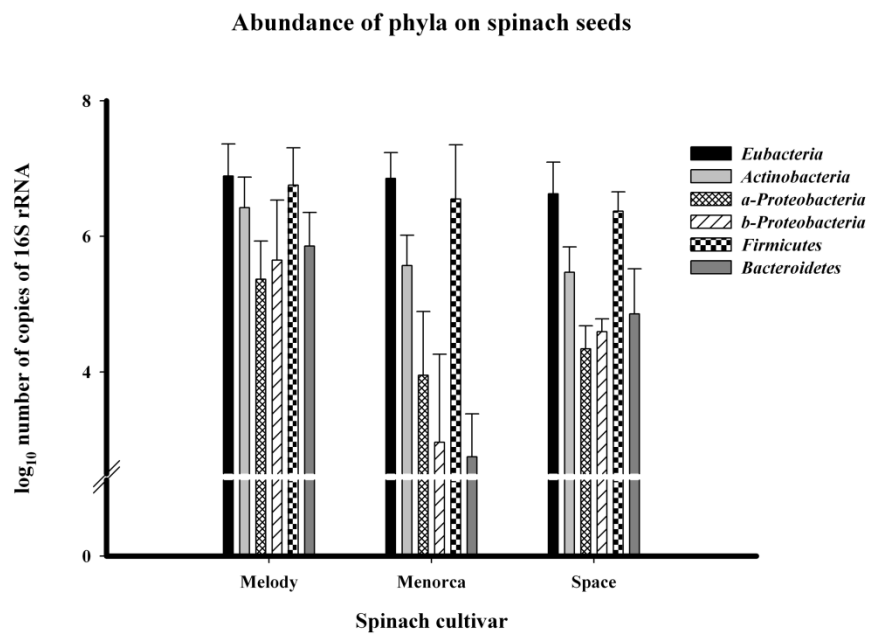


Figure 7: Abundance of bacterial phyla on three cultivars of spinach seed

Table 5: Average (of triplicate) log number of copies grouped by phylum of bacteria according to leaf stage and source for each cultivar

Eubacteria												
Cultivar	Cotyledon				3-4 Leaf				6-8 Leaf			
	Kentland		Growth chamber		Kentland		Growth chamber		Kentland		Growth chamber	
	Avg log # of copies	Std dev	Avg log # of copies	Std dev	Avg log # of copies	Std dev	Avg log # of copies	Std dev	Avg log # of copies	Std dev	Avg log # of copies	Std dev
Melody	7.14 <sup>a</sup>	0.18	7.25 <sup>c</sup>	0.30	7.63 <sup>a,b</sup>	0.08	7.63 <sup>c</sup>	0.11	7.90 <sup>b</sup>	0.12	6.44 <sup>d</sup>	0.28
Menorca	7.56 <sup>a</sup>	0.63	6.69 <sup>c</sup>	0.10	7.63 <sup>a,b</sup>	0.12	7.57 <sup>e</sup>	0.06	8.11 <sup>b</sup>	0.09	6.20 <sup>d</sup>	0.36
Space	7.16 <sup>a</sup>	0.17	7.56 <sup>c</sup>	0.22	7.73 <sup>a,b</sup>	0.70	7.49 <sup>e</sup>	0.20	7.79 <sup>b</sup>	0.36	6.56 <sup>d</sup>	0.13
<i>Actinobacteria</i>												
Melody	5.97 <sup>a</sup>	0.24	5.71 <sup>d</sup>	0.52	5.42 <sup>b,c</sup>	0.26	5.29 <sup>d</sup>	0.15	5.87 <sup>a</sup>	0.16	4.36 <sup>e</sup>	0.38
Menorca	6.06 <sup>a</sup>	0.51	5.30 <sup>d</sup>	0.08	5.35 <sup>b,c</sup>	0.23	5.48 <sup>d</sup>	0.04	6.07 <sup>a</sup>	0.07	4.02 <sup>e</sup>	0.54
Space	5.76 <sup>a</sup>	0.19	5.18 <sup>d</sup>	0.32	5.41 <sup>b,c</sup>	0.21	4.95 <sup>d</sup>	0.13	5.80 <sup>a</sup>	0.16	3.74 <sup>e</sup>	0.26
<i>Bacteroidetes</i>												
Melody	4.11 <sup>a</sup>	0.33	4.37 <sup>d</sup>	0.47	4.63 <sup>c</sup>	0.19	4.04 <sup>d</sup>	0.22	5.36 <sup>b</sup>	0.03	2.41 <sup>e</sup>	0.88
Menorca	4.29 <sup>a</sup>	0.46	3.90 <sup>d</sup>	0.12	4.69 <sup>c</sup>	0.11	4.35 <sup>d</sup>	0.24	5.54 <sup>b</sup>	0.05	2.52 <sup>e</sup>	1.66
Space	3.70 <sup>a</sup>	0.07	4.44 <sup>d</sup>	0.68	4.57 <sup>c</sup>	0.23	3.68 <sup>d</sup>	0.19	5.19 <sup>b</sup>	0.35	3.57 <sup>e</sup>	1.61
<i>Firmicutes</i>												
Melody	5.78 <sup>a</sup>	0.27	5.40 <sup>b</sup>	0.48	5.60 <sup>a</sup>	0.15	5.70 <sup>b</sup>	0.07	5.94 <sup>a</sup>	0.06	3.64 <sup>c</sup>	1.65
Menorca	5.84 <sup>a</sup>	0.55	4.61 <sup>b</sup>	0.25	5.63 <sup>a</sup>	0.07	5.45 <sup>b</sup>	0.19	5.98 <sup>a</sup>	0.17	3.55 <sup>c</sup>	1.47
Space	5.48 <sup>a</sup>	0.12	5.41 <sup>b</sup>	0.43	5.69 <sup>a</sup>	0.27	5.46 <sup>b</sup>	0.24	5.77 <sup>a</sup>	0.18	3.47 <sup>c</sup>	1.01
<i>alpha-Proteobacteria</i>												
Melody	5.57 <sup>a</sup>	0.31	5.42 <sup>c</sup>	0.51	6.11 <sup>b</sup>	0.19	6.03 <sup>c</sup>	0.08	6.46 <sup>b</sup>	0.07	3.94 <sup>d</sup>	1.42
Menorca	5.78 <sup>a</sup>	0.46	4.82 <sup>c</sup>	0.19	6.14 <sup>b</sup>	0.14	5.74 <sup>c</sup>	0.27	6.37 <sup>b</sup>	0.29	3.65 <sup>d</sup>	1.70
Space	5.23 <sup>a</sup>	0.33	5.65 <sup>c</sup>	0.33	5.72 <sup>b</sup>	0.42	5.62 <sup>c</sup>	0.19	6.16 <sup>b</sup>	0.26	3.51 <sup>d</sup>	1.11
<i>beta-Proteobacteria</i>												
Melody	4.27 <sup>a</sup>	0.32	3.60 <sup>c</sup>	0.88	4.62 <sup>b</sup>	0.50	4.06 <sup>c</sup>	0.13	5.53 <sup>b</sup>	0.01	2.56 <sup>d</sup>	0.94
Menorca	4.26 <sup>a</sup>	0.65	2.83 <sup>c</sup>	0.34	4.85 <sup>b</sup>	0.27	3.79 <sup>c</sup>	0.62	5.16 <sup>b</sup>	0.77	2.44 <sup>d</sup>	0.94
Space	3.69 <sup>a</sup>	0.43	4.89 <sup>c</sup>	0.75	4.50 <sup>b</sup>	0.08	4.05 <sup>c</sup>	0.09	4.98 <sup>b</sup>	0.69	2.01 <sup>d</sup>	1.16

A different letter indicates significant difference ( $p < 0.05$ ) within phyla

The leaf data was analyzed using ANOVA and univariate analysis in JMP® Statistical Discovery Software (©SAS Institute Inc., Cary, NC) and applying the Tukey adjustment.



## Identification of Bacterial Antagonists Isolated from Spinach Seeds

Two of the bacterial colonies isolated from the seed were found to inhibit the growth of *E. coli* O157:H7 *in vitro*. No bacteria isolated from the spinach seed showed antagonism towards *Salmonella enterica*. The two antagonists were identified as two different strains of *Pantoea* (also known as *Erwinia*). Members of the genera *Erwinia* are frequently isolated from the phyllosphere of plants, where they typically live non-pathogenic lifestyles. However, some strains of *Erwinia* cause bacterial soft rot in spinach (28). This demonstrates that there are bacteria present on the seed that are antagonistic towards *E. coli* O157:H7. Additionally, this bacterium has been isolated from leaves of mature spinach plants (30); therefore, it has the potential to be a valuable antagonist for *E. coli* O157:H7.

Biological seed treatments have been commercially available for a number of years and applications include plant disease control. Trichoderma fungal seed treatments have been commercially available for approximately 15 years and are among the oldest biological seed treatments on the market (32). BioYield™ is one commercial product that contains two PGPR isolates, *Bacillus subtilis* strain GB03 and *Bacillus amyloliquefaciens* strain GB99, in a formulation including chitin, shown to elicit low levels of resistance responses in tomato (15, 32).

Kodiak®, a *B. subtilis* strain GB03 registered as a seed treatment, shows exceptional rhizosphere competence, colonizing the rhizosphere of monocots and dicots. Though the initial success of strain GB03 has been observed in the production of cotton, other crops have shown positive yield responses following bacterization. Since *B. subtilis* is a spore-forming organism, it

is extremely tolerant of environmental stresses, including seed-treatment pesticides, soil and seed pH, cultivar effects, edaphic factors and long-term storage (9, 32).

AgraQuest has developed a product called Serenade®. Serenade® contains a unique, patented strain of *B. subtilis* (strain QST 713), which provides over 30 different lipopeptides that work synergistically to destroy disease pathogens and provide superior antimicrobial activity. It protects vegetables, fruit, nut and vine crops against diseases such as fire blight, botrytis, sour rot, rust, sclerotinia, powdery mildew, bacterial spot and white mold. Quality controlled formulation and state of the art manufacturing adds to the consistent performance that Serenade® provides (15, 32).

Most research with bacterial seed treatments has focused on prevention/control of plant diseases, but my results demonstrate the potential for microbial seed treatments as a biological control measure for human pathogens as well.

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## CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

This research provided insight into diversity of the bacterial communities on seeds of three spinach cultivars pre and post germination. The bacterial richness and abundance were influenced by the developmental stage of the plant. Vertical transmission of bacteria from the seed to the resulting leaves was evident as DGGE profiles of the community were similar from seed to leaves. DGGE profiles of bacteria present on the leaves at different developmental stages clustered by cultivar, which indicated that each cultivar had its own distinct bacterial community. The environment in which spinach was grown did not affect the richness of the bacterial community. However, the abundance of bacteria increased on field grown plants compared with plants grown in growth chambers. Therefore, bacterial diversity on the leaves is influenced both by the contributions of the seed and the environment in which the plant was grown.

This study examined seeds that were produced in Denmark and the Netherlands. It is possible that region of production affected differences in the microbial community of developing seeds. Future experiments should compare seeds produced in more production areas (Washington, USA, Denmark, Netherlands, and New Zealand). If possible, examining a cultivar grown in two locations but processed using the same post harvest treatments would be informative. Future studies should also examine not only the microbial community of seeds but also plants that develop from these seeds as well and the seeds produced by the mature plants for shifts in the microbial community.

Microbial abundance of different phyla present on the seed and the leaves of the plants at the cotyledon, 3-4 leaf stage, and the 6-8 leaf stage was determined using real-time PCR. Phyla



specific primers were used to determine the abundance of all bacteria, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*,  $\alpha$ -*Proteobacteria*, and  $\beta$ -*Proteobacteria*. Environment clearly affected microbial abundance on the leaves of spinach plants. Abundance was not affected by cultivar for any of the phyla. This study did not quantify members of the  $\gamma$ -*Proteobacteria*, whose members are known to be the most dominant phyla on the phyllosphere. The reason for this was we were unable to identify primers that did not amplify members of other phyla, which would artificially increase their numbers. Future studies should identify another marker besides 16s rDNA that could be targeted to assess the numbers of this important phylum. The abundance of bacteria may be affected by leaf age. However, this study did not separate leaves of different ages, which would allow us to determine if the abundance of bacteria on the older leaves were larger than younger leaves as suggested by this study.

Bacterial seed treatments have traditionally focused on prevention/control of plant diseases. This study also sought to identify a seed-borne microorganism that could inhibit the growth of *E. coli* O157:H7 and/or *Salmonella enterica in vitro*. A large number of bacteria cultured from the spinach seed surface were screened for antagonism of the human pathogens *in vitro* on agar plates. Two strains of the bacterium *Pantoea* (also known as *Erwinia*), were identified that showed antagonism toward *E. coli* O157:H7. *Pantoea* have also been cultured from 6-8 leaf stage plants. This stage is critical because Baby Spinach is often sold in ready-to-eat packages when plants have approximately 6-8 leaves. At present, study of *Pantoea* is limited because no primers are available that are specific only for the genera. Further research is needed to design primers for the antagonistic strains of *Pantoea* and to use these primers to describe the *Pantoea* community present on the seed and the leaves. Further study of this bacterium and

other potential antagonists is needed to determine seed versus leaf abundance and the ability of an antagonist to persist throughout the life cycle of the plant.

Further research is also needed to explore delivery systems of the antagonists from the seed to the leaves. Bio-priming is a controlled seed hydration process in a bacterial solution that has been used to load beneficial microbes onto seeds to control seedling diseases. Bio-priming may have potential for loading antagonists to human pathogens as well. Other possibilities include microencapsulation, biofilms, and seed film coating. More antagonists need to be studied along with their longevity before effective delivery systems can be developed and tested. The results of this study simply demonstrate the potential for control of human pathogens by microbial antagonists.

## APPENDIX A

### Scanning Electron Microscope Surface Images of Spinach Seed Produced at Kentland Research Farm and Spinach Seed Obtained from Commercial Seed Companies

#### Introduction

The objective of this experiment was to visually compare, using scanning electron microscopy (SEM), the surface topography and the surface microbial community of spinach seed produced at Kentland Research Farm to spinach seed obtained from commercial seed companies. Seed companies routinely use hot water baths or chemicals to disinfect the seed surface after harvest. Seed may also be lightly coated with a fungicide. These treatments help to prevent the spread of plant diseases caused by bacteria and fungi (*1*).

#### Methods

Three cultivars of chemically untreated spinach seed (including ‘Imperial Spring’ Lot B259102 Origin Washington State, ‘Menorca’ Lot 847122 Origin Denmark, and ‘5633’ Lot N187101 Origin Washington State) were planted on Kentland Research Farm in Blacksburg, Virginia on 28 April 2009. A pre-emergence herbicide (Dual II Magnum®, Syngenta Crop Protection, Inc, Greensboro, NC) was applied at 0.6 pt/acre two days after planting. The plants were allowed to bolt and produce seed. Seeds were harvested and handled with gloves at all times. The seeds were analyzed on 05 August 2009 using the FEI Quanta™ 600 FEG environmental SEM at 10.0 kV and 136 Pa.

For comparison, three cultivars of chemically untreated spinach seed (including ‘Melody’ Lot 714089 Origin Denmark, ‘Menorca’ Lot 847122 Origin Denmark, and ‘Space’ Lot 34018 Origin Netherlands) obtained from a commercial seed company were analyzed on 11 September 2009 using the FEI Quanta™ 600 FEG environmental SEM at 10.0 kV and 136 Pa. These seed were also handled with gloves at all times.

## **Results and Discussion**

Using the SEM, images of spinach seed at different magnifications were photographed to demonstrate the seed coat topography and various microorganisms present on the seed coat (Figures 8-19). Even though spinach seeds feel smooth to the touch and appear smooth to the eye, the seed coat is made up of ridges and valleys (Figures 8-19), which can harbor bacteria and fungi. SEM images of the seeds that were harvested from Kentland showed an abundance of both bacteria and fungi on the seed coat (Figures 8-13). The physical appearance of the plants indicated a fungal infection at time of seed harvest and it is likely that some of the structures visualized at greater magnifications are fungal spores. Bacteria are much smaller than most fungi, ranging from 200 nm to 1.2 mm with the average length of 600 nm. In contrast, the commercially produced seeds (Figures 14-19) had very little bacteria and fungi, but more debris on the surface (Figures 14, 16, and 18). Pollen was occasionally seen on the seed coat (Figure 14) of the commercially produced seeds. The typical size of spinach pollen is 100-200  $\mu\text{m}$ . The Menorca (Figure 17) seed coat did have some very tiny oblong structures about 500-600 nm in length. These are probably desiccated bacteria that may be viable if cultured.

The hot water treatment of the commercially produced seeds definitely decreased the amount of bacteria and fungi present. Seed can be a rich source of both bacteria and fungi.

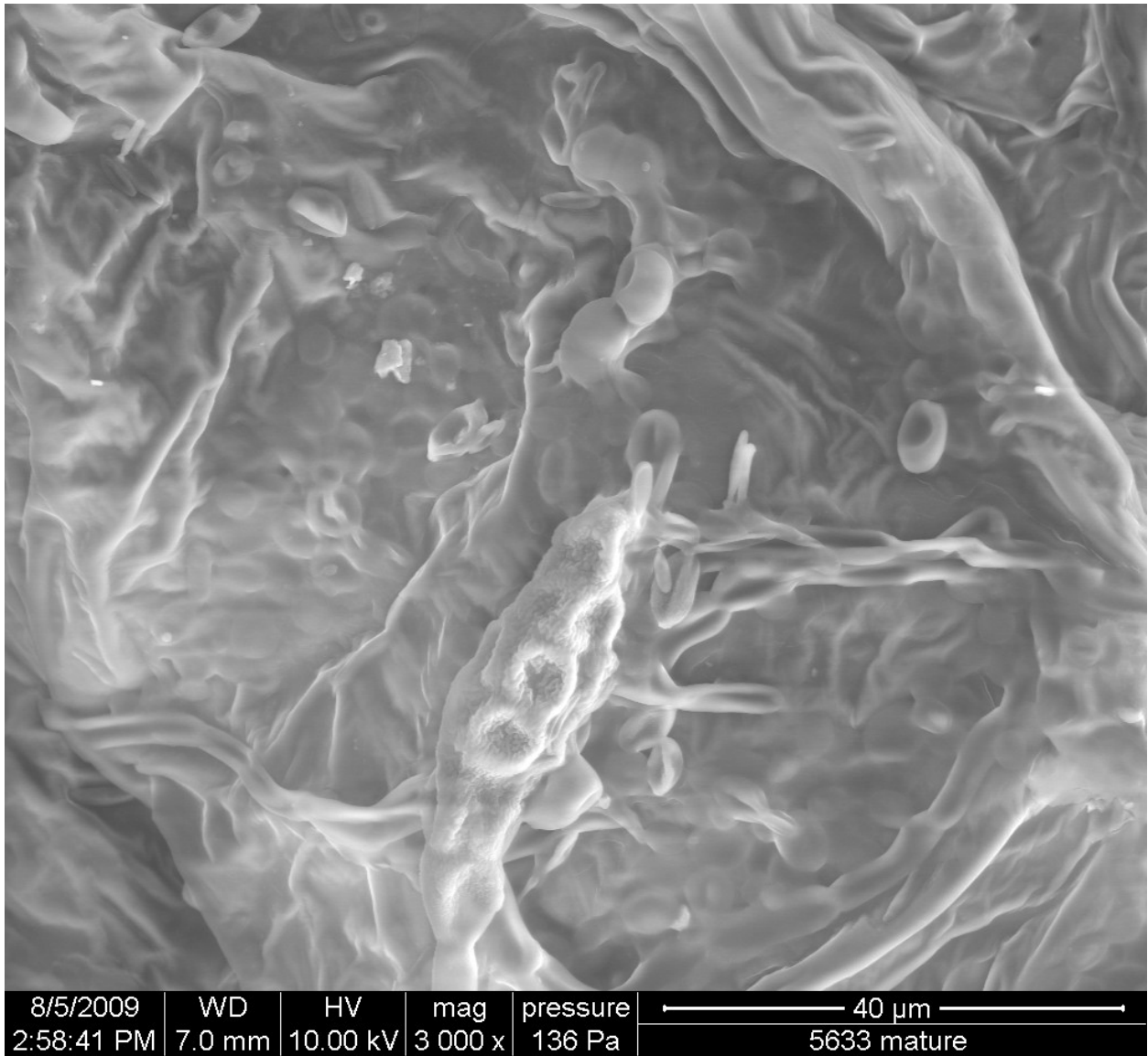


Figure 8: View 1 of spinach seed coat of 5633 Lot N187101 harvested from Kentland Farm

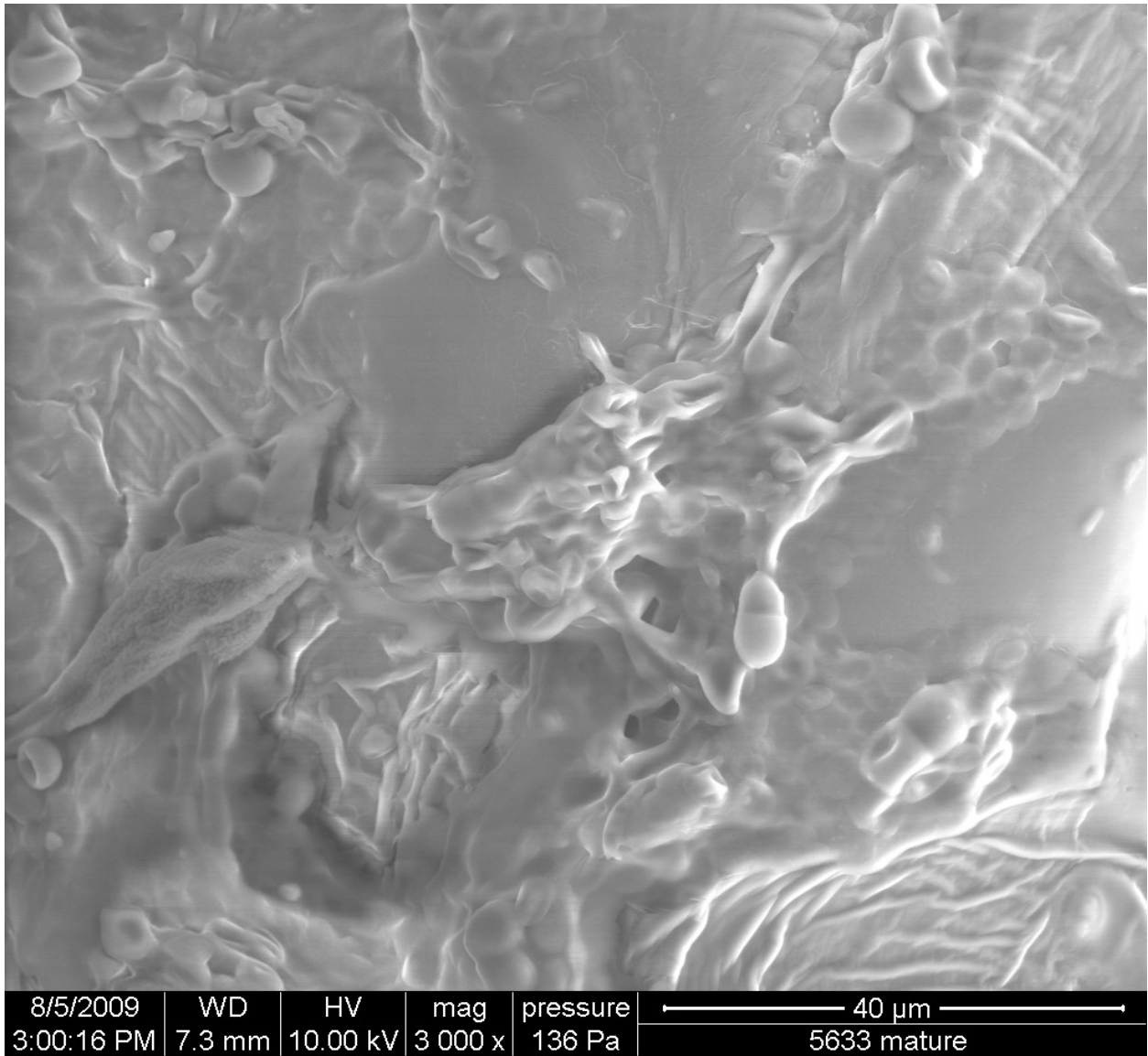


Figure 9: View 2 of spinach seed coat of 5633 Lot N187101 harvested from Kentland Farm



Figure 10: View 1 of spinach seed coat of 'Imperial Spring' Lot B259102 harvested from Kentland Farm showing fungal infection

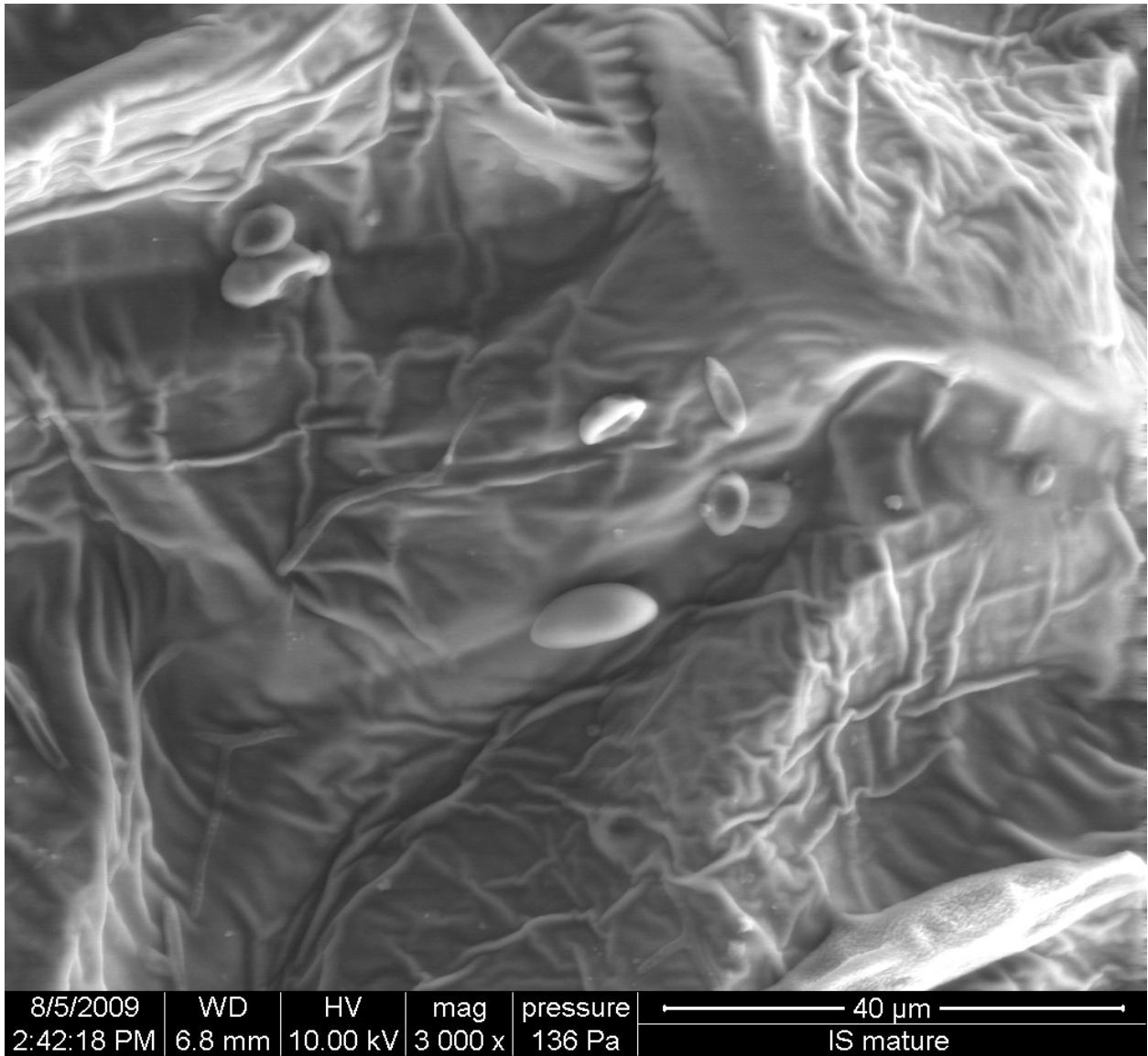


Figure 11: View 2 of spinach seed coat of 'Imperial Spring' Lot B259102 harvested from Kentland Farm showing possible bacteria



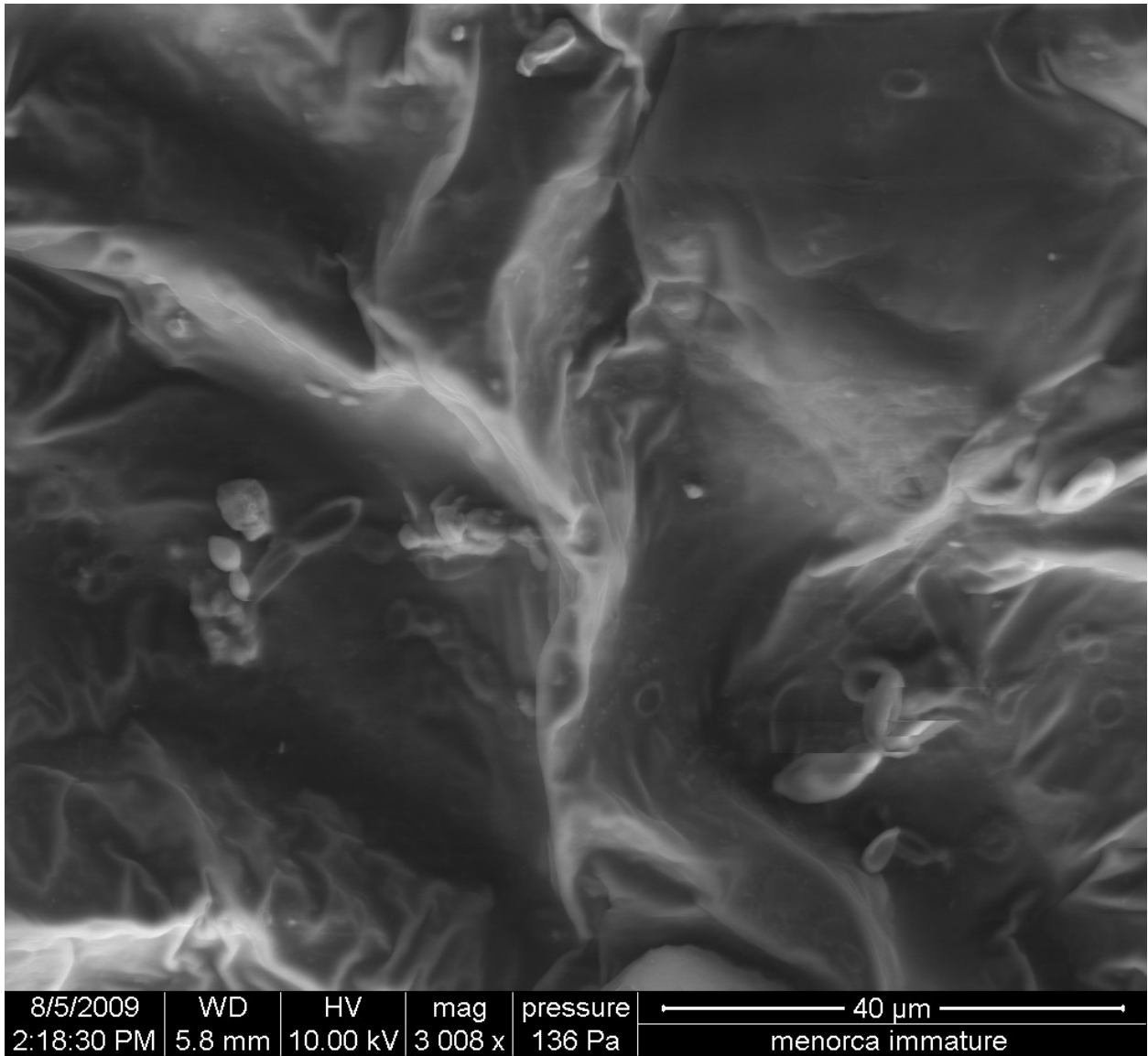


Figure 12: View 1 of spinach seed coat of 'Menorca' Lot 847122 harvested from Kentland Farm

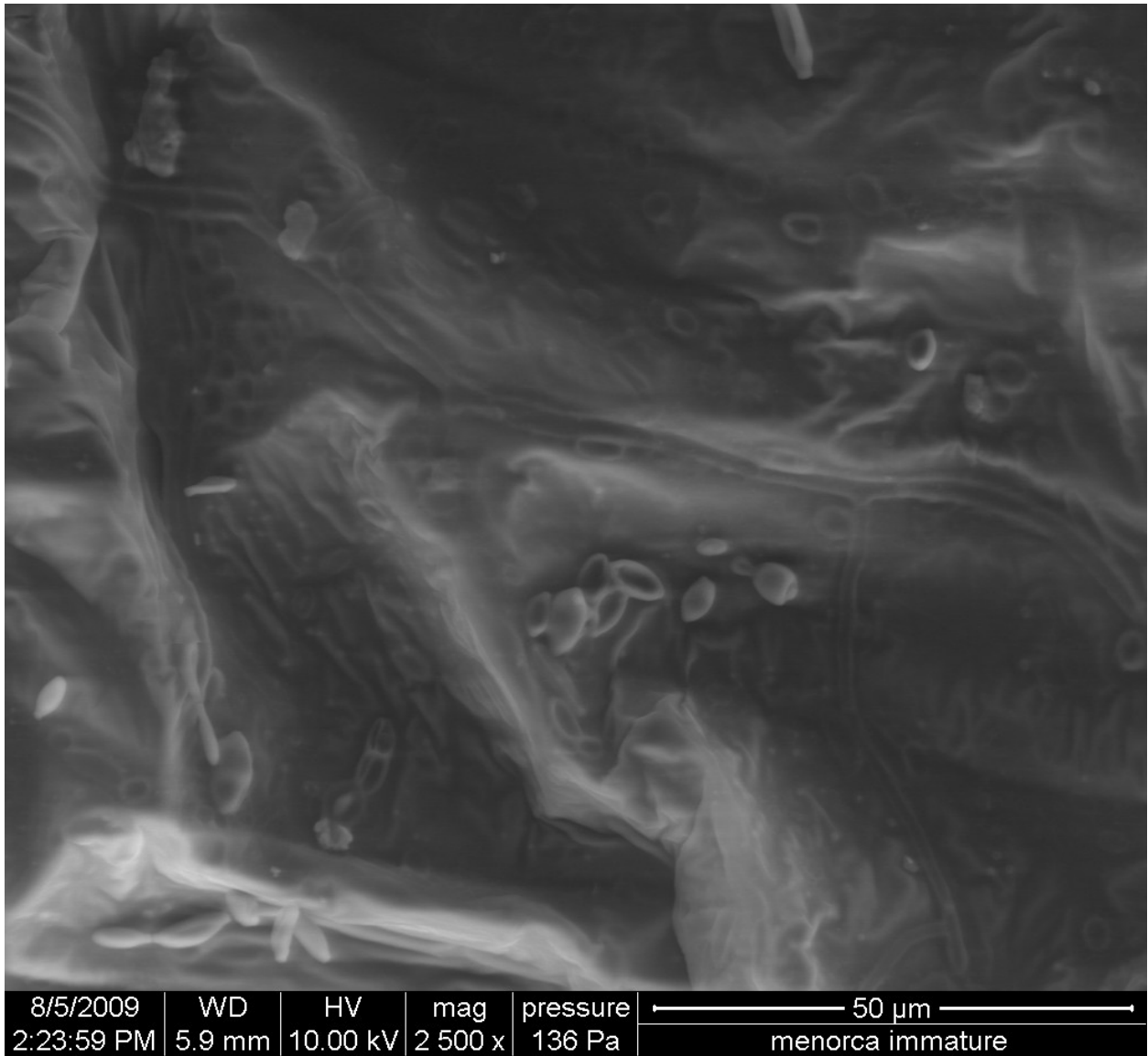


Figure 13: View 2 of spinach seed coat of ‘Menorca’ Lot 847122 harvested from Kentland Farm showing probable bacteria

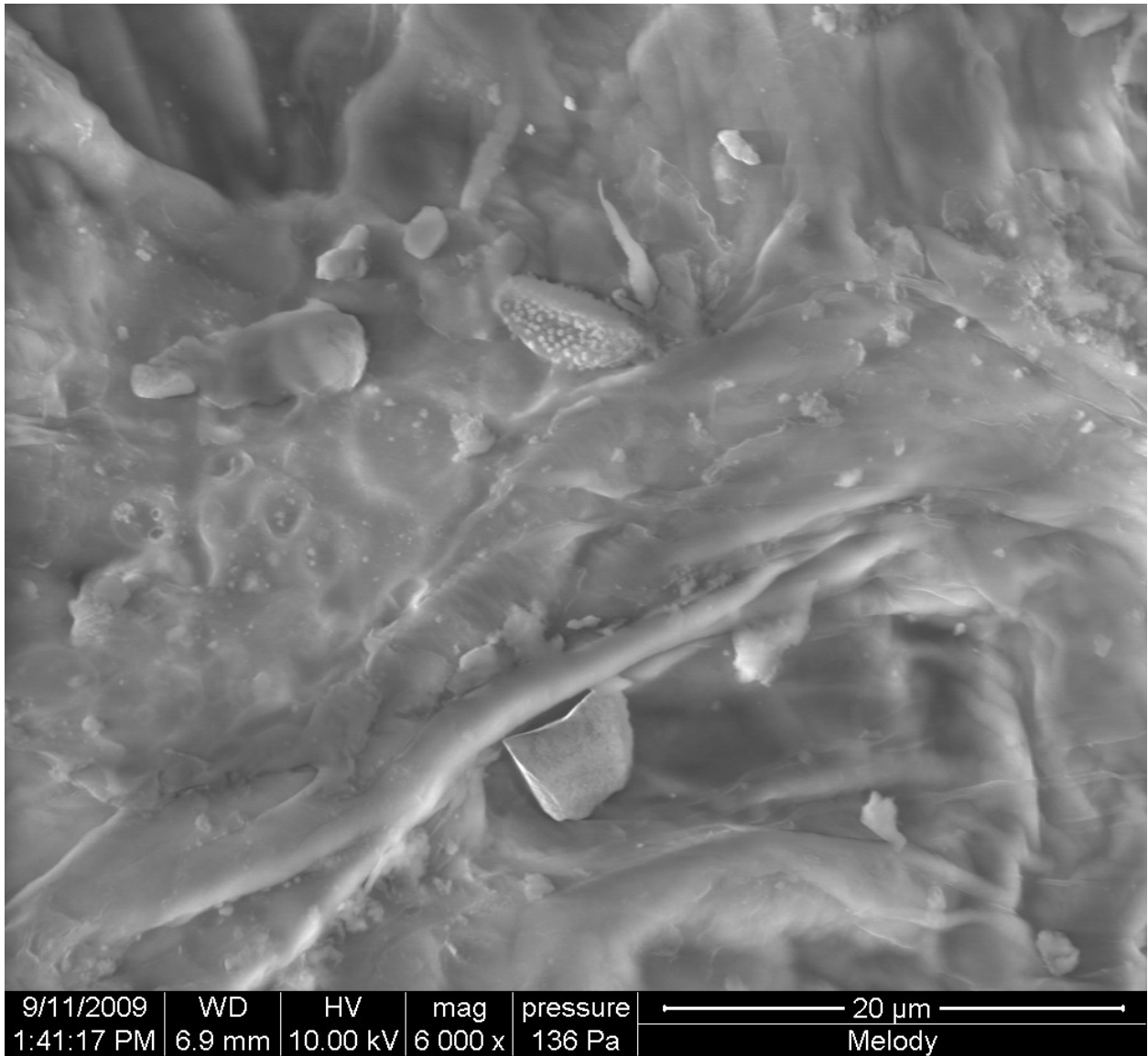


Figure 14: View 1 of spinach seed coat of 'Melody' Lot 714089 obtained from commercial seed company

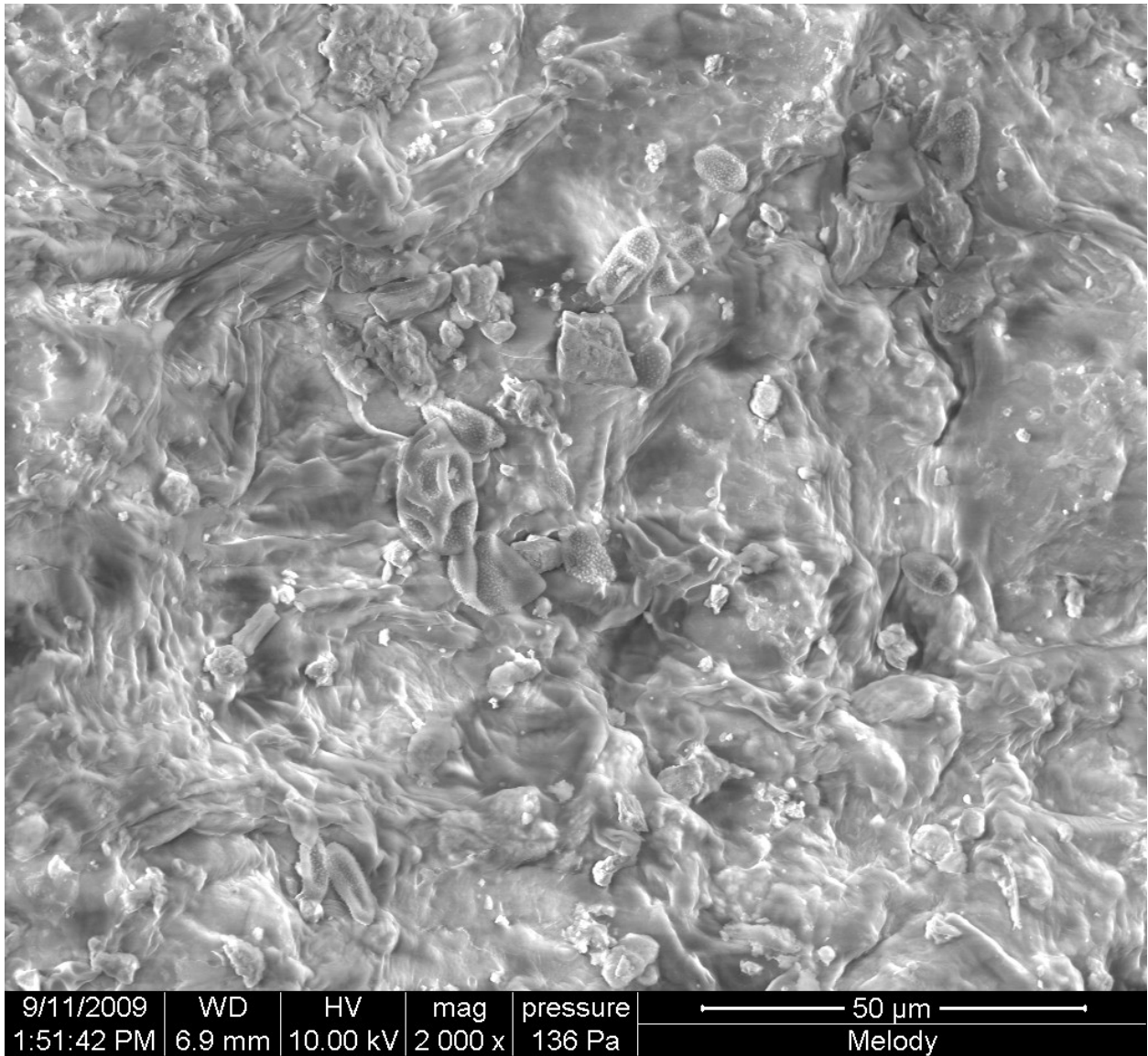


Figure 15: View 2 of spinach seed coat of 'Melody' Lot 714089 obtained from commercial seed company

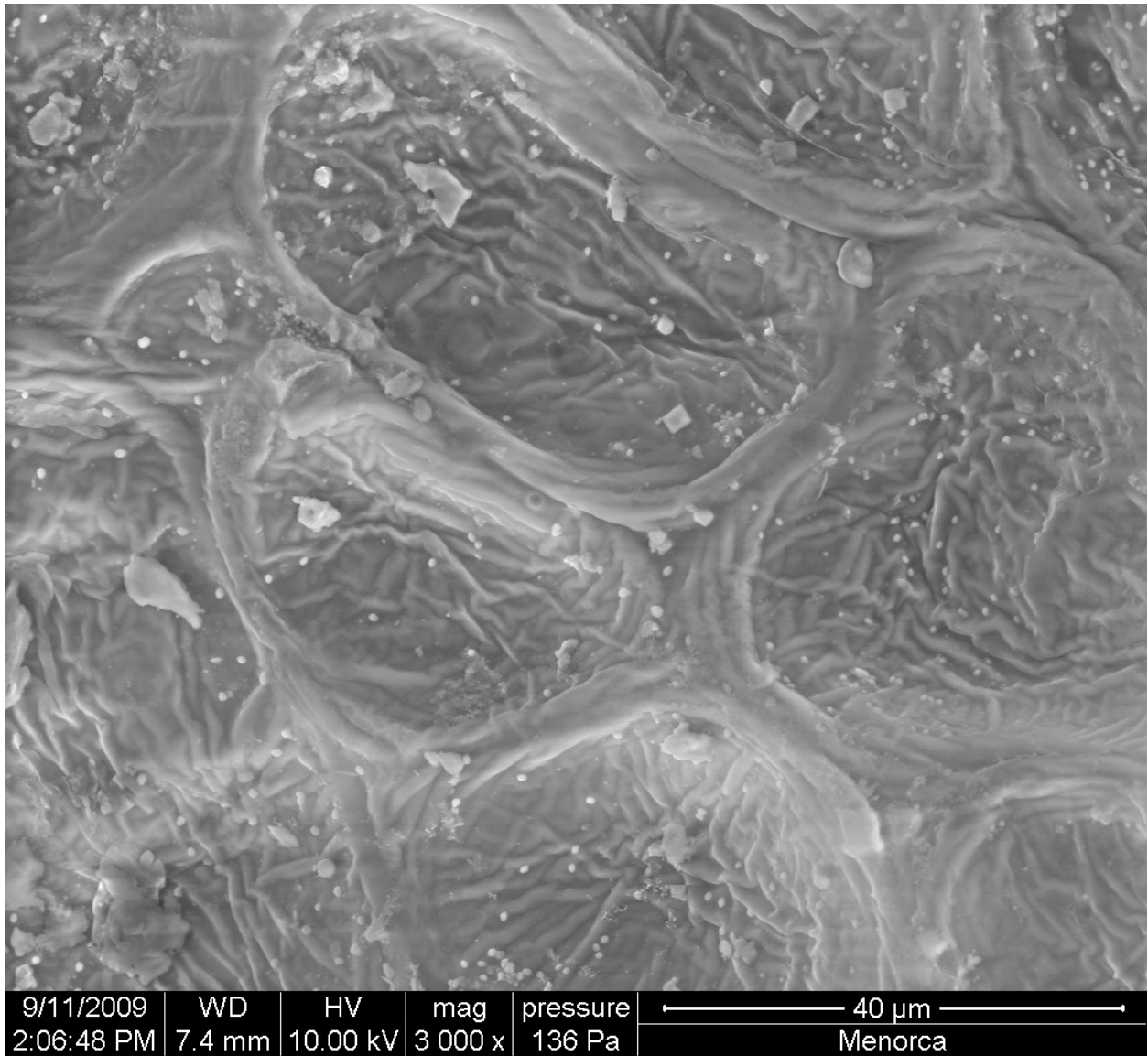


Figure 16: View 1 of spinach seed coat of 'Menorca' Lot 847122 obtained from commercial seed company

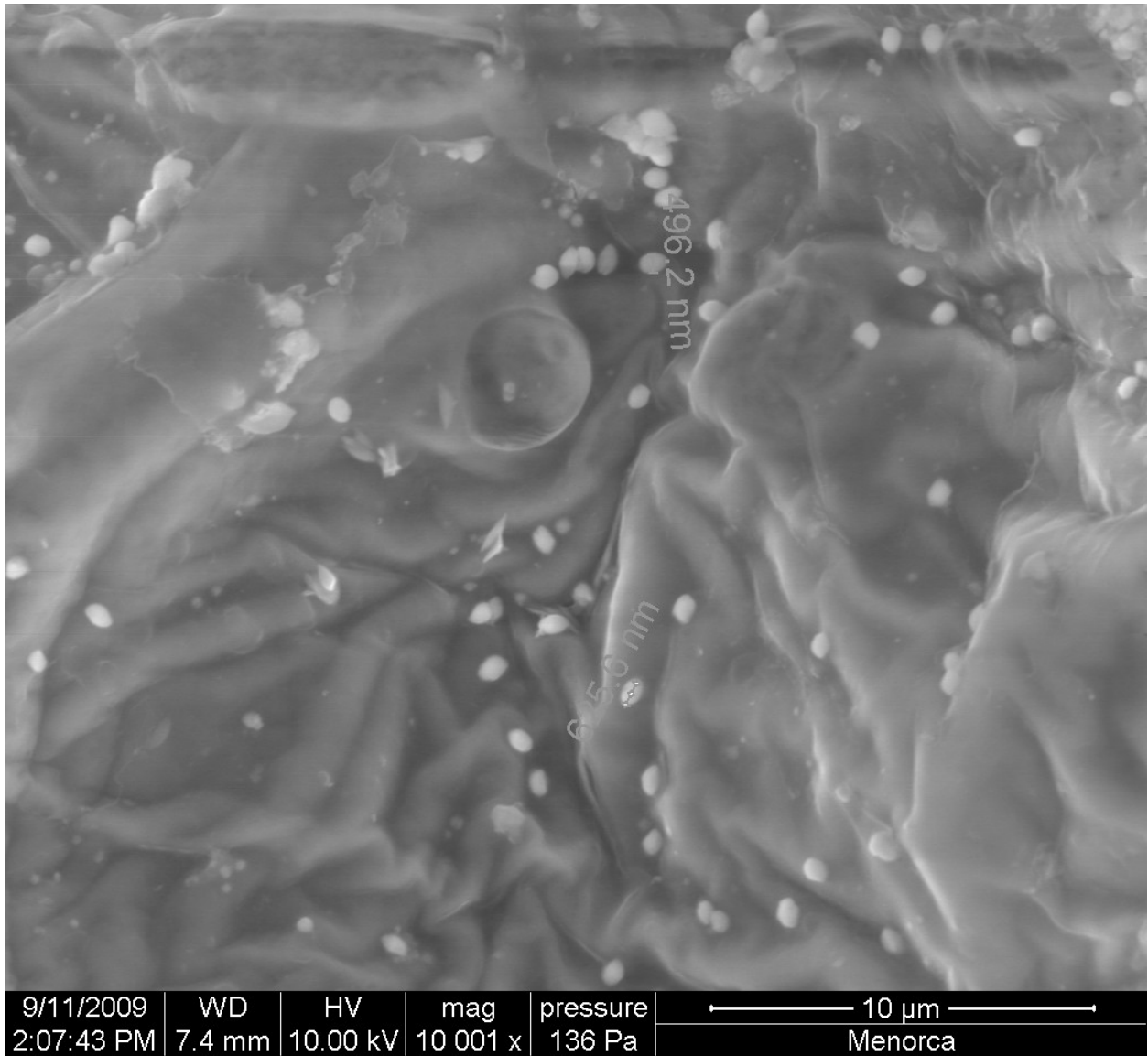


Figure 17: View 2 of spinach seed coat of 'Menorca' Lot 847122 obtained from commercial seed company showing possible desiccated bacteria

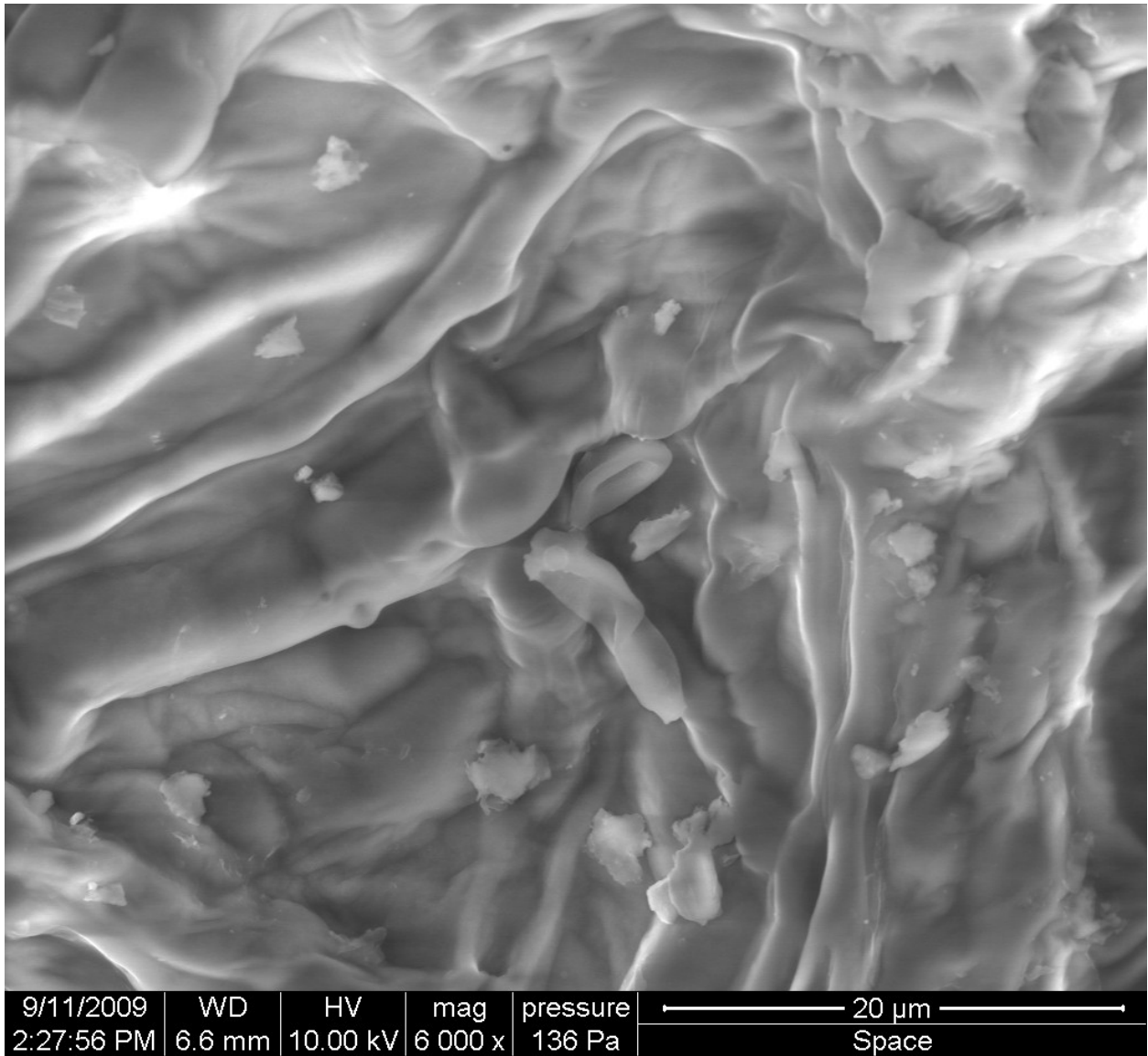


Figure 18: View 1 of spinach seed coat of 'Space' Lot 34018 obtained from commercial seed company showing possible leaf debris

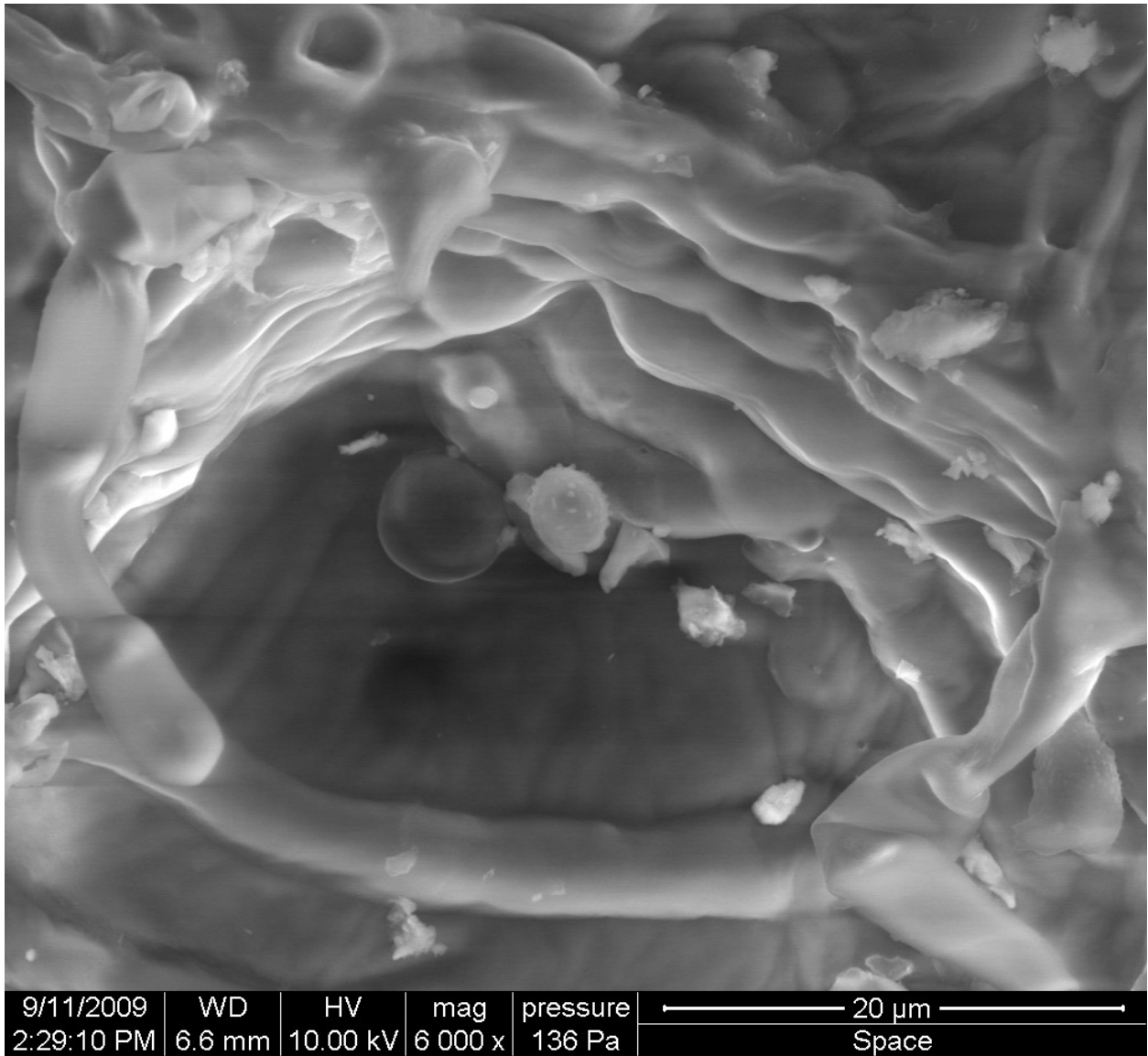


Figure 19: View 2 of Spinach seed coat of 'Space' Lot 34018 obtained from commercial seed company



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