CENTER for PRODUCE SAFETY

CPS – 2009 RFP

FINAL PROJECT REPORT

Project Title
Differential susceptibility of spinach grown under slow- and fast-growth conditions to enteric bacterial colonization

Project Period
October 1, 2009 – September 30, 2011

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Objectives

Objective 1: Using light, transmission, scanning electron microscopy, compare physical parameters of spinach varieties grown under slow- and fast-growing conditions.

Objective 2: Determine susceptibility to bacterial colonization of spinach varieties grown under slow- and fast-growing conditions using a fluorescently-tagged strain of *E. coli* O157:H7.

Objective 3: Compare susceptibility of spinach to *E. coli* colonization after insect damage using fluorescent microscopy.

Abstract

The physical and physiological differences between spinach grown under cool and warm season conditions (slow- and fast-growth conditions) was investigated using growth chamber and field grown plants. Leaves of fast-growth plants examined by light and electron microscopy were found to be 30%-50% thinner than those of slow-growth plants. Increased thickness of slow-growth tissue was due to increased size of palisade layer cells and larger apoplastic spaces. Cell walls of slow growth leaves were significantly thicker than those of fast-growth cell walls. Fatty acid composition was altered between the two types of leaves. Slow growth leaves were stronger than fast-growth leaves, but only after a period of wilting. *Salmonella* attachment experiments suggest that this pathogen attaches more readily to slow growth plant tissues, but the variation between samples and experiments was too high and precludes a definite conclusion. Direct observation of GFP-tagged *Salmonella* on the spinach surface suggested the opposite; more fluorescent bacteria were observed on the surface of fast-growth plant leaves than on slow-growth plant leaves. Overall, the data suggest some leaf property differences that may warrant different processing protocols for spinach grown under different environmental conditions.

Background

Spinach breakage. The Salinas Valley environmental conditions are suitable for the growth and harvest of commercial fresh spinach throughout many months of the year. However, during the warmer summer months of June, July, August, and September, machine harvested spinach that is bulk processed is susceptible to a phenomenon called spinach breakage in which the leaves may become folded, creased, or torn. Normally, these creased leaves of spring-grown spinach can recover and symptoms of water-soaking disappear during wash, processing and packaging within the plant, but sometimes spinach grown in warmer months does not. Broken spinach fails to recover and this results in a lower quality product. Complaints of broken spinach usually start in mid to late June and can continue until early fall.

Susceptibility of spinach to human pathogens. Spinach has been involved in outbreaks of foodborne pathogen contamination, most of these have occurred during the summer or early fall when temperatures are warmer. Because of this loose correlation, it has been suggested that spinach breakage is directly involved with increased pathogen contamination. To determine if there is a direct correlation between the breakage phenomenon and increased susceptibility to pathogen contamination, it needed to be determined that 1) fast-growth and slow growth plants are physically and physiologically different and 2) that pathogen attachment is different between these two types of plants.

Research Methods and Results

Spinach plant growth conditions. For the growth chamber studies, two growth chambers were used to mimic cool season and warm season environmental conditions. The fast-growth chamber (warm season conditions) was set at 75 F daytime, 55 F night time, 12:12 L:D photoperiod. The slow-growth chamber (cool season conditions) was set at 65 F daytime, 45 F night time 10:14 L:D photoperiod. These conditions yielded plants that very strongly resembled field grown spinach (Figure 1). For field grown spinach, NewStar sowed “Silverwhale” spinach in early spring and late spring for early April and late June harvest.

Bacteria. It was determined that *Salmonella enterica* should be substituted for *E. coli* O157:H7. A green fluorescent protein- tagged strain was used in all studies. Fresh culture was streaked before all experiments. For attachment studies, a small loop was resuspended in 0.1% peptone. A cell count was carried out on serially diluted culture using an Olympus BX2 compound microscope. Resuspended bacteria was adjusted to 1-5 x 10^6 cfus/ml for all experiments.

Project Approach

Growth Chamber Studies. Plants were sown in standard potting mix (Metromix 600) in 128 plug trays in the fast-growth chamber and at approx. 3 weeks, plugs were transplanted into 4.5 in pots. The cohort was then separated, half remaining in the fast-growth chamber and half moving to the slow growth chamber. Watering and fertilization was identical for the plants in both chambers. The two first true leaves were measured every third day using a Licor leaf area meter. When leaves in the fast-growth leaves stopped expanding, samples were collected and processed for microscopy. We continued measuring the slow-growth plant leaves until they, too stopped expanding, approximately two weeks later. Both fast- and slow-growth leaves were collected and processed for microscopy. Samples of the same *physiological age* (FG date 1 and SG date 2) and *chronological age* (FG date 2 and SG date 2) were compared.

Light and Transmission Electron Microscopy. Small 2mm2 sections of the growth chamber-grown plants were cut from leaves approximately 2 cm from the distal end adjacent to the midrib. Plant tissues were fixed in 2% glutaraldehyde for 7-10 days at 4 C, then subjected to a standard dehydration sequence before a 2 hr exposure to 1% osmium tetroxide. The tissues were then embedded in Spurr's resin. Samples were thick sectioned and stained with methylene blue and examined with a compound microscope. Thick sections were cut and mounted on carbon coated grids and examined using a Leica digital transmission electron microscope. At least 5-6 leaves per treatment were processed, but 3 samples per treatment were examined, comparing fast and slow growth leaves at the same physiological age and at the same chronological age. This was repeated in its entirety three times. The field grown samples were compared only once. *Results:* Measurements of the thick sections revealed that slow-growth plants were 50% thicker than fast-growth leaves at the same physiological age and 30% thicker than fast-growth leaves at the same chronological age (Figure 3, Table 1). Increased thickness was probably due to the size of the palisade cells being larger in the slow-growth samples than cells of the fast-growth tissues. Examination of the ultrastructure by TEM revealed no major anatomical differences, but the cell walls of both the epidermal cells and the palisade cells were much thicker in slow-growth cells compared to fast-growth cells (Figure 4). This strongly suggests that the slow-growth leaves are structurally more sturdy than fast-growth leaves.

Scanning Electron Microscopy. Fast and slow growth plants at the same chronological age were fixed and dehydrated, then critically point dried. Samples were then mounted on metal stubs and sputter coated with palladium and examined by scanning electron microscopy. *Results:* It was difficult to see any microtears or artificially-induced wounds in both types of plants. There were, however, several ridges present on the surface of the fast-growth plants that were not as evident on the slow-growth plants (Figure 2). These may represent structures for easy attachment of surface bacteria.

Fatty Acid Analysis. Extracted waxes were analyzed by gas chromatography (GC) to determine differences in wax amount and composition. Waxes were extracted from the top and bottom of a 10 mm diameter area near the middle of each leaf using chloroform (30 sec, 2 times). After chloroform

was evaporated under a stream of nitrogen trimethylsilyl derivatives of hydroxyl containing compounds were prepared by adding 50ul each of BTSFA and pyridine and incubating at 70°C for 30 min. A 1 ul sample was then injected onto a 30 m long DB-1 column on a GC equipped with a FID detector. Semi-quantization of wax components was done by comparing to tetracosane and heptacosanol internal standards. Identification of wax component peaks was done using authentic standards and GC-MS.

Results: Cuticular waxes consisted mainly of primary alcohols (C24, C26) and alkanes (C29-C33, with C31 dominant). FG and SG leaves had significant differences in the amount of alkanes (P=0.0092) and primary alcohols (P=0.0148) but not in total wax (P=0.4091). SG leaf waxes consisted mainly of primary alcohols while FG waxes were predominantly alkanes (Figure 5). This appears to be independent of leaf size, but it may be related to chronological age because one set of young FG leaves (42 days) had slightly more alcohols (not significant due to small sample size). Furthermore based on this analysis, cuticular wax thickness does not appear to vary by leaf size or on different sides of the leaves. Wax amount did increase with chronological age although the correlation was poor (R^2=0.4).

Leaf Strength Tests. The 2nd or 3rd pair of leaves from four plants from each growth condition were clipped into two 10 mm wide by 20 mm long test samples. These placed in an Instrong machine for a tensile test at a strain rate of 10mm/min. One leaf from each plant was tested immediately; the second one was stored on a tray at 14C for about 2 hours. The leaves were slightly wilted after 2 hours.
Results: Leaves that were grown under both conditions and tested after a 2 hr wilting period were stronger than leaves tested immediately (Figure). This makes sense because wilted leaves are "stretchier" and less crisp than fresh leaves. Slow growth leaves were stronger than fast-growth leaves (Figures 6 and 7), requiring up to 20% more strain force to break the leaves in the wilted state. This may be very important towards explaining why slow-growth (spring-grown) spinach is less susceptible to breaking.

Field Grown Spinach Comparisons. Field grown spinach, var. "Silverwhale" was harvested by NewStar crews in April 2010 for slow growth plants and late June 2011 for fast growth plants and bulk shipped to Oklahoma State University. Representative leaves were prepared for microscopy using methods outlined below. *Results:* These samples have not yet been sectioned and measured.

Attachment of Salmonella to Fast-growth and Slow-growth Spinach. Spinach leaves were assayed for Salmonella attachment using two methods. We first tried using whole leaves immersed in resuspended bacterial culture at approx. 10^6 cfus/ml. After rinsing and drying, leaves were macerated in 1:10 volume of 0.1% peptone and 100 ul was plated on LB-ampicillin plates. The second method used 2 cm circular punches taken from both types of leaves. Punches were placed on LB agar plates to keep them moist and 50 ul of culture was pipetted onto the adaxial side. After 1 hr, the inoculum was removed, the leaf punches carefully rinsed, and then macerated in 1 ml peptone water using a bead-beater. Samples were plated as above.
Results: Observations of fast- and slow-growth plants exposed to GFP-tagged Salmonella gave inconsistent and highly variable results when comparing attachment between samples and between replications. In some replications, more GFP-tagged bacteria were observed by fluorescent on slow-growth leaves than on fast-growth leaves, whereas other replications resulted in the opposite finding. The leaf punch method gave more consistent results, but variation between numbers of bacteria recovered remained high. On average, 25% more cells attached to the slow-growth leaf tissues (unbroken) than to fast-growth tissues. This was an unexpected result and does not support the hypothesis that fast-growth plant tissue is more susceptible to bacterial contamination.

Several attempts to mimic the damage inflicted upon fast-growth spinach during the packing process were carried out with variable success. Finally, slow wilting accompanied by increased applied weight to mimic the compression and folding that occurs in a field harvesting bin resulted in minor folding and water soaking of fast and slow growth spinach. Using these leaves, an attachment study revealed that 20% more bacteria attached to the slow-growth leaves than to fast-growth, again, this is opposite of what we expected to find and does not support the hypothesis that fast growth leaves are more susceptible to bacterial contamination.

Insect damage and pathogen attachment. This objective was not completed because of difficulties encountered obtaining sufficient numbers of whiteflies for the study.

Outcomes and Accomplishments

Most of the experiments outlined in Objectives 1 and 2 were completed. One major experiment has yet to be completed, that is the measurement of leaf parameters of field-grown spinach. The project was successful in that physical differences were clearly documented between plants grown under these two conditions. We also showed that slow growth plants withstand wilting much better than do fast grown plants. However, our attempts to successfully mimic "breakage" in the laboratory and to test and quantify differences in pathogen attachment fell short of expectation. See recommendations below.

Summary of Findings and Recommendations

1. Leaves of slow growth plants were 30-50% thicker than those of fast-growth plants.
2. Both types of leaves had approximately the same number of cells per unit area, but the slow-growth cells were larger and the cell walls were thicker.
3. The surface of slow growth leaves were smoother than those of fast-growth leaves
4. Cuticular wax composition differed between fast- and slow-growth leaves.
5. Slow growth leaves were stronger than fast-growth leaves, especially after wilting slightly.
6. Salmonella attachment to slow-growth leaves was higher than attachment to fast-growth leaves, but the variation was too high to show significant differences.
7. Similarly, when broken leaves were compared to unbroken leaves, mean number of Salmonella cells attached to the unbroken leaves was higher.
8. Tensile strength tests showed that the slow-growth leaves were stronger (less susceptible to tearing) than fast-growth leaves after a period of wilting.

Recommendations:

We have been able to demonstrate physiological and physical differences in a single variety of spinach (Silverwhale) using growth chamber-grown plants. These plants, while they strongly resemble field grown material, should not be considered equivalent to field-grown spinach. A number of factors that were examined in this study suggest that spinach grown in the summer months is weaker than spring-grown spinach and may benefit from different harvesting/processing protocols to avoid the stresses that result in 'breakage.' This may include management strategies designed to slow down the rapid growth during the summer months or harvesting techniques that minimize physical stresses on the leaves.

The question of increased/decreased pathogen attachment to fast- or slow-growth leaves or to broken vs. unbroken leaves was not adequately answered. In my opinion, this may not be possible to test in the laboratory. It is my suggestion that this question be addressed, but with closer collaboration with a leafy greens processor. NewStar sent me samples whenever requested, but it would have been better if field grown spring and summer spinach could have been processed and tested on site, rather than have the samples shipped to me. A larger scale experiment of contamination and recovery of pathogen would have been better for this particular research objective.

APPENDICES

Publications and Presentations (required)

One manuscript is in preparation at this time pending the outcome of one final tissue analysis "Structural and physiological differences in spinach, *Spinacea oleraceae*, grown under different environmental conditions" Kamenidou, S. , J. Fletcher, J. Dillwith, J. Hardin, R. Madden, K. Brooks, and A. Wayadande.

Two presentations at the Center for Produce Safety symposia in 2010 and 2011 were made.

Budget Summary (required)

As of the end of the granting period, $75,688.34 of the $84,063 awarded was spent. A breakdown of expenditures is as listed:

Salaries and wages:	$37,994.94
Benefits:	$6,550.07
Materials and Supplies	$8,283.51
Equipment	$0.00
Travel:	$6,483.95
Other Direct (EM lab, shipping, growth chambers)	$16,375.87

Tables and Figures (optional)

Figure 1. A. Slow-growth and B. fast-growth spinach grown in growth chambers.

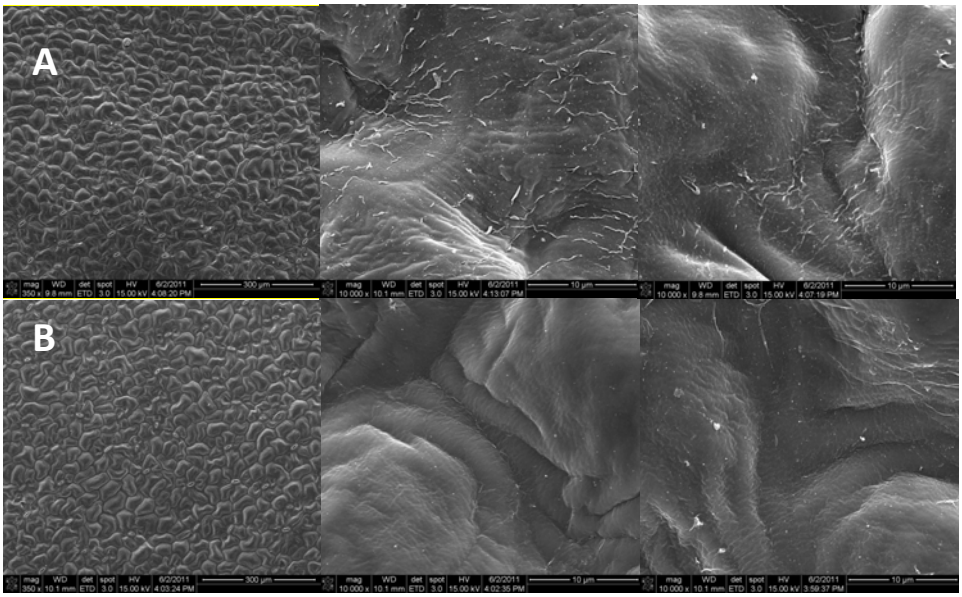

Figure 2. Scanning electron micrographs of A. Fast-growth and B. Slow growth spinach leaves.

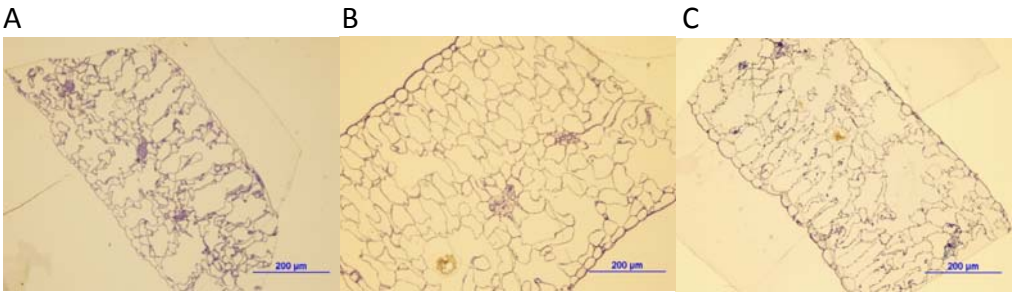

Figure 3. Thick sections of A. fast growth spinach collected at time point I B. slow growth spinach collected at time point II and C. fast-growth spinach collected at time point II.

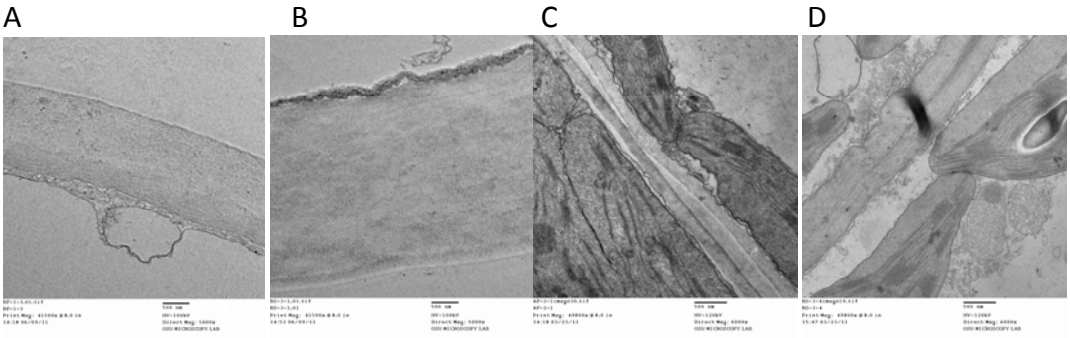

Figure 4. Transmission electron micrographs of A. Fast-growth epidermal cell wall B. Slow-growth epidermal cell wall C. Fast growth palisade cell wall and D. Slow-growth palisade cell wall

Table 1. Mean leaf parameter measurements of spinach grown under fast-growth and slow-growth conditions. Compare slow-growth to same physiological age (11/1) of fast growth or same chronological age (11/15). N= 9 for each treatment.

	Fast (11/1) physiol	Slow (11/15)	Fast (11/15) chronol
Leaf thickness	36.0 un	54.4 un	41.2 un
# epidermal cells	10.3	10.1	11.0
# palisade cells	51	61	51
Length of palisade cells	2.30	3.15	2.75
Epidermal cell wall thickness	1.81 µm	4.16 µm	2.4 µm
Palisade cell wall thickness	0.19-0.23 µm	0.26-0.49 µm	0.35-0.37 µm

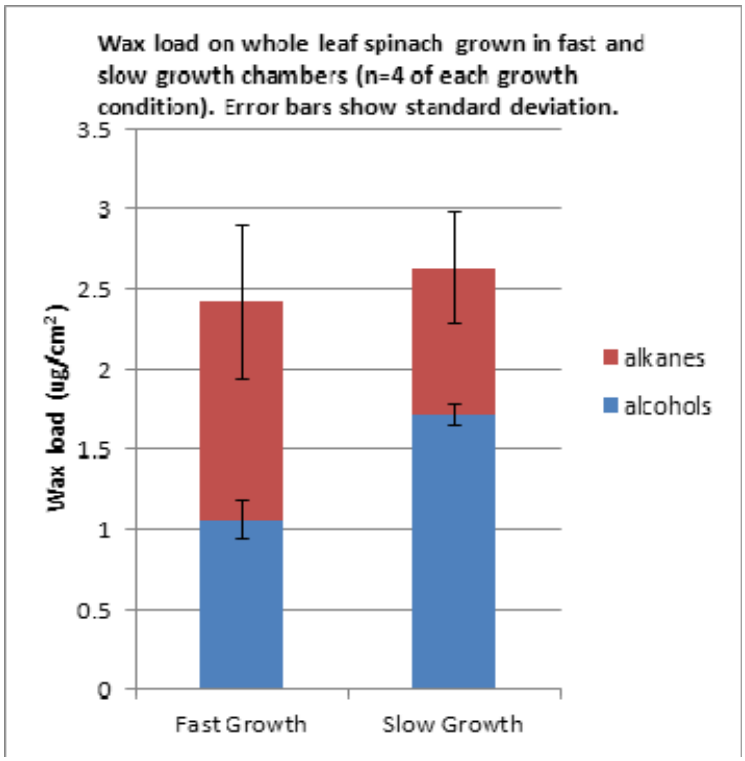

Figure 5. Fatty acid analysis of fast- and slow-growth spinach. Fast growth leaves contained more alkanes on the surface than did slow growth leaves.

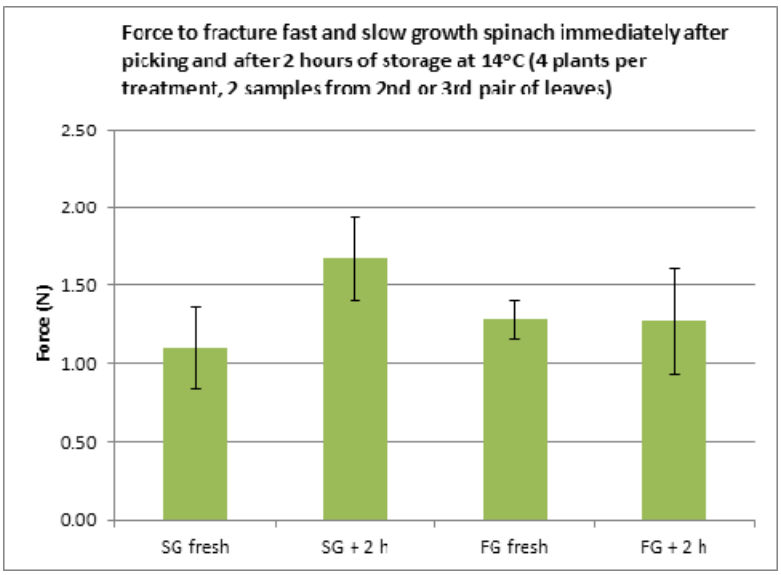

Figure 6. The amount of force needed to break spinach immediately after picking and after a two hour wilting period. Note that it takes almost 50% more force to break wilted spinach than fresh for the slow-growth plants.

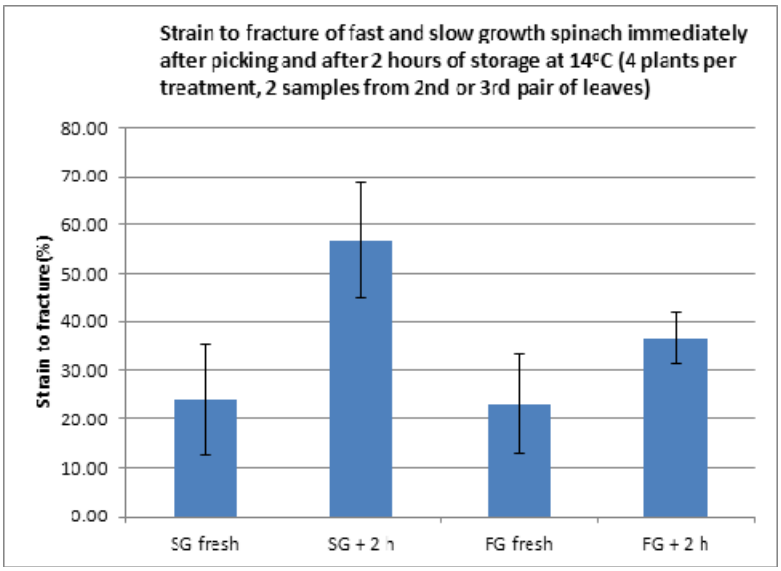

Figure 7. This graph represents how much the leaves "stretch" before breaking. Both slow- growth and fast-growth spinach stretch more after wilting, but the slow-growth leaves are much more flexible in the wilted state than are the fast-growth leaves.