REPORT

Project title: Probing the genetic diversity in lettuce–*Escherichia coli* interactions

Principal investigator:
Maeli Melotto, Department of Plant Sciences, University of California, Davis
Phone: 530-752-1747
E-mail: melotto@ucdavis.edu

Cooperating personnel:
Joseph Student, Department of Plant Sciences, University of California, Davis
Ivan Simko, USDA-ARS, Salinas, CA
Rebecca Zhao, USDA-ARS, Salinas, CA
Alicia Scholler, USDA-ARS, Salinas, CA

Abstract:
Despite extensive efforts to deliver safe and nutritious food, disease outbreaks associated with consumption of leafy greens continue to occur. In 2020, the Center for Disease Control reported the analysis of disease outbreaks that occurred between 2009 and 2018 (https://wwwnc.cdc.gov/eid/article/26/10/19-1418_article) and concluded that: (a) multiple outbreaks may have originated during crop production; (b) leafy greens were the second most common source of *E. coli* O157 disease outbreaks in the US and Canada; and (c) 54% of them were linked to romaine lettuce. Thus, prevention of contamination during pre-harvest is one of the most important steps to reduce risks to human health and improve lettuce safety. We have recently discovered that the persistence of *E. coli* O157:H7 in lettuce leaves varies with the plant genotype. As lettuce germplasm is highly diverse, we reasoned that genetic resistance could be explored as an additional control measure to reduce bacterial persistence in leaves. Thus far, we have screened 28 lettuce commercial cultivars and breeding lines for *E. coli* O157:H7 persistence and found that the bacterium population multiplied significantly in 4 cultivars, remained stable in 11 cultivars, or declined significantly in 13 cultivars. The results of this research confirm that lettuce genetics is a factor that contributes to bacterial growth in leaves. The genes involved in genetic resistance should be identified and used in breeding efforts towards reducing the risks associated with crop safety.

Objective:
To determine the extent of genetic variation that exists in *Escherichia coli* O157:H7 persistence in lettuce leaves.

Procedures:
Plant material: Lettuce genotypes (*Lactuca sativa* L.) representing breeding lines and commercial cultivars were used in this research. Seeds were obtained from lettuce breeding programs at the UC Davis and USDA-ARS Salinas, as well as from donations by the California Leafy Greens

Research Board. Genotypes were coded and their identity will be released upon completion of the research and with permission of the leafy greens board.

Plant growth in environmental rooms: Seeds were sown on water-soaked germination paper and incubated for 5 days at 20°C. Germinated seeds were transferred to 7.62 cm^2 pots (Kord Products, Toronto, Canada) containing a commercial soil mix (Sun Gro® Sunshine® #1 Grower Mix with RESiLIENCE™, MA, USA). Plants were grown under a photosynthetically active light intensity of 240 ± 10 µmol/m^2/sec with a 12-hour photoperiod. Day and night conditions were set at 19 ± 1°C and 75 ± 4% RH, and 18 ± 1°C and 92 ± 2% RH, respectively. At 7 days after transplanting, 0.05 g/plant of fertilizer (Multi-Purpose 19-11-21, Peters®Excel, OH, USA) was dissolved in the irrigation water.

Plant growth in the field: Eleven genotypes were also be grown on outdoor benches with overhead netting. Germinated seeds were transferred to 17cm diameter pots containing soil mix as above, and one-week-old seedlings were transported to the USDA-ARS facility in Salinas, CA. Dr. Ivan Simko's team maintained the plant for three weeks starting on April 19, 2021. After one week, Marathon® 1% Granular (OHP) was added to the topsoil (1g/pot). The plants were watered as needed and fertilized with Peters Professional 20-20-20 once a week per manufacture's recommendation. When plants reached 4-weeks of age (**Fig. 1**), pots were transported to the Melotto laboratory at UC Davis inside of a van. Plants were acclimated in an environmental room for five days prior to inoculation as described below.

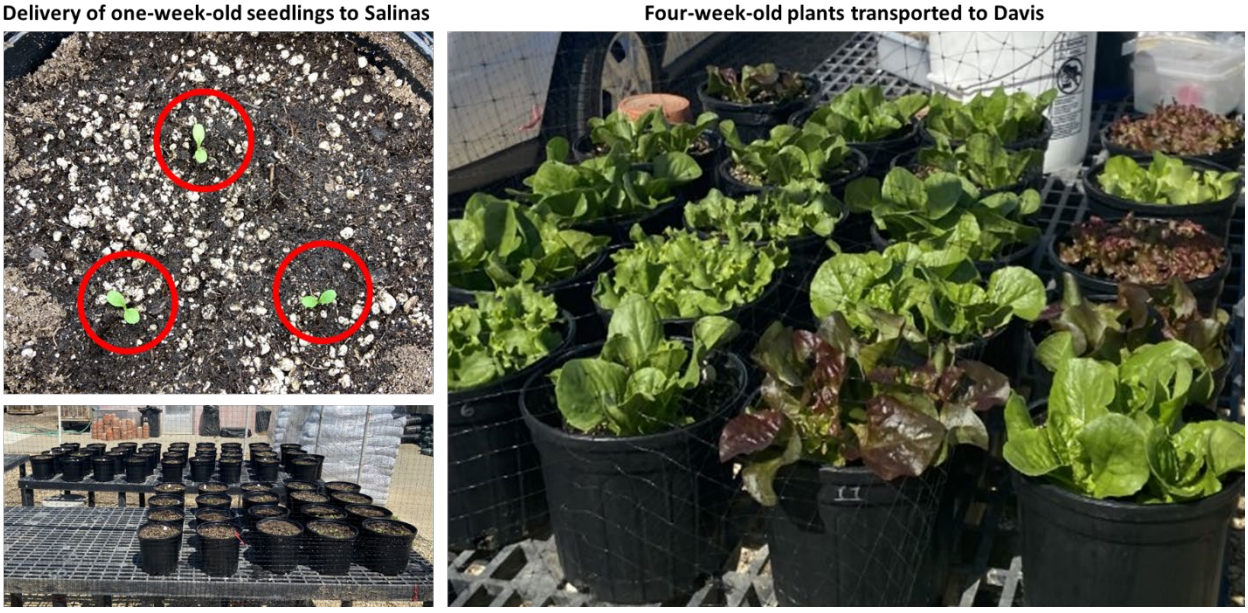

Fig. 1. Lettuce genotypes grown in Salinas, CA. Pictures on the left show seedlings placed on open benches, and the picture on the right shows plants used for bacterial inoculation.

Preparation of bacterial inoculum: The enterohemorrhagic *Escherichia coli* serotype O157:H7 was grown in Low Salt Luria-Bertani (LSLB) medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) at 28°C, supplemented with 50 µg/mL of streptomycin. Bacterial culture for the

preparation of inoculum was obtained by streaking cells from frozen glycerol stocks onto solid LSLB medium and incubating overnight at 28°C. From this culture, a single colony was used to inoculate liquid LSLB medium, which was grown on an orbital shaker-incubator until reaching an OD_{600} of 0.9 to 1. Bacterial cells were collected by centrifugation at 1,250 ×g for 20 min at 20°C (Eppendorf Centrifuge 5810R, Rotor 157 A-4-81, Hamburg, Germany) and suspended in sterile distilled water to obtain an inoculum concentration of 1 x 10^6 CFU/mL.

Bacterial persistence assay: Leaves of four-week-old lettuce plants were inoculated by immersing the aerial parts of the plant into the inoculum and applying vacuum. For the field grown plants, leaves were inoculated with a needleless syringe and dipped into the inoculum. Both procedures ensure that the leaf surface and interior are inoculated uniformly. Total bacterial population was enumerated by sampling the youngest, fully developed leaves at 10 days after inoculation using a standard serial-dilution planting technique. Additionally, total bacterial population was determined immediately after inoculation (day 0) to ensure initial uniform inoculation titer across all plant lines and biological replicates, as well as to calculate the net growth of the bacterium population over time (ratio day 10 / day 0).

Experimental design and statistical analysis: Three leaves collected from three separate plants (n=3) were used for each sample point per genotype. Data points were averaged, the standard error of the mean was calculated, and statistical differences between the means (day 0 versus day 10) was compared using a two-tailed Student's *t*-test.

Results and Discussion:

This project started in April 2020, one week after the University closed due to the COVID-19 pandemic. New research was only allowed to start in September 2020 and since then we have been operating at a 30% capacity. Thus, this report represents a portion of the proposed research. A full, final report will be submitted by the end of September 2021. We believe the research will be completed by then as it is anticipated that the lab will be open at full capacity in July 2021.

Thus far, we have screened 28 lettuce genotypes for *E. coli* O157:H7 growth over the period of 10 days. These plants were grown in environmental rooms and bacterial inoculation caused no visual symptoms in any cultivar tested.

Overall, we observed that the *E. coli* O157:H7 population multiplied significantly in 4 cultivars, remained stable in 11 cultivars, or declined significantly in 13 cultivars (**Fig. 2**). Cultivar 15 was the most susceptible to bacterial growth (~2.5 times), whereas bacteria were almost cleared from cultivars 19, 20, 21, 22, and 24. The average population net growth observed with cultivars 4, 9, 16, and 32 were above 1, indicating bacterial multiplication (**Fig. 2**). However, the statistical analysis indicated non-significant differences between bacterial populations at day 0 and day 10 due to the variation among biological replicates. These cultivars will be screened again to confirm the results.

Nine types of lettuce were included in this first screen to test for the possibility of significant correlation between bacterial net growth and lettuce type. However, there is no indication of such correlation within this initial panel of genotypes. For instance, some romaine and butter lettuce

supported bacterial growth, whereas other cultivars of these types supported bacterial population decline (**Fig. 2**).

In April 2021, eleven cultivars were also grown on open benches in Salinas to test as to whether environmental conditions (controlled environment versus open field) affect bacterial population growth. The plants were inoculated in the last week of May 2021 and data is yet to be analyzed.

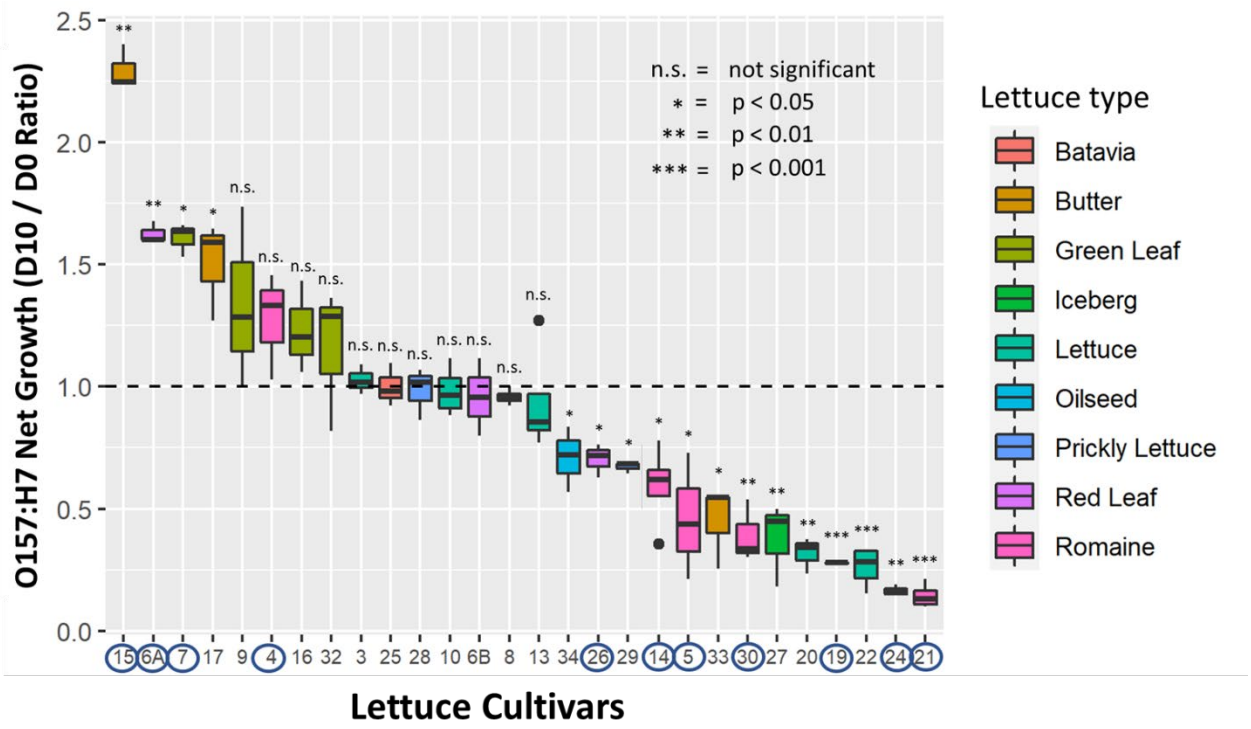

Fig. 2. Bacterial net growth over 10 days post-inoculation (dpi). *Escherichia coli* O157:H7 population size was estimated at the day of inoculation (D0) and 10 dpi (D10) to calculate the net growth ratio. Lettuce cultivars were coded with numbers on the x-axis. Plot center lines show the mean; box limits indicate the 25th and 75th percentiles, and whiskers extend to minimum and maximum data points, n = 3 plants. The fill color of the boxes represent the various commercial types of lettuces. Pairwise mean comparisons (bacterial population at D0 versus D10 for each cultivar) was performed with two-tailed Student's *t*-test. Ratios significantly above 1, approximately 1, and significantly below 1 indicate bacterial multiplication, stable persistence, or decline, respectively, over 10 day-period. Blue circles at the x-axis indicate the cultivars used for the field trial.

In conclusion, this research confirmed that lettuce genetics is a factor that contributes to bacterial growth in leaves; however, it may not be related to the lettuce type. The genes involved in genetic resistance should be identified and used in breeding efforts towards reducing the risks associated with crop safety.