CALIFORNIA LEAFY GREENS RESEARCH PROGRAM REPORT
April 1, 2022-March 31, 2023

Project Title: Lettuce Breeding, Genetics, and Physiology

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Abstract:

The lettuce industry of California requires continued development of improved, adapted cultivars to meet new disease and insect problems, changes in the market, and changes in growing procedures. The lettuce breeding, genetics, and physiology project aims to incorporate valuable traits into crisphead, mixed lettuce, and spring mix cultivars and breeding lines that are adapted to coastal California and low desert production conditions. In parallel, we develop information and tools to increase the utility of our germplasm. The 2022 – 2023 report focuses on postharvest quality, genetics and physiology of yellow spot malady, resistance to bacterial leaf spot, and the effect of silicon on lettuce performance. Field testing continued to assess resistance and to develop breeding lines with improved resistance to lettuce drop, lettuce dieback, downy mildew, bacterial leaf spot, impatiens necrotic spot virus, as well as extending fresh-cut salad shelf-life. In all programs, horticultural traits and adaptation were considered essential.

Objectives:

Develop landmark cultivars, advanced breeding lines, information, and tools for use by other breeders, scientists, producers and growers. Address problems facing the lettuce industry using genetic approaches that are suited to coastal, desert, and interior valley locations, to the various types of lettuce, and to the demands of different production and marketing approaches.

Develop romaine, iceberg, and leaf breeding lines with improved **lettuce drop** resistance for Salinas Valley.
Map genes and loci associated lettuce resistance to **lettuce drop**.
Combine **lettuce dieback** resistance and improved **shelf life** for romaine and leaf type grown in Salinas Valley.
Develop **downy mildew** polygenic resistance in romaine, iceberg, and leaf type grown in Salinas Valley.
Map genes and loci associated with lettuce polygenic resistance to **downy mildew**.
Develop **bacterial leaf spot** R-gene and polygenic resistance in baby leaf grown in Salinas Valley.
Map genes and loci associated with lettuce resistance to **bacterial leaf spot**.
Improve **shelf life** of processed lettuce grown in Salinas Valley.
Map genes and loci associated with **postharvest deterioration** of tissue and **enzymatic browning**.
Identify lettuce germplasm with limited susceptibility to **yellow spot**.
Map genes and loci associated with lettuce susceptibility to **yellow spot**.
Study the impact of **silicon nutrition** on post-harvest quality of fresh-cut lettuce.

Procedures, Results, and Discussion:

ENZYMATIC DISCOLORATION OF LETTUCE RIBS (in collaboration with *H. Peng,* and *Y. Luo*)

Whole lettuce heads and processed salads that develop enzymatic discoloration (pinking/browning) on the cut surface have reduced market acceptance. Identifying the genetic determinants and factors associated with this physiological process can facilitate the development of cost-effective and environmentally friendly methods to minimize the loss of product. We used a diversity panel of 376 lettuce accessions to investigate the relationship between enzymatic discoloration and agronomic traits and to determine the genetic characteristics of the discoloration process based on four field experiments. Among tested accessions, Balady Aswan Green (stem type) and PI 169496 (romaine) were identified as the varieties with the lowest browning potential, thus providing a valuable resource for breeding low-browning cultivars. The discoloration did not show association with plant developmental rate, resistance to yellow spot malady, bacteria leaf spot, or downy mildew but displayed a significant, though not strong association with the initial levels midribs brownness (r = -0.146, $p < 0.05$) and greenness (r = -0.255, $p < 0.001$), and the deterioration rate (r = 0.391, $p < 0.001$) of fresh-cut lettuce packaged in modified atmospheres. The trait exhibited a relatively high broad-sense heritability (H^2 = 0.67) across four trials conducted at three locations in three years. The genome-wide association study (GWAS) revealed the significant effect of six loci (*qDis1.1, qDis4.1*, *qDis6.1*, *qDis8.1*, *qDis8.2*, and *qDis9.1*) defined by eight single nucleotide polymorphism (SNP) markers (Fig. 1). A total of 965 genes were predicted in the identified chromosomal regions, with three quinone oxidation-reduction (redox) catalyzers and 19 transcription factors being potentially the most promising candidate genes involved in regulating the rate of postharvest discoloration. This observation further confirmed that discoloration is a complex trait regulated by multiple genes.

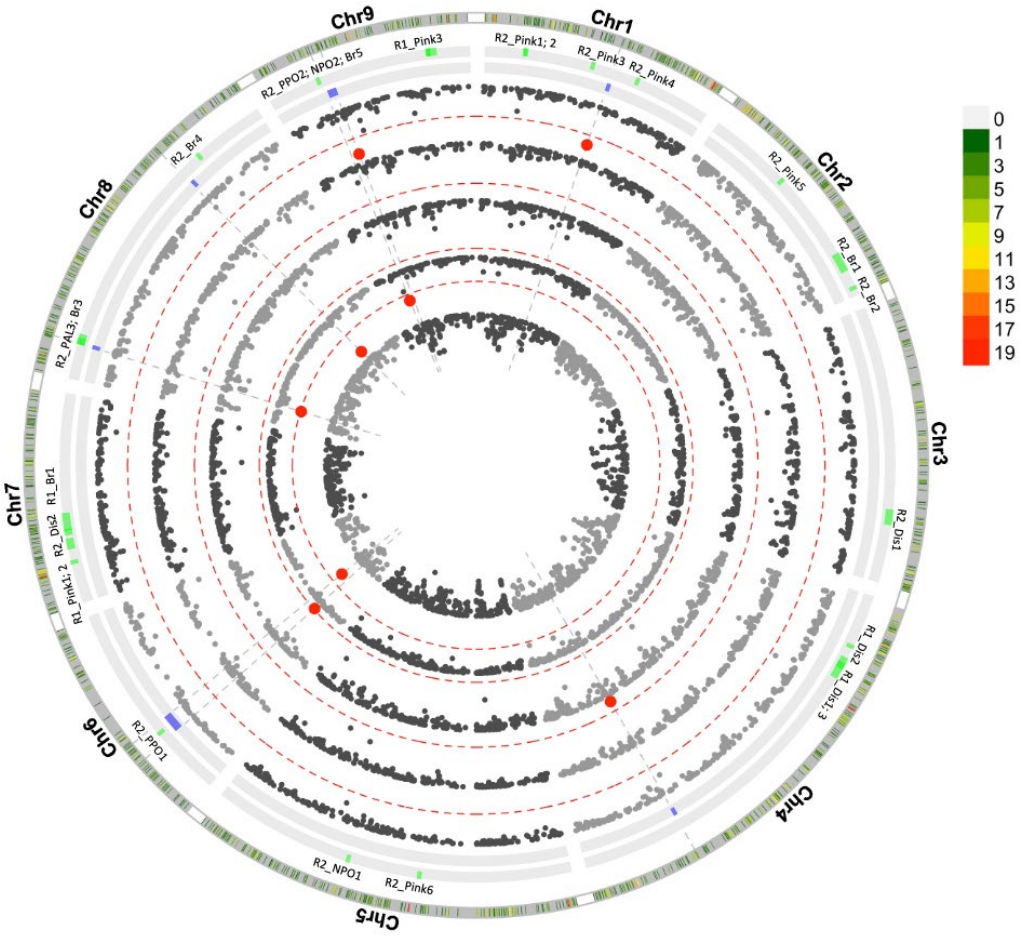

Figure 1. Circular graph of Manhattan plots showing results of the genome-wide association analysis (GWAS) for discoloration level in lettuce. The first four inner circles display results of the experiments performed at Gonzales in 2019 (Gon19), Spence in 2019 (Spe19), Salinas in 2020 (Sal20), and Salinas in 2021 (Sal21). The fifth circle shows the results obtained from the mean values of browning index scores (squared roots) of accessions used in all four experiments. The circle with blue bars shows the location of six QTL identified in this study. The length of QTL consisting of multiple markers is determined by two flanking markers while that of single marker QTL was set at 4 Mbp to make them visible on the graph. The circle with green bars shows locations of loci associated with 'pinking' (Pink), 'browning' (Br), 'overall discoloration' (Dis), phenylalanine ammonia lyase (PAL), polyphenol oxidases (PPO), and NADP-dependent quinone oxidoreductase (NPO), detected in previous studies: R1 (Atkinson et al., 2013) and R2 (Hunter et al. (Fig.3, 2022). The red dash line indicates the genome-wide significance threshold (p = 0.05). The red dots depict locations of significant SNPs. The outermost circle is the diagram of nine chromosomes (chr. 1 to chr. 9) showing locations of SNP markers. The color indicates the density of SNP markers (counts per Mb) as shown by the accompanying scale.

YELLOW SPOT MALADY OF LETTUCE (in collaboration with *H. Peng, R. Zhao,* and *R. Smith*)

Yellow spot (YS) blemishes on leaves have recently become appearing more frequently in commercial fields in California potentially reducing the product marketability. YS occurs on all morphological types of cultivated lettuce regardless of their color, with the highest (0.9) and lowest (0.09) rates observed on stem/oilseed and leaf lettuce, respectively. The spots ranging in diameter from 0.5 to 3.1 mm (depending on genotype) were mostly observed on the adaxial leaf surface of mature or nearly mature plants (Fig.2). YS symptoms began as light-yellow circular spots that later became more intensely yellow and eventually turned necrotic (Fig.2). The YS affected area displayed decreased chlorophyll fluorescence and photosynthesis (Fig.3) that were not caused by the effect of fungi, bacteria, viruses, insects, or foliar nematodes. Among 31 tested chemical elements, four macronutrients (P, Ca, Mg, and S) and eight microminerals (B, Cd, Cu, Li, Mn, Ni, Si, and Sn) had significantly different concentration in the YS affected leaves (Fig.4). The trait exhibited intermediate to high broad-sense heritability (H^2 = 47.9% ~ 68.4%) across four experiments conducted at two locations in three years. The genome-wide association study (GWAS) performed on a set of 479 accessions revealed the effect of eight loci (*qYS2.1*, *qYS3.1*, *qYS3.2*, *qYS4.1*, *qYS5.1*, *qYS5.2*, *qYS6.1*, and *qYS7.1*) defined by 10 significant SNP markers (Fig. 5). A total of 162 genes were predicted in the identified chromosomal regions, with four oxidation-reduction (redox) catalyzers being potentially the most promising candidate genes involved in the regulation of this physiological disorder termed 'Yellow spot malady' (YSM). A more detailed information is provided at the recently published paper https://doi.org/10.1016/j.scienta.2022.111389.

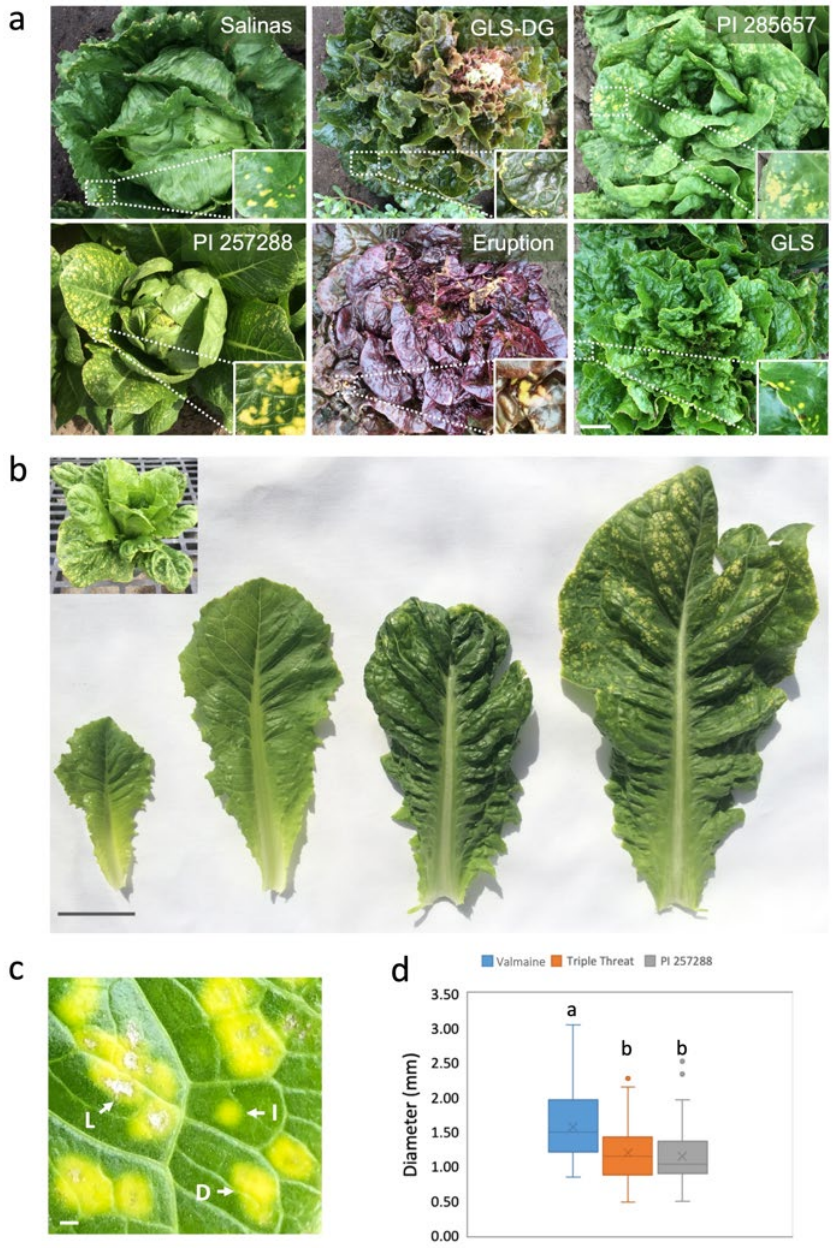

Figure 2. Morphological characterization of lettuce 'Yellow spot malady' (YSM). a, YSM in various lettuce accessions. Photographs were taken on 10-weeks old plants grown in field. bar = 5 cm; b, Distribution of symptoms on lettuce leaves. Four leaves representing different developmental stages of YSM were sampled from a plant (accession PI 211600) that grew in a greenhouse for eight weeks. bar = 5 cm; c, YSM at different developmental stages. The photograph was taken on the leaf of a 10-weeks old plant of cv. Barcarole grown in the field. YSM stages: initial (I), developing (D), and late (L) often appear in the adjacent areas. bar = 5 cm; d, Size of yellow spots. The diameter of spots was measured on 10-weeks old plants grown in field with at least three biological replicates. The size of yellow spots in three accessions was compared with Tukey HSD test ($p < 0.001$), different letters indicate significant difference.

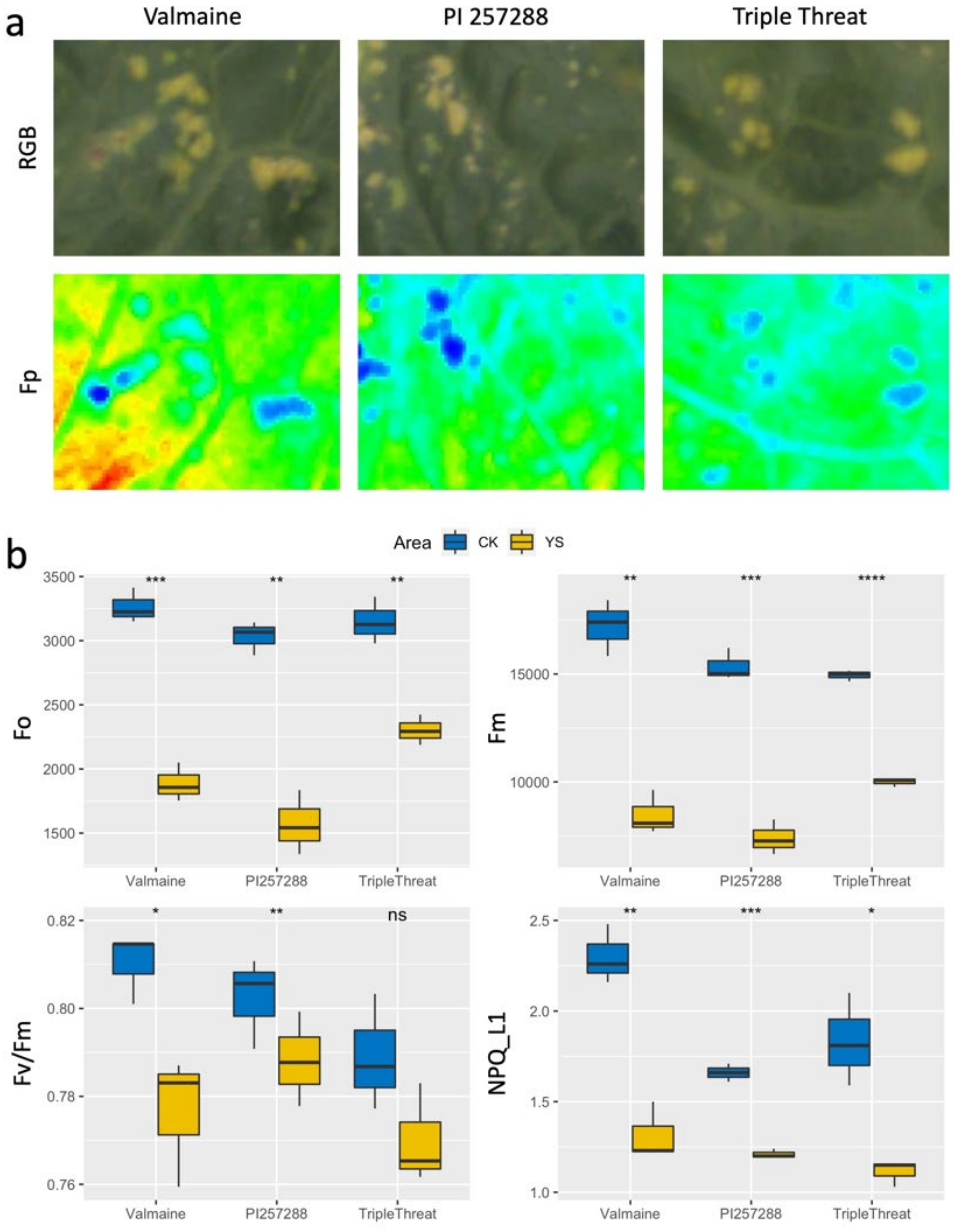

Figure 3. Chlorophyll fluorescence analysis on the lettuce leaves affected by 'Yellow spot malady' (YSM). a, Regular (RGB) and fluorescent images of the YSM affected lettuce leaves. The fluorescent image represents the peak fluorescence (*Fp*) during the initial phase of the fluorescence induction. Color shifting from red to blue indicates the intensity of change in fluorescence from high level to low level. b, Comparison of chlorophyll fluorescence of the YSM affected (YS) and non-affected (CK) leave areas. Displayed values include the minimum (F_0) and maximum (*Fm*) fluorescence in the dark-adapted state, the ratio of variable ($Fv = Fm - F_0$) to maximum fluorescence after dark-adaption (*Fv*/*Fm*), and the non-photochemical quenching at the first light pulse (NPQ_L1) during photosynthesis. Bars represent the mean values from three biological replicates. Asterisks indicate statistical significance as calculated by the paired *t*-test. * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

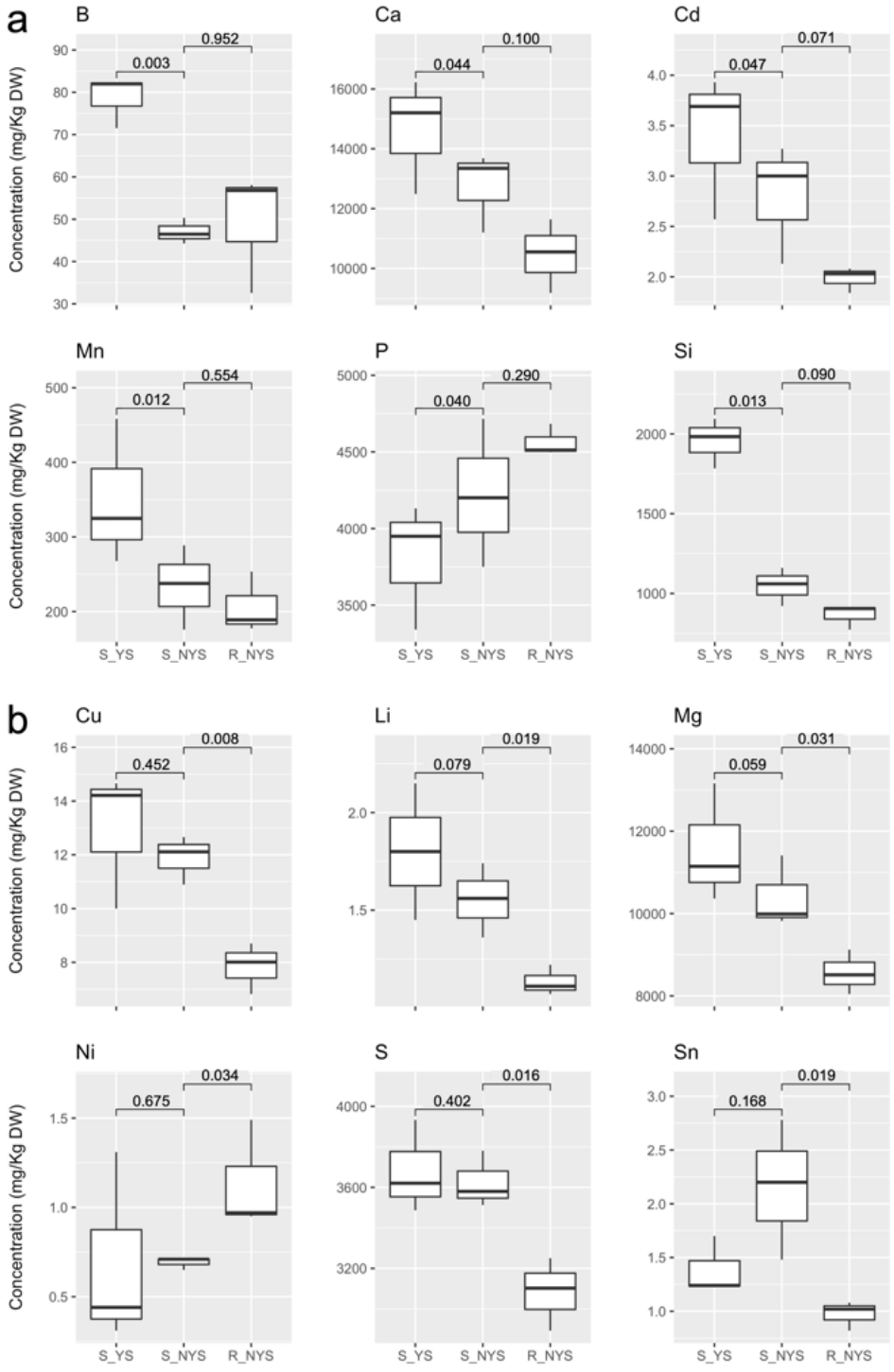

Figure 4. Concentration of macronutrients and microminerals associated with 'Yellow spot malady' (YSM). YS (S_YS) and non-YS (S_NYS) tissues were equally collected from the affected leaves on the plants (> 5 plants per accession) of three susceptible accessions (cv. Balloon, PI 491051, and PI 257288). NYS tissues from the area like that for S_NYS were collected from three

resistant accessions (PI 491015, cv. Green Forest, and cv. Romana Verde Hortelana), designated as R_NYS. Concentration of each element is based on dry weight (DW) of samples. The comparison between S_YS and S_NYS was analyzed with the paired *t*-test, and the comparison between S_NYS and R_NYS was analyzed with the unpaired *t*-test. *p* values are indicated for each comparison with three biological replicates. a, Macronutrients and microminerals showing significant difference in concentration between S_YS and S_NYS samples; b, Macronutrients and microminerals showing significant difference in concentration between S_NYS and R_NYS samples.

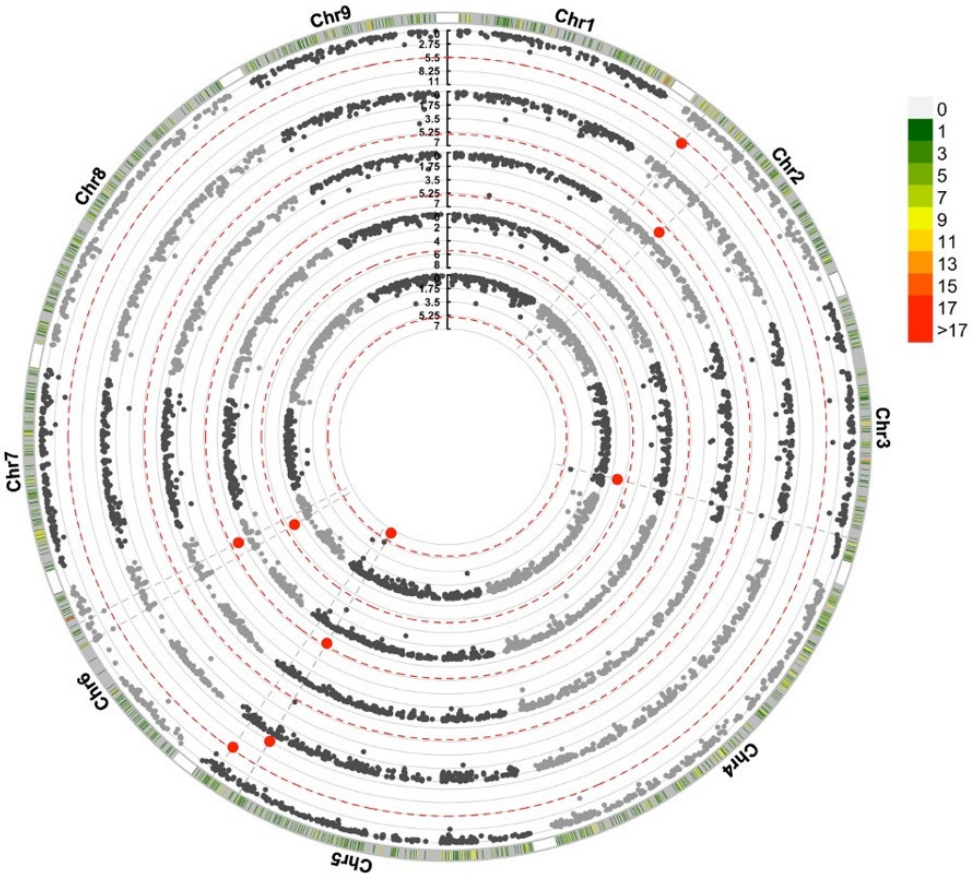

Figure 5. Circular graph of Manhattan plots that show results of the genome-wide association analysis (GWAS) for susceptibility to 'Yellow spot malady' (YSM). The first four inner circles display results of the experiments performed at Gonzales in 2019, Salinas in 2019, Salinas in 2020 and Salinas in 2021. The fifth circle shows the results obtained from the mean values of YSM scores across four experiments. The outermost circle is the diagram of nine chromosomes (Chr. 1 to Chr. 9) with the locations of SNP markers. The color indicates the density of SNP markers (counts per Mb) as shown by the accompanying scale. The red dash line indicates the genome-wide significance threshold ($p = 0.05$). The red dots depict the significant SNPs.

EFFECT OF SILICON ON LETTUCE PERFORMANCE (in collaboration with *H. Peng*)

Lettuce production and its postharvest quality are frequently affected by environmental stresses, causing a substantial food waste and economic loss. The application of exogenous silicon (Si) can enhance growth and quality of diverse crops such as soybean and tomato but its effect on lettuce production has not been sufficiently tested. We initiated a study to determine the effect of Si (in SiO_2 form) on the growth, stress response, and postharvest quality of lettuce. In addition, we also study the factors affecting Si treatment. To identify the optimal concentration of Si application, agronomic traits, including plant weight, anthocyanin content, and chlorophyll content, were evaluated on potted plants of treated with Si at concentrations 0.011%, 0.022%, 0.044%, 0.088%, and 0.176% in two experiments performed in a net house. (Fig. 6). Based on these results, four subsequent experiments were conducted to examine the effect of Si at 0.022% on multiple traits, including plant weight, leaf thickness, anthocyanin content, and chlorophyll content, with potted plants grown in net house. Generally, Si treatment enhanced plant weight, leaf thickness, and anthocyanin and chlorophyll content, however the results were not consistent across all tested cultivars (Fig. 7). More experiments are being performed to examine the effect of Si on plant growth, stress response, and quality of lettuce grown in field.

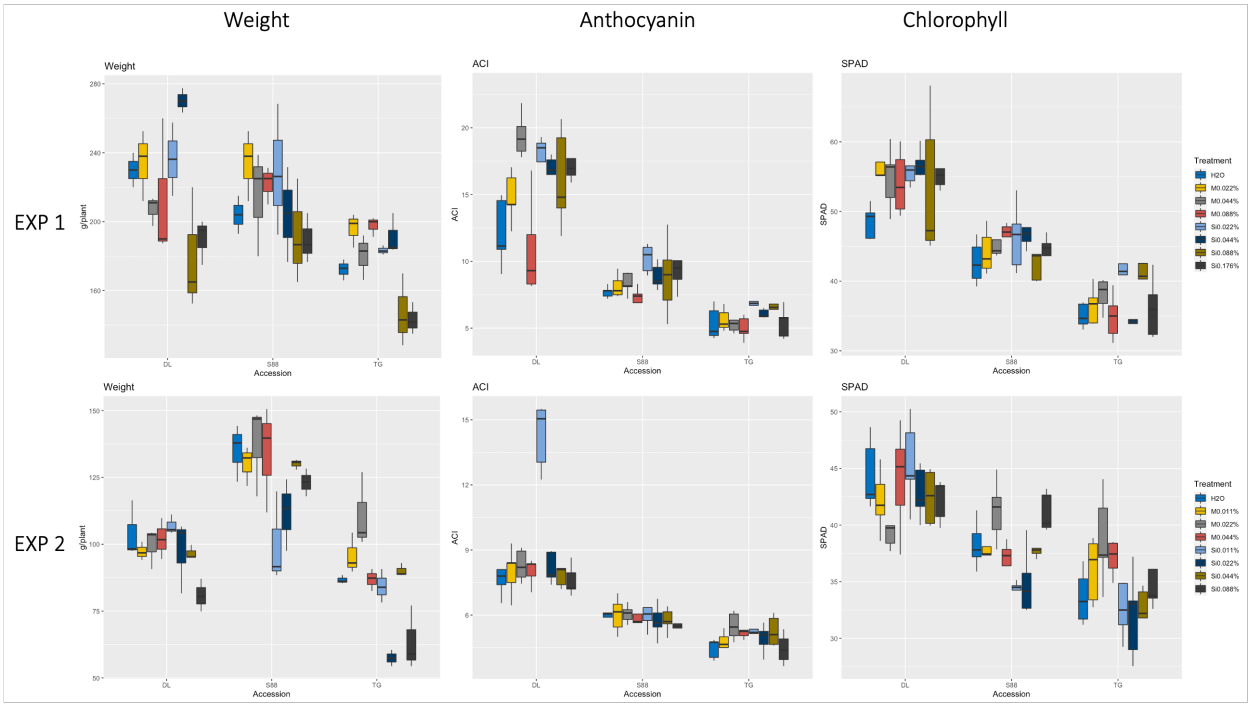

Figure 6. Plant weight and leaf anthocyanin and chlorophyll content of lettuce under silicon (Si) and silicon plus calcium (M) treatments. Foliar spray of Si and M solutions at different concentrations were applied to potted lettuce plants weekly until most plants reached market maturity in two experiments (Exp 1 and Exp 2). Evaluated we cultivars Darkland (DL), Salinas 88 (S88), Tall Guzmaine (TG).

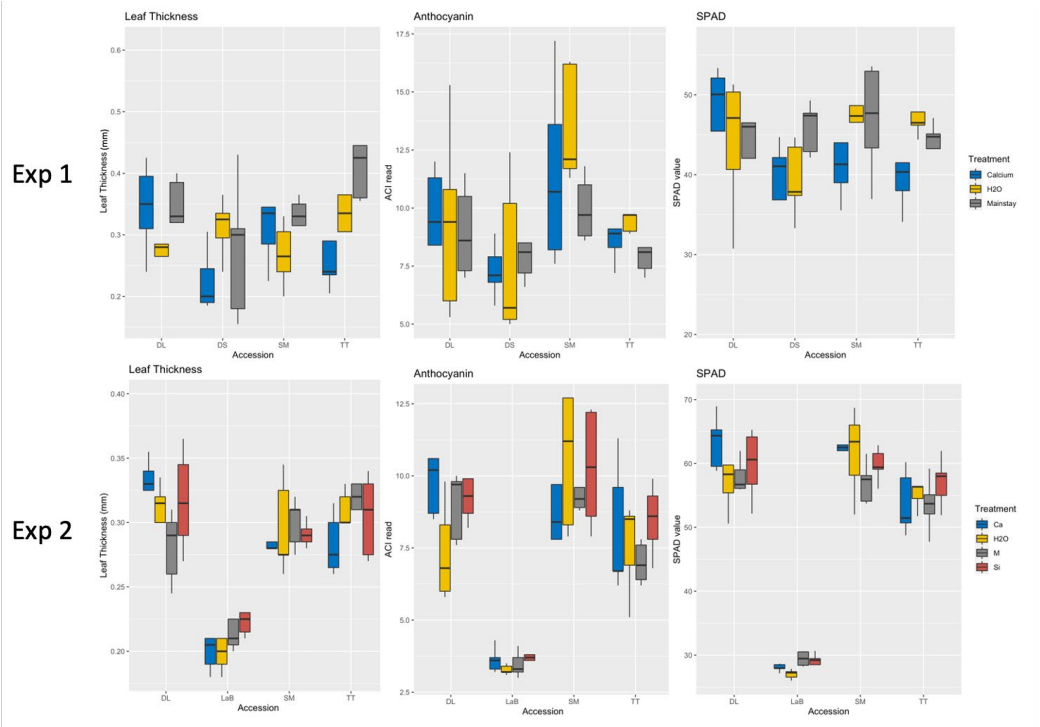

Figure 7. Leaf thickness and anthocyanin and chlorophyll content of lettuce under silicon (Si), calcium (Ca), and silicon plus calcium (M) treatments. Foliar spray of Si (0.022%), Ca (0.01%), and M (0.022% Si, 0.01% Ca), solutions at different concentrations were applied to potted lettuce plants weekly until most plants reached market maturity in two experiments (Exp 1 and Exp 2). Evaluated were cultivars/breeding lines Darkland (DL), La Brillante (LB), SM13-R2 (SM), and Triple Threat (TT).

RESISTANCE TO BACTERIAL LEAF SPOT (in collaboration with *G. Sandoya, J. Sthapit Kandel, C. Bull*, and *B. Mou)*

Bacterial Leaf Spot (BLS) is a disease that affects lettuce production in mild temperatures and high relative humidity, conditions that are conducive to unpredictable disease outbreaks. Plants for baby leaf lettuce production are grown at very high densities, which enhances the occurrence of bacterial leaf spot (BLS) caused by *Xanthomonas hortorum* pv. *vitians* (*Xhv*), a disease that can make the crop unmarketable. Growing cultivars with high resistance to BLS will reduce loss of yield and quality thus minimizing economic detriment to lettuce and spring mix growers. In this study, a collection of 495 lettuce accessions were screened with six isolates (BS0347, BS2861, BS3127, L7, L44, and Sc8B) of *Xhv* (Fig. 8). Accessions showing overall high-level resistance to all tested *Xhv* isolates were 'Bunte Forellen', PI 226514, 'La Brillante', ARM09-161-10-1-4, 'Grenadier', 'Bella', PI 491210, 'Delight', and 'Romana Verde del Mercado'. Genome-wide association studies of BLS resistance by mixed linear model analyses identified significant QTLs on four lettuce chromosomes (2, 4, 6, and 8) (Fig. 9). The most significant QTL

was on chromosome 8 (p = 1.42 x 10^{-7}) that explained 6.7% of total phenotypic variation for the disease severity. Accessions with high level of resistance detected in this study are valuable resources for lettuce germplasm improvement. Molecular markers closely linked to QTLs can be considered for marker-assisted selection to develop new lettuce cultivars with resistance to multiple races of *Xhv*. Additional information can be found at two recently published papers: https://doi.org/10.1094/PDIS-09-21-2087-RE, and https://doi.org/10.1007/s42161-022-01123-0.

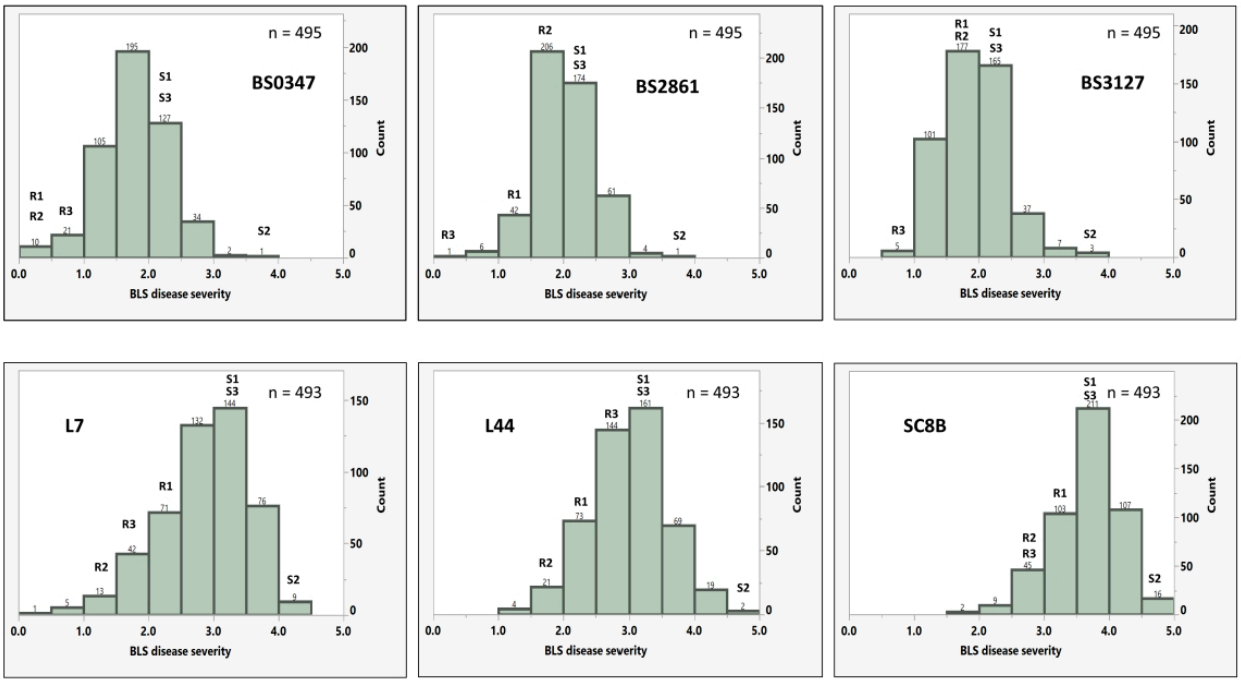

Figure 8. Histograms showing distribution of bacterial leaf spot (BLS) disease severity (DS) ratings evaluated on the diversity panel of 495 lettuce accessions tested with six isolates (BS0347, BS2861, BS3127, L7, L44, Sc8B) of *Xanthomonas hortorum* pv. *vitians*. Isolates BS0347, BS2861, BS3127 were tested in California and isolates L7, L44, Sc8B were tested in Florida. The greenhouse conditions in two locations were not identical and may have contributed to the differences in data distribution. Distribution of three resistant controls: 'Reines des Glaces' (R1), 'La Brillante' (R2), and ARM09-161-10-1-4 (R3), and three susceptible controls: 'Salinas' (S1), PI 251246 (S2), and 'Clemente' (S3) are labeled.

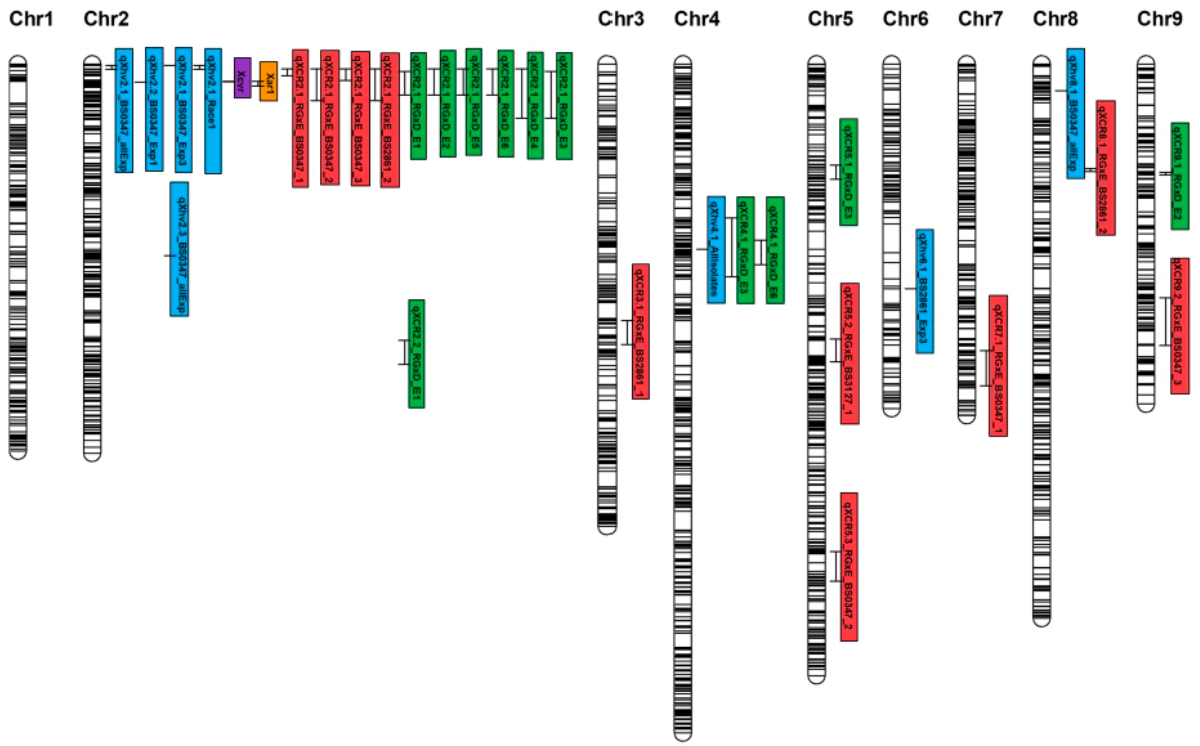

Figure 9. Positions of the quantitative trait loci (QTL) for bacterial leaf spot (BLS) resistance in 495 lettuce accessions screened with six isolates of the BLS pathogen *Xanthomonas hortorum* pv. *vitians* (*Xhv*). The QTL regions identified in the population of 495 accessions are represented by blue boxes. For comparison with the location of the previously reported QTL for BLS resistance, red boxes indicate QTL identified in 'Reines des Glaces' (RG) × 'Eruption' (E) population and green boxes indicate QTL identified in 'Reines des Glaces' (RG) × 'Delsay' (D) population (Sandoya et al. 2019), purple box indicate locus *Xcvr* in PI 358001 × 'Tall Guzmaine' (Wang et al. 2016) and orange box indicates locus *Xar1* in 'Salinas 88' × 'La Brillante' (Hayes et al. 2014). The significant markers from all the populations were aligned to the lettuce genome (Reyes-Chin-Wo et al. 2017).

References:

Atkinson, L.D., McHale, L.K., Truco, M.J., Hilton, H.W., Lynn, J., Schut, J.W., Michelmore, R.W., Hand, P., Pink, D.A.C., 2013b. An intra-specific linkage map of lettuce (*Lactuca sativa*) and genetic analysis of postharvest discolouration traits. Theor. Appl. Genet. 126, 2737–2752.

Hayes, R. J., Trent, M. A., Truco, M. J., Antonise, R., Michelmore, R. W., and Bull, C. T. 2014b. The inheritance of resistance to bacterial leaf spot of lettuce caused by *Xanthomonas campestris* pv. *vitians* in three lettuce cultivars. Hortic. Res. 1:14066.

Hunter, P.J., Chadwick, M., Graceson, A., Hambidge, A., Hand, P., Heath, J., Lignou, S., Oruna-Concha, M.J., Pink, D., Rada, B., Wagstaff, C., Barker, G., Monaghan, J.M., 2022. Elucidation

of the biochemical pathways involved in two distinct cut-surface discolouration phenotypes of lettuce. Postharvest Biol. Technol. 183, 111753.

Reyes-Chin-Wo, S., Wang, Z., Yang, X., Kozik, A., Arikit, S., Song, C., Xia, L., Froenicke, L., Lavelle, D. O., Truco, M.-J., Xia, R., Zhu, S., Xu, C., Xu, H., Xu, X., Cox, K., Korf, I., Meyers, B. C., and Michelmore, R. W. 2017. Genome assembly with in vitro proximity ligation data and whole-genome triplication in lettuce. Nat. Commun. 8:14953.

Sandoya, G. V., Maisonneuve, B., Truco, M. J., Bull, C. T., Simko, I., Trent, M., Hayes, R. J., and Michelmore, R. W. 2019. Genetic analysis of resistance to bacterial leaf spot in the heirloom lettuce cultivar Reine des Glaces. Mol. Breed. 39:160.

Wang, Y., Lu, H., and Hu, J. 2016. Molecular Mapping of high resistance to bacterial leaf spot in lettuce PI 358001-1. Phytopathology 106:1319–1325.

RECENT (2022 – 2023) PUBLICATIONS RELEVANT TO THIS PROJECT

Simko I, Puri KD, Dhar N, Peng H, Subbarao KV. Mapping quantitative trait loci for lettuce resistance to *Verticillium dahliae* race 3, plant development, and leaf color using an ultra-high-density bin map constructed from F_2 progeny. PhytoFrontiers. 2022; 2:257-67. https://doi.org/10.1094/PHYTOFR-11-21-0078-R

Sthapit Kandel J, Sandoya GV, Zhou W, Read QD, Mou B, Simko I. Identification of quantitative trait loci associated with bacterial leaf spot resistance in baby leaf lettuce. Plant Disease. 2022; 106:2583-90. https://doi.org/10.1094/PDIS-09-21-2087-RE

Simko I, Peng H, Sthapit Kandel J, Zhao R. Genome-wide association mapping reveals genomic regions frequently associated with lettuce field resistance to downy mildew. Theoretical and Applied Genetics. 2022; 135:2009-24. https://doi.org/10.1007/s00122-022-04090-3

Sandoya GV, Rosenthal E, Simko I, Rodrigues-Porto LN, Wadlington WH, Bull CT, Carroll A. Lettuce (*Lactuca sativa* L.) germplasm resistant to bacterial leaf spot caused by race 1 of *Xanthomonas hortorum* pv. *vitians* (Brown 1918) Morinière et al. 2020. Journal of Plant Pathology. 2022; 104:993-1008. https://doi.org/10.1007/s42161-022-01123-0

Kumar P, Eriksen RL, Simko I, Shi A, Mou B. Insights into nitrogen metabolism in the wild and cultivated lettuce as revealed by transcriptome and weighted gene co-expression network analysis. Scientific Reports. 2022; 12:9852. https://doi.org/10.1038/s41598-022-13954-z

Peng H, Zhao R, Smith R, Simko I. Phenotypic and genetic analyses of yellow spot malady in lettuce. Scientia Horticulturae. 2022; 305:111389. https://doi.org/10.1016/j.scienta.2022.111389

Brandl MT, Mammel MK, Simko I, Richter TK, Gebru ST, Leonard SR. Weather factors, soil microbiome, and bacteria-fungi interactions as drivers of the epiphytic phyllosphere

communities of romaine lettuce. Food Microbiology. 2023; 113:104260. https://doi.org/10.1016/j.fm.2023.104260

Peng H, Simko I. Extending lettuce shelf life through integrated technologies. Current Opinion in Biotechnology. 2023; 81:102951. https://doi.org/10.1016/j.copbio.2023.102951

Simko I, Hasegawa DK, Peng H, Zhao R. Genetic and physiological determinants of lettuce partial resistance to Impatiens necrotic spot virus. Frontiers in Plant Science. 2023; 14:1163683. https://doi.org/10.3389/fpls.2023.1163683