

## SCRI 2017-51181-26830 SPINACH REPORT

**Project Title:** *Developing Genetic and Molecular Resources to Improve Spinach Production and Management*

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Spinach Portal: <https://spinach.uark.edu/>

### Project Summary

In the last 20 years, the US spinach industry has seen a dramatic increase in the popularity of spinach, and as a result, increased production and consumption. In order to keep up with the demand, the spinach industry requires continuous development of improved and adapted cultivars to meet numerous disease constraints to satisfy consumer demands. Spinach breeding presents some unique challenges, and efforts to improve and expedite the selection process would greatly accelerate the release of new, improved, and durably disease resistant cultivars. The proposed project is a multi-state and multi-agency collaboration to develop tools for molecular breeding and outreach activities directly to benefit stakeholders. The effort will accelerate the introgression of critically important traits into breeding populations for improved spinach cultivar development and establish a set of outreach activities to communicate information and impact industry stakeholders. We propose to develop SNP markers associated with the three most economically important diseases affecting spinach (downy mildew, white rust, and Fusarium wilt) as a tool for spinach breeders, in both public and private sectors, to select resistant germplasm through marker-assisted selection. As a direct consequence, spinach resources for resistance to downy mildew, white rust, and Fusarium wilt will be characterized and developed for release to spinach breeding programs. This project will address the areas of the USDA SCRI program: (1) "Research in plant breeding, genetics, genomics, and other methods to improve crop characteristics"; and (2) "Efforts to identify and address threats from pests and diseases, including threats to specialty crop pollinators".

### Major Goals of the Project

Spinach (*Spinacia oleracea* L.) has become an increasingly important economic vegetable crop worldwide with an estimated annual value of \$11.8 billion (Correll et al. 2011, van Deynze 2015). The US is the second largest producer of spinach after China with over 550,000 tons harvested, valued at over \$300 million annually (Correll et al. 2011; NASS 2015). In addition to its economic importance, spinach is one of the healthiest vegetables in the human diet due to its high concentration of nutrients and health-promoting compounds (Dicoteau 2000; Morelock and Correll 2008). During the last 15 years, the US spinach industry has seen a dramatic increase (double) in fresh market demand (NASS 2015). This requires development of new, improved cultivars to increase production. The major yield-limiting diseases in spinach production in the US are downy mildew (DM), white rust (WR), and Fusarium wilt (FW). The purpose of this project is to build a molecular breeding platform integrated into a classical breeding approach in spinach. With improved next generation sequencing (NGS) and the decreasing cost of sequencing, it is becoming feasible

to discover millions of single-nucleotide-polymorphisms (SNPs) for any plant and connect these markers to desirable phenotypic traits. Spinach is a very popular vegetable crop, which can greatly benefit from the development of molecular tools to improve commercial cultivars. The overall goal of this proposal is to develop genetic, molecular, and management resources to improve US spinach production by combining molecular and conventional breeding to expedite breeding procedures and speed up genetic gains for the release of spinach cultivars adapted to the main regions of spinach production.

The specific research objectives of this project are:

- (1) Genetic mapping and SNP marker identification for downy mildew and white rust resistance in spinach;
- (2) Evaluation, QTL and association mapping and SNP marker identification for Fusarium wilt resistance in spinach;
- (3) Introgression of downy mildew and white rust resistance into spinach lines with diverse leaf quality characteristics to satisfy market demand; and
- (4) Construction of high-density SNP consensus genetic maps of six chromosomes in spinach using whole genome resequencing.

## Major Accomplishments under Major Goals

### I. Genetic mapping and SNP marker identification for downy mildew and white rust resistance in spinach

#### 1. Downy mildew evaluation in commercial cultivars/hybrids:

- (1) 70 spinach cultivars were evaluated for disease reactions to downy mildew (*Peronospora effusa*, syn. *P. farinosa* f. sp. *spinaciae*, *Pfs*) in two locations at the Salinas Valley in California and at Yuma in Arizona during the winters 2022-2021, 2020-2021, 2019-2020, 2018-19, 2017-18, and 2016-17 seasons. The data of downy mildew illustrated a wide range in disease incidence from 0.0 to 100%. The results showed that there were >30 spinach cultivars showed downy mildew resistance with no disease or <1% disease incidence in each season (Correll et al. 2019a, b, 2017; Dhillon et al. 2020a, b; Matheron et al. 2019).
- (2) The 70 spinach cultivars were evaluated their reactions to 10 downy mildew races *Pfs* 13, 15, 16, 18, 19, UA202043A, UA202044A, UA202046, UA202048, and UA202105C under greenhouse/growth chamber conditions and results showed that >10 spinach cultivars were resistant to each race, respectively (Dotun et al. 2020, 2021; Feng et al. 2021).

#### 2. Downy mildew field evaluation in spinach germplasm:

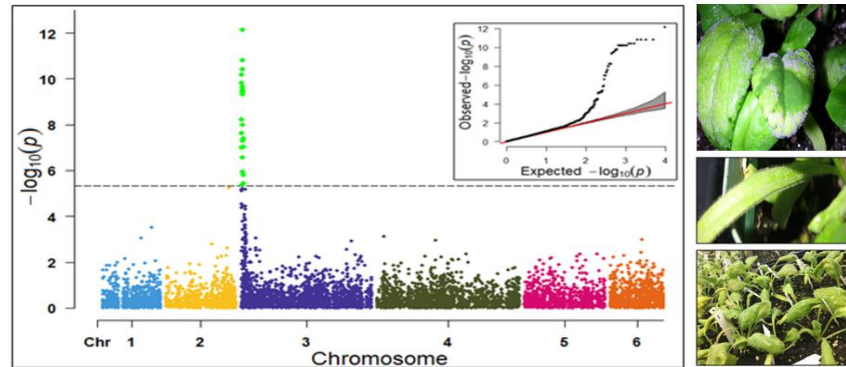
- (1) 440 spinach genotypes, including USDA spinach germplasm, commercial hybrids varieties, and Arkansas lines were evaluated their downy mildew resistance in USDA downy mildew selection nursery at Salinas, CA and Yuma, AZ with two replicates each line and the downy mildew incidence and severity were recorded during winter 2017-2018 and 2018-2019 seasons. A wide variation in downy mildew resistance was observed in spinach germplasm and cultivars. We identified 12-spinach germplasm (Whale, Dikensiz Kuzu, Cornell ID #86, Lokalen, SPI 161/86, 139 x 135, Meerkat, Hydrus, Long Standing Bloomsdale, Palak, Esfinags) showing resistance with disease severity less than 5% (Bhattarai et al. 2019e).
- (2) A panel of 300 USDA spinach germplasm accessions was evaluated for downy mildew disease response under field condition in the Texas Wintergarden area in Crystal City, TX and over 10 spinach lines showed downy mildew tolerance in the field condition in Southern Texas and the spinach lines were also testing in Weslaco, TX but there was no strong downy mildew infected (Bhattarai et al., 2020c).

#### 3. Molecular markers for downy mildew resistance:

Thirteen, two and eight molecular markers have been developed for three loci, RPF1, RPF2 and RPF3, of downy mildew resistance, respectively. One marker was linked to both RPF2 and RPF3 loci, and four markers were linked to all three loci, indicating that these three resistance loci are closely linked (Feng et al. 2018b).

4. *Downy mildew resistance QTL/ gene mapping and genome-wide association study (GWAS):*

(1) GWAS was conducted using 9,783 GBS-generated SNPs on a panel of 174 spinach genotypes to identify the genetic loci governing the resistance to downy mildew pathogen *Pfs* race 13 of downy mildew pathogen and the resistance gene alleles were mapped to spinach chromosome (chr) 3 within a 0.5 M region for resistance to *Pfs* 13 in spinach hybrids T-Bird, Swan, Squirrel, and Tonga (Bhattarai et al. 2020a) (**Figure 1**).



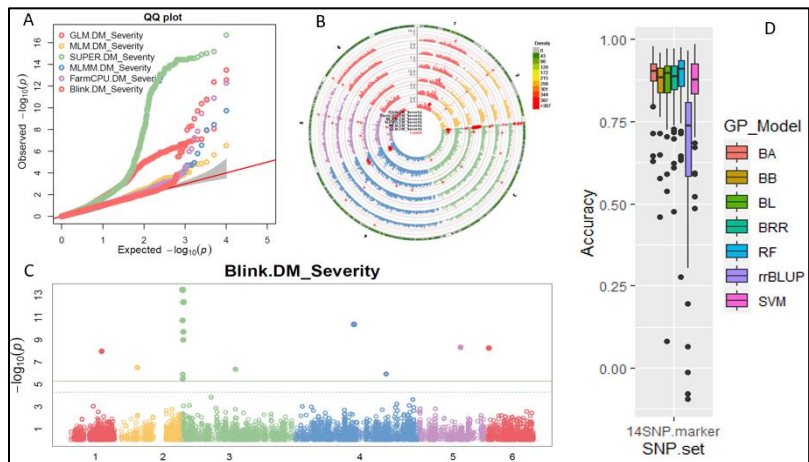
**Figure 1. Manhattan and QQ plots showing significantly associated SNP markers on chromosome 3 to downy mildew race *Pfs* 13 resistance.**

(2) Association analysis was performed in 172 spinach genotypes using 10,788 GBS (genotyping by sequencing) generated SNPs in the population derived from a cross of Whale and Lazio segregating for *Pfs* 16 resistance and SNP markers and genetic alleles were identified in the region of chr 3 for *Pfs* 16 resistance from cultivar Whale as for *Pfs* 13 resistance (Bhattarai et al. 2021a).

(3) The F2 populations derived from Viroflay x Whale inoculated with *Pfs* 5 were genotyped following a low-coverage genome resequencing approach mapped the resistance on the 1.2 Mb region of chr 3 with LOD > 30 in the TASSEL GLM model (Bhattarai et al., in prep).

(4) Our lab is investigating other two F2 populations derived from Viroflay crossed Califlay and Lazio that were phenotyped for *Pfs* 5 resistance.

(5) GWAS performed under greenhouse/growth chamber condition for resistance to *Pfs* 5 in 251 genotypes including 216 USDA spinach germplasm accessions and 35 commercial hybrids/cultivars; the major QTL/alleles was mapped near the previously mapped region on chr. 3; 14 SNP markers were strongly associated with downy mildew resistance; and the prediction accuracy (r-value) was 0.81 – 0.90 when 14 SNP markers were used (Olaoye 2021 MS Thesis; Olaoye et al. 2022b) (**Figure 2**).



**Figure 2. QQ and Manhattan plots based on six GWAS models (A, B); BLINK Manhattan plot (C) showing significantly associated SNP markers on chr 3 and other chr 1, 2, 4, 5, and 6 for downy mildew race *Pfs* 5 resistance; genomic prediction (r-value) of seven models based on 14 SNP markers.**

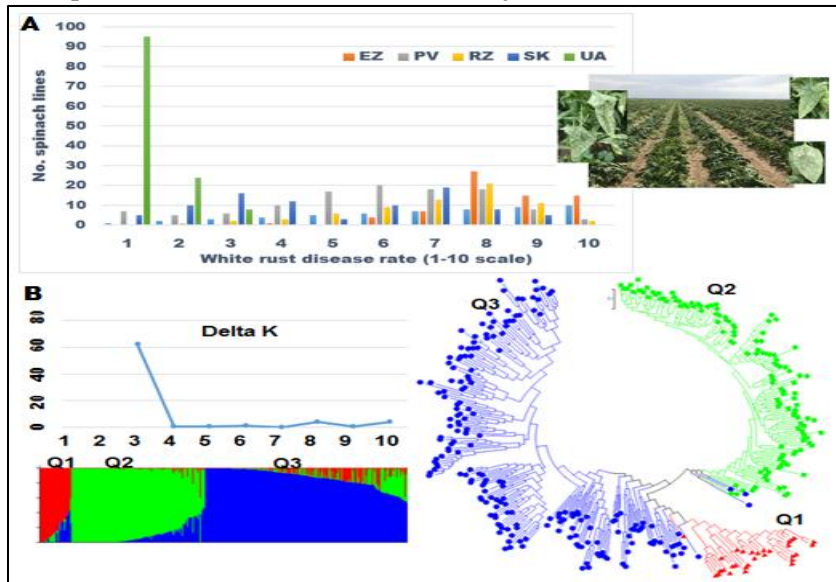
5. GWAS of downy mildew resistance in spinach under field condition:

GWAS has been performed in 434 spinach genotypes using 4,000 SNPs in a multi-year and multi-location DM screening in Salinas, CA and Yuma, AZ (Bhattarai et al. 2020b, Bhattarai et al., 2022) and in Wintergarden area in southern Texas (Bhattarai et al. 2020e). We identified 40 SNP markers were associated with field resistance from Salinas and Yuma trial and eight SNPs were associated in the Texas trial. GP using the GWAS associated markers provided prediction accuracy  $r > 0.6$  for all tested environments (Bhattarai et al., 2022). These SNPs plus new analyses derived SNPs will be used for non-race specific resistance validation Spinach USDA association panel consisted of 246 spinach accessions was evaluated for downy mildew disease response under field condition in the Texas Wintergarden area in Crystal City, TX, and GWAS was performed to identify SNP markers associated with downy mildew tolerance in the field condition in Southern Texas, using 5,400 GBS generated SNPs (Bhattarai et al. 2020c).

6. Evaluation and GWAS for white rust resistance in spinach commercial cultivars/hybrids:

A total of 522 spinach lines from Pop Vriends (PV), Enza Zaden (EZ), Rijk Zwaan (RZ), Sakata (SK), and the University of Arkansas (UA) were grown in the Del Monte White Rust Nursery in Crystal City, Texas during the winter of 2014-15 and white rust disease severity was rated with on a scale of 0-10 (**Figure 3A**).

352 of the 522 spinach lines were evaluated for genetic diversity (**Figure 3B**) and conducted GWAS and nine SNPs were shown to be associated with white rust resistance based on SMR, GLM, and MLM in TASSEL and cMLM in GAPIT with an LOD greater than 3.0. When the nine SNPs were used to do



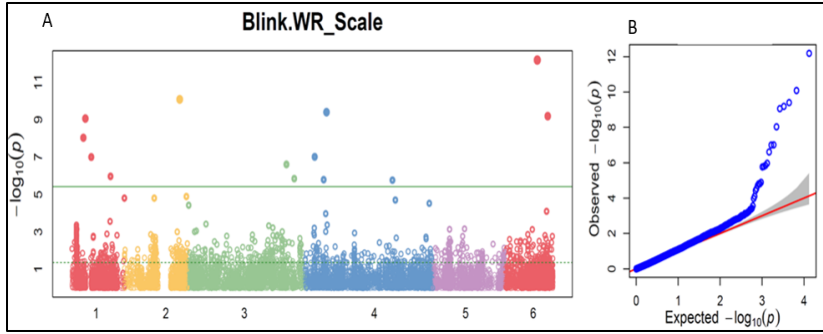
**Figure 3. Screening results for WR resistance: (A) Distribution of disease severity index on evaluated germplasm and (B) Population structure.**

genomic prediction /genomic selection (GP/GS) using RR-BLUP in rrBLUP, the average correlation coefficients ( $r$ ) between genomic estimated breeding values (GEBVs) and observed value was 0.6, indicating the GS will be efficient using the eight SNP markers in GS for selection white rust resistance. The results of spinach resistant lines and the associated SNP markers have been sent and released to the companies. The SNP markers were also validated by PV, RZ and SK using different spinach lines. We have made presentations several times and the final report has been sent to companies (Shi et al. 2020, 2019, 2018a, b).

7. GWAS and Genomic prediction (GP) for white rust resistance in USDA spinach germplasm:

- (1) GWAS was performed in 346 USDA germplasm accessions for white rust resistance (Shi et al. 2022). The results showed that 23 accessions were resistant; 40 and 9 SNPs were associated with WR resistance based on one or nine GWAS models, individually or combined (**Figure 4**); and predictive accuracy was up to 0.84 when 4,836 SNPs were used; 0.75 when the 40 SNP markers used; and 0.61 when the 9 SNP markers used based on nine GP models.

(2) Another set of 322 USDA spinach accessions were screened for white rust resistance at both Rio Grande Valley and Wintergarden, TX during winter 2020-2021. Genotyping by whole genome resequencing (WGR) with 10 x genome size (~10 Gb short-read sequencing data each spinach line) has been done for this panel at BGI. GWAS and GP have been analyzing and manuscript is being written.



**Figure 4. Manhattan and QQ plots showing significantly associated SNP markers on chromosomes 1, 2, 4, and 6 for white rust**

8. White rust susceptibility in spinach germplasm:

Minor alleles were identified to be associated with white rust susceptibility in spinach (Awika et al. 2020a, 2019b, 2018) and a new phenotyping was developed to do white rust phenotyping in the field (Awika et al. 2021b, 2019a, 2018). The manuscript describes the application of allelic mapping to improve market to trait association using white rust as a model. We showed that by segmenting data based on phenotypic strength, the target alleles in individuals showing stronger WR phenotype have a significantly higher minor allele frequency than in those showing weaker WR phenotype. Application of this method could narrow down the population targeted for a detailed study focusing on disease associated with minor alleles. This may be useful in assigning marker significance directly to the important phenotypic tails and quickly for assessing the underlying molecular causative variants and thus provides insight into loci required for susceptibility

9. QTL mapping and GWAS of white rust resistance:

Spinach populations were screened for white rust resistance at the Rio Grande Valley and Wintergarden, TX during winter 2020-2021, including two F2 populations (200 plants each) – (Viroflay x F415) F2 and (Viroflay x F380) F2, and two F2:3 populations (61 and 120 plots respectively). White rust incidence and severity was measured. Genotyping by low-coverage whole genome resequencing (WGR) has been done for these populations. Data have been analyzing and manuscript is in preparation.

**II. Evaluation, QTL and association mapping and SNP marker identification for Fusarium wilt resistance in spinach**

1. Fusarium wilt resistance evaluation:

68 spinach commercial cultivars was evaluated for Fusarium wilt resistance under greenhouse. Bandicoot, Serpens, Galah, Sheep, Spoonbill, Kodiak, PV1452, Baboon, and Minkar showed very strong resistance to inoculum consisting of a mix of three isolates of *F. oxysporum* f. sp. *spinaciae* (*Fos*). In addition, of 48 breeding lines from the University of Arkansas evaluated for resistance, AK17, AK6, and AK25 were partially resistant to *Fos*. Likewise, of 374 USDA spinach germplasm accessions screened, 21 showed partially resistant reactions. Of these, NSL 6099, PI 169686, PI 321020, PI 339545, PI 358247, PI 419004, PI 419218, and PI 648948 had the least severe wilt ratings. Of 75 wild spinach accessions (*S. turkestanica*) evaluated, CGN 24992, CGN 24955, CGN 25138, CGN 24956, CGN 24996, CGN 25086, CGN 25005, CGN 24957, and CGN 25002 showed highly resistant reactions to *Fos*. These resistant genotypes will be useful sources of resistance for breeding Fusarium wilt resistant cultivars (Gyawali et al. 2019 a, b, c, 2021).



## 2. QTL mapping of *Fusarium wilt* resistance:

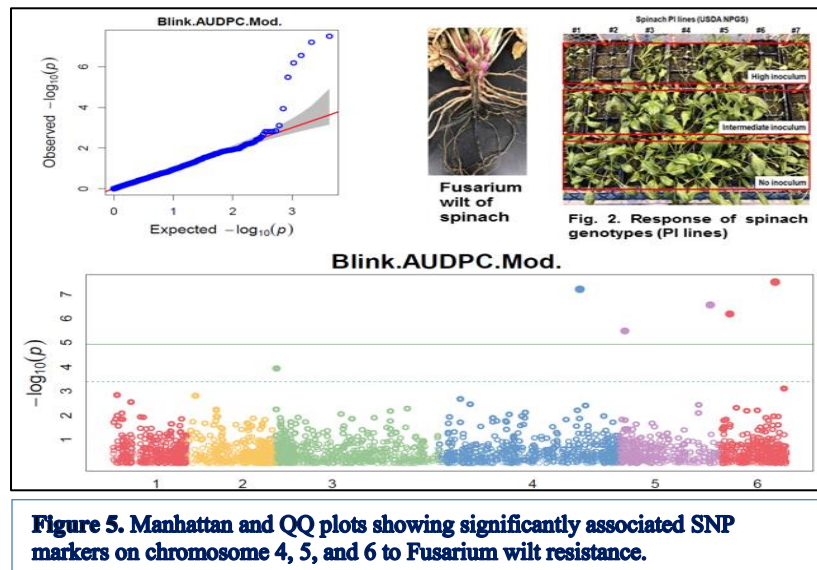
A bi-parental spinach population derived from the susceptible cultivar Viroflay and the partially resistant line AS10-1512F was phenotyped for severity of *Fusarium wilt*. From this, 192 genotypes of Viroflay/AS10-1512F (including the parents) were sequenced at the Genomics and Bioinformatic Center of Texas A&M University, TX, using whole genome sequencing and SNPs have been identified. *Fusarium wilt* resistant QTLs are been analyzing and it is expected SNP markers will be identified by the end of 2021 (Gyawali et al. 2019b).

## 3. GWAS for *Fusarium wilt* resistance:

The reactions of 351 USDA spinach accessions to a mixture of three *Fos* isolates (Fus058, Fus254, and Fus322) representing two pathogenicity groups of the fungus, were utilized for a GWAS of spinach *Fusarium wilt* resistance. In total, 46 resistance QTLs were discovered on six chromosomes, of which 11 QTL were detected in multiple evaluations of *Fos* resistance ratings completed 21, 28, and 35 days after planting the accessions, and for the *Fusarium wilt* resistance index and area under the disease progress curve, at medium and high rates of inoculum of the pathogen. The  $R^2$  of these QTL ranged from 3.5-9.3%. The GWAS for spinach biomass revealed 44 QTLs on chromosomes 2, 3, 4, 5, and 6. The  $R^2$  of these QTL ranged from 3.1-8.8% (Gyawali et al. 2019a).

## 4. Evaluation, GWAS and GP of *Fusarium wilt* resistance in wild spinach species *S. turkestanica*:

So far, 75 *S. turkestanica* accessions were obtained from The Centre for Genetic Resources in the Netherlands (CGN), Wageningen University and Research (WUR). The accessions were phenotyped for *Fusarium wilt* resistance and genotyped with genotyping-by-sequencing (GBS). The GWAS revealed a major QTL on chromosomes 4, 5, and 6 for *Fusarium wilt* resistance and 33-associated SNP markers were identified (Figure 5). The prediction accuracy (r-value) was >0.6 based on six GP models.



**Figure 5. Manhattan and QQ plots showing significantly associated SNP markers on chromosome 4, 5, and 6 to *Fusarium wilt* resistance.**

## III. Introgression of downy mildew and white rust resistance into spinach lines with diverse leaf quality characteristics to satisfy market demand

1. Crosses were made among cultivars with different downy mildew-resistant genes to combine their resistances. Progenies from 34 crosses, along with resistant and susceptible controls, were planted in fields at the USDA-ARS station in Salinas, CA. We have made steady progress in downy mildew resistance through the cycles of recurrent selection. For example, in 2019, ten populations had 0%, and another five populations had < 10% downy mildew incidences, as compared to the susceptible control ('Viroflay') with 98% disease incidence. That is compared to 2018, when only one population had 0% and another five populations had <10% downy mildew incidence. The 2019 populations are progenies of the plants selected from the 2018 populations. Of the 16 populations with data from both 2018 and 2019 seasons, 12 populations (75%) showed decreases of downy mildew incidences, 3

populations had a small increase, and only one population had a large increase in disease incidence. Six of the 16 populations dropped downy mildew incidences to 0%. These results show that the recurrent selection method was very effective to increase the downy mildew resistance in the spinach populations. If the downy mildew resistance is confirmed in future trials, these populations can be released to seed companies to produce seeds or develop new cultivars.

2. For white rust, over 50 spinach lines were evaluated their white rust resistance in the field of Crystal City, TA and showed these lines are white rust resistant; and 30 crosses have been made between AR spinach lines with white rust resistance with commercial spinach cultivars with downy mildew resistance in order to release cultivars resistant to both diseases. Thirty-advanced spinach breeding with white rust resistance were selected.
3. 66 spinach lines have been released to Bowery Farming; 20 lines to 80 Acres Farms, and 30 lines to Clemson University for spinach production testing.
4. Twenty new crosses between Arkansas white rust resistant, USDA white rust resistant, and susceptible lines with different leaf characteristics were made. In addition, five segregating F2 populations between the cross of resistant and susceptible white rust breeding lines was developed for cultivar development and marker validation.

#### **IV. Construction of high-density SNP consensus genetic maps of six chromosomes in spinach using whole genome resequencing**

Ten F2 populations were developed from Viroflay, a spinach cultivar with high susceptibility to all three diseases of downy mildew, white rust, and Fusarium wilt, crossed to ten other spinach lines including seven downy mildew resistant lines (Lazio, Whale, Boeing, Califlay, Campania, NIL1, and NIL3), two white rust resistant lines (F415 and F380), and one Fusarium wilt resistant line (AS10-1512F). So far, five F2 populations with a total of 960 F2 individuals were phenotyped and genotyped using low-coverage WGR in Texas A&M Bioinformatics Center. Over half million SNPs were identified in the seven F2 populations. The consensus genetic maps of the spinach six genomes has been analyzing and manuscript is being written.

#### **VI. Other spinach research**

##### **1. Whole genome sequencing (WGS) and whole genome resequencing (WGR):**

- (1) The spinach line NIL1, a Viroflay-background, near isogenic line with the RPF1 downy mildew resistance gene, was subjected to de novo assembly at Novogene company (<http://en.novogene.com/>) using 10X Genomics Chromium System powered by 10x GemCode Technology. 66.7% whole genome coverage of NIL1 was aligned and assembled from Novogene. The NIL1 and another spinach line, 09-04-103 were also sequenced in Dovetail Genomics and 92.8% and 60.2% whole genome coverage was assembled, respectively.
- (2) 30 spinach genotypes were sequenced with 33X coverage, i.e., 33 Gb sequencing data for each spinach sample. >6 million were new discovered in spinach.
- (3) 480 spinach genotypes have been sequenced using whole genome resequencing at Beijing Genome Institute (BGI) with 10X coverage, and the sequence data is available and the sequence data have been deposited at NCBI SRA with BioProject: PRJNA860974. We now have > 10 millions of genome-wide SNP markers for the spinach association panel available. GWAS has been conducting on all major traits that have been phenotyped, and SNP markers associated with each trait will be identified.

##### **2. Downy mildew resistance evaluation method:**

A new disease evaluation method to characterize the resistance-susceptibility response of spinach plants following inoculation of the detached leaves were developed and standardized on a set of differential cultivars using three *Pfs* races/ isolates. Disease response of the differential cultivars on detached leaves was compared to the standard whole plant inoculation method, and complete correspondence between two methods was confirmed (Bhattarai et al. 2020c).

3. Gene-expression for resistance to downy mildew pathogen (*P. effuse*):

- (1) Transcriptome profile was studied in the downy mildew resistant and susceptible spinach cultivars, Solomon and Viroflay using RNAseq. Both up regulated and downregulated host gene expression were detected resistance to downy mildew pathogen (Kandel et al. 2019).
- (2) The transcriptome sequencing and differential gene expression studies have been performed for selected resistant and susceptible lines that have provided new information on genetic regulations of host-pathogen interactions (Kandel et al. 2020; Zia et al. 2021).
- (3) The resistant (NIL6) and susceptible (Viroflay) spinach cultivars were inoculated with *Pfs* 5, and RNA was sequenced for multiple time-point. Comparative transcriptome analysis of resistant and susceptible cultivars following *Pfs* 5 inoculation has been performed to evaluate gene expression profiles and identify differentially expressed genes between resistant and susceptible spinach cultivars (Bhattarai and Shi 2021c). Genes and pathways regulating defense mechanisms against the *Pfs* pathogen are currently ongoing.

4. Spinach leaf spot evaluation:

271 USDA spinach germplasm accessions, with 35 commercial spinach cultivars and five the University of Arkansas breeding lines, were evaluated with Sb-1-St001 isolate of *S. vesicarium* under the greenhouse at the University of Arkansas. The results showed that 15 lines were resistant and 42 SNP markers significantly associated with the *Stemphylium* leaf spot resistance were identified following GWAS analysis using the disease scores and whole WGR generated SNP markers (Liu et al. 2020, 2021; Bhattarai et al. 2022).

5. Genetic diversity study in spinach:

480 spinach genotypes were sequenced using genotype by sequencing (GBS), 343 out of which were used to do genetic diversity and population structure analysis in spinach. The results showed that genetic background in improved commercial F1 hybrids and in Arkansas, cultivars/lines had different structured populations from the USDA germplasm. In addition, the genetic diversity and population structures were associated with geographic origin and germplasm from the US Arkansas breeding program had a unique genetic background. These data could provide genetic diversity information and the molecular markers for selecting parents in spinach breeding programs (Shi et al. 2017).

6. Genetic diversity in wild spinach species:

The wild spinach accessions of *Spinacia turkestanica* collected from Uzbekistan and Tajikistan were genotyped following GBS approach and the SNP data were used to investigate the genetic diversity, population structure, and domestication history of spinach. The genetic groups were identified among the *S. oleracea* and *S. turkestanica* accessions, while the *S. turkestanica* accession formed two distinct sub-population groups. The two genetic subpopulations of *S. turkestanica* were separated by the mountain range in Uzbekistan. The result revealed the domestication of cultivated *S. oleracea* from the Q2 group of *S. turkestanica* that originated in the western side of the Zarafashan mountain range in Uzbekistan (Gyawali et al. 2021a, b).



7. SSR discovery in spinach:

Genome-wide SSRs in spinach were discovered with a total of 79,347 di-, tri-, tetra-, penta-, and hexa- repeat SSR loci with minimum repeat times of 6, 5, 4, 4, 4 respectively were identified (Bhattarai et al. 2021b). SSR length variation among the genome sequence of 20 spinach cultivars was identified following computational effort. A subset of such variable length SSRs (~ 5 SSR loci from each of the six-spinach chromosomes) were PCR amplified and alleles were determined. Of the 36 SSR loci, two failed to amplify, and the remaining 34 were polymorphic in a set of 48 spinach accessions. This result validated the computationally identified thousands of polymorphic SSRs in this study and eliminated the need for laborious experimental screening for polymorphism saving time and cost.

8. Spinach flowering time:

The USDA spinach accessions and commercial cultivars were evaluated for flowering time in the greenhouse. GWAS performed using GBS markers identified SNP markers associated with slow bolting and late flowering in spinach (Bhattarai et al. 2020d).

9. High-throughput phenotyping in spinach:

A high-throughput phenotyping (HTP) technology has been developed for fast phenotyping in spinach (Avila et al. 2020a, 2019, 2018; Awika et al. 2019a, b). HTP was combined with next generation sequencing to identify molecular markers associated to plant growth parameters. Additional work is being performed to measure biomass (yield), water-use efficiency, and abiotic stress screening using unmanned aerial systems (UAS) and machine learning for spinach management (Awika et al. 2021a, b, c).

10. Downy mildew pathogen race (*P. effusa*, *Pfs*) identification, genetic diversity and disease management:

So far 19 *Pfs* races has been identified and reported (Correll et al. 2021; Feng et al. 2018a, 2018c); the genetic diversity of the *Pfs* pathogens was analyzed (Feng et al. 2021; Lamour et al. 2018); the sexual oospore germination of *Pfs* was observed (Kandel et al. 2019b; Liu et al. 2018b); and the effectiveness of biopesticides in minimizing downy mildew incidence was investigated in field plots and found that the bio pesticide Prosodic significantly reduced the disease incidence (Kandel et al. 2019a).

11. Anthrachnose leaf spot evaluation:

Anthrachnose (*Colletotrichum dematium*) is an important disease in spinach (*Spinacia oleracea*). A diverse collection of 276 spinach accessions was scored for anthracnose disease severity. We then evaluated marker identification approaches by testing how well haplotype-based trait modelling compares to single markers in identifying strong association signals. Alleles in linkage disequilibrium were tagged in haplotype blocks, and anthracnose-associated molecular markers were identified using single-SNP (sSNP), pairwise haplotype (htP) and multi-marker haplotype (htM) SNP tagging approaches. We identified 49 significantly associated markers distributed on several spinach chromosomes using all methods. The sSNP approach identified 13 markers, while htP identified 24 (~63% more) and htM 34 (~162% more). Of these markers, four were uniquely identified by the sSNP approach, nine by htP and nineteen by htM. The results indicate that resistance to anthracnose is polygenic and that haplotype-based analysis may have more power than sSNP (Awika et al 2020a)

## V. Extension

### 1. Spinach field days for downy mildew disease:

Dr. Correll organized the multiple stakeholder Spinach Field Days with over 150 attendees for each meeting. The Spinach Field Days were held on October 9th, 2019 at Salinas, CA; held on February 20, 2019, February 26, 2020, and March 15, 2021 at Yuma, AZ; and at University of Massachusetts Amherst, UMass Extension on Jan 28, 2021

(<https://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=31421>;

<https://desertagsolutions.org/events/377-spinach-field-day>;

<https://desertagsolutions.org/events/427-2020-spinach-field-day>;

<https://extension.arizona.edu/2021-downy-mildew-field-day>;

<https://ag.umass.edu/vegetable/events/field-day-winter-greens-diseases-variety-trials>).

### 2. Spinach field day in Texas:

Two field days were organized by Larry Stein in the winter seasons on February 19, 2020 and February 25, 2021 at Tiro Tres Farms, Crystal City, TX with the presence of more than 50 producers and spinach industry representatives. Field days include fungicide trials against white rust and anthracnose. In addition, commercial cultivars were demonstrated to attendees. Avila and Stein gave talks about spinach white rust evaluation and fungicide tests to local producers and spinach industry representatives in the field days. Avila talked about white rust evaluations, research objectives and potential benefits expected resistant varieties receiving a positive feedback and interest from producers (Avila 2020b). In addition, results were presented including white rust disease evaluation trials on commercial and USDA accessions, *Stemphylium* evaluation, and commercial cultivar trials. A booklet having evaluation results were given to attendees and sent by email to producers and stakeholders that were not able to attend to the field days.

### 3. Extension and outreach for Fusarium wilt:

Several talks by Lindsey du Toit and her lab members were presented at the Western Washington Seed Workshop held at the Washington State University (WSU) Mount Vernon Northwestern Washington Research and Extension Center (NWREC) on Jan. 11, 2019 and Jan. 10, 2020; and in the WSU Mount Vernon NWREC Field Day on July 11, 2019, March 2, 2020, 2021. The information was shared with representatives of all the seed companies that contract with growers in western WA to produce seed crops of these vegetables, as well as seed growers, students, researchers, extension specialists, and public.

### 4. Extension and outreach for white rust:

Carlos Avila and Larry Stein have built two new white rust screening plots (nursery) at the Rio Grande Valley and in Wintergarden area of TX and spinach was planted during 2018-2019, 2019-2020, 2020-2021 winter seasons. The new white evaluation nursery allowed to screen more spinach lines at multiple locations. Outreach included spinach grower/industry meetings to discuss the various spinach white rust and other disease challenges being faced in the Rio Grande Valley and Wintergarden region of TX including *Stemphylium* leaf spot and Anthracnose in spinach production.

### 5. Spinach extension and outreach activities in Larry Stein's lab:

Dr. Stein traveled to California and Washington State on July 7 to 11, 2019 to examine growing practices and seed production fields; worked with Peterson Brother's Nursery in San Antonio to prepare a leafy greens display for the HEB produce convention for their produce buyers; extension was instrumental in making this happen during an odd time of the year to try to grow leafy greens; and plants delivered 31 July 2019; held a series of 7 am breakfast meetings with producers to map out strategies for the upcoming growing season on July 22, August 6, and September 2, 16, and 25

in 2019; and hosted several spinach field days in cooperation with Tiro Tres Farms and the Wintergarden Spinach Producers Board and conducted white rust screening for the various companies in the winter seasons since 2016.

6. Spinach breeding and California Leafy Greens Research Program: Beiquan Mou attended and presented spinach breeding and production ten-times from 2017 to 2022 (Mou, 2020, 2019a, b, c, d, 2018a, b, 2017a, b, c).
7. Economic estimation: Robert Hogan has been working on review trends and seasonality associated with fresh market spinach in 2018 and spinach varieties to be planted in years 2019 and 2020.
8. International Spinach Conference:  
Jim Correll has hosted several meeting of International Spinach Conferences and Field Day (<https://spinach.uark.edu/meetings-2/>). The recent one was hold on February, 2018 on Murcia, Spain and several members at PIs' labs gave presentations in the meeting (<https://spinach.uark.edu/spain-presentations/>). Due to COVID-19, the one planned in 2020 was cancelled and the next one will be in Feb/March 2022 in Belle Glade, FL.
9. So far, 66 spinach lines have been released to Bowery Farming; 20 lines to 80 Acres Farms, and 30 lines to Clemson University for spinach production testing.

## VII. Training and professional development

1. A course Hort6033 "Molecular Plant Breeding (Genetic Techniques in Plant Breeding)" has been developed for training students and scientists in molecular breeding at University of Arkansas, Fayetteville and it has been opened the class in Fall 2017 and Fall 2019 and taught by Ainong Shi.
2. Sanjaya Gyawali, the postdoc in Toit's lab organized a workshop and taught the population structure and genome wide association studies (GWAS) on 23-24 May 2019 at the WSU Mount Vernon NWREC, where there were ten participants and three observers, including graduate students and professional staff from WSU in the workshop.
3. Gyawali participated a workshop as the main instructor to teach the population structure and genome wide association studies (GWAS) from 17-22 June 2019 at the International Center for Agricultural Research in Dry Areas (ICARDA), Rabat, Morocco, where there were nineteen participants, including graduate students (PhD and MS), and research scientists (pathologists, breeders, and gene bank managers) from India, Ghana, Rwanda, Tunisia, and Morocco.
4. Dr. Avila was invited as a panelist in a workshop organized by the Vegetable Breeding interest group of the American Society for Horticultural Sciences (ASHS) during the annual meeting held on August 11<sup>th</sup>, 2020. Avila gave a presentation to attendees (online audience) about current efforts in his program to develop and utilize high-throughput phenotyping in vegetable breeding.
5. Five postdoctoral research associates have been working on this project: one at Shi's lab, one at Correll's lab, one at Avila's lab, one at Mou's lab and one at du Toit's lab. Two PhD students and one MS student Research Assistant were graduated from both Shi's and Correll's labs.

## VIII. PUBLICATION (article, abstract/presentation)

**Refereed Article** (underline for the PI and Co-PI) (\*corresponding author):

1. Awika, H.O., A.K. Mishra, H. Gill, J. DiPiazza, C.A. Avila, and V. Joshi\*. 2021a. Selection of Nitrogen Responsive Root Architectural Traits in Spinach Using Machine Learning and Genetic

- Correlations. *Scientific Reports* 11:9536. <https://doi.org/10.1038/s41598-021-87870-z>
2. Awika, H.O., J. Solorzano, U.C. Rivera, A. Shi, J. Enciso, and C.A. Avila\*. 2021b. Prediction modeling for yield and water-use efficiency in spinach using remote sensing via an unmanned aerial system. *Smart Agriculture Technology* 1 (2021) 100006. <https://doi.org/10.1016/j.atech.2021.100006>
  3. Awika, H, Cochran, V. Joshi, R. Bedre, K.K. Mandadi, C.A. Avila\*. 2020a. Single-marker and haplotype-based association analysis of anthracnose (*Colletotrichum dematium*) resistance in Spinach (*Spinacia oleracea*). *Plant Breeding* 139:402-418. <https://doi.org/10.1111/pbr.12773>
  4. Awika, H, R. Bedre, J. Yeom, T.G. Marconi, J. Enciso, K.K. Mandadi, J. Jung, C.A. Avila\*. 2019a. Developing Growth-Associated Molecular Markers via High-Throughput Phenotyping in Spinach. *The Plant Genome* 12(3):1-19. <https://doi.org/10.3835/plantgenome2019.03.0027>
  5. Awika, H.O., T.G. Marconi, R. Bedre, K.K. Mandadi, C.A. Avila\*. 2019b. Minor Alleles are Associated with White Rust Susceptibility in Spinach. *Horticulture Research* 6:129. <https://doi.org/10.1038/s41438-019-0214-7>
  6. Batson, A.\*, Spawton, K, Katz, R., and du Toit, L.J. 2022. Cladosporium leaf spot, caused by *Cladosporium variabile*, in winter high tunnel production of spinach (*Spinacia oleracea*) in Maine, United States. *Plant Disease*: in press. PDIS-11-21-2424-PDN.R1.
  7. Batson, A.M.\*, Fokkens, L., Rep, M., and du Toit, L.J. 2021. Putative effector genes distinguish two pathogenicity groups of *Fusarium oxysporum* f. sp. *spinaciae*. *Molecular Plant-Microbe Interactions* 34:141-156. <https://doi.org/10.1094/MPMI-06-20-0145-R>
  8. Bhattarai, G.\*, A. Shi\*, B. Mou\*, and J. Correll\*. 2022. Resequencing worldwide spinach germplasm identifies downy mildew field tolerance QTLs and genomic prediction tools. *Horticulture Research*, Published: 13 September 2022, uhac205, <https://doi.org/10.1093/hr/uhac205>; <https://academic.oup.com/hr/advance-article/doi/10.1093/hr/uhac205/6696976>
  9. Bhattarai, G., D. Olaoye, B. Mou\*, J. C. Correll\*, A. Shi\*. 2022. Mapping and selection of downy mildew resistance locus RPF3 in spinach by low coverage whole genome sequencing. *Frontiers in Plant Science*, PUBLISHED 06 October 2022, <https://doi.org/10.3389/fpls.2022.1012923>.
  10. Bhattarai, G., W. Yang, A. Shi\*, C. Feng, B. Dhillon, J.C. Correll\*, and B. Mou\*. 2021a. Mapping and candidate gene identification of downy mildew race 16 resistance in spinach. *BMC Genomics* 22-478. <https://doi.org/10.1186/s12864-021-07788-8>
  11. Bhattarai, G., A. Shi\*, D.R. Kandel, N. Solís-Gracia, J.A. da Silva, and C.A. Avila\*. 2021b. Genome-wide simple sequence repeats (SSR) markers discovered from whole-genome sequence comparisons of multiple spinach accessions. *Scientific Reports*, 11, Article number: 9999. <https://doi.org/10.1038/s41598-021-89473-0>
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  14. Bhattarai, G., C. Feng, B. Dhillon, A. Shi, M. Villarroel-Zeballos, J.C. Correll\*. 2020b. Detached leaf inoculation assay for evaluating resistance to the spinach downy mildew pathogen. *European Journal of Plant Pathology*, 158:511–520. <https://doi.org/10.1007/s10658-020-02096-5>
  15. Bhattarai, G. 2019a. Genetic resistance to the downy mildew pathogen and mapping the RPF resistance loci in spinach (PhD Dissertation at Shi's and Correll's lab). Available at: <https://scholarworks.uark.edu/etd/3442>.

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17. Clark, K. J., Feng, C., Zima, H. V., Poudel-Ward, B., Slinski, S. L., Porchas, P. Klosterman; S. J., and J.C. Correll\*. 2021. Evaluation of spinach cultivars for downy mildew resistance in Yuma, AZ 2021. *Plant Disease Management Reports*. Report No. 15:V112. <https://www.plantmanagementnetwork.org/pub/trial/pdmr/volume15/abstracts/v112.asp>
18. Clark, K.J., Feng, C., Dhillon, B., Kandel, S.L., Poudel, B., Mou, B., Klosterman, S.J., Correll, J.C.\* 2020. Evaluation of spinach cultivars for downy mildew resistance in Yuma, AZ 2020. *Plant Disease Management Reports*. 14. Article V146.
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20. Correll, J.C.\*, Feng, F., Matheron, M.E., Koike, S. T. 2019b. Evaluation of spinach varieties for downy mildew resistance, Monterey County, CA, 2018. *Plant Disease Management Reports*.
21. Correll, J.C.\*, C. Feng, and B. Liu. 2017. First report of white rust (*Albugo occidentalis*) of spinach in Mexico. *Plant Disease* 101(3):511. <https://doi.org/10.1094/PDIS-06-16-0905-PDN>
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23. Dhillon B., C. Feng, G. Bhattarai, B. Poudel, M. E Matheron, and J. C. Correll\*. 2020a. Evaluation of spinach varieties for downy mildew resistance, Yuma, AZ 2019. *Plant Disease Management Reports*.
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36. Liu, B., L. Stein, K. Cochran, [L.J. du Toit](#), [C. Feng](#), and [J.C. Correll\\*](#). 2021a. Three New Fungal Leaf Spot Diseases of Spinach in the United States and the Evaluation of Fungicide Efficacy for Disease Management. *Plant Disease* 105(2):316-323. <https://doi.org/10.1094/pdis-04-20-0918-re>
37. Liu, B., [L. Stein](#), K. Cochran, [L.J. du Toit](#), [C. Feng](#), B. Dhillon, and [J.C. Correll\\*](#). 2020a. Characterization of Leaf Spot Pathogens from Several Spinach Production Areas in the United States. *Plant Disease* 104(7): 1994-2004. <https://doi.org/10.1094/pdis-11-19-2450-re>
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43. Olaoye, D. 2021. Resistance Screening and Association Analysis of Downy Mildew Resistance in Spinach (MS Thesis at [Shi's and Correll's Lab](#)), University of Arkansas.
44. Qin, J., [A. Shi\\*](#), [B. Mou\\*](#), M.A. Grusak, Y. Weng, W. Ravelombola, G. Bhattarai, L. Dong, and W. Yang. 2017. Genetic diversity and association mapping of mineral element concentrations in spinach leaves. *BMC Genomics* 18:941. <https://doi.org/10.1186/s12864-017-4297-y>.
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46. She, H., W. Qian, H. Zhang, Z. Liu, X. Wang, J. Wu, [C. Feng](#), [J.C. Correll\\*](#), and Z. Xu. 2018. Fine mapping and candidate gene screening of the downy mildew resistance gene RPF1 in spinach. *Theoretical and Applied Genetics* 2018 Dec;131(12):2529-2541. <https://doi.org/10.1007/s00122-018-3169-4>
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resistance in USDA GRIN Spinach Germplasm. Horticulture Research, Volume 9, 2022, uhac069, <https://doi.org/10.1093/hr/uhac069>.

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50. Synoground, T., Batson, A., Derie, M., Koenick, L.B., Pethybridge, S.J., and du Toit, L.J.\* 2020. First report of *Cercospora* leaf spot caused by *Cercospora chenopodii* on *Spinacia oleracea* in the USA. Plant Disease 104:976. <https://doi.org/10.1094/PDIS-09-19-1924-PDN>
51. Zia, B. 2021a. Genetic Resistance to the Downy Mildew Pathogen and Breeding towards Durable Disease Management in Spinach (PhD Dissertation at Shi's lab), University of Arkansas.

#### **SUBMISSION/PREPARED**

52. Bhattarai, G., Liu, B., Shi, A.\*, Correll, J.C\*. 2022b. Genome-wide association analysis and genomic selection of *Stemphylium vesicarium* leaf spot resistance in USDA spinach germplasm. Preparing to submit in Frontiers in Plant Science, in an internal review.
53. Gyawali, S., ... L.J. du Toit\*, et al. 2022. Identification of major QTLs associated with Fusarium wilt resistance in wild spinach, *Spinacia turkestanica* (in preparation).
54. Feng, C., K. Lamour, B.D.S, Dhillon, M.I. Villarroel-Zeballos, V.L. Castroagudin, B. Liu, B.H. Bluhm, A. Shi, A. Rojas, and J.C. Correll\*. 2022. Genetic diversity of the spinach downy mildew pathogen based on hierarchical sampling (<https://www.biorxiv.org/content/10.1101/2020.02.18.953661v1>).
55. Olaoye, D., A. Shi\*, J.C. Correll\* et al. 2022a. Resistance Characterization to the Downy Mildew Pathogen in Spinach (Preparation for Euphytica).
56. Olaoye, D., A. Shi\*, J.C. Correll\* et al. 2022b. Genome-wide association study and genomic prediction of resistance to downy mildew race 15 in spinach (preparation for Frontiers in Plant Science).
57. Zia, B., B. Dhillon, C. Feng, J.C. Correll\*, and A. Shi\*. 2022. Gene expression profiling to identify genes associated with resistance to the downy mildew pathogen *Peronospora effusa* in spinach (in preparation).

#### **Abstract/Presentation** (underline for the PI and Co-PI)

58. Avila, C.A. 2020a. High throughput phenotyping in Vegetable breeding. Vegetable Breeding Interest Group Workshop. 2020 ASHS Annual Conference, August 9 – 13 ZOOM meeting <https://ashs.confex.com/ashs/2020/meetingapp.cgi/Paper/33980>
59. Avila C.A. 2020b. Wintergarden field day (Feb 2020). Dr. Avila presented results of white rust resistance evaluation to local producers and spinach industry representatives during field day on February 19th 2020 at Tiro Tres Farms near Crystal City, TX.
60. Avila, C.A. D. Kandel, and H.O. Awika. 2019. Development of Molecular and Phenotyping Selection Tools for Spinach Breeding. American Society for Horticultural Sciences Annual Meeting, Las Vegas, NV July 21-25.
61. Avila, C.A. 2018. Texas A&M AgriLife Spinach Breeding Program: Selection tools for cultivar development. Texas A&M AgriLife Research and Extension Center. Uvalde, TX May 11th, 2018. Target audience: Wintergarden producers, extension specialists, and faculty members (Project PI).
62. Awika, H.O., J. Solorzano, U.C Rivera, A. Laredo, J. Enciso, C.A. Avila. 2020b. Using RGB and multispectral sensors to assess growth rate and water use efficiency in spinach. Annual meeting of the American Society for Horticultural Sciences (ASHS). Orlando, Florida. August, 2020 (Virtual

- Meeting). <https://ashs.confex.com/ashs/2020/meetingapp.cgi/Paper/32819>
63. Awika, H., T.G. Marconi, R. Bedre, K.K. Mandadi and C.A. Avila. 2019c. White Rust Resistance in Spinach Is Multigenic and Non-Chromosome Specific. American Society for Horticultural Sciences Annual Meeting, Las Vegas, NV July 21-25.
  64. Awika, H, T.G. Marconi, J. Enciso, J. Jung, and C.A. Avila. 2018. Development of Molecular Markers Associated to Spinach Growth Parameters. American Society for Horticultural Sciences Annual Meeting, Washington D.C. July 31st to August, 3rd 2018.
  65. Batson, A., Gyawali, S., and du Toit, L. 2020. Evaluation of spinach and Beta vulgaris cultivars for differential susceptibility to two pathogenicity groups of *Fusarium oxysporum* f. sp. *spinaciae*. Phytopathology 110: In press. Abstract of poster presented at Plant Health 2020 Online, the Annual Meeting of American Phytopathological Society, 10-14 Aug. 2020.
  66. Bhattarai, G., A. Shi, B. Mou and J.C. Correll. 2022. Molecular Breeding Effort Using Genome Resequencing in Spinach for Resistance to Economically Important Pathogens. 2022 ASHS annual conference July 30 – August 3, Chicago, IL. <https://ashs.confex.com/ashs/2022/meetingapp.cgi/Paper/37526>
  67. Bhattarai, G., and A. Shi. 2021d. Comparative Transcriptome and Proteome Analysis of Resistant and Susceptible Spinach in Response to Downy Mildew Pathogen. 2021 ASHS International Conference, August 5-9, Denver, Colorado. <https://ashs.confex.com/ashs/2021/meetingapp.cgi/Paper/35991>
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  69. Bhattarai, G., Shi, A., and Correll, J. C. 2020d. Identification of genomic regions associated with bolting and flowering time in spinach. *HortScience* 55, S108. (Abstr.). Available at: <https://ashs.confex.com/ashs/2020/meetingapp.cgi/Paper/33055>.
  70. Bhattarai, G., Shi, A., Avila, C. A., and Stein, L. A. 2020e. GWAS on USDA Spinach Germplasm Evaluated for Downy Mildew in the Texas' Wintergarden Production Area. *HortScience* 55, S227. (Abstr.). Available at: <https://ashs.confex.com/ashs/2020/meetingapp.cgi/Paper/33070>.
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  72. Bhattarai, G., A. Shi, J.C. Correll, and B. Mou. 2019b. Mapping Resistance to Race of Downy Mildew in Multiple Spinach Populations. 2019 ASA-CSSA-SSSA International Annual Meeting, Nov. 10-13, San Antonio, Texas. <https://scisoc.confex.com/scisoc/2019am/meetingapp.cgi/Paper/122853>
  73. Bhattarai, G., C. Feng, B. Dhillon, A. Shi, and J.C. Correll. 2019c. Evaluation of a Detached Leaf Inoculation Method to Screen for Resistance to Downy Mildew in Spinach. ASHS 2019 Annual Conference. July 21-25, Las Vegas Nevada. <https://ashs.confex.com/ashs/2019/meetingapp.cgi/Paper/30872>.
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