

C-REEMS Grant Proposal Number: **2023-05687**

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Grant Title: **Development of germplasm resources and molecular breeding tools to combat endemic and emerging diseases in US spinach production**

Non-Technical Summary

Spinach (*Spinacia oleracea* L.) production is threatened by several diseases that individually or together severely reduce yield and quality. The most effective approach to improving productivity, marketability, and sustainability in spinach cultivation is by developing resistant cultivars. Traditionally, spinach breeding has relied on the time-consuming phenotypic selection that delays cultivar release. Alternatively, molecular breeding offers an advantage by accelerating the development of disease-resistant cultivars. The project builds upon the achievement of a previously awarded NIFA-SCRI project (2017-51181-26830), which focused on molecular breeding through molecular characterization of resistance to the three predominant diseases of spinach: downy mildew (DM), white rust (WR), Fusarium wilt (FW), which laid the foundation for genetic tools, including SNP markers and genetic maps. Building upon this solid groundwork of molecular breeding initiative in spinach, the current project takes on a renewed focus by concentrating on quantitative trait loci mapping, marker validation, and the strategic integration of these findings into cultivar development. This project aims to expedite the development of spinach cultivar resistance to DM, WR, FW, and two emerging leaf spot diseases, namely *Stemphylium* and anthracnose (SLS, ALS). This project aims to generate improved genome-enabled resources to introduce resistant traits into the cultivar development pipelines to effectively manage prevalent and emerging diseases in spinach. The project's long-term goal is to develop new disease-resistant cultivars by utilizing advanced genetic tools and selection methodologies delivered from this project effort that will serve as a valuable resource for spinach improvement. The breeding process will be expedited by integrating newly developed molecular breeding tools benefitting both public and private breeders and growers. The project will address disease threats and contribute to effective disease management strategies by leveraging genetics, genomics, and innovative breeding techniques. This project will address the following 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 "emerging and invasive species."

GOALS / OBJECTIVES

Spinach (*Spinacia oleracea* L.) is an economically important vegetable crop worldwide with an estimated annual value of \$11.8 billion. The US is the second-largest spinach producer globally while leading in the fresh market segment with an annual product value of around \$500 million. Over the past three decades, the increasing interest in health-conscious diets has led to a consistent demand for spinach. Despite this increase in demand and production, the commercial cultivation of spinach faces a significant threat from several diseases. These diseases, individually or in combination, reduce yield and quality. Downy mildew (DM), white rust (WR), Fusarium wilt (FW), *Stemphylium* leaf spot (SLS), and anthracnose leaf spot (ALS) are five key diseases threatening spinach production in the US and worldwide. The spinach industry seeks continued

development of improved, locally adapted germplasm and cultivars with resistance to major pathogens. Thus, the overarching objective of this project is to develop spinach germplasm with resistance to these five key pathogens and develop molecular tools for effectively introducing these traits into the breeding pipeline for cultivar development. The practical long-term goal of this proposal is to generate molecular breeding tools to expedite disease resistance breeding efforts and the continuous release of resistant spinach cultivars against endemic and emerging diseases suitable for both conventional and organic production markets that can dramatically reduce fungicide usage for disease control. To meet this goal, the project aims to develop efficient molecular breeding methods and phenotyping tools to accelerate the cultivar development process and serve the industry's needs. The specific research objectives of this proposal are to:

1. Characterize and validate molecular markers for resistance to endemic and emerging spinach diseases.

Hypothesis: Genetic diversity in spinach germplasm collections includes sources of resistance to these five diseases that can be used for cultivar development.

2. Introgress disease resistance into spinach breeding lines for cultivar development.

Hypothesis: Combining molecular and disease phenotyping approaches will expedite cultivar development in spinach.

3. Develop a spinach grower-oriented outreach program based on experiential learning and economic decision tools for disease management in spinach.

Hypothesis: Interactive and demonstrative methods can increase the adoption of management practices to reduce production risks and increase production profits.

The proposed efforts encompass identifying genomic regions that govern disease-resistant mechanisms, optimizing practical genetic tools to improve selection processes, and developing resistant spinach cultivars. In summary, this project endeavors to extend our ongoing efforts to make advances in spinach breeding, develop genomic and phenotyping tools, generate new knowledge, and expand genetic resources to expedite disease-resistant cultivar development to support sustainable and economically viable spinach production in the US.

METHODS

We will evaluate the germplasm accessions from the USDA-National Plant Germplasm System, and breeding lines from the University of Arkansas, Texas A&M AgriLife Research, USDA-Salinas breeding program, and the Netherlands spinach germplasm collection, as well as commercial cultivars and wild relatives (*S. turkestanica*) for the resistance to all five diseases included in this project. Further, F₂ segregating populations have been and will be developed from F₁ sister-plants crossed between dioecious male and female spinach plants, and BC₁F₂ populations from susceptible parents as the male line backcrossed to all female plants of the BC₁F₁ populations. All these segregating panels will be evaluated for disease resistance.

Phenotyping: Disease evaluation will be conducted in greenhouse and field experiments. (1) DM evaluations: Resistance will be evaluated under field conditions in three locations, Salinas, CA, Yuma, AZ, and Crystal City, TX in winter seasons from 2024 to 2027. We will also test spinach genotypes in growth chamber/greenhouse conditions using DM races Pfs 5, Pfs 13, and Pfs 16, respectively, as we did in our previous SCRI project to identify major QTLs for race-specific DM resistance. After combining field and growth chamber/greenhouse evaluations, we

will identify both major genes/alleles and minor QTLs for DM resistance.

(2) WR evaluation: The evaluation of white rust resistance will be evaluated at White Rust nursery at Crystal City, TX where heavy disease pressure has consistently been observed for ~10 years in winter seasons.

(3) FW evaluation: Fusarium wilt resistance screening will be carried out in the greenhouses at the Washington State University (WSU) Mount Vernon NWREC. The protocol used in the spinach Fusarium wilt soil bioassay and the parent line screening developed by du Toit's program will be used to quantify the level of Fusarium wilt resistance.

(4) SLS greenhouse evaluation: The spinach germplasm panel will be evaluated in a RCBD with three replications, each containing 5-8 plants per replication. Plants will be inoculated with an isolate of *S. vesicarium* obtained from infected spinach plants in Arizona, using our established inoculation and phenotyping methods as described in previous studies (Liu et al., 2021). After inoculation, plants will be incubated in a humidity chamber maintained at 100% relative humidity for 72 h and transferred to a greenhouse with a night/day cycle of 14°C/27°C. Disease incidence (%) and severity will be evaluated three weeks after inoculation on a scale of 0-100.

(5) Field evaluations for SLS and ALS: The 600 spinach genotypes will be evaluated for both SLS and ALS in field experiments at Yuma, AZ and Crystal City, TX in the winters in 2023-24 and 2024-25. The experimental design will be a RCBD with three replications. Disease severity and incidence will be recorded.

Genotyping: A total of 480 spinach consisting of more than 400 USDA accessions and commercial cultivars have been genotyped with whole genome re-sequencing (WGR) at 10x coverage. Other remaining accessions will be genotyped using WGR and genotyping by sequencing (GBS).

Genetic diversity: A model-based clustering method in the program STRUCTURE 2.3.4 (Pritchard et al., 2000) will be used to infer the population structure of the spinach genotypes based on SNPs generated from WGR. Genetic diversity will be assessed and the phylogeny trees will be drawn using MEGA 7 (Kumar et al., 2016).

QTL mapping: Genetic maps for each F₂ population and BC1F₂ will be created using JoinMap 5 and QTL mapping will be done using QTL IciMapping.

Association analysis: The phenotypic data and WGR based SNP genotypic dataset will be used to perform GWAS, using GLM, MLM, SUPER, FarmCPU, and BLINK models in GAPIT 3 (Wang and Zhang, 2020) and TASSEL 5 program (Bradbury et al., 2007). More Pairwise linkage disequilibrium (LD) between SNPs will be calculated using the squared allele-frequency correlations (r^2) in TASSEL 5. The LD plot (r^2) and haplotype blocks will be drawn for each chromosome and trait-associated regions using Haploview (Barrett et al., 2005).

SNP marker validation: The significantly associated SNP markers with major effects on phenotyping identified from multiple greenhouse and field experiments will be validated via KASP SNP genotyping.

Genomic prediction: Using the 600 spinach panels phenotyped at three locations in two growing seasons, BLUP methods (RR-BLUP, gBLUP, and cBLUP) and Bayesian methods (BayesA, BayesB, and

Bayes LASSO) will be used to predict genomic estimated breeding value (GEBV). Genomic prediction (GP) accuracy will be estimated using a 5-fold cross-prediction study for 100 runs and 1-fold to 10-fold studies will also be tested to select the optimized prediction models, as we did in previous studies (Bhattarai et al., 2022a; Shi et al., 2022).

Marker assisted and genomic selection: Molecular markers linked to resistance genes/QTL will be used to select resistant plants from segregating populations. For this, the top 5 resistant lines identified in **Objective 1** for each of the five diseases (DM, WR, FW, SLS, and ALS) will be crossed with our elite spinach lines individually and in groups to generate resistant line-specific populations and a mixed population for each disease. Before implementation, a validation test will be conducted to select the top SNP markers as a marker-assisted selection (MAS) set for resistance to each pathogen of the five diseases. After validation, the top 1-3 SNP markers showing polymorphism among parents will be used in each specific population to select targeted plants without phenotyping through MAS. For genomic selection (GS), the GEBV of each disease resistance trait will be estimated based on associated SNP markers for each disease resistance and validated using testing populations. We will perform MAS and GS with 1,000 samples of DM and WR and 600 samples of each of FW, SLS, and ALS. The top 5% with resistance will be selected for each disease.

Selection for multiple disease resistance: Stacking two to three multiple disease resistance will be done according to regional needs by each participating breeding program in California, Texas, Arkansas, and Washington by utilizing conventional and molecular breeding approaches. For example, DM and leaf spots for California, while WR and leaf spots for Texas could be stacked in parallel. In this project, we plan to use 800 samples for multiple resistance screening, either DM combined (i) WR, (ii) ALS, or (iii) SLS resistance. The spinach lines resistant to the proposed disease will be planted and allowed to inter-cross in isolation chambers to generate next-generation seedlings with recombination between alleles for resistance to different pathogens. These newly developed spinach breeding lines will be tested for disease incidence and severity in greenhouses and field conditions for multiple diseases and resistant lines will be selected. Resistant lines identified from each participating breeding program will be validated at other locations. Resistance germplasm developed will be shared between participating breeding programs under a standard material transfer agreement issued by each institution.

Economic decision tools: A stochastic probabilities approach model will be utilized to estimate economic success for spinach production based on enterprise budget and US target markets.

Field demonstrations: Field trials will be established in growers' fields in CA, AZ, TX, AR, MA, and WA to evaluate developed germplasm under diverse management practices.

TARGET AUDIENCES

Our team of researchers and extension specialists is committed to delivering improved spinach breeding lines and cultivars with greater resistance to key diseases (medium- to long-term goal) to stakeholders to ensure a reliable supply of this healthy vegetable. Our systematic approach and objectives are based on consumer and economic surveys and feedback. Results from the project will be incorporated into comprehensive extension programs, including field days at regional Research and Extension Centers, on-farm demonstrations, workshops, and presentations at the International Spinach Conferences, to encourage the adoption of improved cultivars. Spinach Field Days have been held every winter in Salinas, CA, Yuma, AZ, the Rio Grande Valley and Wintergarden area of TX, Mount Vernon, WA, and the Northeastern USA. In addition, peer-reviewed publications, bulletins, and stories resulting from this research will be communicated through Texas A&M AgriLife Today, Seed World and social media. Prior to the release of improved genotypes, we will conduct extensive, replicated trials using cultivars developed by the participating breeders. Elite inbred lines/experimental hybrids/improved lines selected through on-field evaluations with participating stakeholders will be made available to seed companies or public breeding programs under material transfer agreements (MTAs) to ensure their rapid availability to growers. Each institution involved in this project will utilize their standard license agreements with public breeding programs and seed companies.

Target audience:

- Stakeholders: Stakeholders will be engaged in project development and evaluation through the duration of the project and they have a strong interest to invest in domestic production of leafy greens, including spinach. We will translate our results into useable information for stakeholders.
- Seed Company: New cultivars can be licensed to companies for increasing seeds and then sold to growers.
- Producers and growers: New spinach cultivars can be used directly by producers and growers. Our extension program will benefit producers and growers by establishing demonstration plots and from the field day organized at each participating location.
- Spinach breeders: New spinach cultivars, germplasm, and new breeding lines can be used by other public and private breeders as parents to develop new elite cultivars. Breeders can use SNP markers as a tool for selecting linked to and associated with resistance to downy mildew (DM), white rust (WR), Fusarium wilt (FW), Stemphylium and anthracnose leaf spot (SLS, ALS) through marker-assisted selection (MAS) and genomic selection (GS) in breeding program.
- Scientists: Our research methodologies, new QTL and association mapping results, SNP marker discoveries, articles, abstracts, presentations, and reports are valuable for the scientific society and communities for further research and development in science and technologies.
- Students: Through the project, students will be trained in classic breeding such as crossing, generation advanced, and variety development, and in molecular breeding technology including QTL and association mapping, genome-wide association and genomic prediction, SNP discovery and genotyping, next-generation sequencing such as whole genome

sequencing (WGS), whole genome resequencing (WGR), and genotyping by sequencing (GBS), how to use bioinformatics tools in breeding program and how to molecular breeding through MAS and GS.

PRODUCTS

Our integrated team of researchers and extension specialists is committed to delivering improved spinach breeding lines to stakeholders, releasing cultivars with greater resistance to key diseases (medium- to long-term goal), and providing consumers with a reliable supply of this healthy vegetable. The Spinach Disease Resistance website (<https://spinachdb.org>) and the Portal public website (spinach.uark.edu) will be used to disseminate project research accomplishments and extension efforts to state, regional and national stakeholders, including growers, industry partners, and Master Gardener programs. We will also develop grower-oriented economic and risk decision tools for spinach production to improve producer's adoption of new cultivars and IPM practices. In addition, our outreach effort includes presentations at professional conferences, peer-reviewed publications, bulletins, social media, and webinars.

This project's short-term metrics include population development, phenotyping, and genotyping; QTL and association mapping; SNP marker identification for DM, WR, FW, ALS and SLS resistance. The medium-term metrics include the introgression of spinach lines for disease resistance, field validation of disease resistance for developed germplasm, validation of genomic predictive models, and validation of SNP markers in segregating populations and breeding lines. The long-term metrics should build a spinach breeding system combining classic and molecular breeding through MAS and GS to expedite breeding procedures and release spinach cultivars. Project metrics are listed below:

- Around 10 SNP markers will be validated to have strong associations with resistance to DM, WR, FW, respectively (Year 1-3).
- Around 10 SNP markers will be identified and validated to be strongly associated with ALS and SLS resistance, respectively (Year 1-4).
- More than five DM and WR resistant breeding lines will be developed and released by the end of this project (Year 4).
- More than five advanced breeding lines will be developed for FW, ALS and SLS resistance, respectively (Year 4).
- Around 5 breeding lines will be successfully introgressed with resistance alleles for two or more of the five pathogens (Year 4).
- 1 economic feasibility and risk analysis model for spinach production in USA (Year 3-4).
- More than 10 new articles will be written for publications (Years 1-4).
- 20 field days (One field day per region/ year) will be organized for a variety trial and to demonstrate best production practices to control DM, WR, FW, ALS and SLS in AR, AZ, CA, TX, and WA, and in Northeast Region (Year 1-4).
- 200 spinach producers and stakeholders (50 per region) trained on integrated pest management using resistant germplasm (Years 1-4)
- In addition to the direct benefits to spinach production and consumption, our project will involve training next-generation plant scientists (3 graduate students, 2 undergraduate students, and 4 post-doctoral fellow/research associates) on phenotypic selection, genotyping

by DNA sequencing, molecular marker identification and validation, QTL and association mapping, GWAS and GP, classic breeding and molecular breeding using MAS and GS.

- Furthermore, this project will establish a multi-disciplinary outreach effort involving extension personnel, educational programs, workshops, field demonstrations, and annual meetings for stakeholders. Annual field days will be organized in each cooperating state to educate growers about cultivars being developed and the new method is used to facilitate the adoption of improved cultivars.

OUTCOMES

Outcome 1: Enhance the competitiveness of specialty crops through more sustainable, diverse, and resilient specialty crop systems.

1. New or improved innovation models (biological, economic, business, management, etc.), technologies, networks, products, processes, etc. developed for specialty crop entities, including producers, processors, distributors, etc.: This project will deliver five genotyping models (one for each disease) to support molecular breeding selection will be developed for disease resistance cultivar development.
 - Ten SNP markers will be identified and validated to be strongly associated with each DM, WR, and FW resistance.
 - Ten SNP markers will be identified and validated to be strongly associated with both ALS and SLS resistance.
2. Innovations adopted: Two innovations will be adopted in spinach breeding, including genomic selection in disease breeding and combining of major resistance genes with broad QTL resistance for long-lasting broad resistance. This project will result in the development of GS and MAS tools to enhance selection efficiency in cultivar development. It is expected that upon validation and publication, developed technologies will be used as season-to-season selection tools by public breeding programs participating and non-participating in this proposal. We expect to publish ten or more peer-reviewed scientific journal articles based on the experiments proposed in this project (at least two per disease).

Outcome 2: Enhance the competitiveness of specialty crops through greater capacity of sustainable practices of specialty crop production resulting in increased yield, reduced inputs, increased efficiency, increased economic return, and/or conservation of resources.

1. Plant/seed releases (i.e., cultivars, drought-tolerant plants, organic, enhanced nutritional composition, etc.): breeding lines (medium term) and cultivars (long term) will be developed and released. Developed and characterized breeding lines and accessions will be released for utilization in breeding programs upon material transfer agreement by the end of the project. Cultivar release upon recurring selection using developed tools is a continuous long-term goal.
 - Five DM and WR resistant breeding lines will be developed and released.
 - Five spinach lines will be developed for each of FW, ALS and SLS resistance.
 - Five lines with resistance to 2 or more of the five pathogens (Year 4).

2. Adoption of best practices and technologies resulting in increased yields, reduced inputs, increased efficiency, increased economic return, and conservation of resources.
 - Number of growers/producers indicating adoption of recommended practices: 10 (long term).
 - Number of growers/producers reporting reduction in pesticides, fertilizer, water used/acre: 10 (long term).
3. Upon cultivar release and dissemination of the technology, it is expected that producers adopt new disease resistance cultivars and practices to optimize disease IPM practices, reduce pesticide applications, and increase profit due to reduced risk of production.
 - One economic feasibility and risk analysis model for spinach production in US.
 - Twenty field days (One field day per region/ year) for variety trials and demonstrate best production practices to control DM, WR, FW, ALS and SLS in AR, AZ, CA, TX, and WA, and in Northeast Region.
 - Two hundred spinach producers and stakeholders (50 per region) will be trained on integrated pest management using resistant germplasm.

KEYWORDS:

Spinach, molecular breeding, disease resistance, downy mildew, white rust, Fusarium wilt, leaf spot
