STAKEHOLDER RELEVANCE STATEMENT

1. Title of the project: Development of germplasm resources and molecular breeding tools to combat endemic and emerging diseases in US spinach production

2. **Keywords**: Leafy vegetables

3. Type of project: Standard Research and Extension Project

4. Estimated amount of funding: total project funding: $6,400,000/4 years; federal funding request: $3,200,000.

5. Economic/environmental/social significance of the problem

Spinach (*Spinacia oleracea* L.) production is threatened by several diseases that individually or together, can reduce yield and quality dramatically. The most effective approach to improve spinach productivity and marketability in a sustainable manner is through genetic improvement for disease resistance. However, this process requires screening large populations over multiple breeding cycles that often result in low selection gains using traditional breeding methods. The dioecious nature of spinach with male and female reproductive organs on separate plants, further complicates that delays the release of new cultivars with improved disease resistance.

This proposal aims to expand the efforts of NIFA SCRI project # 2017-51181-26830, titled "Developing Genetic and Molecular Resources to Improve Spinach Production and Management". The previous project focused on transitioning from conventional to molecular breeding by identifying new sources of resistance and developing molecular tools mainly genomic resources, markers, and genetic maps. This current proposal focuses on quantitative trait loci (QTL) mapping, marker validation and implementation, and germplasm and cultivar development.

In the previous project, we worked on the three most important diseases in spinach production in the USA, i.e., downy mildew, white rust, and Fusarium wilt, with significant advances toward molecular marker-assisted cultivar development. Based on spinach producers' feedback, this proposal adds two emerging foliar diseases, Stemphylium leaf spot and anthracnose, which are causing increasingly significant yield losses in the main spinach production regions of the USA. A summary of specific achievements and further needs are delineated below by disease:

Downy mildew (DM): caused by the oomycete *Peronospora effusa* (syn. *P. farinosa* f. sp. *spinaciae*, *Pfs*) (Brandenberger et al. 1994; Correll et al. 2011), is the most economically important disease of spinach. To date, 19 different races of *Pfs* (Pe: 1 to Pe: 19) have been reported (Feng et al. 2018; Ribera et al. 2020; Feng et al. 2020; Smilde et al. 2021). The DM has been a major problem for spinach growers as the pathogen is able to overcome host genetic resistance, which is the primary method for managing this disease (Correll et al. 1994, 2011; Kandel et al. 2019; Morelock and Correll 2008). There are currently 13 known resistance loci in spinach and a series of resistant spinach lines with one or two of these loci have been developed (Dijkstra 2015a, b, 2016; Ribera et al., 2020) and mapped (Feng et al., 2018; Bhattarai et al., 2020, 2021, 2022; She et al., 2018). Unfortunately, a new race (Pe: 19) has emerged that can overcome the resistance in Hydrus (gene RPF11), which is resistant to races 1 to 18 and cause breakdown of R-gene (Feng et al 2020). So far, no single gene has been able to provide resistance to all known races in spinach production. As new races continue to emerge at an alarming rate (Feng et al. 2014, 2018, 2020, 2021; Ribera et al. 2020), the use of QTL for downy mildew resistance is becoming an important alternative approach to disease management. Ideally, the use of a combination of major genes and QTL in commercial cultivars and hybrids may provide the most durable resistance.

White rust (WR): caused by *Albugo occidentalis*, is an oomycete obligate pathogen that can cause severe yield losses (Correll et al 2005). In the previous SCRI project, we conducted a genome-wide association study (GWAS) to identify single nucleotide polymorphism (SNP) markers

associated with white rust resistance in spinach, and to perform genomic prediction (GP) for estimating prediction accuracy (PA). A GWAS panel of 346 USDA germplasm accessions was phenotyped for white rust resistance under field conditions and GWAS was performed using 13,235 whole-genome resequencing (WGR) generated SNPs. Nine SNPs located on chromosomes 2, 3, 4, and 6 were associated with white rust resistance in this GWAS panel. Four scenarios were tested for PA using Pearson's correlation coefficient (r) between the genomic estimation breeding value (GEBV) and the observed values: (1) different ratios between the training set and testing set, (2) different GP models, (3) different SNP numbers in three different SNP sets, and (4) the use of GWAS-derived significant SNP markers. The results indicated that a 2- to 10-fold difference in the various GP models had similar, although not identical, average r values in each SNP set; using GWAS-derived significant SNP markers will increase PA with a high r-value of up to 0.84. The SNP markers and the high PA can provide valuable information for breeders to improve spinach using marker-assisted selection (MAS) and genomic selection (GS). Furthermore, Avila's lab (Co-PI) identified 448 minor alleles associated with white rust susceptibility in spinach (Awika et al. 2019). These results indicated that susceptibility might be highly determined by a disproportionate over-representation of minor alleles. Validation is required to implement the identified markers in spinach breeding programs.

Fusarium wilt (FW): caused by *Fusarium oxysporum* f. sp. *spinaciae*, is a major disease of vegetative spinach production and spinach seed crops in some regions in USA (Correll et al. 1994; Horinouchi et al. 2010). It has become increasingly important issue in California and Washington. Unfortunately, no major genes for resistance to FW have been identified (Laguna 2000; Gyawali et al. 2019), however, multigenic resistance has been identified by duToit's and Correll's labs (Co-PIs). As a result, several populations of FW resistant x susceptible accessions have been developed. QTL mapping and GWAS have been conducted to map FW resistance and identify SNP markers (Gyawali et al. 2019). The genomic resources available for both the host (spinach) and the pathogen (*F. oxysporum* f. sp. *Spinaciae*) can be used for mapping host resistance, pathogen virulence genes, and host-pathogen interactions using QTL. In a previous SCRI study, a panel of 351 USDA spinach accessions was inoculated with a mixture of three Fos isolates (Fus058, Fus254, and Fus322) representing two races of the fungus, resulting in a discovery of 46 resistance QTL on six chromosomes with R^2 ranging from 3.5-9.3% following GWAS (Gyawali et al. 2019). In addition, we identified novel FW resistance sources in wild spinach species *Spinacia turkestanica*, a wild spinach relative, with *75* accessions phenotyped for FW resistance and genotyped with genotyping-by-sequencing (GBS). The GWAS revealed major QTL on chromosomes 4, 5, and 6 and 33-associated SNP markers for FW resistance. The PA (r-value) was >0.6 based on six GP models (Gyawali et al. 2021). These resistance QTL/genes identified in wild spinach *S. turkestanica* need to be introgressed into cultivated spinach to benefit from these novel resistance sources.

Stemphylium leaf spot (SLS): is caused by both *Stemphylium vesicarium* and *S. beticola* (= *S. botryosum; S. botryosum* f. sp. *spinacia*) (Liu et al. 2020). A recent survey has shown that SLS is the predominant leaf spot in the USA in Arizona, California, Florida, South Carolina, and Texas (Liu et al. 2020). Previous research has identified eight SNP markers associated with resistance to SLS by screening USDA collection and commercial cultivars with a *S. botryosum* isolate(Mou et al. 2008; Shi et al. 2016). More recently, Correll's and Shi's labs evaluated 316 USDA spinach accessions and 30 commercial cultivars for SLS resistance in a greenhouse using a *S. vesicarium* isolate collected from Yuma, AZ that showed a significant differences in disease severity among the genotypes tested. A GWAS analysis using disease scores and whole genome resequencing generated SNP markers identified 42 SNP markers significantly associated with SLS resistance (Liu et al. 2020, 2021;

Bhattarai et al. 2022). It will be necessary to evaluate SLS at multiple locations and in multiple seasons to select germplasm with resistance and to identify molecular markers associated with resistance to both species that cause SLS, *S. vesicarium* and *S. beticola*.

Anthracnose leaf spot (ALS): caused by *Colletotrichum dematium*, is another important emerging disease in spinach. Currently, no cultivars are resistant to ALS, and fungicides are ineffective against this pathogen. As a result, there have been increasing outbreaks of ALS in spinach-producing areas in the USA, leading to up to 100% yield loss in the Texas Wintergarden region. In preliminary work, Avila's group (Co-PI) screened a collection of 276 diverse spinach accessions for resistance to anthracnose, with only 5 (2%) being classified as highly resistant and most (61%) being susceptible(Awika et al 2019b). Furthermore, A GWAS analysis identified 49 significantly associated markers for resistance distributed across several spinach chromosomes. The results suggest that resistance to ALS is polygenic. Additional testing is required to validate and use these molecular markers in breeding programs.

6. Potential economic/environmental/social benefit to solving the problem addressed

Developing disease resistant spinach cultivars will reduce losses and production risks, save on farm insurance costs, and potentially increase the acreage of spinach farmed.
Economic impact: About 99% of harvested acres of spinach in the USA in 2018 were in California, Arizona, Texas, and New Jersey. Acreage has been increasing ~8% per year over the last seven years, with 61,450 acres valued at $422.9 M in 2018. California leads the US market with 75% of sales, of which 35-55% is organic (USDA-NASS 2019). Western Oregon and Washington produce 99% of spinach seed crops in the USA, and ~20% of the world's supply of spinach seeds. Spinach producers (>300 in the USA, with >130 in California and >50 in Texas), food processors involved in fresh and processed spinach production (~100 in the USA), spinach seed growers, seed companies, and millions of spinach consumers in the USA will benefit from the development of disease-resistant, high-quality spinach cultivars.
Environmental impact: The use of more resistant spinach cultivars will reduce the amount of fungicide applications, and reduce associated potential effects of fungicides on soil, water, and the environment as well as farm workers and consumers.
Social benefit: This project will contribute to a consistent supply of high quality, healthy spinach for consumption. Spinach is a nutrient-dense leafy green that can contribute to reduced intake of calories and fats as part of the fight against chronic diet-related diseases in the USA. Nutritional content of spinach exceeds that of lettuce, the most popular leafy green, in both vitamins and minerals. Furthermore, spinach has high levels of health-promoting compounds (e.g., lutein, zeaxanthin, ascorbic acid) that reduce oxidative stress.

7. How stakeholders were engaged in defining the problem and project objectives.

Our team at the Univ. of Arkansas has organized national and international spinach meetings approximately every 18 months since 2000, including an international meeting in Murcia, Spain in February 2018 (https://spinach.uark.edu/meetings-2) with approximately 140 participants from the spinach industry, including producers, seed industry representatives, and public sector researchers. Feedback from these meetings has indicated a need for additional breeding efforts, focused on developing resistance to emerging diseases and efficient methods for screening spinach populations.

The USDA-ARS spinach breeding team at Salinas, CA attends and presents spinach research at the semi-annual meetings and field days of the CA Leafy Greens Research Program (CLGRP), attended by >250 growers, producers, shippers, processors, researchers, breeders, seed

companies, and other industry personnel. The CLGRP help to set research priorities and provides feedback and assistance to our research activities and results.

As part of the Texas A&M University's strategic investment in vegetable production solicited input from various stakeholders in the industry, including vegetable growers, agro-industry representatives, seed company personnel, retailers, processors, and distributors, and conducted a SWOT (strengths, weaknesses, opportunities, and threats) analysis to assess the internal and external factors impacting vegetable production. The stakeholders who showed the most interest were invited to join an advisory board, which recommended incorporating resistance to emerging diseases as a priority. A project submitted by our team titled "Improving Spinach Productivity by Developing Cultivars Adapted for Conventional and Organic Production in Texas" was funded in 2017 and has provided the basis for some sections of this SCRI proposal.

The spinach research program at Washington State University, led by of du Toit, directly engages with spinach seed growers and seed industry. For the past 13 years, du Toit's program has offered a spinach soil bioassay to help seed growers quantify the risk of FW in their fields. In addition, seed companies also submit advanced inbred lines for screening each winter to determine their relative susceptibility to FW. The program regularly holds field days, open houses, and workshops to disseminate research progress and information with spinach seed growers. The Puget Sound Seed Growers' Association has funded FW research in du Toit's program for >15 years.

Winter production of spinach in the Northeastern US is an important economic component for many farm businesses. However, spinach production is often threatened by diseases, particularly DM. UMass Extension, New England Vegetable & Berry Grower's Association (NEV&BGA), and Johnny's Selected Seeds have established spinach exhibition fields and hold field days to demonstrate growers and farmers how to manage DM and other diseases in spinach, including utilizing cultivars with resistance. DM is also a severe disease in spinach growing in Yuma, AZ, which is the second largest spinach production region in the USA after Salinas, CA. In the past several years, the Univ. of Arizona in collaboration with the Univ. of Arkansas has grown new spinach cultivars, evaluated DM responses, held field days each winter, and shared new information with growers, farmers, and industry representatives. This will continue for duration of this proposed project.

8. How stakeholders will continue to be engaged in project development and evaluation.

Stakeholders will be involved in all phases of this project by serving as members of our advisory board (AB), which will meet on a semi-annual basis. The AB will include representatives from various sectors of the spinach industry, including leafy green growers, commodity groups, seed companies, and academics (see letters). The AB serves as the steering committee for the project to address stakeholders' concerns and questions and determining the deliveries of the project. The AB will provide guidance input on market trends and focus areas throughout the duration of the project, as well as refine activities as the project evolves. In addition, producers will collaborate directly with researchers and extension specialists by hosting field trials on their farms and providing direct feedback on breeding efforts. The AB will assess the potential market advantage of newly developed germplasm, facilitate the exchange of germplasm, provide directions for achieving project goals, enable on-field evaluations, and monitor the economic feasibility, sustainability and impacts of the project. Annual meetings with the AB and project personnel will summarize progress and refine objectives and activities. Presentation at bi-annual spinach meetings and professional conferences will provide opportunities to discuss progress on the project and obtain feedback. A strong outreach program, including feedback surveys, in-person

discussions, and field days, will also be implemented to ensure strong engagement with stakeholders.

9. How information developed during the project and will be translated into actionable recommendations or products and delivered to end-users.

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.

10. Experience of project director and key co-Investigators

Ainong Shi (PD), Associate Professor at the Univ. of Arkansas. Dr. Shi's breeding program is focused on molecular breeding to expedite spinach cultivar development for high yield, uniformity, slow bolting, and disease resistance. Dr. Shi has over 20 years of experience in plant breeding and genetics (over 9 years in spinach). He has also more than 15 years of experience in molecular breeding including QTL and association mapping, SNP discovery, DNA sequencing data analysis and marker-assisted selection and breeding. Dr. Shi will serve as project director.

Beiquan Mou (co-PI), Research Geneticist at USDA-ARS, Salinas, CA. Dr. Mou conducts breeding and genetic studies of lettuce and spinach for disease and insect resistance, abiotic stress tolerance, improvement of nutritional values, and horticultural traits. He has released 40 spinach and lettuce varieties and genetic stocks possessing unique traits, improved quality, new genes, and disease/insect resistance. Dr. Mou will co-direct the evaluation of spinach to DM, SLS and FW resistance in California and Arizona.

Jim Correll (co-PI), Distinguished Professor of Entomology and Plant Pathology at U. of Arkansas. Dr. Correll has 25+ years of experience in spinach disease research, particularly on pathogen race and host resistance. Dr. Correll works closely with the spinach industry in California/Arizona through both the California Leafy Greens Research Program and the California Seed Association, and he organizes and coordinates the International Spinach Conference. Dr. Correll will co-direct phenotyping for DM, WR, SLS, and FW resistance.

Carlos Avila (co-PI), Associate Professor of Vegetable Breeding at Texas A&M AgriLife. Dr. Avila will direct Texas research efforts on WR and ALS and high throughput phenotyping. Dr. Avila has over 14 years of experience in conventional and molecular breeding (8 years in spinach). His current research program focuses on the development of disease resistant-high-quality spinach cultivars adapted to Texas conditions. His work also involves breeding for biotic and abiotic stress tolerance, post-harvest shelf-life and defensive signaling.

Lindsey du Toit (co-PI), Alfred Christianson Distinguished Professor and Extension Plant Pathologist, Washington State University. Dr. du Toit's program is focused on the etiology, biology and management of diseases that affect vegetable seed crops grown in the Pacific Northwest USA, including spinach seed crops. Dr. du Toit will lead FW resistance efforts. Her program has worked on spinach diseases, including many seed-borne pathogens, for >22 years.
Larry Stein (co-PI), Regents Fellow, Associate Department Head, Professor and Extension Horticulturist, Dept. of Horticultural Sciences, Texas A&M with >30 years in spinach research. Dr. Stein's area of research has been on the control of spinach diseases and the screening of new varieties for tolerance to diseases. Dr. Stein will lead extension efforts for on field demonstrations of the screening of spinach varieties for resistance to various diseases.

11. List of project objectives with corresponding hypotheses to be tested.

i. Characterize and validate molecular markers for resistance to endemic and emerging spinach diseases. ***Hypothesis:*** *Genetic diversity in spinach germplasm includes sources of resistance to various diseases that can be used for developing resistant cultivars.*

ii. Introgress disease resistance into elite spinach breeding lines for cultivar development. ***Hypothesis:*** *Combining conventional and molecular and high-throughput phenotyping approaches will expedite cultivar development in spinach.*

iii. 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 adoption of management practices to reduce production risks and increase growers' profits.*

12. Outline of methods to be used to achieve project objectives

-Plant Material: USDA-National Plant Germplasm System, University of Arkansas, Texas A&M University, USDA-Salinas breeding program, and the Netherlands spinach germplasm collection, as well as commercial cultivars and wild relatives (*S. turkestanica*).

-Population construction: F_2 segregating populations were developed from F_1 sister-plants crossed between dioecious male and female spinach plants, and BC_1F_2 populations from susceptible parents as the male line backcrossed to all female plants of the BC_1F_1 populations.

-Phenotyping: Disease evaluation will be conducted in greenhouse and field experiments.

-Genotyping: SNP genotyping will use whole genome re-sequencing and GBS.

-Association analysis: GWAS conducted with TASSEL 5, GAPIT R and ATOMM packages.

-QTL mapping: Genetic maps for each F_2 population will be created using JoinMap 5 and QTL mapping will be done using QTL IciMapping.

-Genomic prediction: BLUP methods (RR-BLUP, gBLUP, and cBLUP) and Bayesian methods (BayesA, BayesB, and Bayes LASSO) will be used to predict genomic estimated breeding value.

-Marker assisted and genomic selection: Molecular markers linked to resistance genes/QTL will be used to select resistant plants from segregating populations. The predicted breeding values of disease resistance based on SNP markers, will be validated using training populations.

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.

13. Appendix_A._Logic_model_chart.

Situation: Spinach production in the USA accounts for over 550,000 million tons valued at more than $3300 million/year with an annual growth of ~35K acres for fresh consumption and 9K for processed market during the last decade. In order to sustain spinach production, it is necessary to develop regionally adapted resistant varieties with enhanced resistance to crop devastating diseases to improve production, to increase producer's profit and to satisfy consumers' demand.

Inputs

-USDA research funds invested in the project

-Stakeholder advisory feedback (producers,, seed industry, retailers)

-Interdisciplinary, Multistate research and extension team

-Breeding germplasm from public (U. of Ark, TAMU, WSU, USDA) and private sectors

Activities

-Multi-state/year screening for disease resistance using breeding lines and USDA accessions
-Development of conventional and molecular breeding tools to speed up cultivar development
-Introgression of resistance trait into elite breeding lines
-Development of grower-oriented outreach learning economic and agronomic decision tools

Outputs

-New improved genotypes with high resistance to spinach diseases (DM, WR, FW, Leaf spot)

-New breeding tools to optimize and speed up cultivar development (Markers, genomic selection)

-Socio-economic benefits due to high-quality and yield of resistant cultivars

-Increased grower's profitability by reduced inputs and losses for optimum production

-Publications for disseminating research findings to stakeholders to continuing efforts in disease resistance.

-Partnerships with stakeholders to meet consumer demand for nutrient-dense high-quality spinach that exceeds standards.

Impacts

SHORT-TERM

-Validated screening methods for the development of disease resistant spinach cultivars

-Identification and characterization of sources of resistance for cultivar development.

-Spinach breeding lines carrying resistance traits adapted to US production regions.

-Understanding of the positive impact of sustainable business model and profitability for stakeholders.

MEDIUM-TERM

-Reduced use and dependency on pesticide applications

-Increase crop performance and yield protection due to resistance cultivars

-Increased profit and reduced spinach penalties due to low quality

-Documented molecular markers, QTLs, and genomic selection tools for optimized cultivar development

LONG-TERM

Growers, processors, retailers:
-Sustainable spinach production
-Improved quality and productivity

Consumers:
-Improved nutrition and health benefits by consuming nutrient dense spinach
-Continuous high-quality US locally grown spinach

Agricultural industry:
-Reduced dependency on pesticide applications
-Reduced yield and quality loss due to disease incidence

Situation

Increased disease pressure resulting in:
-Low yield
-Reduced quality
-Low profit
-Reduced supply

Participation

-Research scientist, technicians, graduate & undergraduate students

-Extension faculty

-Advisory board & Leafy green commodity groups

-Seed companies

EVALUATION

-Number of identified and improved selections and breeding lines with disease resistance traits for sustainable production.
-Number of selection tools (e.g., molecular markers and genomic selection protocols) for optimized cultivar development
-Economic, social, and environmental benefits
-Public-private partnerships for sustainable production and commercialization of developed germplasm.

Assumptions: a) Spinach genetic diversity can be exploited for genetic improvement of spinach, b) Gain in selection can be improved by using molecular and phenotypic tools **External factors:** a) US policies (e.g., Farm Bill, etc.), b) grower acceptance and willingness to innovate and adapt science-based strategies.

14. Appendix_B._Industry_stakeholder_support_letters.
See attached collaboration letters including: (1) BASF, (2a) Seminis, (2b) Bayer, (3) California Leafy Greens Research Program, (4) CA Seed Association, (5) Johnny's Selected Seeds, (6) JV Farms, (7) Naktuinbouw, (8) New England Vegetable & Berry Grower's Association, (9) Pop Vriend, (10) Puget Sound Seed Growers' Association and Western Washington Small Seed Advisory Comm., (11) Syngenta, (12) Takii & Co., (13) Texas Wintergarden Spinach Producers Board, (14) True Organic Products Inc, (15) Vilmorin, (16) Bejo, (17) DeSeed, (18) Enza Zaden, (19) Rijk Zwaan, (20) Sakata, and (21) United Vegetable Growers Cooperative.