Title
Exploring Root Architecture as a Defense against Soil-Borne Pathogens

Principle investigators and cooperators
Hulbert, Scot
Garland-Campbell, Kim
Paulitz, Tim
Thompson, Alison L.
Mahoney, Aaron

Key words
Soil-borne pathogens, root lignin, quantitative trait loci

Abstract Soil-borne pathogens, particularly root-lesion nematodes (*Pratylenchus* spp.), *Fusarium* spp., and *Rhizoctonia* spp. are a major constraint to grain production in the Pacific Northwest. Yield losses associated with these pathogens range from 20-60% in infested fields. Symptoms often mimic drought or nutrient deficiencies because of plant root damage. The options available to growers to control pathogen populations that are environmentally and economically sustainable are limited. Increased populations of soil-borne pathogens are associated with reduced tillage and need to be controlled in order to expand this soil conserving farming practice. The objectives of this project were to (i) determine if root lignin is a causal factor for *Pratylenchus* resistance by evaluating lignin biosynthesis gene transcription using quantitative real-time PCR (qRT-PCR) on susceptible cultivar Louise, resistant accession PI 621458, and select lines from the recombinant inbred line (RIL) population developed by same, (ii) identify molecular markers associated with increased root lignin content and evaluate the markers for diagnostic use, and (iii) determine if increased root lignin could provide resistance to root pathogens *Fusarium* and *Rhizoctonia* by evaluating the RIL population in greenhouse and field trials for resistance to these pathogens. We were unable to completely accomplish the project directives because the postdoctoral researcher on the project left to take another permanent position. A graduate student, D. Larkin, will complete the remaining objectives by the spring of 2016.

Project description

Objective 1 Primer sequences corresponding to nine lignin biosynthesis genes in wheat were provided by Bi et al. (2011). The primers were evaluated using the recommended annealing temperatures provided by Bi et al. on six different wheat genotypes with simple PCR. The PCR products were viewed with a 2% agarose gel and SYBR safe DNA gel stain (Invitrogen, Carlsbad CA). Root tissue from 12 different genotypes (Louise, PI621458, and 10 RILs) was collected at 3 different time points in root development (2, 4, and 8 weeks). A high through-put method of RNA extraction was needed to for this experiment. Five seedlings each of Louise and PI621458 were grown under controlled conditions on 50% Murashige and Skoog (MS) growth media for two weeks. The roots were collected in 1-ml microtube strips (BioExpress, Kaysville UT) and immediately frozen in liquid nitrogen. Steel ball bearings were added to each sample and ground using a Geno/Grinder 2010 (SPEX, Metuchen NJ) with the 96-well cryo adapter. Software for a BioSprint-96 (Qiagen, Venlo Netherlands) was modified to accommodate a MagJET Plant RNA kit (ThermoScientific, Waltham MA) for 96 sample extractions. Extracted RNA using this method was evaluated for quantity and quality with a bioanalyzer (Agilent, SantaClara CA). Plant tissue was grown and collected for this objective. Completing this accomplishment has been delayed due to changes in personnel but we expect to complete it during 2015.

Objective 2 Molecular markers associated with increased root lignin content were previously identified by Thompson (2013) and obtained for use in kompetative allele specific primer (KASP) PCR (KBiosciences, Beverly MA). Reactions were performed following manufacturer instructions with DNA templates from PI621458 (increased root lignin content), and cultivars Louise, Otis, and Alpowa (lower root lignin content). The KASP marker alleles that were developed on the basis of putative QTLs were not highly associated with the root lignin concentration. The QTL is being re-mapped. A second replication of the root lignin concentration assay was accomplished in spring 2015 and a third rep will be accomplished this fall. The molecular marker map has been re-analyzed. Analysis and verification of QTLs for lignin concentration will be accomplished in 2016.

Objective 3 The RIL population was evaluated for distribution of resistance to *Fusarium culmorum* in two greenhouse experiments as described by Smiley et al. (2005). Each experiment had two known resistant and susceptible controls with five replicates for each line evaluated. One replication of the greenhouse Fusarium assay was accomplished in spring 2015 and a second replication will be conducted in Fall 2015. The population was assayed in a three replication trial in the Field and Lind WA in the summer of 2015. Root rating data will be finished by Dec. 2015.

Output

Objective 1 High throughput RNA extraction: The simple PCR analysis indicates that seven of the nine primer pairs from Bi et al. (2011) were successful and will be used in the qRT-PCR (Figure 1). Evaluation of extracted RNA with the bioanalyzer (Agilent, SantaClara CA) showed an average extraction rate of 38ng/µl from 0.5 g of tissue with an RNA integrity number of 6 (Figure 2). This indicates the high through-put method will provide high quality RNA for the qRT-PCR. The RNA extractions from the stored samples will be performed by May 2016.

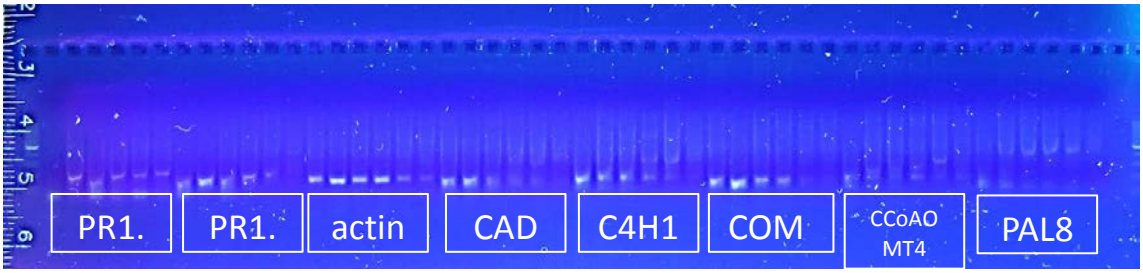

Figure 1. PCR analysis of primer sequences for lignin biosynthesis gene candidates from Bi et al, 2011 as seen on a 2% agarose gel. Abbreviations are as follows pathogenesis related (PR), actin, cinnamyl alcohol dehydrogenase (CAD), cinematic 4-hydoxlase (C4H), coffee acid/5-hydroxyferulic acid (COMT), caffeoyl CoA O-methyltransferase (CCoAOMT), phenylalanine ammonia lyase (PAL), 4-coumarate (Figure 1) CoA ligase (4CL), and ferulate 5-hydroxylase (not shown).

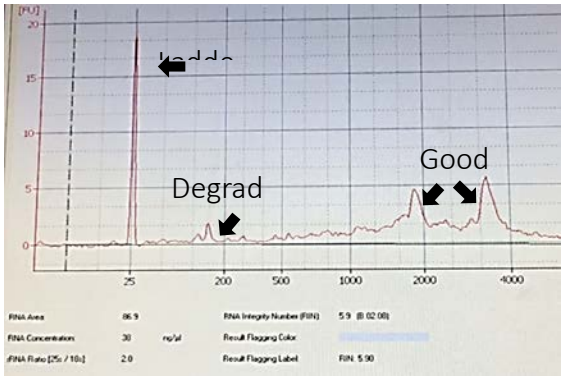

Figure 2. RNA quality and quantity of root tissue as determined by an Agilent bioanalyzer

Objective 2

The evaluation of the previously identified KASP markers associated with increased root lignin content was shown to be non-diagnostic (data not shown). The RIL population exhibited segregation distortion. The linkage maps for the RIL population were re-evaluated and new maps generated using JoinMap4 software (Kyazma, Wageningen Netherlands) (Figure 3), although segregation distortion was still evident.

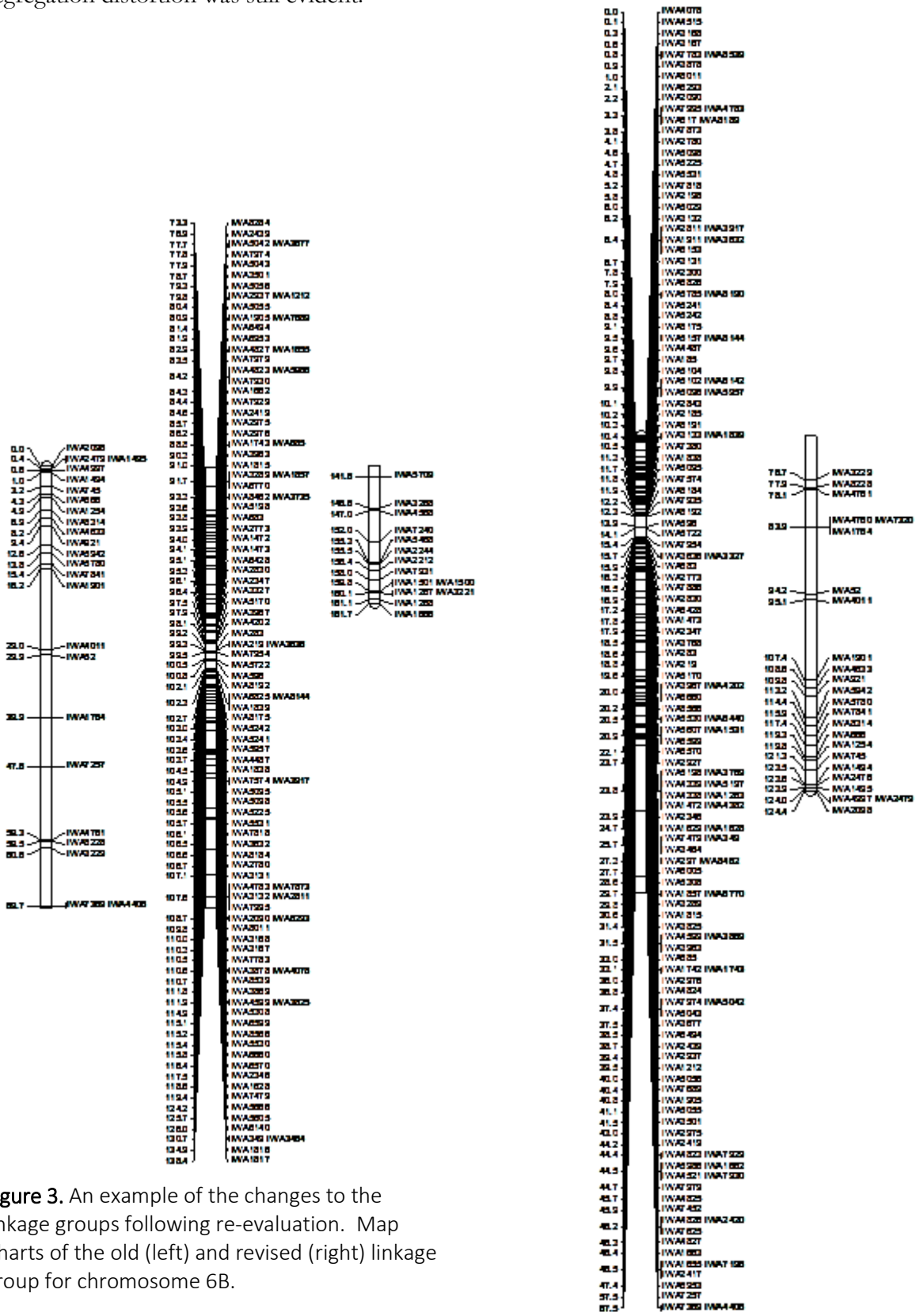

Figure 3. An example of the changes to the linkage groups following re-evaluation. Map Charts of the old (left) and revised (right) linkage group for chromosome 6B.

The RIL population is also being phenotyped again for root lignin content using the thioglycolic acid (TGA) precipitation method described by Brinkmann et al. (2002). When the phenotyping is completed the quantitative trait loci analysis will be performed again with the new linkage maps to better identify molecular markers associated with increased root lignin content

Objective 3 The evaluation of the RIL population for resistance to *Fusarium culmorum* shows resistance is segregating in this population where lower score values indicate resistance. The distribution is skewed towards resistance with average scores at 2.77 for experiment 1 and 2.13 for experiment 2 (Figure 4). The resistant controls, accession PI621458 and Sunco averaged scores of 3.1 and 3.4 respectively and susceptible Louise averaged a score of 5 (data not shown). This indicates quantitative trait loci (QTL) and associated molecular markers can be identified using this population. The analysis will be performed at the same time as the root lignin analysis to identify any overlapping QTL. Overlapping QTL are an indication that genes involved for one trait are also involved in another. In this case overlapping QTL would potentially indicate increased root lignin is involved in resistance to *Fusarium* as was indicated with resistance to *Pratylenchus*.

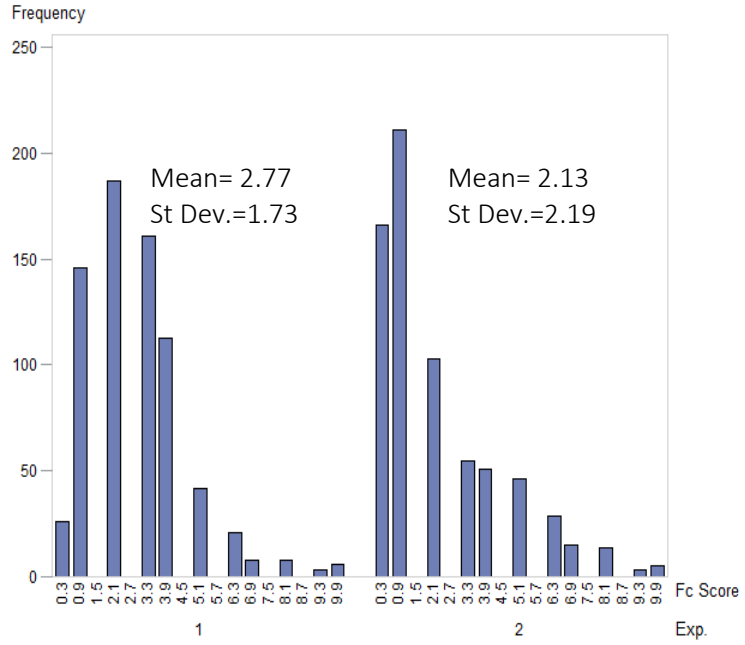

Figure 4. Distribution of *Fusarium culmorum (Fc)* resistance in the RIL population derived from Louise and PI 621458 (CWI57134, AUS28451) from two greenhouse experiments.

Additional related work assessing root disease in the Louise/AUS RILs:

Resistance screening at the Palouse Conservation farm with natural inoculum The first field trial to assess the resistance to natural inoculum was conducted at the Palouse conservation field station (PCFS), Pullman, Washington in the spring of 2014. This site has a history of natural *Rhizoctonia* inoculum. In order to keep the inoculum at high levels, bulk wheat seed was planted in the fall of 2013 and allowed to overwinter. This provided plant residue and a carbon source for the pathogen. In the spring, sixty meter passes spanning the length of the field (Fig. 1) were sprayed either at 21 days (clean treatment) or 3 days (green bridge treatment) before seeding. The 142 F_5 RILs of Louise x AUS28451 and the parents were direct-seeded into a randomized complete block designs for

which there were three replicates. Each plot consisted of four, 1-meter rows sown with five grams of each RIL or parent. Seedlings were harvested at 21 days post direct-seeding. Five plants from each row were harvested. Each seedling was measured from the crown to the longest leaf. To convert to percent stunting, the green treatment was subtracted from the clean treatment and this difference was divided by the clean treatment. This quotient was multiplied by 100 for percentage stunting ((clean-green)/clean)*100.

Resistance screening in a greenhouse trial with artificial inoculum. The greenhouse trials were conducted using the procedure described by Kim et al. (1997). Briefly, wheat seeds were surfaced sterilized in a bleach solution and planted in approximately 110g of sieved and pasteurized Palouse silt loam soil. The seeds were covered with 5 grams of soil amended with *Rhizoctonia* infested oat-kernel inoculum and watered as needed. After 14 days in a controlled growth chamber, the seedlings were scored for root damage using a 0-8 categorical scale and root and shoot fresh weight as well as shoot length were measured.

Results

Assessment of stunted seedlings associated with Rhizoctonia root disease Typical stunted seedlings, which is associated with root damage, was observed for most of the RILs and both parental lines including AUS28451 (Fig. 2). However, in the field trial, AUS28451 showed almost no stunting (0.8%) compared to its clean control treatment. This was in contrast to the greenhouse trial, where AUS28451 showed on average 5.6 percent stunting on average (Fig. 2.). Louise showed significant damage in both the field and greenhouse trials with an average of 18.5 and 22.8 percent, respectively. The percentage stunting of the RILs was distributed continuously over the range of 0.2 and 40 percent. In the greenhouse assay, 3 RILs demonstrated increased tolerance to *Rhizoctonia* compared to AUS28451. In both the field and greenhouse screens, many RILs demonstrated greater susceptibility than Louise, which could demonstrate transgressive segregation for these individuals. An ANOVA from the field trial revealed that the percent stunting variance of RIL genotypes was significantly different ($F>20$ $P<0.0001$; $R^2 > 0.82$). The RILs in both the greenhouse and field assay demonstrated a normal distribution for resistance; this distribution suggests a quantitative resistance in the Louise x AUS28451 population.

Assessment of root damage associated with Rhizoctonia root disease. Root damage produced by *Rhizoctonia* was observed for all RILs and parental genotypes. Parental genotype Louise sustained significant root damage (6 out of 8) compared to AUS28451 ($P< 0.05$), which had a severity rating of 1 out of 8. A regression analysis comparing stunting of each RIL to its root severity ratings identified a significant correlation between stunting and root disease rating ($P <.001$, $R^2 =0.79$, Fig. 3).

Discussion

The *Rhizoctonia* resistance provided by AUS28451 seems to follow a quantitative trait distribution. In both the field and greenhouse assays, this genotype provided good tolerance against seedling stunting, and root damage. This tolerance will provide a much needed durable resistance for minimal tillage systems, which are often infested with soil borne pathogens. As we have only tested *Rhizoctonia*, we cannot assume that this line will provide resistance against other soilborne pathogens. However, previous data suggests this line provides quantitative resistance against nematodes (Thompson et al. *in prep*). Overall, we will need to run the field experiment for a second year to further validate the resistance phenotype. Further analysis using molecular markers to genotype the population and identify QTL providing this resistance will also need to be performed.

Fig.1 Experimental field site for clean-green assay for *Rhizoctonia*. 60 meter passes were sprayed with glyphosate either 21 days low plant debris (left) or 3 days prior to direct-seeding (right).

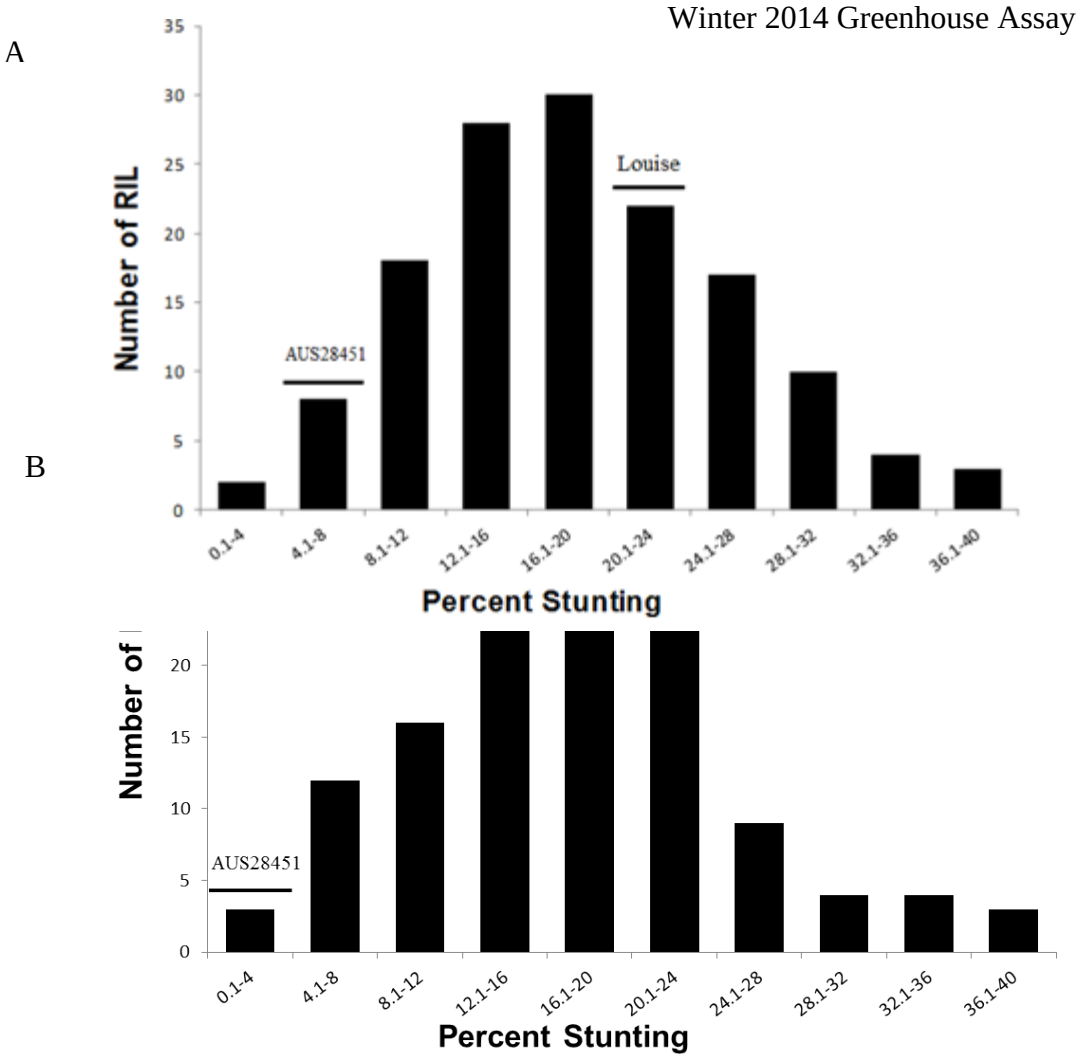

Fig. 2 The phenotypic assessment of Louise x AUS28451 mapping population in Rhizoctonia soils. The distribution of 142 RILs, produced from Louise x AUS28451 spring wheat cross, for the range of *Rhizoctonia* root disease measured by percent stunting. (**A)** In the greenhouse trial RILs were artificially inoculated with *Rhizoctonia* AG8 isolate C1. (**B)** The mapping population was planted at PCFS which has a history of naturally occurring and soil cultured *Rhizoctonia* pathogen.

Impacts

Short term We have identified accession PI621458 as a source of resistance to more than one soil-borne pathogen. This will aid breeding efforts leading to the release of cultivars that will decrease pathogen damage and increase yields.

Mid term Molecular markers associated with resistance to multiple soil-borne pathogens will be identified shortly and will aid in breeding efforts.

Long term A new RNA extraction method has been identified which can aid any research where many samples are being evaluated. After qRT-PCR has been performed on the RIL material it will identify gene specific targets for increased root lignin which has also been associated with improved drought performance.

Publications, Handouts, Other Text & Web Products

a) Thompson, A.L., Smiley, R.W., and Garland-Campbell, K. Registration of LouAu (Louise/IWA8608077) wheat recombinant inbred line mapping population. J. Plant Registrations 9: (doi:10.3198/jpr2015.01.0002crmp).
b) Thompson, A.L. "Breeding program strategies" Seminar for National Program Leader, Maricopa AZ, April 13, 2015.
c) Thompson, A.L. "From drought to soil-borne pathogens, wheat to cotton" Seminar at University of Arizona Tucson AZ, May 26, 2015.

Additional Funding Applied For/Secured

We have used the data generated through funding from this project to apply several grants:

Received:

- K. Sanguinet, A. Thompson, S. Hulbert. Washington Wheat Foundation equipment purchase grant. CID root scanning system and software from CID BioScience.
- K. Sanguient, C. Steber, K. Garland-Campbell Washington Grains Commission: A Genetic Arsenal for Drought Tolerance; Getting to the Root of the Problem. $40,100
- H. Pappu, K.Garland Campbell, T.C. Paulitz Fusarium Crown Rot On Wheat: Prebreeding And Development Of Tools For Genetic Disease Management, $67,761

Applied for:

- K. Sanguinet, T. Sullivan, K Garland-Campbell, T. Paulitz USDA-NIFA-National Needs Fellowship grant: Cross Disciplinary Phytobiome Research and Education, $250,000.

Graduate Students Funded

Post-Doctoral Research Assistant Alison L. Thompson

Recommendations for Future Research

Once molecular markers have been identified for breeders and resistance incorporated into popular breeding lines, field evaluations will be needed to assess if resistance incorporation will have any impact on important agronomic traits. Field studies will also be needed to assess the long term durability of the resistance so management recommendations can be made to growers.

Literature Cited

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3. Smiley, R.W., Gourlie, J.A., Easley, S.A., and Patterson, L-M. 2005. Pathogenicity of fungi associated with the wheat crown rot complex in Oregon and Washington. Plant Disease 89:949-957.
4. Thompson, A.L. 2013. Identifying root-lesion nematode (*Pratylenchus* spp.) resistance and functional mechanisms in wheat. Ph.D. Thesis. Washington State University: USA.