Spring blackstem and leaf spot resistance screening in the USDA-ARS National Plant Germplasm System's *Medicago* spp. genetic resources

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Abstract

Alfalfa (*Medicago sativa*) is the fourth most widely grown agricultural crop and the most significant forage in the U.S. Alfalfa diseases reduce yield and quality impacting production and producers' economic gains. Spring blackstem and leaf spot, caused by *Phoma medicaginis*, is an important fungal leaf spot pathogen for which good resistance is lacking in commercial cultivars. To identify potential sources of resistance in alfalfa germplasm, an optimized greenhouse seedling inoculation protocol was developed. Several isolates were evaluated for pathogenicity and spore concentrations were determined for ideal level of disease pressure. A modified rating scale incorporating additional half values was developed to account for differences observed in disease phenotypes. Following these modifications, a group of 79 standard check alfalfa cultivars and 189 alfalfa-related *Medicago* spp. accessions were screened for disease reaction in replicated randomized complete block designs. In addition, 15 plants for each of 2,834 alfalfa germplasm accessions were screened for disease reaction in non-replicated evaluations. All germplasm evaluated was sourced from the USDA-ARS National Plant Germplasm System temperate-adapted forage legume germplasm collection. Results suggest that an inoculum concentration of approximately $5x10^4$ spores/ml was ideal as at this concentration susceptible 'Lahontan' and moderately resistant 'Ramsey' standard check cultivars performed as expected. Several cultivars appeared to be more resistant than recommended moderately resistant checks, with some related *Medicago* species showing very little disease. Many of the alfalfa accessions screened, especially those originating from colder environments (e.g., northern latitudes), appeared to be more resistant than the reference cultivars. Resistant germplasm selections have been made from screenings for further recurrent selection and the development of advanced populations. The modified Standard Test protocol, summarized data, and resistant germplasm will become publicly available.

Introduction

Alfalfa (*Medicago sativa* L.) is the most important forage legume in the world. Alfalfa forage is known for its high protein content and quality, but also contributes to agricultural sustainability as a cover crop fixing nitrogen and stabilizing soils preventing erosion. In the U.S., it is one of the leading crops with a total of 16.7 million acres harvested producing 54.9 million tons in 2019 (USDA_NASS, 2020). Domestically produced alfalfa is used predominantly by the dairy industry with high-quality hay also destined for export markets.

This plant species is native to Central/Southwestern Asia and regions around the Mediterranean Basin and most likely was domesticated in modern-day Iran (Barnes, 1977; Bolton, 1962; Small,

2011). It was introduced to the U.S. sometime in the mid- to late-1700s from the British Isles and Chile (Clayton et al., 1997). Two important *M. sativa* subspecies (*sativa* and *falcata*) and their hybrid (nothosubspecies *varia*), as well as other major germplasm source introductions have contributed to most modern alfalfa cultivars in the U.S. (Barnes, 1977). As the industry grew, so did the number of cultivars that were adapted to larger growing areas. At this time, breeding efforts also began focusing on the incorporation of disease and insect resistance traits.

Many of the cultivars developed after 1900 can trace back their lineage to original alfalfa germplasm accessions held by the USDA National Plant Germplasm System (NPGS) (Barnes, 1977; Bauchan and Greene, 2001; Caddel et al., 2000). Today the NPGS alfalfa collection conserves and provides access to significant diversity with over 4,090 accessions assembled from diverse origins, some dating back to the early 1900s (Bauchan and Greene, 2001). The collection also includes over 4,500 additional *Medicago* species, some of which are cultivated annual medics, and many are alfalfa wild relatives (Bauchan and Greene, 2001). All NPGS germplasm, including *Medicago* spp., and their associated information is publicly accessible via a web browser and the following link https://npgsweb.ars-grin.gov.

Alfalfa production is limited by a significant number of diseases. Among these, foliar fungal plant pathogens are responsible for considerable losses. To manage many of these diseases, alfalfa breeding efforts have focused on identification and incorporation of resistance into synthetic hybrids through recurrent selection approaches. Resistance has been bred into alfalfa cultivars for important foliar fungal diseases like anthracnose (Elgin et al., 1981; Elgin and Ostazeski, 1985) and downy mildew (Lehman et al., 1988). However, cultivars with high levels of resistance to spring black stem and leaf spot (SBS) are not readily available (Gray and Horton, 1990; Samac et al., 2016).

Spring black stem and leaf spot of alfalfa is caused by the necrotrophic Ascomycete, *Phoma medicaginis* Malbr. & Roum. Infection predominantly affects aboveground portions of the alfalfa plant leading to reductions in quality, severe defoliation and crop losses especially during first cuttings (Samac et al., 2016). The disease has been reported affecting alfalfa in temperate regions of the world where cool wet weather occurs early in the year (Samac et al., 2016). In the U.S. the disease on alfalfa occurs mostly in Midwestern states (Leath and Salter, 1991; Rhodes and Myers, 1986) were ideal infection conditions occur, but has been reported elsewhere (Akamatsu et al., 2008). Considerable diversity in *P. medicaginis* isolate aggressiveness has been reported (Castell-Miller et al., 2008; Ellwood et al., 2006; Gray and Horton, 1990) which should be taken into consideration when evaluating germplasm for disease reaction and/or selection. Research utilizing *Medicago* spp. germplasm on *P. medicaginis* infection processes in plants differing in resistance (Castell-Miller et al., 2007) and screening annual medic core collections (Ellwood et al., 2006; O'Neill et al., 2003) has also been conducted. Only moderate resistance occurs in commercial cultivars (O'Neill et al., 2003; Samac et al., 2016) with management focusing on cultural practices of harvesting early when disease pressure is severe. However, approaches looking at efficacy and cost-effectiveness of fungicide applications have been explored (Samac et al., 2013) with possible transgenic methods to managing disease being proposed (Hipskind and Paiva, 2000).

Because disease management practices for SBS are not completely effective, the current project proposed to identify potential sources of resistance through disease reaction screening experiments. Specifically, project objectives included: 1) the optimization of SBS inoculation protocols; 2) the evaluation of standard check cultivars and inventories for SBS disease reaction; 3) the disease reaction in subsets of representative *Medicago* spp. taxa; and 4) the systematic SBS disease reaction screening of large number alfalfa germplasm accessions. In addition, the proposed project was to make data and associated information collected in these evaluations publicly available through presentations, publications and through the USDA-ARS National Plant Germplasm System's GRIN-Global database.

Materials and Methods

Pathogen isolates and culture. After securing a USDA-APHIS permit to receive and perform research on alfalfa fungal plant pathogens, eight *P. medicaginis* isolates[1] were received as cultures from the USDA-ARS Plant Science Research Unit in St. Paul, MN (**Table 1**). Isolates were transferred and grown on full-strength potato dextrose agar (PDA) for 20 days under 24-hour fluorescent light at 22ºC for sporulation and inoculum preparation.

Germplasm and plant culture. All plant material was requested through the National Plant Germplasm System (NPGS), Germplasm Resources Information Network (GRIN)-Global database. Seed was requested and received separately for each objective as each included large numbers of accessions. Standard Check seed for all current NAAIC standard disease tests (79 cultivars) were requested for optimization purposes as well as to screen cultivars for disease reaction and for subsequent selection. A second group of seeds of 189 accessions from 69 alfalfa wild relatives, covering most of the taxonomic diversity in *Medicago* spp. holdings of the NPGS, were also requested to study disease reaction. In this objective, three accessions of each taxon were included (when available) to better estimate disease response across a representative sample. Lastly, over 2,800 alfalfa (*M. sativa*) cultivars, landraces and wild-collected accessions were obtained for disease assessment and selection. As seed was received, it was scarified for 30 sec with low 320 grit sandpaper in a custom-built air compressor chamber and queued for sowing in greenhouses experimental trials. Individual seed were sown ~1 cm deep into Sunshine Mix #5 (Sun Gro Horticulture, Agawam, MA) in properly labelled standard 1020 greenhouse trays with holes (Hummert International, Earth City, MO). Plants were grown for three to four weeks (20-30 days) or until 3-4 trifoliates had emerged at ~24ºC with 12-hour light/dark cycle. Plants were watered as needed and fertilized once at a rate of 0.55 g/l, two weeks after sowing, with all-purpose (24-08-16) Miracle-Gro (Scotts Miracle-Gro, Marysville, OH) water-soluble fertilizer.

***Inoculation*.** Inoculum was prepared from three-week-old PDA cultures (**Figure 1a**), by breaking free conidia from surface mycelium with a sterilized glass rod into 5 ml sterile water. The 5 ml concentrated spore solution was then transferred to a 100 ml beaker and adjusted in sterile distilled water after counting on a hemocytometer under a compound microscope (400X). Adjusted spore inoculum was then used to atomize seedling foliage, to the point of run off, with an air brush compressor. Two water agar Petri dishes were also atomized with the same inoculum and used for spore germination confirmation (**Figure 1b**). Plants, and one of the water agar Petri dishes, were incubated in a Percival I-36D dew chamber (Percival Scientific Inc.,

[1] In addition to the eight *P. medicaginis* isolates six *Stemphylium* spp. isolates were also received

Perry, IA) at 21-22ºC for 48 h in the dark. The second water agar dish was used for spore verification in the laboratory immediately following inoculations. Following incubation, plants were removed and placed in greenhouse at ~24ºC with a 12-h light/dark cycle for 10 days prior to scoring. The incubated Petri dish was used to confirm spore germination and inoculum viability.

Although fungal isolates received were identified to species, their pathogenicity needed to be confirmed. The eight *P. medicaginis* isolates were inoculated onto a set of 10 Standard Check cultivars including all four of the recommended SBS susceptible ['Ranger' (W6 22328) and 'Lahontan' (W6 22300)] and moderately resistant ['PL-PhR' (W6 22323) and 'Ramsey' (W6 22327)] Standard Test check cultivars. Extra caution was taken to prevent cross contamination by thoroughly cleaning with 95% ethanol and with sterile water atomizing equipment between isolate inoculations.

Spore concentrations for inoculations also required optimization. This was evident following inoculations using the Standard Test suggested concentrations (l x 10^6 to 4 x 10^6 spores per ml) where no clear differences in disease reaction could be observed between SBS susceptible and moderately resistant checks. Therefore, a series of spore concentrations (1 x 10^6, 8 x 10^5, 6 x 10^5, 4 x 10^5, 2 x 10^5, 1 x 10^5, 5 x 10^4, 2.5 x 10^4, 1 x 10^4, and 5 x 10^3 per ml) were tested for ideal disease reaction. Inoculations were performed with and without the recommended 50 ppm Tween®20 surfactant (Sigma-Aldrich, St. Louis, MO).

***Experimental design*.** Isolate pathogenicity and spore concentration optimizations involved non-replicated plantings of 30 plants for each of the four SBS Standard Check cultivars in two 15 plant rows per flat. These plantings included the recommended moderately-resistant ('PL-PhR'/'Ramsey') and susceptible ('Ranger'/'Lahontan') Standard Checks cultivars. For experiments involving the screening of Standard Check cultivars and *Medicago* ssp. taxa, randomized complete block designs with 10 rows of 15 plants per flat were used. A high inoculum concentration equal to the current Standard Test lowest recommended concentration of l x 10^6 spores/ml and a low concentration of l x 10^4 spores/ml were used in screening the Standard Check cultivars. A total of 45 plants for each of the 79 Standard Check cultivars were assessed in three blocks at both concentrations, with the evaluations being repeated once. A similar planting arrangement was used in the *Medicago* ssp. alfalfa taxon screen evaluations with 45 plants for each of 189 accessions in each of three blocks. In the taxon screen, a single concentration of 5 x 10^4 spores/ml was used, and the evaluation was repeated once. For the alfalfa germplasm screening evaluations, a total of 15 plants per accession in 15 rows per flat were sown (**Figure 2a**), inoculated and evaluated at a single 5 x 10^4 spores/ml concentration.

Only twelve flats could be logistically included in each inoculation because of dew chamber capacity and time required for scoring. Therefore, multiple inoculations were needed to evaluate plants in all trials. Both SBS moderately-resistant ('PL-PhR'/'Ramsey') and susceptible ('Ranger'/'Lahontan') cultivars were included in all inoculations for the Standard Check cultivar evaluations, as recommended in the Standard Test. The susceptible 'Lahontan' and moderately resistant 'Rambler' (W6 22326) cultivars were included in the *Medicago* ssp. taxon and germplasm screening evaluations. 'Rambler' was substituted for 'Ramsey' as it consistently was more resistant in the Standard Check evaluations and seed was readily available. A group of close to 800 NPGS alfalfa and a few related species (*M. cacellata*, *M. platycarpus*, and *M. ruthenica*) accessions had previously been evaluated for SBS reaction and reported by K.T. Leath in the GRIN-Global database under alfalfa descriptors (Alfalfa Descriptors GRIN-Global).

Of these, the five lowest and the five highest mean disease reaction rated accessions were also included in evaluations. These cultivars and germplasm accessions were included to serve as points of reference and comparisons in disease reaction.

Scoring. Inoculated plants were scored ten days post-inoculation, once disease symptoms had developed (**Figure 2b**). An updated and optimized disease rating scale with a total of nine ratings including half scores (e.g., 1.5, 2.5) was developed. This rating scale was similar to the original ranging from 1 to 5, where 1 was resistant and 5 susceptible (**Table 2**). Each plant, that was at an appropriate developmental stage when inoculated (3-4 trifoliates), received a visual rating that was recorded and used for subsequent analyses.

Analyses. Means were calculated for the 15 plants in each of the three blocks for the Standard Check cultivars and *Medicago* ssp. taxa objectives. All analyses were carried out using the statistical program R (R_Core_Team, 2020). Data could not be combined for analyses of variance due to significant interactions across batches within replications. Instead, experimental batches were modeled as a fixed effect with entry, accession nested within entry, and block modeled as random effects in a mixed model using *lme4* and *lmerTest* (Bates et al., 2015). Best linear unbiased predictions (BLUP) were calculated to compare entry performance using the 'ranef' function in *lme4* (Bates et al., 2015). Means comparisons were conducted by comparing 95% confidence intervals (Goldstein, 2011). Data in most cases conformed to normality and where it did not, square root transformations were employed. Data wrangling and plotting were conducted using *dplyr* and *ggplot2* (Wickham, 2016; Wickham et al., 2015).

Mean disease reaction was also determined for the 15 plants for each accession in the germplasm evaluations. Correlations between mean disease reaction ratings and the original accession's source environment were explored. These analyses were performed only on a subset of accessions collected as wild materials or landraces. Analyses were performed in the statistical program R using Pearson and Spearman correlations (R_Core_Team, 2020). Environmental variables included in the analyses were 'mean annual temperature', 'mean temperature coldest quarter', 'mean temperature warmest quarter', 'annual precipitation', and 'elevation' and were sourced from WorldClim dataset (Fick and Hijmans, 2017).

Selections. In both the Standard Check and germplasm screening objectives, resistant plant selections with low disease ratings (<1.5) were made. These plants were appropriately identified, transplanted, isolated and allowed to recover for future crosses and further selections.

Project Objectives and Corresponding Results

1. *Optimize inoculation protocols for fungal species causing SBS disease of alfalfa.*

Optimized inoculations protocols were developed, with suggested modifications to the NAAIC SBS Resistance Standard Test protocol. Modifications included addition of Petri dishes for spore viability assessment, reduced spore concentration for inoculations, substituted resistant Standard Check cultivar, and a modified rating scale.

2. *Evaluate standard check cultivars[2] and inventories for reaction to SBS disease.*

Standard Check cultivars, regularly used in NAAIC Standard Test protocols, were evaluated for SBS disease reaction. A few cultivars showed reduced disease severity ratings when compared to

[2] Substituted the word 'cultivars' for 'variety' as this was more appropriate.

the Standard Test protocol-recommended moderately resistant 'Ramsey' reference cultivar and are suggested as substitutes. In addition, a large group (~500) of disease resistant and genetically diverse Standard Check cultivar plants differing in fall dormancy were selected for improved SBS disease resistant population development.

3. *Define host range for SBS disease in subsets of representative Medicago spp. taxa.*

A collection of 189 accessions from 69 *Medicago* spp. taxa were screened for disease reaction to SBS. Of these, many taxa were considerably more resistant than the moderately resistant 'Rambler' Standard Check cultivar with a few showing little to no disease.

4. *Systematically screen through alfalfa germplasm for disease resistance to SBS.*

More than 2,800 accessions were screened for SBS disease reaction with a large group of plants (~500) selected for their low disease reaction rating (<1.5) and for development of improved populations.

5. *Make data, and associated information, publicly available through presentations, publications and through the USDA-ARS National Plant Germplasm System's GRIN-Global database.*

Results from the experiments were presented at the annual meeting of the American Phytopathological Society. Work was also presented to the annual meeting of the Alfalfa Crop Germplasm Committee (now, Forage CGC). The data for all the preceding objectives will continue to be summarized, analyzed and compiled into a peer-reviewed publication in 2021 and will be loaded into the NPGS GRIN-Global database for public access.

Results and Discussion

Protocol optimization. Many aspects of the NAAIC published SBS Resistance Standard Test protocol (Leath and Salter, 1991) were followed with slight modifications. As this was the first time working with this protocol, optimization of environmental conditions for growing of plants prior to and following inoculations was required. Improved pathogen culture growing conditions, inoculation techniques, adjusted spore concentrations, and incubation conditions were also modified and optimized. Other adjustments were needed for seed scarification as well as for choice of planting vessels and soil type, number of plants and spacing distances, and number of replicates and total plants to be inoculated at a given time. A simple addition of water agar Petri dish atomized with the spore suspension during inoculations to confirm presence and viability (i.e., germination) of spores was added. In addition, a new scoring system was developed that better reflected visual symptoms observed in infected plants and better captured subtle differences in disease reaction. Additional complications encountered during optimization of protocols included variable growing conditions in greenhouses due to differences in daylength (across seasons) as well as soilborne pathogenic and surface growing saprophytic fungal contaminants infecting and affecting plants before and after inoculations.

When confirming isolate pathogenicity, the eight isolates were inoculated onto alfalfa Standard Checks and evaluated for disease reaction. Seven of the eight isolates were pathogenic with an isolate from Waupaca, WI (P Wp 102 H1) showing little to no symptoms of disease in tests. The *P. medicaginis* isolate, P ROC 601-4, from Rochester, MN collected in 2012 was chosen as the isolate for all subsequent SBS evaluations as it produced copious spores (conidia) in a short period of time and was intermediate to highly virulent in pathogenicity tests.

Although the Standard Test protocol suggests that a range of spore concentrations from 1 x 10^6 to 4 x 10^6 spores/ml of water should be used, at these high rates no clear differential disease response was observed with many plants showing extreme susceptibility. A similar observation was made by O'Neill et al. (2003) when screening the NPGS annual medic core collection, where even at reduced spore concentration rates of 3-4 x 10^5 spores/ml, no statistically significant differences were seen between moderately resistant and susceptible check cultivars. Consequently, a series of inoculum serial dilutions were developed to try to optimize spore concentrations in order to best assess diseases reaction. Evaluations were conducted for ranges in spore concertation from a high of 1 x 10^6 spores/ml, which coincided with the low end of the Standard Test protocol, down to 5 x 10^3 spores/ml. In this study, and in O'Neill et al. (2003), ideal spore concentrations were somewhere close to the low end of dilutions tested (e.g., 1 x 10^4 – 5 x 10^4 spores/ml) and found that the Tween 20® surfactant was essential for good disease development. The lowest spore concentration at which clear symptom development was observed was 1 x 10^4 spores/ml. At higher than 1 x 10^5 spores/ml, concentrations differences in disease reaction were more difficult to discern.

Over the course of optimizing the spore concentration protocols, it became evident the original rating scales and descriptions included in the Standard Test protocol were insufficient to capture the true disease reaction being observed. Therefore, a new disease rating scale was proposed (**Table 2**) that more closely reflected observed symptoms on diseased plants. The new scale used a total of nine ratings and ranged from 1-5 (like the original scale), where 1 was resistant and 5 susceptible. The additional ratings came as half-scores (1.5, 2.5, 3.5 and 4.5) to denote intermediate ratings and to add granularity to the scoring/ratings. The need for these intermediate ratings came from observations where for example, the more general current Standard Test rating of 5 would have included all plants with defoliation but would not have considered the severity of defoliation or other symptoms on these plants. Smaller lesion size or lower frequency of lesions even on a defoliated plant might have been useful in considering them more resistant and merit a lower rating.

Standard checks. Following the optimized protocols, a group of 79 alfalfa Standard Check cultivars were evaluated for disease reaction at two spore concentrations. These cultivars are used in NAAIC recommended Standard Tests and are distributed by the Pullman, WA NPGS genebank. When means where compared for the SBS moderately-resistant and susceptible reference cultivars across batches and replications at the lower spore concentration (>1 x 10^4 spore/ml), significant differences, but no interactions, were observed. However, when means for the reference cultivars at the higher concentration were analyzed, a significant interaction across batches within replications was observed. Therefore, data could not be combined for ANOVA. Because of the strong interactions present in the data, an estimation of entry means as random effects in a mixed model via best linear unbiased prediction (BLUP) was used for analyses. This is an approach commonly used in plant breeding for data collected across locations in an unbalanced fashion. Although data collected reflect real means associated with entry as a fixed effect, they never occurred within the same trial batch simultaneously. This may be loosely compared to the idea of correcting for differences among batches using the means of the check cultivars. It is important to note that result is not a true mean and will nearly always be associated with shrinkage towards the intercept.

Significant differences were obtained when comparing estimated mean ratings for Standard Checks cultivars at both concentrations. **Figures 3** and **4** show the estimated means and their

corresponding 95% confidence intervals for all cultivars screened at both concentrations. As would be expected, the overall estimated mean for the cultivars at the high concentration (> 1 x 10^6 spores/ml) was higher (3.8) than the overall estimated mean for cultivars inoculated at the lower concentration (3.0) (1 x 10^4 spore/ml). At both concentrations, moderately-resistant ('PL-PhR'/'Ramsey') and susceptible ('Ranger'/'Lahontan') alfalfa Standard Check cultivars also behaved as anticipated and showed lower and higher disease reactions, respectively. In general, the spread in estimated mean disease reaction rating values was more at the lower concentration with the higher concentration data showing less of a spread (i.e., compressed). This confirms initial observations during the optimization processes, where plants that appeared to be resistant at lower concentrations did not seem to be as resistant and were 'masked' in their reaction at higher concentrations. In spite of this 'masking', generally the alfalfa cultivars that were resistant (<2.5 rating) at the low concentration also rated low (<3.0) in their disease reaction at the higher concentration. A similar pattern was observed for susceptible cultivars with higher ratings at both concentrations. Although, the recommended moderately resistant Standard Checks ('PL-PhR'/'Ramsey') rated low, other cultivars showed lower mean disease ratings. For example, 'Travois' averaged a 1.9 at 1 x 10^5 spores/ml and potentially could be substituted in the Standard Test as a more effective resistant reference. In addition, a link between cultivars with low fall dormancy ratings and low mean disease reactions was observed. Cultivars 'Travois', 'Rambler' and 'Ramsey' that are fall dormant and contain *M. s.* subsp. *falcata* in their pedigrees (Barnes, 1977), showed consistently low mean disease ratings at both concentrations and produced many resistant plant selections (*discussed below*).

A product of this objective generated a resistant group of plants (~500) that were selected for initial crosses and improvement through a recurrent selection approach. The resistant plant selections have been subdivided by reported fall dormancies (NAAIC – Fall Dormancy Standard Test) into five groups with similar plant numbers: FD1-2; FD3; FD4; FD5-8; FD9-11. Of these groups, the lower FD rated groups (i.e., FD1-2; FD3 and FD4) were fall transplanted (2020) to field plots for crossing, seed increase and subsequent disease screening and selection (**Figure 5**). The remaining groups, with higher fall dormancy ratings, will be clonally propagated to reach an optimal number of plants and transplanted to the field for seed increase and subsequent selection in spring 2021. Although outside of the scope of the original proposed work, this extended prebreeding effort will produce SBS-resistant plants providing novel sources of freely accessible germplasm that could be incorporated into improved cultivars.

Host range/Taxon screen. Because of likely interactions in the data across batches, an estimation of entry means was also used for data analyses in this objective. The moderately-resistant reference used in these evaluations was 'Rambler', as it turned out to have a lower mean disease rating (i.e., more resistant) than 'Ramsey' in the Standard Check evaluations and seed was readily available. The 1 x 10^4 spores/ml concentration, which was used during optimizations and in screening Standard Check cultivars, was on the low end of the dilutions tested and was a concentration tested on alfalfa (*M. sativa*). Therefore, the decision was made to increase the inoculum concentration to 5 x 10^4 spores/ml to ensure infection, to identify taxa or accessions within taxa that were most resistant and to avoid possible escapes.

Based on estimated mean disease reaction ratings and their 95% confidence intervals, significant differences were also observed between *Medicago* spp. taxa (**Figure 6**). Both susceptible and moderately resistant alfalfa reference cultivars performed as anticipated with an estimated mean of 3.6 for 'Lahontan' and a lower mean rating of 2.7 for 'Rambler', respectively. Many other

Medicago spp. taxa with low disease reaction ratings were observed when compared to 'Rambler'. Mean ratings for many of the *Medicago* spp. taxa were lower when compared to means for most Standard Check cultivars screened in the previous objective at the lower 1 x 10^4 spore/ml concentration. A group of four taxa including *M. monspeliaca* (2.0), *M. marina* (1.9), *M. cretaceae* (1.7) and *M. pironae* (1.7) all showed low estimated mean ratings (**Figure 6**) with little to no disease symptoms. When comparing results obtained here with matching taxa evaluated by O'Neill et al. (2003), results generally agreed. For example, in both studies the *M. orbicularis*, *M. tenoreana*, *M. heyniana*, *M. doliata*, *M. soleriolii*, *M. constricta* and *M. praecox* were all identified as having higher levels of resistance when compared to other taxa. Similarly, several taxa were also identified as being susceptible in both studies including *M. scutelata*, *M. lanigera*, *M. arabica*, *M. mínima* and *M. rotata*. Although in most situations results were congruent, a few exceptions were apparent. O'Neill et al. (2003) found that *M. laciniat*a and *M. murex* were resistant, while in the current study they were susceptible. Both *M. sauvagei* and *M. rugosa* were moderately resistant in the current findings, but reported as susceptible by O'Neill et al. (2003). Discrepancies across the two studies might have been due to having screened different accessions within taxa being compared or the use of distinct pathogen isolates varying in virulence. Another likely possibility for the observed differences was the lower inoculum spore concentration used in this study (1 x 10^4) vs. the higher rate by O'Neill et al. (2003) (3-4 x 10^5). It is also probable that the modified rating scales used in both studies played a role in differences observed. Lastly, the current study included close to 30 additional *Medicago* taxa not evaluated by O'Neill et al. (2003), with varying disease mean disease reaction ratings.

Some of these taxa evaluated in this objective are alfalfa crop wild relatives in secondary and tertiary genepools with successful hybrids reported (Bingham et al., 2013; McCoy and Bingham, 1988) and could potentially serve as sources of SBS disease resistance. If related taxa with high disease resistance cannot be hybridized with alfalfa, it is possible that these may serve as a tool in genetic analyses to study inheritance and/or as tools in possible gene discovery (Yang et al., 2008). Any genetic control elucidation to SBS disease resistance in related taxa could potentially aid in discovery of similar mechanisms in alfalfa.

Germplasm screen. Initial analyses of mean disease ratings indicate that, as with other objectives, both the susceptible and moderately resistant Standard Check cultivars behaved as expected. For 'Lahontan', the mean across inoculation batches was 4.2 and for 'Rambler' 2.5. Comparisons in mean disease ratings from the current study to the five most resistant and susceptible germplasm accessions in the germplasm screening reported by Leath, showed a close correspondence (**Table 3**). All low (2.87) and high (4.90) mean disease rated accessions identified by Leath also showed similarly low (2.40) and high (4.31) overall mean ratings in the current results. Even ranking of accessions according to disease reaction within the two groups (high/low) was mostly maintained. Overall mean disease ratings for both groups was higher for accessions screened by Leath as most likely a higher inoculum concentration than the current 5 x 10^5 spores /ml was used in those evaluations.

Close to 19.7% (559 of 2,834) of the accessions screened showed lower disease ratings when compared to the moderately resistant 'Rambler' reference. Of these, 162 showed a disease reaction rating lower than 2.0 with 33 accessions rating below a 1.5. Thirteen of the 19 accessions screened with the lowest disease reactions (<1.4) belonged to the *falcata* subspecies. Mean disease ratings for each taxon evaluated was compared (**Table 4)**. The number of

accessions screened for the *viscosa* (12), *glomerata* (11) and especially *tunetana* (6) subspecies was low, so means might not reflect true disease reaction when evaluating larger sample sizes. For subspecies where a greater number of accessions (>100) were evaluated, a clear delineation in disease reaction was observed. The *falcata* subspecies generally was more resistant with the *sativa* subspecies being most susceptible and *varia* (a hybrid between these two subspecies) showing an intermediate reaction.

Using alfalfa germplasm accession original source information, based on latitude and longitude coordinates from GRIN-Global, correlations between mean disease reaction and environment were explored. Unfortunately, only a subset of accessions had useful and accurate latitude and longitude coordinates. An interesting moderate negative correlation was observed for the *caerulea* (r = -0.59) and *falcata* (r = -0.43) subspecies. It appears that accessions of these two subspecies originally sourced from wetter or higher precipitation environments showed lower mean disease ratings and were more resistant (**Figure 7**). This could be explained by assuming the disease is more prevalent in these 'wetter' environments and plant disease resistance has evolved in native alfalfa in these regions.

These analyses were carried out to show possible linkages between accession with low (or high) mean disease reactions and specific environmental conditions in order to select germplasm for additional screening and/or possible breeding. This approach, 'homing in' on specific germplasm based on environmental conditions, is termed focused identification of germplasm strategy (FIGS). FIGS has been used successfully in other germplasm collections to identify and source specific traits for breeding objectives in grain (Bari et al., 2011) and legume crops (Dadu et al., 2018; El haddad et al., 2020). Knowing if clear associations exist between source environmental conditions and possible disease resistance can be leveraged to target future germplasm acquisitions.

Like with the Standard Check objective above, a group of more than 500 individual plants with low disease ratings <1.5 were kept and will be used for developing improved disease resistant populations (**Figure 8**). In this case, the selection of resistant plants (523) in several different taxa (**Table 4**) will be further screened and evaluated for their disease resistance to an inoculum 'cocktail' of all seven pathogenic *P. medicaginis* isolates. This will be done to confirm selection of resistant plants to a geographically diverse set of isolates and not just the P ROC 601-4 isolate initially chosen for screening. As fall dormancy ratings were not known for many of these accessions, plants will be established in spring 2021 field plots for seed/generation increase. It is likely the resistant plants from each unique taxonomic background will also be kept separate in the development of resistant populations. A second generation will be recurrently selected for disease resistance using a similar approach and released as improved germplasm. As with objective 2, this prebreeding effort is outside the scope of the original proposal but will provide freely available sources of disease resistant germplasm.

In objectives where resistant plant selections were produced, a couple of cautionary statements should be made. Selections were made using a single *P. medicaginis* isolate (P ROC 601-4). Care must be taken to screen developed populations at some point before release with a genetically diverse group of isolates (Castell-Miller et al., 2008; Ellwood et al., 2006; Gray and Horton, 1990) to confirm broad and durable resistance. Also, in breeding programs, it still could be useful to use a high spore concentration (e.g., =/> 1 x 10^6 spores per/ml) when inoculating plants if the objective is to only identify highly resistant plants. However, using this approach the

possibility of narrowing genetic diversity by keeping only a minimal number of plants of a common genetic background exists.

Another side product of this large germplasm screening objective was that an assessment on overall viability of a large proportion (>2,800) of the collection could be made. Of the total screened (2,844 accessions), ten failed to produce viable seedlings with other accessions indicating reduced viability. Forthcoming efforts will focus on attempting to germinate these difficult-to-replace accessions using in vitro assays for subsequent field regeneration.

Acknowledgements

Funding for this study was provided by the U.S. Alfalfa Farmer Research Initiative of the National Alfalfa & Forage Alliance. Additional acknowledgements go to technical staff including E. Cervantes, S. Shellenberger, J. Prieto, E. Caskey and K. McCord for help in generating information it this report.

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Keywords

Alfalfa, *Medicago*, germplasm, disease resistance, fungi

Tables and Figures

Table 1. *Phoma medicaginis*, causal agent of spring blackstem and leafspot (SBS) in alfalfa (*Medicago sativa*), fungal isolate designation and source/location.

#	Designation	Source	#	Designation	Source
1	P Roc 302-1	Rochester, MN	5	StC P 306-10	St Cloud, MN
2	P ROC 601-4[1]	Rochester, MN	6	StC P 306-2	St Cloud, MN
3	StC P 306-5	St Cloud, MN	7	P Roc 302-4	Rochester, MN
4	StC P 306-6	St Cloud, MN	8	P Wp 102 H1[2]	Waupaca, WI

[1] Isolate chosen for initial SBS evaluations. [2] Not pathogenic in initial tests.

Table 2. Proposed new disease reaction rating scale for spring blackstem and leafspot, caused by *Phoma medicaginis*, in alfalfa (*Medicago sativa*).

Score	Class	Description[1]
1.0	Resistant	No lesions, chlorosis, or defoliation. Plant is symptom-free.
1.5	Resistant	5 or less pinpoint lesions (~0.25 mm), with <5% leaflet area affected. No chlorosis, stem/petiole lesions or defoliation. Plant is mostly symptom-free.
2.0	Resistant	>5 pinpoint to small lesions (~0.25 to 0.5 mm), with 5-10% leaflet area affected. None to very mild chlorosis. No lesions on stem/petioles or defoliation.
2.5	Moderately resistant	Small lesions (~0.5 mm), 11-20% of leaflet area affected. None to mild chlorosis. Few small stem/petiole lesions may be present. No defoliation. Plant rated overall between resistant and susceptible.
3.0	*Susceptible*	Small to medium sized lesions (0.5-2 mm), 21-30% leaf area affected. Mild to moderate chlorosis. Small stem/petiole lesions may be present. Single leaflet defoliation. Overall, plant is mildly susceptible.
3.5	*Susceptible*	Medium sized lesions (1-2 mm), 31-40% leaflet area affected. Mild to moderate chlorosis. Mild stem/petiole lesions, no girdling of stem, but may have petiole girdling. One/two leaflet and/or petiole defoliations. Overall plant is moderately susceptible.
4.0	*Susceptible*	Medium to large sized lesions (>3 mm), with 41-50% leaflet area affected. Moderate chlorosis. Mild to moderate stem/petiole lesions, girdling of petioles may occur. Mild to moderate defoliation of leaflets and abscission of petioles. Overall plant is above average in susceptibility.
4.5	*Susceptible*	Medium to large-sized lesions with >50% leaflet area affected. Extensive chlorosis. Moderate to severe stem and/or petiole lesions. Moderate defoliation of leaves and/or girding on multiple petioles. Overall plant is highly susceptible.
5.0	*Susceptible*	Many large lesions, extensive chlorosis, severe defoliation. Overall plant is in extremely susceptible or dead.

[1] New rating scale and description takes into consideration lesions size, % leaflet area affected, chlorosis, stem/petiole lesions as well as petiole and leaflet abscission.

Table 3. Comparison between mean disease ratings in the current study and those for the five most resistant/five most susceptible (*Medicago sativa*) germplasm accessions reported by K.T. Leath evaluated against *Phoma medicaginis*, causal agent of alfalfa (*Medicago sativa*) spring blackstem and leafspot.

Acc. #	Taxon	Geography	Current[1]	Leath[2]
		---------- *Resistant* ----------		
PI 325409	*M. s.* subsp. *falcata*	Russian Federation	1.69	2.83
PI 325387	*M. s.* subsp. *falcata*	Stavropol, Russian Federation	2.19	2.84
PI 315461	*M. s.* subsp. *sativa*	Russian Federation	2.35	2.79
PI 315479	*M. s.* subsp. *falcata*	Russian Federation	2.55	2.92
PI 315456	*M. s.* subsp. *sativa*	Russian Federation	3.21	2.99
Mean			**2.40**	**2.87**
		---------- *Susceptible* ----------		
PI 206110	*M. s.* subsp. *sativa*	France	3.97	4.87
PI 86696	*M. s.* subsp. *sativa*	Turkistan	4.27	4.89
PI 196243	*M. s.* subsp. *sativa*	India	4.37	4.87
PI 212612	*M. s.* subsp. *sativa*	Afghanistan	4.40	4.88
PI 179702	*M. s.* subsp. *sativa*	India	4.53	4.97
Mean			**4.31**	**4.90**

[1] Current study using proposed new rating 1-5 rating scale. [2] Reported by K.T. Leath in alfalfa descriptors online (GRIN-Global).

Table 4. Number of accessions screened, mean disease reaction and resistant plant selections for alfalfa (*Medicago sativa*) subspecies evaluated against *Phoma medicaginis*, causal agent of spring blackstem and leafspot.

Taxon	N[1]	Mean[2]	Selections	
			Plants[3]	%[4]
M. s. subsp. *falcata* var. *viscosa*	12	2.47	17 (4)	9.4
M. s. subsp. *glomerata*	11	2.50	20 (4)	12.1
M. s. subsp. *falcata*	371	2.67	187 (70)	3.4
M. s. subsp. *caerulea*	71	2.98	13 (13)	1.2
M. s. subsp. *varia*	347	3.00	128 (75)	2.5
M. s. subsp. *sativa*	2,016	3.18	539 (356)	1.8
M. s. subsp. *tunetana*	6	3.39	2 (1)	2.2
Total	**2,834**	**-**	**523**	

[1] Number of accessions screened per taxon. [2] Using proposed new rating 1-5 rating scale developed in this research. [3] # resistant plant selections (rating <1.5); (*n*) = # accessions resistant plants were selected from. [4] Proportion (%) of resistant plants selected from total plants evaluated for each subspecies (e.g., *viscosa* [(17 plant selections) / (12 accessions x 15 plants/accession) x (100) = 9.4%])

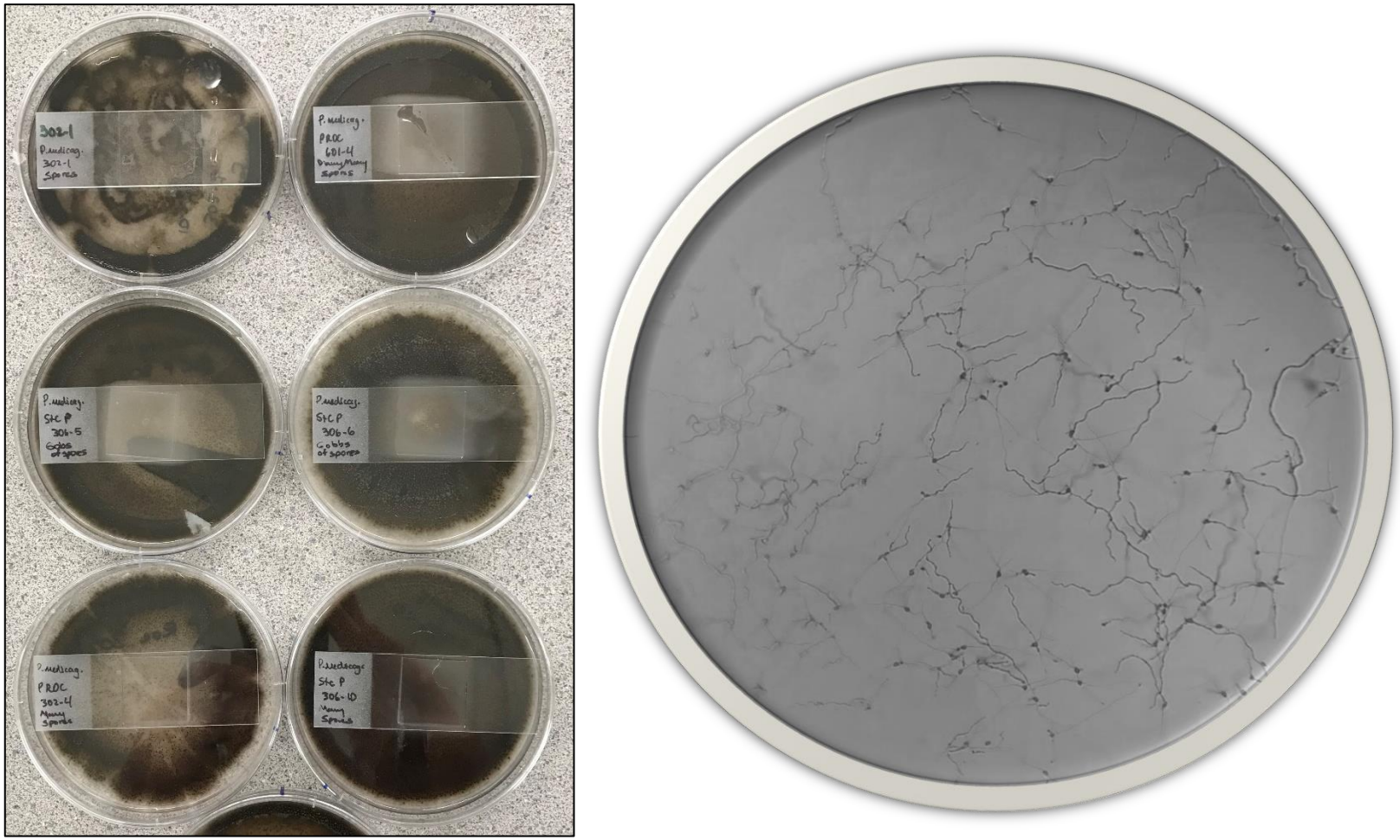

Figure 1. a) Three-week-old *Phoma medicaginis* isolates in culture on potato dextrose agar; b) Microscopic field of view (40X) of a water agar Petri dish following a 48-hour dew chamber incubation period at 21°C (notice conidia germinating and hyphae branching indicating spore viability).

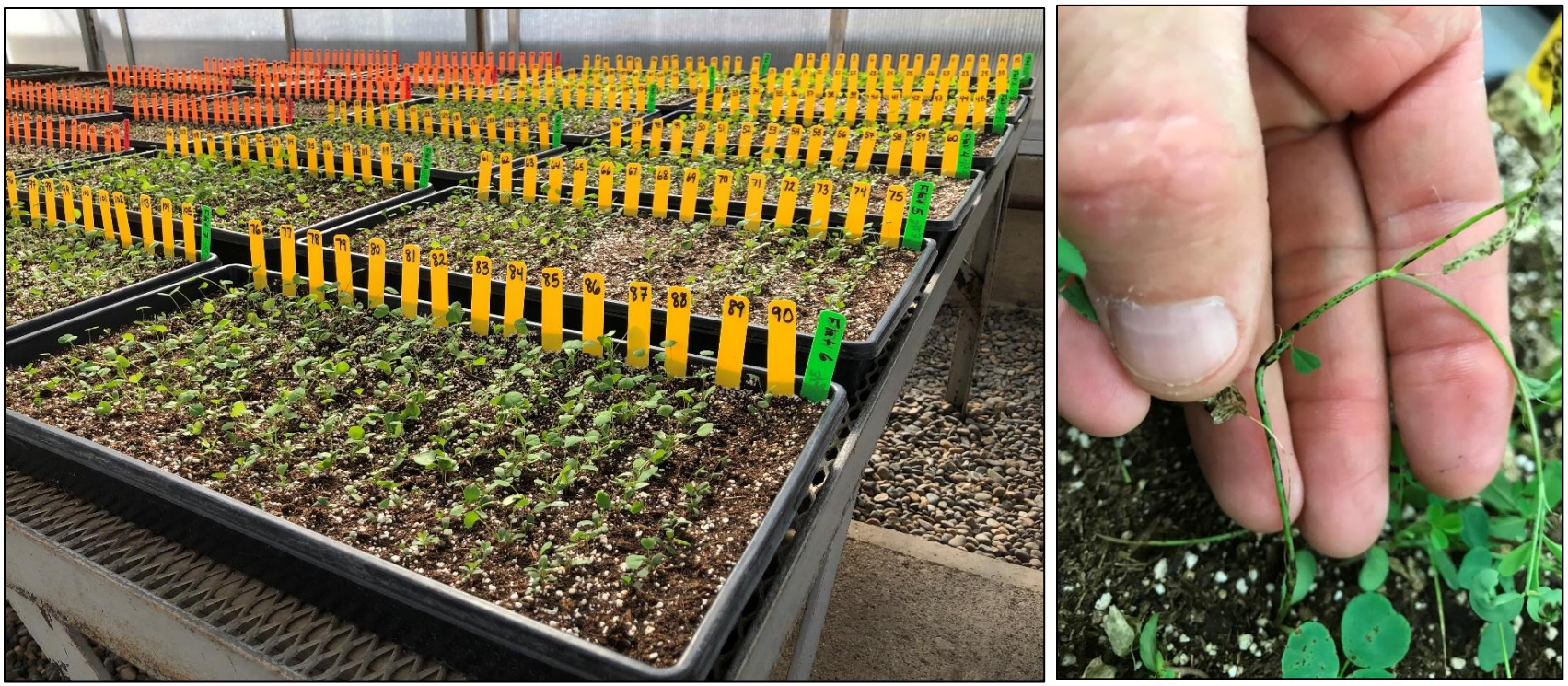

Figure 2. a) Approximately two-week-old greenhouse planting of *M. sativa* germplasm accession seedlings prior to inoculations; b) Characteristic *Phoma medicaginis* disease reaction (stem and leaf symptoms) on susceptible plant at scoring using isolate P ROC 601-4.

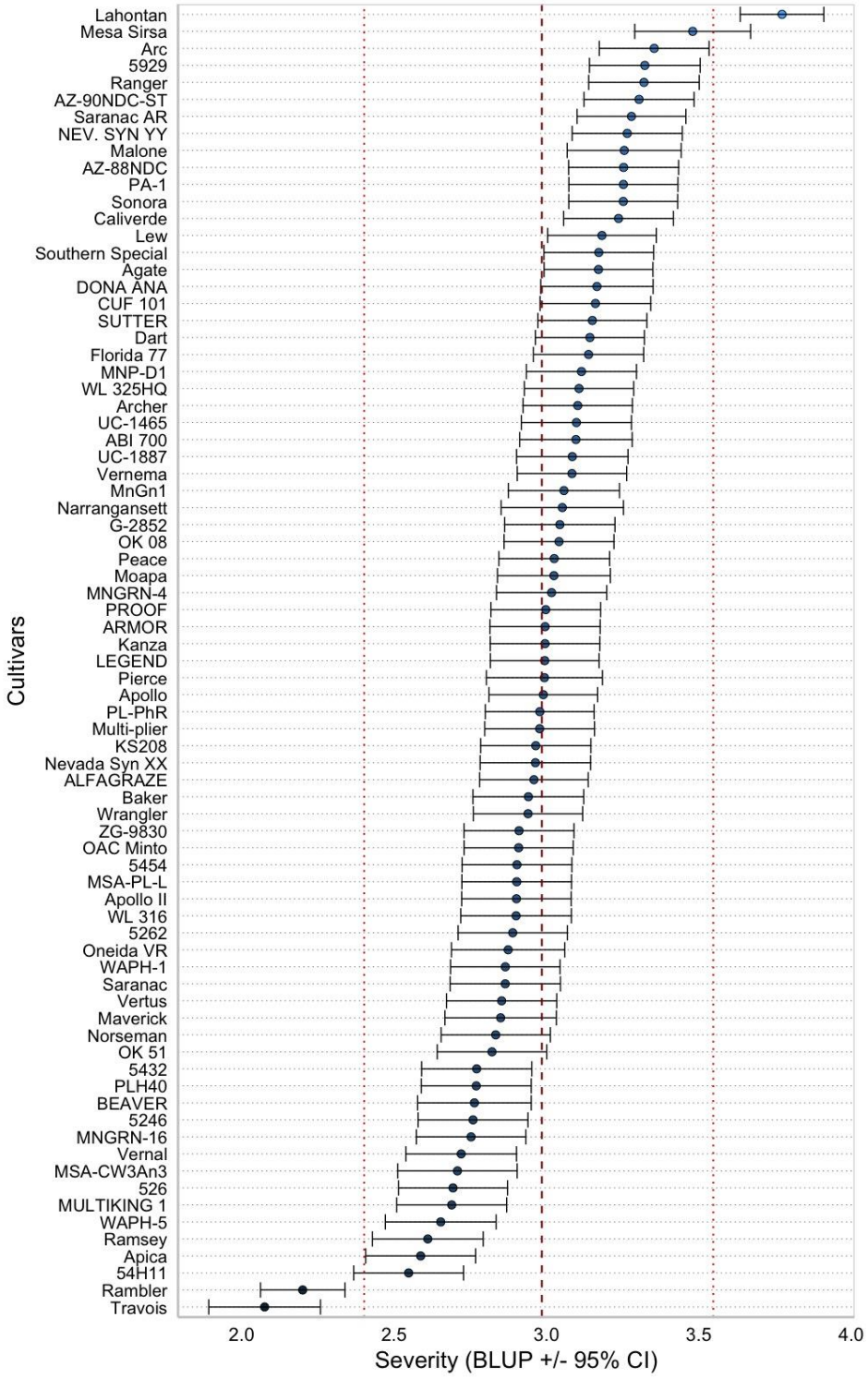

Figure 1. Estimated mean disease rating using best linear unbiased predictions (BLUP) and a modified 1-5 rating scale along with 95% confidence intervals for alfalfa (*Medicago sativa*) Standard Check cultivars evaluated for disease reaction to *Phoma medicaginis*, causal agent of spring blackstem and leaf spot (Concentration 1 x 10^4 spores/ml)

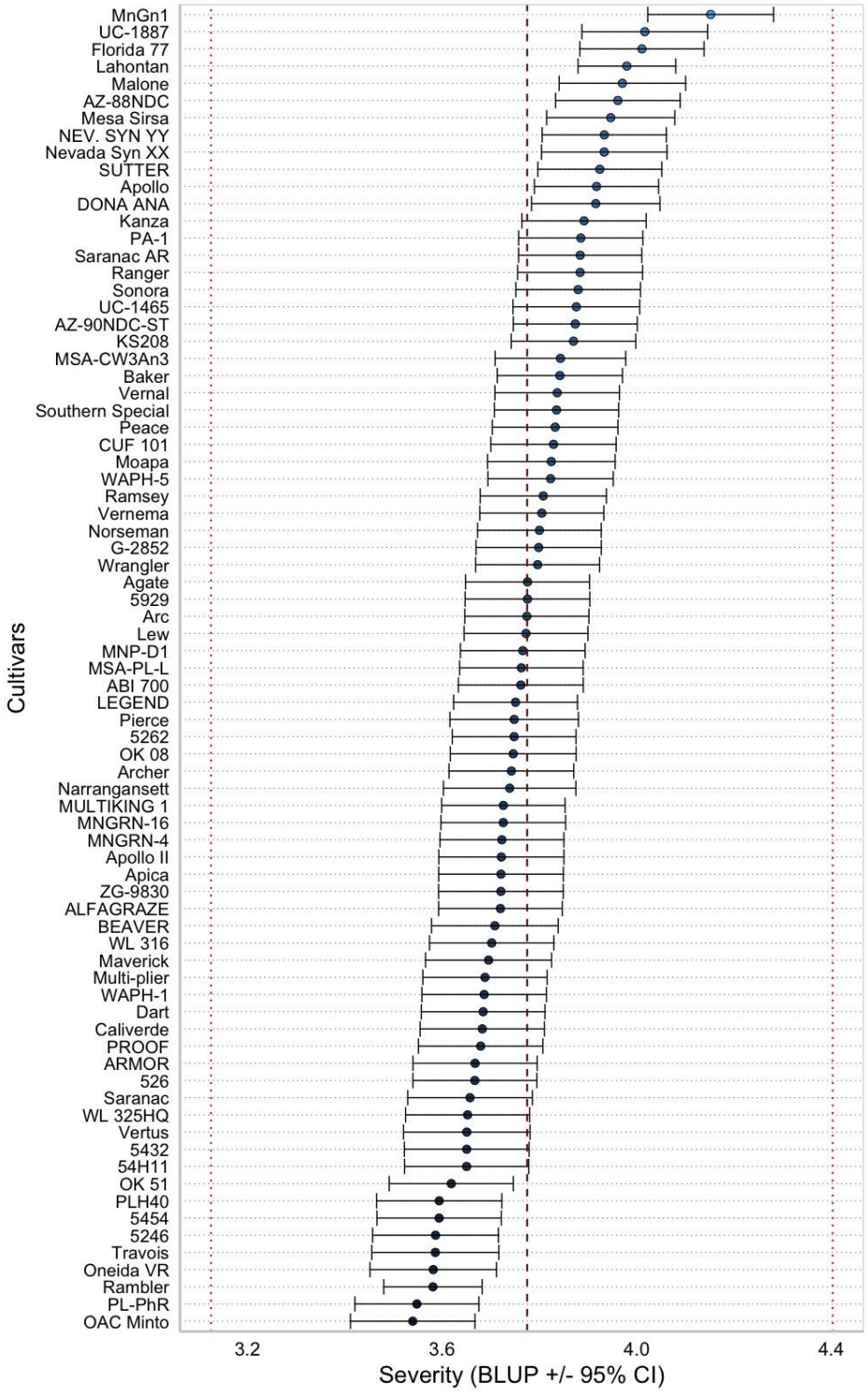

Figure 4. Estimated mean disease rating using best linear unbiased predictions (BLUP) and a modified 1-5 rating scale along with 95% confidence intervals for alfalfa (*Medicago sativa*) Standard Check cultivars evaluated for disease reaction to *Phoma medicaginis*, causal agent of spring blackstem and leaf spot (Concentration 1 x 10^6 spores/ml).

Figure 5. Fall field transplant establishment of alfalfa (*Medicago sativa*) crossing block plots for selected spring blackstem and leaf spot (*Phoma medicaginis*) resistant Standard Check germplasm.

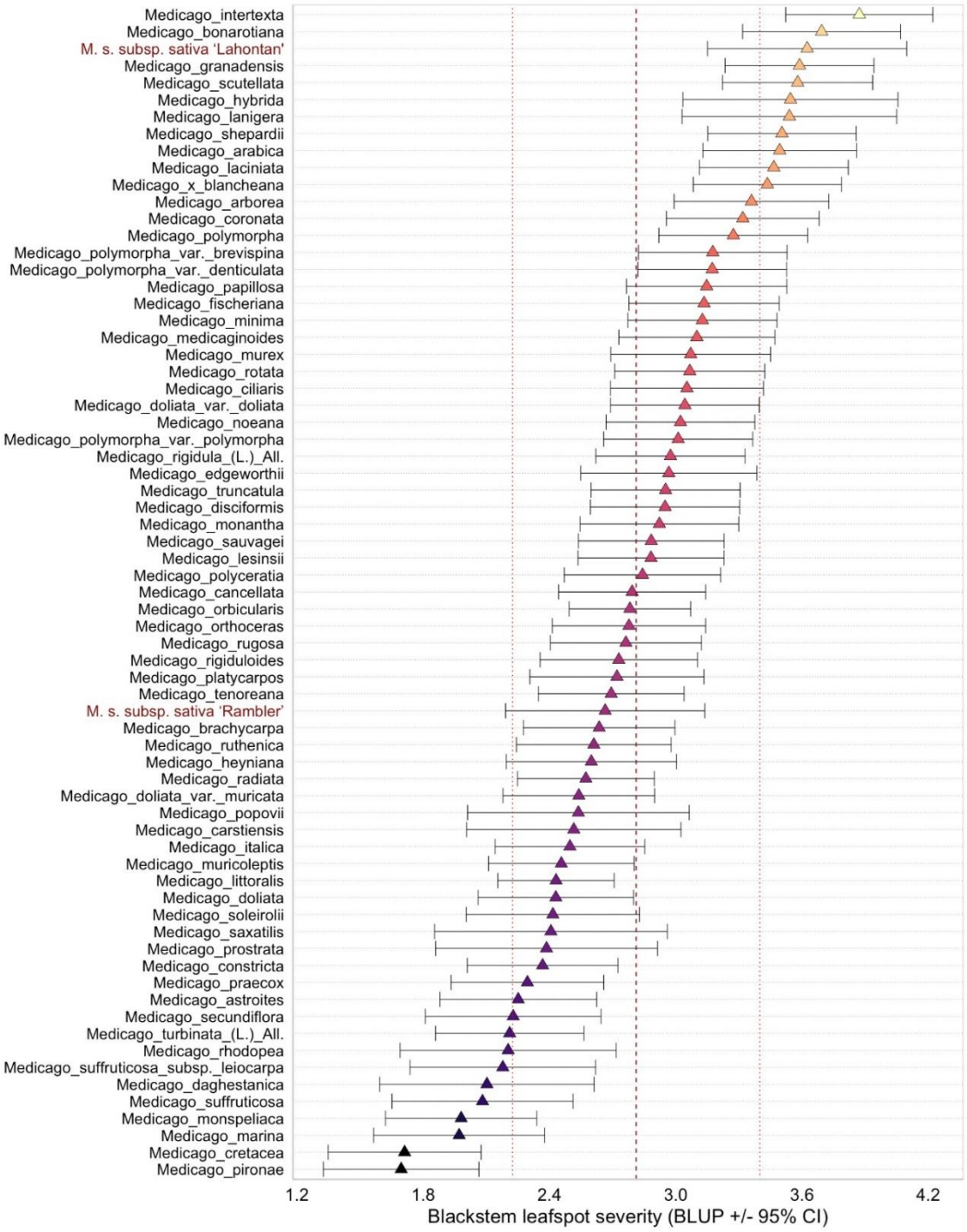

Figure 6. Estimated mean disease rating using best linear unbiased predictions (BLUP) and a modified 1-5 rating scale along with 95% confidence intervals for *Medicago* spp. taxa evaluated for disease reaction to *Phoma medicaginis*, causal agent of spring blackstem and leaf spot (Concentration 5 x 10^4 spores/ml).

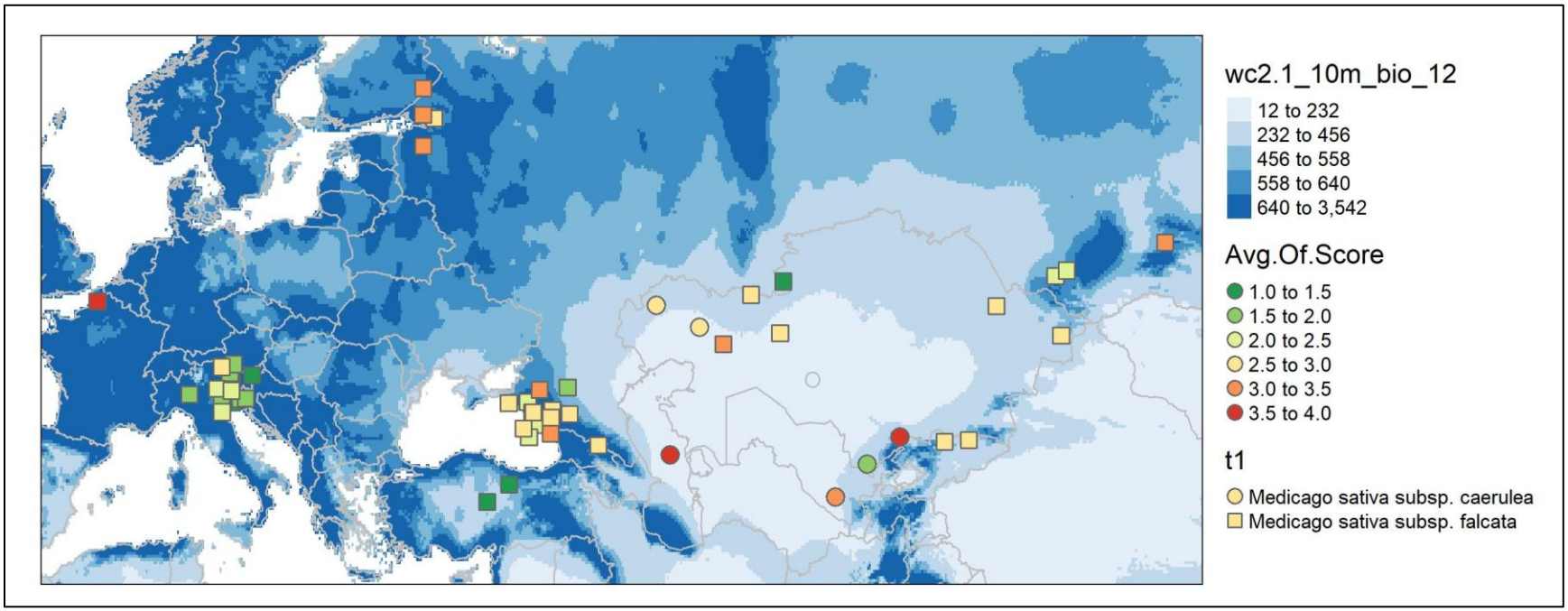

Figure 7. Source location for *Medicago sativa* subsp. *falcata* and subsp. *caerulea* germplasm accessions showing correlation between region's precipitation and mean disease resistance reactions to *Phoma medicaginis*, causal agent of spring blackstem and leaf spot.

Figure 8. Greenhouse transplanted alfalfa (*Medicago sativa*) germplasm selections resistant to *Phoma medicaginis*, causal agent of spring blackstem and leaf spot.