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Research

Development of *Glycine max* Germplasm Highly Resistant to *Sclerotinia sclerotiorum*

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Abstract

Sclerotinia stem rot (SSR) of soybean caused by *Sclerotinia sclerotiorum* is a devastating disease of soybean, especially in the Upper Midwest region of the United States. To mitigate yield losses due to this disease, many control methods are available for producers, including cultural control practices, chemical control, and cultivars with quantitative resistance. However, due to there being few commercial cultivars with high levels of resistance, producers are often limited in their seed selection. The aim of this study was to develop novel conventional soybean cultivars with high levels of resistance to SSR, favorable agronomic traits, and resistance to additional economically important diseases. Initial crosses were conducted in 2016 with two different sources of SSR resistance. Across multiple generations of screening for resistance to SSR, three highly resistant soybean lines were identified as the elite lines. These elite lines were demonstrated to be highly resistant across multiple years in both greenhouse and field trials, including high levels of resistance to multiple diverse *S. sclerotiorum* isolates. The three selected elite lines also resulted in moderately high yields and favorable agronomic traits, such as low lodging and moderate branching, indicating their viability to be released for production. In addition to SSR resistance, these three elite lines demonstrated resistance to other economically important soybean diseases, such as frogeye leaf spot, anthracnose, Cercospora leaf blight, and brown stem rot. Overall, this work has led to three SSR-resistant soybean lines that could be useful for future breeding efforts or commercial soybean production.

Keywords: genetic resistance, plant breeding, soybean, white mold

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Soybean (*Glycine max* [L.] Merrill) production in the United States and Ontario, Canada, averages 115.9 million metric tons annually, and this crop is of global importance for feed, food, and oil (Bradley et al. 2021). The Upper Midwest region of the United States and Canada is consistently threatened by Sclerotinia stem rot (SSR),

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caused by the fungal pathogen *Sclerotinia sclerotiorum*. Across this region, yield losses can reach up to 1.8 million metric tons in a single season (Bradley et al. 2021).

Management of SSR has been well studied, and many effective options are available to producers, such as the use of differing cultural practices (wide row spacings or low seeding rates; Webster et al. 2022), cover cropping (Euteneuer et al. 2021; Pethybridge et al. 2020), or pesticide applications (Willbur et al. 2019). The use of genetic resistance is another highly effective tool for managing SSR and does not require additional inputs, which may ultimately be beneficial for ecological preservation and long-term prevention of fungicide resistance (McCaghey et al. 2019). The integration of SSR resistance into soybeans can be categorized into two groups, conventional breeding and biotechnology based, both demonstrating promising results. Conventional breeding has resulted in many soybean genotypes with improved quantitative resistance, but this method has many barriers (Antwi-Boasiako et al. 2021). Some of these barriers could include the high cost of screening a large number of progeny lines from each cross, the high labor and space requirements for resistance screenings under greenhouse conditions, and the variable spatial distribution of pathogen populations in field screenings, resulting in inconsistent disease pressure. The use of biotechnology has resulted in the development of transgenic soybeans with highly improved resistance (Cober et al. 2003; Donaldson et al. 2001; Yang et al. 2019, 2020). One study reported a 96% reduction in SSR severity after the transgenic insertion of an oxalate decarboxylase (Cunha et al. 2010). Recent work has also led to the improvement of SSR resistance by utilizing host-induced gene silencing of oxaloacetate acetylhydrolase (*Ssoah1*) in soybean (McCaghey et al. 2021; Rana et al. 2022).

Being partial in nature, resistance to SSR in soybean has been associated with many minor-effect quantitative trait loci (Arahana et al. 2001; Bastien et al. 2014; Kandel et al. 2018; McCaghey et al. 2017; Vuong et al. 2008). Due to this quantitative nature, marker-assisted selection has yet to become a viable option (Schuster 2011). Investigations into the mechanisms of SSR resistance have revealed the importance of specific phenylpropanoid pathway intermediates, which were correlated with decreased accumulation of lignin and have anecdotally been associated with increased lodging (Ranjan et al. 2019). Furthermore, field resistance has been explored, examining escape mechanisms such as improved standability, decreased plant height, and differing crop maturities (Kim and Diers 2000). Despite the complicated and multifaceted nature of SSR resistance, successful efforts to breed soybean genotypes with high levels of resistance have been achieved (McCaghey et al. 2017; Polloni-Barros et al. 2022; Webster et al. 2021), but there is still a lack of commercial cultivars with high levels of resistance available to growers.

Screening soybean genotypes for resistance to SSR has been problematic due to difficulties in assessing the ranking of resistance between genotypes. Therefore, a group of four soybean genotypes was developed with standardized levels of resistance to SSR (Webster et al. 2021). These four lines ranged from highly susceptible to highly resistant and were validated under both greenhouse and field conditions. These four lines were called the check lines and were developed with the intent to screen a large number of soybean lines quickly and accurately for their level of resistance to SSR.

To further complement the four soybean check lines, a panel of nine *S. sclerotiorum* isolates was identified to refine the accuracy of selection during the soybean screening process (Willbur et al. 2017). These nine isolates range in aggressiveness from highly aggressive to mildly aggressive. This panel was developed for the ability to screen soybean genotypes for resistance to many potential *S. sclerotiorum* populations that may be present in producer fields in the Midwest United States and beyond. Previous reports have demonstrated that field populations of *S. sclerotiorum* differ across wide geographic regions, leading to potential differences in host responses. For example, one soybean genotype from a statewide variety trial study in Wisconsin displayed different relative degrees of susceptibility to SSR depending on the location (Shawn Conley, University of Wisconsin-Madison, *personal communication*). Therefore, the use of such a diverse panel is highly important for assessing the levels of resistance to multiple isolates of *S. sclerotiorum*. As a result of the high number of isolates in this panel, screening many soybean genotypes would quickly become laborious and time consuming. Therefore, this panel was designed to be used for screening a few late-generation soybean genotypes with known levels of resistance to SSR to ensure resistance in the candidate soybean genotypes was effective under pressure from a diverse population of *S. sclerotiorum*.

Although great potential exists in having standardized groups of soybean genotypes and *S. sclerotiorum* isolates for screening and selection of resistant soybean genotypes, to date, no study has examined their use in tandem. This present study aimed to do that by (i) screening many soybean breeding lines for resistance to SSR alongside soybean check lines and (ii) screening late-generation and SSR-resistant soybean breeding lines with a nine-isolate panel of diverse *S. sclerotiorum* isolates.

MATERIALS AND METHODS

Standardized panels

All disease screenings were performed with standardized check lines of soybean genotypes with known levels of resistance to SSR unless noted otherwise. These check lines consisted of a public cultivar, Dwight (susceptible; MG 2.9), and three lines previously developed from our program, SSR51-70 (moderately resistant; MG 2.0), 51-23 (moderately resistant; MG 2.3), and 52-82B (resistant; MG 2.8). Dwight is a public cultivar previously released by the University of Illinois in 1997 (Nickell et al. 1998). SSR51-70, 51-23, and 52-82B were all developed at the University of Wisconsin-Madison (McCaghey et al. 2017). These four soybean lines have been assessed for their consistent response to inoculations of multiple diverse isolates of *S. sclerotiorum* and are called the check lines (Webster et al. 2021).

A standardized panel of nine *S. sclerotiorum* isolates was used to screen for resistance to SSR under greenhouse conditions during the F_9 generation (Supplementary Table S1). This panel was developed by Willbur et al. (2017) as a tool to assess soybean resistance to a diverse range of *S. sclerotiorum* isolates with varying degrees of aggressiveness. Isolates also vary in their geographic origin, as well as their host of origin at isolation. Due to viability issues with one isolate (#62), the panel used in this study consisted of eight isolates.

Field disease screenings

Between the R5 (pods contain 3.2-mm-long seeds at one of the four uppermost nodes; Fehr et al. 1971) and R6 (seeds filling the entire pod at one of the four uppermost nodes; Fehr et al. 1971) growth stages, soybeans were rated for SSR development. SSR incidence percentage was measured by counting the total number of plants with SSR present within the two center rows of each plot and dividing by the total number of plants in the assessed rows. Disease severity was measured by taking 30 independent 0.3-m sections of the inner two rows of each plot and determining the mean SSR severity on a scale of 0 to 3 (0 = no SSR present, 1 =

SSR present on a lateral branch, 2 = SSR present on the main stem but not fully girdling, and 3 = SSR present on the main stem and fully girdling) (Grau et al. 1982). An average disease severity score was determined by summing all disease severity scores and dividing by the total number of 0.3-m sections that contained SSR. An SSR disease severity index (DIX, %) was then calculated by dividing the average disease severity by three and multiplying the resulting value by the disease incidence percentage (Willbur et al. 2019).

Additional diseases assessed in the field included frogeye leaf spot (causal agent: *Cercospora sojina*), Cercospora leaf blight (causal agent: *Cercospora kikuchii*), and anthracnose (causal agent: *Colletotrichum truncatum*). Both frogeye leaf spot severity scores (%) and Cercospora leaf blight severity scores (%) were determined by visually estimating the mean foliar severity (percentage of leaf area with lesions) for each respective disease in the upper canopy of each plot assessed at the R6 growth stage. Anthracnose severity scores (%) were determined by visually estimating the mean disease severity (percentage of stem area with lesions) on soybean stems for each plot assessed at the R8 growth stage (full maturity; Fehr et al. 1971).

Assessing agronomic traits

Agronomic traits were assessed in multiple generations (F_6, F_7, and F_8). These traits included lodging, branching, plant height, and pubescence color. Lodging was assessed on a 1-to-5 scale based on the degree angle of main stems relative to the ground (1 = no lodging, 2 = 20° lean, 3 = 45° lean, 4 = >45° lean, and 5 = 90° lean or fully lodged). Branching was assessed by estimating the average number of lateral branches for plants in each plot. Plant height was assessed for each plot. Pubescence color for each plot was recorded as being tawny, light tawny, or gray.

Greenhouse conditions

All greenhouse screenings were performed under the same conditions at West Madison Agricultural Research Station (WMGH), Madison, WI, unless noted otherwise. Light was supplemented with 1,000-watt high-pressure sodium lights at a height of 2 m above the bench top. Three lights were evenly spaced across each 4.9-m length of bench space, and each light produced between 250 and 320 μmol of photosynthetically active radiation energy at a height of 45 cm above the benchtop. Plants were grown under a 15-h photoperiod between 5:00 a.m. and 8:00 p.m. Temperatures within the greenhouse ranged from 20°C at night to 28°C during the day. Pots (15.25 cm in diameter) were filled with moist soil (ProMix HP Biofungicide and Mycorrhizae; Premier Tech Horticulture, Quakertown, PA) and planted with five seeds. After germination, pots were thinned to three plants, but due to differences in seed germination, some pots only had one or two plants. Pots were watered daily and were supplemented with fertilizer (Scotts Peters Professional Peat-Lite Special 20-10-20; Scotts-Sierra Horticultural Products, Marysville, OH) twice per week. Each pot served as a single experimental unit, and each treatment was replicated four times.

S. sclerotiorum inoculations and greenhouse screening

All *S. sclerotiorum* inoculations were performed following these methods unless otherwise noted. Prior to inoculation, sclerotia were retrieved from long-term storage. These were stored at 4°C in capped 15-ml polypropylene conical tubes (Becton Dickinson, Franklin Lakes, NJ) within sealed plastic bags containing desiccant packets (Humidity Sponge; Traceable Products, Webster, TX) (Pottinger et al. 2008). Prior to long-term storage, these isolates were grown and harvested from autoclaved carrot discs and allowed to develop to full maturity. Sclerotia from each isolate were retrieved from storage and surface disinfested for 45 s in 95% EtOH, followed by 45 s in a 0.06% sodium hypochlorite solution. Each sclerotium was washed in sterile water, dried, and cut using a sterile scalpel. Pieces of cut sclerotium were inserted into potato dextrose agar (PDA; Thermo Fisher Scientific, Waltham, MA) on Petri plates. Once hyphal growth had been initiated, 5-mm plugs were taken from the leading edge of culture and transferred to thick PDA plates (100 × 25-mm Petri plates). These cultures were allowed to grow until they reached the edge of the plate, which took 72 to 120 h depending on the isolate.

S. sclerotiorum inoculations followed the cut-petiole protocol described by Peltier and Grau (2008). Soybean plants were grown to the V5 growth stage (five open trifoliate leaves; Fehr et al. 1971), and the second trifoliate was cut leaving a 2.5-cm-long petiole. Mycelial plugs were removed from the outer edge of each culture by inserting an inverted 1,000-ml pipette tip. These plugs were then inserted onto the cut petioles so that the mycelial cultures were in close contact with the cut end of the petiole. These inoculations were performed on each plant of each experimental unit.

Unless noted otherwise, all experiments were measured for lesion lengths at 7, 11, and 14 days postinoculation (dpi). At each time point, lesion lengths were measured for each plant within a pot, and these lesion lengths were averaged for a mean lesion length for each experimental unit. The lesion length measurements at each of the three time points were then used to calculate area under the disease progress curve (AUDPC) values (Madden et al. 2007).

Cadophora gregata inoculations and greenhouse screening

Cadophora gregata inoculations were performed at WMGH during the spring of 2021 under similar greenhouse conditions to those mentioned above. The 10 F_8 soybean lines were grown alongside the four soybean check lines and the public line, CorSoy79. Corsoy79 was included as a susceptible check, and Dwight served as the resistant check, as previously demonstrated by Impullitti et al. (2009). Seeds for all tested lines were grown in individual cells of trays (3.8 cm^2 and 5.7 cm deep), and seedlings were allowed to grow until the V1 growth stage (one open trifoliate leaf; Fehr et al. 1971). Due to soybean genotypes differing in their response to either type A or type B isolates of *C. gregata* (Gray 1971), both isolate types were tested in this study. The *C. gregata* type A isolate was originally collected from soybean in 2018 from a plant grown in Livingston, WI, and the *C. gregata* type B isolate was originally isolated from a soybean plant collected in 2018 from Oregon, WI. This study used a 15 × 3 factorial design with all 15 of the assessed soybean genotypes tested against both *C. gregata* isolate types (type A and type B) and a nontreated control. Each treatment combination was replicated four times using a randomized complete block design (RCBD), and only one experimental run was performed.

Both isolates of *C. gregata* were retrieved from long-term storage at −20°C, and isolates were grown in green bean broth amended with streptomycin for 7 to 10 days. Isolate cultures were then blended and added to 0.1% water agar solutions. Inoculations were performed by removing individual plants from the cell trays and gently washing the roots with water. Then, roots were trimmed (1/3 of the root system removed from the end) and allowed to soak in the inoculum solution for 20 min. Two plants of the same treatment were then repotted into 15.25-cm

pots with new, moist soil. Plants were allowed to grow for 6 weeks postinoculation prior to disease assessments. Plants were destructively assessed by splitting the main stems vertically and measuring the length of vascular browning. All soybean genotypes assessed within this experiment demonstrated similar final plant heights. The measurements for each of the two plants in a single pot were averaged, and this value served as one observation. Each treatment was replicated four times, and only one experimental run was performed.

Diaporthe caulivora inoculations and greenhouse screening

Diaporthe caulivora inoculations were conducted in Brookings, SD, at South Dakota State University following the toothpick inoculation method as described by Ghimire et al. (2019). Flat wooden toothpicks (Diamond; Hearthmark, Rye, NY) were first autoclaved and then placed onto the *Diaporthe* culture grown on PDA plates. The plates were then incubated for 15 days at 22°C under 12-h alternating light and dark conditions. After 15 days, the toothpicks colonized by *Diaporthe* were inserted into stem tissue of each soybean plant at a 45-degree angle and around 50 mm below the first trifoliate node. Noninoculated plants were treated with a noninfested toothpick to serve as the control. Each inoculation site was then sealed with petroleum jelly. Plants were then misted for 3 s every 5 min for 3 dpi, and then for 10 s every 3 h until the end of the experiment at 21 dpi. At 21 dpi, disease severity was measured using the following scale: 0 = no lesions, 0.5 = elongated lesions present on the stem (length of lesion was >1 cm compared with noninoculated plants), and 1 = plant death (Benavidez et al. 2010; Campbell 2016; Chiesa et al. 2009; Pioli et al. 2003). Only one experimental run was performed for this assay, with four biological replicates per soybean line.

Parental genotypes and initial crosses

Three soybean genotypes with either moderate or high levels of resistance to SSR were identified from germplasm screenings by McCaghey et al. (2017), including the lines 51-23 (MG: 2.3), SSR51-70 (MG: 2.0), and 52-82B (MG: 2.8). 51-23 and SSR51-70 are both derived from the same initial cross (W04-680 × W04-1002), and 52-82B is derived from the cross of W04-680 × AxN-1-55. All three genotypes were originally derived from the same female soybean genotype, W04-680 (Dwight × PI 567479) with known resistance to soybean cyst nematode caused by *Heterodera glycines* and brown stem rot caused by *C. gregata*. 51-23 and SSR51-70 share the same male parent, W04-1002 (PI 567157A), and the 52-82B male parent is AxN-1-55 (PI 640911; Diers et al. 2006). Despite originating from unique sources, both W04-1002 (Peltier and Grau 2008) and AxN-1-55 exhibit high levels of resistance to SSR.

In the summer of 2016, two crosses were performed in research fields at Iowa State University. The first cross was 51-23 × 52-82B, and this population was designated as 16201. The second cross was SSR51-70 × 51-23, and this population was designated as 16202. In the 16201 population, the female donor was 51-23, and the male donor was 52-82B. In the 16202 population, the female donor was SSR51-70, and the male donor was 51-23.

Early generation selections

F_1 seed was collected from successful crosses and was planted at WMGH in Madison, WI, under greenhouse conditions in the winter between 2016 and 2017. Plants were allowed to mature, and the F_2 seeds were collected at harvest. As it was previously demonstrated that the heritability of SSR resistance can differ between environments (Kim and Diers 2000), three differing environments were used to screen for F_2 SSR resistance. These environments included field conditions at Arlington Agricultural Research Station (ARS) in Arlington, WI; field conditions at Hancock Agricultural Research Station (HARS) in Hancock, WI; or greenhouse conditions at WMGH. ARS field locations were composed of a silt-loam soil type, and HARS field locations were compose of a sandy soil type. ARS is the more southern location, with Hancock being located approximately 110 km north of ARS.

At both ARS and HARS, F_2 seed was bulk planted under field conditions. At the end of the season for each location, 30 individual plants were selected from each population based on the following observations: the absence of SSR infection, a high amount of lateral branching, short internodes, and a high pod count. Lateral branching is a highly desirable trait in soybean as it allows for the capacity to compensate for missing plants in poor stand establishments, leading to greater yield stability (Agudamu et al. 2016). Short internode lengths are an important trait in soybeans as they result in shorter plant height and increased resistance to lodging (Oki et al. 2018). At WMGH, F_2 plants were inoculated following cut-petiole inoculations (Peltier and Grau 2008) with *S. sclerotiorum* isolate #20, previously determined to be highly aggressive on soybeans (Webster et al. 2021; Willbur et al. 2017). Lines that did not develop girdling lesions due to *S. sclerotiorum* infection were progressed to maturity at WMGH, and F_3 seed was collected. Each population name was then given a number to designate the environment in which F_2 selections were made, representing subpopulations. The subpopulation identifications are as follows: ARS = 1, HARS = 2, and WMGH = 3 (example: 201-3 = 51-23 × 52-82B, selected at WMGH in F_2). All selected lines are described by their subpopulation identification in Supplementary Table S2.

F_3 seed for each subpopulation was sent to Rancagua, Chile, between 2017 and 2018 for bulk increase. A total of 60 plants were selected for each subpopulation, and seed was harvested and bulked. The F_4 seed was then sent back to Wisconsin, and each subpopulation was planted at HARS in the summer of 2018. Around 50 individual plants were selected from each subpopulation based on the absence of SSR infection, high amount of lateral branching, short plant height, and high pod count.

The resulting F_5 seed from each selected plant then represented individual families. Each line was then given a new number, which indicated the family (example: 201-3-21 = the 21st plant selected from the subpopulation 201-3). The roughly 300 families were grown and increased in Chile between 2018 and 2019. Selections were made by assessing for favorable agronomic traits such as low lodging, high number of lateral branches, and high pod count. Three to five plants were selected from the most agronomically favorable families, and these selections represented individual lines.

In total, 501 F_6 individual lines were returned to Wisconsin and planted at ARS in 2019. Between the R1 (one open flower at any node; Fehr et al. 1971) and R2 (one open flower at one of the two uppermost nodes; Fehr et al. 1971) growth stages, the flower color of each line was recorded, and at the end of the season, lines were assessed for the date of maturity on a weekly basis. Once all lines had reached full maturity, lines were assessed for agronomic traits including lodging (less than a 45-degree lean), a branching threshold of at least three lateral branches, and plant height. Only lines with a maturity group of III or less were progressed. Based on these agronomic traits, the 160 best-performing lines were selected.

Screening F_7 generation

In the spring of 2020, 25 of the 160 F_7 lines were selected as the most agronomically favorable lines, having demonstrated no lodging and the greatest number of lateral branches during the previous season. Line W19-1321 was noted for segregating for a yellow and a black seed coat, and these were separated and treated as individual lines. The resulting 26 lines were screened for resistance to *S. sclerotiorum* isolate #20. Greenhouse SSR screening assays were performed at WMGH, but due to limited greenhouse space, the 26 lines needed to be screened as two independent groups. The first group consisted of 14 breeding lines, and the second group consisted of 12 breeding lines. Both groups were tested alongside four standardized soybean check lines described below to accurately assess resistance levels. Within each group, all genotypes were replicated four times, following an RCBD, and only one experimental run was performed for each group.

In the summer of 2020, the 25 F_7 lines were grown under field conditions at ARS and at HARS. Only the seed of the black-coated W19-1321 line was included in all further screenings due to limited seed supply of the yellow-coated W19-1321. The four check lines were not included in these trials. Each line was planted in two-row plots with a 76-cm row spacing for a length of 6.1 m and a width of 1.5 m. Each line was replicated four times, following an RCBD, at each location. Due to unfavorable planting conditions at ARS (wet and cold), plots were severely impacted by seedling diseases and final plant stands were significantly reduced. SSR only developed at HARS, and measurements were taken following the protocol described below. Additionally, frogeye leaf spot and Cercospora leaf blight developed at ARS, and anthracnose developed at HARS. These diseases were assessed following the protocols listed below. Flower colors were recorded between the R1 and R2 growth stages, and prior to harvest, agronomic characteristics such as pubescence color, lodging scores, branching, and plant height were measured following the protocols listed below. Plots were harvested using a small plot harvester, and yields were calculated after standardizing to 13% moisture. After all data had been collected, these 25 soybean lines were selected for high levels of SSR resistance and favorable agronomic traits. A total of 10 F_8 soybean lines were selected and chosen to be screened in the next generations.

Screening F_8 generation

In the winter between 2020 and 2021, the 10 selected F_8 soybean lines were again screened for resistance to *S. sclerotiorum* under greenhouse conditions, following the same protocol as described below. Each line was replicated four times, following an RCBD, and two experimental runs were performed. Following inoculations, lesion lengths were measured at 7, 11, and 14 dpi in the first experimental run and 5, 7, and 9 dpi in the second experimental run. The resulting AUDPC values were divided by the number of days at the last measurement to determine a standardized AUDPC as described by Madden et al. (2007). Additionally, the 10 F_8 lines were screened for resistance to *C. gregata* (causal agent of brown stem rot) and *D. caulivora* (causal agent of stem canker) under greenhouse conditions following the protocols described below.

In the summer of 2021, the 10 F_8 lines were planted at ARS and HARS alongside the four check lines. All 14 lines were planted as four-row plots with a 76-cm row spacing and a length of 6.1 m. Each line was replicated four times at each location and grown in an RCBD. SSR developed at HARS and was assessed following the previously discussed protocol. At both ARS and HARS, the dates of full maturity were recorded, and upon full maturity, agronomic traits (branching, lodging, and plant height) were recorded following the protocol described below. At harvest, each plot was measured for yield, and grain subsamples were taken from each plot. Each subsample was tested for quality traits such as the percentage of protein and oil using an Inframatic 9500 grain near infrared analyzer meter (Perten Instruments, Stockholm, Sweden). Protein and oil were measured by taking the average of 10 independent measurements. Hundred-seed weight was recorded by taking the average of three independent measurements.

Screening F_9 generation

In the spring of 2022, the three selected F_9 elite lines were screened in the greenhouse against a panel of eight diverse *S. sclerotiorum* isolates (#3, 10, 15, 19, 20, 30, 47, and 60) with varying levels of aggressiveness (Willbur et al. 2017). The soybean genotypes, Dwight and 52-82B, were included in these trials to serve as experimental controls. Two experimental runs were performed, with four replications in each run. In the first experimental run, isolate #30 was not assessed due to viability issues. However, isolate #30 was included in the second run. Inoculations were performed using the cut-petiole inoculation technique described below.

Statistical analysis

All data were analyzed using generalized linear mixed models with the GLIMMIX procedure in SAS (v 9.4; SAS Institute, Cary, NC) unless noted otherwise. Individual experiments and their respective generalized linear mixed models are described below. Differences between treatment means for significant main effects and interactions were determined by Fisher's least significant difference ($\alpha = 0.05$) using the 'mult' macro (Piepho 2004). All logarithmic transformed treatment means and standard errors were back-transformed using the omega method (Michelle Edwards 2017).

F_7 field trial

These trials were analyzed using SSR DIX, frogeye leaf spot severity (%), Cercospora leaf blight severity (%), and anthracnose severity (%) as the response variables. Soybean genotypes were treated as the fixed effects, and experimental replicate was a random effect in the model. Due to each disease only being identified at one of the two locations, there were no environmental effects assessed.

F_8 field trial

These trials were analyzed using SSR DIX, yield, branching, plant height, oil content, protein content, and hundred-seed weight as the response variables. This analysis designated soybean genotype, environment, and their interaction as the fixed effects and experimental replicate nested within the environment as the random effect. Normality of each response variable was confirmed, with SSR DIX requiring a logarithmic transformation to meet the assumptions of normality. SSR DIX was then back-transformed for visualization using the omega method. Due to lodging being rated on an ordinal scale, these data were analyzed using a Friedman test in RStudio (v. 4.1.2, R. RStudio, Boston, MA), where the soybean genotype was set as the response variable, and experimental replicate nested within the environment was set as the blocking factor.

F_7 greenhouse SSR screening

These assays were composed of two distinct subgroups of breeding lines, and each group was analyzed separately. Both analyses consisted of AUDPC as the response variable, soybean genotype as the sole fixed effect, and experimental replicate as the random effect. Both groups required a logarithmic transformation to confirm normality.

F_8 greenhouse SSR screening

These assays were analyzed using the standardized AUDPC as the response variable and soybean genotype as the fixed effect, and the random effects included the experimental run and replicate nested within a run. Data were confirmed to be normally distributed without a transformation.

F_9 greenhouse SSR screening

Due to an unbalanced design of the experiments because of differing numbers of *S. sclerotiorum* isolates, each of the two experimental runs was analyzed separately. Both runs were modeled similarly, with AUDPC set as the response variable; the fixed effects including soybean genotype, *S. sclerotiorum* isolate, and their interactions; and the random effect being the replicate. Normality was confirmed for the first run without the need for a transformation, and the second run required a logarithmic transformation.

F_8 greenhouse SSR screening

This assay was analyzed using the mean lesion length as the response variable. The fixed effects included the soybean genotype, the *C. gregata* isolate type, and their interactions, and the only random effect was replicate. Data were confirmed to be normally distributed without the need for a transformation. Due to the lack of lesion formation on the noninoculated plants, disease rating data were not included in this analysis.

RESULTS

F_7 generation SSR screening

In the winter between 2019 and 2020, the 25 most agronomically favorable breeding lines were selected to be screened for resistance to *S. sclerotiorum*. From two greenhouse experiments, soybean genotypes differed in their development of SSR. Eleven of the 14 breeding lines from the first group had greater resistance levels compared with the susceptible check, Dwight ($P = 0.03$; Fig. 1A), and 10 of the 12 breeding lines from the second group had improved resistance ($P < 0.01$; Fig. 1B).

To investigate field resistance, the 25 soybean lines were grown under field conditions during the summer of 2020. From this field trial, 16 of the breeding lines exhibited high levels of resistance to *S. sclerotiorum* ($P < 0.01$; Fig. 1C). However, eight lines had similarly high levels of susceptibility (Fig. 1C). From both greenhouse and field trials, 10 breeding lines were selected based on high levels of resistance to SSR for testing in the F_8 generation.

F_8 generation SSR screening

The 10 selected F_8 lines were again screened for resistance to *S. sclerotiorum* under both greenhouse and field conditions. Experiments under greenhouse conditions resulted in four soybean lines displaying high levels of resistance, such as 52-82B, the resistant check ($P < 0.01$; Fig. 2A). Conversely, one soybean line, W19-2409, was as susceptible as Dwight, the susceptible check (Fig. 2A). Under field conditions, the check line SSR51-70 resulted in the lowest levels of SSR, and four breeding lines were similarly resistant ($P < 0.01$; Fig. 2B). The breeding lines W19-1331 and W19-2409 were as susceptible as Dwight (Fig. 2B), which was consistent with the previous greenhouse screening results (Fig. 2A).

In addition to SSR data, agronomic and quality traits (branching, lodging, plant height, hundred-seed weight, protein content, oil content, and yield) were also assessed under field conditions. All agronomic and quality traits were influenced by soybean genotype ($P < 0.01$). Interactions between genotype and the two environments were observed for plant height ($P = 0.03$), hundred-seed weight ($P = 0.01$), protein content ($P < 0.01$), oil content ($P < 0.01$), and yield ($P < 0.01$). Two breeding lines had the highest levels of branching (W19-1321 and W19-1273), with plot means around two lateral branches for each individual plant (Supplementary Table S3). Three breeding lines exhibited low levels of lodging (W19-1190, W19-1191, and W19-2484), with mean lodging scores below 2 (Supplementary Fig. S2). The breeding line W19-1273 had the highest protein content in both environments, and W19-2379 had similarly high levels at HARS (Supplementary Table S3). When considering oil content, W19-2409 had the highest levels of the breeding lines (Supplementary Table S3). The four highest-yielding breeding lines at ARS were W19-1190, W19-1191, W19-1321, and W19-2484 (Supplementary Table S3), and the highest-yielding lines at HARS were W19-1273 and W19-1321 (Supplementary Table S3).

F_9 generation SSR screening

Three of the most elite breeding lines were selected for further screenings in the F_9 generation (W19-1190, W19-1321, and W19-2484). These three lines were then subjected to SSR resistance assays to multiple unique isolates of *S. sclerotiorum*, representing possible isolates that can be found across the soybean-growing region. Each experimental run was analyzed separately due to differences in the number of isolates used between experimental runs. The first experimental run used seven *S. sclerotiorum* isolates, and the analysis revealed a significant interaction between soybean genotypes and isolate ($P < 0.01$; Fig. 3A). Overall, the three soybean breeding lines demonstrated either resistance levels similar to that of 52-82B (W19-1321 and W19-2484) or improved resistance (W19-1190; Fig. 3A). The second experimental run used eight *S. sclerotiorum* isolates, and the analysis identified significant effects due to both soybean genotype ($P < 0.01$; Fig. 3B) and isolate ($P < 0.01$; Fig. 3C). All three soybean breeding lines had high levels of resistance, with W19-1321 being similarly resistant when compared with 52-82B.

Additional disease screenings

When screening the F_7 generation in field conditions, additional diseases screened included Cercospora leaf blight, frogeye leaf blight, and anthracnose. All three diseases were found to be influenced by soybean genotypes ($P < 0.01$, Supplementary Figs. S2, S3, and S4). When screening the F_8 generation for resistance to *C. gregata*, soybean genotypes differed in their disease response dependent on the fungal type (either type A or type B, $P = 0.03$, Supplementary Fig. S5). Furthermore, screenings for resistance to *D. caulivora* using the protocol reported by Ghimire et al. (2019) demonstrated that all 10 of the F_8 breeding lines were highly susceptible (Supplementary Table S4).

DISCUSSION

Breeding for resistance to SSR has been difficult to achieve due to the highly quantitative nature of this type of resistance (Arahana et al. 2001; McCaghey et al. 2017; Vuong et al. 2008). Despite challenges, breeding efforts have made strides to improve resistance compared with susceptible lines and widely used commercial varieties. The study presented here builds on previous breeding and research efforts to develop three novel soybean germplasm lines with high levels of resistance to SSR. Furthermore, this research examined the combination of two established methods for screening soybean breeding lines for resistance to SSR throughout multiple generations.

Breeding efforts for SSR resistance have been slow and burdensome due to a lack of efficient methods for evaluating soybean germplasm and breeding populations (Antwi-Boasiako et al. 2021). Many methods have been previously studied for assessing soybean genotypes for resistance to SSR (Kim et al. 2000; Kull et al. 2003; Peltier and Grau 2008), but all methods are quite laborious and require large amounts of space. By identifying a targeted approach to screen such soybean lines, assessing large populations could be more efficient and effective at identifying lines with high resistance.

The research presented here demonstrates the development of three highly resistant soybean genotypes by using two previously developed standardized screening panels of both soybean and *S. sclerotiorum* isolates. By combining both panels into one breeding pipeline, soybean lines will be assessed for resistance to SSR across multiple generations in an efficient and targeted approach. The three elite soybean genotypes selected at the completion of this study (W19-1190, W19-1321, and W19-2484) consistently resulted in low levels of SSR development in both greenhouse and field studies (Figs. 1, 2, and 3). The importance of screening for SSR resistance under both conditions assesses a soybean line's physiological and field resistance, which has been shown to differ (Kim and Diers 2000; Kim et al. 1999; Nelson et al. 1991). For example, the line SSR51-70 displays moderate resistance under greenhouse conditions, whereas it displays the highest relative

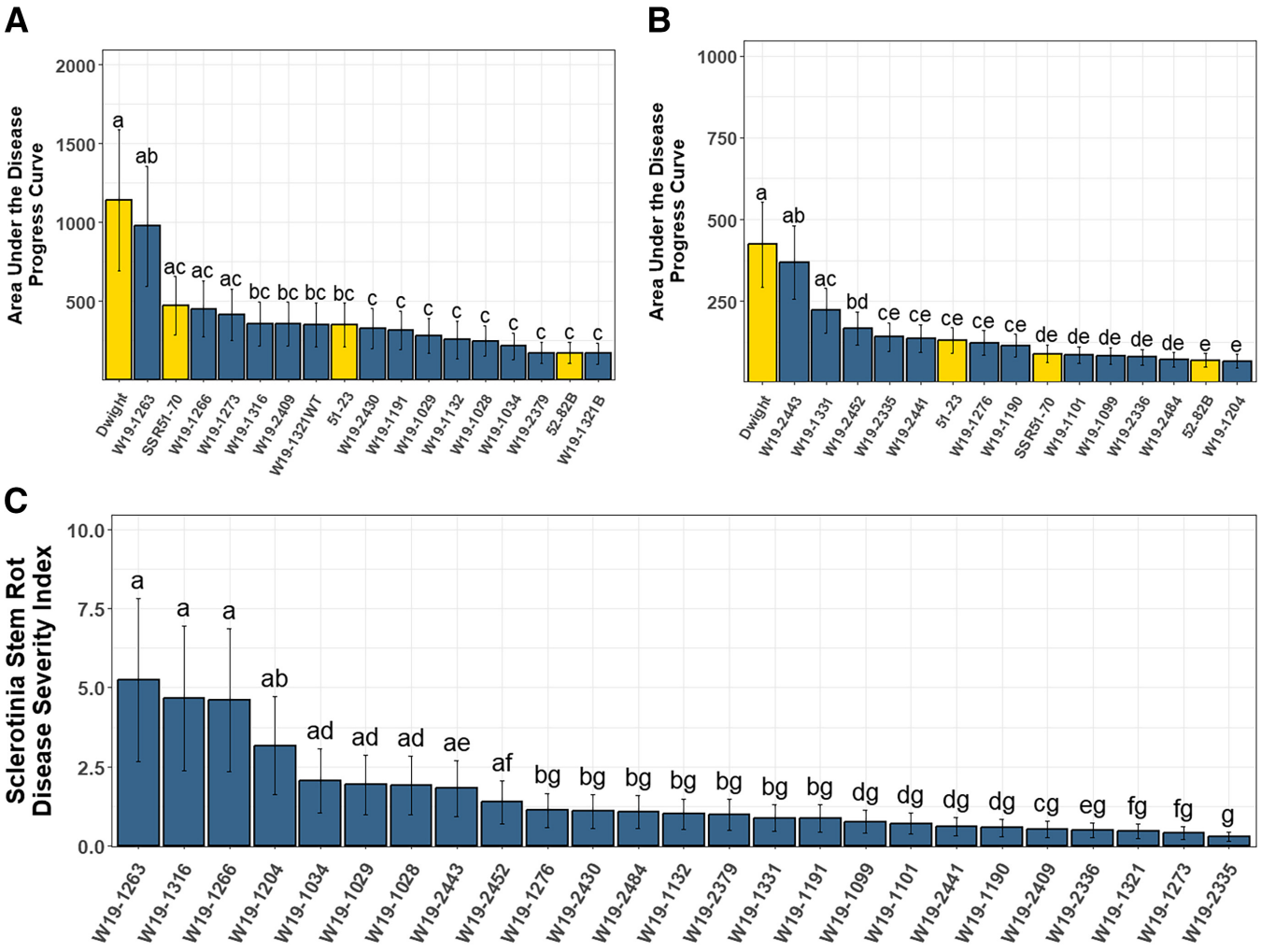

FIGURE 1
Screening F_7 soybean breeding lines for resistance to *Sclerotinia sclerotiorum* under both **A and B,** greenhouse conditions in Madison, WI, between 2019 and 2020 and **C,** field conditions in Hancock, WI, in 2020. Greenhouse trials were conducted by inoculating A, 14 and B, 12 F_7 soybean breeding lines and four soybean check lines with standardized resistance levels to *S. sclerotiorum* with a single isolate of *S. sclerotiorum* ($n = 4$). Blue bars represent the breeding lines, and gold bars represent the four check lines. Area under the disease progress curve values were calculated for each treatment based on three lesion length measurements taken at 7, 11, and 14 days postinoculation. The field trial assessed the disease severity index of natural infections of Sclerotinia stem rot on 25 F_7 soybean breeding lines ($n = 4$). Error bars represent the standard error of the mean. Soybean lines sharing the same letter do not statistically differ as determined by Fisher's least significant difference ($\alpha = 0.05$).

resistance under field conditions. This could be explained by the earlier relative maturity of this line, allowing the soybeans to escape infection due to a shorter flowering period, the stage when soybeans are most susceptible to SSR infection.

In addition to all three elite soybean lines having high levels of SSR resistance, these three lines were highly resistant to lodging and yielded moderately high in the Arlington, WI, location in 2021 (Supplementary Fig. S1; Supplementary Table S3). However, at the Hancock, WI, trial in 2021, weed pressure was severe, and yields of W19-1190 and W19-2484 were low, suggesting that these two lines are poor competitors against weeds. This could be due to the low branching potential of these two lines. Conversely, W19-1321 yielded moderately in Hancock, WI, in 2021, potentially a result of the greater branching and thus greater competition against weeds (Supplementary Table S3). Despite these three lines all sharing high levels of SSR resistance and important agronomic traits, these lines are also unique in many distinct ways.

W19-1190 is a yellow-seeded soybean with a clear hilum, and, therefore, this line shows potential as a food-grade soybean for human consumption. However, due to low protein levels in this line (Supplementary Table S3), it may also be suited for an oilseed-type soybean. This line was also found to have the highest level of resistance to frogeye leaf spot (Supplementary Fig. S3) and high levels of resistance to *C. gregata* type A (Supplementary Fig. S5A). However, this line was found to be highly susceptible to anthracnose (Supplementary Fig. S2), *C. gregata* type B (Supplementary Fig. S5B), and *D. caulivora* (Supplementary Table S4).

W19-1321 is a black-seeded soybean with a black hilum and shows potential as a specialty food-grade soybean, specifically for East Asian markets. This line was also found to have high levels of resistance to anthracnose (Supplementary Fig. S2), Cercospora leaf blight (Supplementary Fig. S4), and both types of *C. gregata* (Supplementary Fig. S5) but only moderate levels of resistance to frogeye leaf spot (Supplementary Fig. S3). Conversely, this line demonstrated high susceptibility to *D. caulivora* (Supplementary Table S4).

W19-2484 is a yellow-seeded soybean with a black hilum and shows potential as an oilseed-type soybean for oil and animal feed. This line was also found to have moderate resistance to Cercospora leaf blight (Supplementary Fig. S4) and *C. gregata* type B (Supplementary Fig. S5B) but was found to have susceptibility to anthracnose (Supplementary Fig. S2), frogeye leaf spot (Supplementary Fig. S3), *C. gregata* type A (Supplementary Fig. S5A), and *D. caulivora* (Supplementary Table S4).

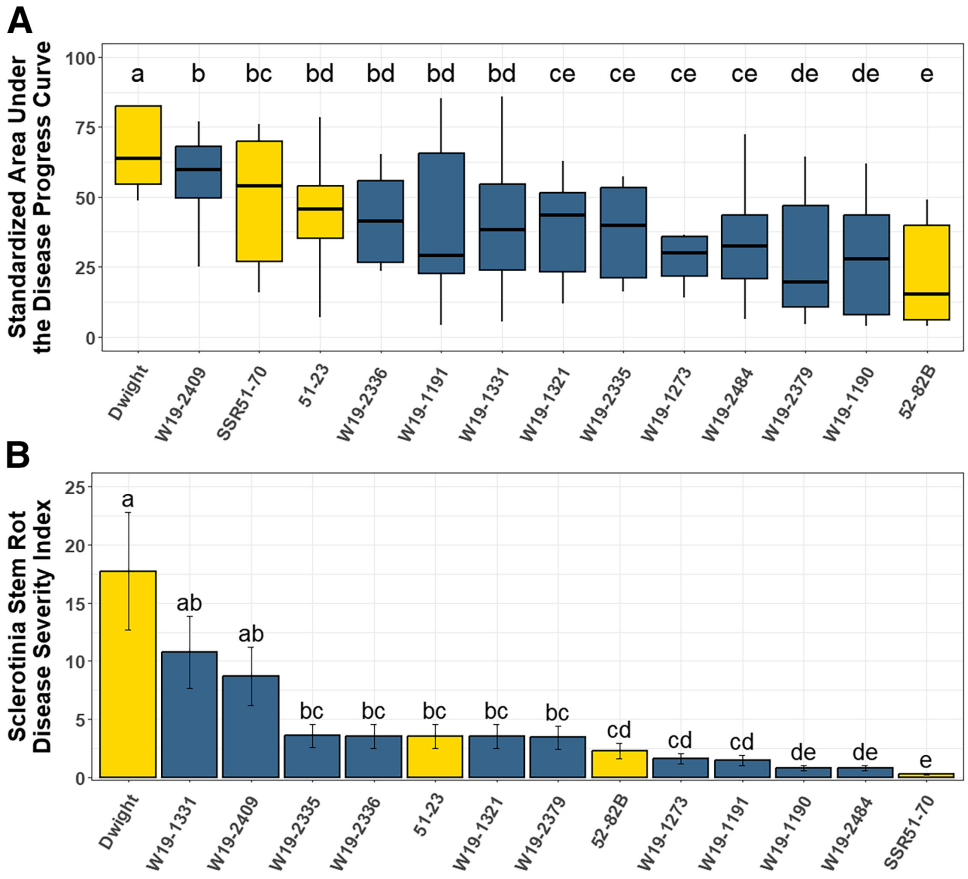

FIGURE 2

Screening F_8 soybean breeding lines for resistance to *Sclerotinia sclerotiorum* under **A,** greenhouse conditions in Madison, WI, between 2020 and 2021 and **B,** field conditions in Hancock, WI, in 2021. Greenhouse trials were conducted by inoculating soybean breeding lines and four soybean check lines with standardized resistance levels to *S. sclerotiorum* with a single isolate of *S. sclerotiorum* ($n = 8$). Blue bars represent the breeding lines, and gold bars represent the four check lines. Standardized area under the disease progress curve values were calculated for each treatment based on three lesion length measurements at three independent time points. The field trial assessed the disease severity index of natural infections of Sclerotinia stem rot on 10 F_7 soybean breeding lines and four soybean check lines ($n = 4$). Whiskers from the box and whisker plots represent the upper and lower quartiles, and error bars represent the standard error of the mean. Soybean lines sharing the same letter do not statistically differ as determined by Fisher's least significant difference ($\alpha = 0.05$).

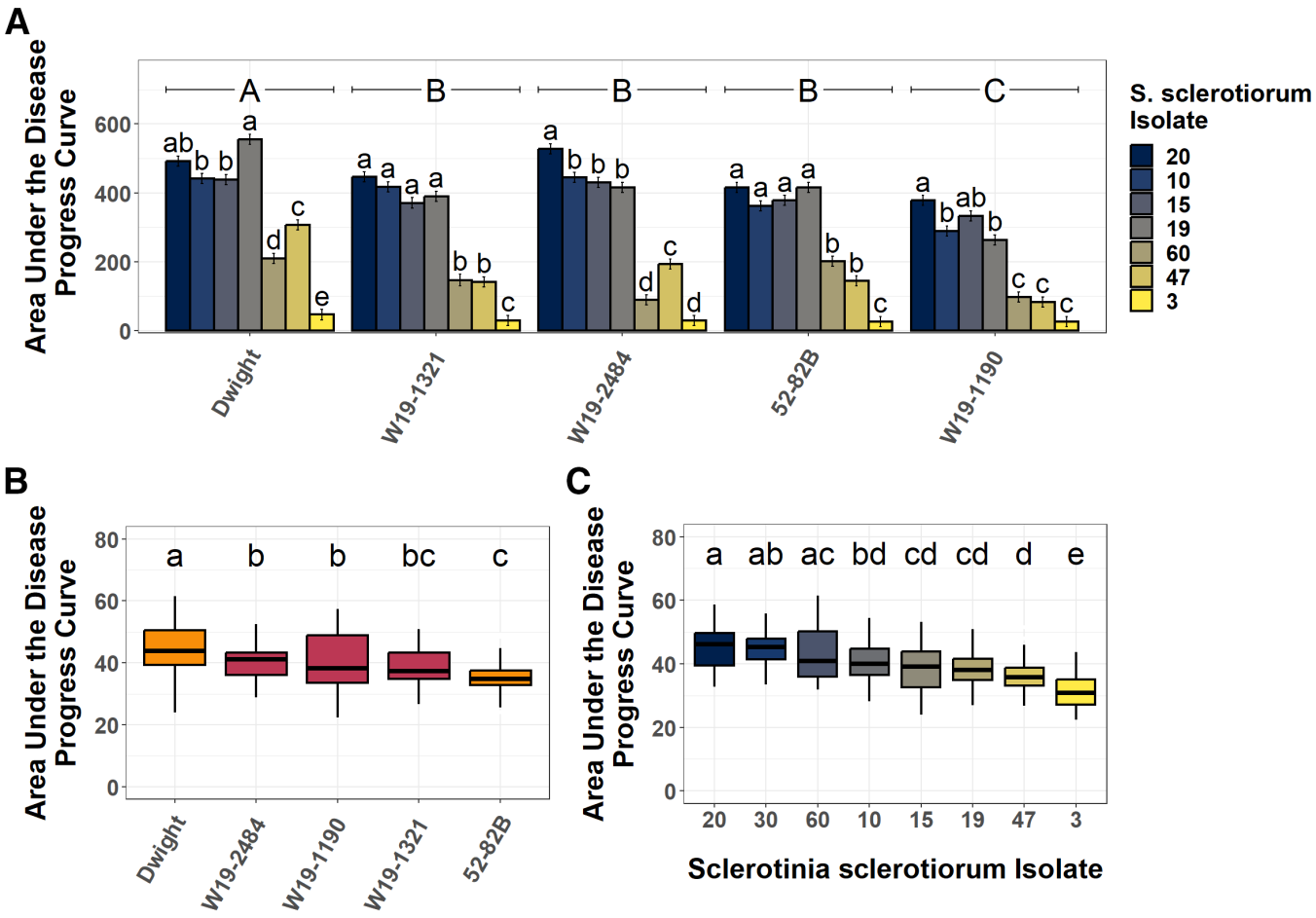

FIGURE 3

Three F_9 soybean breeding lines and two soybean check lines with standardized resistance levels to *Sclerotinia sclerotiorum* were screened for resistance to *S. sclerotiorum* under greenhouse conditions in Madison, WI, during the spring of 2022. **A,** The first experiment screened these five soybean lines against seven *S. sclerotiorum* isolates ($n = 4$), and **B and C,** the second experiment screened these five soybean lines against eight *S. sclerotiorum* isolates ($n = 4$). B, Red boxes represent the three breeding lines, and orange boxes represent the two check lines. In the first experiment, soybean lines sharing similar uppercase letters do not statistically differ as determined by Fisher's least significant difference ($\alpha = 0.05$), and *S. sclerotiorum* isolates sharing the same lowercase letter within each soybean line do not statistically differ as determined by Fisher's least significant difference ($\alpha = 0.05$). Error bars represent the standard error of the mean. B and C, In the second experiment, soybean lines or *S. sclerotiorum* isolates sharing the same letter do not statistically differ as determined by Fisher's least significant difference ($\alpha = 0.05$). Whiskers from the box and whisker plots represent the upper and lower quartiles.

It should also be noted that line W19-1191, a sibling line to W19-1190, had high levels of SSR resistance through all screenings in the F_7 and F_8 generations and high yields in Arlington, WI, in 2021 (Supplementary Table S3). However, due to the mixed seed hilum color (gray and clear) within the seed supply, this line was excluded from the F_9 SSR screenings, but it will be assessed in the future due to the high levels of resistance displayed throughout this study (Figs. 1 and 2).

Breeding for disease-resistant crops is of utmost importance for protecting yield and reducing the quantity of pesticide inputs in production systems. Through the combination of two distinct screening panels, two soybean breeding populations were assessed for SSR resistance across multiple generations. This work resulted in the development of three soybean lines with high levels of resistance to SSR, as well as favorable agronomic traits. These lines have potential to be released as public cultivars for regional producers to utilize in their production systems, or they could serve as parental germplasm in future breeding endeavors. Through the development of improved resistance screening methods and collaborative soybean breeding efforts, both plant pathologists and plant breeders will be better equipped in the future to prevent yield losses to diseases such as SSR and protect soybean crops.

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