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Genome-Wide Association Study Reveals Marker–Trait Associations with Resistance to *Pythium irregulare* from Soybean Germplasm

Christopher Detranaltes [1], Jianxin Ma [1,2] and Guohong Cai [1,3,*]

[1] Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA
[2] Department of Agronomy, Purdue University, West Lafayette, IN 47907, USA
[3] Crop Production and Pest Control Research Unit, Agricultural Research Service, United States Department of Agriculture, West Lafayette, IN 47907, USA
* Correspondence: guohong.cai@usda.gov

Abstract: Soybean (*Glycine max* (L.) Merr.) ranks as the second-largest crop by total production in the United States, despite its production experiencing significant constraints from plant pathogens, including those causing seedling diseases. *Pythium irregulare* Buisman stands out as a predominant driver of yield loss associated with the seedling disease complex. There is currently a lack of public or commercial varieties available to growers with adequate genetic resistance to manage this pathogen. To address the pressing need for germplasm resources and molecular markers associated with *P. irregulare* resistance, we conducted a screening of 208 genetically diverse soybean accessions from the United States Department of Agriculture Soybean Germplasm Collection (USDA-SGC) against two geographically and temporally distinct isolates under controlled greenhouse conditions. Disease severity was assessed through comparisons of the root weight and stand count ratios of inoculated plants to mock-inoculated controls. Employing linear mixed modeling, we identified ten accessions (PI 548520, PI 548360, PI 548362, PI 490766, PI 547459, PI 591511, PI 547460, PI 84946-2, PI 578503, FC 29333) with resistance significantly above the population average to one or both of two isolates originating from Ohio or Indiana. Previously curated genotyping data, publicly accessible via the SoyBase database, was subsequently utilized for conducting a genome-wide association study. This analysis led to the discovery of two significant marker–trait associations (MTAs) located on chromosomes 10 and 15 and accounting for 9.3% and 17.2% of the phenotypic variance, respectively. The resistant germplasm and MTAs uncovered through this study provide additional resources and tools for the genetic improvement of soybean resistance to seedling disease caused by *P. irregulare*.

Keywords: soybean seedling disease; pythium irregulare; host resistance; genome-wide association study

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1. Introduction

Soybean (*Glycine max* (L.) Merr.) is a crucial source of the world's supply of protein and oil for human consumption, livestock feed, and industrial purposes. The global production of soybean faces a persistent threat from plant pathogens, including those causing seedling diseases, which severely limit yield in the United States [1–4]. Seedling diseases are caused by multiple pathogenic species from the genera *Pythium*, *Fusarium*, *Rhizoctonia*, and *Phytophthora*. Effectively managing pathogenic species within these four prominent genera necessitates distinct approaches, particularly when choosing appropriate chemical controls and resistant varieties. This, coupled with both the need for and difficulty of diagnosing which pathogenic species is the predominant causative agent on a case-by-case basis, poses a challenge to achieving consistent and dependable disease management.

The current best practices for seedling disease management largely depend on improving soil drainage and the application of large-scale preventative seed treatments when disease pressure is forecast to be high, particularly during wet seasons [5]. Concerns about the impacts of agrichemicals on human and environmental health have led to growing consumer concern about large-scale applications of preventative pesticides [6]. Furthermore, the discovery of fungicide insensitivity observed amongst major drivers of seedling diseases in soybean [7–10], as well as the associated financial costs to growers, threatens the sustainability of depending on pesticides for disease management going into the future. Currently, resistant varieties are unavailable for many of the predominant pathogens in the seedling disease complex. Without suitable alternatives for disease management, continued and potentially worse yield and profit losses attributable to seedling diseases should be expected across soybean production regions.

Seedling diseases caused by *Pythium* spp. are a multifaceted disease management challenge, due to the many causative pathogenic species in this genus each with unique fungicidal sensitivities and interactions with resistant varieties [11,12]. Within this genus, *Pythium irregulare* is an especially significant threat to soybean seeds and seedlings and is globally distributed. In addition to causing disease in soybean, *P. irregulare* has demonstrated pathogenicity in corn and wheat, crops commonly rotated with soybean, especially in the North Central USA [13,14]. The prevalence of *P. irregulare* as a predominant driver of soybean seedling disease was noted as far back as 1982 in a soybean–oat rotation under both conventional and minimum tillage [15]. Numerous publications have since corroborated both the prevalence and aggressiveness of *P. irregulare* in soybean production regions across North and South America [11–13,16–18], where seedling diseases have been especially problematic [1–4,19–21]. *P. irregulare* has been shown to thrive in poorly drained to flooded, sour silt soils with low organic matter and high concentrations of magnesium [22,23]. Strains with reduced sensitivity to azoxystrobin, trifloxystrobin, mefenoxam [8,13], and oxathiapiprolin [9] have been reported, suggesting potential for the development of fungicide resistance in field populations.

Adding to the complexity of managing *P. irregulare* is the genetic variation that has been observed within the species, leading to the division of several intra-specific groupings [24]. At least two of these groups have been shown to differ in growth rate; however, both have the same demonstrated growth range of 12 to 36 °C, with an optimal growth temperature of 27 °C and exhibit variations in aggressiveness on an isolate-by-isolate basis [25]. The relative impact this genetic variation has on fungicide sensitivity and the selection of resistant host varieties remains to be determined.

Variation in soybean germplasm resistance to *P. irregulare* has been previously reported. In comparisons between the cultivars 'Archer' and ' Hutcheson', 'Archer' was shown to be more resistant to *P. irregulare* based on visual disease rating scores [26]. Partial resistance was subsequently observed in 'Archer' in a screening of 102 major ancestors of North American cultivars for plant stand and shoot and dry root weight ratios compared to non-inoculated controls [27]. Additionally, PI 84637, 'Maple Isle', 'Fiskeby III', and 'Fiskeby 840-7-3' were identified as partially resistant. A screening of six North American pure lines revealed that 'Archer', 'Maple Isle', 'Maple Glen', 'Conrad', 'Sloan', and 'Williams' showed varying levels of resistance and significant isolate interactions by accession on measures of germination, root, and total weights when challenged with *P. irregulare* [28]. Nine pure lines adapted to southern Brazil were identified with partial resistance to *P. irregulare* (referred to in the cited text as syn. *Globisporangium irregulare*) using visual severity, total dry matter mass, and emergence measures compared to the controls [29]. Lastly, a screening of 65 diverse soybean pure lines, including 'Archer', revealed that PI 424354 had the highest mean root weight compared to the controls and the lowest visual severity of the total population [30].

Several quantitative trait loci (QTL) have been mapped for *P. irregulare* resistance from resistant germplasm [30–35]. Ellis et al. [30] identified six QTL across chromosomes 1, 6, 8, 10, 11, and 13, contributing between 4.4 and 17.8% of the variation in standardized root weights and visual severity after inoculation with *P. irregulare*. Stasko et al. [35] uncovered two QTL from chromosomes 14 and 19, explaining 5.5–6.6% of the variation in standardized root weights. Additionally, Scott et al. [34] mapped 12 suggestive QTL, explaining variation in visual severity, percent gemination, and standardized root weight scores across chromosomes 1–5, 10, 11, 13, and 16–18. Importantly, the three aforementioned studies used the same isolate, Br 2-3-5, and greenhouse screening procedures across separate recombinant inbred line (RIL) populations. Two publications also mapped three separate QTL on chromosomes 11, 16, and 20, explaining 5.72–15.4% of variation in standardized root weights from separate RILs generated from Michigan breeding lines [32,33]. Finally, Clevinger et al. [31] mapped five QTL from chromosomes 1, 5, 6, 8, and 11 explaining between 4.8 and 26.6% of the variation in visual severity and percentage of rotted seeds in a Petri plate assay. The consistent absence of major gene immune-type interactions and the lack of QTL consensus across studies suggests that resistance to *P. irregulare* is likely to be quantitative in nature and scattered across multiple genetic backgrounds.

Despite the identification of these QTL, public and commercial varieties have yet to be developed with genetic resistance to *P. irregulare*, likely owing to the difficulty and limited return of breeding for small-effect QTL as compared to major genes and large-effect QTL. An alternative approach involves utilizing marker-assisted selection to simultaneously target multiple marker–trait associations (MTAs) at once. This approach may lead to cumulative effects significant enough to justify breeding efforts. Thus, advancing our understanding of genetic resistance against *P. irregulare* through the characterization of resistance sources from within public germplasm networks and the identification of markers linked with resistance loci remains important and warrants continued efforts. This study aimed to achieve two primary objectives: (a) assess phenotypic resistance to *P. irregulare* in a diverse panel of soybean accessions originating from international sources, including landraces, cultivars, and elite varieties (hereafter accessions), suitable for growth in the North Central USA; and (b) conduct a genome-wide association study (GWAS) to pinpoint molecular markers associated with the observed resistance. We first screened 208 soybean accessions for resistance against two *P. irregulare* isolates using a greenhouse assay. Following this, we executed a genome-wide association study (GWAS) by merging our phenotypic data with single-nucleotide polymorphism (SNP) genotyping information from SoyBase, publicly available as of August 2024 (www.soybase.org).

2. Materials and Methods

2.1. Plant Material

The germplasm chosen for resistance screening against *P. irregulare* included 207 accessions of *G. max* and a single *G. soja* accession (total 208) selected from the USDA-ARS Germplasm Resources Information Network (GRIN, https://npgsweb.ars-grin.gov/ accessed on 13th August 2024)) from maturity groups 0-VI. These accessions encompassed multiple geographic origins and were selected with the aim of maximizing the diversity of their genetic backgrounds (Supplementary Table S1). The accession PI 424354 was previously identified as resistant to *P. irregulare* and exhibited a stronger phenotypic resistance than the previously identified resistant check 'Archer' [30]. PI 424354 served as a benchmark throughout this study to allow for comparison of the relative resistance of our accessions to a known resistant line in a single set of standardized experimental conditions. The accessions PI 548631 (cv. 'Williams') and PI 525453 (cv. 'Conrad') were selected as susceptible checks because of their relative phenotypic susceptibility reported in previous publications [28,30]. Seeds were requested through the GRIN service and subsequently increased in the field in 2020 at the Purdue University Agronomy Center for Research and Education. The plants were manually harvested, and each accession was processed using a

single-plant belt thresher, ensuring thorough cleaning between each batch. The seeds were kept in cold storage set to 5 °C until they were utilized in the resistance screenings.

2.2. Inoculum Preparation

Two isolates of *P. irregulare* were obtained for the screening for resistance. Isolate Br 2-3-5, which has been used in several previous studies, was baited from a clay loam soil collected from Brown County, OH, USA, in 2006 [22,30,34,35]. Isolate SE21_0607_01 was collected from a soybean seedling sample from the Southeast Purdue Agricultural Center of Purdue University in June of 2021 and isolated on 1.5% water agar. The pathogen was identified morphologically as *Pythium* and confirmed to be *P. irregulare* using the cytochrome oxidase subunit 1 gene sequence [36] and nucleotide BLAST comparison to deposited specimens in GenBank (100% match to accession no. HQ708665). The isolates were maintained on corn meal agar (CMA) medium and additionally preserved cryogenically in 20% glycerol in a liquid nitrogen cryotank for long-term storage.

The isolates were grown for 7 days on a benchtop at an ambient temperature of 21 ± 2 °C on CMA in 100 × 15 mm Petri plates and increased using a rice medium, as described in a previous resistance study [33]. Briefly, a 1:1 ratio of water and parboiled brown rice was mixed and autoclaved in an autoclave-safe plastic spawn bag for 40 min at 121 °C twice, with one 24 h interval between sterilizations. In a biosafety cabinet, one plate was aseptically sectioned into pieces roughly 1 cm² in size and subsequently incorporated per 700 g of sterile rice medium. A control treatment was made by adding sterile CMA medium plates to the rice media in the same manner. The inoculum bags were heat-sealed, grown on the benchtop for 14 days, and shaken every other day to promote even colonization of the rice grains. On the day of planting, the spawn bags were grouped by treatment, bulked into separate bags, and thoroughly shaken to achieve uniformity in both inoculum treatments.

2.3. Resistance Evaluation and Assessment

Phenotypic data from each accession were collected across two temporally separated screenings. The initial screening was performed with all 208 accessions using the isolate Br 2-3-5. After assessing the results of this screening, the accessions with the 30 highest mean relative root weight (RRW) scores (elaborated below) and 95% confidence intervals not overlapping with zero, as well as the accessions with the lowest 10 RRW scores, were screened for resistance again with the isolate SE21_0607_01. The resistance and susceptibility checks were included, in addition to the 40 accessions screened in the second experiments for a total of 43 accessions in the second screening.

The inoculations were performed in a greenhouse in April–May of 2021 for the initial screening and November of 2021 for the second screening, following a procedure used in previous studies [33,37]. Briefly, seeds were carefully chosen from each of the seed lots of each accession based on uniform size, color, and the absence of visible damage or disease symptoms. Perforated plastic greenhouse flats with 48-cell inserts were filled with double-autoclaved fine vermiculite, and each cell was dibbled with a hole to 4 cm depth. Before planting the seed, 1.5 mL of either inoculated or mock-inoculated rice medium was added to the bottom of each hole. One seed was placed per cell on top and in direct contact with the inoculum and covered with sterile vermiculite. Each accession was replicated six times in both the inoculated and mock-inoculated treatments. The inoculum was left in place for the duration of the experiment. Trays were then arranged according to a randomized complete block design on tables flooded to the level of inoculum. Phenotypic assessments were replicated three times per isolate, with the greenhouse maintained at a temperature of 24–28 °C with a 16:8 photoperiod of supplemental overhead lighting throughout the experiments.

At 14 days after planting, measurements of the stand counts and fresh root weights were taken from all of the plants within the same accession across both treatment groups. To assess the resistance to pre-emergence damping off, adjusted stand counts for each accession were calculated by dividing the number of germinated seeds in an inoculated replicate by those in its corresponding mock-inoculated control. Seeds were deemed germinated when their cotyledons fully emerged above the surface of the vermiculite. For precise evaluations of subtle variations in the severity of root rot that otherwise might be challenging to discern visually, the RRW score proposed by Lin et al. was used as a quantitative disease severity index [33]. Briefly, the baseline germination of each accession was calculated by taking the average stand count across all mock-inoculated control treatments. Subsequently, an RRW score was generated for each accession per replicate, determined by using the following formula:

$$\mathrm{RRW} = \frac{\left[\dfrac{\text{Total fresh root weight of the inoculated replicate}}{\text{Baseline germination of all three control replicates}}\right]}{\left[\dfrac{\text{Total fresh root weight of the control replicate}}{\text{Stand count of the same control replicate}}\right]}$$

2.4. Statistical Analysis

To determine significantly resistant accessions, linear mixed models were fitted with accessions designated as fixed effects and replicates assigned as random effects, using the 'lme4' package [38] in R, implemented using the following formula:

$$Y_{ij} = \mu + Acc_i + Rep_j + \varepsilon_{ij}$$

where Y_{ij} is the observed RRW score of the ith accession in the jth replicate, μ is the intercept and estimated baseline mean before any genotypic effects, Acc_i is the ith accessional genotypic fixed effect, Rep_j is the jth replicate random effect, and ε_{ij} is the residual variance associated with the observation of the ith accession in the jth replicate. p-Values for each accession's estimated genotypic effect were generated using 'lmerTest' using Satterthwaite's approximation for the degrees of freedom at an $\alpha = 0.05$ [39]. Mixed models were fit separately to the RRW scores and accessions first from the initial screening against Br 2-3-5 with all 208 accessions, then from the screening against SE21_0607_01 with 43 tested accessions, and with pooled data across all accessions and both isolates. In the pooled dataset, accessions not included in the second screening were imputed as missing data for three replicates and the isolate used to generate each RRW was added to the mixed model as an additional random effect.

Broad-sense heritability (H^2) for the RRW score was calculated by decomposing both the Br 2-3-5 and SE21_0607_01 models with all factors fitted as random effects to generate variance component estimates and using them as follows:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{r}}$$

where σ_g^2 is the genotypic variance, σ_e^2 is the error variance, and r is the number of replicates. Broad-sense heritability was estimated using the variance components estimated for the measure of the RRW score for both isolates separately and averaged.

2.5. Genome-Wide Association Study

SNP data generated using the SoySNP50K iSelect BeadChip were obtained for each accession from SoyBase [40]. Only markers mapped to the 20 soybean chromosomes were considered for a total of 42,449 genome-wide SNP markers. For the GWAS, the GAPIT3 package (version 3.1.0) was utilized in R by employing the statistical method 'BLINK' [41]. Significantly associated markers represented in less than 5% of the total number of acces-

sions, or a minor allele frequency (MAF) < 0.05, were discarded as potential false positives. The pedigrees for this population were largely unavailable, as a substantial portion of the accessions were landraces of unknown heritage. To address the potential cryptic population structure, principal component analysis (PCA) was employed to analyze the SNP data for clustering using the function 'prcomp' in base R [42]. Principal components that accounted for more than 5% of the variance in SNP inheritance among the 208 accessions were incorporated into the GWAS. The pooled RRW scores across all replicates and accessions were used as the phenotype for the association analysis. Accessions not included in the second screening were imputed as missing data for the three SE21_0607_01 replicates.

3. Results

3.1. Phenotypic Evaluation of Soybean Germplasm for P. irregulare Resistance

The screening of soybean accessions revealed substantial variation in their response to *P. irregulare* infection. None of the accessions showed symptomless immunity after inoculation. By 14 days after planting (DAP), symptoms of seedling diseases, such as the presence of necrotic root lesions, root system stunting, and damping off, were clearly evident in the inoculated treatments but not in the controls.

In the screening against isolate Br 2-3-5, the mean RRW scores of the 208 accessions were continuously distributed between 0 and 0.647 (Supplementary Table S1). Thirty-seven accessions failed to germinate across all three replicates. The data were left-skewed on both measures of the RRW score and the adjusted stand counts, with all accessions showing some degree of susceptibility (scores < 1) to this isolate in both measures (Figure 1). The mean RRW score for the population was 0.124, with a standard deviation of 0.215. Nine accessions (PI 490766, PI 547459, PI 591511, PI 547460, PI 548520, FC 29333, PI 548360, PI 84946-2, and PI 548362) had RRW scores significantly greater than the population mean (Table 1). The mean adjusted stand count of the population was 0.162, with a standard deviation of 0.23. The resistant check PI 424354 had a mean RRW score of 0.312 but was nonsignificant compared to the population mean. The susceptible check PI 525453 ('Conrad') failed to germinate across all three replicates, indicating complete susceptibility under the conditions of our experiment. The susceptible check PI 548631 ('Williams') had a mean RRW score of 0.039.

Table 1. Summary of the estimated fixed effects and population mean for the accessions with the top 10 RRW scores in the datasets of each isolate individually and in the pooled dataset encompassing both isolates.

	Isolate Br 2-3-5			**Isolate SE21_0607_01**			**Pooled**	
Estimator	**Effect**	***p*-Value [a]**	**Estimator**	**Effect**	***p*-Value [a]**	**Estimator**	**Effect**	***p*-Value [a]**
Intercept	0.126	3.16×10^{-1}	*Intercept*	0.176	5.74×10^{-3}	*Intercept*	0.114	2.89×10^{-1}
PI 490766	0.521	4.54×10^{-4} *	**PI 548520**	0.256	1.08×10^{-3} *	**PI 548520**	0.358	4.02×10^{-3} *
PI 547459	0.488	1.00×10^{-3} *	PI 578503	0.253	1.28×10^{-3} *	FC 29333	0.373	9.14×10^{-3} *
PI 591511	0.401	6.82×10^{-3} *	PI 84987 A	0.139	6.96×10^{-2}	PI 84946-2	0.321	2.49×10^{-2} *
PI 547460	0.390	8.51×10^{-3} *	PI 632418	0.135	7.75×10^{-2}	PI 490766	0.273	2.81×10^{-2} *
PI 548520	0.385	9.33×10^{-3} *	PI 548521	0.131	8.80×10^{-2}	PI 547459	0.271	2.94×10^{-2} *
FC 29333	0.381	1.01×10^{-2} *	PI 603549	0.108	1.58×10^{-1}	PI 548362	0.310	3.00×10^{-2} *
PI 548360	0.331	2.52×10^{-2} *	PI 567258	0.104	1.74×10^{-1}	**PI 548360**	0.245	4.82×10^{-2} *
PI 84946-2	0.329	2.63×10^{-2} *	PI 438239 B	0.088	2.46×10^{-1}	PI 591511	0.242	5.14×10^{-2}
PI 548362	0.318	3.15×10^{-2} *	PI 88468	0.085	2.63×10^{-1}	PI 547460	0.237	5.64×10^{-2}
PI 639559 B	0.268	6.97×10^{-2}	**PI 548360**	0.085	2.66×10^{-1}	PI 639559 B	0.260	6.86×10^{-2}

[a] *p*-Values calculated using Satterthwaite's approximation for the degrees of freedom. * Statistically significant from the population mean at $\alpha = 0.05$. The two accessions among the top ten resistant across all three datasets are in bold.

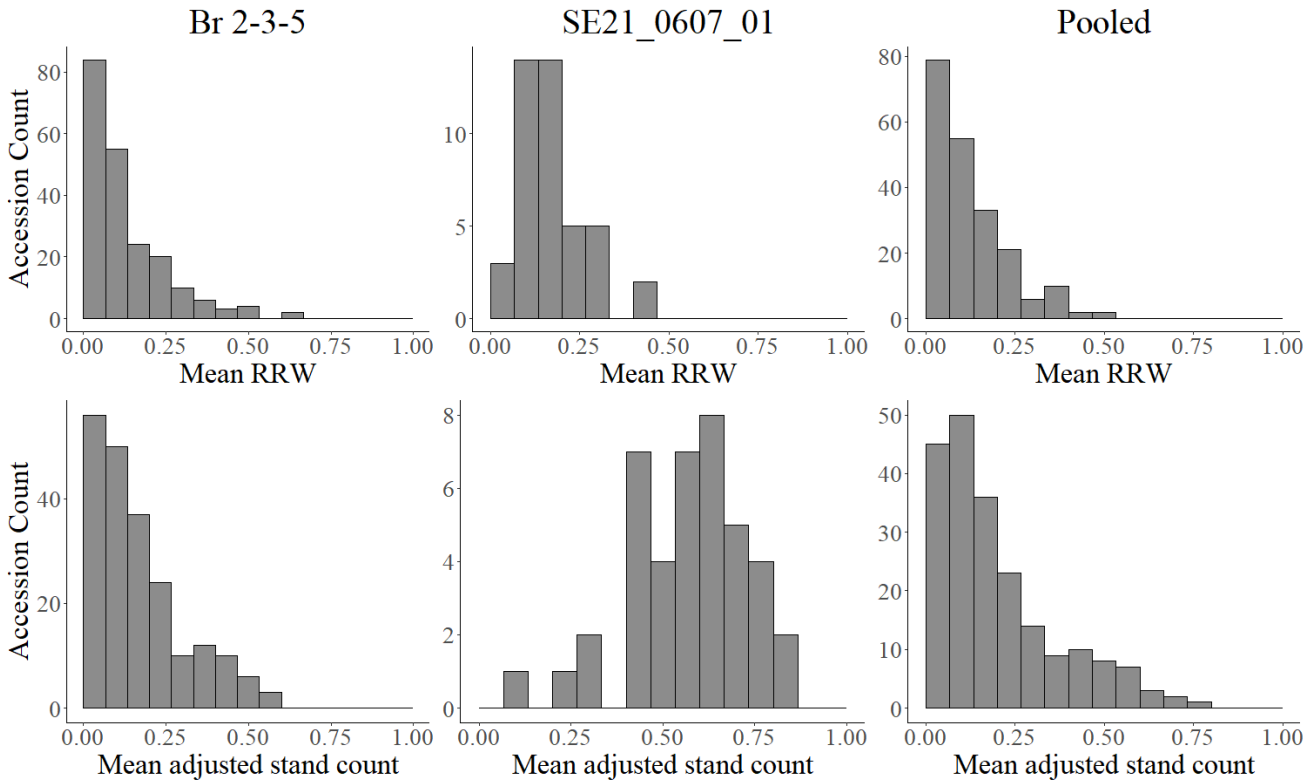

Figure 1. Frequency histograms of the mean RRW scores and adjusted stand counts in each of the three datasets.

The accessions with the 30 highest mean RRW scores with 95% confidence intervals not overlapping with zero and the lowest 10 RRW scores in the initial screening, as well as the three checks, were included in an additional screening against a geographically and temporally distinct isolate. When screened against isolate SE21_0607_01, the RRW scores remained left-skewed, while the adjusted stand counts became normally distributed around a mean of 0.584 with a standard deviation of 0.27. The population mean RRW score, when challenged with this isolate, was 0.178, showing a standard deviation of 0.13 (Supplementary Table S1). The mean RRW scores of the checks PI 424354, PI 525453, and PI 548631 were nonsignificant but lower than the population mean at 0.101, 0.159, and 0.132, respectively. Two accessions (PI 548520 and PI 578503) had RRW scores that were significantly greater than the population mean for this isolate (Table 1).

Within the pooled dataset across both isolates, PI 548520, FC 29333, PI 84946-2, PI 490766, PI 547459, PI 548362, and PI 548360 all had RRW scores significantly greater than the population mean of 0.134 (Table 1). The resistant check PI 424354 had a mean RRW score of 0.206, while the susceptible checks PI 548631 and PI 525453 had mean scores of 0.086 and 0.080, respectively. The RRW scores of all checks were nonsignificant compared to the population mean. PI 548520 was found to be significantly resistant to both isolates independently and in the pooled dataset (Table 2). PI 548360 was significantly resistant to Br 2-3-5 and additionally among the top ten resistant lines to SE21_0607_01 with lower standard errors than PI 548520 across both experiments. In all three datasets, accession was a significant predictor of the RRW score (Table 3). A significant interaction between accession and isolate was observed ($p = 0.013$). H^2 for the RRW scores was estimated to be 33.1% when challenged with Br 2-3-5 and 66.6% against SE21_0607_01. The mean H^2 of the RRW scores, measuring resistance across both isolates of *P. irregulare*, was calculated to be 49.9%.

Table 2. Analysis of variance of the linear mixed models applied to each of the two datasets encompassing each isolate individually, and the pooled dataset encompassing both isolates.

Isolate	Predictor	SS	MS	NDF	DDF	F-Value	*p*-Value [a]
Br 2-3-5	Accession	10.089	0.04874	207	414	1.4958	3.120×10^{-4} *
SE21_0607_01	Accession	1.083	0.0258	42	84	2.9961	9.530×10^{-6} *
Pooled	Accession	9.057	0.0438	207	539	1.4333	6.728×10^{-4} *

[a] *p*-Values calculated using Satterthwaite's approximation for the degrees of freedom. * Significant predictor at $\alpha = 0.05$.

Table 3. SNP markers associated with the RRW score disease severity rating after inoculation with *P. irregulare*, genome position, and their estimated effect sizes.

Marker Name	Chromosome	Position (bp) [a]	Major/Minor Allele	MAF [b]	*p*-Value [c]	RRW Effect [d]
ss715621416	15	26,089,343	C/**A**	0.087	3.56×10^{-15}	0.172
ss715606444	10	36,778,506	C/**T**	0.082	1.53×10^{-6}	0.093

[a] According to the Wm82.a1 reference genome. [b] Minor allele frequency. [c] FDR-adjusted *p*-value. [d] Estimated degree of effect of favorable allele substitution on the RRW, relative to the mean RRW of the population. The favorable allele associated with resistant accessions is in bold.

3.2. Genome-Wide Association Study and Candidate Gene Analysis of Pythium irregulare Resistance

The population structure of the 208 accessions was estimated using PCA (Figure 2). Two principal components (PCs) were identified, contributing 15.7 and 6.0% of the variance in the SNP data, and the number of PCs called through BLINK was correspondingly set to two. Two significant marker–trait associations (MTAs) were identified, with *p*-values below the false discovery rate (FDR) adjusted α of 0.05. The MTAs were identified with SNP markers ss715606444 on chromosome Gm10 and ss715621416 on chromosome Gm15 (Figure 2 and Table 3). The RRW effect sizes were 0.093 and 0.172, respectively (Table 3). Candidate gene analysis, compared to the available reference genome sequences deposited in SoyBase, revealed that ss715606444 on chromosome 10 is located within the gene model Glyma10g27960 (Wm82.a1) or Glyma.10g139200 (Wm82.a2 and Wm82.a4), which is predicted to encode a pectin acetylesterase. The MTA on chromosome 15, identified with ss715621416, is not located within any annotated genes in the Wm82.a1, Wm82.a2, or Wm82.a4 reference genomes. The closest annotation is a predicted gene encoding an alpha/beta hydrolase carboxylesterase, Glyma15g25010 (Wm82.a1), or Glyma.15g206300 (Wm82.a2 and Wm82.a4), located roughly 60–70 kbp away from ss715621416. The physical location of this gene is between the MTA and its nearest flanking SNP marker (ss715621418), which was also included in the GWAS. Comparisons to the 'Lee' published genome within the 200 kbp regions flanking the physical positions of both MTAs are not concordant with the predicted genes identified from 'Williams82'; however, the SoySNP50K iSelect BeadChip markers are not annotated in this reference genome, so precise locations of the MTAs could not be determined in this accession.

Additional markers were found to be significantly associated with resistance but had minor allele frequencies less than 5% and were discarded from further analysis as potential false positives. Of the seven accessions identified as resistant from the pooled dataset (Table 4), PI 490766 and PI 547459 carried homozygous copies of the favorable alleles at both markers. PI 548520 and PI 548362 carried the favorable allele at the MTA site identified on chromosome 10, while FC 29333 carried the favorable allele on chromosome 15. PI 84946-2 and PI 548360 carried neither of the favorable alleles at either MTA.

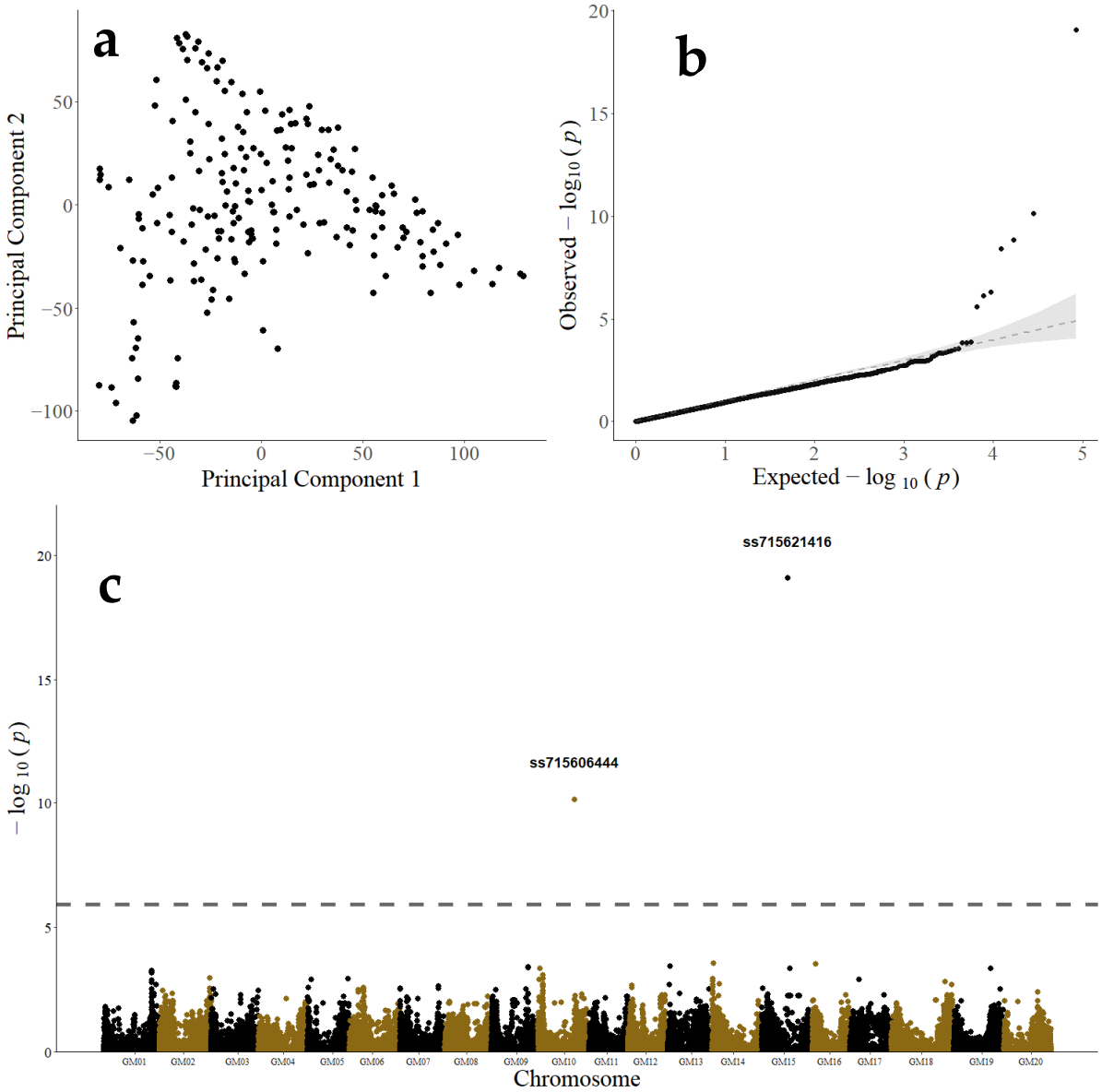

Figure 2. (**a**) Two-dimensional scatterplot of the first two principal components of SNP-estimated relatedness; (**b**) Q-Q plot of all *p*-values from the marker association tests, with dashed line showing the estimated linear relationship between the expected and observed negative decimal logarithm transformed *p*-values for null association and the 95% confidence interval shaded in gray; and (**c**) genome-wide Manhattan plot displaying SNP marker association transformed *p*-values with the RRW score measure. Color is alternated between chromosomes for visual contrast, the two significant markers are annotated, and the dashed line shows the FDR-adjusted transformed significance threshold (an additional 5 significant MTAs with MAF < 0.05 visible in the Q-Q plot were removed from this plot).

Table 4. Favorable SNP allele variants found in each of the seven resistant accessions identified from the pooled dataset, with favorable resistance alleles in bold and summary of their phenotypic performance averaged across both isolates of *P. irregulare*.

SNP Marker	Major/Minor Allele	Effect	PI 548520	FC 29333	PI 84946-2	PI 490766	PI 547459	PI 548362	PI 548360
ss715621416	C/**A**	0.172	C	**A**	C	**A**	**A**	C	C
ss715606444	C/**T**	0.093	**T**	C	C	**T**	**T**	**T**	C
Sum of allelic effects:		0.265	0.093	0.172	0	0.265	0.265	0.093	0
Pooled mean RRW:			0.472 ± 0.12	0.507 ± 0.35	0.455 ± 0.37	0.386 ± 0.16	0.384 ± 0.16	0.444 ± 0.30	0.359 ± 0.06
Pooled mean adjusted stand count:			0.794 ± 0.15	0.467 ± 0.24	0.222 ± 0.18	0.597 ± 0.10	0.594 ± 0.17	0.444 ± 0.24	0.581 ± 0.09

4. Discussion

A total of 208 soybean accessions were evaluated for their resistance against two isolates of *P. irregulare*. After an initial screening of all 208 accessions against one isolate, 30 accessions exhibiting superior performance and 10 showing inferior performance were assessed in a second screening against an additional isolate. A total of 10 accessions across both screenings were identified with significant resistance to *P. irregulare*, nine of which showed resistance to just one isolate, while a single accession exhibited resistance to both isolates. A subsequent GWAS resulted in the identification of two MTAs on chromosomes 10 and 15 underlying resistant versus susceptible accessions.

The statistically significant interaction between accessions and isolates in the mixed linear model analysis suggests that soybean accessions screened in this study responded to infections by the two tested isolates differently. This is highlighted by the contrasting performance observed across several of both the best and worst scoring accessions between screenings with Br 2-3-5 and SE21_0607_01. For example, PI 490766, PI 547459, PI 591511, and PI 547460 all performed with mean RRW scores above 0.5 after inoculation with Br 2-3-5; however, these same lines had mean RRW scores below 0.2 when inoculated with SE21_0607_01 (Supplementary Table S1). On the other hand, the 10 accessions with inferior performance when inoculated with Br 2-3-5 (no germination across three replicates) all germinated when inoculated with SE21_0607_01, and four of the accessions, PI 438500, PI 548402 S, PI 632418, and PI 84987 A, had mean adjusted stand counts over 0.5. Population studies have shown that significant genotypic differences have been observed across isolates of *P. irregulare* that are correlated with a number of different characteristics among isolates, including aggressiveness [25]. The genotypic differences between the two isolates used in this study were not examined. Previous studies looking for quantitative resistance to *P. irregulare* have been limited by the use of single isolates to generate phenotypes when gene mapping [30–35]. The strength of this study is that it utilized two isolates isolated decades apart from two separate geographical locations in a pooled dataset. Subsequently, the accessions and MTAs identified in this study are expected to provide broader resistance to *P. irregulare* and may, therefore, provide more durable resistance against pathogen populations in field environments, where many genetically distinct isolates may be present at once.

Two MTAs, namely SoySNP50k markers ss715606444 and ss715621416 on chromosomes 10 and 15, respectively, were shown to be significantly associated with the observed resistance against two geographically and temporally distinct isolates of *P. irregulare*. The SNP marker ss715621416 had a major estimated effect, increasing the mean RRW scores of all accessions carrying the favorable allele by an average of 0.172, while marker ss715606444 affected the mean RRW score by just over half that at 0.093. The estimated population mean RRW score from the pooled dataset was 0.114, and accessions carrying favorable alleles at both markers would be predicted to have, on average, RRW scores of 0.379, which was observed to be true in the accessions PI 490766 and PI 547459 (Table 4). However, the other five significant accessions produced higher mean RRWs despite lacking one or both of these MTAs. Several explanations may account for the performance of these accessions despite the absence of the identified resistance markers. Structural variations, such as insertion/deletions or copy number variations that may contribute to *P. irregulare* resistance, cannot be detected by the SoySNP50k chip assay, and thus, can remain undetected in an association analysis. It is also important to highlight that while a GWAS is a powerful tool for finding genomic markers linked to genes controlling traits of interest, it often lacks the statistical power necessary to identify numerous small polygenic effects underlying complex traits. Thus, additional minor-effect MTAs accounting for the superior performance of the other five accessions may have been overlooked. Furthermore, other MTAs may be present in these five accessions but underrepresented in the population as a whole, reducing the power for GWAS to detect them. For instance, rare alleles with low minor allele frequency are discarded for their potential to introduce false positive associations.

The MTA represented by ss715606444 is physically located in a predicted gene encoding a pectin acetylesterase. Pectin acetylesterases hydrolyze pectin acetylester bonds, thereby modulating the distribution of pectin *O*-acetylation within the plant cell wall [43]. *PMR5*, which confers broad-spectrum resistance to powdery mildew in Arabidopsis, relies on a conserved esterase domain essential for the acetylation of cell wall oligogalacturonides [44]. Cell wall acetylation is implicated in both plant defense signaling pathways as well as providing physical resistance against plant pathogens and may play a role in soybean resistance to *P. irregulare*. The MTA on chromosome 10 is also within 50 kbp of a gene predicted to encode a *myb* transcription factor, which may also be involved in defense pathway signaling [45]. The MTA identified with SNP marker ss715621416 on chromosome 15 was not physically located in any predicted genes across three versions of the 'Williams82' reference genome. The only predicted gene between ss715621416 on chromosome 15 and its nearest flanking SNP markers is predicted to encode an alpha/beta hydrolase fold containing carboxylesterase. Carboxylesterases of this kind have been demonstrated to play roles in secondary metabolite synthesis, plant defense activation signaling, as well as a wide variety of hydrolytic reactions that degrade proteins from a diversity of pathogens [46–50]. It is important to note that association analysis lacks the resolution necessary to statistically infer the estimated boundaries of specific genomic intervals around the MTAs where potential candidate genes are most likely to reside. Many promising candidate genes potentially involved in the mechanisms underpinning resistance to *P. irregulare* exist in the regions flanking the MTAs discovered here. Further physical mapping using traditional QTL mapping methods is warranted to begin exploring the specific intervals associated with resistance in the flanking regions of these MTAs.

The two MTAs that were detected in this study, to the best of our knowledge, represent novel molecular markers for *P. irregulare* resistance in soybean. In prior publications, quantitative trait loci associated with *P. irregulare* resistance have been documented on chromosome 10. Of the two QTL reported on chromosome 10, both peak markers were a minimum of 6.9 Mbp away from the MTA linking the ss715606444 SNP and *P. irregulare* resistance reported here [30,34]. This comparison was made using two physical maps. Wm82.a1 and Wm82.a2, of the soybean reference genome. As of the present writing, no published report has indicated the presence of resistance QTLs to *P. irregulare* associated on chromosome 15.

The MTAs on chromosomes 10 and 15 coincide with the interval ranges previously reported for the loci RpsSu, qRps10–01, and qRfv15–02, conferring resistance to two other soilborne root rot pathogens of soybean, *Phytophthora sojae* and *Fusarium virguliforme* [51–57]. However, the reported interval ranges of these loci all span over 33 Mbp, leaving uncertainty regarding whether our MTAs are directly linked to the genes or QTL responsible for the observed resistance to these two other pathogens. Two other *P. sojae* and a single *F. virguliforme* resistance QTL are reported on chromosome 15, which are each located at a minimum distance of 13 Mbp away from our MTA [54,57–59]. Additionally, the MTA detected on chromosome 10 was situated 8.7 Mbp away from a QTL for resistance to *Diaporthe longicolla* and 23 Mbp away from a QTL related to *F. graminearum* resistance [60,61]. Finally, two additional QTL linked to resistance against *F. graminearum* and *M. phaseolina* were found on chromosome 15 and are 40 and 18 Mbp away from our MTA, respectively [62,63].

The resistant varieties identified in this study, along with the molecular markers linked to their resistance, may be of immediate use to breeders and researchers interested in further interrogating the underlying defense mechanisms and breeding for *P. irregulare* resistance. Notably, PI 548520 demonstrated significant resistance against two isolates of *P. irregulare* from separate geographic origins and is an improved pure line. The combination of these characteristics may be desirable to breeders looking to introgress *P. irregulare* resistance into soybean cultivars while minimizing the potential introduction of undesirable agronomic traits. Additionally, PI 548360 is significantly resistant to Br 2-3-5 and shows high stability of the RRW score phenotype in the presence of both isolates of *P. irregulare*, with very

low deviation across replicates. The two MTAs identified in this study hold immediate applicability for marker-assisted selection in untested germplasm.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb15030056/s1, Table S1: Mean RRW scores and adjusted stand counts from the accessions when challenged by two isolates of *P. irregulare*.

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