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Technical Advance

The Use of Detached Leaf Inoculation for Selecting *Cercospora kikuchii* Resistance in Soybean Genotypes

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Abstract

Cercospora leaf blight (CLB) causes extensive losses in soybean production in worldwide, including major soybean-producing countries such as Argentina. *Cercospora kikuchii*, *C*. cf. *sigesbeckiae*, *C*. cf. *flagellaris*, and *C*. cf. *nicotianae* are identified as pathogens of CLB. Soybean resistance against CLB is still unknown. Also, chemical control for CLB is losing effectiveness because of fungicide resistance of pathogens such as *C. kikuchii*. We urgently need to breed a CLB-resistant cultivar. Unfortunately, efficient methods for the screening of a resistant soybean genotype have not yet been established. In this study, we designed a new, high-throughput inoculation method for identifying resistance against one of the CLB pathogens, *C. kikuchii*. We used liquid-cultured mycelia of the pathogen *C. kikuchii* on detached soybean leaves. Lesions on soybean leaflets appeared 9 days postinoculation by this method. We used this method to select four *C. kikuchii*-resistant genotypes from 80 genotypes in the World Soybean Core Collection. The high-throughput screening method developed in this study can contribute to the research about *C. kikuchii* resistance by facilitating identification of resistant varieties.

Keywords: *Cercospora kikuchii*, Cercospora leaf blight, fungal pathogens, inoculation method, soybean

Cercospora leaf blight (CLB), caused by fungal pathogens classified in the genus *Cercospora*, is a global threat in the production of soybean (*Glycine max* (L.) Merr.). The disease has been reported in three major soybean-producing countries in South America; namely, Argentina, Brazil, and Paraguay (Wrather et al. 2010). *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M. W. Gardner was identified as a pathogen of CLB (Matsumoto and Tomoyasu 1925). Recently, other *Cercospora* spp. such as *C*. cf. *sigesbeckiae*, *C*. cf. *flagellaris*, and *C*. cf. *nicotianae* also became known as CLB pathogens (Albu et al. 2016; Sautua et al. 2020; Soares et al. 2015). Among the four CLB pathogens, profiles for *C. kikuchii* are well studied. The pathogen causes dark-purple-colored lesions on the leaves and the petiole, and premature

Author contributions
The experimental design was conceived and conducted by T.K. and N.Y. based on the information provided from M.A.L., A.D.P., and A.J.G.I. Fungal isolates were isolated, identified, and provided for the research by M.A.L., A.D.P., and A.J.G.I. Data were analyzed and the manuscript was written by T.K. and N.Y. with valuable input from M.A.L., A.D.P., and A.J.G.I.

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defoliation (Matsumoto and Tomoyasu 1925; Walters 1980). The disease diminishes soybean production by a loss of leaves at the final stage of crop development (Walters 1980). *C. kikuchii* also causes a problem on seed called purple seed stain (PSS) (Roy and Abney 1976; Suzuki 1921). A key pathogenicity factor of the pathogen is the purple-colored pigment cercosporin (Daub and Hangarter 1983; Kuyama and Tamura 1957; Yamazaki et al. 1975). Fungicide application is one of the main strategies to manage this disease. However, the long-term use of such chemicals can result in fungicide resistance in pathogenic fungus in the field (Price et al. 2015). To manage this disease, it is advisable to utilize soybean varieties resistant against *C. kikuchii*.

Genetic resistance against PSS has been reported in some varieties (Alloatti et al. 2015; Jackson et al. 2006, 2008; Wilcox et al. 1975). However, it has been suggested that there is no correlation between resistance against PSS and resistance to CLB (Li et al. 2019; Ward-Gauthier et al. 2015). Soybean varieties resistant against CLB pathogens such as *C. kikuchii* remain unknown. The objective of this study was to establish a practical method for screening soybean varieties based on resistance against *C. kikuchii*.

Several barriers exist in establishing an effective screening method for *C. kikuchii* resistance. The disease development of *C. kikuchii* on soybean leaves is slow compared with other foliar fungal diseases such as Asian soybean rust (pathogen *Phakopsora pachyrhizi*). In general, CLB severity can be assessed 2 to 3 weeks after fungal treatment (Cai et al. 2009). To ensure extensive lesions on the leaflets, plants should be kept under conditions preferable for the fungal development. Optimum conditions include high humidity for several weeks before clear observation of the first lesions. Keeping inoculated soybean plants for long periods produces a lot of biomass. Thus, it is essential to consider time and space efficiency when studying a new screening method for this disease. Difficulties in maintaining spore production of *Cercospora* isolates on artificial culture are well known (Goode and Brown 1970). Furthermore, spore production on artificial culture such as potato dextrose agar (PDA) of some *C. kikuchii* isolates is insufficient for screening of soybean genotypes. Alternatively, sufficient amounts of mycelia of *C. kikuchii* can be obtained from liquid media such as potato dextrose broth (PDB) in less than a week. Hence, we used mycelia for inoculating *C. kikuchii*.

In this research, we developed a high-throughput screening method for *C. kikuchii* resistance, termed the leaf culture inoculation method. Screening the worldwide soybean collection, we identified varieties with a low incidence of *C. kikuchii* lesions on the leaves. This process can contribute to controlling this devastating disease by identifying resistant genotypes.

MATERIALS AND METHODS

Fungal isolates and inoculum preparation

It has been suggested that the population of *C. kikuchii* in South America was different from that in Japan (Imazaki et al. 2006). Thus, we obtained three *C. kikuchii* isolates from different provinces of Argentina: #9 (Pergamino, Province of Buenos Aires), #40 (San Borgita, Province of Corrientes), and #121 (San Miguel de Tucumán, Province of Tucumán). The fungi were isolated from diseased soybean and identified as *C. kikuchii* by morphology. Observations for mycelial colony and conidia of the isolates were consistent with the literature (Chupp 1953; Ward-Gauthier et al. 2015). The three isolates #9, #40, and #121 were grouped with ex-type CPC 5068 and other isolates of *C. kikuchii* in a phylogenetic tree containing *C.* cf. *flagellaris*, *C.* cf. *sigesbeckiae*, and *C.* cf. *nicotianae* (unpublished data). The isolates were maintained on PDA (BD Biosciences, San Jose, CA, U.S.A.) at 25°C and kept in the dark to obtain mycelial colonies. Agar with mycelia recovered from the PDA plates were soaked in 20% glycerol and kept at temperatures below −80°C for long-term storage.

To obtain fresh mycelia, we inoculated *C. kikuchii* into 50 to 100 ml of PDB (BD Biosciences) in 300-ml Erlenmeyer flasks with baffles (e.g., 016310-300A; Shibata Scientific Technology, Saitama, Japan). An approximately 5-mm^2 agar piece with mycelia excised from the PDA plate was transferred to each flask. The flasks were shaken on a rotary shaker at 140 rpm for 4 to 5 days at 25°C. Then, PDB cultures of *C. kikuchii* were roughly disrupted using a hand blender (THM311; Tescom Denki, Tokyo, Japan) for 10 s. This step was repeated three times. The culture was filtered using single-layered gauze to remove aggregated mycelial pieces. The filtrated culture was centrifuged at 2,330 × *g* for 10 min, and the resulting supernatant was discarded. Autoclaved distilled water with 0.02% (vol/vol) Tween 20 was used to prepare the mycelial suspension. The concentration of mycelial pieces (approximately 0.05 mm in size or larger) was counted with a hemocytometer and adjusted to 1.0×10^5 mycelia/ml.

Screening for *C. kikuchii* resistance on soybean varieties

The list of soybean varieties used for inoculation is provided in Table 1. Soybean seed of the World Soybean Core Collection (WC) were obtained from the National Agriculture and Food Research Organization (NARO) Genebank. WC contains 96 varieties representing the various worldwide characteristics of soybean (Kaga et al. 2012). The varieties have been selected from approximately 4,000 soybean varieties based on their passport data, evaluation data, agromorphological traits, and single-nucleotide polymorphisms (Kaga et al. 2012). The accessible 80 of the 96 varieties of soybean were used in this study. Soybean seed were sown on hydrated vermiculite. Seed germination, plant growth promotion, and leaf culture inoculation took place in growth chambers LH-350, LH-410S, or LH411-SPC (Nippon Medical & Chemical Instruments, Osaka, Japan). Germination was promoted at 28°C, under a photoperiod of 14 h/day using white fluorescent lamps (100% of light intensity). One week after sowing, the most vigorous three or four seedlings were transplanted to a 113-mm-diameter and 140-mm-depth pot with soil collected from the experimental field of Japan International Research Center for Agricultural Sciences (JIRCAS, Tsukuba, Ibaraki, Japan). Plants were maintained at 24°C with 14 h of daily photoperiod (100% of light intensity).

The leaflets were incubated in disposable plastic cases (width = 170 mm, height = 115 mm, and depth = 45 mm) containing a hydrated paper towel (Kimtowel; Nippon Paper Crecia, Tokyo, Japan) to maintain the saturation of water vapor inside the case during inoculation (Fig. 1A). Leaflets were obtained from the first trifoliate leaves of 3-week-old plants (V2 to V3 growth stage). Then, up to three leaflets were placed in one case prior to inoculation with mycelia. The leaflets harvested from different plants for each were placed abaxial-side-up on hydrated paper towel. A hydrated laboratory wipe (Kimwipes; Nippon Paper Crecia) was placed at the edge of each leaflet to prevent them from drying. Approximately 2 ml of mycelial suspension was sprayed into each

TABLE 1

List of soybean varieties used in this study

Code[a]	ID[b]	Name of variety	Status	Origin
WC1	GmWMC001	FISKEBY V	Developed variety	Sweden
WC2	GmWMC006	KS 1034	Landrace	Malaysia
WC3	GmWMC011	SEITA	Landrace	Republic of Korea
WC4	GmWMC012	MANSHUU	Landrace	China
WC5	GmWMC014	KLS 203	Landrace	Republic of Korea
WC6	GmWMC015	CHUUHOKU 2	Landrace	Republic of Korea
WC7	GmWMC018	RIGAI SEITOU	Landrace	China
WC8	GmWMC019	CHOUSENSHU (CHA)	Landrace	Korean Peninsula
WC9	GmWMC020	POCHAL	Landrace	Taiwan
WC10	GmWMC022	NEZUMI META	Landrace	Korean Peninsula
WC11	GmWMC024	CHIENEUM KONG	Landrace	Republic of Korea
WC12	GmWMC027	KONGNAMUL KONG	Landrace	Republic of Korea
WC13	GmWMC029	SHIROSOTA	Landrace	Korean Peninsula
WC14	GmWMC035	PEKIN DAI OUTOU	Landrace	China
WC15	GmWMC036	MASSHOKUTOU (KOU 502)	Landrace	China
WC16	GmWMC038	ICHIGUUHOU	Landrace	China
WC17	GmWMC042	MASSHOKUTOU (KOU 503)	Landrace	China
WC18	GmWMC045	OKJO	Landrace	Republic of Korea
WC19	GmWMC046	KE 32	Landrace	Philippines
WC20	GmWMC048	HEAMNAM	Landrace	Republic of Korea
WC21	GmWMC066	HEUKDAELIP	Landrace	Republic of Korea
WC22	GmWMC070	CHOYOUTOU	Landrace	China
WC23	GmWMC071	PK 73-54	Landrace	India
WC24	GmWMC072	M 581	Landrace	India
WC25	GmWMC073	URONKON	Landrace	Korean Peninsula
WC26	GmWMC075	CHEONGYE MYONGTAE	Landrace	Republic of Korea
WC27	GmWMC083	KEUMDU	Landrace	Republic of Korea
WC28	GmWMC084	PEKING	Landrace	China
WC29	GmWMC086	ANTO SHOUKOKUTOU	Landrace	China
WC30	GmWMC089	BONGCHUNBAEKJAM	Landrace	China
WC31	GmWMC094	JEOKGAK	Landrace	Republic of Korea
WC32	GmWMC103	SENYOUTOU	Landrace	China
WC33	GmWMC107	HAKKA ZASHI	Landrace	China
WC34	GmWMC108	KARASUMAME	Landrace	China
WC35	GmWMC113	BARITOU 3 A	Landrace	Indonesia
WC36	GmWMC115	WILLIAMS 82	Developed variety	U.S.A.
WC37	GmWMC118	OUDU	Landrace	Republic of Korea
WC38	GmWMC119	HAKUBI	Landrace	China
WC39	GmWMC120	U 1416	Landrace	Nepal
WC40	GmWMC122	GAPSANJAELAE (I)	Landrace	Republic of Korea
WC41	GmWMC123	N 2295	Landrace	Nepal
WC42	GmWMC125	BHATMAS	Landrace	Nepal
WC43	GmWMC129	AOKI MAME	Landrace	China
WC44	GmWMC132	L 2A	Landrace	Philippines
WC45	GmWMC136	LOCAL VAR (SEPUTIH RAMAN)	Landrace	Indonesia
WC46	GmWMC138	COL/PAK/1989/IBPGR/2326 (1)	Landrace	Pakistan
WC47	GmWMC141	PETEK	Landrace	Indonesia
WC48	GmWMC142	JAVA 5	Landrace	Indonesia
WC49	GmWMC143	M 44	Landrace	India
WC50	GmWMC144	M 918	Landrace	India
WC51	GmWMC146	HM 39	Landrace	India
WC52	GmWMC147	COL/THAI/1986/THAI-78	Landrace	Thailand
WC53	GmWMC148	M 42	Landrace	India
WC54	GmWMC150	U 1042-1	Landrace	Nepal
WC55	GmWMC151	JAVA 7	Landrace	Indonesia
WC56	GmWMC152	U 1290-1	Landrace	Nepal
WC57	GmWMC154	MANSHUU MASSHOKUTOU	Landrace	China
WC58	GmWMC156	U 8006-3	Landrace	Nepal
WC59	GmWMC159	COL/PAK/1989/IBPGR/2323 (2)	Landrace	Pakistan
WC60	GmWMC160	N 2392	Landrace	Nepal
WC61	GmWMC162	COL/THAI/1986/THAI-80	Landrace	Thailand
WC62	GmWMC163	N 2491	Landrace	Nepal

(*Continued on next page*)

[a] Code for the varieties based on the previous study (Aoyagi et al. 2020).
[b] Identifier for World Soybean Core Collection, NARO Genebank Project (https://www.gene.affrc.go.jp/databases-core_collections_wg_en.php).

TABLE 1
(*Continued from previous page*)

Code[a]	ID[b]	Name of variety	Status	Origin
WC63	GmWMC165	KARASUMAME (SHINCHIKU)	Landrace	Taiwan
WC64	GmWMC166	MERAPI	Developed variety	Indonesia
WC65	GmWMC168	L 317	Landrace	India
WC66	GmWMC169	HAKUCHIKOU	Landrace	China
WC67	GmWMC170	M 652	Landrace	India
WC68	GmWMC171	U-1741-2-2 NO.3	Landrace	Nepal
WC69	GmWMC173	KARASUMAME (NAIHOU)	Landrace	Taiwan
WC70	GmWMC175	BISHUU DAIZU	Landrace	China
WC71	GmWMC176	SANDEK SIENG	Landrace	Cambodia
WC72	GmWMC181	CHIENGMAI PALMETTO	Landrace	Thailand
WC73	GmWMC182	LOCAL VAR. (TEGINENENG)	Landrace	Indonesia
WC74	GmWMC183	KARASUMAME (HEITOU)	Landrace	Taiwan
WC75	GmWMC186	RINGGIT	Developed variety	Indonesia
WC76	GmWMC187	KADI BHATTO	Landrace	Nepal
WC77	GmWMC188	E C 112828	Landrace	India
WC78	GmWMC190	SAN SAI	Landrace	Thailand
WC79	GmWMC191	MISS 33 DIXI	Landrace	Philippines
WC80	GmWMC192	U 1155-4	Landrace	Nepal

plastic case (Fig. 1B) using a 0.5-mm needle airbrush (SX0.5D; TAMIYA, Shizuoka, Japan) and air compressor (PS251; GSI Creos, Tokyo, Japan). Inoculated leaflets were kept at 25°C in the dark for 48 h to promote infection of *C. kikuchii*. The cases were then kept at 25°C under 12 h of daily photoperiod (25% of light intensity). The percentage of the lesion area (%LA) on each leaflet was evaluated at 9 days postinoculation (dpi). Leaflets were scanned by an image scanner (CanoScan 9000F Mark II; Canon, Tokyo, Japan) and %LA was evaluated. The %LA was calculated from successfully scanned parts of the leaflets. It was not practicable to distinguish *C. kikuchii* lesions on excessively damaged leaflets. In that case, %LA was not determined.

The first and second screenings were performed using all of the 80 varieties in a single inoculation experiment. Results were analyzed as two independent inoculations to select *C. kikuchii*-resistant or -susceptible variety candidates. At least three leaflets per variety were prepared in each screening. The average %LA ± standard error of the mean was calculated using GraphPad Prism 8 (GraphPad Software, San Diego, CA, U.S.A.). Candidates for the resistant and susceptible varieties were selected based on the average %LA. For resistant candidates, %LA was under 5% in every three *C. kikuchii* isolates for both replications. We also selected candidates for *C. kikuchii*-susceptible varieties for the comparison with the *C. kikuchii*-resistant varieties. The susceptible candidates consistently produced over 10% for %LA. To confirm the selection, a third screening was done by using 12 leaflets per selected variety for each inoculation. One-way analysis of variance and TukeyÕs multiple comparisons test as post hoc analysis was done by GraphPad Prism 8 to compare results between varieties in the third screening. Also, Pearson correlation analyses between average %LA for each inoculation were conducted by GraphPad Prism 8.

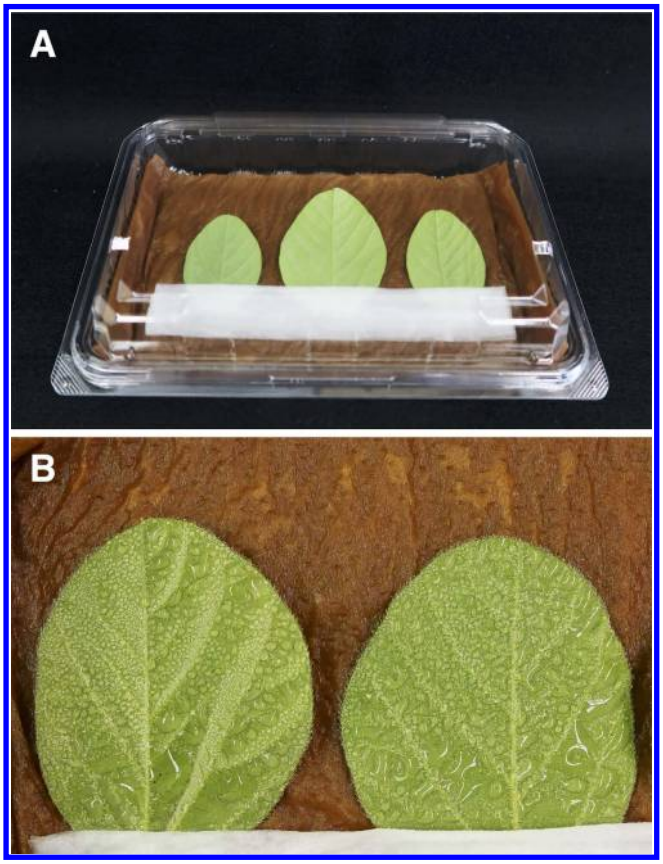

FIGURE 1
Leaf culture inoculation method for *Cercospora kikuchii*. Leaflets were placed in a disposable plastic case and kept in high humidity for 9 days to promote infection. **A,** Picture of the plastic case with soybean leaflets. **B,** Leaf pieces after spray inoculation.

RESULTS AND DISCUSSION

The first and second screenings were performed using the 80 varieties. At least three leaflets per variety were prepared for each inoculation. However, inoculation of WC5, WC8, WC16, WC17, WC18, WC19, WC29, WC30, WC50, WC51, WC60, WC71, and WC79 was performed with less than three leaflets per inoculation due to low germination rate of seed or damage to leaflets prior to inoculation. Pictures of representative examples of leaflets for %LA levels are shown in Figure 2. In this screening, five varieties, including WC3, WC7, WC26, WC37, and WC43, presented unclear lesions on the

leaves. They were excluded from further research. These varieties retained a green-cotyledon or stay-green character that made it difficult to definitively determine the extent of lesions on the leaves (Fig. 3). Results are presented in Figure 4. The average of %LA for all assessed leaflets of each variety was used in the selection criteria of candidates for *C. kikuchii*-resistant varieties. WC32, WC36, WC39, and WC54 were selected as resistant variety candidates. The average %LA for these resistant candidates was under 5% in every *C. kikuchii* isolate for both replications. We also selected WC6, WC73, and WC76 as *C. kikuchii*-susceptible varieties for comparison with the *C. kikuchii*-resistant varieties. The three susceptible candidates consistently produced over 10% for %LA. The experiment continued for the seven mentioned varieties with a third inoculation test.

The inoculation test on the seven selected varieties was performed using *C. kikuchii* isolates #9, #40, and #121. Twelve leaflets per variety were prepared for each inoculum. The results agreed with those of the former two screenings: WC32, WC36, WC39, and WC54 exhibited lower %LA (0.17 to 1.08% for #9, 2.58 to 7.96% for #40, and 1.71 to 3.08% for #121) compared with the susceptible variety candidates (Fig. 5A). %LA of the four varieties (WC32, WC36, WC39, and WC54) was significantly lower than %LA of the susceptible variety candidate WC73 in all inoculums (Fig. 5B). Unexpectedly, the other two susceptible candidates, WC6 and WC76, exhibited moderate susceptibility to the inoculation of strains #9 and #121. Our results demonstrate that WC32, WC36, WC39, and WC54 are varieties resistant against *C. kikuchii*. Also, WC73 is demonstrated as a variety susceptible to *C. kikuchii* based on our results. These results were confirmed by a whole-plant inoculation test (Supplementary Fig. S1). The average %LA for resistant varieties (0.02 to 0.20 for WC32, WC36, WC39 and WC54) was significantly lower than the susceptible variety (2.33 for WC73).

As described, WC6 and WC76 exhibited slightly different susceptible responses to the different isolates. The average %LA of the three resistant varieties inoculated with #40 were higher than 5% (WC32 = 6.38%, WC36 = 7.96% and WC39 = 6.83%). Our analysis comparing the isolates revealed that the results of #9 and #121 were highly correlated ($r = 0.9772$) but the correlations between #9 and #40 ($r = 0.7739$), and #40 and #121 ($r = 0.8186$) were lower (Fig. 6). These isolates originated from different provinces in Argentina. This result implies that the *C. kikuchii* population in Argentina harbors geographic diversity in pathogenicity against soybean varieties.

There were some differences in pathogenicity against the soybean varieties of the three *C. kikuchii* isolates. However, WC54 exhibited a high level of resistance with all of the tested isolates. This suggested that it is a good candidate as a genetic source of resistance against *C. kikuchii* in Argentina (Fig. 5B).

Disease development of *C. kikuchii* is slow. It usually takes 3 weeks after inoculation to determine disease severity (Cai et al. 2009). To screen *C. kikuchii* resistance on a large number of soybean varieties, it is difficult to keep uniform conditions for each inoculated leaflet on a whole plant because the light and humidity surrounding individual leaflets can vary. Lighting is important for cercosporin toxicity (Daub and Hangarter 1983;

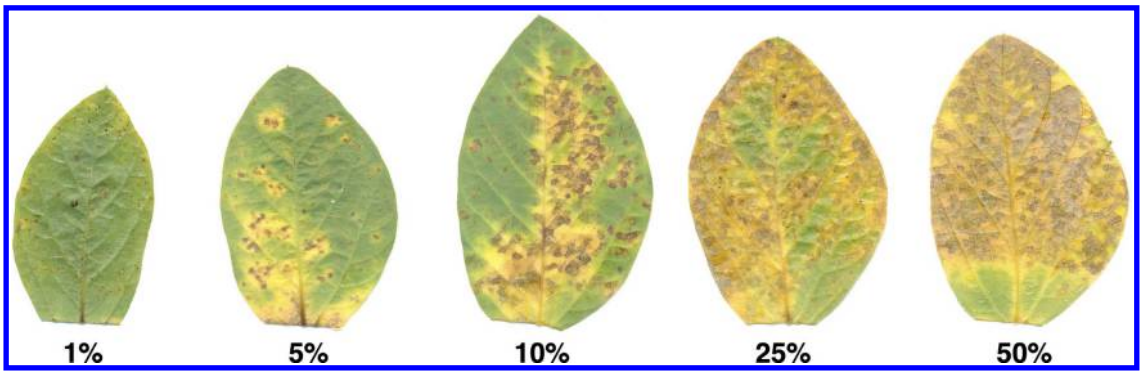

FIGURE 2
Representative examples of leaflets for percentage of the lesion area (%LA) levels. Leaflets of WC11 (1%), WC1 (5%), WC49 (10%), WC38 (25%), and WC40 (50%) in the first inoculation trial of isolate #121 are provided.

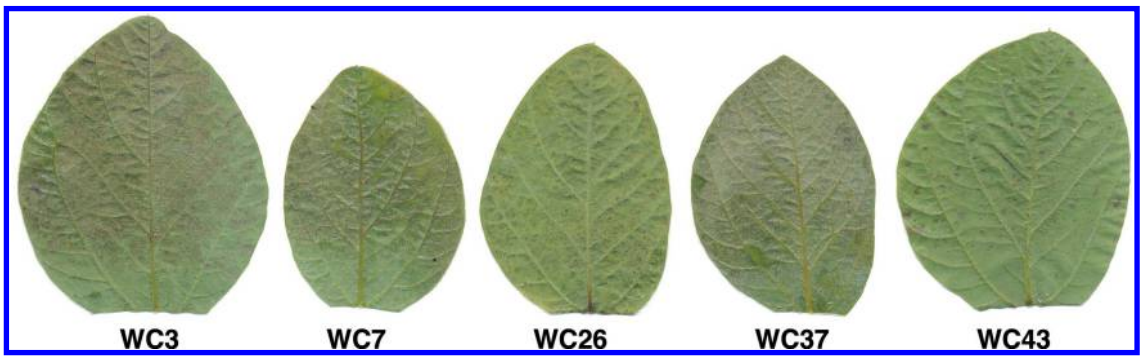

FIGURE 3
Lesions on soybean varieties WC3, WC7, WC26, WC37, and WC43. *Cercospora kikuchii* isolate #121 was inoculated; pictures represent leaflets 9 days postinoculation (first inoculation trial). *C. kikuchii* lesions on the leaflets of these varieties were unclear. We were unable to estimate percentage of the lesion area (%LA) due to the strain characteristics of the green-cotyledon or stay-green phenotype.

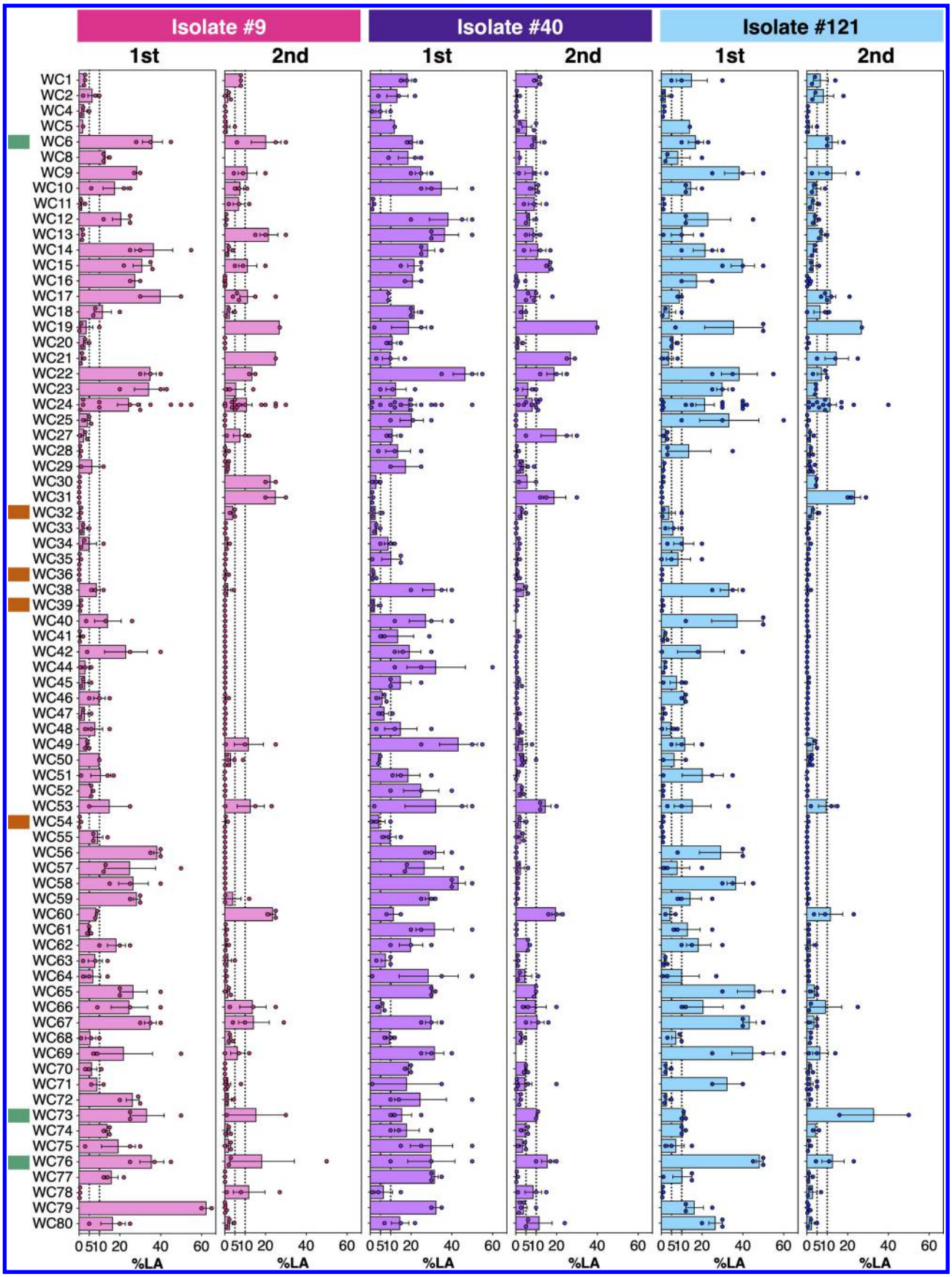

FIGURE 4

Cercospora kikuchii disease severity on soybean varieties from the world core collection. Percentage of the lesion area (%LA) on leaflets as evaluated 9 days postinoculation. Inoculation was duplicated (first and second). Plots on the graph represent the %LA on the assessed leaflets. Bars indicate standard error of the mean. Candidates for *C. kikuchii*-resistant and -susceptible varieties are highlighted in vermilion and green, respectively.

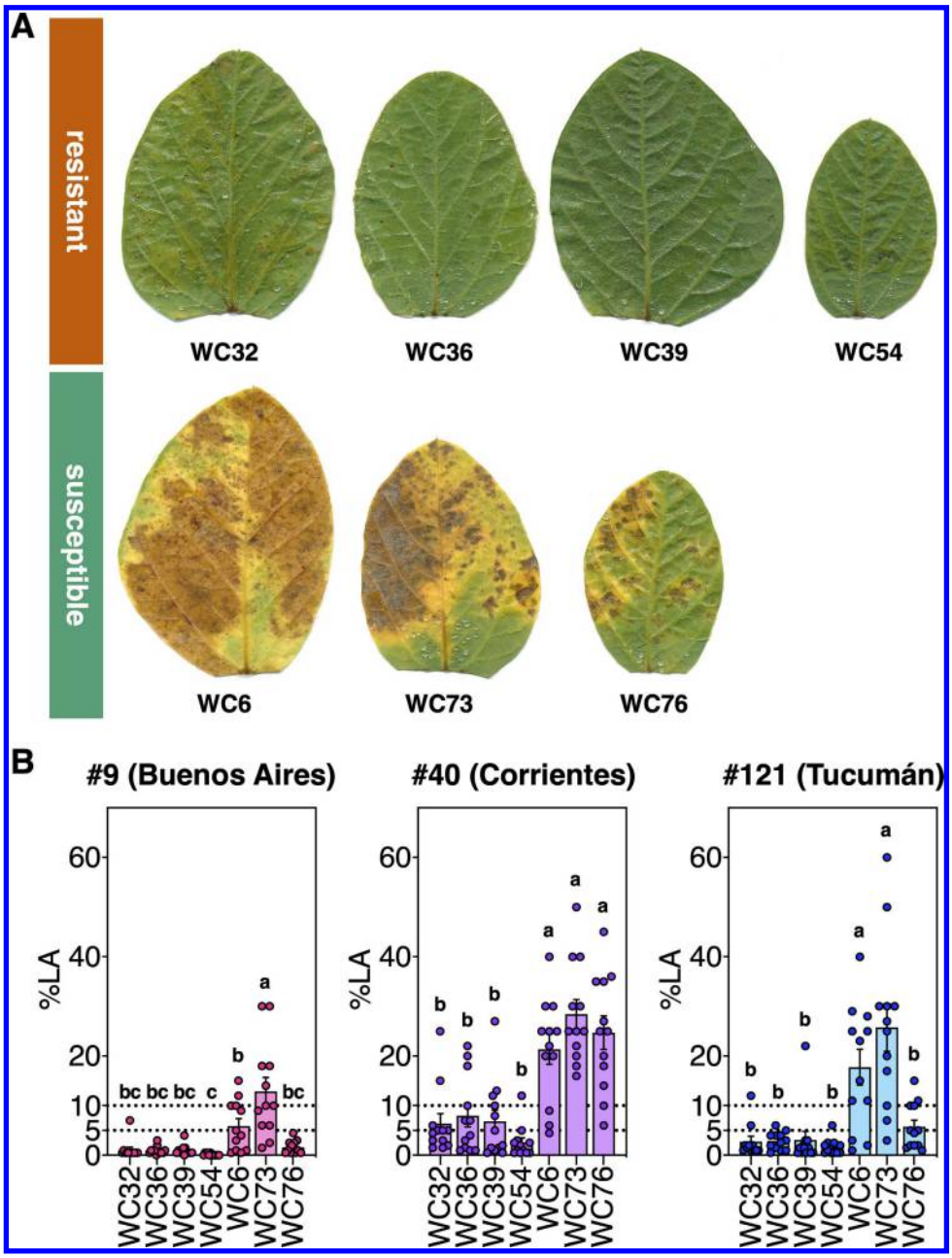

FIGURE 5
Disease severity in the *Cercospora kikuchii*-resistant and -susceptible variety candidates. **A,** Representative photograph of the abaxial side of leaflets at 9 days postinoculation inoculated with *C. kikuchii* isolate #121. **B,** Average percentage of the lesion area (%LA) calculated from 12 leaflets for each inoculum. Plots on the graph represent %LA on the assessed leaflets. Bars indicate standard error of the mean. Significance was determined for each variety via analysis of variance; letters correspond to a Tukey post hoc test ($P < 0.05$).

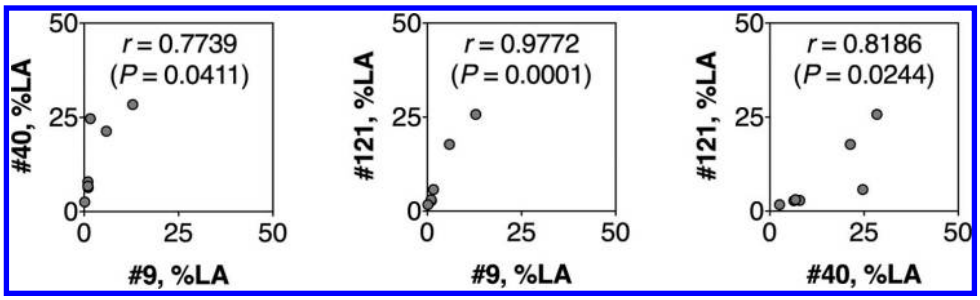

FIGURE 6
Correlation between the results of the inoculation test for the three *Cercospora kikuchii* isolates (#9, #40, and #121). Plots on the graph show average percentage of the lesion area (%LA) of seven selected varieties inoculated with three isolates. Values indicate Pearson correlation coefficient (*r*) and two-tailed *P* value (*P*).

Yamazaki et al. 1975). High humidity is also necessary to promote infection. To investigate resistance against this pathogen, high-throughput and uniform screening methods are required. Our method was able to successfully select both *C. kikuchii*-resistant and -susceptible varieties (Fig. 5). Continuous high humidity in stable conditions for inoculum on leaflets shortened the period required for *C. kikuchii* disease development. Lesions appeared within 9 dpi by our method. Our method also achieved a greater percentage of lesion formation on the leaflets in less than half the time of a previous report (Cai et al. 2009). Additionally, the compact design allowed the screening location to be in a temperature- and light-controlled growth chamber in a conventional laboratory. The leaf culture inoculation method for *C. kikuchii* that we have developed is appropriate for screening *C. kikuchii* resistance in soybean varieties cultivated worldwide. Results of the screening were supported by an inoculation test using whole soybean plants (Supplementary Fig. S1). Furthermore, resistance against *C. kikuchii* of the varieties in field has yet to be validated. Cai et al. (2009) compared results obtained from *C. kikuchii* inoculations performed in the controlled environment and the field. Some varieties represent correlated results between two conditions, but the correlation was not confirmed for all varieties tested in that study. To investigate correlation between the disease severity of *C. kikuchii* in the field and the controlled environment, further research is needed by using a large set of soybean varieties in field. As a result of the leaf culture inoculation, in some varieties, inoculation results varied between leaflets and replication. To use this screening method, the number of biological and technical replications of each inoculation is important to avoid misidentification of *C. kikuchii* resistance. Also, destruction of leaflets during inoculation should be considered for further improvement of this method.

A high-throughput screening method for *C. kikuchii* resistance is a long-sought-after tool for research (Cai et al. 2009). Our method can contribute to achieving high-throughput screening for disease resistance.

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