Article

Evaluation of *Glycine max* and *Glycine soja* for Resistance to *Calonectria ilicicola*

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**Abstract:** Breeding for resistance to soybean red crown rot (*Calonectria ilicicola*) has long been hampered by the lack of genetic sources of adequate levels of resistance to use as parents. Mini core collections of soybean (*Glycine max*) originating from Japan (79 accessions), from around the world (80 accessions), and a collection of wild soybeans (*Glycine soja*) consisting 54 accessions were evaluated for resistance to *C. ilicicola* (isolate UH2-1). In the first two sets, average disease severity scores of 4.2 ± 0.28 and 4.6 ± 0.31 on a rating scale from zero for no symptom to 5.0 for seedling death were recorded from the set from Japan and the world. No high levels of resistance were observed in these two sets. On the other hand, disease severity score of 3.8 ± 0.35 for the wild soybean accessions was somewhat lower and exhibited higher levels of resistance compared to the soybean cultivars. Three accessions in the wild soybean collection (Gs-7, Gs-9, and Gs-27) had disease severity score ≤2.5 and showed >70% reduction in fungal growth in the roots compared to soybean control cv. “Enrei”. Further analysis using 10 *C. ilicicola* isolates revealed that accession Gs-9 overall had a wide range of resistance to all isolates tested, with 37% to 93% reduction in fungal growth relative to the cv. Enrei. These highly resistant wild soybean lines may serve as valuable genetic resources for developing *C. ilicicola*-resistant soybean cultivars.

**Keywords:** soybean; wild soybean; *Calonectria ilicicola*; red crown rot; resistance

1. Introduction

Soybeans (*Glycine max*) are an important source of vegetable protein and edible oil, and provide various nutrients and bioactive components with potential health benefits [1]. With an ever-growing population and stronger economies around the world, the demand for soybeans is rapidly increasing, and accordingly, soybean global annual-production has remarkably increased over the last several decades: 100 Mt in 1987, 144 Mt 1997, 219 Mt in 2007, and 352.6 Mt in 2017 [2]. This increase has been closely associated with increase in seed yield; from 1.91 t ha⁻¹ in 1987 to 2.85 t ha⁻¹ in 2017 [2]. However, this has been not the case for Japan, in which case both annual production and yield have remained unaltered at approximately 250,000 t annually and 1.65 t ha⁻¹, respectively, during the last 30 years [2]. An investigation into why production remained stagnant revealed that red crown rot (RCR) caused by the root-colonizing pathogenic fungus *Calonectria ilicicola* (syn. *Cylindrocladium parasiticum*) is one of the major limiting factors responsible for low soybean seed yield in Japan [3].
Plants that are infected with *C. ilicicola* produce symptoms of root rot, damping-off of young seedlings, and early defoliation [4–11]. Estimated maximum soybean yield losses caused by RCR may be as high as 50% [12,13]. RCR incidence is most commonly associated with poor soil drainage, such as occurs in lower areas in the field. In Japan, ≥80% of soybeans are grown in fields converted from rice paddies, which often results in particularly high levels of RCR incidence [3]. The fungus is capable of infecting a wide range of plant species including soybean, wild soybean, peanut, alfalfa and avocado, and represent over 15 species in seven families [9]. The fungus survives as microsclerotia in the soil for long periods [14], with pathogenicity reportedly retained even after seven years in the soil under natural conditions [15]. In addition, the pathogen can infect soybean roots at any stage during the growing season [6,16]. Because of the multi-host pathogenicity and long survival in soil, control of *C. ilicicola* is extremely difficult once it has established.

Currently, methods to manage this disease are lacking. Several agricultural strategies have been adopted for the control of this disease; for example, inter-crop rotation, late planting, and sowing seeds on ridges [6,7,12,17,18]. However, none of these strategies are sufficiently effective. An agrochemical, tebuconazole (Silvacur flowable, Bayer CropScience K.K., Tokyo), was reported to reduce the RCR incidence, but it also caused a delayed plant growth. Soybean breeding may be effective if sources of resistance to *C. ilicicola* available. However, development of resistant cultivars has been hampered by the lack of resistance sources to use as parents in breeding programs. Indeed, not one soybean cultivar possessing an acceptably high level of resistance to *C. ilicicola* has been identified [19,20].

Wild soybean (*Glycine soja* Sieb. and Zucc.), the ancestor of domesticated soybean, has been demonstrated to show significantly wider genetic variation for many traits than cultivated soybeans [21–23]. Several genes or alleles associated with stress-response traits have been identified and characterized in wild soybean [21], some of which have been successfully introduced into cultivated soybeans including resistance to common cutworm (*Spodoptera litura* Fabricius) [24] and soybean cyst nematode [25–27], and tolerance to salt [28,29] and alkaline salt [30]. These studies suggest that wild soybeans might serve as an important genetic resource for disease-resistance breeding of soybean.

This study aimed to identify genetic sources of resistance to *C. ilicicola* in wild and cultivated soybean accessions to provide the genetic resources for developing *C. ilicicola*-resistant soybean cultivars and studies of the resistance mechanism.

2. Materials and Methods

2.1. Plant Materials

Soybean mini core collections (MC) [31,32] consisting of 79 (Table S1) and 80 (Table S2) accessions from Japan (JMC) and worldwide (WMC) accessions, respectively, were provided by the NARO Genebank, Tsukuba, Japan (formerly NIAS Genebank; http://www.nias.afric.go.jp/). Soybean cultivar “Jack” (PI 540556, IL, USA), not included in the mini core collection, was also used for evaluation of resistance to *C. ilicicola*. Soybean cultivar “Enrei” (included in JMC, NARO Genebank accession number JP28862), an RCR-susceptible elite cultivar in Hokuriku Japan whose genome sequence has been determined at the NIAS, Japan (http://www.nias.afric.go.jp/eng/genome/daizu/index2.html), was used as control in the *C. ilicicola*-resistance tests throughout this study.

The NARO Genebank (https://gene.afric.go.jp/index_en.php) conserves over 2200 accessions of wild soybeans. Among them, we selected 54 accessions for resistance screening: 10 from China, 3 from USA and 41 from 39 prefectures of Japan (Table S3).
2.2. Plant Preparation

For wild soybean seeds, a small portion of the seed coat on the distal end to the hilum was scraped off with a flat file to enable water-permeability. Both cultivated soybean and wild soybean seeds were pre-conditioned in a moisture-saturated plastic box for 24–48 h at 25 °C before sowing to improve and synchronize seed germination. Seeds were sown in commercially available pre-fertilized and granulated soil (Nippi No.1, Nippon Hiryo, Japan) in 65-mm² plastic pots with a depth of 50 mm (180-mL) and a drainage hole. Five seeds were sown per pot and the top of the pot was covered with a two-mm layer of pre-fertilized peaty soil Supermix-A (Sakata Seed Corporation, Japan). All the soils used in this work were autoclaved to eliminate any effect from other soil pathogens. Seeded pots were watered just enough to fully wet the soil and placed in a container. Seedlings were grown in a greenhouse at 26 °C and 50% RH. Water was added to the container as necessary to maintain the soil wetness until the termination of the assay.

2.3. Pathogen Culture

Ten C. ilicicola isolates obtained from different prefectures in Japan were used in this study including, four isolates from the NARO Genebank, namely, MAFF102001 (S1), MAFF102004 (S4), MAFF102005 (S5), and MAFF102006 (S6). Six more isolates were isolated by single hyphal tip culture from RCR-diseased soybean roots; these included, UH2-1 from Sasayama, Hyogo; SN2-1 from Shinano, Nagano; NI1-3-1 from Koshi, Kumamoto; AID1-12 from Aizumisato, Fukushima; KA1-52 from Tsukuba and Ibaraki, and Y11-1b from Tsukubamirai, Ibaraki. These isolates were confirmed by PCR-amplification of ribosomal DNA (rDNA) using a primer set specific to C. ilicicola (CiIGSF = 5′-TCCATTGCTCTATTATCTGCG-3′ and CiIGSR = 5′-GCGTAAGATTTTCCAACCCG-3′) [18], and verified for their pathogenicity on soybean cultivar Enrei. In our pretests, the UH2-1 showed a stable and relatively strong pathogenicity, whereby it was selected for use on the experimental soybean mini core collections and the wild soybean accessions for evaluation of C. ilicicola-resistance in the tests reported herein (Figures 1–4). Ten isolates were used for evaluation of the resistance spectrum of wild soybean Gs-9 to C. ilicicola. The fungus was grown on potato dextrose agar (PDA) plates (90 mm) at 26 °C and long-term stored on barley grains at −80 °C as described previously [33].
between mean values of two independent sets were assessed by non-parametric t-test using the statistic module of Excel software 2016. A P-value of less than 0.05 was considered statistically significant.

3. Results
3.1. Evaluation of \textit{C. ilicicola}-Resistance in Soybean Mini Core Collections

We first observed RCR incidence of \textit{C. ilicicola}-infection in four soybean elite cultivars: Enrei and "Fukuyutaka", the two major JMC varieties cultivated in the Hokuriku and the Kyushu-Tokai regions of Japan, respectively. In turn, Jack (PI 540556, IL, USA) and "Peking" (WMC) are varieties originated from USA and China, respectively. As shown in Figure 2, these cultivars were all severely infected by \textit{C. ilicicola}(UH2-1) as manifested by brown necrosis all over the roots, and root rot and loss. No disease symptoms were observed in the mock-infected control plants in our experimental environment.

![Figure 2. Incidence of red crown rot in four elite soybean cultivars. The photograph was taken 14 days after inoculation with \textit{Calonectria ilicicola}(UH2-1, 1%). Mock plants (Enrei) were grown without \textit{C. ilicicola}-inoculation. The visual rating scores of red crown rot (RCR) severity are shown in parentheses (means ± SD, n = 3 replicates of 5 individuals each).](image)

Next, we evaluated resistance to \textit{C. ilicicola}(UH2-1) in two soybean mini core collections [31]. As shown in Table S1 (JMC) and Table S2 (WMC), high variability for \textit{C. ilicicola}-resistance was observed among individual soybean accessions, with disease severity ranging from 3.2 to 5.0 within JMC (Figure 3A) and from 3.6 to 5.0 within WMC, respectively (Figure 3). Interestingly, in average, JMC showed slightly higher resistance than WMC, with an average disease severity score of 4.2 ± 0.28 in JMC, compared with 4.6 ± 0.31 in WMC. Moreover, there were eight accessions that showed moderate resistance with disease severity scores ranging from 3.01 to 3.5 in JMC, whereas there was none in WMC. However, no accession showing outstandingly high resistance was identified in either mini core collection.

![Figure 3. Frequency distribution of red crown rot severities in mini core collection from Japan (A, JMC), and worldwide (B, WMC) and wild soybean collections (C). Three replicates of 5 individuals of each accession were scored for disease severity after 14 days of inoculation with \textit{Calonectria ilicicola}(UH2-1, 1%).](image)
Figure 4. Identification of high resistance to *Calonectria ilicicola* in wild soybean (*Glycine soja*) lines. (A) Images of disease incidence in control soybean cultivar “Enrei” and wild soybeans Gs-7 (JP30157), Gs-9 (JP30159) and Gs-27 (JP36084). (B) Relative fungal growth in roots of soybean “Enrei” and the *C. ilicicola*-resistant wild soybeans. Data were recorded 14 days after inoculation with *C. ilicicola* (UH2-1, 1%). Values are means ± SD, *n* = 3 replicates of 5 individuals each. Asterisks denote a significant difference to the Enrei control plants (non-parametric *t*-test, ** *p* < 0.01).

2.4. Pathogen Inoculation

Pathogen inoculation was performed as outlined by Nishi et al. [17], with slight modifications. Briefly, five to eight pieces (~5-mm cubes) of PDA with vigorously growing *C. ilicicola* mycelia were placed in a 500-mL flask containing 200 g of wheat bran-vermiculite medium (wheat bran/vermiculite/water 1:1:3, w/w/v) and incubated at 26 °C for 14 days, until the medium was fully covered by the fungus. This culture was used as inoculum, and an inoculum–soil mixture was prepared by mixing the inoculum with Nippi No.1 soil to a strength of 1–3% (w/v) and filled into a plastic pot (180 mL). Fourteen days after seed sowing (days post inoculation, dpi), seedlings were removed from the pots and the roots were washed gently with running tap water to remove the adhering soil. Disease severity was evaluated either by visual severity scoring (see below) or by determining *C. ilicicola* genomic 28S rDNA (relative fungal growth, see below).
2.5. Visual Evaluation of Disease Severity

In this study, the evaluation of *C. ilicicola*-resistance was based on root rot at seedling stage (14 dpi). Disease severity was visually scored based on a six-point rating scale (0 to 5) according to Nishi et al. [17], with some modifications to fit our greenhouse inoculation assays (Figure 1). Accordingly, 0 = no visible symptom, 1 = small brown necrotic lesions on the primary root, 2 = brown necrotic lesions extending all over the primary root and some lateral roots, 3 = ≥50% root-loss by rot and severe brown necrosis on subterranean stem, 4 = almost all roots rotted and lost, and 5 = seedlings are dead. Individual seedlings were considered dead if they had withered and fallen down. For the purpose of this study, accessions with disease severity scores ≤2.5, ≤3.5, and >3.5 were considered resistant, moderately resistant and susceptible to *C. ilicicola*, respectively.

2.6. Genomic DNA Preparation

Genomic DNA was extracted from roots by the cetyltrimethylammonium bromide (CTAB) method as described previously [34], with some modifications. Briefly, about 300 mg of fresh roots was grinded in liquid nitrogen with a mortar and a pestle to a fine powder, and transferred into a microtube containing 500 µl of preheated CTAB isolation buffer (2% CTAB (Sigma-Aldrich, Tokyo, Japan), 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0) and 20 mM EDTA). After incubation for 30 min, the microtube was centrifuged at 13,000 rpm for 10 min at room temperature in a microcentrifuge (Eppendorf, model 5424R, Hamburg, Germany). The supernatant was vortexed with 200 µl of chloroform and centrifuged as above, and the resultant supernatant was mixed with the same volume of isopropanol to precipitate DNA by centrifugation as above. The resultant DNA pellet was washed with 500 µl of 70% ethanol, air-dried, and dissolved in 200 µl distilled water. A 1-µl aliquot of the extracted DNA was used for the RT-qPCR reaction (see below).

2.7. Relative Fungal Growth Determination by Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Primer sets used for RT-qPCR-determination were: (1) primers targeting the intergenic spacer region of the *C. ilicicola* rDNA, CiIGSF (forward) = 5′-TCCATTGCCTCTATTTATCCTGC-3′ and CiIGSR (reverse) = 5′-GCGTAAAGATTTTCCAACCCG-3′ [18]; (2) primers for soybean actin gene 11 (Glyma.15G050200), GmActinF (forward) = 5′-GAG CTATGAATTGCCTGATGG-3′, and GmActinR (reverse) = 5′-CGTTTCATGA ATTCCAGTAGC-3′ [35]. RT-qPCR was run on a Thermal Cycler Dice TP800 system (Takara), using SYBR premix Ex Taq mixture (Takara), with cycles of 95 °C for 5 s, 55 °C for 20 s and 72 °C for 20 s [35].

Relative fungal growth was expressed as *C. ilicicola* rDNA amplification folds relative to soybean actin gene amplification [36,37].

2.8. Experimental Design and Data Analysis

All experiments were conducted with three replicates, each consisting of two pots with five plants per pot for each inoculation. For some accessions with low germination rate, three pots with five plants per pot were inoculated. Plants for each replicate were placed in a separate container, and the three replicate containers were rotated within the greenhouse every two to three days to minimize any location effects.

Calculation of mean values and standard deviations, and graph plotting were performed using Excel software 2016 (Microsoft Corporation, Tokyo, Japan). Based on the results of *F*-test, differences between mean values of two independent sets were assessed by non-parametric *t*-test using the statistic module of Excel software 2016. A *P*-value of less than 0.05 was considered statistically significant.
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3.2. Identification of C. ilicicola-Resistance in Wild Soybeans (G. soja)

As shown in Figure 3C, wild soybeans generally showed higher resistance to C. ilicicola (UH2-1), with an average disease severity score of 3.8 ± 0.35 (Table S3). Particularly, there were three wild soybean accessions, JP30157 (Gs-7), JP30159 (Gs-9), and JP36084 (Gs-27), which exhibited a resistance to C. ilicicola, with disease severity scores ≤2.5 (Figures 3C and 4).

It should be noted that the resistance observed in these wild soybean accessions was not complete resistance, as some necrotic lesions developed along the roots, although to a much lesser extent than in our soybean control, Enrei (Figure 4A). The relative fungal growth in these wild soybeans was about 3.0–3.82, one fifth to one fourth of that observed in Enrei (i.e., 16.63) (Figure 4B).

3.3. Evaluation of the Resistance Spectrum of Wild Soybean Gs-9 to C. ilicicola

Among the three C. ilicicola-resistant wild soybeans (Figure 4), a sufficient amount of seed of Gs-9 (Genbank accession number JP30159) was available from the NARO Genebank for further characterization. We used these seeds to examine the resistance spectrum of this accession to 10 different isolates of C. ilicicola originated from different regions of Japan. The inoculation assay was carried out at two different inoculation strengths (i.e., 1% and 3%) of each isolate.

As shown in Figure 5A, Gs-9 seedlings displayed higher resistance than control Enrei to all the ten C. ilicicola isolates at 1% inoculum strength. Different C. ilicicola isolates resulted in different disease severities on the roots; relative fungal growth in Gs-9 was reduced by 41% (SN2-1) to 94% (S6) compared with Enrei (Figure 5C). Isolate AID1-12 and Y11-1b showed a particularly high pathogenicity to both Enrei and Gs-9, followed by UH2-1 and SN2-1. In contrast, isolates NI1-3-1, S1 and S5 caused almost no disease symptoms even at 3% inoculum strength (Figure 5B). At the higher inoculation strength (3%), the disease severity was further increased regardless of C. ilicicola isolates, and the relative fungal growth in Gs-9 was reduced by about 38% (SN2-1) to 87% (S6) compared with Enrei (Figure 5C).
Figure 5. Evaluation of the resistance of wild soybean accession Gs-9 (JP30159) to 10 different isolates of Calonectria ilicicola. (A,B) Images of disease incidence at 1% (A) and 3% (B) of inoculum concentrations, respectively, in control soybean “Enrei” (left of the vertical dotted line) and wild soybean accession Gs-9 (right of the vertical dotted line, JP30159). (C) Relative fungal growth in roots of soybean Enrei (grey bars) and the C. ilicicola-resistant wild soybean Gs-9 (black bars) at 1% and 3% inoculum strengths respectively. Data were recorded 14 days after inoculation with 10 different isolates of C. ilicicola, as indicated on the figures. Values are means ± SD, n = 3 replicates of 5 individuals each. Asterisks denote a significant difference to the Enrei control plants (non-parametric *t*-test, * p < 0.05, ** p < 0.01).
4. Discussion

Red crown rot (RCR) caused by C. ilicicola has been identified as one of the major factors that currently limit soybean yield in Japan [3]. Therefore, the development of C. ilicicola-resistant cultivars warrants effort to improve soybean productivity; however, to date such end has been hampered essentially by the lack of genetic resources with sufficiently high levels of resistance to C. ilicicola. Thus, the identification of C. ilicicola-resistant wild soybean accessions in this study should contribute significantly to the development of C. ilicicola-resistant soybean cultivars.

Considerable efforts have been previously made to identify resistance to C. ilicicola in soybeans. Kim [20] observed a range of susceptibility to C. ilicicola among 18 cultivars in a field test, but none of them was completely resistant. Similarly, Nakajima et al. [19] observed strong genetic variability for resistance to C. ilicicola within a germplasm sample comprising 150 soybean cultivars and 7 wild soybean accessions; however, no complete resistance was detected in this case either. These results are consistent with our finding; no complete resistance was found among 159 accessions of the soybean mini-core collections or in the 54 accessions of the wild soybean collection. Nevertheless, the levels of resistance to C. ilicicola among wild soybeans were higher than those of cultivated-soybean accessions (Figure 3). Among wild soybeans, three accessions, Gs-7 (JP30157), Gs-9 (JP30159) and Gs-27 (JP36084), showed particularly high resistance to C. ilicicola with disease severity scores ≤ 2.5 (Figures 3 and 4). These accessions should be valuable sources of resistance for developing new C. ilicicola-resistant soybean cultivars. Thus, each of these accessions has been crossed with soybean cultivar Enrei to produce recombinant inbred lines for quantitative trait loci (QTL) analysis. Field trials are also underway to assess the resistance efficacy of the three wild soybeans to C. ilicicola.

Interestingly, a difference in resistance levels to C. ilicicola (isolate UH2-1) was also evident among soybean accessions in JMC and WMC. Particularly, eight varieties from JMC showed moderate resistance with disease severity scores ≤ 3.5, whereas not a single accession from WMC did. The nature of the biogeographical difference between these two collections is yet unknown, but it may provide some reference for selection of suitable genotypes in breeding programs and cultivation areas.

All C. ilicicola isolates tested in this study caused root rot in both cultivar Enrei and wild soybean accession Gs-9, albeit to a different extent, suggesting that C. ilicicola isolates do not cause a differential response at least to the two genotypes tested. On the other hand, a marked difference in disease severity and symptoms was observed for RCR caused by different C. ilicicola isolates, suggesting a physiological differentiation of this fungus. These results are consistent with previous reports of physiological differentiation, but not race differentiation, in C. ilicicola–soybean pathosystems [19,20]. Moreover, it has been reported that C. ilicicola isolates derived from either soybean or peanut infected both soybean and peanut; however, each isolate exhibited a preferential virulence to its original host plant [38]. This physiological differentiation among C. ilicicola isolates might further lead to race development in the future [17]. Furthermore, the disease severity was further increased regardless of C. ilicicola isolates when applied at a high inoculation strength (Figure 5B), which further confirms that the resistance is partial (horizontal, quantitative), rather than complete resistance. Indeed, there is an increasing interest in using partial resistance as genetic source of resistance in breeding programs, as it generally confers durable and wide spectrum resistance, in contrast to complete resistance, which is race-specific and often short-lived (Grau et al. 2004).

In summary, we succeeded for the first time in identifying three novel wild-soybean accessions that possess a wide range and high-levels partial resistance to C. ilicicola. These wild soybean lines should serve as valuable genetic resources for developing C. ilicicola-resistant soybean cultivars and for further elucidating the molecular mechanism of C. ilicicola-interaction.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/6/887/s1, Table S1: RCR-severities in JMC varieties. Table S2: RCR-severities in WMC varieties. Table S3: RCR-severities in WMC varieties.
Author Contributions: C.-J.J. and S.S. conceived, designed and performed the experiments; S.O. isolated fungus Calonectria ilicicola; A.K. and M.I. prepared soybean seeds of mini core collections; C.J., analyzed the data, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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