A fresh weight-based method for evaluating soybean resistance to red crown rot

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Soybean red crown rot (RCR) caused by Calonectria ilicicola is a serious soil-borne disease affecting soybean production and quality. The current visual necrosis-based method for the measurement of RCR severity is prone to subjectivity as well as time consuming and laborious as it requires digging out and washing the roots to remove adhering soil prior to the visual scoring. Using cultivar Enrei, we show that, upon C. ilicicola infection, relative fresh weights (RFW; fresh weights relative to non-inoculated control plants) showed a significant negative correlation with visual RCR severity in apical shoot (trifoliate and above, $R^2 = 0.96$), shoot (unifoliate and above, $R^2 = 0.82$) and roots ($R^2 = 0.89$). Furthermore, apical shoot RFW efficiently correlated with varying levels of C. ilicicola resistance in two test sets containing 37 soybean cultivars and three wild soybean accessions, exhibiting a significant correlation with visual severity ($R^2 = 0.72$ and $0.79$, $p < 0.01$). Taken together, our results suggest that RFW can serve as an index of soybean RCR severity, providing a simple, rapid, consistent, and cost-effective method for evaluating C. ilicicola resistance in soybeans.

Key Words: soybean, red crown rot, disease severity, fresh weight, resistance screening.

Introduction

Soybean red crown rot (RCR) caused by the soil-borne fungal pathogen Calonectria ilicicola (Anamorph: Cylindrocladium parasiticum) is one of the most serious soil-borne diseases worldwide (Crous et al. 1993, Padgett et al. 2015). Root infection by C. ilicicola can occur soon after soybean planting (Kuruppu et al. 2004), but initial symptoms in soybean fields appear during mid to late reproductive growth stages (Akamatsu et al. 2020, Roy et al. 1989, Yamamoto et al. 2017). The typical symptoms include leaf chlorosis/necrosis, root and stem decay with reddish-brown or orange-colored perithecia developing on the basal parts of main stems and roots, and precocious defoliation (Akamatsu et al. 2020, Roy et al. 1989, Yamamoto et al. 2017). In severe cases, most lateral roots are lost from the taproots, resulting in poor plant growth and reduction of seed yield and quality (Akamatsu et al. 2020, Roy et al. 1989, Yamamoto et al. 2017). It has been implicated that a toxic metabolite (PF1070A) of the fungus is associated with the virulence of C. ilicicola and disease development (Ochi et al. 2011). The estimated maximum yield loss in soybean caused by C. ilicicola may be as high as 50% (Berggren and Snow 1989, Gao et al. 2012, Padgett et al. 2015).

In Japan, more than 80% of soybean crop is grown after paddy (MAFF 2014, Sugimoto et al. 2012). The clayed nature of such soybean fields, which is favorable for paddy cultivation, makes them prone to short-term waterlogging due to poor drainage (Bajgain et al. 2015). These conditions are favorable for RCR development. RCR has thus been identified as one of the factors limiting soybean production in Japan (MAFF 2014). To date, no practically effective fungicides exist for the control of this disease. Therefore, sustainable agricultural management practices and an efficient ecological strategy to control RCR are urgently required.

Several agricultural practices have been employed to handle RCR in soybean, such as delaying sowing, intercropping with non-host crops, biological control, and conventional tillage to reduce inoculum and spore dissemination by C. ilicicola (Gao et al. 2012, 2014, Kuruppu et al. 2004). However, environmental conditions in the field affect the consistency and effectiveness of these practices. Thus, the use of host genetic resistance is considered the most effective and eco-friendly way of reducing yield losses due to RCR incidences in soybeans. Moreover, resistant cultivars can provide disease protection at no additional cost to the grower. While variation in resistance to C. ilicicola has long been reported among soybean cultivars, no complete or high-level resistance to C. ilicicola has yet been identified (Jiang et al. 2020, Kim et al. 1998, Nakajima et al. 1994). Recently, we reported identification of three wild-soybean accessions that possess a high-level resistance to C. ilicicola (Jiang et al. 2020). These wild soybean lines
should serve as valuable genetic resources for developing *C. ilicicola*-resistant soybean cultivars.

A simple and reliable assessment method is essential for genetic screening and identifying resistance to *C. ilicicola* from a large number of soybean germplasms in order to use them in resistance breeding programs. For quantifying plant disease, visual assessment is probably still the most widely used method (Bani et al. 2012, Bock and Nutter Jr. 2012, Pilet-Nayel et al. 2002), and the current method for RCR evaluation also relies on visual scoring of necrosis (browning) of the roots. However, visual scoring by naked eyes, especially in a large-scale assessment, can result in bias arising from differences in individual judgments, as well as between experimental repeats (Bock et al. 2010). This method is also laborious and time-consuming and costly, because it requires digging out and washing the roots to remove adhering soil prior to the visual scoring.

In view of this, the purpose of this study was to develop a simple and consistent laboratory method for measurement of soybean RCR severity. We present a new method that involves simply measuring apical shoot fresh weights of inoculated and non-inoculated control soybean plants.

### Materials and Methods

**Cultivars**

The soybean cultivar Enrei was used in all the experiments. Additionally, 36 local soybean cultivars (kindly provided by Drs. Youhei Nanjou and Fumio Taguchi-Shiobara at Institute of Crop Science, NARO, Japan), as well as three wild soybean accessions (*Glycine soja* Sieb. and Zucc; JP30157, JP30159, and JP36084, NARO Genebank, http://www.nias.affrc.go.jp/) recently identified to be highly resistant to *C. ilicicola* (Jiang et al. 2020), were used in two validation experiments: the first validation experiment involved 24 soybean cultivars including Enrei and three wild soybeans ([Supplemental Table 1](#)); and the second experiment included 14 cultivars including Enrei and three wild soybeans ([Supplemental Table 2](#)).

**Plant growth**

Both cultivated and wild soybean seeds were preconditioned in a moisture-saturated plastic box for 24–48 h at 25°C before sowing, to improve and synchronize seed germination. To increase the water permeability of wild soybean seeds, a small portion of the seed coat on the distal end to the hilum was scraped off with a flat file. Seeds were sown in commercially available, pre-fertilized and granulated soil (Nippi No. 1, Nippon Hiryo, Tokyo, Japan) at a depth of 2–3 mm 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 2 mm layer of pre-fertilized peaty soil Supermix-A (Sakata Seed Corporation, Yokohama, Japan). All the soils used in this work were autoclaved one day before the seed sowing to eliminate any effect from other soil pathogens. Seeded pots were watered just enough to fully wet the soil and placed in a container. Water was added to the container as necessary to maintain the soil wetness until the termination of the assay.

**Pathogen culture and inoculation**

The fungus *C. ilicicola* (isolate UH2-1) was kindly provided by Dr. Sunao Ochi (Research Center for Agricultural Information Technology, NARO, Japan), which was isolated from RCR-diseased soybean roots from Sasayama, Hyogo (Jiang et al. 2020). Fungal mycelia were grown on potato-dextrose agar (PDA) plates at 25°C for 1–2 weeks or until the fungal mycelial growth reached the edges of the petri plates.

Pathogen inoculation was performed as described previously (Jiang et al. 2020). 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 10–14 d, until the medium was fully covered by the fungal mycelia. This culture was used as inoculum, and an inoculum-soil mixture was prepared by mixing the inoculum with Nippi No. 1 soil to a density of 0.2–3.2% as stated in the results and figure legends. The mixture was used to fill plastic pot (180 ml) in which five seeds per pot were sown as described above. Seeds were germinated and the seedlings were grown in a greenhouse at 26°C and 50% relative humidity.

Two validation experiments were done under temperature-controlled growth cabinet at 26°C with 16 h of daytime under 250 to 350 μmol photons m⁻² s⁻¹ of light intensity and 50% relative humidity for the remainder of the experimental period. The well-controlled conditions in growth cabinet generally resulted in more stable assay results than in the greenhouse. However, on the other hand, it was found that the disease severity tended to be slightly lower than that obtained under greenhouse conditions. Therefore, in these experiments, the inoculum density was increased to 1.2% v/v to obtain disease levels similar to those in the greenhouse assays.

**Measurement of disease severity**

Fourteen days after seed sowing (days post inoculation, dpi), the shoot (unifoliate and above) and apical shoot (first trifoliate and above) of soybean seedlings ([Fig. 1B](#)) were cut and their fresh weights were measured. Subsequently, the seedlings were taken out from the pots and the roots were washed gently with running tap water to remove adhering soil. The roots were cut off at the base of the stem (hypocotyl) and blotted on a towel paper to remove excess water, and their fresh weights were measured. The relative fresh weight (RFW) was calculated as fresh weight of inoculated plants relative to that of the non-inoculated control plants.

The visual disease severity of roots was scored based on...
Development of a fresh weight-based method for RCR severity measurement

Compared with the non-inoculated control plants, *C. ilicicola* inoculation at 1% density resulted in a remarkable growth reduction in cultivar Enrei (Fig. 1A), manifesting reductions of up to 50% in plant height (Fig. 1C), 33% in shoot fresh weight (Fig. 1D), and 23% in root fresh weight (Fig. 1E). These results demonstrated that the root rot caused by *C. ilicicola* resulted in biomass reduction. Thus, we investigated how the extent of biomass reduction is correlated to the degree of RCR severity. Plant height was not included in the further investigation as it is generally highly affected by growth conditions other than fungal infection, like light, temperature and soil water conditions.

The Enrei seeds were sown in different doses of *C. ilicicola* inoculum ranging from 0.2 to 3.2% (w/v) to produce varying disease severities (Fig. 2). The results showed a dose dependent visual disease severity (Fig. 2A). The concentrations of 0, 0.2, 0.4, 0.8, 1.6, and 3.2% inoculum resulted in disease severity score of 0, 3.0, 3.3, 3.4, 4.0, and 4.5, respectively (Fig. 2B). On the other hand, the RFW of apical shoot, shoot, and roots were significantly reduced in inoculated plants compared to that in non-inoculated plants. The RFW ranged from 1.0 to 0.81, 0.50, 0.51, 0.39, and 0.29 in apical shoot, 1.0 to 0.84, 0.59, 0.63, 0.51, and 0.39 in shoot, and 1.0 to 0.77, 0.67, 0.70, 0.63, and 0.49 in roots, respectively, with the increasing dose of inoculum. In agreement with the previous studies, the incremental plant growth decreased, and root discoloration increased as inoculum concentration of the pathogen increased (Jackson et al. 2005, Jiang et al. 2016, Raftoyannis and Dick 2002, Shakiba et al. 2012). The RFW reduction was most prominent in apical shoot, followed by shoot and roots (Fig. 2B).

Correlation analysis revealed that the reduction in RFW of all the three tissues is significantly negatively correlated with the increase in visual RCR severity, with the correlation coefficients ($R^2$) of 0.96 ($p < 0.01$), 0.82 ($p < 0.05$), and 0.89 ($p < 0.05$) for apical shoot, shoot, and roots, respectively (Fig. 3). The finding that RFW is significantly correlated to RCR severity, strongly suggests that the RFW can serve as a new alternative index to current visual disease severity.

Among RFWs examined in this study, the RFW of apical shoot showed the highest reduction in response to RCR incidence, as well as the highest correlation coefficient to RCR severity ($R^2 = 0.96$, $p < 0.01$). These results suggest that the RFW of apical shoot should be the first choice for use in the new method for assessment of RCR severity. The RFW of roots also showed a high correlation coefficient.
Validation of fresh weight-based measurement in genetic differentiation of *C. ilicicola* resistance

Two experiments, each containing 27 and 17 genotypes, respectively, were conducted using 1.2% inoculum density to validate the RFW of apical shoot as an index of RCR severity. The results of one-way ANOVA indicated a significant genotypic variation (\(p < 0.002\)) in RFW of apical shoot and visual disease severity (\(p < 0.000\)). According to one-way ANOVA results, visual disease severity and RFW of apical shoot for the first and second validation experiments are presented in Supplemental Tables 1 and 2.

The validation experiment using 24 soybean cultivars together with the three wild soybean accessions (Supplemental Table 1) showed that the RFW of apical shoot was clearly different between *C. ilicicola* resistant wild soybean accessions (Jiang et al. 2020) and cultivated soybean cultivars (Fig. 4). Based on Tukey’s Honest Significant Difference (HSD) Test (\(p < 0.05\)), the greatest values of RFW were detected for wild soybeans, while their visual disease severities were the lowest among the tested accessions. The *C. ilicicola*-infection caused significant changes in RFW of the soybean cultivars, whereas it was not the case for *C. ilicicola* resistant wild soybeans. Among the soybean cultivars, Nakasennari and Murayutaka exhibited relatively high RFW of apical shoot (≥0.45) and low disease severity (≤2.77) compared to the others. The correlation coefficient (\(R^2\)) of RFW-based measurement to visual disease severities was 0.72 (\(p < 0.001\)) (Fig. 4). However, in both visual

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**Fig. 2.** Impact of *Calonectria ilicicola* inoculum density (0.2–3.2%) on growth of soybean plants (cultivar Enrei) at 14 days post-inoculation (dpi). (A) Representative photograph of the whole plant and (B) relative fresh weight (RFW) of root, shoot, and apical shoot, and visual disease severity. Different letters indicate significant difference at the 5% level according to Tukey’s Honest Significant Difference (HSD) Test (\(p < 0.05\)) test. Values are means ± SD, \(n = 30\). There were three replications and each replicate consisted of 10 plants, \(n = 30\).

**Fig. 3.** The correlation analysis between visual disease severity and relative fresh weight of shoot (blue square), root (grey triangle), and apical shoot (white circle) of Enrei.

\[R^2 = 0.89, p < 0.05\]; however, determination of root RFW is very laborious and time consuming, and so is less advantageous compared to the others.

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**Fig. 4.** The relationship between visual disease severity and relative fresh weight (RFW) of apical shoot of 24 soybean cultivars and three wild soybean accessions at 14 days post-inoculation (dpi) with *Calonectria ilicicola* (1.2%). The soybean cultivars and wild soybean accessions are shown in square filled with grey (Satonohohoemi), black (LD00-3309), purple (Otsuzu), brown (UA4805), white (Nemashirazu), red (Ayakogane), yellow (Gendenshirazu), blue (Tachinagaha), and green (Yukishizuka), circle filled with blue (Murayutaka), green (Nakasennari), red (Akimaro), black (Tamahomare), pink (Peking), grey (Shuurei), purple (Ottsuru), and yellow (Yukishizuka), triangle filled with green (wild 9), blue (wild 7), and purple (wild 27), and rhombus filled with blue (Nattosyoryu), green (Miyagishimo), yellow (Nanbushirome), black (NC-Raleigh), brown (Enreinosora), grey (Ryuhou) and red (Enrei). There were three replicates per genotype, each replicate consisted of 5 plants, \(n = 15\).
and RFW methods, it appears difficult to determine minor differences in *C. ilicicola* resistance among the susceptible soybean cultivars.

Further validation using 14 soybean cultivars together with the three wild soybean accessions (Supplemental Table 2) gave similar results, which clearly distinguished the *C. ilicicola* resistant wild soybean accessions from the soybean cultivars. In addition, the cultivars Tanbakuro, Horokanai Zairai and Kitamishiro showed higher RFW of apical shoot (≥0.7) and lower visual disease severity scores (<2.59) compared with the other soybean cultivars (Fig. 5, Supplemental Table 2). The correlation coefficients (R²) of RFW-based measurement to visual disease severities were 0.79 (p < 0.001) (Fig. 5).

It should be noted that the degree of disease severity in both visual and RFW measurements can vary between experiments depending on pathogen growth, and environmental conditions like light and temperature. Therefore, the assessment of resistance between different cultivars should be done in the same experimental set. Moreover, a genetic variation for resistance to *C. ilicicola* was observed among soybean and wild soybean accessions, however, no complete resistance was detected; which is consistent with previous screenings for *C. ilicicola* resistance in large populations of soybean and wild soybean accessions (Jiang et al. 2020, Kim 1994, Nakajima et al. 1994).

In summary, we present a new method for the evaluation of *C. ilicicola* resistance that involves simply measuring the RFW of the apical shoot, which should greatly facilitate evaluation and screening for resistance to *C. ilicicola*, especially in large-scale population studies. This method showed favorable consistency over the traditional visual scoring method for RCR severity, and efficiently differentiated *C. ilicicola* resistance among soybean and wild soybean accessions. The new method provides advantages over the traditional visual evaluation; these include simplicity, ease of performance, high uniformity and high sample throughput, and cost effectiveness. Whether this method is also applicable to field evaluation of *C. ilicicola* resistance remains unknown and deserves further investigation in the future studies.

**Author Contribution Statement**

KW performed experiments and wrote the manuscript; CJ conceived and designed the research, and revised the manuscript.

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