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A Hypovirulent Strain of *Calonectria ilicicola* for Biological Control of Soybean Red Crown Rot

Mitsunori TAKANASHI[1], Michie KOBAYASHI[1]* and Chang-Jie JIANG[1,2]*

[1] Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), Tsukuba, Japan

Abstract

Soybean red crown rot (RCR), caused by the soil-borne fungus *Calonectria ilicicola*, significantly reduces soybean yield and quality. A hypovirulent strain of *C. ilicicola*, designated UH2-1A, was obtained by repeated subculturing of the virulent strain UH2-1. UH2-1A exhibited significantly reduced pathogenicity after 20 and 40 subcultures, with RCR symptom scores of 0.8 and 1.0, respectively, compared to 3.0 and 4.0 for UH2-1. No differences in growth rate or direct antagonistic effects were observed between UH2-1A and UH2-1. Notably, incorporating UH2-1A into the UH2-1 inoculum mixture significantly reduced the RCR incidence at UH2-1A/UH2-1 ratios of ≥ 1.0 in greenhouse experiments. The suppressive effect of UH2-1A was also observed against four additional virulent *C. ilicicola* strains, suggesting broad control potential. These results highlight UH2-1A as a promising biological control agent for soybean RCR and provide an effective method for generating hypovirulent pathogen strains to control diseases caused by intraspecific pathogens.

Discipline: Agricultural Environment
Additional key words: *Glycine max*, long-term subculture, soil-borne disease, soybean RCR

Introduction

Soybean (*Glycine max* L. Merr.) is a globally important crop, serving as a primary source of vegetable oil and protein for human and animal consumption. However, its production is frequently threatened by various diseases, among which red crown rot (RCR), caused by the soil-borne fungal pathogen *Calonectria ilicicola* (anamorph: *Cylindrocladium parasiticum*), poses a significant challenge (Jiang & Xie 2023). This is particularly true in Japan, where soybeans are predominantly cultivated in paddy-converted fields with high soil moisture content. This exacerbates crop vulnerability to soil-borne pathogens (Akamatsu et al. 2020, Jiang & Xie 2023, Nishi 2007). *C. ilicicola* infects the root system and basal stem of soybean plants, leading to distinctive symptoms, including red-brown discoloration of the crown and root tissues, leaf chlorosis, premature defoliation, and plant death in severe cases (Kleczewski et al. 2023). These symptoms are particularly damaging during reproductive stages, resulting in significant reductions in both yield and seed quality (Ochi et al. 2022).

The management of RCR is complicated by several factors, including its wide host range and persistence in soil as microsclerotia that can survive for seven years or more (Nishi 2007, Jiang & Xie 2023). Current approaches to RCR management include various strategies, each with significant limitations. Cultural practices such as late planting (Kuruppu et al. 2004, Ochi et al. 2013), flooding fields during winter (Ochi et al. 2013), and crop rotation (e.g., maize-soybean intercropping) (Gao et al. 2014) can reduce disease severity but are often constrained by labor-intensive regional climatic conditions or inconsistent efficacy. Chemical control with fungicides such as tebuconazole, fludioxonil, and azoxystrobin can inhibit fungal growth (Kleczewski & Geisler 2022, Nakagawa & Ochi 2006, Wu et al. 2022). However, some treatments may suppress plant growth, and repeated use risks fostering pathogen resistance (Nakagawa & Ochi 2006). Although some wild soybean (*Glycine soja*) accessions exhibit resistance (Antwi-Boasiako et al.

*Corresponding authors: Michie Kobayashi, kobayashi.michie713@naro.go.jp; Chang-Jie Jiang, cypa44@hotmail.com
[2] Present address: Shandong Academy of Agricultural Sciences, Jinan, China
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2024, Jiang et al. 2020), breeding fully resistant cultivars remains a long-term goal.

Biological control is a promising alternative for sustainable agricultural principles. Various biological control agents (BCAs), including antagonistic microorganisms, such as *Trichoderma* spp. (Nakagawa 2004), *Bacillus subtilis* (Tsurumi et al. 2020), *Bacillus altitudinis* (Wu & Chang 2024), and *Pseudomonas* spp. (Win et al. 2022), have been investigated for their potential to suppress soil-borne pathogens. However, the effectiveness of these BCAs against *C. ilicicola* needs to be confirmed under field conditions because cost, propagation, and performance challenges limit their widespread adoption.

Given the limitations of the existing methods, innovative approaches are required to enhance RCR management. One promising strategy involves using nonpathogenic or virulence-attenuated (hypovirulent) strains of the pathogen itself, leveraging mechanisms such as competitive exclusion or interference to suppress the wild-type pathogen. Hypovirulence, characterized by reduced pathogenicity in the pathogen, has been successfully used to manage several diseases, including chestnut blight caused by *Cryphonectria parasitica* (Heiniger & Rigling 1994, Milgroom & Cortesi 2004), wheat take-all disease caused by *Gaeumannomyces graminis* (Duffy & Weller 1996), root rot diseases caused by *Rhizoctonia* spp., *Fusarium* spp., and *Pythium* spp. (Alabouvette et al. 2009, Sneh 1998), rice blast caused by *Magnaporthe oryzae* (Deng et al. 2024, Jiao et al. 2023), and white mold caused by *Sclerotinia sclerotiorum* (Fu et al. 2024). The mechanisms underlying this protection may involve competition for infection sites, induced resistance, or interference with pathogenic factors (Nuss 2005).

Hypovirulent strains offer several advantages when used in biocontrol strategies. Unlike the introduced BCAs, hypovirulent strains derived from the pathogen itself are likely to be well-adapted to the ecological niche of the target pathogen, potentially leading to better establishment and persistence in the field (Ghabrial et al. 2015). Additionally, the ecological risks associated with the introduction of non-native microorganisms are minimized when attenuated strains of indigenous pathogens are used (Collatz et al. 2021, Lahlali et al. 2022). This approach aligns with the growing demand for environmentally friendly disease management practices that minimize ecological impacts and reduce the reliance on chemical inputs. Despite these potential advantages, the application of hypovirulent strains in RCR management has not been extensively explored.

In our research on soybean RCR using the *C. ilicicola* strain UH2-1, a highly virulent strain that provides stable inoculation, we sometimes failed to inoculate soybean seedling roots. After examining the various factors affecting infection, we hypothesized that a decrease in virulence due to repeated subculturing of the fungus may be the cause. In this study, we investigated the potential of a virulence-attenuated strain of *C. ilicicola*, designated UH2-1A, as a biological control agent for soybean RCR. These findings contribute significantly to integrating hypovirulent strains into existing disease management practices, offering a novel and eco-friendly solution to persistent agricultural challenges.

Materials and methods

1. Pathogen culture

Three *C. ilicicola* strains (UH2-1 from Tambaasayama City, Hyogo; SN2-1 from Shinano Town, Nagano; and Y11-1b from Tsukubamirai City, Ibaraki) were isolated from RCR-diseased soybean roots using single hyphal tip culture (Jiang et al. 2020). Two strains, MAFF102004 (S4) and MAFF102006 (S6), were obtained from the NARO Genebank. The fungus was grown on potato-dextrose agar (PDA) plates at 25°C, and subcultures were prepared with fungal mycelia. For long-term storage, the fungus was grown on barley grains and stored at −80°C as previously described (Ochi & Nakagawa 2010).

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 25°C for 10-14 days until the fungal mycelia entirely covered the medium. This culture was used as the inoculum.

2. Plant material, inoculation, and growth conditions

The soybean cultivar Enrei was used in this study. The seeds were pre-conditioned in a moisture-saturated plastic box for 2-14 days at 4°C. The soil medium (Nippi No.1; Nippon Hiryo, Tokyo, Japan) was autoclaved and mixed with the inoculum at a concentration of 0.5%-4% (*w*/*v*). Five seeds were sown per 180 mL pot or one seed per 1,500 mL pot, and each pot was covered with a 2 mm layer of pre-fertilized peaty soil Supermix-A (Sakata Seed Corporation, Yokohama, Japan). Plants were then grown in a climate-controlled plant growth chamber (Nippon Light Metal Company, Ltd., Tokyo, Japan) under 16 h light conditions at 25°C and 50% relative humidity, or in a controlled greenhouse at 26°C-28°C.

The visual severity of the root disease was scored

based on a six-point rating scale (0-5) as previously described (Jiang et al. 2020, Nishi et al. 1999): 0 = no visible symptoms, 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 due to rot and severe brown necrosis on the subterranean stem, 4 = almost all roots rotted and lost, and 5 = seedling death. Individual seedlings were considered dead if they wilted or fell. The progression of disease severity was not constant across experiments, so we evaluated it at 14-21 days after inoculation, when the Enrei control cultivar reached a disease severity of at least 3.

3. Sequencing of the internal transcriber spacer (ITS) region

The genomic DNA of isolates UH2-1 and UH2-1A was extracted using a MagExtractor (Toyobo, Osaka, Japan) following the manufacturer's instructions. These isolates were confirmed using PCR-amplification of the ITS region using the primer pair ITS1 (5′-TTCCGAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Chen et al. 2022, White et al. 1990).

4. Experimental design and statistical analysis

The inoculation tests of UH2-1 and UH2-1A were conducted in a greenhouse as a single-factor experiment (randomized complete block design) with four treatments (UH2-1 and UH2-1A were mixed at ratios of 1:0 (control), 1:0.5, 1:1, and 1:4) with three replicates. Then, a two-factorial experiment (completely randomized design) with four treatments (UH2-1 and UH2-1A mixed at ratios of 0:0, 1:0, 0:1, and 1:1) and three replicates was conducted twice on different days in the greenhouse. The inoculum concentrations of UH2-1 and UH2-1A were designated Factors A and B, respectively. Finally, a 5 × 2 factorial experiment was conducted in a randomized complete block design with three replicates. In this experiment, five *C. ilicicola* strains were assigned Factor A, and the presence or absence of inoculation with UH2-1A was assigned Factor B. Disease severity was used as the dependent variable, and a linear mixed model was applied with treatments, Factors A and B, and their interactions as fixed effects. The block, pot, and experiment date were included as random effects. After the analysis, non-significant fixed and random effects with variance components estimated to be zero were excluded from the model. For this experiment, 95% confidence intervals (CIs) for the estimated marginal means or predicted values were calculated for significant fixed effects. Multiple comparisons between the levels of fixed effects were performed using Tukey's method. The degrees of freedom were calculated using the Kenward–Roger method.

Statistical analyses were performed using the lme4, lmerTest, pbkrtest, ggeffects, multcomp, multcompView, and emmeans packages in R software (ver. 4.3.2).

Results and discussion

1. Generation of a hypovirulent *C. ilicicola* strain

We repeatedly subcultured the mycelium of the UH2-1 strain on PDA plates for 20 and 40 generations. These UH2-1 subcultures were identified as UH2-1A.

Inoculation tests (180 mL pot) showed that the culture of the UH2-1A strain had significantly reduced pathogenicity, as evidenced by RCR severity scores of 0.8 and 1.0 compared with scores of 3.0 and 4.0 for the original UH2-1 strain ($p < 0.05$) after 20 and 40 passages of subculture, respectively (Fig. 1A, B).

The decline or loss of virulence (hypovirulence) of microbial pathogens during long-term *in vitro* subcultures in artificial media under laboratory conditions has been reported (Breen et al. 2022, Bridle et al. 2015, Ishiguro et al. 1981, Jirakkakul et al. 2018, Petrillo et al. 2006, Shimizu et al. 1999). Our results also confirmed that repeated subculturing resulted in a decline in the virulence of *C. ilicicola* (Fig. 1A, B).

The mechanisms underlying hypovirulence often involve mycoviruses, which reduce the pathogen's ability to colonize the host and cause disease (Castro et al. 2003, Chu et al. 2002, Deng et al. 2024, Nuss 2005), genetic mutations (Akamatsu et al. 1999, Hatta et al. 2002), or epigenetic modifications (Breen et al. 2022), leading to a reduced ability of the pathogen to colonize the host and cause disease. In this study, the diameter of mycelial growth of UH2-1A on PDA plates was 7.5 cm, slightly smaller than 8.5 cm for UH2-1. However, this difference was not statistically significant (Fig. 1C). No antagonistic interactions were observed between UH2-1A and UH2-1 in the confrontation culture test (Fig. 1D). These results suggest that growth retardation and antagonism are unlikely to be the primary cause of the attenuated virulence of UH2-1A. Furthermore, the sequence of the ITS region of UH2-1A was identical to that of the original UH2-1 strain (data not shown), indicating that no significant genomic change occurred during continuous subculturing. Therefore, comparative whole-genome analysis will be necessary to identify the gene(s) responsible for the attenuated virulence of UH2-1A.

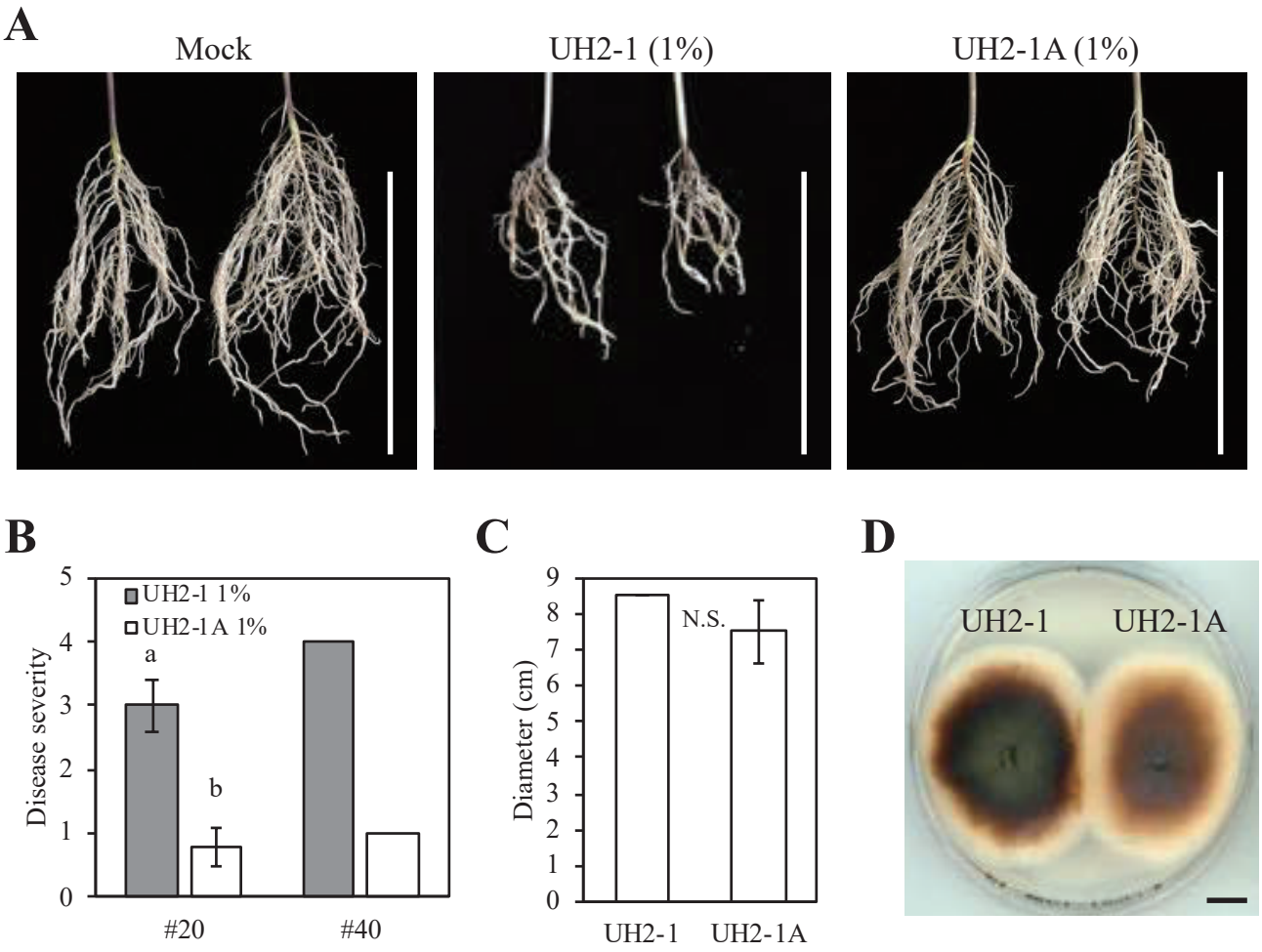

Fig. 1. Generation of the hypovirulent strain UH2-1A of *C. ilicicola*

(A, B) Pathogenicity tests of UH2-1 and UH2-1A. Soybean seeds were inoculated with the original virulent strain UH2-1 and the hypovirulent UH2-1A obtained after 20 (#20) and 40 (#40) passages of subculture of UH2-1 at an inoculum concentration of 1% (*w*/*v*). (A) Red crown rot (RCR) symptoms taken at 14 days post-inoculation (dpi). Bars = 5 cm. (B) Disease severity of RCR was scored at 14 dpi. Bars represent mean ± SD (n = 4 for #20 and n = 1 for #40). Different letters above columns indicate significant differences according to Welch's t-test ($p < 0.05$). (C) Mycelial growth of UH2-1 and UH2-1A strains on potato-dextrose agar (PDA) plates. Growth diameter was measured after 13 days of subculture. "N.S." indicates no significant difference by Welch's t-test (n = 3; $p > 0.05$). (D) Antagonism test of UH2-1A against UH2-1. Bar = 1 cm.

2. Biological control efficacy of UH2-1A against soybean RCR

The reduced virulence of UH2-1A in soybean roots prompted us to investigate its potential as a biological control agent against its wild-type counterpart, UH2-1 (in 180 mL pots). Since the 20th and 40th subcultures exhibited similar levels of virulence, the 20th subculture was used in subsequent experiments.

As shown in Figure 2, co-inoculation of UH2-1A at concentrations of 0.5%, 1.0%, and 4.0% (*w*/*v*) with UH2-1 at 1% (*w*/*v*) resulted in disease severity reductions of 0.8, 1.0, and 1.1 scores, respectively, compared to that in the control (UH2-1, 1% alone). Statistical analysis revealed significant differences between treatments (F (3, 7.9) = 5.68, p = 0.022), and multiple comparisons showed significant disease suppression ($p < 0.05$) at both 1.0% and 4.0% UH2-1A concentrations relative to that in the control. Notably, the minimal difference (0.1) between the 1.0% and 4.0% UH2-1A treatments was not statistically significant, indicating that increasing UH2-1A concentration beyond 1.0% did not substantially enhance disease suppression. These results demonstrated that 1.0% (*w*/*v*) is the optimal concentration of UH2-1A for the effective biological control of UH2-1-induced disease in soybean.

Disease severity served as the response variable for the linear mixed model analysis, with inoculum concentrations of strains UH2-1 and UH2-1A and their

interaction as explanatory variables (1,500 mL pot). The results revealed that, while individual inoculum concentrations of both strains positively affected disease severity, their interaction produced a significant negative effect, indicating reduced disease severity under mixed inoculation conditions (Table 1). Specifically, the predicted disease severity values were 4.2 for UH2-1 at 1% (*w*/*v*) and 1.2 for UH2-1A at 1% (*w*/*v*). Notably, the value was only 3.3 for mixed inoculation at 1% (*w*/*v*) of each strain (Table 1). These findings confirmed both the reduced pathogenicity of the UH2-1A strain and its disease-suppressive effect against soybean RCR when co-inoculated with the more virulent UH2-1 strain. This demonstrated a protective interaction between these strains under conditions that are more representative of field environments.

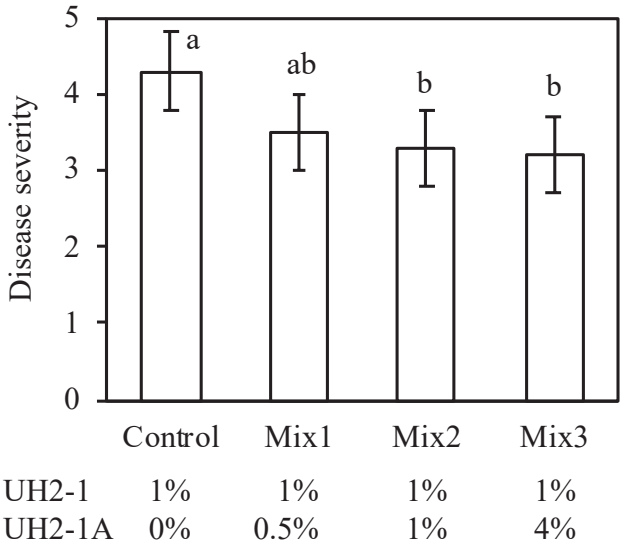

Fig. 2. Suppression of RCR incidence by UH2-1A
UH2-1A was mixed with the inoculum of the original virulent strain (UH2-1) at ratios of 1:0, 1:0.5, 1:1, and 1:4, and co-inoculated with soybean seeds. The disease severity was scored at 14 dpi. Bars represent mean ± 95% CI (n = 3). Different letters above the columns indicate significant differences according to Tukey's test ($p < 0.05$).

3. Broad-spectrum suppression of UH2-1A against *C. ilicicola* strains

To verify the disease-suppressing effect of UH2-1A against *C. ilicicola* strains other than UH2-1, we evaluated its effectiveness against four virulent strains, SN2-1, S6, S4, and Y11-1b, in addition to UH2-1 (Jiang et al. 2020). As shown in Figure 3, co-inoculation with UH2-1A significantly reduced the severity of RCR against all *C. ilicicola* strains tested. However, the degree of reduction varied (Fig. 3), demonstrating broad-spectrum biocontrol potential.

For the linear mixed model analysis, a 5 × 2 factorial experiment was performed with the five strains as Factor A and the presence or absence of inoculation with the UH2-1A strain as Factor B. No significant interaction (F (4, 20.1) = 0.31, p = 0.871) between strain type (Factor A) and UH2-1A inoculation status (Factor B) was observed, although both factors independently showed significant effects (Factor A, F (4, 20.1) = 17.35, $p < 0.001$; Factor B, F (1, 20.2) = 22.48, $p < 0.001$); therefore, a main-effects model was adopted (Table 2). This model was used to calculate the predicted disease severity values (Table 2). The results revealed significant variations in pathogenicity among the *C. ilicicola* strains, with Y11-1b exhibiting the highest disease severity and SN2-1 the lowest (Table 2; Fig. 3). Notably, UH2-1A inoculation significantly reduced disease severity across all strains ($p < 0.001$) regardless of their inherent pathogenicity

Table 1. Coefficient estimates from linear mixed models and predicted disease severity

Factor	Coefficient	Standard error	df [a]	t value	Pr (>|t|)
UH2-1 (A)	4.17	0.31	14.01	13.63	1.8×10^{-9}
UH2-1A (B)	1.17	0.31	14.01	3.82	1.9×10^{-3}
A × B	−2.00	0.43	20.77	−4.62	1.5×10^{-4}
Model [b]		A (%)	B (%)	Predicted value [c]	95% CI [d]
$y = 4.17x_A + 1.17x_B - 2x_{A \times B}$		0	0	0.0	0.0-0.0
		1.0	0	4.2	3.6-4.7
		0	1.0	1.2	0.6-1.7
		1.0	1.0	3.3	2.8-3.9

[a] Degrees of freedom calculated using the Kenward–Roger method.
[b] This model has no intercept.
[c] Values at 21 days post-inoculation
[d] CI: Confidence intervals

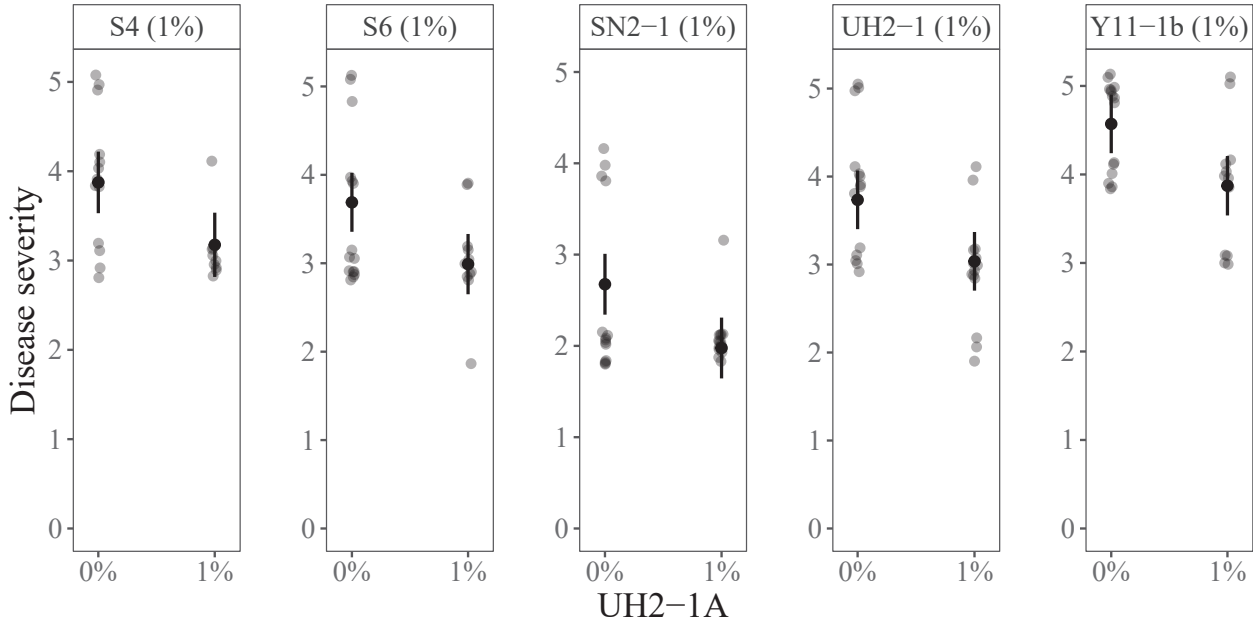

Fig. 3. Broad-spectrum RCR-suppression effects of UH2-1A

Soybean seeds were inoculated with 1% virulent *C. ilicicola* strains SN2-1, S6, UH2-1, S4, and Y11-1b, either with (1%) or without (0%) UH2-1A. Black circles and error bars represent the predicted disease severity at 14 dpi and corresponding 95% CI, calculated using the main-effects model. Grey circles represent observed values. The treatment had three replicates, with 3–5 plants per replicate (n = 9~15).

Table 2. Coefficient estimates from linear mixed models and predicted disease severity for each strain with or without UH2-1A

Factor	Coefficient	Standard error	df[a]	t value	Pr(>|t|)
Intercept	3.87	0.17	25.73	22.22	2.0×10^{-16}
A					
S6	−0.19	0.23	25.79	−0.82	0.41
SN2-1	−1.20	0.22	25.50	−5.34	1.5×10^{-5}
UH2-1	−0.14	0.22	25.50	−0.62	0.54
Y11-1b	0.70	0.22	25.41	3.10	4.7×10^{-3}
B					
UH2-1A	−0.70	0.14	24.02	−5.01	5.0×10^{-5}

Model	Strain (1%)	UH2-1A (%)	Predicted value[b]	95% CI[c]
$y = -0.70x_B + 3.87$	S4	0	3.9	3.5-4.2
		1.0	3.2	2.8-3.6
$y = -0.19x_A - 0.70x_B + 3.87$	S6	0	3.7	3.3-4.0
		1.0	3.0	2.6-3.4
$y = -1.20x_A - 0.70x_B + 3.87$	SN2-1	0	2.7	2.3-3.0
		1.0	2.0	1.6-2.3
$y = -0.14x_A - 0.70x_B + 3.87$	UH2-1	0	3.7	3.4-4.1
		1.0	3.0	2.7-3.4
$y = 0.70x_A - 0.70x_B + 3.87$	Y11-1b	0	4.6	4.2-4.9
		1.0	3.9	3.5-4.2

[a] Degrees of freedom, which were calculated by the Kenward–Roger method
[b] Values at 14 days post-inoculation
[c] Confidence intervals

levels (Table 2; Fig. 3). These findings demonstrated that, despite considerable variation in pathogenicity among the tested *C. ilicicola* strains, UH2-1A consistently suppressed disease development, suggesting its broad-spectrum effectiveness as a biocontrol agent against this pathogen.

Conclusion

In this study, we successfully generated a hypovirulent strain of *C. ilicicola*, UH2-1A, by repeated *in vitro* subculturing of the highly virulent UH2-1 strain. Co-inoculation experiments revealed that UH2-1A exhibited broad-spectrum disease-suppressing activity against multiple virulent *C. ilicicola* strains, regardless of their inherent pathogenicity. Importantly, although the underlying mechanisms of hypovirulence and RCR suppression in UH2-1A remain to be elucidated, these results highlight its promising application as a biocontrol strategy to manage RCR in soybeans, offering a promising alternative or complement to chemical fungicides in agricultural disease management.

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