Identification of *Pseudomonas* strains for the biological control of soybean red crown root rot

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Soybean red crown root rot (RCR), caused by the soil-borne fungal pathogen, *Calonectria ilicicola*, is the most destructive disease affecting soybean production in Japan. To date, no resistant cultivars or effective fungicides have been developed to control this disease. In this study, we evaluated 13 bacterial strains to determine their efficacy in controlling *C. ilicicola*. We first investigated whether the volatile organic compounds (VOCs) emitted by the bacterial strains exhibited any antifungal activity against *C. ilicicola* using the double-plate chamber method. The results showed that VOCs from three *Pseudomonas* bacterial strains, OFT2 (*Pseudomonas* sp.), OFT5 (*Pseudomonas* sp.), and Cab57 (*Pseudomonas protegens*), exhibited strong inhibitory activity against *C. ilicicola* mycelial growth. Some antifungal activity was also observed in the culture supernatants of these *Pseudomonas* strains. Greenhouse soil inoculation tests showed that application of OFT2, OFT5, and Cab57 cultures around soybean seeds after seed sowing significantly reduced the severity of RCR, as shown by up to 40% reduction in *C. ilicicola* fungal growth in the roots and 180–200% increase in shoot and root fresh weights compared to the water control. Our results suggest that OFT2, Cab57, and OFT5 produce potent antifungal compounds against *C. ilicicola*, thereby showing considerable potential for the biological control of *C. ilicicola* during soybean production.

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and/or induction of systemic resistance in host plants\(^{17,18}\). BCAs can directly suppress pathogen growth via the production of inhibitory antibiotic chemicals and competition for nutritional resources\(^{14,17-19}\). Various antibiotic chemicals, including iron-chelating compounds (siderophores) and antibiotics, have been identified\(^{14,18,19}\). In over 400 plant species including soybean\(^{26}\). Gao et al.\(^{34}\) reported that the application of the rhizobium strain, a fungal pathogen with a broad host range of found to produce antifungal mVOCs against S. sclerotiorum\(^{14}\). The two rhizosphere matum Bradyrhizobium the root exudates of soybean plants inoculated with \(\text{ACC} \) deaminase\(^{35}\). The two rhizosphere strains were found to produce and emit microbial volatile organic compounds (mVOCs) that are directly toxic to soil pathogens\(^{20-21}\). For instance, several cyanogenic Pseudomonas strains have been found to inhibit the tobacco black root rot-causing fungal agent Thielaviopsis basicola\(^{22}\) and potato late blight-causing oomycete agent Phytophthora infestans\(^{23,24}\) by producing antifungal mVOCs including the volatile respiratory inhibitor hydrogen cyanide (HCN). So far, more than 1300 mVOCs have been identified, with the major chemical classes being alcohols, ketones, aromatic compounds, terpenes, organic acids, esters, aldehydes, sulfur compounds, alkanes, and nitrogen compounds\(^{19}\). Among them, dimethyl disulfide has been the most extensively studied and successfully patented and commercialized as a soil fumigant (Paladin\(^{\text{TM}}\)) in greenhouses and open fields (Paladin Technical US EPA Reg. No. 55050-3)\(^{19,20}\). In soybean, several bacterial and fungal strains have been reported for their biological control effects against Phytophthora sojae\(^{25}\), Sclerotinia sclerotiorum\(^{6,27}\), Fusarium solani\(^{28}\), Rhizoctonia solani\(^{28-30}\), Pythium aphanidermatum\(^{31}\), Phytophthora nicotianae\(^{32}\), and Sclerotium rolfsii\(^{33}\). Several Pseudomonas bacterial strains have been found to produce antifungal mVOCs against S. sclerotiorum, a fungal pathogen with a broad host range of over 400 plant species including soybean\(^{36}\). Gao et al.\(^{34}\) reported that the application of the rhizobium strain, Bradyrhizobium sp. BXYD3, or the arbuscular mycorrhizal fungus (AMF), Glomus mosseae, from maize roots (Zea mays L.) significantly decreased the occurrence and development of RCR in soybean roots. Interestingly, the root exudates of soybean plants inoculated with Rhizobium and/or AMF significantly inhibited C. ilicicola mycelial growth, suggesting that inoculation with these microbes promotes the production of antibiotic substances in soybean plants\(^{34}\). We have previously reported the isolation of endophytic\(^{35}\) and rhizosphere\(^{36,37}\) bacteria belonging to diverse genera from different plant species inhabiting Japan. In this study, we evaluated the antifungal activities of these bacterial strains against the fungal pathogen, C. ilicicola, and identified three Pseudomonas bacterial strains (OFT2, OFT5, and Cab57) with strong antifungal activity, which may aid in the development of BCAs for the effective and eco-friendly management of RCR in soybean production.

**Results**

**Identification of bacterial strains with antifungal activity against C. ilicicola.** Thirteen bacterial strains (11 endophyte and two rhizosphere bacteria) were tested in this study, which were previously isolated from various plant species in different prefectures (Table 1)\(^{35-37}\). All 11 endophytic bacteria, including four Pseudomonas strains (OFT2, OFT5, RH6, and RH7), possess a gene encoding 1-aminocyclopropane-1-carboxylate (ACC) deaminase\(^{35}\). The two rhizosphere Pseudomonas strains Cab57\(^{37}\) and Os17\(^{36}\) exhibited biocontrol activity against damping-off and root rot caused by Pythium ultimum in cucumber plants\(^{36}\). The impact of bacterial VOCs on the mycelial growth of nine different C. ilicicola isolates with different pathogenic properties obtained from different prefectures in Japan\(^{10}\) was tested using the double-plate chamber method by growing the bacteria and fungi in the same atmosphere, but physically separated from each other, which is the most widely used method for the in vitro assessment of VOC-mediated microbial interactions. The 13 bacterial strains showed significantly different inhibitory effects on C. ilicicola growth (Fig. 1; Table 2). Among the 13 bacterial strains tested, two Pseudomonas sp. strains (OFT2 and OFT5) and one Pseudomonas protegens strain (Cab57) showed particularly high average inhibition rates (≥35%) against mycelial growth of all nine C. ilicicola isolates: 13–84% and average 46% for OFT2; 17–86% and average 35% for OFT5; and 30–86% and average 47% for Cab57. Interestingly, the growth inhibition effects of the three Pseudomonas strains were more evident against the highly virulent C. ilicicola isolates, UH2-1, AID1-12, and Y11-1b\(^{10}\), with inhibition rates of 57, 58, and 84% by OFT2, 36, 36, and 86% by OFT5, and 50, 53, and 86% by Cab57, respectively (Fig. 1;

| Strains | Species | Inhabiting types | Host plants | Localities | Accession no |
|---------|---------|------------------|-------------|------------|--------------|
| Cab57   | Pseudomonas protegens | Rhizosphere | Shepherd’s purse | Hokkaido | AP014522 |
| Os17    | Pseudomonas sp. | Rhizosphere | Rice | Ibaraki | AP014627 |
| HA3     | Streptomycyes sp. | Endophyte | Apple (fruit) | Aomori | LC075701 |
| HK1     | Pantoos sp. | Endophyte | Apple(fruit) | Aomori | LC075700 |
| HK3     | Nocardia sp. | Endophyte | Apple(fruit) | Aomori | LC075702 |
| MF6     | Streptomycyes sp. | Endophyte | Apple(fruit) | Iwate | LC075711 |
| MF7     | Streptomycyes sp. | Endophyte | Apple(fruit) | Iwate | LC075703 |
| OFT2    | Pseudomonas sp. | Endophyte | Carrot (root) | Ibaraki | LC075708 |
| OFT5    | Pseudomonas sp. | Endophyte | Turnip (root) | Ibaraki | LC075709 |
| RH10    | Mycobacterium sp. | Endophyte | Sweet pepper (fruit) | Mie | LC075704 |
| RH2     | Mycobacterium sp. | Endophyte | Sweet pepper (fruit) | Mie | LC075705 |
| RH16    | Pseudomonas sp. | Endophyte | Sweet pepper (fruit) | Mie | LC075706 |
| RH3     | Pseudomonas sp. | Endophyte | Sweet pepper (fruit) | Mie | LC075707 |

Table 1. Bacterial strains used in this study, which were isolated from various plant species in different prefectures (localities) in Japan.
Table 2). All remaining bacterial strains showed an average inhibition rate of ≤31% (Fig. 1; Table 2). Meanwhile, a Pantoea sp. strain (HK1) and a Streptomyces sp. strain (MF7) showed high inhibition activity against the C. ilicicola isolate Y11-1b of 78 and 64%, respectively.

We prepared bacterial culture supernatants of the three Pseudomonas strains (Cab57, OFT2, and OFT5) to examine whether these bacteria also produce and secrete antifungal substance(s) into the surrounding environment using the C. ilicicola isolate, UH2-1. The results showed that the culture supernatants of all three bacterial strains significantly inhibited C. ilicicola mycelial growth compared with the mock control (Fig. 2).

Evaluation of the biological control activities of the Pseudomonas strains against C. ilicicola infection. As the Pseudomonas strains, OFT2, OFT5, and Cab57, showed strong antifungal activity against C. ilicicola (Figs. 1 and 2; Table 2), we further investigated whether these bacterial strains exhibited any biological control activity in soybean plants against RCR caused by C. ilicicola (UH2-1). Compared with the disease-free mock control, a marked reduction in plant growth parameters, including plant height and fresh weight, was observed in C. ilicicola-inoculated water control plants at both 2-WPI (Figs. 3A, 4A–C) and 4-WPI (Figs. 3B, 4D–F). In contrast, seed application with OFT2, OFT5, and Cab57 significantly alleviated the negative impact of C. ilicicola infection on soybean plant growth at both sampling time points (Figs. 3A,B; 4A–C,D–F), although it could not restore the growth to the levels of the mock control. The negative impact of C. ilicicola infection was most drastic in the roots, as shown by the short height and small volume of the roots compared to the water control (Figs. 3A,B, 4C,F). Moreover, seed application of OFT2, OFT5, and Cab57 significantly reduced the root damage, as shown by the significantly recovered root volume and (Fig. 3A,B) fresh weight (Fig. 4C,F).
Consistently, the relative fungal growth of *C. ilicicola* was significantly reduced by the application of the *Pseudomonas* strains when compared with the water treatment (Fig. 3C,D). The highest reduction was observed for OFT2 (60%), followed by Cab57 (54%) and OFT5 (34%) at 2-WPI (Fig. 3C). No significant differences in the reduction of relative fungal growth were observed between the *Pseudomonas* strains. A similar trend was observed at 4-WPI, where the *Pseudomonas* strains reduced the relative fungal growth by 42% for Cab57, 27% for OFT5, and 26% for OFT2 (Fig. 3D).

**Effects of the *Pseudomonas* strains on plant growth.** In the control plants (not inoculated with *C. ilicicola*), no negative effects of the *Pseudomonas* strains were observed at either 2 or 4 weeks after seed sowing (Fig. 4). A significant increase in the fresh weights of the roots by OFT2 and the roots and shoots by OFT5 was observed at 4 weeks after seed sowing (Fig. 4E,F). No such effect was observed with Cab57.

**Discussion**

Biological control is important as an eco-friendly and practical approach for plant disease management in various crops, particularly for controlling soil-borne pathogens. Several bacterial and fungal isolates have been isolated and studied for the biological control of various soybean diseases, but no BCAs with practical and industrial use have been identified.
commercial potential for *C. ilicicola* control have been reported. Application of rhizobia and/or AMF alleviates RCR severity in soybean roots38. However, further investigation is needed on the overall RCR control effect to use these microbes as BCAs, as *C. ilicicola* can also invade the roots via the nodules, which may lead to even more severe RCR symptoms39. Moreover, it is technically challenging and expensive to in vitro propagate obligate biotrophs, including AMF, for practical use40. The success of this study in identifying the *Pseudomonas* strains (OFT2, OFT5, and Cab57) with strong biological control activity against *C. ilicicola* will aid in the development of effective BCAs to control soybean RCR. These findings may be particularly significant for soybean production in Japan, where RCR is one of the major limiting factors for soybean grain yield4.

The *Pseudomonas* sp. OFR2 and OFT5 are endophytic bacteria isolated from carrot and turnip, respectively, and both express ACC deaminase35. Inoculation of OFT5 into tomato seedlings can enhance their salt stress tolerance by reducing stress-related ethylene production41. *Pseudomonas protegens* Cab57 is isolated from the rhizosphere of Shepherd’s purse37, and exerts biocontrol activity against damping-off and root rot caused by *Pythium ultimum* in cucumber plants36. In this study, three *Pseudomonas* strains produced and emitted mVOCs with potent antifungal activity against nine different *C. ilicicola* isolates (Fig. 1; Table 2). Each bacterial strain exhibited different growth inhibitory effects on different *C. ilicicola* isolates, and some bacterial strains, HK1 and MF7, showed a high inhibition rate for a particular *C. ilicicola* isolate (Y11-bb) (Table 2). In addition, the three selected *Pseudomonas* strains (OFT2, OFT5, and Cab57) had high growth inhibitory effects against *C. ilicicola* isolates with high virulence (UH2-1, AID1-12, and Y11-1b) in soybean plants10 (Fig. 1; Table 2). Whether this biased inhibitory effect on different *C. ilicicola* isolates has any biological significance remains to be clarified.

Taken together, these results indicate some genetic and biochemical variations among the *Pseudomonas* and *C. ilicicola* isolates, which determine the outcomes of the interactions between individual bacterial and fungal isolates. The *Pseudomonas* strains (OFT2, OFT5, and Cab57) were also shown to produce and secrete antifungal substance(s) against *C. ilicicola* (UH2-1) (Fig. 2), indicating that these bacteria can suppress *C. ilicicola* growth by producing antifungal mVOCs and secretory metabolites. In support of this notion, genomic analysis of strain Cab57 revealed that it harbors the gene clusters for production of HCN37, a potent antifungal mVOC22–24, and the

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**Figure 3.** Plant growth features of soybean seedlings (A) 2 and (B) 4 weeks post-inoculation (WPI) with *C. ilicicola* (UH2-1), and the relative fungal growth (Ci-rDNA/Gm-b-Act) in the roots at (C) 2-WPI and (D) 4-WPI (n = 20). Different letters next to error bars indicate that the means are significantly different from each other as per Tukey’s HSD test (p < 0.05).
antibiotics 2,4-diacetylphloroglucinol, pyrrolnitrin and pyoluteorin etc.\textsuperscript{16,37}. It would be important and interesting to assess the role of these chemical compounds in the inhibition activity on \emph{C. ilicicola} growth.

In soil inoculation experiments, \emph{C. ilicicola} inoculation caused severe root rot and growth retardation in the soybean seedlings. In contrast, co-inoculation of \emph{C. ilicicola} with a \emph{Pseudomonas} strains (OFT2, OFT5, or Cab57) significantly reduced \emph{C. ilicicola} proliferation in the roots (Fig. 3C,D) and rescued the plant growth inhibition caused by \emph{C. ilicicola} infection to some extent (Fig. 3A,B; Table 2). These results demonstrate that the \emph{Pseudomonas} strains, OFT2, OFT5, and Cab57, have strong biocontrol activities against \emph{C. ilicicola}, which may be used for the development of BCAs to manage RCR during soybean production. Our results suggest that the mechanism of biocontrol activity of these bacteria is at least partly associated with the antagonistic suppression of \emph{C. ilicicola} growth via the production and release of antifungal mVOCs (Fig. 1; Table 2) and secretory metabolites (Fig. 2). Whether these bacterial strains are also capable of inducing host resistance remains to be determined in future studies.

No negative effects of the \emph{Pseudomonas} strains (OFT2, OFT5, and Cab57) on soybean plant growth were observed (Figs. 3A,B, 4), which is important for their practical use as BCAs in RCR management. Rather, an increase in the fresh weight of the roots by OFT2 and the roots and shoots by OFT5 was observed (Fig. 4E,F). These growth-promoting effects of OFT2 and OFT5 may be attributed to their ACC deaminase activity, as
ethylenegenerally reduces the plant growth\textsuperscript{35,41}. On the other hand, the strain Cab57 showed no significant plant growth promoting effect, even though it also contains a homologue of ACC deaminase gene in its genome (PPC\_RS20245)\textsuperscript{37}. Therefore, further study remains to clarify the contribution of ACC deaminase activity to soybean plant growth.

In summary, we found that three \textit{Pseudomonas} strains, OFT2, OFT5, and Cab57, significantly inhibited the development of soybean RCR caused by the fungal pathogen, \textit{C. ilicicola}. This biological control effect relies on the antagonistic suppression of \textit{C. ilicicola} growth via the production and release of antifungal substances. These bacterial strains may provide a basis for the development of BCAs for the effective management of soybean RCR. However, the specific substance(s) responsible for the suppression of \textit{C. ilicicola} growth and the efficacy of these bacterial strains in controlling RCR in actual soybean fields require further elucidation in future studies.

\textbf{Methods}

\textbf{Plant material and growth conditions.} Soyabean (\textit{Glycine max}) cv. Enrei was used for all experiments in this study. Enrei is a \textit{C. ilicicola}-susceptible cultivar popularly cultivated in Hokuriku and Northeast regions of Japan. The seeds of Enrei were obtained from the Institute of Agrobiological Sciences, NARO, Japan. All the experimental procedures including the collection of plant material complied with institutional, national and international guidelines and legislations.

The seeds were pre-conditioned in a moisture-saturated plastic box for 24–48 h at 25 °C. The seeds were then sown in commercially available pre-fertilized and granulated soil (Nippi No.1, Nippon Hiyori, Tokyo, Japan) in 144-cm\textsuperscript{2} plastic pots at a depth of 20 cm. Five seeds were sown per pot (12 cm × 12 cm × 20 cm; 1500 mL) and grown in a greenhouse at 25 °C and 50% relative humidity. All soils used in this study were autoclaved at 120 °C for 1 h one day before seed sowing to eliminate any effects of other soil pathogens.

\textit{C. ilicicola} culture and inoculation. Fungal mycelia of nine \textit{C. ilicicola} isolates (Table 2) were cultured on potato dextrose agar (PDA) plates at 25 °C for 1–2 weeks or until fungal mycelial growth reached the edges of the Petri plates (9 cm)\textsuperscript{2,10}.

The \textit{C. ilicicola} isolate, UH2-1, was used for the inoculation of soybean (Enrei) as described previously\textsuperscript{10,42}. Briefly, 5–8 pieces (~5-mm cubes) of PDA with vigorously growing \textit{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 days, until the fungal mycelia fully covered the medium\textsuperscript{2}. This culture was used as the inoculum, and an inoculum-soil mixture was prepared by mixing the inoculum with Nippi No.1 soil to generate a concentration of 1% (w/v). The soil mixture was then filled in plastic pots (12 × 12 × 20 cm; 1500 mL), into which five seeds were sown per pot.

\textbf{Culture of bacterial strains.} The bacteria (Table 1) were cultured overnight on tryptic soy agar (TSA) plates at 28 °C. For biological control assays, bacteria were cultured in tryptic soy broth (TSB) medium with shaking (150 rpm) at 28 °C for 24 h.

\textbf{Measurement of antifungal effects of bacterial culture supernatant.} The bacterial culture supernatants of the three \textit{Pseudomonas} strains (Cab57, OFT2, and OFT5) were investigated for their antifungal activities against \textit{C. ilicicola}. Bacterial culture supernatants were prepared according to the method described by Pethani\textsuperscript{44}, with slight modifications. The \textit{Pseudomonas} strains (Cab57, OFT2, and OFT5) were cultured on TSA plates at 28 °C for 24 h. The TSA medium containing the bacterial culture was homogenized by passing through a syringe several times and mixed with an equal volume of sterilized water. The slurry mixture was centrifuged at 10,000 g for 60 min, and the supernatant was filtered through a 0.22-μm Millipore filter (Whatman\textsuperscript{9911-1302 Syringe filter}) to remove any remaining bacteria. A filter paper (Whatman) was soaked in 4 mL of bacterial supernatant and placed in a petri plate (9-cm diameter). A small agar plug of \textit{C. ilicicola} culture was inoculated onto the filter paper at the center of the plate and incubated at 28 °C for 7 days. The supernatant prepared from the TSA medium without bacterial culture was used as the control (mock).

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Evaluation of the biological control activities of the *Pseudomonas* strains against *C. ilicicola*. The three *Pseudomonas* strains, Cab57, OFT2, and OFT5, were evaluated for their biocontrol activities against *C. ilicicola*. The bacteria were cultured in 30 mL of TSB medium with shaking (150 rpm) at 28 °C for 24 h and collected by centrifuging at 10,000 rpm for 10 min at 4 °C (TOMY MX-301 high-speed refrigerated micro-centrifuge). The resultant bacterial pellets were washed twice via resuspension in sterile water, and the density was adjusted to 0.4 at OD600 (approximately 10^7 cells mL^-1_) in sterile water.

After seed sowing, as described in section “Plant material and growth conditions”, 1 mL of the bacterial suspension was poured concentrically around each seed, and the top of the pot was covered with a 2-mm layer of autoclaved pre-fertilized peaty soil Supermix-A (Sakata Seed Corporation, Yokohama, Japan). The pots were arranged in a completely randomized design with four replicates in a greenhouse maintained at 25 °C and 50% relative humidity. Pot positions were randomly changed daily to minimize positional effects in the greenhouse, and plant density and size were small enough to induce mutual shading among different plants. Two and four weeks post-inoculation (WPI), plant growth parameters, including plant height and shoot and root fresh weights, were recorded.

Real-time quantitative polymerase chain reaction (qPCR) for examination of relative fungal growth. Relative fungal growth of *C. ilicicola* (UH2-1) was detected using qPCR, as described previously10. Briefly, genomic DNA was extracted from the whole root system using a MagExtractor (Toyobo, Osaka, Japan), following the manufacturer's instructions. Three root samples were represented for each replicate, and there were four replicates for each treatment and three biological replicates (n = 36). Real-time qPCR was performed on a Thermal Cycler Dice TP800 system (Takara Bio. Inc., Otsu, Japan) 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. Relative fungal growth was expressed as *C. ilicicola* rDNA amplification fold-relative to host β-actin gene amplification. The PCR primers used were (1) primers targeting the intergenic spacer region of the *C. ilicicola* rDNA: CiIGSF (forward) = 5′-TCCATTTGGCCTATTTATCCGTG-3′ and CiIGSR (reverse) = 5′-GGGATAGTTCCAAACCG-3′; (2) primers for soybean β-actin gene 11 (Glyma.15G050200): Gm-β-ActinF (forward) = 5′-GAGCGTATGAATGCCGATTG-3′ and Gm-β-ActinR (reverse) = 5′-CGTTTTGTAATTCCATGAC-3′.

Experimental design and data analysis. Antifungal assays were performed in three independent replicates, each consisting of three culture plates. Biological control assays were performed using three independent biological replicates, each consisting of four pots with five plants per pot for each treatment. All experiments were performed twice and representative data from one experiment are shown.

The mean values were compared using Tukey's honest significant difference test (p < 0.05) with XLSTAT Version 2017 (Addinsoft).

Data availability
The complete or partial genome sequences of 13 bacteria used in the present study are available in the DDBJ/EMBL/GenBank database under accession numbers as indicated in Table 1.

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The authors declare no competing interests.

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Author contributions

C.-J.J. and K.T.W. conceived the research ideas, designed the experiments, and wrote the article; K.T.W., M.K., F.T., K.T. and A.Z.O. performed the experiments and data analyses. All authors have read and agreed to the publish the manuscript.

Competing interests

The authors declare no competing interests.
Additional information

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