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Biological activity of *Paenibacillus polymyxa* GT2 isolate from soil in Japan against anthracnose caused by *Colletotrichum orbiculare* in cucumber

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Abstract: Cucumber anthracnose is a destructive fungal disease caused by *Colletotrichum orbiculare*. Common control strategies include chemical fungicides. However, this can lead to the development of pathogenic resistance. Therefore, it is necessary to identify natural compounds or microorganisms to develop new chemicals and the biological control of fungal pathogens. Isolate GT2, a bacterial isolate from soil samples collected in Shimane Prefecture, Japan, significantly inhibited *in vitro* mycelial growth and conidial germination of *C. orbiculare*, indicating a fungicidal effect against this pathogen. Furthermore, anthracnose lesion formation was significantly suppressed without phytotoxicity when cucumber leaves were pretreated with a cell culture suspension of the isolate GT2 before inoculation with *C. orbiculare*. Bioautography of the culture filtrate (CF) of the isolate GT2 using thin-layer chromatography showed that the compound inhibiting *C. orbiculare* growth had an Rf value of 0.38. The effective compound in GT2-CF was ethyl acetate insoluble and heat-stable at 121 °C and has a molecular weight larger than 1 000 Da. In conclusion, *Paenibacillus polymyxa* GT2 demonstrated the potential for developing a new fungicide and biological agent against anthracnose disease caused by *C. orbiculare*.

Keywords: biological control; disease control; fungicidal activity; inhibitory compound

Biological control has been extensively studied as an alternative to chemical control of fungal and bacterial diseases in many crops, including grains, vegetables, and fruit trees (Chinchoikar & Mukerji 2010). Various genera, including *Bacillus*, *Erwinia*, *Fusarium*, *Pseudomonas*, and *Trichoderma*, have been used to develop microbial fungicides, although the diversity of these genera used in this process is low. Chemical control has contributed to cost reductions, labour savings, and a stable supply of crops. However, chemical control has notable disadvantages, including detrimental effects on the ecosystem (Tase et al. 1989) and non-target beneficial microorganisms (Channabasava et al.

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2015), as well as the potential to promote fungicidal resistance [Fungicide resistance action committee (FRAC), available at: https://www.frac.info/#open-tour]. However, the development of resistance to biological control by antagonistic bacteria has not yet been reported. Secondary metabolites produced by microorganisms have attracted attention as potential biological and chemical control agents for the treatment of fungal diseases in various crops (Shimizu et al. 2000; Joshi et al. 2022). Different microorganisms, including strains within the same species, have different physiological characteristics and thus produce different compounds. Therefore, it is important to identify novel microorganisms and compounds that can effectively control plant diseases.

Therefore, we constructed a microorganism library [Shimane Microbial Library (SML)] to catalogue the microbes originally isolated from Shimane soil. We previously characterised the diversity of microorganisms isolated from soil in Shimane Prefecture (Lemtukei et al. 2016; Ganphung et al. 2019, 2021; Abdullah et al. 2021; Ino et al. 2022). We also investigated the biological activity of these microorganisms on plant pathogens, including *C. orbiculare*, *Pyricularia oryzae*, *Podosphaera xanthii*, and *Pythium aphanidermatum*, which are suppressed by microbial isolates catalogued in the SML (Lemtukei et al. 2016; Ganphung et al. 2019, 2021; Abdullah et al. 2021; Ino et al. 2022). These results demonstrated the usefulness of SML microorganisms.

Anthracnose is caused by the hemibiotrophic fungal pathogen *C. orbiculare* (Berk. and Mont), Arx [syn. *C. lagenarium* (Pass.) Ellis & Halst.]. It is a major disease of cucumber and other cucurbit crops and can lead to yield losses of up to 60%, depending on the environmental conditions (Thompson & Jenkins 1985; Wasilwa et al. 1993; Agrios 2005; Hyde et al. 2009). It has been established that *C. orbiculare* has a wide host range (Sato & Moriwaki 2009), and more than 290 fungicides are commercially available in Japan for controlling this fungus (FAMIC). However, resistance to benzimidazoles, quinone external inhibitors (QoIs), and sterol demethylation inhibitors (DMIs) has been reported in *Colletotrichum* (Chung et al. 2006; Wong & Midland 2007; Inada et al. 2008).

In a preliminary experiment at the beginning of this research, we investigated the dual culture of the strain isolated in Gotsu City, Shimane Prefecture, Japan, with the *C. orbiculare* and conidia germination of the *C. orbiculare* in the culture filtrate. We found that the infectious behaviour of *C. orbiculare* was inhibited by the SML microbial isolate GT2. The objectives of this study were to: *(i)* investigate the inhibitory activity of GT2 against *C. orbiculare* in cultured cells, *(ii)* investigate the suppressive effect of the isolate GT2 on lesion formation by *C. orbiculare* in cucumber plants, and *(iii)* characterise the inhibitory compounds of GT2.

MATERIAL AND METHODS

Microorganisms. As previously described, GT2 was isolated from soil collected in Gotsu City, Shimane Prefecture, Japan (Lemtukei et al. 2016). It was suspended in a 15–20% glycerol solution and stored at −80 ℃ until further use. To prepare cell cultures, isolate GT2 was grown in Luria-Bertani agar (LBA: 10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, 20 g/L agar) medium for 72 h. Subsequently, a single colony was used to inoculate into test tubes containing 3 mL Potato sucrose (PS) liquid media (200 g/L potato extract and 20 g/L sucrose) and incubated at 25 ± 2 °C for 1–7 days with constant shaking on a rotary shaker at 130 rpm (MMS-3010, Tokyo Rikakiki, Japan). This cell culture was used as the cell culture suspension for GT2 cells (GT2-CS). The concentration of the GT2 cell suspension was confirmed to be above an OD_{600} of 2.0 using a turbidimeter before use. Cell cultures were prepared using the above method and incubated at various temperatures to determine the optimal growth temperature of GT2 and to examine the production of inhibitory compounds at different temperatures (4, 20, 25, 28, and 37 °C). The culture suspension was filtered through 0.22 μm micropore membrane filters (0.22 μm/ϕ32 mm Nylon Syringe Filter; AS ONE Corp., Japan), and the resulting solution was used as a culture filtrate from isolate GT2.

Pathogen and Plant. Strain CO-01 of *C. orbiculare*, a pathogen of the cucumber plant, was used as the test pathogen; it was maintained on PS agar slants until use. The strain was isolated from the leaves of diseased cucumber plants cultivated in a field in Shimane Prefecture and is preserved at the Plant Pathology Laboratory at Shimane University. *C. orbiculare* was inoculated onto rice bran agar (50 g/L rice bran, 20 g/L sucrose, 20 g/L agar) and incubated at 25 ± 2 °C for 7 days to produce abundant conidia for inoculum.

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Cucumber plants of the cultivar Hokushin (Takii & Co., Ltd., Japan) were grown in plastic pots (diameter 6 cm) containing commercial garden soil (Kumiai Nippi Engeibaido No. 1; Nihon Hiryo Co., Ltd., Japan) at 25 ± 2 °C under a 12 h light/12 h dark photoperiod. Seedlings in the first true-leaf stage were used for the inoculation experiments.

Dual culture assay. The inhibitory activity of GT2 on the mycelial growth of *C. orbiculare* was investigated using the dual-culture method on the PS agar medium. Mycelial plugs (diameter: 7.0 mm) of *C. orbiculare* and paper disks (8.0 mm; Advantech Toyo Kaisha, Ltd., Japan) were placed on PSA plates 4.0 cm apart. Subsequently, the paper disks were inoculated with a cell culture suspension of the isolate GT2 (7-day-old culture, 30 µL) cultured in a PS liquid medium. The PS liquid medium was inoculated onto paper disks as a control. All experiments were performed in triplicate. All petri dishes were incubated at 25 ± 2 °C for 14 days. The inhibition area (mm^2) of C. orbiculare was measured using LIA 32 software (version 0.378), and a mycelium tip of *C. orbiculare* near the GT2 colony was observed under a light microscope (BA210E-T3M, Shimadzu Rika Ltd., Japan).

Measurement of the inhibitory activity of *C. orbiculare* conidia germination. The isolate GT2 was sub-cultured in a PS liquid medium for seven days. An isolated GT2 cell suspension was prepared as previously described. *C. orbiculare* conidia (1.0×10^5 conidia/mL) were suspended in the isolate GT2 cell culture suspension (1 mL, OD:2.0), placed on glass slides (3 drops, 10 µL/drop), and incubated in a moist chamber at 25 ± 2 °C. Liquid PS medium was used as the control. After 24 h, the percentage of germinated conidia was determined by counting the regions in a total of 450 conidia under a light microscope, and the following formula was applied:

% germinated conidia = (number of germinated conidia/total number of conidia) × 100

All experiments were performed in triplicate.

Investigation of the fungicidal activity of microorganisms on *C. orbiculare*. A conidial suspension of *C. orbiculare* strain CO-01 was prepared as follows. Sub-cultured plates prepared as described above were flooded with distilled water, and the colony surfaces were gently brushed with a small paintbrush. The resulting suspension was filtered through two layers of tissue paper to remove mycelial, and then centrifuged at 20 400 × g for 10 min. The conidial concentration was adjusted using a Thoma hemocytometer (Hirschmann Laborgeräte GmbH & Co. KG, Germany). *C. orbiculare* strain CO-01 conidia (1.0×10^5 conidia/mL) were suspended in 1 mL of cell culture suspension of isolate GT2 (7-day-old culture), while PS liquid medium was used as the control and maintained at 25 ± 2 °C. After 24 h, the suspension was centrifuged at 20 600 × g for 10 min, the supernatant was discarded to remove inhibitory compounds from the culture medium, and 1 mL of distilled water was added. The suspension was pipetted onto PSA plates containing 60 ppm chloramphenicol to suppress the GT2 growth. After five days at 25 ± 2 °C, the mycelial area (mm^2) of *C. orbiculare* was measured using LIA32 software. The experiments were repeated in triplicates with six petri dishes (9 cm) per experiment.

Suppressive effect of the microorganism on anthracnose in cucumbers. At the first leaf stage, whole cucumber seedlings were sprayed once with 5 mL of the GT2 cell culture suspension (7-day-old culture). Liquid PS medium was used as a control. The pretreated cucumber plants were maintained at 25 ± 2 °C for 24 h before a suspension of *C. orbiculare* conidia was applied to the leaves of these plants with a dropper (1.0×10^5 conidia/mL; 10 µL/drop) at six different positions on a leaf. The inoculated cucumber plants were maintained under natural light conditions for seven days at 25–27 °C, and the area of anthracnose lesions on the cucumber leaves was measured using LIA 32 software (version 0.378). Data were collected from five cucumber plants per treatment in each experiment. The experiments were independently repeated in triplicate.

Detection of the inhibitory compound on thin-layer chromatography (TLC). Isolate GT2 grown on LBA medium was incubated in an Erlenmeyer flask containing PS liquid medium at 25 ± 2 °C for seven days with constant shaking on a rotary shaker (120 rpm). The culture suspension was filtered through 0.22 µm micropore membrane filters (Syringe Filter Nylon 0.22 µm/ϕ32 mm: AS ONE Corp., Japan), and the obtained solution was used as culture filtrate. The filtrate (500 µL) was extracted twice with *n*-butanol (1 mL). The *n*-butanol fraction was evaporated at 50 °C under reduced pressure until dry. The precipitate was dissolved in methanol (500 µL) and used as a 10-fold n-butanol extract of the GT2 culture filtrate. The *n*-butanol extract of the PS liquid medium was used as a control. The extracted

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culture filtrate of isolate GT2 (7-day-old culture, 1-fold, 300 µL) was spotted onto Silica Gel thin layer chromatography (TLC) plates (Silica Gel 60, Merck KgaA, Germany) and separated using a chloroform and methanol solvent system (6.5:3.5, *v/v*). A conidial suspension of *C. orbiculare* (> 1.0×10^7 conidia/mL) was sprayed onto plates containing molten PSA and 20 ppm chloramphenicol. The plates were examined for growth inhibition zones after 4 days in a moist chamber at 25 ± 2 °C.

Identification of the candidate microorganism. To identify isolate GT2 based on its 16S rRNA gene, genomic DNA was extracted, and a specific region of the gene was amplified via PCR using previously described primers (Huong et al. 2007; Matsui et al. 2009). Genomic DNA was extracted from the bacterial colony, following the methods described by Suzuki et al. (2006). It was used as a template for PCR amplification of the 16S rDNA region, which was performed under the following temperature profile: initial denaturation at 95 °C for 30 s; 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, elongation at 72 °C for 105 s; and final extension at 72 °C for 10 min. The 16S rRNA gene was then amplified using the universal primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RP2 (5'-ACGGCTACCTTGTTACGACTT-3'). Amplicons were purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Germany). Purified amplicons were sent to Fasmac Co., Ltd. (Kanagawa, Japan) for nucleotide sequencing. Sequence homology was determined using the BLAST suite of programs from the DNA Data Bank of Japan. The 16S rDNA sequences of the reference strains were obtained from the NITE Biological Resource Center database (https://www.nite.go.jp/nbrc/catalogue/?lang=en). Multiple sequence alignments were generated using CLUSTAL W (Thompson et al. 1994). Aligned sequences were calculated using Kimura's two-parameter model (Kimura 1980) and analysed using the neighbour-joining method (Saitou & Nei 1987) in GENETYX (version 13). Bootstrap percentages were calculated using 1 000 replicates. *Bacillus subtilis* NBRC13719(AB271744) was used as the outgroup.

Separation of culture filtrates. To characterise the compounds in the GT2 culture filtrate, GT2 culture filtrate (7-day-old culture) samples were separated with ethyl acetate following previously described methods (Ino et al. 2022). Samples (20 mL) were extracted twice with ethyl acetate (40 mL of ethyl acetate). The ethyl acetate soluble fraction was added to distilled water and evaporated at 45 °C under reduced pressure until only the water fraction remained, and the aqueous volume was adjusted to 20 mL. The ethyl acetate insoluble fraction was evaporated at 45 °C under reduced pressure until only the water fraction remained, and the aqueous volume was adjusted to 20 mL. GT2 culture filtrate (7-day-old culture, 0.5 mL) was also separated into two molecular weight fractions (< 1 000 and ≥ 1 000 Da) by ultrafiltration using an Amicon Ultra 0.5 mL 1 K centrifugal filter (MilliporeSigma, USA). The volume of each sample was adjusted to 0.5 mL. GT2 culture filtrate samples were also subjected to heat treatment at 121 °C for 20 min. Inhibitory activity was assayed using the "Measurement of inhibitory activity of *C. orbiculare* conidia germination" described above. The experiments were repeated three times with five Petri dishes per experiment.

Data analysis. Data are reported as the means ± standard deviation. The polarity data for the germinated conidia were arcsine square root-transformed before analysis to improve the homogeneity of variance. The t-test and Tukey–Kramer test using SPSS Statistics for Windows (version 28.0) determined significant differences between groups' experimental values. Statistical significance was set at $P < 0.05$.

RESULTS

Inhibitory activity of isolate GT2 on mycelial growth of *C. orbiculare* by dual culture assay. The inhibitory activity of GT2 against the mycelial growth of *C. orbiculare* was observed using a dual-culture analysis method. The isolate GT2 inhibited the mycelial growth of *C. orbiculare* in the presence of GT2 compared to the control (Figure 1A). The inhibition area (mean ± SD) of *C. orbiculare* mycelia was 1 569.0 ± 181.1 mm² in the medium with the cell culture suspension of isolate GT2 (Figure 1B). In the control group, no inhibition was observed (0.0 ± 0.0 mm²). We also investigated the effects of GT2 on the hyphal morphology of *C. orbiculare* using light microscopy. The presence of isolate GT2 did not disrupt the hyphal tip of *C. orbiculare* (Figure 1C).

Inhibitory effect of isolate GT2 cell culture suspension on conidial germination of *C. or-*

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Table 1. Time course of the inhibitory activity of isolate GT2 culture suspension (CS) and culture filtrates (CF) on the germination of conidia of *Colletotrichum orbiculare*

Variable	Incubation period (day)						
	1	2	3	4	5	6	7
IR of GT2-CS	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]
IR of GT2-CF	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]

IR – Inhibition rate of conidia germination; IR (%) = [(conidial germination rate in control – conidial germination rate in treatment) / conidial germination rate in control] × 100; mean values followed by different letters were significantly different according to the Tukey-Kramer test ($P < 0.05$)

biculare. Conidial germination of *C. orbiculare* was significantly inhibited in the presence of the GT2 cell culture suspension (Figure 2A). The germination percentage of these conidia was 0% in the cell culture suspension of isolate GT2 and 98.8 ± 1.6% in the control (Figure 2B).

Fungicidal activity of isolate GT2 cell culture suspension against *C. orbiculare.* The direct effects of GT2 on *C. orbiculare* were also investigated. After 24 h of pretreatment with the GT2 cell culture suspension, the number of *C. orbiculare* colonies was significantly reduced compared to the control (Figure 3A). The area of mycelia in the control was 3 964.4 ± 671.0 mm^2 (Figure 3B), while that of *C. orbiculare* pretreated with the cell culture suspension was 771.3 ± 604.4 mm^2 (Figure 3B).

Suppression effect of cucumber anthracnose disease on the cucumber plant by pretreatment with cell culture suspension of isolate GT2. The effect of the cell culture suspension on cucumber anthracnose disease was investigated by pretreating cucumber leaves for 24 h. As shown in Figure 4, anthracnose disease lesion formation was significantly suppressed by pretreatment with the cell culture suspension of isolate GT2 compared to that in the control group (Figure 4A). The area of the lesions on cucumber leaves inoculated with *C. orbiculare* after pretreatment with the isolated GT2 cell culture suspension was 0 mm^2 (Figure 4B). In contrast, the area of the anthracnose lesions was 36.3 ± 19.0 mm^2 in leaves that were treated with PS liquid media 24 h after inoculation (Figure 4B).

Detection of an inhibitory compound produced by isolate GT2 using the thin-layer chromatography (TLC) technique. Growth inhibition zones of *C. orbiculare* colonies were investigated using TLC to detect the presence of inhibitory compounds in the culture filtrate obtained from GT2. A zone where *C. orbiculare* growth was inhibited was observed at an Rf value of 0.38 (Figure 5).

Identification of isolate GT2. The 16S rDNA sequence was analysed by PCR using specific primers to identify the bacterial isolate, GT2. Molecular phylogenetic analysis revealed that GT2 was most closely related to *P. polymyxa* NBRC15309

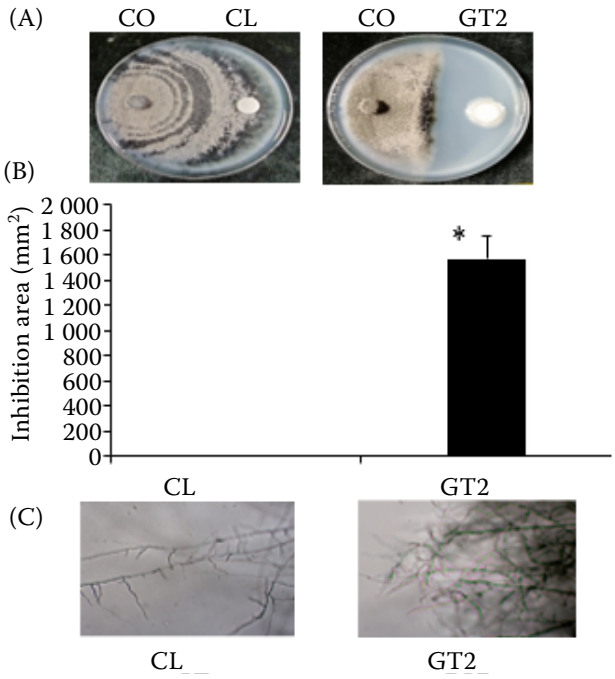

Figure 1. Dual-culture assay for *in vitro* inhibition of *Colletotrichum orbiculare* mycelia by the isolate GT2 on potato sucrose agar (PSA) medium

Mycelial plugs (7 mm) of *C. orbiculare* (CO) and paper disks were placed on PSA plates 4.0 cm apart. The paper disks were inoculated with a suspension (30 µL) of the isolate GT2 cultured in PS liquid medium (GT2). The PS liquid medium was inoculated onto paper disks as a control (CL). All petri dishes were incubated at 25 ± 2 °C for 14 days (A) and then a mycelial area of *C. orbiculare* was measured (B). In addition, the tip of *C. orbiculare* near the GT2 colony was observed under a light microscope (C). The asterisk indicates a significant difference compared with the control (Student's *t*-test, $P < 0.05$)

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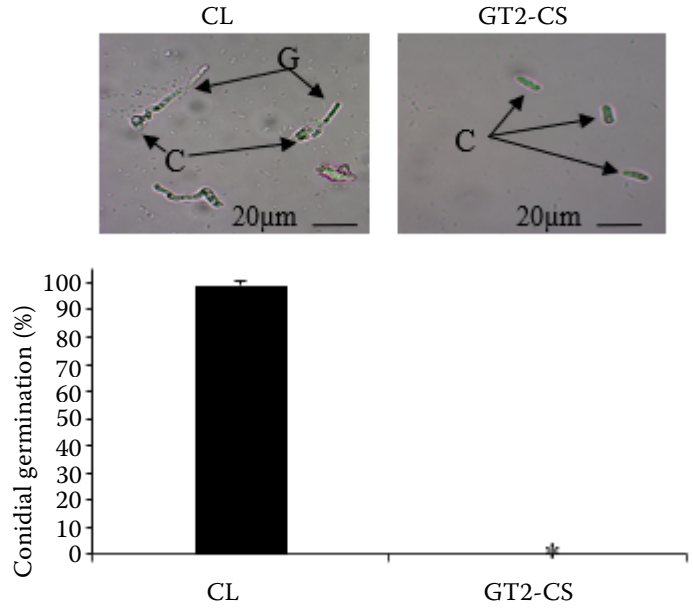

Figure 2. Effect of the isolate GT2 on inhibiting conidial germination of *Colletotrichum orbiculare*
C. orbiculare conidia suspension (1 × 10^5 conidia/mL) was dropped onto slides in the absence (CL) or presence (GT2-CS) of isolate GT2 and incubated in a moist chamber at 25 ± 2 °C in the dark; C – conidia, G – germ tube; after 24 h, the percentage of germinated conidia was determined by light microscopy. The experiments were repeated thrice, and 450 conidia per experiment were examined. The error bars represent the standard deviation of the mean. Asterisks indicate a significant difference (Student's *t*-test, $P < 0.05$)

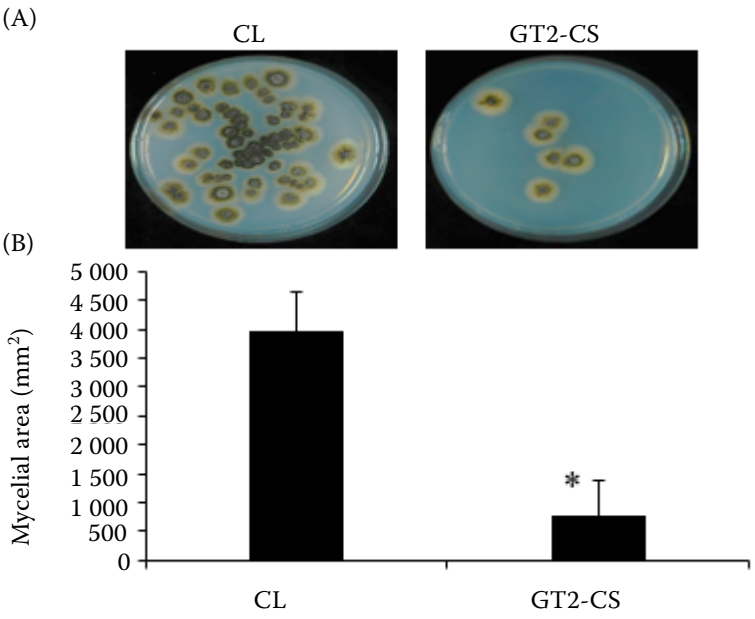

Figure 3. Fungicidal activity of GT2 against *Colletotrichum orbiculare*
(A) Conidial suspensions (1 × 105 conidia/mL) of *C. orbiculare* were treated without (CL) or with (GT2-CS) a cell culture suspension of isolate GT2 at 25 ± 2 °C. After 24 h, the supernatant was removed, and distilled water was added. Each conidial suspension was inoculated onto potato sucrose agar plates containing chloramphenicol (60 ppm). (B) The inoculated plates were incubated at 25 ± 2 °C for 5 days before the mycelial area of *C. orbiculare* was measured. The experiments were repeated in triplicate, and six petri dishes were examined per experiment. The error bars represent the standard deviation of the mean. Asterisks indicate a significant difference (Student's *t*-test, $P < 0.05$)

(AB271758) (Figure 6). Isolate GT2 (accession number LC789558) shared 99% similarity with *P. polymyxa* NBRC15309 in the 16S rDNA region (accession number AB271758), corresponding to a two-nucleotide difference of 1 479 bp. Therefore, strain GT2 was identified as a member of the genus *Paenibacillus*. The nucleotide sequence data reported here are available in the DDBJ/EMBL/GenBank databases under accession number LC789558.

Characterisation of GT2 cell culture suspension cell and culture filtrate. As shown in Table 1,

Table 2. Effect of temperature on the growth of isolate GT2 and its inhibition against *Colletotrichum orbiculare*

Variable	Temperature (°C)				
	4	20	25	28	37
Growth of GT2	−	+	+	+	+
Inhibition rate of conidial germination (%)	ND	100[a]	100[a]	100[a]	100[a]

(−): no growth; (+): growth, ND − experiments were not performed; mean values followed by different letters were significantly different according to the Tukey-Kramer test ($P < 0.05$)

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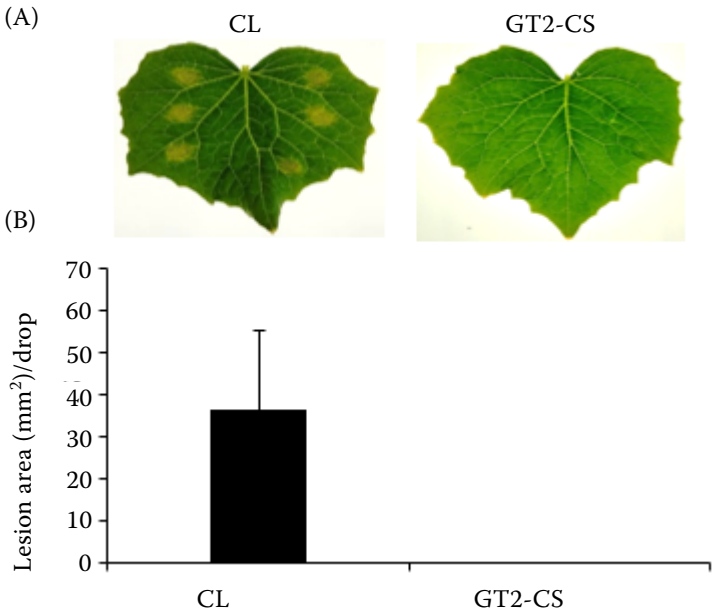

Figure 4. Suppressive effect of pretreatment with a cell culture suspension of isolate GT2 on lesion formation by *Colletotrichum orbiculare*

Cucumber leaves were pretreated with a cell suspension of the isolate GT2 (GT2-CS). The PS liquid medium was used as a control for pretreatment (CL). After 24 h, pretreated cucumber leaves were inoculated with a conidial suspension of *C. orbiculare* (1.0 × 105 conidia/mL). After 7 days, anthracnose lesions were observed (A), and lesion areas (B) were recorded. The bars at the top of each column represent the standard deviations of the means. Means followed by different letters indicate significant differences (Student's *t*-test, $P < 0.05$).

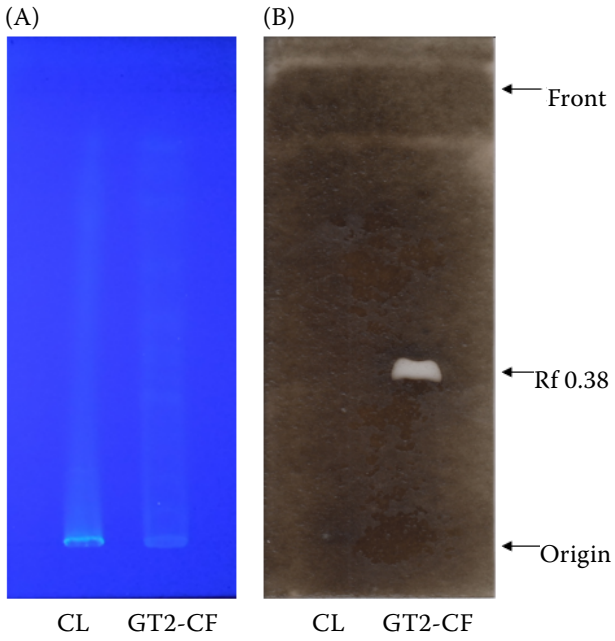

Figure 5. Thin layer chromatography (TLC) was used to detect inhibitory compounds in the culture filtrate of isolate GT2

The silica gel TLC plate was spotted with the extract of culture filtrate of GT2 (GT2-CF) and developed using a chloroform and methanol solvent system (6.5 : 3.5, *v/v*). The PS liquid medium extract was used as a control (CL). The plate was sprayed with molten potato sucrose agar containing *Colletotrichum orbiculare* (> 1 × 107 conidia/mL) and incubated at 25 ± 2 °C for 4 days. A – UV irradiation before spray with *C. orbiculare*; B – after spray with *C. orbiculare*

the GT2-cell culture and GT2-culture filtrate completely inhibited the germination of *C. orbiculare* conidia after one day of cultivation (Table 1). The GT2 isolate grew between 20 °C and 37 °C but did not grow at 4 °C (Table 2). Additionally, the cell culture fraction of GT2 cultured at 20–37 °C inhibited *C. orbiculare* conidial germination (Table 2). The inhibitory activity of ethyl acetate-soluble and ethyl acetate-insoluble fractions of the GT2 culture filtrate (7-day-old culture) on conidial germination was assessed. When *C. orbiculare* was exposed to the ethyl acetate-soluble fraction, the conidial germination rate was 74.0 ± 7.9%. On the other hand, the germination rate was 0% in the presence of the ethyl acetate-insoluble fraction (Table 3). The germination rates of conidia treated with PS and the original GT2 culture filtrate were 96.3 ± 3.6% and 0%, respectively. The molecular weights of the inhibitory compounds in the GT2 culture filtrate (7-day-old cultures) were also investigated. When *C. orbiculare* was treated with the fraction containing compounds with a molecular weight less than 1 000 Da, the conidial germination rate was 67.4 ± 6.2%; however, treatment with the fraction containing compounds with a molecular weight of ≥ 1 000 Da resulted in a conidial germination rate of 0% (Table 3). Finally, the effect of heat-treated (121 °C for 20 min) GT2- culture filtrate (7-day-old culture) on *C. orbiculare* conidial germination was examined, resulting in a conidial germination rate of 0% (mean ± SD) in the presence of heat-treated GT2-CF (Table 3).

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Table 3. Characterization of the inhibitory compounds of culture filtrates of the isolate GT2 to conidia germination of *Colletotrichum orbiculare*

Variable	PS (control)	Culture filtrates of the isolate GT2					
		original	heat	Ethyl acetate		Molecular weight	
				in-soluble	soluble	< 1 000	≥ 1 000
Conidia germination (%)	96.3 ± 3.6^{c}	0.0^{a}	0.0^{a}	0.0^{a}	74.0 ± 7.9^{b}	67.4 ± 6.2^{b}	0.0^{a}

Mean values followed by different letters were significantly different according to the Tukey-Kramer test ($P < 0.05$)

DISCUSSION

The Cucurbitaceae family includes cucumbers, melons, watermelons, pumpkins, and squash. It is taxonomically divided into 96 genera and includes over 1 000 species (Renner & Schaefer 2016). Cucurbits are popular food crops consumed worldwide, but are susceptible to several diseases (Agrios 2005).

C. orbiculare causes major diseases in cucurbits and other crops. Previously, it was reported that a species in the genus *Paenibacillus* acts as a biocontrol agent and inhibits the growth of *Botrytis cinerea*, *C. gloeosporioides*, *C. acutatum*, *F. oxysporum*, *Magnaporthe oryzae*, *Phytophthora palmivora*, *P. aphanidermatum*, *Rhizoctonia solani*, and *Sclerotinia sclerotium* (Son et al. 2009; Timmusk et al. 2009; Nguyen et al. 2015; Kim et al. 2016; Ali et al. 2021; Zhai et al. 2021; Abd-El-Kareem et al. 2022). However, the effective control of *C. orbiculare* by *P. polymyxa* in cucumber plants has not yet been elucidated. In this study, we found that GT2, which was identified as the most closely related to *P. polymyxa* in our phylogenetic analysis, showed inhibitory activity against the growth of mycelia (Figure 1), germination of conidia (Figure 2, Table 1), and lesion formation in *C. orbiculare* (Figure 4). Our results indicated that GT2 might contribute to the biological control of cucumber anthracnose caused by *C. orbiculare*.

The characteristics of isolate GT2 were investigated to further explore the possibility of controlling cucumber anthracnose using isolate GT2. Generally, the optimum temperature range for cucumber growth is 28–35 °C (Grimstad & Frimanslund 1993). In addition, the optimum temperature for generating cucumber fungal diseases is 20–30 °C (Kishi 1998). In this study, the isolate GT2 was grown at 20–37 °C (Table 2). Conidi-

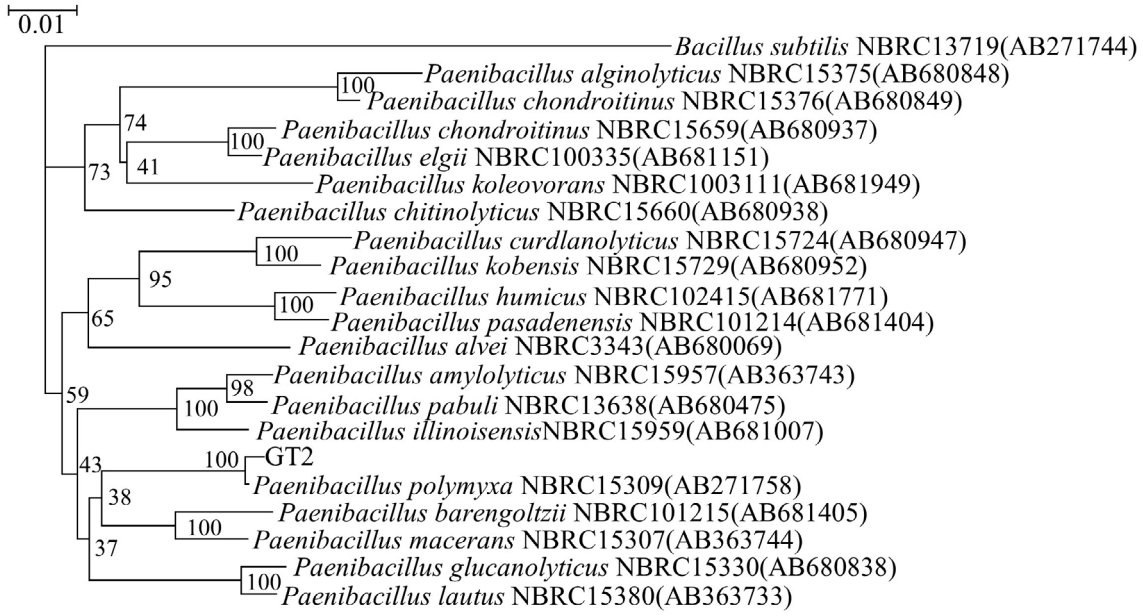

Figure 6. Phylogenetic tree based on 16S rDNA from *Paenibacillus* sp. and isolate GT2

A bootstrap consensus neighbor-joining tree of isolate GT2 was created using the Kimura 2-parameter distance matrix (1000 replicates). *Bacillus subtilis* NBRC13719(AB271744) was used as the outgroup, and the scale bar represents 1% sequence dissimilarity

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al germination was inhibited at 20–37 °C in the presence of GT2 (Table 2). These results suggest that GT2 may be useful for controlling anthracnose caused by *C. orbiculare* in greenhouse-grown cucumbers. In addition, GT2 growth was observed in the presence of fungicides, such as Banrot WP, Bellkute WP, and Top jin M WP, which are used to treat cucumber diseases (data not shown). Ons et al. (2020) demonstrated that combining biological control agents with synthetic chemical pesticides improved disease control for various plant pathogens. However, they indicated that compatibility with fungicides is important because fungicides can adversely affect biological control agents. These results indicate that the GT2 isolate can be combined with fungicides typically used to control diseases in cucumbers chemically.

However, the ability of GT2 to suppress other pathogenic fungi and bacteria that infect greenhouse-grown cucumber plants has not yet been investigated. Therefore, further studies are required to investigate the inhibitory activity of GT2 against other pathogenic fungi and bacteria that infect cucurbits to control multiple diseases in greenhouse-grown cucumber plants.

In the present study, the TLC analysis indicated that the culture filtrate of the isolate GT2 contained a compound that suppressed the growth of *C. orbiculare.* In addition, it was shown that isolate GT2 releases a compound that inhibits *C. orbiculare* (Table 1). Therefore, we investigated the inhibitory substances that GT2 released from these cells. The effective compound in GT2-CF was ethyl acetate insoluble, heat-stable up to 121 °C, and had a molecular weight larger than 1 000 Da (Table 3). *Paenibacillus* sp. has been reported to produce various types of inhibitory compounds against plant diseases, such as butyl 2,3-dihydroxybenzoate, fusaricidins A, B, and C, and pelgipeptins A and B (Wu et al. 2010; Nguyen et al. 2015; Mikkola et al. 2017). However, the inhibitory compounds demonstrating this activity have not yet been identified. Hence, further studies are required to analyse and characterise the nature of the inhibitory compounds in GT2-CF. In addition, it is well known that the genus *Paenibacillus* induces plant growth by biological nitrogen fixation, phosphate solubilisation, production of the phytohormone indole-3-acetic acid (IAA), and release of siderophores (Grady et al. 2016; Yuan et al. 2017). It also promoted resistance against plant diseases (Grady et al. 2016). Therefore, future studies should investigate plant growth promotion and disease resistance induction using GT2 isolates.

CONCLUSION

This study indicates that GT2 exhibits biocontrol activity and that GT2-CF contains inhibitory compounds that suppress *C. orbiculare*. In particular, demonstrating the fungicidal effect of the *P. polymyxa* GT2 against *C. orbiculare* represents a significant finding in developing biological and chemical control agents. *P. polymyxa* suppresses plant pathogens, promotes plant growth, and reduces plant resistance. However, our study is the first to demonstrate suppression of *C. orbiculare* by *P. polymyxa*. Given the numerous plant diseases caused by *Colletotrichum* sp., the inhibitory effects of the *P. polymyxa* GT2 on *Colletotrichum* sp. may contribute to managing various diseases induced by *Colletotrichum* in the future. In conclusion, GT2 isolate may contribute to developing new biological and chemical fungicidal agents to protect plants against anthracnose caused by *C. orbiculare*.

Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

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