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A novel strain of endophytic *Streptomyces* for the biocontrol of strawberry anthracnose caused by *Glomerella cingulata*

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ABSTRACT

Anthracnose caused by *Glomerella cingulata* is one of the most devastating diseases of strawberry in Japan, particularly during its nursery period in the summer. In this study, we aimed to isolate and screen endophytic actinobacteria, to identify potential biocontrol agents capable of suppressing strawberry anthracnose. A total of 226 actinobacteria were successfully isolated from surface-sterilized strawberry tissues. In the first screening, 217 out of 226 actinobacteria isolates were studied for their suppression effect on strawberry anthracnose using a detached leaflet assay. It was discovered that isolates MBFA-172 and MBFA-227 markedly suppressed the development of anthracnose lesions. The efficacy of both isolates was then tested on two-month-old strawberry plug seedlings in a controlled environmental chamber. It was found that isolate MBFA-172 provided consistent disease suppression and was thus selected as a final candidate for further evaluation in a glasshouse experiment. Results showed that the severity as well as incidence rate of strawberry anthracnose was significantly reduced by treatment with isolate MBFA-172 compared with that of untreated control. Accordingly, the disease control efficacy provided by MBFA-172 was statistically comparable to the chemical fungicide propineb. A re-isolation experiment using a spontaneous thiostrepton-resistant mutated strain of isolate MBFA-172 revealed that it efficiently colonized the above-ground tissues of strawberry plants for at least three weeks after spray treatment. Using cultural, morphological, and physiological tests combined with 16S rRNA-based molecular analysis, MBFA-172 was identified as a moderately thermophilic *Streptomyces thermocarboxydus*-related species. Upon review, our results strongly indicated that MBFA-172 is a promising biocontrol agent for strawberry anthracnose.

1. Introduction

Anthracnose caused by two species complexes of the fungi referred to as *Colletotrichum gloeosporioides* (Penzig) Penzig & Saccardo (teleomorph: *Glomerella cingulata* [Stoneman] Spaulding & Schrenk) and *Colletotrichum acutatum* JH Simmonds (teleomorph: *G. acutata* JC Guerber & JC Correll) (Smith and Black, 1990; Weir et al., 2012) is a major disease of the cultivated strawberry, responsible for up to 80 % of plant deaths in strawberry nurseries and over 50 % of yield losses in production fields (Sreenivasaprasad and Talhinhas, 2005). *G. cingulata* and its anamorph (*C. gloeosporioides*) have been the dominant and most devastating species in many parts of the United States and in Asian countries, including Japan (Kao et al., 2019; Okayama and Tsujimoto, 1994; Smith, 2008), whereas *C. acutatum* is the most prevalent species complex in Europe (Garrido et al., 2008). According to the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF), strawberry production in Japan is valued at more than 170 billion JPY, making it the third most valuable crop after rice and tomatoes (MAFF, 2018), and the economic loss of Japanese strawberry production owing to anthracnose caused by *G. cingulata* is estimated to be over 3.5 billion JPY each year (Sato and Moriwaki, 2009).

Most strawberry varieties grown in Japan are the winter type. Therefore, new strawberry seedlings propagated by removing rooted runners from mother plants are raised in pots inside nursery glasshouses

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during the summer season (between June and September). Strawberry plants are then transplanted to production beds in around mid-September and harvested in mid-November, where they can last up to 6 months (Yamasaki, 2013; Yoshida, 2013). During the nursery period in summer, *G. cingulata* often seriously injures and kills the seedlings, and thus leads to considerable economic loss (Ishikawa, 2003; Okayama, 1993).

In Japan, management of strawberry anthracnose is highly reliant upon numerous fungicide applications that follow a calendar-based application schedule (Hirayama et al., 2010). However, it is increasingly difficult to prevent and control the disease with chemical fungicides due to the development of resistances in the pathogen against commonly used fungicides such as benzimidazoles, diethofencarb, iminoctadine triacetate, and strobilurins (Chung et al., 2006; Inada et al., 2008; Kikuchi et al., 2010). Although several anthracnose-resistant cultivars, such as Kaorino, Karenberry, Ohkimi, and Sanchi-go, have been developed (Kitamura et al., 2015; Yoshida, 2013), susceptible cultivars are still widely grown in many production areas (Hirayama et al., 2018). This is perhaps due to the consumer's preference for high-quality strawberry fruits, particularly certain sensory characteristics (e.g., smell, appearance, taste, and texture) found in many susceptible cultivars. Therefore, other control countermeasures like sub-irrigation, drip watering, and biocontrol have been employed by strawberry growers (Ishikawa, 2013; Okayama, 1993).

Currently, only one microbial formulation containing *Talaromyces flavus*, SAY-Y-94-01 (Biotrust®; Idemitsu Kosan Co., Ltd, Tokyo, Japan), has been registered as an effective biological fungicide for controlling strawberry anthracnose in Japan. However, as highlighted by Berg et al. (2017), there is a demand nowadays for more diversity among and within biocontrol products. Thus, it would be rational to develop other biological fungicides for the management of strawberry anthracnose.

Recently, endophytic filamentous actinobacteria have been attracting intensive attention in research on the biocontrol of plant pathogens (Álvarez-Pérez et al., 2017; Goudjal et al., 2014; Hassan et al., 2017; Himaman et al., 2016; Hyakumachi et al., 2014). Actinobacteria are well-known as prolific producers of antimicrobial compounds and enzymes (Jogaiah et al., 2016; Salwan and Sharma, 2020; Supong et al., 2016). Moreover, previous studies analyzing plants' microbial communities revealed that actinobacteria are one of the dominant taxa among the endophytic bacterial communities of various agriculturally important crops including strawberries (Cardinale et al., 2015; de Melo Pereira et al., 2012d; Li et al., 2019; Okubo et al., 2014). This indicates that endophytic actinobacteria have a high symbiotic compatibility with a wide range of plants, and thus may possess various beneficial traits for the plant including biocontrol ability against plant pathogens. However, so far, there are no reports regarding the utilization of endophytic actinobacteria for controlling strawberry anthracnose.

The aim of this study is to isolate and screen endophytic actinobacteria for their biocontrol ability against strawberry anthracnose caused by *G. cingulata*, in order to develop a new biological fungicide.

2. Materials and methods

2.1. Isolation of endophytic filamentous actinobacteria from strawberry plants

Endophytic filamentous actinobacteria were isolated from surface-sterilized strawberry plants (cvs. Akihime and Nyoho) as described in an earlier study (Hassan et al., 2017). In brief, leaflets and petioles were sampled from strawberry plants which were grown in a vinyl greenhouse, and cut into small segments. The surface of tissue segments was then sterilized with 1 % (v/v) sodium hypochlorite and 70 % (v/v) ethanol, rinsed three times with sterile distilled water (SDW), and air-dried for 30 min in a laminar flow cabinet. These samples were incubated at 30 °C on a 1.5 % (w/v) water agar supplemented with antifungal agents (amphotericin B, 50 mg; rifampicin–viccillin solution [rifampicin, 20 mg; viccillin, 1 g; 100 % ethanol, 16 mL; SDW, 4 mL], 10 mL; ×30 Heritage® [Syngenta Japan, Tokyo, Japan], 10 mL in 1 L). Approximately one to two months after incubation, colonies of spore-forming filamentous actinobacteria that had emerged from the tissue segments were isolated and purified by the membrane filter method described in an earlier study (Shimizu et al., 2000). Pure isolates were cultured on a mannitol-soya agar (MSA; Hobbs et al., 1989) at 30 °C until sporulation. Spores and mycelia were harvested in a 10 % (v/v) glycerol solution containing 10 % dimethyl sulfoxide and maintained at −80 °C until use.

To check whether the actinobacteria appeared from the samples were endophytic; the surface-sterilized tissue segments were stamped onto the above isolation medium, and the water used for the last rinsing of the surface-sterilized samples were spread onto the same medium. These plates were incubated at 30 °C for two weeks and examined for the growth of actinobacteria.

2.2. Preparation of actinobacteria and fungal pathogen inocula

The actinobacteria inoculum was prepared as described previously (Hassan et al., 2017). In brief, each isolate was cultured on an MSA plate at 30 °C. Spores and mycelia were harvested in SDW from a two-week-old culture and filtered through sterile cotton to remove mycelia. The spore concentration in the filtrate was determined using a hemocytometer and adjusted to 10^8 spores mL^{-1}. The spore suspension was supplemented with 0.01 % (v/v) Silwet L-77 (Nuc-silicon L-77; Nippon Unicar, Kawasaki, Japan) and used as an inoculum.

G. cingulata isolate 06080405 which was isolated from a naturally infected strawberry and which has been kept at Mie Prefecture Agricultural Research Institute was cultured in a potato sucrose broth at 27 °C and shaken at 120 rpm for 10 days. After incubation, the culture broth was filtered through two layers of sterile gauze to remove mycelia and then centrifuged. The precipitated conidia were suspended in SDW and adjusted to 10^5 conidia mL^{-1} using a hemocytometer, and the suspension was used for inoculation.

2.3. Screening of candidate biocontrol isolates

2.3.1. First screening by detached leaflet assay

Fully expanded leaflets were detached from hydroponically grown strawberry plants (anthracnose susceptible cv. Nyoho) and washed gently with tap water, followed by air-drying for about 30 min. Subsequently, a detached leaflet was placed adaxial side up on a moist paper towel in a square dish (11 × 14 cm), and then spray-treated with 1 mL of spore suspension from each actinobacteria isolate. These dishes were placed in clear polyethylene bags and incubated in a controlled environmental chamber (30 °C, 12 h daylight) for 24 h. After incubation, the leaflet was spray-inoculated with 1 mL of the *G. cingulata* conidial suspension and incubated under the same conditions for another seven days. As a control, the leaflets treated with the sterile 0.01 % (v/v) Silwet L-77 solution were challenged with the pathogen. In this experiment, four leaflets were used for each treatment. The lesion area was measured with the public domain program Image J (developed by the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/) as described in an earlier study (Shimizu et al., 2009), and then the percent lesion area and control efficacy was calculated using the following formulae: percent lesion area = total lesion area / total leaflet area × 100, and control efficacy = [1 − (mean percent lesion area in the actinobacteria treatment)/(mean percent lesion area in the control treatment)] × 100.

2.3.2. Second screening by seedling assay

The seeds of a hybrid strawberry (anthracnose-resistant parental line Mie No.2 × anthracnose susceptible cv. Beni-hoppe) were sown in 288-plug trays containing a commercial soil mixture "Ichigo Ikubyo Baido" (Takii seed, Kyoto, Japan) and grown under glasshouse

conditions for about two months. These plug seedlings were spray-treated until drip-off with a spore suspension of the selected actinobacteria isolates, which exhibited remarkable biocontrol effects in the first screening, and incubated in a controlled environmental chamber (30 °C, 12 h daylight) for seven days. As a control, seedlings were spray-treated with a 0.01 % Silwet L-77 solution. After incubation, the treated seedlings were spray-inoculated until drip-off with the conidial suspension of the anthracnose pathogen. The inoculated seedlings were incubated in a moist chamber (30 °C, relative humidity 100 %, 12 h daylight) for 24 h, and then placed in a controlled environmental chamber (30 °C, 12 h daylight) for 13 days. Each treatment comprised four replicates and each replicate consisted of 24 seedlings; the experiment was repeated twice. Disease severity of the seedlings was visually scored on a scale of 0–5, where 0 = no visible lesions, 1 = only one true leaf showed anthracnose symptoms, 2 = two to three true leaves showed symptoms, 3 = four to five true leaves showed symptoms, 4 = the entire seedling showed severe symptoms, and 5 = the seedling was dead. The disease severity index and control efficacy were calculated using the following formulae: disease severity index = [Σ (the number of diseased seedlings in each scale × disease scale)/(total number of seedlings investigated × the highest disease scale)] × 100, and control efficacy = [1 − (mean disease severity index of the actinobacteria treatment) / (mean disease severity index of the control treatment)] × 100.

2.4. Efficacy of the isolate MBFA-172 under glasshouse conditions

This experiment was conducted in a glasshouse at the Mie Prefecture Agricultural Research Institute (Ureshino Kawakita-cho, Matsusaka city, Mie, Japan). The average temperature and humidity inside the glasshouse during the experimental period were 28.4 °C and 64.8 %, respectively. Strawberry plants (cv. Nyoho) used in this experiment were propagated by runners and grown in pots containing a commercial potting mix inside the glasshouse for two months. To prepare the source of infection, strawberry plants were spray-inoculated with the conidial suspension of the anthracnose pathogen and kept in a glasshouse for one week. The resulting diseased plants were used as an infection source. In this experiment, the following three treatments were applied: (1) control, (2) MBFA-172, and (3) fungicide. Twenty healthy strawberry plants were arranged on a steel bench in two concentric circles around one diseased plant. For the MBFA-172 treatment, these healthy plants were spray-treated with a spore suspension of the isolate MBFA-172 in the morning at weekly intervals (10 mL per plant). For the fungicide treatment, 500-fold diluted propineb (Antracol® WG; Bayer Crop Science, Leverkusen, Germany), which is commonly used for the management of strawberry anthracnose in Japan, was sprayed onto the strawberry plants at weekly intervals. Plants treated with tap water served as a control. Twenty-one days after the onset of the experiment, an additional diseased plant was placed as a new infection source at the center of each plot. During the experiment, plants were irrigated from above twice daily with tap water. Thirty-five days after the onset of the experiment, the disease severity of the strawberry plants was visually scored on a scale of 0–4, where 0 = no visible symptoms, 1 = ≤25 % of petioles (including compound leaves) showed symptoms, 2 = 26%–50% of petioles showed symptoms, 3 = 51%–75% of petioles showed symptoms, and 4 = ≥76 % of petioles showed symptoms or were dead. The disease severity index, disease incidence, and control efficacy were calculated using the following formulae: disease severity index = [Σ (the number of diseased plants in each scale × disease scale)/(total number of seedlings investigated × the highest disease scale)] × 100, disease incidence = (total number of diseased plants in the MBFA-172 or propineb treatment/total number of plants investigated) × 100, and control efficacy = [1 − (mean disease severity index or disease incidence of the actinobacteria treatment or fungicide treatment)/(mean disease severity index or disease incidence of the control treatment)] × 100. The experimental design was completely randomized with three replicates per treatment.

2.5. Colonization capacity of MBFA-172 on strawberry plants

A spontaneous and stable thiostrepton (tsr)-resistant mutated strain of the isolate MBFA-172 (designated as MBFA-172^{tsr+}) was used for the colonization study. The MBFA-172^{tsr+} was created as follows. A 100 µL of spore suspension of the wild type MBFA-172 was spread-plated on a yeast-starch agar (YSA; Emerson, 1958) containing 1 ppm tsr and incubated at 30 °C for one week. A tsr-resistant colony appeared on the plate and was then streak-plated on a YSA containing 2 ppm tsr and incubated for one week. This procedure was repeated until the mutant acquired resistance to 5 ppm tsr. The resulting MBFA-172^{tsr+} was maintained on an MSA plate containing 5 ppm tsr. A spore suspension of MBFA-172^{tsr+} was prepared as described above and used for the following experiment.

Two-month-old strawberry plants (cv. Nyoho) with four fully expanded compound leaves (remaining elder leaves were thinned) were spray-treated with the spore suspension of MBFA-172^{tsr+} (10 mL per plant) and grown in a controlled environmental chamber (30 °C, 12 h daylight). Untreated plants served as a control. After one and three weeks of treatment, mature leaflets (already expanded at the day of spray treatment), young leaflets (newly emerged after the treatment), and crowns were sampled from both treated and untreated plants, and then washed in running tap water. These tissues were cut into 24 small segments (leaf, ca. 1 cm^2; crown, ca. 1 cm^3) and immersed in 0.1 % (v/v) Tween 20 for 30 s. They were then submerged in an antibiotic solution (rifampicin, 10 mg; viccillin, 500 mg; SDW, 1000 mL) for 3 min to kill epiphytic bacteria and washed three times in SDW. After air-drying for about 30 min in a laminar flow cabinet, 1 g of the segments (around 20–22 leaf segments and 4–5 crown segments) were homogenized and then serially diluted with SDW. The dilution of the homogenates was spread-plated on a humic-vitamin agar (Hayakawa et al., 1996), a selective medium for actinobacteria isolation, containing 5 ppm tsr and incubated at 30 °C for one week. After incubation, the number of MBFA-172^{tsr+} colonies was counted and the population density of the mutated strain in the original samples was calculated (expressed as Log CFU g^{-1} fresh tissue). In this experiment, two strawberry plants for each treatment were investigated at each time point.

2.6. Sensitivity of MBFA-172 to commercial fungicides

In order to develop an effective and practical management program for strawberry anthracnose, it is important to know the compatibility of the selected biocontrol isolate with chemical fungicides. Therefore, we investigated the sensitivity of the isolate MBFA-172 to five commercial fungicides (viz., azoxystrobin [Amistar® 20 flowable; Syngenta Japan, Tokyo, Japan], iminoctadine tris [albesilate] [Bellkute® WP; Nippon Soda, Tokyo, Japan], captan [Osocide® WP; Nihon Nohyaku, Tokyo, Japan], propineb [Antracol® WG; Bayer Crop Science, Leverkusen, Germany], and benomyl [Benlate® WP; Sumitomo Chemical, Osaka, Japan]), which are commonly used to control strawberry anthracnose in Japan. These fungicides were diluted with SDW according to their label directions. A 50-µl aliquot of each diluted fungicide was applied to a sterilized paper disk (paper disk for antibiotic assay; 8 mm diameter, Toyo Roshi Kaisha, Tokyo, Japan).

The isolate MBFA-172 was cultured in 10 mL of glucose-yeast liquid medium (1 g of glucose and 1 g of yeast extract per liter, pH 7.2) at 30 °C and shaken at 200 rpm for 24 h. After incubation, 2 mL of the culture broth containing mycelial fragments of the isolate was added to 100 mL of IMA-2 agar medium (Shimizu et al., 2000) melted at 45 °C and mixed well, then poured into a square dish (11 × 14 cm). The paper disks saturated with each fungicide suspension were then placed on the solidified agar plate and incubated at 30 °C for 48 h. After

incubation, the diameter of the growth inhibition zones around the paper disks was measured to evaluate the sensitivity of MBFA-172 to fungicides.

2.7. Antifungal activity and mycoparasitic ability of the isolate MBFA-172

The antifungal activity of the isolate MBFA-172 towards *G. cingulata* was assessed using a dual culture assay. The following media were used: potato dextrose agar (PDA), IMA-2, 1.5 % water agar (WA), 1 % strawberry leaf agar (composed of 1 % strawberry leaf homogenate and 1.5 % agar), and Bennett medium (Li et al., 2004). The cell suspension of the isolate MBFA-172 (5 μL) was streaked perpendicularly on one side of a series of 9-cm Petri dishes, each containing one of the above media. Subsequently, an agar plug of *G. cingulata* was placed on the other side of the dish, at a distance of 6 cm from the MBFA-172. The plates were incubated in the dark for 14 days at 27 °C and then the inhibition zone was determined. Additionally, the mycoparasitic ability of MBFA-172 was investigated by microscopic observation of the interacting site between MBFA-172 and the pathogen grown on the same media as above. The experiment was repeated three times.

2.8. Identification of the isolate MBFA-172

The isolate MBFA-172 was preliminarily identified based on taxonomic criteria, including its cultural and morphological characteristics on the International *Streptomyces* Project (ISP) No.2–5 media (Daigo No. 2–7; Wako, Osaka, Japan) recommended by Shirling and Gottlieb (1966).

The morphological characteristics of the isolate cultured on an ISP-4 medium for two weeks were observed by a scanning electron microscope as described previously (Shimizu et al., 2009).

Additionally, the utilization of carbon sources was tested by culturing the isolate on an ISP-9 medium containing sterilized 1 % (w/v) carbon sources (viz., L-arabinose, *myo*-inositol, D-xylose, sucrose, D-fructose, D-mannitol, raffinose, and α-L-ramnose) at 30 °C for one week. The growth of the colony on each medium was compared with that on ISP-9 medium with D-glucose and without sugars as positive and negative controls, respectively. Furthermore, the temperature sensitivity was determined by culturing the isolate (50 μL) on an ISP-3 medium at temperatures from 30 °C to 45 °C, at intervals of 5 °C, for two weeks.

The 16S rRNA gene was sequenced as described by Coombs and Franco (2003). The nucleotide sequence of the 16S rRNA gene was determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence of the 16S rRNA gene for the isolate has been deposited in the GenBank database under accession no. MN733203. The 16S rRNA gene sequence was compared with those of type strains in the EzBioCloud database (https://www.ezbiocloud.net/) (Yoon et al., 2017). A phylogenetic tree based on neighbor-joining method with Kimura's two-parameter model was constructed using MEGA X (Kumar et al., 2018).

2.9. Data analysis

The data was analyzed using Fisher's LSD test ($P < 0.05$). All analyses were performed using BellCurve for Excel (version 3.2.0; Social Survey Research Information, Tokyo, Japan).

3. Results

3.1. Isolation of endophytic filamentous actinobacteria

Many filamentous actinobacteria appeared from the segments of surface-sterilized strawberry tissues after one to two months of incubation (Fig. S1). These actinobacteria were presumed endophytic, because no actinobacteria could grow after stamping or spreading the last washing solution on agar medium plates. These actinobacteria endophytes were purified by the membrane filter method. Accordingly, a total of 226 endophytic actinobacteria isolates were successfully isolated without contamination from other undesired microbes (Table S1). In total, 130 and 96 isolates were successfully obtained from cv. Akihime and cv. Nyoho, respectively. However, 9 of 130 isolates from cv. Akihime exhibited slow growth and poor sporulation on agar medium. Since those isolates were considered unsuitable as biocontrol agents, they were excluded. Consequently, the remaining 217 isolates with active growth and sporulation were subjected to the following first screening.

3.2. First screening

In the first screening, 217 endophytic actinobacteria isolates were divided into 16 groups and tested for their suppression effect on the development of anthracnose lesions on detached strawberry leaflets in separate trials. After seven days of challenge inoculation with *G. cingulata*, most of the control leaflets were covered with large necrotic lesions, and the lesion area reached an average of 48.9 % (median = 50.7) of a leaflet's total area (Figs. S2 and S3A).

On the other hand, the lesion size on the leaflets treated with many of the actinobacteria isolates was smaller (median = 34.4) than that on the control leaflets (Fig. S2). Particularly, the isolates MBFA-172 and MBFA-227 (originally isolated from cv. Nyoho), tested in trial 16th, markedly suppressed the development of the anthracnose lesions (Table 1 and Fig. S3B and S3C). Accordingly, the treatment with MBFA-172 and MBFA-227 gave a 96.0 % and 90.2 % reduction in percent lesion area, respectively, compared with the control. Based on this result, these two isolates were selected for the subsequent second screening.

3.3. Second screening

The efficacy of the two isolates, MBFA-172 and MBFA-227, was tested on two-month-old strawberry plug seedlings in two repeated trials conducted in a controlled environmental chamber. As shown in Fig. S4A, the seedlings in the control treatment were intensively infected with *G. cingulata* and showed severe necrotic symptoms. However, the symptoms of the seedlings treated with the isolate MBFA-172 were apparently milder than those of the control seedlings (Fig. S4B). The symptom severity of MBFA-227-treated seedlings was slightly milder than that of the control seedlings (Fig. S4C). In the first trial, the

Table 1
Suppression effects of actinobacteria isolates against the development of anthracnose lesions on strawberry leaflets (the result of 16th trial).

Treatment	Percent lesion area [a]	Control efficacy [b]
Control	61.1 ± 48.5	–
MBFA-162	54.8 ± 35.5	10.3
MBFA-165	52.8 ± 54.7	13.5
MBFA-166	26.6 ± 49.0	56.4
MBFA-172	2.4 ± 2.9	96.0
MBFA-185	29.1 ± 47.9	52.3
MBFA-187	59.2 ± 48.2	3.1
MBFA-192	25.5 ± 49.7	58.3
MBFA-193	34.8 ± 47.2	43.0
MBFA-195	75.3 ± 49.4	−23.3
MBFA-206	26.2 ± 49.2	57.0
MBFA-207	50.5 ± 57.1	17.3
MBFA-209	36.4 ± 44.0	40.4
MBFA-212	27.7 ± 48.5	54.6
MBFA-227	6.0 ± 8.8	90.2
MBFA-228	26.5 ± 49.1	56.6

[a] Percent lesion area = total lesion area / total leaflet area × 100. Data represented as mean ± standard deviation of four replications.

[b] Control efficacy = [1 − (mean percent lesion area in the actinobacteria treatment)/(mean percent lesion area in the control treatment)] × 100.

Table 2
Effect of the isolates MBFA-172 and MBFA-227 on the severity of anthracnose disease on strawberry seedlings.

Treatment	Trial 1		Trial 2	
	Disease severity index [a]	Control efficacy [b]	Disease severity index	Control efficacy
Control	58.9 ± 2.2 a	–	56.7 ± 6.1 a	–
MBFA-172	43.5 ± 6.6 b	26.2	37.9 ± 17.4 a	33.2
MBFA-227	53.7 ± 8.1 a	8.9	53.1 ± 10.5 a	6.4

[a] Disease severity index = [Σ (the number of diseased seedlings in each scale × disease scale)/(total number of seedlings investigated × the highest disease scale)] × 100. Data represented as mean ± standard deviation of four replications. Different lowercase letters in the same row indicate significant difference at $P < 0.05$ level by Fisher's LSD test.
[b] Control efficacy = [1 − (mean disease severity index of the actinobacteria treatment)/(mean disease severity index of the control treatment)] × 100.

disease severity index of the control seedlings reached 58.9 % (Table 2). However, treatment with the isolate MBFA-172 significantly ($P < 0.05$) reduced the disease severity index to 43.5 %, resulting in a control efficacy of 26.2 %, while treatment with the isolate MBFA-227 showed a slight reduction in the disease severity (53.7 %), resulting in a control efficacy of 8.9 %. Similarly, in the second trial, the disease severity index of the control seedlings reached 56.7 % (Table 2). Conversely, treatment with the isolate MBFA-172 considerably reduced the disease severity index to 37.9 %, resulting in a control efficacy of 33.2 %, whereas the control efficacy of the isolate MBFA-227 was only 6.4 %. Based on its consistent disease suppression in two trials, the isolate MBFA-172 was selected as the final candidate isolate and subjected to the glasshouse experiment.

3.4. Efficacy of the isolate MBFA-172 under glasshouse conditions

Around two weeks after the onset of the experiment, dark or black necrotic spots, which are a typical sign and symptom of *G. cingulata* infection, gradually became visible on several leaves and petioles of the control plants placed around the inoculum plants. At the end of the experiment (35 days after the onset), the number of leaves and petioles with anthracnose lesions increased and several plants were severely wilted due to the infection of crown tissues with the pathogen (Fig. 1A). Accordingly, an average of 26.7 % plants were diseased and the disease severity index reached an average of 13.0 in the control treatment (Table 3). In contrast, treatment with the isolate MBFA-172 significantly ($P < 0.05$) reduced the disease incidence and severity index to an average of 8.3 % and 3.4, respectively, resulting in a control efficacy of 68.9 % (based on disease incidence) and 73.8 % (based on disease severity index) (Fig. 1B, Table 3). Treatment with the fungicide propineb also showed consistent suppression of anthracnose on strawberry plants and resulted in a significant reduction ($P < 0.05$) in disease incidence (an average of 1.7 %) and of the severity index (an average of 0.4) compared with the control (Fig. 1C, Table 3). There were no significant differences in the level of disease incidence and severity between the MBFA-172 and fungicide treatments.

3.5. Colonization capacity of MBFA-172 on strawberry plants

The isolate MBFA-172^{tsr+} was successfully recovered from most examined regions (mature leaflets, young leaflets, and crown) of the strawberry plants, while none of actinobacteria were recovered from untreated control plants. In mature leaflets, the population of MBFA-172^{tsr+} was relatively stable (about 10^6 CFU g^{-1} fresh tissue) for up to three weeks post treatment in both trials (Table 4). From the young leaflets, MBFA-172^{tsr+} was not detected one week post inoculation; however, it was detected (about 3.4×10^5 CFU g^{-1} fresh tissue) at three weeks post inoculation in the first trial. In the second trial, however, MBFA-172^{tsr+} was successfully recovered (about 10^4 CFU g^{-1} fresh tissue) at both sampling times (one and three weeks post inoculation). MBFA-172^{tsr+} was detected (10^3 to 10^5 CFU g^{-1} fresh tissue) in the crown at both sampling times in both trials.

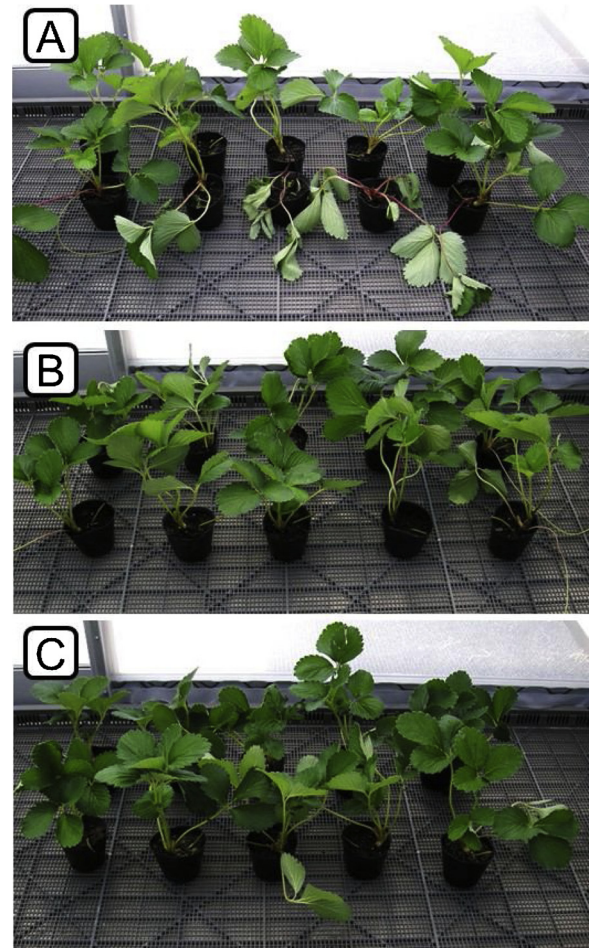

Fig. 1. Strawberry plants untreated (A) or pretreated with the endophytic *Streptomyces* sp. MBFA-172 (B) and fungicide propineb (C). Photographs were taken 35 days after the onset of the experiment.

Table 3
Biocontrol effect of *Streptomyces* sp. MBFA-172 on strawberry anthracnose under glasshouse conditions.

Treatment	Disease severity index [a]	Control efficacy (%)	Disease incidence (%) [b]	Control efficacy (%) [c]
Control	13.0 ± 3.8 a	–	26.7 a	–
MBFA-172	3.4 ± 1.9 b	73.8	8.3 b	68.9
Propineb	0.4 ± 2.9 b	96.9	1.7 b	93.6

[a] Disease severity index = [Σ (the number of diseased plants in each scale × disease scale) / (total number of plants investigated × the highest disease scale)] × 100. Data represented as mean ± standard deviation of three replications. Different lowercase letters indicate significant difference at $P < 0.05$ level by Fisher's LSD test.
[b] Disease incidence = (total number of diseased plants in the MBFA-172 or propineb treatment/total number of plants investigated) × 100.
[c] Control efficacy = [1 − (mean disease severity index or disease incidence of the MBFA-172 or propineb treatment)/(mean disease severity index or disease incidence of the control treatment)] × 100.

Table 4
Population dynamics of the *Streptomyces* sp. MBFA-172^{tsr+} mutant on the strawberry plants grown in a controlled environmental chamber.

	Population (CFU g^{-1} fresh weight)			
	Trial 1		Trial 2	
	One week post treatment	Three weeks post treatment	One week post treatment	Three weeks post treatment
Mature leaflets	8.3×10^4	7.0×10^5	1.0×10^6	7.3×10^5
Young leaflets	ND*	3.4×10^5	3.7×10^4	3.7×10^4
Crown	1.0×10^4	1.1×10^3	3.0×10^5	5.3×10^3

* ND: not detected.

Table 5
Sensitivity of the *Streptomyces* sp. MBFA-172 to commercial fungicides.

Fungicide	Diameter of growth inhibition zone (mm)
Azoxystrobin	ND*
Iminoctadine tris	9.26
Captan	ND
Propineb	ND
Benomyl	ND

* ND: Growth inhibition zone was not detected.

3.6. Sensitivity of MBFA-172 to commercial fungicides

The sensitivity of MBFA-172 towards commonly used commercial fungicides is shown in Table 5. The isolate was found to be insensitive to most tested fungicides (azoxystrobin, captan, propineb, and benomyl). In contrast, the isolate was sensitive to iminoctadine tris (9.26 mm inhibition zone) at the predetermined concentration.

3.7. In vitro antifungal activity and mycoparasitic ability of the isolate MBFA-172

Antifungal activity of the isolate MBFA-172 towards *G. cingulata* was tested with a dual culture technique using five different media (viz., PDA, IMA-2, 1.5 % WA, 1 % strawberry leaf agar, and Bennett medium). The isolate MBFA-172 did not inhibit the mycelial growth of *G. cingulata* on any of the agar media tested (Table S2), indicating that the isolate does not produce any antifungal compounds that inhibit *G. cingulata.* Furthermore, the mycoparasitic ability of MBFA-172 against *G. cingulata* was examined on the same agar media. Light microscopic observation (magnification: x400) of the site of colony interaction between *G. cingulata* and MBFA-172 revealed no obvious morphological changes related to mycoparasitism such as coiling of MBFA-172 mycelia around *G. cingulata* hyphae and lysis of *G. cingulata* hyphae (Fig. S5).

3.8. Identification of the biocontrol isolate MBFA-172

The cultural characteristics of the isolate MBFA-172 are summarized in Table 6. The isolate grew well on each ISP medium to form flat and powdery colonies. The aerial mycelia were grayish in color. The substrate hyphae were brownish in color. The isolate did not produce diffusible pigments on any medium.

Scanning electron microscope observation revealed that spore chains of MBFA-172 formed on an ISP-4 medium were simple spirals with about 30–45 spores per chain, and the surface of these spores was spiny (Fig. 2).

As a result of a carbon source utilization test, it was found that the isolate MBFA-172 could utilize L-arabinose, D-xylose, D-fructose, α-L-rhamnose, *myo*-inositol, and D-mannitol as carbon sources (Table S3). In addition, MBFA-172 was able to grow at 45 °C, with optimal growth in the temperature range 30 °C – 40 °C (Fig. S6).

Table 6
Cultural characteristics of *Streptomyces* sp. MBF-172 colonies on four different International *Streptomyces* Project (ISP) media.

Medium	Feature	Characteristics
ISP-2	Aerial mycelium	Slightly abundant, powdery, light gray
	Substrate hyphae	Light brown
	Reverse of the substrate mycelium	Yellowish brown
	Diffusible pigment	None
ISP-3	Aerial mycelium	Slightly abundant, powdery, light gray
	Substrate hyphae	Light grayish brown
	Reverse of the substrate mycelium	Light grayish brown
	Diffusible pigment	None
ISP-4	Aerial mycelium	Abundant, powdery, light gray
	Substrate hyphae	Yellowish brown
	Reverse of the substrate mycelium	Grayish brown
	Diffusible pigment	None
ISP-5	Aerial mycelium	Scant, powdery, light bluish gray
	Substrate hyphae	Dark yellowish brown
	Reverse of the substrate mycelium	Grayish brown
	Diffusible pigment	None

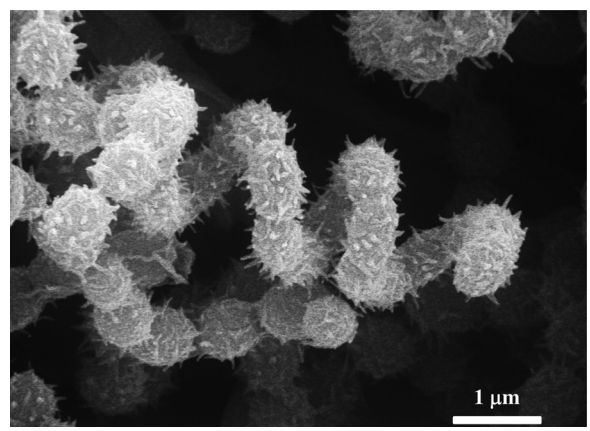

Fig. 2. Scanning electron micrograph of spore chains in *Streptomyces* sp. MBFA-172. The bar represents 1 µm.

The analysis of a full-length 16S rRNA sequence of the isolate MBFA-172 1238 bp accession number: MN733203 indicated that the isolate shared the greatest similarity with that of *Streptomyces thermocarboxydus* DSM 44293^T (99.92 %) (accession number: U94490). To clarify the phylogenetic position of the isolate, a phylogenetic tree was constructed on the basis of the complete 16S rRNA gene sequence (Fig. 3). As a result, the isolate MBFA-172 showed a clear distinction from the type strain of *S. thermocarboxydus*.

A comparison of the cultural and morphological characteristics of the isolate MBFA-172 with those of the type strain *S. thermocarboxydus* AT37^T (Kim et al., 1998) further indicated that some characteristics of MBFA-172 are different from those of *S. thermocarboxydus.* For example, the substrate hyphae of MBFA-172 were brownish in color, while there is no distinctive mycelium color for those of AT37^T. As described above, spore chains of MBFA-172 were a simple spiral type and the surface of these spores was spiny, whereas those of AT37^T were warty in an open-spiral or looped (*retinaculiaperti*) chains (Kim et al., 1998).

Based on these results, we concluded that the isolate MBFA-172 is closely related to, but distinct from, *S. thermocarboxydus;* therefore, we identified it as a *Streptomyces* sp.

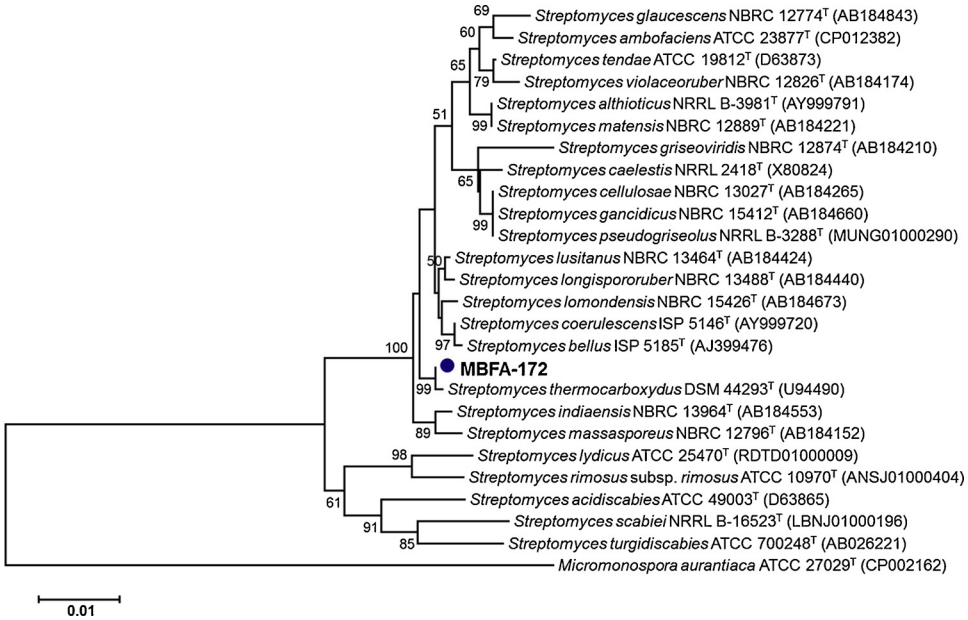

Fig. 3. Phylogenetic position of *Streptomyces* sp. MBFA-172 based on a complete 16S rRNA gene sequence analysis obtained by the neighbor-joining method. The dendrogram shows the position of the isolate MBFA-172 among its other closely-related species. Bootstrap values of ≥50 % (1000 replicates) are shown next to the branches. Accession numbers for sequences of each strain are shown in parentheses. The scale bar shows the number of base substitutions per site.

4. Discussion

With the aim of developing a new biological fungicide, we explored the biocontrol potential of 217 endophytic actinobacteria isolates recovered from the above-ground surface-sterilized tissues of strawberry plants as an agent to guard against strawberry anthracnose disease.

Screening based on *in vitro* antagonistic activity has the possibility of overlooking the beneficial isolates that can suppress plant diseases by indirect mechanisms such as the stimulation of plant immunity and competition with pathogens for nutrients or ecological niches. Actually, several studies have reported that non-antagonistic actinobacteria display excellent biocontrol efficacy in the crop environment (Crawford et al., 1993; Hassan et al., 2017; Shimizu et al., 2006). Therefore, we directly assessed the protective effect of actinobacteria isolates against *G. cingulata* on the strawberry plants in large-scale screening process under laboratory conditions. Accordingly, the *Streptomyces* sp. isolate MBFA-172 was selected as a possible BCA of strawberry anthracnose based on its consistent biocontrol effect throughout the two-step screening tests (i.e., detached leaflet assay and seedling assay). As if proving the effectiveness of our screening strategy, this isolate was shown to be incapable of inhibiting the mycelial growth of *G. cingulata* on all of the agar media tested (Table S2).

Surprisingly, the isolate MBFA-172 had a remarkable control efficacy towards strawberry anthracnose under glasshouse conditions that was statistically comparable to that of the chemical fungicide propineb (Table 3 and Fig. 1). Moreover, it was found that isolate MBFA-172 could grow even in the presence of some fungicides (azoxystrobin, captan, propineb, and benomyl) (Table 5) commonly used for the management of strawberry anthracnose in Japan. These results strongly indicated that the isolate MBFA-172 has great potential for use as an alternative, or complementary, fungicide to conventional chemical fungicides. To date, several studies have reported the biocontrol potential of *Streptomyces* isolates against anthracnose disease of various plants such as cucumber, yam, chili, and banana (Palaniyandi et al., 2011; Shimizu, et al., 2009; Shu et al., 2017; Thilagam and Hemalatha, 2019). As far as we know, this is the first study reporting an endophytic *Streptomyces* isolate as a beneficial BCA for the control of strawberry anthracnose.

The results of a re-isolation experiment using a spontaneous thiostrepton mutant of MBFA-172 demonstrated that this isolate has the ability to efficiently colonize the leaf and crown tissues of strawberry plants for at least three weeks after spray treatment and expand its territory from mature tissues to younger leaf tissues that emerge after the treatment (Table 4). The ability of microbial BCAs including *Streptomyces* to colonize the same ecological niche favored by the target pathogens enables them to exert a long-lasting and consistent biocontrol effect on plant diseases (Chen et al., 2016; Salvatierra-Martinez et al., 2018; Sang and Kim, 2014). Therefore, the capacity of the isolate MBFA-172 to colonize the above-ground tissues of strawberry plants was considered one of the reasons for its superior biocontrol efficacy. Endophytic *Streptomyces* have been reported to enter their host plants through natural openings (e.g., stomata) or by penetrating the leaf cuticle and expanding into the underlying epidermal cells through hyphal growth (Hassan et al., 2017; Shimizu et al., 2009; Suzuki et al., 2005; van der Meij et al., 2018v). Moreover, Patel et al. (2018) suggested that following the entry of endophytic *Streptomyces* sp. into the root system of a rice plant, it could migrate upwards into the stems and even reach the leaf tissues. Very recently, it was also reported that endophytic *Streptomyces* was able to move within the strawberry vascular system from both the flowers and rhizosphere (Kim et al., 2019). In this study, we did not observe how the isolate MBFA-172 colonized strawberry plants. Therefore, in the future, we will investigate the mode of penetration, manner of dissemination, and colonization pattern of MBFA-172 in strawberry plants by using, for example, fluorescent proteins in combination with confocal laser scanning microscope.

Environmental factors such as temperature strongly influence the persistence of BCAs after application, thereby affecting their biocontrol performance (Hjeljord et al., 2000; Marian and Shimizu, 2019). Most of the *Streptomyces* species are mesophilic and can grow at temperatures below 37 °C (Hasani et al., 2014), but the isolate MBFA-172 was considered to belong to the moderately thermophilic species because it could grow actively even at 45 °C (Fig. S6). This was also evident from the result of the phylogenetic analysis. Phylogenetic analysis based on a 16S rRNA gene sequence revealed that the isolate MBFA-172 is most closely related to *S. thermocarboxydus*, which is among the moderately thermophilic *Streptomyces* species that can thrive at 28–60 °C (Shivlata and Tulasi, 2015). The epidemic of strawberry anthracnose occurs mainly during summer in Japan (Ishikawa, 2003). In the main strawberry production areas such as Tochigi, Fukuoka, Kumamoto, and Shizuoka prefectures, the average maximum air temperature during summer is usually in the range of 26–33 °C and the daytime temperature sometimes exceeds 37 °C (Japan Meteorological Agency, 2019). Therefore, the natural ability of MBFA-172 to withstand high temperatures may also be an advantageous trait that ensures the consistent

biocontrol effect of the isolate against strawberry anthracnose during the nursery period in summer.

Several mechanisms have been reported to play a significant role in the biocontrol activity of endophytic actinobacteria against fungal pathogens, including mycoparasitism, antibiosis, and the induction of host resistance (Shimizu, 2011). Microscopic observation of the site of colony interaction between the isolate MBFA-172 and *G. cingulata* on the agar media showed that isolate MBFA-172 did not parasitized on growing hyphae of the pathogen (Fig. S5). *S. thermocarboxydus*, which is phylogenetically the closest to isolate MBFA-172, has been found to show antimicrobial activity and produce several antifungal antibiotics (Passari et al., 2017). However, as mentioned above, the isolate MBFA-172 did not show antifungal activity towards *G. cingulata* (Table S2), implying that mechanisms other than antibiosis, such as induced disease resistance, may be involved in the biocontrol effect of this isolate. Several studies have reported the ability of endophytic actinobacteria to protect host plants from pathogen infections by enhancing disease resistance. For example, Shimizu et al. (2005) reported that the endophytic *Streptomyces galbus* R-5 (renamed MBR-5) induced jasmonate-mediated disease resistance in *Arabidopsis thaliana* against *Colletotrichum higginsianum*. Similarly, Martínez-Hidalgo et al. (2015) demonstrated that the endophytic *Micromonospora* sp. ALFpr18c controlled *Botrytis cinerea* by priming the expression of jasmonate-mediated defense responses in tomato plants.

In conclusion, the findings from the present study clearly demonstrate that the *Streptomyces* sp. isolate MBFA-172 is a potential biocontrol agent capable of suppressing strawberry anthracnose, and thus would contribute to the development of a practical biocontrol approach to the anthracnose disease. More studies are still necessary to better understand the detailed mechanisms of the disease suppression capabilities of our isolate.

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CRediT authorship contribution statement

Malek Marian: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Teppei Ohno:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization. **Hirofumi Suzuki:** Formal analysis, Investigation, Resources. **Hatsuyoshi Kitamura:** Formal analysis, Investigation, Resources. **Katsutoshi Kuroda:** Investigation, Resources. **Masafumi Shimizu:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors confirm that this article has no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126428.

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