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Inhibitory Activity of *Paenibacillus* sp. Isolated from Soil in Gotsu City, Shimane Prefecture, Against *Xanthomas oryzae* pv. *oryzae,* the Causal Agent of Rice Bacterial Leaf Blight

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Abstract

Microorganism isolates (n = 49) were obtained from the soil samples collected from field in Gotsu city (Kawahira), Shimane. Isolate GT2-E culture inhibited the growth of *Xanthomonas oryzae* pv. *oryzae* in disk diffusion method. Rice bacterial leaf blight was suppressed by GT2-E culture in the pre- and post-treated rice leaves. Sequence analysis of 16S rDNA region of the GT2-E isolate indicated that it shared 99% similarity with *Paenibacillus polymyxa*. The growth of GT2-E on LB medium was observed at 15˚C, 28˚C, 37˚C, and 45˚C, but not at 4˚C. GT2-E isolate could be grown even in the presence of agrochemicals (Amister, Blasin and Kasumin). Furthermore, the growth of *X. oryzae* pv. *oryzae* was inhibited by the culture filtrate of GT2-E isolate in disk diffusion method. However, the inhibitory activity of the culture filtrate was heat-unstable. This result suggested that GT2-E isolate can produce heat-unstable inhibitory compound(s). In conclusion, GT2-E isolate might contribute to the development of a new bactericide and biological agent against rice bacterial leaf blight.

Keywords

Rice Bacterial Leaf Blight, *Xanthomonas oryzae* pv. *oryzae*, Microorganisms, *Paenibacillus polymyxa*

1. Introduction

Rice (*Oryzae sativa* L.) is one of the major staple food crops in the world. Annually, more than 40% of the world rice crop is lost owing to biotic stresses like insects, pests, pathogens, and weeds [1]. Diseases cause by bacterial, fungal, and

viral pathogens. Among several other diseases, rice bacterial leaf blight have devastated rice yield all over the world. Rice bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most destructive disease of rice around the globe. This disease reduces the grain yield to varying levels depending on the stage of the crop, degree of cultivar susceptibility, and a great extent to the conduciveness of the environment in which it occurs [2]. Rice bacterial leaf blight appears on the infected leaves as pale green to grey green water soaked streaks near leaf tips and margins after a while, the lesions coalesce and turn yellowish. Finally, the whole leaves may become whitish or grayish and eventually dies [3]. Several chemicals have been developed to control the disease. However, none of them has been fully effective under severe condition [4] [5]. In addition, a wide range chemicals and broad-spectrum antibiotics have been recommended for control of rice bacterial leaf blight [6]. However, chemical control has been known to have several disadvantages, e.g., causing detrimental effects on the ecosystem and human health, being costly, and potentially leading to pathogen resistance [7]. Biological control using antagonistic bacteria, particularly plant growth-promoting rhizobacteria has been considered an attractive alternative for control of rice bacterial leaf blight [8].

In fact, control of plant disease by using microorganisms and microbial antagonists is an important component of integrated pest management. Some studies on biological control of rice bacterial leaf blight have been reported from several countries. Most of the studies attempted to screen microorganisms from phyllplane or other sources for antibacterial activity against the bacterial pathogen *in vitro*. However, their practical use in the field still remains largely unknown, because to provide a clear demonstration of the biological control, considerable knowledge on the growth and survival of the microbial antagonists in the phyllplane of rice and the development of valid experimental techniques are required. Therefore, more extensive testing of potential microbial antagonists against *X. oryzae* pv. *oryzae* is required for effective biological control of rice bacterial leaf blight. It is well known that microorganisms produce different compounds even in the same species. Furthermore, microorganisms have different characteristics even in the same species. Therefore, it is necessary to examine a collection of wide biological diversity to find new inhibitory compounds and microorganisms for plant disease control. In the past ten years, advances in molecular genetics, monoclonal antibody technology, and biological control of plant disease have been made. In order to develop an ecofriendly technology, agrochemicals are not recommended for disease control, because they may be toxic to living beings and might lead to the development of pesticide-resistant pathogen. Therefore, control of plant disease by microorganisms is an alternative approach to prevent chemical control of plant disease. Geographically, Shimane prefecture is elongated from east to west and has various characteristic climatic diversities. Consequently, the soil of Shimane prefecture is expected to have diverse microbial resources. However, there is a limited report to microbial communities found in Shimane, or on their utility in the pathogen control for rice

bacterial leaf blight.

In this article, we report the biological control of rice bacterial leaf blight with microorganism (GT2-E) isolated from soil in Gotsu City, Shimane Prefecture, Japan.

2. Materials and Methods

2.1. Microorganism, *Xanthomonas oryzae* and Rice Plant

The microorganism (GT2-E) were isolated and obtained from the soil samples collected from field in Gotsu city (Kawahira), Shimane in accordance with previously reported procedures [9]. It was suspended in 15% - 20% glycerol solution and stored at – 80˚C until use. A single colony of the isolate was transferred to PC-1 medium (5 g starch, 5 g polypeptone, 5 g molasses, 1200 µL 2N NaOH, and final volume of 1000 mL distilled water). The liquid cultures were incubated at 26˚C ± 2˚C for 3 - 4 days with constant shaking on a rotary shaker (130 rpm). *X. oryzae* pv. *oryzae* (strain MAFF 210616) was grown on peptone sucrose agar (PeSA; 5 g peptone, 20 g sucrose, 20 g agar, and final volume of 1000 mL distilled water) or PeS broth medium at 26˚C ± 2˚C. The liquid cultures were incubated at 26˚C ± 2˚C for 3 - 4 days with constant shaking on a rotary shaker (130 rpm). Seedlings of *Oryza sativa* L. "Asahi" and "Koshihikari" were used in this study. Germinated seeds were soaked in water for 4 days, and then germinated seeds were sown in plastic pots. Rice seedlings were grown to six- to seven-leaf stage under glasshouse conditions.

2.2. Disk Diffusion Method

The antibacterial activity of GT2-E isolate against *X. oryzae* pv. *oryzae* was investigated by disk diffusion method using PeSA medium. *X. oryzae* pv. *oryzae* (100 µL) was subcultured on PeSA and paper disc (8 mm), evaluated for antibiotic testing, and placed on petri dishes containing agar. Subsequently, the paper disc were inoculated with culture medium (50 µL) containing the isolate. All petri dishes were incubated at 28˚C for 7 days and the diameter of inhibition zone was measured using a scale.

2.3. Pre-Treatment Test

In this investigation, six- to seven-leaf stage of rice seeding was treated once with the culture suspension of the isolated microorganism by clipping method [10]. PeS was treated on a set of seedlings as a control. The pretreated rice plants were maintained in a natural condition for 3 days, and then inoculated with the culture suspension of *X. oryzae* pv. *oryzae* by clipping method [10]. Inoculated rice plants were maintained under natural light condition. The lesion length of rice bacterial leaf blight on rice leaves were measured 2 weeks after inoculation. The experiments were repeated three times independently.

2.4. Post-Treatment Test

In this investigation, six- to seven-leaf stage of rice seeding was inoculated with

the culture suspension of *X. oryzae* pv. *oryzae* by clipping method [10]. The inoculated rice plants were maintained in a natural condition for 3 days, and then treated with the culture suspension of the isolated microorganism by clipping method [10]. PeS was treated on a set of seedlings as a control. Inoculated rice plants were maintained under natural light condition. The lesion length of rice bacterial leaf blight on the rice leaves were measured 2 weeks after inoculation. The experiments were repeated three times independently.

2.5. DNA Extraction, PCR Amplification, Sequencing, and Generation of Phylogenetic Tree

To identify the GT2-E isolate, the 16S rDNA sequence was determined by PCR using the primers mentioned in **Table 1**. The bacterial genomic DNA was extracted by the method described previously [11]. PCR amplification of the 16S rDNA region was performed by the PCR enzyme kit: KOD FX (Toyobo), which included the following steps: An initial step at 95˚C for 30 s, followed by 30 cycles of denaturation at 95˚C for 30 s, annealing at 53˚C for 30 s, and extension at 72˚C for 1.45 min, and a final step at 72˚C for 10 min. The PCR-amplified fragments were purified using HiYield Gel/PCR DNA fragment extraction kit (RBC Bioscience, Taipei, Taiwan). DNA sequencing was performed using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA). DNA sequence analysis was performed on an ABI PRIZM 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequence homology was determined using the BLAST suite of programs (DNA Data Bank, Japan). The phylogenetic tree was constructed using the neighbor-joining method.

2.6. Heat Stability Test

To evaluate heat stability, culture filtrate of GT2-E was heated at 121˚C for 20 min. The treated culture (heated culture filtrate) and unheated culture filtrate were exposed to the plates against rice bacterial leaf blight. The antibacterial activity of heated culture filtrate and unheated culture filtrate against *X. oryzae* pv. *oryzae* was investigated by the disk diffusion method using PeSA medium. *X. oryzae* pv. *oryzae* (100 μL) was subcultured on PeSA and paper disc (8 mm), evaluated for inhibitory activity test, and placed on petri dishes containing agar.

Table 1. The sequence of primers used for the isolate sequence determination.

Primers	Sequence (5’ → 3’)
fD1	AGAGTTTGATCCTGGCTCAG
rP2	ACGGCTACCTTGTTACGACTT
EUB906F	AAACTCAAAGGAATTGRCGG
EUB532R	CACGGTCGKCGGCGCCATT
1115R	GTTGCTCGCGTTGGGA
802R	CCTAATCTATGGGACCAT
926F	AAACTCAAAGGAATTGACGG
785F	GGATTAGATACCCTGGTAGTC
536R	GTCGTCGGCGCCATTATG

Subsequently, the paper discs were inoculated with heated culture filtrate and unheated culture filtrate (50 µL). PC-1 medium was used as a control. All the petri dishes were incubated at 28˚C for 7 days and the diameter of inhibition zone was measured using a scale.

2.7. Temperature Stability Test

To investigate the temperature stability, GT2-E isolate was incubated at different temperatures. The culture suspension of the GT2-E isolate (50 µL) was spread on surface of LB medium and then incubated at 4˚C, 15˚C, 28˚C, 37˚C, and 45˚C. After 3 days, the growth of GT2-E isolate was observed.

2.8. Agrochemical Sensitivity Test

We determined the agrochemical sensitivity of the GT2-E isolate. Amistar (Azoxystrobin, Syngenta Japan K.K.), Blasin (Ferimzone, Phthalide, Hokko Chemical Industry Co., LTD) and Kasumin (Kasugamycin, Hokko Chemical Industry Co., LTD) were used in this experiment. Each agrochemical was diluted (1000 fold) in molten LB medium and mixed thoroughly before dispensing to the respective, labeled 7-cm petri plates. The culture suspension of the GT2-E isolate (50 µL) was spread on LB medium in the presence of agrochemicals and then incubated at 28˚C. After 3 days, the growth of GT2-E isolate was observed. LB medium without agrochemical addition was used as a control.

2.9. Data Analysis

Data are represented in terms of the mean ± standard deviation values. Statistically significant differences were determined by t-test ($P < 0.05$) using Statistical Package for the Social Sciences (IBM SPSS version 22.0).

3. Results

3.1. Inhibitory Effect of Culture Suspension of GT2-E on Growth of *X. oryzae* pv. *oryzae*

The inhibitory effect of the microbial isolates from soil against rice bacterial leaf blight diseases was evaluated using the disk diffusion method. Forty-nine microbial isolates were obtained from the soil in Gotsu city (Kawahira), Shimane, Japan (date not shown). Among the 49 isolates, GT2-E isolate significantly inhibited the growth of rice bacterial leaf blight compared to control plates (**Figure 1(a)**). The diameter of the inhibition zone in the petri dish containing disc inoculated with GT2-E isolate was 13.8 ± 0.5 mm (**Figure 1(b)**). In contrast, inhibition zone was not observed in the control plates in the absence of isolated microorganism (**Figure 1(b)**).

3.2. Suppression of Rice Bacterial Leaf Blight by the Pre-Treatment with Culture Suspension of GT2-E

To investigate the suppression effect of rice bacterial leaf blight by the pre-treatment

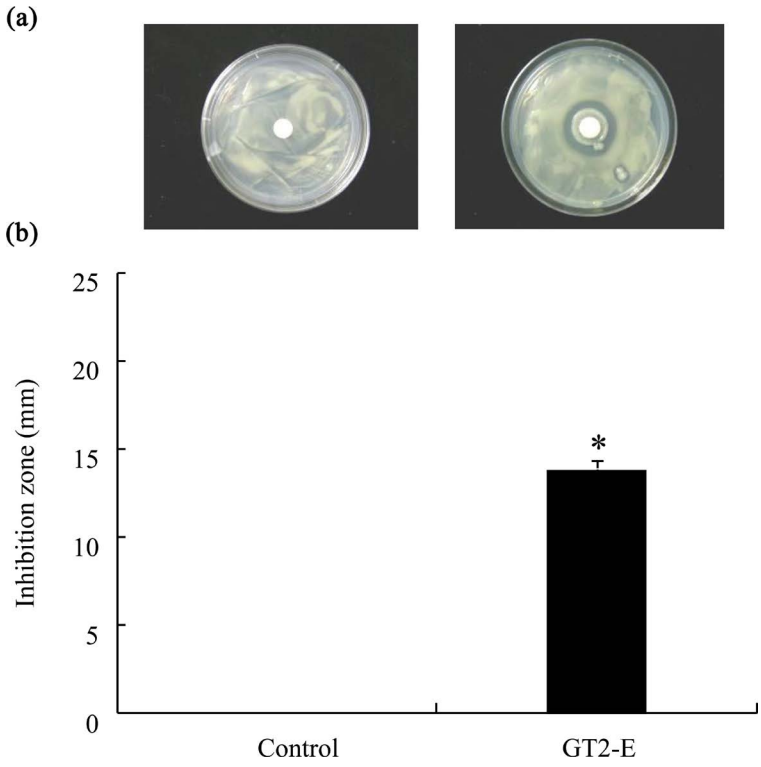

Figure 1. Inhibitory activity of isolate GT2-E to the growth of *Xanthomonas oryzae* pv. *oryzae* observed by disk diffusion method on peptone sucrose agar plate (a) and the diameter of inhibition zone to *X. oryzae* pv. *oryzae* without or with the GT2-E isolate (b). Data are represent the mean values of the results from three separate experiments. The bar at the top of each column represent the standard deviation of the mean. The asterisk indicates a significant difference compared to the result obtained in the control (*t*-test, $P < 0.05$).

with culture suspension of GT2-E, rice plant cultivars "Asahi" and "Koshihikari" were pre-treated with the culture suspension of GT2-E and were inoculated with *X. oryzae* pv. *oryzae* for 3 days. The result showed that the lesion formation in the two cultivars by *X. oryzae* pv. *oryzae* was inhibited by GT2-E (**Figure 2(a)**). In control, the length of the lesion on the leaves of cultivars "Asahi" and "Koshihikari" were 11.0 ± 2.9 and 12.1 ± 3.8 cm, respectively (**Figure 2(b)**), whereas the length of the lesion in both cultivars with pre-treated culture suspension of GT2-E were 2.5 ± 1.8 and 3.6 ± 2.1 cm, respectively (**Figure 2(b)**).

3.3. Suppression of Rice Bacterial Leaf Blight by Post-Treatment with the Culture Suspension of GT2-E

To investigate the therapeutic effect of rice bacterial leaf blight by the post-treatment with the culture suspension of GT2-E, rice plant cultivars "Asahi" and "Koshihikari" were inoculated with *X. oryzae* pv. *oryzae* and were post-treated with the culture suspension of GT2-E 3 days. The result showed that lesion formation in the two cultivars by *X. oryzae* pv. *oryzae* was inhibited by GT2-E post-treatment (**Figure 3(a)**). In control, the length of the lesion on the leaves of cultivars "Asahi" and "Koshihikari" were 12.1 ± 2.6 and 16.3 ± 4.3 cm, respec-

tively (Figure 3(b)), whereas the length of the lesion in both cultivars with post-treated culture suspension of GT2-E were 4.3 ± 1.8 and 7.0 ± 3.0 cm, respectively (Figure 3(b)).

3.4. Identification and Characterization of GT2-E

To identify the GT2-E isolate, we used specific PCR primers to amplify the 16S rDNA. The phylogenetic analyses showed that the microorganism was most

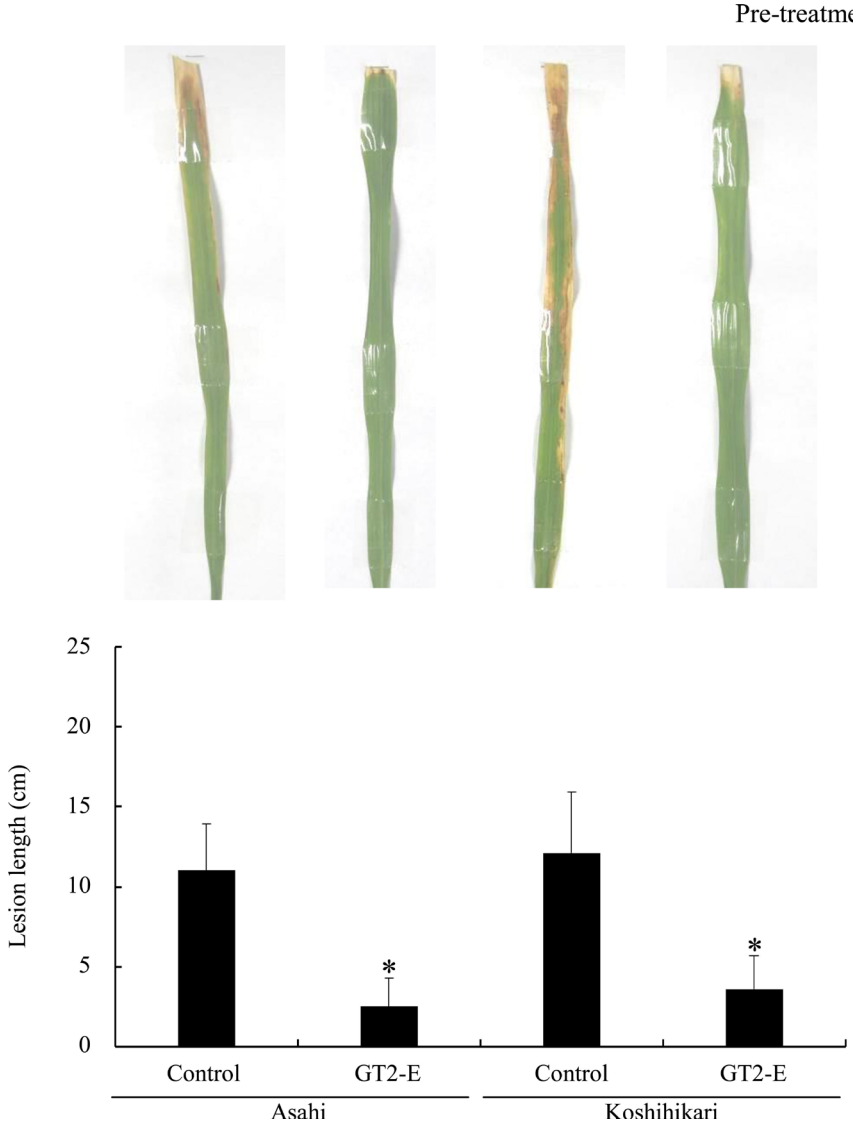

Figure 2. Effect of pre-inoculation with isolate GT2-E culture on lesion length development by *Xanthomonas oryzae* pv. *oryzae* in the rice cv. Asahi and Koshihikari. The rice leaves were pre-inoculated with isolate GT2-E culture (GT2-E) or with PC-I medium (Control) for 3 days, inoculated with *X. oryzae* pv. *oryzae* and maintained in glass house. Two weeks after inoculation, the length of lesion per leaf (a) was measured (b). Data are represent the mean values of the results from three separate experiments. The bar at the top of each column represent the standard deviation of the mean. The asterisk indicates a significant difference compared to the result obtained in the control (*t*-test, $P < 0.05$).

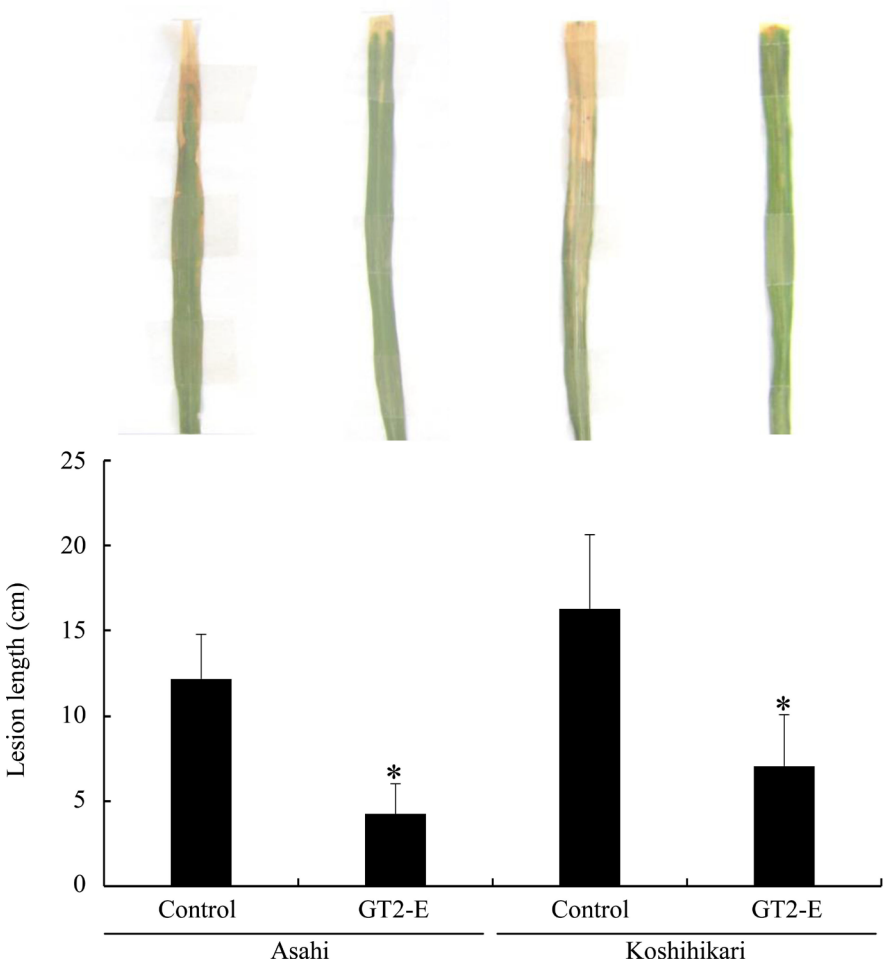

Figure 3. Effect of post-inoculation with isolate GT2-E culture on lesion length development by *Xanthomonas oryzae* pv. *oryzae* in the rice cv. Asahi and Koshihikari. The rice leaves were inoculated with *X. oryzae* pv. *oryzae* for three days, inoculated with isolate GT2-E culture (GT2-E) or with PC-I medium (Control) and maintained in glass house. Two weeks after inoculation, the length of lesion per leaf (a) was measured (b). Data are represent the mean values of the results from three separate experiments. The bar at the top of each column represent the standard deviation of the mean. The asterisk indicates a significant difference compared to the result obtained in the control (*t*-test, $P < 0.05$).

closely related to the strain type of *Paenibacillus polymyxa* NBRC 15309 (Figure 4) In the present study, the growth of GT2-E was observed at 15˚C, 28˚C, 37˚C, and 45˚C, but not at 4˚C. The optimum temperature of the growth of GT2-E was 28˚C - 37˚C (Table 2). Furthermore, no inhibitory activity on the growth of GT2-E was observed in the presence of agrochemical against rice diseases, such as Amister, Blasin, and Kasumin (Table 3).

3.5. Effect of the Culture Filtrate of GT2-E on the Growth of *X. oryzae* pv. *oryzae*

For evaluating the inhibitory effect of the culture filtrate of GT2-E isolate, we

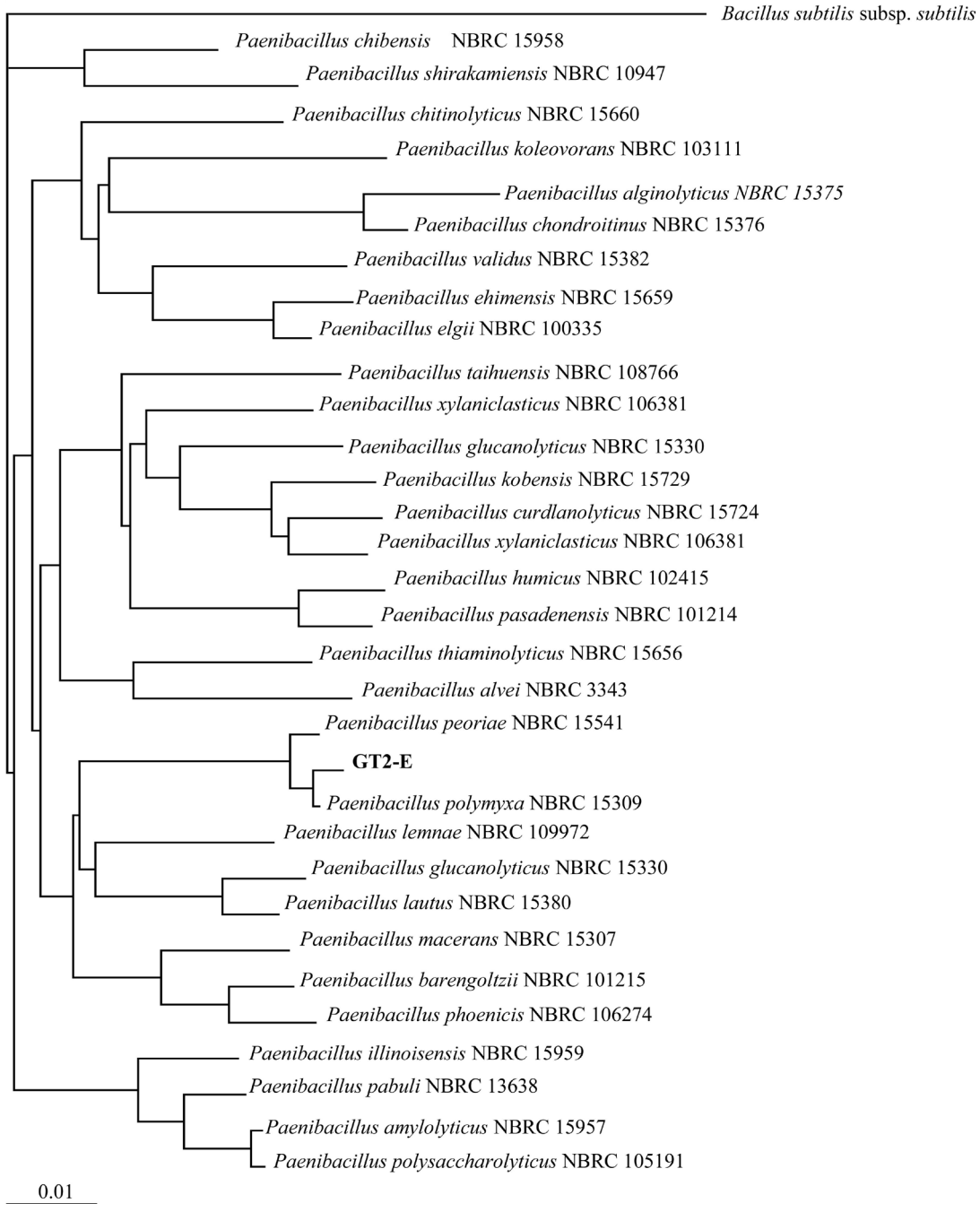

Figure 4. Phylogenetic tree constructed basis on 16S rDNA sequences of the isolate GT2-E. A bootstrap consensus neighbor-joining tree for the isolate GT2-E was created based on the Kimura 2-Parameter distance matrix (1000 replicates). *Bacillus subtilis* subsp. *subtilis* was used as an out-group. The scale bar represents 1% sequence dissimilarity.

Table 2. Effect of temperature on growth of isolate GT2-E under different incubation temperature.

Isolate	Temperature(˚C)				
GT2-E	4	15	28	37	45
	-	+	++	++	+

-: means no growth, +: means moderate growth, ++: means maximum growth.

Table 3. Effect of agrochemical on growth of isolate GT2-E on agrochemical amended LB medium.

Isolate	Agrochemical			
GT2-E	Control	Blasin	Kasumin	Amister
	++	++	++	++

++: means maximum growth was observed.

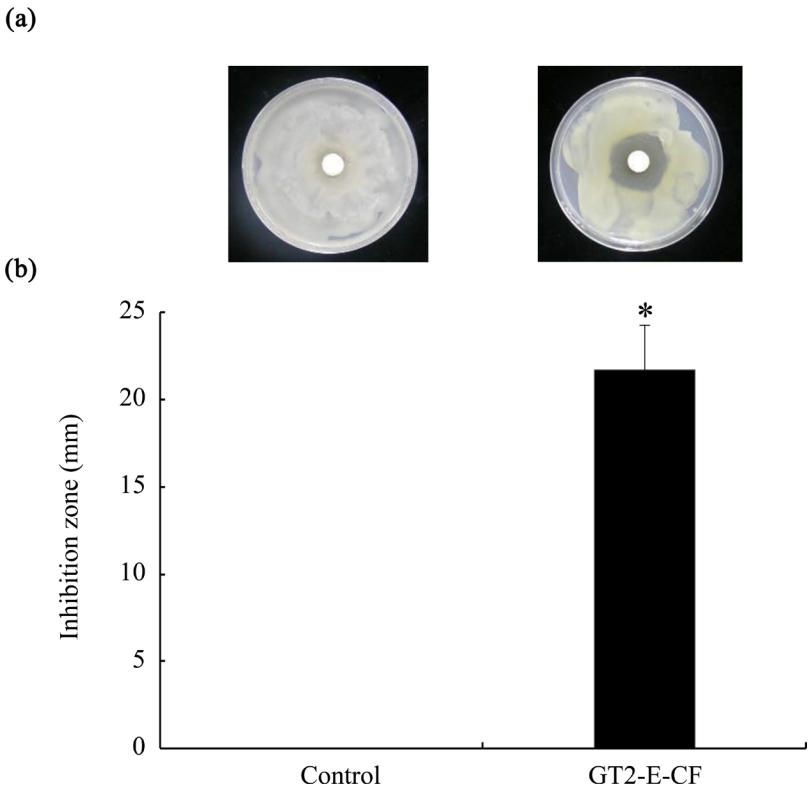

Figure 5. Inhibitory activity of culture filtrate of isolate GT2-E to the growth of *Xanthomonas oryzae* pv. *oryzae* observed by disk diffusion method on peptone sucrose agar plate (a) and the diameter of inhibition zone without (Control) or with culture filtrate of isolate GT2-E (GT2-E-CF) to *X. oryzae* pv. *oryzae* (b). Data are represent the mean values of the results from three separate experiments. The bar at the top of each column represents the standard deviation of the mean. The asterisk indicates a significant difference compared to the result obtained in the control (*t*-test, $P < 0.05$).

investigated the effects of the culture filtrate of GT2-E isolate on the growth of *X. oryzae* pv. *oryzae* by the disk diffusion method. Culture filtrate of GT2-E inhibited *X. oryzae* pv. *oryzae* growth compared to control (**Figure 5(a)**). The diameter of the inhibition zone in the petri dish containing disc inoculated with the culture filtrate of GT2-E isolate was 21.7 ± 2.4 mm (**Figure 5(b)**). In contrast, the inhibition zone was not observed in the control plates in the absence of the isolated microorganism (**Figure 5(b)**). We also determined the inhibitory effect of heat-treated GT2-E culture filtrate. The result showed that heat-treated GT2-E culture filtrate did not inhibit the growth of *X. oryzae* pv. *oryzae* (**Figure 6(a)**). The diameter of the inhibition zone in the petri dish containing discs

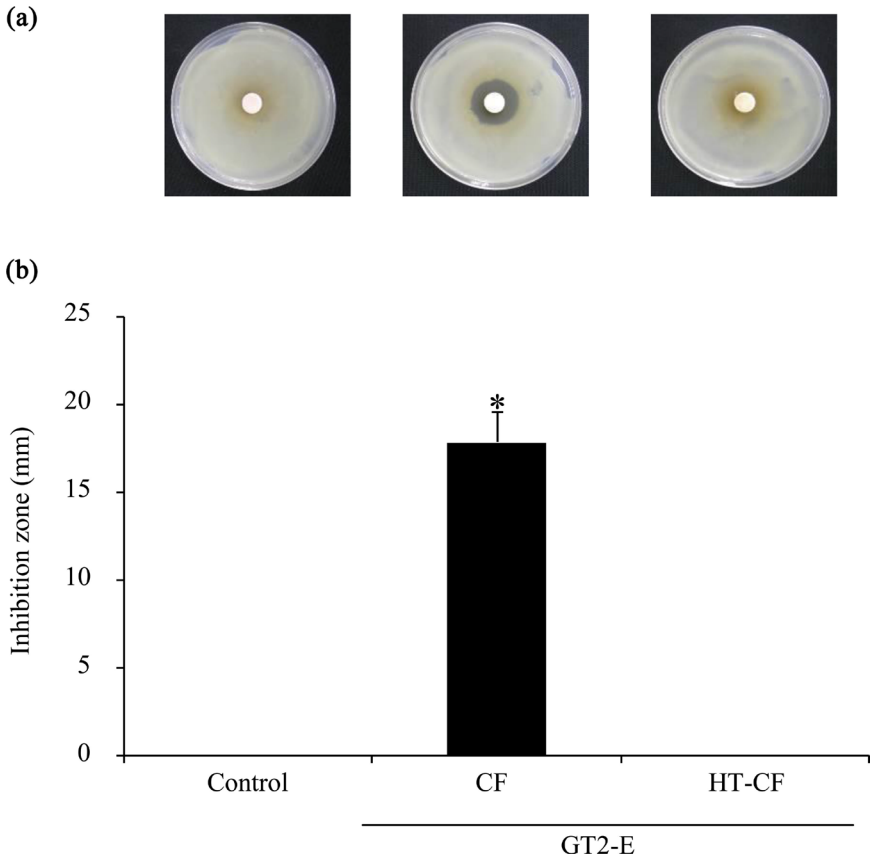

Figure 6. Inhibitory activity of heat treated culture filtrate of isolate GT2-E to the growth of *Xanthomonas oryzae* pv. *oryzae* observed by disk diffusion method on peptone sucrose agar plate (a) and the diameter of inhibition zone without (Control) or with heat treated (HT) culture filtrate (CF) of isolate GT2-E to *X. oryzae* pv. *oryzae* (b). Data are represent the mean values of the results from three separate experiments. The bar at the top of each column represent the standard deviation of the mean.

inoculated with the culture filtrate and heat-treated culture filtrate of GT2-E isolate was 17.9 ± 1.8 and 0.0 ± 0.0 mm, respectively (**Figure 6(b)**).

4. Discussion

Rice (*Oryzae sativa* L.) is one of the second staple food crops after wheat across the world. Rice plants are under constant threats owing to many diseases. Rice bacterial leaf blight is one of the most destructive bacterial disease that rendered considerable yield and economics losses. Control strategies applied against rice bacterial leaf blight mainly involve the use of chemicals and resistance cultivars. Several chemicals and broad-spectrum antibiotics have been recommended for the control of rice bacterial leaf blight [6]. However, chemical control has been known to have several disadvantages, e.g., causing detrimental effects on the ecosystem and human health, is costly, and use of chemical control potentially leads to pathogen resistance [7]. In addition, host resistance gene deployment is one of the solutions for rice bacterial leaf blight. However, the rapid adaptation of rice bacterial leaf blight population may affect the durability of the resistance

genes [12] [13] [14]. Biological control might assume a special significance in being an environmental friendly and cost effective alternative for the management of rice bacterial leaf blight. Furthermore, development of resistance to biological control by antagonistic bacteria has not been reported. Several antagonistic bacteria such as *Pseudomonas fluorescens*, *Bacillus* sp. and *Burkholderia* sp. against rice bacterial leaf blight have been identified and extensively studied. Geographically, Shimane prefecture is extended from the east to west and has various characteristic diversities of climate in each region. Consequently, soil of the Shimane prefecture is expected to have diverse microbial resources. We reported that microorganisms isolated from the soil of Gotsu city (Shimane prefecture) suppress rice blast disease caused by *Magnaporthe oryzae* [11]. In this study, it was observed that GT2-E had inhibitory activity against *X. oryzae* pv. *oryzae*. The phylogenetic analysis of the GT2-E isolate revealed that the organism was most closely related to *P. polymyxa*.

Previously, it was reported that the genus *Paenibacillus* acts as a biocontrol agent and inhibits the growth of *Botrytis cinerea*, *Fusarium oxysporum*, *Phytophthora palmivora, Pythium, aphanidermatum*, and *Rhizoctonia solani* [15] [16] [17]. Furthermore, it is well known that the genus *Paenibacillus* is not only plant growth-promoting rhizobacteria, but also induces resistance against plant diseases [18].

However, the effective control of *Xanthomas oryzae* pv. *oryzae* by *P. polymyxa* in rice plants has not yet been elucidated and the inhibitory substances are not yet to be detected.

In this study, rice bacterial leaf blight (*X. oryzae* pv. *oryzae*) was significantly suppressed by GT2-E culture suspension in pre- and post-treated rice plants. This result suggested that GT2-E has prevention and therapeutic effects against rice bacterial leaf blight. Moreover, plant growth promotion activity and induced resistance against plant diseases by GT2-E isolate has not been investigated up to date. Therefore, it will require further investigations in the plant.

In the present study, the culture filtrate of GT2-E inhibited the growth of *X. oryzae* pv. *oryzae*. However, the inhibitory activity of heat-treated culture filtrate was significantly decreased compared to that of non-heat-treated culture filtrate. These results suggested that the GT2-E isolate produced heat-unstable compound(s) against *X. oryzae* pv. *oryzae*. It was reported that *Paenibacillus* sp. produce various types of inhibitory compounds against plant diseases, such as butyl 2,3-dihydroxybenzoate [19], fusaricidins A, B, and C [20], pelgipeptins A and B [21]. Therefore, further studies are required to identify the inhibitory compound(s) secreted in the culture filtrate of GT2-E isolate.

Remarkably, GT2-E isolate was tolerant to several agrochemicals such as Amistar (Azoxystrobin), Blasin (Ferimzone, Phthalide), and Kasumin (Kasugamycin). It is well known that these agrochemicals are used against rice diseases. This result indicated that these agrochemicals can be used for the control of rice diseases along with GT2-E. In addition, the growth of GT2-E isolate was observed at 15˚C - 45˚C. Generally, the disease development of rice bacterial leaf

blight caused by *X.oryzae* pv *oryzae* occurs at the optimum temperature range from 25˚C - 30˚C. This result indicated that GT2-E will be useful for the control of rice bacterial leaf blight under field conditions. Suppression effect of rice bacterial leaf blight by GT2-E will require further investigation in the field condition.

5. Conclusion

In conclusion, these results suggested that GT2-E might contribute to the development of a new biocontrol agent against rice disease, such as rice bacterial leaf blight.

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