Bull. Fac. Life Env. Sci. Shimane Univ., 27 : 1, December31, 2022

Secondary metabolites produced by actinomycetes affect appressorium formation and melanin synthesis of *Pyricularia oryzae* causing rice blast disease

Ibuki Kamada[1], Masatoshi Ino[2], Junichi Kihara[1,2], Michihiro Ito[3], Naoya Shinzato[3], Makoto Ueno[1,2]*

Abstract Rice blast disease, caused by *Pyricularia oryzae*, is the most important disease concerning rice worldwide. Control strategies against *P. oryzae* in rice mainly involve the application of chemical fungicides and resistant cultivars. However, new strains of the pathogen appear to be able to overcome the resistance genes in currently grown cultivars. Therefore, there is a need to identify natural compounds and develop new agents to control *P. oryzae*. This study evaluated the inhibitory activity of the isolate 1-86, isolated from soil in Kumejima islamd. in Okinawa prefecture against *P. oryzae*. The isolate 1-86 did not inhibit the mycelial growth of *P. oryzae* by dual culture method. However, its presence induced abnormal spherical structure of the hyphal tip of *P. oryzae*. When the direct effects of the cell extract of isolate 1-86 (1-86-CE) was determined by investigating the germination of *P. oryzae* conidia, induction of abnormal formation and melanization suppresion of appressorium were observed in the presence of 1-86-CE. Sequence analysis of the 16S rDNA region of isolate 1-86 revealed high similarity with the genus *Streptomyces*. The active compounds in 1-86-CE was ethyl acetate-insoluble and heat-stable. Futhermore, 1-86-CE significantly suppressed blast lesion development in rice plants. These results strongly suggest that the inhibitory effects of 1-86-CE on plant pathogenic fungi may contribute to the development of new fungicide agents to control plant diseases caused by plant pathogens such as *P. oryzae*.

Keywords actinomycetes, *Pyricularia oryzae*, rice blast disease, secondary metabolites

Introduction

Chemical and biological fungicides are an important disease control tool to obtain a stable yield. Currently, control strategies against plant diseases mainly involve the use of chemical fungicides. However, resistance development to chemical fungicide has been reported in instances of extensive use (FRAC, 2018).

Additionally, non-target beneficial microorganisms are affected by chemical control. (Channabasava et al., 2015). Therefore, it is necessary to identify other antifungal compounds with a different mode of action that can be developed into new fungicides. Natural microbial products have attracted attention as potential biological and chemical control agents against fungal diseases in various crops (Shimizu et al., 2000; Joshi et al., 2008). Different microorganisms, even different strains within the same species, have different physiological characteristics and thus produce different compounds. Therefore, a wide range of taxa need to be tested to identify new microorganisms and compounds that can effectively control plant diseases. Members of *Streptomyces*, a genus of Actinobacteria, have potential as control agents due to their inhibitory ability against various plant pathogens (Shimizu et al., 2000; Shimizu et al., 2009; Kim et al., 2011), and several studies have reported the isolation of inhibitory compounds from *Streptomyces* species (Lee et al., 2005; Park et al., 2006). Okinawa—the only subtropical area in Japan with numerous island ecosystems—is expected to have a wide diversity of microbial resources. Recently, we reported the

[1]Graduate school of Natural Science and Technology, Shimane University, [2]The United Graduate School of Agricultural Sciences, Totori University, [3]Tropical Biosphere Research Center, Centre of Molecular Biosciences, University of the Ryukyus *Corresponding author. E-mail address: makoto-u@life.shimane-u.ac.jp

construction of a culture filtrate library with microbes originally isolated from the soil in Okinawa [Okinawa Microbial Library (OML), http://omlus.jimdo.com/], including the Yaeyama Archipelago, and validated its phylogenetic diversity (Ueno et al., 2016). The dominant phylum in the OML was Actinobacteria (Ueno et al., 2016). We also reported that the cell extract of isolate 3-45 present in the OML induced the formation of an abnormal appressorium, inhibition of melanin production, and suppression of the development of rice blast disease (Tamura et al., 2019). Herein, we report the inhibitory effect of isolate 1-86 which demonstrates similar activity against rice blast fungi present in the OML.

Materials and Methods

Bacteria and plant pathogen

Isolate 1-86 was obtained from soil samples collected from fields in Kumejima, Okinawa Prefecture, Japan, using humic acid-vitamin (HV) agar medium as previously described (Hayakawa & Nonomura, 1987; Ueno et al., 2016). Isolated bacteria were suspended in 15–20% glycerol solution and stored at −80°C until use. *Pyricularia oryzae* (strain Naga 69–150, race 007) was used as the test pathogen. It was grown on rice-bran agar (50 g/L rice-bran, 20 g/L sucrose, 20 g/L agar, and distilled water) at 26 ± 2°C for 14 d, washed with distilled water to remove aerial hyphae, and maintained at 26 ± 2°C for a further 3-4 d under near-ultraviolet radiation provided by fluorescent lamps (FL20S・BLB; Panasonic, Osaka, Japan) to induce abundant conidiation. Synchronously formed conidia were used as the inoculum.

Preapration of bacteria cell extraction and investigation of infection behaviour of *P. oryzae*

Isolate 1-86 was grown on LB medium, and individually inoculated in Erlenmeyer flasks containing of an LB liquid medium. The liquid cultures were incubated at 28 ± 2°C in the dark with constant shaking (130 rpm). Isolated LB-grown 1-86 cells were extracted with acetone of different concentation. The acetone extract was added to distilled water and subjected to evaporation at 50°C under reduced pressure until only the distilled water remained. The aqueous volume was adjusted to 1 mL/g of isolated 1-86 cells (1-86-CE). Conidia of *P. oryzae* were dropped onto glass slides in the presence of 1-86-CE and maintained in a moist chamber at 26 ± 2°C. Distilled water was used as the control. After 24 h, diameter and melanization of appressoria were determined.

Dual culture assay

The inhibitory activity of the isolate 1-86 on the mycelial growth of *P. oryzae* was investigated using the dual culture method with potato sucrose agar (PSA) medium. Mycelial plugs (8 mm diameter) of *P. oryzae* and a paper disc (8 mm; Advantec Toyo Kaisha, Ltd., Tokyo, Japan) containing 30 µL of the isolate 1-86 suspension culture were placed on PSA plates, 4 cm apart. Subsequently, the LB broth was inoculated on the same kind of paper disc and used as a control. All petri dishes were incubated at 26 ± 2°C for 10 d. The mycelial area (mm^2) of *P. oryzae* was then measured using LIA 32 ver.0.378 software (https://www.agr.nagoya-u.ac.jp/~shinkan/LIA32/index-e.html). Experiments were replicated three times with five plates per treatment.

Identification of isolated bacteria

To identify isolate 1-86, its 16S rDNA sequence was determined by PCR (Tamura et al., 2019), using previously described primers (Huong et al., 2007; Matsui et al., 2009). Genomic DNA was extracted from the bacteria colony, following the method described by Suzuki et al. (2006) and was used as a template for the PCR amplification of the 16S rDNA region. The following PCR protocol was used: initial denaturation at 95°C for 30 s; 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and elongation at 72°C for 1.45 min; and final extension at 72°C for 10 min. Amplicons were purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co., KG, Düren, Germany). The purified amplicons were sent to FASMAC (Fasmac Co., Ltd., Kanagawa, Japan) for nucleotide sequencing. Sequence homology was determined by using BLAST from the GenBank database http://www.ncbi.nlm.nih.gov/BLAST/). The aligned

sequences were calculated using Kimura's two-parameter model (Kimura, 1980) and analyzed using the neighbor-joining method (Saitou & Nei, 1987) with GENETYX ver. 13 (https://www.genetyx.co.jp). Bootstrap percentages were calculated with 1000 replicates. *Actinoalloteichus cyanogriseus* (NBRC 14455) was used as the outgroup. The morphological types of the mycelium and conidia of isolate 1-86 were observed using scanning electron microscopy (S4800, Hitachi, Tokyo, Japan).

Infection-inhibiting activity of 1-86-CE

Rice leaf sheaths were inoculated with *P. oryzae* conidia in the presence or absence of 1-86-CE. Each inoculated leaf sheath was maintained in a moist chamber at 26 ± 2°C. After 48 h, the percentage of infection as indicated by formation of hyphae and the index of infectious hyphae were determined. The rate of infectious hyphae formation per 50 appressorium per experiment were calculated. The data are presented as means of three experiments with three replicates. The percentage of infectious hyphae was calculated as: Percentage of infectious hyphae (%) = (number of infectious hyphae/total number of appressorium) ×100. The index of infectious hyphae in rice leaf sheaths was calculated according to Takahashi et al. (1958), as observed under a light microscope.

Inoculation test

Seedlings of *Oryza sativa* L. 'Koshihikari' were grown to the 5-6-leaf stage in a glasshouse as previously described (Fujita et al., 1994). *P. oryzae* conidia (1×10^5 conidia/mL) suspended in the absence (Control) or presence of 1-86-CE were sprayed on the rice plant (2 mL/plant). The inoculated rice plants were incubated in a moist chamber for 24 h in the dark, and then maintained under natural light conditions. The number of blast lesions on top of the expanded leaves was measured 7 d after inoculation. The experiments were independently repeated in triplicate. A total of 10 leaves per experiment were examined.

Statistical analysis

Data are reported as the mean(s) ± standard deviation (SD). Differences in the experimental values between groups were determined by the *t*-test or Tukey-Kramer test using SPSS Statistics ver. 22.0 for Windows (IBM, Armonk, NY, USA). Differences were considred significant when $P < 0.05$.

Results and Discussion

To assess the inhibitory activity of the isolate 1-86, its inhibitory activity against *P. oryzae* was tested using the dual culture method. The isolate 1-86 did not inhibit the mycelial growth of *P. oryzae* compared to the control (Fig. 1). The mycelial area in the control or isolate 1-86 was 2505.9 ± 407.2 mm^2 or 2273.2 ± 318.8 mm^2, respectively (Fig. 1). We also investigated the influence of the isolate 1-86 on the morphology of the *P. oryzae* hyphae using light microscopy. The presence of the isolate 1-86 induced spherical structure of the hyphal tip of *P. oryzae* (Fig. 1).

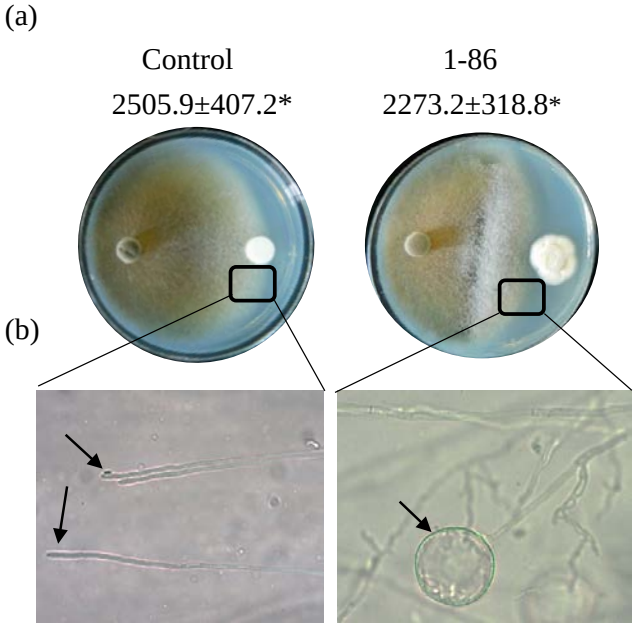

Fig. 1 Dual culture assay for inhibition of the mycelial growth of *Pyricularia oryzae* with the isolate 1-86 on potato sucrose agar (PSA) medium. Mycelial plugs (8 mm) of *P. oryzae* and paper disc was placed on PSA plates, 4.0 cm apart from each other. The paper disc was inoculated with a suspension (30 µL) of the isolate 1-86 cultured in LB liquid medium. LB liquid medium was inoculated on the paper disc as a control. All petri dishes were incubated at 26 ± 2°C for 10 d (a) and then a mycelial tip (arrow) of *P. oryzae* near the 1-86 colony was observed under light mycroscopy (b). *Mycelial area (mean ± standard deviation) of *P. oryzae*.

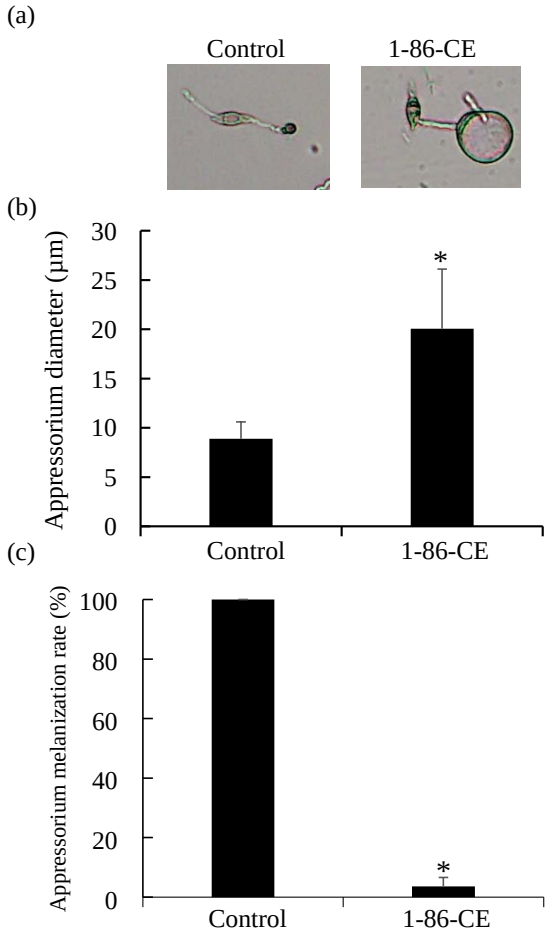

Fig. 2 Effect of cell extract (CE) from isolate 1-86 on appressorium formation and melanization in *Pyricularia oryzae* (a). Isolate 1-86 was grown in LB medium and then extracted with 50% acetone. The acetone extract was added to distilled water and subjected to evaporation at 50°C under reduced pressure until only the distilled water and extract remained. The aqueous volume was adjusted to 1 mL/g of isolate 1-86 cells (1-86-CE). Conidia of *P. oryzae* were dropped onto glass slides in the presence of isolate 1-86-CE and maintained in a moist chamber at 26 ± 2°C. Distilled water was used as the control. After 24 h, the diameters (b) and melanization of appressoria (c) were determined. Experiments were independently repeated in triplicate. At least 150 conidia per experiment were examined. Bars represent mean ± standard deviation. Means followed by the same letters are not significantly different ($P < 0.05$) according to the *t*-test.

Therefore, we next investigated that effect of 1-86-CE on infection behaviour of *P. oryzae*. In presence of distilled water (Control), normal appressoria were formed (8.9 ± 1.7 μm) (Fig. 2a, b). However, abnormal appressorium formed in 1-86-CE (20.1 ± 6.4 μm) (Fig. 2a, b). Also, appressorium melanization was significantly inhibited in the presence of 1-86-CE (3.6 ± 3.1%), but not in the absence of 1-86-CE (100%) (Fig. 2c). In this study, the optimal condition for the extraction of the active compounds from the cells of isolate 1-86 was 50% acetone (data not shown). Culture filtrate of isolate 1-86 did not indicate abnormal appressorium formation and melanization. In our previous report, isolate 3-45 produced active compounds that induced abnormal appressorium formation and suppressed appressorium melanization only under oligotrophic conditions. On the other hand, isolate 1-86 produced active compounds that induced abnormal appressorium formation and supressed appressorium melanization even under nutrient-rich conditions. This characteristic of 1-86 would be useful for easy preparation and utilization of a large amount of the active compound(s). In the future, it is necessary to identify the active compounds of 1-86-CE.

For the characterization of the active compounds of isolate 1-86-CE, we investigated the ability of both the ethyl acetate soluble and ethyl acetate insoluble fractions of 1-86-CE to induce abnormal appressorium formation of *P. oryzae*. When *P. oryzae* was exposed to the distilled water (Control) and 1-86-CE (Original), the appressorium diameter was 9.4 ± 1.5 μm and 19.2 ± 5.8 μm, respectively. On the other hand, in the presence of the ethyl acetate soluble and insoluble fractions, it was 9.2 ± 1.3 μm and 19.2 ± 6.1 μm, respectively (Table 1). Furthermore, we investigated the heat stablity of the active compounds of isolate 1-86-CE. The appressorium diameter values were as follows: Original (non-heat), 21.9 ± 7.9 μm; 60°C, 21.5 ± 7.5 μm; 100°C, 20.8 ± 7.0 μm; 121°C, 21.5 ± 8.4 μm; (Table 2). In the control, the appressorium diameter was 9.5 ± 1.3 μm (Table 2). Active compounds of isolate 1-86-CE was acid-tolerance, with a molecular weight of more than 50 kDa (data not shown). These characteristics were the same as the active compounds of isolate 3-45, which produced compounds exhibiting similar activity (Tamura et al., 2019).

To identify isolate 1-86, its 16S rDNA was analyzed. Based on phylogenetic analysis, results showed that isolate 1-86 was most closely related to members of the genus *Streptomyces* (data not shown). In addition, conidia had smooth surfaces and fine structures of spore chain

Table 1 Characterization of the extract of 1-86-CE to abnormal appressorium formation of *Pyricularia oryzae.*

		1-86-CE		
			Ethyl acetate	
Variable	Control	Original	In-soluble	Soluble
Appressorium diameter (μm)	9.4±1.5[a]	19.2±5.8[b]	19.2±6.1[b]	9.2±1.3[a]

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

Table 2 Heat stability of the extract of 1-86-CE to abnormal appressorium formation of *Pyricularia oryzae.*

		1-86-CE			
			Heat treatment (°C)		
Variable	Control	Original	60	100	121
Appressorium diameter (μm)	9.5±1.3[a]	21.9±7.9[b]	21.5±7.5[b]	20.8±7.0[b]	21.5±8.4[b]

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

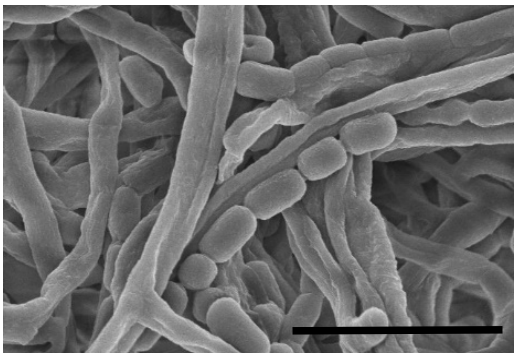

Fig. 3 Scanning electron microscopy of isolate 1-86. The scale bar represents 4 μm.

morphology (Fig. 3). Therefore, isolate 1-86 was considered to be a member of the genus *Streptomyces.* The isolate 1-86 was in a different clade to the plant pathogen *S. scabiei* and human pathogen *S. Somaliensis.* Polyoxin, produced by *S. cacaoi* var. *asoensis* is known to induce swelling of germination tubes and to the tip of hyphae (Endo et al.,1970). However, isolate 1-86 was in a different clade than *S. cacaoi* var. *Asoensis.* Recently, we reported that the cell extract of *S. erythrochromogenes* isolate 3-45 present in the OML induced the formation of abnormal appressorium and inhibition of melanization, and subsequently suppressed the development of rice blast disease. However, isolate 1-86 was in a different clade to *S. erythrochromogenes* (isolate 3-45). These results suggested that different *Streptomyces* species might produce compounds with similar activity, although the two species produce compounds under quite different conditions as described above. In the future, it is necessary to identify these active compounds.

To study the infective behaviour of *P. oryzae*, rice leaf sheaths were inoculated with *P. oryzae* in the presence of 1-86-CE. Abnormal appressoria formation and melanization inhibition of *P. oryzae* were observed in rice leaf sheaths inoculated with *P. oryzae* in the presence of 1-86-CE (Fig. 4a). The infection rate by *P. oryzae* in the absence and presence of 1-86-CE was 99.3 ± 11.6 and 3.3 ± 18.0, respectively (Fig. 4b). In addition, we investigated the index of infectious hyphae in rice leaf sheaths inoculated with *P. oryzae*. Hyphae formation by *P. oryzae* was significantly inhibited in leaf sheaths subjected to inoculation with *P. oryzae* in the presence of 1-86-CE, but not in sheaths inoculated with *P. oryzae* in the absence of 1-86-CE (Fig. 4c). The index of infectious hyphae in the absence and presence of 1-86-CE was 7.7 ± 3.6 and 0.1 ± 0.6, respectively (Fig. 4c). Furthermore, to determine the inhibitory activity of isolate 1-86-CE against *P. oryzae* in rice, leaves were inoculated with conidia of *P. oryzae* in the presence of 1-86-CE. As a result, the number of blast lesions formed was suppressed compared to the control treatment (Fig. 5). The number of blast lesions formed by *P. oryzae* in the absence and presence of 1-86-CE was 17.1 ± 9.6 and 9.7 ± 6.3, respectively (Fig. 5). The formation of an appressorium and its melanization are required for infection by *P. oryzae*, the causal agent of rice blast disease. In the future, it is necessary to investigate whether 1-86-CE suppresses the gene expression of melanin synthesis and affects the gene expression of appressorium formation-related genes of *P. oryzae.*

In conclusion, this study on the inhibitory effects of 1-86-CE on plant pathogenic fungi may contribute to the development of a new fungicide for the control of rice blast disease caused by *P. oryzae.*

Acknowledgements

The authors gratefully acknowledge the faculty of Life and Environmental Science in Shimane University for financial support to publish this report. This study was also supported by the Collaborative Research of Tropical Biosphere Research Center, University of the Ryukyus.

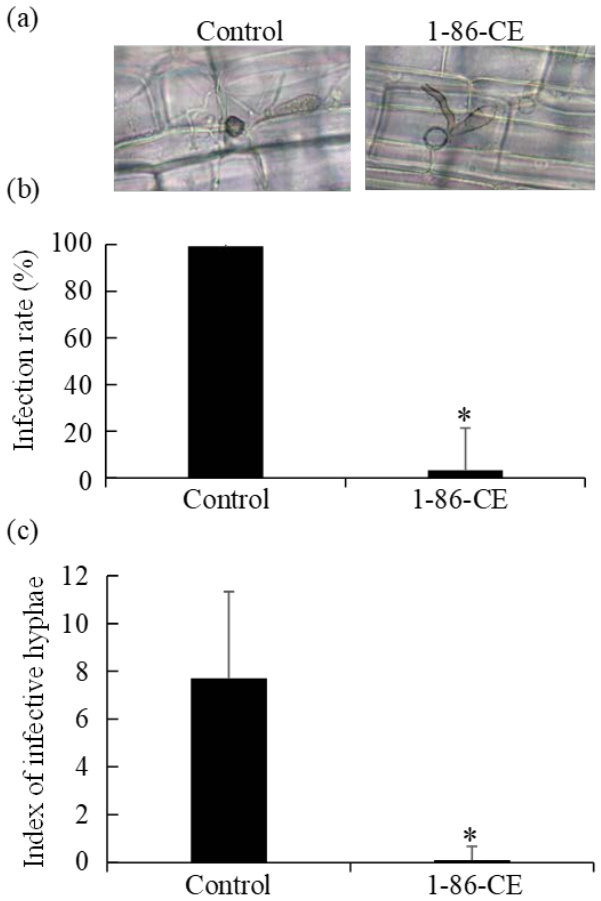

Fig. 4 Effect of cell extract (CE) from isolate1-86 on the infectious behaviour of *Pyricularia oryzae* in rice leaf sheaths. These were inoculated with a conidia suspension of *P. oryzae* in the presence or absence of 1-86-CE and maintained in a moist chamber at 26 ± 2°C. Forty-eight hours after inoculation (a), infectious hyphae formation rate (as indicated by hyphae formation) (b) and index of infectious hyphae (c) were determined. The rate of infectious hyphae formation per appressorium was calculated. The data are the means of the three experiments with three replications each. Fifty infected sites were examined per experiment. Bars represent the mean ± standard deviation. The asterisk indicates a significant difference compared to controls (*t*-test, $P < 0.05$).

Fig. 5 Effect of cell extract (CE) from isolate 1-86 on blast lesion formation by *Pyricularia oryzae* on rice leaves. Rice leaves were inoculated with a *P. oryzae* conidia suspension in the presence or absence of 1-86-CE and maintained in a moist chamber for 24 h at 26 ± 2°C. Seven days after inoculation, disease development and the number of blast lesions were measured. Experiments were independently repeated three times. A total of 10 leaves per experiment were examined. Bars represent mean ± standard deviation. The asterisk indicates the number of blast lesions formed (mean ± standard deviation) by *P. oryzae* per leaf.

References

Channabasava, A., Lakshman, H.C. and Jorquera, M. A. (2015) Effect of fungicides on association of arbuscular mycorrhiza fungus *Rhizophagus fasciculatus* and growth of Proso millet (*Panicum miliaceum* L.). Journal of Soil Science and Plant Nutrition, **15**: 35–45.

Endo, A., Kakiki, K. and Misato, T. (1970) Mechanism of action of the antifugal agent Polyoxin D. Journal of Bacteriology, **104**: 189–196.

Fujita, K., Arase, S., Hiratsuka, H., Honda, Y. and Nozu, M. (1994) The role of toxin(s) produced by germinating conidia of *Pyricularia oryzae* in pathogenesis. Journal of Phytopathology, **142**: 245–252.

Fungicide resistance action committee (FRAC) (2018) http://www. frac.info/publications/downloads.

Hayakawa, M. and Nonomura, H. (1987) Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. Journal of Fermentation Technology, **65**: 501–509.

Huong, N. L., Itoh, K. and Suyama, K. (2007) Diversity of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading bacteria in Vietnamese soils. Microbes and Environments, **22**: 243–256.

Joshi, S., Bharucha, C. and Desai, A. J. (2008) Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B. Bioresource Technology, **99:** 4603–4608.

Kim, J. D., Han, J. W., Lee, S. C., Lee, D., Hwang, I. C. and Kim, B. S. (2011) Disease control effect of strevertenes produced by *Streptomyces psammoticus* against tomato fusarium wilt. Journal of Agricultural and Food Chemistry, **59**: 1893–1899.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, **16**: 111–120.

Lee, J.Y., Lee, J. Y., Moon, S. S. and Hwang, B. K. (2005) Isolation and antifungal activity of 4-phenyl-3-butenoic acid from *Streptomyces koyangensis* strain VK-A60. Journal of Agricultural and Food Chemistry, **53**: 7696–7700.

Matsui, T., Kato, K., Namihira, T., Shinzato, N. and Semba, H. (2009) Stereospecific degradation of phenylsuccinate by actinomycetes. Chemosphere, **76**: 1278–1282.

Park, H. J., Lee, J. Y., Hwang, I. S., Yun, B. S., Kim, B. S. and Hwang, B. K. (2006) Isolation and antifungal and antioomycete activities of staurosporine from *Streptomyces roseoflavus* strain LS-A24. Journal of Agricultural and Food Chemistry, **54**: 3041–3046.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, **4**: 406–425.

Shimizu, M., Nakagawa, Y., Sato, Y., Furumai, T., Igarashi, Y., Onaka, H., Yoshida, R. and Kunoh, H. (2000) Studies on endophytic actinomycetes (I) and *Streptomyces* sp. isolated from Rhododendron and its antifungal activity. Journal of General Plant Pathology, **66**: 360–366.

Shimizu, M., Yazawa, S. and Ushijima, Y. (2009) A promising strain of endophytic *Streptomyces* sp. for biological control of cucumber anthracnose. Journal of General Plant Pathology, **75**: 27–36.

Suzuki, S., Taketani, H., Kusumoto, K. and Kashiwagi, Y. (2006) High-throughput genotyping of filamentous fungus *Aspergillus oryzae* based on colony direct polymerase chain reaction. Journal of Bioscience and Bioengineering, **102**: 572–574.

Takahashi, Y. (1958) A method for forecasting blast disease using the leaf sheath inoculation technique (in Japanese). Plant Protection, **12**: 339–345.

Tamura, T., Shinzato, N., Ito, M. and Ueno, M. (2019) Microbial secondary metabolite induction of abnormal appressoria formation mediates control of rice blast disease caused by *Magnaporthe oryzae*. Journal of Phytopathology, **167**: 156–162

Ueno, M., Nguyen, T. Q., Shinzato, N. and Matsui, T. (2016). Antifungal activity of collected in subtropical region, Okinawa, against *Magnaporthe oryzae*. Tropical Agriculture and Development, **60**: 48–52.