Development of vermicompost from moso-bamboo, and analysis of its mechanisms of plant-disease suppressiveness

Development of vermicompost from moso-bamboo, and analysis of its mechanisms of plant-disease suppressiveness

（モウソウチク由来ミミズ堆肥の作出とその植物病害抑制機構の解明）

By

XIAODONG YOU

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of a

Doctor of Philosophy

in

Applied Life Sciences

2020

Division of Applied Life Sciences

Graduate School of Life and Environmental Sciences

OSAKA PREFECTURE UNIVERSITY

JAPAN

ACKNOWLEDGEMENTS

I am very thankful to all of those people who gave me the support and guidance throughout my doctoral studies. First, I would like to thank my supervisor, Prof. Motoaki Tojo, who has always been very supportive from the start to the end of my studies. You have been more than my supervisor, not only have taught me how to set up experiments, analyze data, make a PPT for conferences, write proposal for applying for the JSPS Research Fellowship for Young Scientists (DC 2) and more, but also help me for job hunting, attend my wedding ceremony, and act as the guarantor for my mother's visa applications. Without your help and support, sitting here and writing down these words would never have happened. Next, I would like to thank Dr. Koon-hui Wang of Department of Plant and Environmental Protection Sciences University of Hawai'i at Mānoa. When I studied in your lab as an exchange student from April to July 2017, you were always helpful, gave me stimulation suggestions and encouragements. Without your help and support, I would not have finished submitting the paper to Journal of Nematology. I have found myself so lucky to have such a wonderful opportunity to work with you. I am also sincerely thankful to Prof. Tomoo Hosoe and Assist. Prof. Daigo Wakana, Department of Organic Chemistry, Hoshi University, Tokyo, Japan. Without your technical support and valuable suggestions, I would not have learned technical skills like isolation compounds from vermicompost, and identification of the compounds, and submitted the paper to Advance in Microbiology. To my mentor, Prof. Tetsuo Kawakita at the Graduate Program for System-inspired Leaders in Material Science (SiMS) of Osaka Prefecture University, it was a rare opportunity for someone to have such a mentor like you and I was blessed to have you as a research advisor. I am also grateful to Prof. Satoshi T. Ohki and Dr. Tomofumi Mochizuki for the kind advices and all supports during the period of my work. I would like to express my sincere gratitude to Prof. Takashi Inui and Prof. Junpei Takano for your valuable advises and thoughtful criticisims on my

Ph.D. dissertation. I am also grateful to all my student colleagues Teppei Okura, Shinpei Murakami, Han Zhao, Naohiro Kimura, Kazuki Ishikawa, Mouyna Bastien, Joffroy Barraud, Philip Waisen, Josiah Marquez, Shelby Ching, Franciele Carneiro, Basith Cader for your technical assistance and advice. Special thanks are due to Mr. Yukihiro Shimogami of Kawachinagano City Office and members of Eboshi-yama Satoyama Conservation Club for providing me the experiment materials, moso-bamboo powder. I also thank Mr. Raymond Uchida and Mr. Russell Yost for assistance in shipping vermicompost from Japan to Hawaii, and Dr. Janice Uchida, Mrs. Donna Meyer for technical assistance with advice. I would also like to thank the Graduate Program for System-inspired Leaders in Material Science (SiMS) of Osaka Prefecture University by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Japan Society for the Promotion of Science (JSPS) for sponsoring whole of my master and doctoral degree programs. I would also like to thank subsistent financial supports on this research by JA Bank Osaka Industry-University Cooperation Project, and Mr. Yuji Okamura of the president of Geol Cosmetics Co. Ltd., and Mr. Tomoaki Mori of the Geol Cosmetics Co. Ltd. Finally, I want to thank my father, my mother, my wife (Shasha) and my sweet daughter (Yuetong) for their unconditional love and support.

LIST OF ORIGINAL PUBLICATIONS AND PRESENTATIONS

The thesis is based on the following articles, which are referred to, in the text, by their Arabic numerals.

Published papers

(1) **You, X.D.** and Tojo, M. (2020) Temperature dependency of *Pythium* and *Globisporangium* spp. to cause soybean damping-off in Japan. Annual Report of The Kansai Plant Protection Society, 62: (in press).

(2) **You, X.D.,** Wakana, D., Ishikawa, K., Hosoe, T., Tojo, M. (2019) Antifungal Activity of Compounds Isolated from Bamboo vermicompost against *Rhizoctonia solani* AG1-IB. Advances in Microbiology, 9: 957-970.

(3) **You, X.D.,** Barraud, J. and Tojo, M. (2019) Suppressive effects of *Pythium oligandrum* on soybean damping off caused by *P. aphanidermatum* and *P. myriotylum*. Annual Report of The Kansai Plant Protection Society, 61: 9-13.

(4) **You, X.D.,** Zhao, H. and Tojo, M. (2019) First Report of *Pythium irregulare* Causing Damping-off on Soybean in Japan. Plant Disease, 103: 2696-2696.

(5) **You, X.D.,** Kimura, N., Okura, T., Murakami, S., Okano, R., Shimogami, Y., Matsumura, A., Tokumoto, H., Ogata, Y. and Tojo, M. (2019) Suppressive effects of vermicomposted-bamboo powder on cucumber damping-off. JARQ, 53: 13-19.

(6) **You, X.D.,** Tojo, M., Ching, S., and Wang, K.-H. (2018) Effects of vermicompost water extract prepared from bamboo and kudzu against *Meloidogyne incognita* and *Rotylenchulus reniformis*. Journal of Nematology, 50: 569-578.

(7) **You, X.D.** and Tojo, M. (2017) First report of soybean damping off caused by *Pythium aphanidermatum* and *P. coloratum* in Japan. New Disease Report, 36: 14.

(8) **You, X.D.,** Park, J.E., Takase, M., Wada, T. and Tojo, M. (2015) First report of *Pythium aphanidermatum* causing root rot on common ice plant (*Mesembryanthemum crystallinum*). New Disease Report, 32: 36

Conference abstracts

1. **You, X.D.**, Shimogami, Y., Matsumura, A. and Tojo, M. (2015). Suppressive effect of vermi-composted bamboo-powder on *Pythium ultimum* var. *ultimum*. Proceedings of the Australasian Plant Pathology Conference. Page no. 176.

2. **You, X.D.**, Park, J.-E., Takase, M., Wada, T. and Tojo, M. (2015). First Report of Root Rot of Ice Plant (*Mesembryanthemum crystallinum*) Caused by *Pythium aphanidermatum* in a Hydroponic Greenhouse. Kansai Division Meeting of Phytopathological Society of Japan. 82(1):61.

3. Nagashima, S., **You, X.D.** and Tojo, M. (2015). Stem and root rot of *Hydrangea macrophylla* caused by *Pythium intermedium*. Kansai Division Meeting of Phytopathological Society of Japan. 82(1):55.

4. **You, X.D.**, Shimogami, Y., Matsumura, A. and Tojo, M. (2016) Preparation of vermicompost from bamboo powder and kudzu plant mixtures, and its suppressive effect on *Pythium ultimum*. Annual Meeting of Phytopathological Society of Japan. 82(3):215.

5. **You, X.D.**, Shimogami, Y., Matsumura, A. and Tojo, M. (2016) Suppressiveness of vermicompost made from bamboo powder and kudzu plant on soilborne phytopathogens, and its partial mechanisms. Annual Meeting of Phytopathological Society of Japan. 82(3):215.

6. Nagashima, S., **You, X.D.** and Tojo, M. (2017) Occurrences of stem and root rot of *Hydrangea macrophylla* caused by three *Pythium* species in Japan. Annual Meeting of Phytopathological Society of Japan. 83(3):183.

7. **You, X.D.**, Wakana, D., Hosoe, T., Shimogami, Y. and Tojo, M. (2017) Analysis of compounds associating soilborne-phytopathogen suppressiveness of vermicompost made from bamboo powder and kudzu plant. Annual Meeting of Phytopathological Society of Japan. 83(3):201.

8. **You, X.D.**, Wang, K. -H., and Tojo, M. (2017) Nematode suppressive effects of vermicompost

teas prepared from bamboo and kudzu vs that from vegetable food waste, Society of Nematologists Annual Meeting. 49(4):540.

9. **You, X.D.**, Wang, K.-H., and Tojo, M. (2017) Nematode-suppressive effect of vermicompost tea prepared from bamboo and kudzu. Kansai Division Meeting of Phytopathological Society of Japan. 84(1):36.

10. **You, X.D.**, Wang, K. -H., Ching, S., Shimogami, Y. and Tojo, M. (2017) Suppressiveness of vermicompost and its water extractprepared from moso-bamboo and kudzu mixture, against soil-borne plant pathogens and plant-parasitic nematodes. Asian Conference on Plant Pathology.

11. **You, X.D.**, Kimura, N., Okano, R., Shimogami, Y., Tokumoto, H., Ogata, Y., and Tojo, M. (2018) Suppressiveness of cucumber damping-off by vermicomposted bamboo powder and its microbial associations. Annual Meeting of Phytopathological Society of Japan. 84(3): 262.

12. Murakami, S., **You, X.D.** and Tojo, M. (2018) Evaluation of vermicomposted leaf debris from a golf course on suppressiveness of *Pythium* red blight of bentgrass. Annual Meeting of Phytopathological Society of Japan. 84(3): 225.

13. **You, X.D.**, Wang, K. -H., Ching, S. and Tojo, M., Suppressiveness of vermicompost prepared from moso-bamboo and its water extract against soil-borne plant pathogens and plant-parasitic nematodes. Annual Meeting of Chinese Society for Plant Pathology.

14. **You, X.D.**, Barraud, J. and Tojo, M.: Suppressive effects of *Pythium oligandrum* on soybean damping-off caused by *P. aphanidermatum* and *P. myriotylum*. Kansai Division Meeting of Phytopathological Society of Japan. 85(1): 79.

15. **You, X.D.**, Furukawa, H., Matsumura, A., Shimogami, Y., and Tojo, M. (2018) Effects of vermicompost produced from bamboo powder on growth, yield and seed quality of green soybean (*Glycine max* (L.) Merr.) . The 246th Meeting of Crop Science Society of Japan. p. 91.

16. **You, X.D.** and Tojo, M. (2019) First report of *Pythium aphanidermatum*, *P. coloratum*, and *P.*

irregulare causing damping-off on soybean in Japan. Annual Meeting of Phytopathological Society of Japan. 85 (3): 257.

17. **You, X.D.**, Mouyna, B. Y., Murai, H., Mochizuki, T. and Tojo, M. (2019) Suppressive effect of *Bacillus amyloliquefaciens* isolated from bamboo vermicompost against cucumber damping-off. Kansai Division Meeting of Phytopathological Society of Japan.

18. **You, X.D.**, Wang, K.-H., and Tojo, M. (2019) Suppressive effects of vermicompost produced from moso-bamboo and kudzu mixture on plant pathogens. International Conference on Agricultural and Food Science (3rd ICAFS 2019). p. 14.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS…….i

LIST OF ORIGINAL PUBLICATION AND PRESENTATIONS…….....iv

TABLE OF CONTENTS……...vii

INTRODUCTION…….……...1

CHAPTER 1 Production of moso-bamboo vermicompost and evaluation of its suppressive effects on cucumber damping-off

1.1 Introduction…….....5

1.2 Materials and methods…….....6

1.3 Results

1.3.1 Vermicompost maturity assessment using komatsuna-seed germination test……...9

1.3.2 Physical and chemical properties…….….9

1.3.3 Suppressive effects of VB against cucumber damping-off……12

1.3.4 Concentration dependence of VB against cucumber damping-off……..14

1.4 Discussion……...14

CHAPTER 2 Determination of the mechanisms for the suppressive effects of VB against cucumber damping-off pathogens

2.1 Introduction……...16

2.2 Material and methods…….17

2.3 Results

2.3.1 Microbial activity, populations and diversity in VB, aVB and CNM…….....23

2.3.2 Isolation and identification of *Bacillus* and *Flavobacterium* from VB……...25

2.3.3 Inhibition effects of *Flavobacterium* spp. and *Bacillus* spp. on *Rhizoctonia solani* AG1-IB and *Globisporangium ultimum* var. *ultimum* mycelial growth……...25

2.3.4 Suppressive effect of *Flavobacterium* spp. and *Bacillus* spp. on cucumber damping-off caused by *Rhizoctonia solani* AG1-IB and *Globisporangium ultimum* var. *ultimum*…...29

2.3.5 Microbial diversity at different times after vermicomposting and earthworm intestinal....30

2.3.6 Effect of SWE treatment on mycelium growth rate of *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum* and *Rhizoctonia solani* AG1-IB…….....31

2.3.7 Isolation of antifungal compounds from VB…..……...32

2.3.8 Inhibitory effect of ergosterol peroxide (1) isolated from VB on mycelium growth of *Rhizoctonia solani* AG1-IB……37

2.3.9 Inhibitory effect of EtOAc extracts from initial substrate materials of VB on mycelium growth of *Rhizoctonia solani* AG1-IB…….……..38

2.4 Discussion……..38

CHAPTER 3 Effects of vermicompost water extract prepared from moso-bamboo against *Meloidogyne incognita* and *Rotylenchulus reniformis*

3.1 Introduction……....42

3.2 Material and methods…….44

3.3 Results

3.3.1 Nutrient analysis……..49

3.3.2 Hatching experiment……...49

3.3.2 Mobility experiment……....51

3.3.3 Root penetration experiment using split-root assay……....51

3.3.4 Bioindicators of biological activities of vermicompost……...52

3.3.5 Cowpea field experiment…….....52

3.4 Discussion……...55

CHAPTER 4 Suppressive effects of VB combined with *Pythium oligandrum* against plant damping-off caused by *Globisporangium ultimum var. ultimum*

4.1 Introduction……....58

4.2 Materials and methods……59

4.3 Results

4.3.1 The suppressive effects of a combination of VB with *Pythium oligandrum* on soybean damping-off caused by *Globisporangium ultimum* var. *ultimum*…….…..63

4.3.2 The suppressive effects of a combination of VB with *Pythium oligandrum* on cucumber damping-off caused by *Globisporangium ultimum* var. *ultimum*…….64

4.3.3 Effect of VB on density of *Pythium oligandrum* in soil…….....64

4.4 Discussion…….…..65

CHAPTER 5 General discussion and conclusions…….....67

REFERENCES……...70

SUMMARY…….81

SUMMARY IN JAPANESE……..85

Introduction

Vermicomposting is an agricultural recycling process for biodegradable solid waste that is facilitated by the decomposition and digestion of earthworms and associated microorganisms (Elvira et al. 1998). The feedstocks that are commonly used for vermicomposting include animal manure and vegetable or fruit scraps from kitchens or farms (Atiyeh et al. 2000; Garg et al. 2006). Many kinds of vermicompost are known to be used as a component of nursery potting media (Scheuerell et al. 2005) owing to their ability to control plant pathogens such as *Pythium ultimum* Trow var. *ultimum*, *Rhizoctonia solani* Kuhn, and *Verticillium* sp. (Chaoui et al. 2002). Vermicompost water extract is generally at 1:10 or 1:20 dilution ratio of vermicompost to water aerated over a certain period of time, pending on usage, for the purpose of numerous bioactive molecules as well as microbial populations of the vermicompost (Edwards et al. 2006). Applying vermicompost water extract to crops is easier than applying vermicompost, which is bulky and heavier, and needs soil incorporation that makes post-plant treatment impractical in many cases. Many scientists reported that drenching vermicompost water extract suppressed plant-parasitic fungi (Singh et al. 2003; Scheuerell and Mahaffee 2004), or plant-parasitic nematodes such as *Meloidogyne* spp. in different crops (Arancon et al. 2002; Edwards et al. 2007; Mishra et al. 2017). Thus, vermicompost can be used in either solid or liquid.

Rhizoctonia solani and *Pythium* spp. are widespread soilborne pathogens which cause damping–off and root rot on a wide variety of plants (Baker 1970; You et al. 2015; You and Tojo 2017; You et al. 2019a). They do not only cause pre-emergence or post-emergence damping-off of many crop species (Callan et al. 1990), but also they can cause severe root rot on older plants (Larsson 1994). Annual tremendous economic loss was reported by the infection with these pathogens (Abdelzaher 2003; Tanina et al. 2004). They are usually difficult to control, because of their ability to persist under adverse soil conditions, as it contains structures such as sclerotia

or oospores that survive for several years in the absence of host crops and exhibits saprophytic activity, a wide host range, and versatility (Ogoshi 1996). Although fungicide application is effective for suppressing such diseases, frequent use of fungicides can result in the emergence of fungicide-resistance strains, leading to undesirable effects on human health and environmental safety (Becker et al. 1998; Campion et al. 2003). Therefore, alternative strategies for controlling this disease are urgently needed. Owing to its effective disease suppression, vermicompost is thought to be a promising alternative to chemical pesticides. However, vermicompost produced from different feedstocks vary in disease suppressiveness. Szczech & Smolińska (2001), for example, showed that vermicompost produced from manures of cattle, sheep, or horse significantly suppressed *Phytophthora nicotianae* Breda de Haan var. *nicotianae*, but that produced from sewage sludge had no effect against the same pathogen.

Bamboos, especially moso bamboos (*Phyllostachys edulis* (Carrière) J. Houz.), grow vigorously in the vicinity of populated areas in Japan, causing substantial damage to agricultural production and rural ecosystems (Isagi et al. 1997; Imaji et al. 2013; Kajisa et al. 2011). Since moso bamboos are an abundant and sustainable resource throughout the world (FAO 2010), they have the potential to be an ideal feedstock for composting for agricultural use. Considering that bamboo powder has relatively homogeneous qualities, it may generate a vermicompost superior to conventional vermicompost for suppressing plant pathogens. However, as far as we know, vermicomposting of bamboo powder has never been studied, and consequently no information is available on its suppressive effects against plant pathogens.

The bamboo wastes from abandoned forests converted into vermicompost can achieve a win-win result, including bamboo wastes can be reduce and recycled and the resulting vermicompost can be used as a promising alternative to chemical pesticide in suppressing plant pathogens. Based on this aspect, present study aimed to develop the vermicompost from moso-

bamboo, and to clarify its mechanisms on plant-disease suppressiveness. Each of the chapters was intended to followings.

The objective of the **chapter 1** were to produce the vermicompost from bamboo powder and to identify its suppressive effects against the damping-off of cucumber caused by *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum*, and *Rhizoctonia solani* AG1-IB under greenhouse conditions.

Although the suppressive effects of many kinds of vermicompost on plant pathogens have been reported in previous studies, the mechanisms of how vermicompost activity in plant disease suppression are not well-understood (Simsek-Ersahin 2011). The action of microbial antagonists present in vermicompost was considered as an important mechanism for the disease suppression. Several antagonistic bacteria and fungi, such as *Bacillus subtilis*, *Streptomyces* spp., *Trichoderma* sp., and *Aspergillus* sp., were isolated from vermicompost and shown to suppress soil-borne plant pathogens (Mu et al. 2017; Gopalakrishnan et al. 2011; Barocio-Ceja et al. 2013). These microbes are well-known to produce diversity bioactive compounds including antifungal compounds. Release of antifungal compounds by these microbes into the vermicompost may play an important role in inhibiting plant pathogens, which has not been widely examined. Understanding the microbial and chemical factors of how vermicompost suppresses soil-borne plant pathogens may promote effective utilization of the vermicompost. The objectives of the **chapter 2** were to (1) characterize and evaluate the antagonistic bacteria isolated from bamboo vermicompost for their ability to suppress cucumber damping-off caused by *P. aphanidermatum*, *G. ultimum* var. *ultimum*, and *R. solani* AG1-IB and (2) isolate and characterize antifungal compounds against *P. aphanidermatum*, *G. ultimum* var. *ultimum*, and *R. solani* AG1-IB present in the vermicompost.

Disease complex involving plant-parasitic nematodes and soil-borne plant pathogens has been documented. The damping-off disease caused by *R. solani* or *Pythium* spp. became more

severe when plant-parasitic nematodes were present, compared to single infections showing a synergistic interaction between them (Morris et al. 2016; Ahmad et al. 2019). Thus, to control of the nematodes may be also important for controlling damping-off disease caused by *R. solani* or *Pythium* spp. In **chapter 3**, the nematode suppressive effects of vermicompost water extract prepared from moso-bamboo against two common plant-parasitic nematodes, the southern root-knot nematode (*Meloidogyne incognita*) and the reniform nematode (*Rotylenchulus reniformis*) were examined.

Management of plant pathogens by a single approach is not always effective, especially in the field conditions. Recently the integrated disease management (IDM) strategies, combining two or more appropriate techniques, are recommended. *Pythium oligandrum* is an effective biological control agent of damping off and root diseases caused by several soil-borne pathogens, including phytopathogenic *Pythium* species (Martin and Hancock 1987; Hase et al. 2006). It interacts directly with the fungal pathogens through mycoparasitism, antibiosis, nutrient and space competition, and/or indirectly by inducing resistance in the plants (Benhamou et al. 1999; Takenaka et al. 2003). In **chapter 4**, the effects of integrated use of *Pythium oligandrum* with moso-bamboo vermicompost in suppressing damping-off of cucumber and soybean caused by phytopathogenic *Pythium* spp. were evaluated.

CHAPTER 1

Production of moso-bamboo vermicompost and evaluation of its suppressive effects on cucumber damping-off

1.1 INTRODUCTION

Vermicompost, as the final product of vermicomposting, can suppress some soil-borne pathogens (Chaoui et al., 2002) such as *Pythium*, *Rhizoctonia solani*, *Verticillium* (Choui et al, 2002) and *Fusarium* (Szczech, 1999). Previous studies have demonstrated the ability of some earthworm species to consume a wide range of organic wastes such as sewage sludge, animal dung, crop residues and industrial refuse (Mitchell et al. 1980; Chan and Griffiths 1988; Hartenstein and Bisesi 1989; Edwards 1998).

Moso-bamboo (*Phyllostachys edulis* (Carrière) J. Houz.) was considered as an indispensable natural resource for the life worldwide especially in Asia. It was used as a useful material for a variety of beneficial tools, such as building material, basket, musical instruments, and umbrella. Also, bamboo shoot was recognized as important food for their daily lives. In Japan, however, as the rapid economic growth after 1960s, cheap bamboo materials and products are imported from overseas. Meanwhile, most of the products which were used to be made of bamboo were replaced by the plastic products. This has discouraged the landowners from managing their bamboo forests (Tokunaga and Ariki 2007). With other factors such as drain problem on the labor force and aging problem in the countryside, bamboo forest has been abandoned. Today, most of those abandoned forests have become an over-density forest since nobody has taken care of them. Besides, unmanaged bamboo forests are invading vigorously into agricultural lands and other forests (Qian et al. 2012). The increasing bamboo forests may cause some changes in terrestrial water and carbon cycles (Isagi et al. 1997; Onozawa et al. 2009; Komatsu et al. 2010). Thus, it is

important to take some measures to manage the abandoned bamboo forests. Since moso bamboos are an abundant and sustainable resource throughout the world (FAO 2010), they have the potential to be an ideal feedstock for vermicomposting for agricultural use. However, there is no information available on demonstrating the feasibility of vermicomposting of bamboo, and its suppressive effects against plant pathogens.

The objective of the present work was to evaluate the ability of the earthworm *Eisenia foetida* to transform moso-bamboo into vermicompost; and to evaluate the ability of vermicomposting products to suppress cucumber damping-off caused by *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum* and *Rhizoctonia solani* AG1-IB (You et al., 2019b).

1.2 MATERIALS AND METHODS

Vermicomposted bamboo (VB) production

The production scheme of VB is shown in **Fig. 1-1A**. One-year-old shoots of moso-bamboo (*Phyllostachys edulis* (Carrière) J. Houz.) were ground into powder using a grinder and soaked in 10 times the volume of tap water for 24 hours. Water soluble phenolic compounds can be toxic to earthworm (Roberts & Dorough 1984; Abe et al. 2007), and they are considerably contained in bamboo shoots (Chongtham et al. 2011). Therefore, the powder was soaked in water before vermicomposting. Ten kilograms of the water-soaked bamboo powder was mixed with 100 g of pieces of kudzu vine (*Pueraria lobata* (Willd) Ohwi., < 10 cm in length) as a nitrogen source, along with 100 g of earthworms (*Eisenia fetida* Savigny, commercial name ‘Kumataro-futomushi’, Fishing Azumino, Azumino, Japan). Kudzu was chosen as the nitrogen source because it is abundant close to moso-bamboo habits (Kajisa et al. 2011). The earthworms were starved for at least 2 h to evacuate the intestinal residuals in advance. A small amount of a commercial horse

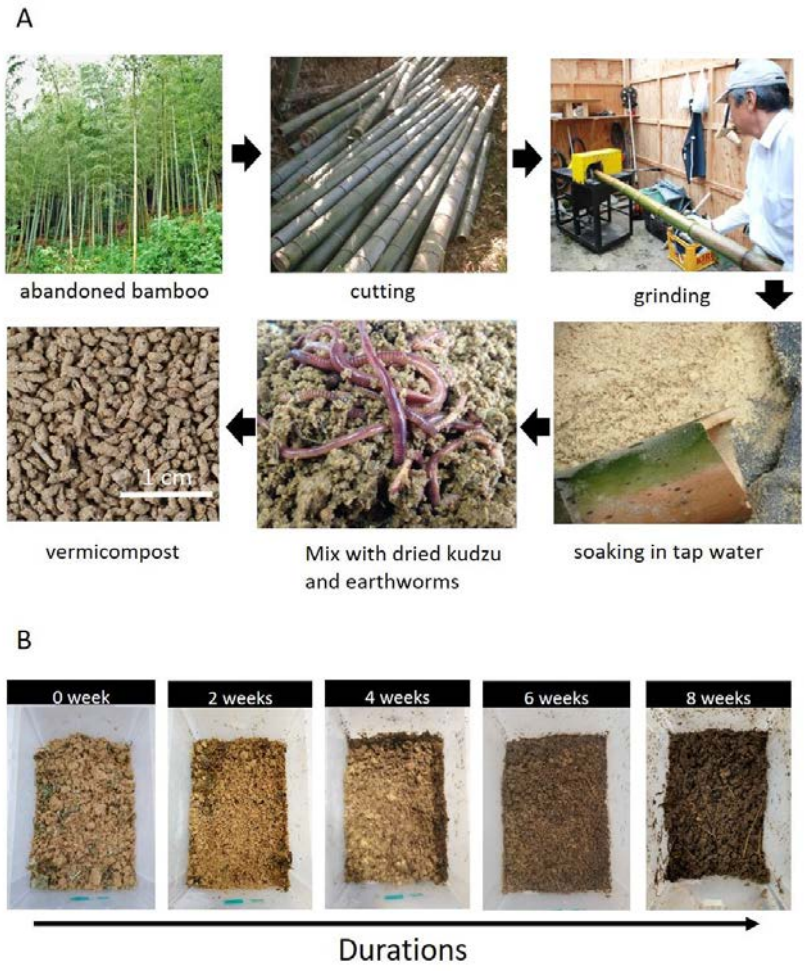

Figure 1-1. (A) The procedure of producing vermicompost from moso-bamboo. (B) Vermicompost at different vermicomposting stages from 0 week to 8 weeks.

manure/wheat straw compost (20 g of dry weight, Iris Ohyama Inc., Sendai, Japan) was added, because it can provide suitable living conditions for earthworms (Huang et al. 2013). The mixture was placed in a plastic box covered with a lid, kept at 28 ± 2°C, and watered once a week to maintain moisture at approximately 80% (w/w). Samples were collected every 2 weeks after starting vermicomposting for vermicompost maturity assessment using komatsuna-seed germination test as described previously (Hase and Kawamura 2012). After 8 weeks' incubation, earthworms and their eggs were removed manually and via a sieve (4 mm mesh), and the mixture was divided into two subsamples. One was used for physical and chemical properties analysis,

and the other was used for the evaluation of cucumber damping-off suppressiveness as described later. Prior to the analysis of physicochemical and chemical properties, the samples were dried at 70°C for 2 days. NH_4^+, NO_3^-, PO_4^{3-} and K^+ were detected using an RQ-flex reflect meter (Reflectoquant ammonium test, nitrate test, phosphate test and potassium test; Merck KGaA, Darmstadt, Germany). Ca^{2+} and pH were tested using handheld meters (LAQUA twin series, model B-751 for Ca^{2+}, and B-71X for pH, Horiba Ltd., Kyoto, Japan). Mg^{2+} was measured using portable photometers (HI96752, Hanna Instruments, Woonsocket, RI, USA). The autoclaved VB (aVB) (autoclaving at 121°C for 1h, and kept in a laboratory room for 1 day), and a commercial nursery medium (CNM, Aisai-1, Katakura Chikkarin Co., Ltd., Tsuchiura, Japan), were used as comparisons. The microstructure of dried vermicompost was determined by scanning electron microscope (SU-1510; Hitachi HighTechnologies Corp., Tokyo, Japan). The dried bamboo powder was used as a control.

Suppressive effects of VB against cucumber damping-off

The suppressive effects of VB on cucumber damping-off was evaluated under greenhouse conditions, using aVB and CNM as controls. *Pythium aphanidermatum* isolate MAFF245234, *Globisporangium ultimum* var. *ultimum* isolate MAFF240023, and *Rhizoctonia solani* AG1-IB isolate SLS1 (= MAFF244980) were used. Each isolate was cultured on autoclaved bentgrass seeds at 25°C in darkness for one week as described by Tojo et al. (1993). One gram of the culture of each isolate was suspended in 20 ml or 200 ml of 0.35% water agar and used as inoculum. Seven-day-old cucumber (*Cucumis sativus* L., 'Aonagakei-jibai'), seedlings grown on the CNM were transplanted to plastic pots each containing VB, aVB and CNM. Each seedling received 1 ml of inoculum at the plant base (avoiding direct contact of the inoculum on the plants), and was irrigated daily with tap water via hand irrigation. Uninoculated (pathogen-free) treatments

included uninoculated VB, uninoculated aVB, and uninoculated CNM. The number of plants showing damping-off were recorded 0, 5, 10, and 15 days after the inoculation. In all experiments, replicates of each treatment were arranged in a randomized block, with three replications for each treatment. Each treatment per replication consisted of 30 cucumber seedlings. The temperature of the greenhouse was maintained in the range of 23-33°C for the experimental period.

Concentration dependence of VB against cucumber damping-off

The experiment was conducted under greenhouse conditions as the same method as above. Five treatments were tested and the treatments consisted of CNM (Soil control), VB25 (VB substitution at 25% v/v), VB50 (VB substitution at 50% v/v), VB75 (VB substitution at 75% v/v), VB. *G. ultimum var. ultimum* isolate MAFF240023 was used.

1.3 RESULTS

1.3.1 Vermicompost maturity assessment using komatsuna-seed germination test

The plant germination and root elongation were found to increase gradually in response to vermicomposting duration from 2 weeks to 8 weeks (**Fig. 1-2A**). The vermicompost after 8 weeks' incubation showed the highest germination index, approximately 100% (**Fig. 1-2B**).

1.3.2 Physical and chemical properties

VB exhibited a distinct physical appearance as compared with the bamboo powder after gut transit process, as evidenced from bumpy surface obtained (**Fig. 1-3**). VB contained lower concentrations of NO^{3-}, PO_4^{3-} and Ca^{2+}, but similar Mg^{2+} levels, and higher NH^{4+} and, K^+ levels than CNM. In comparison with aVB, VB contained lower concentrations of Mg^{2+} and Ca^{2+}, but similar levels of NO^{3-}, PO_4^{3-}, and K^+, and higher levels of NH_4^+. Both VB and aVB had higher

pH levels than CNM (**Table 1-1**). Because of the lower concentrations of total nitrogen and phosphorus in VB than in CNM, leaf yellowing and slightly poorer growth were observed in the seedlings grown in VB (**Fig. 1-4B**).

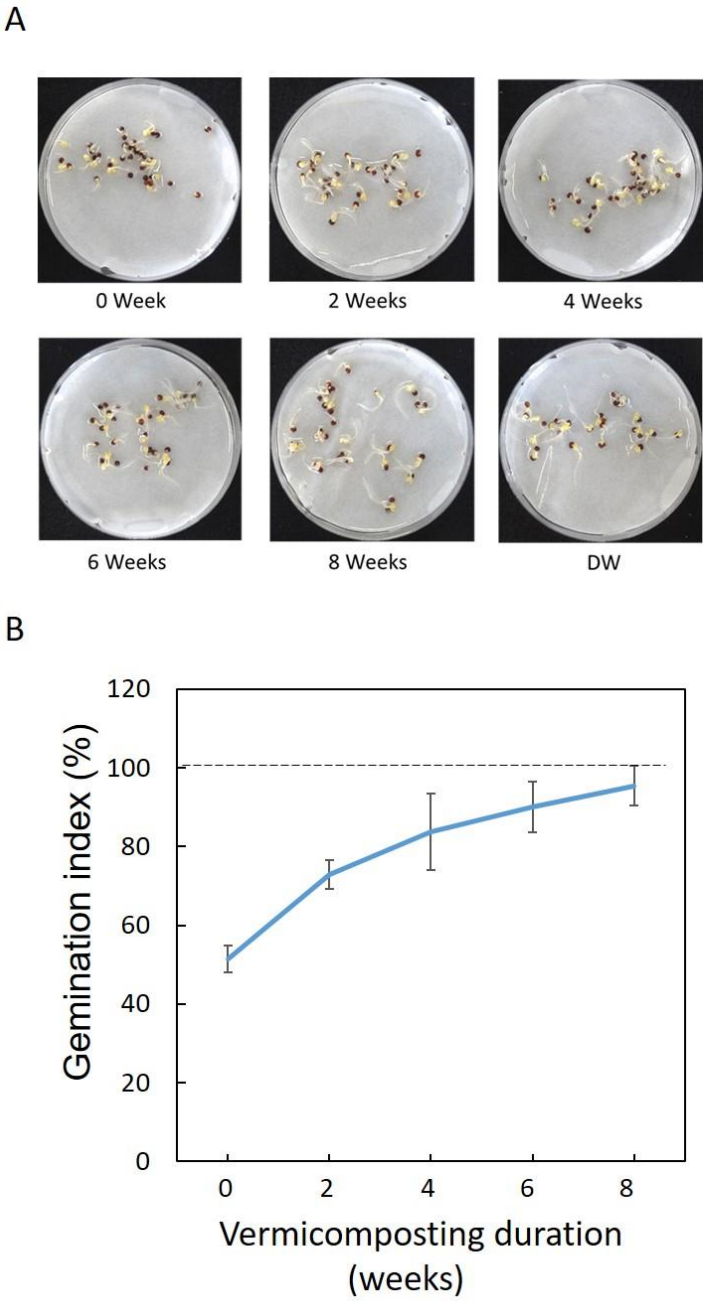

Figure 1-2. (A) The germinating seeds in each vermicompost water extracts and distilled water as a control. (B) Change of germination index in each vermicompost water extracts during the maturing period of manure vermicomposting.

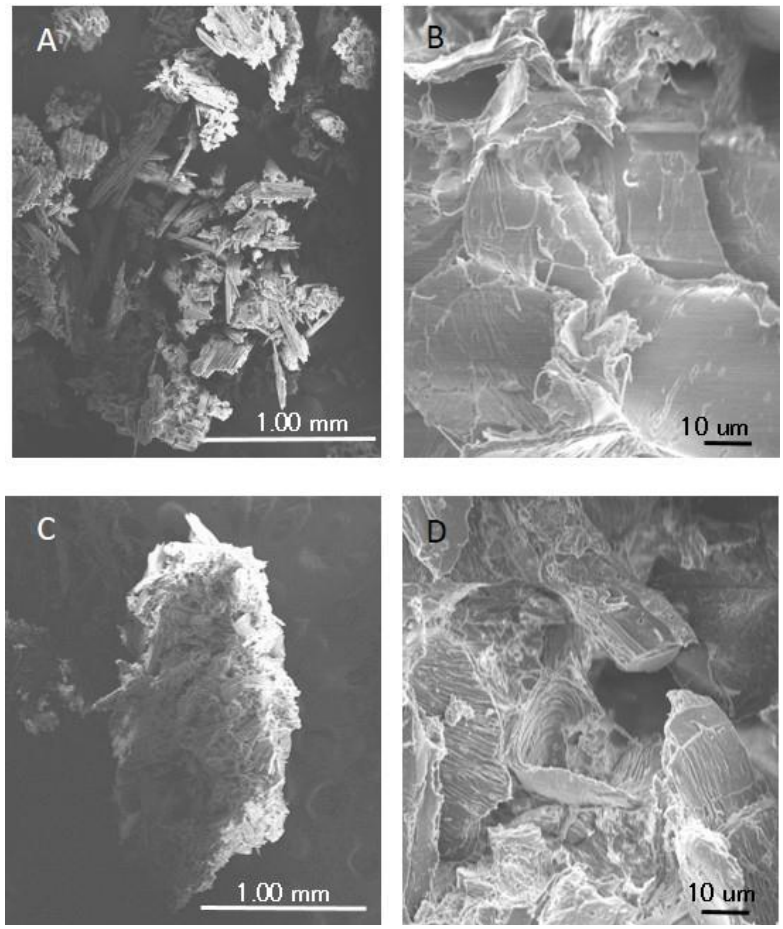

Fig. 1-3. The scanning electron micrographs. (A), (B) bamboo powder, (C), (D) vermicomposted moso-bamboo.

Table 1-1. Comparison of chemical properties of vermicomposted moso-bamboo (VB), the autoclaved VB (aVB), and a commercial nursery medium (CNM).

Parameters	VB	aVB	CNM
NO_3^- (mg/g)	0.6±0.12 b	0.3±0.05 b	5.5±0.45 a
NH_4^+ (mg/g)	0.7±0.08 a	0.2±0.00 b	0.2±0.00 b
PO_4^{3-} (mg/g)	2.7±0.14 b	3.3±0.27 ab	4.6±0.48 a
K^+ (mg/g)	34.0±2.66 a	36.8±0.27a	0.8±0.07 b
Mg^{2+} (mg/kg)	6.6±0.80 b	12.2±0.67 a	7.3±0.29 b
Ca^{2+} (mg/kg)	111.0±6.06 c	420.0±4.47 a	282.0±11.88 b
pH	7.8±0.20 a	7.7±0.01 a	5.6±0.03 b

Data are mean ± standard error (N = 3). Different letters indicate significant difference according to Tukey's HSD test (P < 0.05).

1.3.3. Suppressive effects of VB against cucumber damping-off

The damping-off caused by isolates of *P. aphanidermatum*, *G. ultimum* var. *ultimum*, and *R. solani* AG1-IB, was significantly ($P < 0.05$) suppressed by VB as compared to CNM (**Fig. 1-4A**). After 15 days of inoculation, the damping-off severities of the three pathogens were reduced by 41%, 26%, and 47% in VB, as compared to CNM, respectively (**Fig. 1-5**). The disease suppressiveness appeared from 5 and 10 days after the inoculation (**Fig. 1-5**). This effect was lost when VB was autoclaved. All pathogens were re-isolated from the diseased seedlings by *Pythium* selective NARM medium (Morita & Tojo 2007), for the isolates of *P. aphanidermatum* and *G. ultimum* var. *ultimum* or 1.5% water agar for the *R. solani* AG1-IB isolate.

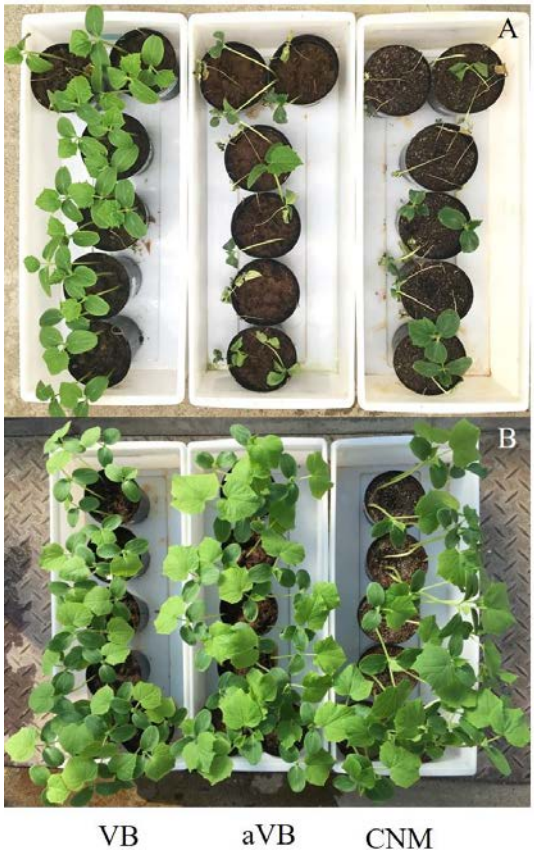

Figure 1-4. Suppressive effects of VB on damping-off of cucumber caused by *Pythium aphanidermatum* under greenhouse conditions. (A) Damping-off appeared in VB five days after inoculation and transplanting. Autoclaved VB (aVB) and CNM were used for comparison. (B) The cucumber seedlings' growth in the noninoculated controls including VB, aVB, and CNM in 10 days after transplanting.

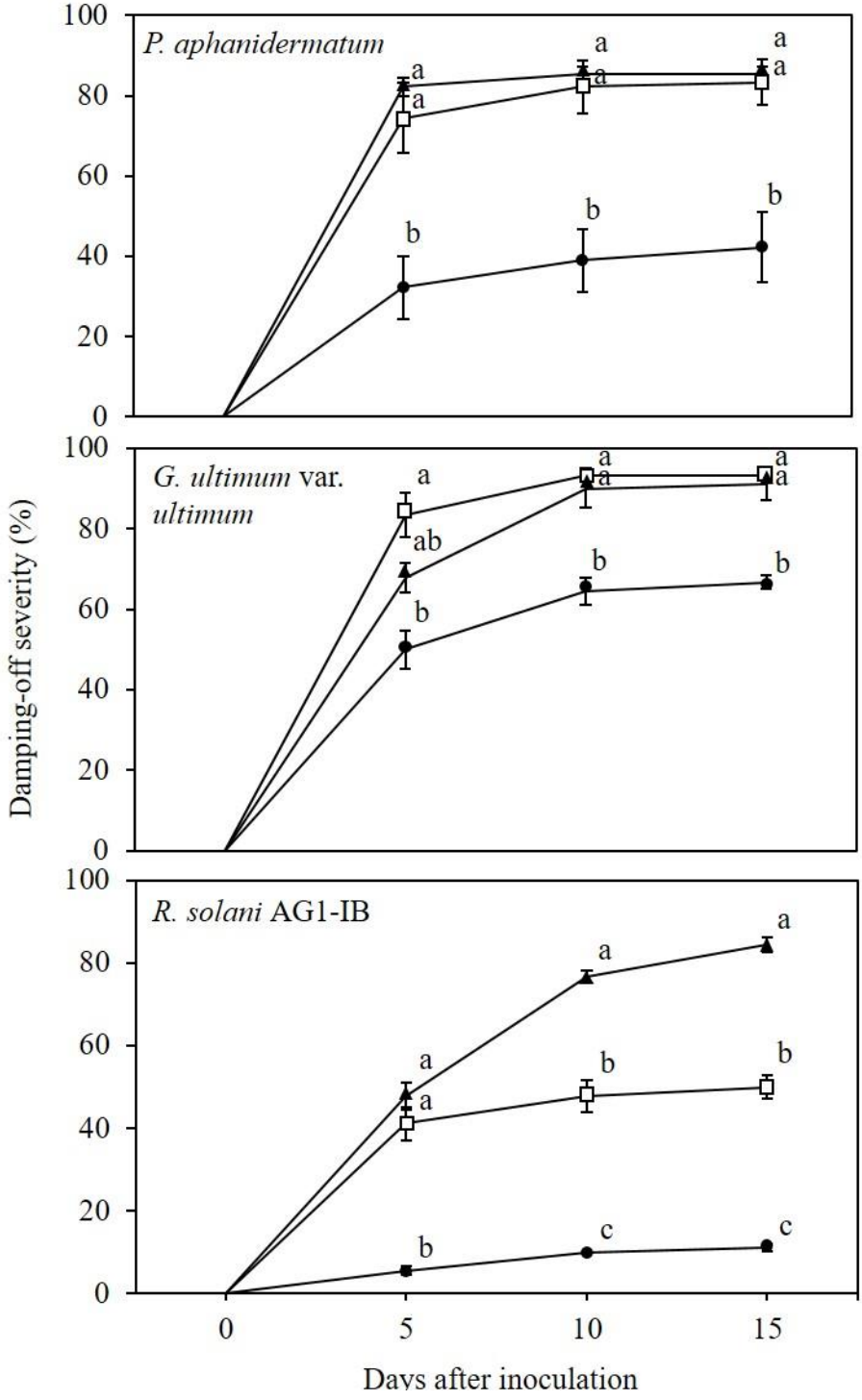

Figure 1-5. The effect of VB on the damping-off severity of cucumber, caused by *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum*, and *Rhizoctonia solani* AG1-IB under greenhouse conditions. Autoclaved VB (aVB) and CNM were used for comparison. VB (●), aVB (▲), and CNM (□). Bars indicate standard error ($N = 3$). Treatments with different letters indicate a significant difference at the 0.05 level, according to Tukey's test ($P < 0.05$).

1.3.4. Concentration dependence of VB against cucumber damping-off

The suppressive effect of bamboo vermicompost against cucumber damping-off caused by *G. ultimum* var. *ultimum* was found to decrease gradually in response to decreasing concentrations of bamboo vermicompost (**Fig. 1-6**).

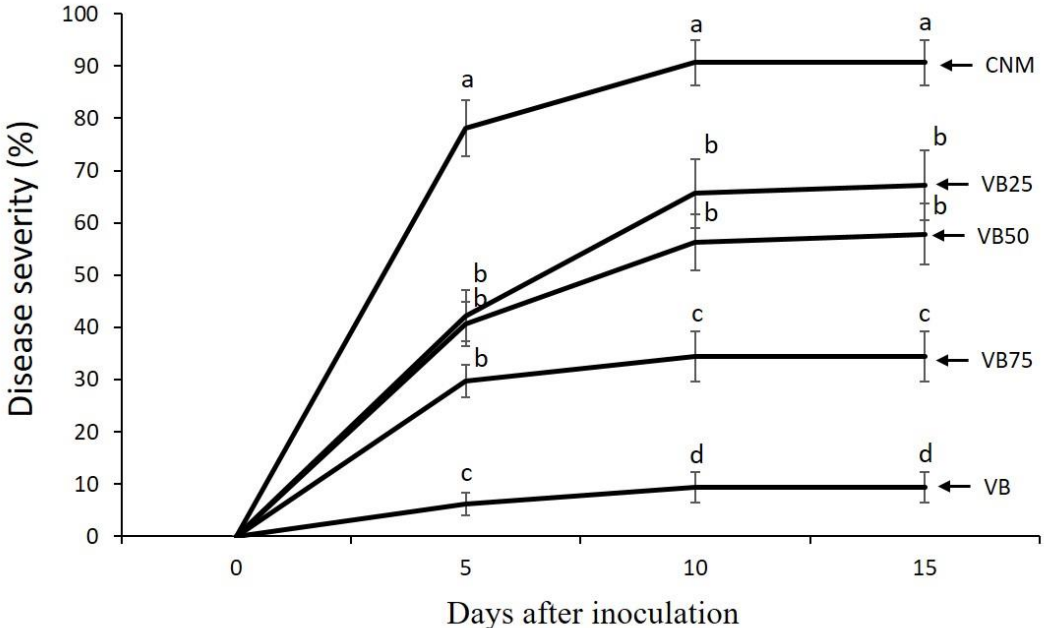

Figure 1-6. The effect of VB at different concentrations on the damping-off severity of cucumber, caused by *Globisporangium ultimum* var. *ultimum* under greenhouse conditions. Five treatments were tested, including CNM (Soil control), VB25 (VB substitution at 25% v/v), VB50 (VB substitution at 50% v/v), VB75 (VB substitution at 75% v/v), and VB. Bars indicate standard error (N = 3). Treatments with different letters indicate a significant difference at the 0.05 level, according to Tukey's test ($P < 0.05$).

1.4 DISCUSSION

In this study, we demonstrated that the vermicompost produced from moso bamboos had potential to suppress cucumber damping-off pathogens, including *P. aphanidermatum*, *G. ultimum* var. *ultimum*, and *R. solani* AG1-IB, under greenhouse conditions. The suppressiveness of VB on these pathogens is thought to be mainly due to living microorganisms, because the effect was nullified by the autoclave treatment. The highest ammonia concentration is probably also one of

the factors of the suppression of VB to *Pythium*, because the suppression of *Pythium* damping-off is related to ammonia volatilization of composts (Scheuerell et al. 2005). As far as we are concerned, this is the first report showing the suppressive effects of VB on plant diseases. The suppressive effects of VB were found concentration dependency. It agreed with a previous study which showed that substituting the soil with different amounts of vermicompost resulted in a significant reduction in chickpea mortality, which increased progressively in response to the increase in substitution concentration (Sahni et al. 2008)

The present results also demonstrated that VB had more valuable characteristics with respect to equivalent or higher inorganic nutrients than CNM did. The surface area of bamboo powder was increased after vermicomposting, which can offer more sites for microbial activities. The observations agreed with the previous results that the appearance of humic acid fractions in substrate was shifted to more closed-grained and lumpy after vermicomposting (Li et al., 2011). Leaf yellowing and slightly poorer growth were observed in the seedlings grown in VB, as compared with those grown in CNM. However, the amendments of organic fertilizers, such as oil cakes, resolved these problems without resulting in a loss of disease suppressiveness of VB (You unpublished). The pH of the VB was 7.8 ± 0.20, which is higher than that of CNM but is an acceptable range for cucumber growth (Old Farmer's Almanac 2016).

Moso-bamboo grows vigorously in the vicinity of populated areas in Japan and causes substantial damage to agricultural production and rural ecosystems (Isagi et al. 1997, Imaji et al. 2013, Kajisa et al. 2011). The resent results suggested that the moso-bamboo waste can be recycled as an agricultural material through its powdering and vermicomposting.

CHAPTER 2

Determination of the mechanisms for the suppressive effects of VB against cucumber damping-off pathogens

2.1 INTRODUCTION

Vermicompost is a composting product of accelerated biodegradation of organic matter by earthworms and their associated microbes through non-thermophilic decomposition. Recently several studies have focused on the potential of vermicompost to suppress soil-borne plant pathogens including *Pythium* spp., *Globisporangium* spp. and *Rhizoctonia solani* (Chaoui et al. 2002; Ersahin et al. 2009). The activities of microbial antagonists present in vermicompost are considered as an important mechanism for disease suppression. Several antagonistic bacteria and fungi, such as *Bacillus subtilis*, *Streptomyces* spp., *Trichoderma* sp., and *Aspergillus* sp., were isolated from vermicompost and shown to suppress soil-borne plant pathogens (Mu et al. 2017; Gopalakrishnan et al. 2011; Barocio-Ceja et al. 2013). Recently, sequencing technologies and bioinformatic tools have become widely available. Analysis of 16S ribosomal DNA (rDNA) has been used to determine the microbial communities in various samples including anaerobic sludge (Riviere et al. 2009), dairy farm or natural environment (Mulder et al. 2005, Øvreås et al. 2000, Pandey et al. 2018), and vermicompost (Cai et al. 2018). Although some studies have investigated the bacterial and/or fungal communities changes during the vermicomposting processes (Cai et al. 2018; Chen et al. 2015), the specific microbes responsible for the plant pathogen suppression are not completely clear.

The antagonistic bacteria and fungi isolated from vermicompost are well-known to produce diversity bioactive compounds including antifungal compounds. Release of antifungal compounds by these microbes into the vermicompost may play an important role in inhibiting

plant pathogens, which has not been widely examined. Understanding the microbial and chemical factors of how vermicompost suppresses soil-borne plant pathogens may promote effective utilization of the vermicompost.

In the present study, we first identified the predominant bacteria in the vermicompost by the Illumina MiSeq sequencing of 16S rRNA gene amplicons. The identified bacteria were then isolated and assessed for their ability to suppress cucumber damping-off pathogens by culture-dependent measures to elucidate the importance of the specific bacteria in cucumber damping-off suppression. We further investigated whether antifungal compounds present in the vermicompost contribute to the suppression of cucumber damping-off pathogens (You et al. 2019c).

2.2 MATERIALS AND METHODS

Microbial activity, populations and diversity in VB

Microbial activity in VB, aVB and CNM was estimated using the rate of hydrolysis of fluorescein diacetate (FDA) as described by Adam & Duncan (2001) with a slight modification. Briefly, 2 grams of samples (fresh weight; $N = 4$) were put in 50 mL centrifuge tubes and mixed with 15 mL of potassium phosphate buffer (60 mM, pH 7.6). After 20 min of incubation, 15 mL chloroform/methanol (2:1 v/v) was added to terminate the reaction. The absorbance (490 nm) of the filtered solutions was determined spectrophotometrically. The number of bacteria and fungi in VB and CNM were determined using the plate count technique employing selective media as described by Waksman & Fred (1922) and Martin (1950), respectively. The bacterial communities of VB, aVB and CNM were further examined using next generation sequencing. VB, aVB and CNM were collected for three samples respectively, with each sample corresponding to 1 L of organic matter. After the samples were homogenized by manual mixing, 4 grams of the subsample was subjected to DNA extraction by MORA-EXTRACT (Kyokutoseiyaku Co. Ltd., Tokyo,

Japan). The bacterial V3-V4 region of the 16S rDNA of each sample was amplified by PCR as described by Nishioka et al. (2019). The PCR products were sequenced using the Illumina MiSeq platform, before bacterial communities of each sample were identified by MagicSuite.

Isolation and identification of *Bacillus* and *Flavobacterium* spp. from VB

The *Bacillus* and *Flavobacterium* spp. were isolated according to the method as described by Mu et al. (2017) using *Flavobacterium* selective culture medium (Nishioka et al. 2016) and Luria-Bertani Agar (LBA), respectively. The 16S rRNA gene of the bacterium was amplified and sequenced with bacterial universal primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) for *Flavobacterium* spp. (Lane, 1991). The partial sequence of DNA gyrase subunit B gene (gyrB) was also amplified by the primer set gyrB-F (5´-TTGRCGGHRGYGGHTATAAAGT-3´) and gyrB-R (5´-TCCDCCSTC AGARTCWCCCTC-3´) for *Bacillus* spp. (Abdallah et al. 2018). The 16S rRNA gene and gyrB gene sequences were aligned and compared with similar sequences from GenBank database using the DNADynamo software. The phylogenetic tree was built by the neighbor joining method using the MEGA 6. Biochemical characteristics were determined using the bioMerieuxTM APITM system (Bio-Mérieux, Marcy L'Etoile, France) with API 50CHB strips that are usually used to identify genus *Bacillus* strains following the manufacturer's instructions. The analyses were performed using Apiweb software (version 4.0; bioMérieux).

Inhibition effects of *Flavobacterium* spp. and *Bacillus* spp. on *Rhizoctonia solani* AG1-IB and *Globisporangium ultimum* var. *ultimum* mycelial growth

A mycelial plug of *R. solani* AG1-IB or *G. ultimum* var. *ultimum* was cut from the colony margin by a 0.4 cm diameter cork borer and placed on one edge 1 cm apart from the corner of PDA plate,

and then each bacterial isolate was streaked on the other edge 1 cm apart from of plate. After 4 days incubation at 28℃ in darkness, percent inhibition of mycelial growth of the pathogens and clear zone caused by bacterial isolates were recorded. A PDA plate without any bacteria antagonist was also used as a control.

Suppressive effect of *Flavobacterium* spp. and *Bacillus* spp. on cucumber damping-off caused by *Rhizoctonia solani* AG1-IB and *Globisporangium ultimum* var. *ultimum*

Bacillus thuringiensis YM1, *B. amyloliquefaciens* YM3, *B. pumilus* YM4, and *Flavobacterium akiainvivens* FL1 were prepared on LBA and transferred to LB after one day. The suspension was shaken at 160 rpm in a shaker for 48 h at 28℃ and concentration adjusted to 1.5×10^{8} CFU/ml. *R. solani* AG1-IB was cultured on autoclaved bentgrass seeds at 28℃ in the dark for one week as described by Tojo et al. (1993). One gram of the culture was suspended in 100 ml of 0.35% water agar and used as an inoculum. Eight cucumber (*Cucumis sativus* L., "Aonagakei-jibai") seeds were sown on each pot containing commercial nursery soil (Takii-ikubyoubaido; Takii Seed Co., Ltd., Kyoto, Japan), and thinned to 6 seedlings after germinated. Seven days after sowing, each pot received 25 ml of each bacterial suspension. After 3 days, each pot received 3 ml of the inoculum at the plant base (avoiding direct contact of the inoculum on the seedlings) and was irrigated daily with tap water via hand irrigation. The pots received the inoculum without adding bacterial suspension were used as controls. In order to determine the effect of each bacterial suspension on plant, uninoculated (pathogen-free) treatments were also prepared. Each treatment per replication consisted of 5 pots and repeated 3 times. The experiment was conducted in an incubator at 28℃ (12h daytime/12h nighttime).

Microbial diversity at different times after vermicomposting and earthworm intestinal

Samples of vermicompost were collected on weeks 0, 2, 4, 6, and 8 and stored at -20˚C to analyze the microbial community composition during the compost process. The microbial DNA of vermicompost samples and earthworm intestinal extraction was conducted as above.

Preparation of sterilized water extract of VB (SWE)

The water extracts of VB were prepared by mixing the samples with distilled water at a ratio of 2:3 (v/v) followed by active aeration with a small air pump at 20℃ for 24 h according to a previous study (Ebrahimi et al. 2018) with some modifications. The supernatant of the water was filtered through a filter paper (Whatman No.1, 9 cm in diameter, GE Healthcare UK Ltd., Little Chalfont, UK) to obtain water extract of VB, followed by filter-sterilization through a 0.22 μm cellulose acetate filter (Corning Inc., Corning, NY, USA) to obtain sterilized water extracts of VB (SWE).

Effect of SWE treatment on mycelium growth rate of *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum* and *R. solani* AG1-IB

One milliliter of SWE was pipetted onto a PDA plate and expanded over the entire surface with a sterilized glass rod according to a previous study (Ebrahimi et al. 2018) with some modifications. A 5 mm-diameter agar plug with 7-day-old mycelium of each pathogen was placed on the center of the PDA plate and incubated in the dark at 25°C. Sterilized distilled water was used as a control. Mycelium growth was evaluated by visual measurement of the colony radius at 24 h intervals for 2 days. The experiments were repeated five times.

Isolation of antifungal compounds from VB

The separation scheme of the antifungal compounds from VB is shown in **Fig. 2-1**. Ten liters of

VB was extracted with acetone, and the organic layer was evaporated. The resulting extracts were suspended in H_2O and extracted with EtOAc, and then the organic layer was evaporated *in*

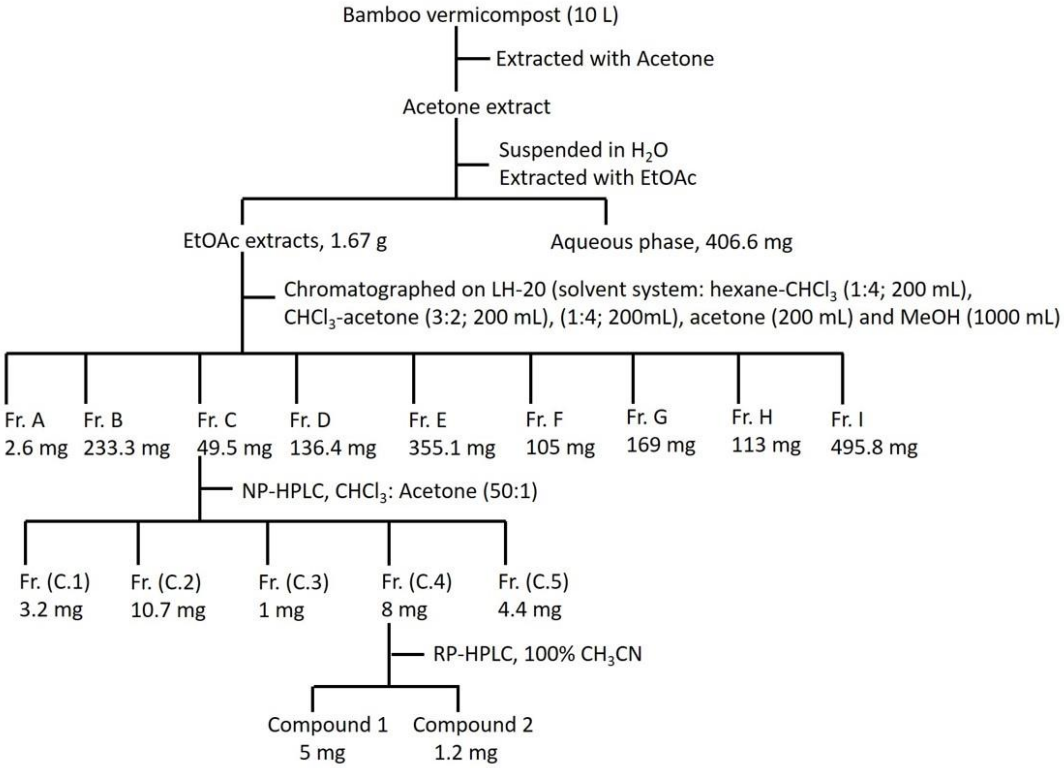

Figure 2-1. Separation scheme of compounds from vermicomposted moso-bamboo (VB).

vacuo for further analysis. The EtOAc extracts (1.67 g) were used to determine their antifungal activities against *R. solani* AG1-IB by the paper disc diffusion method (Nguyen et al., 2009). The EtOAc extracts were then chromatographed on Sephadex LH-20 (GE Healthcare Bio-Sciences, Uppsala, Sweden; solvent system: hexane-$CHCl_3$ (1:4; 200 mL), $CHCl_3$-acetone (3:2; 200 mL), (1:4; 200 mL), acetone (200 mL) and MeOH (1000 mL) to collect nine fractions: 2.6 mg for Fr. A, 233.3 mg for Fr. B, 57 mg for Fr. C, 136.4 mg for Fr. D 355.1 mg for Fr. E, 105 mg for Fr. F, 169 mg for Fr. G, 113 mg for Fr. H, 495.8 mg for Fr. I. The nine fractions were used to determinate the antifungal activities of these samples against *R. solani* AG1-IB by paper disc diffusion method (Nguyen et al., 2009).

The fraction (C) showing two major peaks was further isolated by normal-phase-HPLC with $CHCl_3$: acetone to produce five fractions (Fr. C.1-C.5). The fraction (C.4, 8 mg) which contained the two major peaks was further purified by HPLC on octadecylsilyl (ODS) and eluted with 100% CH_3CN to obtain compounds **1** (5.0 mg) and **2** (1.2 mg). Their chemical structures and mass spectra were determined by NMR and mass spectrometry as described previously (Ishikawa et al. 2019).

Inhibitory effect of ergosterol peroxide (1) isolated from VB on mycelium growth of *Rhizoctonia solani* AG1-IB

Antifungal activity of ergosterol peroxide (**1**) against *R. solani* AG1-IB was tested as described previously (Vinale et al. 2009)) with some modifications. Briefly, pathogen plugs (5-mm diameter) from the growing edges of colonies were placed at the center of Petri dishes containing PDA. Samples of 10 μL of the compound at concentrations of 150, 300, 600, and 900 μg $plug^{-1}$ were applied on the top of each plug. The negative controls were obtained by applying 10 μL of solvent (ethyl acetate) alone. The solvent was evaporated in a laminar flow cabinet, and the plates were incubated at 25°C for 48 h. Pathogen growth was measured daily as the colony diameter (mm). Each treatment consisted of three replicates. The percentage of inhibition was calculated based on the following formula: Relative efficacy of inhibition (%) = (mycelial growth of the control-mycelial growth of the treatment) / mycelial growth of the control × 100.

Inhibitory effect of EtOAc extracts from initial substrate materials of VB on mycelium growth of *Rhizoctonia solani* AG1-IB

The initial substrate materials of VB contains bamboo shoots powder and kudzu vine pieces. In order to determine whether the antifungal compounds presented in VB were originally in the

initial substrate materials or not. The bamboo shoots powder or kudzu vine pieces were extracted in 100% methanol at a ratio of 1:2 (v/v, dry plant material/solvent), then the organic layer was evaporated. The resultant extracts were suspended in H_2O and extracted with EtOAc, and then the organic layer was evaporated in vacuo. VB was used as a positive control. The EtOAc extracts used to determinate their antifungal activities against *R. solani* AG1-IB by paper disc diffusion method (Nguyen et al., 2009).

Data Analysis

Data are represented in terms of the mean ± standard error. Comparison between the effect of treatment with SWE and sterilized distilled water on *R. solani* AG1-IB mycelium growth was carried out according to Student's *t*-test ($P < 0.05$). The other data were evaluated by Tukey's HSD test ($P < 0.05$) using with IBM SPSS Statistics 25 (IBM Corp., Armonk, NY, USA).

2.3. RESULTS

2.3.1 Microbial activity, populations and diversity in VB, aVB and CNM

Microbial activity and populations estimated by FDA hydrolysis and the soil plating technique were significantly ($P < 0.05$) higher in VB than in aVB and CNM (**Table 2-1**). The NGS analysis revealed that among the predominant bacterial genera (relative abundance of more than 1.0%), *Flavobacterium* and *Luteolibacter* were only presented in VB, but not in aVB and CNM (**Table 2-2**). Pathma and Sakthivel (2013) showed that among vermicompost bacteria that exhibit antagonistic and biofertilizing potential isolated from straw and goat manure based vermicompost, genus *Bacillus* was predominant bacteria. Mu et al. (2017) showed that *Bacillus subtilis* isolated from cow dung based vermicompost exhibited antifungal ability against ten plant pathogenic fungi by producing antifungal volatiles. In the present study, the relative abundance of genus

Bacillus was significantly abundant in VB than in aVB and CNM (**Table 2-2**).

Table 2-1. Comparison of the activity and population of microbes between the vermicomposted moso-bamboo (VB), autoclaved VB (aVB) and a commercial nursery medium (CNM).

	VB	aVB	CNM
FDA hydrolysis (μg/0.5 h)	8.2 a	4.9 b	1.7 c
Bacteria (cfu)	6.2×10^{10} a	—	1.5×10^{9} b
Fungi (cfu)	2.7×10^{6} a	—	4.0×10^{4} b

Means (N = 4) with different letters within a row indicate significant difference ($P < 0.05$) based on Tukey's HSD test for FDA hydrolysis and Student's *t*-test for the number of bacteria and fungi. The number of bacteria and fungi in VB and CNM were determined by a plate count technique using media described by Waksman & Fred (1922) and Martin (1950), respectively. Microbial activity was measured by FDA hydrolysis described by Adam & Duncan (2001). "—" no data.

Table 2-2. Relative abundances of genera comprising more than 1.0% of genera in VB, aVB and CNM.

Genus	VB	aVB	CNM
Terracidiphilus	0	0	1.67725
Cellulomonas	4.533919	4.899962	0.302168
Demequina	1.766715	0	0.026275
Actinocatenispora	0	0	6.367418
Nocardioides	0.151293	0	11.00066
Streptomyces	0.014641	0	36.79877
Conexibacter	0.082967	0	1.199912
Flavobacterium	1.825281	0	0
Lactobacillus	4.88E-03	18.41468	0
Leuconostoc	0.014641	50.02549	0
Bradyrhizobium	1.268912	0	0.240858
Mesorhizobium	0.707662	0	2.220276
Rhizobium	2.118106	0	0.34596
Altererythrobacter	1.849683	0	0.035034
Sphingobium	1.283553	0	0.218962
Escherichia	0	1.937046	0
Kluyvera	0.541728	5.454314	0.249617
Brenneria	0	2.899197	0
Rahnella	0.039043	6.779661	0
Pseudomonas	1.634944	1.529247	0.026275
Pseudoxanthomonas	1.527574	0	0.078826
Rhodanobacter	0	0	2.119553
Stenotrophomonas	0.883358	7.149229	0.070068
Luteolibacter	2.284041	0	0
Bacillus	0.4539	0	0.1314

2.3.2 Isolation and identification of *Bacillus* and *Flavobacterium* from VB

A total of 10 isolates of *Bacillus* and 1 isolate of *Flavobacterium* were obtained. A phylogenetic tree using gyrB gene sequences was constructed, YM1 and YM2 were grouped together with *B. thuringiensis* and *B. cereus*, YM3 was grouped together with *B. amyloliquefaciens*, and YM4~YM10 were grouped together with *B. pumilus* (**Fig. 2-1A**). A phylogenetic tree using 16S rRNA gene sequences was constructed, FL1 was grouped together with *Flavobacterium akiainvivens* (**Fig. 2-1B**). The biochemical properties of YM1 and YM2 showed 94.9% and 94.7% consistency with that of *B. thuringiensis*, respectively, in the database of the Apiweb software (bioMérieux). The biochemical properties of YM3 showed 99.2% consistency with that of *B. amyloliquefaciens* in the database of the Apiweb software (bioMérieux). The biochemical properties of YM4~YM10 showed 99.9% consistency with that of *B. pumilus* in the database of the Apiweb software (bioMérieux). Based on the consistency between the results of the gyrB and 16s rRNA gene sequences analysis and the biochemical characterization, these strains of *Bacillus* and *Flavobacterium* spp. isolated from VB were identified as *B. amyloliquefaciens*, *B. pumilus*, *B. thuringiensis* and *Flavobacterium akiainvivens*, respectively.

2.3.3 Inhibition effects of *Flavobacterium* spp. and *Bacillus* spp. on *Rhizoctonia solani* AG1-IB and *Globisporangium ultimum* var. *ultimum* mycelial growth

In this study, all the 11 bacterial isolates were tested for antagonistic effects *in vitro* against *R. solani* AG1-IB and *G. ultimum* var. *ultimum*. Antagonistic effects were confirmed by the formation of inhibition zones between the bacteria isolates and the fungal isolates. All 11 bacterial isolates had antagonistic effects on mycelial growth of *R. solani* AG1-IB and *G. ultimum* var. *ultimum*. Among them, *B. amyloliquefaciens* YM3 showed the highest antifungal activity (**Fig. 2-2 and 2-3**).

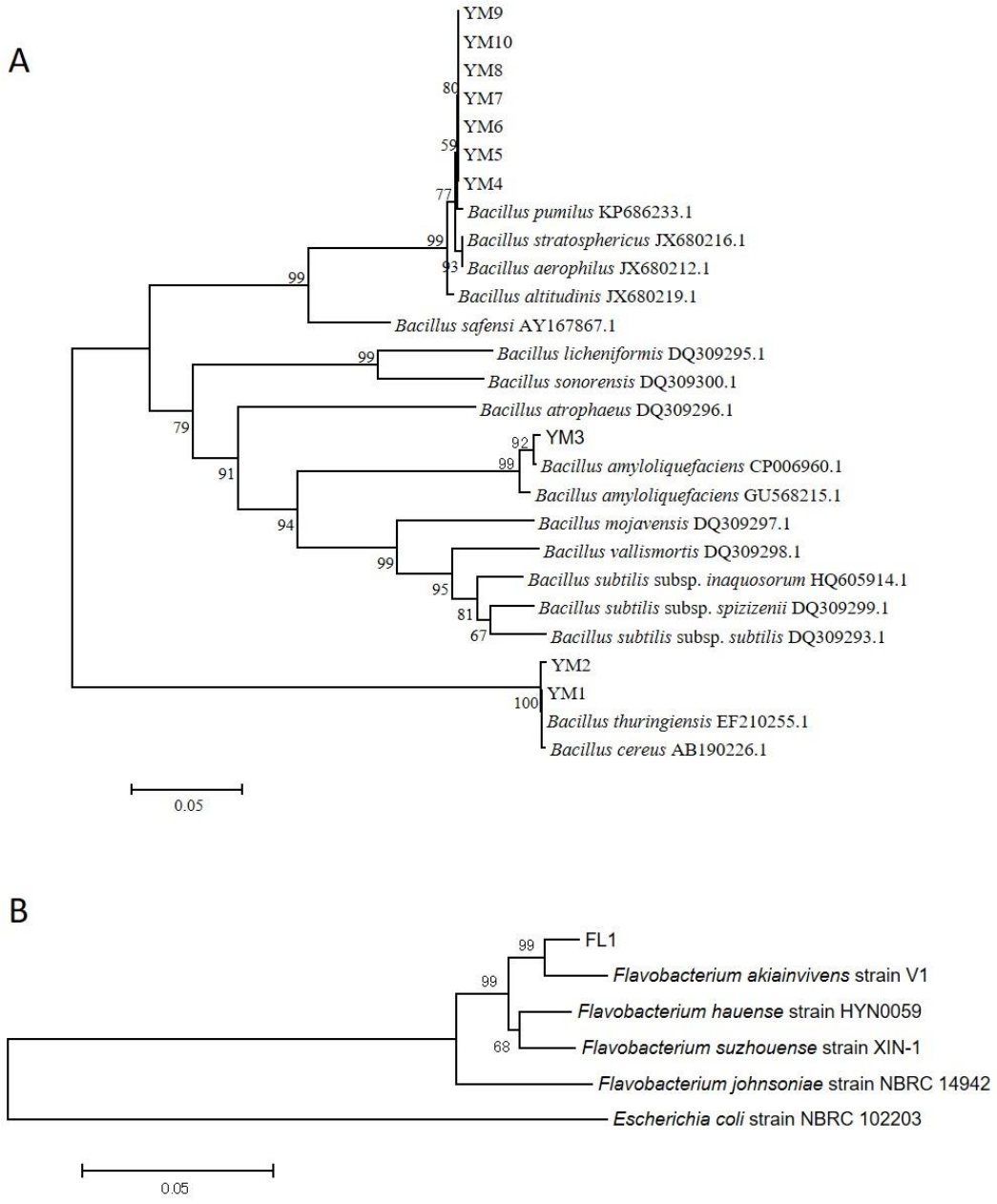

Figure 2-2. Neighbour-joining tree showing the phylogenetic positions of the bacterial isolates from VB and other related taxa based on gyrB for *Bacillus* (A) and 16S rRNA for *Flavobacterium* (B) gene sequences. Bootstrap support values resulted from 1000 replicates. Bootstrap values below 50% are not shown.

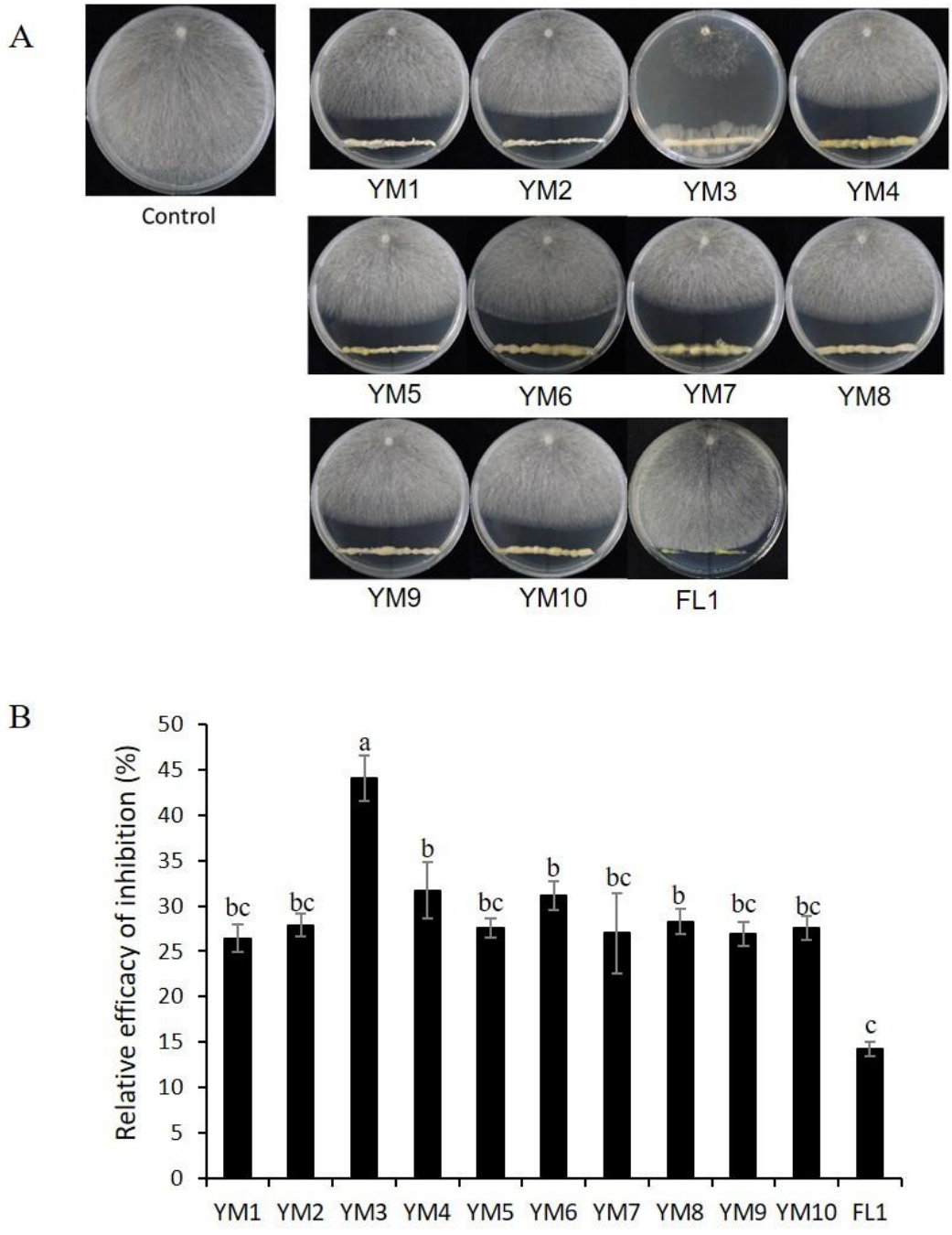

Figure 2-3. Antifungal activities of bacterial isolates on mycelial growth of *Rhizoctonia solani* AG1-IB. These antifungal activity tests were repeated three times (N = 3).

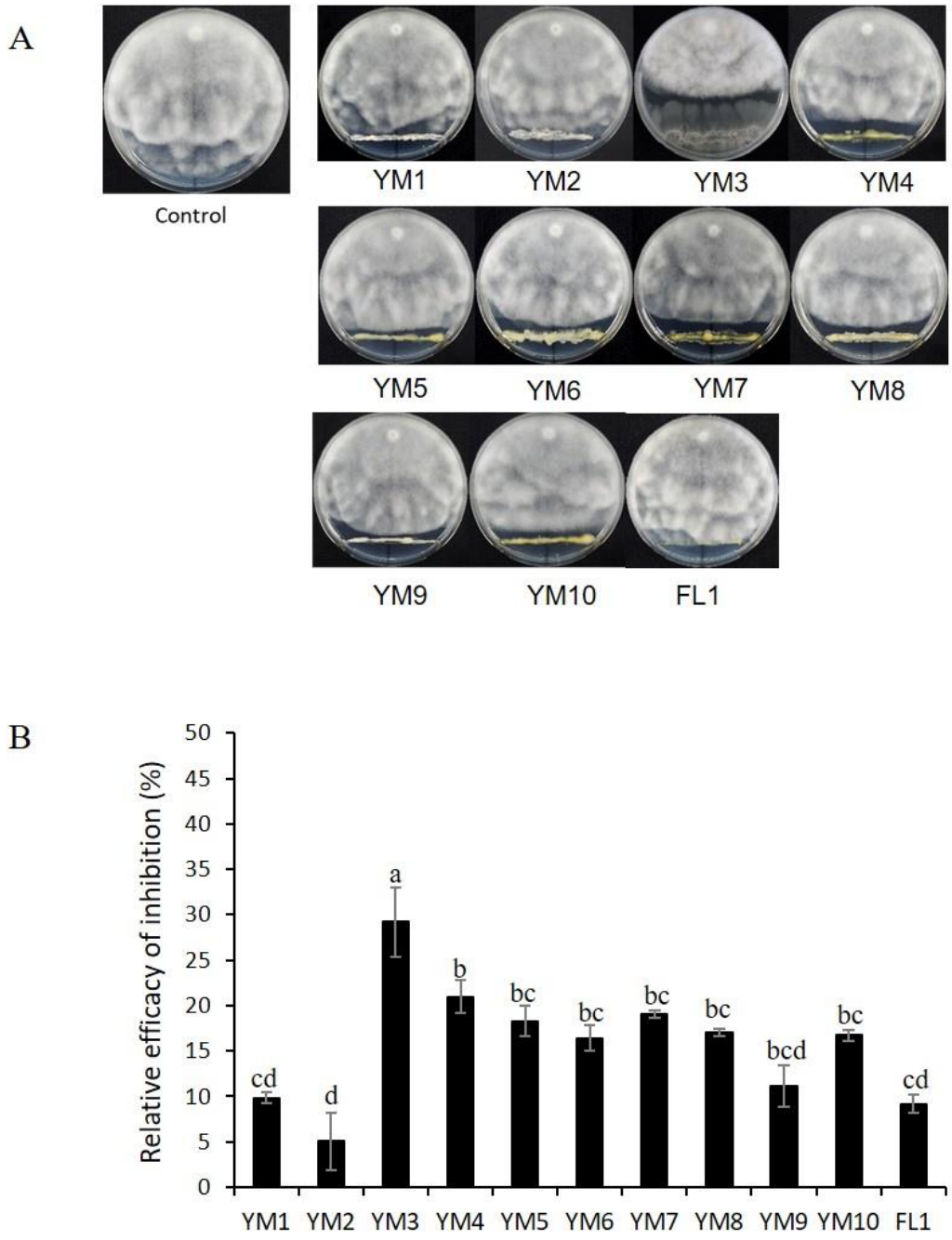

Figure 2-4. Antifungal activities of bacterial isolates on mycelial growth of *Globisporangium ultimum* var. *ultimum*. These antifungal activity tests were repeated three times (N = 3).

2.3.4 Suppressive effect of *Flavobacterium* spp. and *Bacillus* spp. on cucumber damping-off caused by *Rhizoctonia solani* AG1-IB and *Globisporangium ultimum* var. *ultimum*

All the *Flavobacterium* spp. and *Bacillus* spp. suppressed the cucumber damping-off caused by *R. solani* AG1-IB significantly, but none of the bacterial stains suppressed the cucumber damping-off caused by *G. ultimum* var. *ultimum* (**Figs. 2-5 and 2-6**).

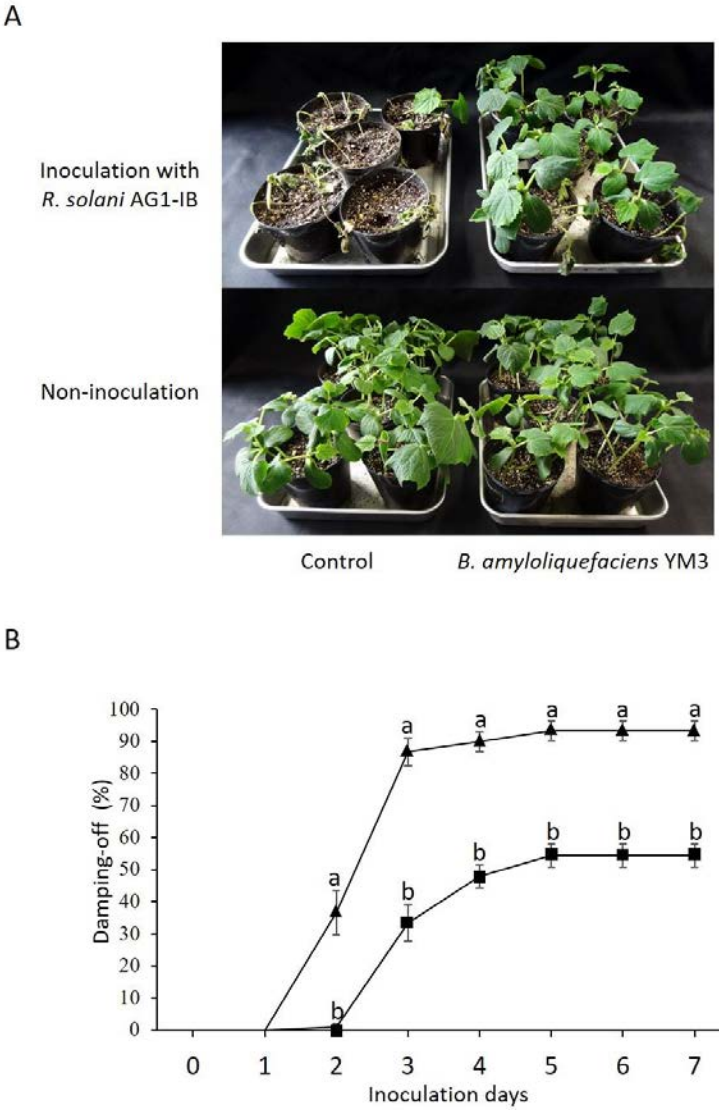

Figure 2-5. Suppressive effects of *Bacillus amyloliquefaciens* YM3 on damping-off of cucumber caused by *Rhizoctonia solani* AG1-IB in an incubator at 28℃ (12h daytime/12h nighttime). (A) Photos (B) The damping-off rate with different treatments. *R. solani* AG1-IB (▲) and *R. solani* AG1-IB + *B. amyloliquefaciens* YM3 (■). Bars indicate standard error (N = 3). Treatments with different letters indicate a significant difference at the 0.05 level, according to Tukey's test ($P < 0.05$).

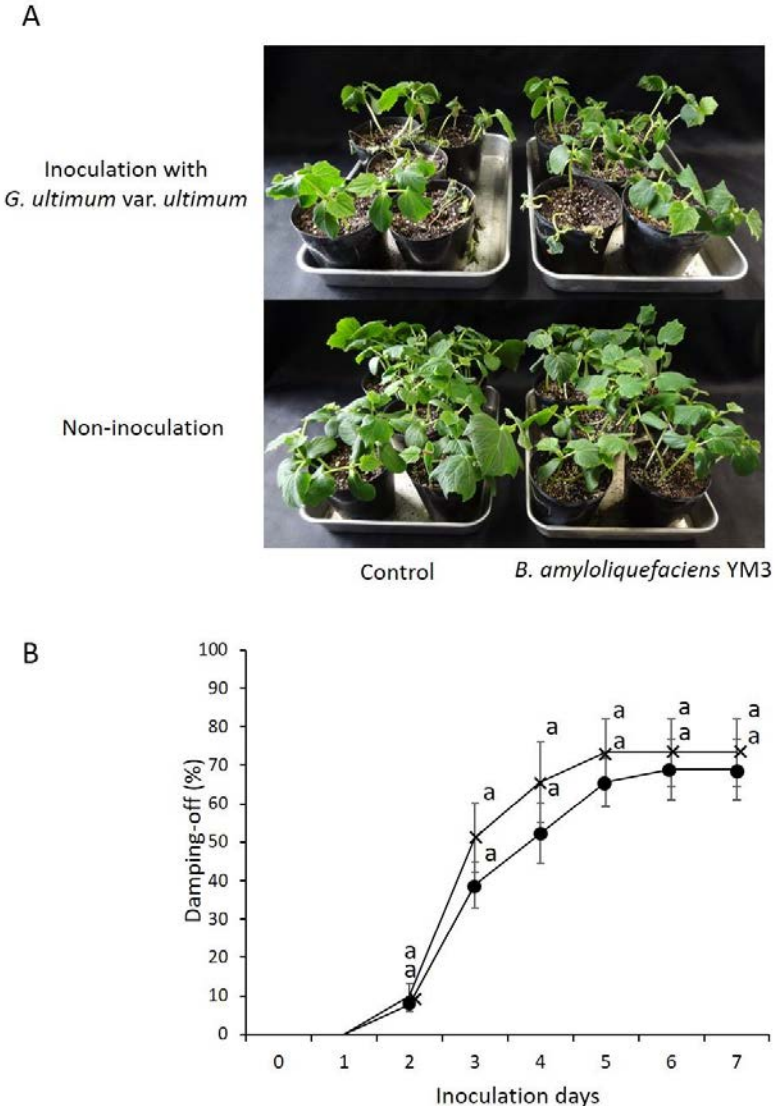

Figure 2-6. Suppressive effects of *amyloliquefaciens* YM3 on damping-off of cucumber caused by *Globisporangium ultimum* var. *ultimum* in an incubator at 28℃ (12h daytime/12h nighttime). (A) Photos (B) The damping-off rate with different treatments. *G. ultimum* var. *ultimum* (●) and *G. ultimum* var. *ultimum* + *B. amyloliquefaciens* YM3 (✖). Bars indicate standard error (N = 3). Treatments with different letters indicate a significant

2.3.5 Microbial diversity at different times after vermicomposting and earthworm intestinal

The relative abundance of the dominant bacterial genera in the vermicompost at different sample times and earthworm intestinal were shown in **Table 2-3**. *Flavobacterium* and *Luteolibacter* in the vermicompost were from earthworm intestinal, and *Bacillus* was from the original materials of vermicompost including bamboo and kudzu.

Table 2-3. Relative abundances of genera comprising more than 1.0% of genera in VB0, VB2, VB4, VB6, VB8, and earthworm intestinal.

Genus	VB-0	VB-2	VB-4	VB-6	VB-8	Earthworm intestinal
Flavobacterium	0	1.354523	0.88304	0.428345	1.825281	0.158183
Aquihabitans	0	9.78E-03	0.358913	0.446969	0.531967	3.183938
Mycobacterium	0	0.479218	1.219165	0.744948	0.478282	0.081119
Demequina	0	9.78E-03	0.205093	1.312971	1.766715	7.710404
Leucobacter	0	0.547677	0.15382	1.042928	1.454368	0.012168
Nocardioides	0	0.01956	0.091153	0.195549	0.151293	2.324072
Dyadobacter	0	2.298289	1.538199	0.158302	0.014641	0.048672
Bradyrhizobium	0	0.05379	0.512733	1.145358	1.268912	0.105455
Rhodopseudomonas	0	0.498778	1.053951	0.428345	0.082967	0.016224
Hyphomicrobium	0	0.05868	0.432974	1.340907	1.615422	0.073008
Kaistia	0	0.523227	0.347519	0.442313	0.463641	3.21233
Phyllobacterium	0	0.01467	0.017091	0.046559	0.024402	2.39708
Shinella	0	2.713936	0.94001	0.689077	0.483163	0.259582
Paracoccus	0	0.02934	0.039879	0.041903	0.063446	1.10728
Terrimicrobium	0	0.562347	1.230559	0.162957	0.048804	2.10505
Luteolibacter	0	2.176039	1.606563	1.024304	2.284041	0.640844
Roseimicrobium	0	0.352078	0.199396	0.02328	0.014641	1.886027
Novosphingobium	0	3.061125	1.429955	0.735636	0.90776	0.064896
Bacillus	4.09E-03	0.112469	0.301943	0.265388	0.45388	0
Chryseobacterium	1.398087	0.278729	0.119638	1.05224	0.268424	0
Paenibacillus	0.314774	1.300733	1.355894	0.87997	0.746706	0
Lactobacillus	8.102363	0.07824	0.034182	0.032591	4.88E-03	0
Leuconostoc	50.7808	0.312958	0.074061	0.013968	0.014641	0
Brevundimonas	4.09E-03	6.303178	0.94001	0.325915	1.351879	0
Sphingomonas	0.012264	0.753056	1.310317	0.679765	0.556369	0
Achromobacter	0.392445	9.550122	2.005355	1.415402	1.698389	0
Rahnella	11.13564	0.112469	0.022788	0.013968	0.039043	0
Acinetobacter	0.347478	0.308068	0.205093	0.931185	3.870181	0
Stenotrophomonas	2.698062	4.640587	3.178944	1.410746	0.883358	0
Rhodococcus	4.09E-03	0.02934	0.034182	0.069839	0.11713	1.508822
Cellulomonas	6.360886	10.37164	7.787843	6.974579	4.533919	3.435409
Salinibacterium	0.130815	0.787286	1.259044	1.299004	0.780869	22.59582
Lactococcus	4.406835	0.01467	0.017091	0.083807	0.073206	0.089231
Devosia	0.024528	3.530562	6.807953	5.335692	5.661298	0.068952
Rhizobium	0.085847	13.59902	12.59614	4.674551	2.118106	1.257351
Kluyvera	4.374131	1.887531	0.820373	1.168638	0.541728	0.101399
Hafnia	5.007767	0.107579	0.028485	9.31E-03	0.053685	0.04056
Pseudomonas	0.977026	2.924205	0.865949	1.047584	1.634944	0.121679
Bdellovibrio	4.09E-03	1.486553	0.837464	0.730981	0.531967	0.044616
Saccharimonas	4.09E-03	0.308068	0.541218	1.75994	0.780869	1.265463

The bacterial genera from earthworm intestinal, the original materials of vermicompost including bamboo and kudzu or both of them were highlighted with yellow, green and blue, respectively.

2.3.6 Effect of SWE treatment on mycelium growth rate of *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum* and *Rhizoctonia solani* AG1-IB

SWE significantly ($P < 0.05$) inhibited the mycelium growth of *R. solani* AG1-IB but not *P. aphanidermatum* and *G. ultimum* var. *ultimum* on PDA medium as compared to the control (**Fig. 2-7a**). The presence of SWE in PDA medium decreased the fungal growth rate from 23.8 mm/24 h to 20.3 mm/24 h (**Fig. 2-7b**).

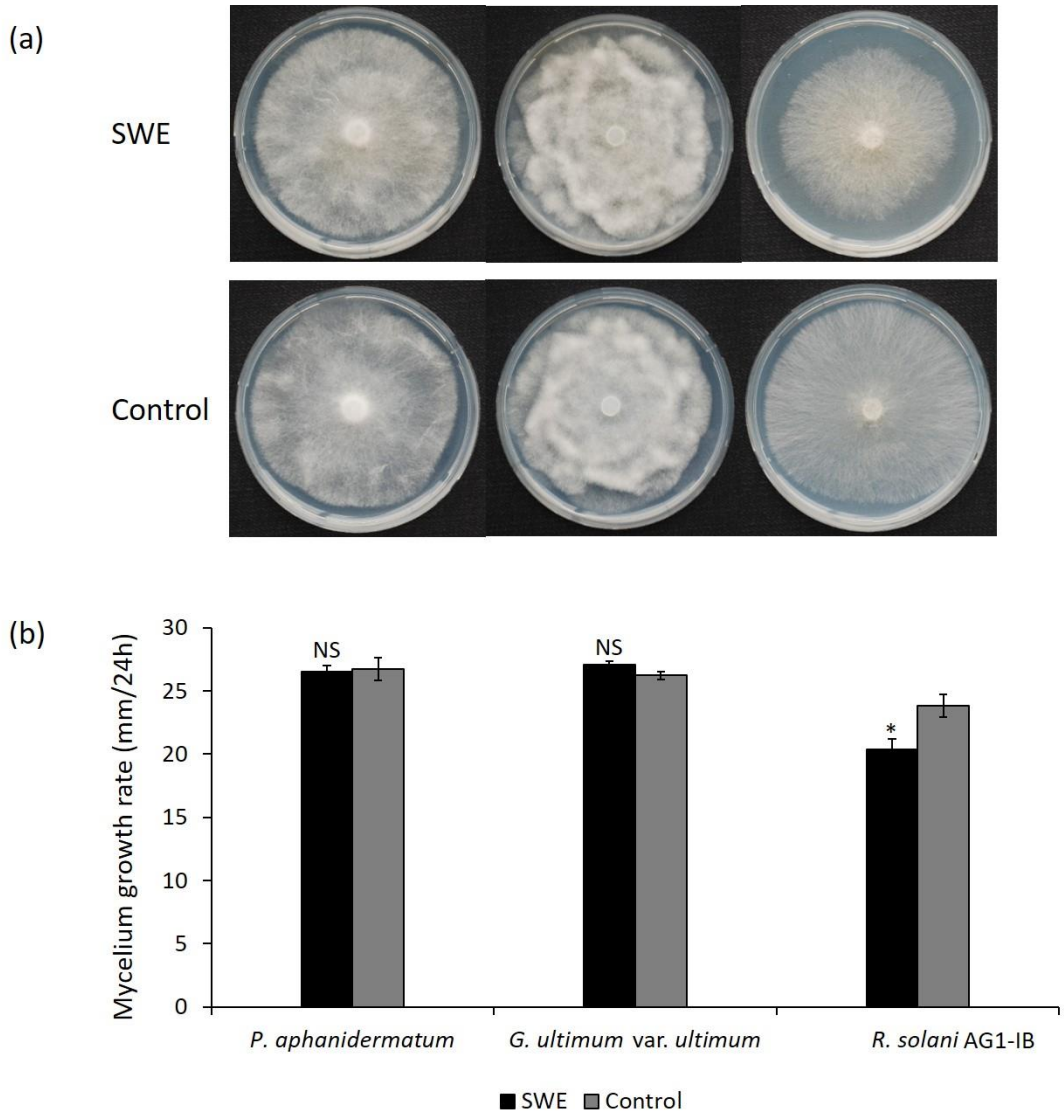

Figure 2-7. (a) Effect of treatment with SWE on mycelium growth of *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum* and *Rhizoctonia solani* AG1-IB observed on a potato dextrose agar plate. (b) Mycelium growth rate (mm/24h) of *P. aphanidermatum*, *G. ultimum* var. *ultimum* and *R. solani* AG1-IB when treated with or without the SWE. Data are the mean values of the results (N = 5). The bar at the top of each column represent the standard error of the mean. The asterisk indicates a significant difference compared to the result obtained in the control (t-test, $P < 0.05$). NS: no significant difference.

2.3.7 Isolation of antifungal compounds from VB

The fractions (C, D, E, F and G) showed antifungal activities, whereas the other fractions did not show these effects (**Fig. 2-8**). The fraction C was chosen for further fractioned as it showed two

major peaks (Figure 4). However, no major peaks were observed on the fractions (D, E, F and G) (data not shown). Two compounds, **1** and **2**, were obtained from the fraction C. For compound **1**, appearing as the major peak (tR 45.78 min) shown in **Fig. 2-9** was determined as ergosterol peroxide by detailed analysis of the ^{1}H and ^{13}C NMR spectra (**Table 2-5**). Compound **2**, the minor peak (tR 44.48 min) in **Fig. 2-9** was determined a (22*E*,24*R*)-5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol by detailed analysis of the ^{1}H and ^{13}C NMR spectra (**Table 2-6**). The physical and chemical properties of the two isolated compounds were as follows:

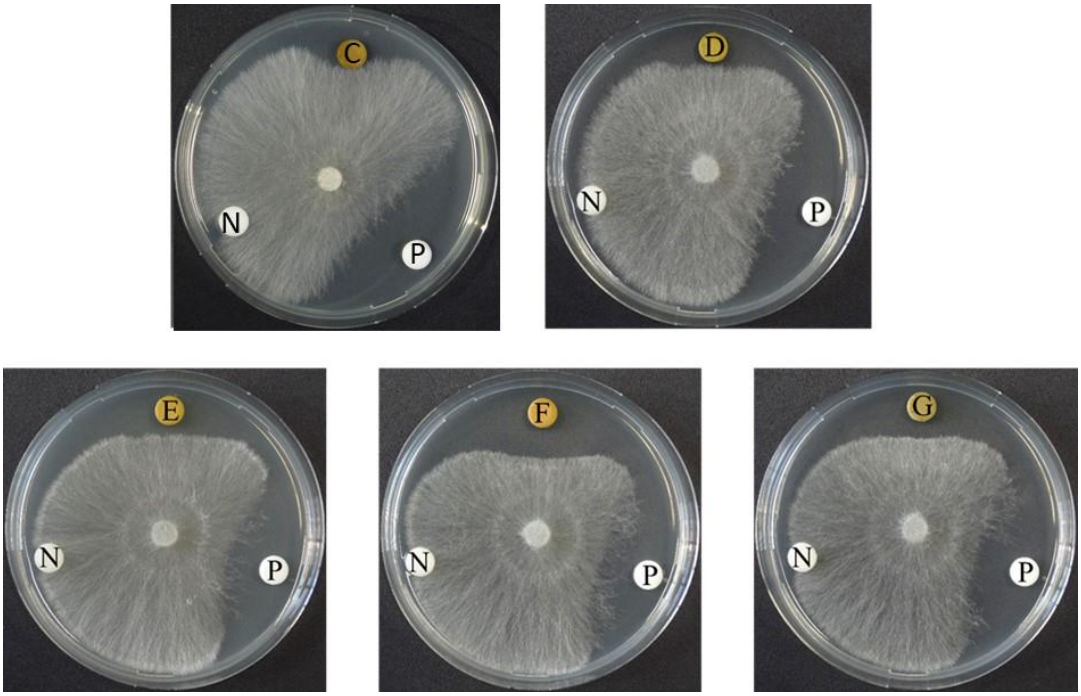

Figure 2-8. Antifungal activity of the Fr. C, D, E, F, G of EtOAc extracts of bamboo vermicompost acetone extracts against the mycelia growth of *Rhizoctonia solani* AG1-IB on PDA using the paper disc diffusion method. The mycelial growth was observed after 48 hours incubation in the dark at 25°C. P: Positive control (cycloheximide), N: Negative control (EtOAc alone).

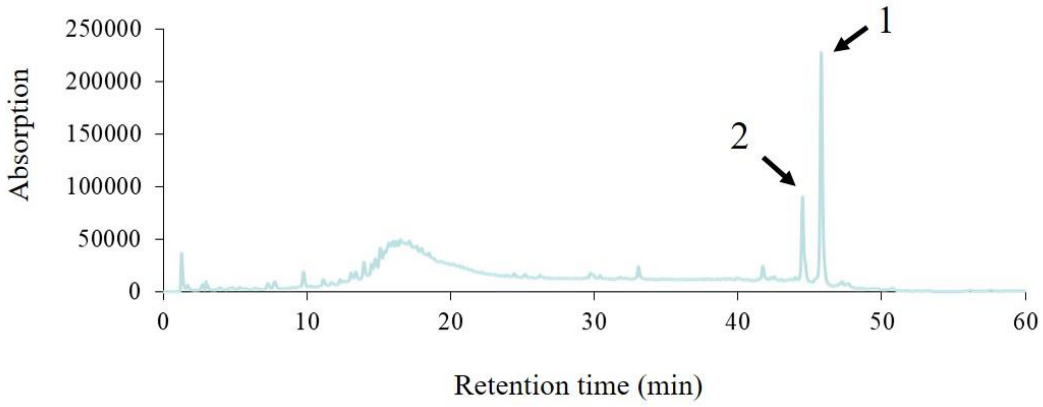

Figure 2-9. HPLC analysis of the EtOAc fraction C obtained by silica gel column chromatography of the acetone layer from the vermicomposted moso-bamboo (VB).

Compound **1** was obtained as a colorless amorphous powder and the molecular formula was determined as $C_{28}H_{44}O_3$ by electrospray ionization-mass spectrometry. The ^{1}H-NMR spectrum of compound **1** showed four olefinic protons, an oxygenated proton, four doublet methyl protons, and two singlet methyl protons. The number of these methyl groups and coupling patterns suggested that the carbon flame of **1** was ergostane. The ^{13}C-NMR spectrum showed four sp^2 carbons, three oxygenated carbons, and 21 aliphatic carbons, for a total of 28 carbons. From these data compound **1** was assumed to be ergosterol peroxide. Therefore, compound **1** was confirmed to be ergosterol peroxide, as the ^{1}H- and ^{13}C-NMR data of compound **1** matched previous data (Kim et al. 1997).

Compound **2** was obtained as a colorless amorphous powder and the molecular formula was confirmed to be $C_{28}H_{42}O_3$ by electrospray ionization-mass spectrometry. ^{1}H- and ^{13}C-NMR spectra of compound **2** were very similar to those of compound **1** except for the appearance of an olefinic proton in the ^{1}H-NMR-spectrum and appear-ance of two sp^2 carbons and disappearance of two aliphatic carbons. Therefore, compound **2** was considered to be an ergosterol peroxide derivative with one additional dou-ble bond. The compound (22E,24R)-5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol satis-fied the above data. Therefore, the ^{1}H- and ^{13}C-NMR data of

compound 2 were com-pared to previous data (Du and Shen 2016). Compound **2** was identified as (22E,24R)-5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol, based on these data showing very good agreement.

Table 2-5. Comparison of the compound **1** obtained in this study and ergosterol peroxide in ^{1}H- and ^{13}C-NMR data in $CDCl_3$.

	1		ergosterol peroxide	
atom	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	35.1	1.73m 1.97m	35.1	1.71(dd, 13.5, 3.1), 1.98(dd, 13.5, 3.1)
2	30.5	1.54m, 1.84m	30.5	1.55m, 1.85m
3	66.8	3.97m	66.8	3.98m
4	37.3	1.92m, 2.11m	37.3	1.94m, 2.11m
5	82.5		83.1	
6	135.8	6.25(d, 8.6)	135.8	6.25(d, 8.6)
7	131.1	6.51(d, 8.6)	131.1	6.51(d, 8.6)
8	79.8		79.8	
9	51.4	1.50m	51.4	1.50m
10	37.3		37.3	
11	23.8	1.23m, 1.55m	23.8	1.22m, 1.53m
12	39.7	1.25m, 1.98m	39.7	1.25m, 1.96m
13	44.9		44.9	
14	52.1	1.59m	52.1	1.57m
15	21.0	1.42m, 1.66m	21.0	1.40m, 1.66m
16	29.0	1.35m, 1.79m	29.1	1.35m, 1.80m
17	56.6	1.23m	56.6	1.24m
18	13.1	0.82s	13.3	0.83s
19	18.6	0.88s	18.6	0.89s
20	40.1	2.01m	40.1	2.05m
21	21.3	1.00(d, 6.9)	21.3	1.00(d, 6.6)
22	135.6	5.14(dd, 15.2, 8.3)	135.6	5.15(dd, 15.2, 7.7)
23	132.7	5.22(dd, 15.2, 7.6)	132.4	5.22(dd, 15.2, 8.2)
24	43.1	1.86m	43.1	1.85m
25	33.4	1.46m	33.4	1.50m
26	20.0	0.82(d, 6.9)	20.0	0.82(d, 6.7)
27	20.3	0.83(d, 6.9)	20.3	0.84(d, 6.7)
28	17.9	0.91(d, 6.9)	18.0	0.91(d, 6.7)

Table 2-6. Comparison of compound **2** and (22*E*,24*R*)-5α,8α-epidioxyergosta 6,9(11),22-trien-3β-ol in ^{1}H- and ^{13}C-NMR data in $CDCl_3$.

	2		(22*E*,24*R*)-5a,8a-epidioxyergosta-6,9(11),22-trien-3β-ol	
atom	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	32.5		32.5	
2	30.6		30.5	
3	66.3	4.01m	66.3	4.01m
4	36.0		36.0	
5	82.7		82.7	
6	135.4	6.29(d, 8.3)	135.4	6.27(d, 8.4)
7	130.7	6.60(d, 8.3)	130.7	6.58(d, 8.4)
8	78.3		78.3	
9	142.5		142.4	
10	37.9		37.9	
11	119.7	5.43(dd, 5.8, 1.5)	11.97	5.41(dd, 6.0, 0.8)
12	41.2		41.1	
13	43.6		43.6	
14	48.1		48.1	
15	20.9		20.8	
16	28.6		28.7	
17	55.8		55.8	
18	12.9	0.74s	12.9	0.71s
19	25.5	1.09s	25.5	1.06s
20	39.9		39.9	
21	20.7	1.00(d, 6.9)	20.7	0.98(d, 6.4)
22	135.1	5.17(dd, 15.5, 8.3)	135.1	5.15(dd, 15.2, 7.2)
23	132.4	5.25(dd, 15.5, 8.3)	132.4	5.27(dd, 15.2, 8.0)
24	42.7		42.7	
25	33.0		33.0	
26	19.9	0.84(d, 6.9)	19.9	0.81(d, 6.8)
27	19.6	0.82(d, 6.9)	19.6	0.80(d, 6.4)
28	17.5	0.92(d, 6.9)	17.5	0.89(d, 6.8)

The structures of the compounds 1 and 2 are shown in **Fig. 2-10**. Compound **1** was then assayed for antifungal activity against *R. solani* AG1-IB, but compound **2** was not tested because of its insufficient amount.

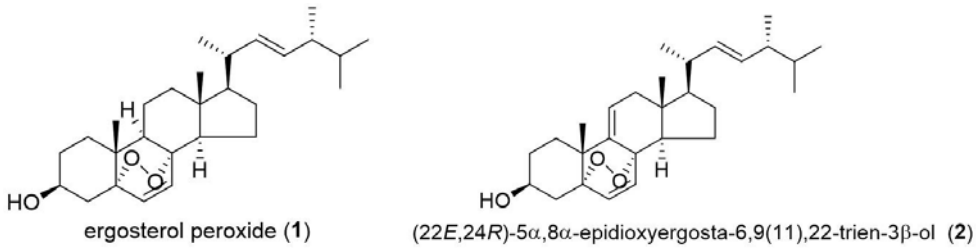

Figure 2-10. Structures of compounds **1** and **2** isolated from vermicomposted moso-bamboo.

2.3.8 Inhibitory effect of ergosterol peroxide (1) isolated from VB on mycelium growth of *Rhizoctonia solani* AG1-IB

Mycelial growth by *R. solani* AG1-IB was found to decrease gradually in response to increasing doses of ergosterol peroxide from 150 to 900 μg (**Fig. 2-11a**). Ergosterol peroxide at 150, 300, 600, and 900 μg showed 13%, 22%, 34%, and 53% mycelial growth inhibition of *R. solani* AG1-IB, respectively, as compared to control (**Fig. 2-11b**).

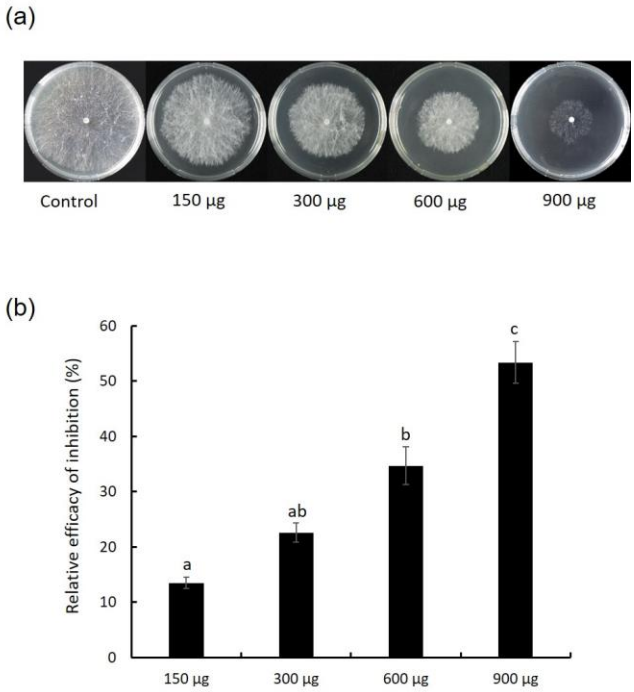

Figure 2-11. Inhibitory effects of the ergosterol peroxide on the mycelial growth of *Rhizoctonia solani* AG1-IB on PDA media. Radial growth of mycelia was measured after 48h of inoculation. (a) Photos; (b) Relative efficacy of inhibition (%). Samples of 10 μL of ergosterol peroxide at concentrations of 150, 300, 600 and 900 μg plug^{-1} were applied on top of each plug (N = 3). Bars with different letters were significant according Tukey's HSD test (P < 0.05).

2.3.9 Inhibitory effect of EtOAc extracts from initial substrate materials of VB on mycelium growth of *Rhizoctonia solani* AG1-IB

The EtOAc extracts from VB inhibited the mycelium growth of *R. solani* AG1-IB, whereas those from bamboo shoots powder and kudzu vines did not show these effects (**Fig. 2-12**).

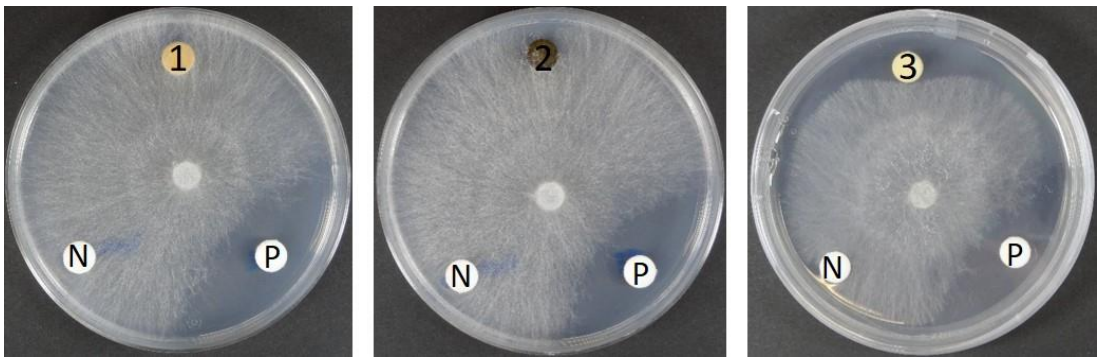

Figure 2-12. Antifungal activity of crude EtOAc extracts from bamboo shoots powder (1), kudzu vines (2), and vermicomposted moso-bamboo (VB) (3). The crude EtOAc extracts were placed on paper discs. P: Positive control (cycloheximide), N: Negative control (EtOAc alone).

2.4 DISCUSSION

Damping-off pathogens such as *Pythium* spp., *Globisporangium* spp. and *Rhizoctonia solani* causes serious economic losses of different crops and their control strategies rely mainly on chemical fungicide applications. An ecofriendly disease management strategy is urgently needed. Vermicompost has become a promising alternative to chemical pesticide because of its disease suppression effects. However, the mechanisms of how suppression of vermicompost activity in plant disease are not well-understood (Simsek-Ersahin 2011). Cluster analysis based on the data from the Illumina MiSeq sequencing of 16S rRNA gene amplicons demonstrated that the bacterial community structures of moso bamboo vermicompost (VB) were different from those of autoclaved vermicompost (aVB) and CNM (commercial nursery medium). Among the predominant bacterial genera, the genus *Flavobacterium* was only presented in VB but not aVB

and CNM. Nishioka et al. (2019) showed that the suppression of Fusarium wilt by *Allium* cultivation is mainly due to the accumulation of antagonistic *Flavobacterium* species. Carrión et al. (2019) showed that in the suppressive soils, which can reduce disease incidence despite pathogen presence, *Flavobacterium* isolate was critical for full disease suppression through site-directed mutagenesis. In addition, the relative abundance of genus *Bacillus* was significantly abundant in VB than in aVB and CNM. Pathma and Sakthivel (2013) showed that among vermicompost bacteria that exhibit antagonistic potential isolated from straw and goat manure based vermicompost, genus *Bacillus* was predominant bacteria. Mu et al. (2017) showed that *Bacillus subtilis* isolated from cow dung based vermicompost exhibited antifungal ability against ten plant pathogenic fungi by producing antifungal volatiles. Therefore, we hypothesized that the accumulation of *Flavobacterium* and *Bacillus* species is a key component of cucumber damping-off suppressiveness of vermicompost. Indeed, *Flavobacterium* and *Bacillus* isolates recovered from the vermicomposted moso-bamboo exhibited significant suppressive effects against cucumber damping-off caused by *R. solani* AG1-IB. However, none of the *Flavobacterium* and *Bacillus* isolates suppressed the cucumber damping-off caused by *G. ultimum* var. *ultimum*. The microbes in VB were higher activity and populations than in aVB and CNM. Many soil-borne pathogens including *Pythium* spp. can grow saprophytically in the rhizosphere to reach and infect their host, and their success in the colonization of roots is influenced by competition with the native rhizosphere microbiome (Bakker et al. 2012; Chaparro et al. 2012). Thus, the suppressive effects of VB against *G. ultimum* var. *ultimum* should be attributed to the higher activity and population of the rhizosphere microbiome, which limits the growth and activity of soil-borne pathogens, and consequently plant diseases. This agrees with several previous studies demonstrating that the total microbial activity and diversity of soils or composts are highly positively correlated with *Pythium* spp. and *Rhizoctonia solani* suppression (Craft and Nelson

1996; Ghini and Morandi 2006).

In addition, antifungal compounds, which are released by beneficial organisms in the vermicompost, may play an important role in inhibiting plant pathogens; however, these mechanisms have not been widely examined. Although Mu et al. (2017) showed that some volatile organic compounds, such as 3-methyl-3-hexanol, released by *Bacillus subtilis* isolated from a cow dung based vermicompost showed significant inhibitory activity against *Botrytis cinerea*, no antifungal compounds have been isolated directly from vermicompost.

In this study, five EtOAc fractions (C, D, E, F and G), obtained by silica gel column chromatography of the acetone layer from the bamboo vermicompost showed antifungal activities against *R. solani* AG1-IB. Two known compounds were obtained from the fraction (C) showing two major peaks and characterized as ergosterol peroxide (**1**) and (22*E*,24*R*)-5α and 8α-epidioxyergosta-6, 9(11), 22-trien-3β-ol (**2**). This is the first study to isolate these two compounds from vermicompost. The mycelium growth of *R. solani* AG1-IB was significantly suppressed by ergosterol peroxide. The antifungal capacity of ergosterol peroxide against *R. solani* was demonstrated in this study. The EtOAc extracts from vermicomposted moso-bamboo inhibited *R. solani* AG1-IB mycelium growth, whereas those from the original substrates did not, including bamboo shoots powder and kudzu vines. This result suggests that the antifungal compounds in vermicompost were released by microbes in the vermicompost during vermicomposting, but not from the original substrates. This agrees with a previous study demonstrating that filter sterilized aqueous extract of vermicompost prepared from paper sludge and dairy sludge significantly inhibited the spore germination of *Fusarium moniliforme* as compared to the aqueous extract of filter sterilized aqueous extract of the fresh sludge (Yasir et al. 2009).

Ergosterol peroxide is a non-volatile compound that has been isolated from various species of mushrooms, microscopic fungi such as *Trichoderma longibrachiatum*, and yeasts, and has been

reported to exhibit antitumor, anti-inflammatory and antimicrobial activities in vitro (Krzyczkowski et al. 2009; Ji et al. 2014). Some of the producers of ergosterol peroxide likely actively grow in the vermicompost. However, the antifungal compounds in the other EtOAc fractions, including D, E, F, and G, have not yet been identified. Further studies are required to identify these antifungal compounds and determine their suppressive effects on seedling damping-off diseases caused by *R. solani*. In this study, we demonstrated that vermicompost inhibited the development of fungal pathogens through the antifungal compounds present in it, which may promote effective utilization of vermicompost. We also demonstrated that antifungal compounds present in the vermicomposted moso-bamboo were released by microbes but not from the original materials of vermicompost.

Based on above mentioned, the mechanisms for the suppressive effects of vermicomposted moso-bamboo against *Globisporangium* spp. and *Rhizoctonia solani* are different. The suppressive effects of vermicomposted moso-bamboo on cucumber damping-off caused by *Globisporangium* spp. can be attributed to higher activity and population of the rhizosphere microbes, which limits the growth and activity of soil-borne pathogens, and consequently plant diseases. The suppressive effects of bamboo vermicompost on cucumber damping-off caused by *Rhizoctonia solani* can be attributed to the specific microbial groups including *Flavobacterium* and *Bacillus* spp., and antifungal compounds released by microbes in the vermicompost.

CHAPTER 3

Effects of vermicompost water extract prepared from moso-bamboo against *Meloidogyne incognita* and *Rotylenchulus reniformis*

3.1 INTRODUCTION

Plant-parasitic nematodes are economically damaging pests of many vegetable crops, causing about 100 billion dollars crop loss worldwide (Chitwood 2003). The southern root-knot nematode (*Meloidogyne incognita*) and the reniform nematode (*Rotylenchulus reniformis*) are two of the most destructive species in the tropics and subtropics (Perry et al. 2009; Heald 1975). These nematodes are not only pathogenic to crops alone, when they were co-infested with soil-borne plant pathogens, they caused more severe disease. For example, the presence of root knot nematode increased the cucumber damping-off caused by *Pythium aphanidermatum* (Morris et al. 2016). Although chemical pesticides are effective in suppressing plant-parasitic nematodes, most of them have been banned due to environmental and health concerns. Recently, many researches have focus on the potential of organic amendments for plant-parasitic nematode management (Oka 2010).

Vermicompost is a material, which is produced from biodegradable solid wastes through the decomposition and digestion of earthworms and its associated microorganisms (Elvira et al. 1998; Tajbakhsh et al. 2011). Generally, a minimum of two months are needed to produce vermicompost suitable to be used as organic fertilizer (Radovich and Arancon 2011). Various feed stock commonly used for vermicomposting include animal manure, shredded paper, vegetable or fruit scrap from kitchens or farms (Atiyeh et al. 2000; Garg et al. 2006). Vermicompost water extract is generally at 1:10 or 1:20 dilution ratio of vermicompost to water aerated over a certain

period of time, pending on usage, for the purpose of numerous bioactive molecules as well as microbial populations of the vermicompost (Edwards et al. 2006). Applying Vermicompost water extract to crops is easier than applying vermicompost, which is bulky and heavier, and needs soil incorporation that makes post-plant treatment impractical in many cases. Many scientists reported that drenching vermicompost water extract suppressed plant-parasitic fungi (Singh et al. 2003; Scheuerell and Mahaffee 2004), or plant-parasitic nematodes such as *Meloidogyne* spp. in different crops (Arancon et al. 2002; Edwards et al. 2007; Mishra et al. 2017). Although the mechanisms on how vermicompost water extract drench suppresses plant-parasitic fungi or plant-parasitic nematodes are not completely known, there are evidence showing its consistent effect against various diseases. For example, Mishra et al. (2018) showed that vermicompost water extract could induce host-plant resistance in cucumber against *Meloidogyne* spp. through split-root experiments. Moreover, some studies suggested that the abundant organic acids substances such as humic acids, hormones such as N-indole-3-acetic acid (IAA), cytokinin, and gibberellins found in VCT could suppress nematode infestation (Oka, 2010; Arancon et al. 2012).

Owing to the effective disease suppression, vermicompost water extract has become a potential alternative to chemical pesticides in agriculture. Previous research by the authors has demonstrated that vermicompost produced from moso-bamboo showed (VB) showed promising suppressive effects on several soil-borne plant pathogens including *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum*, and *Rhizoctonia solani* AG1-IB (You et al. 2019), but its potential effects against plant-parasitic nematodes has not been examined. A comparison of VB water extract versus a conventional vermicompost water extract produced from vegetable food waste as feed stock (VV) was conducted to examine for their nematode suppressive effects against two common plant-parasitic nematodes in the tropics, the southern root-knot nematode (*Meloidogyne incognita*) and the reniform nematode (*Rotylenchulus reniformis*). Previously, VV

water extract has been demonstrated to suppress root penetration and egg hatching, but not the reproduction, of *M. incognita*.

Besides suppressing plant-parasitic nematodes, another advantage of drenching vermicompost water extract to plant rhizosphere is to improve soil and plant health. Free-living nematodes have been used as soil health bioindicators as they can be used to determine dominant nutrient decomposition pathways, soil food web structure and ecosystem functions in soil (Ingham et al. 1985; Bongers and Ferris 1999; Wang and McSorley 2005). A healthy soil food web should sustain nematodes with different life strategies and feeding behaviours ranging from fast growing and reproducing bacteria-feeding nematodes (colonizers) at the bottom of the soil food web to slow reproducing but longer living predaceous nematodes at the top of the soil food web (Bongers 1990). This research project aimed to also evaluate the soil health benefits of drenching roots with water extract of VB vs VV.

Specific objectives of this project were to compare the ability of water extracts of VB and VV on mitigating (i) egg hatching, (ii) vermiform stages mobility, (iii) root penetration, and (iv) damage of *M. incognita* and *R. reniformis* on cowpea, as well as their ability to improve soil health (You et al. 2018).

3.2 MATERIALS AND METHODS

Vermicompost water extract preparation

Vermicomposted bamboo (VB) was prepared from 10 kg of the bamboo shoots powder (*Phyllostachys edulis* (Carrière) J. Houz.) mixed with 100 g of air dried kudzu vine pieces (< 10 cm in length) and 20 g of a commercial horse manure/wheat straw compost (Iris Ohyama Inc., Sendai, Japan) and adding 100 g of red wiggler (*Eisenia fetida* Savigny) (Commercial name ‘Kumataro-futomushi’, Yokomizo-shokai Inc., Mito, Japan) in a closed container to conduct

vermicomposting for 2 months (You et al. 2019). Vermicomposted vegetable waste (VV) was initiated 3 years prior to this experiment with approximately 100g of commercial mix of red wiggler (*E. fetida*) and blue worms (*Perionyx excavatus* Perrier) (Waikiki Worms Co., Honolulu, HI). The worms were fed weekly with vegetable food waste such as lettuce, kale, papaya, and banana peel (Mishra et al. 2017). All earthworms were removed from the vermicompost right before vermicompost water extracts preparation. The vermicompost water extracts were prepared fresh for each experiments, and were prepared by mixing each type of vermicompost in water at 1:10 (v/v) ratio, and aerated for 24 h using 2.5 W Elite 800 air pumps (Rolf C. Hagen Inc., Montreal, Canada) at room temperature (24±1℃). The vermicompost water extract was filtered using a kitchen strainer to separate the solid from the liquid prior to application. Samples of water extracts of VB and VV were submitted to the Agriculture Diagnostic Services Center (ADSC) at the University of Hawaii at Manoa to assay for concentrations of macro-nutrients (nitrogen, phosphorus, potassium, calcium, magnesium and boron) and micro-nutrients (Fe, Mn, Zn and Cu).

Hatching experiment

A laboratory assay was conducted to examine the effects of VB and VV water extracts on the hatching of *M. incognita* and *R. reniformis* compared to that of water control. *Meloidogyne incognita* eggs were extracted from coleus (*Plectranthus scutellarioides* (L.) R. Br.) roots and *R. reniformis* eggs were extracted from pineapple (*Ananas comosus* (L.) Merr.) roots using NaOCl and centrifugal flotation methods (Hussey and Barker 1973). Water, VB or VV water extract was contained in 60-ml plastic cups at 15 ml/cup. The experiment was arranged in complete randomized design with 4 replications. Each plastic cup served as a hatching chamber where approximately 200 freshly extracted *M. incognita* eggs or *R. reniformis* eggs were suspended in 200 µl of water over a 60.33-µm pore size screen (**Fig. 3-1**). This mesh size kept nematode eggs

on the screen but allowed second stage juveniles (J2s) to pass through. To avoid bacterial growth, VB or VV water extract or water was replaced everyday with fresh one. Hatched J2s were collected every day for 7 days and total hatching was counted under an inverted microscope (Leica DMIL LED, Wetzlar, Germany). The egg hatching experiment was repeated once.

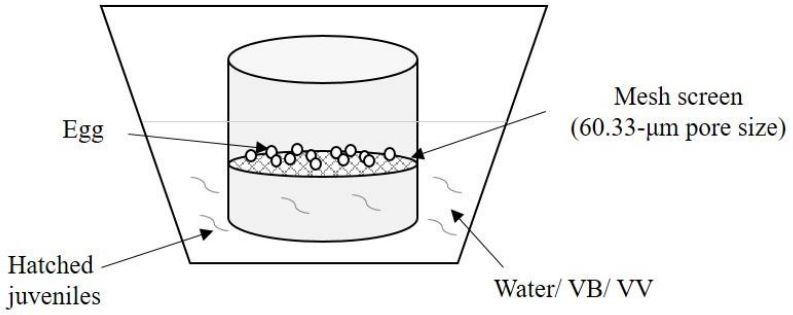

Figure 3-1. Hatching chamber used to allow harvesting of hatch juveniles over a 7 day period.

Mobility experiment

A laboratory assay was established to examine the effects of VB and VV water extracts on the mobility of *M. incognita* J2s. After adding 10 ml of water, VB or VV water extract into individual 60-mm diameter Petri plates, 0.5 ml of water suspension containing approximately 100 freshly hatched *M. incognita* J2s were added to each plate. Treatments were replicated 4 times and the experiments were repeated once. Mobility of *M. incognita* J2s was examined by probing with a dental probe after 24 hours incubation of the nematodes in the solutions. Percentile of immobilized nematodes was calculated for each Petri dish.

Root penetration experiment using split-root assay

Two greenhouse trials were conducted in the Gilmore Greenhouse at the University of Hawaii at Manoa, Honolulu, HI from April to July 2017 to examine the effects of VB and VV water extracts on root penetration of *M. incognita* on 'Bush Champion' cucumber (*Cucumis sativus* L.), and that

of *R. reniformis* on 'Blackeye #5' cowpea (*Vigna unguiculata* (L.) Walp.) by inducing host-plant resistance against nematodes. Roots of cucumber or cowpea seedlings were split into two parts and transplanted into two conjoined pots (**Fig. 3-2**). The purpose of using split-root assay was to avoid direct contact of VB or VV water extract on the tested nematodes that would lead to parasitism or immobilization of the nematodes by the chemical compounds or microbes in the vermicompost water extracts. One side of the root system was drenched with VB or VV water extract or water 3 days prior to inoculation of the targeted nematode (**Fig. 3-2**). Two hundred J2s of *M. incognita* or 100 J2s of *R. reniformis* were introduced into the untreated conjoined pot. One week after *M. incognita* inoculation, or 3 weeks after *R. reniformis* inoculation, roots from the nematode inoculated side were stained with Acid Fuchsin (Daykin and Hussey, 1985) and quantified for nematode penetration under the microscope.

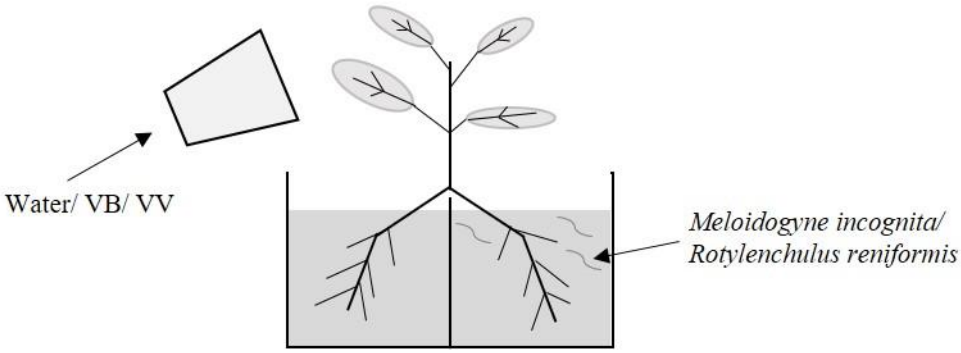

Figure 3-2. Split-root experiment constructed by two conjoint pots where one side of the roots will be drenched with vermicompost water extract and the other side of the roots will be inoculated with the designated plant-parasitic nematodes.

Bioindicators of biological activities of vermicompost

To examine biological activities of VB or VV, free-living nematodes were used as bioindicators. Nematodes were extracted from 30 cm^3 VB or VV by immersing the vermicompost into 300 ml water using Baermann trays for 24 hours (Southey, 1986). Each treatment was replicated 3 times.

Bacterivorous and omnivorous nematodes, the two most dominant nematode trophic groups present, were counted under an inverted microscope (Leica DMIL LED, Wetzlar, Germany).

Cowpea field experiment

Two field trials were conducted at the Poamoho Research Station in Waialua, Oahu, HI to compare the mitigation of nematode damage on 'Black Eye #5' cowpea by VB or VV water extract compared to water control using cowpea as a bioassay crop in field naturally infested with *M. incognita* and *R. reniformis*. Cowpea plants were drenched with VB or VV water extract, or water weekly at 50 ml per plant during the first two weeks, followed by 250 ml per plant for the rest of the crop over a 3-month growing period from 27 April to 12 July, 2017. Each experimental plot had 8 cowpea plants in a 1×3 m^2 -area. The three treatments were arranged in randomized complete block design with 4 replications. Soil nematode population densities were monitored at pre-plant, and at 1 and 2 months after planting. Shoot and root weights, and root gall index (RGI) were measured from 3 plants randomly selected in each plot at 2 month after planting. Root galling was rated using a root gall index based on a scale of 0 to 5, where 0 = 0, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 ≥ 100 galls (Taylor and Sasser, 1978). Cowpea pods from 5 plants per plot were harvested and weighted weekly from 21 June to 12 July, 2017.

Nematode assay

Soil samples were collected 1 and 2 months after cowpea planting in both trials. Four 20-cm deep soil samples were collected from each plot and combined into one bag. Nematodes were extracted from 250-cm^3 soil by elutriation and centrifugal floatation (Jenkins, 1964; Byrd et al., 1976). All nematodes extracted were identified and assigned to a trophic group of bacterivores, fungivores, omnivores, or predators (Yeates et al., 1993), but herbivores were identified to the genus level

with the aid of the inverted microscope described above.

Statistical analysis

Differences in macro- and micro-nutrient content between the VB and VV water extracts were analyzed by Student's *t*-test. The other data were checked for normality, nematode data were log transformed [$\log_{10}(x+1)$] if needed and subjected to one-way analysis of variance (ANOVA) using SAS (SAS Inc., Cary, NC). Repeated measures of nematode abundance from the cowpea field experiment were subjected to homogeneity of variance test over time. If there was no significant interaction between sampling date and treatment effect, data were subjected to repeated measures analysis. Means were separated using Waller-Duncan *k*-ration (*k*=100) *t*-test. Only true means were presented.

3.3 RESULTS

3.3.1 Nutrient analysis

VB contained lower concentrations of nitrogen, phosphorus, potassium, boron, Fe and Cu than VV, but both vermicomposts contained similar concentration of calcium, magnesium, Mn and Zn levels (**Table 3-1**).

3.3.2 Hatching experiment

Both VB and VV water extracts suppressed *M. incognita* egg hatching compared to the water control ($P \leq 0.05$, **Fig. 3-3A**). In the first trial of *R. reniformis* egg hatching test, both VB and VV water extracts reduced *R. reniformis* hatching compared to the water control. However, in the second trial, only VB water extract ($P \leq 0.05$) reduced the hatching of *R. reniformis* compared to the water control (**Fig. 3-3B**).

Table 3-1. Macro- and micro-nutrients content of vermicompost water extracts prepared from vermicompost composed of moso-bamboo (VB) and vegetable food waste (VV).

Content (mg/l)	VB	VV
N	27.60 b	280.00 a
P	0.80 b	5.62 a
K	53.59 b	213.24 a
Ca	20.86 a	25.74 a
Ma	17.21 a	18.89 a
B	0.06 b	0.47 a
Fe	0.03 b	0.27 a
Mn	0.01 a	0.02 a
Zn	0.03 a	0.03 a
Cu	0.01 b	0.02 a

Means (n=3) with same letters within a row were not different ($P > 0.05$) based on Student's *t* test).

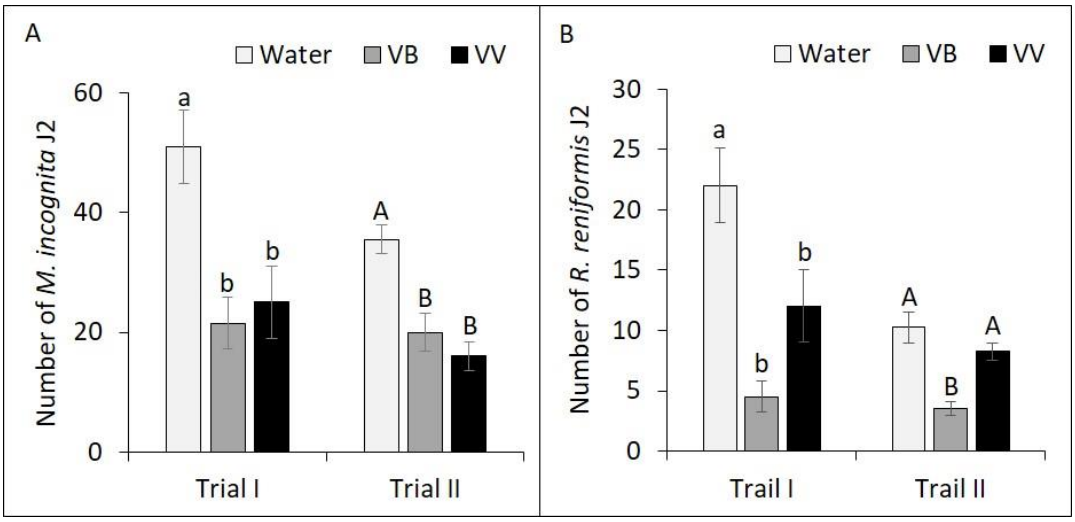

Figure 3-3. Numbers of juveniles of (A) *Meloidogyne incognita* and (B) *Rotylenchulus reniformis* hatched after incubating their eggs in vermicompost water extracts prepared from vermicompost composed of moso-bamboo (VB) and vegetable food waste (VV), and water control for 7 days. Columns (n=4) with same letter(s) are not different according to Waller-Duncan *k*-ration (*k*=100) *t*-test.

3.3.2 Mobility experiment

Although VV water extract immobilized *M. incognita* J2s more effectively than VB water extract in Trial I ($P \leq 0.05$), both VB and VV water extracts suppress the J2s mobility equally in Trial II compared to the water control ($P \leq 0.05$, **Fig. 3-4**).

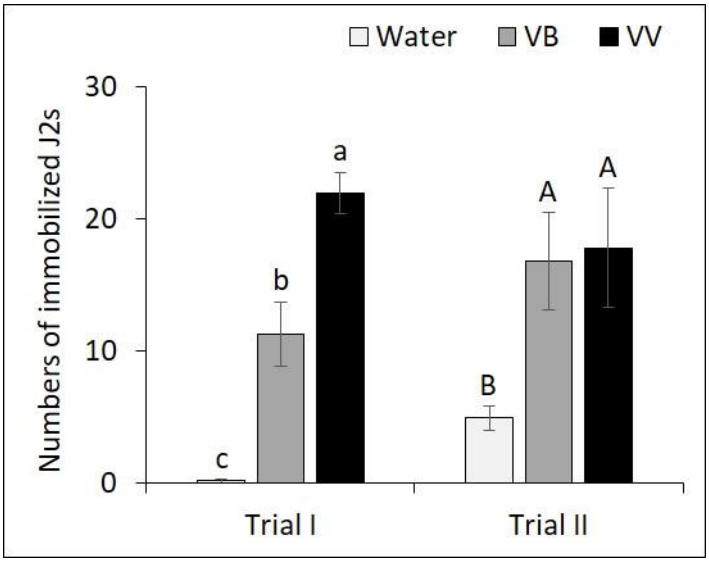

Figure 3-4. Numbers of *Meloidogyne incognita* J2 immobilized after incubating in vermicompost water extracts prepared from vermicompost composed of moso-bamboo (VB) and vegetable food waste (VV) and water for 24 hours. Columns (n=4) followed by the same letter(s) are not different according to Waller-Duncan *k*-ration (*k*=100) *t*-test.

3.3.3 Root penetration experiment using split-root assay

VB water extract suppressed root penetration of *M. incognita* consistently in both split-root trials ($P \leq 0.05$), but VV water extract was only effective in Trial II (**Fig. 3-5A**). However, neither VB nor VV water extract suppressed *R. reniformis* root penetration ($P > 0.05$, **Fig. 3-5B**) despite showing a trend of suppression compared to the water control.

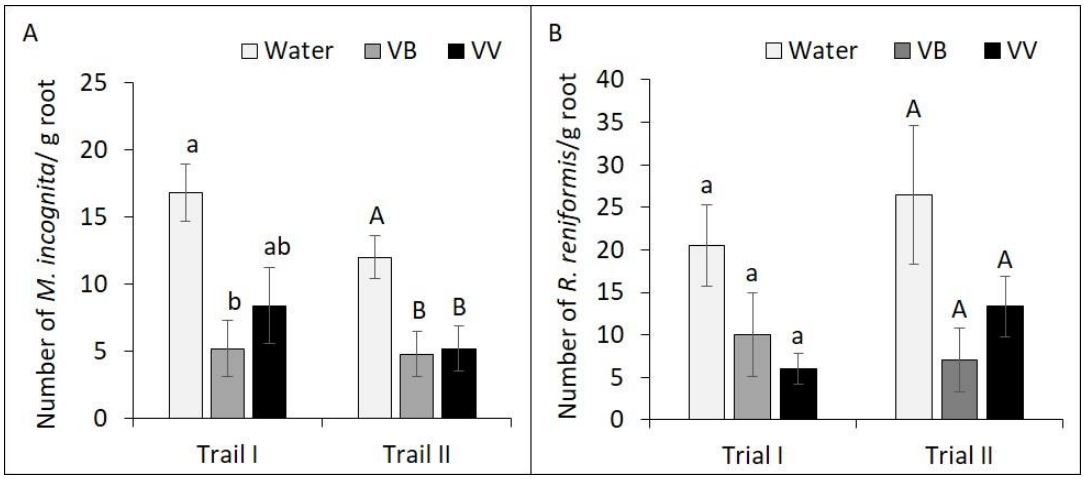

Figure 3-5. Effect of vermicompost water extracts prepared from vermicompost composed of moso-bamboo (VB) and vegetable food waste (VV) compared to water control on root penetration of (A) *Meloidogyne incognita* in cucumber, and (B) *Rotylenchulus reniformis* in cowpea using split-root assays. Means are average of 5 and 4 replications for *M. incognita* and *R. reniformis*, respectively. Column followed by same letter(s) are not different according to Waller-Duncan *k*-ration (*k*=100) *t*-test based on log transformed values, log (x+1).

3.3.4 Bioindicators of biological activities of vermicompost

No nematodes were found in the water control. There were more bacterivorous nematodes in VB than VV water extract in Trial I ($P \leq 0.05$, **Fig. 3-6A**), but more omnivorous nematodes were found in VB than VV water extract in Trial II ($P \leq 0.05$, **Fig. 3-6B**). No fungivorous, herbivorous or predatory nematodes were detected in either vermicompost examined.

3.3.5 Cowpea field experiment

Drenching of both types of vermicompost water extract did not affect shoot, root, and pod weights of cowpea ($P > 0.05$, data not presented). However, VB water extract reduced root-gall index compared to the water control in both trials ($P \leq 0.05$, **Fig. 3-7**). Both vermicompost water extracts did not reduce the number of *M. incognita* and *R. reniformis* in the soil in neither of the trials (**Table 3-2**). In fact, the VB water extract treatment increased the abundance of *Meloidogyne* spp.

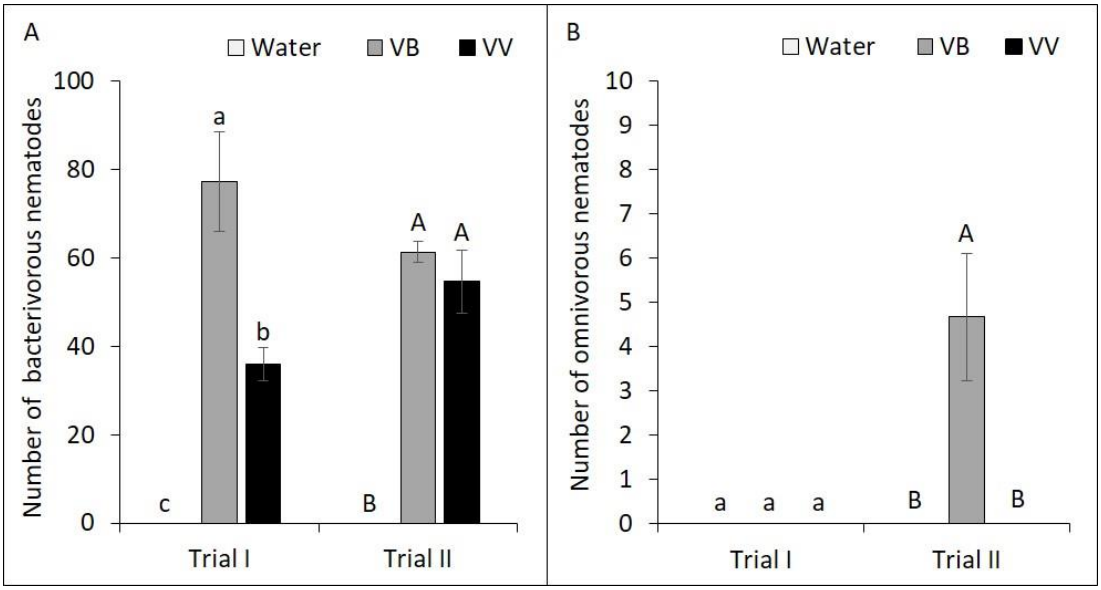

Figure 3-6. Abundance of (A) bacterivorous and (B) omnivorous nematodes in water or vermicompost water extracts prepared from 30 cm^3 of vermicompost composed of moso-bamboo (VB) and vegetable food waste (VV) incubated in Baermann trays. Columns (n=3) followed by the same letter(s) are not different according to Waller-Duncan *k*-ration (*k*=100) *t*-test.

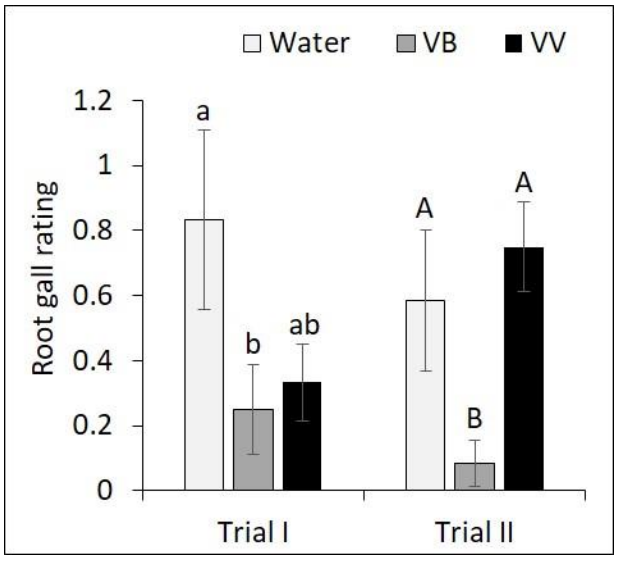

Figure 3-7. Effect of vermicompost water extracts prepared from vermicompost composed of moso-bamboo (VB) and vegetable food waste (VV) compared to water control on root gall index (in a scale of 0-5) of cowpea in two field trials. Columns (n=4) followed by same letter(s) are not different according to Waller-Duncan *k*-ration (*k*=100)

in Trial II compared to the water control at the end of the experiment. Although abundance of bacterivores and fungivores were not affected by vermicompost water extracts drenching compared to the water control on all sampling dates in both field trials ($P > 0.05$), VB water extract increased omnivorous nematodes in Trial II by >5-fold at two months after cowpea planting ($P \leq 0.05$, **Table 3-2**).

Table 3-2. Effect of vermicompost water extracts on plant-parasitic nematodes and percent trophic groups of free-living nematodes in a cowpea agroecosystem.

	Trial I			Trial II		
Nematodes	Water	VB	VV	Water	VB	VV
				5/25/17		
M. incognita	62 a	90 a	120 a	155 A	132 A	35 A
R. reniformis	312 a	465 a	435 a	728 A	450 A	402 A
% Bacterivores	40.45 a	27.11 a	27.12 a	20.82 A	21.18 A	25.00 A
% Fungivores	22.22 a	15.77 a	12.19 a	17.53 A	17.91 A	12.37 A
% Omnivores	0.28 a	0.35 a	0.00 a	0.39 A	0.10 A	0.61 A
				6/21/17		
M. incognita[z]	25 a	95 a	60 a	28 B	195 A	30 B
R. reniformis	402 a	285 a	495 a	338 A	385 A	502 A
% Bacterivores[y]	18.89 a	17.84 a	12.21 a	17.99 A	12.68 A	13.38 A
% Fungivores	10.62 a	10.92 a	6.53 a	9.82 A	10.76 A	6.89 A
% Omnivores	3.62 a	1.42 a	0.96 a	0.45 B	2.90 A	1.75 AB

[z] Nematode abundance (numbers/250 cm^3 soil) was log transformed, log(x+1) prior to analysis of variance.

[y] Percent nematode in trophic groups was square-root transformed √(x+0.1) whenever needed to normalize the data prior to analysis of variance.

Means (n=4) followed by the same letter (s) are not different according to Waller-Duncan *k*-ratio (*k*=100) *t*-test.

3.4 DISSCUSSION

Both VB and VV water extracts showed promising results in reducing mobility and root penetration of *M. incognita* J2s, and egg hatching of both nematodes in the laboratory and greenhouse experiments. These results were consistent with the findings of Mishra (2017) on vermicompost water extract prepared from vegetable waste against *M. incognita*, and that of Wang et al. (2014) on vermicompost water extract prepared from chicken manure against *R. reniformis*. Performance of VB water extract was more consistent in suppressing *M. incognita* compared to VV water extract, possibly due to higher carbon content that supported more abundant beneficial bacteria growth, as suggested by higher abundance of bacterivorous in the laboratory study and omnivorous nematodes in the Field Trial II. In our previous study, *Bacillus amyloliquefaciens, B. pumilus, B. thuringiensis* isolated from the vermicompost prepared from moso-bamboo, showed antagonistic effects on soil-borne plant pathogens. All the three *Bacillus* species have also been demonstrated their suppressive effects on plant parasitic nematodes, through nematicidal compounds and enzymes (Lee and Kim 2016; Wei et al. 2003) and/or indirectly by inducing resistance in the plants (Burkett-Cadena et al. 2008).

Suppression of root penetration of *M. incognita* by both VB and VV water extracts in the cucumber split-root assays indicated that this suppression is not due to direct antagonistic effects or nematicidal effects imposed by vermicompost but rather a host plant response. Mishra (2018) reported that cucumber plants drenched with vermicompost water extract prepared from vegetable waste showed an up-regulation of defense related genes such as CHIT-1, PAL-1 and LOX-1 encoding for chitinase, phenylalanine ammonialyase, and lipoxygenase protein 1, respectively. This result is supporting the hypothesis that vermicompost water extract stimulated Induced Systemic Resistance (ISR) in cucumber. Similar induction of host plant resistance (ISR) by vermicompost water extract against plant-parasitic nematode has also been suggested by Xiao et

al. (2016). It is encouraging to see VB water extract reduced root-gall index on cowpea compared to the water control in both cowpea field trials. However, due to the partial resistance possessed by 'Black Eye #5' cowpea against *M. incognita*, only minimal *Meloidogyne* spp. were recovered in both cowpea field trials.

Rotylenchulus reniformis was more abundant than *Meloidogyne* spp. in the cowpea field trials. Lack of induction of host plant resistance against *R. reniformis* in cowpea in both the greenhouse split-root experiment as well as the field experiment could be due to the cowpea lack of response to ISR compared to the cucumber. In addition, interference from multiple pests or pathogens attacking cowpea plants in the field could also have disrupted the induction of ISR as suggested by Pangesti et al. (2013). Aphids and whiteflies were abundant pests on cowpea in these cowpea trials (especially towards harvesting), but no attempt was taken to take these data as it was not originally expected to interfere with vermicompost water extract root drenching treatments. However, antagonistic crosstalk between jasmonic acid (JA) induced ISR and salicylic acid (SA) induced SAR can occur when sucking insects (aphids and whiteflies) are attacking a plant that was expressing ISR (Rodriquez-Saona et al. 2010). As suggested by Spoel and Dong (2012), crosstalk between plant defense hormone signaling pathways and pathogen invasion is at the expense of energy used for plant growth. It is possible that crosstalk-induced ISR may be the reason that VCT did not improve the growth and yield of cowpea, nor reduce the number of plant-parasitic nematodes in the field experiment.

Overall, drenching plant roots with VB water extract introduced high biological activities leading to high abundance of bacterivorous and omnivorous nematodes, suppression of egg hatch and mobility of *M. incognita* and *R. reniformis*, and induction of ISR that reduced root penetration of *M. incognita*. Although neither vermicompost water extract examined reduced population densities of plant-parasitic nematodes in the cowpea field, nor improved cowpea growth and yield,

drenching VB water extract increased the abundance of omnivorous nematodes in one of the field trial towards the end of the second month of cowpea growth, indicating a gradual improvement of soil food web structure and thus soil health. Wang et al. (2014) also reported that continuous drenching of vermicompost water extract prepared from chicken manure based vermicompost increased abundance of predatory nematodes toward the end of a second zucchini crop. Nico et al. (2004) showed that vermicompost could contain nematicidal compounds, depending on the feed stocks used. Future research should examine if potential nematicidal compounds such as tannins or phenolic compounds are associated with nematode suppressive effects of VB water extract, and whether the use of this VB water extract can be improved by integrating with other nematode management practices.

CHAPTER 4

Suppressive effects of VB combined with *Pythium oligandrum* against plant damping-off caused by *Globisporangium ultimum* var. *ultimum*

4.1 INTRODUCTION

The integrated use of biocontrol microorganisms with vermicompost has been documented as effective biocontrol agent for disease control in various host-pathogen systems as well as in field trials. Sahni et al. (2008) showed that the combined effect of 25% vermicompost substitution along with seed bacterization with *Pseudomonas syringae* was more effective in suppressing *Sclerotium rolfsii* than vermicompost or *P. syringae* alone under field conditions. Rao et al. (2017) demonstrated that *Bacillus subtilis* can multiply in vermicompost and soil application of such biological control agent enriched organic material increases the yield and decreases the nematode and associated disease complex in carrot.

Pythium oligandrum is an effective biological control agent of damping off and root diseases caused by several soil-borne pathogens, including phytopathogenic *Pythium* species (Hase et al., 2006; You et al. 2019d). It interacts directly with the fungal pathogens through mycoparasitism, antibiosis, nutrient and space competition, and/or indirectly by inducing resistance in the plants (Benhamou et al., 1999; Takenaka et al., 2003). Although its great potential as a biological control agent for a wide range of diseases suppression, *P. oligandrum* isolated from Japan has not yet been practical application in agricultural field. One of the main reasons is a large scale production of *P. oligandrum* oospores at low cost have never established (Takenaka and Takahashi 2012).

Considering that the combined application of biocontrol microorganisms with vermicompost can significantly enhance the process of colonization and survival of the inoculants,

thus enhancing the biocontrol activity (Sahni et al., 2008; Singhai et al., 2011). This study was carried out to determine the suppressive effects of a combination of VB with *P. oligandrum* on plant damping-off caused by *Globisporangium ultimum* var. *ultimum.*

4.2 MATERIALS AND METHODS

The suppressive effects of a combination of VB with *Pythium oligandrum* on soybean damping-off caused by *Globisporangium ultimum* var. *ultimum*

The experiment was conducted under a growth chamber at 28 ℃ (12 h day) / 25 ℃ (12 h night). Seven treatments were tested and the treatments consisted of Con (Soil control), VB25 (VB substitution at 25% v/v), PO (Soil infested with *P. oligandrum*).

Globisporangium ultimum var. ultimum were cultured on autoclaved bentgrass seeds at 25℃ in darkness for 7 days. One g of the culture was mixed with 1L of each treatment, and put into ceramic pots where the pots were filled with 700 ml of soil, VB and soil substituted with VB25 in a growth chamber at 28 ℃ (12 h day) / 25 ℃ (12 h night). Each isolate was cultured on autoclaved bentgrass seeds at 25 ℃ in darkness for one week; 2.5 g of the colonized seeds were thoroughly mixed with 1 L of commercial nursery soil (Takii Co. Ltd., Kyoto, Japan) using a mortar and pestle, and 200 ml of the mixture was put in a ceramic pot. Eight soybean seeds were sown per pot and incubated in one of the two temperature treatments; a low temperature growth chamber at 15 ℃ (12 h day) / 12 ℃ (12 h night) or a high temperature growth chamber at 28 ℃ (12 h day) / 25 ℃ (12 h night). Non-inoculated pots were used as controls. Before the experiment, the seeds were surface sterilized in 0.05 % sodium hypochlorite (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) for 5 minutes, then rinsed in sterile distilled water. Each pot was watered daily with tap water. After 14 days, the pathogenic aggressiveness of the strains was determined using a 0 - 4 scale as in John et al. (2010), with some modifications: 0 =

healthy, seed germinated without visible infection; 1 = germinated with short, discolored roots; 2 = germinated with short, severely discolored roots; 3 =died after germination; and 4 = died before germination (**Fig. 4-1**). The aggressiveness of the pathogen strain on the soybean seeds was calculated as a disease index from the following equation according to Abdel-Monaim et al. (2011), with some modifications:

$$\Sigma\{1A+2B+3C+4D)\}/3(T)\times 100$$

where *A*, *B*, *C* and *D* are the number of plants corresponding to the numerical grade 1, 2 3 and 4, respectively, and 3*T* is the total number of plants (*T*) multiplied by the maximum disease grade 3, where $T = A + B + C + D$.

The pathogens were re-isolated from the symptomatic roots on NARM medium (Morita and Tojo, 2007) and identification by the formation of sexual organs. The experiments were repeated five times, using one pot per repetition. Data were evaluated by Tukey's HSD test ($P < 0.05$) in IBM SPSS Statistics 25 (IBM Corp., Armonk, NY, USA).

Figure 4-1. The rating scale for aggressiveness on soybean was: 0 = seed germinated without visible infection; 1 = germinated with light discoloration on roots; 2 = germinated with short, severely discolored roots; 3 = died after germination; and 4 = died before germination. Disease index = $\Sigma x_i/N$ where x_i is disease rating of its replicate (i = 0-4) and N is the total number of seedlings examined.

The suppressive effects of a combination of VB with *Pythium oligandrum* on cucumber damping-off caused by *Globisporangium ultimum var. ultimum*

The experiment to determine the suppressive effects of a combination of VB substitution of concentrations (25% and 50%) with *P. oligandrum* on damping-off of cucumber caused by *G. ultimum var. ultimum* was conducted in ceramic pots where the pots were filled with 700 ml of soil, VB and soil substituted with different amounts of VB (25% and 50%) under greenhouse conditions. *P. oligandrum* were cultured on autoclaved bentgrass seeds for 7 days at 25℃ in darkness, then one gram of the colonized seeds was mixed with 1 L soil. Seven treatments were tested and the treatments consisted of Con (Soil control), VB25 (VB substitution at 25% v/v), VB50 (VB substitution at 50% v/v), PO (Soil infested with *P. oligandrum*), VB25+PO (VB substitution at 25% v/v+ *P. oligandrum*), VB50+PO (VB substitution at 50% v/v+ *P. oligandrum*) and fungicide Tachigaren®.

G. ultimum var. ultimum were cultured on autoclaved bentgrass seeds at 25℃ in darkness for 7 days. One g of the culture was suspended in 20 ml of 0.35% water agar used as inoculum. Eight 7-days-old cucumber seedlings (*Cucumis sativus* L. cv. Jibai) were transplanted into each pot of treatments. Each treatment consisted of 8 pots and each pot served as single replication. Each pot received 5 ml of inoculum at the plant base (avoiding direct contact of the inoculum on plants) two days after transplanting and irrigated daily with tap water. For fungicide treatment, Tachigaren® which was diluted 1000-fold by tap water was applied 200 mL to each pot just before the inoculation. Damping-off (percentage of collapsed plants) was recorded at 5, 10, 15 days after inoculation. The temperature of greenhouse was maintained in the range 23-33 ℃ for the experimental period. Data were compared using one-way analysis of variance (ANOVA) in the IBM SPSS 22 software program (SPSS Inc., USA), and significant differences between means were determined by using Tukey's HSD test ($P < 0.05$).

Effect of VB on density of *Pythium oligandrum* in soil

PO (Soil infested with *P. oligandrum*), VB25+PO (VB substitution at 25% v/v+ *P. oligandrum*), VB50+PO (VB substitution at 50% v/v+ *P. oligandrum*) prepared as described above without growing plants, were incubated in a growth chamber at 28 ℃ (12 h day) / 25 ℃ (12 h night). The populations of *P. oligandrum* in each treatment were measured at 0, 7 and 14 days by dilution plate method. 10 g of each sample was diluted in 500 ml of autoclaved 0.35% agar, and then shaken at 200 rpm on a rotary shaker (NR-30; Taitec, Saitama, Japan) at 25 ºC for 30 min. A 1-ml aliquot of the soil dilution was plated onto each of *Pythium*-selective NARM plates (Morita and Tojo 2007), and a bent glass rod was used to spread the aliquot over the plate. After incubation for 48 h at 25 ºC in the darkness, the soil suspension was washed from the surface of each plate under a slow stream of water and the colonies were counted under a stereoscope at ×10 and ×20 magnifications. The colonies were re-identification based on morphology by microscope (**Fig. 4-2**). The density of *P. oligandrum* was expressed as the number of colonies per gram of dry soil.

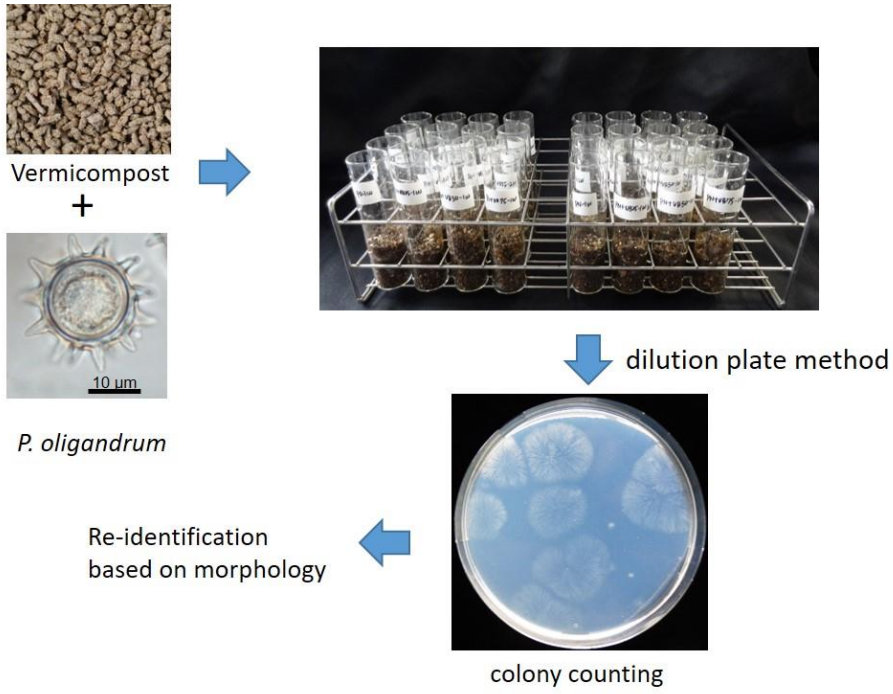

Figure 4-2. Effect of vermicompost on the activity of *Pythium oligandrum* in the soil.

4.3 RESULTS

4.3.1 The suppressive effects of a combination of VB with *Pythium oligandrum* on soybean damping-off caused by *Globisporangium ultimum var. ultimum*

All the treatments reduced the disease severity of soybean significantly as compared with the control, ranged from 44% to 94% in case of fungicide (**Fig. 4-3**). In addition, VB25+PO caused higher of reduction in disease severity as compared with VB25 or PO alone (**Fig. 4-3**).

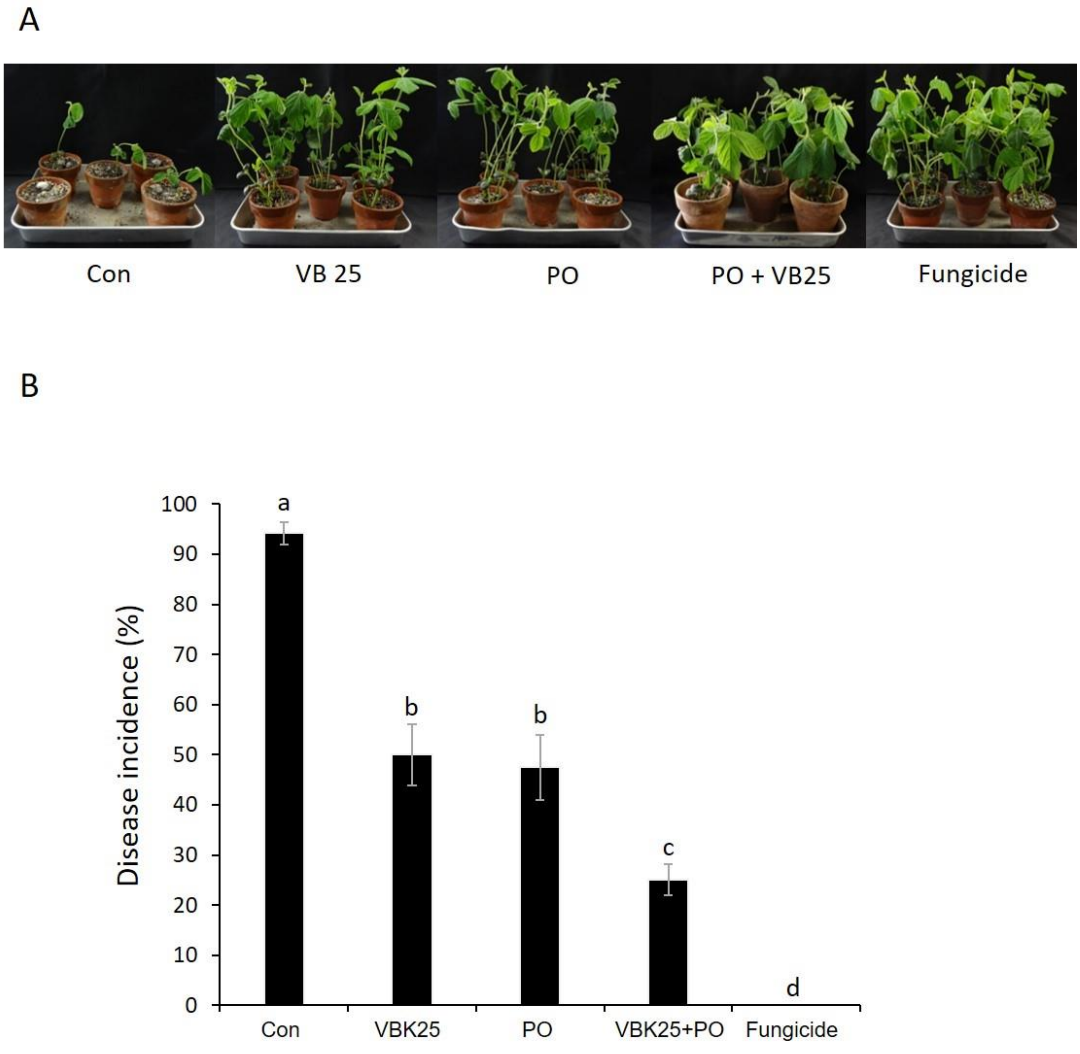

Figure 4-3. The suppressive effects of a combination of VB with *Pythium oligandrum* on soybean damping-off caused by *Globisporangium ultimum* var. *ultimum*. (A) Photos; (B) Disease incidence (%). Bars with different letters were significant according to Tukey's HSD test ($P < 0.05$).

4.3.2 The suppressive effects of a combination of VB with *Pythium oligandrum* on cucumber damping-off caused by *Globisporangium ultimum var. ultimum*

All the treatments, except VB25 alone, reduced the disease severity of soybean significantly as compared with the control, ranged from 36% to 73% (**Fig. 4-4**). VB25+PO and VB50+PO caused the highest of reduction in disease severity, and were comparable to the conventional fungicide Tachigaren®. In addition, VB50+PO caused higher of reduction in disease severity as compared with VB50 or PO alone.

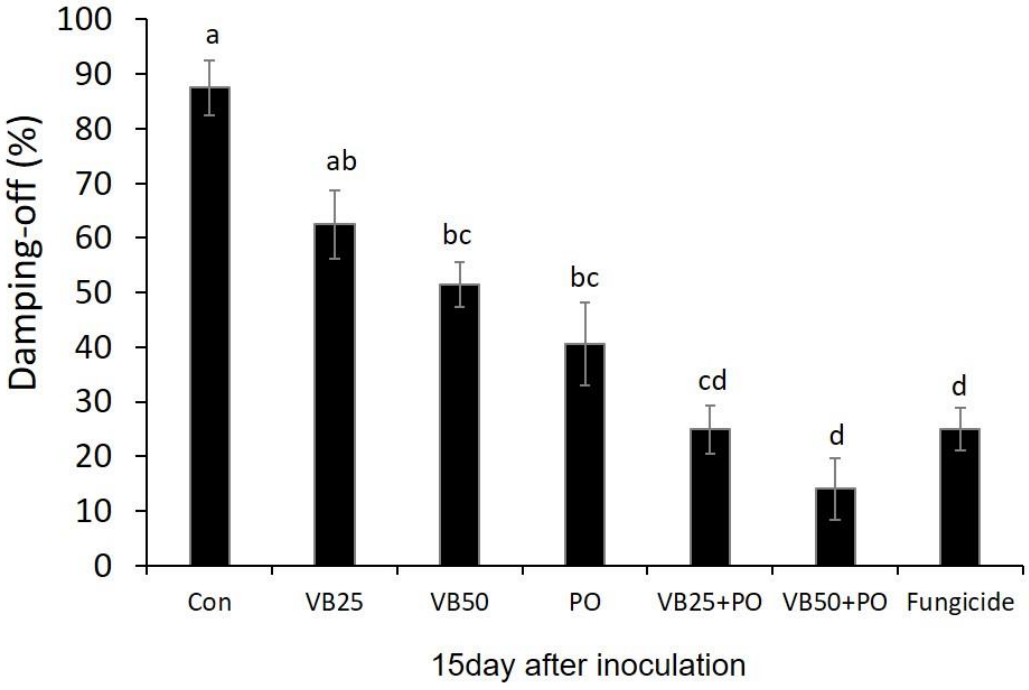

Figure 4-4. The suppressive effects of a combination of VB with *Pythium oligandrum* on cucumber damping-off caused by *Globisporangium ultimum* var. *ultimum*. Bars with different letters were significant according to Tukey's HSD test ($P < 0.05$).

4.3.3 Effect of VB on density of *Pythium oligandrum* in soil

The density of *P. oligandrum* in the soil was increased gradually by addition of VB from 0 day to 14 days (**Fig. 4-5**). The representative colonies were re-identification as *P. oligandrum* based on morphology (data not shown).

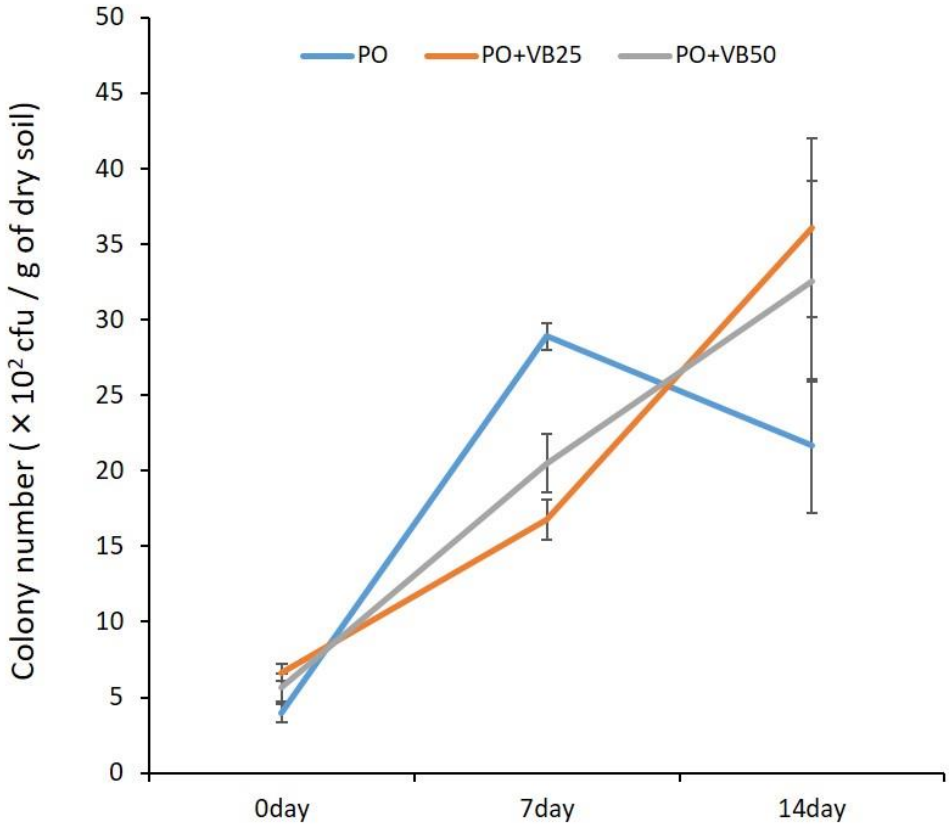

Figure 4-5. Effect of VB on density of *Pythium oligandrum* in soil.

4.4 DISCUSSION

Although chemical treatments, such as fungicide-seed coating and/or soil fumigations, are effective in managing the soil-borne plant pathogens, frequent use of chemical treatments cause the emergence of fungicide-resistant strains, and risk human health as well as the surrounding environment (Bradley 2008; Becker et al. 1998). An alternative strategy to control soil-borne plant diseases is the application of biocontrol agents, such as rhizobacteria and rhizofungi (Léon et al. 2009; John et al. 2010). However, the biocontrol effect of such biocontrol agents can sometimes be low and/or short-lasting effects on plant diseases in the field (Yuliar and Toyota 2015). The main reason is when applying such biocontrol agents in the field, they have to compete for nutrients with other native microbes and resist to the field conditions such as soil temperature

and moisture as well as exposure to ultraviolet radiation on soil surfaces. Recently a combination of antagonistic microbes with vermicompost was shown higher efficacy in suppressing plant diseases than using single antagonistic microbes or vermicompost alone (Sahni et al. 2008; Rao et al. 2017). In this study, a combination of VB with *P. oligandrum* showed significantly higher suppressive effects on damping-off diseases than using VB or *P. oligandrum* alone. VB25+PO and VB50+PO were comparable to the conventional fungicide Tachigaren® in suppressing cucumber damping-off. To our knowledge, this is the first report showed that the combination of vermicompost with *P. oligandrum* increased the suppressive effects on plant disease. There are several possible mechanisms including (1) some essential source such as C source for *P. oligandrum* growth present in the vermicompost, (2) the synergism of antagonists and (3) the change in the composition of soil microflora. In our research, the density of *P. oligandrum* in soil was increased gradually by addition of vermicompost, agreed with a previous study showed that vermicompost can sustain more biological control agents.

Although *P. oligandrum* has great potential as a biological control agent for a wide range of diseases suppression, the strains from Japan has not yet been practical application in agricultural field. One of the main reasons is a large scale production of *P. oligandrum* oospores at low cost have never established (Takenaka and Takahashi 2012). Soil usually does not contain sufficient organic substrates for the introduced microorganisms. The formulation of *P. oligandrum* using vermicompost can contribute to the control of soilborne pathogens in a variety of crops by acting as a C source for *P. oligandrum*, which may reduce the amount to use, and consequently cost down for using *P. oligandrum*. Thus we propose to produce a mixture of vermicompost and *P. oligandrum* as a new biocontrol agent for soil-borne pathogens management. Our further work will determine whether the *P. oligandrum* combined with vermicompost is effective in suppressing plant damping-off in fields.

CHAPTER 5

General discussion and conclusions

Chemical pesticides have played a major role in securing food supplies the world over. However, excessive use has led to cause the emergence of fungicide-resistant strains, and risk human health as well as the surrounding environment (Bradley. 2008; Becker et al. 1998). In recent years, increasing consumer concern about issues such as food quality, environmental safety and soil conservation has led to a substantial increase in the use of sustainable agricultural practices. This has driven the search for less harmful alternative strategy to control plant diseases.

Vermicomposting is a low-technology, environmentally-friendly process used to treat organic waste. The resulting vermicompost has been shown to have positive impacts on plant growth and health. Many kinds of vermicompost are known to be used as a component of nursery potting media (Scheuerell et al. 2005) owing to their ability to control plant pathogens such as *Pythium ultimum* Trow var. *ultimum*, *Rhizoctonia solani* Kuhn, and *Verticillium* sp. (Chaoui et al. 2002). Owing to its effective disease suppression, vermicompost has become a promising alternative to chemical pesticides. However, vermicompost produced from different feedstocks vary in disease suppressiveness (Szczech and Smolińska 2001). The mechanisms of plant disease suppression are still not well understood. Understanding the details of how vermicompost suppresses plant pathogens may promote effective utilization of the vermicompost in agricultures.

In Japan, wild moso-bamboo (*Phyllostachys edulis*) is extremely destructive to natural and agricultural ecosystems. Over the years little success has been achieved in combatting the continuing spread of moso-bamboo. Researchers have tried finding uses for these wild plants to encourage their removal from the ecosystems. In this study we are successfully in converting moso-bamboo into vermicompost, which has the ability to suppress several plant pathogens and plant parasitic nematodes (**Chapter I and III**). The mechanisms of bamboo vermicompost for

the suppression are clearly clarified in this study (**Chapter II and III**). The mechanisms of bamboo vermicompost for the suppression differ significantly among these plant pathogens and plant parasitic nematodes (**Fig. 5-1**). The suppressive effects on *Globisporangium ultimum* var. *ultimum* are mainly attributed to the higher activity and population of the rhizosphere microbiome, which limits the growth and activity of soil-borne pathogens, and consequently plant diseases (**Table 2-1**). The suppressive effects on *Rhizoctonia solani* AG1-IB are mainly attributed to the specific bacteria, including antagonistic *Bacillus* and *Flavobacterium* species, and antifungal compounds released by microbes in the vermicompost (**Figs. 2-3, 2-4, 2-5, 2-6, 2-11 and 2-12**). The suppressive effects on plant parasitic nematodes interact directly in reducing mobility and egg hatching of nematodes by beneficial bacteria and indirectly by inducing resistance in the host plant (**Figs. 3-3, 3-4 and 3-5**). To improve the biocontrol activity of bamboo vermicompost against plant pathogens, a biocontrol agent *Pythium oligandrum* was introduced into the vermicompost. It is revealed that the vermicompost can significantly enhance the process of colonization and survival of the inoculants, thus enhancing the biocontrol activity (**Chapter IV**). Based on these findings in this study, some points were highlighted below to use bamboo vermicompost for plant disease suppression.

Soaking of bamboo powder in tap water before vermicomposting

The lethal compounds on earthworms were demonstrated presented in the bamboo powder. Since they are water-soluble, soaking in tap water before vermicomposting for 24 hours in room temperature is enough to remove them from bamboo powder.

Amending of organic fertilizers with bamboo vermicompost before growing plants

Bamboo vermicompost contains lower nitrogen compared with a commercial nursery soil. Leaf

yellowing and slightly poorer growth were observed in the seedlings grown in bamboo vermicompost as a potting medium, as compared with those grown in commercial nursery soil. However, the amendments of organic fertilizers, such as oil cakes, resolved these problems without resulting in a loss of disease suppressiveness of the vermicompost. Thus bamboo vermicompost amended with organic fertilizers are recommended for practical applications.

Introducing a biocontrol agent such as *Pythium oligandrum* into the vermicompost

The suppressive effect of bamboo vermicompost against cucumber damping-off caused by *G. ultimum* var. *ultimum* was found to decrease gradually in response to decreasing concentration of bamboo vermicompost. To improve the biocontrol activity of bamboo vermicompost against plant pathogens, introducing a biocontrol agent such as *Pythium oligandrum* into the vermicompost is effective.

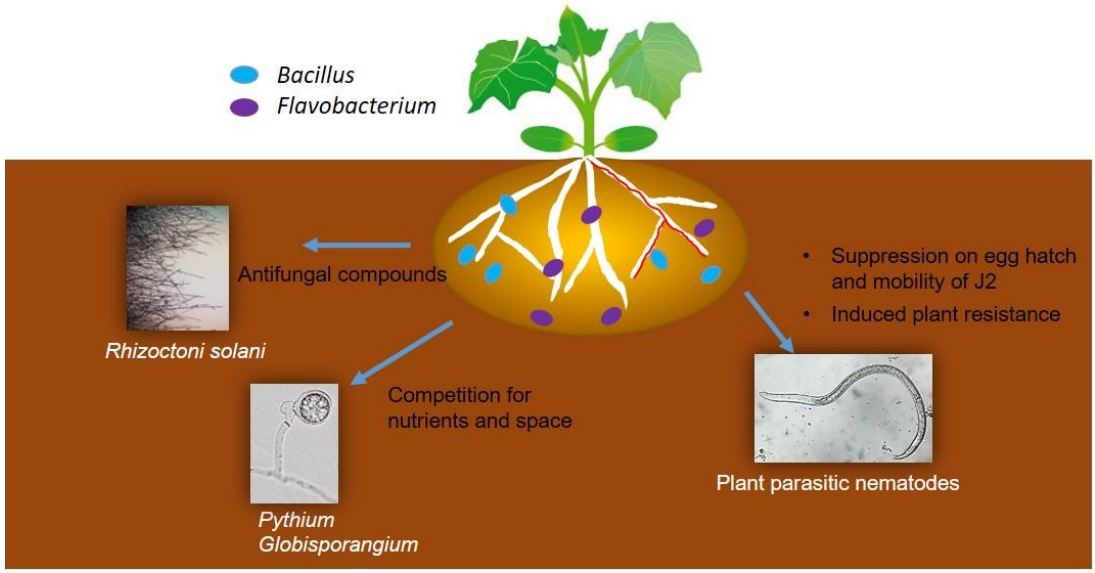

Figure 5-1. A model explaining the mechanisms of the vermicomposted moso-bamboo for plant disease suppression.

REFERENCES

Abbasi, P.A., Conn, K.L. and Lazarovits, G. (2004) Suppression of *Rhizoctonia* and *Pythium* damping-off of radish and cucumber seedlings by addition of fish emulsion to peat mix or soil. *Canadian Journal of Plant Pathology* **26**, 177-87.

Abdallah, D. B., Frikha-Gargouri, O. and Tounsi, S. (2018). Rizhospheric competence, plant growth promotion and biocontrol efficacy of *Bacillus amyloliquefaciens* subsp. *plantarum* strain 32a. *Biological Control* **124**, 61–67.

Abdel-Monaim, M. F., Ismail, M. E. and Morsy, K. M. (2011) Induction of systemic resistance of benzothiadiazole and humic acid in soybean plants against *Fusarium* wilt disease. *Mycobiology* **39**, 290-298.

Abe, M., Yamada, M. and Inoue, Y. (2007) Earthworm avoidance test for evaluation of environmental hazard of landfill leachate. *Environmental Science* **20**, 503-510.

Adam, G. and Duncan, H. (2001) Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biology and Biochemistry* **33**, 943-951.

Arancon, N.Q., Edwards, C.A., Yardim, F. and Lee, S. (2002) Management of plant parasitic nematodes by use of vermicomposts. *Proceedings of Brighton Crop Protection Conference – Pests and Disease* **8B-2**, 705-710.

Arancon, N.Q., Pant, A., Radovich, T., Hue, N.V., Potter, J.K. and Converse, C.E. (2012) Seed germination and seedling growth of tomato and lettuce as affected by vermicompost water extracts (teas). *HortScience* **47**, 1722-1728.

Atiyeh, R. M., Domínguez, J., Subler, S. and Edwards, C. A. (2000) Changes in biochemical properties of cow manure during processing by earthworms (*Eisenia andrei*, Bouché) and the effects on seedling growth. *Pedobiologia* **44**, 709-724.

Baker KF. (1970) Types of *Rhizoctonia* diseases and their occurrence. In: Parmeter Jr JR, editor. *Rhizoctonia solani*, biology and pathology. Berkeley, California: University of California Press; p. 125–48.

Bakker, M. G., Manter, D. K., Sheflin, A. M., Weir, T. L. and Vivanco, J. M. (2012) Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* **360**, 1-13.

Barocio-Ceja, N.B., Ceja-Torres, L.F., Morales-García, J.L., Silva-Rojas, H.V., Flo-res-Magallón, R. and Ochoa-Estrada, S. (2013) In vitro biocontrol of tomato pathogens using antagonists

isolated from chicken-manure vermicompost. *Phyton, International Journal of Ex-perimental Botany* **82**, 15-22.

Becker, J.O., Ohr, H.D., Grech, N.M., McGiffen, M.E.J. and Sims, J.J. (1998) Evaluation of methyl iodide as a soil fumigant in container and small field plot studies. *Pesticide Science* **52**, 56-62.

Benhamou, N., Rey, P. Picard, K. and Tirilly, Y. (1999) Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soilborne plant Pathogens. *Phytopathology* **89**, 506-517.

Bongers, T. (1990) The maturity index: an ecological measure of environmental disturbance based on nematode species composition. *Oecologia* **83**, 14-19.

Bongers, T. and Ferris, H. (1999) Nematode community structure as a bioindicator in environmental monitoring. *Trends in Ecology & Evolution* **14**, 224-228.

Bradley, C. A. (2008) Effect of fungicide seed treatments on stand establishment, seedling disease, and yield of soybean in North Dakota. *Plant Disease* **92**, 120-125.

Burkett-Cadena, M., Kokalis-Burelle, N., Lawrence, K. S., van Santen, E., Kloepper, J. W. (2008) Suppressiveness of root-knot nematodes mediated by rhizobacteria. *Biological Control* **47**, 55-59.

Byrd, D. W., Barker, K. R., Ferris, H., Nusbaum, C. J., Griffin, W. E., Small, R. H. and Stone, C. A. (1976) Two semi-automatic elutriators for extracting nematodes and certain fungi from soil. *Journal of Nematology* **8**, 206-212.

Cai, L., Gong X., Sun, X., Li, S. and Yu, X. (2018) Comparison of chemical and microbiological changes during the aerobic composting and vermicomposting of green waste. *PLoS One*, **13**, e0207494.

Campion, C., Chatot, C., Perraton, B. and Andrivon, D. (2003) Anastomosis groups, pathogenicity and sensitivity to fungicides of *Rhizoctonia solani* isolates collected on potato crops in France. *European Journal of Plant Pathology* **109**, 983-992.

Carrión, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., de Hollander, M., Ruiz-Buck, D., Mendes L. W., van Ijcken, W. F.J., Gomez-Exposito R., Elsayed, S. S., Mohanraju, P., Arifah, A., van der Oost, J., Paulson, J. N., Mendes, R., van Wezel, G. P., Medema, M. H., Raaijmakers J. M. (2019) Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science* **366**, 606-612.

Chaoui, H., Edwards, C.A., Brickner, A., Lee, S. and Arancon, N.Q. (2002) Suppression of the plant diseases, *Pythium* (damping-off), *Rhizoctonia* (root rot) and *Verticillium* (wilt) by vermicomposts. *Brighton Crop Protection Conference– Pests and diseases* **8**, 711-716.

Chaparro, J. M., Sheflin, A. M., Manter, D. K. and Vivanco, J. M. (2012) Manipulating the soil microbiome to increase soil health and plant fertility. *Biology and Fertility of Soils* **48**, 489-499.

Chen Y., Zhang, Y., Zhang, Q., Xu, L. and Li, R. (2015) Earthworms modify microbial community structure and accelerate maize stover decomposition during vermicomposting. *Environmental Science and Pollution Research* 22, 17161-17170.

Chitwood, D. J. (2003) Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture–Agricultural Research Service. *Pest Management Science* **59**, 748-753.

Chongtham, N., Bisht, M. S. and Haorongbam S. (2011) Nutritional properties of bamboo shoots: potential and prospects for utilization as a health food. *Comprehensive Reviews in Food Science and Food Safety* **10**, 153-168.

Craft, C. M. and Nelson E. B. (1996) Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by *Pythium graminicola*. *Applied and Environmental Microbiology* **62**, 1550-1557.

Daykin, M.E. and Hussey, R.S. (1985) Staining and histopathological techniques in nematology. Pp. 39-48 in Barker, K.R., Carter, C.C., and Sasser, J.N., eds. An advanced treatise on *Meloidogyne*, vol. II. Methology. Raleigh, NC: North Carolina State University Graphics.

Du, Z.Z. and Shen Y.M. (2016) A Rare New Cleistanthane Diterpene from the Pericarp of *Trewia nudiflora*. *Helvetica Chimica Acta* **89**, 2841-2845.

Ebrahimi, E., Werren, D. and Niemsdorff, P.V.F. (2018) Suppressive effect of composts from residual biomass on *Pythium ultimum*. *Journal of Plant Diseases and Protection*, **125**, 443-449.

Edwards, C.A., Arancon, N. Q. and Greytak, S. (2006) Effects of vermicompost teas on plant growth and disease. *Biocycle* **47**, 28-31.

Edwards, C.A., Arancon, N.Q., Emerson, E. and Pulliman, R. (2007) Suppression of plant-parasitic nematodes and arthropod pests with vermicompost tea. *Biocycle*, **48**, 38-39.

Elvira, C., Sampedro, L., Benitez, E. and Nogales, R. (1998) Vermicomposting of sludges from paper mill and dairy industries with *Eisenia andrei*: A pilot-scale study. *Bioresource Technology* **63**, 205-211.

Ersahin, Y.S., Haktanir, K. and Yanar, Y. (2009) Vermicompost suppresses *Rhizoctonia solani* Kühn in cucumber seedlings. *Journal of Plant Diseases and Protection*, **116**, 182-188.

FAO (2010) Global Forest Resource Assessment 2010. Rome, Italy: Food and Agriculture Organization of the United Nations. http://www.fao.org/docrep/013/i1757e/i1757e.pdf

Garg, P., Gupta, A. and Satya, S. (2006) Vermicomposting of different types of waste using *Eisentia foetida*: A comparative study. *Bioresource Technology* **97**, 391-395.

Ghini, R. and Morandi, M. A. B. (2006) Biotic and abiotic factors associated with soil suppressiveness to *Rhizoctonia solani*. *Scientia Agricola* **63**, 153-160.

Gopalakrishnan, S., Pande, S., Sharma, M., Humayun, P., Kiran, B.K., Sandeep, D., Vidya, M.S., Deepthi, K. and Rupela, O. (2011) Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of *Fusarium* wilt of chickpea. *Crop Protection* **30**, 1070-1078.

Hase, S., Shimizu, A., Nakaho, K., Takenaka, S. and Takahashi, H. (2006) Induction of transient ethylene and reduction in severity of tomato bacterial wilt by *Pythium oligandrum*. *Plant Pathology* **55**, 537-543.

Heald, C. M. (1975) Pathogenicity and Histopathology of *Rotylenchulus reniformis* Infecting Cantaloup. *Journal of Nematology* **7**, 149-152.

Huang, K., Li, F., Wei, Y., Chen, X. and Fu, X. (2013) Changes of bacterial and fungal community compositions during vermicomposting of vegetable wastes by *Eisenia foetida*. *Bioresource Technology* **150**, 235-241.

Hussey, R.S. and Barker, K.R. (1973) A comparison of methods of collecting eggs of *Meloidogyne* spp., including a new technique. *The Plant Disease Reporter*, **57**, 1025-1028.

Imaji, A., Ueda, M., Waguchi, Y., Tanaka, M., Uematsu, A., Kasuya, N. and Ikeda T. (2013) Effects of bamboo colonization on water relations of Japanese cedar (*Cryptomeria japonica*) and hinoki cypress (*Chamaecyparis obtusa*). *Nippon Shinrin-gakkaishi (J. Jpn. For. Soc.)* **95**, 145-146 [In Japanese with English Summary].

Ingham, R. E., Trofymow, J. A., Ingham, E. R. and Coleman, D. C. (1985) Interactions of bacteria, fungi, and their nematode grazers: Effects on nutrient cycling and plant growth. *Ecological Monographs* **55**, 119-140.

Isagi, Y., Kawahara, T., Kamo, K. and Ito, H. (1997) Net production and carbon cycling in a bamboo *Phyllostachys pubescens* stand. *Plant Ecology* **130**, 41-52.

Ishikawa, K., Takahashi, K., Hosoi, S., Takeda, H., Yoshida, H., Wakana, D., Tsubuki, M., Sato, F., Tojo, M. and Hosoe T. (2019) Antimicrobial agent isolated from *Coptidis rhizome* extract incubated with *Rhodococcus* sp. strain BD7100. *The Journal of Antibiotics* **72**, 71-78.

Jenkins, W.R. (1964) A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Disease Reporter* **48**, 692.

Ji, Z.L., Ma, D.S. and Miao, F.P. (2014) Chemical constituents from *Trichoderma longibrachiatum*, an endophytic fungus derived from marine green alga *codium fragile*. *Journal of Shenyang University* **26**, 277-280,319.

John, R. P., Tyagi, R. D., Prévost, D., Brar, S. K., Pouleur, S. and Surampalli, R. Y. (2010) Mycoparasitic *Trichoderma viride* as a biocontrol agent against *Fusarium oxysporum* f. sp. *adzuki* and *Pythium arrhenomanes* and as a growth promoter of soybean. *Crop Protection* **29**, 1452-1459.

Kajisa, T., Yoshida, S., Nagashima, K., Murakami, T., Mizoue, N., Sasaki, S., Kuwano, Y., Saho, K., Shimizu, M., Miyazaki, J., Fukuzato, K., Oda, M. and Shimozono, H.(2011) Situation of erosion, landslide, and limiting factors of vegetation recovery on abandoned clear-cut sites in Kyushu region. *Nippon Shinrin-gakkaishi (Journal of the Japanese Society for Horticultural Science)* **93**, 288-293 [In Japanese with English Summary].

Kameyama, A. (1978) Characterization of the kudzu colonies in vegetation successions on the highway slopes. *Journal of the Japanese Society of Revegetation Technology* **5**, 36-42.

Kim, D.S., Baek, N.I., Oh, S.R., Jung, K.Y., Lee, I.S., Hee, J. and Lee, H.K. (1997) Anticomplementary activity of ergosterol peroxide from *Naematoloma fasciculare* and reassignment of NMR data. *Archives of Pharmacal Research* **20**, 201.

Krzyczkowski, W., Malinowska, E., Suchocki, P., Kleps, J., Olejnik, M. and Herold, F. (2009) Isolation and quantitative determination of ergosterol peroxide in various edible mushroom species. *Food Chemistry* **113**, 351-355.

Lane, D.J. (1991) 16S/23S rRNA sequencing, p. 115–175. In E. Stackebrandt and, M. Goodfellow (ed.), Nucleic Acid Techniques in Bacterial Systematics. Wiley & Sons, Chichester.

Lee, Y. S. and Kim, K. Y. (2016) Antagonistic potential of *Bacillus pumilus* L1 against root-knot nematode, *Meloidogyne arenaria*. *Journal of Phytopathology* **164**, 29-39.

León, M., P. M. Yaryura, M. S. Montecchia, A. I. Hernandez, O. S. Correa, N. L. Pucheu, N. L. Kerber, A. F. García (2009) Antifungal activity of selected indigenous *Pseudomonas* and *Bacillus* from the soybean rhizosphere. *International Journal of Microbiology* ID 572049, 9 pages.

Lewis, J.A. & Lumsden, R.D. (2001) Biocontrol of damping-off greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. *Crop Protection*, **20**, 49-56.

Li, X., Xing, M., Yang, J. and Huang, Z. (2011) Compositional and functional features of humic acid-like fractions from vermicomposting of sewage sludge and cow dung. *Journal of hazardous materials* **185**, 740-748.

Martin, J. P. (1950) Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. *Soil Science* **69**, 215-232.

Misawa, T., Hagiwara, A., Ohhira, M. and Yamaguchi, T. (2016) Subgroup identification of *Rhizoctonia solani*, the causal agent of spinach foot root, and pathogenicity comparison of the pathogens with the isolates belonging to related subgroups to spinach. *Annual report of the Society of Plant Protection of North Japan* **67**, 94-99.

Mishra, S., Wang, K.-H., Sipes, B. S. and Tian M. Y. (2017) Suppression of root-knot nematode by vermicompost tea prepared from different curing ages of vermicompost. *Plant disease* **101**, 1-4.

Mishra, S., Wang, K.-H., Sipes, B.S. and Tian, M.Y. (2018) Induction of host-plant resistance in cucumber by vermicompost tea against root-knot nematode. *Nematropica* **48**, 164-171.

Morita, Y. and Tojo, M. (2007) Modifications of PARP medium using fluazinam, miconazole, and nystatin for detection of *Pythium* spp. in soil. *Plant Disease* **91**, 1591-1599.

Morris, K. A., Langston, D. B., Dutta, B., Davis, R. F., Timper, P., Noe, J. P. and Dickson, D. W. (2016) Evidence for a Disease Complex Between *Pythium aphanidermatum* and Root-knot Nematodes in Cucumber. *Plant Health Progress* **17**, 200-201.

Mu, J., Li, X., Jiao, J., Ji, G., Wu, J., Hu, F. and Li, H. (2017) Biocontrol potential of vermicompost through antifungal volatiles produced by indigenous bacteria. *Biological Control* **112**, 49-54.

Mulder, C., Cohen, J. E., Setälä, H., Bloem, J. and Breure, A. (2005) Bacterial traits, organism mass, and numerical abundance in the detrital soil food web of Dutch agricultural grasslands. *Ecology Lett*ers **8**, 80-90.

Nakhshiniev, B., Perera, C., Biddinika, M.K., Gonzales, H.B., Sumida, H. and Yoshikawa, K. (2014) Reducing ammonia volatilization during composting of organic waste through addition of hydrothermally treated lignocellulose. *International Biodeterioration & Biodegradation* **94**, 58-62.

Nguyen, V.N., Nguyen, D.M.C., Seo, D.J., Park, R.D. and Jung, W.J. (2009) Antimycotic activities of Cinnamon-derived compounds against *Rhizoctonia solani* in vitro. *BioControl* **54**, 697-707.

Nico, A.I., Jiménez-Díaz R.M. and Castillo, P. (2004) Control of root-knot nematodes by composted agro-industrial wasters in potting mixtures. *Crop Protection* **23**, 581-587.

Nishioka, T., Elsharkawy, M. M., Suga, H., Kageyama, K., Hyakumachi, M. and Shimizu, M. (2016) Development of culture medium for the isolation of *Flavobacterium* and *Chryseobacterium* from rhizosphere soil. *Microbes and Environments* **31**, 104-110.

Nishioka, T., Marian, M., Kobayashi, I., Kobayashi, Y., Yamamoto, K., Yamaki, H., Suga, H. and Shimizu, M. (2019) Microbial basis of *Fusarium* wilt suppression by Allium cultivation. *Scientific Reports* **9,** 1715.

Ogoshi, A. (1996) Introduction – the genus *Rhizoctonia solani*. In: Sneh B, Jabaji-Hare S, Neate SM, Dijst G (eds), *Rhizoctonia* species, Taxonomy, Molecular Biology, Ecology; Pathology and Disease Control. Kluwer, Dordrecht, 1-9.

Oka, Y. (2010) Mechanisms of nematode suppression by organic amendments – a review. *Applied Soil Ecology* **44**, 101-115.

Old Farmer's Almanac (2016) "Cucumbers: Planting, growing, and harvesting cucumbers". Yankee Publishing, Inc., Dublin, NH. 2016. http://homeguides.sfgate.com/soil-ph-cucumbers-73822.html

Øvreås, L. (2000) Population and community level approaches for analysing microbial diversity in natural environments. *Ecology Lett*ers **3**, 236-251.

Pandey, P., Chiu, C., Miao, M., Wang, Y., Settles, M., Rio N. S. D., Castillo, A. Souza, A., Pereira, R. and Jeannotte, R. (2018) 16S rRNA analysis of diversity of manure microbial community in dairy farm environment, *PLoS One*, **13**, e0190126.

Pangesti, N., Pineda, A., Pieterse, C. M. J., Dicke, M. and van Loon, J. J. A. (2013) Two-way plant-mediated interactions between root-associated microbes and insects: from ecology to mechanisms. *Frontier of Plant Science* **23**, https://doi.org/10.3389/fpls.2013.00414.

Pathma, J. and Sakthivel, N. (2013) Molecular and functional characterization of bacteria isolated from straw and goat manure based vermicompost. *Applied Soil Ecology* **70**, 33-47.

Pattnaik, S. and Reddy, M.V. (2012) Microbial characterisation of vermicompost and compost of urban waste processed by three earthworm species- *Eudrilus eugeniae*, *Eisenia fetida* and

Perionyx excavates. *International Journal of Environmental Technology and Management* **15**, 466-500.

Rao M.S., Kamalnath, M., Umamaheswari, R., Rajinikanth, R., Prabu, P., Priti, K., Grace, G.N., Chaya, M.K. and Gopalakrishnan, C. (2017) *Bacillus subtilis* IIHR BS-2 enriched vermicompost controls root knot nematode and soft rot disease complex in carrot. *Scientia Horticulturae* **218**, 56-62.

Radovich, T. J. K. and Arancon, N. (2011) Tea time in the tropics: A handbook for compost tea production and use. College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu, HI. 70 pp. ISBN: 978-1-929325-24-5.

Perry, R. N., Moens, M. and Starr, J. L. (eds.) (2009) Root-Knot Nematodes. CAB International, Wallingford, UK.

Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P. and Sghir, A. (2009) Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J* **3**, 700-714.

Roberts, B. L. and Dorough, H. W. (1984) Relative toxicities of chemicals to the earthworm *Eisenia foetida*. *Environmental Toxicology and Chemistry* **3**, 67-78.

Rodriguez-Saona, C., Musser, R.O., Vogel, H., Hum-Musser, S.M and Thaler, J.S. (2010) Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. *Journal of Chemical Ecology* **36**, 1043-1057.

Sahni, S., Sarma, B. K., Singh, D. P., Singh, H. B. and Singh, K. P. (2008) Vermicompost enhances performance of plant growth-promoting rhizobacteria in *Cicer arietinum* rhizosphere against *Sclerotium rolfsii*. *Crop Protection* **27**, 369-376.

Scheuerell, S. J., Sullivan, D. M. and Mahaffee, W. F. (2005) Suppression of seedling damping-off caused by *Pythium ultimum*, *P. irregulare*, and *Rhizoctonia solani* in container media amended with a diverse range of Pacific Northwest compost sources. *Phytopathology* **95**, 306-315.

Scheuerell, S.J. and Mahaffee, W.F. (2004) Compost tea as a container medium drench for suppressing seedling damping-off caused by *Pythium ultimum*. *Phytopathology* **94**, 1156-1163.

Shannon, C. (1948) A mathematical theory of communication. *Bell Labs Technical Journal* **27**, 379-423.

Simpson, E. H. (1949) Measurement of diversity. *Nature* **163**, 688.

Simsek-Ersahin, Y. (2011) The use of vermicompost products to control plant diseases and pests. In: Karaca, A. (Ed.), Biology of Earthworms. Springer, 191-213.

Singh, U.P., Maurya, S. and Singh, D.P. (2003) Antifungal activity and induced resistance in pea by aqueous extract of vermicompost and for control of powdery mildew of pea and balsam. *Journal of Plant Diseases and Protection*, **110**, 544-553.

Singhai, P. K., Sarma, B. K. and Srivastava, J. S. (2011) Biological management of common scab of potato through *Pseudomonas* species and vermicompost. *Biological Control* **57**, 150-157.

Southey, J.F. (1986) Laboratory methods for work with plant and soil nematodes. Ministry of Agriculture, Fisheries and Food reference book 402, London.

Spoel, S.H. and Dong, X. (2012) How do plants achieve immunity? Defense without specialized immune cells. *Nature Reviews Immunology* **12**, 89-100.

Szczech, M. and Smolińska, U. (2001) Comparison of suppressiveness of vermicomposts produced from animal manures and sewage sludge against *Phytophthora nicotianae* Breda de Haan var. *nicotianae*. *Journal of Phytopathology* **149**, 77-82.

Tajbakhsh, J., Goltapeh, E.M. and Varma, A. (2011) Vermicompost as a biological soil amendment. pp. 215-228 *in* Karaca A ed. Soil biology: Biology of Earthworms. Springer, Berlin-Heidelberg, Germany.

Takenaka, S. and Takahashi, H. (2012) Present status of studies on a resistance-inducing beneficial microbe, *Pythium oligandrum* in Japan. *Plant Protection* **66**, 332-336.

Taylor, A.L. and Sasser, J.N. (1978) Biology, Identification and Control of Root-knot Nematodes (*Meloidogyne* species). North Carolina State University Graphics, Raleigh, NC, USA.

Tojo, M., Fujita, Y., Awad, H. M. and Ichitani, T. (1993) Preparation of *Pythium* inocula using bentgrass seeds for glasshouse studies. *Proceedings of the Kansai Plant Protection Society* **35**, 1-5 [In Japanese with English Summary].

Vinale, F., Flematti, G., Sivasithamparam, K., Lorito, M., Marra, R., Skelton, B.W. and Ghisalberti, E. L. (2009) Harzianic Acid, an Antifungal and Plant Growth Promoting Metabolite from *Trichoderma harzianum*. *Journal of Natural Products* **72**, 2032-2035.

Waksman, S. A. and Fred, E. B. (1922) A tentative outline of the plate method for determining the number of microorganisms in the soil. *Soil Science* **14**, 27-28

Wang, B., Wei, W.J., Liu, C.J., You, W.Z., Niu, X. and Man, R.Z. (2013) Biomass and carbon stock in moso bamboo forests in subtropical China: characteristics and implications. *Journal of Tropical Forest Science*, **25**, 137-148.

Wang, K.-H. and McSorley, R. (2005) Effects of soil ecosystem management on nematode pests, nutrient cycling, and plant health. APSnet Features article. doi: 10.1094/APSnetFeatures/2005-0105.

Wang, K.-H., Radovich, T., Pant, A. and Cheng, Z.Q. (2014) Integration of cover crops and vermicompost tea for soil and plant health management in a short-term vegetable cropping system. *Applied Soil Ecology* **82**, 26-37.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C. and Aroian, R.V. (2003) *Bacillus thuringiensis* crystal proteins that target nematodes. *Proceedings of the National Academy of Sciences* **100**, 2760–2765.

Xiao, Z., Liu, M., Jiang L., Chen, X., Griffiths B.S., Li, H. and Hu, F. (2016) Vermicompost increases defense against root-knot nematode (*Meloidogyne incognita*) in tomato plants. *Applied Soil Ecology* **105**, 177-186.

Yasir, M., Aslam, Z., Kim, S.W., Lee S.W., Jeon, C.O. and Chung, Y.R. (2009) Bacterial community composition and chitinase gene diversity of vermicompost with antifungal activity. *Bioresource Technology* **100**, 4396-4403.

Yeates, G.W., Bongers, T., De Goede, R.G.M., Freckman, D.W. and Georgieva, S.S. (1993) Feeding habits in soil nematode families and genera- an outline for soil ecologists. *Journal of Nematology* **25**, 315-331.

You, X.D., Park, J.E., Takase, M., Wada, T. and Tojo, M. (2015) First report of *Pythium aphanidermatum* causing root rot on common ice plant (*Mesembryanthemum crystallinum*). New Disease Report, 32: 36

You, X.D. and Tojo, M. (2017) First report of soybean damping off caused by *Pythium aphanidermatum* and *P. coloratum* in Japan. New Disease Report 36: 14.

You, X.D., Tojo, M., Ching, S. and Wang, K.-H. (2018) Effects of vermicompost water extract prepared from bamboo and kudzu against *Meloidogyne incognita* and *Rotylenchulus reniformis*. *Journal of Nematology* **50**, 569-578.

You, X.D., Zhao, H. and Tojo, M. (2019a) First Report of *Pythium irregulare* Causing Damping-off on Soybean in Japan. *Plant Disease* **103**, 2696-2696.

You, X.D., Kimura, N., Okura, T., Murakami, S., Okano, R., Shimogami, Y., Matsumura, A., Tokumoto, H., Ogata, Y. and Tojo, M. (2019b) Suppressive effects of vermicomposted-bamboo powder on cucumber damping-off. *JARQ* **53**, 13-19.

You, X.D., Wakana, D., Ishikawa, K., Hosoe, T. and Tojo, M. (2019c) Antifungal Activity of Compounds Isolated from Bamboo vermicompost against *Rhizoctonia solani* AG1-IB. *Advances in Microbiology* **9**, 957-970.

You, X.D., Barraud, J. and Tojo, M. (2019d) Suppressive effects of *Pythium oligandrum* on soybean damping off caused by *P. aphanidermatum* and *P. myriotylum*. *Annual Report of The Kansai Plant Protection Society* **61**, 9-13.

SUMMARY

Vermicomposting is an agricultural recycling process for biodegradable solid waste that is facilitated by the decomposition and digestion of earthworms and associated microorganisms. The feedstocks that are commonly used for vermicomposting include animal manure and vegetable or fruit scraps from kitchens or farms. Many kinds of vermicompost are known to be used as a component of nursery potting media owing to their ability to control plant pathogens. Bamboos, especially moso-bamboos (*Phyllostachys edulis* (Carrière) J. Houz.), grow vigorously in the vicinity of populated areas in Japan, causing substantial damage to agricultural production and rural ecosystems. Since moso-bamboos are an abundant and sustainable resource throughout the world, they have the potential to be an ideal feedstock for vermicomposting for agricultural use. The bamboo wastes from abandoned forests converted into vermicompost can achieve a win-win result, including bamboo wastes can be reduce and recycled and the resulting vermicompost can be used as a promising alternative to chemical pesticide in suppressing plant pathogens. Based on this aspect, present study aimed to develop the vermicompost from moso-bamboo, to determine its suppressive effects on plant pathogens and plant parasitic nematodes, and to clarify its mechanisms on plant-disease suppressiveness. To improve the efficacy and reliability of disease control obtained, the inoculation of vermicompost with biological control agents was also evaluated.

CHAPTER 1. Production of moso-bamboo vermicompost and evaluation of its suppressive effects on cucumber damping-off

One-year-old shoots of moso-bamboo were ground into powder using a grinder and soaked in the 10 times-volume of tap water for 24 hours to remove the toxic compounds to earthworm. Ten kilograms of the wet bamboo powder was mixed with 100 g of pieces of kudzu vine (*Pueraria lobata* (Willd) Ohwi., < 10 cm in length) as a nitrogen source, along with 100 g of earthworms (*Eisenia fetida* Savigny). The mixture was placed in a plastic box covered with a lid, kept at 28 ± 2°C, and watered once a week to maintain moisture at approximately 80% (w/w). Samples were collected every 2 weeks after starting vermicomposting for vermicompost maturity assessment using the komatsuna-seed germination test. The plant germination and root elongation were found to increase gradually in response to vermicomposting duration from 2 to 8 weeks. The vermicompost after 8 weeks' incubation showed the highest germination index, approximately

100%.

The suppressive effects of vermicomposted bamboo (VB) on damping-off disease of cucumber was evaluated under greenhouse conditions, using autoclaved VB (aVB) and a commercial nursery medium (CNM) as controls. *Pythium aphanidermatum*, *Globisporangium ultimum* var. *ultimum*, and *Rhizoctonia solani* AG1-IB were used. The damping-off disease caused by these pathogens was significantly ($P < 0.05$) suppressed by VB as compared to aVB and CNM. The concentration denpendency of VB against cucumber damping-off was further determined by the above method with the following mixtures; VB (VB:CNM = 100:0 in volume), VB75 (VB:CNM = 75:25 in volume), VB50 (VB:CNM = 50:50 in volume), VB25 (VB:CNM = 25:75 in volume) and CNM (VB:CNM = 0:100 in volume). The suppressive effect of VB against cucumber damping-off was found to increase gradually in response to increasing concentrations of VB.

CHAPTER 2 Determination of the mechanisms for the suppressive effects of VB against cucumber damping-off pathogens

The suppressiveness of VB on these pathogens is thought to be mainly due to living microorganisms or their secondary metabolites, because the effect was nullified by the autoclave treatment. Thus, the microbial activity and the number of bacteria and fungi in VB was determined using the rate of hydrolysis of fluorescein diacetate (FDA), and the plate count technique, respectively. Autoclaved VB (aVB) and CNM were used as controls. Microbial activity and populations were significantly higher ($P < 0.05$) in VB than in aVB and CNM. The bacterial communities of VB, aVB and CNM were further examined using the next generation sequencing (NGS). The NGS analysis revealed that *Flavobacterium* was only presented in VB, but not in aVB and CNM. The relative abundance of genus *Bacillus* was significantly larger in VB than in aVB and CNM. *Bacillus amyloliquefaciens*, *B. pumilus*, *B. thuringiensis* and *Flavobacterium akiainvivens* were isolated from VB, and all of them showed inhibition effects on *R. solani* AG1-IB and *G. ultimum* var. *ultimum* mycelial growth. All the *Flavobacterium* spp. and *Bacillus* spp. suppressed the cucumber damping-off caused by *R. solani* AG1-IB significantly, but none of the bacterial strains suppressed the cucumber damping-off caused by *G. ultimum* var. *ultimum*. In addition, the NGS analysis revealed that *Flavobacterium* originally existed in earthworm intestinal, and *Bacillus* was from the VB substrates including moso-bamboo and kudzu.

Sterilized water extracts of VB (SWE), using a 0.22-μm cellulose acetate filter, significantly ($P < 0.05$) inhibited the mycelium growth of *R. solani* AG1-IB on a potato dextrose agar plate. This suggests that antifungal compounds are present in VB. The ethanol acetate (EtOAc) crude extracts of VB showing antifungal activity were further separated. Two compounds were isolated from the EtOAc fraction of VB and characterized as ergosterol peroxide (1) and (22E,24R)-5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol (2). Their chemical structures and mass spectra were determined by nuclear magnetic resonance and mass spectrometry analyses. Ergosterol peroxide tested at 150, 300, 600 and 900 μg showed 13, 22, 34 and 53% mycelial growth inhibition against *R. solani* AG1-IB, respectively. Because EtOAc crude extracts of the initial substrate materials of VB did not inhibit mycelium growth of *R. solani* AG1-IB, antifungal compounds in the vermicompost may be released by microbes but not from the original substrates during vermicomposting.

CHAPTER 3 Effects of vermicompost water extract prepared from moso-bamboo against *Meloidogyne incognita* and *Rotylenchulus reniformis*

A series of experiments in laboratory, greenhouse, and field were conducted to determine the nematode suppressive effect of VB against *Meloidogyne incognita* and *Rotylenchulus reniformis*. The water extract of VB, prepared at 1:10 dilution ratio of VB to water aerated over 24h, was used in the experiments due to its easier applying to crops in the field. The water extract of a vermicompost prepared from vegetable food waste (VV) and tap water were used as controls. Two laboratory trials were conducted by incubating eggs of *M. incognita* and *R. reniformis* in VB or VV over 1 week. These trials revealed that although both VB and VV suppressed *M. incognita* egg hatching compared to water control, only VB suppressed *R. reniformis* egg hatching. In addition, both VB and VV suppressed the mobility of second stage juveniles (J2s) of *M. incognita* equally compared to water control though suppression from VB performed inconsistently between the trials. When root penetration of *M. incognita* on cucumber drenched with VCT on one side of a split-root system in a greenhouse sterile sand-soil mix was examined, VB suppressed root penetration of *M. incognita* on the other side of the root in two trials, but VV was only effective in one trial. However, both VB and VV did not suppress *R. reniformis* root penetration. When the effects of the VB and VV were examined in two cowpea (*Vigna unguiculata*) field trials, drenching of VB or VV did not affect cowpea growth and yield, but VB reduced root-gall index

compared to the water control in both trials. Overall, performance of VB was more consistent than VV for plant-parasitic nematodes suppression.

CHAPTER 4 Suppressive effects of VB combined with *Pythium oligandrum* against damping-off disease caused by *Globisporangium ultimum* var. *ultimum*

The integrated use of biocontrol microorganisms with vermicompost has been documented as effective biocontrol agents for disease control in various host-pathogen systems as well as in field trials. *Pythium oligandrum* (PO) is an effective biological control agent of damping-off and root diseases caused by plant pathogens. The suppressive effects of a combination of VB with *P. oligandrum* on cucumber and soybean damping-off disease caused by *G. ultimum* var. *ultimum* was investigated. VB50+PO showed higher reduction in cucumber damping-off comparing with VB50 or PO alone. This effectiveness was comparable with the treatment of the conventional fungicide Tachigaren®. VB25+PO also caused higher of reduction in disease severity as compared with VB25 or PO alone. The propagule density of *P. oligandrum* was found to increase gradually by addition of VB from 0 day to 14 days in VB25 and VB50.

CHAPTER 5 General discussion and conclusions

In this study we are successfully converting moso-bamboo into vermicompost, which has the ability to suppress several plant pathogens and plant parasitic nematodes. The mechanisms of VB for the suppression are clearly defined in this study. The suppressive effects on *G. ultimum* var. *ultimum* are mainly attributed to the high activity and population of the rhizosphere microbiome, which limits the growth and activity of soil-borne pathogens, and consequently plant diseases. The suppressive effects on *R. solani* AG1-IB are mainly attributed to the specific bacteria, including antagonistic *Bacillus* and *Flavobacterium* species, and antifungal compounds released by microbes in the vermicompost. A biocontrol agent *P. oligandrum* was successfully introduced into VB to improve its biocontrol activity against plant pathogens. Present study demonstrated that the bamboo waste from abandoned forests can be recycled by converting to vermicompost, and has potential to use as an alternative of chemical pesticides for suppressing plant pathogens.

SUMMARY IN JAPANESE（要旨）

ミミズによる堆肥化は家畜し尿・敷きわらなど固体廃棄物リサイクルの有効な手段であり、様々な植物病害に抑制効果を示すことがこれまでに報告されている。日本のタケの主要種であるモウソウチクは、かつては食用や建材などに広く利用されていたが、生産者の高齢化によって現在その多くが放置され農地や森林崩壊の原因になっている。モウソウチクを植物病害に抑制効果をもつミミズ堆肥に変えて植物生産現場で利用すれば、植物病害の軽減と放置竹林の有効活用を同時に図ることができる。しかしタケ材をミミズ堆肥化したという報告はこれまでに見られず、その植物病害抑制効果については全く不明である。モウソウチク由来ミミズ堆肥を植物生産で利用するためには、その作出法を開発して植物病害に対する発病抑制効果を確かめるとともに、発病抑制効果に関わる要因を明らかにする必要がある。

そこで本研究では、モウソウチクから植物病害に抑制効果をもつミミズ堆肥を作出して植物生産に利用することを目的として、堆肥の作出方法の確立、植物病原糸状菌および植物寄生性線虫に対する抑制効果の評価、植物病原糸状菌への抑制に関わる生物的および化学的要因の特定、およびこの堆肥への生物防除微生物添加による病害抑制効果向上の検討を行った。

第 1 章　モウソウチク由来ミミズ堆肥の作出と植物病原糸状菌に対する抑制効果の評価

モウソウチクをパウダー状の細粉にした後に水道水に約 12 時間浸漬することにより、シマミミズがモウソウチクを食べて糞化することがわかった。シマミミズは水道水に浸漬しないモウソウチク細粉中では生存できなかった。モウソウチクからミミズに毒性を示す水溶性物質の存在が確認され、水道水への短時間の浸漬で除去可能であった。また、シマミミズがモウソウチク細粉中で長期間生存するためには、マメ科植物等による有機体窒素の供給も必要であることがわかった。これらの結果に基づき、水道水に一晩浸漬したモウソウチク粉末 10 kg（湿重）に窒素源としてのクズ乾燥茎葉 100 g を添加したものにシマミミズ 100 g を投入し、含水率約 80%、温度約 28℃に静置した。2 週間毎に堆肥の熟度をコマツナ発芽試験で調べた結果、シマミミズ投入の 8 週間目に完熟することがわかった。

この方法で作出したモウソウチク由来ミミズ堆肥（vermicomposted bamboo、以下 VB）を育苗土として用いた場合のキュウリ苗立枯病に対する抑制効果を調べた。その結果、市販育苗培土 commercial nursery medium、以下 CNM）に比べて VB では苗立枯病を起こす糸状菌3種（*Pythium aphanidermatum*、*Globisporangium ultimum* var. *ultimum* および *Rhizoctonia solani* AG1-IB）による発病が有意に抑制された。VB と CNM を、0、25、50、75 および 100% (v/v)になるように混合し、上記と同様に接種実験を行った結果、VB の発病抑制効果は濃度依存的に高くなることがわかった。

第 2 章　植物病原糸状菌に対する抑制効果の要因の解析

VB をオートクレーブしたもの（aVB）では発病抑制力が完全に消失したことから、VB の発病抑制効果には微生物やその代謝産物が関わっていることが示唆された。そこで VB 中の微生物の活性と密度を FDA 加水分解活性法と希釈平板法により aVB や CNM と比較した。その結果、VB の微生物活性と微生物密度は aVB や CNM に比べて有意に高かった。次に Miseq を用いたアンプリコンシーケンス解析により細菌叢を網羅的に調べた結果、VB にのみ *Flavobacterium* 属菌が存在し、また *Bacillus* 属菌が aVB や CNM に比べて高い割合で存在することがわかった。そこで *F. akiainvivens*、*B. amyloliquefaciens*、*B. pumilus* および *B. thuringiensis* の計 4 種について、植物病原糸状菌の活性と発病に及ぼす影響を検定した。植物病原糸状菌としてキュウリ苗立枯病菌 *Rhizoctonia solani* AG1-IB と *Globisporangium ultimum* var. *ultimum* を用いた。その結果、いずれの細菌株も *R. solani* AG1-IB に対して顕著な抗菌活性と発病抑制力を示した。一方で、*G. ultimum* var. *ultimum* に対してはいずれの細菌株も発病抑制力を示さなかった。*F. akiainvivens* と *Bacillus* 属菌3種の由来を明らかにするためにミミズ腸内および VB の原料であるモウソウチクとクズに含まれる細菌叢を網羅的に調べたところ、*F. akiainvivens* はミミズ腸内に、*Bacillus* 属菌3種はモウソウチクとクズに由来することがわかった。またこれらの *Bacillus* 属菌はミミズの腸内を通ることで約 100 倍に密度を増加させることがわかった。

次に VB 中に含まれる抗糸状菌物質について調べた。糸状菌として植物病原菌の *R. solani* AG1-IB を供試した。酢酸エチル等による抽出やペーパーディスク検定を経て VB 中の主要な抗糸状菌物質の成分を単離し構造解析を行った結果、VB の酢酸エチルエキスの 5 つの画分で抗糸状菌活性が確認された。これらの内の 1 つから主成分を単離して構造解析を行った結果、ergosterol peroxide とそのアナログと同定された。原料であるモウソウチク粉末の酢酸エチルエキスにはこのような活性が見られなかったことから、この物質はモウソウチクが堆肥される過程で産生されたと考えられた。

第 3 章　植物寄生性線虫に対する抑制効果の評価

植物寄生性線虫のネコブセンチュウとニセフクロセンチュウに対する VB の防除効果を、ササゲを用いた温室および圃場レベルで調べた。この実験では、圃場で施用しやすい水抽出液にして VB を供試した。比較として、野菜残渣ミミズ堆肥（vermicomposted vegetable waste、以下 VV）の水抽出液と水道水を用いた。評価項目は、1）二期幼虫への殺効果と卵の孵化抑制、2）線虫の根への侵入の抑制、および 3）ササゲの生長・収量の影響およびネコブ形成の抑制とした。その結果、VB と VV で殺線虫効果や卵の孵化抑制および線虫の根への侵入の抑制が見られた。VB と VV との比較では、ニセフクロセンチュウの卵の孵化で VB の方が高い抑制効果が見られたが、ネコブセンチュウの孵化では有意な差が見られなかった。また VB は両線虫の根侵入に対して VV より高い抑制効果を示したが、ネコブセンチュウの二期幼虫

に対する殺効果は VV よりも低かった。さらに VB と VV のいずれも、ササゲの生長・収量への影響が見られなかったが、VB ではササゲの線虫によるネコブ形成において、時期が異なる2つの試験で有意な抑制が見られた。このように VB と VV の両方で線虫に対する抑制効果が見られたが、VB の方が、試験の時期に関わらず安定した抑制を示した。これはモウソウチク由来ミミズ堆肥では材料が時期によらず均質であるのに対し、野菜残渣由来ミミズ堆肥では時期によって材料の種類や質が異なることが原因と考えられた。

第 4 章　生物防除微生物 *Pythium oligandrum* の添加による VB の発病抑制効果の向上

上述のように VB は植物病原糸状菌や植物寄生性線虫に対する抑制効果を示すことが明らかになったが、その効果は殺菌剤と比較すると低い。そこで VB の発病抑制効果の向上を目的として、苗立枯病を抑制する生物防除微生物として既に良く知られている *P. oligandrum* の添加の影響を調べた。VB を 25 および 50% (v/v)の比率になるように CNM に混合し、*P. oligandrum* の培養物を 1% (v/v)になるように添加した。これらを用いてキュウリおよびダイズを育成し、各植物の苗立枯病菌を接種して発病抑制効果を評価した。比較として標準的な殺菌剤を供試した。その結果、VB に *P. oligandrum* を添加した試験区では、VB や *P. oligandrum* の単独で処理した場合よりもキュウリ苗立枯病に対する発病抑制効果が高く見られ、さらに殺菌剤と同等のレベルに抑制効果が向上することがわかった。またダイズ苗立枯病に対しても *P. oligandrum* の添加による発病抑制効果の向上が確認された。育苗土中での *P. oligandrum* の菌密度を調べたところ、VB が一定割合で培土に存在する場合に有意な菌密度の増加が見られた。これらの結果から、VB は植物病原糸状菌や植物寄生性線虫を抑制するが、生物防除微生物の *P. oligandrum* に対してはその活性を促進することがわかった。

結論

本研究では、まずモウソウチク由来ミミズ堆肥の作出法を確立し、その植物病原糸状菌に対する抑制効果を確認した。そして、この抑制効果をもたらす主要な要因が、VB 中に多様かつ高密度の微生物が存在することによる栄養や生育場所をめぐる競合、*F. akiainvivens* や *B. amyloliquefaciens* 等の拮抗作用、および ergosterol peroxide などの抗菌活性物質の存在であることを明らかにした。また温室および圃場レベルで VB が 2 種の植物寄生性線虫に対して抑制効果を示すことも確認した。さらに生物防除微生物 *Pythium oligandrum* を VB に添加することによってキュウリ等の苗立枯病に対して、標準的な殺菌剤と同等のレベルにまで発病抑制効果が向上することを明らかにした。今後、VB の植物病害抑制力をさらなる圃場試験で評価することにより、有機栽培や減農薬栽培などを行う作物生産現場で VB を活用することが期待される。