Proceedings of International Symposium on Innovative Crop Protection for Sustainable Agriculture

2018

Date: March 7-8, 2018
Venue: 6th Floor, UGSAS Building, Gifu University, Japan

The United Graduate School of Agricultural Science, Gifu University

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Gifu University

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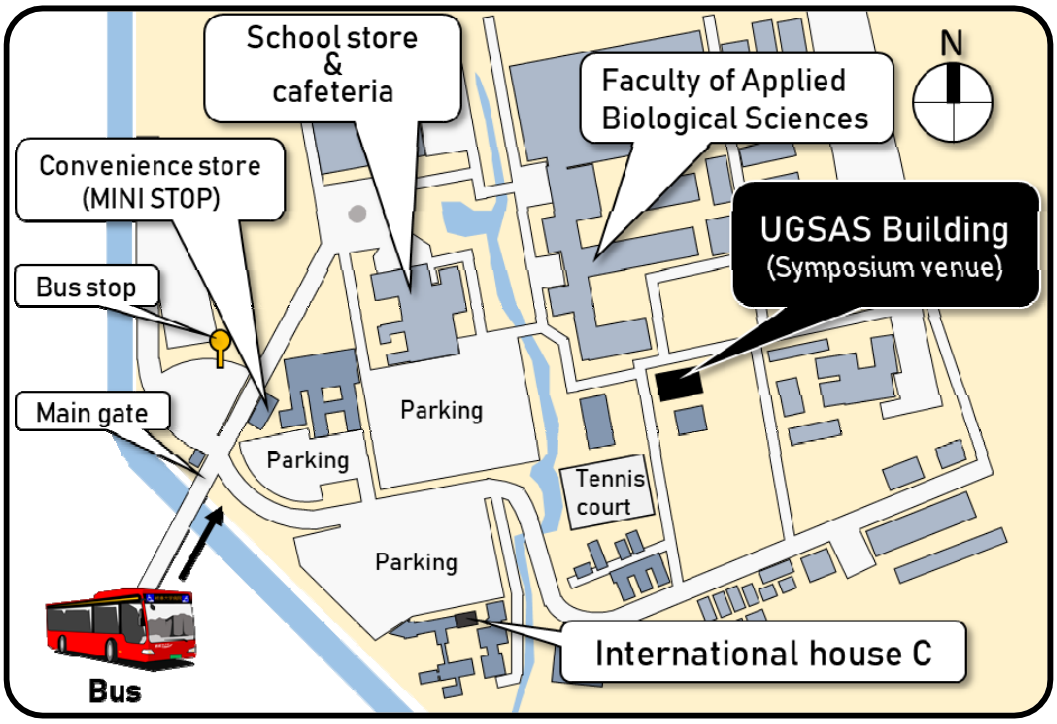
* This symposium is supported by IC-GU12.

General Information

Venue

The United Graduated School of Agricultural Science (UGSAS), Gifu University

1-1 Yanagido, Gifu 501-1193, Japan
Tel: +81-58-293-2984 (or, 2985)
E-mail: renno@gifu-u.ac.jp
Home page: http://www.ugsas.gifu-u.ac.jp/eng/



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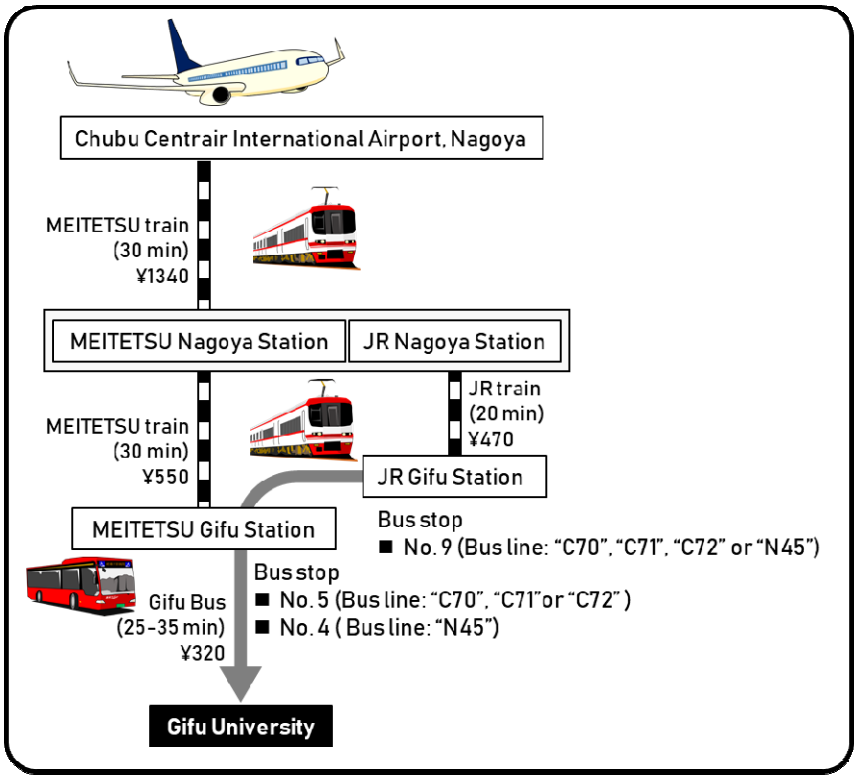

Daily schedule

March 7th (Wed)	9:30–10:00	Registration
	10:00–10:05	Opening remarks
		Dr. Masateru Senge (Dean of UGSAS, Gifu University)
	10:05–10:10	Welcome speech
		Dr. Fumiaki Suzuki (Executive Director and Vice President of Gifu University)
	10:10–10:30	Special guest speech
		Dr. Shirley C. Agrupis (President of Mariano Marcos State University)
	10:30–10:40	Photo session
	10:40–11:40	Poster presentation & free discussion
	11:40–12:10	Plenary lecture 1
	12:10–13:30	Lunch break & poster viewing
	13:30–14:00	Plenary lecture 2
	14:00–15:15	Oral session 1
	15:15–15:30	Coffee break& poster presentation
	15:30–17:10	Oral session 2
	17:40–19:00	Dinner meeting
March 8th (Thu)	9:30–10:00	Registration
	10:00–10:30	Plenary lecture 3
	10:30–11:45	Oral session 3
	11:45–12:45	Lunch break & poster viewing
	12:45–13:15	Plenary lecture 4
	13:15–14:55	Oral session 4
	14:55–15:10	Coffee break & poster presentation
	15:10–16:50	Oral session 5
	16:50–17:00	Closing remarks
		Dr. Kohei Nakano (Gifu University)

Plenary lectures (Main seminar room, 6th floor of UGSAS-GU Building)

PL-1 (March 7th, 11:40–12:10)

Chair: Dr. Haruhisa Suga (Gifu University)

Dr. Sotaro Chiba (Nagoya University)
"Farmer Field Schools leading to sustainable management of insect pests in Cambodian rice fields"

PL-2 (March 7th, 13:30–14:00)

Chair: Dr. Koji Kageyama (Gifu University)

Dr. Masafumi Shimizu (Gifu University)
"Endophytic *Streptomyces*: attractive biocontrol agents"

PL-3 (March 8th, 10:00–10:30)

Chair: Dr. Koji Kageyama (Gifu University)

Dr. Haruhisa Suga (Gifu University)
"Molecular characterization of *Fusarium fujikuroi* in Japan"

PL-4 (March 8th, 12:45–13:15)

Chair: Dr. Masafumi Shimizu (Gifu University)

Dr. Shigenobu Yoshida (National Agriculture and Food Research Organization)
"Perspective on the development of biopesticides applicable to both agricultural insect pests and disease"

Oral sessions –Day 1– (Main seminar room, 6th floor of UGSAS-GU Building)

OS I : Current status and management of crop diseases in Indonesia

March 7th, 14:00–15:15
Chair: Dr. Yuyun Fitriana (Lampung Univ.)

OS I-1 (14:00–14:25)	**Dr. Achmadi Priyatmojo** (Gadjah Mada University) "Current status and management of *Rhizoctonia solani,* the causal pathogen of sheath blight disease on rice and maize in Indonesia"
OS I-2 (14:25–14:50)	**Ms. Hanifah Ihsaniyati** (Sebelas Maret University) "Indonesian farmers problems in implementing integrated pest management (IPM)"
OS I-3 (14:50–15:15)	**Ms. Dwiwiyati Nurul Septariani** (Sebelas Maret University) "Taxonomical studies of blood disease bacterium of banana"

OS II : Plant probiotic bacteria

March 7th, 15:30–17:10
Chair: Dr. Md. Motaher Hossain (BSMRA Univ.)

OS II-1 (15:30–15:55)	**Dr. Tri Joko** (Gadjah Mada University) "Bacterial endophytes isolated from orchids and their influence on plant health"
OS II-2 (15:55–16:20)	**Dr. Radix Suharjo** (Lampung University) "Potential of endophytic bacteria as plant growth promoter and antagonist against pineapple-fungal plant pathogen in Indonesia"
OS II-3 (16:20–16:45)	**Dr. Hadiwiyono** (Sebelas Maret University) "Endophytic Bacillus as biological control agent of banana wilt"
OS II-4 (16:45–17:10)	**Dr. Md. Rashidul Islam** (Bangladesh Agricultural University) "Molecular based identification and formulation of cyanogenic *Pseudomonas* spp. controlling *Phytophthora infestans*"

Oral sessions –Day 2– (Main seminar room, 6th floor of UGSAS-GU Building)

OS III : Natural product-based pesticides and physical control measures

March 8th, 10:30–11:45
Chair: Dr. Tri Joko (Gadjah Mada Univ.)

OS III-1 (10:30–10:55)
Dr. Siti Subandiyah (Gadjah Mada University)
"Utilization of chitosan and glukomanan for fruit coating of chili againts antrachnose disease"

OS III-2 (10:55–11:20)
Dr. Pongphen Jitreerat (King Mongkut's University of Technology Thonburi)
"Antifungal effects of ethanolic shellac - Modified coconut oil (ES-MCO) combined with physical treatments against postharvest diseases of mango and mangosteen"

OS III-3 (11:20–11:45)
Dr. Kanlaya Sripong (King Mongkut's University of Technology Thonburi)
"Enhancing plant defense in mango fruit by hot water and UV-C treatments"

OS IV : Plant probiotic fungi

March 8th, 13:15–14:55
Chair: Dr. Achmadi Priyatmojo (Gadjah Mada Univ.)

OS IV-1 (13:15–13:40)
Dr. Moslama Aktar Maya (British American Tobacco Bangladesh Limited)
"Management of fusarium wilt in cyclamen plants using multiple soil microbes (AMF and *Piriformospora indica*)"

OS IV-2 (13:40–14:05)
Dr. Maria Viva Rini (Lampung University)
"Mycorrhizal oil palms seedlings response to different sources of *Ganoderma boninense* as the causal agent of basal stem rot disease"

OS IV-3 (14:05–14:30)
Dr. Purnomo (Lampung University)
"Potency of watery extract compost plus *Beauveria* sp. after storage for controlling planthopper and rice bug"

OS IV-4 (14:30–14:55)
Dr. Yuyun Fitriana (Lampung University)
"Low pH-tolerant mutant of *Trichoderma* spp. induced by EMS, gamma rays and UV irradiation"

Oral sessions –Day 2– (Main seminar room, 6th floor of UGSAS-GU Building)

OS V : Resistant cultivars

March 8th, 15:10–16:50

Chair: Dr. Pongphen Jitareerat (KMUTT)

OS V-1 (15:10–15:35)	**Dr. Abu Shamim Mohammad Nahiyan** (Advanced Seed Research & Biotech Centre) "Management of rice and wheat blast pathogen in Bangladesh"
OS V-2 (15:35–16:00)	**Dr. Triwidodo Arwiyanto** (Gadjah Mada University) "Control of eggplant and tomato bacterial wilt by grafting in Indonesia"
OS V-3 (16:00–16:25)	**Dr. Md. Motaher Hossain** (Bangabandhu Sheikh Mujibur Rahman Agricultural University) "Detection and characterization of Asia soybean rust in Bangladesh"
OS V-4 (16:25–16:50)	**Dr. Nandariyah** (Sebelas Maret University) "In vitro selection of sugarcane (*Saccharum officinarum* L) for Fusarium-pokah bung (Pb) resistance"

Poster session (Seminar room, 6th floor of UGSAS-GU Building)

P-1 Fumonisin production recovery in a *Fusarium fujikuroi* strain by complementation of *FUM21, FUM6* and *FUM7* genes
Sharmin Sultana, Hironori Kobayashi, Ryuou Yamaguchi, Masafumi Shimizu, Koji Kageyama, Haruhisa Suga

P-2 Genetic mapping of chromosome No.1 region associated with pathogenicity in Fusarium head blight pathogen
Rina Okumura, Maho Ikawa, Yuki Hirata, Masafumi Shimizu, Koji Kageyama, Haruhisa Suga

P-3 Isolation of plant probiotic *Bacillus* spp. from tea rhizosphere
Nusrat Ahsan, Tomoki Nishioka, Haruhisa Suga, Hiroyuki Koyama, Masafumi Shimizu

P-4 Microbial basis of Fusarium wilt suppression by *Allium*-cultivated soils
Tomoki Nishioka, Malek Marian, Haruhisa Suga, Masafumi Shimizu

P-5 Isolation of novel deoxynivalenol-degrading microorganisms from Poaceae planted soils
Hiroyuki Morimura, Sotaro Chiba, Daigo Takemoto, Kazuhito Kawakita, Ikuo Sato

P-6 Plant growth-promoting traits of rhizospheric *Flavobacterium* and *Chryseobacterium*
Fumiya Mizutani, Tomoki Nishioka, Haruhisa Suga, Koji Kageyama, Masafumi Shimizu

P-7 Establishment of global *Phytophthora* database for quarantine control
Ayaka Hieno, Mingzhu Li, Kayoko Otsubo, Haruhisa Suga, Koji Kageyama

P-8 Morphological and molecular identification of causal agent of cocoa pod rot disease in Indonesia
Masanto, Ayaka Hieno, Arif Wibowo, Siti Subandiyah, Masafumi Shimizu, Haruhisa Suga, Koji Kageyama

P-9 Biocontrol of tomato bacterial wilt using *Ralstonia* and *Mitsuaria* species
Malek Marian, Tomoki Nishioka, Hiroyuki Koyama, Haruhisa Suga, Masafumi Shimizu

P-10 Comprehensive evaluation of the resistance of root-stock-used *Cucumis melo* stock to *Meloidogyne incongnita*
Wanxue BAO

P-11 Population genetics analysis of *Phytopythium helicoides* in Japan
Auliana Afandi, Emi Murayama, Ayaka Hieno, Haruhisa Suga, Koji Kageyama

P-12 Study of a transcriptional regulator of plant pathogenic genes in a soft rot disease causing bacterium, *Dickeya dadantii*
Dina Istiqomah, Naoto Ogawa

Poster session (Seminar room, 6th floor of UGSAS-GU Building)

P-13 **Identification of freshness marker of stored soybean sprouts**
Syukri, D., Thammawong, M., Kuroki, S., Tsuta, M., Yoshida, M., Nakano, K.

P-14 **Studies on acetaldehyde tolerance system in the budding yeast using *myo*-inositol**
Annisyia Zarina Putri, Mizuho Inagaki, Masaya Shimada, Takashi Hayakawa, Tomoyuki Nakagawa

P-15 **Identification of bioaerosols from environmental samples in the AIST, Tsukuba, Japan**
Panyapon Pumkaeo, Wenhao Lu, Youki Endou, Tomohiro Mizuno, Junko Takahashi, Hitoshi Iwahashi

P-16 **The effect of persimmon (*Diospyros kaki*) on the prevention of sarcopenia**
Nayla Majeda Alfarafisa, Tomio Yabe

P-17 **Transcriptional biomarkers for managing pulse crop production in acid soil region**
Raj kishan Agrahari, Hiroyuki Koyama

P-18 **The accumulation of carotenoid in mango during fruit maturation**
W. Yungyuen, T.T. Vo, G. Ma, L.C. Zhang, P. Jitareerat, A. Uthairatanakij, M. Kato

P-19 **Augmented nuclease resistance and gene silencing with 3'-end modified small interfering RNAs and dendrimer based drug delivery**
Akash Chandela, Yoshihito Ueno

P-20 **Protein-based functional analysis of renin and (pro)renin receptor genes in hypertensive and diabetic Bangladeshi population: Pursuing the environment-induced molecular traits**
Jobaida Akther, A. H. M. Nurun Nabi, Tsutomu Nakagawa, Fumiaki Suzuki, Akio Ebihara

P-21 **Proposals for countermeasures to reduce risk of hydraulic fracturing adjacent to culvert – A case study**
Duy Quan Tran, Shinichi Nishimura, Masateru Senge, Tatsuro Nishiyama, Fumitoshi Imaizumi

P-22 **Droughts hotspot distribution by long term assessment the Standardized Precipitation Index (SPI) in Indonesia**
Yudhi Pramudya, Takeo Onishi

P-23 **The role of floral volatiles for attracting pollinators and reproductive isolation in *Mimulus* species**
Muhammad Arifin, Tomoko Okamoto

Plenary Lecture Abstracts

Farmer Field Schools leading to sustainable management of insect pests in Cambodian rice fields

Sotaro CHIBA[1,2], Chhay NGIN[2,3], Seng SUON[4], Toshiharu TANAKA[1,2], Editha C CEDICOL[2], Akira YAMAUCHI[1,5], Kazuhito KAWAKITA[1]

([1]Graduate School of Bioagricultural Sciences, Nagoya University, [2]Asian Satellite Campuses Institute, Nagoya University, [3]General Directorate of Agriculture, Ministory of Agriculture, Forestry and Fisheries of Cambodia, [4]Center for Development-Oriented Research in Agriculture and Livelihood Systems, [5]International Cooperation Center for Agricultural Education, Nagoya University)

SUMMARY

Agro-chemicals has brought benefit in rice productivity and management, especially for easy control of insect pests by intensive treatments of insecticides. However, undesired side-effects of agro-chemicals are appeared problematic: environmental degradation, elimination of natural biocontrol agents, poisoning pesticide applicators, and declined food safety. This study demonstrates effectiveness of Farmer Field School (FFS) as a farmer education system for implementing Integrated Pest Management (IPM) in Cambodian rice fields, to accomplish improved profitability by reducing insecticide cost and to sustain field natural enemies for insect pest control. Field observation leveled an intriguing fact that most of Cambodian rice fields are rich enough in natural enemy population to autonomously control insect pest. The IPM-FFS also contributed to decrease farmers' exposure risk to toxic chemicals. This paper is based on the thesis of Dr. Chhay Ngin who had studied under the Transnational Doctoral Program for Leading Professionals in Asia, Nagoya University Asian Satellite Campuses Institute.

Introduction

Rice is the staple food of Asia and is central to the food security of about half of the world's population. Rice production is an important source of livelihood for approximately 140 million rice-farming households. As Cambodia's staple food crop, rice has provided income to majority of the rural population and has contributed to the national economy. It is predominantly grown on more than three million hectares comprising 75% of the agricultural land in Cambodia.

Major challenges include the need to produce more rice to meet the rising demand from world population growth that is expected to reach 9.6 billion in 2050; environmental degradation; decline in rice biodiversity; climate change and increasing competition for land, labour and water. Increase in crop yield has often been attained through the exhaustive application of pesticides and fertilizers. However, the indiscriminate use of these chemicals damages the environment and human health while jeopardising agricultural production.

For increasing crop production profitability while reducing the risks from pesticides to humans and the environment, new ways need to be explored. One of the ways to achieve this, we bereave, is by implementing suitable Integrated Pest Management (IPM) practices to reduce reliance on pesticide use and moving towards food production methods based on the effective use of ecosystem services that are regenerative and that minimize negative impacts. Such an implementation of IPM in Cambodian farmers has been attempted by the government-leading farmers' education program called 'Farmer Field School' (FFS).

In this study, we assessed impacts of IPM-FFS in terms of natural enemy populations in the rice fields, rice farmers' profitability (cost effectiveness), and reduction in farmers' risk of exposure to toxic pesticides. To this end, three independent but co-related subjects were established and conducted as follows: 1) investigation on effect of insecticide-treatments in rice filed on arthropod population and on economic balance, 2) elucidating effectiveness of technology intervention by FFS-training on rice production, cost performance, and farmers' profitability, and 3) unveiling long-term impact of FFS by interviewing FFS-participants whether they retain their capability developed during FFS, those briefly schematically represented in Fig. 1.

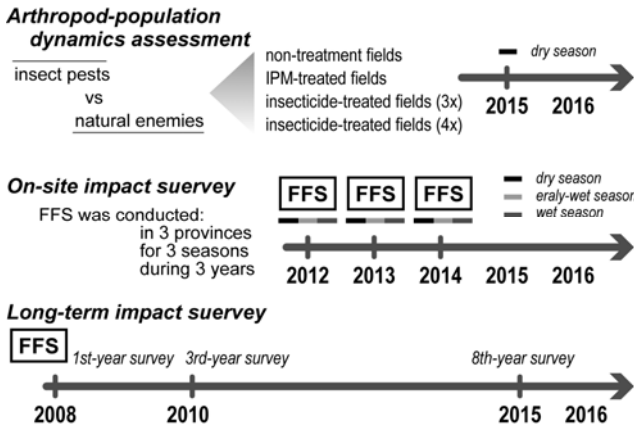

Fig. 1 Research scheme of three independent but related studies.

Rich natural enemy population controlling insect pests in Cambodian rice fields

In three provincial research stations, IR66 rice variety was planted during dry season (January-April) in 2015 with either of treatments: non-treatment (control), IPM-treatment, and insecticide- (Cypermethrin; 2 L/ha) treatments with 3- or 4-times spray. Then major pest insects (caseworms, stem borers, leaffolders, gall midges, brown planthoppers, and rice bugs) and natural enemies (spiders, dragonflies/damselflies, lady beetles, ground beetles/earwigs, long-horned grasshoppers/crickets, mirid bugs, and water bugs) were each counted weekly from 15 to 67 days after transplanting (DAT) (Fig. 1).

In brief, total numbers of insect pests were always below that of natural enemies, and rice productivities were at similar levels between non-/IPM-treated plots and insecticide-treated plots regardless of spray times in two provinces. An incident of caseworm was occurred and damaged rice plants in another province, and this decreased production compared with those in insecticide-treated plots. However, IPM-based coping to caseworm successfully controlled the damage and gained equivalent harvest with insecticide-treated plots. Overall, representative rice fields in Cambodia are covered by rich population of natural enemies and pest insects are under autonomous control of them. Even though some pest outbreak occurs, an appropriate IMP-treatment can manage this and sustain rice production. Nevertheless, saving the cost for insecticides directly reflected higher profitability in non-/IPM-treated plots than in insecticide-treated plots. This encouraging fact highlights the benefit of conservation of natural enemies in Cambodian rice fields, as classically stated this important natural resource and function 'ecosystem services.'

IPM practice directed in Cambodian FFSs

The FFS is used as an extension tool to facilitate farmers' skill on IPM and modern agriculture. In FFS, farmers learn about knowledge-intensive management techniques/approaches with on-site participatory learning style. In Cambodia, the first implementation of FFS was in 1996. While it was said that the FFS program in Cambodia has been successfully improved farmers, there was no scientific evidence provided before. Thus, the study assessed effectiveness of IPM–FFS training on the production efficiencies (yields and profitability) of rice farming in three provinces by comparing the technically recommended practices (TRs) with the traditional farmer practices (FPs). Data from 270 randomly selected FFSs, operated during dry, early-wet, and wet seasons in 2012-2014 were used for statistical analyses (Fig. 1).

In general, TR-plot received higher rice yield per ha than FP-plots in all provinces, seasons and years, and it showed reverse trends for total expenses. This resulted in greater net profit in TR-plot, i.e., maximum difference between TR- and FP-plots was 399 USD/ha in dry season (785 vs 386, USD/ha). The cost factors that FP-plots expensed more were seeds (15-45 USD/ha difference) and pesticide (22-42 USD/ha dif.), and TR-plots conversely fairly did for fertilizers (5-13 USD/ha dif.). To determine cost factors contributing to rice yields in TR-plots, multiple regression analysis was performed, and it revealed that the costs for field management (land leveling, watering, etc.) and fertilizer application (fertilizer and labour hiring costs) were significantly contributed, but not pesticide application costs. Thus, together with right management of fields and fertilizers, the IPM-based insect pest control contributed to gain higher profit. These results suggested that FFSs conducted in Cambodia 2012-2014 were effective enough to convincingly encourage participants for applying IPM technology in their own farms.

IPM practices taken root in Cambodian FFS-participants

The development of a sustainable agricultural program is a challenging task. Although abovementioned FFS program in Cambodia was effective to demonstrate a merit of IPM and modern agricultural practice to farmers, whether this leads to capacity building of farmers has not been evaluated since the first introduced of IPM-FFS in Cambodia in 1996. As poisoning of farmers by toxic pesticides have long been serious concern, the IPM-FFS

is expected to be one of solutions which guide farmers to reduce pesticide use and protect themselves as well. In this study, we examined short-term (2-year, 2010) and long-term (7-year, 2015) impacts of an FFS in Prey Veng province, by comparing baseline data obtained during FFS in 2008 (Fig. 1). To this end, three farmers groups (30 farmers each) were interviewed and comparably analyzed: FFS-trained farmers, non-trained farmers in the same village and in a distant village where FFS has never been implemented.

By looking at trends of farmers' behavior on protection of themselves from toxic pesticides, all farmers groups showed significant increases in the rate of using protective ware (groves and boots; head-protective items are almost 100% used from the beginning). Likewise, rate of farmers adopting the practices of burying/burning pesticide waste, the rate of pesticide applicators experienced the pesticide poisoning, and calculated potential risk of pesticide applicators were also commonly decrease year by year. Of note is, these four parameters were always better in FFS-trained farmers than non-trained farmers in the same or outer village, with one exception of FFS-trained farmers that experienced poisoning at baseline survey. This exception might come from fear to pesticide toxicity after taking education in the FFS.

A remarkable difference was observed in the amount of total pesticide use. That is, while FFS-trained farmers had a reduced pesticide usage over the short-term and continued to reduce their usage over the long-term, two control farmers groups had a reduced pesticide usage over the short-term but showed an increased usage at the long-term survey. In the meantime, FFS-trained farmers sustained greater net profit which gradually increased 2008-2015, seemingly by decreased pesticide usage. Therefore, working towards a non-toxic environment is a viable option for achieving sustainable production, maintaining a healthy environment, protecting human health and attaining income security for rice producers in Cambodia.

Concluding remarks

IPM provides a knowledge-intensive integrated crop and pest management approach, whereas FFS training provides opportunities for farmers to learn and develop their technical capacity and informed decision-making skills to become local experts to build economically viable and environmentally friendly crop management techniques based on the field realities. In this study, we evidenced that the IPM-FFS is one of the best educational paradigms for improving rice production in Cambodia in a sustainable way which includes technical, economic, social and environmental aspects. This combined approach can be empowered by appropriate strategy and conducive policy on ecology-based agriculture production. We strongly hope that the Cambodian government pays attention to the research outcome and leads to a more sustainable and cost-effective rice production, a risk reduction to public health in terms of reduced toxic residues in food, an improvement in field biodiversity and marketability of the products contributing to enhanced rice productivity, an improvement in the farmers' livelihoods and, eventually, acceleration of national economic growth.

Acknowledgment

This study is supported in part by Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA, Philippines), the Transnational doctoral program for leading professionals in Asian countries (Nagoya University ASCI), and a grant-in-aid for Scientific Research (A) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (KAKENHI). We express sincere thanks to Dr. Buyung Hadi (IRRI, Philippines), Mr. Jan Willem Ketelaar (FAO, Thailand), Dr. Ikuo Sato, Dr. Daigo Takemoto (Nagoya University), and Provincial IPM trainers (Cambodian National IPM Program) for their kind support and fruitful discussions.

Related articles

Ngin C, Suon S, Tanaka T, Yamauchi A, Cedicol CE, Kawakita K, Chiba S. (2017) Rice productivity improvement in Cambodia through application of technical recommendation in Farmer Field School. International Journal of Agricultural Sustainability Vol. 15 (1), 54-69.

Ngin C, Suon S, Tanaka T, Yamauchi A, Kawakita K, Chiba S. (2017) Effects of mechanical defoliation and detillering at different growth stages on rice yield in dry season in Cambodia. International Journal of Agriculture and Environmental Research 3 (4), 3452-3470.

Ngin C, Suon S, Tanaka T, Yamauchi A, Kawakita K, Chiba S. (2017) Impact of insecticide applications on arthropod predators and plant feeders in Cambodian rice fields. Phytobiomes journal (in press, published on-line ahead of print: https://doi.org/10.1094/PBIOMES-01-17-0002-R)

Ngin C, Suon S, Chou C, Tanaka T, Kawakita K, Chiba S. Long term impact of Farmer Field School on pesticide reduction and productivity improvement in Cambodian rice cropping. (submitted)

Endophytic *Streptomyces*: Attractive biocontrol agents

Masafumi SHIMIZU

(Faculty of Applied Biological Sciences, Gifu University)

Abstract

Almost all terrestrial plants are known to harbor large and diverse microbial communities within their bodies. Microbes that have an ability to endophytically colonize plant tissues play important roles in the growth and health of their host plants. Recent metagenomics analysis revealed that actinobacteria, particularly the genus *Streptomyces*, are one of the most abundant groups in the endophytic bacterial communities present in a wide variety of plant species. *Streptomyces* species are ubiquitous in the natural environment, particularly in soils, and are well-known producers of bioactive secondary metabolites, including antimicrobials, that are effective against plant pathogens. Therefore, since a long time, antagonistic *Streptomyces* species have often been isolated from soils and used for the biocontrol of soil-borne diseases. Over the last two decades, the global attention on the biocontrol of plant diseases using mutualistic endophytic *Streptomyces* has been increasing. Although the importance of a vast majority of the endophytic *Streptomyces* species in plant growth, development and ecological fitness is still unclear, some of them have been found to have a great potential to defend host plants from pathogens. We isolated and characterized several endophytic *Streptomyces* species that can effectively suppress various plant diseases.

In this lecture, the speaker will describe an example of the biocontrol of plant diseases using our isolate of endophytic *Streptomyces* and its possible mode of action.

Molecular characterization of *Fusarium fujikuroi* in Japan

Haruhisa SUGA

(Life Science Research Center, Gifu University)

SUMMARY

Rice bakanae is a serious plant disease in rice growing country. *Fusarium fujikuroi* is known as a fungal pathogen of the rice bakanae disease and also as an important mycotoxin producer such as fumonisins. This fungal species has been isolated not only from rice but also from other crops. We obtained 95 strains of *F. fujikuroi* most of which were from various source in Japan and performed phylogenetic analyses based on AFLP finger printing pattern to demonstrate genetic relationship of individual strains. *F. fujikuroi* strains used in this study were divided into two genetically different groups and difference of fumonisin and gibberellin producibility was observed between them. These result indicates *F. fujikuroi* comprises two different hazardous groups in the sense of plant pathogens and food safety.

Introduction

Rice is one of the most important grains and approximately 480 million tons of rice is produced in the world every year. Rice bakanae disease occurs in almost all rice growing area. Diseased rice are slender, a pale yellowish color leaves bending and taller than undiseased plants.

Fusarium fujikuroi, a fungal pathogen of the rice bakanae disease, is one of the species in *Fusarium fujikuroi* species complex (*Ff* complex). *Ff* complex includes more than 50 phylogenetical species, in which thirteen independent mating populations (MPs) have been discovered and MP-C corresponds to *F. fujikuroi*. *F. fujikuroi* produce a large variety of secondary metabolites such as bikaberin, fusaric acid and fusarbin. Among them, gibberellins and fumonisins have been intensively studied. Gibberellin currently well known as a plant hormone was originally discovered as a virulence factor of *F. fujikuroi* to rice. Clustering of seven genes involving gibberellin biosynthesis has been revealed in *F. fujikuroi* (GIB cluster) (Wiemann et al. 2013). A similar cluster has been detected from other species in *Ff* complex though many genes are putatively inactive and actual gibberellin production was observed only in several strains belong to *Fusarium proliferatum*, *Fusarim konzum* and *Fusarim sacchari* (Studt and Tudzynski 2014)

Fumonisin, a polyketide mycotoxin, is associated with human esophageal cancer in South Africa and causes leucoencephalomalacia in horses and lung edema in swine. *F. fujikuroi* that is frequently isolated from rice is known to produce fumonisin though its closely related species *Fusarium verticillioides* is the predominant fungal contaminant in the corn in the world. Basically, fumonisin production can be detected in all *F. verticillioides* strains while in case of *F. fujikuroi*, fumonisin production can be detected only in some strains (Suga et al. 2014). In this study, strains that do not produce detectable level of fumonisins under *in vitro* culturing are tentatively referred to as fumonisin non-producers. Clustering of sixteen genes involving fumonisin biosynthesis has been revealed in *F. verticillioides* (FUM cluster) and a similar cluster was detected in the whole genome sequence of *F. fujikuroi* (Wiemann et al. 2013). However, the FUM cluster was detected not only in fumonisin producers but also in non-producers. Therefore, fumonisin production of non-producers of *F. fujikuroi* is still ambiguous and genetic elucidation of fumonisin non-production is required for accurate assessment of fumonisin producibility in *F. fujikuroi*.

Material and Method

Phylogenetic analysis

AFLP Microbial Fingerprinting kit was used for obtaining binary matrix for phylogenetic analysis. Binary data of totally 66 AFLP markers from 96 taxa including an outgroup, *F. proliferatum* MAFF236459 and SUF1207 were compiled into a single data matrix. Neighbor-joining analysis was performed with PAUP. The strength of the internal branches from the resulting tree was tested by bootstrap analysis with 1,000 replications.

Gibberellin analysis

The strains were grown in 10 % ICI medium for 7 days on a reciprocal shaker at 25 °C. GA_3 and $GA_4/_7$ in culture filtrate were analyzed with a TLC silicagel 60F254 plate. GA_1, GA_3, GA_4 and GA_7 concentrations was quantified by LC-MS/MS analysis using an ACQUITY UPLC^{TM} system coupled to a Xevo QTof mass spectrometer. Ionization was conducted with ESI probe in negative polarity.

Fumonisin analysis

The strains were grown in sterile cracked maize seeds at 25 °C for 10 days. Fumonisin in cracked maize cultures were extracted with methanol:water (3:1, v/v). A ELISA using RIDASCREEN®FAST Fumonisin kit (R-Biopharm, Darmstadt, Germany) was performed and fumonisin concentrations were determined on the basis of standard curves made with authentic fumonisin (0.2 to 6.0 ppm), as provided with the kit. Fumonisin purification was performed by a strong anion exchange column before LC-MS/MS analysis. FB_1, FB_2 and FB_3 concentrations was quantified by LC-MS/MS analysis. Ionization was conducted with ESI probe in positive polarity.

Crossing

Crossing progenies between a F-group strain Gfc0825009 (MAT 1-1 type) and a G-group strain Gfc0801001 (MAT 1-2 type) was obtained. They were transplanted to sterilized rice straws in a beaker. Perithecthicia developed in two weeks later were thoroughly rinsed by sterile water. Ascospores discharged on a glass slide were collected by several hundred μl of sterile water and spread to a MMTS medium.

Linkage analysis

SNPs between Gfc0825009 and Gfc0801001 were obtained by PCR and sequencing for *CPR* , *P450-4* in GIB cluster, *FUM1* and *FUM18* including *FUM18* / *FUM19* intergenic region in FUM cluster. Data of five SNP markers (TEF_T618G in Suga et al. 2014, CPR_C1152A, P4504_C842T, FUM1_G423A, FUM18_G51T) were obtained by allele-specific primer extension reactions. Reaction products were finally detected with a Luminex 100 flow cytometer for determination of SNPs. MAT type data was obtained by PCR.

Result and Discussions

Phylogenetic tree

In order to investigate intraspecific differentiation of *F. fujikuroi*, phylogenetic analysis of 95 strains based on AFLP markers was conducted. *F. fujikuroi* strains divided into two phylogenetic groups. Fumonisin production was detected only from one group by ELISA and we designated it as F-group (Fig. 1).

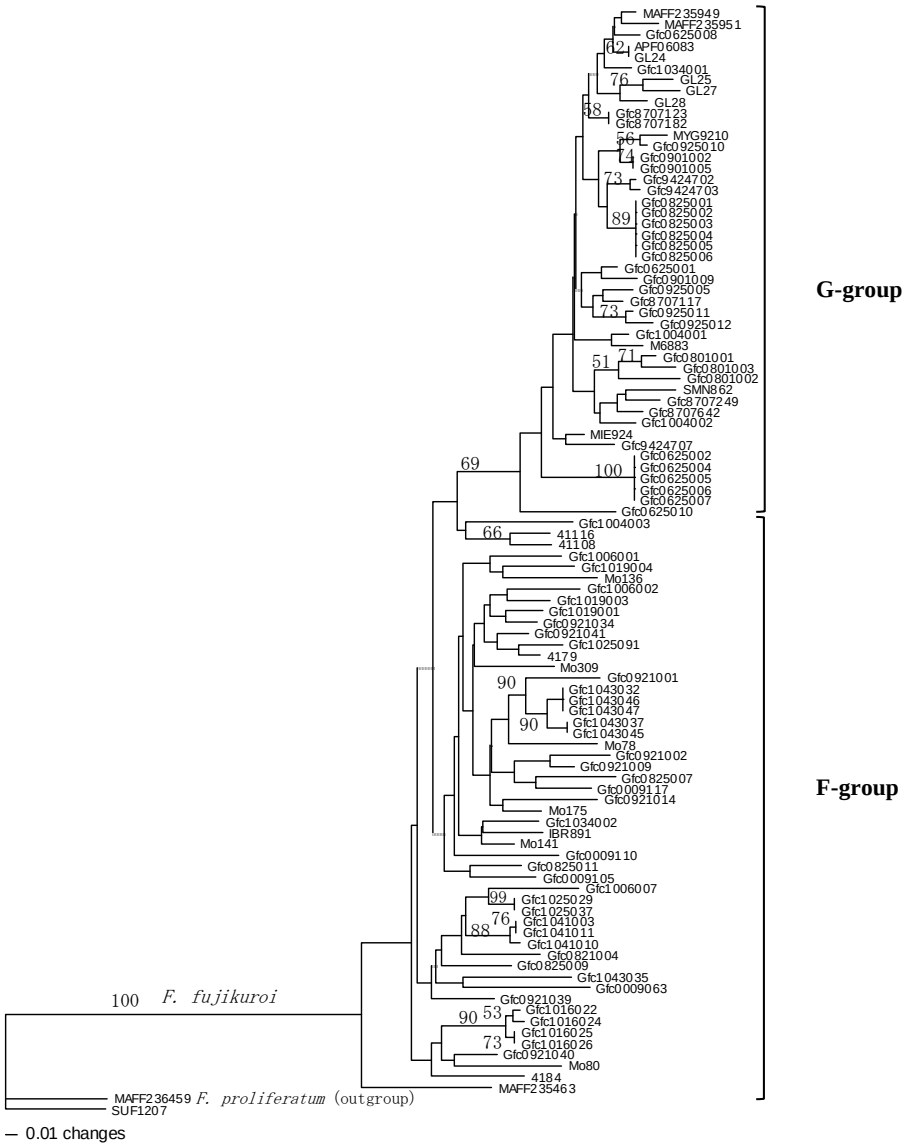

Fig. 1 Phylogenetic tree of *F. fujikuroi* based on 66 AFLP markers (NJ method)

Gibberellin producibility

All 21 strains from bakanae diseased rice were fumonisin non-producers (Suga et al 2014) and, therefore, gibberellin production of 95 stains was investigated by *in vitro* culturing. Gibberellin was not detected in F-group by TLC, while it was detected from all the strains belonging to another group.We designated it as G-group (Fig. 1).

LC-MS/MS analyses of a representative strain of G-group and F-group

Preliminary results of fumonisin and gibberellin producibility obtained by ELISA and TLC were further confirmed by LC-MS/MS analyses using strain Gfc0801001 and strain Gfc0825009 as a representative of G-group and F-group, respectively.

As for fumonisins, FB_1 (182.2 ppm), FB_2 (121.5 ppm) and FB_3 (3.7 ppm) were detected from the F-group strain Gfc0825009 but they are undetectable level in the G-group strain Gfc0801001. As for gibberellins, GA_3 (37.8 ppm), GA_7 (2.0 ppm), GA_4 (1.2 ppm) and GA_1 (1.2 ppm) were detected from the G-group strain Gfc0801001. Though GA_3 and GA_7 /GA_4 spots were not detected from the F-group strain Gfc0825009 by TLC, low level of GA_3 (2.2 ppm), GA_7 (0.4 ppm) were detected from this strain.

Based on these results, it was considered that F-group has fumonisin producibility / gibberellin low producibility and G-group has lack of fumonisin producibility / gibberellin high producibility.

Linkage analysis

In order to reveal the cause of gibberellin low producibility in the F-group strain Gfc0825009 and the lack of fumonisin producibility in the G-group strain Gfc0801001, linkage analysis was conducted using crossing progenies between Gfc0825009 and Gfc0801001. Complete linkage was observed between a SNP marker (P4504_C842T) in the GIB cluster and gibberellin low producibility (Table 1). The lack of fumonisin producibility showed complete linkage to the SNP markers (FUM1_G423A and FUM18_G51T) in the FUM cluster (Table 1).

Conclusion

F. fujikuroi strains divided into F-group with fumonisin producibility / gibberellin low producibility and and G-group with the lack of fumonisin producibility / gibberellin high producibility. Linkage analysis indicated that gibberellin low producibility attributes to the GIB cluster in Gfc0825009 and the lack of fumonisin producibility attributes to the FUM cluster in Gfc0801001.

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Table 1 Result of SNP analyses of a part of 42 progenies between Gfc0825009 and Gfc0801001

Progeny	SNP data					MAT type	GA_3 prodetection by TLC	Fumonisin detection by ELISA
	TEF_T618G	CPR_C1152A	P4504_C842T	FUM1_G423A	FUM18_G51T			
Gfc0825009 (parent)	T	C	C	G	G	1-1	-	+
Gfc0801001 (parent)	G	A	T	A	T	1-2	+	-
Gfc①CP91002	G	A	C	G	G	1-2	-	+
Gfc①CP91005	T	C	C	A	T	1-1	-	-
Gfc①CP91007	T	C	T	A	T	1-1	+	-
Gfc①CP91008	G	A	C	A	T	1-2	-	-
Gfc①CP91009	G	A	T	G	G	1-1	+	+
Gfc①CP91011	T	C	T	G	G	1-1	+	+
Gfc①CP91017	T	C	T	G	G	1-1	+	+
Gfc①CP91019	T	A	T	A	T	1-1	+	-
Gfc①CP91020	G	C	C	G	G	1-2	-	+
Gfc①CP91022	T	C	C	A	T	1-1	-	-

Perspectives on the development of biopesticides applicable to both agricultural insect pests and diseases

Shigenobu YOSHIDA

(Central region of agricultural research center, NARO)

SUMMARY

Biopestiscides effective against both agricultural insect pests and diseases have been implicated as promising materials to promote IPM practices and environmental friendly agriculture with cost- and labor-saving in Japan. We recently found that several microbes hitherto known to control agricultural insects also showed inhibitory effects against soilborne plant diseases such as bacterial wilt of tomato. Here we will mention outline on the research development on biocontrol using of such 'dual control' agents.

Biocontrol is a promising control measure to solve issues on agricultural chemical residues and emergence of drug resistant insects and microbes. For biocontrol against agricultural insects, bioinsecticides have been developed and various products have been on the market so far. Although these insecticides include microorganisms or their derivatives effective against agricultural insects, reports that such microbes also show beneficial functions against plant diseases caused by various plant pathogenic microbes have been increased. For example, a famous bioinsecticidial bacterium, *Bacillus thuringiensis*, has been reported to show inhibitory effects against plant pathogenic fungi, such as *Fusarium oxysporum* [1)] and *Phytophthora megasperma* f. sp. *medicaginis* [2)], based on secretion of antifungal substances. In addition to such insecticidial bacteria, similar inhibitory effects have also been reported on several insecticidial fungi; *Beauveria bassiana* has been reported to suppress damping off of tomato seedling caused by *Pythium myriotylum* and *Rhizoctonia solani* [3,4)], and several isolates belonging to genus *Lecanicillium* inhibited occurrence of various diseases such as powdery mildew of cucumber, tomato and strawberry [5)]. Thus, application of insecticidial microbes against plant diseases is feasible, and development of the control agents suppressing both agricultural insects and plant diseases contribute cost and labor-saving pest managements, which is recently more necessary to promote in Japanese agriculture system.

From 2011 to 2014, we carried out a research project funded by Ministry of Agriculture, Forestry and Fisheries to develop novel microbial agents which can lead to biopesticides, and methods for application of the microbes against significant diseases of Solanaceae, such as bacterial wilt, Fusarium wilt, and crown and root rot. Through the research project, our research group found *Bacillus thuringiensis*, which is known as an effective bio-insecticide, has an ability to activate plant defense system on tomato plants, leading to suppression of bacterial wilt. Hyakumachi et al. [6)] reported that when root of tomato seedlings were treated with *B. thuringiensis* culture and challenge-inoculated with *Ralstonia solanacearum*, development of wilt symptoms were suppressed to less than one third of the control. This disease suppression in tomato plants was reproduced by pretreating their roots with a cell-free filtrate (CF) that had been fractionated from *B. thuringiensis* culture by centrifugation and filtration. They also found that, in tomato plants challenge-inoculated with *R. solanacearum* after pretreatment with CF, the growth of *R. solanacearum* in stem tissues clearly decreased, and expression of defense-related genes such as PR-1, acidic chitinase, and β-1,3-glucanase was induced in stem and leaf tissues. Furthermore, the stem tissues of tomato plants with their roots were pretreated with CF exhibited resistance against direct inoculation with *R. solanacearum*. Taken together, they suggested treatment of tomato roots with the CF of *B. thuringiensis* systemically suppresses bacterial wilt through systemic activation of the plant defense system.

In addition to the above report, Takahashi et al. [7)] further elucidated the details of the systemic activation of the plant defense system by *B. thuringiensis*. When they carried out comparative analysis of the expression of a marker gene for induced resistance to pathogens, in

various tissues of tomato seedlings treated with CF on their roots, *B. thuringiensis*-induced defense system was activated in the leaf, stem, and main root tissues, but not in the lateral root tissue. In addition, the growth of the causal pathogen was significantly suppressed in the CF-treated main roots but not in the CF-treated lateral roots. This distinct activation of the defense reaction and suppression of *R. solanacearum* were reflected by the differences in the transcriptional profiles of the main and lateral tissues in response to the CF. In CF-treated main roots, but not CF-treated lateral roots, the expression of several salicylic acid (SA)-responsive defense-related genes was specifically induced, whereas jasmonic acid (JA)-related gene expression was either down-regulated or not induced in response to the CF. From these results, they concluded the co-activation of SA dependent signaling pathway with ET-dependent signaling pathway and suppression of JA-dependent signaling pathway might play key roles in *B. thuringiensis*-induced resistance to *R. solanacearum* in tomato.

Because the insecticidial bacterium, *B. thuringiensis* has verified to show another inhibitory potential against the plant disease, we further screened to find novel biocontrol agents effective against both agricultural insects and plant diseases from bioinsecticide products on the market in Japan. When dilutions of each candidate product were irrigated into the potted tomato seedlings and *Ralstonia solanacearum* was challenge-inoculated, several bioinsectisides showed inhibitory activity against the bacterial disease. Particularly, commercial product of *Paecilomyces tenuipes* (Gottsu A) showed the superior inhibitory activity. The product contains *P. tenuipes* strain T1 isolated from soil in Japan, and is known to be effective against whiteflies and aphids of vegetables. Gottsu A has been registered as a bioinsecticide since 2008.

To verify the mode of actions in the disease suppression by the strain T1, experiments using potted tomato seedlings were carried out. Consequently, the following results were obtained: 1) tomato seedlings irrigated with CF of strain T1, as well as the fungal propagules, showed inhibitory effects against bacterial wilt, while the CF did not inhibit the growth of the causal pathogen in vitro. 2) expression of SA dependent marker genes (PR-1 and PR-2), responsible for induced resistance to pathogens of tomato, was increased on the main roots of tomato seedlings dipped with the CF. From these results, the disease inhibitory activity by the strain T1 was suggested to be based on the systemic activation of the plant defense system.

Such disease inhibitory activity was confirmed in several field experiments. When the tomato seedlings drenched with 500-folds dilution of the commercial Gottsu A were transplanted in bacterial wilt-infested test fields, the disease occurrence was significantly suppressed on the transplanted plants by the treatment. Also, the suppression tended to be more stable by additional drenching of the dilution into the base of the plants every two weeks. Thus, the *P. tenuipes* bioinsecticide is also applicable as a promising novel biopesticide for bacterial wilt, and further field demonstration on the disease suppression by the insecticidial fungal product are ongoing to lead to registration as the biopesticide.

Concept that plural control of both agricultural insects and plant diseases is generally called as 'dual control' [5]. As described above, this concept could be suitable to solve issues arising in the recent Japanese agricultural systems, cost and labor-saving pest managements, since using products containing such wider spectral biocontrol agents reduce the kinds of products farmers should prepare for pest managements. Although here we mentioned *B. thuringiensis* and *P. tenuipes* are promising to be used as biopesticides against a soilborne disease, bacterial wilt of tomato, biopesticides against soilborne diseases have less been developed in Japan, rather than in other countries, such as Europe and USA. Hence, more commercialization of such dual control agents would also contribute to more provision of biological control measures against soilborne diseases.

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Oral Session Abstracts

Current status and management of *Rhizoctonia solani,* the causal pathogen of sheath blight disease on rice and maize in Indonesia

Achmadi PRIYATMOJO

(Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia)

SUMMARY

Rhizoctonia solani, a fungal soil-borne pathogen, has vary wide host range. In Indonesia, *R. solani* has been reported causing significantly yield losses on rice and maize. The causal agent was mostly identified as *R. solani* anastomosis group 1-IA (AG 1-IA) on the basis of colony cultural morphology on PDA. However other *Rhizoctonia* spp. were also isolated from rice and maize but anastomosis grouping could not be established because of lack of the AG tester isolates of *R. solani* for anastomosis test. Moreover, determination by colony cultural morphology is very difficult because of its high variation even in isolate belonging to the same AG or subgroup of AG. At present in Indonesia, many of isolates of *R. solani* obtained from other crops have not been differentiated into AG due to lack of the tester isolates. Precise identification of *R. solani* into AG is very useful for control recommendation. Currently, sheath blight on rice and maize caused by *R. solani* has been managed by controlling water for irrigation, applying proper fertilizers, utilizing biological control agents, and and spraying chemical fungicides. For both enviromental friendly and healthy product reasons, the use of chemical fungicides should not be as a primary control strategy.

Introduction

Rice is an important staple food in Indonesia. Rice production is dominated by the small-holder farmers which each farmer holding an average of land area less than 0.5 hectares. Small-holder farmers account for approximately 90 percent of Indonesia's rice production (20). In some regions of Indonesia, maize is chosen as staple food for some reasons such as climate and price factors. Moreover, maize is also used for animal feed (46).

Many decades Indonesia Government has been created many programs to reach rice and maize self-sufficiency in order to maintain political, social and economic stability. However, many constraints have been appeared for increasing rice and maize production in most of the rice and maize growing area of Indonesia, for examples low educational of farmers, unfavorable climate condition, attacking pests, and occurring diseases. One of the most important pathogens on rice and maize is *Rhizoctonia solani* causing sheath blight disease (34). Therefore disease control measures are necessary to prevent sheath blight disease development.

Grouping of *Rhizoctonia* Species

Rhizoctonia species is a universal filamentous fungal soil exist as saprophytes, mycorrhizal symbionts and pathogens of many plant species (7,24,40). The fungus survives in unfavorable conditions by forming dormant mycelia or sclerotia (43). Sclerotia can survive for 2 years in soil and are spread during field preparation and irrigation. These sclerotia can become source of disease inoculum for the next planting (21).

Rhizoctonia sp. is rare to produce sexual spore or basidiospore in field. Many attempts have been developed to induce sexual state of *Rhizoctonia* species in the laboratory (1,12,49). However, some of AGs or subgroups of *Rhizoctonia* sp. remain could not be induced the formation of basidiospores by applying these artificial techniques.

On the basis of number of nuclei per hyphal cell, *Rhizoctonia* sp. is divided into uninucleate, binucleate, and multinucleate which contains one, two, and more than two nuclei per hyphal cell, respectively. At present, uninucleate *Rhizoctonia* isolates have been reported only from Norwegian and Finnish conifer seedlings (8,39).

Moreover, both binucleate and multinucleate *Rhizoctonia* are grouped under species level based on hyphal fusion or hyphal anastomosis reaction (3,5,23,25). Currently, 21 anastomosis groups (AGs) of binucleate *Rhizoctonia* and 13 AGs of multinucleate *Rhizoctonia solani* have been reported (6,10,18,37). On the other hand, multinucleate *Rhizoctonia circinata* is divided into

R. circinata var. *circinata*, *R. circinata* var. *oryzae*, and *R. circinata* var. *zeae* based on differences of colony morphology of the vegetative state (15,26).

Some AGs of binucleate *Rhizoctonia* and multinucleate *Rhizoctonia solani* are further divided into subgroups or types based on cultural morphology, pathogenicity, hyphal fusion frequency, DNA complementarity, fatty acid analysis, and zymogram patterns (9,11,16,27-32).

Occurrence of *Rhizoctonia* Species in Indonesia

Rhizoctonia sp. obtained from various plants has been reported from many places in Indonesia. These isolates of *Rhizoctonia* sp. belong to binucleate and multinucleate. None of isolates belong to uninucleate *Rhizoctonia*. The pathogen attacks rice, maize, wheat, sorghum, peanut, soybean, pepper, tomato, potato, tobacco, cotton, cabbage, lettuce, strawberry, cacao, coffee, pine and other economically crops (33-35,44).

In addition to pathogenic *Rhizoctonia* sp., many beneficial *Rhizoctonia* sp. have been obtained from many places in Indonesia. These beneficial *Rhizoctonia* sp. are predominantly by binucleate *Rhizoctonia* (7,40,45).

Due to the lack of AG tester isolates of *Rhizoctonia* sp. in Indonesia, most of *Rhizoctonia* sp. isolates from various places and crops have not been grouped yet into AG system (41), except for isolates of mycorrhizal *Rhizoctonia* obtained from healthy vanilla roots which reported belong to binucleate *Rhizoctonia* AG-F based on anastomosis reaction and PCR-RFLP. The study was held in Gifu University, Gifu, Japan in 2009 (7). The remaining isolates of *Rhizoctonia* sp. are still unknown AG. However, other approaches applying cultural morphology, pathogenesis, and molecular techniques have been taken in order to placing isolates of *Rhizoctonia* sp. into proper AG. Recent study reveal that *R. solani* collected from rice showing sheath blight symptom and from potato showing black scurf symptom could be placed into AG 1-1A and AG 3, respectively (2,4).

Rhizoctonia solani on Rice

Rhizoctonia solani, the causal agent of sheath blight disease on rice, is often occur in rice fields and easily isolated from diseased tissue plant or from sclerotia that produced around disease symptom. Symptoms are usually observed from tillering to milk stage (generative stadium) in a rice crop and include oval or ellipsoidal greenish gray lesions, usually 1-3 cm long, on the leaf sheath, under favorable conditions, these initial lesions multiply and expand to the upper part of the sheaths (Figure 1) (22,34). In the absence of protection measures, sheath blight disease on rice causes 10-30% yield loss and may reach up 50% under favorable environmental condition for disease development (17,51).

Figure 1. Sheath blight symptom on rice.

Rhizoctonia solani on Maize

Banded leaf and sheath blight (BLSB) on maize are commonly found in the center of maize production in Indonesia. *Rhizoctonia solani*, a soil-borne disease, has been reported as causal pathogen of BLSB. It is a major disease on maize and can cause serious damage reach up to 100% yield loss (34,38,47). The initially infection is from the basal sheath and then spread to the developing ear under favorable environmental conditions. The developing ear is completely damaged and dries up prematurely (Figure 2).

Figure 1. Sheath blight symptom on maize.

Sheath Blight Disease Management

Sheath blight disease on rice and maize in Indonesia is very difficult to be controlled because of continuing rice or maize planting a whole year, favorable environmental condition, almost lack of resistant variety of rice or maize to the disease, and existing of sclerotia which can survive over 2 years. Resistant variety of rice and maize to sheath blight has not been reported in Indonesia (19).

Cultural Control

As a tropical country, rice can be planted 2 or 3 times a year depend on water availability for irrigation. This condition is favorable for sheath blight disease development because many sclerotia will be produced as a source of inoculum for the next successive rice planting (21). Therefore, in the rice field with the history of occurring sheath blight disease, it is not recommended to cultivate rice or other crop belonging to Poaceae family continuously (34). Study on the host range of *R. solani* obtained from rice, wheat, and maize showed that *R. solani* from each crop could infect each other crops (36).

Chemical Control

After establishment of integrated pest management (IPM) program in 1995, chemical control by applying fungicides should be the last choice after other control techniques cannot protect or prevent disease development (50). Some of active ingredients of fungicide used to control *R. solani* on rice and maize are propiconazole, difeconazole, benomyl, mancozeb, validamycin, and methyl thiophanate.

Biological Control

Biological control technique using antagonistic microorganisms including fungi, bacteria and actinomyces has been increased in recent year. Compare to chemical control, biological control is more environmental friendly and provide quality health for human. *Bacillus* sp., *Trichoderma* sp. and *Gliocladium* sp. are among the most biological control agents used to control sheath blight disease on rice and maize (14,19,41,42).

Conclusion

Sheath blight disease on rice and maize is an important disease causing serious damage and can reach up to 50-100% yield loss. Many compatible control strategies should be applied and creating resistant variety should be strongly supported in controlling the disease.

Acknowledgement

The author is grateful to UGSAS, Gifu University, Gifu Japan for providing financial support to attend International Conference on Innovative Crop Protection for Sustainable Agriculture.

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Indonesian farmers problems in implementing integrated pest management (IPM)

Hanifah IHSANIYATI

(Faculty of Agriculture, Sebelas Maret University)

SUMMARY

Food occupies an important position in Indonesian agricultural sector. As food producers, farmers are often faced with problems caused by pests. As the enemy of farmers, pests can damage food crops and eventually decrease production. The best solution for pest problems is Integrated Pest Management (IPM). Although some research indicates that information related to IPM has already been familiar to farmers, the practice of IPM has not been implemented by all farmers in Indonesia. There are several problems faced by Indonesian farmers in implementing IPM. This article aims at providing an overview of the problems faced by Indonesian farmers in the implementation of IPM. This paper is presented descriptively based on several articles and previous research on IPM.

Keywords. Farmers, problems, pests, management, food

Introduction

Integrated Pest Management (IPM) has been a dominant crop protection paradigm promoted globally since the 1960s (Parsa et al., 2014). The fundamental principles of pest management include 1) understanding the ecology of the agroecosystem and the population dynamics of the species in the ecosystem, 2) recognizing and using economic-injury thresholds, and 3) integrating all appropriate control tactics into a rational system of management (Robbins et al., 1974 in Minnick , 1976); (Hammond et al., 2006); (Chandler, 2011); (Minnick, 1976). An analysis of 62 IPM research and development projects in 26 countries, covering over 5 million farm households, showed that IPM leads to substantial reductions in pesticide applications (Chandler, 2011). However, its adoption by developing country farmers is surprisingly low (Parsa et al., 2014).

An understanding of the various methods employed in managing pests is essential in obtaining a perspective of integrated pest management. An inventory of the more prominent control tactics includes chemical control, attractants and growth regulators, preventive measures, autodial control, host-plant resistance, cultural and physical control, and biological control. Cultural control are usually associated with such standard agricultural practices as site selection, irrigation, fertilization, tilling, crop rotation, mixed or trap crops, sanitation, and drying (Minnick, 1976); (Effendi, 2009).

The effort to introduce IPM started in the early 1979, after Indonesia had experienced its first nationwide outbreak of brown planthopper in 1975-1977 (Roling and Elske van de Fliert, 1994). It was then declared as a national policy in 1986, and its implementation has had a profound effect on national agricultural production (SEAMEO, 1999 in Utama, 2003). The concept of IPM in Indonesia is also described in the Law Number 12 of 1992 (Indonesian : Undang-Undang Nomor 12 Tahun 1992) on Agricultural Cultivation System and the Decree of the Minister of Agriculture/the Chairman of BIMAS Controlling Agency Number 14/ SK/Mentan/Bimas.XII/1990 on the guidelines of the implementation of integrated pest control (Sulistiyono et al. , 2008).

In the beginning, farmers have made efforts to control Plant Pest Organisms (Indonesian: *Organisme Pengganggu Tanaman or OPT*) both in physical and mechanical ways. However, as the development of science and technology, the use of pesticides is considered more effective in controlling pests (the Directorate General of Agricultural Facility (Indonesian : Ditjen Bina Sarana Pertanian), 2005 in Sulistiyono et al., 2008). There is a common view that the success of agricultural development cannot be separated from the use of pesticides. It is better to use more pesticides because it can increase the production of agricultural products. This is a general perception that still prevails in today's world, including in Indonesia (Sulistiyono, 2004). It is supported by the significant increase of pesticides registered at the Directorate General of Agricultural Production Facility of the Ministry of Agriculture.

Looking at the data of registered trademark, there were 770 formulations in 2000, it then increased to 1,298 formulations in 2005 (the Directorate General of Agricultural Facility, 2005 in Sulistiyono et al, 2008).

The constraints in the implementation of IPM in Indonesia are mainly due to the actions of farmers themselves, such as the use of uncontrolled pesticides. Focusing on the use of pesticides, some of the problematic actions that farmers undertake in the implementation of IPM in Indonesia will be described in this paper, including the attitudes of farmers as a motive that affects the action. Attitude is an important key to understanding long-term behaviour and it affects behaviour (Newcomb et al., 1985); (Gibson et al., 1994). Therefore, the attitude formation as a stage in the adoption of technology needs to be given serious attention to the coaches because it determines the speed of farmers in adopting technology (Herman et al., 2006).
There have been a number of reviews on the issues of IPM implementation and pesticide use by farmers; however, it is still necessary to re-sharpen our knowledge and awareness of IPM and the underlying factors that affect farmers' lack of actions in supporting the implementation of IPM. It is hoped that this paper can be a reference in determining the program of assistance and empowerment of Indonesian farmers in the implementation of IPM.

Material and Method

This paper is arranged based on the compilation of several research papers and books written by the experts in the field of IPM and attitudes. It is presented descriptively in discussing the problems of farmers in the implementation of IPM, which focuses on the attitudes of farmers in Indonesia as a fundamental factor that affects farmers' lack of actions in supporting the implementation of IPM

Result and Discussions

Although conceptually the use of pesticides is positioned as the last alternative in controlling Plant Pest Organisms (OPT) in the IPM concept, in reality, pesticides are often used as the main and most common choice of farmers. Pesticides have been widely used by farmers in overcoming pests. This condition has become a tradition and existed up to the present day among farmers in running their system of farming (Sulistiyono, 2004); (Untung, 1996 in Effendi, 2009); (Effendi, 2009). In Asia, Indonesia is a country that uses a lot of pesticides after China and India (Soerjani, 1996 in Wahyuni, 2010).

Generally, the high use pesticides in handling pests and diseases cannot be separated from the old paradigm seeing that the success of agricultural production is a result of the role of pesticides. The support from government policy has encouraged farmers to use pesticides through a subsidy regulation of 80% of the price of pesticides in 1987. In addition, this condition is reinforced by the creation of a chance circle between knowledge gaps of farmers in pest control and the incessant promotion of pesticides efficacy, also the weak supervision and law enforcement and the policy to achieve the target of agricultural production programs (Sulistiyono, 2004).

The most intensive and intensive use of pesticides in agricultural activities is in the types of holticulture and palawija (such as corn, sorghum, green beans, soybeans) cultivation activities (Abadi et al, 1993 in Rario et al, 2005).

The benefits of pesticides are proven to be so large that they become the determinant factor in increasing crop productivity and improving product quality, as reflected in every package of programs or agricultural activities that always include pesticides as part of production inputs (Wahyuni, 2010).

Pesticides are considered as the saviour of agricultural production (Sulistiyono, 2004). However, pesticides not only provide benefits to agriculture, but also have negative impacts. The negative effects of pesticide use have been widely reported in various studies. These impacts may include ecosystem instability, residuals on crops and their processed materials, environmental pollution and poisoning, and even death in humans (Wahyuni, 2010).

D.R.Minnick (1976) stated that chemical compounds are indiscriminate in their pesticidal properties; they kill many non-target species that help to control pest populations. The environmental effects of pesticide residues have been well documented (Graham 1970; Reese 1972; Robbins et al. 1974; Turk et al. 1975). Some pesticides, when introduced in large quantities and on a continual basis into the biosphere, are responsible for disrupting life processes in non-target food chains. Residues incorporated into human tissues, for example, may prove harmful (EPA 1975; Malibach et al. 1971). Several insecticides used extensively in rice in numerous

countries are extremely toxic to birds and are expected to cause frequent and largely unavoidable mortality (Parsons et al., 2010).

Having a closer look at its impacts on human lives, the WHO data also photograph at least 20,000 people per year died from pesticide poisoning. About 5,000 to 10,000 people per year are affected by its side effects, such as cancer, disability, infertility, and liver disease. Pesticides are one of the examples of Hazardous and Toxic Materials (Indonesian: *Bahan Berbahaya dan Beracun or B3*) that are often used by farmers (Riyadi, 1995 in Wahyuni, 2010). FAO surveys in Tegal and Brebes conducted in 1991-1992 found 69 pain symptoms in which 43 of them were known as typical symptoms of pesticide poisoning, such as shortness of breath, dizziness, nausea, and vomiting. The symptoms were apparent after farmers performed 21% of the frequency of spraying. Unfortunately, such a phenomenon is ignored by the farmers (Wahyuni, 2010).

Experts state that in its practice, farmers are spraying chemical pesticides on schedule, inaccurately targeted, with inaccurate doses (they likely tend to mix some pesticides and use more chemical pesticides than they should), without using self-protection equipment and lack of environmental awareness (Sulistiyono et al., 2008); (Ningrum, 2012); (Ruhs et al., 1999 and Kartaatmadja et al., 1997 in Wahyuni, 2010). The appropriate dosage of pesticide use by onion farmers who have attended Integrated Pest Management School (Indonesian: *Sekolah Lapang Pengendaian Hama Terpadu or SLPHT*) in Nganjuk District were; 4.17% appropriate and 95.83% not appropriate, whereas the data garnered in the non-SLPHT participants showed that 1.04% appropriate and 98.96% not appropriate. This way of using pesticides has generated very serious ecological impacts (Sulistiyono, 2004).

Another issue of concern according to Pimentel and Khan (1997) is a consumer demand that makes farmers consider pesticides as the main alternative. Until today, consumers assess the quality of horticultural products especially from the enchanting appearance of the products, usually known as “Cosmetic Appearance”. Because “Cosmetic Appearance” is still serving as the main assessment of consumers, it is necessary to create a climate of competition among horticultural crop farmers to keep the appearance of the products that attract attention. The farmers' efforts are to use pesticides in protecting crops from pest attacks; their products can be sold quickly at competitive prices so that maximum profits can be achieved (Sulistiyono, 2004).

Farmers may not realize that pesticides are actually toxic. They even make them as a primary choice and consider it as medicine. Sometimes, farmers are still spraying vegetables that have been harvested and ready to be transported, fearing that their harvests will be attacked by pests. The ease of getting pesticides also encourages farmers to use excessive pesticides (Wahyuni, 2010).

The lack of farmers' support in the implementation of IPM is influenced by their perception and attitude. Sulistyono et al. (2008) stated that the direct application of pesticides in the field is still constrained by several factors, such as knowledge of farmers about pesticides, farmers’ attitudes toward the regulation of pesticide use, and the action of its use. A research conducted by Dwi Sadono (2001) in Kabupaten Karawang West Java revealed that there is a significant relationship between the perception of farmers and the level of its adoption. Sadono (1999) in Herman et al (2006) also said that the internal factors of farmers that correlate significantly with the application of integrated pest control (IPM) are the education and perception of farmers towards IPM.

Elske van de Fliert's (1993) study on IPM in Central Java mentions farmers’ perception about pest management, including: 1) *“Hama jangan sampai merajalela” (*Do not let pests break out), is an often heard expression from rice farmers in the eight villages in Grobogan, which clearly indicates the prevalent attitude towards pests in the rice crop: one insect pest in the crop is the forerunner for the others if the outbreak is uncontrollable. 2) “*Sedia payung sebelum hujan* “ (Have an umbrella ready before it starts raining), though often heard, does not exactly describe the actual perception of many farmers towards (chemical) pest control. A more appropriate expression would be: “Use an umbrella even when it does not rain”, explaining the habits of many farmers in using pesticides as their preventive actions, as a result of the exposure of rice intensification program for over twenty years. Christopher Vaughan (1988); Irham and Joko Maryono (2011); Dwi Sadono, (2001) also stated that farmers were often spraying their fields habitually – regardless of whether the fields were infested – and that overuse of pesticides actually increased the risk and severity of pest infestation in the

rice fields.

A research conducted by Herman et al. (2006) in West Sulawesi showed that the application of pest control technology (cocoa) is influenced by four factors, including farmers' attitudes, level of income, assistance of guide/coach, and space of garden. The attitudes of farmers have a positive effect on farmers' actions in adopting cocoa pod borer pest control technology. Anggi Khoirudin Siregar (2014) study stated that there is still a lack of knowledge and attitude towards integrated pest management (IPM) in District of Rancabungur, Bogor Regency. This is influenced by the perception of farmers who still believe that the use of pesticides is the one and only effective way in controlling pests. Although most of farmers (82.5%) agree that pesticides endanger the environment and users (farmers), as many as 70% of the respondents agree that pesticides are effective in controlling pests and diseases.

Baron and Byrne (2003) also explained that, according to a social psychologist, attitudes are very important factors. Not only because it is difficult to change, but it is also seen as the central issue in the field of social psychology, for the following reasons: 1) attitudes deeply influence social thinking, although they are not always reflected in apparent behaviour, 2) attitudes often affect behaviour, this is especially true when the attitudes are strong and steady.

Luluk Sulistiyono et al. (2008); (Utama, 2003) mentioned that farmers' attitudes in using pesticides are formed from information that is believed to be true and it is inherited either in private way or through communication between farmers. Wahyuni (2010) said that the activity of controlling pest intruders is a work that takes a lot of time, effort, and cost. Besides being easy to use, the efficacy of pesticides is reliable. Furthermore, in addition to its high success rate, pesticides are available and easy to get at relatively low and affordable prices. These conditions allow the farmers to expose their plants with pesticides easily, and even excessively.

Luluk Sulistiyono et al. (2008) stated that there are several factors causing a weak relationship between attitude and action, they are: 1) anxiety, it happens when farmers feel very anxious if there is crop failure so that they cannot return the investment value. 2) forecasting, it is a lack of farmers' ability in predicting what pests and diseases that will attack during the growing season, so that they likely tend to conduct the spraying on schedule. 3) awareness, there is farmers' low awareness in the implementation of IPM, 4) behaviour intention, farmers intend to conduct IPM because of the support of cognitive aspects, but its implementation is strongly influenced by their surroundings so that it will restrict them to act based on the rules. Azwar (2012) explained that a person who is considered important in making decisions will greatly influence the formation of attitudes, including: parents, peers, close friends, co-workers, family, people with a higher social status, neighbours. Individuals tend to have the similar attitudes with those who are considered important. According to Rogers (2003), farmers' innovative decision-making is also influenced by the behaviours or decisions taken by social institutions (groups/organizations) existing within farming communities, such as neighbours, relatives, farmer groups, and religious groups, 5) internal conflict, the most influential internal factors are the fulfilment of needs and the constraints of farming, severe pest attacks raise fears and cause strong feelings of anxiety that encourages farmers to apply pesticides in a reckless way.

Instead of being instantly changed, the development of attitudes likely tends to need a relatively long time and it is commonly accompanied by repetitive efforts that result in positive attitudes toward its newly introduced technology, which will then be followed by the stability in adopting the technology (Tjitropranoto, 2005). Therefore, although it is not easy, farmers' empowerment practitioners need to develop strategies to shape farmers' positive attitudes toward IPM activities and change their mindset of using excessive pesticides so that they will have a right mindset on how to deal with plant pests and diseases.

Conclusion

Integrated Pest Management (IPM) aims at limiting the use of synthetic insecticides by introducing the concept of economic threshold as a basis for determining pest control. This approach encourages the replacement of chemical pesticides with alternative control technologies, which use more biological materials and methods, including natural enemies, biological pesticides, and pheromones. Unfortunately, this still cannot be thoroughly implemented by farmers in Indonesia. There are several obstacles or problems in the implementation of IPM in Indonesia, and they are mainly due to the lack of support from the farmers themselves. These actions

are caused by their attitudes toward IPM, especially their use of pesticides in overcoming pests and plant diseases.

Acknowledgement

Farmers' empowerment practitioners need to realize that the actions of farmers in the implementation of IPM are influenced by their attitudes and mindset. Therefore, although it is not easy, the farmers' coach or empowerment practitioners need to carry out a specific strategy to form farmers' positive attitudes toward IPM so that in the future they are able to use pesticides more wisely.

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Taxonomical studies of blood disease bacterium of banana

Dwiwiyati Nurul Septariani, Hadiwiyono

(Faculty of Agriculture, Sebelas Maret University)

SUMMARY

Ralstonia solanacearum is considered a species complex-a heterogeneoius group of related but genetically distinct strains. The term “species complex” was used first to describe the high genetic variation between isolates, and the concept of *R. solanacearum* species complex was expanded by including two closely related species from Indonesia, *Ralstonia syzygii* (a pathogen of clove trees) and the agent of blood disease of banana, known as the BDB. Within the *R. solanacearum* group, BDB and *R. syzygii* are most similar to the *R. solanacearum* strains from Indonesia, and thus belong to phylotype IV. The four phylotypes encompass three different species, thereby justifying the use of the term “species complex”, defined as a cluster of closely related isolates whose individual members may represent more than one species. However, the genetic basis for this variation is unknown, and original culture deposited as the type strain no longer exists si the name is taxonically invalid. For the past four decades, two different systems, race and biovar, have been widely used to differentiate *R. solanacearum* strains. The race structure of *R. solanacearum* is poorly defined and not taxonomically useful. Comparative genomic analysis is needed to defined phylogenetic of *R. solanacearum* species complex.

Introduction

Banana is a leading food crop in terms of production value. Its total tarde value stood at some USD 8 billion in 2016 with some 15 percent of global production exported and making bananas the largest traded fruit crop in value terms [1]. In Indonesia, that is not only rich in banana diversity, but also is a center of banana’s distribution. Banana production in Indonesia is one of the largest in the world. However, 90% of the products are used on national scale only [2]. It caused productivity particularly in terms of the quality of the fruit. An indication that result in a decerase of quality is pest and disease problems at the time of its growth [3].

Independent of region and production system, pests and diseases have been considered the main constraints responsible for yield losses and low productivity of bananas. The fungal diseases black leaf streak disease (commonly known as black Sigatoka), (*Mycosphaerella fijiensis*) and Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*) have always been considered as the most important banana diseases globally and have therefore received more attention. However, bacterial diseases cause significant impacts on yield globally and management practices are not always well known [4].

Bacterial diseases of banana and enset can be classified into three distinct groups: i) Ralstonia-associated diseases (Moko/Bugtok disease caused by *Ralstonia solanacearum* and banana blood disease caused by *R. syzygii* subsp. *celebesensis*); ii) Xanthomonas wilt of banana and enset, caused by *Xanthomonas campestris* pv. *musacearum* and iii) Erwinia-associated diseases (bacterial head rot or tip-over disease (*Erwinia carotovora* ssp. *carotovora* and *E. chrysanthemi*), bacterial rhizome and pseudostem wet rot (*Dickeya paradisiaca* formerly *E. chrysanthemi* pv. *paradisiaca*). Other bacterial diseases of less widespread importance include: Javanese vascular wilt, bacterial wilt of abaca and bacterial fingertip rot (probably caused by *Ralstonia* spp., unconfirmed) [4].

Banana blood disease is thought to have originated on Salayar Island near Sulawesi, where it was first reported after the introduction of dessert bananas in the early 1900s [5,6]. The disease was confined to Salayar for many years due to the strict quarantine regulations implemented by the Dutch. However, it had become widespread on local cooking banana cultivars in southern Sulawesi (formerly Celebes) by 1920 [7,8], and then probably spread throughout the island until its discovery in Java in the late 1980s [6].

Unfortunately, the pathogen has since continued its spread to most of the larger Indonesian islands, where average yield losses often exceed 35% [9]. These outbreaks were associated with the transmigration of people from Java to less populated islands in Indonesia [10]. The banana blood disease is currently spreading in peninsular Malaysia where it coexists with the Moko and Fusarium wilt diseases [11]. The disease has been first detected in the province of Perak and more recently in the province of Selangor [11,12,13]. Banana blood disease has also been observed on the island of New Guinea [14]. Severe destruction due to banana blood disease was noted in South Sulawesi, where 70–80% of plantations were lost [15], and in West Java, where 27–36% plantation loss was

recorded [16]. In Lampung Province (Sumatra), more than 20,000 tons of banana, with an estimated value of US$1 million, were lost to banana blood disease [17]. Losses will most likely escalate with disease spread. If the disease 1 day arrives on the South-East Asian mainland there would be no barriers to its eventual/gradual movement to the Indian subcontinent [10,15].

One disease have caused production tend to stagnant or decline was blood bacterial. It was seem that blood bacterial wilt disease caused by blood disease bacterium (BDB) have involved in the case of low production of bananas [9]. The national loss of banana production due to blood bacterial wilt disease was estimated around 36% in 1991 [19]. The damage of banana mats was extremely serious in certain districts in where ABB genomic group were planted such as Bondowoso and Lombok, the disease incidence could reach over 80% [9]. Now the pathogen has been distributed in 90% of Provinces in Indonesia with various disease incidences from 10 thousand to millions of banana clusters [20].

BDB is existent in all of the sample tissue point and sistemically distributed in all parts of infected plants. Specific symptoms showed hanging drop of leaves on vegetative stage, dried inflorescence of flower and wilt on generative stage, and dried inflorescence of flower extending to upper parts or fruits on bunch. Internal symptoms showed browning in vessel cells occurred in the most part of plants, pulp, stalk, fruit shelter, pedundle, middle peduncle and pseudostem, basal peduncle, midrib, petiole, corm, and root; and yellowing leaf lamina [21].

Ralstonia solanacearum was first described and classified as *Bacterium solanacearum* by Erwin F. Smith at the end of the 19th century [22]. The causal agent of bacterial wilt was then successively named *P. solanacearum*, and more recently, by application of DNA-based methods, *Burkholderia solanacearum* [23] and finally *R. solanacearum* [24].

Material and Method

Strains and genomic DNA extraction, sequencing and assembly, genomic annotation, and comparative genomic analyses.

Result and Discussions

The genus *Ralstonia* belongs to the family *Burkholderia* (class Betaproteobacteria) that includes nine genera and many humanand plant-pathogenic species and several symbionts. *Ralstonia* is an aerobic, Gram-negative rod with a polar flagella tuft. It is oxidase positive, arginine dihydrolase negative, and accumulates poly-hydroxybutyrate intracellularly. Most strains denitrify and produce a diffusible brown-red pigment on rich medium. It does not grow below 4^0C or above 40^0C, and there is little or no growth in 2% NaCl [25, 26, 27, 28].

Ralstonia solanacearum is a heterogeneous species, as demonstrated by its large host range, pathogenic specialization and physiological and cultural properties, as well as its phylogeny [29]. Despite being classified as a single species, it has been reported that different strains of *R. solanacearum* may have DNA–DNA relatedness values below 70% [30] and therefore could possibly be members of different species. The term ‘species complex,’ which refers to ‘a cluster of closely related bacteria whose individual members may represent more than one species,’ was then proposed for *R. solanacearum* [31].

It is assumed that *R. solanacearum* originated, adapted and evolved in widely different places, resulting in great geographic and pathogenic diversity and translating in variable disease expression and disease potentials for each host/parasite genotype interaction [32,33]. However, recent studies suggest that *R. solanacearum* most likely originated in Oceania/Indonesia, and migrated to Africa, South America and Asia, possibly before the fragmentation of the ancestral continent Gondwana [34,35].

The *R. solanacearum* species complex is subdivided into four distinct phylotypes, largely correlating with the geographic origin and evolutionary past of the strains. Strains are assigned to the Asian (phylotype I), American (II), African (III), and Indonesian (IV) phylotypes [36,37,38]. Phylotype IV hosts the two closely related bacteria *R. syzygii* (the causal agent of Sumatra disease of clove) and the ‘blood disease bacterium (BDB) [28,34,38,39,40].

Using a polyphasic taxonomic approach, the *R. solanacearum* species complex was merged into three species: *R. solanacearum* corresponding to phylotype II strains (including Moko strains); *R. pseudosolanacearum* corresponding to phylotypes I and III; and *R. syzygii* corresponding to phylotype IV. *R. syzygii* sp. nov is further divided into three subspecies: the broad host range strains are *R. syzygii* subsp. *indonesiensis* subsp. nov.; the strains causing Sumatra disease of cloves as *R. syzygii* subsp. s*yzygii* subsp. nov.; and the BDB strains causing the banana blood disease as *R. syzygii* subsp. *celebesensis* subsp. Nov [41]. Comparative analysis of 29 whole genomes by MUMi and the use of protein profiling on a

larger set of bacterial strains by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS), support the division of the *R. solanacearum* species complex into three species consistent with genomic and proteomic data as well as biological differences [42].

Ralstonia syzygii subsp. *celebesensis* (BDB) was historically described and named *P. celebensis* in 1921, but the name became invalid when the original type strain was lost [5,7,43] suggested that the blood disease pathogen coevolved with banana. Buddenhagen (2009) however, indicated that this was unlikely due to differences in when and where the disease first appeared. Blood disease was first observed where wild bananas were not found (Rijks 1916), supporting the suggestion that the bacterium originated on other plant species than banana [33]. Colonies of the 'banana blood disease' strains are smaller than those of *R. solanacearum* causing Moko and are slow-growing, nonfluidal on Kelman's TZC (Triphenyl Tetrazolium Chloride) medium (commonly used for *R. solanacearum*) and have smooth margins with a dark-red center [44]. Genetic analyses, by whole genome RFLP groupings, comparison of partial 16s ribosomal DNA sequences and analysis of tRNA consensus primer amplification products, indicate a close relationship, but distinctly different from other strains of the *R. solanacearum* species complex [5,39,45]. Genetic analyses revealed that there is little diversity among strains of BDB [38,46], suggesting few introductory and founder events as well as a recent evolutionary past on banana [10].

Conclusion

The genomes of the Blood Diseass Bacterium and *R. syzigii* are closely related to *R.solanacearum* strains from Indonesia. Comparative genomic analysis demonstrated that these specialized organisms, which belong to *R. solanacearum* phylotype IV, are part of the same genomic species. *R. solanacearum* species complex should be divided into three genomic species. The pathogenic behavior of *R. syzygii* very unusual in the *R. solanacearum* species complex, may have resulted from ecological adaptation and genomic convergence during vertical evolution. The pathogenic and life-style traits may have been horizontally acquired from uncharacterized microbes. BDB and *R.syzygii* represent the shortest genomes within the *R. solanacearum* species complex although the genomes are relatively large for bacterial genomes. This reduction in genome size may be a step in the evolution of these specialized bacteria via genome decay under selective pressures within the host. The critical questions about the unique biology of these two microorganisms remain to be experimentally addressed. The two genome sequences will be a valuable tool for these subsequent functional studies.

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Bacterial endophytes isolated from orchids and their influence on plant health

Tri JOKO[1,3], Dewi Nur ANGGRAENI[2], Mery Irianti[3], Budi Setiadi DARYONO[2], Jaka WIDADA[1,3], Siti SUBANDIYAH[1,3]

([1]Faculty of Agriculture, Universitas Gadjah Mada, Indonesia; [2]Faculty of Biology, Universitas Gadjah Mada, Indonesia; [3]Research Center for Biotechnology, Universitas Gadjah Mada, Indonesia)

SUMMARY

The intimate relationship between endophytic bacteria and their host may influence the physiological mechanisms of plants. In this study, a hundred and six bacterial endophytes were isolated from tissues of twelve orchid species, and screened according to *in vitro* antagonistic activity, indole-3-acetic acid (IAA) production, and phosphate solubilization. Among 106 isolates able to inhibit soft-rot pathogen, 79 were found to produce IAA and 92 were able to solubilize inorganic phosphate. Thirty selected isolates were then subjected to ERIC and rep-PCR to investigate their genetic diversity. Three promising isolates (AkOc1, DnAr4, and TbPh7) were then applied for *in vitro* growth and challenged against soft rot bacterial pathogen. It was found that introduction of endophytic isolates to plantlets of *Phalaenopsis amabilis* increased significant growth of plantlet as well as disease suppression. Characterization by partial 16S rRNA gene sequences analysis revealed that the isolates were closely related to *Raoultella ornithinolytica*, *Klebsiella michiganensis*, and *Bacillus amyloliquefaciens* subsp. *plantarum* respectively. The ability of the isolates to inhibit soft rot bacterial pathogen as well as to promote plant growth makes them potential for enhancing plant health.

Introduction

Some bacteria were known to live and associate with plants, either live in rhizosphere or inside plants tissues. Bacteria on roots and in the rhizosphere benefit from root exudates, but some are capable of entering the plant as endophytes that could establish a mutualistic association[1]. Endophytic bacteria are defined as associated bacteria found and colonized within the plant tissue without causing harmful effect for host plants. These bacteria can be isolated from surface-sterilized plant tissue. The growth enhancement by the bacterial endophytes can be a consequence of the synthesis of phytohormones, biocontrol of phytopathogens in the root zone (through production of antifungal or antibacterial agents, siderophore production, nutrient competition and induction of systematic acquired host resistance, or immunity) or by enhancing availability of minerals[2].

Soft rot disease is one of the most common and important limiting factors for growing orchid in many nurseries worldwide. Several reports have indicated the bacterial soft rot was the most disastrous disease and made significant economic losses. Soft rot disease in Indonesia exists in many orchid growing regions and the disease intensity reach up to 46%[3,4]. Until recently, the application of chemical spray still the most common disease control by growers, but it was not very successful. Rather, chemical application might impact on environmental problems. Therefore, alternatives to control soft rot disease should be the alternative way out to encounter this problem. The present investigations have been focused on the ability of endophytic bacteria from orchid to growing stimulation of *Phalaenopsis amabilis* plantlets and as an alternative to chemical in plant disease management.

Materials and Methods

Endophytic bacterial strains and growth conditions. Investigated bacterial strains were originally isolated from root, pseudobulb, leaf, stalk, and flower tissues of twelve orchid species collected from nature and nurseries. Isolation and characterization of the strains based on their phenotypic characteristics, as well as their screening for antibiosis were described earlier. The strains were routinely maintained at 4°C after growth at room temperature on Tryptic soy broth (TSB) or Nutrient agar (NA). Cultivation in liquid media was performed in 50 ml shake flasks; 20 ml of experimental medium was inoculated with a 1% of inoculums, grown in the same medium for 48 h. Bacterial growth was estimated by measuring optical density at 600 nm (OD600).

Colorimetric assay for indole acetic acid (IAA) estimation. To confirm the presence of IAA in the supernatant, one hundred and six bacterial isolates were grown in 5 ml liquid medium (YPB) in a sterile flask supplemented with L-Tryptophan. One ml of suspension bacterial from each flask was centrifuge to separate the supernatant and pellet. One ml of the supernatant was removed and mixed with 4 ml of Salkowski's reagent (50 ml of 35% $HClO_4$ + 1 ml of 0.5 M $FeCl_3$ x $6H_2O$) and allowed to stand for 20 min at room temperature. The positive reaction for IAA production was observed as the development of a pink-red color[5]. IAA amounts were determined at 550 nm in a Microplate Manager Bio-Rad Lab, Inc.

Phosphate solubilization (PSB) activity. Quantitative screening of phosphate-solubilizing bacteria was carried out by culturing the bacterial isolates on solid National Botanical Research Institute`s Phosphate Growth (NBRIP) medium containing (per liter): 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl, and 0.1 g of $(NH_4)_2SO_3$, and then incubated for 3 days at room temperature. The presence of clearing zone around associated bacterial spot was measured[6].

Genomic DNA extraction. Genomic DNAs from the bacterial strains were prepared as described previously[7]. The bacterial isolates were grown overnight in 5 ml of TSB in a rotary shaker at 28°C. Total DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA) in accordance with the instructions of the manufacturer.

Rep-PCR analysis. Thirty orchid associated bacteria were selected to characterize by rep-PCR technique[8]. The DNA sequences of the primers used for DNA fingerprinting were as follows:
5′-CTACGGCAAGGCGACGCTGACG-3 (BOX A1R), 5′-CCGCCGTTGCCGCCGTTGCCGCCG-3′ (MBO REP1),5′-AAGTAAGTGACTGGGGTGAGCG-3′(ERIC 2). PCR reactions were carried out in a final volume of 25 µL consisting of GoTaq green master mix (Promega). The reaction conditions were as follows: After the initial activation step (15 min, 95 °C), 35 cycles at 94 °C for 1 minute, at 53 °C for 1 minute, and at 72 °C for 2.5 min were run followed by a final elongation step at 72 °C for 10 min. Ten microliters of the PCR reactions were analyzed on a 1% agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light.

DNA sequencing and phylogenetic analyses. To confirm the identities of the isolates, PCR amplification and sequencing of the 16S rRNA gene were performed. The 16S rRNA genes were PCR-amplified from the genomic DNA using the bacterial universal primer set of 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGC TAC CTT GTT ACG ACT T-3'), which were also used for sequencing[9]. The reaction mixture contained 1µl of the purified genomic DNA, 1µl of each primer and 25µl GoTaq®Green master mix (Promega, USA) in a total volume of 50 µl. Amplification was done in a thermocycler (MyCycler, Bio-Rad). Initial denaturation at 94°C for 2 min was followed by 34 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and elongation at 68°C for 30 sec. After 34 cycles, there was a final 5-min extension at 72°C and then cooled and held at 4°C. The amplified PCR products were analyzed by 1% (w/v) agarose gel electrophoresis[10]. Sequencing of the amplified DNA fragments was performed using the services of 1st BASE (http://base-asia.com/dna-sequencing-services).

Screening of selected bacteria for *in planta* biocontrol activity and growth enhancement. Three selected bacterial endophytes were tested for the ability to protect plants from infection with soft rot bacterial pathogen, as well as to evaluate the effect on plant growth by using the orchid plantlet *P. amabilis*. Plantlets grown in tissue culture were dipped in bacterial endophyte cultures (10^8 cells per ml) and subsequently transplanted onto MS agar[11]. After incubation for 1 week in a growth chamber at 22°C with a light period of 14 h per day, plantlets were dipped in a culture of *Pectobacterium carotovorum* (10^8 cells per ml) and again transplanted. After 3 weeks of incubation in the growth chamber, plants were scored for disease symptoms. Nonbacterized controls were dipped in either a 0.9% sterile NaCl solution or the pathogen only. The effect of endophytic bacterization on the growth of plantlets was evaluated 2 months later.

Results and Discussion

Production of secondary metabolites. All bacterial isolates tested in this study were previously shown to have antagonistic activity against *P. carotovorum* with variation range of inhibition. When testing for the production of metabolites that may facilitate plant growth, 79 isolates of orchids associated bacteria (74.5% of total isolates) were found to produce

IAA and 92 (86.79%) were able to solubilize inorganic phosphate. Bacterial isolates were varied greatly in their efficiency of IAA production. Strain AkOc1 was the highest in producing IAA, whereas strain TbPh7 which showed the most effective in antibiosis assay, was also the most efficient PSB strain.

Rep-PCR characterization of bacterial isolates using BOXA1R, MBO REP1, and Eric-2 primers. The amplification profiles of thirty selected isolates examined by rep-PCR analysis using 3 different primers were described on Fig. 1. Three selected bacterial isolates (no. 22, 29, 30) which showed different unique rep-PCR patterns and promising production of secondary metabolites were identified and used for further assay.

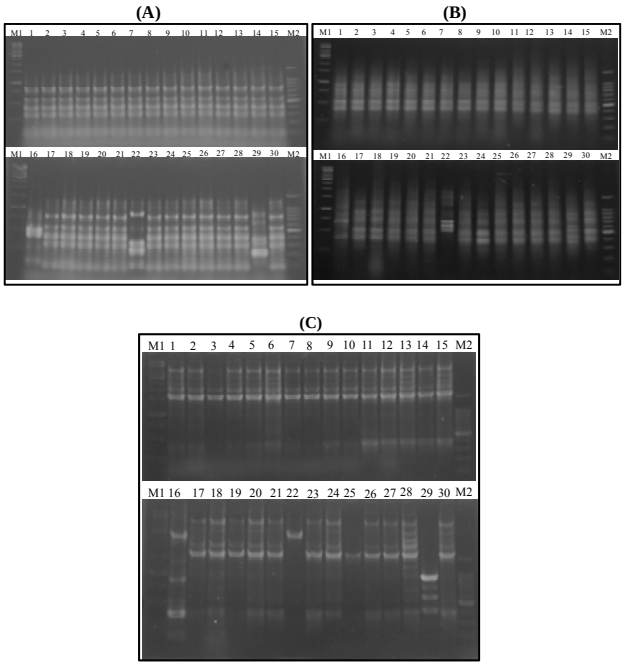

Figure 1. Characteristic rep-PCR fingerprint patterns of endophytic bacteria isolated from different species of orchids in Yogyakarta and west Java, Indonesia using BOX A1R primers (A), MBO-REP1-primers (B), and ERIC2-primers (C).

16S rDNA sequence similarity and phylogenetic analysis. Molecular identification of the isolated strains was carried out based on 16S rDNA sequence analysis. The 1.5 kb sequences obtained from the strains were aligned with all the presently available 16S rDNA sequences in the GenBank database. As a result, a phylogenetic tree was constructed using the neighborjoining method, and is shown in Fig. 2. Phylogenetic analysis using the 16S rDNA sequences indicated that the three bacterial isolates belonged to different genera. From the branching pattern of the tree, isolate AkOc1 determined to be close relatives to *Raoultella ornithinolytica* JCM7251 (GenBank accession number AJ251467) with 98.7% 16S rDNA sequence-similarity. The partial 16S rDNA sequence of isolate DnAr4 showed 98.6% identity with the sequences of *Klebsiella* michiganensis W14 (GenBank accession number JQ070300). In the phylogenetic tree, strain TbPh7 was most closely related to *Bacillus amyloliquefaciens* subsp. *plantarum* SN13 (GenBank accession number KC293995) with 98.05% 16S rDNA sequence-similarity.

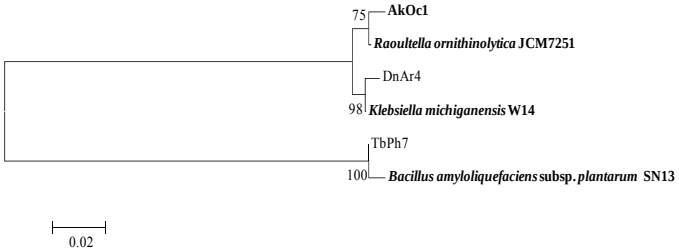

Figure 2. Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between the isolated strains and other species. The tree was constructed using the CLUSTAL-X and neighbour-joining method. Scale bar corresponds to 0.01 subscriptions per nucleotide position. Numbers at nodes indicate levels of bootstrap support (%) determined from 100 re-sampled data.

Monitoring the effect of the isolated bacterial endophytes on disease suppression and plant growth. The beneficial effect of bacterial endophytes was described earlier with potato[11], clove[12], and other crops[13]. In the present study, all the tested bacterial endophytes could suppress soft rot disease on plantlets of *P. amabilis* (Fig. 3). The highest reduction of both disease incidence and intensity was found on *P. ambilis* co-cultured with *B. amyloliquefaciens* subsp. *plantarum* TbPh7 (Table 1). The presence of bacterial endophytes has advantages of escaping microbial competition as well as influencing the host's response to pathogens attack.

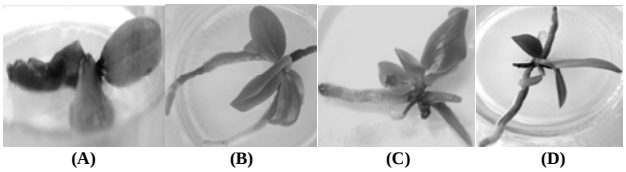

Figure 3. *In vitro* responses of *P. amabilis* plantlets after infection only with *P. carotovorum* (A), or previously co-cultured with *R. ornithinolytica* AkOc1 (B), *K. michiganensis* DnAr4 (C), *B. amyloliquefaciens* subsp. *plantarum* TbPh7 (D).

Table 1. Disease progress after 3 weeks inoculation of *P. carotovorum*

Treatment	Disease incidence (%)	Disease intensity (%)
Control	100	55
AkOc1	20	15
DnAr4	20	5
TbPh7	0	0

Indeed, the growth enhancement of *P. amabilis* plantlets was also influenced by the application of bacterial endophytes. As shown in the Table 2, plantlets co-cultured with *R. ornithinolytica* AkOc1 grew faster and had significantly more roots and length. These effects were not observed when *P. amabilis* plantlets were inoculated with sterile ddH_2O or *K. michiganensis* DnAr4, meaning that not all endophytic introduction will have beneficial effect on growth enhancement.

Table 2. The growth of *P. amabilis* 2 months after inoculation of bacterial endophytes

Treatment	Height (cm)	Root length (cm)	Root number
Control	1.3b	1.33b	2.67ab
AkOc1	1.62a	3.43a	3.00a
DnAr4	1.26ab	2.73ab	2.00ab
TbPh7	1.4ab	1.73ab	2.67ab

Conclusion

In conclusion, from the orchid tissues, we isolated bacterial endophytes that were evaluated as microbial inoculants for disease management and also for stimulation of plant growth. Our data demonstrate that *in vitro* bacterization of orchid plantlets can be used as a new strategy for preventing the spread of soft rot disease; however, a further evaluation of the disease control effectiveness of these strains and the design of a biocontrol formulation and application must be conducted under field conditions.

Acknowledgements

This study was supported by Ministry of Research, Technology and Higher Education of the Republic of Indonesia through national competitive research grants. The authors also thank the United Graduate School of Agricultural Science, Gifu University for providing financial assistance during "International Symposium on Innovative Crop Protection for Sustainable Agriculture" in Gifu, Japan.

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Potential of endophytic bacteria as plant growth promoter and antagonist against pineapple-fungal plant pathogen in Indonesia

Radix SUHARJO[1], Titik Nur AENY[1], Udin HASANUDIN[1], TE SUKMARATRI[2], Ruslan KRISNO[2], Thoriq KHOIRONI[1], Diyan Adinda SAFITRI[1]

([1]Faculty of Agriculture, Lampung University, Indonesia; [2]Great Giant Food Company, Lampung, Indonesia)

SUMMARY

This study was aimed to investigate potential of 15 endophytic bacteria (3C, AK, CH, GKSKK, GBSH, AM, B1, GKSKC, GBSK3, GKSKW, GKSKP, A31, GKSKKn, NS and AP) that were isolated from healthy leaves of pinneaple as plant growth promoter and antagonist of pineapple-fungal plant pathogens. The isolates were investigated on their hipovirulence, ability as plant growth promoter and ability to inhibit three pineapple-fungal pathogens, namely *Phytophtora* sp., *Curvularia* sp. and *Thielaviopsis* sp. The result showed that 10 out of 15 isolates were hipovirulent. Among 10 hipovirulence isolates, 7 isolates had potential as plant growth promoter (3C, AK, GKSKK, AM, B1, GKSKC and GBSK3). In the case of their antagonistic capability, the isolates which were produced the highest percentage of inhibition against *Phytoptora* sp. and *Curvularia* sp. were GKSCK at 72,48% and 66,08% of inhibition, respectively. Meanwhile, the highest percentage inhibition against *Thielaviopsis* sp. was obtained by CH at 64,82% of inhibition. In this study, we found that some of the endophytic bacteria can be plant growth promoter or antagonist or both as plant growth promoter and antagonist.

Introduction

Pineapple is one of the most important fruit comodities in Indonesia. Recently, although not very significant, pineapple production in Indonesia continues to decline. This is due to the decreased of soil fertility and infection of some plant pathogens. Three important plant pathogens that have been reported causing severe economic losses are *Phytophtora* sp., *Curvularia* sp. and *Thielaviopsis* sp.. Application extra chemicals fertilizer and fungicides to solve the problems can cause more severe harms to the environment and future cultivation and efforts to improve pineapple production. Thus, it need to find alternative methods that is safe to be used to solve the complications, and one of which are reducing the chemicals and using bio agents.

Endophytic bacteria is one of the promising bio agents that can be used to improve production of cultivated plants, including pineapple. Endophytic bacteria is bacteria that live internally inside the plant tissue, can be isolated from the plant after surface desinfection and does not cause negative effects on plant growth (Wilson, 1995; Gaiero et al., 2013). It has been reported that endophytic bacteria has capability as plant growth promoter (Gaiero et al., 2013; Santoyo et al., 2016), plant resistance inducer against plant diseases (Costa et al., 2013; Lanna-Filho et al., 2013; Yi et al, 2013; Egamberdieva et al., 2017; Leiwakabessy et al., 2018) and antagonist of many kinds of plant pathogens (Duffy and Defago, 1999; Gaiero et al., 2013).

Fifteen endophytic bacteria were successfully isolated from healthy leaves of pineapple. However, study on their potential as agricultural bio agents has not been performed. This study was conducted in order to investigate virulence, ability as plant growth promoter and antagonist of the fifteen isolates of above mentioned endophytic bacteria against three pineapple fungal pathogens, namely *Phytophtora* sp., *Curvularia* sp., and *Thielaviopsis* sp..

Material and Method

Endophytic bacteria. As much as 15 isolates of endophytic bacteria used in this study. All the strains were isolated from healthy leaves of pineapple.

Hypovirulence test. Hypovirulence test was performed using method developed by Worosuryani (2005). Sprouts of cucumber were used as indicator. Inoculation each of endophytic bacteria was repeated 3 times. Observation was performed until 14 days after inoculation. Disease severity Index (DSI) was calculated using formula : ($\sum$N/Z); N : total score of disease severity on each individu, Z : total individu used. Score of the disease

severity that was used can be explained as follows: 0 : healthy, there was no infection on hypocotyl; 1 : one or two brown spot observed with <0.25 cm of diameter; 2 : brown spot observed with < 0.5 cm of diameter with <10% of wetness area of hypocotyl; 3 : brown spot observed with > 1 cm of diameter with 10%<x<100% of wetness area of hypocotyl; 4 : black spot observed, wilt and sprouts death. The endophytic bacteria with DSI <2 was put in the group of hypovirulent bacteria.

Investigation on its capability as plant growth promoter. Cucumber plant was used as indicator plant. Investigation was conducted using methods developed by Worosuryani (2005). As much as 10 ml of bacterial suspension (~10^8 CFU/ml) was pured into planting medium of plant indicator. Inoculation each of the bacteria was repeated 3 times. Observation of plant height was performed every two days. Greenish leaves level was conducted once at 16 days after inoculation using chlorophyll content meter CCM 200 plus (opsi science) at the 3 of leaves position i.e. top, midle and bottom. Weight of wet and dry of shoot and root was conducted at 21 days after inoculation. In the case of dry weight of shoot and root, the fresh shoot and root were put into envelope and it was incubated at 80^{o}C for 3 days. After incubation, it was weight using digital balance EG 4200-2NM (Kern).

Antagonistic capability agains pineapple fungal-plant pathogens. Three pineapple fungal plant pathogens used in this study i.e. *Phytophtora* sp., *Curvularia* sp., and *Thielaviopsis* sp.. Antagonistic test was performed by scraping the bacteria using inoculating loop with a distance of 2 cm from the edge of petridish (diam 9 cm) contains Potato Sucrose Agar (PSA) medium (Potato extract 1000 ml, Sucrose 20 g, Agar 20 g) in both side. One culture of 7 old days of each of the fungal pathogens (diam 0.5 cm) was placed in the midle of petridish. As control, one culture of each of plant pathogens was put in the midle of petridish contains PSA medium without any endophytic bacteria. All the petridish were incubated at room temperature. Observation was conducted at 1, 3, 5 and 7 days after inoculation on the wide of fungal colony that was measured in milimeter. Percentage of inhibition was calculated using formula : [L1-L2/L1]x100%. L1: wide of fungal colony without endophytic bacteria, L2 : wide of fungal colony with endophytic bacteria.

Result and Discussions

In this study, 15 endophytic bacteria was investigated on their hypovirulence, capability as plant growth promoter and antagonist against 3 pineapple plant pathogens, namely *Phytophtora* sp., *Curvularia* sp. and *Thielaviopsis* sp.. The result showed that 10 out of 15 isolates showed hypovirulent (Table 1). Among those 10 hipovirulent isolates, 7 isolates showed potential as plant growth promoter. Application of the bacterial isolates resulting better growth on indicator plant compared to the untreated plants. Application of endophytic bacteria consistently improve plant height, greenish leaves, wet and dry weight of shoot and root and root length (Fig 1). Gaiero et al (2013) and Santoyo et al. (2016) stating that endophytic bacteria also could promote growth of their host plant. The bacteria release phytohormones (Bloemberg & Lugtenberg, 2001) that can improve plant growth such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Gaiero et al., 2013; Santoyo et al., 2016), jasmonate, indole acetic acid, and abscisic acid (Patten and Glick, 2002; Forchetti et al., 2007). Beside their ability as growth promoter, endophytic bacteria was also reported as plant resistance inducer (Romeiro et al., 2005; Lanna-Filho et al., 2013). Application of endophytic bacteria has been reported can improve plant resistance against plant diseases such as bacterial leaf spot of pepper (Yi et al, 2013), bacterial leaf spot of tomato (Lanna-Filho et al., 2013), bacterial leaf blight of rice (Leiwakabessy et al., 2018), damping off on

Table 1 Disease severity index resulted by inoculation of the bacterial isolates on cucumber sprouts and its role as plant growth promoter

Isolates	Disease Severity Index	Role as plant growth promoter
AP	2.75	Not tested
GKSKKn	2.58	Not tested
NS	2.50	Not tested
A31	2.50	Not tested
GKSKP	2.42	Not tested
3C	2.00	Yes
AK	1.92	Yes
CH	1.83	No
GKSKK	1.67	Yes
GBSH	1.67	No
AM	1.33	Yes
B1	1.17	Yes
GKSKC	1.00	Yes
GBSK3	0.75	Yes
GKSKW	0.33	No
Kontrol	0.00	-

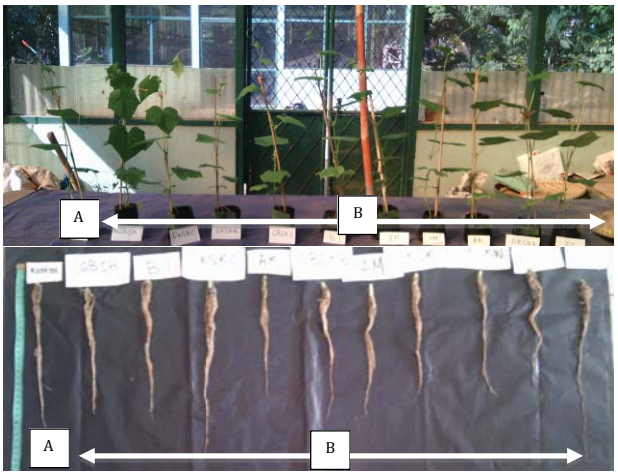

Fig.1 Indicator plant after application of bacterial isolates. A. control, B. treated plants

cucumber (Costa et al., 2013) and root rot of chickpea (Egamberdieva et al., 2017). Some of endophytic bacteria have also been reported produce anti microbial compounds, such as siderophore and antibiotics, that can inhibit growth of plant pathogens (Duffy and Defago, 1999; Gaiero et al., 2013) such as Enterobacter, *Pseudomonas* sp., *Bacillus* sp.. (Muzzamal et al., 2012), *Fusarium oxysporum* f.sp. *lycopersici* (Shahzad et al., 2017), *Phytophtora capsici*, *Alternaria panax* and *Botrytis cinerea* (Paul et al., 2013).

In this study, we found that some of endophytic bacteria used had capability to inhibit *Phytophtora* sp., *Curvularia* sp. and *Thielaviopsis* sp.. (Fig. 2). Inhibition was in the range of 5.13 to 72.48% (*Phytophtora* sp.), 2.33 to 66.08% (*Curvularia* sp.) and 1.33 to 64.82% (*Thielaviopsis* sp.). The best capability to inhibit *Phytophtora* sp., and *Curvularia* sp. was produced by GKSCK. Meanwhile, the highest inhibition of *Thielaviopsis* sp. was produced by CH (Table 2). It was shown that one endophytic bacteria can inhibit more than one pathogens.

Ability of endophytic bacteria to inhibit more than one kinds of pathogens have also been reported. Endophytic bacteria isolated from potato (Berg et al., 2005) and chilli pepper (Paul et al., 2013) have been proven to be antagonist of more than one kinds of pathogens.

Study performed by Berg et al. (2005) revealed that endophytic bacterium isolated from potato could inhibit *Verticillium dahliae* or *Rhizoctonia solani.* Paul et al. (2013) stated that edophytic bacteria isolated from chilli pepper can inhibit *Fusarium oxysporum* or *Alternaria panax* or *Colletotrichum acutatum* or *Phytophtora capsici* or *Botrytis cinerea*.

Conclusion

In conclusion, not all endophytic bacteria used in this study were plant growth promoter and antagonist. There was endophytic bacteria that play a role as plant growth promoter or antagonist or both plant growth promoter and antagonist. The best inhibition to *Phytoptora* sp. and *Curvularia* sp. were produced by GKSCK, meanwhile, the highest inhibition against *Thielaviopsis* sp. was obtained by CH.

Acknowledgement

We thank to Great Giant Food Company for providing financial support and material required for this research. We also thank to Lampung University for many support provide during research.

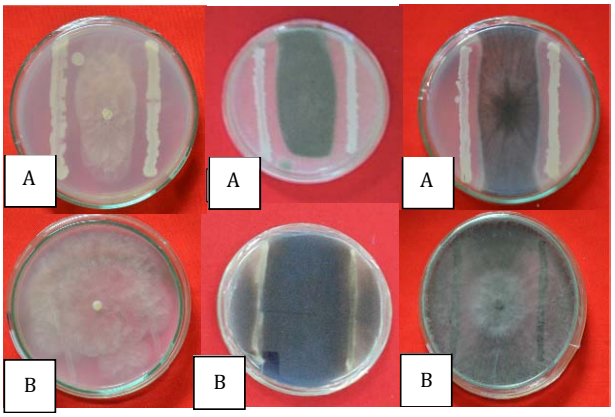

Fig. 2 Antagonist test of endophytic bacteria against 3 pineapple fungal pathogens 7 days after inoculation. From left to right : *Phytophtora* sp., *Curvularia* sp., *Thielaviopsis* sp.. A. Endophytic bacteria that had antagonistic capability, B. Endophytic bacteria that did not has antagonistic capabilitycapability.

Table 2 Percentage of inhibition of endophytic bacteria against thee pineapple fungal pathogens

Isolates	Percentage of inhibition (%)		
	Phytophtora	Curvularia	Thielaviopsis
AP*	14.76	30.18	5.58
GKSKKn*	0.00	27.77	1.53
NS*	0.00	16.73	5.44
A31*	61.85	13.35	46.15
GKSKP*	62.74	6.37	61.85
3C*	0.00	15.40	63.71
AK	64.30	29.94	4.94
CH	67.15	2.40	64.82
GKSKK	0.00	30.53	25.87
GBSH	20.00	14.48	29.68
AM	0.00	5.67	1.50
B1	0.00	9.40	8.72
GKSKC	72.48	66.08	4.88
GBSK3	5.13	2.33	0.00
GKSKW	64.11	24.88	1.33
Kontrol	0.00	0.00	0.00

* Isolates which were virulent (DSI>2) on the result of hypoverulence test

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Endophytic Bacillus as biological control agent of banana wilt

HADIWIYONO

(Faculty of Agriculture, Universitas Sebelas Maret)

SUMMARY

Banana wilt caused by *blood disease bacterium* (BDB) and *Fusarium oxysporum* f.sp. *cubense* (FOC) is a couple important threaten in cultivation of banana in Indonesia. The use of free pathogen seedling from tissue culture is recommended as a component of integrated control of the disease. However, the seedling resulted by tissue culture is more susceptible to pathogen infection due to by loss of bacterial endophyte, the inducer of resistance of banana. One of endophyte bacterial group is *Bacillus* spp. This paper would like to report the potentiality of endophytic Bacillus as biocontrol agent of the banana wilts. Based on the results of some tests in laboratory and screen house, showed that some isolates of Bacillus showed potential antagonistic to the pathogens and could induce resistance of banana plantlet to both banana wilts. The isolates of endophytic Bacillus could release volatile and nonvolatile compound of pathogen growth inhibitor, growth regulator hormone especially IAA, and enzyme of chitinase and pectinase that are considerable to be involved in the antagonism of the Bacillus to the pathogens or inducing resistance of banana planlets to the diseases.

Introduction

Wilt disease is very important in banana cultivation in Indonesia. Banana wilt disease is caused by bacterial blood disease (BDB) and or *Fusarium oxysporum* f.sp. *cubence*. Incidence of wilt disease in the field can reach above 80%. Both pathogens often attack together so that the damage to the plant becomes more severe[1]. So far, both pathogens are still difficult to control. Control of both diseases must be done in an integrated way. One of the most effective and practical integrated control components is the planting of superior variety, to date the resistant banana varieties against both pathogens are not yet available[2,3,4]. Therefore, the use of healthy seedlings becomes an important integrated control component. Healthy seedlings in the field are difficult to detect visually because 20-30% of healthy-looking plants are infected with BDB[5]. For producing pathogen free seedlings have been successfully developed with tissue culture technology[6,7]. Smith et al.[8] reported that the tissue culture seedlings were susceptible to wilt pathogens, due to aseptic conditions during the production in vitro culture process to be free of endophytic bacteria involved in resistance mecahnims of banana. This report is in line with analysis results of endophytic bacterial total based on the pattern of DNA fragments of the asymptomatic banana through ribosomal integenic specer region analysis (RISA) that is different from symptomatic ones[8,9]. Some DNA fragments of certain bacteria are seen predominantly in asymptomatic bananas. It is suspected that the bacteria are as the resistance inducer of the asymptomatic banana. It is popular that one of the most effective endophytic bacteria as plant disease control agents is *Bacillus* spp.[10,11,12]. This paper is a report of some researches results about the effectiveness and mechanism endophytic Bacillus of banana as biological control agent of banana wilt.

Materials and Method

Endophytic Bacillus isolates. A mount of isolate of endophytic Bacillus was isolated from healthy banana sampled from endemic wilt fields. The Bacillus were isolated from 3 peaces ±(0.2 x 0.5 x 10 mm) the bit of internal tissues of peduncle, pseudostem, and rhizome dipped in 5 mL sterile water. The samples were incubated for 12 hours and shaken with Vortex Shaker. A volume of 100 µl suspension was plated on Nutrient Agar (NA) with spread technique on medium in thedish with composition 8 g Nnutrient Broth and 15 g agar Bacto in 1 L waters. The dishes were incubated for 24 hours. Single colony was sub-cultured using the same medium. The pure isolates were streaked on a slant NA medium for stock[13].

Antagonism tests in vitro. Antagonistic Bacillus to the pathogens in vitro was tested on Nutrient Agar (NA) incubated 72 hours to BDB and Potato Dextrose Agar

(PDA) incubated 5 days to FOC. The zone of inhibition was observed as antagonistic variable.

Antagonism tests in vivo. The resistance test of banana seedlings to BDB was performed by placing plantlets having been acclimatized for 3 months on sterilized soil infested by BDB and Bacillus at 10^8cfu g^{-1} in polybags. Banana resistance test to FOC is performed by planting seedlings on infested sterile soils by pathogen spore suspension with density at 10^5 spores g^{-1} of soil. The disease severity was observed weekly for 7 weeks for evaluating the resistance performance of the seedlings.

Antagonistic mechanism of Bacillus to pathogens. One of the possible antagonistic mechanisms of Bacillus to pathogen is antibiosis through production some toxic volatile or nonvolatile compounds released around the medium. The toxic nonvolatile compound was based on toxicity test of filtrate medium. Sterilized filtrate medium harvested from bacterial culture incubated for 4 days was tested in the capability to inhibit the growth of the pathogens. The sterile filtrates were mixed in NA medium for BDB and PDA for FOC. Toxic activity was evaluated by observation of zone inhibition of the pathogens performed by the filtrate after incubating for a week. The inhibition observation was similar with antagonism test procedure. The volatile toxic compound test was prepared by culturing endophytic Bacillus on the NA medium and at the same time filter paper (Φ 5 mm) dyed in BDB inoculum suspention 10^8cfu mL^{-1} and FOC colony (Φ 5 mm) were cultured on the other plate containing the NA or PDA culture medium. The both plate were covered each other with BDB or FOC culture upside down and silted using isolative plastic. The growth colony diameter of BDB and FOC were observed every 2 days for 8 days.

The capability of Bacillus to produce enzim was based on the capability of the bacteria to grow on the medium containing certain enzim. A filter paper used for inoculum was dipped in a suspension of *Bacillus* (10^8 cfu mL^{-1}) prepared using King's B liquid medium incubated with shakering for 24 h. Each isolate was placed in the center of a Petri dish containing chitin and pectin agar as treatment and without chitin and pectin as a control[14].

Results and Discussions

At least 30 isolates of endophytic Bacillus had been collected and 10-15 isolates were tested farther to evaluate the potential antagonism[15,16]. Some researchers also reported that endophytic Bacillus could be isolated from vasculer tissues of veries plants[17,18,19]. Azevedo et al.[20] reported that many endophytic bacteria such Bacillus could be cultured purely from their plant hosts.

The results of antagonism tests of Bacillus to BDB and FOC on agar medium showed that all of isolates were antagosnitic to the pathogens indicated by performing clear zone of growth inhibition to both. The clear zone is formed by toxic compound released by the antagonist. The capability of Bacillus to produce toxic compound was also showed by growth inhibition caused by filtrate of culture medium. It indicates that in the liquid medium, Bacillus released toxic compound into medium.

The results of antagonism test in vivo showed that all of isolates of endophytic Bacillus were antagonistic and could reduce the wilt intensity caused by both the pathogens. Bacillus could reduce wilt intensity caused by BDB with range of 45.08-71.45 and caused by FOC with range of 13.95-72.10[14,15]. Sunaina et al.[21] did a research with *B. subtilis* treatment on tuber that could control bacterial wilt of potato and increase the yields of tuber reaching 160%. Whereas Georg and Kloepper[22] reported that *B. pumilus* strain INR7 and *B. subtilis* strain GB03 had capability to promote the growth of plant and minimizing disease severity

Chen et al.[23] also reported at least 10 isolates of cotton endophytic bacteria that could suppress the disease severity of cotton wilt caused by *Fusarium oxysporum* f.sp. *vasinfectum* through bacterialization of cotton seeds. Haiyan et al.[19] reported that Bacillus could not only induced resistance of plant to disease infection but also to insect pests.

Based on the test of capability of endophytic Bacillus to produce growth regulator hormone showed that all isolates could produce indole acetic acid (IAA). It has been reported that some Bacillus could produce IAA . Patel and Patel[24] revealed that endophytic Bacillus could produce IAA. IAA was involved in promoting growth of plant. Asari et al.[25] found endophytic Bacillus with charater as plant growth promoting bacteria. *Bacillus* spp. was known well as Plant Growth Promoting Rhizobacteria (PGPR) due to by the capability of producing IAA taking a role in biological control mechanism through inducing systemic resistance[11,26].

The capability of Bacillus to produce IAA is believed that the compound involves in promoting the growth and inducing resistant of plant. The role of IAA in inducing resistance of plant is important especially to

weak pathogen such Fusarium that the infection will establish more in weak condition of plant. Waller and Brayford[27] confirmed that Fusarium constituts weak pathogen. It means that FOC will be more infective on weak banana that can be induced by varies biotic or abiotic stresses. Some researchers explain that Bacillus can produce indole-3-acetic acid (IAA), which is fungsional to help nitrogen fixation from the atmosphere, iron fixation through siderophore production, solubilization of potassium (K), zinc (Zn), and phosphate (P) from the soil, and increasing the soil porosity[28,29,30,31]. Along with the micro- and macro-nutrient supply, endophytic Bacillus will protect the plants host from phytopathogenic and take roles in antagonism[28]. It had been also reported that *B. subtilis* isolate A47 could activate biological control to Gram-negative bacterium due to by a metabolite compound the group of iturin being resistant to hydrolysis and thermo-stable[31]. *Bacillus* spp. had been also reported as an important biological control agents through producing toxic volatile and soluble compound of plant[11,24]. Several species of Bacillus have been described to produce different antimicrobial lipopeptides. The compounds including antibiotic subtances and extracellular degradative enzymes[32,35,34,35,36]. Perez et al.[34] confirmed that Bacillus showed the best potential for the production of antifungal and antibacterial substances.

Many Bacillus produce lipopeptides that can be divided into three major families that are surfactins, iturins and fengycins or plispastatins. Surfactins and iturins are composed by cyclic heptapeptides, which contain a β-hydroxy fatty acid and β-amino fatty acid, respectively[35,36,37]. Besides the antimicrobial activity, Surfactin is also an outstanding surface-active property[21,23]. Iturins are a group of lipopeptides that are remarkable antifungal activity to many microorganism including bacterial and fungal plant pathogens[23,26].

All isolates of endophytic Bacillus also could produce extracellular degradative including chitinase and pectinase. Zhao et al.[26] reported that there was positive correlation between pectinase activity and inhibition rate on *Fusarium oxysporum* on the medicinal plant *Lonicera japonica*. It has been revealed that biological control of some soil-borne fungal diseases has been correlated with chitinase production[39].

The play role of chitinase and pectinase in a ntagonism to plant pathogenic bacteria are still poorly understood. The indirect role is possible. It has been reported that production of chitinase and pectinase together with IAA is important in multiplication and colonization of the antagonistic bacteria in or on the hosts. Pectinase has been reported as one of the determinants of induced systemic resistant[40,41,42].

Conclusion

The isolates of endophytic Bacillus could release volatile and nonvolatile compound of pathogen growth inhibitor, growth regulator hormone especially IAA, and enzyme of chitinase and pectinase that are considerable to be involved in the antagonism of the Bacillus to the pathogens or inducing resistance of banana planlets to the diseases.

Acknowledgements

This manuscript was supported effectively by ADP Putra and A.Widyantoro, the student of Agrotechnology Department, Faculty of Agriculture, Universitas Sebelas Maret.

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Molecular based identification and formulation of cyanogenic *Pseudomonas* spp. controlling *Phytophthora infestans*

Md. Rashidul ISLAM, Md. Nizam UDDIN and Mohammad Monirul Hasan TIPU
(Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh-2202)

SUMMARY

Late blight caused by *Phytopthora infestans* is one of the devastating diseases of potato and tomato worldwide. Every year the disease incurs a loss estimated around $16 billion in the developing countries and in Bangladesh the loss estimated around $ 880 million. Recently, bacterial bioagents such as *Pseudomonas* spp. and *Bacillus* spp. have been reported to control *P. infestans*. In this study, a total of 200 bacterial isolates obtained from phylloplane of potato plants were evaluated for the production of hydrogen cyanide and only six bacterial isolates were identified as cyanogenic. The primers PsEG30F (5′-ATYGAAATCGCCAARCG-3′) and PsEG790R (5′-CGGTTGATKTCCTTGA-3′) specific to the conserved sequences of *rpo*D gene (the sigma 70 factor subunit of DNA polymerase) producing a 760bp amplicon confirmed the bacterial isolates are *Pseudomonas* spp. Sequence analyses revealed that these *Pseumonas* spp. are closely related to *Pseudomonas* spp.(SJK83154), *Pseudomonas putida* (CCO02553), *Pseudomonas fulva* (CBI68359), *Pseudomonas parafulva* (CBG22656) and *Pseudomonas monteilii* (CBG22644). *In vitro* growth inhibition assay showed that all cyanogenic *Pseudomonas* spp. inhibited the growth (17.43 to 78.71%) of *P. infestans.* The bacterial isolates were formulated in Talcum powder and the bacterial cells were found viable for up to six months or more. The growing ability of these cyanogenic *Pseudomonas* spp. at cool to warm temperature (8-30°C) suggesting its potentiality in controlling late blight of potato.

Introduction

Potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum*) are the important vegetable crops in Bangladesh where it is primarily grown by resource-poor farmers. Late blight caused by *Phytophthora infestans* (Mont.) De Bary limits the production of potato and tomato especially in cool weather worldwide. *P. infestans* (Mont.) De Bary, is an oomycete which is well known for its explosive development when environmental conditions are suitable and host plants susceptible to infection (Mizubuti and Fry 2006). Estimated worldwide economic losses due to the control cost and disease vary from 3 to 5 billion dollars annually (Judelson and Blanco 2005; Haldar et al. 2006). In Bangladesh, the yield losses have been estimated approximately by 25-57% due to late blight disease of potato (Ali and Dey 1994). Moreover, late blight is considered one of the five highest ranking priorities for potato research in Asia, Africa and Latin America (Fuglie, 2007).

Late blight management has been heavily based on fungicide application and fungicide applications have increased over the last decade due to the introduction of new and more aggressive genotypes of the pathogen (Kato et al. 1997). At the same time, two counter- balancing factors have also grown: societal pressure for reducing pesticide use on crops and acreage of organically-grown food crops, potato and tomato included (Ghorbani et al. 2004). Therefore, innovative and effective control measures are needed if fungicide use is to be reduced or, as in the case of organic production of potato and tomato which is based on some modern and copper-based fungicides for control of late blight. However, there has been increasing pressure to find substitutes for these products because of environmental contamination caused by these chemical residues. Thus, there is enormous interest in finding effective non-chemical alternatives to protect potato and tomato fields against their most threatening foliar disease. The market for non-conventional fungicide products, specifically biological control agent formulations, is growing at a rapid pace. Biological control is a routine part of disease management. Several commercial formulations of biocontrol agents are available and many others are currently being developed (Fravel 2005) and several commercial formulations of biocontrol agents have been tested for efficacy against late blight. Of many trials involving different microorganisms, including *Trichoderma harzianum*, *Bacillus subtilis*, *Streptomyces* sp.,*Coniothy-rium minitans* and a pool of undetermined effective microorganisms (EM 5), the most effective was the *B. subtilis* based-product (Stephen et al. 2005). Cyanogenesis in bacteria seems to be restricted to a relatively small number of species and most hydrogen cyanide-producing bacteria have been identified within the genus *Pseudomonas* belonging to the Gammaproteobacteria (Ryall et al. 2009). Potato associated cyanogenic *Pseudomonas* spp. displaying volatile-mediated high potential against *P.* infestans (De Vrieze et al. 2015; Hunzike et al. 2015). The present study has been undertaken to identify and formulate the effective bioagents against late blight disease of potato and tomato.

Materials and methods

Isolation of *Pseudomonas* spp.: Representative potato plants were collected with their root systems and adhering soil from the farmer's fields. The leaves and stems (referred to here as "shoots") as well as root tissues were separated and treated as follows: shoots was ground in a disinfected ceramic mortar using 5 ml of sterile water; roots and adhering soil will be shaken in sterile water to collect the rhizosphere soil, while the roots

themselves were discarded. The samples were homogenized by shaking and pipetted into a test tube with a cut tip. These suspensions were 10-fold serially diluted in sterile water and plated on King's B medium. All plates were incubated at 20°C for at least 6 days. In order to cover as much of the cultivable diversity as possible, single colonies with different morphologies (rhizosphere versus phyllosphere) were picked for isolation. Strains were then preserved in glycerol stock at
-80˚C.

Analysis of HCN: The emission of volatile hydrogen cyanide (HCN) was assessed qualitatively by growing bacterial isolates in LB medium containing 0.5 g glycine (a precursor for HCN production) per litre medium. Gaseous hydrogen cyanide released by bacterial isolates was detected via a color change from yellow to orange of a filter paper (impregnated with picric acid solution). The filter papers (90 mm Whatman®) were autoclaved, saturated with 1% picric acid solution, let dry in the laminar flow bench overnight and wetted with 10% sodium carbonate solution prior to final use. The filter papers were fixed to the lid of a petridish containing culture medium on which the tested bacteria growon.

Antagonistic assay of *Pseudomonas* spp. against *P. infestans:* The antagonism test was carried out on Luria Bertani (LB) agar medium. Bacterial isolates were streaked on LB agar medium twenty four hours ago in a triangle. Then *P. infestans* isolate was inoculated using a 5 mm cork borer at the centre of the triangle. The plates were incubated at 20ºC for two weeks. This test was done in triplicates. Radial growth inhibition of *P. infestans* was assessed 14 days later by measuring the radial growth of the pathogen in the dual and control plates.

Identification and detection of hydrogen cyanide producing *Pseudomonas* spp.: The *Pseudomonas* spp. were identified using primers PsEG30F (ATYGAAATCGCCAARCG) and 736PsEG790R (CGGTTGATKTCCTTGA) designed based on *rpoD* gene sequence specific to *Pseudomonas* spp. as described previously by Mulet et al. (2009). HCN producing strains were confirmed by PCR using degenerated primers HCNC-F (5' GARTTYYTNTGYGAYCAYCA 3') and HCNC-R (5' ATYTCNCCRTTRTCYTTYTG 3')
designed for the *hcnC* gene, which is part of the *hcnABC* gene cluster that encodes for the HCN synthase (Dürr, 2014). The PCR was carried out with an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 1 minute. The PCR was completed by a final elongation step at 72°C for 10 minutes.

Sequencing of *rpo*D gene: Partial nucleotide sequencing of DNA polymerase sigma factor *rpo*D was performed from the amplified products of different *Pseudomonas* spp.. The PCR products were purified using SV Gel and PCR clean up system (Promega, Madison, USA). The sequencing was done directly from PCR products using primers (Ps30F) according to the standard protocol for ABI3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA) with BigDye Terminator v1.1 and 3.1 Cycle Sequencing Kits. The quality of nucleic acid sequences was evaluated using Chromas (Version 2.6) to avoid the use of low quality bases.

Analysis of nucleotide sequence: The nucleotide sequences were analyzed using online bioinformatics tools. The amino acid sequences translated from the nucleotide sequences were compared with other amino acid sequences of *rpo*D gene of *Pseudomonas* spp. available in the NCBI database using Basic Local Alignment Search Tool (BLAST) algorithm to identify closely related sequences. Multiple sequence alignments were performed using aligner tool from www.justbio.com. The phylogenetic tree was constructed based on *rpo*D gene sequences of different closely isolate of *Pseudomonas* spp. deposited in the NCBI database using CLUSTALW software.

Formulation of cyanogenic *Pseudomonas* spp controlling late blight of potato: The identified potential cyanogenic *Pseudomonas* spp. was formulated on two carrier material -talcum powder and sodium alginate. One hundred grams of each carrier material was placed in a metal tray under aseptic conditions and the pH adjusted to 7.0 by adding $CaCO_3$ at the rate of 15 g/kg. Carboxy methyl cellulose (CMC) was then be added at the rate of 10g/kg and mixed well. Finally, the mixtures were packed into a polythene bag and autoclaved for 1h at 121ºC (15lb/inch^2). After autoclaving, 400 ml of bacterial suspension (1×108 cfu/ml) was added to the sterilized carrier material (1kg) followed by thorough mixing under sterile conditions. The formulation thus prepared was allowed to dry aseptically and was then ground to powder. They were then packed in sterile polythene bags and stored at 4°C. The viability of the formulated bacterial cells was evaluated following dilution plate technique for a period of six months.

Results and Discussions

Identification of *Pseudomonas* spp.: Bacterial colonies were primarily identified as *Pseudomonas* spp. based on colony morphology and gram reactions. All the colonies were yellowish in colour and showed gram negative reactions as tested by KOH solubility test. A total of 200 bacterial colonies were primarily identified as *Pseudomonas* spp..

Analysis of HCN: The emission of volatile hydrogen cyanide (HCN) by the bacterial isolates assessed qualitatively as described above. Gaseous hydrogen cyanide released by bacterial isolates was detected via a

color change from yellow to orange of a filter paper (impregnated with picric acid solution). Six isolates were identified as cyanogenic out of 200 isolates (Data not shown).

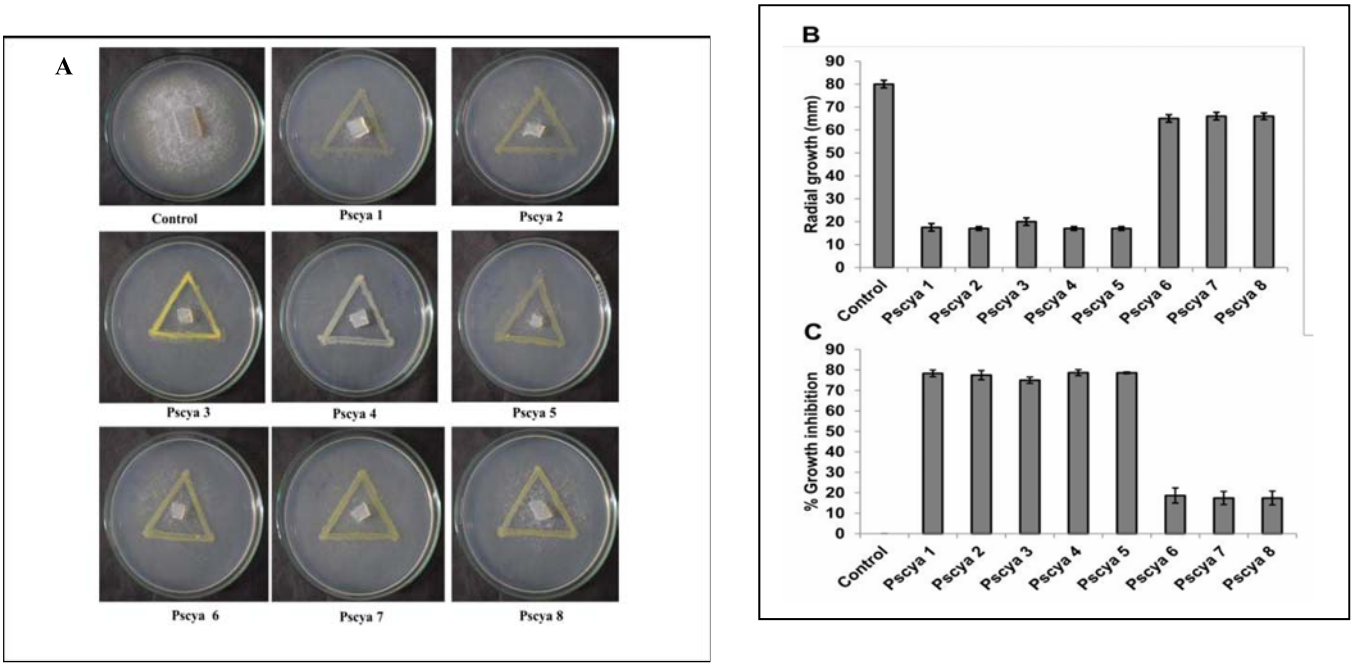


Fig. 1 **A.** *In vitro* growth inhibition of *P. infestans* by different isolates of cyanogenic *Pseudomonas* spp.**, B.** Radial growth of *P. infestans* against different cyanogenic *Pseudomonas* spp. and **C.** Per cent growth inhibition of *P. infestans* by different cyanogenic *Pseudomonas* spp.

***In vitro* growth inhibition of *P. infestans* by *Pseudomonas* spp.:** *In vitro* growth assay showed that the all *Pseudomonas* spp. inhibited the growth of *P. infestans* (Fig. 1A & 1B). The results revealed Pscya 4 isolate inhibited highest (78.71%) growth of *P. infestans* followed by isolate Pscya 1 (78.36%) and Pscya 5 (78.61%) (Fig.1C). The isolates Pscya 2 and Pscya 3 inhibited 77.46 and 75.03% growth of *P. infestans*, respectively.The minimum growth inhibition of *P. infestans* was exhibited by Pscya 6, Pscya 7 and Pscya 8. The variation in growth inhibition of *P. infestans* by different isolates of *Pseudomonas* spp. might be due to their potential in production of some volatile organic compounds. Growth inhibition of *P. infestans* by *Pseudomonas* spp. through synthesis of volatile organic compounds was also observed (De Vrieze et al. 2015; Hunziker et al. 22015).

Molecular based detection: A PCR -based approach was developed for species identification of *Pseudomonas* strains and for the direct detection of *Pseudomonas* populations natural environment (Mulet et al. 2009). A highly selective set of primers (PsEG30F and PsEG790R), giving an amplicon of 760 nucleotides in length, was designed based on the internal conserved sequences of *rpoD* gene sequences (the sigma 70 factor subunit of the DNA polymerase) of *Pseudomonas* type strains. In this study, the cyanide producing *Pseudomonas* spp. was identified by PCR using primers PsEG30F and PsEG790R. An amplicon size 760 bp confirmed the isolates were *Pseudomonas* spp. (Fig. 2A).

PCR based assay for identification of cyanogenic *Pseudomonas* spp.: In addition to the functional qualitative screening methods for cyanogenic bacterial isolates, a PCR based molecular assay was performed. For this purpose, degenerated primers were designed for the *hcnC* gene, which is part of the *hcnABC* gene cluster that encodes for the HCN synthase. All isolates were also tested with the HCNC PCR. Primer design was carried out by Gajender Aleti (AIT) by ClustalW aligning (Thompson et al., 1994) of *Pseudomonas aeruginosa*protein sequences of the HCNC subunit (*P. aeruginosa* strain PAO1 with NCBI accession number NP_250885.1 as starting sequence for initial protein blast). Using wobble codes, degenerate primers were designed for the most conserved regions. With the HCNC PCR set-up described above, amplification was recorded for all isolates, which were detected as producers of hydrogen cyanide with both functional screenings methods. The PCR products could always be detected with a sequence length of approximately 360bp when analyzed with agarose gel electrophoresis. Moreover, no amplification occurred for any of the acyanogenic isolates (Fig. 2B).

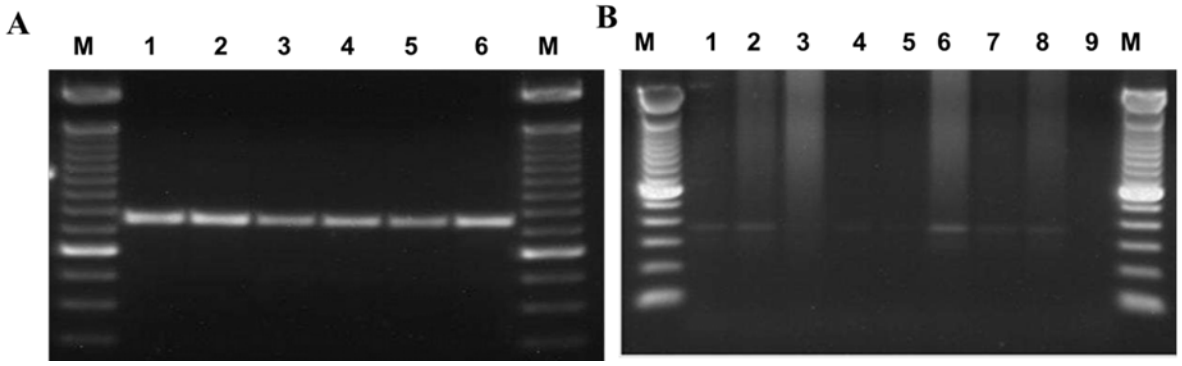


Fig. 2 A. PCR based detection of cyanogenic *Pseudomonas* spp. using primers specific to *rpoD* gene. 1: Pscya 1, 2: Pscya 2, 3: Pscya 4, 4: Pscya 6, 5: Pscya 7 and 6: Pscya 8. **B.** PCR based detection of HCNC genes in *Pseudomonas* spp. 1: Pscya 1, 2: Pscya 2, 3: Pscya 4, 4: Pscya 6, 5: Pscya 7, 6: Pscya 8, 7: Pscya 9 (Negative control), 8: Pscya 10 (Negative control) and 9: Water control

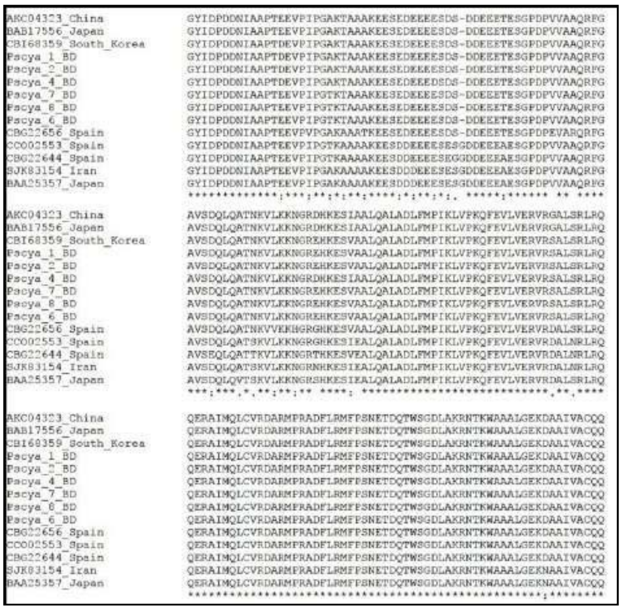
Identification of cyanogenic *Pseudomonas* spp. by sequencing: Nucleotide sequences of *Pseuodomonas* spp. were compared with nucleotide sequences of other *Pseudomonas* spp. available in the NCBI database using Basic Local Alignment search Tool (BLAST) algorithm. Blast homology of nucleotide sequences showed 97-99% sequence identity with the corresponding nucleotide sequences of sigma factor 70 (*rpo*D gene) sequences of *Pseudomonas fulva* (GeneBank accession # FN599525 and CP023048) (Table 1).

Table 1. Closest relatives of cyanogenic *Pseudomonas* spp. with other *Pseudomonas* spp. based on *rpo*D gene sequence

Isolates	Accession no.	Closest relatives (accession no.)	Identity	Homology (%)
Pscya 1		*Pseudomonas fulva* (FN599525)	682/685	99
Pscya 2		*Pseudomonas fulva* (FN599525)	680/687	99
Pscya 4		*Pseudomonas fulva* (CP023048)	690/715	97
Pscya 6		*Pseudomonas fulva* (FN599525)	677/687	99
Pscya 7		*Pseudomonas fulva* (FN599525)	676/685	99
Pscya 8		*Pseudomonas fulva* (FN599525)	677/686	99

Phylogenetic relationship among the isolates of *Pseudomonas* spp.: Blast-P homology search showed amino acid sequence translated from the partial nucleotide sequence of *rpo*D gene of six cyanogenic *Pseudomonas* spp. showed similarity with the RpoD protein of *Pseudomo*nas strains reported in the NCBI database such as 99% homology with *Pseudomonas fulva* (CBI68359), 97% with *P. fulva* (BAB17556) and *P. fulva* (AKC04323), 93% with *P. putida* (CCO02553) and 92% with *P. putida* (BAA25357), *Pseudomonas* sp. (SJK83154) and *P. monteilii* (CBG22644). Phylogenetic relationship of six cyanogenic *Pseudomonas* spp. and their close relatives analyzed using CLUSTALW program based on amino acid sequences of *rpo*D gene revealed that all these *Pseudomonas* spp. were distributed in three different groups. Group I was supported by bootstrap value of 96% and consist of two subgroups supported by bootstrap values of 79 and 83%. Group II includes 7 strains (Six cyanogenic *Pseudomonas* spp. and one *Pseudomonas fulva* (CBI68359) *of* South Korea and was supported by bootstrap value 92%. This group consists of two subgroups and was supported by bootstrap value 100 and 96%. Group III represents two *Pseudomonas* spp. from Japan (BAB17556) and China (AKC04323) These results clearly indicated that the Bangladeshi cyanogenic *Pseudomonas* spp. seems different from other *Pseudomonas* spp. available in the NCBI database (Fig. 3A & B). However, Bangladeshi cyanogenic *Pseudomonas* spp. were distributed in two subgroups. This might be due to their variation in suppressing the growth of *P. infestans* as well as their variation in genetic constituent of RpoD protein.

A



B

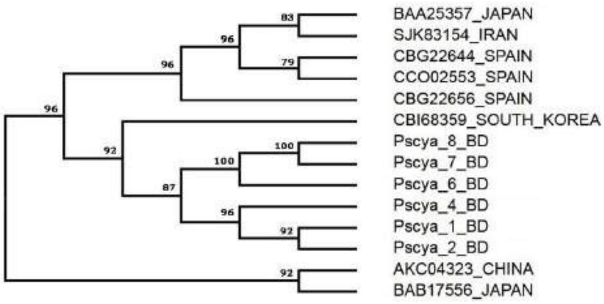


Fig. 3 **A.** Amino acid sequence alignment of conserved region of sigma factor 70 (RpoD) protein of six *Pseudomonas* spp. with other close relatives *Pseudomonas* spp. available in the NCBI database **B.** Phylogenetic trees constructed from partially translated amino acid sequences of sigma factor 70 (*rpo*D gene), using the maximum likelihood method. The evolutionary distances were computed using MEGA (version 5.220). The analysis involved amino acid sequences including six cyanogenic *Pseudomonas* spp. collected from phylloplane of potato and representatives of *Pseudomonas* spp. available in the NCBI database. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in

Formulation of cyangenic *Pseudomonas* spp.: Talc is a natural mineral and chemically it is referred as magnesium silicate ($Mg_3Si_4O_{10}(OH)2$). It has very low moisture equilibrium, relative hydrophobicity, chemical inertness, reduced moisture absorption and prevents the formation of hydrate bridges that enable longer storage periods. Owing to the inert nature of talc and easy availability as raw material from soapstone industries, it is used as a carrier for formulation development. In this study, one of the selected isolates was formulated in talc powder and was stored at 4ºC until six months. The results revealed the survival ability of the formulated bacterial cells up to six month or more at room temperature and at 4-8˚C. Although it was observed that the formulated bacterial cells lost their viability with the increase of storage time at room temperature (Fig. 4). Talc based formulation has been reported for the management of several crop diseases in India (Samiyappan et al., 1998). Talc based formulation of *P. fluorescens* was found to be effective as seed treatment on the control of fusarium wilt of tomato plants (Sarma et al., 2011) and foliar application on the control of rust and leaf spots of groundnut (Meena et al., 2002).

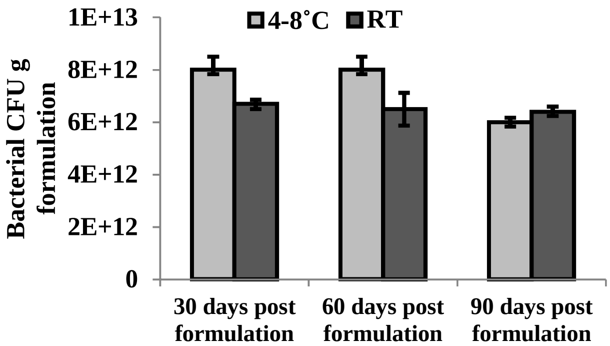


Fig. 4 Colony forming unit (CFU) of cyanogenic *Pseudomonas* spp. in the talcum formulation. The CFU was counted by dilution plate technique at different days post formulation.

Conclusion

Potential cyanogenic *Pseudomonas* spp. was identified from the phylloplane of potato plants. *Pseudomonas* spp. was confirmed by PCR and sequencing of sigma factor 70 DNA polymerase (*rpo*D gene). Sequence analyses of *rpo*D *g*ene revealed the identified *Pseudomonas* spp. are very close to some *Pseudomonas* spp. available in the NCBI database. *In vitro* test confirmed the *Pseudomonas* spp. inhibited the growth of late blight pathogen, ***P. infestans.*** The *Pseudomonas* spp. was then formulated in talcum powder and the bacterial cells become viable up to six month or more at room temperature and at 4-8ºC.

Acknowledgement

This research work was funded by the Bangladesh Academy of Sciences-United States Department of Agriculture (BAS-USDA) (Grant No.: BAS-USDA PALS BAU-CR-38) to Dr. Md. Rashidul Islam.

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Utilization of chitosan and glukomanan for fruit coating of chili againts antrachnose disease

Chaieydha Noer Afiefah[1], Ani Widiastuti[1,2], Arif Wibowo[1,2], and **Siti Subandiyah**[1,2]
([1]Dept. Of Plant Protection, UGM; [2]Research Center for Biotechnology, UGM. Yogyakarta, Indonesia)

ABSTRACT

Chili (*Capsicum* spp.) is the most important vegetable in Indonesia since it is needed for dairy food as chili souce or as hot spice in many culinary cuisines. The cultivation period of chili is mostly in dry season, however the product is needed whole year therefore the supply during rainy season is usually insufficient leading to highly increase of the price in the market. Low production of chili in the wet season is especially due to fungal and bacterial diseases and antrachnose disease is devastating for the fruit product. Antrachnose of Chili is caused by *Colletotrichum* spp., especialy C. *gloeosporioides* and *C. accutatum* group. The fruits may have been infected in the field however the symptom is becoming more severe after harvesting during the ripening period. This research was conducted to protect post harvest chili fruits using nano chitosan, glucomanan, and the mixture of both polymers. Three different chili varieties were used in the experiment, *Capsicum annum* var Hot Chili and var Phoenix and *C. fruitescens* var. RM 28. The antrachnose pathogen of *C. gloeosporioides* was cultivated in the Potato Dextroxa Brorth and the conidium suspension in PBS at the concentration of 10^6/ml was used for spraying inoculation. Disinfected wounded freshly harvested chili fruits were coated before the pathogen inoculation. Fruiit coating application was done using different mixter of 0.1% chitosan, 0.1% gluocoman, and the mixture of 0.1% chitosan and 0.1% glucomanan by immergimg the fruits for 2 min in the solution and air dried before iniculation. The biopolymers were also used for conidium germination and colony growth analysis. The reuslts suggested that chitosan and glucomanan fruit coating of chili could suppress post harvest antrachnose disease. Chitosan gave better protection than glucoman and the mixture of both polymers. While chitosan suppres conidium germination on the other hand glucomanan suppress the colony development *in vitro*.

This research was funded by Australian Center for International Agricultural Research (ACIAR) - HORT 2009/056

Key words: post harvest, biopolymer coating, chili fruit, antrachnose disease

Antifungal effects of ethanolic shellac - Modified coconut oil (ES-MCO) combined with physical treatments against postharvest diseases of mango and mangosteen

Jitreerat PONGPHEN*, Sripong KANYALA*, Vo Thi THOUNG*, Apiradee UTHAIRATANAKIJ*, Sontaya LIMMATVAPIRAT**

(*School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Thailand, ** Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Thailand)

SUMMARY

Postharvest losses has been known a major problems of tropical fruits which resulting to economic losses. Currently control of postharvest diseases is based on fungicide application. However, environmental and health has been now concerned. Therefore, safe alternative methods were studied. This experiment was to evaluate the antifungal properties of ethanolic shellac-modified coconut oil (ES-MCO) in vitro test and to investigate the combined effects of ES-MCO and physical treatments on controlling postharvest disease of mango and mangosteen (in vivo test). Antifungal property of ES-MCO, ES and MCO was measured by paper disc method. 2% MCO and ES-MCO completely inhibited the growth of *Colletotrichum gloeosporioides*, a causal agent of anthracnose disease in mango. In vivo test, antifungal effect of ES-MCO combined with hot water (HW) and UV-C treatments (HW+UV-C+ MCO) against anthracnose disease of mango was tested in compared with treatments of HW+UV-C or ES-MCO. HW+UV-C+ES-MCO treatments showed the most effective in reducing anthracnose disease and delayed fruit ripening. In mangosteen, application of ES-MCO combined with 1-MCP and low density polyethylene (LDPE) bag could delay fruit rot diseases as same as fungicide treatment, and also showed the greatest effective in delaying the changes of calyx and pericarp color, weight loss, hardening of the pericarp and ethylene production. These results suggest that combined effect of ES-MCO and physical treatments may serve as an alternative method for controlling postharvest disease of mango and mangosteen.

Introduction

Mango (*Mangifera indica* L.) and mangosteen (*Garcinia mangostana* L.) are exotic tropical fruit which Thailand produces to serve for the domestic and export markets. However, postharvest diseases is the major loss during storage and marketing period. Anthracnose disease caused by *Colletotrichum gloeosporioides* Penz. is a main problem of mango, whereas fruit rot disease caused by *C. gloeosporioides, Lasiodiplodia theobromae, Pestalotiopsis* sp., and *Phomopsis* sp. are the major problem of mangosteen (Khewkhom et al., 2012). Fungicides are known as the most effective treatment to control plant disease but they have high potentially harmful on human health and environment (Ma and Michailides, 2005). Physical treatments are non-chemical method such as treatments with hot water and UV-C are shown high efficiency to control disease and maintain the fruit quality. Pre-heat treatment and UV-C treatments have directly suppressed pathogens, elicited the natural plant resistance, and also maintained the quality of fruit (Lurie, 1998; Mercier et al., 2001; Sripong et al., 2015b). Otherwise, some physical treatments such as modified atmosphere packaging (MAP) and wax coating has indirectly effect to delay the disease development in several harvested fruits (Pranamornkith et al., 2003; Accaseavorn et al., 2006). In addition, postharvest treatment with 1-methylcyclopropene (1-MCP), which is an ethylene inhibitor (Watkins, 2006) has been shown to prolong the shelf life of many fresh produce and delay postharvest disease development such as mangosteen and jujube (Piriyavinit et al., 2011; Zhang et al., 2012).

Wax coatings are commonly used to maintain the quality of fruit by preventing moisture loss, providing the barrier to oxygen transfer, and also be able to prevent the penetration of microbes resulting in an extend shelf life)Pramod et al., 2016(. Shellac (SH) wax is a natural product from an insect (*Laccifer lacca*) used widely to extend the shelf life of several fresh produce such as

mangosteen and lime)Accaseavorn et al., 2006(. In recent years, wax coatings have been developed with the addition of antimicrobial compounds to against postharvest diseases**.** A mixture of shellac wax with modified coconut oil)ES-MCO(solution could reduce anthracnose disease and maintain the quality of mango fruit (Sripong et al., 2015a).

Coconut oil is edible oil extracted from kernel of mature coconut. In coconut oil contains the fatty acids which have the function of antimicrobial property (DebMandal and Mandal, 2011). Whereas modified coconut oil)MCO(that obtained by glycerolysis of the free fatty acid of coconut oil with glycerol. It contains monolaurin and lauric acid that can inhibit various bacteria)Oyi et al., 2010; Tangwatcharin and Khopaibool, 2012(and fungi)Altieri et al., 2009(. Therefore, this research was to present the effects of ES-MCO combined with various physical treatments and postharvest treatments against postharvest diseases and maintain the quality of harvested mango and mangosteen.

Material and Method

Antifungal property of MCO, SH, ES-MCO against *C. gloeosporioides* by paper disc method

ES-MCO was prepared from 2% MCO and 8% shellac) SH(and dissolved in 95% ethanol (ETOH). Spore suspension of *C. gloeosporioides* was dropped on to black paper disc, dried on air, and then dropped with an equal volume of 2% MCO, 8% SH, ES-MCO and 95% ETOH on the same position. Dropping with sterile distilled water was used as the negative control. All treated discs were incubated in a high moisture chamber, and observed mycelium growth on disc surface after 2 weeks. The survival of spores was detected by placing the treated paper disc onto the surface of PDA plates, and incubated at 25°C for 2 weeks. Paper disc showed mycelial growth on PDA was indicated as the survival of the fungal spores. Each treatment contained three replicates and each replicate consisted of 4 plates.

Combined effects of ES-MCO and physical treatments on anthracnose disease of mango.

Naturally infected mango cv. Chok-Anan at 75-80% were treated as the follows; 1) surface coated with ES-MCO (ES-MCO) for 1 min, 2) dipped in hot water (HW) at 55°C for 5 min followed by irradiating with 6.16 kJ/m^2 UV-C (HW + UV-C), 3) dipped in HW followed by UV-C and then coated with ES-MCO (HW+UV-C+ES-MCO), and 4) untreated as the control. Samples were then placed in plastic basket covered with a polyethylene bag (PE) at 13°C, 85-90%RH for 15 days. Each treatment contained three replicates and each replicate consisted of 4 fruits. Disease incidence, disease severity (Chantrasri et al., 2007), fruit quality (weight loss, firmness, and hue angle color), and respiration rate of mango were determined every 3 days interval.

Combined effect of ES-MCO, 1-MCP and LDPE bag on fruit rot disease of mangosteen

Mangosteen fruits at maturity stage 3)reddish pink(were coated with ES-MCO and/or 500 ml/l carbendazim (fungicide), packed in LDPE bags containing a 1-MCP sachet (ethyl-gone® a.i. 1.7861 µg/g). Samples were then stored at 13°C, 90-95% RH for 42 days. Non-treated and non-packed fruits, carbendazim treated fruits, and ES-MCO coated fruits alone were served as the controls. Fruit quality (color change of pericarp and calyx, weight loss), physiological changes (ethylene prodcution) and disease index were evaluated every 7 days. Each treatment had 4 replicates with 5 fruits in each replicate. The experiment was arranged as completely randomized design)CRD(and the means of each treatment were compared using the Duncan's new multiple range test)DMRT(at $p \leq 0.05$.

Result and Discussions

Antifungal property of MCO, SH, ES-MCO against *C. gloeosporioides*

MCO, SH, ES-MCO and ETOH treatments completely inhibited the mycelail growth of *C. gloeosporioides* on paper disc, whereas the control showed 100% of mycelail growth. The spore survival was tested on PDA plate. It was found that MCO and ES-MCO treatments completely killed the fungal spore, while spore treated with ETOH and SH still grew as same as the control) Table 1(. In general, MCO is obtained by glycerolysis process of fatty acid of coconut oil)Luo et al., 2014(. In this experiment MCO contained with 23. 65% monolaurin and 5. 22% luaric acid that can against various food borne pathogens and postharvest fungal pathogens (Oyi et al., 2010; Tangwatcharin and Khopaibool, 2012; Altieri et al., 2009; Sripong et al. 2015a(. Previous studies, reported that molecule of monoglycerides and fatty acids can penetrate into lipid phase and physically disrupt cell membranes, and inhibit

enzymes involved in energy production and nutrient transfer of microbe, that may lead to the death of microbial cell)Luo et al., 2014(.

Combined effects of ES-MCO and physical treatments on anthracnose disease of mango.

Effect of ES-MCO coating combined with HW and UV-C treatments on anthracnose disease and quality of mango was investigated. This result found that ES-MCO, HW+UV-C, and HW+UV-C+ES-MCO treatments significantly reduced disease incidence and severity (Table 2). Several reports demonstrate that the effect of HW and UV-C on disease reduction could be explained as direct effect of HW and UV-C on the fungi present on the fruit surface, and as indirect effect by eliciting plant defense enzymes in mango, bell pepper and strawberry)Sripong et al., 2015b; Mercier et al., 2001; Pombo et al., 2011(. Moreover, ES-MCO coating may function as a barrier against fungal infection, furthermore MCO also have high antimicrobial property of monoglycerides (Rihakova et al., 2002; Wang et al., 1993(.

Table 1 Mycelial growth and survival of *C. gloeosporioides* spore after treated with sterile distilled water) Control(, ETOH, SH, MCO and ES-MCO by paper dice method

Treatments	Mycelial growth)%(	Survival of spore)%(
Control	100.00[a]	100.00[a]
ETOH	0.00[b]	100.00[a]
SH	0.00[b]	100.00[a]
MCO	0.00[b]	0.00[b]
ES-MCO	0.00[b]	0.00[b]

Table 2 Incidence and severity of anthracnose disease of mango fruit when coated with ES-MCO)ES-MCO(, dipped in hot water followed by UV-C irradiation)HW+UV-C(, dipped in hot water followed by UV-C irradiation and coated with ES-MCO)HW+UV-C+ES-MCO(and untreated)control(during storage at 13°C for 15 days.

Treatment	Dis. incidence (%)	Dis. severity (score)
Control	100a	3.0a
ES-MCO	80b	1.1ab
HWT+UV-C	40c	0.6b
HWT+UV-C+ES-MCO	30c	0.5b

The quality of treated mango shows in Fig.1 and Fig.2. Change of peel color)as indicated by hue angle(and firmness of mango fruit delayed when the fruits were treated with ES-MCO or HWT+UV-C+ES-MCO)Fig.1(. Especially HWT+UV-C+ES-MCO treatment showed the most effective than ES-MCO alone. The lower hue angle changes of coated fruit may be associated with the coating of ES-MCO may help to create modified atmospheres condition within the fruit. Maftoonazad et al.)2007(demonstrated that the presence of CO_2 in the storage atmosphere is an important factor to suppress chlorophyll degradation. In addition, UV-C may retard the activity of chlorophyll degrading enzymes)Costa et al., 2006(and cell wall degrading enzymes)Barka et al., 2000(resulting in the delay of fruit senescence.

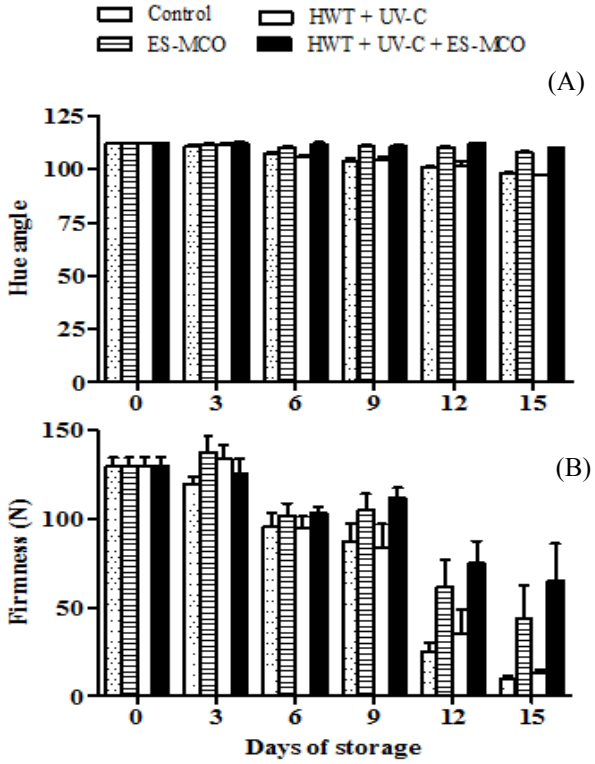

Fig. 1 Hue angle (A) and firmness (B) of mango when coated with ES-MCO, dipped in hot water followed by UV-C irradiation)HW+UV-C(, dipped in hot water followed by UV-C irradiation and coated with ES-MCO)HW+UV-C+ES-MCO(and untreated)Control(during storage at 13°C for 15 days.

Moreover, HW+ UV-C+ES-MCO treatment also had effect to reduce respiration rate which was correlated to lower weight loss in mango fruit when compared with other treatments)Fig. 2(. This may be explained that the coating with ES-MCO forms thin film on mango fruit

which can reduce the respiration rate as be ever reported in strawberry and avocado)Maftoonazad et al., 2007; Perdonesa et al., 2012(

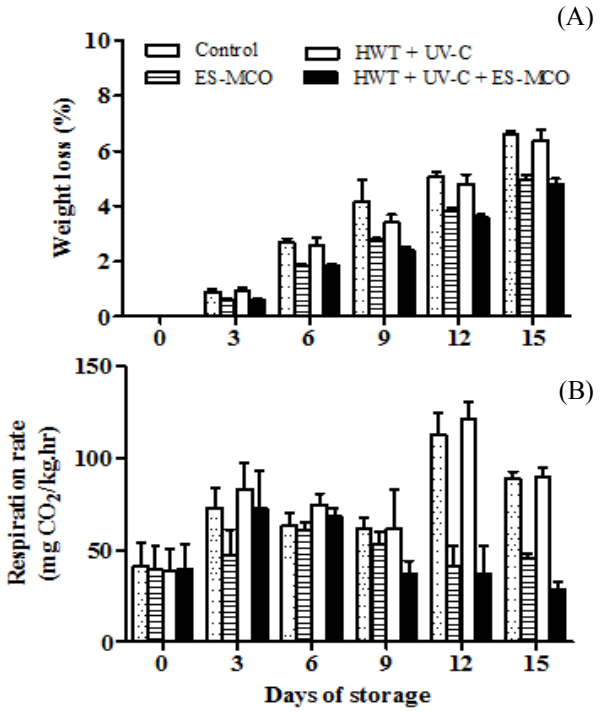

Fig. 2 Weight loss (A) and respiration rate (B) of mango when coated with ES-MCO, dipped in hot water followed by UV-C irradiation)HW+UV-C(, dipped in hot water followed by UV-C irradiation and coated with ES-MCO)HW+UV-C+ES-MCO(and untreated)Control(during storage at 13°C for 15 days.

Combined effect of ES-MCO, 1-MCP and LDPE bag on fruit rot disease of mangosteen.

Rapid increase in pericarp hardening, pericarp color, browning of calyx, and fruit rot disease is the characteristic of senescence of mangosteen fruit which leading to low quality and short shelf life. Our previous studies, we found that 1-MCP+ LDPE could maintain the quality of mangosteen fruit by delaying the ripening process. In this study, ES-MCO coating combined with LDPE+ 1-MCP sachet was used to maintain fruit quality and control fruit rot disease development. The result showed that treatments of ES-MCO and ES-MCO+LDPE bag+ 1-MCP could delay fruit rot diseases as same as fungicide treatments (carbendazime alone and carbendazim+ LDPE+ 1-MCP(. Particularly mangosteen treated with ES-MCO+ LDPE bag+ 1-MCP had a lowest disease index compared with other treatments) Table 4(. Low disease index may result the effect of antifungal activity of MCO (Rihakova, et al. , 2002; Wang et al. , 1993(and 1-MCP.

Table 4 The combined effect of ES-MCO, LDPE bag, 1-MCP sachet on disease index, weight loss (WL), different color (ΔE) change of calyx and pericarp, and ethylene production (µl C_2H_4/kg.hr) of mangosteen during storage at 13ºC for 45 days.

Treatment	Dis. index	WL (%)	ΔE of Calyx	ΔE of Pericarp	C_2H_4 Production
Control	0.4a	5.1a	28.4a	20.6b	10.3bc
CBZ	0.3ab	1.9b	28.4a	22.8b	13.9a
ES-MCO	0.3ab	0.4c	28.9a	27.0a	8.3cd
CBZ+LDPE +1-MCP	0.2b	0.3d	17.9b	17.8c	12.3ab
ES-MCO+LDPE +1-MCP	0.2b	0.2e	23.9ab	15.0c	6.5d

In fact, 1-MCP has been known an effective inhibitor of ethylene action, and can delay a number of genes associated with the ripening process (Watkins, 2006(. However, antifungal activity of 1-MCP against anthracnose disease in harvested mango and apple had been discovered by Xu et al. (2016) and Li et al. (2017). 1-MCP showed the direct suppression effect on the spore germination and mycelial growth of *C. gloeosporioides* and *P. expansum*, and also induced reactive oxygen species (ROS) generation in pathogenic spore. In addition, antifungal effect of 1-MCP was found that it could induce plant disease resistance in jujube fruit against postharvest blue mold and also delayed fruit senescence (Zhang et al., 2012). Similar with our present report, 1-MCP combined with ES-MCO+LDPE bag showed the greatest effective in controlling fruit rot disease of mangosteen and also delaying fruit ripening as indicating by the retarding of color changes of calyx and pericarp, weight loss, ethylene production)Table 4(, and hardening of the mangosteen pericarp)data not shown(.

Conclusion

In conclusion, the data presented here show that MCO and ES-MCO significantly killed *C. gloeosporioides*, a causal agent of anthracnose disease in mangoes. The combination treatment of ES-MCO with other postharvest treatments such as HWT, UV-C, 1-MCP and packaging can enhance the effectiveness to reduce postharvest loss by anthracnose in mango and fruit rot disease in mangosteen. Moreover, the combined

treatments has more effectiveness to delay fruit senescence than the single treatment. Thus, their application could be a promising and alternative approach to the use of chemicals for the control of postharvest diseases and the maintenance of quality in mango and mangosteen.

Acknowledgement

MCO, SH and ES-MCO were obtained Faculty of Pharmacy, Silpakorn University, Thailand. Some part of this work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (RGJ) (Grant No. PHD/00642554).

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Enhancing plant defense in mango fruit by hot water and UV-C treatments

Kanlaya SRIPONG*, Apiradee UTHAIRATAKIJ*, Masaya KATO**, Pongphen JITAREERAT*

(*School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Thailand, **Faculty of Agriculture, Shizuoka University, Japan)

SUMMARY

This research investigated the effects of hot water treatment (HWT), UV-C irradiation and their combination on the induction of resistance to anthracnose disease and on quality improvement in harvested mangoes. Naturally infected mango fruit cv. Chok-Anan was used in this study. Fruit were divided into four treatments; UV-C irradiation at a dose 6.16 kJ m^{-2}, HWT at 55°C for 5 min, HWT followed by UV-C irradiation. Untreated fruit served as the control. All samples were kept at 13°C for 12 days. The combined treatment significantly suppressed the severity of anthracnose symptoms compared with either single treatment. Additionally, the specific activities of key plant defense-related enzymes, such as phenylalanine ammonia lyase (PAL), peroxidase (POD), chitinase (CHI) and β-1,3-glucanase (GLU), increased in peel of the mango fruits, as well as the expressions of the *MI-PAL*, *MI-POD*, *MI-CHI* and *MI-GLU* genes. The combined treatment or UV-C treatment alone significantly delayed fruit ripening by retarding firmness and maintaining peel and pulp color of mango fruit. While, weight loss was not significantly different among the treatments.

Introduction

Mango (*Mangifera indica* L.) is a major tropical fruit in both the domestic and export markets of Thailand. Anthracnose disease caused by *Colletotrichum gloeosporioides* Penz. is a major problem of harvested mango. Due to the fungus is able to infect young fruit and remain latent during fruit growth in the field. Thus, it is hard to prevent disease development after harvested. Control of anthracnose disease is based on fungicides but chemical residues in fruit and development of pathogens to resist chemicals may result. Thus, it is important to search the non-chemical treatments for disease suppression by inducing plant defense mechanisms in fruit before storage.

Hot water treatment (HWT) is a physical method that has been used for the control of postharvest diseases in several fruits such as peach, muskmelon, satsuma mandarin (Liu et al., 2012; Yuan et al., 2013; Hong et al., 2007), and also in mango fruit (Alvindia et al., 2015). HWT showed to be efficient for controlling postharvest diseases by directly inhibiting pathogen and activating the natural resistance of the host (Porat et al., 2000). However, HWT alone cannot completely control postharvest disease of fruit and it has less effective than chemical treatment (Wasker, 2005; Prakash and Pandey, 2000). Therefore, there is a need for a method combining couple of methods together. UV-C irradiation has also emerged as an alternative method to avoid chemical fungicides. Application of UV-C irradiation reduce postharvest disease in several fresh produce such as papaya, strawberry and tomato (Patricia et al., 2007; Marina et al., 20011; Maria et al., 2009). Control of postharvest diseases by UV-C irradiation seems to be depended on two different mechanisms - a direct germicidal effect on pathogens and an indirect effect by inducing defense mechanisms in the plant tissue (Porat et al., 2000). Moreover, HWT and UV-C irradiation also reported as the effective methods to improve the quality of fruits (Pan et al., 2004). In previous studies, the combination of these two treatments has been applied to some crops such as strawberry (Pan et al., 2004) and eggplant (Karasahin et al., 2005), and it could reduce postharvest disease and maintain the quality of fresh produce greater than the results by either one of these treatments. The objectives of the present study was to investigate the combined effects of HWT and UV-C irradiation for inducing enzymes related to the defense mechanism and improving postharvest quality of mango fruit.

Material and Method

Mango fruits cv. Chok-Anan at 75-80% maturity was harvested from commercial orchards located in Ratchaburi province, Thailand. The fruit were selected for uniformity of size, shape and freedom from any

defects, and then disinfected with a solution of 200 ppm sodium hypochlorite and air dried at ambient temperature. Mango fruits were divided into four groups. The first group was subjected to HWT; the HWT was conducted by submersing the fruits in a hot water bath at 55°C for 5 min. After treatment, the fruits were immediately cooled in tap water (10°C) for 15 min and then air dried at ambient temperature. The second group was treated with UV-C irradiation using General Electric 30 W G15T8 germicidal lamps. The mango fruits were placed under the UV-C lamps on aluminum foil for light reflection at a distance of 20 cm from the light source for 5 min to provide a dose of 6.16 kJ m^{-2}. The UV-C dose was measured with a digital radiometer (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). The third group was treated with a combination treatment consisting of hot water and UV-C. They were first treated with hot water at 55°C, cooled, dried and then irradiated with UV-C as described above. A fourth group of fruit was kept untreated as a control. All of the fruit samples were placed in plastic baskets covered within a polyethylene bag (PE) and kept at 13°C under humidified conditions (85-90% RH) for 12 days. Each treatment had 4 replicates with 5 fruits in each replicate. Before and after the treatments, sampling was carried out every 3 days throughout the storage time to evaluate the degree of anthracnose disease. Scores of disease severity were recorded according to Chantrasri (2001), where are 0 = no disease symptoms, 1 = symptoms less than 0.1-10% of overall peel area, 2 = symptoms 10.1-20% of overall peel area, 3 = symptoms 20.1-30% of overall peel area, 4 = symptoms 30.1-40% of overall peel area, 5 = symptoms more than 40% of overall peel area. The specific activities and the expression of defense-related genes were examined, including phenylalanine ammonia lyase (PAL), chitinase (CHI), β-1,3-glucanase (GLU), and peroxidase (POD). The quality of the fruit, including color change of the peel and the pulp, firmness and weight loss were also analyzed. Results were analyzed by conducting analysis of variance using the general linear models procedure of SAS (SAS Institute, Cary, N.C.) for completely randomized design experiments. Data are presented as means ± standard errors of the means.

Result and Discussions

Disease incidence of anthracnose disease

The combination of HW and UV-C treatments significantly ($P \leq 0.05$) reduced both anthracnose disease incidence and severity in mango fruit. The fruit treated with the combined treatment did not show any visible decay until 6 day of storage, while control and fruit treated with UV-C or with HWT alone were 100%, 58.3% and 25.0% of disease incidence, respectively (Table 1). Similarly, disease severity at the end of storage was lowest in the combined treatment fruit, followed by hot water treatment, UV-C treatment and control (Table 1). These result indicated that the combination treatment helped to reduce anthracnose disease in mango fruit better than when either hot water or UV-C alone was used. Similarly, Pan et al. (2004) found that a combination of UV-C and heat treatment enhanced the benefits to control postharvest disease in strawberry and eggplant more than applying of each treatment (Karasahin et al., 2005). In addition, this present result shows that the suppression of anthracnose disease were correlated with the genes expression and activities of enzyme related with plant defense.

Table 1 Incidence and severity of anthracnose disease in mango fruit when treated with UV-C irradiation at a dose of 6.16 kJ m^{-2}, HWT at 55°C for 5 min, HWT followed by UV-C irradiation, and untreated (control) during storage at 13°C.

Treatments	Days of storage				
	0	3	6	9	12
	Disease incidence (%)				
Control	0.0	0.0	100^{a}	100^{a}	100^{a}
UV-C	0.0	0.0	58^{b}	83^{a}	83^{ab}
HWT	0.0	0.0	25^{c}	41^{b}	66^{b}
HWT + UV-C	0.0	0.0	0.0^{c}	33^{b}	33^{c}
F-test	NS	NS	**	**	**
C.V. (%)	0.0	0.0	39.2	19.7	23.5
	Disease severity (score)				
Control	0.0	0.0	1.5^{a}	2.6^{a}	4.1^{a}
UV-C	0.0	0.0	0.6^{b}	1.2^{b}	2.7^{b}
HWT	0.0	0.0	0.2^{c}	0.6^{c}	1.7^{c}
HWT + UV-C	0.0	0.0	0.0^{c}	0.3^{c}	0.6^{d}
F-test	NS	NS	**	**	**
C.V. (%)	0.0	0.0	31.1	35.23	49.14

With a column, means not followed by the same letter are significantly at ** $p \leq 0.01$. The NS indicates statically no-significance. Data are expressed as mean of each replication assays.

The expression of gene and the activity of defence related enzyme

The expression of *MI-PAL* gene increased quickly and

peaked at day 3 of storage, then declined sharply. Its level was significantly higher in the fruit treated with the combined treatment than the fruit treated with HWT or UV-C alone and non-treated fruit (Fig. 1A). This was the same as the activity of PAL which showed highest expression level in the combination treatment (Fig. 1B). These results were closely associated with the levels of disease incidence and severity of anthracnose disease in the mango fruit. The increased activity of PAL was correlated with the compounds synthesized by phenylpropanoid pathway such as phytoalexins, lignin or tannin (Porat et al., 2000). Thus, high activity of PAL is implicated in a protective role against pathogens through reinforcement of plant cell walls and direct inhibition of pathogen growth (Treutter, 2005).

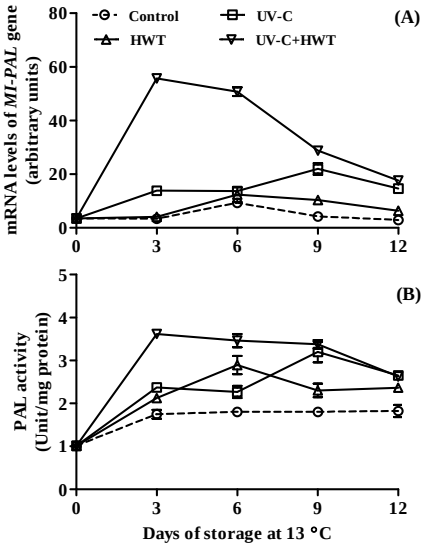

Fig. 1 Expression of *MI-PAL* gene (A) and the activity of PAL in the peel of mango when treated with UV-C irradiation at a dose of 6.16 kJ m^{-2}, HWT at 55°C for 5 min, HWT followed by UV-C irradiation, and untreated (control) during storage at 13°C for 12 days.

The *MI-POD* gene expression, the levels of mRNA increased slightly until 3 days after treatment, and then increased rapidly and peaked at 6 days of storage in the combined treatment. The *MI-POD* expression in the combined treatment fruit was 2-fold higher compared to UV-C alone or HWT alone and 10-fold higher compared to the control (Fig. 2A). Similarly, the activity of POD was the highest in the mango treated with combination treatment (Fig. 2B). POD might contribute to the beneficial effect of reduced anthracnose disease incidence and severity by influencing cell wall lignification. For the expression of the *MI-CHI* gene was low in both treated and control fruit during storage for 24 h, then the levels of mRNA increased rapidly and reached a maximum on day 9 of storage. Highest levels of mRNA were found in the combined treatment followed by HWT, UV-C irradiation and in the non-treated fruit (control). These results were correlated with the enzymatic activities, with the highest activities being found in the combined treated fruit (Fig. 3A and Fig. 3B).

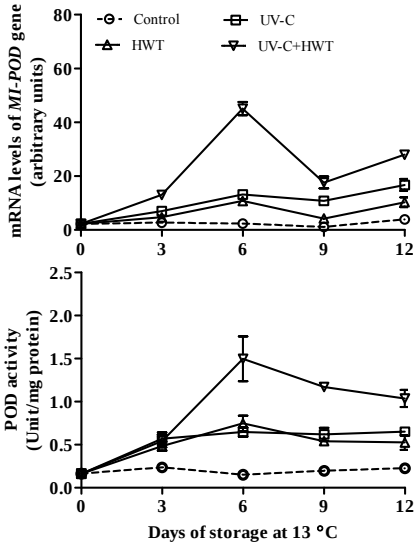

Fig. 2 Expression of *MI-POD* gene (A) and the activity of POD in the peel of mango when treated with UV-C irradiation at a dose of 6.16 kJ m^{-2}, HWT at 55°C for 5 min, HWT followed by UV-C irradiation, and untreated (control) during storage at 13°C for 12 days.

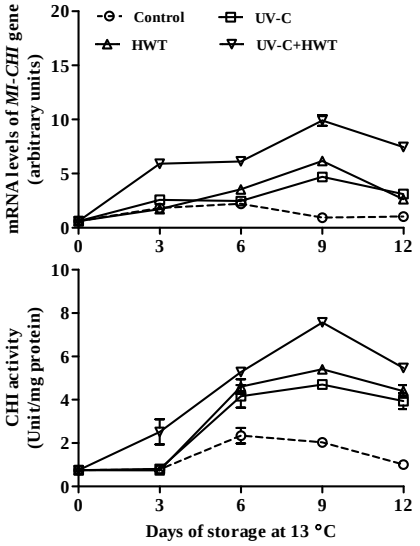

Fig. 3 Expression of *MI-CHI* gene (A) and the activity of CHI in the peel of mango when treated with UV-C

irradiation at a dose of 6.16 kJ m^{-2}, HWT at 55°C for 5 min, HWT followed by UV-C irradiation, and untreated (control) during storage at 13°C for 12 days.

MI-GLU gene expression, the levels of mRNA increased progressively in all treatments and decreased after 3 days of storage, then increased again on day 6 of storage and peaked at the end of storage. The higher expression of *MI-GLU* gene and activity of GLU enzymes were shown in the combined treated fruit (Fig. 4A and Fig. 4B). Which GLU can also inhibit the fungal pathogens indirectly by releasing oligosaccharides and eliciting defense reactions, and then act synergistically with CHI to inhibit fungal growth (Tian et al., 2007).

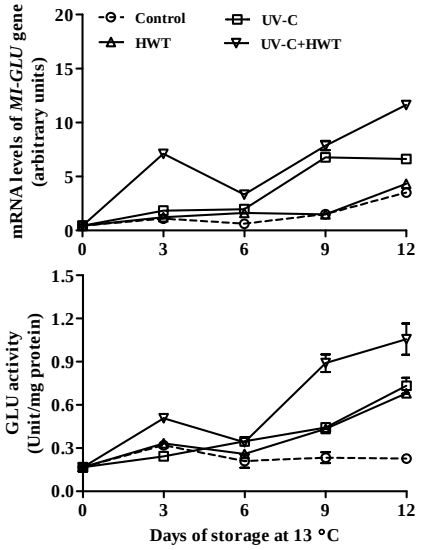

Fig. 4 Expression of *MI-GLU* gene (A) and the activity of GLU in the peel of mango when treated with UV-C irradiation at a dose of 6.16 kJ m^{-2}, HWT at 55°C for 5 min, HWT followed by UV-C irradiation, and untreated (control) during storage at 13°C for 12 days.

The quality of mango during storage

The result found that fruit treated with UV-C irradiation and the combined treatment of UV-C and HW had delayed ripening to a greater degree than fruit treated with HW alone or non-treated fruit (as indicated by fruit firmness, weight loss and change in color peel and pulp of the fruit) (Table 2).

Firmness of mango fruit decreased in all treatments included non-treated fruit. However, after 9 days of storage, the control fruit and HW treated fruit softened more rapidly than those of the other treatments. By the end of the storage, the control fruit and HW treated fruit remained at low level of firmness by 4.96 N and 6.05 N, while UV-C and the combined treatment showed higher firmness by 44.66 N and 41.49 N, respectively. The highest firmness in the UV-C and combined treated fruit, which could be associated with the effect of the radiation on the activity of enzymes involved in cell wall degradation (Barka et al., 2000). In contrast result showed that HW treated mango fruit became soften at a similar rate to that of the control. Therefore, this result indicated that UV-C treatment was the main effect to delay the firmness of mango fruit.

Table 2 Firmness, weight loss, color of peel and color of pulp of mango fruit when treated with UV-C irradiation at a dose of 6.16 kJ m^{-2}, HWT at 55°C for 5 min, HWT followed by UV-C irradiation, and untreated (control) during storage at 13°C.

Treatments	Days of storage				
	0	3	6	9	12
	Firmness (N)				
Control	84.9	83.7	82.5	62.6^{b}	4.9^{b}
UV-C	84.9	80.5	86.7	71.5^{ab}	44.6^{a}
HWT	84.9	88.6	78.2	66.7^{ab}	6.0^{b}
HWT + UV-C	84.9	83.1	78.5	76.4^{a}	41.4^{a}
F-test	NS	NS	NS	*	**
C.V. (%)	2.3	5.0	8.4	9.6	15.6
	a* value of peel				
Control	-17.1	-17.6	-15.7	-12.5^{ab}	-3.0^{a}
UV-C	-17.1	-16.1	-15.0	-15.3^{b}	-14.3^{b}
HWT	-17.1	-16.6	-15.5	-10.6^{a}	-4.2^{a}
HWT + UV-C	-17.1	-16.2	-15.9	-15.1^{b}	-13.5^{b}
F-test	NS	NS	NS	**	**
C.V. (%)	4.0	3.6	3.6	11.6	10.23
	a* value of pulp				
Control	-14.5	-11.7	-11.9^{ab}	-7.1^{a}	-3.2^{a}
UV-C	-14.5	-12.1	-13.3^{b}	-10.3^{b}	-6.7^{b}
HWT	-14.5	-11.5	-13.1^{b}	-6.8^{a}	-3.3^{a}
HWT + UV-C	-14.5	-12.2	-10.6^{a}	-8.2^{a}	-6.8^{b}
F-test	NS	NS	*	*	**
C.V. (%)	1.6	11.5	7.4	13.4	20.8

With a column, means not followed by the same letter are significantly at ** $p \leq 0.01$ and * $p \leq 0.05$. The NS indicates statically no-significance. Data are expressed as mean of each replication assays.

Yellowing of the peel and pulp is one physical manifestation of ripening that can be quantitatively measured based on increases in a* value. These results showed that mango fruit treated with the UV-C and HW combined with UV-C had a delay of color change in peel and pulp of mango which was slower than that in either

the control or HW treated fruit. The effect of UV-C to delayed the color change were recorded in several crops such as tomato, strawberry and red pepper (Obande et al., 2011; Pombo et al., 2011; Cuvi et al., 2011). It was reported that UV-C irradiation can delay the chlorophyll degradation processes (Obande et al., 2011). For the weight loss of the mango fruits in all of the treatments increased progressively during extended storage. However, weight loss was not significantly different among the treatments (data not shown).

Conclusion

The combination of HW and UV-C treatments significantly reduced anthracnose disease and increased the expression and specific activities of the defense related enzymes (PAL, POD, CHI and GLU) of mango fruit when compared with those single treatments and non-treated. The combined treatment of HW and UV-C or UV-C treatment alone delayed fruit ripening significantly by maintaining fruit firmness and color of peel and pulp of mango fruit. While, weight loss was not significantly different among the treatments.

Acknowledgement

This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (RGJ) (Grant No. PHD/00642554).

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Management of fusarium wilt in cyclamen plants using multiple soil microbes (AMF and *Piriformospora indica*)

Moslama Aktar MAYA[1,2*], Iris CAMEHL[1], Philipp FRANKEN[1]

([1]Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Kuehnhaeuser Straße 101, 99090 Erfurt, Germany, [2]Department of Horticulture, EXIM Bank Agricultural University (EBAUB), Chapainawabganj 6300, Bangladesh

*Current affiliation: Agronomy manager, Operations Team, British American Tobacco Bangladesh Limited (BATB), Dhaka 1205, Bangladesh, Email: Ecoagri.Maya@gmail.com)

SUMMARY

Biotic (soil-borne pathogens) and abiotic (high temperatures) stress factors have negative effects on plant growth and development and it limits crop production around the globe. To meet up with the vast demand of food for the increasing population of the world we use hybrids and agrochemicals for more production. To alleviate the environmental hazards due to excessive uses of modern varieties and the agrochemicals, sustainable agricultural production is a current concern. As a part of this approach, a variety of beneficial soil microbes are used as bio-regulating agents in crop production. Bio-regulating agents have been singly being used against biotic and abiotic stresses, but recent studies indicate that mixtures of bio-regulating agents may enhance the efficacy and dependency of the bio-regulating strategies. Based on this perspective, microbe-microbe interactions such as between AM fungi and *Piriformospora indica* and their interaction effects were investigated in Cyclamen crops. Results of this study suggest that single use of AM fungi have promising results in controlling fusarium wilt over combined use of AM fungi and *Piriformospora indica* together.

Introduction

Agriculture is one of the most climate dependent human activities and it is one of the sectors where climate change impacts are expected to be significant [1]. Stress in plants is defined as abnormal changes in physiological processes based on environmental and biological factors or a combination of both. As an abiotic stress factor, high temperatures have a negative effect on plant growth and development, and it limits crop production around the globe. Therefore, research on the mechanism of heat stress in plants is important to develop heat tolerant plants. On the other hand biotic stress agents specially soil-borne pathogens account for enormous losses of agricultural production throughout the world. Current control measures are based on the use of chemical pesticide, and those are ineffective or are banned because of health risks, product safety, and damage to the ecosystem. To alleviate the environmental hazards due to excessive uses of agrochemicals, sustainable agricultural production is a current concern [2]. As a part of this approach, integrated pest management has been conceived and a variety of beneficial soil microbes are also used as biocontrol agents in crop production. Biocontrol agents have been singly being used against biotic and abiotic stresses [3], but recent studies indicate that mixtures of biocontrol agents may enhance the efficacy and dependency of the biocontrol strategy [4]. Based on this perspective, microbe - microbe interactions such as between AM fungi and *Piriformospora indica* and their interaction effects were investigated in Cyclamen.

The association of roots with AM fungi is effective in reducing both shoot and root diseases [5,6] and, also shows bioprotection ability against abiotic stress conditions [7,8,9]. Several studies have been reported concerning interactions between AM fungi and rhizospheric microbes, and only a very few studies have been reported on mycorrhizal interaction with plant growth promoting endophytic *P. indica*. The utilization of microbes and the exploitation of beneficial plant-microbe interactions offer promising and ecofriendly strategies both for conventional and organic agriculture around the globe. Therefore, it is important to understand the interaction effects between AM and *P. indica* fungi for better utilization of these two biocontrol agents in practical agricultural production.

Cyclamen is a herbaceous perennial flowering pot plant growing throughout the temperate regions of the world. Among all the biotic stress agents *Fusarium oxysporum*, causes Fusarium wilt in cyclamen, is one of the highly

destructive soil-borne pathogen which can economically limit the quality cyclamen production [10].

The objective of this study was, therefore, focuses on the following objectives:

1. To observe the overall effect of AM and endophytic fungi on biomass and flower development in cyclamen.
2. To observe the biocontrol efficacy of AM and endophytic fungi and their interactive effects on resistance in cyclamen against Fusarium wilt.
3. To elucidate the molecular mechanisms of resistance mediated by biocontrol agents under pathogen stress condition.

Material and Method

Experimental design

The experimental treatments were conducted using five microbial inoculation (Mock AM and mock *P. indica* (Pi) as control (C); Am with mock Piri as Am; Mock AM with *P. indica* as Pi; AM with *P. indica* as Am+Pi;) along with pathogen *Fusarium oxysporum* inoculation. Two fertilizer treatment was used (Standard phosphorus as +P; Deficient of Phosphorus as –P). A completely randomized design were used and the whole experiment was replicated 4 times. Each replication has 160 plants (10 plants/treatment). The experimental treatments were conducted under both the greenhouse and phytochamber condition. Initially plants were raised in greenhouse condition for 12 weeks. After that plants were transferred to phyto-chamber with 20/18 degree C alternatively 14/10 hrs (day and night) keeping the moisture level at 60% and light intensity 75%. Plants were raised another 4 weeks under phytochamber condition.

Plant materials, growth conditions and microbial inoculation

Three-month-old cyclamen (*C. persicum* Mill cv. Pastel) seedlings (seeds supplied by Hakusan Co., Ltd., Japan) were planted into plastic pots (8 cm in diameter) containing autoclaved (121 °C, 1.2 kg/cm^2, 15 min) commercial potting media. The seedlings were inoculated with (10% of total substrate) AM fungal inoculum for mycorrhizal plants, 50 ml/plant *P. indica* suspension was used as Pi inoculation, and an equal amount of both (Am+Pi) autoclaved inoculum were used for control plants. A commercial mycorrhizal inoculum was used, containing *Rhizophagus irregularis* (spore density unknown). Plants were repotted in 12 cm in diameter after 12 weeks of inoculation. Standard field practices, and regular irrigation was conducted during the entire growth period.

Pathogenic infection

Initially the isolates of *F. oxysporum* MAFF 712100 (Fo) were collected from the Ministry of Agriculture, Forestry and Fisheries, Japan. Isolates were grown in potato dextrose agar (PDA) medium and were incubated at 28°C. The Fo isolates were subcultured into PDA medium and the conidia were harvested in sterile distilled water. The spore concentration was adjusted to 107 cfu/ml and 50 ml suspension was poured in each pot soil for Fusarium wilt challenged treatment after 14 weeks of AMF inoculation under phytochamber condition. Thereafter, the symptoms were checked for Fusarium wilt and the plants were raised another 2 weeks in phytochamber.

Disease incidence and severity

Disease symptoms were checked 2 weeks after pathogen inoculation, both shoots and roots were observed for Fusarium wilt symptoms. The disease severity in individual plants was rated visually on scale of 0 to 5 e.g.

0: no visible symptoms (healthy plants)

1: <20% disease symptom (small discolored leaves lesion covering less than 20% of total leaves of a plant)

2: 20 to 40% disease symptoms (minor small discolored lesions covering 20 to 40% of leaves)

3: 40 to 60% disease symptoms (moderate brown lesions in 40 to 60% of leaves and 15% defoliation)

4: 60 to 80% disease symptoms (mild wilt discoloration covered 60 to 80% of leaves and more than 50% leaf defoliation in case of Fusarium wilt)

5: 80 to 100% disease symptoms (stems and leaves severely affected).

The individual ratings were converted to mean percent infection using {(sum of individual plant rating values*100)/number of plants diseased} and the disease index for *F. oxysporum* was calculated using the following formula:

Disease Index (%) = {Σ (No. of plants * Severity of incidence) / Total no. of plants * no of maximum severity} *100

Result and Discussions

DNA extraction and determination of infection

The specific sequences of *R. irregularis* and *F. oxysporum* were amplified through PCR amplification using total genomic DNA from root system and shoot part as templates. Then, 5 μL of DNA fragments was examined using 1% agarose electrophoresis after amplification (Fig. 1, 2). Specific sequences from *F.*

oxysporum were amplified using total genomic DNA templates extracted from both the root and shoot samples. Bright, clear bands were detected which indicates that the cyclamen root system was successfully infected with *F. oxysporum*.

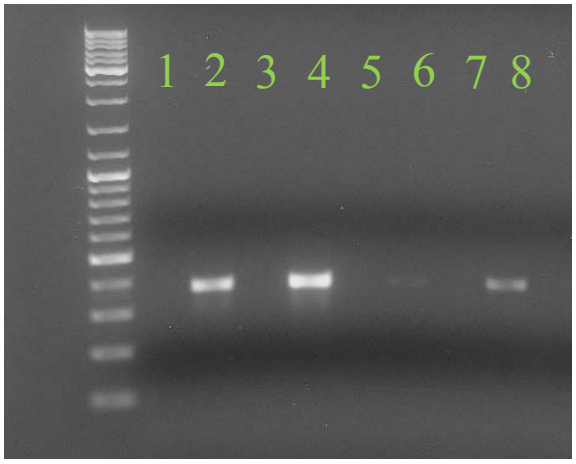

Fig. 1. Molecular quantification of AMF colonization. Note. 1: Control; 2: AMF (M); 3: *P. indica* (Pi); 4: M+Pi; 1-4: Deficient P condition (-P); 5-8: Standard P condition (+P).

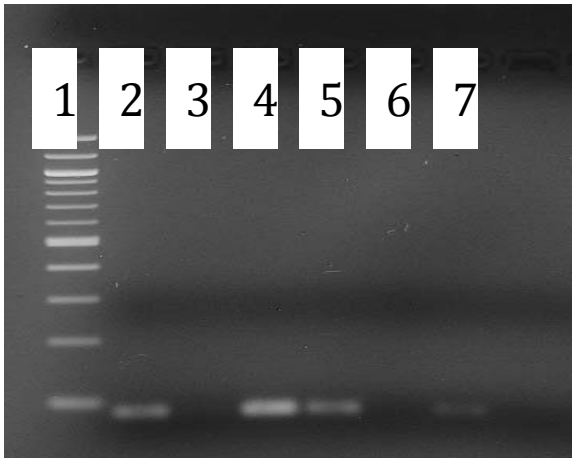

Fig. 2. Molecular quantification of *F. oxysporum.* Note: 1: 100bp DNA ladder; 2-4: C+Fusarium; 5-7: M+Fusarium

Plant growth responses

Average number of leaf and flower bud production was observed throughout the experimental period. The average number of leaf and flower bud production found higher in M treated plant compared to control both before and after pathogen inoculation condition. Whereas, the Pi and MPi inoculated plants have no major differences than the controls in no of total leaves and flower bud production (Fig. 3, 4). The average dry weight (DW) of M treated plants, both before and after pathogen inoculation, was observed higher than the other treatments (fig. 5).

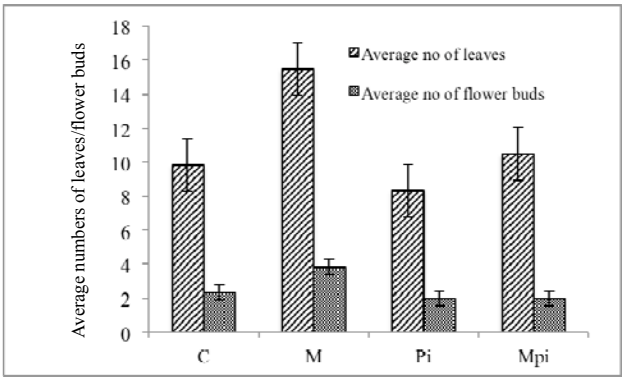

Fig. 3. Average number of leaves and flower buds during 12 weeks after microbial inoculation. Note. C: Control; M: AMF; Pi: *P. indica*; MPi: M+Pi.

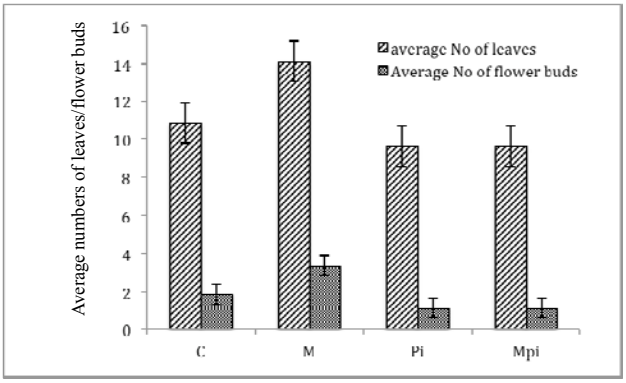

Fig. 4. Average number of leaves and flower buds during 16 weeks after microbial and 2 weeks after pathogen inoculation. Note. C: Control; M: AMF; Pi: *P. indica*; MPi: M+Pi.

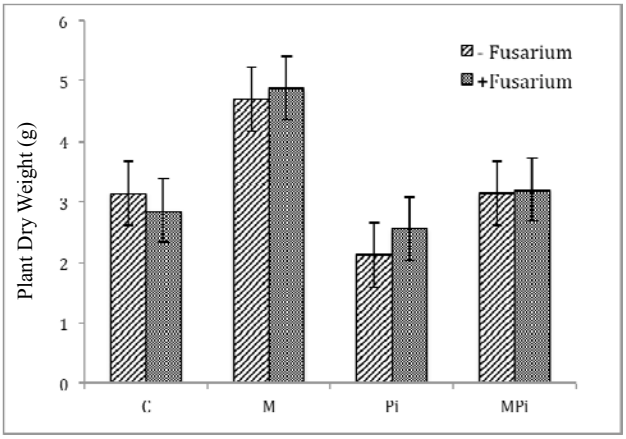

Fig. 5. Average plant dry weights (g) after 16 weeks of microbial inoculation. Note. C: Control; M: AMF; Pi: *P.*

indica; MPi: M+Pi.

Disease incidence and severity

The disease incidence levels for Fusarium wilt were observed higher in control plants (Fig. 6). M treated plants showed more healthy plants (0 severity level) than the controls. Regarding fusarium wilt incidence, the maximum severity level 5 (80 to100% disease symptom) was noticed in Pi and MPi treated plants but not in AMF-colonized plants (Fig. 6). Development of fusarium wilt symptoms in cyclamen plants was affected by the presence of AMF. Controls, Pi and MPi with pathogen association showed maximum disease severity level resulting to higher disease indices than M-colonized plants. Which indicates the microbe-microbe interactions to control fusarium wilt diseases in cyclamen is less effective than the single effects of Mycorrhiza. Pi might have the negative interaction effects with Fusarium and which ultimately have negative impact on to control fusarium wilt symptoms in cyclamen.

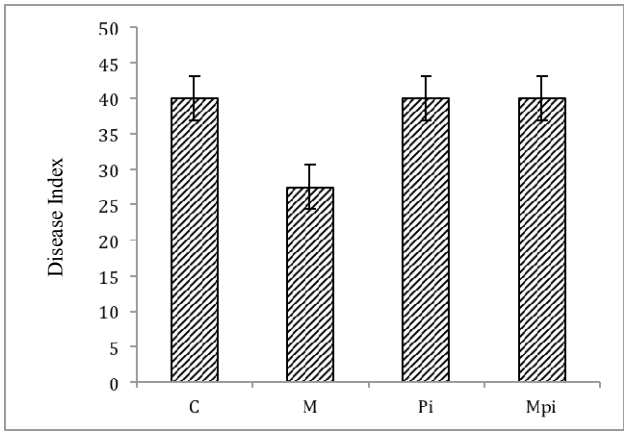

Fig. 6. The disease incidence levels for Fusarium wilt in cyclamen plants in deficient P condition. Note. C: Control; M: AMF; Pi: *P. indica*; MPi: M+Pi.

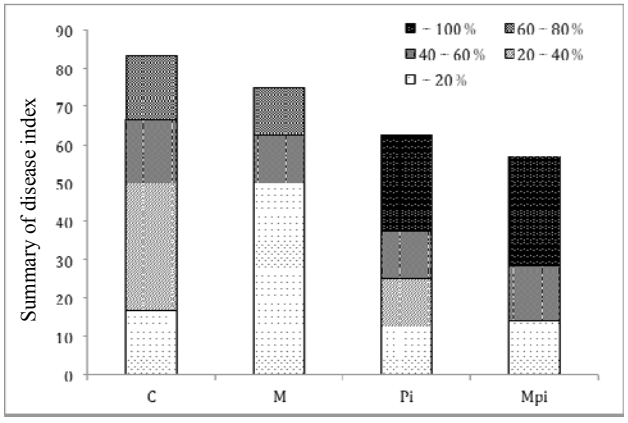

Fig. 7. Distribution of Fusarium wilt disease severity levels in cyclamen plants under different treatments. Note. C: Control; M: AMF; Pi: *P. indica*; MPi: M+Pi.

Molecular observations

RNA accumulation analyses by quantitative RT-PCR based on primer pairs for genes involved in plant defense reactions and to test the ability of AMF and of the non-mycorrhizal root endophyte *P. indica* to increase tolerance and/or resistance against fusarium wilt was observed under this project (data not presented here).

Acknowledgement

The authors sincerely acknowledge the contribution of Dr. Siegfried ZERCHE, Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Kuehnhaeuser Straße 101, 99090 Erfurt, Germany for his generous support in planning and management of phosphoric fertilizers throughout the experimental period.

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Mycorrhizal oil palms seedlings response to different sources of *Ganoderma boninense* as the causal agent of basal stem rot disease

Maria Viva RINI, Syaifudin Nur HASAN, Kuswanta Futas HIDAYAT, Titik Nur AENY

(Faculty of Agriculture, The University of Lampung)

SUMMARY

Basal stem rot (BSR) caused by *Ganoderma boninense* is the important disease of oil palm in Indonesia and Malaysia. BSR is characterized by a decay of roots and bole, production of aerial symptoms such as multiple spears and production of fruit bodies on the base of the trunk. These studies were aimed to evaluate the ability of arbuscular mycorrhiza fungi in controlling the BSR. Two different experiments were carried out. In the first experiment, rubber wood blocks of size 3 x 3 x 6 cm were used to grow the *G. boninense* inoculum for 1 month. The blocks were then inoculated to one primary root of 5 months old mycorrhizal and control oil palm seedlings. In the second experiment, the soil collected from rhizosphere of Infected palm by *G. boninense* was used as media to grow the three months old mycorrhizal and control oil palm seedlings. The first experiment showed that both mycorrhizal and control seedlings were infected by *G. boninense*. However, in the control seedling, the length of primary root that rot by the pathogen was longer than that of mycorrhizal. In the second experiment, no infection of *G. boninense* were observed in mycorrhizal and control seedlings.

Introduction

Basal stem rot (BSR) caused by *Ganoderma* species is the most serious disease of oil palm. Infection by the fungi causes significant loss in yield, often resulting in the palm's death as the disease progressed. The BSR affects the root and basal stem portion of the palm. Infection by the fungus begins in the roots and move into the stem causing a dry rot, which eventually lead to the death of the palm. Infection of living palm occurs through contact of healthy palm root with the infected root mass or bole tissue which serve as the inoculum source (Turner, 1981, Paterson, 2007). Generally, the first visible symptom of infected field palm is the presence of excessive spear leaves, while the foliage appears pale green when compare to that of healthy palm. Progressive yellowing, desiccation and mottling of the lower fronds, followed by necrosis is the characteristic feature of the disease of young palms. In older palms, the typical symptoms are skirting of the lower fronds, production of multiple unopened spears and overall paleness of the canopy (Fee, 2011; Gurmit, 1991).

Mycorrhizal fungi are ubiquitous and form symbiotic relationship with the roots of majority terrestrial plants including oil palm (Sieverding, 1991; Smith and Read, 2008). The mutual symbiosis benefits both the host and the fungus. The largest group, which predominantly associated with agricultural crops is the arbuscular mycorrhiza fungi (AMF). Infection by AMF has been shown to stimulate the growth of many plant species (Smith and Smith, 2011; Zhang *et al*., 2010), increase nutrient uptake especially phosphorus (Rini, 2004; Smith *et al*., 2011), improve the soil structure (through external hyphae that extends into the soil) for better aeration and water percolation, and improve plant physiological processes such as photosynthesis rate and water relation (Lu *et al*., 2007; Rini *et al*., 2000; Ruiz-Lozano and Azcon, 2010). Arbuscular mycorrhiza also has been proposed as an alternative for the management of soil borne pathogen. AM fungi has been proven to impair the development of soil borne pest and pathogens and consequently inhibit or reduce disease severity (Amer and Abou-El-Seoud, 2008; Jung et al., 2012; Tsvetkov *et al*., 2014). Therefore, this study was conducted to evaluate the ability of arbuscular mycorrhiza fungi in controlling the BSR.

Material and Method

The first experiment. A single factor experiment arranged in a completely randomized design was used with ten replications per treatment. The treatment was inoculated with (+M) and without (-M) AM fungi. The *Ganoderma boninense* inoculum was prepared on rubber wood blocks measuring 3 x 3 x 6 cm by inoculating the

block with five 1 cm^2 plugs from 7-10 days old *G. boninense* culture grown on malt extract agar with one plug on each side of the block (done in the air laminar flow). The blocks were incubated at room temperature 27 ± 1 ^oC for ten weeks. A small hole (± 3 cm depth) was made on the top of the blocks using an electric drill. Five month old mycorrhizal (inoculated with inoculum containing mix species of *Glomus mosseae*, *Scutellospora callospora*, and *Acaulospora laevis*) and nonmycorrhizal seedlings were inoculated with these blocks using single root inoculation technique. One of the primary root of the seedling was washed with tap water, the root tip was excised and the cut end of the root was inserted into the hole of the inoculum block. The inoculated seedlings were then put inside a polybag and filled up with the soil (mineral soil:sand = 2 : 1) and water daily. Six month after inoculation, the seedlings were removed from the polybags. The inoculated primary roots were then carefully separated from the bole of the seedlings. The length of inoculated root that rot due to G. *boninense* and AMF root infection were measured. Total phenolic content in the roots was analyzed following the method of Anderson and Ingram (1993). Data obtained were subjected to t-test analysis.

The second experiment. The treatment design used was a factorial design 4 x 2 with 5 replication arranged in completely randomized design. The first factor was application of AMF i.e. without AMF (control, m_0), inoculation with AMF *Glomus* sp. (m_1), *Entrophospora* sp. (m_2) and mixture of *Glomus* sp. and *Entrophospora* sp. (m_3). The second factor was *Ganoderma* i.e. without *Ganoderma* (planting media was sterile soil) and with *Ganoderma* (planting media was rhizosphere soil collected from *Ganoderma* infected palm + fruiting body or sporophore of *Ganoderma*). The one month old oil palm seedlings were inoculated with AMF according to treatment and the seedling were kept in green house for 2 months after which the seedlings were then transferred to bigger polybag according to *Ganoderma* treatments. The seedlings were then kept for another 5 months in green house. At the end of experiment, data on fresh and dry weight of shoot and root and *Ganoderma* infection were recorded.

Result and Discussions

The first experiment. Percentage of AMF colonization 6 month after *Ganoderma* inoculation was in range 42.7—49.0%. Presence of AMF in the root of oil palm significantly reduce the length of the inoculated primary root that rot due to *G. boninense* (Table 1). The length of primary root that rot in nonmycorrhizal seedlings was 11.1 cm compared to only 7.4 cm in mycorrhizal seedling. Length of primary root that rot as a result *Ganoderma* infection was significantly reduced when root was earlier precolonized by AMF. This indicate that the spread of *Ganoderma* infection within primary root of mycorrhizal seedlings was slower compared to that of nonmycorrhizal control. In the present study, the length of rotten root can be estimated 1.23 cm and 1.68 cm per month. The spread of *Ganoderma* infection in the present study was faster compared to Arifin and Idris (1990) who found only 1 cm/month, especially for nonmycorrhizal seedlings (1,68 cm/month). This faster speed could be due to the different size and substrates used to grow *Ganoderma* inoculum. Idris (1999) showed that utilization of different subtrates as source of *Ganoderma* inoculum resulted in different growth rate of the *Ganoderma* mycelia within the primary roots of oil palm.

Table 1. Length of primary roots infected by *G.boninense* after 6 months of inoculation

	+Mycorrhiza	Non-mycorrhiza
Length (cm)	7.4	11.1
P value		<0.05

Total phenolic content in the roots was also significantly higher in mycorrhizal seedlings. The values were 24.43% and 21.75% in mycorrhizal and nonmycorrhizal seedlings respectively (Table 2.)

Table 2. Total phenolic content in mycorrhizal and non mycorrhizal root after 6 months of *G. boninense* inoculation

	+Mycorrhiza	Non-mycorrhiza
Total phenolic in the roots (%)	24.43	21.75
P value		<0.05

Plant phenolic are the most widespread classes of secondary metabolites and are known to be involved in plant microbe interactions. One of the biological functions of phenol is its antimicrobial activity which play an important role in the plant defence mechanism (Morandi, 1996). Result from this study show that total

phenolic content in the roots of mycorrhizal seedlings is higher that that of nonmycorrhizal ones, suggesting that phenolic compounds could be implicated in disease resistance, resulting in slower rate of *Ganoderma* spread within the primary root of seedlings. Devi and Reddy (2002) reported that AMF significantly increase the quantity of phenolics compound in roots and shoots of groundnut. In (1998), Rabie believed that a significant increase in free and total phenolic contents in preinoculation of *G. mosseae* in faba bean contributed to increased resistance of the plant to chocolate spot disease.

The second experiment.

Data obtained from analysis of variance showed that there were no interaction between AMF factor with *Ganoderma* factor for all data recorded. Moreover, results showed that seedlings growth were significantly enhanced by AMF treatment. All AMF inoculated seedlings had better shoot and root fresh weight and dry weight compared to control one. However, no differences were observed within AMF treatment. All AMF treated seedlings whether single (*Glomus* sp. or *Enthrophospora* sp.) or their combination statistically had the same shoot and root fresh weight and dry weight. For *Ganoderma* treatment, no effect were detected in shoot fresh and dry weight. Contrary to shoot, *Ganoderma* treatment increase root fresh and dry weight. Oil palm seedling planted in *Ganoderma* infected soil had higher root fresh weight and root dry weight (Table 3 and Table 4).

Table 3. Fresh weight of shoot and root of 8 months old oil palm seedling treated with AMF and *Ganoderma*.

Treatment	Fresh Weight (g)	
	Shoot	Root
Control	43.3 b	09.7 b
G*lomus* sp. (G)	66.4 a	13.1 a
Entrophospora sp. (E)	64.0 a	13.9 a
G + E	66.7 a	13.5 a
LSD 5%	9.3	2.8
Sterile Soil	56.2 a	10.0 b
Ganoderma Infected Soil	64.0 a	15.0 a
LSD 5%	6.5	2.0

Table 4. Dry weight of shoot and root of 8 months old oil palm seedling treated with AMF and *Ganoderma*.

Treatment	Dry Weight (g)	
	Shoot	Root
Control	13.5 b	3.3 a
G*lomus* sp. (G)	19.3 a	4.2 a
Entrophospora sp. (E)	17.6 a	3.8 a
G + E	19.0 a	4.0 a
LSD 5%	3.5	0.9
Sterile Soil	16.7 a	3.2 b
Ganoderma Infected Soil	18.1 a	4.4 a
LSD 5%	2.5	0.6

In this study, AMF gave the beneficial effects on oil palm seedling growth as indicated by fresh and dry weight of shoot and root. The enhancing in growth could be due to the increase in uptake of nutrient especially phosphorus as mycorrhiza hyphae that developed in the soil can absorb nutrients directly from the soil matrix (Neumann and George, 2010; Rini, 2005) and improve in plant water relation such as increase in water uptake and photosynthesis rate (Ruiz-Lozano and Azcon, 2010; Doubkova *et al*., 2013).

In this study, AMF treatment gave a better impact on plant growth. However, its significance in reducing or control *Ganoderma* infection cannot be examined. All seedling planted in *Ganoderma* infected soil mix with its fruiting body had no *Ganoderma* infection in their root (Table 5).

Table 5. Root infection by AMF and Ganoderma as a result of AMF and Ganoderma treatments

Treatment	Root infection (%) by *Ganoderma*
Control	0
G*lomus* sp. (G)	0
Entrophospora sp. (E)	0
G + E	0
LSD 5%	-
Sterile Soil	0
Ganoderma Infected Soil	0
LSD 5%	-

Contrary to the first study, *Ganoderma* inoculum prepared in rubber wood block successfully infect both mycorrhizal and nonmycorrhizal seedling. Base on this result, it can be suggested that type of inoculum affect the success of *Ganoderma* to infect the root of oil palm seedling. Using rubber wood block to grow the *Ganoderma* inoculum confirmed the statement of Turner (1981) that *Ganoderma* pathogen is a facultative parasite. It is capable of living saprophytically on rotting stumps and roots. When a suitable host like oil palm root becomes available, the pathogen will colonize it and establishes a parasitic relationship. Using infected soil mix with the *Ganoderma* fruiting bodies as *Ganoderma* inoculum failed to cause infection. This result suggest that *Ganoderma* spores that exist in the soil and spores within fruiting body, within the constraints of this study, is not capable in infecting oil palm seedling root.

Conclusion

Base on the results from the study, the following conclusions could be made: (1) arbuscular mycorrhiza fungi improved the oil palm seedling growth and increase the seedling tolerance to *Ganoderma* infection (2) Spores of Ganoderma from the infected soil and *Ganoderma* fruiting bodies were failed to caused disease infection, contrary to the inoculum prepared on rubber wood block that successfully infect the seedling root.

Acknowledgement

The author would like to thank to staff of Labortorium Produksi Perkebunan, Faculty of Agriculture, The University of Lampung for their help in conducting this research.

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Potency of watery extract compost plus *Beauveria* sp. after storage for controlling planthopper and rice bug

PURNOMO, Radix SUHARJO, Ainin NISWATI, Yuyun FITRIANA, and Siti JARLINA

(Faculty of Agriculture, University of Lampung)

SUMMARY

Watery Extract Compost (WEC) is a liquid fertilizer that has been reported can be used as a biological control of plant pests and diseases. One of the entomopathogen which was widely used is *Beauveria* sp. This research was aimed to investigate survival and virulence of *Beauveria* sp. that was added into WEC after storage to planthopper and rice bug. The compost used in this study was made using *Trichoderma* sp. as a starter. The fungus *Beauveria* sp. used in this study was obtained from corn rhizosfer. The fungus was added into WEC before storage. The treatment were WEC suspension which were keep for 1, 2, 3, 4, 5 and 6 months. Each of experimental unit consist of 20 planthopper or rice bug, with ratio 1 : 1 for male and female. The result showed that after 6 months storage, the fungus *Beauveria* sp. which was added into WEC was still had capability to infect and cause mortality of planthopper and rice bug. After 6 months storage, the mortality of planthopper caused by WEC-Beauveria application reach > 50%. Meanwhile, the mortality of rice bug reach 45%.

Introduction

Soil quality degradation as well as pest and plant diseases are two important issues in crop productivity in Indonesia. Therefore, several effort have been done to find environmentally friendly technology. The combination of watery extracts of composted materials and entomopathogenic fungi are one of alternative for solved that problem in which it need to be developed.

Watery Extract Compost (WEC) or commonly call compost tea are fermented watery extracts of composted materials that are used for improving plant nutrition and crop yield including antimicrobial activities (Dionne *et al.*, 2012). Compost tea as a liquid fertilizer were made by mixing compost and water for 2-8 days by adding materials that can increase the microbial population in it (Scheuerell, 2004). Variuous number of stake holder using biofertilizers and biocontrol agents (biopesticide) in an effort to increase crop productivity are a very good step in terms of ecological aspects related to the many bad consequences of using chemical fertilizers continuously (Swastika, 2007) and using chemical pesticides without good control (Untung, 2001). Application of WEC, that were liquid biofertilizer integrated with entomopathogen *Beauveria* sp. fungi, were reported by (Purnomo *et al*., 2017) increased paddy field productivity. The problem is how long entomopathogen *Beauveria* sp. fungi can survive during storage in WEC without lose its capability?.

The purpose of the reaerch were to study ability to survival and virulence of entomopathogen *Beauveria* sp. fungi which is added to the WEC after storage against the planthopper and rice bug. Rice planthopper and rice bug are an important pest of rice plants.

Material and Method

The experiment were conducted at February until October 2017. Compost were made by rice straw mixed with 10% cow dung with starter was *Trichoderma* sp fungi. Preparation and production of WEC were performed at Laboratory of Biotechnology, Faculty of Agriculture, University of Lampung. Rearing and biopesticide (WEC + *Beauveria* sp.) suspension application for rice planthopper and rice bug were held at Crop Protection Laboratory, Trimurjo, Central Lampung. *Beauveria* sp. fungi used were collection of Laboratory of Biotechnology that were isolated from corn rhizosphere. *Beauveria* sp. fungi with density of 10^9 ml^{-1} watery compost were storaged before application with duration of 1, 2, 3, 4, 5 and 6 month in room temperature.

The experiment were arranged with Completely Randomized Design. There were two packages of experiment, one for rice planthopper and one for rice bug. Six replications were used for every treatment. Every experimental unit were given with 20 individual (10 males and 10 females) of rice planthopper and rice bug, respectively. Observation were conducted on rice

planthopper and rice bug mortality attacked by *Beauveria* sp. Infection and emerged of planthopper nymph.

Result and Discussions

The results showed that compost tea is a good medium for the growth of insect pathogenic fungi. It was showed by after stored 6 months in compost tea it still keep continued high capability to kill insects. Table 1 showed that *Beauveria* sp. had ability to kill rice planthopper more that 50% after storage 6 months in compost tea with room temperature. This high virulence was seen at 14 days after application. At 7 days after application, the ability to kill 50% or more is only for storage of 4 months or less.

The symptom of infected planthopper and rice bug by *Beuveria* sp. could be seen two days after application. Four to five days after application, almost the whole body of insect had already covered by white muscardine (Fig. 1)

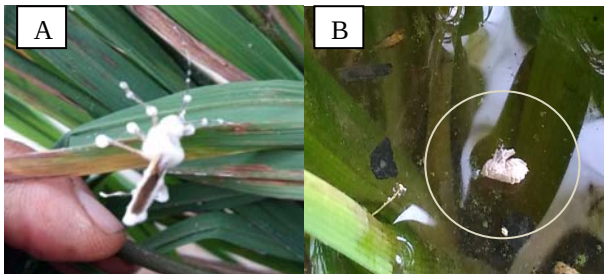

Fig. 1 Rice bug (A), rice planthopper (B) infected by *Beauveria* sp.

Table 1. Percentage of mortality of rice planthopper (%) by application of WEC plus *Beauveria* sp. with different storage duration.

Treatments	*Beauveria* sp.	
	7 days after application	14 days after application
Control	0	0
1 month	71.7	82.50
2 months	67.5	80.00
3 months	58.3	70.83
4 months	60.0	68.33
5 months	41.7	50.83
6 months	40.8	52.50

Table 2. Percentage of mortality of rice bug (%) by application of WEC plus *Beauveria* sp with different storage duration.

Treatments	*Beauveria* sp.	
	7 days after application	14 days after application
Control	0	0
1 month	68.3	85.00
2 months	62.5	78.33
3 months	51.7	70.83
4 months	41.7	55.83
5 months	42.5	56.67
6 months	33.3	45.00

Table 2 showed that storage of *Beauveria* sp. for 5 month in compost tea was be able to kill more than 50% rice bug tested after 14 days application. At the 7 days after application, 50% mortality of rice bug that killed by *Beauveria* sp. which storage less that 3 months.

Entomopathogen fungi that have virulence more than 50% commonly assumed as a potential source for bioinsecticides. Therefore, some researcher worked many effort to increase the virulence of entomopathogen fungi by manipulation of medium for fungi growth (Nuryanti *et al.* 2012; Fan *et al.* 2007; Herlinda *et al.* 2006).

Beside *Beauveria* sp. after storage in tea compost had high virulence infected adult of rice planthopper and rice bug, it also could suppress nymph of rice planthopper that emerge from the egg. Fig. 2 showed that practically storage of *Beauveria* sp. in tea compost for 6 months were not able to suppress emergence nymph of rice planthopper. However, the storage 5 month or less, *Beauveria* sp. could suppress better the emergence nymph of rice planthopper.

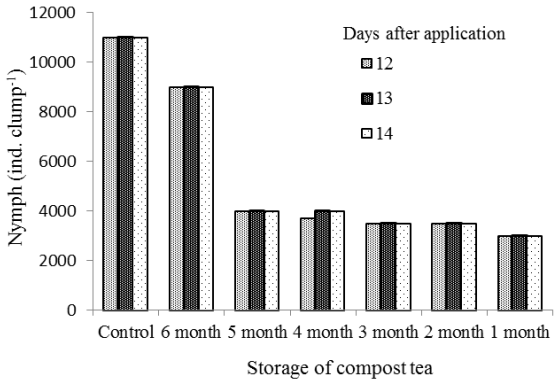

Fig. 2 Population of nymph of rice planthopper emerging after *Beauveria* sp application.

Conclusion

WEC or compost tea can be a good host for insect pathogenic fungi *Beauveria* sp. Application of compost tea has contain *Beauveria* sp. is able to suppress the population of rice planthopper and rice bug, even though the compost tea has been stored for 5 months at room temperature.

It is needed to continous research focused on WEC as a liquid biofertilizer which combined with biocontrol agent for controling plant pest and diseases, for example research of microbial content in compost tea.

Acknowledgement

We thank the staff at Crop Protection Laboratory, Trimurjo, Central Lampung for permitting this study and supporting the green house for experiment. Our study was supported by Product Applied Research Grant *(Penelitian Produk Terapan*), Ministry of Research Technology and Higher Education Republic of Indonesia with contract number 1638/UN26.21/KU/2017.

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Low pH-tolerant mutant of *Trichoderma* spp. induced by EMS, gamma rays and UV irradiation

Yuyun FITRIANA, Radix SUHARJO, Maria Viva RINI, Kuswanta Futas HIDAYAT

(Faculty of Agriculture, Lampung University)

SUMMARY

Basal stem rot caused by *Ganoderma boninense* is one of the most important problems causing severe economic losses in many oil palm industries, including in Indonesia. Recently, *Trichoderma* spp. has been widely used as biological control agent of *G. boninense*, however, this fungus was negatively affected by low pH condition. In Indonesia, oil palm commonly cultivated in marginal soil such us ultisol and peat soil with low pH (acid). In this study, three mutagens (EMS, gamma rays and UV irradiation) were used to generate low pH-tolerant mutant of *Trichoderma* spp. to increase its survival and effectiveness when it is applied in the previous mentioned field circumstance. Four Trichoderma isolates were used as the wild type. The mutant isolates were able to grow and produce spores in the pH 2-Potato Dextrose medium but not for the wild type. Only 1 out of 109 potentially low pH-tolerant mutant of Trichoderma showed better colony growth, sporulation, viability and antagonist to *G. boninense* than the wild type. These results showed that EMS, gamma rays and UV irradiation can be used to improve low-pH tolerant of *Trichoderma* spp.. However, the mutants must be carefully evaluated for any possibility of its negative impacts.

Introduction

Basal stem rot caused by *Ganoderma boninense* is one of the most important problems causing severe economic losses in many oil palm industries, including in Indonesia. Initially, this fungi attacked the roots of oil palm. Over time, it attacks continue to grow toward the base of the stem (Rini, 2003). At the beginning of the attack, the symptoms cannot be seen in the plant canopy. However, when the symptoms have appeared in the plant canopy (unopened shoots, pale green leaves, and old leaves begin to droop, fruiting bodies appear at the base of the stem), the fungal attack on the root and base of the stem is severe. Almost 50% of the base of the stem is damaged (Gurmit, 1991). Therefore, for controlling the *Ganoderma* is more difficult, because early symptoms is occur within the ground that are difficult to detect.

Trichoderma is a fungi that can act as a biological agent and can increase plant growth (Tsvetkov et al., 2014). In Indonesia, most of oil palm is grown on marginal land such as Ultisol and peat soil with low soil pH. In addition, intensive use of chemical fertilizers can further decrease soil pH (Firmansyah and Sumarni, 2013) and decrease the population and microbial effectiveness in soils, including beneficial microbes for plants (Rousk et al., 2009) including Trichoderma.

In this study, four isolates were used as a material for mutation activity to obtain mutant Trichoderma that were resistant to low pH. The mutation techniques used were Gamma rays irradiation, Ultraviolet irradiation (UV), and Ethyl Methane Sulfonate (EMS).

Material and Method

Fungal preparation. Four isolates of wild-type *Trichoderma* spp. (T1, T2, T3 and T4) that had been screened was cultured in Petri dishes (90 mm diameter) containing Potato's dextrose agar (PDA) for 2 weeks. Conidial suspensions were prepared by scraping the conidia/mycelia into sterile 0.1% Tween 80 and then filtering the mixture through sterile cloth (0.2 mm in mesh size) to provide a suspension of conidia.

Gamma rays and UV irradiation. Conidia of the wild-type isolates were irradiated with gamma rays at Badan Tenaga Nuklir Nasional (BATAN), Serpong. UV irradiation and EMS was done at Biotechnology Laboratory, Faculty of Agriculture, Lampung University. Twenty mL of the conidial suspension was injected on membrane filter and then was placed in each replicate plastic Petri dish (60 mm diameter). The dishes were irradiated at a range of doses 0, 30, 100, 300, 1000 and 3000 Gy (Gamma ray). For UV irradiation, wave length that was used was 245 nm with 10, 20 30, 40 and 50 minutes irradiation. All dishes were incubated at 20°C for 24 hours then moved into 3 mL *Potato Dextrose* (PD) liquid medium.

EMS solution. Ten mL of the conidial suspension was centrifused for 10 minutes at 3000 rpm. The pellet was soaked on 1.5 and 2% of EMS suspension for 30 and 50 minutes. Then, it was recentrifuged for 10 minutes at 3000 rpm. The pellet was incubated for 10 minutes at 0°C. Ten mL of *Phosphate buffer salin* steril was added to the pellet then it was centrifuged for 10 minutes at 3000 rpm. This step was replicated twice. The pellet was incubated at 20°C selama 24 jam.

Inoculation of *Trichoderma* isolates that have been treated on medium with low pH conditions. Each of treated isolates grown on 4 mL of PD Broth (liquid medium) at pH 2. Twenty mL of the incubated conidial suspension were entered into each medium. Observation were made daily for 7 days on the growth of fungus, the rate of growth and the formation of conidia. The wild-type were also included as a control.

The growth of colonies diameter, sporulation, viabilitity dan antagonism of *Trichoderma* Mutant Suspects to *Ganoderma* sp. This procedurs were conducted to know whether the mutants have the same ability as its wild type. Measurement methods of growth, sporulation, viability and ability of mutant antagonists were performed according to the same measurement method used in the screening stage to obtain selected Trichoderma fungi. Isolate to be tested previously moved from liquid medium to solid PDA media.

Result and Discussions

Isolates of the Trichoderma Putative Mutants. A total of 109 mutant suspects that capable of growing at pH 2 were obtained in this study. The mutant suspected isolates were obtained from gamma ray irradiation (54 isolates), UV irradiation (8 isolates) and EMS (47 isolates). The first step of determining the mutant was seen from the growing ability and spore production of each isolate that has been treated on each type of media compared to the wild type. If the treated isolate has a different appearance than the wild type, the isolate was determined as putative mutant. On the medium of pH 2, the putative mutant isolates resulting from gamma ray irradiation were able to form spores (marked with green colony color) but not for wild type. Wild type was still able to grow, however it did not produce spores (marked with colony color that remains white). This also occured in UV irradiation.

The growth of colony diameter of Low pH putative mutants.

Gamma rays irradiation. A total of 54 isolate mutants of low pH (pH 2) obtained in this irradiation. Ten mutants of T1 have larger colonies diameter than wild-type and 9 the other putative mutants had smaller colonies diameter than wild-type. The colonies diameter of the mutant resuted from T1 ranges from 4.6 to 7.4 cm. From the isolate T2, 6 mutants had larger colonies diameter than wild-type, while the other 12 isolates have smaller colonies diameter than wild-type. The diameter of mutant isolates from T2 ranges from 4.68 to 8.00 cm. T3 isolate, produced 1 mutant isolate, and has colonies diameter larger than wild type (6.61 cm). For T4 isolates, 14 mutant isolates had larger colonies diameter than wild-type and 2 isolates had colonies diameter smaller than wild-type. The diameter of mutant isolates from T4 ranged from 3.73 to 7.50 cm (Fig. 1).

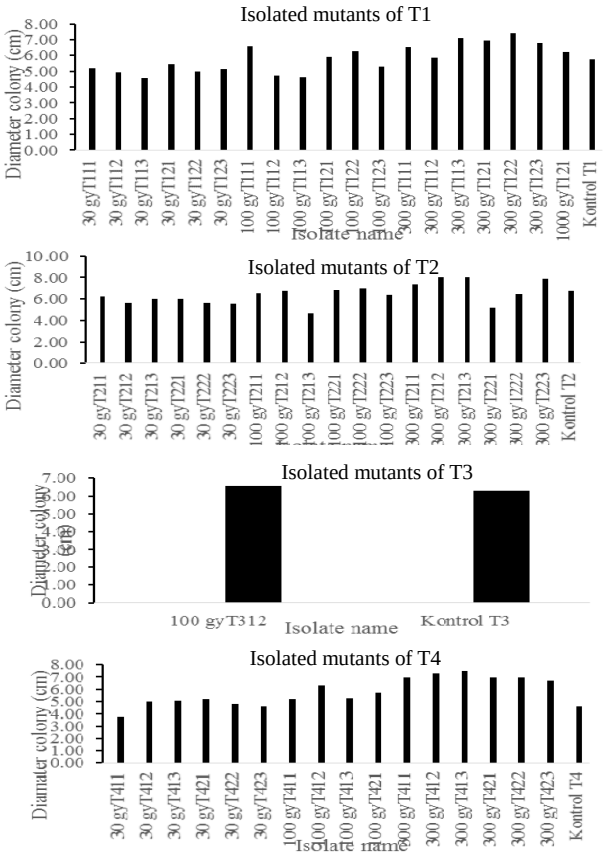

Fig.1 Colonies diameter of low pH mutant irridiated from gamma rays irradiation

UV irradiation. There were 8 isolates of low pH mutants (pH 2), consisting of 6 isolates of T2 and 2 isolates of T3. Two mutants of T2 resulted smaller colonies diameter than wild-type and 6 isolates mutants larger than wild-type. While 2 mutants of T3 had larger colonies diameter than wild-type (Fig. 2).

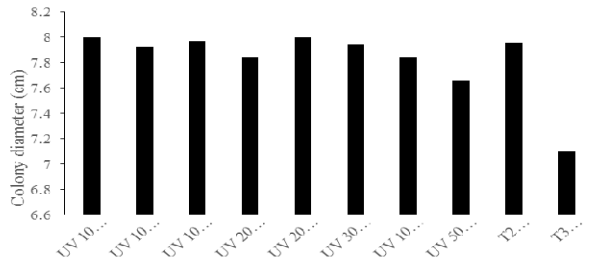

Fig.2 Colonies diameter of low pH mutant irridiated from UV irradiation

EMS solution. Total mutants isolates of low pH of 47 isolates consisted of 12 mutants of T1, 12 mutants of T2, 11 mutants of T3 and 12 mutants of T4. At 2 days after inoculation, 2 mutants of T1 produced larger colonies growth than wild-type meanwhile 10 mutants of T2 produced lower colony diameter than wild-type. Six mutant of T2 had same colonies diameter as wild-type and the other 6 mutants is lower. Five mutants of T3 had same colonies diameter as wild-type but the other 6 mutants were smaller. Only 1 mutant of T4 had larger colonies diameter than wild-type, the other 11 mutants were smaller.

Sporulation.

Gamma rays irradiation. Of all the isolates obtained, 2 mutants of T1 produced spore higher than wild-type and the other 16 mutants have spores lower than wild-type. Spores production of mutants T1 had range from 2.25 to 16.88 x 10^8 spores/ml. One mutant of T2 had spore production higher than wild-type, while 17 mutants had lower spore production than wild-type. Spores production from mutants of T2 ranged from 1.38 to 18.75 x 10^8 spores/ml. The mutant isolates derived from T3 isolates are currently still in the process of analysis. All of the mutants of T4 had lower spore production than wild-type. Spores production of mutants T4 ranged from 2.19 to 13.75 x 10^8 spores/ml (Fig. 3).

UV irradiation. Sporulation of 5 mutants of T2 were smaller than wild-type (15.63 x 10^8 spore/ml) and only 1 mutants equal to wild-type. While 2 mutants of T3, 1 mutant was larger than wild-type (8.13 x 10^8 spores/ml), but 1 mutant had smaller than wild type (Fig.4).

EMS solution. The highest sporulation produced by T2 isolate (6.88 x 10^8 spores/ml) and the lowest was T3 isolate (3.38 x 10^8 spores/ml). Three mutants of T1 produced spore higher than wild-type (> 3.75 x 10^8 spores/ml), while the other 8 mutants have lower spore than wild-type. T2 isolate produced 6 mutants that have higher sporulation than wild-type (> 6.88 x 10^8 spore/ml), while the other 6 mutants had sporulation lower than wild-type. T3 isolate produced 3 mutants that had sporulation smaller than wild-type (> 3.38 x 10^8 spore/ml), while the other 9 mutants were larger than wild-type. T4 isolate produced only 2 mutants that had higher sporulation than wild-type (> 6.25 x 10^8 spore/ml), while the other 10 mutants were smaller than wild-type (Fig.5).

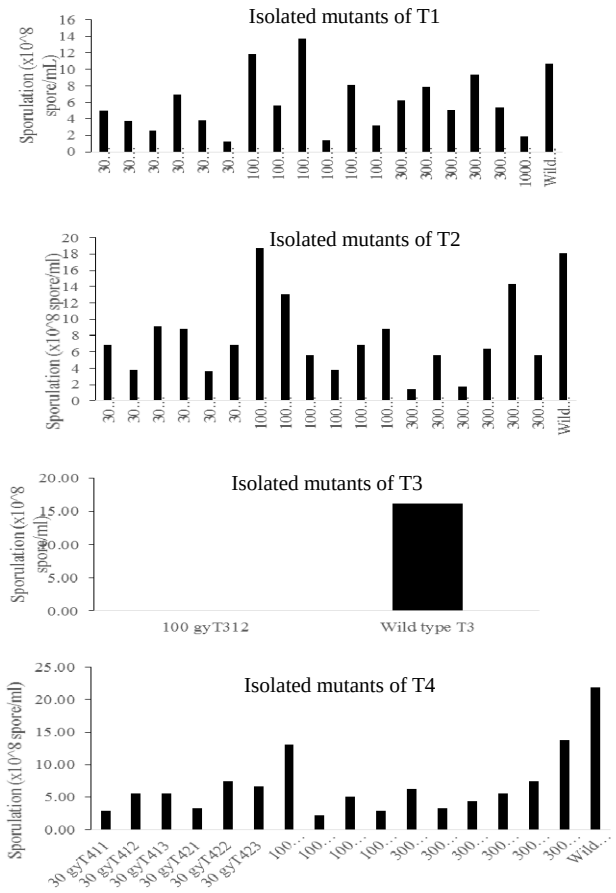

Fig.3 Sporulation of low pH mutant irridiated from gamma rays irradiation.

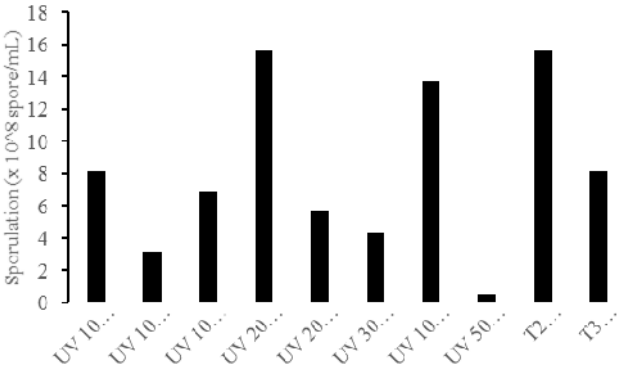

Fig.4 Produksi spora isolat terduga mutan pH rendah hasil iradiasi UV

Spore viability.

Gamma rays irradiation. Seven mutants of T1 produced spore viability higher than wild-type and the other 12 mutants had lower spore viability than wild-type. Spore's viability ranged between 94.17-100%. From T2, 15 mutants produced higher spore viability than wild-type, while the other 3 isolates had lower spore viability than wild-type. The spore's viability of mutants T2 ranged from 95.45-100%. For unexpected isolates of mutants derived from T3, currently is still in a process of analysis. From T4, 9 mutants of T4 had higher spore viability than wild-type, while the other 7 isolates had lower spore viability than wild-type. The spore's viability were between 82.32-100%.

UV irradiation. Five mutants of T2 have spore viability lower than wild-type (<100%) while 1 mutant had spore viability same as wild type. Two mutants of T3 had spore viability higher than wild-type (Fig. 5).

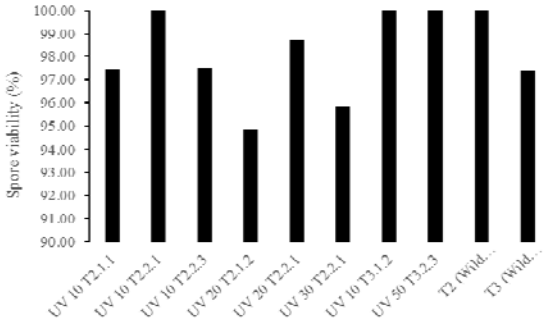

Fig.5 Spore viability of low pH mutant irridiated from UV irradiation

EMS solution. Immersion of EMS solution resulted 2 mutants of T1 that lower than wild-type (<98.52%); 1 mutant was equal to wild-type and 8 mutants were higher spore viability than wild-type. Two mutants of T2 were lower than wild-type while 8 mutants were higher than wild-type. From T3, 2 mutants had lower spore viability than wild-type and 10 mutants were as same as wild-type. Three mutants of T4 had spore viability lower than wild-type and 9 mutants were higher than wild-type (Fig. 6).

The Antagonism of Low pH Mutants to Ganoderma

Gamma rays irradiation. Of the 54 low pH mutants (pH 2) obtained, 5 mutants of T1 had better antagonism capability than wild-type, 1 mutant had same antagonism capability as wild-type and 13 mutants had lower antagonism capability than wild-type. The percentage of inhibition produced by mutants of T1 ranged from 62.17 to 93.48%. From T2, 5 mutants had better antagonism capability than wild-type and 13 mutants had lower antagonism capability than wild-type. The percentage of inhibition produced by mutants of T2 ranged from 54.78 to 96.09%. From T3, the mutant had smaller antagonism capability than wild-type. The resulting percentage is 82.17%. From T4, 4 mutants had better antagonism capability than wild-type and 12 mutants had smaller antagonism capability than wild-type. The resulting percentage ranged from 73.91-94.35%.

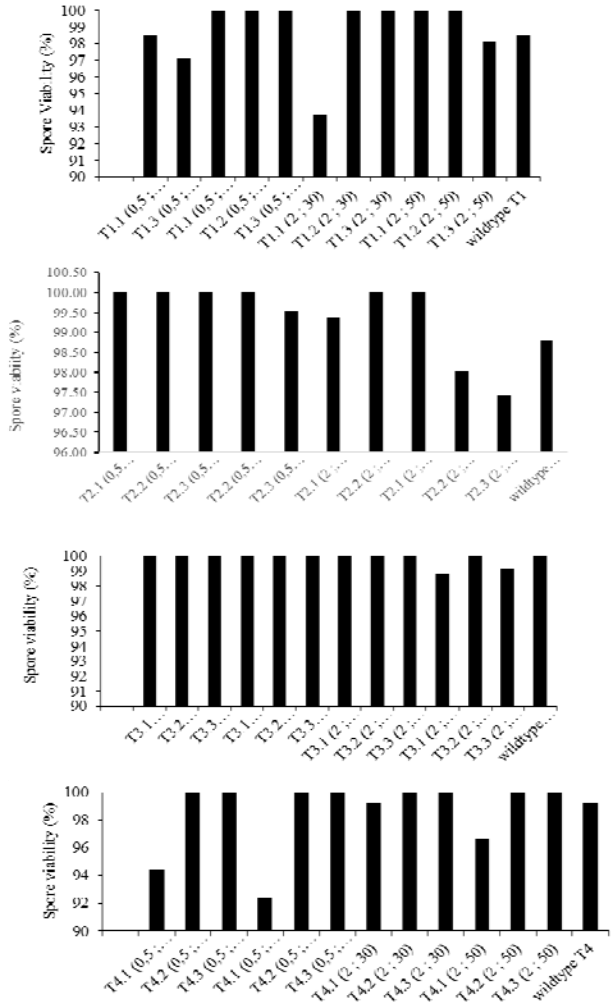

Fig.6 Spore viability of low pH mutant iridiated from EMS

UV irradiation. Six mutants of T2 were not better inhibition to wild-type (92.1%) meanwhile 2 mutants of T3 were better inhibition than wild-type (86.61%) (Fig. 7).

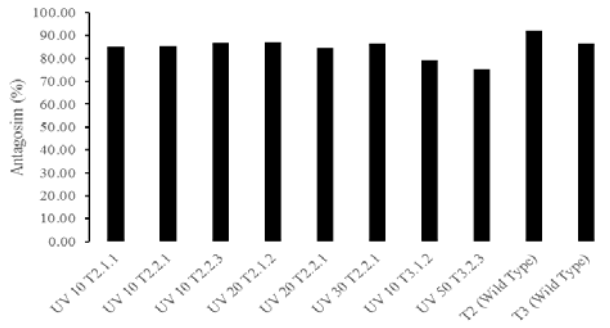

Fig.7 Antagonism to *G. boninense* of Low pH obtained from UV irradiation

EMS solution. Four wild-type produced various mutants. Mutants of T1 and T2 were not able to had better antagonism of wild-type (90.7% and 92.1%, respectively). Whereas 5 mutants of T3 had better antagonism ability than wild-type (86.61%) and the other 7 mutants were smaller than wild-type. Four mutants of T4 were better antagonism than wild-type but the other 8 mutants were smaller antagonism than wild-type (Fig.8).

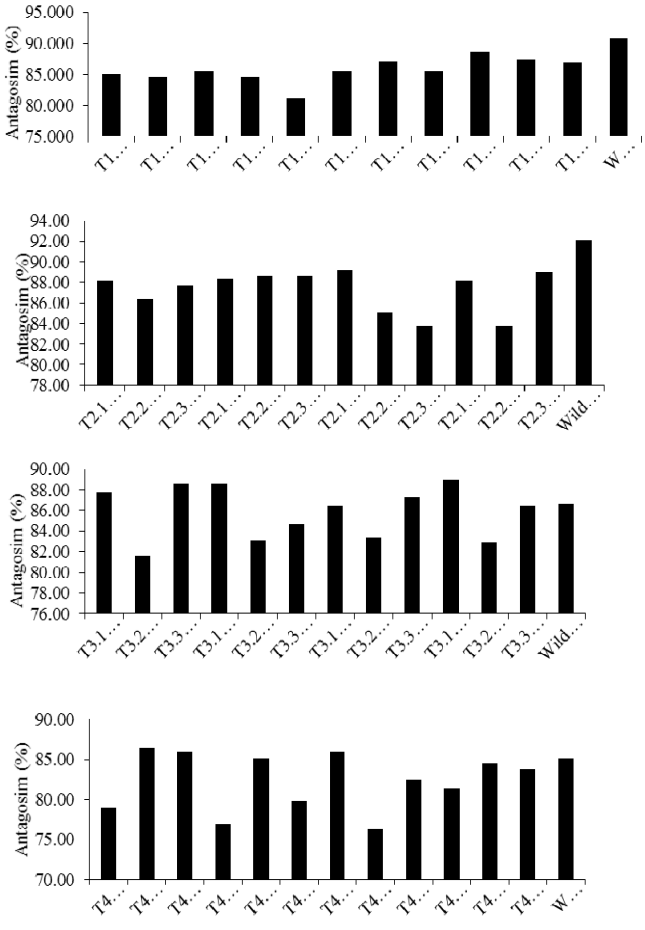

Fig.8 Antagonism to *G. boninense* of Low pH mutants from EMS solution

In this research, we used 3 mutation agents (mutagen) namely gamma ray irradiation, ultraviolet irradiation and Ethyl Methane Sulfonate (EMS). These three mutagens have generally been widely used to generate mutants (Darwis, 2006; Kava et al., 1995). Treatment with these three mutagens will lead to changes in various chemical and molecular bonds of reproductive cells from microorganisms (Darwis, 2006).

Piri et al. (2011) mentioned that gamma ray irradiation has been widely used to produce mutants from plants and microorganisms. Gamma rays irradiation have also been successfully used to produce resistance fungicide of *Isaria fumosorosea* and thermotolerant mutant of *Metarhizium anisopliae* s.l (Shinohara et al., 2013; Fitriana et al., 2014). UV irradiation and EMS have also been reported successfully used to create mutants from microorganisms (Pelczar and Chan, 1986). Radha et al. (2012) reported that the use of EMS can produce *Aspergillus niger* mutants capable of producing proteases 1.5 times higher than wild-type. UV Irradiation was also reported to be able to make mutant of *Aspergillus niger* capable of producing proteases 2 times higher.

From this research, in total, we obtained 109 mutants isolates from three mutagens (UV irradiation, gamma ray irradiation and EMS) that were low pH resistant. All of the mutant isolates have different performance with wild-type. After its treated with mutagen, almost all mutant isolates have better performance than wild-type when it were grown on low pH medium. The results showed that some mutant isolates had different colonies colors compared with wild-type. However, there are also isolates that do not change colonies color either on PDB media or after being transferred back using PDA media.

The ability of mutant isolates to grow, sporulation, spore viability and antagonism to *Ganoderma* sp. were also various. There were some mutant isolates that had performance lower than wild-type, some of they are similar to wild-type and some else had better performance than wild-type.

Najafi and Pezehki (2013) reported that mutations in microorganisms can affect or not affect the nature of these living things. The other research reported that thermotolerant resistant of *Metarhizium anisopliae* obtained from gamma-ray irradiation had lower virulence with wild-type, but others had higher virulence than wild-type (Fitriana, 2015).

Conclusion

The mutant isolates were able to grow and produce spores in the pH 2-Potato Dextrose medium but not for the wild type. Only 1 out of 109 potentially low pH-tolerant mutant of Trichoderma consistently showed better colony growth, sporulation, viability and antagonist to *G. boninense* than the wild type. These results showed that EMS, gamma rays and UV irradiation can be used to improve low-pH tolerant of *Trichoderma* spp.

Acknowledgement

This work was made possible through various facilitations. We thank to BPDP 2015 and Financial Department. We also thank our former students (Fransiska Dina and Fransiskus Ellyando Sinaga) for valuable assistance during research.

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Management of rice and wheat blast pathogen in Bangladesh

Manzur-E-Mohsina FERDOUS, Mohammad Mahmood HASAN , Adeeba RAIHAN, Lutfur RAHMAN, Abu Shamim Mohammad NAHIYAN

(Advanced Seed Research and Biotech Center, ACI Ltd., Dhaka, Bangladesh)

SUMMARY

Rice is a primary source of dietary energy for more than half of the world population. However, one of the most devastating fungal diseases 'rice blast' (caused by *Pyricularia oryzae*) hampers 60-100% yield. In this study, 11 popular rice (*Oryza sativa* Indica) accessions from Bangladesh were screened for blast resistance through artificial inoculation with mixed and single isolates. Disease responses varied among the accessions. Only BR15, BR16 and BRRI dhan 33 showed moderate resistance, with most germplasm susceptible. These accessions were also screened for candidate genes providing horizontal resistance. The gene specific microsatellite markers identified the *Pi 35t* gene in the BR 15 and 16, which is complementary to the disease scale after artificial inoculation and can be further used to develop blast resistant variety through marker-assisted breeding. On the other hand, wheat blast also occurred in Bangladesh recently. This incident is also first time in Asia and very alarming. Resistance breeding is the smart alternative but complex due to combination of several genes. On the basis of above circumstances, an experiment was planned to use biocontrol agent (*Trichoderma* sp.) on 1 mega variety of wheat in Bangladesh BARIGOM 28 and the ASRBC developed 8 mutant lines against wheat blast.

Introduction

Rice (*Oryza sativa* L.) is one of the most important leading food crops of the world which is consumed by 50% of the world population (Fairhurst and Dobermann; 2002). It is also the most important to millions of small farmers and landless workers. In Bangladesh, Rice has a long cultivation history and it is grown all over the country year round except in the south-eastern hilly areas (Shelly *et al*., 2016). It is the staple food for about 156 million people of the country. Although the agro climatic condition is suitable, the national average rice yield is much lower (2.94 t/ha) than the other rice-growing countries (BBS, 2012). The estimated amount of imported rice is 100,000 MT based on the local market demand for 2016/17 marketing year which will be increased to 125,000 for 2017/18 marketing year (Lagos and Hossain, 2017). The population increases at a rate of 2 million per year and concurrently the total cultivable land is decreasing at a rate of more than 1% per year due to urbanization. Moreover, biotic and abiotic constraints create adverse condition, which results serious yield reduction.

Among biotic stresses, rice blast is a very prominent, and widespread disease, which caused by fungal pathogen *Pyricularia oryzae* and reduces crop yield significantly in many rice growing countries over the last decade i.e. 20-100% yield losses have been reported in India, Japan and Kenya (Khush and Jena, 2009; Sharma *et al.*, 2012; Kihoro *et al*., 2013) depending on the rice variety grown. Leaf blast lesions decrease the net photosynthesis rates, which causes more damage than the visible portions of diseased leaves (Bastiaans, 1991). Neck blast is considered the most destructive phase of this trans-boundary disease (Zhu *et al*., 2005) which causes extreme and permanent damage (Biswas, 2017). In Bangladesh, 11,000 MT crop losses have been reported by neck blast on an area of 3000 ha (approx.) at several northern and southern parts of the country (Hossain, 2017) resulted from favorable condition such as adverse weather, high rainfall, prolonged dew and late planting etc (Lagos and Hossain, 2017). High relative humidity plays the most important role in sporulation, germination and release of blast conidia (Ou, 1985; Koutroubas, 2009). Severe outbreak of wheat blast has been taken place last February 2016 in Bangladesh (Callaway, 2016), threatening the food security of major wheat producing neighbouring countries (Islam et al., 2016). In case of rice, 267 blast occurring races have already been identified in Bangladesh, which can become a serious threat for 86 rice growing counties including Bangladesh

in near future. Therefore, attempts should be made to increase the yield per unit area of rice in a sustainable manner for the food and nutritional security of this highly populated country. So, the identification and use of resistant or tolerant cultivars will be the most economic and environment friendly way to manage this blast disease.

Material and Method

Isolation and characterization of the pathogen *Pyricularia oryzae*

The suspected causal agent for neck blast disease, *Pyricularia oryzae* was collected from blast affected plants of rice field. Infected leaves and nodes were cut into small pieces and placed on wet filter papers in sterilized Petri dishes to keep them moisturized. The Petri dishes were covered and kept under light at 24°C. After 24 hours of incubation period, the tissues were examined under stereo microscope to check for the fungal sporulations. The spores were then transferred to water agar media with an inoculation loop and incubated for 3 days at 24°C. Single isolates were identified using morphological characteristics, microscopic studies and final confirmation by ITS sequences. The Petri dishes of purified cultures were stored at 4°C for further use.

Preparation of inoculums

The inoculums were prepared from pre-inoculated plates of single and mixed strains culture. The spores were suspended in distilled water and the final concentration was adjusted to $1x10^6$ spores/ml by using haemocytometer.

Artificial inoculation of *P. oryzae*

Seeds of the rice accessions were sown in several earthen pots and placed on the benches of the greenhouse. After two weeks, the seedlings were inoculated with aqueous suspension containing $1x10^6$ spores/ml of virulent isolates of single and mixed strains of *P. oryzae*. Fresh water was sprinkled on plants three times every day between 11:00 and 16:00 hours to ease the disease development. The greenhouse temperature and humidity were maintained at 28°C and 90-100% respectively.

Screening of rice germplasm against rice blast disease

The seedlings were scored through 'Standard evaluation system of rice on 0-9 scale (IRRI 1996)' based on visual observation of the symptom at 10, 20 and 30 days after inoculation. The experiment screened seedlings in growth chambers and later vegetative stages in a green house. Gene specific microsatellite markers were used to detect genes, which may be providing horizontal resistance against blast.

Result and Discussions

The pathogen *P. oryzae* was successfully isolated with the filter paper method and then and cultured on water agar medium. Microscopic characterization and molecular analysis (Fig 1) were done for final confirmation. Disease symptom was appeared on susceptive plant leaves after inoculation, on the other hand, no visible symptom was found on healthy and disease free plants. Only BR15, BR16 and BRRI dhan33 showed moderate resistance among 13 accessions used in this experiment (Fig. 2 and 3). However, BRRI dhan28 and BRRI dhan29 showed severe susceptibility. The result was evaluated and determined for both single and mixed strain inoculations by scores of disease rating scale provided by IRRI in 1996. The presence of target gene in selected germplasm has been identified by molecular assay. The screened 13 accessions showed horizontal resistance against blast. The gene specific microsatellite markers identified the *Pi35t* gene in the BR15 and BR16 (Fig. 1).

Disease responses varied between the greenhouse and growth chamber as well as between seedling and vegetative stages. Very few reports are available on rice germplasm screening against rice blast in Bangladesh, though huge numbers of rice genotype were screened and found resistant against blast (Ahmed *et al*., 1985; Islam *et al*., 2001) but identification of involved genes has been less practiced to maintain and utilize the known gene pool. In this experiment, only BR15, BR16 and BRRI dhan33 showed moderate resistance, and most germplasm was found susceptible. Mohanta *et al*. (2003) reported to have 3 highly resistant, 12 resistant and 16 moderately susceptible in screening trails between 28 restroed lines and 4 standard checks.

The gene specific microsatellite markers identified the *Pi35t* gene in the BR15 and BR16 in this study, which is complementary to the disease scale after artificial inoculation. In a similar experiment, Khan *et al*. (2014) identified blast resistant genes *Pish*, *Pi9* and *Pita* from resistant varieties chinigura, BRRI dhan50 and Bawaibhog.

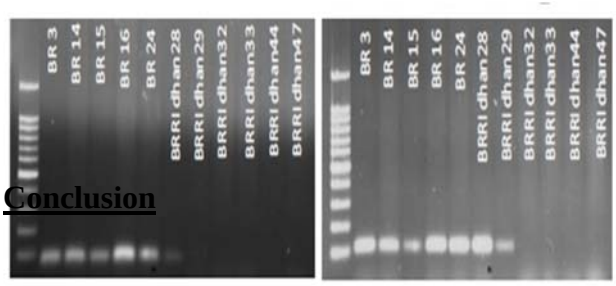

Fig.1. Blast gene detection. (a) *Pi 35t* Gene- RM 1216. (b) *Pi 35t* Gene- RM 1003

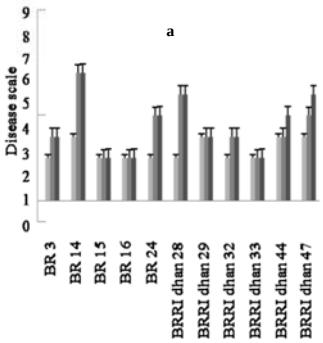

Fig.2. Disease scaling of blast for screening in rice accessions 10, 20 and 30 days after single strain inoculation.
□, 10 days after inoculation; ▨ 20 days after inoculation; ■, 30 days after inoculation

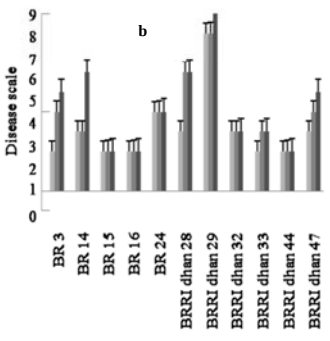

Fig.3. Disease scaling of blast for screening in rice accessions 10, 20 and 30 days after mixed strains inoculation.
□, 10 days after inoculation; ▨, 20 days after inoculation; ■, 30 days after inoculation

Conclusion

Effective evaluation leads breeders to choose advanced germplasm as parental lines for future work and to use as a gene pool in order to improve blast resistance in rice. Host resistance form BR15 and BR16 are being used to develop blast resistant rice varieties through marker-assisted breeding.

Acknowledgement

Special thanks to Dr. FH Ansarey, Managing Director and CEO, ACI Agribusinesses, ACI Ltd. for his continuous support for conducting the research.

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Control of eggplant and tomato bacterial wilt by grafting in Indonesia

Triwidodo ARWIYANTO, YMS MARYUDANI, Kin Lay LWIN, Bellarminus TRIMAN, Sri SULANDARI, Suryanti SURYANTI, Putri LAESHITA

(Faculty of Agriculture, University Of Gadjah Mada)

SUMMARY

Bacterial wilt disease caused by *Ralstonia solanacearum*, reduce production of Solanaceous crops such as potato, tomato, tobacco, and eggplant. The disease was difficult to control by available means. Grafting with resistant rootstock is an alternative control which has been practiced in many countries. In Indonesia, grafting with *Solanum torvum* suppressed eggplant and tomato bacterial wilt in the greenhouse experiments. All grafted plants as well as *S. torvum* did not exhibit wilt symptoms until one month after inoculation. However, non grafted eggplant wilted within the first week after inoculation and at 30 days after inoculation the disease index reached 96.4. The non grafted tomato completely wilted at 30 days after inoculation. Local cultivars of tomato grafted with Eg-203 showed lowest disease index in the experimental field infested with *R. solanacearum*. The grafted plants showed better growth and better fruit production compared with non grafted plants.When the local cultivars were grafted with H-7996, the disease index were higher compared with those grafted with Eg-203, and produced more fruit compared with non grafted plants. Two local tomato cultivars when tested as rootstocks, the grafted plants exhibited lower disease index both in the vinyl polybags and in the field experiments.

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is one of the important diseases in Solanaceous crops such as potato, tomato, tobacco, and eggplant. The disease destroyed cigar tobacco plantation in north Sumatra (Arwiyanto and Hartana, 2001), and along with root knot nemathode devastated keretek tobacco in Temanggung, Central Java (Dalmadiyo, 1991). Although the figure of losses has never been investigated, the disease also reduce significantly the production of potato, eggplant, chilli pepper, and tomato. The disease is difficult to control due to complexity of infection entrance to root and due to genetic variability of the pathogen. Vegetable grafting has been practiced since long time ago in Japan to control soilborne plant pathogen including *R. solanacearum* (Lee et al., 2010). The methods then has been developed and adopted elsewhere in the world. Here we reported the use of *Solanum torvum*, two local varieties of open pollinated tomato, H-7996, and Eg-203 as rootstocks to control bacterial wilt.

Material and Method

Sowing media was a mixture of cocopeat and rice hull charcoal (1:1, v/v). *Solanum torvum* was seeded 3 weeks before scions. Tomato rootstocks were seeded two days before the scion. At the first day until cotyledone emerge, the seeded media was watered daily with ground water. The seedlings then watered with a half strength of Hoagland's solution until ready for grafting. Two methods of grafting were performed. The first methods was cleft grafting as described elsewhere (Oda, 1999) and tube grafting was performed according Arwiyanto *et al*., 2015b. A challenge bacterial strain of *Ralstonia solanacearum* Phylotype 1, race 1, biovar 3 was used in the greenhouse experiments. In the field experiments the artificial inoculation with bacterial strain were not conducted, instead its used natural infection of the disease. In the second experiments, a local tomato cultivar (Martha) was used as scion. The rootstocks used were Solanum torvum (T), H-7996 (H), Eg-203 (E), Amelia (A) and Mawar (M). Thus, TM was denoted to Martha grafted with *Solanum torvum*, HM those which grafted with H-7996, and so on.

Result and Discussions

The first experiments with the used of solely *Solanum torvum* as a rootstock has been reported elsewhere (Arwiyanto et al., 2015a). All grafted plants as well as *S. torvum* did not exhibit wilt symptoms until one month after inoculation. However, non grafted eggplant wilted within the first week after inoculation and at 30 days after inoculation the disease index reached 96.4. The non

grafted tomato completely wilted at 30 days after inoculation (table 1).

Table 1. Average disease index of plants after inoculation with *R. solanacearum*

Treatments	Average disease index at days after inoculatiom			
	7	14	21	28
R1S1	0*	0	0	0a**
R1S2	0	0	0	0a
R1S3	0	0	0	0a
R1S4	0	0	0	0a
R2S1	0	0	0	0a
R2S2	0	0	0	0a
R2S3	0	0	0	0a
R2S4	0	0	0	0a
R3S1	0	0	0	0a
R3S2	0	0	0	0a
R3S3	0	0	0	0a
R3S4	0	0	0	0a
Solanum torvum	0	0	0	0a
'Kalenda' eggplant	29	59	79	96b
'Sanmarino' tomato	31	78	100	100b

*) means of five replications

**) number followed by the same letter were not significantly different (P=0.05) with Duncan's Multiple Range Test.

Source : Arwiyanto *et al*., 2015a.

Several years later, grafting experiments were performed by tube grafting methods. In field experiment, due to low population of *R. solanacearum* in soil, the disease progres was slow (Figure 1).

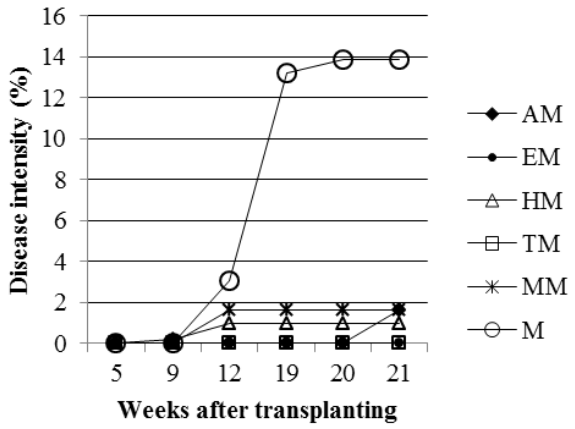

Figure 1. Bacterial wilt disease development of grafted tomato

About ten weeks after transplanting, infection of bacterial wilt were observed on non grafted plants. At 12 weeks after transplanting the disease progres on non grafted plants increase then significantly the disease intensity bcome higher. At the same time, tomato grafted with Mawar (MM) and H-7996 (HM) were infected in low intensity and kept at this point until the end of experiment. While, the non grafted plants reached maximum bacterial wilt intensity in the field. All grafted plants showed lower disease intensity compare with non grafted plants (Table 2). The lower disease intensity in the field experiments indicated that there was low population of the bacterial wilt pathogen. While in the vinyl polybag experiments, the disease intensity was higher because of artificial inoculation with highly virulent of *Ralstonia solanacearum*. In the field experiments, tomato grafted with rootstock Eg-203 (EM) and with *Solanum torvum* (TM) exhibited no disease infection. Again, the resistance of *S. torvum* againts bacterial wilt was proven. The resistance of Eg-203 was broken when challenged with dense population of *R. solanacearum* as showed in the table 2. There was infection of bacterial wilt when artificially inoculated with dense population of highly virulent of *R. solanacearum*, although the figure was small.

Table 2. Disease intensity of grafted tomato plants

Treatment	Disease Intensity (%)	
	Field	Vinyl Polybag
HM	0,95 a	2,67 a
EM	0 a	8,67 a
AM	1,66 a	5,67 a
MM	1,64 a	10,00 a
TM	0 a	-
M	13,86 b	50,67 b

Number followed by the same letter in the same column were not significantly different (P=0.05) with Duncan's Multiple Range Test.

The best yield was obtained when the tomato plants were grafted with Mawar (MM) which produced 2.7 kg/plant followed by those grafted with H-7996 (2.6 kg/plant) then Amelia (2.5 kg/plant) (Table 3). Tomato plants grafted with tomato rootstocks (HM, AM, MM) showed better performance on fruit production compare with those grafted with Eg-203 or *S. torvum*. On the contrary, although there was no infection of bacterial wilt, production of tomato grafted with *S. torvum* showed the

lowest production (1.1 kg/plant), even compared with those non grafted plants (2.0 kg/plant). It is assumed that incompatibility might happen so that the supply of nutrients from the soil was not in maximum capacity.

Table 3. Fruit production of grafted tomato

Treat-ment	Fruit number	Market able fruit	Number of rotten fruit	Production (kg/plant)
HM	33.7d	33.4d	0.3a	2.6c
EM	25.5b	25.3b	0.1a	2.0b
AM	30.5cd	30.3cd	0.2a	2.5c
MM	32.3d	32.0d	0.3a	2.7c
TM	15.1a	14.9a	0.2a	1.1a
M	26.8bc	26.6bc	0.2a	2.0b

Number followed by the same letter in the same column were not significantly different (P=0.05) with Duncan's Multiple Range Test.

Conclusion

Bacterial wilt caused by *Ralstonia solanacearum* can be controlled by grafting with the use of local tomato rootstock. Due to the variability of the pathogen, it is necessary to conduct the same experiment in many different area.

Acknowledgement

This research was financed by the grant from Ministry of Researh, Technology and Higher Education, Republic of Indonesia. The first author thanks to Gifu University for giving oportunity to present this paper by grants for travel and accomodation.

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Detection and characterization of Asia soybean rust in Bangladesh

Md. Motaher Hossain[1], Naoki Yamanaka[2]

([1]Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh, [2]Japan International Research Center for Agricultural Sciences, Japan)

SUMMARY

Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, is one of the most serious diseases of soybean. During April-May 2016, as soybean crops were nearing maturity, the disease was found throughout the region of southwestern Bangladesh, which includes Lakshimpur, Noakhali and Bhola District, where approximately 99% of the soybean crop of Bangladesh is produced. A total of 23 soybean fields in Noakhali, Lakshmipur and Bhola districts of Bangladesh were surveyed for the presence of ASR during April-May in 2016. Disease was detected in all those fields where nearly all plants showed symptoms. Affected crops were mostly in growth stages R4 to R6, except for a few fields that had been visited later and were in a late R7 stage. Leaf lesions were reddish brown, irregularly shaped, and were more abundant on the abaxial surface. Under the dissecting microscope, uredinia were observed as erumpent pustules with a conspicuous central pore. Masses of urediniospores were expelled through the pore and covered the pustules. Urediniospores were hyaline to pale yellow-brown, sub globose to ovoid and an average size of 17 to 36 × 14 to 25 µm µm. ASR susceptible soybean cultivar BRS154 and a local soybean cultivar BARI Soybean-5 inoculated with the spore suspension of three ASR isolates, each from each of three districts yielded symptoms and sign equivalent to those observed in the field. These results confirmed the presence of *P. pachyrhizi* in Lakshimpur, Noakhali and Bhola District, Bangladesh. Inoculation of a range of soybean genotypes was used for diversity and virulence analyses.

Introduction

Bangladesh is one of the countries with huge consumption of soybean. However, the local production of soybean is much lower than the national demand. As a result, large amounts of soybean and soybean products are imported from other countries. Projections indicate that soybean production in Bangladesh has made spectacular progress in the last few years (Shurtleff and Aoyagi, 2013). However, the actual yield is very often lower than most of the soybean growing countries.

To achieve the desirable level of productivity, proper crop management actions should be undertaken. Until some years ago, diseases that imposed important economic losses to Bangladeshi soybean growers were regarded as of insignificant importance. With the global spread of the Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* Sydow & Sydow, the local soybean production system is facing a new challenge that requires a new set of management skills from the soybean researchers and growers. The challenges are enormous since no soybean growing region is free from the occurrence of the ASR and no resistant variety has yet been released to soybean growers. The disease was first reported in Japan and initially limited to tropical and subtropical areas of Asia and Australia, but it has spread to Africa, South America and the USA over the course of the last hundred years. The disease is responsible for premature defoliation and early maturation of pod leading to large yield losses. ASR caused by *P. pachyrhizi* can reduce soybean yields up to 67% (Kumudini et al., 2008).

Several major resistance genes *Rpp1, Rpp2, Rpp3, Rpp4, Rpp5* and *Rpp6* have been identified. However, the durability of the disease resistance genes cannot be predicted because of our lack of understanding about fundamental aspects of the fungus biology. In 2016, a survey was conducted to detect the presence of soybean rust disease in the hot spots of soybean cultivation in Bangladesh. Soybean rust collected from various regions were studied for their morphological and pathological characterization. Characterization of local soybean rust will provide useful information towards designing of better disease management programs in the infected areas.

Material and Method

A total of 23 soybean fields in Noakhali, Lakshmipur and Bhola districts of Bangladesh were surveyed for the presence of ASR during April-May in 2016 (Table 1, Figure 1). In a single locality, soybean field was randomly selected. Depending on the size of the field, 5 to 10 sections of 10-20 ft row length were scouted. Sites were chosen throughout the field and the canopy were examined for

symptoms of the disease. Using a hand lens, the upper and the lower leaf surface were looked for chlorosis or black pinpoint spots and sporulation. Ten to twenty leaves were collected, placed them in a sealed plastic bag and brought to the laboratory. Each leaf let was examined under stereomicroscope for the presence of uredinia and urediniospores. In each filed, urediniospores were collected in 50 ml cryogenic tubes by leaf agitation. The cryogenic tubes were kept in a styrene foam box while completing the collection of the day. The spores were brought to the laboratory 2 days after collection and air-dried overnight at room temperature. On the following day, spores were stored at -80^{O}C.

In order to recover the cultures of *P. pachyrhizi*, spores from each location were multiplied on detached leaves of an ASR susceptible soybean cultivar BRS184 according to Hossain et al., (2015). Pathogenicity of three ASR isolates, ASR-1, ASR-18 and ASR-22 collected from Lakshimpur, Noakhali and Bhola, respectively was performed on susceptible Brazilian cultivar BRS154 and local soybean cultivar BARI Soybean-5. When plants reached V3 to V4 growth stage (approximate 3 weeks after sowing), they were inoculated. The optimal spore concentration used for inoculation of soybean plants was 5×10^4 urediniospores per mL. Plants were first sprayed with distilled water containing 0.04% v/v polyoxyethylenesorbitan monolaurate (Tween 20) using an atomizer. After air-drying for 15 min, plant leaves were inoculated by spraying them with freshly prepared spore suspension until run-off. Plants were allowed to air

dry before being placed in a humid chamber maintaining high humidity and dark conditions overnight and then transferred to a growth chamber with the same conditions as described previously (Yamanaka et al., 2010). Two weeks after inoculation, three infected leaflets were detached from each inoculated plant, and the abaxial side was examined microscopically for examining leaf symptoms and determining sporulation level (SL) and the numbers of uredinia (NoU) per lesion in a maximum of 30 lesions in total, 10 lesions from each leaflet. Additionally, a set of standard soybean genotypes were used to test the pathogenicity of ASR populations. Seeds of these varieties sown in a plastic tray containing vermiculite. One week after, three plants in each genotype were transplanted to pots filled with field soil. When the plants reached V3-V4 stages, three leaflets were derived from the first trifoliate leaf of three independent plants. Leaflets were laid abaxial surface up in the trays placing the lower end on the moistened filter paper. The lower end was covered with triple thickness of moistened filter papers. Frozen urediniospores of each ASR population were thawed in water bath at 39^{O}C for 1 min and suspended in 1 mL of distilled water containing 0.04% polyoxyethylene sorbitan monolaurate (Tween 20). Inoculation was done by spreading spore suspension homogenously on the lower surface of the leaves using a piece of filter paper. Inculcated leaves were kept in the growth chamber in dark overnight and then cultured at 21°C under a 12-h light period of approximately 3,000 lux provided by fluorescent lamps. Sterilized distilled water was pipetted to the moistened filter paper every 2-3 days. Following each inoculation time, aliquots (20 µl) of urediniospores suspension were incubated overnight on a slide glass containing 0.5% water agar for assessment of germination of spores. Two weeks after inoculation, each leaflet was evaluated for SL and NoU per lesion. A maximum of 30 lesions from each leaf let was examined under stereomicroscope to score the SL and NoU. Finally, average NoU and SL were calculated from three replications.

Result and Discussion

Asian soybean rust, caused by *P. pachyrhizi* has been known to occur in the Indian Subcontinent since 1970. More recently, it was reported from Nigeria in 2001, Brazil and Paraguay in 2002 and United States in 2004. Aerobiological models suggests that urediniospores of the pathogen are responsible for widespread dissemination of the soybean rust. In our study, ASR was observed in all the 23 fields in Noakhali, Lakshimpur and Bhola districts surveyed (Fig. 1; Table 1). In each field, all plants showed suspected rust symptoms. Infected plants were at physiological maturity but had not senesced. The crops were mostly in growth stages of R4 to R6, except in Bhola where plants were in a R5-R7 stage. This may be due to two week delay from the original starting date of survey in Bhola 2. Leaf lesions were reddish brown, irregularly shaped, and were more abundant on the abaxial surface. Infection was most found in middle and lower part of the plant canopy. Disease severity index ranged between 3.25 and 3.60, where the lowest and highest were in Noakhali and Bhola, respectively (Table 1). Typical erumpent pustules with a conspicuous central pore on infected leaves were readily apparent under the dissecting microscope. Masses of urediniospores were expelled through the pore and covered the pustules. Urediniospores were hyaline to pale yellow-brown, sub globose to ovoid and an average

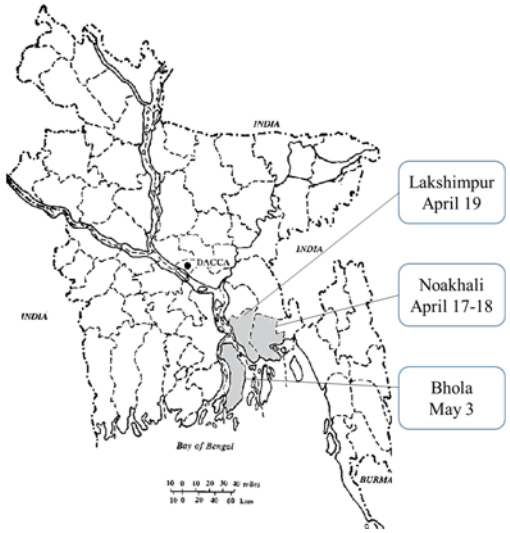

Fig.1 Locations of soybean fields where soybean rust were surveyed in Bangladesh in 2016

size of 17 to 36 × 14 to 25 µm. After germination, spore produced a long germ tube. Inoculation of susceptible soybean cultivar BRS154 with three ASR isolates resulted in development of the expected tan-colored lesions and the production of many uredinia with abundant sporulation. The NoU in BRS154 ranged from 2.76 to 3.93, while the SL were 2.96 to 3.00 (Fig. 2). Inoculation of local soybean cultivar, BARI Soybean-5 with the same ASR populations gave also susceptible infection and induced an abundant uredinia and uredioniospore. The NoU in BARI Soybean-5 ranged from 2.71 to 3.54, while the SL were 2.94 to 3.00. These results confirmed the presence of highly virulent *P. pachyrhizi* in Lakshimpur, Noakhali and Bhola District, Bangladesh.

Soybean rust pathogen has high evolutionary potential. Continuous monitoring for the presence and changes in virulence are essential in order to guide the development of soybean varieties with durable host resistance. In this

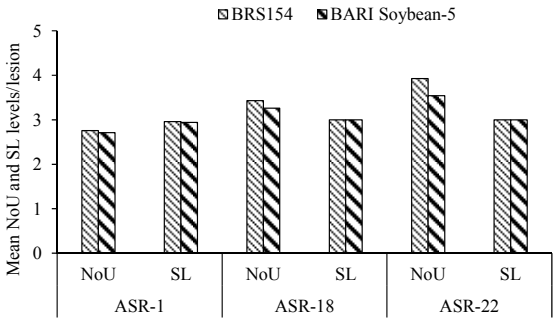

Fig.2 Mean values of number of uredinia per lesion (NoU) and sporulation level (SL) against three ASR isolates in BRS154 and BARI Soybean-5

study, we apprised ASR pathogenic diversity in Bangladesh on soybean host differentials (data were not shown). Several distinct pathotypes were identified. This

Table 1 Number sites, soybean growth stage and disease severity during survey of soybean rust in 2016

Location (District)	No. of sites	Plant growth stage	Mean disease severity
Lakshimpur	10	R4-R6	3.40
Noakhali	8	R4-R6	3.25
Bhola	5	R5-R7	3.60

indicates the existence of pathogenic variation in the ASR populations in Bangladesh. Most *R* genes display specificity with regard to their effectiveness against particular pathogen race. However, some genes in crops have been referred as "broad spectrum" as they recognize the *Avr* genes and confer resistance to a wide array of pathotypes (Ellis et al. 2014). Our study has proven the efficacy of two major genes against diverse pathotypes of ASR fungus in Bangladesh, as they conferred complete resistance against all tested populations. These genes may have valuable use as a source of resistance to rust isolates in Bangladesh. This is the first comprehensive characterization of ASR pathogen in Bangladesh.

Conclusion

In conclusion, our study successfully detected and characterized the ASR pathogen in the soybean hot spots of Bangladesh. The pathogen is highly virulent and variable which infected all cultivable varieties in the field. Identification of most indispensable major genes against the existing ASR pathotypes will help prioritize the deployment *R* gene(s) in soybean varieties through breeding program. Future studies are needed to analyze virulence diversity of ASR pathogen in consecutive years and efficacy of gene-pyramiding lines to confer adequate and durable resistance to soybean cultivars in Bangladesh.

Acknowledgement

This study was partly financially supported by Ministry of Education, Bangladesh (No.-37.20.0000.004.033.013. 2015) and partly by the JIRCAS research project 'Development of technologies for the control of migratory plant pests and transboundary diseases'

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In vitro selection of sugarcane (*Saccharum officinarum* L) for Fusarium-pokah bung (Pb) resistance

NANDARIYAH and Endang YUNIASTUTI
(Study Program of Agrotechnology, Faculty of Agriculture, Sebelas Maret University (UNS), Surakarta Indonesia, E-mail: nandar.suroso@yahoo.com)

SUMMARY

The aim of the research was to obtain mutants of sugarcane callus resistant against *Fusarium-Pb* after treated with Dioxane as mutagen then infected by *Fusarium* filtrate. The research was conducted at the Laboratory of Tissue Culture and consisted of five steps: (1). Growing callus of three clones: Ps-59, Ps-56 and F-154, (2). Treated in Dioxane 2 cc/l in MS medium for 21 days, (3) Treated with Fusarium filtrate: 20% and 50% concentrations. *Fusarium-Pb* was prepared before by isolated of single spore, (4). Selection of resistant callus against *Fusarium-Pb* filtrate, (5) Aclimatization of planlet. This experiment using a Randomized Complete Block Design (RCBD) with treated of three sugarcane clones and each of which was repeated three times. Variables observed were number of growing callus, weight and percentage of survival callus and number of planlet. Data analysis with variance and covariance. The result showed that Dioxane concentration of 20 % give higher number of survival callus and planlets growth than 50% after treated with Fusarium-Pb Filtrat. Sugarcane Clone Ps-56 showed the highest resistant against Fusarium-Pb filtrate than Ps-59 and F-154 clones.

Introduction

Sugarcane is an important agricultural commodity as one of the nine main needs of Indonesia people. The needs of sugar always increase but sugar production has not fulfilled the whole national demand because narrower sugarcane plantation and utilization of seedling contaminated of diseases. One of the disease attack sugarcane is "Pokah bung" (Pb) caused of *Fusarium moniliformae* Sheldon. The disease has 3 stage of symptoms namely Pb1, Pb2 and Pb3. Pb1 is usually identified by chlorotic symptom appearing from young leaves of sugarcane. In the further development the Pb1 can be the Pb2 that shows brownish red lines extending into deeper cavities and bent condition. The last stage is the Pb3 that is the death symptom of sugarcane because the pathogen has attacked the growing point (Pratiwi *et al*, 2013).

Pratiwi *et al*, (2013) said that countermeasures pokah attack bung common is the use of fungicides. However, the use of this fungicide had detrimental effects on the environment, among others, the destruction of predatory animals and have negative effects on the health of foodstuffs. Utilization of resistant varieties is the most effective method. One way that can be applied is the use of resistant varieties and high quality. In the assembly of improved varieties of crops needed because of the high genetic diversity of the population that are available to do the selection of superior properties as desired.

Resistant varieties of sugarcane are obtained through conventional breeding programs through crossbreeding and selection has produced several varieties that are resistant to pokahbung (Yunita, 2009). Breeding methods in vitro by using somaclonal variation combined with the mutations used to produce plants resistant to pokah-bung. Plant tissue regeneration results in in vitro culture is likely to have a tolerant phenotype selection condition. This in vitro method for more efficient mengeuntukan with conditions that can be made homogeneous, does not require space and materials in large quantities, and the effectiveness of the high selection.

Material and Method

The research was conducted in Tissue Culture Laboratory of Biology Faculty University of Gadjah Mada consisted of five steps: (1). Research to know what clone gave the highest growing ability of callus in Murashige and Skoog (MS) medium (Nandariyah, 1990). Research arranged in Randomize Complete Block Design with treated of three sugarcane clones: Ps-59, Ps-56 and F-154 and each of which was repeated three times. (2). Increasing sugarcane variability by treating of callus in Dioxane 2 cc/l as mutagen mixed in MS medium for 21 days. Every clone used of 40 samples compared with control (medium without dioxane). (3) Treating of callus to know the survival callus against Fusarium-Pb by droped of 20% and 50% concentrations of Fusarium-Pb filtrate. The aim of the research was to know resistance of sugarcane callus against Fusarium pb filtrate. (4) Selection of resistant callus against *Fusarium-Pb* filtrate, by calculated

number of survival callus after treatment in *Fusarium*-Pb filtrate. Resistant callus appearing white and clean performed then subculture in MS medium with ad kinetin plant growth regulator to induce planlet growth. (5) After 2 months age planlets then transplanted in sand medium contains humus. Variables observed were number of growing callus, weight and percentage of survival callus and number of growing planlets. Calculated and analysed data with variance and covariance analysis.

Result and Discussions

Number of growing callus

Average number of growing callus after one month showed not significant different among three clones (Table 1).

Table 1. Variance analysis of callus growing ability

Sourch of Variation	Degrees of freedom	Sum of Square	Mean Square	Fcalc	F table 5%	F table 1%
Block	9	15082,66	1675,85	12,76	2,43	3,60
Clone	2	45,60	22,80	0,17 NS	3,55	6,01
Error	18	2363,73	131,32			

Noted: NS = Not significant different at 5% level

Table 1 showed the growing ability of calli of three tested clones, were not different from one to the other. Similarly for number of callus grow from in vitro method, were not different too. This is good for variability of the sugarcane by used mutagen dioxane.

Purnamaningsih (2006) in her research to induced callus of *Musa paradisiaca* showed that the best medium formulation for induction of callus formation was MS + 2,4-D 2 mg/l + casein hidrolisat 3 mg/l, while the best medium formulation for callus regeneration was MS +BA 3 mg/l + thidiazuron 0,1 mg/l.

Increasing variability of callus was done by ad dioxane of 2cc/l media MS compared with media without dioxane. Dioxane is a kind of chemical mutagen that has effected against genetically characters change like Ethyl methane sulfonate (EMS) (Avivi, 2014).

Number of growing callus after 3 weeks subculture in media added dioxane

Sub culture callus then following test for variability by growing callus in medium with dioxane 2 cc/l. As comparison used callus growing in medium without dioxane (Table 2).

Table 2. Analysis of variance number of growing callus after 3 weeks sub culture

Sourch of Variation	Degrees of freedom	Sum of Square	Mean Square	Fcalc	Ftable 5%	Ftable 1%
Block	9	2932,00	265,78	12,48		
Clone	2	281,03	140,52	6,60**	3,39	5,57
Media	1	24,07	24,07	1,13 NS	4,24	7,77
Clone x Media	2	193,63	96,83	4,54*	3,39	5,57
Error	45	958,60	21,30			

Noted: * = Significant different at 5% level
** = Significant different at 1% level
NS= Not Significant different at 5% level

Table 2 showed that there was significant different between three clones of number of callus grow after three weeks of subculture on dioxane was supplemented to media MS and were significant different in interactions between clone and media. Although media MS added dioxane showed were not different on the number of callus life.The use of mutagen to increase variation somaclonal is in line with the effort to done by Lestari *et. Al* (2006). Avivi (2014) in his research to increase variability sugarcane callus for water lodging resistance used EMS of 0,3%. Fig 1. Showed Ps 59 callus after 3 weeks subculture on media without dioxane.

Fig. 1. Ps 59 Callus after 3 weeks subculture on media without dioxane.

Weight of three clone callus in media added dioxane

The ultization of dioxane also able to raising weight of three clones on subculture in media added dioxane (Table 3).

Table 3. Average weight of three clone callus in media is added dioxane after three weeks of subculture

Sourch of Variation	Degrees of freedom	Sum of Square	Mean Square	Fcalc	Ftable 5%	Ftable 1%
Block	9	0,31530	0,035000			
Clone	2	0,15460	0,077300	4,06519*	3,39	5,57
Media	1	0,32269	0,322690	16,96589**	4,24	7,77
Clone x Media	2	0,00227	0,001135	0,05967^{NS}	3,39	5,57
Error	45	0,85590	0,019000			

Noted: * = Significant different at 5% level
** = Significant different at 1% level
NS = Not Significant different at 5% level

Covariance of Surviving Calli of Three Clones After added By Filtrats at 20% and 50% Concentration

From the analysis of covariance obtained (Table 4) turns dioxane use significantly affect the amount of callus life and the analysis proved that the amount of callus life is not significantly different from each other and there is no real interaction between clones and media.

Table 4. Analysis of Covariance of Surviving calli of three clones after added by filtrats at 20% and 50% concentration

Sourch of Variation	Degrees of freedom	Sum of Product			Degrees of freedom	y adjusted for x		F calc	F table
		Xx	xy	Yy		Sum of Square	Mean Square		
Sum	59	801,4	86,6	38,73					
Block	9	97,73	9,43	4,4					
Clone	2	152,1	1,35	0,23					
Media	1	0,6	0,8	1,066					
Clone x Media	2	1,9	-0,25	0,0334					
Error	45	549,0667	75,26	33,0003	44	22,6826	0,5155		
Clone + error	47	701,1667	76,617	33,2333	46	24,8614			
Difference for testing adjusted clone means					2	2,1788	1,0894	2,1133^{NS}	3,39
media + error	46	549,6667	76,0677	34,0669	45	23,5402			
Difference for testing adjusted Media means						0,8576	0,8576	1,6636^{NS}	4,24
Clone + Media + Error	47	550,9667	75,0172	33,0337	46	22,8198			
Difference for testing adjusted Media means					2	0,1372	0,0686	0,1331^{NS}	3,39

Noted : NS= Not Significant different at 5% level

Fig 2. Describe the F154 callus growth after treatment fitrat the concentration of 50% with the overall brown color whereas the callus nearly. Fig.3 callus Ps 59 with all parts brown color after the treatment of the filtrate concentration of 20% and fig.4 showed F 154 on a callus growth medium without treatment dioxane filtrate shows the growth of plantlets.

Use of filtrate concentration of 20% and 50% real decrease the amount of callus life. The higher concentration of the filtrate further reduced the number of resistant callus. By using the filtrate of Fusarium-PB as the selection agent can be obtained subklon resistant.

Fig 2. F154 callus after treatment fitrat the concentration of 50%

Fig 3. Ps 59 after treatment filtrat the concentration 20%

Fig. 4. F 154 on a callus growth medium without treatment dioxane

Jumjunidang *et al*. (2005) on his research showed that concentration of filtrate toxin fusarium culture at 60 and 40% could be used as a selection medium for in vitro screening techniques for *Musa* resistance to *Foc*. The

higher concentration of filtrate toxin of *Foc* culture and fusaric acid, the faster disease incubation periode appeared. There was recovery of attacked plant due to fusaric acid and filtrate toxin of culture *Foc* treatment. This filtrate can be used as an early testing medium for resistance to fusarium wilt of banana.

Conclusion

The results of analysis showed that the growing ability of calli of three tested clones, were not different from one to the other. The 2 cc/l dioxane concentration could not increase the growing ability of the calli after the treatment with 20% and 50% of filtrate concentrations. The higher the filtrate concentration the higher the number of calli became dead. The addition of 20% and 50% filtrate really influence the plantlet growth.

Acknowledgement

The authors are grateful to the Sebelas Maret University and the research team, so this research can be done well.

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Poster Session Abstracts

Fumonisin production recovery in a *Fusarium fujikuroi* strain by complementation of *FUM21, FUM6* and *FUM7* genes

Sharmin SULTANA[1], Hironori KOBAYASHI[1], Ryuou YAMAGUCHI[1], Masafumi SHIMIZU[1], Koji KAGEYAMA[2], Haruhisa SUGA[3]

([1] Faculty of Applied Biological Science, Gifu University, Gifu, Japan; [2] River Basin Research Center, Gifu University; [3] Life Science Research Center, Gifu University)

SUMMARY

A part of *Fusarium fujikuroi* are known to produce a carcinogenic mycotoxin, fumonisin. Sixteen clustered genes associated with fumonisin biosynthesis (FUM cluster) are revealed in the *F. fujikuroi* genome. A FUM cluster was detected not only in fumonisin producers but also in non-producers (the strains do not produce detectable level of fumonisin). Therefore, fumonisin production of non-producers is still ambiguous and genetic elucidation of fumonisin non-production is required for accurate assessment of fumonisin producibility in *F. fujikuroi*. The causative mutations of fumonisin non-production was investigated with a non-producer Gfc0801001 (G1) in this study. *FUM21* is one of the cause of fumonisin non-production in G1. However, a complementation by *FUM21* from a fumonisin producer Gfc0825009 (G9) failed recovery of fumonisin production in G1 and it suggested that G1 has an additional mutation(s) in the FUM cluster. In order to identify the additional mutation(s), simultaneous complementation by *FUM21* and *FUM6*/*FUM7* of G9 was conducted in G1. Fumonisin production was recovered in the transformants and these indicate that additional mutation(s) causing fumonisin non-production in G1 is present in *FUM6*/*FUM7* region.

Introduction

Mycotoxin contamination poses threat to food chains including crop cultivations, storages and distributions. *Fusarium fujikuroi,* an important member of *Fusarium fujikuroi* species complex, is the causal agent of rice bakanae disease. A part of *F. fujikuroi* produces fumonisin that is associated with human esophageal cancer, leukoencephalomalacia in horse and pulmonary edema in swine and its contamination in grains (Marasas 2001; Marasas 1996; Harrison *et al* 1990). *Fusarium verticillioides* and *Fusarium proliferatum* are well known for fumonisin production. *F. fujikuroi* and *F. proliferatum* are sibling species which can occasionally undergo sexual reproduction and produce interspecific hybrid progeny but distinguished by molecular marker and chemotaxonomic criteria (Leslie *et al* 2004; Leslie *et al* 2007*).* They are considered as potential fumonisin producers on rice (Cruz *et al* 2013). Not all *F. fujikuroi* produce fumonisin. Information of the genes affecting fumonisin production can be a target for chemical development to inhibit this mycotoxin production.

The aim of this study is elucidation of the genetic mutations critical for fumonisin production in *F. fujikuroi*. This is not only for specifying key genes for fumonisin production but also for obtaining important information to prevent mycotoxin contamination by inhibition target key gene function. In previous study, genetic mapping using the crossing progenies between a fumonisin producing strain G9 and a non-producing strain G1 indicated that fumonisin non-production in G1 attributes to the FUM cluster (北嶋 2012). PCR results indicated that G1 retain entire FUM cluster. Though *FUM21* expression was detected in G1 by RT-PCR after culturing by fumonisin induction medium, lack of remaining three *FUM* gene expressions suggested dysfunction of FUM21 causes fumonisin non-production in G1. However, complementation by *FUM21* of G9 failed to recover fumonisin production in G1 and further complementation with *FUM6*, *FUM7*, *FUM8*, *FUM3*, *FUM10*, *FUM11*, *FUM2* and *FUM13* recovered fumonisin production in G1. These results suggested additional causative mutation(s) to *FUM21* is present in the FUM cluster in G1. Objective of this study is clarification of the causative mutation(s) of fumonisin non-production in G1.

Material and Method

- Confirmed transcription of some *FUM* gene of G1

and G9 by RT-PCR.

- Plasmids were prepared by cloning the objective gene from G9 and point mutation of plasmids.
- FfT67FUMKOD-1 and FfT21FUMKOD-2, transformants of G1 those carried *FUM6*/*FUM7* and *FUM21* of G9 respectively were used for further transformation. Transformation was performed according to Suga *et al* (2016) and created twenty transformants.
- Fumonisin production recovery of transformants was detected by ELISA by culturing in corn medium for 10 days in 25º C and extracting fumonisin with 75% methanol. Then, fumonisins in three of each transformant series including previous studies (小林 2013) were quantified by LC-MS/MS analyses.

Result and Discussions

Sequence comparison of *FUM* genes between G1 and G9

The distribution and the direction of each *FUM* gene in G1 are the same as in G9. High sequence homology (97.3-99.8 %) was observed in *FUM* genes between G1 and G9 (data not shown). One substitution and 11 amino acid deletion at C-terminal were found in FUM21. These mutations suggested dysfunction of FUM21 in G1 (Fig. 1)

Fig. 1 Comparison of the amino acid sequences of FUM21 of fumonisin producing strain (upper) Gfc0825009 and fumonisin non-producing Gfc0801001 (lower).

RT-PCR of *FUM* genes

Transcription of *Histone H3*, *FUM21*, *FUM1*, *FUM6*, *FUM8* and *FUM10* in G1 and G9 were confirmed by RT-PCR. Amplified DNA with expected size of all genes tested were detected in G9 but only it was detected for *HistoneH3*, *FUM21* and *FUM1* were in G1 (Fig. 2A and 2B). Lack of transcription of multiple *FUM* genes suggested dysfunction of the transcription factor FUM21 in G1 because fumonisin production recovery was failed by complementation of *FUM21* in G1 (小林 2013) (Fig. 3).

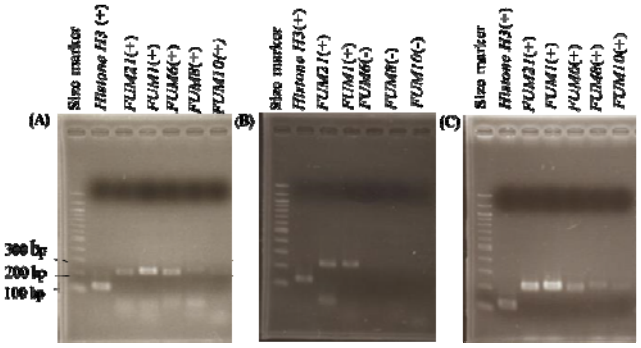


Fig. 2 RT-PCR result of (A) Gfc0825009, (B) Gfc0801001 and (C) FfT21FUMKOD (#2). RT-PCR product was subjected to 2 % agarose gel electrophoresis. (+) means that expected size of RT-PCR product was detected, (-) not detected.

***FUM* gene complementation by transformation**

Fumonisin production recovery was failed by complementation of only *FUM21* but it was succeeded by complementation of *FUM21* and *FUM6*/ *FUM7*/ *FUM8*/ *FUM10*/ *FUM11*/ *FUM2 FUM13* (小林 2013, FfDTFUM21_6_13 in Fig. 3). In order to identify which of g.888G>C (p.D261H) or g.2551G>T (p.G678*) (Fig. 1) is the causative mutation of fumonisin non-production in G1, complementation of *FUM21* with either of these point mutation was conducted in G1.The plasmid that carry a point mutation at 888th (pDT21G888C-1) and 2551th (pDT21G2551T-2) nucleotide in *FUM21* of G9 was created and transformed into FfT67831011213-30 (a transformant of G1 carrying *FUM6*/ *FUM7*/ *FUM8*/ *FUM10*/ *FUM11*/ *FUM2*/ *FUM13* regions of G9) (Fig. 3). None of the transformants recovered fumonisin production in case of pDT21G2551T-2 (FfDTFUM21G2551T_6_13 in Fig. 3) while five transformants recovered fumonisin production in case of pDT21G888C-1 (FfDTFUM21G88C_6_13 Fig. 3). These results suggested that g.2551G>T (p.G678*)

rather than g888G>C (p.D261H) is one of the cause of fumonisin non-production in G1 (Fig. 1). We sequenced the terminal portion of *FUM21* of additional three fumonisin non-producing strains (GL-24, Gfc0625008 and Gfc1034001) and three fumonisin producing strains (Gfc0821004, Gfc0009063 and 41-79) to reveal specificity of g.2551G>T substitution in fumonisin non-producing strains (data not shown) and observed that all fumonisin non-producing strains carried g.2551G>T (p.G678*) in *FUM21* as G1.

In order to identify the additional mutation(s) in *FUM6*/ *FUM7*/ *FUM8*/ *FUM3*/ *FUM10*/ *FUM11*/ *FUM2*/ *FUM13*, simultaneous complementation of *FUM21* and *FUM6*/*FUM7* regions was conducted in G1. Previous studies showed that fumonisin production recovery was failed by independent complementation of *FUM21* and *FUM6*/*FUM7* regions in G1 (Fig. 3).

transformant of G1 FfT21FUMKOD (#2) in that functioning of integrated *FUM21* was confirmed by transcription of *FUM6*, *FUM8* and *FUM10* in G1 (Fig. 2C). *FUM21*/ *FUM6*/ *FUM7* complementary transformant series were also created by transformation of FfT67FUMKOD (#1) with plasmid carrying *FUM21* of G9 (FfDT21T67KOD-1 in Fig. 3). Fumonisin production was recovered in both transformant series (Fig. 3). Multiple mutation(s) causing non-production of secondary metabolite in the biosynthetic gene cluster were reported as aflatoxin non-production in *Aspergillus oryzae* (Tominaga *et al* 2006) and gibberellin non-production in *F. proliferatum* (Malonek *et al* 2005). These results suggested that mutations causing fumonisin non-production in G1 were distributed in *FUM21*, *FUM6* and *FUM7*.

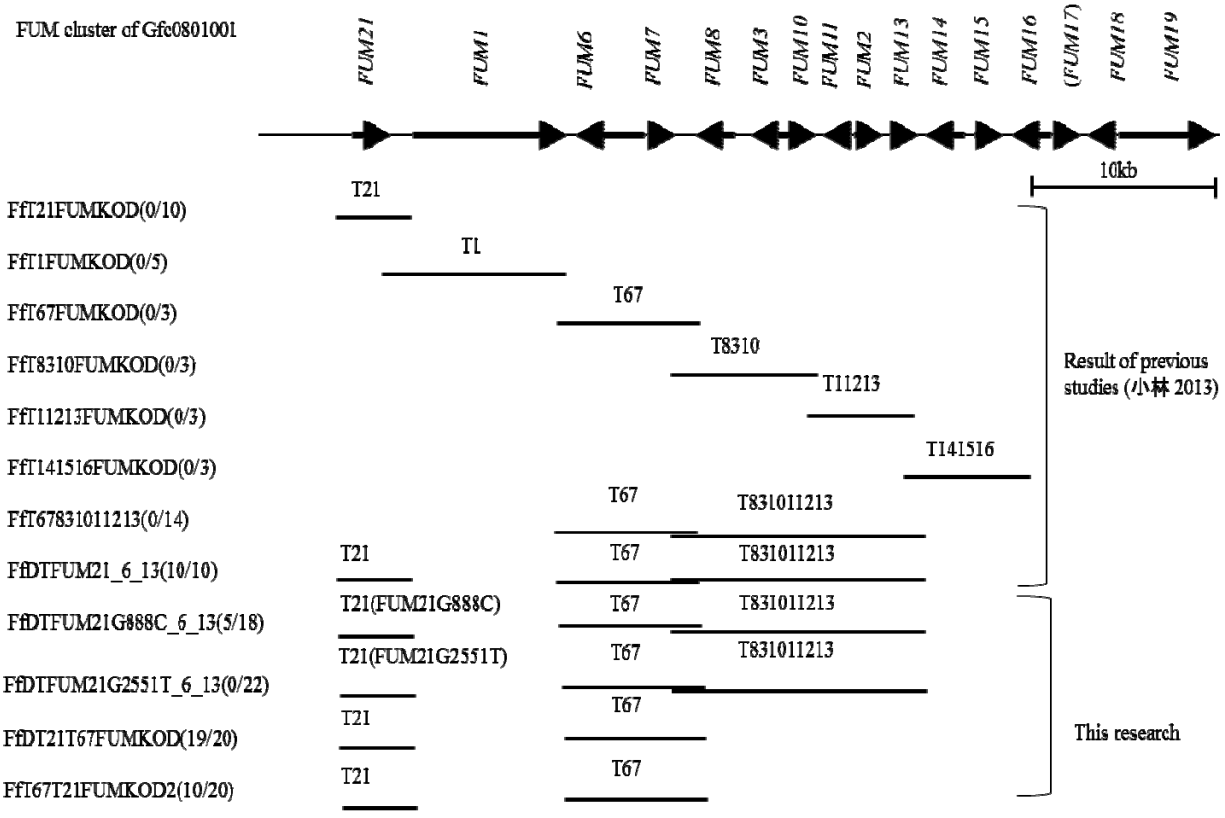
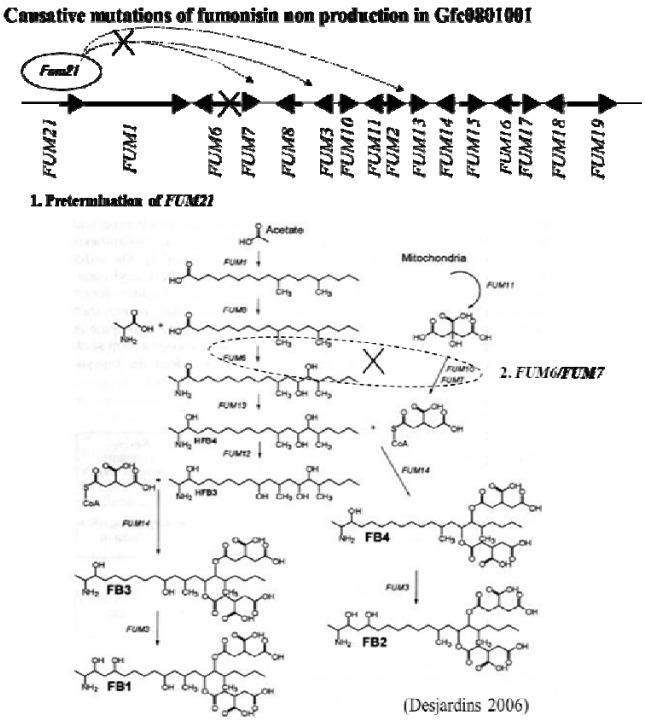


Fig. 3 Result of *FUM* gene complementation in Gfc0801001 by transformation (the number of fumonisin producing transformants/ the number of investigated transformants). Here, "T" is DNA fragment from fumonisin producing strain Gfc0825009. Fumonisin production was determined by detection of fumonisin by ELISA (detection limit is 0.22 ppm). *FUM17* in both Gfc0801001 and Gfc0825009 is a pseudogene indicated in parenthesis.

The plasmid carrying *FUM6*/*FUM7* region of G9 was transformed into a *FUM21* complementary

Conclusion



Acknowledgement

A grant from Ministry of Agriculture, Forestry and Fisheries of Japan (Research project for improving food safety and animal health).

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Genetic mapping of chromosome No.1 region associated with pathogenicity in Fusarium head blight pathogen

Rina OKUMURA[1], Maho IKAWA[1], Yuki HIRATA[1], Masafumi SHIMIZU[1], Koji KAGEYAMA[2], Haruhisa SUGA[3]

([1]Faculty of Applied Biological Sciences, [2]River Basin Research Center, [3]Life Science Resarch Center, Gifu University)

SUMMARY

Members of the *Fusarium graminearum* species complex (*Fg* complex) are the primary pathogens causing Fusarium head blight (FHB) in wheat and barley worldwide. A natural pathogenicity mutant (*Fusarium asiaticum* strain 0233007) was found in a sample of the *Fg* complex collected in Japan. The mutant strain did not induce symptoms in wheat spikes beyond the point of inoculation, and did not form perithecia. No segregation of phenotypic deficiencies occurred in the progenies of a cross between the mutant and a fully pathogenic wild-type strain, which suggested that a single genetic locus controlled both traits. The locus was mapped to ca. 200 kbp region in the chromosome No.1 by using sequence-tagged markers. This suggested the gene involving pathogenicity and perithecium formation is present in this region.

Introduction

In previous studies, several *F. asiaticum* strains that show non-pathogenicity to wheat and non-perithecium formation have been discovered in Japanese collection. One of the strains, 0225022 lacks ca. 2 kbp on chromosome No.2 and loss of *FGSG02810* gene in that region causes lost of both traits (Suga *et al* 2016). On the other hand, another *F. asiaticum* strain 023307 with non-pathogenicity and non-perithecium formation has no deletion on chromosome No.2, and introduction of wild-type *FGSG02810* gene by transformation failed recovery of both traits in the strain 0233007. Therefore, the causative mutation in this strain is expected to be different from that of 0225022. In previous studies, it was estimated that the causative genomic region in this strain is approximately 3,480 kbp (between VNHK909 and VHNK917 markers) on chromosome No.1 (井川 2016). Further mapping was performed in this study.

Material and Method

Strain―The wild-type strain *F. graminearum* s. str. 0407011, showing pathogenicity and perithecium formation, and the mutant-type strain *F. asiaticum* 0233007, showing non-pathogenicity and non-perithecium formation, were used (Suga *et al* 2008). Forty progenies obtained by their crossing (⑩67P7101-⑩67P7147 in 井川(2016)) were also used.

Pathogenicity test ― The pathogenicity test was performed using wheat cultibar Apogee, which has high susceptibility to FHB. Each plant was inoculated about 10^2 conidia added to the wounded lower spikelet. The disease symptoms were evaluated 14 days after inoculation. Pathogenicity was scored as pathogenic or non-pathogenic based on whether the symptoms were confined in the initially inoculated spikelet, as for 0233007, or whether they spread to the neighboring florets, as for 0407011 (Fig.1).

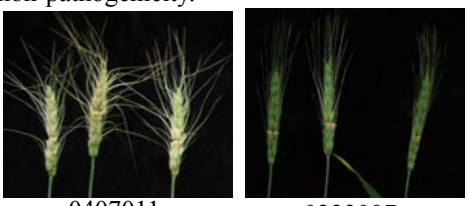
Perithecium formation ― Perithecium formation was assayed on carrot agar plates (Klittich and Leslie 1998) as described above, expect that the mycelial plug of progeny was placed in the center of the plate. Perithecium formation was observed until 3 weeks after aerial mycelium knockdown.

DNA extraction and PCR―Genomic DNA for PCR was extacted from 3-day-old mycelium cultured on potato dextrose broth (Suga *et al* 2008). The final DNA pllet was dissolved in 200 µl of water.

Mapping―For setting markers at arbitrary positions, the National Center for Biotechnology Information (NCBI) website was used. A gene with more than one intron around in the target region was selected and PCR-RFLP marker was developed. Data obtained with PCR-RFLP markers were processed using JoinMap®4 to detect a significant association between pathogenicity and perithecium formation; the markers were processed using logarithm of odds (LOD) scores.

Result and Discussions

Pathogenicity test—In 0407011, whole spike whitened after 14 days, whereas in 0233007, symptom was observed only in the spike injected with the spore suspension or only in the adjacent spike 14 days after the inoculation (Fig.1). Twenty two progenies showed pathogenicity, and 18 progenies showed non-pathogenicity.



0407011 0233007

Fig. 1 Result of pathogenicity test of the parent strains

Perithecium formation—A parent strain 0407011 and 22 progenies showed perithecium formation. A parent strain 0233007 and 18 progenies showed non-perithecium formation (Fig.2). Perithecia formation ability and pathogenic strains were completely identical in progenies.

0407011 0233007

Fig. 2 Result of perithecium formartion assay of the parent strains

Mapping—Nineteen progenies were analyzed with 27 markers. Six markers (VNHK909 ～ VNHK917) in choromosome No.1 showed high LOD values (井川 2016). Therefore, PCR-RFLP markers (PRHS857/Hae□, PRHS859/XhoI, PRHS861/Hinc□, PRHS863/AluI, PRHS865/RsaI, PRHS867/RsaI, PRHS869/Hpa□) was set to be approximately equal positions between the markers. Among these, the PRHS859/XhoI marker showed the highest LOD value (LOD=11.95). Then, additional PCR-RFLP markers were developed for the middle region between PRHS859/XhoI and adjacent PCR-RFLP markers and obtained LOD value. This task was repeated and finally, four markers (PRHS892/NsiI, PRHS859/XhoI, PRHS894/MseI, PRHS896/HhaI) with perfect match between marker type and phenotype in the 40 progenies were obtained (Fig.3). According to the information of chromosome No.1 of *F. graminearum* s. str. strain PH-1 on NCBI (accession number HG970332.1, total length 11,760,950 bp), there are 82 genes in ca. 200 kbp from PRHS890/MseI to PRHS 886/HaeIII adjacent to PRHS892/NsiI and PRHS896/HhaI, respectively.

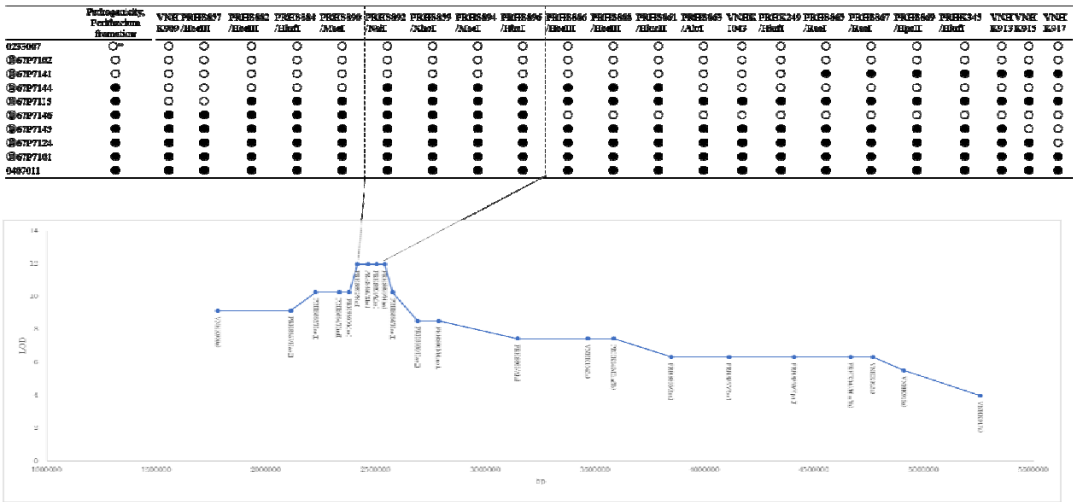


Fig. 3 Mapping of non-pathogenicity and non-perithecium formation in the mutant strain 0233007

The result of a part of progenies is indicated above. The logarithm of odds (LOD) value obtained by analyzing 40 progenies and the position in chromosome No.1 of each maker were indicated below. White and black circles on lines indicate the 0233007 and 0407011-type allele, respectively; white and black circles next to the progeny's name indicate mutant phenotype (non-pathogenic and non-perithecium formation) and wild-type phenotype (pathogenic and perithecium formation), respectively. The marker within the vertical dotted lines showed a perfect match between allele-type and phenotype in the 40 progenies.

Conclusion

It was presumed that a gene involving pathogenicity and prithecium formation is present in ca. 200 kbp in chromosome No.1 by linkage analysis using 40 progenies. There are 82 genes in this region. In the future, gene complematation analysis would be able to identify the gene from them.

Acknowledgement

This work was supported by JSPS KAKENHI Grant Number JP16K07612.

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Isolation of plant probiotic *Bacillus* spp. from tea rhizosphere

Nusrat Ahsan[1], Tomoki Nishioka[1], Haruhisa Suga[2], Hiroyuki Koyama[1],
Masafumi Shimizu[1]

([1] Faculty of Applied Biological Sciences, Gifu University; [2] Life Science Research Center, Gifu University)

Abstract

The aim of this study was to isolate and characterize rhizosphere spore-forming bacteria with plant probiotic activities. Plant probiotic is a general term for microbes which exert health promoting and nutritional benefits on plants.

In this study, a total of 80 isolates of heat-tolerant spore-forming bacteria were isolated from rhizosphere soil of organically cultivated tea plants. Phylogenetic analysis based on 16S rRNA gene sequences showed that 78 of them belonged to genus *Bacillus* and remaining 2 isolates belonged to genus *Lysinibacillus*. In order to evaluate the plant probiotic potential, the bacteria were screened *in vitro* for the siderophore production, indole-3-acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase production, phosphate solubilisation, and antagonistic effect against *Sclerotinia sclerotiorum*. The results showed that all isolates produced IAA, ranged from 0.2 to 12.7 μg/ml, in the presence of L-tryptophan. The ability to solubilize tricalcium phosphate was displayed by 67 isolates of *Bacillus* spp. and 1 isolate of *Lysinibacillus* sp. (GUCS32). Seventy two isolates of *Bacillus* spp. and the isolate GUCS32 of *Lysinibacillus* exhibited the ability to produce siderophore. Additionally, 71 isolates of *Bacillus* spp. and *Lysinibacillus* isolate GUCS32 were found to have antagonistic activity against *S. sclerotiorum*. On the other hands, none of the isolates have an ability to produce ACC deaminase.

For further *in planta* experiments, seven *Bacillus* isolates (GUCS 17, GUCS 18, GUCS 22, GUCS 42, GUCS 58, GUCS 70, and GUCS 78) and *Lysinibacillus* isolate GUCS32 were randomly selected. The plant growth promotion study showed that the inoculation of these isolates significantly increased shoot dry weight of spinach seedlings under climate chamber conditions. Furthermore, the inoculation of soil with the isolates GUCS 22, GUCS 32, GUCS 42, GUCS 58, and GUCS 78 reduced the severity of cabbage damping-off caused by *S. sclerotiorum*. The highest reduction (63%) was recorded for the isolate GUCS42 which was most closely related to the type strain of *Bacillus cereus*.

In order to understand the importance of chemotaxis in the growth promoting and biocontrol behaviours of the selected bacterial isolates, their chemotactic activity towards oxalic acid, which was known to be produced by spinach and *S. sclerotiorum*, was investigated by a capillary assay. However, none of the isolates exhibited chemotaxis towards oxalic acid.

This study demonstrated that a selected isolate GUCS42 could be used for the development of a novel plant probiotic product having growth promoting and disease suppressive effects.

Microbial basis of Fusarium wilt suppression by *Allium*-cultivated soils

Tomoki NISHIOKA[1], Malek MARIAN[1], Haruhisa SUGA[2], Masafumi SHIMIZU[1]

([1]The United Graduate school of Agricultural Science, Gifu University;

[2]Life Science Research Center, Gifu University)

Abstract

Crop rotation and intercropping with *Allium* plants has been reported to suppress the incidence of Fusarium wilt (FW) in various crops caused by *Fusarium oxysporum*. However, the mechanisms responsible for FW suppression in *Allium*-cultivated soil have not been fully elucidated. In this study, we revealed the underlying mechanism of the FW suppressiveness of *Allium*-cultivated soil using culture-dependent and independent approaches.

The *Allium*-cultivated soils exhibited an inhibitory effect on cucumber FW (CFW) caused by *F. oxysporum* f. sp. *cucumerinum* (Focu). However, the suppressive effects of cultivated soils were drastically eliminated by soil pasteurization at more than 60°C. These results suggest that gram-negative bacteria play a key role in the FW suppression by *Allium*-cultivated soils. Furthermore, the characterization and comparison of rhizobacterial communities by 16S rRNA amplicon sequencing revealed that four gram-negative genera, *Burkholderia*, *Pseudomonas*, *Flavobacterium*, and *Chryseobacterium*, were the predominant groups that preferentially accumulated in the rhizospheres of *Allium* plants. The isolates of *Burkholderia*, *Pseudomonas*, and *Flavobacterium* recovered from rhizosphere soils of *Allium* plants tended to exhibit suppressive effects against CFW. Therefore, we speculate that the accumulation of these antagonistic gram-negative bacteria plays a key role in FW suppression by *Allium*-cultivated soil.

Several *Burkholderia* and *Pseudomonas* isolates recovered from *Allium* rhizosphere soils exhibited antagonistic activity toward Focu on agar medium. In comparison with non-antagonistic *Burkholderia* and *Pseudomonas* isolates, antagonistic isolates showed significantly higher suppressive effects on CFW, suggesting that the CFW suppressiveness of these antagonistic isolates may be attributed to the production of antifungal compounds.

Acknowledgments

This work was supported by a Grant-in-Aid for Young Scientists (B) (grant number 24780317) and for JSPS Fellows (grant number 953381) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Isolation of novel deoxynivalenol-degrading microorganisms from Poaceae planted soils

Hiroyuki MORIMURA, Sotaro CHIBA, Daigo TAKEMOTO, Kazuhito KAWAKITA, Ikuo SATO

(Graduate School of Bioagricultural Sciences School of Agricultural Sciences, Nagoya University)

SUMMARY

The mycotoxin deoxynivalenol (DON) is produced by several plant pathogen *Fusarium* species and is the result of Fusarium Head Blight (FHB) which is known as worldwide serious problems in cereal crops. A biological control for the degradation of DON using DON-degrading microorganisms is seem as the one of remarked method in livestock stage, although the species of DON-degrading microbial have been isolated limitedly and known slightly where they habit. In this study, we isolated some DON-degrading microbes from Poaceae (*Oryza sativa*, *Eleusine indica*, *Avena sativa*) planted soils. Collected samples were inoculated into mineral salt medium with 100 μg mL^{-1} DON and inoculated media were cultured several times. The concentration of DON in the media were monitored by using HPLC. The media culture decreasing DON was selected and plated onto 3-fold-diluted R2A gellan gum. Finally, we succeeded in isolate three microbes degrading DON from the randomly selected microbial grown on the gellan gum media.

Introduction

The several plant pathogen *Fusarium* species infect a lot of cereal crops and cause Fusarium Head Blight (FHB). FHB is known as the one of most hazardous plant disease around the world and accumulates several mycotoxin deoxynivalenol (DON), and results in huge economic losses. For example, the wheat and barley losses in U.S.A and Canada during the 1990s are estimated in about $3 billion and $220 million respectively (10). DON, a secondary metabolite produced by the pathogens is not only essential to promote the infection spread against host plants, but also poisonous toward eukaryotes (1, 6, 7, 9). The main toxicity of DON is to inhibit the protein synthesis caused by binding the ribosome in the cell both humans and crops (1, 6, 7, 9). Ingesting DON during long period results in serious symptoms included vomiting, diarrhea, leukopenia, hemorrhage (6). These reasons force us to protect our cereal livestock from the pathogen producing DON by using sustainable and effective methods. A biological management using DON-degrading microorganisms and these microbe's enzymes are drawing attention as a way to solve the contamination problem in food supply chain. Our laboratory has 16 strains, of DON degrading bacteria including 4 *Devosia* spp., *Sphingomonas* sp., *Marmoricola* sp. and 10 *Nocardioides* spp. that have been isolated from a variety of environment samples (3, 4, 5, 8) although the information about DON-degrading microbial is limited. Therefore, we tried experiment with isolating new promising DON-degrading microorganisms. As a result, we isolated 3 microorganisms which are able to degrade DON from Poaceae (*Oryza sativa*, *Eleusine indica*, *Avena sativa*) planted soils.

Material and Method

Fig. 1 describes a detail process of enrichment culture. To isolate DON-degrading microbe, a variety of samples were collected from natural environment. The Poaceae plants including these leaves, stems, spikelets and these planted soils, were mainly collected for isolating, because there is possibility to capture DON-degrading microbes for the reason that the many microbes have already been isolated from wheat or rice planting environments. For enrichment of the microbes, approximately 0.03-0.04g of the collected each samples was suspended in 600-800μL mineral salt medium (MM) (4, 8) containing 100 μg mL^{-1} DON as a carbon source. And the cultures were incubated in a shaker with the condition of shaking 150rpm and temperature 28□ for 7 days. After that, 6-8μL of the cultures was added into 600-800μL same medium and the process was repeated 2-4 times.

In order to determine whether the microorganism degraded DON in the cultures or not, the cultures filtered through 0.45-μm membrane were analyzed by HPLC.

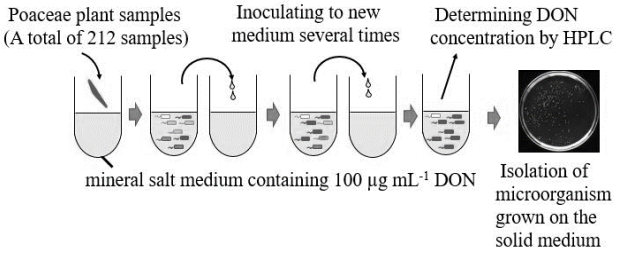

Fig. 1 Enrichment culture of DON-degrading microorganism.

The culture samples with decreasing DON were selected and inoculated on 3-fold-diluted R2A gellan gum (4). And then, the colonies grown on the media were randomly selected and inoculated to MM with 100 μg mL^{-1} DON. The inoculated MM were analyzed by HPLC to determine DON decrease after 7 days incubation as described previously (8).

Result and Discussions

Table 1. Microbes of isolated from Poaceae planted soils.

Strain	Isolation soil	Location	Date
IMS	*Oryza sativa*	Mie	13.06.2017
KOM	*Eleusine indica*	Saitama	07.10.2017
HEM	*Avena sativa*	Hokkaido	07.09.2017

There were 8 cultures that decreased DON in the culture among 212 samples collected from Poaceae planted environments including 77 leaves, 37 stems, 68 spikelets and 30 soils. We isolated 3 DON-degrading microbial strains from the 3 out of the 8 cultures originated from soils planted *Oryza sativa*, *Eleusine indica* and *Avena sativa* named IMS, HEM and KOM, respectively. (Table 1). Figure 2 showed the HPLC elution profiles of DON and its DON metabolites expected. The existence of DON was identified by the peak at 4.0 min in MM containing 100 μg mL^{-1} DON. The profiles of both microorganisms named IMS and KOM showed same peak at 9.1 min, but the profile of HEM showed unique peak at 8.5 min. The HEM also demonstrated peaks at 3.6 min around DON peak. And common peaks were indicated at 1.8 and 2.4 min in all treatments, but the peaks increased in DON-degrading microbial treatments, so this result suggests unidentified compounds and the components of MM be mingled. These above peaks illustrated there are possible to be metabolites of microbes although further analyses are needed.

In fact, 5 samples of enrichment cultures other the 3 DON-degrading microbes have could decompose DON. This result implies more DON-degrading microbes will be unveiled from Poaceae plants. But, these 5 samples were not isolated what microbe had the capacity of degrading DON. As a reason for that there is possible that a few of DON-degrading microbial increased on the media because of multiplying more slowly than other microbes in the MM, or the solid media were bad condition for the microbial colonization. In addition, another mycotoxin degradation by two bacteria was already reported (2), so it is necessary to think about the

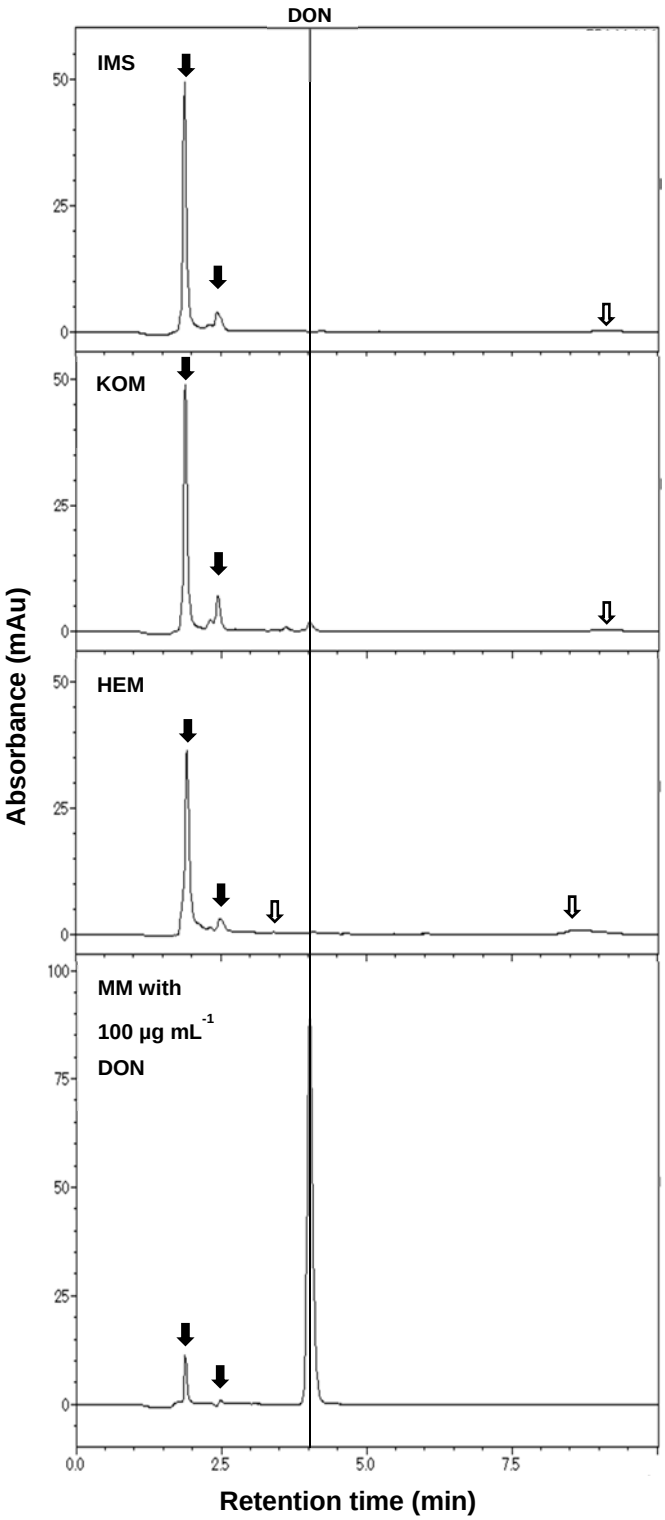

Fig. 2 DON-degrading by microorganisms derived from Poaceae planted soils. This figure shows HPLC profiles of DON and DON metabolites in MM containing 100 μg mL^{-1} DON with IMS (first panel) and with KOM (second panel) and with HEM (third panel) and with neither (forth panel) from top to bottom. The white arrows indicate unidentified compounds except for only MM. The black arrow indicates unidentified compounds or the components of MM. The bar shows the peak of DON.

degradation of consecutive action of several bacteria. Thus, the unknown microbes in the samples should be carefully sought.

Our research group has 16 strains bacteria containing 4 *Devosia* spp. *Sphingomonas* sp. *Marmoricola* sp. 10 *Nocardioides* spp. which are isolated from wheat or rice plants and aquatic environment (3, 4, 5, 8). The *Marmoricola* sp. strain MIM116 derived from wheat heads has been reported already as it is able to remove DON on the wheat kernel (4). The report suggested it be capable to decrease DON in harvested wheat grain affected by FHB with applying other DON-degrading bacteria. Moreover, *Sphingomonas* sp. strain KSM1 derived from lake water has the ability for both DON and another mycotoxin nivalenol (NIV) to assimilate these mycotoxins (5). This study indicated DON-degrading bacteria exist in a wide variety of environments and unknown will be newly discovered in the future.

Finally, we should continue exploring new unidentified DON-degrading microorganisms. And so, we consider hereafter microorganism using method establishing as sustainable and effective way.

Conclusion

We present the isolation of novel 3 DON-degrading microorganisms from Poaceae including *Oryza sativa*, *Eleusine indica*, *Avena sativa*, planted soils. This result implies more DON-degrading microbes will be unveiled from Poaceae plants. Furthermore, it is possible to exist some DON metabolites by the microorganisms. However, the information of DON-degrading microorganisms is limited. Therefore, we ought to run the experiment with searching DON-degrading microorganism from a large variety of environments and analyzing the compounds produced by the microorganisms.

Acknowledgement

This work was supported by JSPS KAKENHI Grant Number 17K15233. We are grateful to Prof. Seiya Tsushima (Tokyo Agricultural University, Japan), Dr. Motoo Koitabashi (NARO, Japan), Dr. Shigenobu Yoshida (NARO, Japan) and Dr. Michihiro Ito (Ryukyu University, Japan) for valuable suggestions and for technical supports.

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Plant growth-promoting traits of rhizospheric *Flavobacterium* and *Chryseobacterium*

Fumiya MIZUTANI[1], Tomoki NISHIOKA[2], Haruhisa SUGA[3], Koji KAGEYAMA[4], Masafumi SHIMIZU[1]

([1]Faculty of Applied Biological Sciences, Gifu University, [2]The United Graduate school of Agricultural Science, Gifu University, [3]Life Science Research Center, Gifu University, [4]River Bacin Research Center, Gifu University)

Introduction

Recent advances in molecular techniques to study microbiota has led to considerable progress in understanding the rhizosphere bacterial communities. The phylum *Bacteroidetes* is one of the abundant bacterial lineages in the rhizospheres of various plants. Among them, the family *Flavobacteriaceae*, especially genera *Flavobacterium* and *Chryseobacteium*, were found to be the most abundant bacteria in the rhizospheres of a wide variety of plants. Accordingly, we hypothesized that *Flavobacterium* and *Chryseobacteium* species might play important roles in the health and development of host plants. However, it was difficult to analyze the function of *Flavobacterium* and *Chryseobacteium* species because there was no suitable medium for the isolation of these bacteria. Recently, we developed the optimized medium for the isolation of *Flavobacterium* and *Chryseobacterium* species from rhizosphere soil (PSR2A-C/T medium, Nishioka *et al*., 2016). In the present study, we isolated rhizospheric *Flavobacterium* and *Chryseobacterium* species with PSR2A-C/T medium, and evaluated their plant growth-promoting traits.

Materials and Methods

Bacteria were isolated from rhizosphere soil of pot-grown Welsh onion (*Allium fistulosum* L.) and onion (*A. cepa* L.) by dilution plating on PSR2A-C/T medium. Yellow or orange colored colonies were purified and then identified by 16S rRNA gene sequence analysis to ascertain their taxonomic positions. Subsequently, bacterial isolates were evaluated in vitro for their plant growth-promoting traits like production of indole acetic acid (IAA), siderophore, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, hydrogen cyanide (HCN), and phosphate solubilization.

Results and Discussion

In this study, six isolates of *Flavobacterium* and 37 isolates of *Chryseobacterium* were successfully obtained from the rhizosphere soils of Welsh onion and onion. By phylogenetic analysis based on 16S rRNA gene sequences, all *Flavobacterium* isolates were grouped into one clade containing *F. anhuience* which was originally isolated from field soil. On the other hands, *Chryseobacterium* isolates were fell into five distinct clades containing various species that were isolated from soils, freshwater lake and waste water. Results of in vitro assays showed that all of the isolates did not have abilities to solubilize insoluble phosphate, produce siderophore, ACC deaminase and HCN but produced IAA in liquid medium, ranged from 0.2 to 5 μg/ml. IAA-producing rhizobateria are known to directly promote plant growth. Therefore, this study may underline the importance of rhizospheric *Flavobacterium* and *Chryseobacterium* species in the development of plants under natural environments.

Establishment of global *Phytophthora* database for quarantine control

Ayaka Hieno[1], Mingzhu Li[2], Kayoko Otsubo[1], Haruhisa Suga[3] and Koji Kageyama[1]

([1]River Basin Research Center, Gifu University; [2]College of Life Science, Shaanxi Normal University; [3]Life Science Research Center, Gifu University)

Abstract

Plant pathogenic *Phytophthora* species cause destructive diseases in agriculture and forest worldwide. For effective quarantine control of *Phytophthora* species, alternative detection methods based on accurate database are highly required. For development of quarantine control, there is a problem that the type isolates of some *Phytophthora* species remain unclear and complicated, for example, two different isolates are enlisted as type of one species. Therefore, we established an accurate list of type strains based on new species description papers, and reliable web databases, such as *Phytophthora* database (http://www.phytophthoradb.org/), and Q-bank (http://www.q-bank.eu/Fungi/) (Fig. 1). According to the list, we collected DNA sequences of 8 genomic regions, rDNA-ITS, rDNA-LSU, β-tubulin, elongation factor 1α, *cox*1, *cox*2, *cox* spacer, and *Ypt*1 from public database. In case that there is no available sequence information in type strain, we collected the ex-type isolates from the researchers and the public bioresource institutes such as CBS-KNAW, WPC and ATCC for analyzing sequences. The coverage of the sequence data reached 75.7~98.5% in 8 genomic regions (Fig. 1).

Based on the collected DNA sequences, we selected *Ypt*1 gene for designing genus specific PCR primer, one primer pair was selected from 22 pair of designed primers by checking of specificity. The selected primer pair was able to detect 104 out of the tested 105 species. Only one species, *Ph. aquimorbida*, was not able to be detected. We confirmed no amplification of 35 species of *Pythium*, 15 species of *Phytopythium*, 1 species of *Aphanomyces* and 6 species of soil-borne pathogens. Only 4 minor species of *Pythium* and 1 species of *Phytopythium* were showed unspecific detection.

Extracted DNA from inoculated and naturally infected plants were subjected to PCR detection using the primer pair. Detection of the target DNA were successful in all DNA extracted from inoculated plants; tomato with *P. nicotianae*, ivy with *P. citrophthora*, hydrangea with *P. hedraiandra*, and natural infected plants; *P. nicotianae* to periwinkle and tobacco (Fig. 2). The PCR-based detection method will contribute to establish an effective quarantine system for *Phytophthora* species.

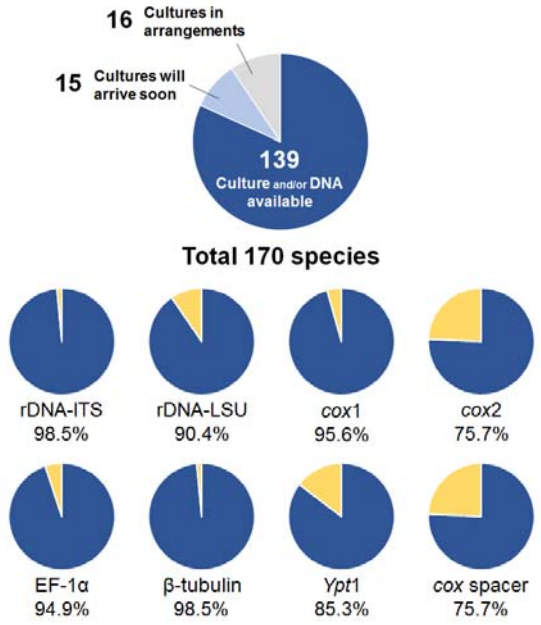

Fig. 1 Coverage of culture, DNA and sequence data of type strain

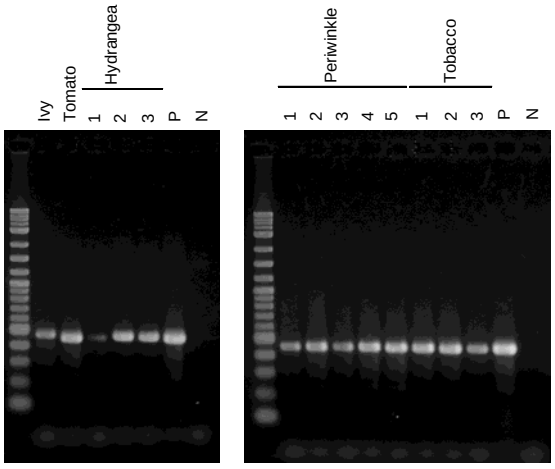

Fig. 2 *Phytophthora* genus specific detection from inoculated (ivy, tomato and hydrangea) and natural infected (periwinkle and tobacco) plants

Acknowledgements

Phytophthora isolates used in this study were kindly provided from Dr. Treena Burgess, CPSM, Australia, Prof. Michael D. Coffey, WPC culture collection, USA, Dr. Everett Hansen, Oregon Univ., USA, Dr. Chuanxue Hong, Hampton Roads AREC, USA, Dr. Thomas Jung, Mendel Univ., Czech Republic, Dr. Paul W. Tooley, USDA ARS, USA, Dr. Bevan S. Weir, ICMP culture collection, New Zealand.

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Morphological and molecular identification of causal agent of cocoa pod rot disease in Indonesia

Masanto[1,2], Ayaka Hieno[3], Arif Wibowo[2], Siti Subandiyah[2], Masafumi Shimizu[4], Haruhisa Suga[5], Koji Kageyama[3]

([1]Faculty of Agriculture, University of Gadjah Mada; [2]UGSAS, Gifu University; [3]River Basin Research Center, Gifu University; [4]Faculty of Applied Biological Science, Gifu University; [5]Life Science Research Center, Gifu University)

Abstract

Cocoa pod rot disease is considered as one of the most important problems in Indonesia as the third cocoa producing country in the world. In this study, 55 isolates infecting cocoa pod rot were collected from 23 cocoa growing provinces in Indonesia. The pathogen was recovered from the adjacent of the infected and healthy part of symptomatic cocoa pod on semi-selective agar medium for oomycetes. Identification was conducted by observing the morphological characteristics (hyphae, sporangium and chlamydospores) under the microscope; while the molecular assay was carried out by multiplex PCR with species-specific primers. The results showed that the collected isolates produced clumsy, coenocytic, and hyaline hyphae, pappillate sporangium (with range of length about 32.82-76.18 µm, range of breadth between 21.61-41.60 µm, l/b ratio around 1.16-2.14, as well as length of pedicel and pappillate ranging between 1.92-9.38 µm and 1.93-8.05 µm, respectively); and chlamydospores (with range of 18.30-48.87 µm in diameter). These features were recognized as the characteristics of *Phytophthora palmivora*. Then, molecular analysis confirmed that all isolates were positively detected with the PCR. The study on genetic diversity of these isolates is still in progress.

Table 1 Morphological features of collected isolates

Features	Remarks
Sporangium Type	Distorted, ellipsoidal, globose, obpyriform, ovoid
Length (L) (µm)	32.82 – 76.18
Breadth (B) (µm)	21.61 – 41.60
L/B ratio	1.16 – 2.14
Length of pedicel (µm)	1.92 – 9.38
Length of pappillate (µm)	
Semi-pappillate	1.93 – 2.43
Full-pappillate	2.77 – 8.05
Chlamydospores	
Shape	Spherical
Production site	Intercalary and terminal
Diameter (µm)	18.30–48.87

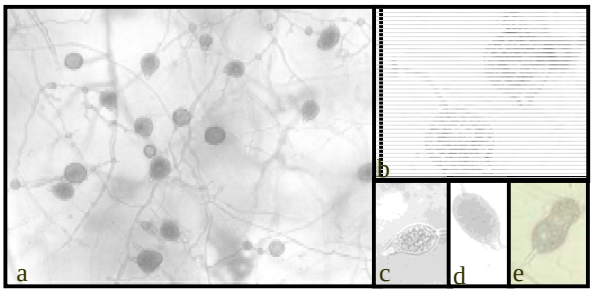

Fig. 1 Characteristics of morphological features of pathogenic agents isolated from symptomatic cocoa pod rot in cacao growing areas of Indonesia (a) appearance of hyphae, sporangia and chlamydospores on V8 agar medium, (b) globose- (top) ovoid- (bottom), (c) obpyriform-, (d) ellipsoidal- (e) distorted-shaped sporangia.

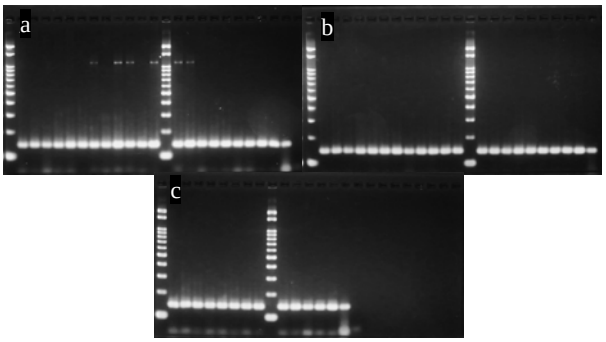

Fig. 2 Performance of DNA band from all isolates after molecular assay using multiplex PCR method with universal (18S-69F and 18S-111R) and specific (GUPal6fw and GUPal8rv) primers (a) isolates no. 1 – 21, (b) isolates no. 22 – 42, (c) isolates no. 43 – 55 with (+) and (-) control were P0633 and SDW, respectively.

Acknowledgements

This research was conducted under collaboration of Faculty of Agriculture, University of Gadjah Mada, Yogyakarta (Indonesia) and River Basin Research Center, Gifu University, Gifu (Japan) within "6-months Sandwich Program in UGSAS-GU".

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Biocontrol of tomato bacterial wilt using *Ralstonia* and *Mitsuaria* species

Malek MARIAN[1], Tomoki NISHIOKA[1], Hiroyuki KOYAMA[1], Haruhisa SUGA[2], Masafumi SHIMIZU[1]

([1]The United Graduate School of Agricultural Science, Gifu University; [2]Life Science Research Center, Gifu University)

Abstract

Bacterial wilt caused by the soil-borne pathogen *Ralstonia solanacearum* is one of the most destructive bacterial diseases of solanaceous species worldwide. The management of bacterial wilt is difficult owing to the viability, adaptability, and genetic diversity of the responsible pathogen. The current countermeasures used against bacterial wilt include biological controls, chemical controls, and cultural practices. However, chemical controls using soil fumigants are potentially harmful to the environment and have not been efficient in eradicating *R. solanacearum*. Moreover, cultural practices through commercially grafted seedlings is expensive and labor intensive and new virulent races of the pathogen might overcome the resistance, resulting in colonization and migration of the pathogen into susceptible scions and causing wilt symptoms. Thus, other alternative methods for controlling bacterial wilt are required. The biological control method of using beneficial microbes has been proposed as an effective, safe, and sustainable approach. Additionally, beneficial bacteria which are capable of colonizing the rhizosphere may provide the first line of defense against the attack of various soil-borne pathogens including *R. solanacearum*. Thus, the aim of this study was to isolate rhizobacteria and evaluate their biocontrol potential against tomato bacterial wilt.

A total of 442 bacteria were isolated from the rhizosphere soil of tomato (*Solanum lycopersicum* cv. Ohgata-Fukuju), Chinese chive (*Allium tuberosum* Rottler ex Spreng., cv. Super green belt), and Welsh onion (*Allium fistulosum* L., cv. Kujo-hoso), which were grown at Gifu University (Yanagido, Gifu city, Gifu prefecture, Japan). Of these isolates, 276 exhibited *in vitro* antibacterial activity against *R. solanacearum*. By analyzing a partial sequence of 16S rRNA gene, these antibacterial isolates were assigned to 24 genera, including *Burkholderia*, *Pseudomonas*, *Acinetobacter*, *Arthrobacter*, *Achromobacter*, *Ralstonia*, and *Mitsuaria*. The suppressive effect of the selected antibacterial isolates against tomato bacterial wilt was further examined using the tomato seedling bioassay. In the first trial, 15 of 276 isolates showed >50% reduction in disease severity. Therefore, the disease suppressive effect of these 15 isolates which belonged to the genera *Ralstonia* and *Mitsuaria* were further evaluated in a second trial of tomato seedling bioassay. All of the tested isolates exhibited strong suppressive effects (ranging from 68.5% to 95.9% reduction in disease severity). Because none of the isolates belonging to genus *Mitsuaria* has been reported as a biocontrol agent against *R. solanacearum*, we evaluated the biocontrol efficacy of all of our *Mitsuaria* isolates, against tomato bacterial wilt in the first pot experiment. As a result, an isolate TWR114 was selected as final candidate for the second pot experiment based on its strong biocontrol effect (60% reduction in disease incidence). Furthermore, an isolate TCR112 was selected as final candidate from eight *Ralstonia* isolates for the second pot experiment because it showed the highest suppressive effect in both trials of seedling bioassay. In the second pot experiment, the final candidate isolates TCR112 and TWR114 significantly reduced the area under disease incidence progress curve by 63% and 48%, respectively. Furthermore, in field experiment, the wilt incidence at 50 days after transplanting was significantly reduced by 57% and 86% in plots drench-treated with TCR112 and TWR114 at weekly intervals, respectively. The isolates effectively reduced the pathogen population in the rhizosphere and above ground stem (crown) of pot grown tomato plants. Monitoring the population dynamics of the biocontrol isolates revealed that both isolates have stable rhizosphere and endophytic colonization capacities. The findings from the current study demonstrate that the isolates TCR112 and TWR114 are promising biocontrol agents against tomato bacterial wilt.

Comprehensive evaluation of the resistance of root-stock-used *Cucumis melo* stock to *Meloidogyne incongnita*

Wanxue BAO

(Life Science Research Center, Gifu University)

SUMMARY

In this study, using *Meloidogyne Incongnita* separated from local pathogenic tomato plants in Guangxi of China as pathogen, I inoculated the pathogen to 9 shares of homozygous root-stock-used muskmelon to evaluate their resistance to root-knot nematode by measuring growth index of experimental rootstocks after inoculation. The result showed the resistance rank of 9 shares of root-stock-used muskmelons, which from top to bottom indicated that T05, TKH1, T06, T03, T07T, T02, T04, T03T, and T07.

Introduction

Root-knot nematode are common diseases which influence production and quality of melon in Guangxi, China. Cultivating by grafting seedling is an effective way to solve this problem, but selecting root-stocks with resistance of root-knot nematode is faced with huge difficulty now. Nowadays, although Yunnan *Cucurbita ficifolia Bouché* is widely used in melon grafting cultivation due to its high grafting efficiency, but it has been proved that its resistance to root-knot nematode is poor. Therefore, selecting rootstocks with high resistance to root-knot nematode is an effective way to increase melon production and economic benefits of farmers.

In this study, using *Meloidogyne incongnita* separated from local pathogenic tomato plants in Guangxi of China as pathogen, I inoculated the pathogen to 9 shares of homozygous root-stock-used muskmelon to evaluate their resistance to root-knot nematode by measuring growth index of experimental rootstocks after inoculation. The evaluated method was new discovered and the result may be more comprehensive and reliable. The result showed the resistance rank of 9 shares of root-stock-used muskmelons. The conclusion can not only provide reference to root-stock breeding, but also guide to complete criterion to evaluate disease resistance of melon.

Material and Method

Expanding propagation of *Meloidogyne incongnita*: Growing seedlings of susceptible tomato Y4 before 20 days of inoculation, then transplant them to nutrition pot 15 days later. I chose seedling substrate mixed with sand as volume ratio 1:1. I poured *Meloidogyne incongnita* egg liquid near tomato root. 6ml per seedling. The concentration of liquid was 1000 egg per ml. The temperature in daylight should not be over 28□, and at night it should not be under 15□. The soil temperature should not be under 5□. When tomato seedlings were infected for 40 days, I dig the roots and collected root nematode.

Preparation of *Meloidogyne incongnita* egg liquid: Cleaning and cutting pathogentic into pieces, and putting them into distilled water mixed with NaHClO as volume ratio 1:1, stirring fast for 1min. Putting the mixture onto 3 sifters which from top to bottom were 50 holes, 350 holes and 500 holes. Watering the back of 500 holes sifter and collecting liquid in the beaker. Putting distilled water in to the beaker to adjust the concentration to 1000 egg per ml.

Inoculation: The method was same to Expanding propagation. Putting 6 ml distilled water to seedlings' root as the control group(CK).

By counting root numbers method, disease level of root-stock nematode was set to 0 to 5(table 1). Disease index(DI)=∑(disease level × plant numbers of this level)/(plant numbers × the highest disease level)×100%. Resistance evaluation criterion was set to 6 levels by calculating DI.

Besides calculating DI, measuring growth characters such as plant height, stem diameter, fresh weight of over ground part, fresh weight of underground part, dry weight of over ground part, dry weight of underground part, and root surface area. Then I calculated relative growth of infected rootstocks. Relative growth=(growth character number of tested group-growth character number of CK)/(growth character number of CK). And I measured and calculated resistance characters such as nodules number index, egg index, propagation coefficient, and disease index.

Nodules number index=nodule number of one seedling/root fresh weight

Egg index=egg number of one seedling/root fresh weight

Propagation coefficient=egg number of one seedling/ infected egg number

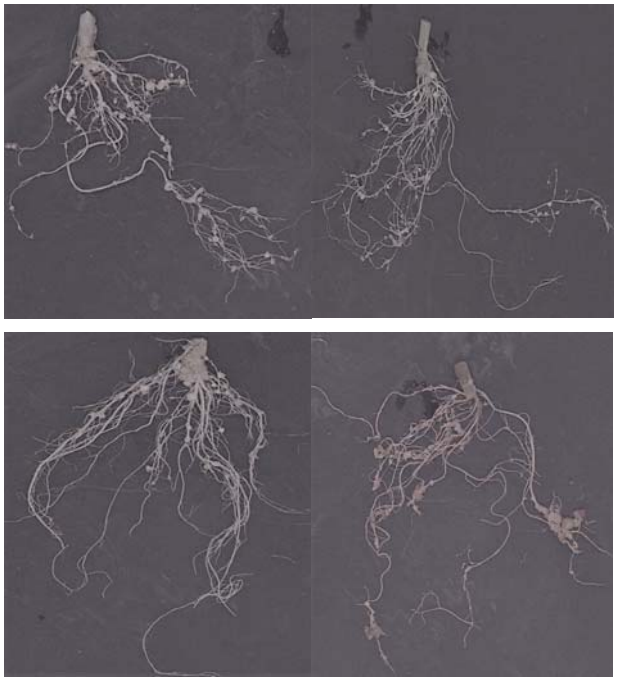
Table 1 Classification criteria of root-knot nematode

Disease level	Disease symptom
0	No root knot
1	1-2 root knots
2	3-10 root knots
3	11-30 root knots
4	31-100 root-knots
5	More than 100 root knots

Table 2 Evaluation criteria of resistance of root-knot

Resistance level	Evaluation criteria
（I） Immunity	DI=0
（HR）High resistance	0.0≤DI≤10.0
（R）Resistance	10.1≤DI≤30.0
（MR）Medium resistance	30.1≤DI≤50.0
（S）Susceptible	50.1≤DI≤75.0
（HS）High susceptible	DI≥75.1

Result and Discussion



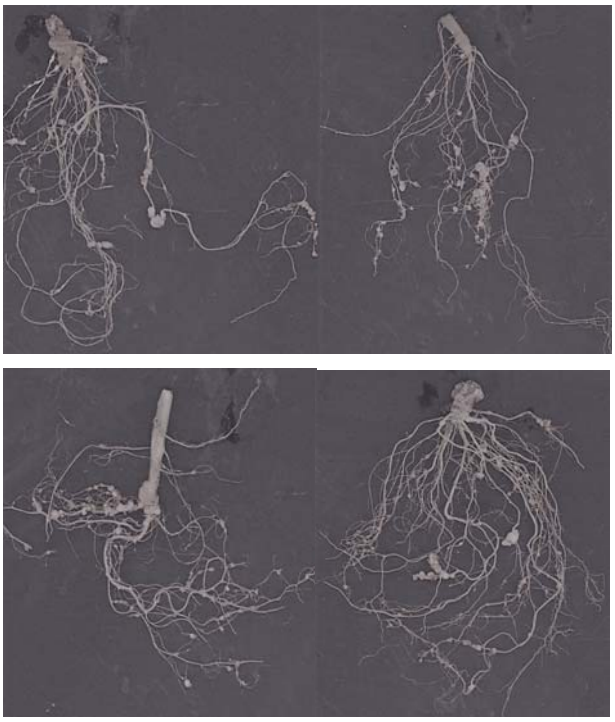


Fig 1 Root scan photos of infected muskmelon under Founder Z2400 scanner(From top to bottom, and each line's right to left are T02, T03T, T04, T05, T06, T07, T07-T, and TKH1).

These photos elucidated that when were inoculated by *Meloidogyne incongnita*, root-knot formed on experimented muskmelon. The size and numbers of root-knots varied among different varieties. The results verified that experimented pathogen had intensive pathogenicity muskmelons could be infected by the pathogen. The quantity of root-knots on each root was huge, and some small root-knot taking group amounts into consideration, it results to difficulties for counting root-knot numbers, and the deviation may be generated during the counting process. Hence, it is necessary to choose an applicable way to evaluate resistance on root-knot nematode of root-stock-used plants.

Table 3 Relative growth rate of root-stock-used muskmelon

NO.	H	D	FW of OP	FW of UP	DW of OP	DW of UP	Root SA
T02	-37.15	-25.96	-27.95	-25.88	-34.76	-20.00	-40.90
T03	-22.89	-28.42	-26.05	-46.80	-16.48	-40.80	-47.74
T04	-19.62	-30.08	-39.45	-45.05	-37.65	-45.56	-63.92
T05	-13.92	-25.64	-13.55	-37.94	-19.26	-41.98	-60.84
T06	-14.02	-29.89	-26.50	-49.10	-23.20	-46.28	-62.17
T07	-14.61	-31.22	-16.57	-43.34	-34.21	-41.67	-59.00
T03T	-23.30	-35.67	-27.68	-40.87	-28.39	-39.87	-63.67
T07T	-18.55	-31.82	-19.96	-43.28	-25.31	-44.15	-63.68
TKH1	-24.05	-20.07	-27.15	-50.68	-23.29	-49.07	-69.12
CV(%)	34.93	15.52	30.36	17.36	27.18	41.04	15.14

H means plant height, D means stem diameter, FW means fresh weight, DW means dry weight, OP means over-ground part of plant, UP means under-ground part of plant. The following tables was as the same. SA means surface area.

Relative growth rate revealed that after been inoculated by *Meloidogyne incongnita*, the strength of activity that kept its own growth potential. It changed more slightly, the negative impact which root-stock-used muskmelon got was slighter, and the resistance or tolerance may be stronger. It(Table 3) showed that the growth indexes of inoculated group declined, the relative growth rate of inoculated groups to control groups was minus number, indicating that *Meloidogyne incongnita* restrained experimented root-stock-used muskmelons, and different rootstocks showed diverse descent degrees. T05 and T03 showed the least descent degrees of 9 root-stock-used muskmelons on relative growth rate. These two rootstocks had resistance or tolerance to the pathogen. Relative growth rate of T04 and TKH1 was lower than others. It may be the sigh that *Meloidogyne incongnita* had strong suppression of these two root-stocks, the resistance or tolerance to the pathogen was the weakest among the experimented root-stock-used muskmelons. Counting CV(coefficient of variation) of these relative growth rates, the result showed that CV of dry weight of underground part and plant height was higher, respectively were 41.04% and 34.93%. The result suggested that after inoculation, the growth index that plant height and dry weight of underground were affected severely by the pathogen, and could taken as measurable indexes of evaluating resistance on *Meloidogyne incongnita* of root-stock-used muskmelons.

Table 4 Change of resistance index of root-stock-used muskmelon after been infected *Meloidogyne incongnita*

NO.	Nodules number (/g·FW)	Egg number (/g·FW)	Propagation coefficient	Disease index (%)
T02	56.62	41419.24	87.60	70.0
T03	9.79	24065.91	40.40	48.0
T04	18.34	17455.10	32.55	54.0
T05	27.73	23722.01	41.63	58.0
T06	33.27	29576.29	48.79	60.0
T07	54.22	49166.83	80.76	76.0
T03T	37.97	37169.31	60.57	64.0
T07T	60.65	56029.86	104.02	72.0
TKH1	73.56	65600.08	123.21	86.0
CV(%)	51.28	42.51	46.01	18.11

After inoculation, the resistance index have changed to different degree. Among 9 experimented materials, the nodules number of per gram fresh weight and disease index of T03 was the lowest, the egg number of per gram fresh weight of T05 was the lowest, and the propagation coefficient of T04 was the lowest. However, these four resistance indexes of TKH1 was the highest. The CV of nodules number and egg number of per gram fresh weight and propagation coefficient were higher, counting as 51.28%, 42.51% and 46.01%. By contrast, the CV of disease index was the lowest, indicated 18.11%. We could get conclusion that if the resistance was evaluated only by disease index, the consequence might be there was no difference of resistance among these rootstocks. But by calculating resistance indexes, the otherness appeared although the disease index seemed similar. Therefore, it may be circumscribed if only taking disease index into consideration when we evaluate resistance of *Meloidogyne incongnita*.

Table 5 Subordinate function value of 5 indexes of root-knot nematode resistance of root-stock-used muskmelon

NO.	H	DW of root	NN	EN	PC	Mean	R
T02	0.5695	0.4332	0.6823	0.5682	0.7032	0.5682	6
T03	0.5056	0.5874	0.6506	0.6314	0.6788	0.6117	4
T04	0.4124	0.6002	0.6584	0.5509	0.6674	0.5399	7
T05	0.5956	0.5201	0.8131	0.7341	0.7319	0.6652	1
T06	0.4401	0.5279	0.7474	0.7253	0.7630	0.6141	3
T07	0.3261	0.6501	0.5100	0.4708	0.5472	0.4367	9
T03T	0.4436	0.5000	0.5916	0.5609	0.5817	0.5390	8
T07T	0.3699	0.5000	0.6377	0.6852	0.5950	0.5720	5
TKH1	0.5547	0.6666	0.6838	0.6617	0.5826	0.6164	2

Doing subordinate function analysis of 5 indexes which had huge CV of inoculated root-stock-used muskmelons, as plant height(H), dry weight of root(DW of root), nodules number(NN), eggs number(EN) and propagation coefficient(PC). By calculating the mean value of these 5 indexes of each rootstock, we got the rank. T05 had the highest mean value, as 0.6652, among all the experimented rootstocks. It proved that T05 had strong resistance or tolerance to *Meloidogyne incongnita*. Where as T07 had the lowest mean value, as 0.4367, suggesting that T07 had the weakest resistance of *Meloidogyne incongnita*. The resistance rank from top to bottom was T05, TKH1, T06, T03, T07T, T02, T04, T03T and T07.

Conclusion

In the experiment, it is discovered that after nine specimens of rootstock-used melons are inoculated with southern root-knot nematodes, significant changes happen to their growth indexes. Compared with the control group injected with clear water, rootstock height, stem thickness, aboveground and underground dry and fresh weights decline to different extent, which demonstrates that the growth of rootstock specimens are impacted by the southern root-knot nematodes, and some differences in resistance indexes of the strains. It is empirically proven that there would be great errors in merely determining disease grades of root-knot nematode based on number of root-knots by human visual observations and counting. After calculating membership functions of indexes of tested rootstocks with higher coefficient of variation after grafting, including stem length, underground dry weight, number of root-knots per gram of fresh weight, number of eggs per gram of fresh weight and reproduction coefficient, the resistance of the rootstocks to southern root-knot nematode is comprehensively evaluated, and T05 rootstocks are found to be the most resistant to the nematode, followed by TKH1, T06, T03, T07T, T02, T04, T03T and T07 respectively.

Acknowledgement

This study was guided by professor Wenjin Yu in Guangxi University. It is honored to work with professor Yu and our group members.

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Population genetics analysis of *Phytopyhtium helicoides* in Japan

Auliana AFANDI[1], Emi MURAYAMA[2], Ayaka HIENO[2], Haruhisa SUGA[3], Koji KAGEYAMA[2]

([1]United Graduate School of Agricultural Science, Gifu University; [2]River Basin Research Center, Gifu University; [3]Life Science Research Center, Gifu University)

Abstract

Phytopythium is a recently established genus containing organisms formerly classified as *Pythium* clade K species. The species belong to this clade were morphologically look like *Pythium* but genetically closer to *Phytophthora* (deCock et al., 2015). *Phytopythium helicoides* was first isolated on 1930 by Drechsler from Dahlia in USA. This high temperature pathogen were not popular until recently. In Japan, it was first isolated on the miniature rose in Gifu in 1996 (Kageyama et al., 2002). The pathogen is able to produce large number of zoospores as secondary inoculum source. Thus, it can rapidly spread on the hydroponic farming system.

Understanding the genetic variability on a pathogen could help in developing effective disease management strategy. The observation on the microevolution and population structure are necessary to predict the adaptation and migration ability of a pathogen. The pathogen that has high genetic diversity and high mobility will perform better adaptation on the environmental change. Study on the population genetics can be done by several methods such as RAPD, RFLP, AFLP, SNP, and microsatellites. The microsatellites are the most convenience due to their codominant, multiallelic, highly polymorphism and require only small amount of DNA for PCR analysis (Yin-Ling et al., 2009). In this study, microsatellite markers containing 4 loci (EM-AGC1, EM-AGGCA, EM-GGA1 and EM-TCA1) with 37 alleles were used for the population genetics analysis.

One hundred fifty five isolates from 15 prefectures in Japan and four overseas isolates were applied to GenAlEx software in Microsoft Excel. The overseas isolates were originated from South Africa, USA, Netherland and Spain. The Hardy- Weinberg Equilibrium were significantly differed from the equilibrium for all loci, indicating a low number of population or occurrence of genetic drift. The analysis of molecular variance showed the significant genetic differentiation between populations (19%) (Table 1). The phylogenetic analysis using neighbor joining algorithm revealed four main clusters. Gifu and Aichi population were clustered by their geographical origin. While Shizuoka populations were divided into two major clusters. One cluster were shared with the isolates from Iriomote-Jima which have similar asexual reproductive behaviour. Another were shared with several isolates from different prefecture but has same host plants (Fig.1). The results suggest that geographical origin will play important role on the population structure of *P. helicoides*.

Table 1. The analysis of molecular variance (AMOVA) of *P. helicoides* population

Source	df*	Est.Var**	%	Fst***
Among Pops	14	0.453	19	0.187
Among Indv	139	0.744	31	
Within indv	154	1.221	50	
Total	307	2.418	100	

*degree of freedom, **estimated variance, ***fixation index

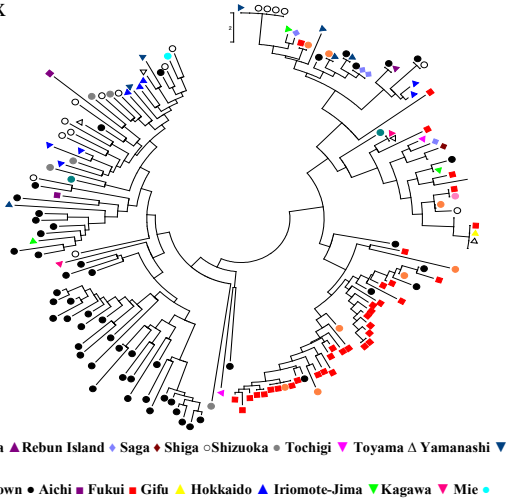

Fig. 1 Phylogenetic Analysis on *P. helicoides* population using Neighbor Joining algorithm

Acknowledgement

We thank Mr. Mikihiko Suzuki, Dr. Hideki Watanabe, and Dr. Noriyuki Miyake for providing the isolates used in this study.

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Study of a transcriptional regulator of plant pathogenic genes in a soft rot disease causing bacterium, *Dickeya dadantii*

Dina Istiqomah[1] and Naoto Ogawa[1, 2]

([1]The United Graduate School of Agricultural Science, Gifu University; [2]Faculty of Agriculture, Shizuoka University)

Abstract

The soft rot pathogenic bacteria, *Dickeya dadantii*, causes soft-rot-disease in plants of various host range including economically important crops. The virulence of *D. dadantii* is known with its ability to synthesize and secrete pectinase (Pel) enzymes which act as plant cell wall degrading enzymes. PelA, PelD and PelE are among the most important Pels for virulence. Among the *pel* genes, *pelD* expression is most strongly affected by pectin derivatives, which makes it a key element in the induction of *D. dadantii* virulence.

The pathogenicity level of pathogen may be affected by involvement of several regulatory protein. Previous study revealed that a gene of LacI transcriptional repressor family, *ltR*, was involved in the expression of virulent factors and pathogenicity of *D. dadantii* in negative manner. To study the function of *ltR*, we conducted experiments including cloning of the gene, expression of the protein and then His-tag purification of the protein. By electrophoretic mobility shift assay (EMSA) using the purified protein, we analyzed the binding of LtR protein with promoter regions of related virulence genes of *D. dadantii.*

EMSA showed that LtR protein could bind to each of the promoter regions of the *pelD*, *pelA*, and *pelE*. This result indicated that LtR protein has a potential to be a transcriptional regulator for the three genes. The affinity of the binding by LtR seemed to be different among the three promoters, suggesting different manner in the regulation by LtR for the promoters. In vivo analysis of the strain using Reverse-Transcriptase PCR is being conducted to analyze the expression of the virulence genes.

Keywords: *Dickeya dadantii*, soft rot disease, transcriptional regulator, virulence gene.

Identification of freshness marker of stored soybean sprouts

Syukri, D.[1], Thammawong, M.[1], Kuroki, S.[2], Tsuta, M.[3], Yoshida, M.[4], Nakano, K.[1]

([1]The United Graduate School of Agricultural Science, Gifu University; [2] Faculty of Agriculture, Kobe University; [3]Food Research Institute, National Agriculture and Food Research Organization, [4]Kanagawa Agricultural Technology Center)

Abstract

The objective of this study was to discover a freshness marker of fruits and vegetables from small reactive carbonyl compound which is well known as a second product of lipid peroxidation. Self-cultivated soybean sprout was used as a sample material, and was stored various temperatures. The sprout was sampled periodically, and was divided into cotyledon and hypocotyl. At the same time, the respiratory CO_2 production was also measured by a flow-through method using a gas chromatography. The carbonyl compounds were extracted from each part by homogenizing with polar-semi polar mixture solvent. Then, they were derivatized with specific carbonyl compounds labeling agent. High performance liquid chromatography/ electrospray ionization tandem mass spectrometry with multiplexed multiple reactions monitoring was used for the comprehensive detection of carbonyl compounds. A large variety of carbonyl compounds were observed in MRM chromatogram from both cotyledon and hypocotyl part of stored soybean sprouts. Several *m/z* values were selected as candidates of the freshness maker of soybean sprout by means of principle component analysis-discriminant analysis (PCA-DA) by relating with the accumulation CO_2 production as a reference freshness indicator. The structure elucidation of these freshness marker candidates will be conducted in future study.

Keywords: freshness evaluation, lipid peroxidation, senescence, soybean sprout

Studies on acetaldehyde tolerance system in the budding yeast using *myo*-inositol

Annisyia Zarina Putri[1], Mizuho Inagaki[1,2], Masaya Shimada[1,2], Takashi Hayakawa[1,2], Tomoyuki Nakagawa[1,2]

([1] The United Graduate School of Agricultural Science, Gifu University; [2] Faculty of Applied Biological Sciences, Gifu University)

Abstract

Acetaldehyde is a toxic compound that up to now is still problem during fermentation in yeast. In recent years many study have been carried out for improving the fermentation capability especially using yeast *Saccharomyces cerevisiae*. According to Furukawa *et al.* (2004), in *S. cerevisiae*, the intracellular *myo*-inositol content was found to affect strain tolerance against ethanol stress. In *S. cerevisiae*, *myo*-inositol is produced from glucose 6-phosphate via the reactions catalyzed by the *INO1*-encoded inositol-3-phosphate synthase and *INM1*-encoded inositol monophosphatase, and then participated in the biosynthesis of phosphatidylinositol. In the present study, decreasing inositol by simultaneous deletion of *INM1* and *INM2* enhanced ethanol tolerance. It was shown that, under high ethanol condition (12-20%) with limited inositol in the medium, yeast cells leak intracellular metabolites such as nucleotides, phosphate and potassium. Under high ethanol concentration, addition of inositol to medium improves ethanol tolerance (Chi *et al.* 1999). From this background, we want to try to do the experiment for acetaldehyde tolerance in yeast *Saccharomyces cerevisiae* using *myo*-inostiol to improve fermentation capability.

Keywords: Acetaldehyde tolerance, *Saccharomyces cerevisiae*, *myo*-inositol, *INO1*

Identification of bioaerosols from environmental samples in the AIST, Tsukuba, Japan

Panyapon Pumkaeo, Wenhao Lu, Youki Endou, Tomohiro Mizuno,
Junko Takahashi, Hitoshi Iwahashi
(The United Graduate School of Agricultural Science, Gifu University;
Faculty of Applied Biological Sciences, Gifu University)

Abstract

The Bioaerosols are the atmosphere particles, mists or dust of μm range, associated with metabolically active or inactive viable particles. They contain living organism's included microorganisms such as viruses, bacteria, and fungi also plant material as well as pollen. Next Generation Sequencing (NGS) is a novel method of DNA sequencing that quickly and efficiently read the underlying sequence of an organism by means of massively parallel sequencing. The aim of this study is identifying organisms which contained in environmental samples by using NGS

This study monitored the environmental sample (Bioaerosols) from November 2013 to January 2015 for 50 days using air samples were collected at AIST, Tsukuba, Japan. Samples were bio-analyzed using a next-generation sequencing method. In this study, we used two NGS platform, GS FLX+ (Roche 454 sequencing) and Illumina Misep. The sample was detected plants, eukaryotes, and bacteria. The sample was divided into two subgroup subgroups according to the size of its bioaerosols, large subgroup contains bioaerosols whose diameter is bigger than 3.3μm, and small subgroup contains those smaller than 3.3μm.

Most of the plant cells were included in large subgroups; while back filter also contained much plant-derived debris. Samples whose size ranges from 0.43μm to 3.3μm showed least plant DNA. Regarding the relevance of plant DNA quantities and season, samples collected in spring showed the highest expression, next was autumn, followed by summer, and winter samples appeared the least DNA expression. Bacteria did not show a correlation between DNA expressions with the collected data. Components of large subgroups showed no obvious difference compared with small subgroups. *Medicago papillosa* is the dominant species in AIST, Tsukuba. Rare, endangered species such as wild rice were traced. The *rbcL* universal primer showed high effective for NGS of the environmental sample than other primers that used in this study.

Keywords: Bioareosols, Next-Generation Sequencing, Bacteria, Plant

The effect of persimmon (*Diospyros kaki*) on the prevention of sarcopenia

Nayla Majeda Alfarafisa, Tomio Yabe

(The United Graduate School of Agricultural Science, Gifu University)

Abstract

Sarcopenia is an age-related syndrome that characterized by progressive loss of mass, strength, and function of skeletal muscle. The loss of skeletal muscle fibers resulted from imbalance between the protein synthesis and degradation, called atrophy, is a common feature in sarcopenia. Until now, the underlying mechanisms and pathophysiology of sarcopenia remains unclear. There is now increasing evidence which suggests that muscle wasting has a strong relationships with increased levels of circulating inflammatory components in elder people. Some proinflammatory cytokines could enhance the proteolysis and decreasing protein synthesis. Persimmon (*Diospyros kaki*), kaki fruit, is a popular fruit and well distributed, especially among Asian countries. Persimmon is enriched with several bioactive compounds that have beneficial effects on human health. For example, flavonoid is one of the most dominant phytochemical that can be found on persimmon peel and fruit. Recent study showed that flavonoid had a positive effect on the prevention of skeletal muscle atrophy *in vitro*, hypothetically via TNF-α counteracted mechanism.In this study, the effect of extracts from persimmon on the progression of skeletal muscle atrophy *in vitro*will be investigated. TNF-α level, the expression of anabolic targets (Akt, mTOR, p70S6k, and 4E-BP1), and catabolic targets (FoxO1, FoxO3a, MAFbx, MuRF1) would be checked after extracts treatment to evaluate the effect of persimmon fruit to the muscle protein turnover and atrophy development. In addition, the effects of extracts on the production of pro-inflammatory will also be investigated, persimmon bioactive compoundswill be expected to prevent the progression of atrophy not only by balanced protein turnover but also by suppress the production of inflammatory cytokines. After treatment, the levels of inflammatory cytokines (NF- κβ, I-κβ, p50, p65, and IL-6) to evaluate the anti-inflammatory effects of persimmon. A better understanding of cellular mechanism of sarcopenia is required to prevent the progression of sarcopenia based on research evidence.

Keywords: Sarcopenia, Atrophy, Inflammatory Response, Flavonoid, *Diospyros kaki*

Transcriptional biomarkers for managing pulse crop production in acid soil region

Raj kishan Agrahari[1], Hiroyuki Koyama[2]

([1]The United Graduate School of Agricultural Science, Gifu University;

[2] Faculty of Applied Biological Sciences, Gifu University)

Abstract

Crop Production decreased by excessive soil minerals and salts, such as sodium chloride, heavy metals, and Aluminum (Al).These stress induces specific damage and stress-responses in the shoots, which can be evaluated by Molecular level. Al specific responses in shoots was analyzed by Sawaki et al.(2016) and they found shoot specific Al responsive gene both in *Arabidopsis thaliana* and *Nicotiana tabacum* out of these it is found that *ALS3* and *PGIP1* are shoot specific Al responsive gene. *ALS3* is ABC transporter that reported in Arabidopsis and it is regulated by *STOP1* (sensitive to proton rhizotoxicity1) proteins for Al specific response but its molecular mechanism and Signal Transduction is still not clear, similarly gene *PGIP1* (polygalacturonase inhibiting protein) is a cell wall pectin degrading enzymes such as those produced by fungal pathogens (Nguema-Ona et al. 2013) but it is reported under low pH (acid soil) expressed in shoot (Sawaki et al. 2016). The existence of microarray data for Al stresses in Arabidopsis provides the opportunity to identify and characterize key conserved genes downstream of Al stress responses. Some of these genes might be eventually used as biomarkers for the evaluation of stress responses in crop. They could also expand our knowledge about the genetic mechanisms underlying the responses to Al stresses in non-model crops. So from this scenario my research area focus on understanding the molecular mechanism of the genes in shoot under Al stress and application of the gene signaling in crops as a biomarker.

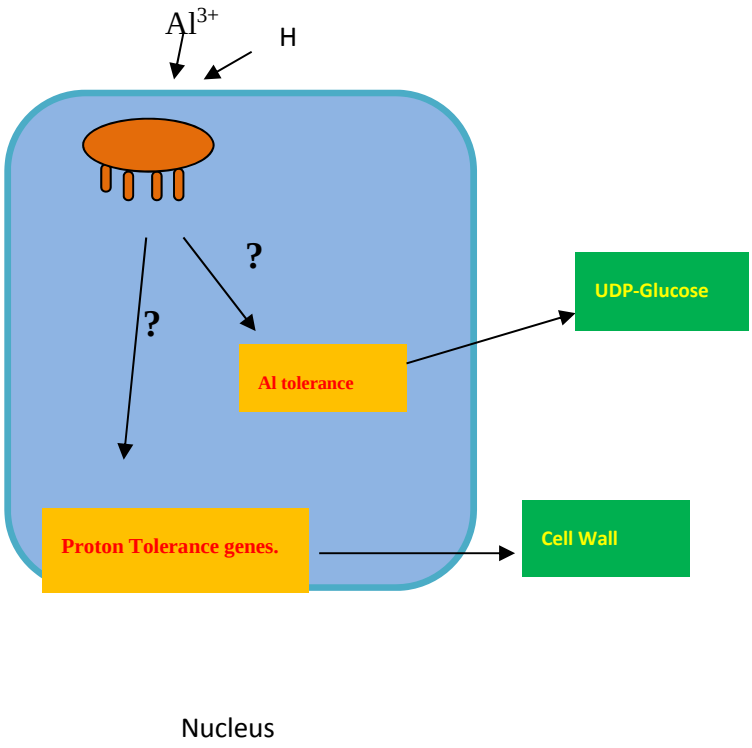

Fig.1: Model representing Research of Interest

The accumulation of carotenoid in mango during fruit maturation

W. Yungyuen[1,2], T.T. Vo[3], G. Ma[2], L.C. Zhang[2], P. Jitareerat[3], A. Uthairatanakij[3], M. Kato[2]

([1] The United Graduate School of Agricultural Science, Gifu University; [2] Graduate School of Agriculture, Shizuoka University; [3] King Mongkut's University of Technology Thonburi)

Abstract

Among tropical fruits, mango is one of the most popular fruits worldwide because of its tasty and attractive color. Carotenoids are considered as an important bioactive compound in mango fruits responsible for bright yellow pigment, which determine quality and appearance of fruits. In addition, carotenoids accumulated in mango fruits can act as bioactive antioxidants, promoting benefits to human health. To date, the accumulation of carotenoids in mango fruits during the maturation process is still unclear. Therefore, the objective of this research was to investigate the changes in carotenoid content and composition during fruit maturation in the three mango varieties from Thailand. The highest carotenoids level was observed in 'Kaituk', follow by, 'Namdokmai No.4', and 'Namdokmai Srithong' in both peel and pulp. The accumulations of six carotenoids were found, including all-*trans*-violaxanthin, 9-*cis*-violaxanthin, lutein, β-cryptoxanthin, α-carotene, and β-carotene. Those carotenoids were highly accumulated in the peel, but their contents were lower in the pulp. Among them, β–carotene was observed as a major carotenoid accumulated in the peel and pulp of three mango varieties. During mango fruits maturation, the carotenoids were accumulated at low level in the green stage fruits. Then, the carotenoid content was significantly increased in the transition stage from green to yellow at 81 days after anthesis, reaching a maximum in the full ripening stage at 98 days after anthesis in the three mango varieties. These results showed different levels of carotenoid accumulated in the three mango varieties, which is useful materials for elucidating the regulation of carotenoid metabolism during mango fruit maturation in the future research.

Keywords: carotenoid metabolism, β-carotene, mango peel, mango pulp

Augmented nuclease resistance and gene silencing with 3'-end modified small interfering RNAs and dendrimer based drug delivery

Akash Chandela[1], Yoshihito Ueno[1,2]

([1]United Graduate School of Agricultural Science, Gifu University; [2]Course of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University)

Abstract

RNA interference (RNAi) is a naturally occurring mechanism where non-coding double stranded RNA regulates the sequence specific posttranscriptional gene silencing. With thousands of druggable target genes, this tool provides a revolution in gene regulation and treating numerous epigenetic disorders. Two major obstacles in this phenomenon are instability of natural RNA (nuclease degradation) and inefficient drug delivery. Therefore, this study is aimed at synthesizing stable siRNA with modified 3'-end and designing branched siRNA for dendrimer based delivery.

siRNA is a key moiety in this pathway that contains two signature characteristics: a two nucleotide 3'-end overhang and a phosphorylated 5'-end. The functional strand, referred as guide strand, interacts directly with the multi domain AGO proteins to form RNA induced silencing complex (RISC). The 3'-end is recognized by PAZ domain housing a hydrophobic pocket, whereas the 5'-phosphorylated end is recognized by MID domain. This RISC recognizes target messenger RNAs with sequences complementary to the guide strand, cleaves them and regulate gene expression. Therefore, we have modified the base at 3'-end with trifluoroethyl and trichloroethyl analogs. These analogs were synthesized by β-selective introduction at 1-position of D-ribofuranose via an acetal linkage. They have been found to exhibit better nuclease resistance and comparable gene silencing to the native. Binding assay have shown lower dissociation energy of these analogs complex with PAZ domain. Hence, we hypothesize upon these findings that it's the optimal interaction which drives this mechanism forward.

Lately, cholesterol conjugated passenger strand with analog modified antisense are also being analyzed as previous literatures have reviewed this combination as more productive to increase interaction with PAZ domain. Furthermore, we have synthesized branched siRNA using a trebler synthon as solid support. This trebler synthon have been synthesized using pentaerythritol.

Thus, our current study postulates that modification with hydrophobic analogs at 3'-end of guide strand yields better nuclease resistance and gene silencing. And, we look forward to analyze the dendrimer based approach for drug delivery with these tripod siRNA.

Protein-based functional analysis of renin and (pro)renin receptor genes in hypertensive and diabetic Bangladeshi population: Pursuing the environment-induced molecular traits

Jobaida Akther[1], A. H. M. Nurun Nabi[2], Tsutomu Nakagawa[3], Fumiaki Suzuki[3], Akio Ebihara[3]

([1]United Graduate School of Agricultural Science, Gifu University; [2]Department of Biochemistry and Molecular Biology, University of Dhaka; [3]Faculty of Applied Biological Sciences, Gifu University)

Abstract

Both renin and (pro)renin receptor [(P)RR] proteins as well as their genetic variations have been found to be associated with the risk of hypertension and diabetes; and no extensive study has been performed so far in Bangladeshi population regarding renin and (pro)renin receptor. In addition, environmental factors, which are sometimes imposed to Bangladeshi workers, can make a large influence on the plasma levels of renin and (P)RR. Their effects can be observed as environmental-induced molecular traits. Thus, this study aims to develop the following methods-

1. Better ELISA for measuring plasma renin concentration (PRC) and soluble (P)RR [s(P)RR]
2. Storage of proteins in solid state using pullulan (a polysaccharide polymer) and their activity check

1. Angiontensin-I ELISA (AI-ELISA) for measuring PRC

Recombinant ovine angiotensinogen (oANG) was prepared using *E.coli* cells according to the method described by Yamashita et al. (2016). The rate of angiotensin-I (AI) generation was determined by AI-ELISA (Suzuki F et al. 1990). Prorenin produced in mammalian cell was treated with trypsin to get renin, and its concentration was determined from trypsin treatment time vs renin activity plot. Again, unknown concentration of renin was determined from renin activity vs renin concentration plot. This assay can be utilized to measure PRC as a biomarker.

2. Formation of oANG tablets using pullulan

Pullulan is a polysaccharide produced from starch by the fungus *Aureobasidium pullulans* and has been used for encapsulation of biomolecules and solidifying enzymes. Pullulan solution was mixed with oANG protein to make tablets. The effect of pullulan on oANG has been examined by measuring renin activity varying storage periods.

Keywords: oANG, AI-ELISA, PRC, Pullulan

Proposals for countermeasures to reduce risk of hydraulic fracturing adjacent to culvert – A case study

Duy Quan Tran[1], Shinichi Nishimura[2], Masateru Senge[2], Tatsuro Nishiyama[2], Fumitoshi Imaizumi[3]

([1] The United Graduate School of Agricultural Science, Gifu University; [2] Faculty of Applied Biological Sciences, Gifu University; [3] Faculty of Agricultural, Shizuoka University)

Abstract

Hydraulic fracturing is the propagation and development of cracks under the effect of water pressure when the stress in the dam is lower than the water pressure. Hydraulic fracturing has been also considered as one of possible causes leading to concentrated leakage and failure of many fill dams especially at the first reservoir filling. Sherard (1972) pointed out that hydraulic fracturing was the most likely cause in seven of eleven dam failures in Oklahoma and all three of the Mississippi dam failures that location of the failures occurred directly adjacent to culverts. Ngambi et al. (1997), according to results of in-situ observations combined with numerical analyses concluded that hydraulic fracturing is the most likely cause of concentrated leakage and of failure of many low dams along the outlet conduit especially after heavy rain.

From such experiences, in this study, a case study of dam failure is introduced. The case study is called KE 2/20 REC dam - an agricultural dam in Central Vietnam that was broken just under one-year in operation. Previous research revealed that the dam failure was related to hydraulic fracturing mechanism (Tran et el. 2017). Whereby, there were stress decreases at both sides of the pipe culvert by arching action that was induced by effects of culvert shape and a steep excavation slope. Based on these findings, in this study, the authors focused on proposal of two countermeasures to prevent risk of hydraulic fracturing adjacent to the culvert of the case study dam. These countermeasures are combinations created by changing the culvert shape and either shifting the position of the excavation slope 5.0 meters away from the former position or replacing the fill soil between the culvert and the excavation slope with a concrete block. Effectiveness of the countermeasures are then verified by numerical models using finite element method (FEM). The results showed that around the proposal culvert, normal stress is always higher than water pressure, and hence, there is no possibility of hydraulic fracturing occurred adjacent to the culvert.

Keywords: Dam failure, Hydraulic fracturing, countermeasure, Finite Element Method (FEM)

Droughts hotspot distribution by long term assessment the Standardized Precipitation Index (SPI) in Indonesia

Yudhi Pramudya, Takeo Onishi

(Faculty of Applied Biological Sciences, Gifu University)

Abstract

Differences in weather and climate conditions in Indonesia influenced by the ITCZ (Inter Tropical Convergence Zone) on January to March and Asian Monsoon in November to December. As a result, this area is frequent experiencing anomalies or climate irregularities primarily associated with the natural phenomenon of La-Nina and El-Nino, which can cause a disaster nature like a drought disaster. Rainfall data used to calculate the value of meteorological drought index in Indonesia using SPI method (Standardized Precipitation Index) to identify drought events means rainfall deficit during peak three months wet season period (SPI-3 during December-February 2000 to 2015). APHRODITE (1961-2007) and CHIRPS (1981-2015) provides estimated rainfall data that can be used to calculate the value of meteorological drought index in a region. Drought index ranged from -2.51 to 2.67 indicating the criteria very dry to very wet, indicates the dominant drought hotspot (extreme climate condition) occurred in the peak of wet season (December–February) has wider extent and higher severity respectively in the West Borneo, Banten and Near Jakarta in 2005, Central Java, Southwest Celebes, Ambon, Maluku, and Papua in 2006, Belitung and South part of West Borneo in 2010, West Sumatera, West Borneo, West Sulawesi 2013, and West Sumatera in 2014. The index also describes dry region in wet months caused flood disaster in Dec-Feb 2012-2013. Spatial analysis information of drought hotspot distribution important to described as one of the efforts to help warning a farmer not to be failed in harvesting, also an anticipation and evaluation for future conditions, so it can be known patterns, severity and frequency of occurrence of drought disaster. From the analysis results predicted areas prone to droughts and floods, then compared with topographical aspects and characteristics of the region (eg agricultural areas, urban areas, etc.) to hydrological planning to face possible disasters (such as drainage, surface water storage etc.). Areas that often experience drought hotspot of the mapping are West Sumatera, West Celebes and Central Java.

Keywords: Drought, Index, SPI-3, Climate, Rainfall

The role of floral volatiles for attracting pollinators and reproductive isolation in *Mimulus* species

Muhammad Arifin, Tomoko Okamoto

(Faculty of Applied Biological Sciences, Gifu University)

Abstract

Flowering plants have a wide variety of signals to attract pollinators. Visual signals, including flower size, color and shape have great influence for preference of pollinator. In addition, flower also emit a large array of volatile compounds, assuming a variety of roles, e.g. attracting effective pollinators and avoiding inappropriate flower visitors. Although many studies have been made to understand plant-pollinator interaction, most of them either focused on visual and olfactory cues. Previous study revealed that visual floral diversity in genus *Mimulus* (family Phrymaceae) correlated with the type of pollinator-animals, leading to reproductive isolation between related species. However, the effect of floral volatiles in *Mimulus* to attract different of pollinators is unknown. In this study, I try to reveal the ecological and evolutionary role of floral volatiles of *Mimulus* species growing in Japan. This study is aimed to (1) clarify the type of pollinator in *Mimulus* flowers, (2) analyze volatile compounds emitted from *Mimulus* flowers and (3) identify the most important volatile compounds to attract specific pollinator animals. After achieving this study, I can understand more clearly about the roles of floral volatiles in plant-pollinator interaction and reproductive isolation in *Mimulus* species in Japan. The sample of all *Mimulus* species will be collected from various area in Japan. Observations of flower visitors will be recorded for a total of 120 hours in several populations to reveal pollinators. Floral volatiles will be collected by head-space sampling in wild. Floral volatiles will be analyzed with a gas chromatography mass spectrometry. Behavioral test using flower volatiles and pollinator animals will be conducted to identify the most important volatiles compounds for pollinator attraction in *Mimulus*.

Keywords: floral scent, plant-pollinator interaction, pollination, chemical ecology, reproductive isolation

The United Graduated School of Agricultural Science, Gifu University

1-1 Yanagido, Gifu 501-1193, Japan

Tel: +81-58-293-2984 (or, 2985)

E-mail: renno@gifu-u.ac.jp

Home page: http://www.ugsas.gifu-u.ac.jp/eng/

ISBN978-4-909365-02-6

Supporting foundation: Gifu Convention and Visitors Bureau, Japan