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Application of biological fungicides to control citrus root rot under field condition in Cambodia

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Pythium ultimum was recorded to be the first time to cause citrus root rot in Cambodia. The pathogen causes a serious damage almost everywhere planted to citrus in Battambang province. The pathogen infects the plants starting from seedlings which show yellow leaves, die back, stem rot, root rot and die. Mainly, the citrus trees express slowly decline from the second year and slowly die which starting from six to seven years old, even using the chemical fungicides. In laboratory test, the detached leave method has shown that only three days after inoculation of pathogen, the citrus leaves turning completely dark brown showing aggressive pathogen and proved for pathogenicity. In field trials, the chemical and biological fungicides namely Chaetomium and Trichoderma biological products were peroidically applied to four year old citrus trees in one year. All products were spayed above plants and to rhizosphere soil every month (metalaxyl-10g/20L of water in combination with chemical fertilizers, Chaetomium-20g/20 L of water and Trichoderma-20g/20L of water in combination with biocompost). Result showed that all treated citrus trees recovered significantly within 3-4 months of applications. The new flashes of leaves and root were emerged and citrus tress recovered. It is proved that the biological products of Chaetomium and Trichoderma gave significantly disease control as equal as the chemical fungicide (metalyxyl) when compared to the non-treated control.

Key words: *Chaetomium cupreum, C. globosum, Trichoderma harzianum,* Metalaxyl, Citrus

Introduction

Citrus is one of the major cash crops for farmers in Cambodia. The citrus plantation is also one of the major fruit commodities to support the household economic for small farmers, which are directed at increasing income, employment opportunities and nutritional status through high value of commercial and nutritional crop. However, orchards and plantation are being damaged by diseases and insects, especially root rot disease. One factor

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associated with citrus production is the most serious disease distributed worldwide, that is foot rot, brown-rot, gummosis, root rot or similar types of gum disease which are caused by either *Pythium* spp or *Phytophthora* spp. These have been reported in every country where citrus is grown (Timmer and Menge, 2000). Ohazuruike and Obi (2000) implicated *Pythium, Phytophthora, Corticium* and *Fusarium* as specific pathogens causing damping off diseases of citrus. *P. ultimum* is widely distributed throughout the world and has a wide range of hosts including many other important crops. *P. ultimum* has been found to cause diseases in Australia, Brazil, Canada, China, Japan, Korea, South Africa and many other countries in the world. In the United States, the occurrence of *P. ultimum* has been reported in most states. Studies have shown that *P. ultimum* is common in most soils of agronomic crops and forests (Hendrix and Campbell, 1973). Other diseases which also seriously damage citrus plants are greening and tristeza virus diseases, found together with *Phytophthora* rot as a disease complex. In the case of root rot, this disease cannot be treated once the plant has become infected. The only choice is that diseased plants need to be removed and replanted. However, infection is so widespread that many mother plants and nursery stocks are also infected. In general, citrus trees are attacked not only by diseases but also by insect pests and nutrient deficiency (Molina *et al*., 1998).

So far, there has been much used of chemical fungicides to control all kind of insects and diseases. But the use of chemical fungicides has resulted in an increased degree of pathogen resistance (Levy *et al*., 1983). Recently, there has been numerous reports that the research development to control the disease problem using microbial antagonists e.g. *Trichoderma harzianum* and *T. viride* to control *P. infestans* causing late blight of potato (Singh, 1986; Mukerji and Garg, 1988). Other promising microbial antagonists are *Chaetomium* spp. As they can degrade cellulolytic plant debris to increase high organic matter in soil and specific isolate can inhibit several pathogens (Soytong and Quimio, 1989). For example, *C. globosum* and *C. cochlioides* can inhibit the growth of *Fusarium* spp. and *Helminthosporium* spp. (Tveit and Moore, 1954). It has also been reported that *C. globosum* produces metabolites that inhibit the growth of *Pythium ultimum* which causes damping-off of sugar beet (Di-Pietro *et al*., 1991), *Rhizoctonia solani* (Walter and Gindrat, 1988), leaf blight of brassicas caused by *Alternaria brassicicola* (Vannacci and Harman, 1987) and can reduce the pathogen inocula of *Botrytis cinerea* on deadly lily leaves in the field (Kohl *et al*., 1995). *Chaetomium cupreum* has been reported to control soybean plant pathogens e.g. *Phomopsis* and *Colletotrichum* spp. (Manandhar *et al*., 1986). Strains of *C. cupreum* and *C. globosum* have also been reported to reduce leaf spot disease of corn caused by *Curvularia lunata*, rice blast caused by

Magnaporthe grisea (*Pyricularia oryzae*) and sheath blight of rice caused by *Rhizoctonia oryzae* (Soytong, 1989, 1992a).

The objectives of this study were tested the biological fungicides formulated from *Chaetomium* spp and *Trichoderma* spp to control the citrus root rot caused by *Pythium ultimum* in the field.

Materials and methods

Isolation of pathogen

Soil samples were collected at random in citrus infected fields in Battambang province, which have been seriously destroyed by root rot pathogen. Hundred sixty soil samples at a soil depth of 10 – 20 cm were collected and brought to laboratory for isolation work at General Directorate of Agriculture, Phnom Penh and confirmation of isolates at Biocontrol Research Unit, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Soil samples were air-dried and ground to fine particles, 50 g each sample was then placed into Sterilized Petri Dish, 20 ml of Sterilized distilled water and leaf disks of citrus (15 mm x 15 mm) were added and incubated at 27^{o}C for 2- 3 days. Under light microscopy (10X), the presence of sporangia of pathogen growing out from the citrus leaf disks in each sample was transferred to the Water Agar (WA) Petri dishes for 2 or 3 days. The Petri dishes were examined daily by using a dissecting microscope to the location of hyphae and marked their locations on the bottom of the Petri dishes. After 1 to 2 days, cut out small blocks of mycelia on WA along the advancing margin of colony and immediately transferred to Potato Dextrose Agar (PDA) plates and isolated to pure culture. Cultures were transferred to PDA slants and stored with sterile paraffin oil for further experiments and maintenance.

Identification

The isolates of *Pythium* spp were grown on PDA and Potato-Carrot Agar (PCA) according to Van der Plaats-Niterink(1981) in 90 mm Petri dishes, and incubated in room temperature (27-30^{o}C). The growth of colony was recorded every 24 hr for 3 days. Each isolate was transferred to PDA plates and observed after incubation for 7 days at room temperature. The hyphal tips of young colony on PDA was cut and floated in sterile distilled water for 24 hr at room temperature for investigation the sporangial development. Oogonia, antheridia and oospore characteristics were determined after 3 days of incubation at room temperature on PCA. The morphological identification was based on the works

of Waterhouse (1968), Trow (1901), Johnson (1971) and van der Plaats-Niterink (1981).

Pathogenicity test

The isolates of *Pythium ultimum* were tested in the detached citrus leaf assays. Completely Randomized Design (CRD) with 4 replications was used for the experiment. Each mycelia plug (5 mm diameter) was taken from young fungal colony of the growing pathogen by sterilized cork borer and artificially inoculated onto wounded leaves. A needle was used to wound leaves before placing 5 mm diameter of mycelia discs from each isolate.

The inoculated leaves were incubated in moist chamber (a moist tissue paper in Petri dish) at room temperature (28-30^{o}C) for 5-7 days. The non-inoculated leaves were treated with sterile agar discs and served as controls. The Petri dishes were examined daily as the infected leaf was rated using an index of 1-4, where 1=green (normal and non-aggressive), 2 = pale green (low aggressive), 3=pale brown (medium aggressive) and 4 = dark brown (high aggressive). The rating was measured from 5-7 days after inoculation. After 7 days, the reisolation was performed to confirm the isolate, then the infected leaf area was cut at the advanced margin between healthy and diseased parts; and transferred to Water Agar Petri dish. After 2 days, cut out small blocks of WA along the peripheral colony and immediately transferred to PDA Petri dish to recover the fungus.

Field trial

The Integrated Pest Management (IPM) in combination of biological fungicides was set up as a field experiment in the infested field planted to citrus at orchard of farmer approximately 0.75 hectares which was covered about 100 of 4-5 year-old citrus trees. The experiments were carried out using a Randomized Complete Block Design (RCBD) with four replications and treatments were set up as follows:- T1= control was non-treated control, T2=Chemical Fungicide:-treatment was applied to rhizosphere soil at 300 g/tree of chemical fertilizers at every month for 1 year, sprayed chemical fungicide (metalaxyl) 20g/20 L of water around the rhizosphere soil and above plants every month for 1 year, T3=GAP (good agricultural practice):-treatment was in combination with chemical fungicide (metalyxyl) and biological fungicide (Ketomium):- treatment was applied to rhizosphere soil at 150 g/tree of chemical fertilizer together with 5 kg/tree of biofertilizer (mud compost from Tale sap), every month for 1 year, sprayed the mixtures of fungicide (metalaxyl) 10g/20 L of water together with Ketomium-biofungicide 10 g/ 20 L

of water into rhizosphere soil and above plants every month for 1 year. T4= Ketomium-biofungicide:- treatment was applied to rhizosphere soil at 150 g/tree of chemical fertilizer together with 5 kg/tree of biofertilizer (mud compost from Talesap) every month for 1 year, sprayed only Ketomium-biofungicide 20 g/20 L of water around rhizosphere soil and above plants every month for 1 year. T5= Trichoderma-biofungicide:- treatment was applied to rhizosphere soil at 150 g/tree of chemical fertilizer together with 5 kg/tree of biofertilizer (mud compost from Talesap) every month for 1 year, sprayed only Trichoderma –biofungicide 20 g/20 L of water around rhizosphere soil and above plants every month for 1 year. Each treatment consisted of 10 citrus trees. Then, all tested citrus trees were 200 trees. All treatments were mulched with rice straws under the canopy. Disease index was recorded.

Soil samples were collected before and after treatments. Measurement was done before and after experiment. The symptom of yellow leaves, die back and root rot was scored as disease index (DI) and evaluated monthly during the experiment. The five citrus trees in each treatment were randomly selected for data collection. Disease Index was recorded and classified into 5 levels as follows:- Level 1=0%, no symptom (healthy plant), Level 2=1–25% of yellow lesions on leaves, Level 3=26–50% of yellow lesions on leaves, Level 4=51-75% of yellow lesions on leaves and Level 5=76–100% of yellow lesions on leaves.

Data was statistical calculated analysis of variance (ANOV), treatment means were compared using Duncan's Multiple Range Test (DMRT) at P=0.05 and P=0.01. Disease Reduction was computed as disease index in control – disease index in treatment / disease index in control × 100.

Results

Isolation, identification and pathogenicity test

Pythium ultimum was isolated from soil baiting technique. Isolates were identified as *Pythium* spp. based on the morphological characteristics of sporangia, antheridia, and oogonia of the genus. Isolates of *P. ultimum* obtained from citrus root rot disease. With this, it showed the hyphal swelling in different sizes. Oogonia smooth with occasionally a few papilla, globose intercalary as well as terminal. Antheridia 1-2 per oogonium, crook necked, closely monoclinous, occasionally diclinous, mostly emerging very close to the oogonial stalk. Oospore single, globose aplerotic and Oospore wall thin. The isolates were confirmed to be the causative agent of citrus root rot by pathogenicity test. The detached leaves showed that 5 isolates are the most

virulence which leaves became dark brown within 3 days. It was clearly seen that its ability to infect detached leave in the laboratory test (Fig. 1).

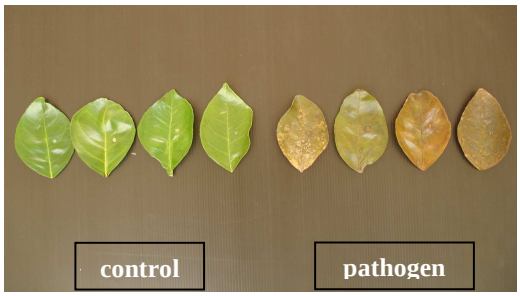

Fig. 1. Pathogenicity test on detached leaves of *Pythium ultimum* isolate T41.

Evaluation of biofungicides to control citrus root rot in the field

The field trial was carried out in 1 ha of 200 trees of citrus orchard in Battambang province where the soil was infested with root rot disease which caused by *Pythium ultimum* and almost citrus trees completely destroyed which the sign of yellowing and small leaves and dieback. The 4 year-old of citrus trees were selected for experiment as the same disease level.

Due to poor management of citrus orchard at the beginning of experiment the situation of citrus tress were very bad. There were not only symptoms of die back, root rot but various fungal and bacteria diseases appeared on leaves, twigs and stems. In addition to this the symptom of nutrient deficiency was also occurred implies as disease complex. The complexity of the symptoms and problem were clearly shown that the citrus condition was not healthy and led to slow recovery.

After treatments for 4 months, the citrus trees have started to recover by producing more new leave flashes and reducing the symptom on leaves and twigs together with the new roots, but it was apparently seen in an improvement of the plants within the 4-month period. The new roots were appeared above rhizosphere soil under the canopy.

Data showed that all treatments were not significantly different in term of disease index for 4 months, thereafter it showed significantly lower disease index than the non-treated control. After 8 months of treatments, the treatments of metalaxyl, GAP, Ketomium and Trichoderma were not significantly different in disease index and gave a less disease index than the no-treated control. As seen in Table1. The was similar result of Usuwan and Soytong (2000) who reported that applied Ketomium and Trichoderma biological

products to control citrus root rot that was not significant different from chemical treatment (metalaxyl) in the fields.

However, the non-treated control was steadily declined from month to month by showing the symptom of yellow leave, die back and root rot. The disease reduction started clearly to recover the citrus tress after treatments for 5 months. Thereafter, the disease reduction was continuously occurred until 9 months approximately over 60% as seen in table 2. It is revealed that the recovered citrus trees from seriously disease that take time. Since then, the cost the agricultural inputs and good soil management must be considered in this matter. It is noticed that the chemical fungicide-metalaxyl was not different in disease reduction when compared to GAP, Ketomium and Trichderma biological fungicides. It is indicated that biological fungicides expressed a curative effect to control diseases plant and it would be good if the application of biological fungicides be applied for protection in the healthly plants.

Table 1. Disease index after application of biological fungicides in the field.

	Disease index (months)					
Treatments	4	5	6	7	8	9
control	2.91a	3.15a	3.36a	3.58a	3.71a	3.90a
metalaxyl	2.92a	2.56ab	2.27a	1.96a	1.70b	1.46b
GAP	2.81a	2.48a	2.18a	1.85a	1.56b	1.33b
Ketomium	2.53a	2.25a	1.98a	1.71b	1.55b	1.31b
Trichoderma	2.86a	2.66ab	2.41b	1.84b	1.61b	1.48b
P.	0.01	0.05	0.01	0.01	0.01	0.01
CV (%)	9.30	12.56	10.14	11.62	13.90	16.03

Table 2. Disease reduction after application of biological fungicides in the field.

	Disease reduction (%)				
Treatments	**5**	**6**	**7**	**8**	**9**
metalyxyl	18.73	32.44	45.25	54.17	62.56
GAP	21.26	35.11	48.32	57.95	65.89
Ketomium	28.57	41.07	52.25	58.22	66.41
Trichoderma	15.35	28.27	48.60	56.60	62.48

Discussion

The research finding is differed from Usuwan and Soytong (1998) which reported *Phytophthora parasitica* is the most serious root rot disease of citrus in Thailand at that time. However, *Pythium ultimum* var. *ultimum* was first isolated and described by Trow (1901), who considered it non-parasitic. But Kucharek and Mitchell (2000) reported that *Pythium* spp. such as *P.*

aphanidermatum, *P. debaryanum*, *P. myriotylum* and *P. ultimum* cause damping off, root rot, seedling blight and stem rot of many plants. The result is confirmed by pathogenicity test that this species is caused citrus root rot in Cambodia. Moreover, Lehman and Wolf (1926) identified it as a soybean parasite. All soil samples collected from diseased citrus trees encountered this pathogen in our study that indicated as soil-borne pathogen. *P. ultimum* var. *ultimum* is reported to be one of the most common soil borne species that has a worldwide distribution and causes diseases on a number of plants (Plaats-Niterink, 1981; Hancock, 1977; Johnson, 1971). It is concluded in this study that *P. ultimum* is found to be the first time as a serious pathogen causes citrus root rot in Cambodia.

In this study, the chemical fungicide and biofungicide products were compared to investigate for their effectiveness in controlling citrus root rot under field condition. This experiment was used both chemical fertilizer and biofertilizers (biocompost) to improve the soil condition for plant growth and at the same time to reduce disease incidence by applying both chemical fungicide and biological fungicides. The experiment was started at the beginning of November 2007. After two months of fertilization and application of chemical fungicide and biological fungicides of Ketomium and Trichoderma. The new leave flashes have gradually emerged for all treatments, except non-treated control. Noticeably, within this period the new root system was also developed. After 3-4 months it fully flashes and along with flowering. However, there were some plants had seriously damaged in the root system, so the recovery was difficult or slowly. It was clearly shown that the plant improvement or management should start earlier not waiting until old age. The disease incidence was also rapidly declined after treated with both chemical fungicide and biological fungicides. Most of the leaves, branches and stems were healthier and developed by applying its above plants and rhizosphere soil. These results are similar to those found in previous work on biological control of *Phytophthora* root rot of durian in the field which demonstrate that the *Phytophthora* pathogen could be reduced after applying *Chaetomium* into infested soil (Prechprome and Soytong, 1997). The application of bio-compost every month to the plants was very important not only to improve the soil fertility but also to maintain the population of the antagonisms in the soil. In Thailand both *Chaetomium globosum* and *Trichoderma harzianum* have been proved to be effective antagonistic fungi by many researchers as Chamswang and Roungwist (1992) reported that when *Trichoderma* was used together with micronutrients and organic matter showed good control for *Phytophthora palmivora* root rot of durian as well as using *Trichoderma* with Ridomil 5G. *Trichoderma harzianum* was reported as an effective biocontrol agent against

Sclerotium rolfsii and *Rhizoctonia solani* (Elad *et.al*., 1980). Our research findings proved that Ketomimum biological fungicide formulated from *C. globosum* and *C. cupreum* gave a good control of infected citrus trees in the field as Kohl *et al*. (1995) reported that *C. globosum* has high competitive ability which is a prerequisite for successful saprobic antagonists introduced to senescing or necrotic leaf tissue under field conditions. *Chaetomium spp.* are strongly competitive and have ability to colonize organic compost and suppress pathogen in the soil.

Similarly, in previous work the results also showed that the application of *Chaetomium* biological products could reduce the pathogen inoculum and disease incidence of *Phytophthora* rot of citrus in the field (Usuwan and Soytong, 1998). The formulated biological fungicides in this study were in power form that used to spray into rhizosphere soil under plant canopy and above plants to infected citrus trees as the work of Soytong *et al*. (2001) stated that Ketomium in the form of biopellets and biopowders were applied to *Fusarium*-infested soils where tomatoes were growing and it was found that Ketomium suppressed pathogen growth and thus disease symptoms. The biological product of Ketomium have been also successfully suppressed the soil pathogen as *Fusarium*-suppressive soils, it was reported that the tomato plants treated with Ketomium biopowder and the chemical fungicide Pentachloronitrobenzen (PCNB) completely prevented damage by *F. oxysporum* f. sp. *lycopersici*. Inoculum of *Phytophthora palmivora* in rhizosphere soil of Black paper was also significantly lower following Ketomium and metalaxyl treatments than in non-treated soils that was reported by Soytong *et al*. (2001). Moreover, it is revealed that the tested biological product of Ketomium has been proved to control several disease in the fields as in this study in Cambodia for the first time e.g. Ketomium-mycofungicide completely prevented root rot caused by *Phythophthora* spp., e.g. root rot of durian (Prechprome and Soytong, 1997), black paper (Sodsaard and Soytong, 1999) and tangerine (Soytong *et al*., 1999).

The mechanism control of biological fungicide produced from *Chaetomium* spp which used as living ascospores to formulate in the powder form, it is reported the same isolates of these species could release some antibiotic substances as reported by Soytong *et al.* (2001) that *C. globosum* can be release antibiotic substance namely Chaetoglobosin C and C. cupreum could produce Rotiorinol as antibiotic substances. These bioactive compounds may possible to kill the pathogen implies antibiosis. Moreover, it had also been reported that *C. globosum* produces metabolites that inhibited the growth of *P. ultimum* which causes damping-off of sugar beet (Di-Pietro *et al*., 1991) and *Rhizoctonia solani* (Walter and Gindrat, 1988). Results from this study suggest

that biological products may have promise for reducing *Pythium* root rot and recover above plant in citrus trees in field condition. Our study indicated that the colonization of citrus roots by *P. ultimum* was reduced by either metalaxyl or biological products of Ketomium and Trichoderma and GAP (combination between chemical and biofungicides) when compared to the roots from non-treated control. Our experiment showed that application of systemic fungicide, metalayxl could control *Pythium* root rot of citrus as effectly as the biological products of Ketomium and Trichoderma. With this, Timmer *et al*. (1989) reported that the application of the fungicides, fosetyl-Al and metalaxyl could control fungal infection of roots and increase fibrous root density (Timmer *et al*., 1989). However, although fungicides can increase yields and fruit size of orange and grapefruit trees on disease susceptible rootstocks but yield response is often variable. However, soil-applied pesticides are proved to loss of efficacy after prolonged usage and it is water soluble and run off to the groundwater after excessive irrigation or rainfall as a pollutants contaminated in the surrounding environment (Kookna *et al*., 1995). As in our study, the success control rot root of citrus in the field using chemical fungicide and biological products of Ketomium and Trichoderma are then sprayed into the rhizosphere soil with applied biocompost that it was also reported by Hoitink and Fahey (1986) and Hoitink and Grebus (1994) who pointed out that the an alternative strategy to fungicides for sustained control of soil borne diseases is to periodically apply composted organic materials as amendments to suppress fungal root pathogens. Composted bark, when incorporated into potting media, suppresses soil borne diseases of ornamental plants. Moreover, there are reported that composted municipal waste reduced the incidence of infection by *P. nicotianae* on citrus seedlings in the greenhouse and improved growth of newly planted trees in several field trials in spite of inoculation with *P. nicotianae* at transplant (Widmer *et al.,* 1996; Widmer *et al.,* 1998a, b). With this, according to our field study, it is suggested the application of biological fungicides of Ketomium and Trichoderma can be applied and promoted to the growers to control plant disease to avoid the toxic chemicals deposited in the soil and contaminated in plant products. Our research finding is also recommended that the biological products can be used to replace the chemical fungicide (metalaxyl) and safety for the human health and environment as well.

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