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Development of Biological Control of *Ralstonia solanacearum* Through Antagonistic Microbial Populations

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ABSTRACT

Potential antagonists were screened out and evaluated *in-vitro* and *in-vivo* as bio-control agents against the bacterial wilt pathogen. All the bio-control agents tested reduced the bacterial wilt disease to various degrees. Among the bio-control agents tested, EM instant was the most effective, while the degree of disease suppression by other microbes varied with the time of application. The effects of EM-FPE and Bokashi were not different from EM instant. Among the antagonists isolated from infested soils LR 10 showed a higher potential of disease suppression. The antagonists isolated from EM sources were effective to suppress against bacterial wilt pathogen. It means that EM contains some microbes, which can suppress the disease. Application method of bio-control agent should be suitable for antagonist's action and prior to pathogen attack.

Key Words: Biological control; Antagonist; Effective microorganisms (EM); *Ralstonia solanacearum;* Bacterial wilt

INTRODUCTION

Bacterial wilt, caused by *Ralstonia solanacearum* E. Yabuuchi formerly known as *Pseudomonas solanacearum* E.F. Smith is one of the most devastating, important and wide-spread bacterial diseases of crops in tropical environments (He *et al.*, 1983). The pathogen has a wide host range representing 44 families (He *et al*., 1983). Highly susceptible crops are potato, tomato, egg plant, chili, bell pepper and peanut. The disease has limited both commercial and domestic level production (Somodi *et al*., 1993).

As the disease is widely distributed, it has a wide host range and is mainly soil-borne; it is difficult to control with chemicals and cultural practices (Grimault *et al.*, 1993). There seems to be a shift to the idea that biological control can have an important role in the management of bacterial wilt (Akiew *et al*., 1993). Biological control strategies may either help development of alternative management measures or be integrated with other practices for effective disease management at the field level. Biological control not only suppresses the disease and increases the crop yield but will be important in preventing the environmental pollution due to pesticides.

Several microorganisms have been tried out with variable success for biological control of bacterial wilt (Shekhawat *et al*., 1993). Effective microorganisms (EM) is a mixture of beneficial microorganisms, which can increase the crop yield and also protect against plant pathogens (Higa, 1999). It is a mixed culture of photosynthetic bacteria, *Azotobacter, Streptomyces* and *Lactobacillus* spp., which improve crop yield by increasing photosynthesis, nitrogen fixation, controlling soil diseases and accelerating decomposition of lignin material in the soil (Hussain *et al*., 1993). EM have been reported to protect against *Pseudomonas* bacteria (Castro *et al*., 1993).

Although the potential to suppress the pathogenic organisms through biological means has been revealed, sufficient information has not been generated so far to fully support the development of biological control measures against *R. solanacearum*. Therefore, the current study was conducted to evaluate the effect of potential antagonists on *R. solanacearum in vivo* and *vitro* and develop effective bio-control measure.

MATERIALS AND METHODS

This study was conducted at the Agricultural system laboratories of the Asian Institute of Technology, Thailand during October 2004 to May 2005. The study included the screening of potential antagonists and their evaluation *in vitro* and *vivo.*

a) Screening of potential antagonists. Bacterial wilt diseased tomato plant parts and infested soils were collected from the farmers' fields in the Pakchong district, Nakorn Ratchasima province of the North-east Thailand where the disease was prevalent. The pathogenic bacterium, *R. solanacearum* was isolated from diseased tissue and pathogenicity was confirmed by root dipped inoculation method (Winstead & Kelman, 1952). Seven suspected antagonists from bacterial disease infected soils and three isolates from the commercially available EM preparations were isolated using the agar layer method (Dhingra & Sinclair, 1995). The degree of antagonism of these antagonists was determined by both cross-culture method and filter-paper disk method (Dhingra & Sinclair, 1995). Of these antagonists, three most effective antagonists were selected based on the degree of inhibition of pathogen and growth rate of antagonist for *in-vitro* evaluation studies.

***In-vitro* evaluation of potential antagonists.** Three selected antagonists (viz. LR 3, LR 6 & LR 10) and four commercial antagonistic microorganisms [viz. EM concentrate, EM instant, EM -fermented plant extract (EM-FPE) and *Bacillus subtilis*] were evaluated against the bacterial wilt pathogen *in vitro*. The experimental designs were complete randomized design (CRD) with four replications. Cross culture method and filter paper disk method were used in first experiment and second experiment, respectively. PDA medium was used in both experiments in order to favor the growth of *R. solanacearum* and the potential antagonists.

***In-vivo* evaluation of potential bio-control agents.** The three selected potential antagonists and five-commercial bio-control agents [viz., EM instant, EM5 (EM preserved with vinegar & distilled ethyl alcohol), EM-FPE, Bokashi (EM fermented compost) and *B. subtilis*] were evaluated *in-vivo* against *R. solanacearum* in the greenhouse using susceptible tomato variety Sida. The experimental designs were complete randomized design (CRD) with five replications. The temperature and relative humidity of the greenhouse were set at 30ºC and 80%, respectively in order to favour the disease development.

In pot experiment I, both antagonists and pathogen were introduced at the same time. Three selected antagonists and four commercial bio-control agents were evaluated the effect of antagonists on the pathogenicity of the causal organism. Each of the antagonist suspensions was mixed with pathogen suspension in a screwed cap bottle equally and allowed for one hour to react each other. After one hour, 15-days-old tomato seedlings were inoculated with the mixture using root dipping method and then transplanted in the pots filled with sterilized soil.

In pot experiment II, the antagonists were introduced one week before the pathogen inoculation. Three selected antagonists and four commercial bio-control agents were applied to 21-days-old tomato seedlings growing in separate pots filled with sterilized soils. Antagonists were applied regularly up to 6 times at one-week interval. To apply antagonists, 15 mL of suspension at a concentration of 10^9 cfu/mL of each of the three selected antagonists and 100 mL of 1:100 diluted solutions from the four commercially available bio-control agents were used.

In pot experiment III, the antagonists were applied after the pathogen was established in the soil. Sick pot method was used to inoculate tomato plants. In this inoculation method, soils in pots were made to be fully infested with the *R. solanacearum* and then the test plants were transplanted in the pots. In the preparation of sick pots, all the pots were first filled with bacterial wilt disease infested soils collected from Pakchong district. The seedlings of the same susceptible tomato variety were transplanted in these pots and allowed to grow. When the plants in the pots died due to bacterial wilt disease, the pots were re-transplanted with another set of new tomato seedlings. When the second set of tomato plants also died due to the disease, the pots containing such soils were considered as sick pots. There were eight bio-control agents (viz. three isolated antagonist suspensions, four commercial antagonist suspensions & Bokashi compost) and a control treatment evaluated. Soils in the sick pots were treated with corresponding antagonist suspensions as in pot experiment 2. Bokashi was applied as one time application at 15 days before transplanting at 1:20 with bokashi: soil ratio. The control treatment received no antagonists. The 21-days old-tomato seedlings were transplanted in the pots 15 days after the first application of antagonists.

Disease severity was recorded 2 to 8 weeks after inoculation at weekly intervals. A 5 point visual scale, 1 being un-damaged and 5 being completely wilted, was used for recording disease development as adopted by Kelman and Person (1961). Standard ANOVA procedure was applied to test the significance differences in the distance of inhibition of pathogen by antagonists in the *in-vitro* evaluation experiment and Fisher's protected least significant difference was used to compare the mean performance among treatments (Steel & Torrie, 1980). The visual scores were ranked based on their performance. IRRI-Stat software was used for data analysis.

RESULTS AND DISCUSSION

Initial screening of potential antagonists. Among the ten antagonists, LR 3 had the highest distance of inhibition of the pathogen growth (Table I). LR 10 was the best among the antagonists isolated from soil. Antagonists isolated from EM products showed a higher potential for inhibition of the pathogen (Table I). Filter paper disc method showed different results to cross-culture method. The antagonistic ability of LR 3 was lower in the filter paper disk method compared to that of cross-streak method. It may be due to its slow growth rate. This could be due to the fact that in the cross-streak method, antagonists were introduced into the media two days before the inoculation of pathogen and hence it had sometime to produce specific toxins or suppressants by the time pathogen was introduced.

***In-vitro* evaluation of potential antagonists.** EM based isolates as well as EM products explicitly showed their ability to inhibit the *R. solanacearum* with the distance of inhibition zone (Table II). Antagonistic potential among the direct EM products showed that EM-FPE was able to significantly reduce the growth of pathogen and by far superior to others. When the ranking was done on the potential of inhibition of the pathogen, EM products were appeared in the highest. The bacterial wilt disease was also observed to decrease under EM-FPE in tomatoes and egg plant (Margarita & Dengel, 2003). Kyan *et al*. (1999) also reported that EM contains antagonistic microorganisms and hence can suppress the plant pathogens. However, the current study shows the significant role and potential of EM products in the suppression of *R. solanacearum.* Growth inhibition zones on agar media may be due to chemical

Table I. Determination of the degree of antagonism of ten isolated antagonists

Antagonist	Distance of inhibition zone (mm)		Source[1/]
	Cross culture method	Filter paper disc method	
LR1	3.00	0.00	Soil
LR 2	3.00	0.00	Soil
LR 3	11.50	2.12	EM.FPE [2/]
LR 4	3.25	0.00	Soil
LR 5	3.25	0.00	Soil
LR 6	5.50	3.37	EM instant[3/]
LR 7	5.00	3.62	EM Concentrate[4/]
LR 8	2.50	0.62	Soil
LR 9	2.50	0.37	Soil
LR 10	3.50	0.75	Soil

1/ Source indicates where the suspected antagonists were extracted from.
2/ EM-FPE – Effective microorganisms in fermented plant extracts (Kyan *et al.*, 1999);
3/ EM instant – Effective microorganisms activated with molasses (Kyan *et al.*, 1999);
4/ EM concentrate – Effective microorganism solutions available (Kyan *et al.*, 1999);

Table II. Inhibition of *R. solanacearum* by potential antagonists in *in-vitro* studies

Treatments	Distance of inhibition zone (mm)			
	Experiment I (Crossstreak method)	Experiment II (Filter paper disc method)	Mean	Rank
EM-FPE	18.25	3.87	11.06	1
EM instant	8.00	3.70	5.88	4
EM concentrate	8.25	3.62	5.94	3
B. substilis	1.25	0.00	0.63	7
LR3	12.25	1.75	7.00	2
LR6	5.25	3.25	4.25	5
LR10	3.25	0.00	1.63	6
PROB	0.000	0.000		
SE	0.659	0.159		
LSD5%	1.939	0.468		
CV%	16.300	13.700		

factors (low pH), to antibiotic substances with a broad spectrum of activity or to more specific bacteriocins, or to the presence of bacteirophages (Gross & Vidaver, 1990).

***In-vivo* evaluation of potential bio-control agents.** In the pot experiment I, the disease severity was highest in EM 5 and *B. substilis* as the control (Table III). EM 4 and LR 3 had the lowest disease condition. EM-FPE, LR 6 and LR 10 had moderate disease conditions. When the antagonists were introduced prior to pathogen, i.e. in the Experiment II, all EM-based antagonists suppressed pathogens better than the rest of the antagonists. In the experiment III, where the antagonists were introduced after the pathogen, the highest disease control was found in EM-instant and Bokashi treatments, while all other antagonists had almost the same degree of moderate disease severity when compared with the control (Table III).

The response of antagonists in terms of disease suppression varied with the antagonist and time of application. Both EM 5 and *B. subtillis* have improved their performance when they were introduced one week before

Table III. Effect of bio-control agents on the severity of bacterial wilt disease

Treatments	Disease severity (Final scoring scale)		
	Experiment I Antagonist and Pathogen applied at the same time	Experiment II Antagonist established before the pathogen	Experiment III Antagonist established after the pathogen
Pathogen only (control)	5.0 (4) */	5.0 (8) */	4.6 (7)*/
EM4 instant	1.6 (1)	1.2 (1)	1.4 (1)
EM5[1]	5.0 (4)	1.6 (2)	2.8 (4)
EM-FPE	2.2 (3)	1.8 (3)	2.4 (3)
Bacillus substilis	5.0 (4)	2.4 (4)	2.8 (4)
LR3	1.8 (2)	3.0 (6)	3.4 (6)
LR6	2.2 (3)	2.8 (5)	3.2 (5)
LR10	2.2 (3)	3.8 (7)	3.4 (6)
Bokashi	-	-	1.6 (2)

*/ Value within parenthesis indicates the rank of performance of the antagonists: 1 being the highest disease suppression (lowest disease incidence) and increasing the number shows the increasing disease infestation; 1/ EM5 – EM preserved with vinegar and distilled ethyl alcohol (Kyan *et al.*, 1999)

pathogen inoculation as compared with the introduction of the antagonists simultaneously with pathogen. But, there was a slight reduction in the disease suppression when the antagonists were introduced one week later, using sick pot technique. The failure *B. substilis* to suppress the pathogen in the pot experiment 1 could be due to its less active or inactive form, as the commercial product of *B. substilis* is available in the spore form, which may require a few days in the soil to activate its action. This has been shown in its enhanced performance in the pot experiment II and III. Various Actinomycetes and bacteria including *Bacillus mesenteriacus*, *B. megaterium*, *B. subtilis*, *B. mycoides* and *Erwinia* have been reported to be active biological control agents (Kelman, 1953). EM 5 which was not effective in pot experiment I, was effective in the other experiments. This may be due to the different application procedure. In the experiment II and III, 1:100 diluted EM 5 solution was used but in experiment I, EM 5 concentrated solution was used. EM 5 contains alcohol and vinegar. It may damage the roots of the test plant when they were dipped in EM 5 and pathogen suspension mixture and cause wilt symptom. Therefore, application method of bio-control agent should be suitable for antagonist's action and prior to pathogen attack.

Bokashi and EM instant showed greater suppression of bacterial wilt pathogen due to its effective combination of microorganisms. In addition, Bokashi contained nutrients (i.e. organic resources) for microbes; thus, EM microbes could multiply properly and react efficiently. Therefore, it needs only one application although the others needs 6 times. For these reasons, Bokashi may be reliable bio-control agent in sustainable management of bacterial wilt.

All bio-control agents tested showed their ability to reduce the severity of bacterial wilt disease in all pot experiments. Among the bio-control agents tested, EM-instant and Bokashi were the best performers in the

suppression of *R. solanacearum.* Milagrosa and Balaki (1999) also reported that the incidence of wilting caused by *P. solanacearum* in potato was lower with the application of Bokashi and EM concentrate alone or combined with inorganic fertilizer than un-treated control. The results of the present study confirm that EM contains antagonists against *R. solonacearum* and hence, it has the ability to suppress the bacterial wilt disease. Furthermore, EM has also been shown to promote the colonization and infection of VAM (Javaid *et al*., 2000). This may be another way EM helps reducing the symptoms of bacterial wilt disease, as it helps plant to acquire its requirements from soil through the access of the VAM fungi even when a part of the root system has been damaged by *R. solanacearum.*

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