Greenhouse evaluations of harpin protein and microbial fungicides in controlling *Curvularia lunata*, *Fusarium moniliforme*, and *Phythopthora palmivora*, major causes of orchid diseases in Thailand

Chan Maketon[1], Yoswadee Tongjib[2], Tanutcha Patipong[2], Nienwan Meechareon[2], Thitima Rungratanaubon[1] and Monchan Maketon[2],*

[1]Department of Environmental Science, Faculty of Environment, Kasetsart University, Ngamwongwan Road, Bangkok 10900, Thailand.
[2]Department of Zoology, Faculty of Science, Kasetsart University, Ngamwongwan Road, Bangkok 10900, Thailand.
*Corresponding author e-mail: fscimcm@hotmail.com; fscimcm@ku.ac.th
Tel: 662 562 5444 ext 3256
Fax: 662 942 8695

Abstract: This study tested the efficacy of harpin proteins in controlling three orchid diseases: flower rust spots on the *Dendrobium* Visa peach hybrid, column blight on the *Dendrobium* Visa peach hybrid, and black rot on *Vanda* Robert's delight. For controlling flower rust spots, harpin protein (3% a.i.) showed the best performance followed by a mixture of *Bacillus subtilis* and *Trichoderma harzianum*, and mancozeb 80% WP. For column blight, harpin protein showed inferior efficacy compared to both bioagents and a mixture of chemicals. However, for black rot, harpin protein at the higher concentration exhibited slightly better control than the bioagents and the chemicals.
[Chan Maketon, Yoswadee Tongjib, Tanutcha Patipong, Nienwan Meechareon, Thitima Rungratanaubon and Monchan Maketon. **Greenhouse evaluations of harpin protein and microbial fungicides in controlling *Curvularia lunata*, *Fusarium moniliforme*, and *Phythopthora palmivora*, major causes of orchid diseases in Thailand.** *Life Sci J* 2015;12(4):125-132]. (ISSN:1097-8135). http://www.lifesciencesite.com. 16

Keywords: Harpin protein; *Bacillus subtilis*; *Trichoderma harzianum*; flowers rust spot disease; column blight disease; black rot disease.

1. Introduction

Several orchid species are important commodities in South American and Asian countries. In Asia, Thailand exports high quality cut orchid flowers to EU, USA, China and Japan (Department of Agriculture, 2010). Orchid cultivation is beset by several diseases that can affect the entire crop. However, early symptom of plant disease can be treated or eradicated upon first appearance, but late symptoms, especially those that appear after harvest and are found once the plant has been exported, are unacceptable. Synthetic chemical fungicides are the most common tool used to prevent such problems, but because of their effect on humans and the environment, alternate methods are often utilized. Microbial fungicides have been introduced for controlling certain plant diseases through colonization and/or hyperparasitism modes of action (Copping and Menn, 2000). However, a newer method of eliciting natural defense mechanisms in the host plant, which is referred to as systemic acquired resistance (SAR), has recently been introduced (Wei et al., 1992; Bednarz et al., 2002). Harpin protein, derived from harp-n-gene fragment, is a SAR molecule that is produced naturally by *Erwinia amylovora*, a bacterium that causes the fire blight disease in apple and pear trees (Wei and Beer, 1996). It does not act directly on the disease organism or alter the DNA of the treated plants. It is currently marketed as a commercially available, broad- spectrum proteinaceous elicitor of SAR (Wei and Betz, 2007).

In this study, harpin protein and a mixture of *Bacillus subtilis* AP-01 and *Trichoderma harzianum* AP-001 (Maketon et al., 2008a) were tested using chemical fungicides for comparison in controlling three orchid diseases: *Curvularia lunata* (Wakker) Boedijin, which causes flowers rust spot, *Fusarium moniliforme* (Sheldon) Wineland, which causes column blight, and *Phytophthora palmivora* Butler, which causes black rot in two orchid types.

2. Material and Methods

The fungal isolates of *C. lunata*, *F. moniliforme*, and *P. palmivora* were obtained from Plant Pathology Department, Kasetsart University, Campangsean Campus, Nakorn Phatom.

Test against flowers rust spot disease caused by *Curvularia lunata*

The *Dendrobium* hybrid type Visa peach was used to test for flowers rust spot disease, and each spike had approximately 6-8 blooms. The following chemicals and biological control agents were used: Tween 80™ (ICI America, USA), harpin protein 3% a.i. (Messenger™, Eden Bioscience Corp., USA), mancozeb 80% WP (Dithane™, Dow Chemical Corp., USA). *B. subtilis* AP-01 and *T. harzianum* AP-001 were obtained from our laboratory and prepared in wettable powder form at $1x10^9$ cfu/g and $2x10^8$ cfu/g,

respectively (Maketon et al., 2008b). Both pure cultures were deposited in the culture collection center at the Thailand Institute of Scientific and Technological Research (TISTR). Table 1 illustrates the dosages of the tested materials. Twenty-four *Dendrobium* hybrid (Visa peach) pots were divided into six treatments with four replications each. All tested materials were thoroughly sprayed on *Dendrobium* flowers according to each treatment (see Table 1). After 7 days, the plants were inoculated by a suspension of *C. lunata*, prepared at $1x10^5$ spores/ml, which was thoroughly sprayed on every flower in every treatment and covered with plastic bag for 1 day to facilitate the disease infection. After 7 days, each testing material was sprayed on the flowers, with the process repeating one week later. Rust spots on petals from both the back and front were counted from the top four blooms on each spike after 28 days.

The percent of disease reduction was calculated according to the method of Tienkao (2007) as follows:

$$\text{Disease reduction (\%)} = \frac{\text{number of spots in each treatment}}{\text{number of spots in the water control treatment}} \times 100$$

Test against column blight disease caused by *Fusarium moniliforme*

For the column blight disease, all tested materials and the *Dendrobium* hybrid were the same as for the flowers rust spot disease test except for the chemical fungicide, with etridiazole + quintozene 6% + 24% w/v EC (Terraclor Super-X, Crompton Corp., USA) used instead of Dithane (Table 1). The suspension of *F. moniliforme* was prepared at $1x10^6$ spores/ml. All spraying and inoculating procedures were same as above. Symptoms of the column blight disease were evaluated and divided into 6 levels:

No disease symptoms (level 0); column blight noticed and adjacent petals have started withering (level 1); the petals have started folding (level 2); the petals have folded and are wilting around the edge (level 3); the petals are wilting (level 4); and the petals have fallen (level 5).

The disease index was modified from Pongpitak (1994) and was calculated according to the following formula:

$$\text{Disease index (\%)} = \frac{\text{number of diseases flowers} \times \text{level damaged}}{\text{total flowers} \times \text{the highest level damaged}} \times 100$$

The disease symptoms were observed and summarized at 18 days after the final test application.

Test against black rot disease caused by *Phytophthora palmivora*

The *Vanda* hybrid type used was Robert's delight. All tested materials were the same as for the column blight disease test (Table 1) except for the inoculation procedure. After the first application of the tested materials, *P. palmivora* that was cultured on potato dextrose agar was transferred using a 3 mm cork borer onto the *Vanda* leaf surface, which had already had the cuticle punched off, was moistened with cotton wool and wrapped. After several days, the orchids were inoculated, then all the wrapping materials were removed and the tested materials were applied. Disease lesions were measured at 9 days after the last application.

Statistical Analysis

The data were analyzed using SAS version 9.3 by one-way analysis of variance (ANOVA), and the means were compared by least significant difference (LSD) at 95% confidence levels.

3. Results

Test against flowers rust spot disease caused by *Curvularia lunata*

For the flowers rust spot disease, harpin protein at both concentrations provided superior protection against the disease, with a statistically significant difference from *B. subtilis* AP-01 + *T. harzianum* AP-001, mancozeb 80% WP and the two control treatments ($F = 40.8$; df = 5,18; $p < 0.05$) (Figure 1a). However, the microbial control agents and the chemical fungicide still provided disease protection. Figure 2 illustrates the rust spot disease severity for each treatment after 28 days.

Test against column blight disease caused by *Fusarium moniliforme*

For the column blight disease, both harpin protein concentrations failed to offer protection. There were no statistically significant differences from the control treatments, however, *B. subtilis* AP-01 + *T. harzianum* AP-001 and etridiazole + quintozene 6 + 24% EC showed better disease protection ($F = 2.61$; df = 5,18; $p < 0.05$) (Figure 1b). Figure 3 illustrates the severity levels of the column blight disease.

Test against black rot disease caused by *Phytophthora palmivora*

For the black rot disease, only harpin protein at 0.75 g/ liter water and Tween 80 showed slightly better disease protection with a statistically significant difference from the control groups. The lower dosages of harpin protein, microbial control agents and chemical fungicide did not control the disease ($F = 6.57$; df = 5, 18; $p < 0.05$) (Figure 1c). Symptoms of the black rot disease are shown in Figure 4.

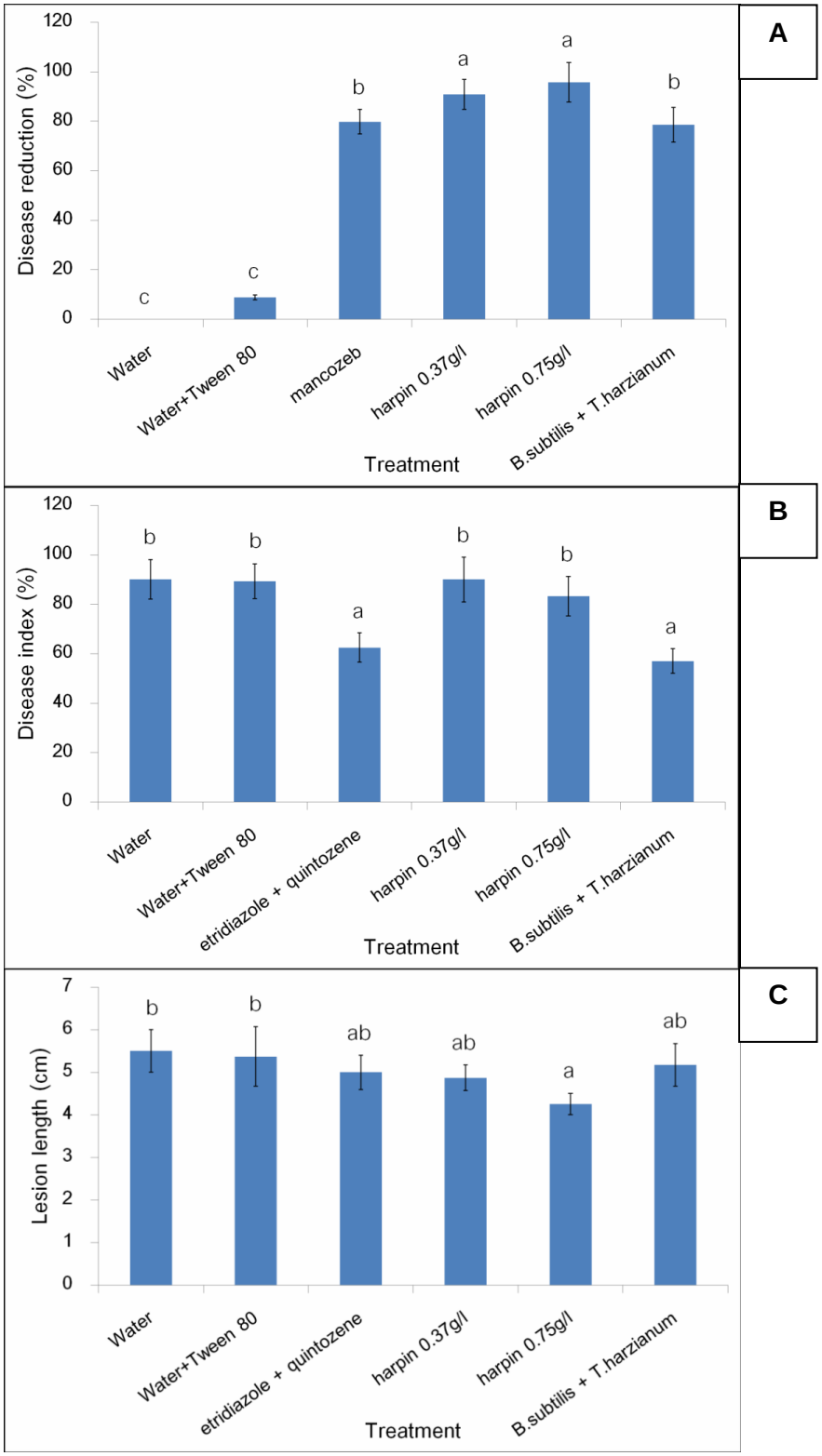


Figure 1: Disease reduction (%) of the flowers rust spot disease on *Dendrobium* Visa peach hybrid versus the six tested materials (a); the Disease index (%) of the column blight disease on *Dendrobium* Visa peach hybrid versus the six tested materials (b); and the lesion length (cm) of the black rot disease on *Vanda* Robert's delight hybrid versus the six tested materials (c).

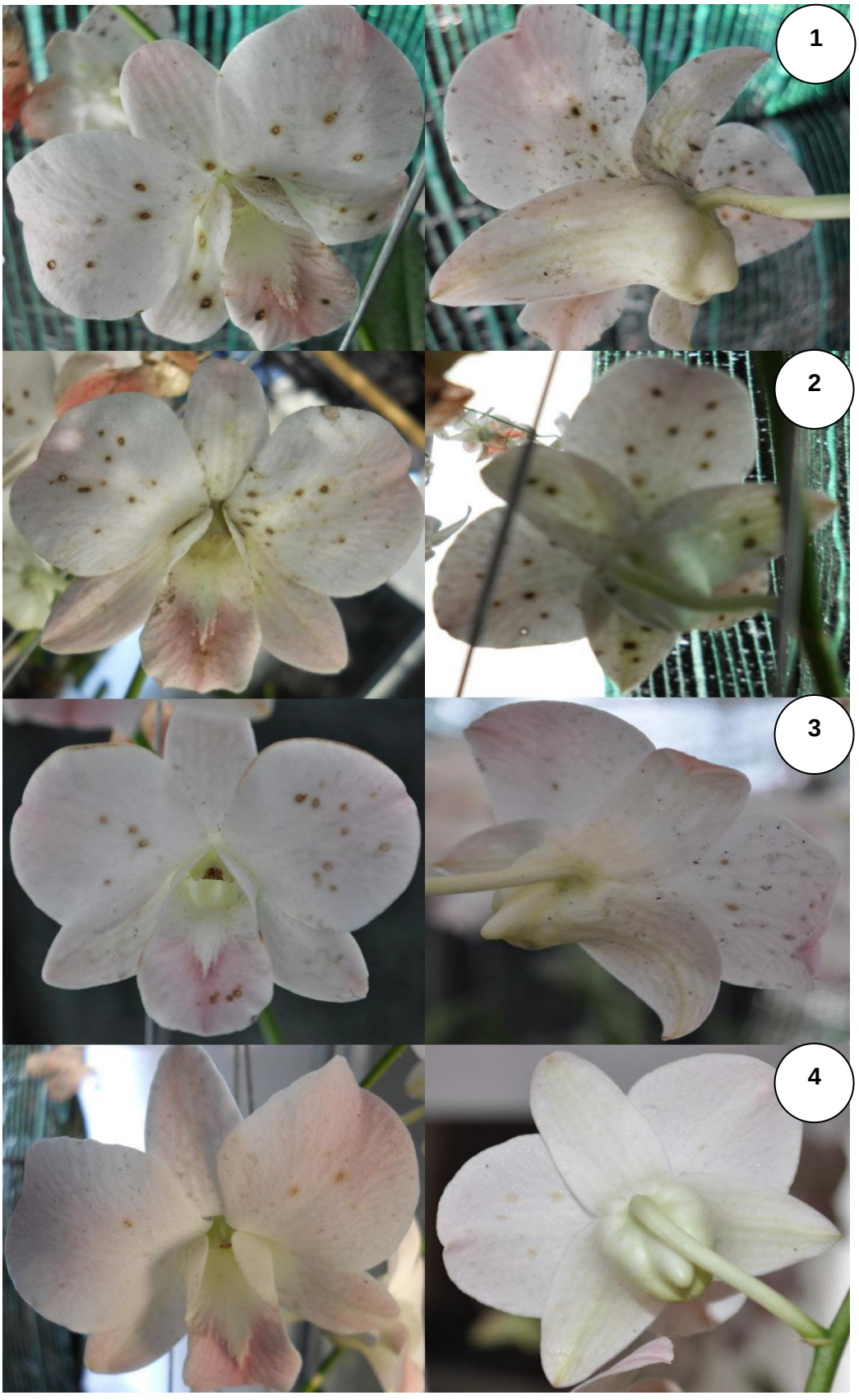

1
2
3
4

Figure 2: Symptoms of the flowers rust spot disease on *Dendrobium* Visa peach hybrid on both front and back petal; sprayed with water (1); water + Tween 80 (2); mancozeb 80% WP (3); harpin (3% a.i.) 0.37 g/l (4); harpin (3% a.i.) 0.75 g/l (5); and *B. subtilis* AP-01 + *T. harzianum* AP-001(6).

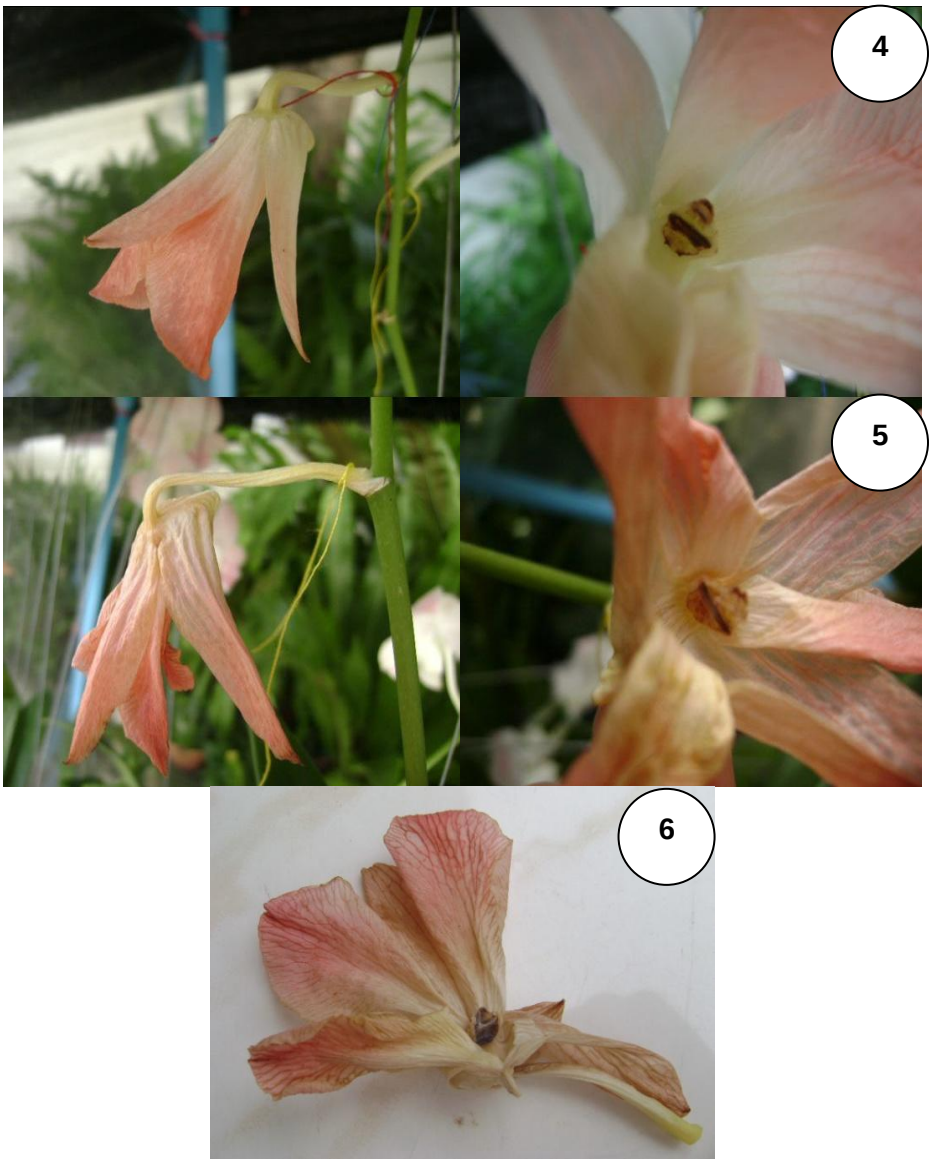
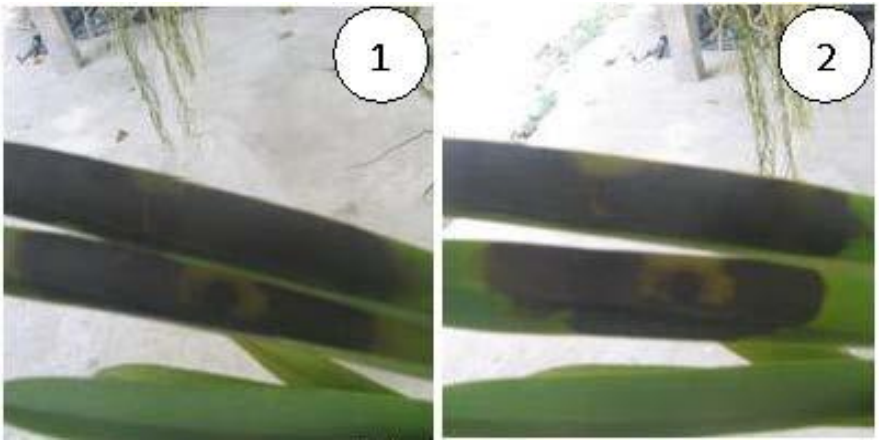


Figure 3: Symptoms of the column blight disease on *Dendrobium* Visa peach hybrid; no symptom (1); column blight noticed and adjacent petals have started withering (2); the petals have started folding (3); the petals have folded and there is wilting around the edge (4); the petals are wilting (5); the petals have fallen (6).



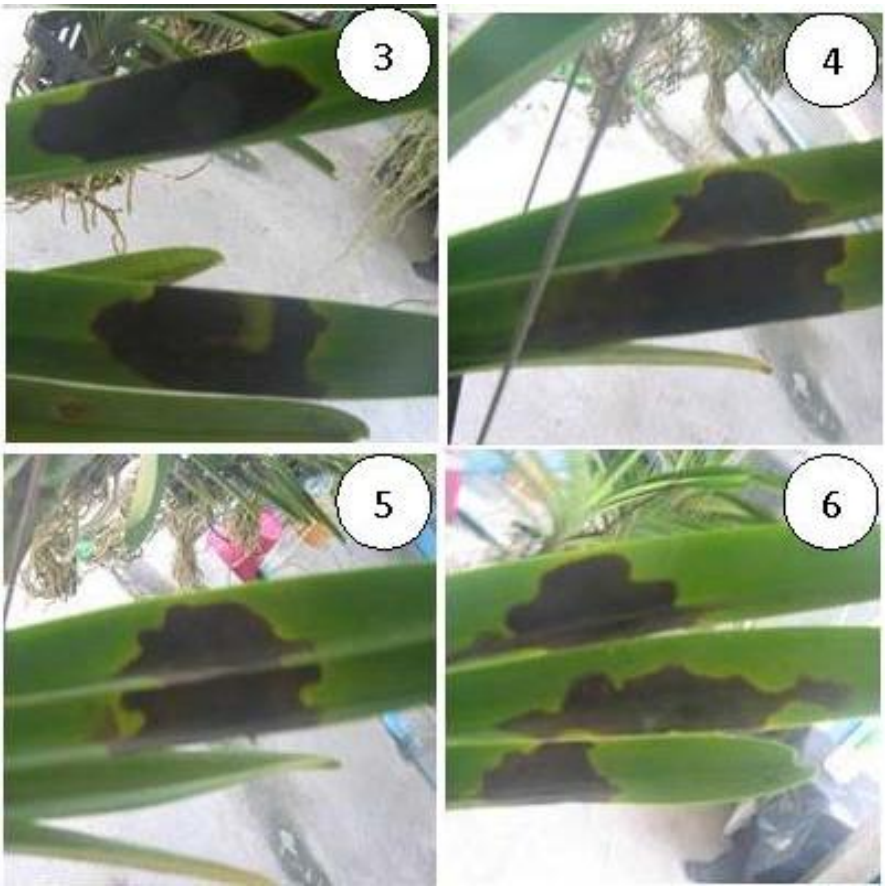


Figure 4: Symptoms of the black rot disease on Vanda Robert's delight hybrid; sprayed with water (1); water + Tween 80 (2); etridiazole + quintozene 6 + 24% EC (3); harpin (3% a.i.) 0.37 g/l (4); harpin (3% a.i.) 0.75 g/l (5); and *B. subtilis* AP-01 + *T. harzianum* AP-001 (6).

Table 1. Testing material and dosage

Testing material and dosage			
Treatment	**For controlling flowers rust spot (*Curvularia lunata*)**	**For controlling column blight (*Fusarium moniliforme*)**	**For controlling black rot (*Phythopthora palmivora*)**
1.	Control #1 sprayed with water	Control #1 sprayed with water	Control #1 sprayed with water
2.	Control #2 sprayed with water + 0.02% Tween 80	Control #2 sprayed with water + 0.02% Tween 80	Control #2 sprayed with water + 0.02% Tween 80
3.	Mancozeb 80%WP Dosage 1.5g/liter water +0.02% Tween 80	Etridiazole+quintozene 6+24% w/v EC Dosage 2.5 ml/liter + 0.02% Tween 80	Etridiazole+quintozene 6+24% w/v EC Dosage 2.5 ml/liter + 0.02% Tween 80
4.	Harpin (3% a.i.) Dosage 0.37 g/liter water +0.02% Tween 80	Harpin (3% a.i.) Dosage 0.37 g/liter water +0.02% Tween 80	Harpin (3% a.i.) Dosage 0.37 g/liter water +0.02% Tween 80
5.	Harpin (3% a.i.) Dosage 0.75 g/liter water +0.02% Tween 80	Harpin (3% a.i.) Dosage 0.75 g/liter water +0.02% Tween 80	Harpin (3% a.i.) Dosage 0.75 g/liter water +0.02% Tween 80
6.	*B. subtilis* AP-01 + *T. harzianum* AP-001 Dosage 2.5 g each/liter water + 0.02% Tween 80	*B. subtilis* AP-01 + *T. harzianum* AP-001 Dosage 2.5 g each/liter water + 0.02% Tween 80	*B. subtilis* AP-01 + *T. harzianum* AP-001 Dosage 2.5 g each/liter water + 0.02% Tween 80

4. Discussions

The SAR molecule harpin protein did not provide broad-spectrum disease control as expected. Obviously, harpin protein was effective in controlling the flowers rust spot disease, but it showed poor disease control for both the column blight and the black rot diseases. This result was most likely caused by harpin protein being a specific-gene elicitor rather than a broad-spectrum elicitor. In addition, the microbial control agents and the chemical fungicides were only able to control the flowers rust spot and column blight diseases and failed to protect against the black rot disease. This result might have been caused by *P. palmivora* having developed resistance against the etridiazole + quintozene fungicide, a lack of synergistic effect by the microbial control agents in controlling the disease or additional applications and/or higher dosages might be required.

Acknowledgements:

The authors would like to thank Appliedchem (Thailand) Co., Ltd for financial support.

References

1. Department of Agriculture. Flowers and ornamentals export data. Bangkok. 2010; 103 p. (in Thai).
2. Copping LG and Menn JJ. Biopesticides: a review of their actions, applications and efficacies. Pest Manag. Sci 2000; 56: 651–676.
3. Wei ZM, Laby R, Zumoff C, Bauer D, Ho SY, Collmer A, Beer S. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 1992; 257: 85–87.
4. Bednarz CW, Brown SN, Flanders TJ, Tankersley TB & Brown SM. Effects of foliar applied harpin protein on cotton lint yield, fiber quality, and crop maturity. Comm. Soil Sci. Plant Anal 2002; 33: 933–945.
5. Wei ZM and Beer SV. Harpin from *Erwinia amylovora* induces plant resistance. Acta Horticulture 1996; 411: 223–225.
6. Wei ZM and Betz FS. Messenger: an environmentally sound solution for crop production and protection. ACS Symp Ser 2007; 957: 195–211.
7. Maketon M, Apisitsantikul J, Siriraweekul C. Greenhouse Evaluation of *Bacillus subtilis* AP-01 and *Trichoderma harzianum* AP-001 in Controlling Tobacco Diseases. Braz J Microbiol 2008a; 39: 296-300.
8. Maketon M, Coghlan PO, Hotaga D. Field Evaluation of Metschnikoff (*Metarhizium anisopliae*) Sorokin in Controlling Cotton Jassid (*Amrasca biguttula biguttula*) in Aubergine (*Solanum aculeatissimum*). Int J Agri & Biol 2008b; 10: 47-51.
9. Tienkao A. Efficacy of *Trichoderma* spp. in inhibiting *Curvularia eragostidis* and controlling the flowers rust spot on *Dendrobium* sp. MS Thesis, Kasetsart University, Bangkok. 2007; 97 p. (in Thai).
10. Pongpitak S. Comparison between the characteristic of the column blight disease caused by *Fusarium moniliforme* and *Penicillium* sp. MS Thesis, Kasetsart University, Bangkok. 1994; 86 p. (in Thai).

4/2/2015