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Efficiency of antifungal compounds against powdery mildew disease of rose (*Podosphaera pannosa*)

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Abstract The Powdery mildew on rose caused by the fungus *Podosphaera pannosa* is a serious problem in comercial rose garden. All aerial parts of the plant can be infected with the production of white mycelium containing conidiospores. Conidiospores can be dispersed by wind or rain to healthy plants. Infection is promoted by high humidity or where poor air movement occur in dryer environments. In this experiment, we observed a rose powdery mildew that was found in rose cultivar "Queen Sirikit" using a light microscope. The morphological characteristics of the anamorph revealed mycelium with indistinct or nipple-shaped appressoria, conidia with fibrosin bodies that measured approximately 6 to 8 x 17 to18 µm and fuliginea-type germtubes. Conidiophores were straight, ca. 5 to 7 x 41 to 80 µm with cylindrical foot-cells. The efficacy of antifungal compounds salicylic acid (SA), fresh cow's milk (10%/V.) and some biocontrol agents: the bacterium *Bacillus subtilis* (BS) and the hyperparasitic fungus *Ampelomyces* sp. were compared with the conventional fungicide carbendazim using an onion tissue bioassay. The results showed that SA inhibited conidia germination by 99.72% followed by BS (98.99%), *Ampelomyces* sp. (95.41%), carbendazim (83.24%) and the last was fresh cow's milk by 80.81%. The control treatment had 100% spore germination. The potential of the antifungal compounds was assessed in a greenhouse experiment and SA was a superior treatment to reduce the severity of disease (57.78%).

Keywords: rose powdery mildew, *Podosphaera pannosa*, salicylic acid, *Bacillus subtilis*, *Ampelomyces* sp., Biological control

Introduction

Powdery mildew (*Podosphaera pannosa*) on rose cultivar "Queen Sirikit" is the most serious disease problem at the Bhubing Palace in Chiang Mai province, Thailand. The mycelium and conidiophores first appear on young parts of rose plants (Carlo *et al*., 1997). The disease is very severe on susceptible rose cultivars. Infection is notably severe at low temperatures and optimal humidity. Temperature seems to be the most important factor affecting several stages of the development of *P. pannosa* on leaves. Given favourable

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conditions, mycelia and conidiospores spread and cover the youngest leaves, often causing distortion, curling, and premature leaf fall. Patches of fungal growth may develop initially on the young stems, especially at the bases of the thorns, a growth that tends to persist when the stems mature. The fungus may also attack the flowers.

The commercial management of powdery mildew relies on fungicide such as carbendazim, triazoles, and chlorothalonil (Linde and Shishkoff, 2003). The use of these fungicides can lead to phytotoxicity and development of fungal resistance. Furthermore, high levels of fungicide can be hazardous to humans and agricultural ecology. Antifungal compounds with low toxicity and low potential risk to the environment can help reduce the use of chemical fungicides as alternative methods for controlling powdery mildews and reducing the severity of powder mildew in greenhouses (Paulitz and Bélanger, 2001). The use of microbial biocontrol agents has potential to effectively replace fungicides in integrated disease management (McGrath, 1991).

The biological control agents (BCAs) were widely applied to control plant disease, as hyperparasitic fungi are specific to host and present no residue in the environment (Belanger and Labbe, 1994). The hyperparasitic fungus *Ampelomyces* was among the first mycoparasites to be studied in detail and infects several genera of powdery mildew (Yarwood, 1932 and Kiss, 1998). Hashioka and Nakai (1980) indicated that *Ampelomyces quisqualis* requires humidity to infect hyphae and grow within powdery mildew hosts. Pycnidia formed within the hyphae, conidiophores, and immature cleistothecia of the host, and its development suppressed asexual and sexual sporulation of the powdery mildew. *Bacillus subtilis* (BS) was also tested against cucurbit powdery mildew caused by *Podosphaera fusca* in Spain (Diego *et al.*, 2007). It was also applied as a supplement to fungicides due to its value in powdery mildew management (Shishkoff and McGrath, 2002).

Another interesting strategy for the control of powdery midew disease is the use of low toxicity antifungal compounds of different origin. Salicylic acid (SA) is a signaling molecule naturally found in plants which is a simple phenolic compound related to signaling pathways in plant cells (Anderson *et al.*, 1998). SA has been shown to induce resistance to infection by fungal, bacterial and viral diseases (Horvath *et al.*, 2007).

The efficacy of fresh cow's milk in controlling powery mildew in several crops including cucumber, zucchini squash, pepper, grape wine or other organic plants has also been researched (Bettiol *et al.*, 2008). Bettiol *et al.* (1999) showed that fresh cow's milk at 5 or 10% whole milk in water was suitable for use against powdery mildew in commercial cucumber crops. The activity of fresh cow's milk mechanisms can interrupt conidia germination of powdery milew or presence of salts (Pasini *et al.*, 1997). Fresh cow's milk can cause indirect effects such as inducing host resistance and enhancing some antagonistic microorganisms on leaf surfaces. Stadnik and Bettiol (2001) reported the increase of microorganisms, especially bacteria, on cucumber when sprayed with milk. In addition, some saprophytic fungi such as *Cladosporium* and *Fusarium* was found but did not affect germination when conidia of *Podosphaera xanthii* infection was present on leaves. Crisp *et al*. (2006) described the application of a solution of milk at 1:10 or 45 g/l and spraying combination with potassium bicarbonate with oil and whey every 10-14 d was as efficient as sulphur in controlling grapevine powdery mildew which was similar to applied whole cow's milk concentration at 5, 10, 20 and 30% when considering the preventive control of downy mildew (Sudisha *et al*., 2011).

The main objective of this study was to assess the efficacy of BCAs and antifungal compounds in controlling powdery mildew in rose.

Materials and methods

Sample sources

Rose powdery mildew (*Podosphaera pannosa*) samples were collected at Bhubing Palace, Chiang Mai Province, Thailand during 2018. The herbarium specimens were deposited at the Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Thailand.

Morphological observation

Light microscopy

The fresh colonies of powdery mildew were stripped off by adhesive tap then mounted in distilled water. Light microscopy with 40X objective phase contrast lenses was used for morphological characteristics observation. For each structure: size and shape of conidia, conidiophore, position of the basal septum, shape and position of hyphal appressoria and presence or absence of fibrosin bodies were measured in 30 replicates (To-anun *et al*., 2007). The conidial germ tubes were recorded follow by method of Hirata (1942).

Laboratory experiments

Effect of the antifungal compounds on spore germination of powdery mildew

Young spores of powdery mildew were inoculated on onion tissue **(**size**:** 1×1 cm^2**)** by the method of Hirata (1942) and 1 mL of suspension of *Bacillus* *subtilis* (10^7cfu/ml), *Ampelomyces* sp. (10^7spore/ml), salicylic acid (SA) 2.5mM or fresh cow's milk 10% was dropped on each sample. Treatments were compared with 1 ml of sterile distilled water which served as the negative control and a conventional fungicide, carbendazim as the positive control. The inoculated treatments were floated on distilled water in a petri dish at room temperature for 24 h. Four replicates were used for each treatment and a light microscope was used to observe spore germination. The percentages of germinated spores were estimated according to the following formula:

$$\text{Percentage of germination} = \frac{\text{No. of germinated spores}}{\text{Total number of spores}} \times 100$$

Percentage of germination inhibition = 100 – Percentage of germination

Greenhouse experiments

Assessment of powdery mildew severity on leaves

The rose cultivar Queen Sirikit naturally infected by *P. pannosa* were examined to estimate disease severity. When the primary infection of powdery mildew is present on leaves they were subjected to six treatments where 1:control (distilled water), 2: *Bacillus subtilis* (10^7cfu/ml), 3: *Ampelomyces* sp. (10^7spore/ml), 4: Salicylic acid (SA) 2.5mM, 5: Fresh cow's milk (10%) and 6: Carbendazim (10cc/20L) and were applied weekly for 8 w finally the disease was estimated in the last week. A randomized complete block design with four replicates was used for data collection. Each treatment had 120 leaflets (30 leaflets/replicate) which were examined to assess the disease severity based on the percentage of infected area using a 0–5 scale divised by Townsend and Heuberger (1943) and Biswas *et al*. (1992) (Figure 1).

Where:

0 = No powdery mildew colonies observed,
1 = 1–20% of infected leaf area,
2 = 21–40% of infected leaf area,
3 = 41–60% of infected leaf area,
4 = 61–80% of infected leaf area,
5 = 81–100% of infected leaf area.

The severity of the disease was calculated using the following formula:

$$\text{Disease severity \%} = \frac{\Sigma\,(n \times v)}{5N} \times 100$$

Where:

n = Number of the infected leaves in each category,
v = Numerical values of each category,
N = Total number of the examined leaves.

The efficiency of the treatments was calculated according to the following formula:

$$\% \text{ Efficiency} = \frac{\% \text{ Infection in the control} - \% \text{ Infection in the treatment}}{\% \text{ Infection in the control}} \times 100$$

Figure 1. Diagrammatic scale of *P. pannosa* showing increasing disease percentages for assessment of disease severity

Effect of antifungal compounds on rose powdery mildew severity in the greenhouse

Greenhouse experiments were carried out on January to March 2018 at the Bhubing Palace, Chiang Mai Province, Thailand. The plants were subjected to the natural infection by powdery mildew. The experiment was laid out using a randomized complete block design (RCBD). Host plants were sprayed with all antifungal compounds as six treatments where 1:control (distilled water), 2: *Bacillus subtilis* (10^7cfu/ml), 3:*Ampelomyces* sp. (10^7spore/ml), 4:Salicylic acid (SA) 2.5mM, 5: Fresh cow milk (10%) and 6: Carbendazim (10cc./20L). All treatments were applied weekly for 8 w and disease was estimated in the last week. Randomized complete block design with four replicates was used for data collection. Each treatment had 120 collected leaflets (30 leaflets/replicate).

Statistical analysis

All experiments were conducted using a randomized complete block design and each treatment was replicated four times. The data from all the experiments were subjected to analysis of variance (ANOVA). Means were compared by using the DMRT ($P \leq 0.05$).

Results

Symptoms

P. pannosa mycelia and conidiophores first appeared as discrete patches on the surface of young leaves. Mycelium on young tissue expanded to the surface of epidermal cells quickly and caused stunting or distortion. In addition, the fungal growth also developed initially on the young stems especially at the bases of the thorns and also attacked the flowers. After infection, an indistinct or nipple-shaped appressorium attached the mycelium to the host cells. Conidiophores produced conidia in chains from their tips and expanded its characteristic whitish colony (Figure 2).

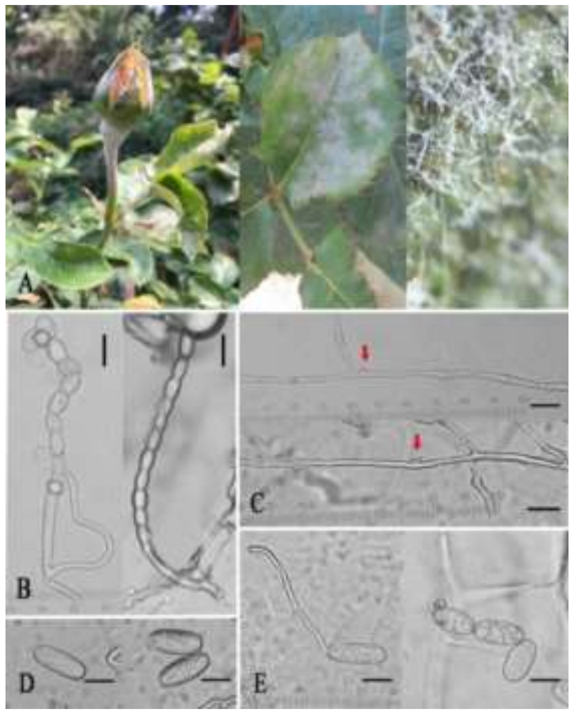

Figure 2. Powdery mildew infections of rose by *P. Pannosa* (A) severe infection on young plant and fungal infections on a flower and leaves, (B) conidiophores produced conidia in chain type, (C) appressoria, (D) conidia with fibrosin bodies, (E) conidia penetrated with germ tubes of the fuliginea-type and pannosa-type. – Bars =10 μm

Laboratory experiments

Effect of antifungal compounds on spore germination of powdery mildew

All antifungal compounds were highly effective in reducing spore germination when compared with distilled water which served as the control. Percentages of spore germination inhibition showed that SA inhibited conidial germination by 99.72%, followed by BS (98.99%), *Ampelomyces* sp. (95.41%) and carbendazim (83.24%). Fresh cow's milk was the least efficient in inhibiting spore germination at 80.81% (Table 1).

Table 1. Effect of antifungal compounds on spore germination of powdery mildew, 24 h after incubation at room temperature

Treatments	Percentage of spore germination inhibition [/1,2]
1.control[*] (distilled water)	0.00 [c]
2.*Bacillus subtilis* (10^7cfu/ml)	98.99[a]
3.*Ampelomyces* sp. (10^7spore/ml)	95.41[a]
4.Salicylic acid (SA) 2.5mM	99.72[a]
5.Fresh cow milk (10%)	80.81[b]
6.Carbendazim[**](10cc./20L)	83.24[b]

[*]1 ml of distilled water (negative control) was dropped on powdery mildew conidia. The number of germinated spores was calculated as 100% severity.
[**]Systemic fungicide (positive control)
[1/]The average was calculated using data from four replications.
[2/] Means within a column, followed by the same letter are not significantly different at the 95% level according to DMRT.

Greenhouse experiments

Effect of antifungal compounds on P. pannosa powdery mildew severity

Initial disease severity was high in all the trials. Application times were weekly for 8 w and estimated at the last week. All treatments significantly reduced the severity of disease when compared to the control treatment. In addition, salicylic acid (SA) was the superior treatment in this regard, being 57.78% efficient, followed by *Bacillus subtilis* (50.00%), carbendazim (46.11%), and fresh cow's milk (19.34%). *Ampelomyces* sp. had the lowest efficiency in reducing the severity of the disease (14.94%) (Table 2, Figure 3).

Table 2. Effect of different spray treatments on *P. pannosa*, powdery mildew disease severity on leaves

	Powdery mildew severity (% leaf coverage)		
Treatments (application time)[a]	Initial severity	8 wk	Efficiency% [/1,2]
1.Control (water)	37.50[b]	83.75[f]	9.38 [c]
2.*Bacillus subtilis* (10^7cfu/ml)	35.00[a]	41.25[b]	50.00 [a]
3.*Ampelomyces* sp. (10^7spore/ml)	38.75[c]	76.25[e]	14.94 [b]
4. Salicylic acid (SA) (2.5mM)	42.56[e]	35.00[a]	57.78 [a]
5. Fresh cow's milk (10%)	38.75[c]	70.00[d]	19.34 [b]
6.Carbendazim (10cc./20L).	40.00[d]	45.00[c]	46.11 [a]

[a/]Application times were weekly for 8 w after initial powdery mildew infection.
[1/]The average was calculated using data from 24 replications.
[2/] Means within a column, followed by the same letter are not significantly different at the 95% level according to DMRT.

Figure 3. Effect of foliar spraying antifungal compounds on severity of powdery mildew on *P. pannosa* after 8 w (A) control, (B) *Bacillus subtilis*, (C) *Ampelomyces* sp, (D) Salicylic acid (SA), (E) Fresh cow's milk, (F) Carbendazim

Discussion

Powdery mildew species on rose (*Podosphaera pannosa*) is a serious problem especially for the susceptible rose cultivar "Queen Sirikit" in Thailand. The fungus spread quickly by wind. The infection occurs in optimal conditions with cool temperature around 22 ℃ and dry weather. The morphological characteristics of rose powdery mildew at anamorphic stage can be viewed by using light microscopy. However, those for telemorphic stages (Chasmothecium) cannot be found. The conidia if present were chains with fibrosin bodies. The appressorium developed an indistinct or nipple-shape that identified the pathogen as *Podosphaera* (Braun *et al*., 2002). In addition, germ tubes branched out from the sides of the conidia which is a characterictic of *Podosphaera* sp. (Cook and Braun, 2009).

Salicylic acid (SA) (2.5mM) was the most effective for conidia inhibition. Argus *et al*. (2015) studied the efficacy of SA on inhibition of conidial germination and effect of *Penicillium expansum* on mycelium growth. The use of salicylic acid solutions adjusted to pH $\leq$ 3 most effectively inhibited conidia germination of *P. Expansum* including blue mold on apples, even at a low concentration, *i.e*., 2.5 mM.

Bacillus subtilis (10^7spore/ml) was more effective than the conventional fungicide carbendazim (10mL/20L). Spraying the treatments significantly reduced the severity of rose powdery mildew. Spraying SA was a superior treatment in this regard, being 57.78% efficient, followed by *Bacillus subtilis* (50.00%), carbendazim (46.11%) and fresh cow's milk (19.34%). Meanwhile, *Ampelomyces* sp. (10^7 spore/ml) was the least efficient with just 14.94% in reducing disease. The *Ampelomyces* sp. activity depended on relative humidity or optimal environmental conditions and host fungi. Yarwood (1939) reported free water can effectively reduce powdery mildew disease. However, spraying water to interrupt spore germination on rose plants, although effective, is not recommened for commercial use, since spraying water causes high humidity on the leaves, leading to further risk of downy mildew and black spot infection.

In conclusion, the results indicated that the use of antifungal compounds SA and the BCAs, especially *Bacillus subtilis*, are effective alternative for powdery mildew disease management that can reduce the use of conventional fungicides. Furthermore, reduction on the use of harmful fungicides and integrated pest management strategies can help reach a better understanding of preventing powdery mildew. Finally, the result of this study can be of help in making decision on the optimal conditions for BCAs application.

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