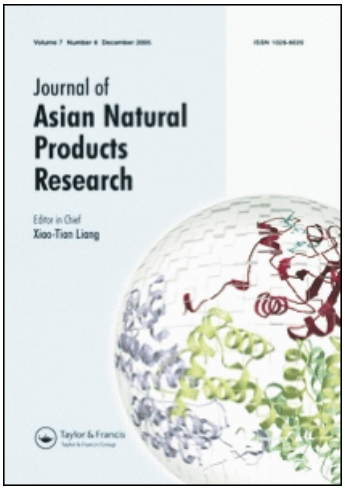

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Efficacy of osthol, a potent coumarin compound, in controlling powdery mildew caused by *Sphaerotheca fuliginea*

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The efficacy of osthol, a natural coumarin compound, in controlling powdery mildew was evaluated in 2004–2005 in Anhui and Hebei Provinces of China. In both years, the treatments (osthol 15.0 and 18.0 g ai ha^{-1}) showed a stable control efficiency of 75.42, 81.24% and 76.36, 84.84%, respectively, at the Institutes of Plant Protection of Hebei Academy of Agricultural Sciences. In field experiments, osthol was as effective as difenoconazole in controlling powdery mildew and was more effective than triadimefon against *Sphaerotheca fuliginea*. Protection was expressed as a significant reduction (up to 87% compared with the control) in the mildewed leaf area in young pumpkin plants. Osthol strongly inhibited spore germination and mycelial growth of *S. fuliginea in vitro*, damaged the cell wall and the organelles of the pathogen. At 48 h after incubation, 50 μg ml^{-1} osthol could completely inhibit spore germination. These findings suggested that the effect of osthol on powdery mildew may be associated with the direct fungitoxic property against the pathogen. We conclude that osthol would be an attractive natural compound for practical agronomic use against powdery mildew.

Keywords: osthol; natural coumarin compound; powdery mildew; *Sphaerotheca fuliginea*

1. Introduction

Powdery mildew, caused by *Sphaerotheca fuliginea*, is a widely distributed and a major foliar disease of greenhouse- and field-grown cucumbers [1]. When commercially acceptable resistant cultivars are not available, the disease control is generally achieved by the use of fungicides including strobilurins, benzimidazoles, demethylation inhibitors (DMIs), β-methoxyacrylates, and sulfur [2–4]. However, the powdery mildew fungus has a great capacity to develop resistance to synthetic fungicides such as DMIs, strobilurins, benomyl, and other inhibitors [5–9]. Once resistant strains appear, most of them survive for several years, therefore further applications of fungicides from the same group increase the risk of reinforcing resistant populations of the pathogen [10,11]. To cope with the need to safeguard cultivated crops without imposing deleterious or even catastrophic impacts on the environment, there is a constant plea worldwide for the development of environmentally safe agricultural fungicides for practical application [12,13]. This intensifies the need for new compounds, with different modes of actions for disease

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control and for minimizing the development of fungicide resistance. Among the alternative methods developed for controlling the disease, the use of plant extracts has been the focus of considerable interest.

Cnidium monnieri (L.) Cusson, found in China, is one of the medicinal plants belonging to the Umbelliferae family. Compound **1**, a natural coumarin compound [$C_{15}H_{16}O_3$, 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one (**1**), Figure 1] isolated from the dried fruits of *C. monnieri*, has been widely used as a traditional Chinese medicine for many human diseases and a series of studies on pharmacological effects have been conducted earlier [14–17]. However, few studies on the effect of **1** on fungi have been conducted so far, although several *in vitro* experiments on fungi suggest that **1** may be effective in the suppression of phytopathogenic fungi [18]. In this regard, a series of studies have been conducted to explore the antifungal activities of **1** as well as the mode of action for plant disease control in this study. Our previous experiments showed that **1** had a wide spectrum of antifungal activity against important plant pathogens such as *Rhizoctonia solani*, *Phytophtora capsici*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Fusarium graminearum* [19].

Our objectives for the present study were to: (1) assess the preventative effect of **1** against *S. fuliginea in vitro*, (2) test the efficacy of **1** in controlling powdery mildew on cucumber, and (3) determine the related mode of action.

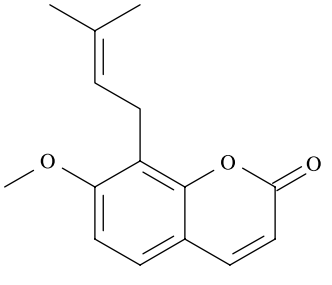

Figure 1. Structure of **1**.

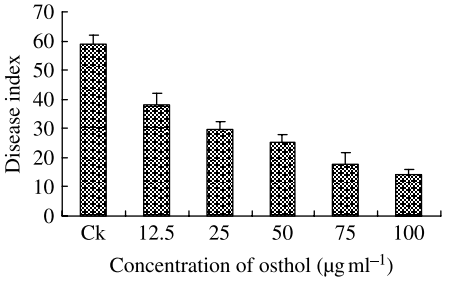

Figure 2. Sensitivity of *S. fuliginea* to **1**. The infected pumpkin seedlings were sprayed with different concentrations (12.5, 25, 50, 75, 100 $\mu g\,ml^{-1}$) of **1**. Disease grades of different treatments were recorded. Sensitivity of *S. fuliginea* to **1** was valued by the control efficacy shown by the disease index. Each value represents the means of the disease index made on nine plants. Error bars indicate the standard error of the means.

2. Results and discussion

2.1 The preventative effect of 1 against S. fuliginea

Previous researches indicated that **1** had a broad spectrum of antifungal activity [19]. However, the effects of **1** on obligate parasite fungi have not been reported till now. Here, we tested the preventative effect of **1** against *S. fuliginea*. Results (Figure 2) showed that **1** could decrease the disease index of powdery mildew by restricting pathogen growth and its spread. Moreover, disease severities correlated negatively with application concentrations of **1**. In addition, the EC_{50} value (effective concentration that causes 50% disease control) of **1** against powdery mildew was close to 50 $\mu g\,ml^{-1}$. Also, the growth and spread of powdery mildew fungi were effectively controlled by 200 $\mu g\,ml^{-1}$ of **1** (results not shown).

2.2 Effect of 1 on the germination of conidia of S. fuliginea

We tested the effect of **1** on spore germination of powdery mildew on 1% water agar plates, and the results (Figure 3) showed that **1** could inhibit spore germination compared with the control. At 48 h

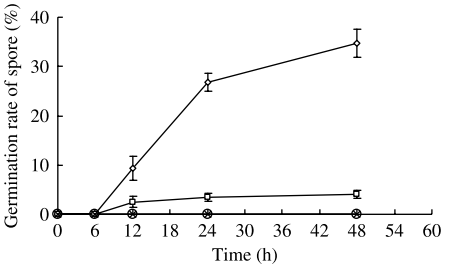

Figure 3. Inhibition effect of **1** on the spore germination of *S. fuliginea*. Compound **1** was dissolved within 1% water agar plates and fresh spores of *S. fuliginea* were shaken from the pumpkin leaves onto the surface of the agar with 0 (–◇–), 25 μg ml^{-1} (–□–), 50 μg ml^{-1}(–○–), and 100 μg ml^{-1} (–×–) of **1**. Spore germination rates were calculated at 0, 6, 12, 24, and 48 h after inoculation. In the untreated control, there was 34.8% of spore germination of *S. fuliginea*. Spores were considered to have germinated if the length of the sperm tube exceeded the diameter of the spore. Four experiments with three replicates for each value. Error bars indicate the standard error of the means.

after incubation, 50 μg ml^{-1} of **1** could completely inhibit spore germination and less than 5% conidia treated with 25 μg ml^{-1} of **1** germinated, while the germination rates of the control were both beyond 30%. Moreover, we tested spore germination on detached leaves and living leaves, and the results were similar to the assay conducted on water agar plates (data not shown).

2.3 Effect of 1 on the morphology of S. fuliginea

SEM and TEM observation revealed that treatment with **1** could cause the breakage of spores and abnormal hyphal morphology. The normal conidia of *S. fuliginea* had a smooth surface (Figure 4(a)) and intact cell wall and the organelles in the cell were clearly seen (Figure 4(c)). However, the **1**-treated spores were wizened and the surface of the cell was rough and shrinking (Figure 4(b)). In particular, parts of the cell wall were blurry and melting. These

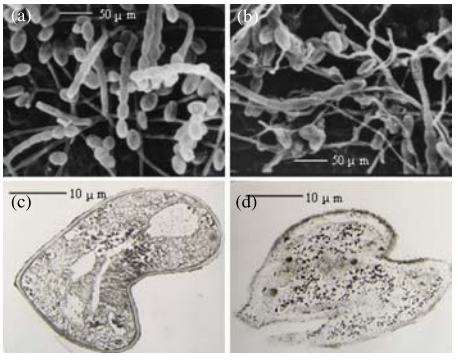

Figure 4. Effect of **1** on the morphology of *S. fuliginea*. (a) Blank control of SEM; (b) SEM of *S. fuliginea* 96 h after the spray of 50 μg ml^{-1} of **1**; (c) blank control of TEM; (d) TEM of *S. fuliginea* 96 h after the spray of 50 μg ml^{-1} of **1**.

dispelling phenomena were also observed in the cytoplasm where organelles were melting and not clearly identified. The contents of the **1**-treated fungus had become homogeneous and the integral morphology of fungi was greatly changed (Figure 4(d)).

2.4 Suppression of 1 on powdery mildew on leaf disks

There was a significant reduction (up to 83.1% in the percentage of germinated conidia with multiple germ tubes, relative to the control) when leaf disks were treated with 100 μg ml^{-1} of **1** after 72 h. The average hyphae length was approximately 410 μm at 48 h after inoculation in the control, however it was only 120 μm at a concentration of 100 μg ml^{-1} of **1**, showing a reduction of 70% in the hyphae length (Figures 5 and 6).

2.5 Effect of powdery mildew control

During the 2004–2005 seasons, there was a good disease development in the experimental fields. In the field trial at the Institutes of Plant Protection of Hebei Academy of Agricultural Sciences (IPP-HAAS), there was little visual disease before the first spraying in 2004 and 2005. The treatment (**1**, 22.5 g ai ha^{-1}) provided

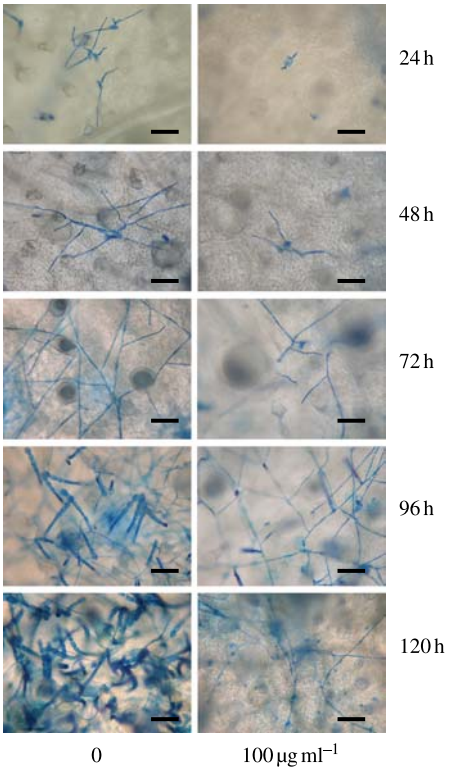

Figure 5. Effect of **1** on the mycelial growth and conidiation at 24, 48, 72, 96, and 120 h after inoculation. Scale bar = 100 μm.

a higher control efficiency superior to that of triadimefon ($p < 0.05$) in both years (Table 1). The control efficacy of treatments of **1** at 22.5 g ai ha^{-1} was slightly lower than that of difenoconazole treatment. In both years, the treatments (**1**, 15.0 and 18.0 g ai ha^{-1}) showed a stable control efficiency of 75.42, 81.24% and 76.36, 84.84% at the IPPHAAS, respectively. In 2004 and 2005, similar results were obtained at both experimental sites.

The field trials showed that **1** applied at 22.5 g ai ha^{-1} provided better efficiency than triadimefon applied at 225 g ai ha^{-1}, and similar efficiency to difenoconazole applied at 45 g ai ha^{-1}, implying that **1** is a good candidate for controlling powdery mildew.

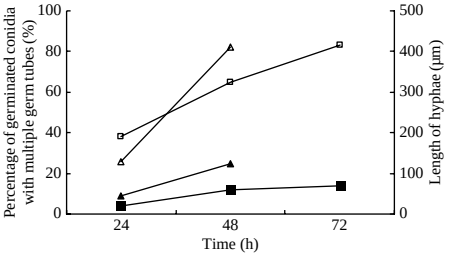

Figure 6. Suppression of **1** (100 μg ml^{-1}) on powdery mildew on leaf disks. Based on at least 100 independent observations. Length of hyphae at 72 h was too long to be measured. Length of hyphae 0 (—△—) and 100 μg ml^{-1} (—▲—). Percentage of germinated conidia with multiple germ tubes 0 (—□—) and 100 μg ml^{-1} (—■—).

2.6 *Discussion*

The major novel findings of the present study were that **1**, a natural coumarin compound isolated from the dried fruits of *C. monnieri*, which has been widely used as a traditional Chinese medicine, can control powdery mildew. Compound **1** has been widely used as an antimicrobial medicine for human diseases [14–17]. However, little attention has been paid to its antifungal activity and related mode of action. To the best of our knowledge, this is the first report to describe the efficacy of **1** on phytopathogenic fungi and the mode of action for powdery mildew.

The field data presented here demonstrated that **1** was as effective as difenoconazole in controlling powdery mildew on cucumber and more effective than triadimefon against *S. fuliginea*. Overall, observations during the 2004–2005 growing seasons showed that increasing the concentration of **1** from 15.0 to 22.5 g ai ha^{-1} improved its efficacy against powdery mildew, and caused no phytotoxicity on plant foliage. The data in the present paper, on the control of powdery mildew, as observed on field-grown cucumber, make **1** an attractive compound for practical agronomic use against *S. fuliginea*. This indicated that **1**, a natural coumarin compound, might have a unique mode of action.

Table 1. Efficacy of 1% osthol EW in controlling powdery mildew of cucumber at the AICA and IPPHAAS, China in 2004 and 2005.

			2004			2005		
Treatment	Cucumber cultivar	Dosage (g ai ha^{-1})	Initial disease index*	Final disease index**	Control efficacy (%)***	Initial disease index*	Final disease index**	Control efficacy (%)***
AICA	JinYou1							
1% Osthol (EW)		15.0	0.43	3.06	71.36 ± 1.92Cb	1.40	6.05	64.96 ± 4.86Bc
		18.0	0.47	2.78	76.19 ± 1.79Bab	1.52	4.53	75.83 ± 2.25Ab
		22.5	0.35	1.78	79.53 ± 2.87Aa	1.45	2.96	83.45 ± 4.12Aa
15% Triadimefon		225	0.40	2.81	71.72 ± 3.18Bc	2.09	6.59	74.43 ± 5.53ABb
Ck			0.45	11.18		1.54	18.99	
IPPHAAS	JinLv3							
1% Osthol (EW)		15.0	0	4.19	75.42 ± 3.18Bc	0	5.26	76.36 ± 3.09Cc
		18.0	0	3.20	81.24 ± 4.09Bb	0	3.40	84.84 ± 3.24Bb
		22.5	0	1.94	88.43 ± 3.51Aa	0	2.20	90.14 ± 1.32ABa
10% Difenoconazole		45.0	0	1.13	93.34 ± 1.65Aa	0	1.47	93.40 ± 2.15Aa
Ck			0	16.96		0	22.21	

Notes: *Disease index before the first spraying.
** Disease index after three foliar sprays at 7-day intervals.
*** Means with columns followed by the same letters are not significantly different at $p = 0.05$ and 0.01 according to Duncan's multiple range test (a: $p = 0.05$; A: $p = 0.01$).

Investigation of **1** on different stages of life cycle of *S. fuliginea* may partially explain the mode of action of foliar-applied **1** in controlling powdery mildew. Compound **1** at 25 or 50 $\mu g\,ml^{-1}$ could inhibit germination of *S. fuliginea* conidia by 85.7 and 100%, respectively (Figure 3). Spraying of 100 $\mu g\,ml^{-1}$ of **1** on potted-grown pumpkin leaves before inoculation with *S. fuliginea* provided 75% protection against powdery mildew development (Figure 2). This indicated that **1** had a better inhibitory effect on spore germinability than mycelial growth of *S. fuliginea*. Spraying **1** at the same concentration on leaf disks before dusting fresh conidia of *S. fuliginea*, only 14% of conidia formed multiple germ tubes 72 h after inoculation (Figure 6). SEM and TEM studies have been carried out for understanding the morphological changes of *S. fuliginea* after treating with 100 $\mu g\,ml^{-1}$ of **1**. Visible symptoms of the colonies and shrinkage of conidia and hyphae have been observed (Figure 4). These findings indicate that the preventive action of **1** against the mycelial growth and spore germination is quite powerful; the unique modes of action may exist in the antifungal mechanism of **1** and it may have the potential to reduce initial infection and spread of the pathogen.

Management of the resistance relies on reducing selection pressure by limiting the plant's exposure to fungicides with the same mode of action, or using replacement fungicides with different biochemical mechanisms of action [20,21]. Compound **1** did exhibit strong fungicidal activity against the plant pathogen *S. fuliginea in vitro* and had the expected effect in field trials at different sites. Furthermore, a lack of any cross-resistance between **1** and other tested fungicides suggests that the mode of action and resistance mechanism of **1** was unique from other fungicides (data not shown). The novel mode of action of **1** may prevent or delay the development of fungicide-resistant pathogens, because there is no cross-reactivity with other fungicides currently used on the market. Since selection pressure on plant pathogens may increase upon the introduction of additional **1**, more research is required to study **1** target sites and to investigate the likelihood of target mutations and the occurrence of **1** cross-resistance. Alternating **1** in spray programs, or its use in mixtures with other fungicides, such as DMIs, should reduce the development of populations of *S. fuliginea* resistant to other fungicides. This practice could also enhance the performance of **1** and provide greater timing flexibility. It will be evaluated as an important subject in our following researches.

In conclusion, the present study shows that **1**, as a natural compound, could directly inhibit the germination of conidia of *S. fuliginea* and the mycelial growth of powdery mildew. Furthermore, **1** has a beneficial effect on controlling powdery mildew in the field. These suggest that **1** is promising as a natural fungicide to partially substitute for the practical use of synthetic fungicides for powdery mildew control.

3. Materials and methods

3.1 Experimental fungicides

Compound **1**, formulated as a 1% emulsion in water (EW), which was registered in 2006 for powdery mildew control in China, was used in field experiments. The DMI fungicide triadimefon (15% WP; Kesheng Group Co., Yancheng, Jiangsu, China) and difenoconazole (10% WG; Syngenta Company, Basel, Switzerland) were used as standard fungicides. Compound **1** (99.5%) used in laboratory experiments was of technical grade and purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, P.R. China). Compound **1** was dissolved in ethanol at 2×10^4 $\mu g\,ml^{-1}$ for stock solutions and stored in the dark at 4°C.

3.2 Laboratory experiments

3.2.1 Pathogen isolation and its cultivation

A single isolate of *S. fuliginea*, collected on pumpkin seedling (Youfeng1) grown in a glasshouse of the Jiangsu Academy of Agricultural Science (China), was used for experiments [22]. Susceptible pumpkin plants (Jinxinmici) placed under plastic covers in a growth chamber at 25°C and 12 h photoperiod were used for the pathogen maintenance and multiplication [23].

3.2.2 Plant material and S. fuliginea inoculation

Seeds of pumpkin were sown in plastic boxes containing Perlite. Seedlings with fully developed cotyledons were transferred to plastic plots of 7 cm in diameter containing garden soil. Plants were grown in a growth chamber at 25°C and 12 h photoperiod and used for experiments at six-leaf stage. Leaves of pumpkin, which were 80–100% covered by fresh sporulating mycelium of powdery mildew, were used as a source of inoculum. The pumpkin plants were inoculated with conidia of *S. fuliginea* by surface contact (dusting/tapping).

3.2.3 The preventative effect of *1* against S. fuliginea

To test the preventative effect of **1** against *S. fuliginea*, **1** was sprayed at concentrations of 0, 12.5, 25, 50, 75, and 100 $\mu g\,ml^{-1}$ on the upper surfaces of each of six-leaf plants. Plants were allowed to dry and inoculated with *S. fuliginea*. After inoculation, plants were kept in a growth chamber at 25°C and 12 h photoperiod for disease development. Disease was rated on total leaves of nine plants per treatment 7 days after inoculation. The percentage of leaf area covered with mildew was visually estimated and recorded according to a 0–5 scale [24]: 0, no symptom; 1, 1–5% of the leaf area infected with mildew; 2, 6–15%; 3, 16–30%; 4, 31–60%; 5, >60%. Disease index was assessed by the following formula:

$$\text{Disease index} = \frac{\sum[\text{disease rating} \times \text{no. of leaves of that specific rating}]}{\text{maximum possible score} \times \text{total number of leaves examined}} \times 100.$$

3.2.4 Effect of *1* on the germination of conidia of S. fuliginea

Compound **1** was dissolved in 1 ml ethanol and mixed with sterile distilled water to give a stock solution of a known concentration. Compound **1** was mixed with pre-autoclaved 1% water agar to give final concentrations of 0, 25, 50, and 100 $\mu g\,ml^{-1}$ of **1**. Conidia were shaken onto glass slides previously coated with water agar containing **1** (four slides for each concentration). Slides were then placed in Petri dishes containing wet filter paper and kept in the dark at 25°C. After intervals of 0, 6, 12, 24, and 48 h after inoculation, the number of germinated conidia was counted under a microscope in three microscope fields, each containing 40–60 spores. Conidia were considered germinated only if the newly grown hyphae were at least twice the length of the conidia [25].

3.2.5 Effect of *1* on the morphology of S. fuliginea

A stock solution of **1** was freshly diluted to 50 $\mu g\,ml^{-1}$ to spray on pumpkin leaves infected by *S. fuliginea*. For scanning and TEM, infected leaves were sampled 4 days after the spray, and untreated leaves were also sampled as the control. Leaf samples were fixed with 3% (v/v) glutaraldehyde in 50 $mmol\,l^{-1}$ phosphate buffer (pH 6.8) for 3–6 h at 4°C. Thereafter, the samples were thoroughly rinsed with 50 $mmol\,l^{-1}$

phosphate buffer (pH 6.8) and post-fixed with 1% (w/v) osmium teroxide in the same buffer for 2 h at 4°C. After rinsing with the buffer, the samples were then dehydrated in a graded acetone series. Part of samples were critical-point dried, mounted on stubs, sputter coated with gold–palladium, and examined with a Philip SEM-505 scanning electron microscope operating at 20 kV. The other samples were infiltrated, embedded with Epon-Araldite, and polymerized at 60°C for 24 h for TEM observations. Ultrathin sections of the samples were cut with a diamond knife and collected on 200-mesh copper grids. After contrasting with uranyl acetate and lead citrate, the grids were examined with a HITACHI H-600 electron microscope at 75 kV [26].

3.2.6 *Suppression of **1** on powdery mildew on leaf disks*

Ten leaf disks (each 5 mm in diameter) were cut from the healthy leaves of the same position (third from the tip) and sprayed with 100 $\mu g\,ml^{-1}$ of **1**. The fog drop bestrewed the surface of the leaves. The experiment was conducted twice, with 20 leaf disks for each treatment. Fresh conidia were dusted onto leaf disks through a copper grid with a 0.8 mm pore size. This allowed the deposition of approximately 100 spores per inoculum site. Leaf disks were then incubated at 100% relative humidity and 25°C. After intervals of 24, 48, 72, 96, and 120 h, leaf disks were laid adaxial surface up on filter paper moistened with an ethanol:glacial acetic acid mixture (3:1, v/v) for 24–48 h until the chlorophyll had been removed. The leaves were then stained in lactophenol-cotton blue (0.05% trypan blue in equal volumes of lactic acid, glycerin, and phenol) for another 24 h to show fungal structure [27,28]. Stained samples were examined under a light microscope. The percentage of conidia with multiple germ tubes and the average hyphae length were determined. These measurements were made on at least 100 colonies. Leaf disks for the controls were prepared in the same way using water. Individual colonies were photographed using a Nikon YS100 camera.

3.3 *Field experiments*

Field experiments using cucumber were conducted in greenhouses located at the IPPHAAS and Anhui Institute for the Control of Agrochemicals (AICA), China in 2004 and 2005 growing seasons. All test cultivars (Table 1) were found to be susceptible to the pathogen, and diseases had been evident in these fields in previous years. The cultivars were planted following normal agronomic practice. The five treatments at these sites were: (1–3) experimental formulation of **1** applied at 22.5, 18.0, and 15.0 g ai ha^{-1}, (4) triadimefon applied at 225 g ai ha^{-1} or difenoconazole applied at 45 g ai ha^{-1}, and (5) the control treated with water. Treatments in each trial were arranged in a randomized complete block design with four replicates. Each plot had an area of 16 m^2. Fungicides were applied after the appearance of the disease on leaves. Spraying was carried out with a plot sprayer at a volume rate of 900 liters ha^{-1}. Three foliar sprays of the fungicides were applied to cucumber at 7-day interval. All experiments were conducted in fields naturally infested with the powdery mildew fungus. No other fungicides were applied to the experimental plots. Methods of fertilization, irrigation, and other cultural practices for tested cucumber were in accordance with standard form products. Visual disease assessment was made before spraying and 10 days after spraying. Disease severity was evaluated on each leaf of each of the eight plants from each plot, based on the percentage of the mildewed leaf area. Disease index was assessed using a five-class rating scale (described as in Section 3.2.3). Efficacy percentage (%) was determined by the formula: [1 − (disease index

of Ck_0 × disease index of Pt_1/disease index of Ck_1 × disease index of Pt_0)] × 100, where Ck_0 is the control before spraying water; Ck_1, the control after spraying water; Pt_0, the treatment before spraying the fungicide; and Pt_1, the treatment after spraying the fungicide.

3.4 Statistical analysis

Duncan's multiple range test was applied to determine whether the differences between treatments were significant.

Acknowledgements

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