Front. Agric. China 2009, 3(4): 425–430
DOI 10.1007/s11703-009-0066-2

RESEARCH ARTICLE

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Establishment and application of bioassay method for screening fungicides against cucumber powdery mildew

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Abstract *Sphaerotheca fuliginea* Poll., a pathogen causing powdery mildew in cucumber, was used as a target for establishing a bioassay method for screening fungicides. Different cucumber varieties, seedling ages, spore culturing times, and inoculation concentrations were tested. The results showed that a highly susceptible variety "Xinchangchunmici" was a suitable cultivar for bioassay test, with the appropriate seedling age of 5–10 d, the spore culturing-time of 10–15 d and the spore concentration of 30–40 spores per field of vision (10 × 10 times under microscope) in the suspension. Cotyledon-spraying method, and leaf-disc method were compared for bioassay and Trypan Blue staining method was used for detecting the development of *S. fuliginea* on the leaf surface. Based on the bioassay system established above, the protective effect of chrysophanol on the development of *S. fuliginea* was evaluated using cotyledon-spraying method. The results showed that chrysophanol reduced the disease index effectively and the protective EC_{50} value was 33.36 $\mu g \cdot mL^{-1}$.

Keywords *Sphaerotheca fuliginea* Poll., cucumber powdery mildew, bioassay system, chrysophanol

1 Introduction

Powdery mildew, caused by *Sphaerotheca fuliginea* Poll., is one of the most important diseases in cucumbers (Dikl et al., 1998). Fungicides and resistant cultivars are the major means to control the disease on cucumber (Cohen, 1993). However, with intensive and long-term use of fungicides, pathogens have developed different degrees of resistance, and control was significantly reduced (Reuveni et al., 1996; Zhou et al., 2001; Ma et al., 2007). Therefore, it is necessary to develop new fungicides with high efficiency, low toxicity and low-residue to control powdery mildew of cucurbit crops. High-throughput screening technology was adopted abroad, which can conduct a large amount of biological activity screening over a short period of time, and this method is micro-quantitative, quick and highly efficient. As an obligate parasite, *S. fuliginea* can not grow and reproduce on the medium (Zhang, 2005), and it is difficult to adopt high-throughput screening methods. Hence, it is important to establish a scientific, standardized and rapid bioassay system to screen active ingredients against powdery mildew (Zhang, 2004).

The cotyledon-spraying method (Jia et al., 2006) was used as a basis, while many aspects of hosts, pathogens and inoculation methods need to be optimized. The objective of our study was to establish a more standardized fungicide screening system for cucumber powdery mildew, therefore providing a reliable test technology for screening and evaluating new fungicides in the future.

Chrysophanol is a free anthraquinone compound and is the secondary metabolite of rhubarb. Tang reported that the crude extract from Chinese rhubarb had good control against cucumber powdery mildew (Tang et al., 2003) and the chrysophanol could be viewed as one of the active ingredients in rhubarb (Tang et al., 2005). In our study, the biological activity of *in vivo* chrysophanol to cucumber powdery mildew was measured by the improved bioassay system.

Received January 4, 2009; accepted April 22, 2009

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2 Materials and methods

2.1 Materials

Cucumber varieties were "Xinchangchunmici", a highly susceptible variety and produced by Xiangfang Seed Co., Ltd. (Harbin, China), "Shennongchunwu" provided by

Jinnan District Seed Company (Tianjin, China), "Funong 3" provided by Fubon Agricultural Products Co., Ltd. (Jinan, Shandong Province, China), "Shennongchunsi" by Shen Nong Seed Industry Co., Ltd. (Tianjin, China) and "Xinchun 4" by the New Fu Shan Seed Industry Co. Ltd. (Xintai City, Shandong Province, China). The *S. fuliginea* was provided by the laboratory of Plant Disease Epidemiology and Integrated Control, Agricultural University of Hebei.

Active ingredient tested and staining agent were 98% Chrysophanol (Ronghe Medicine Technology Development Co., Ltd., Shanghai, China) and Trypan Blue (Sigma).

2.2 Methods

2.2.1 Seedling cultivation

Dry seeds were soaked in a flask filled with 55–60°C warm water for about 10 min, then transferred to a water bath pot of 30°C to soak for another 4–6 h. The treated seeds were placed in Petri dishes with filter paper at the bottom and two layers of gauze above. Seed germination was done in an incubator at a temperature of 30°C for 24 h. When the buds were about 5 mm long, every two seeds were planted about 3 cm apart into one plastic cup (6.9 cm in diameter) containing vermiculite and perlite (1:1, v:v). When the cotyledons were fully expanded in the greenhouse, they were ready for inoculation.

2.2.2 Culturing of *Sphaerotheca fuliginea*

The cucumber cotyledons with fresh disease spots were cut off and put into a solution of 10 $\mu g \cdot mL^{-1}$ sodium dodecyl sulfate (SDS) to wash the conidia off and the spore suspension was diluted to 30–40 spores per field of vision (10 × 10 times under microscope). The cucumber cotyledons were uniformly inoculated using a miniature sprayer, and then cultured in a growth chamber at (25±2)°C with a relative humidity of 60% and a 14 h photoperiodic regime. When the disease symptoms appeared, the spores were ready to use for inoculation.

2.2.3 Comparison of bioassay methods

Trypan Blue staining method: The whole plant was inoculated during its cotyledon stage with the conidia suspension mentioned above, and then cultured in the growth chamber at (25±2)°C with 60% relative humidity and a 14 h photoperiodic regime. According to Kuzuya and others (Frye and Innes, 1998; Yao et al., 1998; Kuzuya et al., 2003; Zhu et al., 2006; Li et al., 2007), leaf disks (9 mm in diameter) were removed from cotyledons by means of a cork borer 18, 24, 48, 72, 96, 120, and 144 h after inoculation, respectively. Then the leaf disks were decolorized in 99% ethanol for 20 min at 95°C and stained with 0.05% trypan blue in equal volumes of lactic acid, glycerin, phenol, and water for 5 min at 95°C. Then, the stained leaf disks were examined under a light microscope.

Leaf disc method: According to Yang et al. (2007a) and Cohen (1993) with some modifications used in our test, two kinds of culture media were prepared including (A) 1% agar + 1% sucrose + 5 $\mu g \cdot mL^{-1}$ benzimidazole and (B) 0.5% water agar amended with 25 $\mu g \cdot mL^{-1}$ benzimidazole. Leaf disks (9 mm in diameter) were removed from cotyledons by means of a cork borer after inoculation. Then they were placed on the culture medium in Petri dishes and cultured under the condition described in the section 2.2.2. Disease severity was investigated when disease symptoms were fully observed and the preservative effects of the two types of media were compared. Disease severity assessment was conducted meeting the following requirements of 0 = disk central should be standard green, noninfected; 1 = a small amount of mycelium or lesion is visible, with an area of no more than 10% of the entire disks; 2 = lesion area amounts to 30% of the entire disks; and 3 = lesions or spores cover the entire disks (Chen et al., 2003). Disease index was calculated using the following equation of Fang (1998):

$$\text{Disease index} = \frac{\sum(\text{the number of leaf disks on this level} \times \text{grade level})}{\text{the total number of leaf disks} \times 3} \times 100. \quad (1)$$

Cotyledon-spraying method: The spore suspension was prepared as mentioned in 2.2.2, then the cucumber cotyledons were uniformly inoculated using a miniature sprayer, and then cultured in the growth chamber at (25±2)°C with 60% relative humidity and a 14 h photoperiodic regime. Disease severity was investigated when disease symptoms were fully observed. And the 5 cucumber varieties mentioned above with different seedling ages (5–12 d after planting) were tested for susceptibility. Simultaneously, spores at different cultivation ages from 9 to 21 d and spore suspension concentration containing 10–20, 20–30, 30–40, 40–50, 50–60, 60–80, 80–100 spores per field of vision (10 × 10 times under microscope) respectively, were tested for pathogenicity. Disease severity assessment was conducted meeting the following requirements of 0 = noninfected; 1 = a small amount of mycelium or lesion visible, with an area of no more than 5% of the entire cotyledon; 3 = lesion area amounting to 6%–25% of the entire disks; 5 = lesion area amounting to 11%–20% of the entire disks; 7 = lesion area amounting to 21%–40% of the entire disks; and 9 = lesion area more than 40% of the entire disks (Pesticide). Disease index was calculated based on Equation (1) and the relative control efficacy was calculated using the following equation:

$$\text{Relative control efficacy(\%)} = \frac{\text{Disease index(CK)} - \text{Disease index(Treated)}}{\text{Disease index(CK)}} \times 100. \quad (2)$$

2.3 Application of this system

The protective effects of 98% chrysophanol on the development of *S. fuliginea* were determined by our optimized system above.

3 Results

3.1 Susceptibility of different cucumber cultivars

The susceptibilities of different cucumber cultivars were tested. Table 1 shows that Xinchangchunmici was significantly more susceptible than the other varieties, and hence it is suitable to use it as a standard cultivar for bioassay test.

Table 1 Susceptibility difference of different cucumber cultivars

variety	disease index
Xinchangchunmici	96.7a
Xinchun 4	72.2b
Funong 3	68.9bc
Shennongchunsi	63.3bc
Shennongchunwu	55.6c

Note: The different letters in the same column represent significance at 0.05 probability level.

3.2 Susceptibility of different seedling ages

Disease severity was different when cucumber cotyledons were inoculated at different seedling ages (Table 2), but there was no significant difference between 5- to 10-day-old cotyledons. As cotyledons were getting older, the disease level decreased. Therefore, the 5- to 10-day-old cotyledons were suitable for bioassay test.

Table 2 Susceptibility of cucumber cotyledons at different seedling ages

seedling age/d	disease index
6	97.7a
5	97.7a
7	96.7ab
9	96.6ab
8	96.6ab
10	95.5abc
11	87.7bc
12	68.9d

Note: The different letters in the same column represent significance at 0.05 probability level.

3.3 Pathogenicity of *S. fuliginea* at different growing stages

Figure 1 shows that there were no significant differences for disease index inoculated with the conidia brushed from the cotyledons which were inoculated for 9–17 days. Because only a small amount of new conidia could be collected on the 9th day after inoculation, and the cotyledons withered 15 days after inoculation, this indicates that it was suitable to use the conidia after 10–15 d after inoculation.

3.4 Spore suspension concentration tested

Based on disease index tested, there were no significant differences for spore suspensions between 30 and 100 spores per field of vision (10 × 10 times under microscope) (Table 3). When the spore concentration was less than 30, the disease index decreased. From a practical point of view, 30–40 spores per field of vision could be used for inoculation.

3.5 Comparison of bioassay methods

Three bioassay methods including Trypan Blue staining method, leaf disc method and cotyledon-spraying method were tested and compared. Each of them had its own advantages and disadvantages. Trypan Blue staining method can show the processes of spore germination,

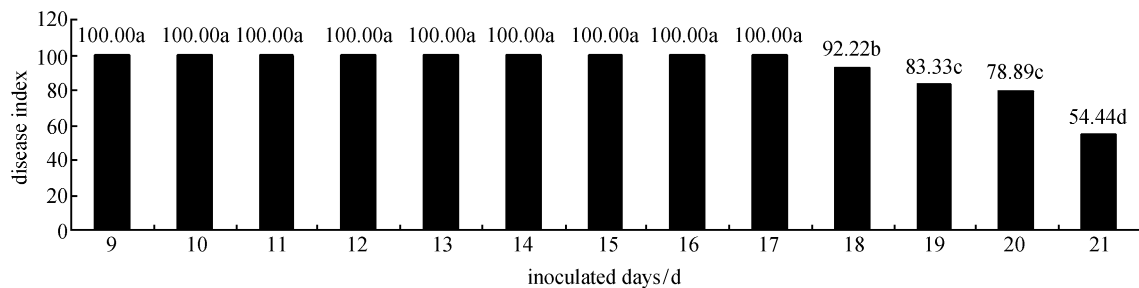

Fig. 1 Pathogenicity of *S. fuliginea* at different growing stages
Note: The different letters represent significance at 0.05 probability level.

Table 3 Disease indexes inoculated by different concentrations of spore suspensions

spore concentration per field of vision /10 × 10 times under microscope	disease index
100–80	98.9a
80–60	97.8a
60–50	97.8a
50–40	96.7a
40–30	91.1ab
30–20	81.1b
20–10	45.0c

Note: The different letters in the same column represent significance at 0.05 probability level.

germ tube formation and the formation of conidia clearly (Fig. 2), providing a reliable way to illustrate the mechanism of fungicides (Li et al., 2007). As for the leaf disc method, temperature and humidity are easier to regulate and the space used for testing is rather limited. The leaf disks on the culture medium B remained green for a longer time than those on medium A and were not as easily polluted as those on medium A (Fig. 3). The cotyledon-spraying method is relatively easy and feasible to operate, but it needs a larger space when a great deal of bioassay tests or disease resistance identification must be done. Besides, it is relatively time-consuming and laborious.

3.6 Application of the bioassay system

Using the optimized bioassay system above, the protective virulence equation of chrysophanol to powdery mildew was obtained: $y = 1.4342x + 2.8154$ ($R^2 = 0.9998$), the protective EC_{50} value was 33.36 $\mu g \cdot mL^{-1}$, which indicated that the chrysophanol had a protective effect against cucumber powdery mildew.

4 Discussion

Bioassay is an indispensable step for screening new compounds with a good inhibiting effect against plant pathogens, and it is very important to establish a standardized and rapid bioassay system for each host-parasite system. As an obligate parasite, *S. fuliginea* can not grow and reproduce on media (Zhang, 2005) because of difficulties in applying the high-throughput screening technology on pathogens. Therefore, it has an important practical value for establishing a scientific, standardized and rapid bioassay system for powdery mildew. Our results showed that cucumber varieties, seedling ages, culturing times of *S. fuliginea* spores, inoculation concentrations, and bioassay methods all had significant effects on the powdery mildew infection of cucumber plants; therefore, much attention should be given to these factors to conduct an accurate analysis of the bioassay results.

The Trypan Blue staining method was confirmed appropriate for staining different phases in the life cycle of *S. fuliginea*, which would accelerate fungicide screening, especially those fungicides inhibiting spore germination and come to an ideal conclusion in 24 h. At the same time this method provides a reliable method for illustrating the inhibiting mechanism of fungicide (Li et al., 2007). However, it also had some limitations (Yang et al., 2007b);

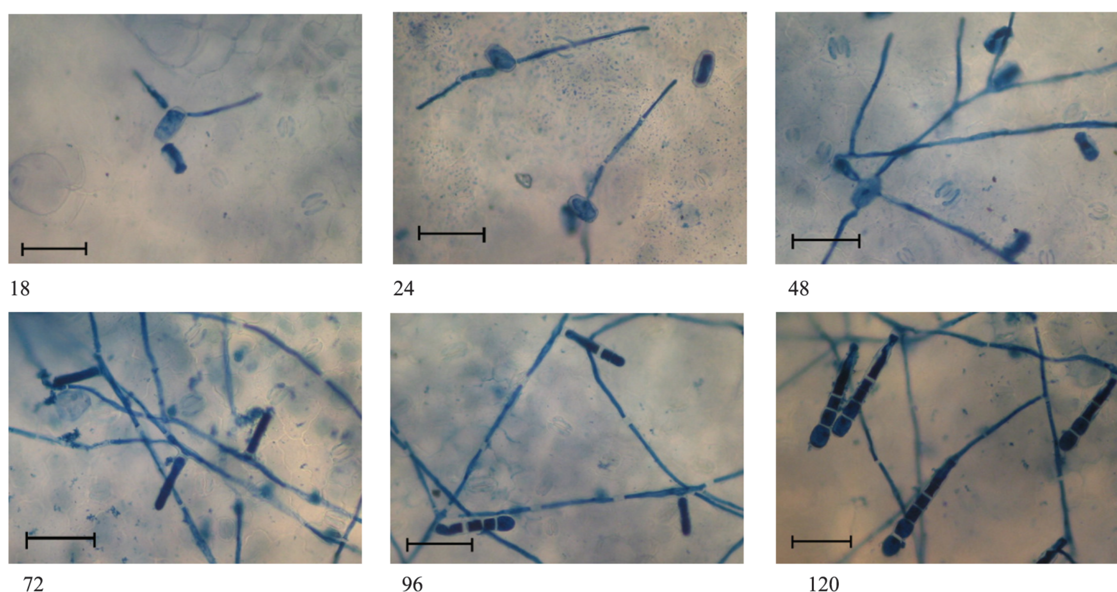

Fig. 2 Spore germination and formation of *S. fuliginea* after different hours of inoculation (h)
Note: bar = 50 μm.

Fig. 3 Leaf disc method on different culture media

different fungicides have different control mechanisms, such as BTH, which inhibits haustorium formation rather than spore germination and appressorium formation (Jorn et al., 1996). Hence, it is inappropriate to adopt this bioassay method for such fungicides (Yang et al., 2007b).

Spore contamination is always a problem during bioassay process. The leaf disc method is easy, effective and accurate for resolving the problem; and the infection conditions, such as spore concentration, humidity, temperature and illumination, can be artificially regulated. One kind of culture medium obtained can keep the leaf disk green for a long time, with the leaf disk fully infected (Hao et al., 2006). The method is expected to develop into a main method for cucumber powdery mildew resistance screening (Chen et al., 2000), simultaneously providing us a new idea for purification and preservation of cucumber powdery mildew because it could avoid cross-contamination, which will be studied in the next step (Cao et al., 2005). However, its use is limited to testing systemic fungicides.

The cotyledon-spraying method was established based on other researches and was further optimized in our study. The method is easy, feasible and reliable to fully infect plants. Although it needs larger space and is relatively laborious when a great deal of bioassay test is on, it is the most efficient way for specific compound testing.

The bioactivity of chrysophanol on *S. fuliginea* was determined by the cotyledon-spraying method. The result showed that the chrysophanol had a significant protective effect on cucumber powdery mildew and it could be developed as a fungicide for powdery mildew control in the future.

Acknowledgements We thank the Science Committee of Haidian District, Beijing for their support of the research project (No. K20090052).

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