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Leaf-disk method for assessment of disease severity of lily leaf blight caused by *Botrytis elliptica*

Ting-Fang Hsieh[1] and Jenn-Wen Huang[2,3]

1. Department of Plant Pathology, Taiwan Agricultural Research Institute, Council of Agriculture, Wu-feng, Taichung 413, Taiwan, ROC.
2. Department of Plant Pathology, National Chung Hsing University, Taichung 402, Taiwan, ROC.
3. Corresponding author, E-mail: jwhuang@dragon.nchu.edu.tw; FAX: 04-22851676
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ABSTRACT

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A leaf-disk assay was developed for disease assessment of lily leaf blight caused by *Botrytis elliptica*. Oriental hybrid lily (cv. Star Gazer) grown from bulbs in a greenhouse for 1.5 to 2 months was used to prepare leaf disks. The fifth to tenth leaves below the apex were surface-disinfested by dipping entire leaves into 0.1% (v/v) sodium hypochlorite three times and rinsed in sterile water after each dip. Leaf disks (15-mm diameter) were then perforated from surface-disinfested leaves with a cork borer, and fully submerged in fungicide or antagonist solutions. The disks (8 per replication) were placed abaxial surface side up in petri dishes containing a piece of 60-mm-diameter sterilized filter paper. Two milliliters of sterile water were added to the filter paper to maintain moisture. Spore suspension of *B. elliptica* was adjusted to 10^5 spores per ml and was sprayed over the disks as inoculum, then the disks were incubated in 20℃ incubator without light. After 3 days incubation, disease severity was assessed as percent necrotic area. The results of the leaf-disk assay were consistent and agree with that of whole plant assays in the greenhouse.

Key words : antagonists, *Botrytis elliptica*, fungicides, leaf-disk method, Lily, *Lilium hybridium*

INTRODUCTION

Leaf blight or fire blight, caused by *Botrytis elliptica* (Berk.) Cooke, is one of the most serious diseases of lily, *Lilium hybridium* Hort., under cool and wet conditions [6, 8, 16]. In 1997, approximately 315 ha of lilies started from bulbs were imported from Netherlands and grown for the cut flowers were estimated to have NT$ 836 million of market value in Taiwan [1]. The central of Taiwan, especially in the Nan-tou and Taichung areas, is a major region for production of lilies. Hsieh and Huang (1998) [16] demonstrated that, under favorable environmental conditions in winter and spring, economic losses due to Botrytis leaf blight disease could reach 100%. Fungicide treatment is a major mean of disease control techniques in lilies. However, after intensive chemical use, dicarboximide and benzimidazole resistant strains of *B. elliptica* have appeared [4,15,19,24]. New strategies to control this disease are highly desirable.

Conventional screening for fungicide resistance and efficacy requires labor intensive methods in the greenhouse. The methods for screening and selecting of fungicides and antagonists could be facilitated. Gould *et al*. (1996) described a petal disk assay for screening antagonists to control petunia gray mold caused by *B. cinerea* [13]. Hsiang and Chastagner (1991) also used a leaf disk assay to assess virulence of *B. cinerea* and *B. tulipae* on tulip [15]. The objective of this study was to establish a leaf-disk assay to screen chemical fungicides and biological antagonists for control of the lily leaf blight.

MATERIALS AND METHODS

Isolates of *Botrytis elliptica*

Two single-spore isolates (B061 and B066) of *B. elliptica* were obtained from leaves of diseased oriental lily cv. Casa Blanca at Tien-Chung and cv. Star Gazer at Hsin-She, respectively, in the central areas of Taiwan. Isolates were then maintained on potato dextrose agar (PDA; Difco Laboratory, Detroit, MI, USA) and stored at 4℃. To maintain its pathogenicity, the spores were inoculated onto surface-sterilized lily leaves, and then reisolated by harvesting freshly-formed spores from diseased tissues incubated under near UV light (Black Light Blue, F10T8BLB, Sankyo Denki, Japan) [6]. These two isolates were used in the following assays.

Inoculum preparation

To prepare fungal inoculum, the two isolates of *B. elliptica* were grown on autoclaved lily leaves or petals for 7 to 10 days at 20 ℃ under near UV light. Spores were harvested from fungal colonies with sterile forceps, which were then swirled in 5 ml sterile water to release the spores. The spore suspensions were centrifuged at 3000x g for 10 min, supernatant was discarded, and 5 ml sterile water was added to the tube. This collection step was repeated three times. Spores were then counted with a hemacytometer, and spore suspension was adjusted to a desired concentration [16].

Isolation and preparation of bacterial antagonists

Lily leaves used for the isolation of bacterial antagonists were obtained from several commercial nurseries in central areas of Taiwan. Four to five grams of whole leaves were cut into 0.5 x 1 cm pieces, rinsed in distilled water and shaken at 100 rpm by shaker for 1 h at room temperature (approximately 25℃). Ten-fold dilutions of the leaf-washes were prepared immediately, and 100μl of the dilutions were plated onto nutrient agar (NA; Difco Laboratory, Detroit, MI, USA) with four replicates. After 4 days, single colonies were isolated and streaked on NA to obtain pure cultures. After preliminary trials with dual cultures of bacteria confronting *B. elliptica* mycelium, the unidentified bacterial isolates 24-2-2, 49-2-2, B-NA-12, B-PDAS-21, D-NYDA-602, and Y-NYDA-21 were selected for leaf-disk tests. The bacterial isolates were cultured in nutrient broth (NB; Difco Laboratory, Detroit, MI, USA) at room temperature for 4 days on shaker with shaking speed at 100 rpm. Bacterial concentrations were adjusted by adding sterile nutrient broth to attain an optical density of 0.38 at 600 nm with spectrophotometer (UV-1201, Shimadzu Co., Kyoto, Japan), which approximates a concentration of 10^8 cfu of bacteria ml^{-1} (as determined by a standard curve).

Fungicide preparation

The following seven fungicides recommended for control of gray mold in Taiwan were chosen to determine the control efficacy of lily leaf blight. Those were procymidone, 50% wettable powder (WP) [N-(3',5'-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide, Sumitomo Chemical Taiwan, Taipei], vinclozolin, 50% WP [3-(3,5-dichlorophenyl)-5-etyenyl-5-methyl-2,4-oxazolidinedione, BASF, Ludwigshafen, F. R. Germany], thiophanate-methyl, 70% WP [dimethyl-4,4'-O-phenylene-bis (3-thioallophanate), BASF, Ludwigshafen, Germany], iprodione, 23.7% WP [3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide, Rhone-Poulenc, Paris, France], dichlofluanid, 50% WP [N-dichlorofluoromethylthio-N', N'-dimethyl-N'-phenylsulfamide, Euparen, Bayer AG, Leverkusen-Bayerwerk, Germany], mancozeb, 80% WP [Mn^{++} 16%, Zn^{++} 2%, ethylene-bis (dithiocarbamate) polymeric complex 62%, Du Pont, Wilmington, DE 19898, USA], and carbendazim, 50% WP [methyl-2-benzimidazole carbamate, BASF, Ludwigshafen, F. R. Germany]. All fungicides were diluted to field recommended rates. Procymidone, vinclozolin and dichlofluanid were diluted to 1500 fold. Thiophanate-methyl, iprodione and carbendazim were diluted to 1000 fold and mancozeb was diluted to 500 fold.

Effects of spore concentration, temperature, inoculum droplet size and leaf position on the disease severity of Botrytis leaf blight using a leaf disk assay

Oriental hybrid lily (*Lilium oriental*) cv. Star Gazer was grown from bulbs in the greenhouse for 1.5 to 2 months and used to prepare leaf disks. The fifth to tenth leaves below the apex were surface-disinfested by dipping entire leaves in 0.1% sodium hypochlorite three times for 3 min, rinsed in three changes of sterile water and blotted dry on sterilized paper towels. Leaf disks (15-mm diameter) were cut from surface-disinfested leaves with a cork borer. The disks (eight pieces per dish) were placed adaxial surface down in petri dishes (90 mm in diameter) containing a 60-mm-diameter piece of sterilized filter paper. Two milliliters of sterile water were added to the filter paper to maintain moisture. A series of experiments were conducted to determine the influence of spore concentration, temperature, inoculum droplet size and leaf position on disease development. A spore suspension prepared as described above and adjusted to 10, 10^2, 10^3, 10^4 and 10^5 spores ml^{-1} was applied (Sigma spray unit, Sigma Chemical Co, St. Louis, MO, USA) to leaf disks (about 25μl per disk). Leaf disks were incubated at 20℃ in darkness for 3, 4 and 5 days and percent infected area per leaf disk was recorded. The effect of temperature was evaluated by incubating leaf disks which had been inoculated with 25μl of 10^5 spores ml^{-1} per leaf at 8 to 32℃, interval 4℃, in darkness for 3 days, and each was examined for the percent infected area. To determine the effect of the droplet size of the inoculum on the disease development, spore suspension (10^5 spores ml^{-1}) was applied to leaf disks with the Sigma spray unit (Sigma Chemical Co., St. Louis, MO, USA) to form droplet sizes of approximately 0.1, 0.2, 0.25, 0.3 and 0.4 mm in diameter. The leaf disks were then incubated at 20℃ in darkness, and infected area per leaf disk was assessed 3 days later. Since leaf position was found to influence disease development in a preliminary test, all leaves of each plant were stripped and leaf disks were prepared. After inoculation and incubation as described above, disks were evaluated by counting percent-infected area. All experiments were repeated at least twice.

Leaf disk assays for screening antagonists and fungicides

Leaf disks were dipped thoroughly either in seven fungicides or six solutions of bacterial antagonists prepared as described above for 10 min. After the surfaces were air dried,

the leaf disks were placed with adaxial surface down in petri dishes containing a piece of 60-mm-diameter sterilized filter paper. Two milliliters of sterile water were added onto the filter paper to maintain moisture. A spore suspension adjusted to 10^5 spores ml^{-1} was sprayed over the disks to form inoculum droplets of up to 0.3 mm in diameter. The leaf disks were then incubated at 20℃ in darkness, and the percentage of infected area was evaluated after 3 days.

Whole plant assays

To compare the relationship of the response of leaf disks assays and that of whole plant assays, the fungicides and antagonists (prepared as above) were tested for control of Botrytis leaf blight on whole lily plants (cv. Star Gazer). Single lily bulbs were grown in 18-cm-diameter plastic pots with non-sterilized nursery soil. The plants from lily bulbs were kept in a greenhouse at 24±4℃. After growth of 1.5 to 2 months, entire lily plants were sprayed with fungicides and antagonists prepared as described above. After air-dried, plants were sprayed by spore suspension of *B. elliptica* with concentration of 10^5 spores ml^{-1}. Each treatment had four replicates and both tap water and nutrient broth sprays served as controls. The inoculated plants were sealed in plastic bags to maintain humidity for 2 days and then were moved into growth chambers of 20℃ with 16 h of light period at light intensity of 3500 lux (Digital illumination meter, DX-200, Jing Chen Instruments Co., Taichung 404, Taiwan). Disease severity was analyzed 7 days after inoculation. The lesion numbers of the fifth to eighth leaves were counted. The experiment was repeated twice.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA), and the means were separated by Duncan's multiple range test.

RESULTS

Effects of spore concentration, temperature, inoculated droplet size and leaf position on the disease severity of Botrytis leaf blight with leaf disk assays

In spore concentration tests, a rating of 80% lesion area on leaf disks was observed 3 days after inoculation with 10^5 spores ml^{-1}. This result was equivalent to 5 days after inoculation with 10^4 spores ml^{-1} (Fig. 1). However, the data of lesion area were less variable when leaf disks were inoculated with 10^5 spores ml^{-1} spore concentration. Most serious disease development was observed in inoculated leaf disks incubated under lower temperatures (12-24℃). No lesions were observed at 32℃. The optimum temperature for disease development was 20℃ (Fig. 2). Droplets of inoculum up to 0.4 mm in diameter were positively related to lesion area expansion. The infected area on leaf disks up to 80% was obtained at the drop size of inoculum up to 0.3 mm in diameter after 3 days inoculation (Fig. 3). When the diameter of inoculum droplets were up to 0.4 mm or over, the droplets would easily fuse together. In this condition, the inoculum was difficult to distribute uniformly on the leaf disks. Leaf disks from leaves of any position on plant could be infected by *B. elliptica*. The fifth to tenth leaf below the apex were less variable in disease development (Fig. 4). The percentage of lesion area on leaf disks was positively related ($r=0.717$, $p<0.01$) to lesion number per leaf on whole plants in greenhouse tests (Fig. 5).

After evaluation of all factors affecting disease development, a standardized leaf-disk assay was devised as follows (Fig. 6). Lilies grew from bulbs in a greenhouse for 1.5 to 2 months were used to prepare leaf disks. The fifth to tenth leaves below the apex were surface-disinfested by dipping entire leaves in 0.1% sodium hypochlorite three times and rinsing it in three changes of sterile water. Leaf disks (15-mm diameter) were cut from surface-disinfested leaves by means of a cork borer for advanced treatment. The disks (8 per replication) were placed abaxial up in petri dishes (90 mm in diameter) containing a piece of 60 mm in diameter of sterilized filter paper. Two milliliters sterile water was added to the filter paper to keep moisture. A spore suspension adjusted to 1 x 10^5 spores ml^{-1} was sprayed over the disks to form inoculum droplets of up to 0.3 mm in diameter. They were then incubated at 20℃ in darkness, with disease severity assessed after 3 days. The percentage of necrotic area on each leaf disk was recorded.

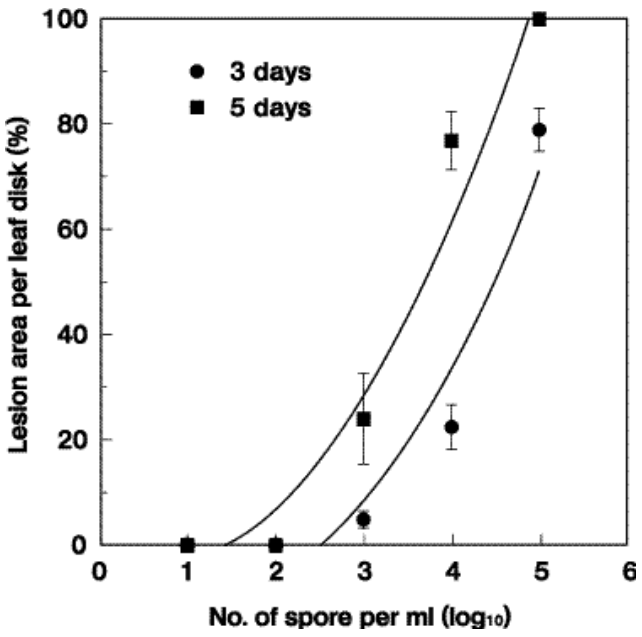

Fig. 1. The relationships between the inoculated concentrations of *Botrytis elliptica* and disease development of lily leaf blight on leaf disks at 20℃ for 3 and 5 days.

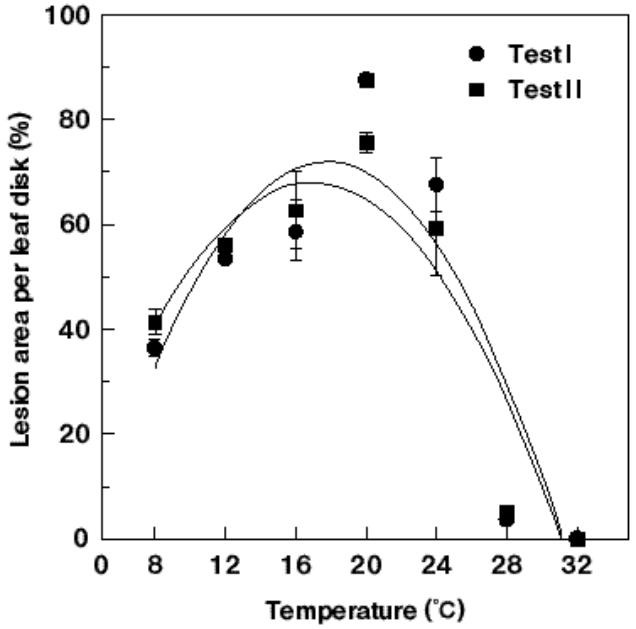

Fig. 2. The effect of temperatures on disease development of lily leaf blight caused by *Botrytis elliptica* for 3 days after 10^5 spores ml^{-1} inoculation. The trials were carried out twice (Test I and II).

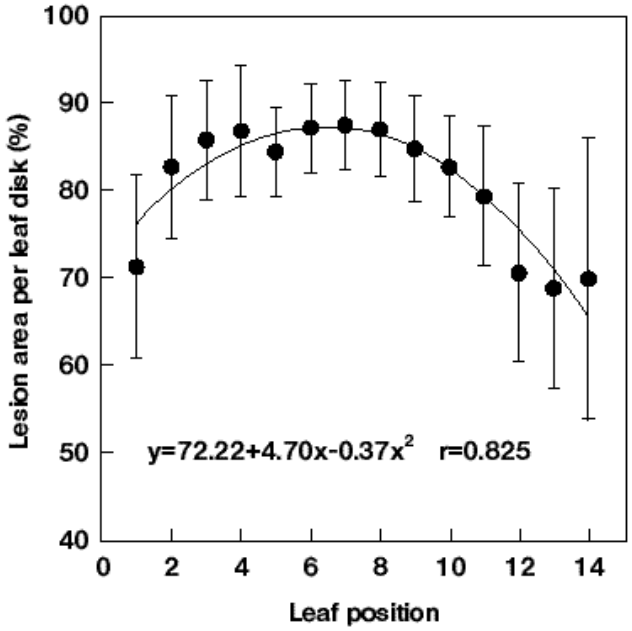

Fig. 4. The influence of leaf position of lily (*L. oriental* hybrid cv. Star Gazer) on disease development of lily leaf blight caused by *Botrytis elliptica* at 20℃ for 3 days.

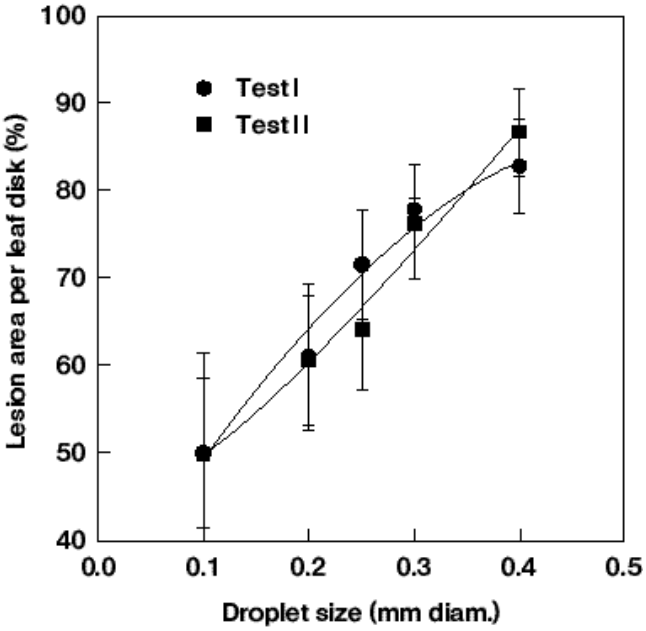

Fig. 3. The relationships between droplet size of inoculum (10^5 spores ml^{-1}) and infected area by *Botrytis elliptica* on lily leaf disks at 20℃ after 3 days incubation. The trials were carried out twice (Test I and II).

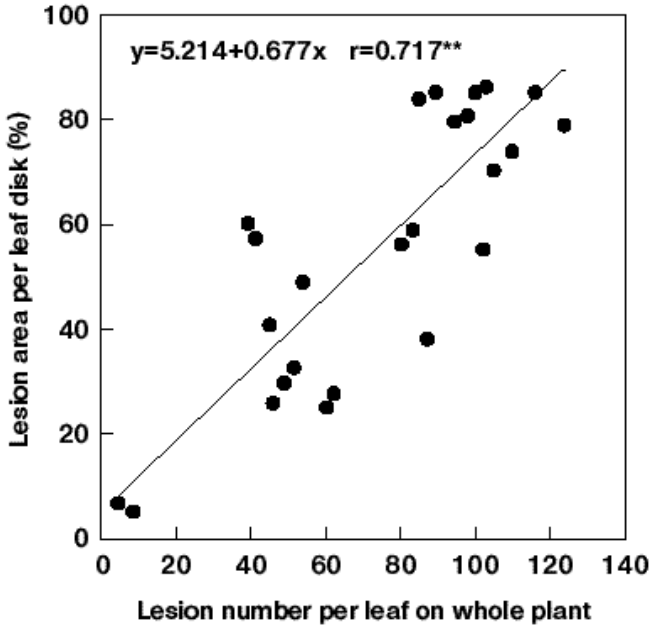

Fig. 5. The relationship between infected area on leaf disks (3 days) and lesion number per leaf on whole plant assays (7 days) after inoculation with *Botrytis elliptica* at 20℃.

Leaf disk assays for screening antagonists and fungicides

In screening antagonists, the percentage of infected area per leaf disk was lower after leaf disks were treated with 10^8 cfu ml^{-1} suspension of bacteria such as B-NA-12, B-PDAS-21, D-NYDA-602 or Y-NYDA-21, and tested against two isolates of *B. elliptica* (Table 1). In fungicides trials compared to water-treated leaf disks inoculated with B061 or B066 isolates of *B. elliptica*, all fungicides, especially iprodione, dichlofluanid and mancozeb, significantly reduced the percentage of necrotic area on leaf disks (Table 2). Among

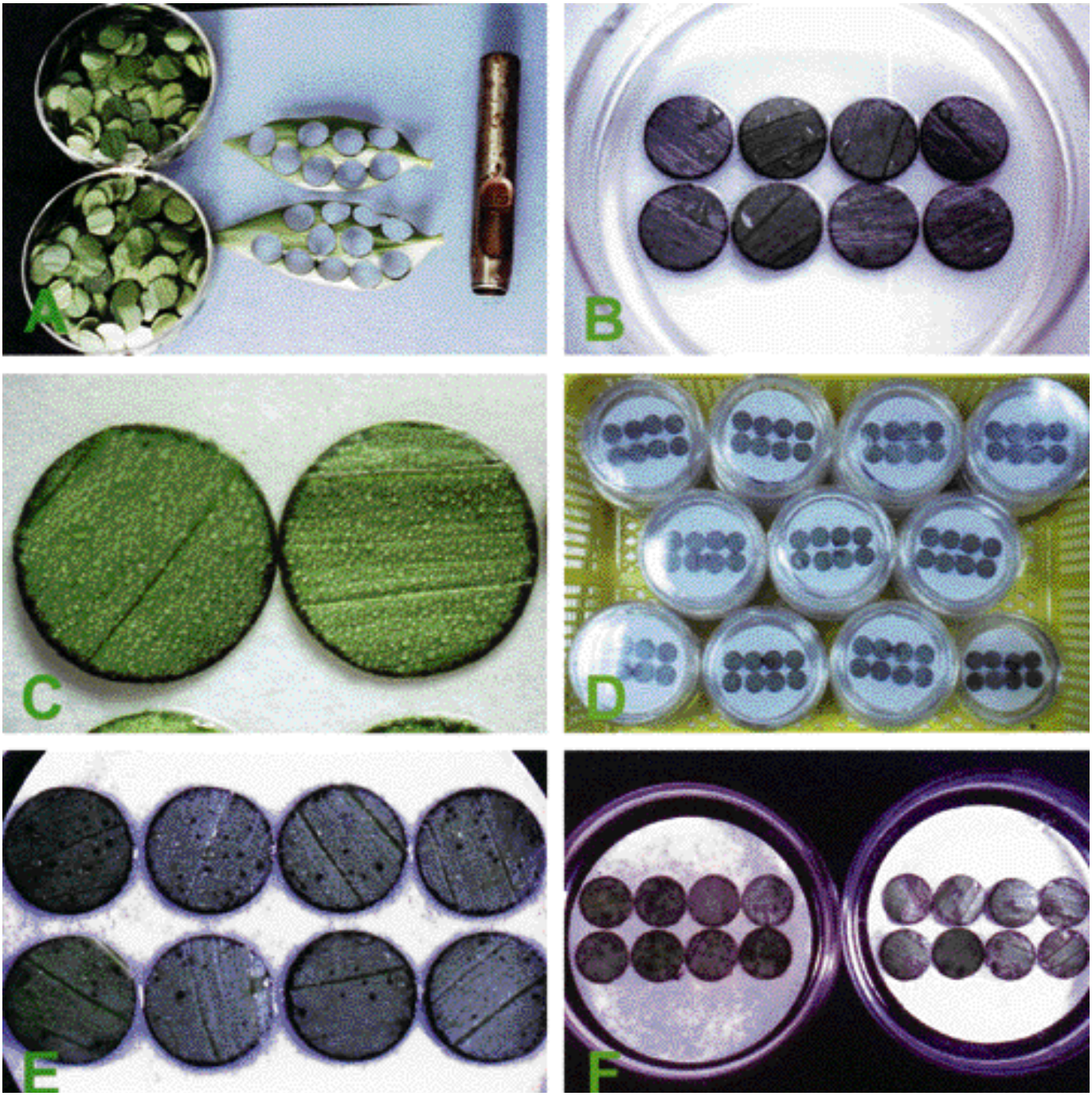

Fig. 6. Leaf disk method. (A) The fifth to tenth leaves below the apex of Oriental hybrid lily (cv. StarGazer) grown from bulbs in a greenhouse for 1.5 to 2 months were used to prepare leaf disks. Leaf disks (15-mm diameter) were cut from surface-disinfested leaves with a cork borer. (B) The disks (8 per dish) were placed abaxial surface up in petri dishes containing a piece of 60-mm-diameter sterilized filter paper. Two milliliters of sterile water were added to the filter paper to maintain moisture. (C) Spore suspension was sprayed over the disks to form inoculum droplets of up to 0.3 mm in diameter. (D) The inoculated disks were incubated at 20℃ in darkness. (E) Water soaked lesions appeared on the surface of leaf disks 16 h after inoculation. (F) Disease severity was assessed as percentage of necrotic area 3 days after inoculation.

Table 1. Disease severity of *Botrytis elliptica* on leaf disks and whole plants of lily after challenged with different microorganisms

Microorganism (Isolate designation)	Leaf disk assay [1] Lesion area (%)		Whole plant test [2] Lesion numbers per leaf	
	B061[3]	B066	B061	B066
24-2-2	79.38 a[4]	83.75 a	94.5 a	85.0 abc
49-2-4	78.75 a	70.00 b	83.0 a	105.4 a
B-NA-12	48.75 b	32.50 cd	53.7 b	51.3 bc
B-PDAS-21	55.63 b	29.38 cd	31.4 c	48.8 c
D-NYDA-602	56.88 b	40.63 c	40.6 bc	44.8 c
Y-NYDA-21	60.00 b	25.63 d	38.9 bc	45.7 c
CK1(Nutrient broth)	85.63 a	85.00 a	102.5 a	89.4 ab
CK2 (Water)	80.50 a	85.00 a	97.7 a	116.2 a

1. A spore suspension adjusted to 10^5 spores ml^{-1} was sprayed over the disks to make the droplet size of inoculation about 0.3 mm in diameter, then petri dishes were incubated at 20℃ in darkness and the percentage of infected area on leaf disks was recorded at the 3rd day.
2. The average numbers of disease lesion on fifth to eighth leaves of the whole plant were counted 7 days after inoculation in the greenhouse.
3. Isolates B061 and B066 of *B. elliptica* were obtained from diseased lily plants.
4. Data followed by the same letter in each column do not differ significantly (p=0.05) according to Duncan's multiple range test.

Table 2. Comparison of leaf disks and whole plant assays on evaluating the efficiency of fungicides on control of lily leaf blight caused by *Botrytis elliptica*.

Fungicide	Diluted fold	Leaf-disk assay [1] Lesion area (%)		Whole plant test [2] Lesion number per leaf	
		B061[3]	B066	B061	B066
Carbendazim, 50% WP	1000	59.3 b[4]	56.0 b	115 b	80 bc
Dichlofluanid, 50% WP	1500	1.8 d	0.0 c	9 d	9 d
Iprodione, 23.7% WP	1000	10.4 d	2.8 c	60 c	55 c
Mancozeb, 80% WP	500	6.5 d	4.6 c	4 d	8 d
Procymidone, 50% WP	1500	32.6 c	13.5 c	77 c	61 bc
Thiophanate-methyl, 70% WP	1000	55.2 b	38.1 b	102 b	87 bc
Vinclozolin, 50% WP	1500	27.5 c	9.4 c	62 c	95 ab
Water		82.4 a	78.7 a	152 a	124 a

Footnotes are the same as Table 1.

tested fungicides, thiophanate-methyl (dicarboximide fungicide) and carbendazim (benzimidazole fungicide) showed lowest efficacy in decreasing the percentage of lesion area on leaf disks inoculated with B061 and B066 isolates (Table 2).

Whole plant assays

Antagonists such as B-NA-12, B-PDAS-21, D-NYDA-602 and Y-NYDA-21 effectively reduced the lesion number of lily leaf blight on leaves (Table 1), but isolates 24-2-2 and 49-2-2 were not effective in decreasing lesion number. In fungicide trials, dichlofluanid 50% WP and mancozeb 80% WP were the most effective for controlling lily leaf blight (Table 2). The effect to reduce the lesion number on whole plants by dicarboximide (procymidone, vinclozolin and iprodione) and benzimidazole (carbendazim and thiophanate-methyl) fungicides was less effective than that of dithiocarbamate (mancozeb) and sulphamide (dichlofluanid) fungicides (Table 2). The response of whole plants assay was in agreement with the results obtained from the leaf disk assays, except for two treatments (bacterium 49-2-4 and fungicide vinclozolin 50% WP) against isolate B066.

DISCUSSION

Lily leaf blight, caused by *Botrytis elliptica*, is a severe disease on ornamental lily in Taiwan as well as many other countries during low temperature season [6,16]. Doss and his colleagues produced a series of reports on spore production of *B. elliptica* [6], infection process of the pathogen on Easter lily [8] and screening varieties resistance of lily to the pathogen with a detached leaf method [7]. In an earlier study, we found that preparation of materials and lily plants were laborious and expensive [16]. For this reason, we hope to develop a bioassay afforded to save materials and allow screening of many treatments at one time be possible. Thus, leaf disks have been used instead of whole plants in many previous experiments such as detection of disease resistance [2,3,5,11,14,18,21], detection of herbicide tolerance [12], determination of host-pathogen interactions [22], genetic transformation systems [20], entomological researches [17, 23] and botanical researches [26,27]. In this study, we demonstrated that the leaf disk assay might accurately predict the response of lily to *B. elliptica* under control conditions. Result showed that using leaf disks to screen antagonists and fungicides for

control of lily leaf blight was highly correlated with results from whole plant assays.

We used a leaf disk assay to evaluate the efficacy of fungicides rapidly. Our results showed that the fungicides recommended for grey mould control such as procymidone, vinclozolin, iprodione (dicarboximide fungicides), thiophanate-methyl and carbendazim (benzimidazole fungicides) had lower efficacy in controlling lily leaf blight (Table 2). In fungicides tested, only dichlofluanid and mancozeb showed high efficacy in controlling lily leaf blight. However, more isolates of *B. elliptica* would have to be screened for their sensitivity. The leaf disk assay presented here could provide a quick and simplified method to pre-screen the fungicides for *B. elliptica* control.

Biological control is one of several approaches to decrease the usage of chemicals. Bacterial antagonists have been used to suppress the disease progress of grey mould on many crops [10,13,25]. Elad (1990) demonstrated that potential antagonists could be isolated from native habitats of pathogens and could survive, grow and show antagonism to pathogens in that habitat [9]. Therefore, The candidate isolates of potential antagonists in this study were obtained from leaves of field-grown lily plants. Some of them showed the potential in controlling lily leaf blight in the leaf disk assays and whole plant trials. Using leaf-disk assays could minimize the number of samples needed, and still reflect the response of whole plant assays. Leaf-disk assays not only saved plant material and trial space, but also shortened the time required to screen fungicides and potential antagonists.

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摘 要

謝廷芳[1]、黃振文[2,3]. 2001. 利用葉片圓盤法評估百合灰黴病的罹病度 植病會刊10:37-44. ([1] 台中縣霧峰鄉 行政院農委會農業試驗所； [2] 台中市 國立中興大學植物病理系； [3] 聯絡作者，電子郵件：jwhuang@dragon.nchu.edu.tw ; 傳真 : 04-22851676)

採取露蕾期之葵百合 (cv. Star Gazer) 葉片，以口徑15 mm之打孔器切取葉片圓盤，評估灰黴病菌 [*Botrytis elliptica* (Berk.) Cooke] 為害百合葉片的情形，包括決定接種源的濃度、溫度對病害發生之影響、噴霧接種之水珠大小、植株葉位之感病性，及葉片圓盤分析法與整株百合罹病之相關性。建立一個葉片圓盤分析的標準方法：即摘取成熟百合植株之第五至十位葉，以 0.1%次氯酸鈉表面消毒後，經無菌水漂洗三次，晾乾後以口徑15 mm打孔器切取葉片圓盤，置於平舖一張6公分之濾紙且含2毫升無菌水之9公分培養皿中，每皿置放8片葉片圓盤，然後接種每毫升含1×10^5個灰黴病菌之胞子懸浮液，使圓盤上覆一層大小約 0.3 mm之水珠，覆以皿蓋，移置於 20℃之定溫箱中觀察病勢的進展。在室內利用葉片圓盤分析法篩選百合灰黴病菌的防治藥劑與拮抗微生物，結果與溫室測試整株植物之結果相當一致。葉片圓盤分析法不但可減少植材與葉片用量外，並可節省試驗空間與時間，且可預先篩選出防治百合灰黴病菌的藥劑與拮抗微生物，以供溫室或田間進一步試驗之用。

關鍵詞：百合、灰黴病、葉片圓盤法、拮抗微生物、藥劑