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Early Stages of Infection of Lily Leaves by *Botrytis elliptica* and *B. cinerea*

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ABSTRACT

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According to our observations, infection of lily leaves (Oriental hybrid cv. Star Gazer) by *Botrytis elliptica* resulted in the development of necrotic lesions, but inoculation with *B. cinerea* only caused tiny specks. A comparative study was conducted herein to investigate early stage of infection of 'Star Gazer' leaves by these pathogens. Microscopical examination 12 hr after inoculation showed that the percentage of germinated conidia of both *B. elliptica* and *B. cinerea* on the abaxial surfaces of lily leaves reached to 95%, but the number of appressoria produced by the germ tubes of *B. elliptica* was significantly higher than that produced by *B. cinerea*. In addition, the penetration of lily leaves by *B. elliptica* was nearly 54.5%, but it was nil by *B. cinerea*. During the attempted penetration by *B. elliptica* and *B. cinerea*, some of the epidermal cells at penetration sites became dead. Among these dead cells, some were associated with fluorescence. Although some of the penetration hyphae of *B. elliptica* were retarded by the dead cells of lily leaf epidermis, some still expanded and resulted in the development of necrotic lesion. Fluorescent depositions as indicated by aniline blue staining appeared in lily leaf tissues infected by *B. elliptica*. However, this phenomenon was not observed in lily leaves inoculated with *B. cinerea*.

Key words : *Botrytis*, infection, lily, plant response

INTRODUCTION

Botrytis elliptica (Berk.) Cooke, a pathogen with narrow host-range, mainly infects stems, leaves and flowers of lily plants (*Lilium* spp.) [10,11,15]. *Botrytis cinerea* Pers.: Fr. is a pathogen with broad host-range of over 200 species of plants, including several floral crops [2,8,13]. *B. elliptica* and *B. cinerea* are known to form appressoria as swollen germ tubes in single-lobe or digitate shape to facilitate the penetration of plants [2,3,5,9]. As well as mechanical force, secretion of esterase by *B. elliptica* and *B. cinerea* on the leaf surface have been noted, that may play a role in the fungal penetration into plants [9,12,17].

It has been noted that *B. cinerea* causes lesions on the flowers and wounded leaf tissues of lily plants [15,20], but the infection of intact leaf tissues of lily by *B. cinerea* is rarely reported. In order to get better understanding on the interactions between different *Botrytis* species and lily, in this study, the plant responses including symptom development and histological alterations of lily leaves (Oriental hybrid cv. Star Gazer) after inoculation with *B. cinerea* and *B. elliptica* were examined. In addition, conidial germination, appressorium formation and penetration of 'Star Gazer' leaves by these pathogens were compared to find out which step(s) of the infection by *B. elliptica* and *B. cinerea* might be different.

MATERIALS AND METHODS

Preparation of *B. elliptica* and *B. cinerea* inocula

B. elliptica strain B061-1-1 and *B. cinerea* Pers.: Fr. strain B071-1-1 were single spore cultures of strains B061 and B071, respectively. Strains B061 and B071 were provided by Dr. T. F. Hsieh of Taiwan Agricultural Research Institute, Taichung, Taiwan. Both strains were maintained on V-8 agar slants (20% V-8 vegetable juice, 0.3% $CaCO_3$, 1.5% agar) at 4 ℃ after sporulation. For maintaining sporulation of *B. elliptica*, it was required to transfer stock culture to autoclaved lily leaves periodically and re-establish new single spore cultures. The inoculum of each strain was prepared using V-8 agar slant cultures incubated at 20℃ under near UV light for five days [14]. Conidia were removed from V-8 agar slant cultures and suspended in sterile distilled water

containing 0.05% Tween 20. Conidia in suspension was collected by centrifugation (5,000 rpm, 10 min) using a high speed micro refrigerated centrifuge (MRX-150, Tomy Seiko Co., Ltd., Japan). Conidia were resuspended in sterile distilled water containing 0.05% glucose and 0.05% K_2HPO_4 [12] and adjusted to approximate 5×10^4 conidia/ml of *B. elliptica* and 3×10^5 conidia/ml of *B. cinerea*.

Plant materials

Bulbs of lily cv. Star Gazer were planted in a mixture of pot medium (Bas Van Buren, No.2) and perlite (3:1 v/v). They were placed under a semi-open house covered with a shading screen. Full-expanded leaves of lily plants (25-45 days old) were used for experiments. The middle leaves (eighth to 16th from the top) were removed and placed in moist glass Petri dishes (two leaves per dish). A piece of wet cotton was placed on the cutting end of leaf petiole to keep moisture.

Examination of symptom development caused by *Botrytis elliptica* and *B. cinerea*

The abaxial surfaces of detached lily leaves were inoculated by depositing droplets of conidial suspensions of *B. elliptica* and *B. cinerea* (20 μl per droplet; six droplets per leaf). The inoculated leaves were incubated at 20-23℃ room condition and examined daily for symptom development. In each trial, fifty inoculation sites were examined for each pathogen. The experiment was repeated three times.

Examination of conidial germination and appressorium formation on the surface of lily leaves

The abaxial surfaces of lily leaves were inoculated as described above. The inoculated leaves were peeled 6 and 12 hr after inoculation and stained with 0.05% trypan blue in lactophenol for 2-3 minutes. Germination of conidia and appressorium formation by germ tubes were examined under a light microscope (Leica DMR, Wetzlar, Germany) with a photographic attachment (COOLPIX990, Nikon). A total of 100 spores and 70 germ tubes were examined for the germination of conidia and appressorium formation, respectively.

Fluorescence microscopy

For the examination of penetration of lily leaves and induced responses of lily leaf tissues caused by both *Botrytis* species, the abaxial surfaces of lily leaves were inoculated as described above and examined 12 hr and 24 hr after inoculation by a procedure of fluorescence microscopy. The β-glucan materials in fungal cell walls and plant callose depositions could be detected as fluorescent materials after aniline blue staining [7,9,19]. Firstly, lily leaves were immersed in 30-50 ml of 1 M KOH and autoclaved at 121℃ for 15 min, then rinsed in deionized water three times. The specimens were mounted in staining solution (0.05% aniline blue in 0.067 M K_2HPO_4 at pH 9.0) and examined under a fluorescence microscope (Leica DM IL, Wetzlar, Germany). The fluorescence microscope was equipped with a Mercury burner and G340-380 nm exciter:LP (long pass) 425 nm fluorescence filters. The photographs were taken with a digital camera (COOLPIX990, Nikon). About 50 sites were examined for the penetration of lily leaves by each *Botrytis* species 24 hr after inoculation.

RESULTS

Symptom development on lily leaves

On lily leaves inoculated with *B. elliptica*, 63.5% of inoculation sites developed brown necrotic lesions 12 hr after inoculation and the percentage of brown necrotic lesion increased to 100% 24 hr after inoculation. In contrast, lily leaves inoculated with *B. cinerea* only developed needle-point specks 12 hr and even 24 hr after inoculation. The tiny specks did not expand further during the incubation period of eight days.

Conidial germination of *B. elliptica* and *B. cinerea* on lily leaves

In order to clarify whether the germination percentage of conidia on lily leaves would be related to the lesion formation, germinated conidia of *B. elliptica* and *B. cinerea* at inoculation sites were counted. Results showed that 80.4% of conidia of *B. elliptica*, but only 5% of conidia of *B. cinerea* germinated 6 hr after inoculation. However, all the examined conidia of *B. elliptica* and 96.1% of conidia of *B. cinerea* germinated 12 hr after inoculation.

Appressorium formation and penetration of lily leaves by *B. elliptica* and *B. cinerea*

Results of further observations on appressorium formation showed that 38.7% and 26% of germ tubes from conidia of *B. elliptica* and *B. cinerea*, respectively, formed appressoria on lily leaves 6 hr after inoculation (Fig. 1). The percentages of appressoria of *B. elliptica* and *B. cinerea* increased to 44% and 29.1%, respectively, 12 hr after inoculation. The differences between *B. elliptica* and *B. cinerea* were significant at 95% of confidence level as shown by Student *t* test. The percentage of penetration sites on lily leaves was 54.5% by *B. elliptica* 24 hr after inoculation, but it was none by *B. cinerea* even after prolonged incubation for eight days.

Microscopical examination on plant responses of inoculation sites

In many attempted penetration sites of *B. cinerea*, plant cells became dead and some of the dead cells were associated with fluorescence as observed 12 and 24 hr after inoculation

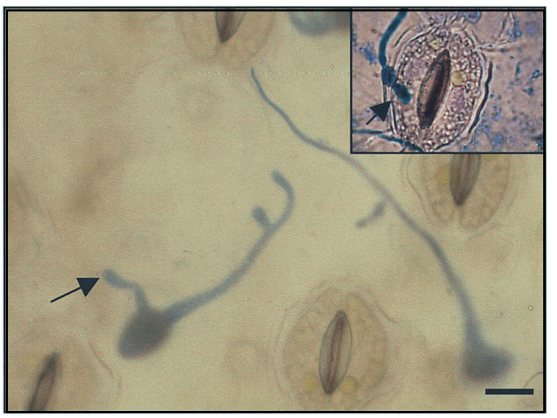

Fig. 1. Appressorium formation of *Botrytis elliptica* and *B. cinerea* on lily leaves. *B. elliptica* and *B. cinerea* (insert picture) produced appressoria on lily leaves that were photographed 6 hr and 12 hr after inoculation, respectively. Arrows indicate appressoria of *B. elliptica* and *B. cinerea*. Scale bar = 24.5 μm.

(Fig. 2). Similar phenomena were observed on the leaf epidermis inoculated with *B. elliptica*. However, in the case of *B. elliptica*, some of the infection hyphae penetrated through leaf epidermis and grew into lily leaf tissues (Fig. 2). In addition, fluorescent depositions appeared in *B. elliptica*-infected leaf tissues as stained with aniline blue 12 hr and 24 hr after inoculation, but not in lily leaves inoculated with *B. cinerea* (Fig. 3).

DISCUSSION

B. cinerea is a broad-host-range pathogen. In lilies, it has been found to infect leaves and bulbs following a wound or injury [15]. A preliminary experiment showed that *B. cinerea* cause tiny speck on 'Star Gazer' leaves, but caused expanding necrotic lesion when inoculated on wounded leaves [20]. The former trait was confirmed in this study. A presence of difference in the early interactions of different *Botrytis* pathogens and 'Star Gazer' leaves was proposed. Since the conidial germination of *B. cinerea* was near 100% as that of *B. elliptica*, limited symptom development caused by *B. cinerea* was unlikely due to non-or less germination of *B. cinerea* conidia on 'Star Gazer' leaves; but was possibly

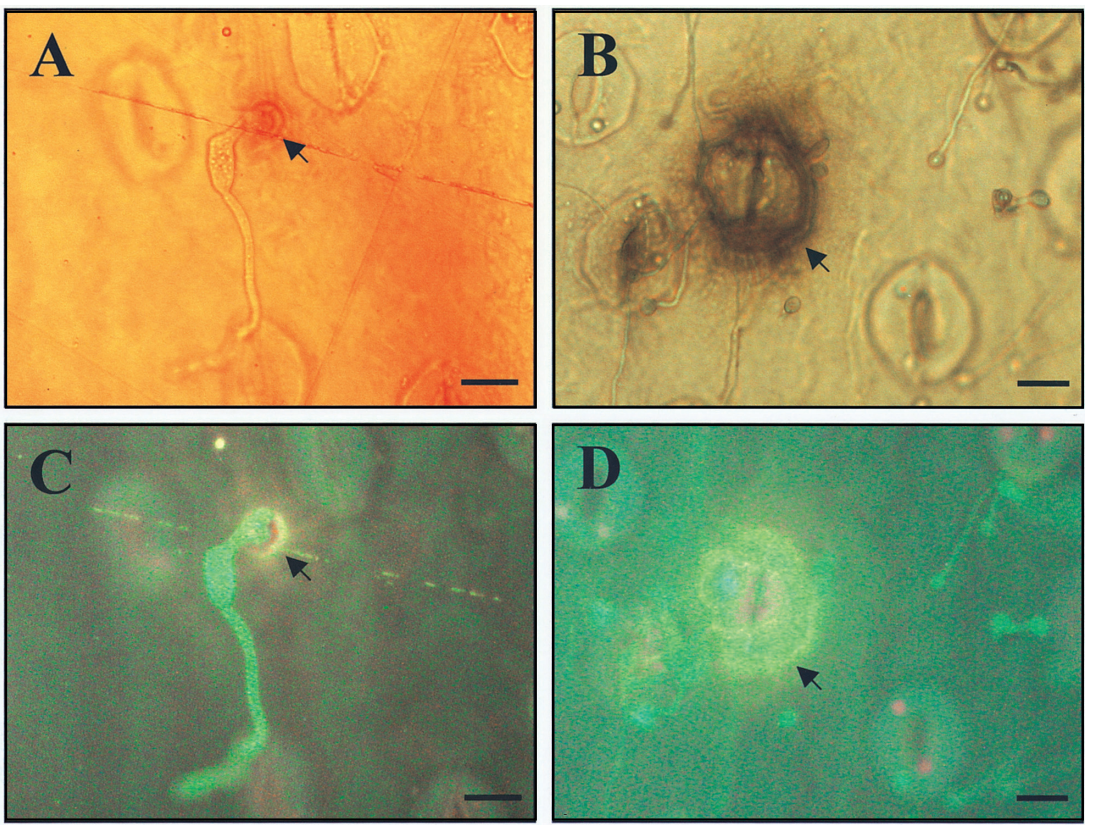

Fig. 2. Lily leaf epidermis inoculated with *B. elliptica* and *B. cinerea*. A germ tube from the conidium of *B. elliptica* was fully constrained by the dead cells of lily leaf, which appeared fluorescence (A). However, an infection hyphae successfully penetrated lily leaf epidermis (A and C). A guard cell at the inoculation site of *B. cinerea* became browning and fluorescence (B and D). Photographs were taken under light (A and B) and fluorescence (C and D) microscopes 24 hr after inoculation. Arrows indicate dead cells of lily leaves. Scale bar = 24.5 μm.

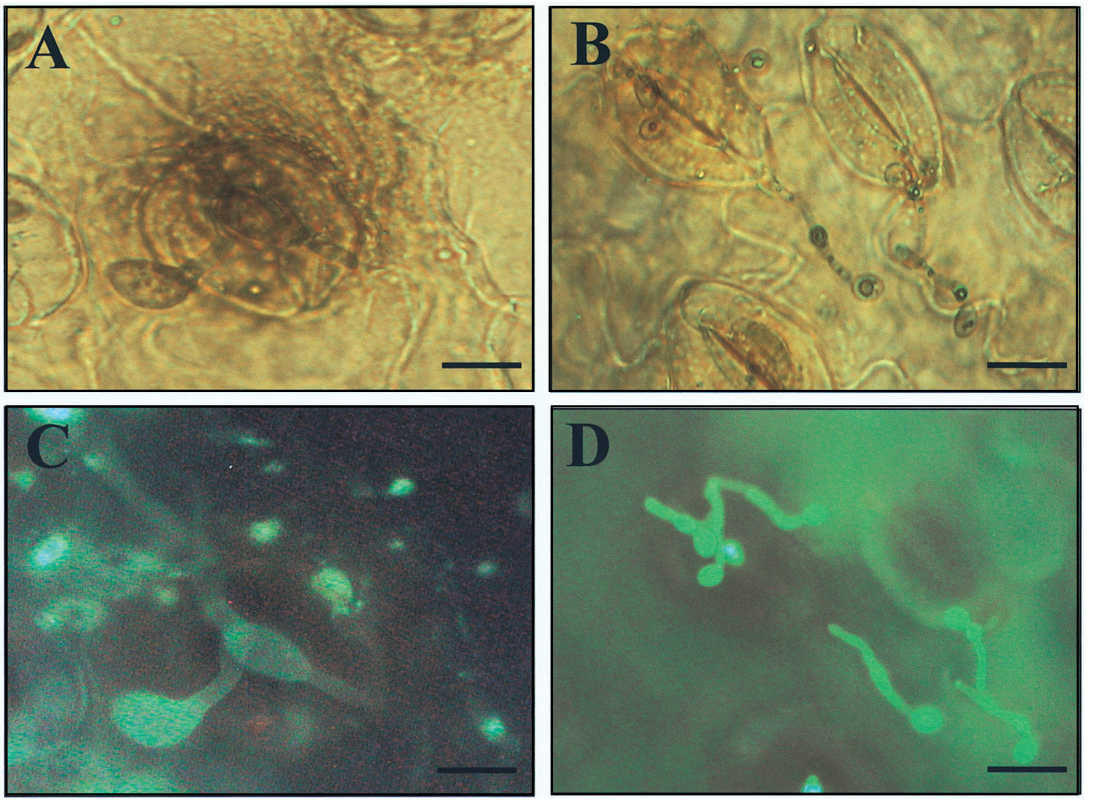

Fig. 3. Fluorescent depositions in lily leaf infected by *Botrytis elliptica*. Penetration of stomata of lily leaf by *B. elliptica* resulted in the development of necrosis (A) and the accumulation of fluorescent materials (C) in the infected tissues 12 hr after inoculation. *B. cinerea* could not penetrate lily leaf (B) and cause accumulation of fluorescent materials (D) eight days after inoculation. Photographs were taken under light (A and B) and fluorescence (C and D) microscopes. Scale bar = 32 μm.

related to appressorium formation or fungal penetration of leaf epidermis.

Exogenous nutrients requisite for appressorium formation by *B. cinerea* on onion leaves has been reported [1]. In this study, nutrients were added in the inoculation fluid, which might facilitate the appressorium formation by *B. cinerea* on 'Star Gazer' leaves. However, *B. cinerea* produced less appressoria than *B. elliptica*. Although appressoria formed by fungi could assist fungal penetration into plants [4], appressorium formation did not appear to facilitate the penetration of lily leaves by *B. cinerea*. Thus, penetration of 'Star Gazer' leaves by *B. cinerea* was not observed. Factors interferring penetration of lily leaves by *B. cinerea* remained unclear.

Both the attempted penetration sites of *B. elliptica* and *B. cinerea* had dead plant cells and some of them were associated with fluorescence. The reddish epidermal cells and the cell death have been observed on the leaves of broad bean, onion and tulip inoculated with different *Botrytis* species [16,18] and green fruits of nectarine and plum infected by *B. cinerea* [5]. These phenomena are attributed to early defense responses of plants in response to pathogen infection [5,16,19]. However, some of the germ tubes from conidia of *B. elliptica* could successfully penetrate lily leaf epidermis although some were constrained by the dead cells of leaf epidermis. This phenomenon was similar to that exhibited in partial host resistance [19].

In addition, fluorescent depositions that were detected and indicated as callose formation [19], appeared in *B. elliptica*-infected leaf tissues. Plant defense elicited by invading *B. elliptica* was presumed. As known, plant cell death was capable of enhancing the growth of *B. cinerea* in plants [6]; however, this effect was not exhibited when 'Star Gazer' leaves were inoculated with *B. cinerea*. Presumably, *B. elliptica* might have this kind of strategy to grow in lily leaves.

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摘 要

侯秉賦[1]、陳昭瑩[1,2]. 2003. 灰黴病菌 *Botrytis elliptica* 與 *B. cinerea* 於百合葉片之感染初期. 植病會刊 12:103-108. ([1.] 臺北市 國立臺灣大學植物病理學系；[2.] 聯絡作者：電子郵件 cychen@ccms.ntu.edu.tw，傳眞：+886-2-23636490)

據所觀察，*Botrytis elliptica* 感染葵百合 (Oriental hybrid cv. Star Gazer) 葉片造成壞疽病斑；*B. cinerea* 則僅在葵百合葉片上造成針狀斑點。本研究即在比較二灰黴病菌於葵百合葉片上早期的感染情形。接種後 12 小時，*B. elliptica* 與 *B. cinerea* 孢子在葵百合葉背上的發芽率達 95%；*B. elliptica* 的附著器形成率則明顯地高於 *B. cinerea*。然而，在接種後 24 小時，百合受 *B. elliptica* 的侵入率可達 54.5%；*B. cinerea* 則無法成功地侵入葵百合葉片組織。在 *B. elliptica* 及 *B. cinerea* 嘗試侵入的部位，可見死亡的葵百合葉片細胞，有些並呈現螢光。*B. elliptica* 之嘗試侵入可能受阻於此等死亡細胞；但有些感染菌絲則繼續生長。此外，*B. elliptica* 之侵染並在葵百合葉片造成螢光物質的累積；但接種 *B. cinerea* 的葵百合葉片則無類似的反應。

關鍵詞：灰黴病菌、感染、百合、植物反應