# Techniques for Inoculum Production and Inoculation of Lily Leaves with *Botrytis elliptica*

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**ABSTRACT**

Doss, R. P., Chastagner, G. A, and Riley, K. L. 1984. Techniques for inoculum production and inoculation of leaves with *Botrytis elliptica*. Plant Disease 68: 854-856.

Sporulation by selected isolates of *Botrytis elliptica* occurred readily when the fungus was cultured on potato-dextrose agar under ultraviolet light at 20 C. Microconidia were present after 12 days in cultures grown under the same conditions. Application of conidial suspensions to the undersides of detached *Lilium longiflorum* cv. Ace leaves followed by incubation for 48 hr at 100% relative humidity and 20 C was nearly optimal for infection and subsequent lesion development. Probit analysis indicated that a spore concentration of 10,000/ml should result in lesion formation in about 50% of the treated leaves. Uninjured leaves became infected only when spores were applied to the undersides. Fungal germ tubes did not penetrate cells on the upper leaf surfaces. Incidence of infection of Ace lily leaves did not vary with plant age or leaf position.

Fire, or Botrytis blight, caused by *B. elliptica* (Berk.) Cooke, is an important foliar disease of Easter lilies (*Lilium longiflorum* Thunb.) and other ornamental lilies (1,2,4,10,13,16,18,19). Little experimental work has been done on this disease, although the behavior of *B. elliptica* on nonhost plants has been the subject of recent basic research (11,12,15).

Lily breeders and growers have observed that some selections and cultivars of lilies are less visibly damaged by fire than others. This indicates that a procedure for screening lilies for resistance to *B. elliptica* could be useful. Several obstacles to development of a screening procedure have been overcome. First, a number of isolates have been obtained. Second, methods for inducing conidium formation by this usually poorly sporulating fungus (19) were developed. Finally, an inoculation procedure for screening lily selections for resistance to *B. elliptica* has been devised. The methods used and the results obtained in carrying out this work are reported.

## MATERIALS AND METHODS

Easter lily leaves showing symptoms of fire were collected throughout the 1981 growing season from five commercial lily fields and one experimental plot near Brookings, OR. Segments cut from the margins of lesions were removed from leaves after surface-sterilization for 1 min with 1.2% NaOCl and transferred to petri dishes containing potato-dextrose agar (PDA) (Difco). In cases where *Botrytis*-like mycelia grew onto the agar, mycelia were transferred to tubes of PDA that were hardened as slants. The slants were held at 20 C under continuous fluorescent light (about 2,000 lux).

A sample of isolates that formed conidia under these conditions was used to find conditions that would result in optimal sporulation. Mycelium was comminuted and used to inoculate slants (25 × 150 mm) containing 10 ml of either PDA, medium-X (7), or a lily decoction agar (aqueous lily leaf extract obtained by autoclaving 80 g of fresh Ace lily leaves in deionized water, 10 g of glucose, and 15 g of Difco-Bacto agar per liter). Cultures were grown at 20 C under four radiation regimes: continuous fluorescent light (about 2,000 lux), continuous darkness, photoperiodic cycles consisting of 12 hr of fluorescent light (about 2,000 lux) and 12 hr of darkness, or continuous ultraviolet (UV) light delivered by two F15T8 BLB tubes (General Electric) suspended about 7 cm above the cultures. After 10 days, conidial suspensions were prepared and spores counted with a hemacytometer. One isolate that sporulated well was grown at 20 C on PDA under UV light to find the optimal incubation period for spore production.

Because continuous subculturing using spore suspensions resulted in poor sporulation after several cycles, isolates to be used for inoculation of lily leaves were stored in a medium made of of 3% (v/v) Gerber oatmeal in fine silica sand. Twelve to 13 g of this mixture was placed in a culture tube (25 × 150 mm), moistened with 1 ml of deionized water, and autoclaved. About 0.5 ml of spore suspension was added to the sterile sand medium. After several days of growth at room temperature under continuous fluorescent light, the cultures were sealed with labeling tape and stored in darkness at about 3 C. To obtain a spore suspension, a few grains of sand were placed on 10 ml of PDA hardened as a slant in a culture tube (25 × 150 mm). After 1 wk of growth at 20 C under continuous UV light, these cultures contained enough spores to prepare a spore suspension with which to start additional slants. After 10–14 days of growth under UV light at 20 C, these slants had numerous spores.

To test the influence of incubation conditions on infection of lily leaves by *B. elliptica*, spore suspensions containing $8 \times 10^5$/ml were prepared. Tween 20 (about 0.02%, v/v) was added to the sterile water used to prepare spore suspensions. A 5-μl drop of the suspension was placed on the undersides of mature, healthy Ace Easter lily leaves that had been removed from mature greenhouse-grown plants. After the spore suspension dried, the leaf bases were recut and the leaves were inserted through a slit in a Parafilm membrane into water contained in either 50-ml Erlenmeyer flasks or in standard glass scintillation vials.

The leaves were then placed in a dew chamber maintained at 100% relative humidity with a photoperiodic cycle of 12 hr of light (3,000–4,000 lux) and 12 hr of darkness. The influence of incubation period (24–96 hr) and incubation temperature (12.5–25 C) was examined. After removal from the dew chamber, leaves were placed in a growth chamber maintained at 15 C, with about 70% relative humidity and a photoperiodic cycle of 16 hr of light (4,000–4,500 lux) and 8 hr of darkness. After 1 wk, incidence of infection was noted. Lesions appeared as dry, brownish, sunken areas visible on the upper surfaces of the leaves at the points of inoculation.

In a separate study, the relationship between spore concentration ($10^3$–$10^6$/ml) and incidence of infection was investigated. This work was carried out as described before, with the dew chamber set at 20 C. Leaves were removed from the dew chamber and placed in the growth

Scientific Paper 6737, Project 0496, of the College of Agriculture, Washington State University, Pullman 99164.

Accepted for publication 16 June 1984 (submitted for electronic processing).

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chamber after 48 hr of incubation.

Incidence of infection as a function of leaf position and plant age was studied by testing leaves on intact Ace plants at 3-wk intervals. Inoculation with a 5-$\mu$l drop of spore suspension containing $10^4$/ml was followed by incubation for 48 hr at 20 C and 100% relative humidity, with a photoperiodic cycle of 12 hr of light and 12 hr of darkness. Plants were returned to the greenhouse after incubation and lesion development was scored 1 wk after inoculation. Percentage infection values were transformed (14) before carrying out analysis of variance.

Location of infection courts was studied by spraying either the upper or lower surfaces of detached Ace lily leaves with a conidial suspension. After various periods of incubation in the dew chamber, leaves were cleared in saturated chloral hydrate, stained with either acid fuchsin or cotton blue in lactophenol (17), and examined under a microscope to determine where penetration had occurred.

Most of the work in this report was repeated two or more times with similar results. Statistical procedures used with various studies are described in the figure legend and tables.

## RESULTS AND DISCUSSION

Attempts were made to isolate *B. elliptica* from about 750 infected leaves collected four times during the 1981 growing season. Isolates similar to *B. elliptica* were obtained with 332 leaves. From these, 193 isolates produced enough spores to allow confirmation as *B. elliptica* on the basis of conidial shape and average conidial dimensions (Table 1). Wright (19) reported a greater variability in conidial dimensions than did other investigators (2,16,18), with lengths ranging from 18 to 32 $\mu$m and widths from 13 to 24 $\mu$m. The even larger range in conidial dimensions noted in this study was probably due to examination of a larger sample size. With the exception of isolate 30, spore shape and appearance were as reported for *B. elliptica*. Inoculation of lily leaves with spores from DWII 3-3-81 No. 3 and several other isolates listed in Table 1 (excluding isolate 30) produced lesions identical to those found on diseased leaves.

No organisms were obtained from 418 of the leaves from which isolation was attempted. In subsequent work, it was found that to isolate fungi from a high percentage of infected leaves, it was necessary to take a leaf segment extending well into a lesion. Leaf segments that extended only slightly beyond the margin of a lesion often failed to yield organisms.

In a factorial experiment examining the influence of isolate, culture medium, and lighting on sporulation, only one of 10 isolates yielded measurable numbers of spores after 10 days of growth under continuous fluorescent light. None of the isolates sporulated when grown under photoperiodic cycles consisting of 12 hr of light and 12 hr of darkness or under complete darkness. All 10 isolates yielded measurable numbers of spores when cultured on either PDA or medium-X under UV light. There was no significant difference between the number of spores per culture tube when isolates were cultured on these two media (averaging $3.7 \times 10^5$ spores per isolate on medium-X vs. $3.5 \times 10^5$ on PDA). Sporulation was poor on lily decoction agar (averaging $2.4 \times 10^4$ spores per tube), probably because of poor growth on this medium. The ability of UV light to promote sporulation of *B. elliptica* is not surprising in view of results with a number of fungi (8). *B. elliptica* is apparently more sensitive to UV treatment than *B. cinerea*. The latter has been called a slightly sensitive species (9) because it sporulates fairly well in the dark. Medium-X has been used to culture *B. elliptica* (14) and UV light has been used with medium-X to obtain good sporulation (J. W. Mansfield, *personal communication*). In our study, isolates sporulated as well on PDA as on medium-X. Hence, the more easily formulated PDA was used in all subsequent studies.

Maximum numbers of conidia were formed after 12 days of incubation at 20 C under UV light (Table 2). At 15 days, conidial numbers decreased, perhaps because of germination of conidia that had been dislodged from the conidiophores. Spore suspensions from 10- to 14-day-old cultures were used in studies with lily leaves.

Microconidia were also formed under these conditions but appeared only after 12 days. Microconidia were hyaline, unicellular, averaging 2.7 $\mu$m (range 2.0–3.4 $\mu$m) in diameter with a conspicuous lipid droplet. Microconidia were borne on short phialides, averaging 7.6 $\mu$m (range 5.9–10.7 $\mu$m) long, that were acolarette and inflated toward the base. Although Jarvis (5) indicated that all *Botrytis* species produce microconidia, their formation by *B. elliptica* has not been reported before (19).

Temperature and incubation period influenced the incidence of infection of detached Ace lily leaves (Table 3). Infection took place slowly at 25 C and was most rapid at 20 C. At this temperature, not all leaves showed lesions at the end of the 48-hr incubation period; however, all leaves did have lesions after 5 more days in the growth chamber. On the basis of these results, a standard incubation temperature of 20 C and an incubation period of 48 hr were used in all subsequent studies.

Standard incubation conditions were used in five experiments carried out to determine the influence of spore concentration on infection. The sigmoid curve best relating probability of infection to spore concentration was

**Table 1.** Dimensions of conidia for 10 *Botrytis elliptica* isolates grown at 20 C under ultraviolet light

| Isolate | Spore length × width[a] ($\mu$m) | Range[a] ($\mu$m) |
|---|---|---|
| DW II, 3-3-81, No. 3 | $38.6 \pm 1.8 \times 20.8 \pm 0.5$[b] | 33–50 × 20–23 |
| 30 | $23.6 \pm 0.7 \times 9.5 \pm 0.5$[c] | 20–28 × 7.5–10 |
| DW III, 3-3-81, No. 1 | $28.9 \pm 2.0 \times 16.4 \pm 0.8$ | 18–38 × 15–20 |
| HHHR IV, 3-20-81, No. 3 | $27.9 \pm 1.7 \times 16.9 \pm 0.7$ | 18–33 × 13–18 |
| CH II, 3-4-81, No. 4 | $28.1 \pm 0.7 \times 15.6 \pm 0.9$ | 25–30 × 13–20 |
| HHHF V, 5-19-81, No. 1 | $31.8 \pm 2.2 \times 18.4 \pm 0.9$ | 25–45 × 15–23 |
| CHR III, 5-19-81, No. 5 | $33.0 \pm 1.5 \times 16.7 \pm 0.8$ | 25–40 × 13–20 |
| HSR I, 5-19-81, No. 8 | $30.1 \pm 1.4 \times 16.9 \pm 0.7$ | 23–38 × 15–20 |
| HHHF I, 5-19-81, No. 9 | $28.0 \pm 1.9 \times 17.0 \pm 1.6$ | 20–33 × 10–23 |
| HSR V, 5-19-81, No. 8 | $30.0 \pm 1.3 \times 18.1 \pm 0.9$ | 25–35 × 13–23 |

[a] Means ± standard errors for 10-spore samples grown on medium-X (7).
[b] A second measurement by a different investigator of a sample of 25 conidia of DW II, 3-31-81, No. 3, yielded $33.7 \pm 0.9 \times 19.8 \pm 0.5$ $\mu$m.
[c] Isolate 30 was an atypical isolate obtained from a hybrid lily planting in Mt. Vernon, WA. The remainder of the isolates were obtained from Easter lily leaves collected near Brookings, OR.

**Table 2.** Conidial and microconidial production as a function of duration of the incubation period for isolate DW II, 3-3-81, No. 3, grown on PDA at 20 C under ultraviolet light

| Incubation period (days) | Conidia/culture tube[a] | Microconidia/culture tube[a] |
|---|---|---|
| 3 | $(4.9 \pm 1.6) \times 10^4$ | ... |
| 6 | $(3.2 \pm 0.3) \times 10^5$ | ... |
| 9 | $(4.6 \pm 0.3) \times 10^5$ | ... |
| 12 | $(7.9 \pm 0.6) \times 10^5$ | $(1.8 \pm 0.5) \times 10^5$ |
| 15 | $(5.9 \pm 0.5) \times 10^5$ | $(1.1 \pm 0.1) \times 10^6$ |
| 18 | $(5.3 \pm 0.5) \times 10^5$ | $(3.9 \pm 0.8) \times 10^6$ |
| 21 | $(5.3 \pm 0.7) \times 10^5$ | $(3.4 \pm 0.7) \times 10^6$ |

[a] Means ± standard errors for five cultures.

**Table 3.** Percentages of Ace lily leaves (n = 7) possessing lesions 7 days after inoculation with *Botrytis elliptica* as a function of incubation temperature and duration of free moisture on leaf surface[a]

| Temperature (C) | Duration of free moisture (hr) | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| 12.5 | 0 | 29 | 100 | 100 |
| 15.0 | 29 | 71 | 100 | 100 |
| 20.0 | 43 | 100 | 100 | 100 |
| 25.0 | 0 | 0 | 43 | 57 |

[a] Isolate DW II, 3-3-81, No. 3; 800,000 spores per milliliter.

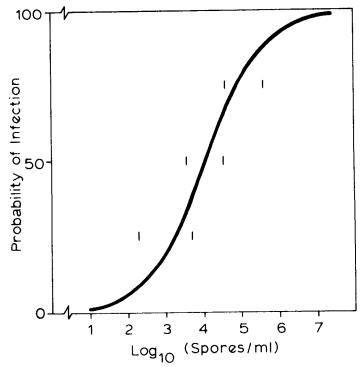


**Fig. 1.** Probability of infection of detached Ace lily leaves as a function of spore concentration, determined using a probit procedure to analyze data from five trials that explored the relationship between spore concentration and incidence of infection. Brackets indicate 95% fiducial limits. $\chi^2 = 16.03$ with 10 df; probability of a larger $\chi^2$ attributable to chance = 0.09.

determined using probit analysis (3,6) (Fig. 1). Fiducial limits (95%) are plotted as bracketed lines for probabilities of 0.25, 0.50, and 0.75.

When intact Ace plants were inoculated with spores of *B. elliptica*, there were no significant differences in incidence of infection with plant age. This was true with inoculations ($1 \times 10^4$ spores per milliliter) carried out at 3-wk intervals beginning 69 days before and extending 37 days past full anthesis (10-plant sample per inoculation).

There was no significant difference in incidence of infection with leaf position. Leaves from the lower, middle, or upper one-third of the plant showed similar rates of infection even though the lower leaves yellowed earlier than the upper leaves. Most of the lower one-third of leaves were dead by full anthesis.

Leaves became infected only when inoculated on the undersides. This was true even with concentrations of up to $10^6$ spores per milliliter. Leaves of *L. longiflorum* possess stomata only on the lower surfaces. A histological study showed that penetration occurred through stomatal apertures, guard cells, and epidermal cells. Ward (18) reported germ tube penetration through the cuticle of *L. candidum* but failed to note (but did not rule out) stomatal penetration. It is not clear whether Ward inoculated both upper and lower leaf surfaces. Conidia germinated when placed on upper surfaces, but penetration through the upper epidermis was not observed.

Sufficient information is now available to allow development of procedures for screening lily plants for resistance to *B. elliptica*. An effort to screen a number of ornamental lily clones is in progress.


## ACKNOWLEDGMENTS

We thank Lee Riddle of the Pacific Bulb Growers Research and Development Station for collecting Easter lily leaves and providing lily bulbs, James Christian for technical assistance, and the Pacific Bulb Growers' Association and the Fred C. Gloeckner Foundation, Inc., for providing financial assistance and support.


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