Infection of Photinia Leaves by *Entomosporium mespili*

A. B. A. M. BAUDOIN, Assistant Professor, Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg 24061

ABSTRACT

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Young, expanding leaves of *Photinia × fraseri* were highly susceptible to Entomosporium leaf spot, but few spots formed on fully expanded leaves. Infection took place on both surfaces of the young leaf, the abaxial surface being penetrated about twice as frequently as the adaxial surface. The fungus usually penetrated directly through the cuticle and cell wall; stomatal penetration was uncommon. At 25 C, penetration into epidermal cells could be detected within 12 hr of inoculation. Conidia germinated and produced appressoria on mature leaves, but penetration through the thick cuticle and cell wall was uncommon. Most penetrations of mature leaves occurred on the abaxial surface, through guard cells or stomata.

Additional key words: *Diplocarpon maculatum, Entomosporium maculatum, Fabraea maculata*

Entomosporium leaf spot is a widespread and sometimes destructive disease of ornamental shrubs of the genus *Photinia* and a number of other genera in the subfamily Pomoideae of the Rosaceae (5,12). The conidial state of the fungus causing this disease has usually been referred to as *Entomosporium maculatum* Lév.; however, Sivanesan and Gibson (12) gave *E. mespili* (DC. ex Duby) Sacc. as the name having priority. The teleomorph names *Fabraea maculata* Atk. and *Diplocarpon maculatum* (Atk.) Jorstad are both used in recent literature. Leaf spots on photinia render the plants unsightly and unsalable as nursery stock, and heavy infection may defoliate plants (8). The disease can be controlled by fungicides (4,7), but frequent sprays are needed to protect susceptible new growth (4,10). No quantitative data on the susceptibility of young and older foliage of photinia have been published. Because new foliage appears in flushes only during certain times of the year, knowledge of changes in the susceptibility of the foliage may help to design more efficient spray schedules.

Although several authors have described the pathological histology of the disease and the formation of fruiting bodies by the pathogen on several hosts (6,9,13), information on the infection process itself is limited. Piehl and Hildebrand (9) stated that germ tubes penetrated the cuticle of pear leaves and developed into a compact network above

the epidermal layer. They did not illustrate this process, however, and the subcuticular network was not found by Stowell and Backus (13). A recent report (14) provides a brief description of the infection process by cuticular penetration of young pear leaves. However, a direct correlation between stomatal number and susceptibility of pear varieties and species to Entomosporium leaf spot (2) suggests that stomatal penetration might play a role. Therefore, this study on leaf susceptibility to infection was combined with a study of the infection process on young and mature photinia leaves.

MATERIALS AND METHODS

Healthy softwood cuttings of *Photinia × fraseri* Dress. were rooted and grown in a greenhouse. The plants used were 1–2 yr old and were grown in milled pine bark medium (pH 4.7) in 473- or 946-ml containers. They were fertilized with a slow-release fertilizer (Osmocote 14-14-14) and pruned when necessary to keep the plants small and to encourage development of new leaves.

New leaves of photinia usually appear in flushes of three to seven leaves. For experiments on leaf age, the length of the emerging leaves was measured every 3 or 4 days. Length and width were measured just before inoculation and again at symptom evaluation 14 days later. This final leaf size represented the fully expanded leaf for all leaves except those that were very young at the time of inoculation. Leaf age was expressed as the number of days since the leaf reached 50% of its final length and was estimated to the nearest day by linear interpolation between measurements. This stage could be determined more accurately than other reference points such as leaf emergence from the bud or leaf unfolding. Consequently, very young

leaves were assigned a negative age.

Leaf surface areas were calculated from measurements of leaf length and width, using a relationship determined as follows (1): The length and width of 50 leaves were measured to the nearest millimeter, and leaf area was measured with an electronic leaf area meter (model LI-3000, Li-Cor, Inc., Lincoln, NE). The best-fitting linear regression equation was: area = $0.6777 \times$ length $\times$ width ($r^2 = 0.9975$, for leaf areas ranging from 5 to 25 cm^2).

Cultures of *E. mespili*, isolated from leaf spots on photinia and grown on potato-dextrose or V-8 juice agar either in the dark or under 14 hr of light (about $50 \mu E m^{-2} s^{-1}$) each day, did not sporulate consistently (see also 14). Therefore, conidia for inoculation were collected from leaves with sporulating lesions. Leaves were immersed in tap water, and the lesions were gently rubbed with a glass rod to liberate conidia from acervuli, or leaves were placed in a capped jar with water and the jar was shaken vigorously. The conidial suspension was filtered through glass wool, and the spore concentration was estimated with a hemacytometer and adjusted to 10^4 conidia per milliliter unless otherwise indicated. Spore suspensions were sprayed over the plants with a hand-operated atomizer (DeVilbiss Co., Toledo, OH) or with a Chromist spray unit (Gelman Sciences, Ann Arbor, MI). Plants were held in a dark dew chamber at 25 C for 24 hr, then placed in a growth chamber at 25 C and 14 hr of light per day. In some experiments, plants were placed in a greenhouse (20–32 C) after the dew period. Symptoms were evaluated 14 days after inoculation.

Several experiments were conducted with detached leaves. Shoots were clipped from the plants and held under water while the leaves were cut from the stem. The leaves were measured and placed on wet filter paper in petri dishes, then the exposed surface was blotted dry with absorbent paper. Detached, young leaves expanded little (less than 20% in surface area), so their age could not be calculated by the method used for attached leaves and had to be estimated from their appearance.

Detached leaves were inoculated by spraying a conidial suspension over the leaves with a Chromist spray unit. The petri dishes were closed and incubated at 25 C in the dark in a plastic box lined with wet paper towels. After 24 hr, the leaves were blotted dry with absorbent paper

and placed in holes in a 19-mm-thick Styrofoam plate floating on water so that the petioles were immersed in water. Air was removed from the petioles by floating the plate on water in a vacuum desiccator and subjecting the leaves to a partial vacuum. The plate with leaves was then floated on water in a plastic box, which was covered loosely and held at 25 C and 14 hr of low-level light ($15 \mu E m^{-2} s^{-1}$) per day. In this fashion, leaves could be kept turgid at a relative humidity lower than 100%. Spots were counted after 9 and 14 days. Leaves that were inoculated, allowed to dry immediately, and held under these conditions did not develop leaf spots.

For observation of spore germination and infection, detached leaves were inoculated with drops of a more concentrated conidial suspension ($5-20 \times 10^4$ conidia per milliliter). To determine percent germination, inoculum drops were allowed to dry after various periods of incubation, then the leaf surface was covered with a thin layer of clear nail polish. After drying, the nail polish films were removed and mounted in lactophenol with 0.05% trypan blue. In addition, inoculated leaf pieces of about 10–25 mm^2 were excised after various periods of incubation. Some leaf pieces were fixed and cleared in lactic acid-absolute ethanol (1:3, v/v) for several days or several hours at 60 C, stained with trypan blue (0.05%) in lactophenol, and mounted in clear lactophenol. Structures within the leaf were observed by interference-contrast optics in cleared leaf pieces that had been boiled in 10% KOH for 10 min and mounted in lacto-phenol. Other leaf samples were fixed in formalin-acetic acid-ethanol-water

(13:8:95:95, v/v), dehydrated in an ethanol-*t*-butanol series (3) and embedded in Paraplast. Sections were cut 10 μm thick with a rotary microtome and stained with safranin-fast green (3).

RESULTS AND DISCUSSION

When inoculated plants were held at 25 C, the first symptoms were tiny red flecks on the young leaves visible to the unaided eye 5 days after inoculation. Spots were easily visible after 6 days, and acervuli appeared as small, raised blisters after 8 days. When the spots were kept wet, ruptured acervuli releasing conidia were observed after 9 or 10 days. Very few additional spots appeared after more than 14 days. At lower temperatures (15–20 C), the sequence required several additional days. Spots on mature leaves were uncommon but developed at the same rate. This incubation period is shorter than the incubation period of 3 wk reported by Raabe and Hansen (11) for Entomosporium leaf spot of *Raphiolepis* and comparable to the 4–7 days reported for Entomosporium leaf spot of pear (14).

Under greenhouse conditions (20–32 C), leaves reached 50% of their final length 5–8 days after they emerged from the bud and were fully expanded after about 14 additional days. Leaf age was a major determinant of susceptibility (Fig. 1). In three experiments, the average leaf spot density on leaves 0–7 days old was 521, 240, and 616 spots, respectively, per 100 cm^2 of inoculated leaf surface, possibly because of differences in viability of the conidia or differences in amounts of leaf exudates (14) in the inoculum. Leaf spot densities on individual leaves were expressed as a

percentage of these average densities, allowing the calculation of standard errors based on the combined experiments. Leaves were very susceptible as long as they were expanding rapidly. Susceptibility decreased as the expansion rate declined, and fully expanded leaves developed very few spots. Leaves less than 3 days old appeared to be slightly less susceptible than leaves 4–7 days old. This may be due to the more upright posture of very young leaves, which may therefore retain less inoculum per unit of surface area than the more horizontal leaves in intermediate stages of expansion.

Although leaf age, as defined here, could be calculated only after completion of the experiment, distinct changes in leaf appearance and texture coincided with the time when leaves became resistant. Young leaves were reddish, glossy, thin, and tender. Maturing leaves that had reached more than 90% of their final surface area became green, dull, firm, and stiff.

The leaf spot density resulting from inoculating the lower (abaxial) surfaces of young, detached leaves with a conidial spray was 2.2 times higher than when the upper (adaxial) surfaces were inoculated. A similar difference was found with attached leaves that were inoculated on one side with a directed spray of the Chromist unit so that no wetting occurred on the other side. On mature leaves inoculated with 5×10^4 conidia per milliliter, the difference was larger: three to 25 times more lesions resulted from inoculation of the lower leaf surface than of the upper surface. In these experiments, plants were allowed to dry after inoculation before being placed in the mist chamber. In one experiment, in which the inoculated young leaves were still wet when placed in the mist chamber, there was no difference associated with leaf surface inoculated. Conidia in droplets on the lower surface of the leaf may not become attached to the leaf surface as rapidly as those in drops on the upper leaf surface and thus may be more likely to run off the leaf with the accumulating moisture. In the field, this effect would counteract the difference in susceptibility of the two leaf surfaces.

Some conidia germinated within 6 hr of inoculation on both surfaces of the leaf on young as well as mature leaves. Germ tubes were about 5 μm in diameter, and their average length 24 hr after inoculation was 7.4 μm with a standard deviation of 6.2 μm (range 0–53 μm) (Fig 2A). Sometimes, no lateral germ tube formed, but an appressorium formed directly beneath a conidium (Fig. 2D,G). Percent germination and mean germ tube length differed considerably among inoculation droplets and leaves; there was little difference in germination on the upper and lower surface and on young and mature leaves. Inoculum used in these experiments presumably contained
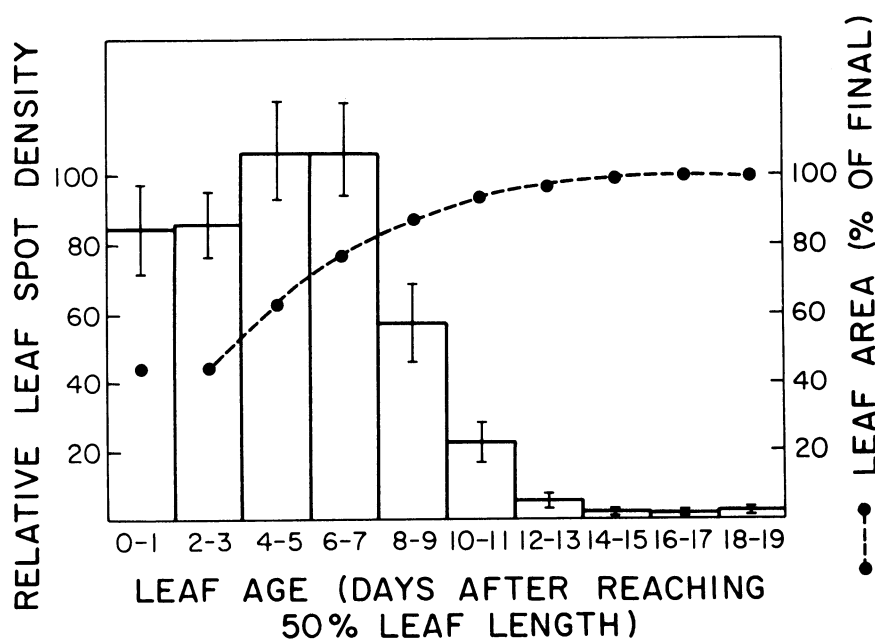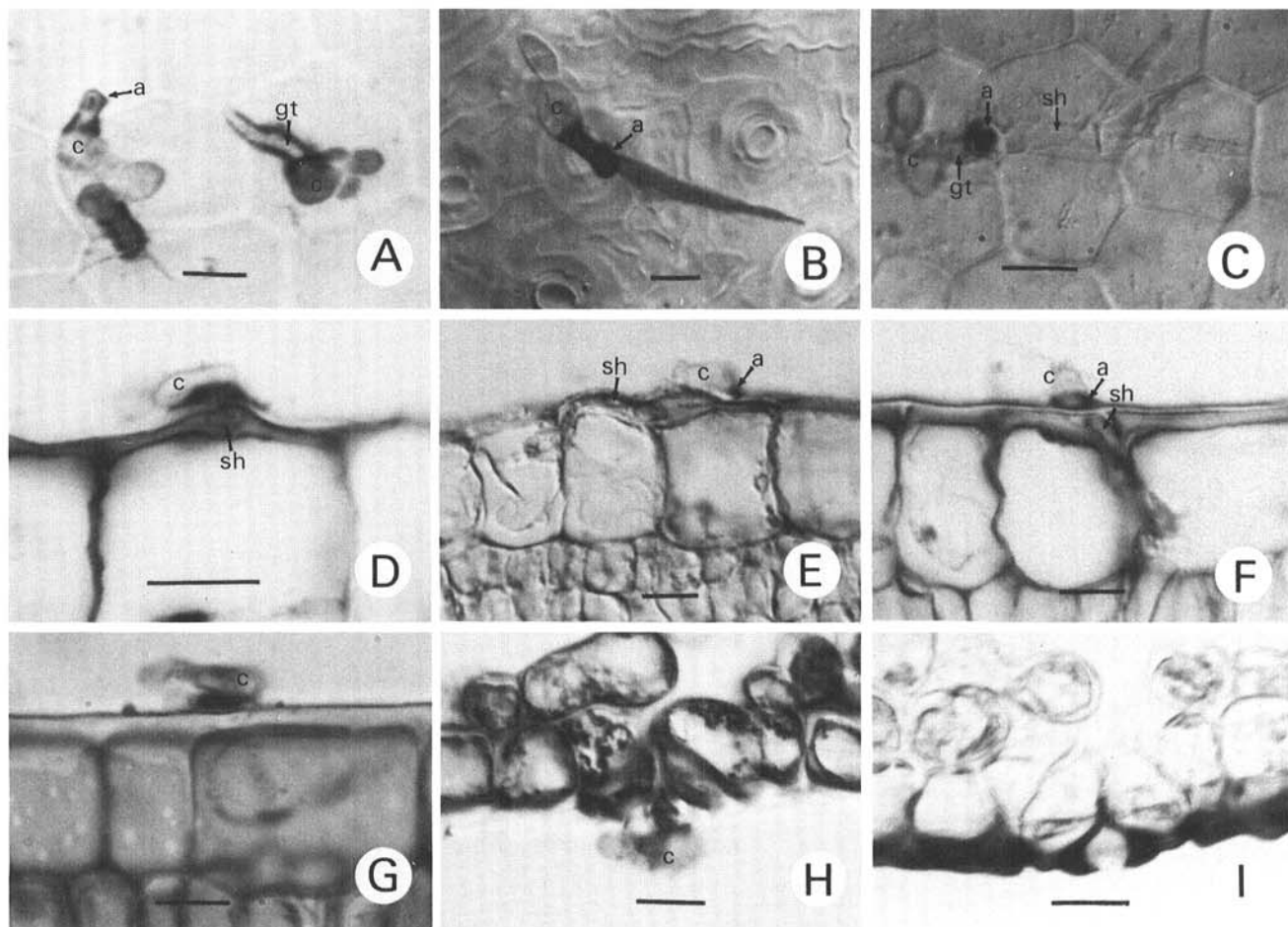

Fig. 1. Effect of leaf age on susceptibility of photinia leaves to Entomosporium leaf spot. Leaf age is expressed as days after reaching 50% of the final length. Relative leaf spot density is expressed as a percentage of the average leaf spot density on 0- to 7-day-old leaves. Data are the averages of three experiments.

Fig. 2. Infection of photinia leaves by *Entomosporium mespili*. **(A-F)** Young leaves. **(A)** Germinated conidia showing presence and absence of appressoria. **(B)** Germinated conidium with germ tube that has resumed growth after forming an appressorium. **(C)** Germinated conidium with subcuticular hypha. **(D)** Cross section through subcuticular hypha. **(E)** Longitudinal section through subcuticular hypha. **(F)** Longitudinal section of penetrating hypha growing directly into leaf interior. **(G-I)** Mature leaves. **(G)** Germinated conidium with appressorium, showing absence of penetration through thick cuticle and cell wall. **(H)** Penetration attempt through stoma. **(I)** Section through stoma showing thinner cuticle over guard cells than over other epidermal cells, stained with Sudan IV. **(A and C-G)** Adaxial leaf surface, **(B, H, and I)** abaxial leaf surface. All figures 24 hr after inoculation except D and G, which are 48 hr after inoculation. a = Appressorium, sh = subcuticular hypha, and gt = germ tube. Scale bars = 10 μm.

significant amounts of leaf leachates from the (usually mature) leaves from which the conidial suspensions were prepared, which may have affected germination rates (14). When conidia were filtered through a membrane filter, rinsed, and resuspended in distilled water, their germination percentage on mature leaves was about half of that on young leaves. The longest germ tubes were found on the upper surfaces of young leaves (mean length 14.0 μm); the shortest were found on the upper surfaces of mature leaves (mean length 4.2 μm). Twelve hours after inoculation, many germ tubes had appressoria consisting of a rounded germ tube apex with a darkly staining center (Fig. 2A,C). Other germ tubes were still tapered at the apices (Fig. 2A). Sometimes, a germ tube appeared to have resumed growth after formation of an appressorium (Fig. 2B). No patterns were evident with respect to the direction of the germ tubes or the location of penetration attempts. On the upper leaf surface, which lacked stomata, appressoria occurred both above anticlinal walls and above the cell lumen; on the lower

surface, appressoria were found on stomata as well as on epidermal cells. There was no evidence that germ tubes preferentially grew towards stomata (Fig. 2B).

It was not possible to reliably detect the earliest penetration in whole leaf mounts, but after 12 hr, mycelium was observed inside necrotic cells beneath some germinated conidia. The penetrating hypha often grew in the outer epidermal cell wall across several epidermal cells before penetrating the cell lumen (Fig. 2C-E), although penetration through the cell wall was sometimes rather direct (Fig. 2F). Subcuticular hyphae have been observed in *Entomosporium* infection of pear (14). Further penetration was mostly intercellular, with haustoria penetrating into cells as described by Stowell and Backus (13). Penetration of mature leaves was studied by inoculating detached leaves with 5×10^4 conidia per milliliter and incubating them on moist filter paper in petri dishes until lesions became visible after 5–6 days. Lesions were then excised, cleared, and stained. Among 41 spots where the germinated conidium could be

located, 2 penetrations took place on the upper surface, 18 penetrations were associated with the stomatal opening (Fig. 2H), 17 penetrated through a guard cell, and 4 were on the lower surface but not associated with stomata. Guard cells may be susceptible to penetration because their cuticles are thinner than those of other epidermal cells. Cuticle thickness, determined by staining with Sudan IV, was about 1 μm on young leaves, 7 μm on the upper surfaces of mature leaves, and 6 μm on the lower surfaces of mature leaves. The cuticle covering the guard cells, however, was only 3 μm thick (Fig. 2I). Thus the number of stomata appears to affect the susceptibility of mature leaves but not of young leaves.

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