Effects of Cultural Conditions on Sporulation, Germination, and Pathogenicity of *Entomosporium maculatum*

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ABSTRACT

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Basic physiological studies of the pear leaf blight fungus, *Entomosporium maculatum*, resulted in development of a sucrose-casein culture medium that allowed significantly more growth and sporulation than media used previously. *E. maculatum* was found to require an exogenous source of thiamine, and the sucrose-casein medium allowed sufficient spore production for development of a mass screening program for seedlings. When thiamine was added to potato-dextrose agar, fungal growth rate increased fivefold and conidial production 30-fold. Maximum spore production occurred at 100 lux. Higher light intensities inhibited early growth of colonies but did not affect sporulation if spores were allowed to germinate and grow initially in darkness. Germination in culture was stimulated by glucose but was inhibited by ammonium nitrate and by pear leaf extracts. However, leachates from leaves susceptible to infection stimulated conidial germination in vitro while leachates from resistant leaves had no effect. Cultures lost pathogenicity rapidly, becoming almost avirulent after three transfers. Conidia from colonies maintained in culture for 3 mo were less virulent than conidia kept on diseased leaves at 2 C, and the loss in virulence was inversely related to increased germination and growth in culture.

Additional key words: brown spot, *Fabraea maculata*, fruit spot, *Pyrus*.

Pear leaf spot, leaf blight, or fruit spot is known to occur in most countries of the world where pears (*Pyrus* spp.) and quince (*Cydonia* spp.) are grown. Depending upon plant tissue susceptibility of a cultivar, the causal organism, *Fabraea maculata* (Lev.) Atk. [= *Diplocarpon maculatum* (Atk.) Jorstad = *D. sorauri* (Kleb.) Nannf.; anamorph = *Entomosporium maculatum* Lev. (imperfect stage)] causes a leaf spot and/or fruit spot. Other hosts include hawthorn (*Crataegus* spp.), cotoneaster, photinia, mountain-ash (*Sorbus* spp.), and occasionally apple (*Malus silvestris*) (4,5,13–15). Though generally controlled by fungicide sprays, the organism can cause reduced fruit quality and extensive defoliation, particularly in nursery trees (1).

Generally, leaf and fruit infections show symptoms 4–7 days after inoculation, developing from red and purple pinpoint dots to black spots, 1–3 mm in diameter. Acervuli form at the center of these spots and white masses of conidia (*E. maculatum*) are observed after 2–4 wk. Current-season shoots can also be infected, and some twig lesions develop into cankers on 1-yr-old wood that produce conidia the following spring. Our observations agree with those of others (6,8) that these twig cankers represent the primary inoculum source. Conidia and ascospores (*F. maculata*) can be produced on overwintering leaves (15), but twig cankers may be more important under different climatic conditions or on some cultivars (10,11).

Few cultural studies have been done on *E. maculatum*. An optimum temperature of 22–26 C was determined and a pH range of 4.0–7.0 was established (7). Potato-dextrose agar (PDA) has been the most successful medium for culturing the fungus (7,9). However, we found that the organism grows very slowly and loses the ability to sporulate on PDA after two or three transfers. Even first-generation cultures have poor yields of conidia after 24–30 days.

Controversy exists regarding the initial infection stages of the disease, though no etiological studies have been done. Piehl and Hildebrand (9) believed the germ tube of the pathogen directly penetrated the cuticle of the upper leaf surface, growing subcuticularly at first. However, Stowell and Backus (15,16) found mycelium in incipient lesions in the interior of leaves of *Crataegus*, but not directly beneath the leaf cuticle. Both authors also found haustoria invading host cells.

Resistance to leaf spot has been noted within the genus *Pyrus* (18). Incorporating resistance to this disease in pear cultivars could reduce the number of fungicide sprays needed to grow pears. However, no screening program has been attempted and there are few, often conflicting, reports of field resistance among different cultivars (2,4,19). The major obstacle to a screening program has been the poor sporulation of the fungus in culture. Optimum conditions for artificial inoculations are known, and results from such inoculations are encouraging (11), but the poor growth of the

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fungus in culture has made a mass screening program unfeasible.

Therefore, our first objective was to increase sporulation in vitro by studying the effects of nutritional and environmental factors. Spore suspensions from cultures were then used to study the infection process, loss of virulence, and the effects of nutrients and leaf leachates on spore germination. Preliminary information on these studies was reported earlier (17).

MATERIALS AND METHODS

Growth and sporulation. Initially, the fungus was grown under continuous light at 26 C on a vitamin-free basal medium, which contained (per liter): glucose (20 g), casein hydrolysate (Difco, 5 g), agar (15 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KH_2PO_4 (1.0 g), $FeSO_4 \cdot 7H_2O$ (9.2 mg Fe), $ZnSO_4$ (0.2 mg Zn), and $MnSO_4$ (0.1 mg Mn). To this medium, the following growth factors were added in separate tests: thiamine HCl (100 mg), biotin (100 mg), riboflavin (50 mg), choline chloride (100 mg), Ca pantothanate (100 mg), nicotinic acid (100 mg), pyridoxine HCl (200 mg), folic acid (50 mg), cyanocobalamin (15 mg), *p*-aminobenzoic acid (50 mg), or myo-inositol (1 g).

To test various nitrogen (N) sources, casein hydrolysate (10% N), beef peptone (16% N), yeast extract (10.5% N), asparagine, $Ca(NO_3)_2$, and NH_4-tartrate were added to the basal medium plus 1 μg of thiamine per milliliter in amounts necessary to attain 0.5 g N per liter. To test carbon (C) sources, glucose, mannitol, sucrose, sorbitol, fructose, maltose, and Na-citrate were added to attain the equivalent of 20 g of glucose per liter. The effect of pH was tested by adding appropriate amounts of HCl or KOH after autoclaving.

Light intensity effects were determined by placing the cultures at various distances from a cool-white fluorescent lamp which was left on continuously in an otherwise dark room. Optimum pH was determined by growing the fungus on the basal medium plus thiamine (substituting 20 g of sucrose for glucose) at 100 lux after allowing spore suspensions to germinate in complete darkness for 1 wk. Unless noted otherwise, spores for all experiments were obtained from first-generation cultures grown on PDA.

Dry weights were determined by melting the agar medium and trapping colonies on cheesecloth. Colonies were dried at 100 C for

TABLE 1. Effect of thiamine concentration on growth and sporulation of *Entomosporium maculatum* cultured on a basal medium

Thiamine concentration (ppm)	Mean dry weight/colony (mg)	Numbers of conidia/colony ($\times 10^3$)
0.00	0.3 a[z]	2.6 a
0.25	4.1 b	19.6 b
0.50	8.0 c	47.2 c
1.00	8.2 c	83.4 d
2.00	9.9 c	98.8 d
3.00	9.2 c	101.2 d

[z] Means of four colonies; values within a column and followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 2. Sporulation by *Entomosporium maculatum* on potato-dextrose agar with thiamine (1 μg/ml) and casein hydrolysate added

Casein hydrolysate (g/L)	Conidia ($\times 10^3$) per colony: Without supplemental thiamine	With supplemental thiamine
0	1.9 a[z]	33.7 a
1	2.5 a	54.7 b
3	2.8 a	74.6 c
5	3.0 a	89.8 d

[z] Means of four colonies; values within a column and followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 3. Effect of various nitrogen sources on the growth of *Entomosporium maculatum* cultured on a basal medium

Nitrogen source	Maximum dry weight (mg)
Casein hydrolysate	23.6 a[z]
Calcium nitrate	18.0 b
Yeast extract	12.4 c
Ammonium tartrate	10.4 c
Beef peptone	10.4 c
Asparagine	3.8 d

[z] Means of four colonies; values within a column and followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

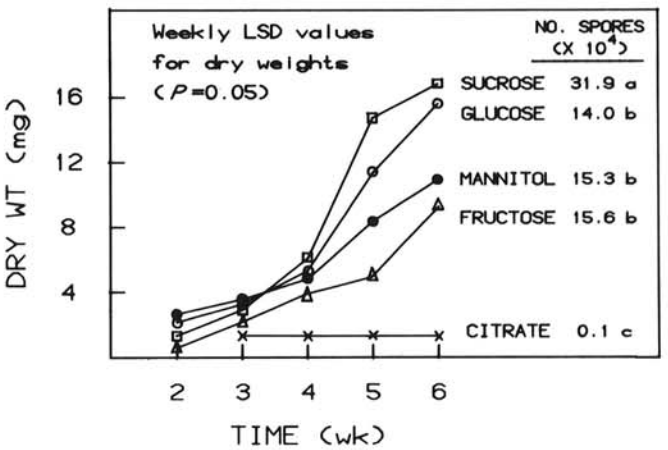

Fig. 1. Effect of some carbon sources on growth and sporulation by *Entomosporium maculatum* grown on a basal medium including thiamine.

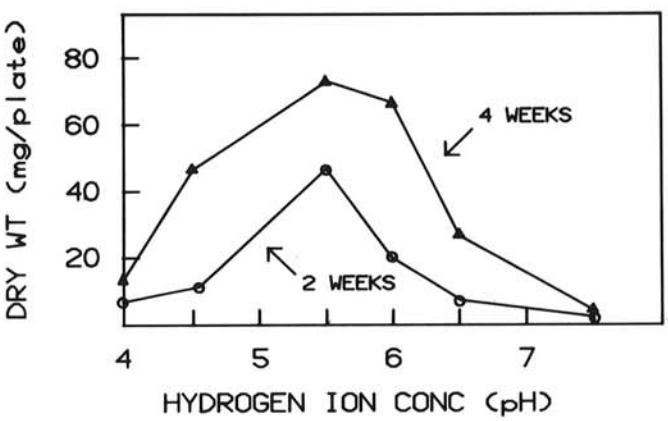

Fig. 2. Effect of initial pH of sucrose-casein-thiamine medium on growth of colonies of *Entomosporium maculatum* after 2 and 4 wk.

TABLE 4. Effect of glucose concentration on growth and sporulation of *Entomosporium maculatum*, cultured on a basal medium including thiamine

Glucose concentration (%)	Mean dry weight/colony (mg)	Conidia per colony ($\times 10^4$)
0.0	0.4 a[z]	0.6 a
0.5	7.9 b	12.8 bc
1.0	12.6 c	18.3 c
2.0	14.0 d	14.0 bc
2.5	16.9 e	30.7 d
4.0	15.6 de	11.8 b

[z] Means of four colonies; values within a column and followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

24 hr and weighed to the nearest 0.1 mg. Spores were counted by removing colonies from agar, blending them in 2 ml of sterile distilled water, and performing two counts per colony with a hemacytometer. All values represent the average of two to three replicates.

Spore germination. One-tenth milliliter of leaf leachates or liquid basal medium (plus 1 μg of thiamine per milliliter) were added to individual wells of microtiter plates (Lab Tek). Leaf leachates were obtained from leaves that had been surface-sterilized for 3 min in 10% sodium hypochlorite, then rinsed for 3 min in sterile distilled water. These leaves were immersed in distilled water for 48 hr and the solution was autoclaved after the leaves were removed. A concentration gradient was obtained by dilution of the original solution with sterile distilled water. Young leaves were defined as the uppermost four pairs of leaves on actively growing shoots, whereas older leaves were lower, dark-green leaves that were no longer expanding.

To these wells, 0.1 ml of a 10^3 conidia per milliliter of spore suspension was added. The plates were sealed with Parafilm and transferred to an incubation chamber maintained at 26 C. After 48 hr, they were observed with an inverted microscope and the percent germination and average germ tube length were recorded. Two counts per well (approximately 50 spores per count) were averaged and four replicate wells per treatment were used.

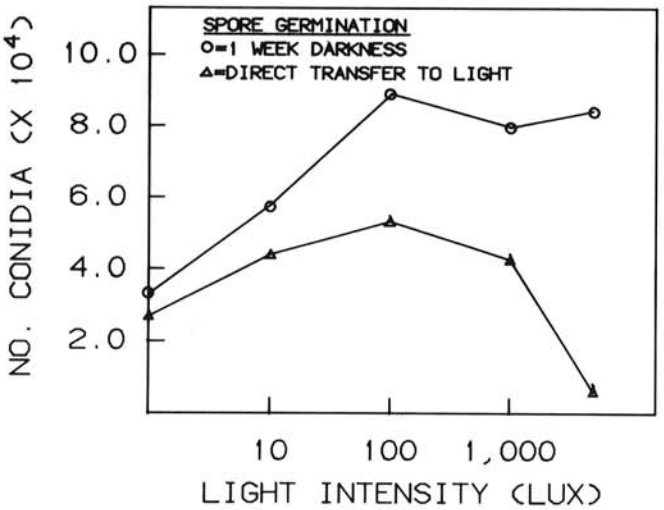

Fig. 3. Effect of light intensity on spore production by colonies of *Entomosporium maculatum* either transferred directly to continuous light or allowed to germinate in darkness for 1 wk.

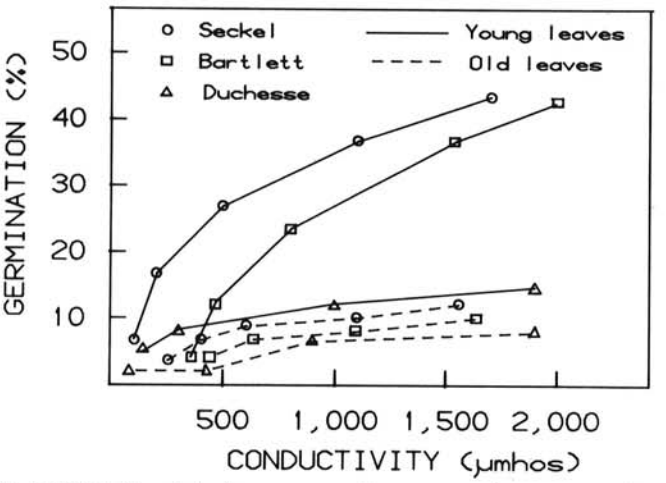

Fig. 4. Effect of leachates from young and mature pear leaves, susceptible (Seckel), moderately susceptible (Bartlett), and relatively resistant (Duchesse) to leaf spot, on germination of *Entomosporium maculatum* conidia.

Etiology and pathogenicity. To study the initial stages of infection, uppermost leaves of propagated pear trees were surface-sterilized and rinsed twice as described before. Leaves were then floated in sterile distilled water in petri dishes with either the upper or lower surface upward, and were inoculated by placing 1-ml drops of a conidial suspension on the surface with a syringe. Leaves were removed at intervals of 18, 24, 48, and 72 hr and boiled in 90% ethanol until all chlorophyll was removed, left overnight in lactophenol-cotton blue, destained in 5% lactophenol, and placed on a sterile microscope slide. One drop of cyanoacrylate glue (20) was added, a coverslip was placed over the leaf, and the slides were viewed and photographed.

For inoculation studies, conidial suspensions were taken from colonies in PDA plus 1 μg of thiamine per milliliter and 5 g of casein per liter or from diseased leaves and transferred to an atomizer. A spore suspension (100 ml) contained approximately 10^4 conidia per milliliter in sterile distilled water and two drops of Tween-20. The suspension was sprayed on budded pear trees in the greenhouse until run-off. Plants were then placed in an inoculation chamber (polyethylene-enclosed) and mist was sprayed from a humidifier for 48 hr to maintain 100% relative humidity. Temperature in the greenhouse was maintained at 26 C ± 5 C and light intensity in the chamber was about 50% of bright daylight, lasting about 12 hr. Pathogenicity was tested on potted, 2-yr-old cultivar Bartlett seedlings with four replicate trees per treatment.

RESULTS AND DISCUSSION

Growth and sporulation in vitro. Of all growth factors tested, only thiamine increased growth and sporulation (Table 1). No significant increase in growth and sporulation occurred when thiamine levels were increased beyond 0.5 and 1.0 μg/ml, respectively. Addition of other growth factors to the media that included thiamine had no significant effects. The related rose leaf spot fungus, *Diplocarpon rosae*, has also been reported to be deficient for thiamine (12). Addition of thiamine to PDA produced a fivefold increase in dry weight and a 30-fold increase in spore production (Table 2), indicating that lack of thiamine was largely responsible for poor growth and low conidia yields on PDA. Addition of casein hydrolysate to PDA resulted in a threefold increase in spore production, but only when thiamine was also present.

Of the various N sources used with the basal medium, only casein hydrolysate yielded significant numbers of spores. It also supported the highest dry weights per culture (Table 3). Nitrate-and ammonium-N were both used, with nitrate-N supporting better growth. Asparagine supported poor growth, indicating that the effect of casein hydrolysate represented a specific requirement for one or more amino acids.

E. maculatum did not grow on citrate and grew poorly on maltose and sorbitol. Sucrose supported the best growth and sporulation (Fig. 1). With fructose as the carbon source, the fungus grew slowly but sporulated well. Combined with the results for

TABLE 5. Germinability and infectivity of *Entomosporium maculatum* conidia from diseased leaves and from colonies maintained in culture for varied amounts of time

Spore source	Germination (%)[x] Distilled water	Water + 2% glucose	Basal medium[x]	Basal + Pear leaf extract	Leaves infected[y] (%)
Leaf lesions	0.0 a[z]	4.4 a	25.0 a	13.4 a	95.1 c
First generation	14.4 b	25.7 b	44.5 b	14.1 a	73.5 b
Third generation	19.3 c	27.7 b	56.2 c	33.5 b	9.6 a

[x] Basal medium = basal glucose-casein medium excluding agar.
[y] Averages of eight counts per treatment and four seedlings per source.
[z] Means of four colonies; values within a column and followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

sucrose vs. glucose, these data suggest that sporulation may be specifically enhanced by fructose. The fructose concentration in pear fruit is relatively high and varies widely among cultivars and *Pyrus* species. This may explain the observed variation in leaf spot resistance among *P. calleryana* seedling progenies (18). The optimum glucose concentration was 2.5% (Table 4), and the optimum C:N ratio was approximately 20:1.

A pH of 5.5 was optimum when the fungus was grown on a sucrose-casein amended basal medium and no growth was observed at pH 7.5 (Fig. 2). These data are in contrast to the broad pH optimum found by Horie and Kobayashi (7), probably because they used potato-sucrose agar on which fungal growth is much poorer than on our medium.

Maximum sporulation occurred at a low light intensity (100 lux), and spore production was greatest when spores were allowed to germinate and grow for 1 wk in darkness and then transferred to continuous light for 17 days (Fig. 3). High light intensity (5,000 lux) inhibited germination and initial growth of colonies, but did not directly affect spore production. When colonies were grown at 25 C on the optimal medium containing (per liter) 20 g of sucrose, 1 mg of thiamine, 5 g of casein hydrolysate, minerals as specified in the basal medium, and a pH of 5.5 at low light intensity for 3 wk after 1 wk of initial growth in darkness, we achieved a 200-fold increase in spore production over that produced on PDA, making a future attempt for a mass-screening program feasible.

Germination and infection. Conidia typically germinate within 18 hr, and then form appressoria and penetrate the leaf cuticle within 48 hr. Hyphae then spread subcuticularly at first, later invading host cells by means of haustoria. This is similar to the infection process observed in *D. rosae* (3). Conidia could infect both upper and lower surfaces of leaves, but infection of the lower surface was rare.

Results from infection tests on detached leaves of several pear cultivars suggested that conidia did not germinate equally well on all leaves and that spores produced in culture were not as infective as those obtained from diseased leaves. To test these hypotheses, leachates from leaves of susceptible (cultivar Seckel), moderately susceptible (cultivar Bartlett) and resistant (cultivar Duchesse) pear trees were compared for effects on spore germination. Leachates from different cultivars increased the germination rate and cultivar differences corresponded to differences in susceptibility observed in the field (Fig. 4). The fact that extracts from young leaves stimulated germination more than those from older leaves also coincides with the observed pattern of susceptibility on the pear cultivars that were studied. Germination increased as the concentration of leachate from susceptible leaves was increased, whereas increasing the concentration of leachate from old or resistant leaves had no effect. These data suggest that susceptibility involves leaching of a stimulatory compound(s) into spore-containing water droplets and that resistance is at least partially due to the lack of the leached compound(s) and therefore a low rate of spore germination on leaf surfaces.

Germination of spores from cultures and from diseased leaves was enhanced by glucose and minerals contained in the basal medium (Table 5), but addition of extracts obtained by blending Bartlett pear leaves reduced germination. Increased germination by spores produced in culture was associated with lower infection rates. Spores lost virulence rapidly in culture, and were nearly noninfective after three transfers. An effective screening program must use inoculum from diseased leaves (which may be contaminated with other pathogens) or from first-generation cultures, emphasizing the importance of rapid production of large numbers of spores.

Spores incubated in closed containers with detached leaves of different cultivars did not differ in germination rates, suggesting that the factor(s) responsible is not volatile, but is water-soluble. The stimulating effects of leaf leachates from susceptible leaves may be useful as a screening technique. It has the advantages of requiring little plant material and being nondestructive to the whole plant and can therefore be incorporated into the existing fire blight resistance screening program. Such a program would result in a significant addition to the USDA pear breeding project to develop disease resistant cultivars.

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