Optimal Conditions for In Vitro Growth, Asexual Spore Release, and Germination of *Thyronectria austro-americana*

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ABSTRACT

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Growth of the honeylocust pathogen *Thyronectria austro-americana* on solid and liquid media was assessed at various temperatures, on carbon and nitrogen sources, and on other nutrients. Spore release from excised naturally formed pycnidia was practically instantaneous when water was added at temperatures between −15 and 40 C after storage at 25 C and 35–45% RH for 12 mo. Spore germination occurred from 15 to 40 C with optimal germination at 25–35 C. Germination was delayed at 96–75% RH over that found at 100% RH and completely inhibited at 62%. Carbon and organic nitrogen compounds promoted, but were not necessary for, germination. Water extracts of honeylocust wood stimulated germination. The fungus appears to be very well adapted for growth and dissemination in the varied climates where the host is planted.

Honeylocusts (*Gleditsia triacanthos* L.) are popular shade and ornamental trees in much of North America. A canker disease, which is induced by the fungus *Thyronectria austro-americana* (Spegazzini) Seeler, has become a widespread problem on many honeylocusts. The disease causes an estimated 1% mortality per year in Colorado and the extensive planting of honeylocusts may result in more serious losses in the future (7). The disease was first described by Seeler (9) on honeylocusts in Massachusetts. Later reports place the disease in Alabama, Tennessee, Mississippi (4), and Illinois (1,2); in the urban areas of Colorado (7); and in the windbreaks, rest stops, native forests, and urban areas of Kansas and Oklahoma (3,5). The fungus was also described as a common saprophyte of honeylocusts in Nebraska (8).

The taxonomic characteristics and developmental aspects of the pathogen's fruiting bodies were described by Lieneman (8) and Seeler (10). The fungus produces abundant irregularly shaped pycnidia (stromatic conidiomata) and occasionally perithecia in stromata on tree surfaces. Perithecia are rare in Colorado (7) but common in Kansas (5). Ascospores are assumed to be wind disseminated, whereas the conidia are thought to need rain, wind driven rain, or insects for dissemination (9).

The epidemiology and dissemination mechanisms are not adequately described for this canker disease. Progress in managing this disease is hampered by limited knowledge about the growth requirements of the fungus and the effects the environment has on release of conidia from pycnidia and on conidial germination.

This paper reports optimal cultural conditions for fungal growth and the effects of the environment on in vitro release and germination of conidia.

MATERIALS AND METHODS

Fungal cultures. Three single conidial isolates obtained from individual pycnidia on three trees in Denver, CO, in 1981 were used throughout these studies. The isolates were routinely maintained at 25 C on a defined medium of glucose and asparagine (6). All nutritional studies utilized this basic medium in solid and liquid form. Solid media were adjusted to a pH of 5.5 and 20 g of agar per liter was added before autoclaving. For liquid cultures, the media were adjusted to a pH of 5.5, and 125 ml was placed in 250-ml flasks before autoclaving. The agar plates were kept under fluorescent light (80 $\mu E \cdot m^{-2} \cdot s^{-1}$) 7 hr/day at 25 C. Liquid cultures were placed on a rotary shaker at 22–25 C under fluorescent light (40 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 24 hr/day. Plates and flasks with media were inoculated with 5-mm-diameter agar disks containing mycelium and conidia.

Fungal growth on solid media was assessed after 10 days by measuring and averaging two diameters at right angles for each colony. Colony mass was subjectively rated in some experiments on a scale of 1–3: 1 = slight, 2 = moderate, and 3 = heavy development. On liquid media, dry weight was determined after 8 days by filtering out the fungus on Whatman No. 4 filter paper and drying for 48 hr at 100 C. The pH of liquid cultures was recorded at harvest time.

Each experiment was repeated two or three times with five to 10 plates or four to five flasks per treatment for each isolate. All experiments were in a randomized complete block design and the data were subjected to analysis of variance.

Optimal culture conditions. Solid media cultures were used to assess optimal temperatures for vegetative growth at 5, 10, 15, 20, 25, 30, 35, 40, and 45 C.

Fungal growth on eight carbon sources (dextrin, fructose, galactose, glucose, maltose, mannose, sorbose, and sucrose) was assessed on liquid and solid media cultures. Each carbon source was tested by replacing glucose in the basic medium at 4 g of elemental carbon per liter.

Fungal growth on seven nitrogen sources (L-asparagine, ammonium tartrate, ammonium nitrate, ammonium sulfate, casamino acid, L-glutamic acid, and potassium nitrate) was assessed on liquid and solid media cultures. Sucrose was used as the carbon source, and nitrogen sources replaced asparagine in the basic medium at the rate of 0.424 g of elemental nitrogen per liter.

Cultures on liquid and solid media were used to assess fungal requirements for other nutrients in the basic medium. Two separate experiments assessed growth on the basic medium and on series of six basic media each lacking one of the following: KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), Fe (0.2 mg/L), biotin (5 μg/L), thiamine (100 μg/L) and a combination of Zn (0.2 mg/L) and Mn (0.1 mg/L).

Spore release. Environmental effects on spore release were assessed by using naturally formed pycnidia obtained from three different honeylocust trees. Pycnidia with attached bark were stored at 25 C and 35–45% RH in sealed glass jars for 1–12 mo prior to use. Individual clusters of the pycnidia were placed on double-

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stick tape on a microscope slide supported on a bent glass rod in a glass petri plate. Pycnidia were observed under a dissecting microscope at ×40, and spore release was recorded when spores were found discharged from pycnidia. Water drops were placed on pycnidia to provide free moisture. Temperature effects were determined by placing pycnidia in incubators without light at 5, 10, 15, 20, 25, 30, 35, and 40 C at 100% RH for 5 or 30 hr, at −15 C for 11 mo, and at 25 C at 35–45% RH for 12 mo. To determine if spore release would occur without free water, saturated salt solutions (11) were used to maintain RHs of 100, 96, 92, 85, 75, and 62% in petri plate chambers at 25 C without light for up to 48 hr.

Spore germination. Environmental effects on germination of conidia were assessed by using unwashed spores exuded from and still located on pycnidia formed in vitro on solid cultures. A spore suspension of ~15,000 spores per milliliter of distilled water was used in all experiments. Conidia were streaked on microscope slides coated with basal medium and placed on glass rods in petri plates. One slide was used for each reading since dye and cover slips were needed to observe the spores at a ×450 magnification. Four to eight counts of 100 spores were made at each reading time with at least two repetitions of the experiment. Temperature effects were assessed at 5, 10, 15, 20, 25, 30, 35, and 40 C at 100% RH without light. The effects of relative humidities of 100, 96, 92, 85, 75, and 62% were assessed by using saturated salt solutions (11). The petri plate chambers were allowed to equilibrate 6–8 hr before spores were streaked on the agar. To determine if spores could survive a period of low humidity, spores were placed on microscope slides in chambers at ~50, 46, and 43% RH for 24 hr before testing at 100% RH.

Nutrient requirements for germination were determined by using the complete basic medium, basic medium minus carbon or nitrogen, and water agar on microscope slides at 100% RH.

The effect of light on spore germination was determined by exposing conidia on slides with complete medium for 24 hr at 100% RH and 25 C in darkness or fluorescent light (80 $\mu E \cdot m^{-2} \cdot s^{-1}$).

The effect on conidial germination of water extracts of ground honeylocust twigs with the bark peeled or not was assessed by combining filter sterilized extracts with double strength basal medium or water agar (1:1, v/v). Extracts were prepared from ground wood (2 mm-mesh sieve) by soaking for 6 hr in distilled water (1 g of wood per 50 ml of H_2O) held at 60 C in a water bath. The extracts were collected by vacuum filtration.

There was no difference between the analysis of arcsin transformed and untransformed percent germination data so the untransformed data are presented. There was no significant difference among the three isolates in any of the previous experiments so the results were combined.

TABLE 1. Mycelial growth of *Thyronectria austro-americana* on a defined medium[x] amended with various carbon sources

Liquid media		Solid media	
Carbon source	Mean growth/day[y] (mg)	Carbon source	Mean diameter[z] growth/day (mm)
Dextrin	40.9 a	Dextrin	5.9 a
Mannose	39.9 ab	Galactose	5.3 b
Glucose	30.7 bc	Mannose	5.3 b
Sucrose	30.5 bc	Fructose	5.2 b
Maltose	30.3 bc	Maltose	5.1 b
Fructose	27.3 c	Sucrose	5.0 b
Galactose	27.0 c	Glucose	4.9 b
Sorbose	5.5 d	No carbon	3.0 c
No carbon	1.4 d	Water agar	2.4 d
Water agar	1.0 d	Sorbose	0.2 e

[x] Defined medium based on Helton and Konicek (6).

[y] Mean growth per day based on dry weights of three isolates, five replications, and two repetitions. Means within columns with common letters were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

[z] Mean colony diameter growth based on two diameters at right angles, of three isolates, ten replications, and two repetitions. Means within columns with common letters were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

RESULTS

All three isolates grew at temperatures from 10 to 40 C with no growth at 5 and 45 C. Optimal growth occurred at the temperatures of 25 and 30 C.

Growth was similar on both solid and liquid cultures with all carbon sources except sorbose (Table 1). Dextrin was the only carbon source on which significantly greater growth occurred on both liquid and solid media than any of the other carbon sources.

Significantly more growth occurred on liquid and solid media amended with the organic nitrogen sources glutamic acid, casamino acid, ammonium tartrate, and L-asparagine than with ammonium sulfate, ammonium nitrate, and potassium nitrate

TABLE 2. Mycelial growth of *Thyronectria austro-americana* on a defined medium[w] amended with various nitrogen sources

Liquid media		Solid media	
Nitrogen source	Mean growth/day[x] (mg)	Nitrogen source	Mean diameter[y] growth/day (mm)
L-glutamic acid	58.1 a	L-asparagine	5.5 a
Casamino acid	45.4 b	L-glutamic acid	5.1 ab
Ammonium tartrate	44.0 b	Potassium nitrate	4.9 ab[z]
L-asparagine	39.5 b	No nitrogen	4.8 b[z]
Ammonium sulfate	17.7 c	Ammonium tartrate	4.1 c
Ammonium nitrate	16.2 c	Casamino acid	3.8 cd
Potassium nitrate	2.0 d	Ammonium nitrate	3.5 cd
No nitrogen	1.7 d	Ammonium sulfate	3.4 d

[w] Defined medium based on Helton and Konicek (6).

[x] Mean growth per day based on dry weights of three isolates, five replications, and two repetitions. Means within columns with common letters were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

[y] Mean colony diameter growth per day based on two diameters at right angles, of three isolates, ten replications, and two repetitions. Means within columns with common letters were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

[z] Rapid growth of a few hyphae giving large diameter readings, but very little fungus mass present.

TABLE 3. Mycelial growth of *Thyronectria austro-americana* on a defined medium[w] amended with various nutrients

Liquid media		Solid media	
Nutrient	Mean growth/day[x] (mg)	Mean diameter[y] growth/day (mm)	Mass rating[z]
Experiment I			
Complete (=C)	58.0 a	5.7 a	2.7 a
C minus KH_2PO_4	2.1 b	5.9 a	1.0 b
C minus $MgSO_4$	2.7 b	4.6 b	2.7 a
C minus KH_2PO_4 + $MgSO_4$	1.1 c	6.2 a	1.0 b
Experiment II			
Complete (=C)	45.3 a	5.6 a	2.8 a
C minus Fe	33.1 b	5.3 a	2.6 a
C minus thiamine	32.7 b	5.5 a	2.5 a
C minus Mn + Zn	21.2 c	5.5 a	2.6 a
C minus biotin	2.4 d	6.4 a	1.0 b
C minus Fe, Mn + Zn, thiamine, and biotin	1.4 d	5.6 a	1.0 b

[w] Defined medium based on Helton and Konicek (6).

[x] Mean growth per day based on dry weights of three isolates, four replications, and four repetitions. Means with common letters within columns and experiments were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

[y] Mean colony diameter growth per day (based on two diameters at right angles) of three isolates, eight replications, and two repetitions. Means with common letters within columns and experiments were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

[z] Mass of fungus on solid cultures was subjectively rated 1 = slight, 2 = moderate, and 3 = heavy.

TABLE 4. Effect of temperature on percentage of germination of conidia of *Thyronectria austro-americana* obtained from pycnidia grown in vitro

Temperature (C)	Mean percentage germination[x] after incubation: 16 hr	20 hr	24 hr
5	0.0	0.0	0.0
10	0.0	0.0	0.0
15	0.0	0.0	1.0
20	2.2 b	9.6 b	43.9 b
25	32.5 a	58.7 a	75.8 a
30	40.9 a	51.9 a	73.9 a
35	29.1 a	59.9 a	82.6 a
40	4.5 b	6.0 b	10.6 c

[x]Mean percentage germination for test period based on four counts of 100 spores per temperature and two repetitions. Means with common letters within columns were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

TABLE 5. Effect of relative humidity (RH) on percentage germination of conidia of *Thyronectria austro-americana* obtained from pycnidia grown in vitro

RH (%)	Mean percentage germination[z] after incubation: 16 hr	20 hr	24 hr
100	80.9 a	94.8 a	95.5 a
96	35.5 b	67.8 b	83.9 ab
92	24.6 bc	75.1 ab	77.4 ab
85	9.9 cd	59.6 b	73.4 b
75	3.0 d	37.0 c	67.0 b
62	0.0 d	0.0 d	0.4 c

[z]Mean percentage germination for test period based on eight counts of 100 spores per relative humidity and two repetitions. Means with a common letter within columns were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

TABLE 6. Rates of germination on various media of *Thyronectria austro-americana* conidia obtained from pycnidia grown in vitro

Media[y]	Mean percentage germination[z] after incubation: 16 hr	20 hr	24 hr
Complete	10.3 a	32.2 a	68.5 a
Complete minus carbon	1.2 a	17.1 b	31.6 b
Complete minus nitrogen	10.4 a	16.1 b	28.0 b
Water agar	2.0 a	7.8 b	16.1 b

[y]Media consisted of H_2O agar or complete medium based on Helton and Konicek (6) with or without a nitrogen or carbon source.

[z]Mean percentage germination for test period based on six counts of 100 spores per medium and two repetitions. Means with a common letter within columns were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

TABLE 7. Effect of extracts of peeled and nonpeeled honey locust twigs on percentage germination of conidia of *Thyronectria austro-americana* obtained from pycnidia grown in vitro

Media[y]	Mean percentage germination[z] after incubation: 16 hr	20 hr	24 hr
H_2O agar + H_2O	6.7 a	21.8 b	80.4 a
H_2O agar + nonpeeled	9.0 a	70.8 a	98.4 a
H_2O agar + peeled	18.9 a	75.5 a	98.9 a
Complete + H_2O	14.1 a	24.2 b	94.2 a
Complete + nonpeeled	15.9 a	58.6 a	98.6 a
Complete + peeled	12.2 a	65.4 a	99.9 a

[y]Media consisted of H_2O agar or complete medium based on Helton and Konicek (6) with water extracts of ground peeled or nonpeeled honey locust branch wood.

[z]Mean percentage germination of test period based on eight counts of 100 spores per medium and three repetitions. Means with a common letter within columns were not significantly different at $P = 0.05$ based on Duncan's new multiple range test.

(Table 2). There was a significant decrease in pH of the liquid media at the end of each experiment for each nitrogen source except L-glutamic acid and potassium nitrate.

Growth of the fungus in liquid culture was significantly decreased when magnesium, potassium, iron, thiamine, biotin, or a solution of manganese and zinc were absent from the basic medium (Table 3). Fungal growth on solid media responded similarly to the absence of these nutrients if both diameter growth and mass were considered. Diameter growth was an inadequate measurement of growth in this case. A comparison of liquid media containing dextrin and glutamic acid or glucose and asparagine indicated fungal growth was significantly improved on dextrin and glutamic acid, but pH values also were significantly higher (7.6 versus 4.9).

Spore release from pycnidia occurred within 5–50 sec following the addition of water after the pycnidia and water were held for 5 or 30 hr at 5–40 C. Pycnidia frozen for 11 mo or stored at 25 C for 12 mo released spores within 10 sec after the addition of water. Spore release occurred at 100% RH without free water, but not at any RH below 100%.

Spores germinated at temperatures from 15 to 40 C (Table 4). Maximum germination occurred at temperatures from 25 to 35 C. Germination did not occur at 5 and 10 C and was significantly reduced at 20 and 40 C.

Spore germination was reduced or delayed at RHs <100% (75–96% RH) and was totally inhibited at 62% RH ($P = 0.05$) (Table 5). No germination occurred at 100% RH after a 24-hr pretreatment at 50, 46, or 43% RH.

There was a significant reduction but not complete inhibition in germination of spores placed on water agar or media lacking carbon or nitrogen compared to a complete medium (Table 6). Germination percentages were similar in light or dark. Water extracts of honeylocust wood increased germination after 20 hr of incubation over that on water agar or complete medium alone, but there were no significant differences at 16 or 24 hr (Table 7). There was no difference between germination on media with extracts of peeled and nonpeeled wood.

DISCUSSION

It is important that the fungal growth, spore release, and spore germination of this pathogen occur over a wide range of conditions. This versatility of the fungus may explain its ubiquitous occurrence through a wide range of climates from Colorado to Massachusetts.

The fungus grows well over a wide range of temperatures which may allow it to damage trees after they become dormant and to grow under the temperature extremes often experienced in Colorado. The ability of the fungus to utilize a wide range of carbon and nitrogen sources may allow it to colonize various tissues of living and dead trees.

Spore release from pycnidia of *T. austro-americana* is impressive since it can occur over a wide range of temperatures and even after storage in a laboratory or freezing for a year. This ability to release spores would enable the fungus to persist and spread in the relatively dry climate of Colorado. Prompt removal of infected trees and disposal of the wood might reduce the amount of potential inoculum, since the pycnidia remain viable for a long time and the saprophytic nature of the fungus will allow it to produce more pycnidia on dead or cut wood.

Free moisture, or 100% RH, is needed for spore release, so rain and irrigation probably play an important role in spore release. Although the mode of spore dispersal is unknown, it could be assumed that these spores are disseminated by rain splash, because free water or 100% RH is required for spore release.

Disease management is further complicated by lack of information on environmental effects on spore germination and infection. If the environmental conditions required for infection are similar to those required for germination, it might be difficult to prevent infection through wounds. The RH in the microclimate of the wound is likely to exceed the 62–75% required for germination. This would be especially important on days when free moisture or high humidity is lacking. Germination may also be stimulated by the presence on wounds of carbon and nitrogen compounds and

other water-soluble substances similar to those that caused stimulation in the in vitro tests. Since spores are sensitive to a 24 hr "drying," infection may be reduced by keeping pruning and other wounds dry by proper tree maintenance.

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