Thyronectria Canker of Honeylocust: Influence of Temperature and Wound Age on Disease Development

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Accepted for publication 2 October 1985.

ABSTRACT

Riffle, J. W., and Peterson, G. W. 1986. Thyronectria canker of honeylocust: Influence of temperature and wound age on disease development. Phytopathology 76:313-316.

The number of days for three isolates of *Thyronectria austro-americana* to girdle stems and wilt foliage of 20-wk-old seedlings of *Gleditsia triacanthos* decreased as temperatures increased from 16 to 28 C. Inoculation of freshly made wounds, and 7-, 14-, and 21-day-old wounds with *T. austro-americana* produced cankers on 100, 62, 10, and 6% of the seedlings, respectively. Growth in culture was optimum at 28–32 C for three Nebraska isolates.

Honeylocust, *Gleditsia triacanthos* L., was planted extensively in windbreaks in the central and southern Great Plains during the Prairie States Forestry Project (1935–1942) and still is used widely in windbreak and other types of plantings throughout the Great Plains (8). More than 115,000 of the 3.9 million broadleaf seedlings distributed for outplanting in 1980 in the Great Plains were honeylocust (11). This species also is commonly planted as a landscape tree in urban areas where *Ulmus americana* L. has been killed by *Ceratocystis ulmi* (Buism.) C. Moreau and elm yellows.

Several canker pathogens have been reported on honeylocust (2,17,18,22). *Thyronectria austro-americana* (Spegazzini) Seeler, the most widely reported cause of cankers on honeylocust in Alabama, Illinois, Massachusetts, Mississippi, and Tennessee (5,18,20), is the most damaging canker pathogen of honeylocust in windbreaks and urban plantings in the Great Plains (4,6,9,21). Dieback and mortality of honeylocust, initially reported in 12- to 19-yr-old windbreak plantings in southern Kansas and north-central Oklahoma (16), have been observed in windbreak and urban plantings with increasing frequency within the last 5 yr. *T. austro-americana* was found on nearly 70% of the dead or dying honeylocust in a windbreak in west-central Oklahoma (4), and on honeylocust in plantings, woodlands, and naturalized stands in 39 counties of Kansas (6). Trunk cankers and tree mortality were associated with pruning wounds, sunburn damage, and insect borer wounds in newly established windbreaks and open landscape sites in Kansas. Cankers on lower branches of trees in older windbreaks and native stands appeared to have little significance because these branches are shaded out before spread of *T. austro-americana* to the stem can occur.

The potential threat of *T. austro-americana* to honeylocust in windbreak and urban plantings is of concern to landowners. Some biological information on *T. austro-americana* is available (2,13,14,18,19), but more information is needed before a sound technology can be developed for assessing and predicting the damage it causes and for improving methods for its control. Studies have been conducted to determine conditions optimum for growth of *T. austro-americana* in vitro, for spore release, and for spore germination (10).

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This paper reports the effect of temperature on mycelial growth of *T. austro-americana* in vitro, and the effect of temperature and stem wound age on canker development caused by *T. austro-americana* in honeylocust seedlings.

MATERIALS AND METHODS

Temperature effects on mycelial growth. Three isolates of *T. austro-americana* were obtained from margins of active cankers on honeylocust. Isolate 429 was obtained from a windbreak tree at the University of Nebraska Horning State Farm near Plattsmouth in August 1981; isolate 433 was obtained from a windbreak tree at the University of Nebraska Horticultural Gardens, Lincoln, in May 1982; and isolate 434 was obtained from a landscape tree at East Campus, University of Nebraska, Lincoln, in May 1982. These isolates were maintained on Difco potato-dextrose agar (PDA).

Nine petri dishes with PDA were placed in each of seven incubators at 12, 16, 20, 24, 28, 32, and 36 C for 24 hr before adding disks of agar from cultures of the isolates. Disks (5-mm-diameter) were cut from the margins of 3-day-old cultures and were placed on the medium in the center of each plate, with the mycelial side down. Three dishes were prepared for each temperature and each isolate. The plates were placed randomly in each incubator and incubated for 5 days; then colony diameters were determined by two measurements of each colony made along lines on the bottom of the plates at right angles to each other. The test was repeated two additional times. A balanced incomplete block design with incubators as the blocking factor was used to control randomization across the three tests (3).

Data on colony diameters were analyzed using a two-factor analysis of variance with temperatures and isolates as variables, after removal of the influence of the incubators. An analysis for interaction of isolates and temperatures also was made to determine if isolates responded similarly to temperatures. Tukey's multiple comparison test with experiment-wide $\alpha = 0.05$ was used to determine significant differences among means of variables.

Temperature effects on disease development. Seeds of *G. triacanthos* collected in 1980 in Lincoln, NE, were treated with concentrated sulfuric acid for 1.5 hr then washed in flowing tap water for 30 min. The seeds were planted in 1:1:1 soil-peat moss-vermiculite growing medium in 25 40-cell Spencer Lemaire Rootrainer (Spencer-Lemaire Industries Ltd., Edmonton, Alberta, Canada) book-type containers on 22 October 1982. Each cell had a volume of 410 ml. The containers were kept for 20 wk in a greenhouse with ambient temperature of 19–26 C and a 16-hr photoperiod. Seedlings with 2.2 mm and larger stem diameters 5 cm above the root collar were selected for the experiment.

Temperatures. Temperatures of 16, 20, 24, or 28 C, respectively, were maintained in four plant growth chambers. All chambers were kept at 70% RH, 16-hr photoperiod (0600–2200 hours), and 80 μM photons$\cdot m^{-2}\cdot a^{-1}$ light intensity. Two hundred honeylocust seedlings were placed in each chamber 48 hr prior to inoculation.

Inoculum and inoculation. Isolates 429, 433, and 434, whose pathogenicity to honeylocust was established in a greenhouse test in May 1982, were used as inoculum. The isolates were maintained on PDA at 24 C.

The bark from 1 to 12 cm above the root collar on 800 seedlings was surface disinfested with 70% ethanol and was wounded at 5 cm above the root collar immediately before inoculation. Bark was wounded by making one horizontal cut into the stem to the xylem, followed by a second cut at a 45-degree angle to and above the first cut. This procedure resulted in removal of a wedge of bark approximately 3 mm long × 2 mm wide × 1 mm deep.

The wound on each of 600 seedlings was inoculated with one 5-mm-diameter mycelial disk from 5- to 7-day-old cultures of the three isolates on 8–10 March 1983; 200 seedlings were inoculated with each isolate. The disks were secured in place with a strip of Parafilm. Each of 200 seedlings received one 5-mm-diameter disk of PDA medium as a control on the fresh wound, which was secured in place with a strip of Parafilm on 7 March 1983.

Within each growth chamber 50 seedlings from each of the four inoculation treatments were placed randomly in the environmental regimes previously noted. Seedlings were watered with tap water as needed.

Data collection. Parafilm around inoculation sites was removed at 7 days after inoculation, and the number of infected seedlings with wilted foliage, resulting from stem girdling by *T. austro-americana*, was recorded daily for the next 35 days. At 42 days after inoculation, necrotic bark was removed to the xylem, and the lengths of cankers were measured from the inoculation site downward to the junction between live (white, nondiscolored) and dead or dying (reddish brown) bark tissues. Wounds made at inoculation sites were not included in canker measurements. Lengths of cankers above the inoculation sites on infected seedlings were not measured, because at 24 and 28 C, stems of seedlings were girdled rapidly and portions of stems distal to the girdle died. Reisolations were made from 10 random seedlings from each isolate and temperature treatment to verify the presence of *T. austro-americana* at canker margins.

Data on number of days required for foliage to wilt and on lengths of cankers were analyzed by a two-factor analysis of variance with temperatures and isolates as variables. An inherent weakness for estimating the effects of specific temperatures on disease development was variation associated with the four growth chambers. Analysis of data of the experiment on stem wound age, in which temperatures among the chambers were not varied, found significant effects associated with the chambers for length of cankers. Thus, the effects of temperature on disease development could have biased results because of differential performance of the growth chambers. However, results of this experiment were sufficiently strong to perform the statistical analysis and to emphasize trends in disease development associated with changes in temperature. All tests of significance were performed at $\alpha = 0.05$.

Stem wound age. Four hundred 1-yr-old seedlings of *G. triacanthos* obtained from a nursery in Nebraska were transplanted into Spencer Lemaire Rootrainer book-type containers containing soil-peat moss-vermiculite (1:1:1, v/v) growing medium that had been pasteurized in flowing steam at 93 C for 2 hr. Seedlings were kept in a greenhouse for 8 mo at 16–32 C and 16-hr photoperiod prior to wounding and inoculation.

Twenty-month-old seedlings in groups of 100 were wounded by removing bark disks to the xylem at 21, 14, 7, and 0 days before inoculation. Stems were surface disinfected with 70% ethanol, and a bark disk (4-mm-diameter) was removed aseptically with a No. 1 cork borer at 5 cm above the root collar of each of 100 seedlings on 7, 14, 21, and 28 December 1983. Wounds were covered with Parafilm to reduce possible infection by *T. austro-americana*. The seedlings were kept in a greenhouse until inoculation.

Inoculum and inoculation. Isolate 429 was used as inoculum. Parafilm was removed from wounded stems, and the wound sites of each of 100 seedlings with freshly made wounds and wounds of age 7, 14, and 21 days were surface disinfected with 70% ethanol. Disks (5-mm-diameter) were cut from the margins of 5-day-old cultures grown on PDA, and 50 seedlings with wounds of each age were inoculated on 28 December 1983 by placing an agar disk on the wound and securing it in place with Parafilm. Similarly, the remaining 50 seedlings with wounds of each age each received a disk of PDA and served as controls. Thus, eight treatments, each represented by 50 seedlings, were used.

Inoculated seedlings were placed randomly in four growth chambers so that each treatment was represented by 12 or 13 seedlings in each of the chambers. The chambers were programmed at constant 28 C, 70% RH, 16-hr photoperiod (0600–2200 hours), and light intensity of 175 μM photons$\cdot m^{-2}\cdot s^{-1}$. Seedlings were watered as needed.

Data collection. The number of seedlings cankered by *T. austro-americana* was recorded at 14, 21, and 28 days after inoculation. The total lengths of cankers and the lengths of cankers above and below the inoculation sites at 14 and 21 days after inoculation were determined by measuring discolored bark from the inoculation site to the margin of the discoloration. The lengths of cankers at 28 days after inoculation were measured as previously described. The percentage of stem circumference girdled was determined at 28 days after inoculation by use of Kessler's (12) technique for measurement of cankers on small woody stems. Reisolations were made from each of 10 random seedlings from each treatment to verify the presence of *T. austro-americana* at canker margins.

A contingency table was used to test differences in the percentage of infected seedlings by wound age (7). Differences in lengths of cankers and in percent stem circumference girdled for each wound age were analyzed using a randomized block analysis of variance with growth chambers as the blocking factor and $\alpha = 0.05$.

RESULTS

Temperature effects on mycelial growth. Colony diameter was influenced significantly by temperature and isolate. All isolates of *T. austro-americana* grew at the temperatures tested, and growth was optimum at 28–32 C (Fig. 1). Colony diameter of isolate 429 grew larger (47 mm) than colony diameters of isolate 433 (43 mm) and 434 (44 mm), but this difference was not significant according to Tukey's multiple comparison test. The interaction between isolates and temperatures for colony diameters was not significant.

Temperature effects on disease development. All 600 inoculated seedlings became infected during the 42-day experiment; no control seedlings became infected. The three isolates caused wilt symptoms in seedlings in the same time period and there was no isolate ×temperature interaction; thus the isolate data were combined for presentation. The number of days for the isolates of *T. austro-americana* to girdle stems and wilt foliage of inoculated seedlings at the four temperatures decreased as temperatures increased (Fig. 2). Stems of the inoculated seedlings at 28 C were girdled rapidly, and foliage subsequently wilted. Wilted foliage was first observed 3 days after inoculation, and by 13 days, all seedlings had girdled stems and wilted foliage (Fig. 2). Foliage of seedlings at 16 C started to wilt by 13 days after inoculation, and all 150 seedlings had girdled stems and wilted foliage by 42 days after inoculation.

All inoculated seedlings developed cankers. Cankers were flat to distinctly sunken; infected bark was red-orange. The interaction between isolates and temperatures for canker length was significant, indicating that isolates did not respond similarly to temperatures. Among temperatures, canker lengths on seedlings at 24 and 28 C were larger than those at 16 and 20 C for isolates 429 and 433 (Fig. 3). Canker lengths for isolate 434 did not differ at 20, 24, and 28 C, and were larger than those on seedlings at 16 C. Among isolates, there were no differences in canker lengths at 16, 20, and 24 C, but at 28 C isolate 433 produced greater canker length than isolate 434 (Fig. 3).

After removal of bark, the junction of live and dead bark at canker margins on seedlings at 20, 24, and 28 C was readily

discernible, but the junction was not readily discernible on seedlings at 16 C. Adventitious sprouts readily developed below margins of cankers on seedlings at 28 C during the experiment.

T. austro-americana was recovered from tissues at canker margins from 94% of the 120 infected seedlings from which isolations were made but was not recovered from wounds of 40 control seedlings.

Stem wound age. Nearly all infections of wounded stems occurred within 2 wk after inoculation. The 50 seedlings that were wounded and inoculated simultaneously (freshly made wounds) with *T. austro-americana* were cankered by 14 days after inoculation. Nearly 60% of the seedlings with 7-day-old inoculated wounds were cankered by 14 days after inoculation, and 62% were cankered by 28 days after inoculation (Table 1). This percentage

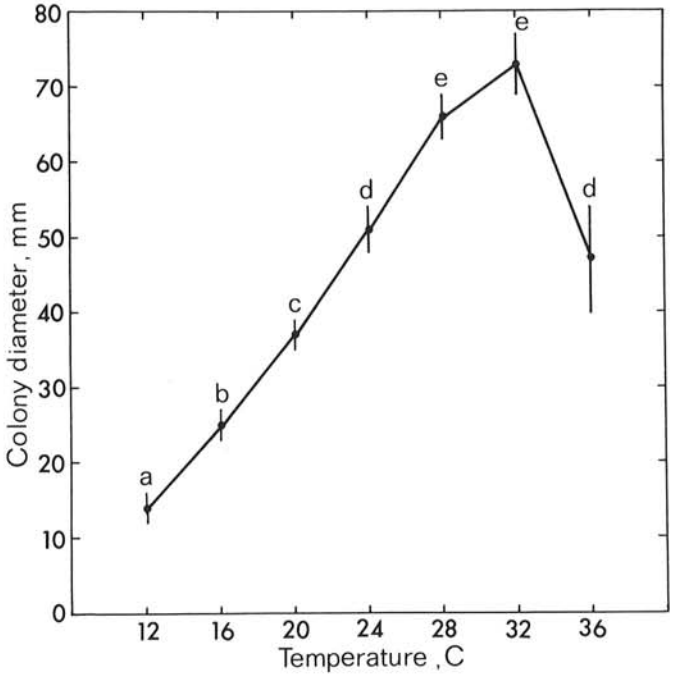

Fig. 1. Effect of temperature on colony diameter of *Thyronectria austro-americana* grown for 5 days on potato-dextrose agar medium. Data on three isolates were combined for presentation. Values followed by a common letter are not statistically different according to Tukey's multiple comparison test ($\alpha = 0.05$).

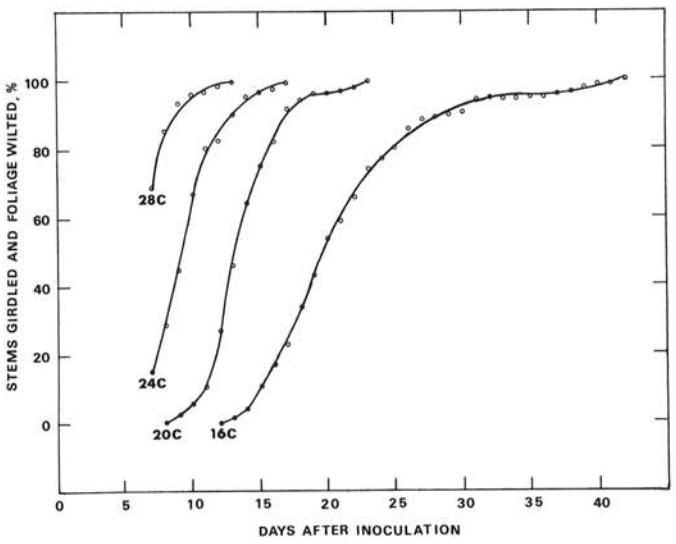

Fig. 2. Percentage of honeylocust stems girdled and foliage wilted on 20-wk-old seedlings at four temperatures from 7 to 42 days after inoculation with *Thyronectria austro-americana*. Data on three isolates were combined for presentation. (Basis = 150 seedlings per temperature.)

with cankers was significantly higher than for seedlings with 14- and 21-day-old inoculated wounds (Table 1). None of the 200 control seedlings became cankered.

The length of cankers for seedlings wounded and inoculated simultaneously was significantly greater than length of cankers for seedlings with 7-day-old inoculated wounds (Table 1). So few wounds became infected for wound ages 14 and 21 days when inoculated that a statistical analysis was impossible for these two treatments. In general, the fungus advanced farther above than below inoculation sites.

Upon removal of bark tissue, red-brown streaking in the xylem commonly was observed above and below wound sites both of control and of inoculated seedlings with wounds of all ages. Streaking was visible on the surface of the xylem and extended to the pith of some seedlings. This streaking apparently was a response to wounding, because it was observed in both control and inoculated seedlings.

The mean stem diameter of seedlings at inoculation sites was 5.5 mm and did not differ among treatments. The percentage of the

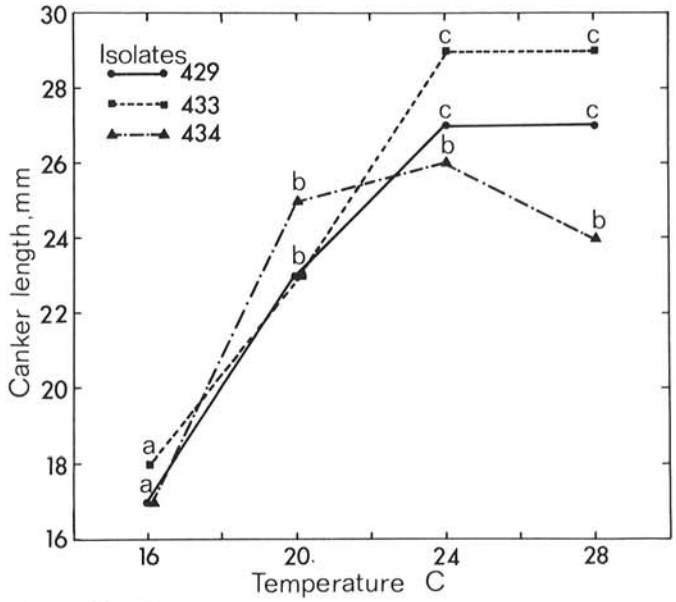

Fig. 3. Effect of temperature on canker lengths below inoculation wounds on 20-mo-old honeylocust seedlings following inoculation with three isolates of *Thyronectria austro-americana* and growth for 42 days. (Basis = 50 seedlings per isolate per temperature.)

TABLE 1. Percentage of honeylocust seedlings cankered; length of cankers at 14, 21, and 28 days after inoculation; and percentage of circumference of stems girdled at 28 days after inoculation of different-aged stem wounds with *Thyronectria austro-americana*[x]

Wound age when inoculated (days)	Seedlings cankered (%) at 28 days[y]	Length of cankers in mm[z]			Circumference of stem girdled (%)
		14 days	21 days	28 days	
0 (freshly made)	100 a	30 ± 7 a	37 ± 9 a	44 ± 12 a	91 ± 19 a
7	62 b	11 ± 1 b	12 ± 9 b	16 ± 10 b	52 ± 28 b
14	10 c	17	21	25	64
21	6 d	6	16	22	58

[x] Seedlings were grown at 28 C for 28 days.

[y] Fifty seedlings were inoculated per wound age. Values followed by a common letter are not significantly different at $\alpha = 0.05$ according to contingency table test.

[z] Values in columns three through five represent means ± standard deviations for canker lengths on seedlings infected per wound age. So few wounds became infected for wound ages 14 and 21 days when inoculated that a statistical analysis was impossible for these two treatments. Values followed by a common letter are not statistically different at $\alpha = 0.05$ according to Tukey's multiple comparison test.

circumference of stems girdled on seedlings which were wounded and inoculated simultaneously was more than 90% at 28 days after inoculation, significantly greater than for seedlings with 7-day-old inoculated wounds (Table 1).

T. austro-americana was recovered from margins of cankers of all inoculated seedlings, but was not isolated from 40 control seedlings with different-aged wounds.

DISCUSSION

T. austro-americana grew at temperatures of 12–36 C, with optimum growth occurring at 28–32 C on PDA medium. Other investigators report that this fungus is relatively thermotolerant, with growth occurring from 10 to 40 C (2,10). Growth of isolates from Colorado and Illinois was optimum at 30 C on PDA (2). Three other isolates of *T. austro-americana* from Colorado grew at temperatures from 10 to 40 C; growth was optimum at 25–30 C on a defined medium of glucose and asparagine (10).

Results from honeylocust seedling inoculation experiments show that disease development from 16 to 28 C was related to growth of *T. austro-americana* in culture. Infection and girdling of stems was enhanced at 28 C, a temperature optimum for growth of *T. austro-americana*. At 28 C, the fungus rapidly invaded and killed bark tissues of 20-wk-old seedlings, resulting in foliage wilt distal to the girdled cambium of all inoculated seedlings in 13 days after inoculation. In contrast, wilting of foliage of inoculated seedlings at 16 C did not begin until 13 days after inoculation.

Previous investigators reported that wounds are necessary for infection of honeylocust by *T. austro-americana* (5,9). Results of our studies show that age of wounds influences infection of honeylocust seedlings. Fresh stem wounds are necessary for maximum infection of seedlings. The three Nebraska isolates of *T. austro-americana* infected all fresh bark wounds made on 650 honeylocust seedlings in two experiments and 62% of the 7-day-old inoculated wounds made on 50 additional seedlings. In a related study on a canker disease of honeylocust caused by *Nectria cinnabarina* (Tode:Fr.) Fr., all experimental trees were wounded at the same time and inoculated at various time intervals after wounding; wounds remained susceptible to infection by *N. cinnabarina* for 1 wk or less (1).

Susceptibility of wounds of trees to canker pathogens is influenced by wound closure, which may partly explain why 7-, 14-, and 21-day-old inoculated wounds on honeylocust seedlings were less susceptible to infection by *T. austro-americana* than were fresh wounds. The experimental seedlings were growing vigorously and callus tissues were developing at wound margins on many seedlings, especially those with 14- and 21-day-old wounds at the time of inoculation. Whether the Parafilm used to cover wounds provided a favorable microclimate that enhanced development of callus on wounds of seedlings is not known. The percentages of seedlings with callus at wound or canker margins at 14 days after inoculation for 0-, 7-, 14-, and 21-day-old inoculated wounds were 0, 0, 24, and 56, respectively; comparable percentages of seedlings with callus at 21 days after inoculation were 0, 16, 78, and 96. Observations on development of cankers and callus on field-inoculated 4-yr-old honeylocust in eastern Nebraska show that trees respond quickly to inoculation and that *T. austro-americana* is compartmentalized by formation of callus tissue. Honeylocust seedlings or trees growing under stress are less able to respond to wounding than vigorously growing trees, and subsequent infection results in canker development (15).

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