Changes in Dehydrogenase and Peroxidase Activities of Aspen Infected with Hypoxylon mammatum

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ABSTRACT

Hypoxylon mammatum infection of aspen reduced glyceraldehyde-3-phosphate dehydrogenase activity and increased glucose-6-phosphate dehydrogenase activity. Glucose-6-phosphate dehydrogenase activity and callus formation in wood peaked 4 weeks after wounding in uninoculated trees, but not in inoculated trees. Despite large increases in peroxidase activity, inoculated host tissue did not exhibit resistance to *H. mammatum*. *H. mammatum* apparently interferes with wound-healing during the infection stage of parasitism.

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Additional key words: tree disease, canker, *Populus tremuloides*.

Mycelium of *Hypoxylon mammatum* (Wahl.) Mill. (Hypoxylon canker of aspen) can be used to reproducibly infect aspen (*Populus tremuloides* Michx.), but successful infection seldom occurs when ascospores or conidia are used (17). When mycelium is used, visible symptoms appear 3- 4 weeks after inoculation.

Hypoxylon mammatum infects aspen by an unknown means under natural conditions, grows through the wood, and subsequently invades the bark from within (8, 15). Thus, the fungus is well established before symptoms appear in the bark, which contains high levels of materials toxic to *H. mammatum* (9). Theoretically, therefore,

invaded bark tissue must undergo significant changes before invasion by *H. mammatum* is successful.

Metabolic activity affords one measure of changes that occur in plant tissue. These changes in metabolic activity can be measured either manometrically or by the activity of various enzymes that participate in the pathways. Alteration of specific enzymic activity occurs in many host-parasite combinations; for example, changes have been observed in enzymes of the Embden-Meyerhof-Parnas (E-M-P) and the pentose phosphate (PP) pathways in infected tissue. In addition, changes have been observed in peroxidase activity in infected tissue.

Increased glucose-6-phosphate dehydrogenase (G-6-PD, E.C. 1.1.1.49) occurs in a variety of plant-parasite combinations (7, 10, 12, 16). Scott (16) found that both host and parasite contributed to increased G-6-PD activity in mildewed barley. Koenigs (10) found G-6-PD activity in cells of white pine blister rust cankers although most G-6-PD activity was in fungus tissue. Changes in G-6-PD activity can be induced by a variety of factors in addition to plant disease (4). Scott (16) found a slight increase in enolase but not in hexoseisomerase or phosphofructokinase, all enzymes of the E-M-P-pathway.

Peroxidase activity (E.C. 1.11.1.7) affords a second measure of changes that occur in plant tissue. Peroxidase activity can be markedly altered by factors such as wounding (3), onset of senescence (11), and infection (3). During the time before onset of symptoms, metabolic changes may occur in the inoculated trees that might serve as markers to indicate the establishment of a successful infection. Because monitoring of activity changes may indicate a successful infection before symptoms become apparent, I monitored the activity of glyceraldehyde-3-phosphate dehydrogenase (G-3-PD, E.C. 1.2.1.11), G-6-PD, and peroxidase in wounded, wound-inoculated, and healthy aspen trees.

MATERIALS AND METHODS.—From an aspen stand covering 0.26 hectares(ha) (about 500 trees), 330 healthy trees were assigned to one of three groups: (i) wounded; (ii) wound-inoculated; and (iii) healthy (untreated). Treated trees were wounded by drilling a hole (9.5 mm inner diam) through the bark; wounds were filled with either sterile or *H. mammatum* overgrown rye grain. At the time of treatment and at biweekly intervals thereafter, 15 inoculated, 10 wounded, and 10 healthy trees were randomly selected for removal of a sample (1.0 cm outer diam by about 1 cm thick) of bark and wood

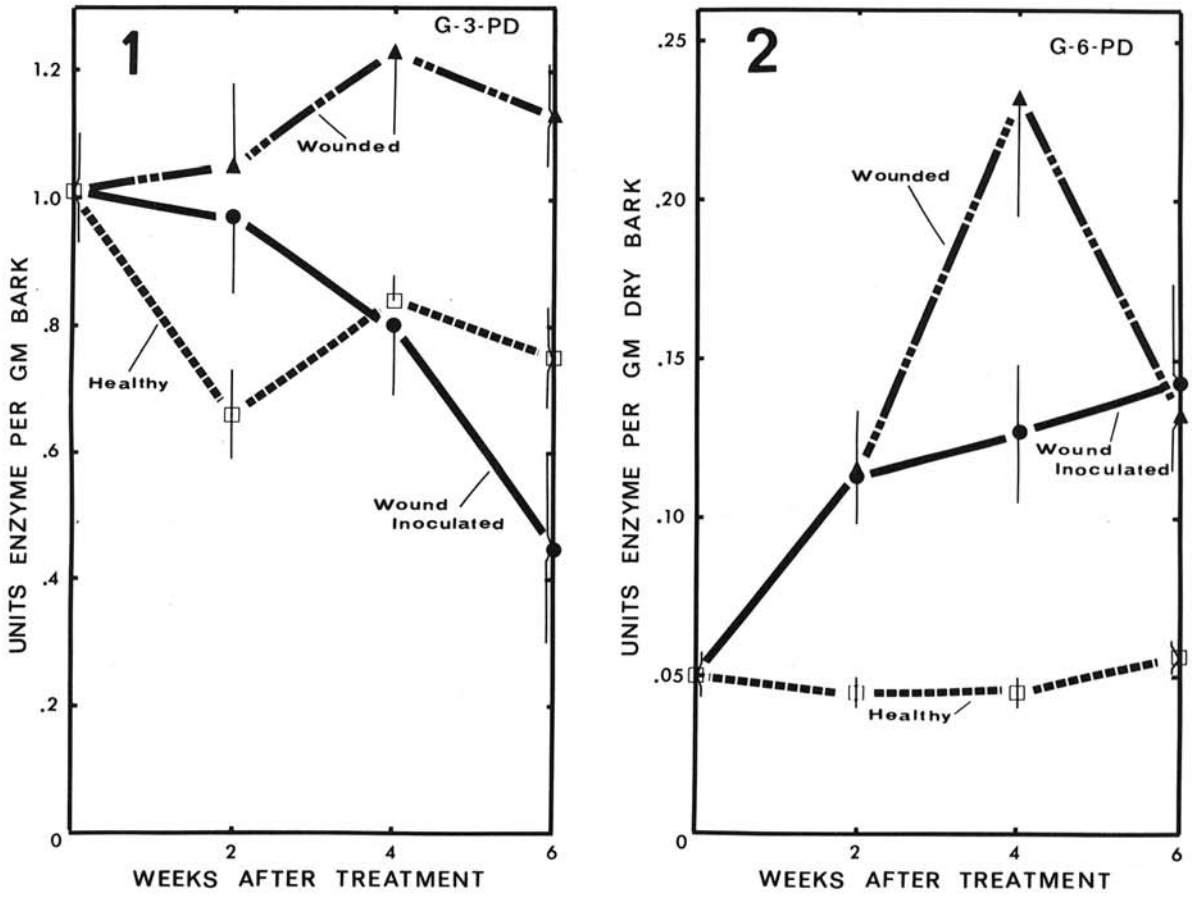

Fig. 1-2. 1) Glyceraldehyde-3-phosphate dehydrogenase activity in aspen bark that is healthy (control), wounded, or wound inoculated with *Hypoxylon mammatum*. Vertical bars denote standard errors of the mean. 2) Glucose-6-phosphate dehydrogenase activity in aspen bark that is healthy (control), wounded, or wound inoculated with *H. mammatum*. Vertical bars denote standard errors of the mean.

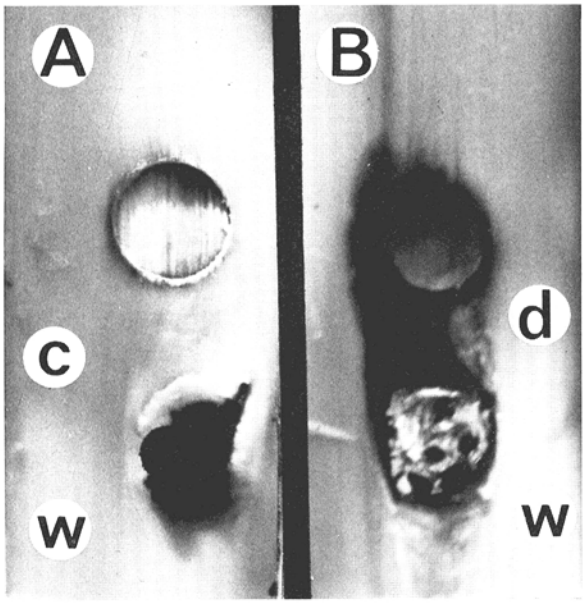

Fig. 3-(A, B). Aspen wounds 4 weeks after treatment: **A)** Uninoculated wound. The lower wound was treated with sterile rye grain. A sample for enzyme assay was removed from the upper hole, c - callus, w - wound. **B)** Inoculated wound. The lower wound was treated with *Hypoxylon mammatum* on rye grain. A sample for enzyme assay was removed from the upper hole, d- discoloration, w- wound.

from a point 2.0 cm above the wound using a hollow punch (15). For each sample the wood and bark were separated, placed in separate 11-ml vials, and immediately frozen using dry ice.

As described by Haissig and Schipper (6), the tissue was freeze-dried, and ground in a Wiley mill so that it would pass a 20-mesh screen, and 100-mg subsamples were homogenized in buffer using a motor-driven Duall homogenizer (Kontes Glass Co.). After centrifugation, dehydrogenases were assayed spectrophotometrically by monitoring the reduction of NAD or NADP, and peroxidase was assayed spectrophotometrically using *o*-dianisidine as a free radical trap. The enzyme assays and units of activity are those given in the Worthington Enzyme Manual (18). Peroxidase isozymes were separated electrophoretically and stained with *o*-dianisidine and H_2O_2 (5).

Eight additional 50-mg samples from healthy aspen stems were re-extracted 12 times (6). When the activity of peroxidase recovered by each re-extraction reached a low but constant amount, the cellular debris was subjected to 32.2 units/ml cellulase (Calbiochem) in 3 ml 0.1 M citrate buffer (pH 5.0) for 26 hours so that any peroxidase trapped during cell wall formation would be released as described by Ridge and Osborne (13). After centrifugation, the supernatant liquid from the treatment was assayed for peroxidase.

RESULTS.—During the first 2 weeks after inoculation, symptoms were not visible. By the end of 4 weeks, symptoms typical of *H. mammatum* infection appeared. These included orange discoloration and necrosis of the bark. The canker margins extended 1.5 cm above and below the inoculated wound; cankers averaged 3.6 cm long at 6 weeks, 4.3 cm at 8 weeks, and 7.1 cm at 10 weeks. In wounded trees that were not inoculated, neither bark discoloration nor necrosis appeared; however, callus had begun to form in the wound at the end of 4 weeks.

In bark from wounded trees, G-3-PD activity increased slowly during the first 2 weeks, and then rose rapidly 4 weeks after treatment as shown in Fig. 1. In healthy trees, it dropped rapidly the first 2 weeks, then rose after 4 weeks. In inoculated trees, it remained about constant during the first 2 weeks, and then declined rapidly after 4 weeks.

In bark from wounded trees, G-6-PD activity in the bark from wounded trees increased rapidly at 2 weeks and then peaked at 4 weeks, as shown in Fig. 2. This peak coincided with proliferation of callus tissue in the wounds (Fig. 3-A). In bark from inoculated trees, the increase during the first 2 weeks was identical to that in the bark from wounded trees, after which the increase slowed so drastically that it did not peak within the experimental period. However, callus tissue did not form in the wounds of inoculated trees (Fig. 3-B). In bark from healthy trees, it remained about constant during the entire period.

The greatest changes in activity occurred in the peroxidase enzyme as shown in Fig. 4 and 5. In both bark and wood, activity increased slightly during the first 2 weeks after treatment. Between the second and fourth week, it increased rapidly in inoculated trees, but much slower in wounded trees. In wood, it declined rapidly after the fourth week, but in bark it continued to increase after the fourth week, and only began to decline after the sixth week in inoculated trees. It remained low throughout the experiment in healthy trees. High peroxidase activity in wood of inoculated trees was not an artifact caused by high levels in the bark because the level of peroxidase in wood dropped while such activity in bark remained high. It could not be attributed to the release of peroxidase previously trapped in cell walls (13) because treatment of peroxidase-poor aspen cell wall debris with cellulase did not release large amounts of additional peroxidase (Fig. 6).

Six peroxidase isozymes were present in healthy, wounded, and inoculated trees throughout the period of this experiment (Fig. 7). In addition, three new isozymes appeared in wounded and inoculated aspen bark 2 weeks after treatment, apparently because the total amount of peroxidase present increased, not because of increased synthesis of specific isozymes.

In wounded trees, the three new isozymes were present during weeks 2, 4, and 6, but were not present by week 8; in inoculated trees, they remained present throughout the experiment. Two more new isozymes were found in both wounded and inoculated aspen bark 4 weeks after treatment. A twelfth isozyme appeared in the bark of inoculated trees; it remained through the remainder of the experiment. None of the new isozymes appeared in quantities sufficient to account for the large increases in peroxidase activity.

DISCUSSION.—None of the enzymes gave indication of successful infection before visible symptoms could be seen, probably because the enzyme changes all occurred

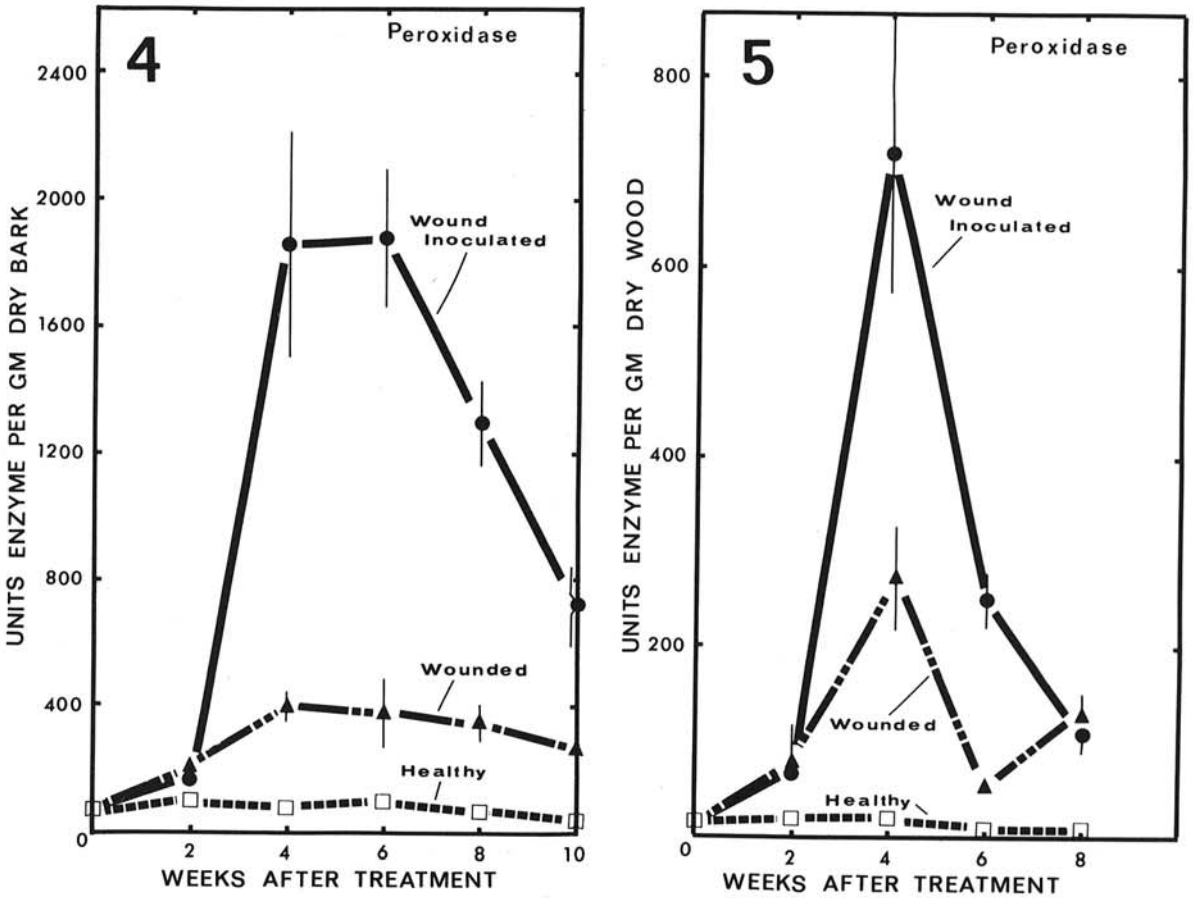

Fig. 4-5. 4) Peroxidase activity in aspen bark that is healthy (control), wounded, or wound inoculated with *Hypoxylon mammatum*. Vertical bars denote standard errors of the mean. Errors smaller than the symbols used are not shown. 5) Peroxidase activity in aspen wood that is healthy (control), wounded, or wound inoculated with *H. mammatum*. Vertical bars denote standard errors of the mean. Errors smaller than the symbols used are not shown.

in the bark rather than in the wood. If the tissue samples had been taken from the wounded area rather than adjacent to it, the activity changes might have appeared before symptoms. In a subsequent study in which we treated aspen seedling stem cuttings with cell-free extracts of *H. mammatum* cultures (14), we found peroxidase activity increased significantly as early as 48 hours after treatment (Schipper and Harrell, *unpublished*).

The best indicator of a successful infection among the enzymes monitored is a comparison of G-6-PD activity in wounded and inoculated tissue. The large increase in G-6-PD activity in wounded aspen bark seems to correlate with wound healing, because the peak of activity occurred when wound callus was rapidly forming. In inoculated trees no wound callus formed and G-6-PD activity did not peak. Furthermore, it seems necessary that the fungus must interfere with the wound-healing process if infection is to occur. Apparently, Bier (2) and others are correct when they describe *H. mammatum* as a wound parasite because *H. mammatum* has never been found to penetrate intact bark.

G-3-PD activity appears to be a much less reliable indicator of successful infection because the pattern of activity decline in the inoculated tissue does not significantly differ from this pattern in healthy tissue until 6 weeks after inoculation. This decline in the inoculated tissue probably reflects the increased amount of necrotic tissue at each sampling time.

More G-3-PD activity was present on the day the experiment was started than in subsequent samples of healthy trees. In dormant aspen bark G-3-PD activity is higher than when growth begins in the spring (Haissig and Schipper, *unpublished*). The drop in activity that occurs during the first 2 weeks in healthy tissue may reflect the decrease in activity of G-3-PD; apparently, G-3-PD is not as stable in samples from growing aspen as it is in samples from dormant aspen. Nevertheless, 2 weeks after treatment, more G-3-PD activity was present in wounded and in inoculated tissue than in healthy tissue. Between 2 and 4 weeks after treatment the activity of G-3-PD in wounded tissue rose, while the activity in wound-inoculated tissue declined. This increase in metabolic activity in wounded tissue occurred at the same time as maximum callus development in the wound. At this time G-3-PD activity in inoculated tissue was diminished, which indicates that the wound healing process is stopped in inoculated tissue.

The increase in peroxidase activity occurs at the same time as symptoms appear on the bark. All of the peroxidase measured had to be of host origin because *H. mammatum* mycelium does not contain measurable peroxidase. Infection could not be correlated with appearance or disappearance of any of the isozymes because only one isozyme appeared in extracts of infected tissue that did not appear in wounded tissue. This apparently was caused by increases in concentration of peroxidase isozymes in infected tissue, rather than appearance of a new isozyme. If the peroxidase in wounded tissue had been concentrated to the specific activity in inoculated tissue, identical isozyme complements probably would have been detected.

Whereas cellulase treatment of aspen cell wall material released some cell-wall-bound peroxidase, the amount released was not sufficient to account for the quantity of peroxidase present in the *H. mammatum*-infected aspen. Apparently, the increased activity in infected aspen is the result of increased peroxidase synthesis by the host plant, or by activation or release of stored peroxidase subunits in response to Hypoxylon infection.

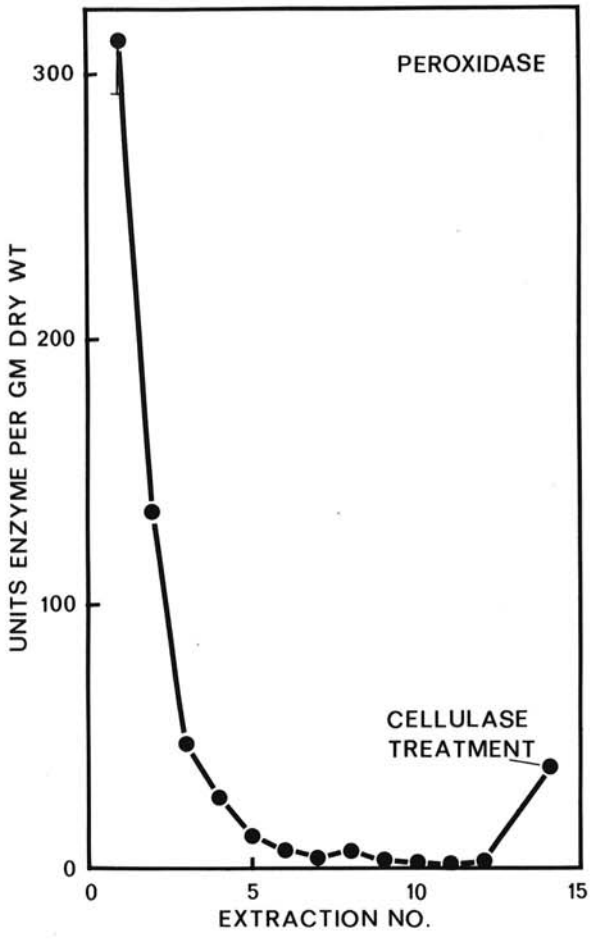

Fig. 6. Peroxidase release by cellulase treatment of peroxidase-poor aspen bark. The amount of peroxidase released is not enough to be a factor in the large increases in peroxidase found in inoculated aspen bark.

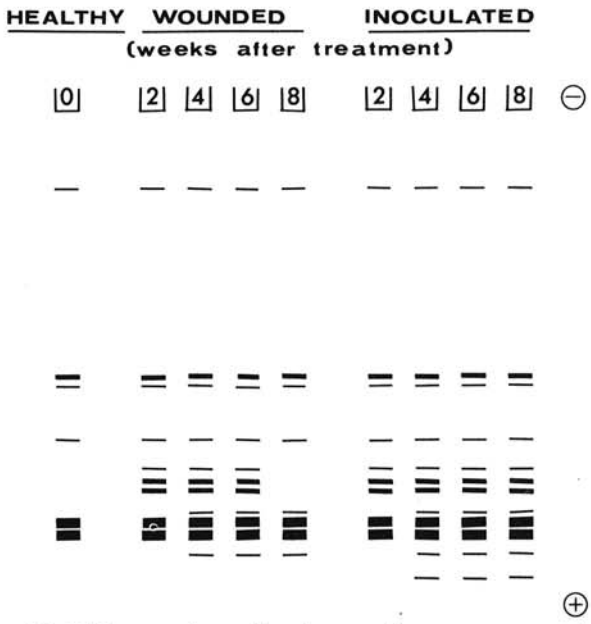

Fig. 7. Diagram of peroxidase isozymes from aspen separated by electrophoresis.

Because all of the inoculated trees became infected, the large increase in peroxidase activity does not appear to be correlated with any form of resistance in aspen trees. More likely, this increase signals the onset of injury to host tissue in both the bark and the wood, and the decline in activity indicates tissue necrosis, which is in agreement with those who have found that *H. mammatum* advances first in the wood and only subsequently into the bark (8, 15). Peroxidase activity declined in wood before it did in bark because the hyphal tips had passed through and killed the wood before they killed the adjacent bark.

All three enzymes responded in the same way in wounded and in inoculated tissue during the first 2 weeks after treatment. The acceleration of enzyme activity in the wounded tissue indicates that the wound-healing process started in both wounded and in inoculated tissues, but that shortly thereafter it was inhibited in the inoculated tissue. Wound-healing interference by *H. mammatum* does not occur in all *Populus* spp. that I have inoculated with *H. mammatum*. For example, I did not observe such interference in *H. mammatum*-inoculated cottonwood (*P. deltoides* Marsh.), and the plants did not become infected. Berbee and Rogers (1) found that cottonwood is immune to *H. mammatum* infection, as are a number of other poplar species. They also found that most poplar hybrids in which quaking aspen is one of the parents are susceptible to *H. mammatum*. Therefore, interference with wound healing may be a characteristic factor in Hypoxylon canker disease. In another study (14) I found (i) that *H. mammatum* produced a toxin in culture and in cankered quaking aspen, and (ii) that this toxin causes both the wound-healing interference and the bark necrosis symptoms of the Hypoxylon canker disease. I think that this *H. mammatum* toxin is important both in the infection and disease development stages of the Hypoxylon canker of quaking aspen.

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