Hypoxylon mammatum Ascospore Infection of *Populus tremuloides* Clones: Effects of Moisture Stress in Tissue Culture

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ABSTRACT

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Tissues from six aspen clones were cultured to produce plantlets from dormant buds. Plantlets of 1–2 cm were moisture stressed by adding various concentrations of mannitol to the growth medium. Inoculation of unwounded plantlets with ascospores of *Hypoxylon mammatum* resulted in visible signs of mycelium after 3–4 days. After 10 days, mycelial growth on controls and moderately stressed plants remained superficial; in contrast, highly stressed plants were invaded by the mycelium and exhibited necrotic lesions at the site of inoculation. The level of moisture stress needed for mycelium invasion and lesion development varied (−0.45 to −1.2 MPa) among the clones. Clonal differences observed could be applied in basic physiological studies or in aspen breeding programs for hypoxylon canker resistance.

Attempts to consistently induce hypoxylon cankers (*Hypoxylon mammatum* (Wahl.) Mill.) in living aspen (*Populus tremuloides* Michx.) by ascospore inoculation have been unsuccessful (11,18,22). Anderson and French (3) attributed this problem to the inability of the mycelium to expand following spore germination. Griffin et al (14) hypothesized that environmental variables, especially drought stress, could influence the growth of the fungus. Bier and Rowat (8) found that it was possible to induce cankers on *Populus trichocarpa* Torr. & Gray by reducing the water content of the bark. Bagga and Smalley (5) reported that any factor inducing water stress increased the susceptibility of aspen to hypoxylon canker, but they did not quantify the amount of drought stress required to promote levels of infection.

The ability to reproduce aspen in tissue culture (1,2) offers the opportunity to generate a large number of genetically identical plants maintained in similar environmental conditions. Such conditions provide a system where the osmotic potential of the medium can be modified while other variables are kept constant (24).

In this study, we hypothesized that water-stressed plantlets should be more sensitive to infection by ascospores of *H. mammatum*. Our specific objectives were to develop a reliable ascospore inoculation assay on aspen plantlets and to assess the role of water stress on the response of aspen plantlets to inoculation with ascospores. To achieve this, we precisely adjusted different levels of osmotic potential under which the plantlets were tested. A preliminary report of this study has been presented (6).

MATERIALS AND METHODS

Tissue culture of aspen clones. The procedure of Ahuja (2) was used for the culture of aspen plantlets. Axillary buds from six aspen clones (A00, A10, A40, B10, B70, and D10) selected from a collection of 29 clones studied by Falk (12) were collected and surface sterilized and their outer scales were removed. These buds were sterilized and transferred directly onto a growth medium. As shoots developed from the apical meristem, the clusters were subdivided and transferred to fresh growth medium. This process was continued until sufficient numbers of plantlets were obtained for each clone.

Induction of water stress. Four osmotic agents were tested for their ability to induce a water stress in the growth medium: mannitol, polyethylene glycol, sodium chloride, and agar. Osmotic potential measurements were made with a Decagon SC-10 thermocouple psychrometer. Samples of the medium were placed directly in the psychrometer cups, and readings were taken at 21 C. Readings in microvolts were converted to corresponding megapascal (MPa) values by comparisons to KCl solutions used as standards.

Mannitol was retained for the ascospore inoculation assay. Eight samples of seven to eight aspen plantlets (approximately 200 mg fresh weight) were grown for 2 wk in Magenta culture vessels, four samples in mannitol-free medium, and four in medium containing 0.30 M mannitol, to determine mannitol absorption by the plantlets. At the end of the 2-wk period, the samples were ground in a mortar with 2 ml of water. The larger tissues were sedimented, and the supernatant solution was filtered and dried in vacuo. The dried portion was dissolved in 20 μl of ethanol and spotted on paper for chromatography; standards of 1, 0.1, and 0.02 μmole mannitol were spotted separately for comparison. The ascending paper chromatography method described by Gordon et al (13) with periodic acid and benzidine as reagents was used.

Ascospore inoculation assay. Spores were collected following the methods of Mahoney (17). Perithecial stromata from naturally occurring cankers were collected and spore discharge was induced by placing pieces of stroma on wet filter paper in sterile petri plates at 10 C. The spores were discharged on a sterile glass microscope slide placed over the stroma. After 48 hr, the slides bearing spores were stored in a sterile case at 10 C. Spore inoculation was carried out under aseptic conditions. A small sterile paintbrush was used to pick up a few spores from the slides. The spores were gently brushed on the midsection of each stem of plantlets taken out of optimal growth conditions. For each of the six clones, 20 plantlets were inoculated and four were transferred into each of five Magenta culture vessels adjusted to the following levels of water stress with mannitol: −0.45 (control), −0.6, −0.8, −1.0, and −1.2 MPa. Osmotic potentials were remeasured at the end of the experiment. The experiment was repeated three times for a total of 12 aspen plantlets per treatment per clone. Concurrently, the same experiment was run with plantlets of black cherry (*Prunus serotina* Ehrh.) to assess host specificity of *H. mammatum*.

Spore germination and mycelial growth were observed daily

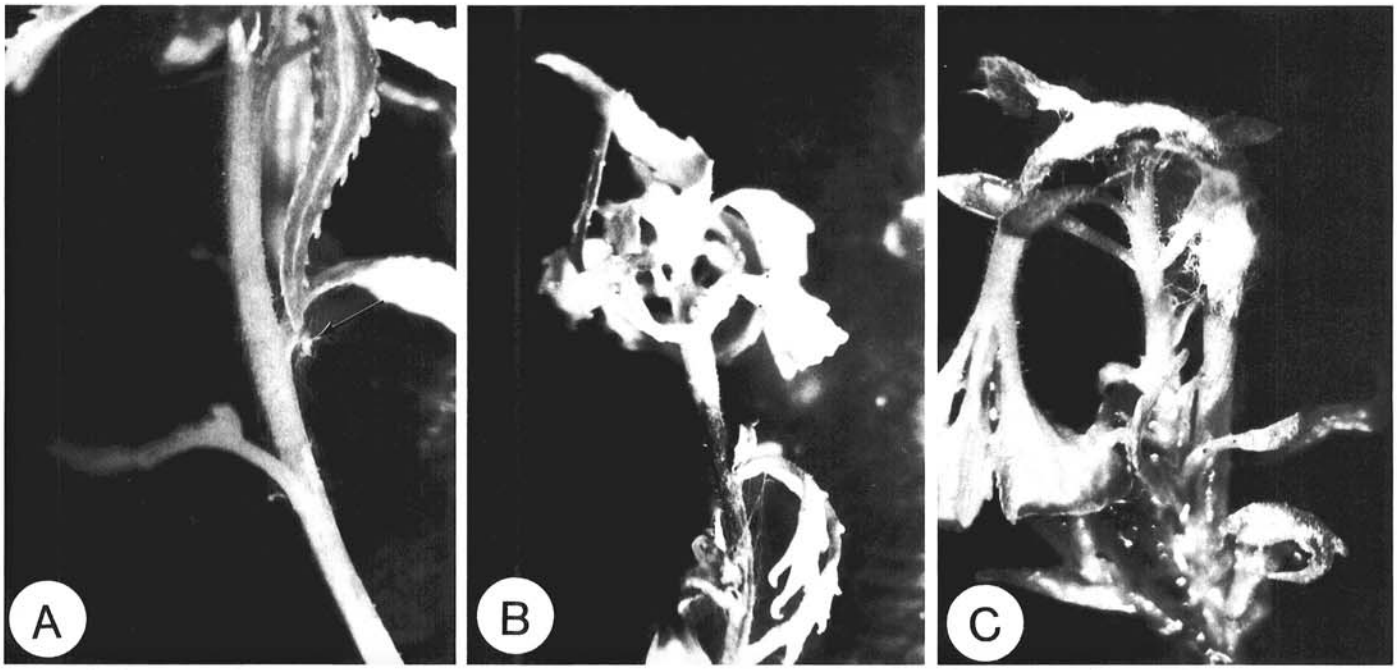

Fig. 1. Infection by mycelia and associated symptoms on plantlets of *Populus tremuloides* inoculated with ascospores of *Hypoxylon mammatum* after **A,** 5 days, **B,** 10 days, and **C,** 14 days.

under a dissecting microscope. At the end of a 2-wk period, the response was quantified based on a scale of 1 to 5 as follows: 1, spores did not germinate; 2, spore germination with limited mycelial growth; 3, spore germination with good mycelial growth but no apparent symptoms on the plantlet; 4, necrosis; and 5, death of the plantlet. Results were analyzed with the Waller and Duncan *k*-ratio test (27,28) provided by the computer program package of the Statistical Analysis Systems Institute, release 82.3 (b) (19).

For reisolation of *H. mammatum*, 12 infected aspen plantlets were randomly selected. Six of them were directly plated on a defined medium (14), and mycelial filaments taken on the six others also were plated. Microscopic features of the fungus were used for identification.

RESULTS

Induction of water stress. The four osmotic agents tested exhibited different properties. Polyethylene glycol was unacceptable for our purposes because high concentrations prevented agar from solidifying. The addition of sodium chloride to the growth medium had a harmful effect on the plantlets. The use of agar also was rejected because of its inability to modify the osmotic potential to the desired levels. Mannitol concentrations of 0.11, 0.16, 0.22, and 0.30 M induced in the growth medium osmotic potentials of −0.6, −0.8, −1.0, and −1.2 MPa, respectively. These osmotic potentials remained constant over a 2-wk period. Plantlets did not survive potentials exceeding −1.2 MPa.

Mannitol was not detected at 0.02 μmole sensitivity in four samples of plantlets grown in media containing 0.30 M mannitol.

Ascospore inoculation assay. The first signs of spore germination appeared after approximately 5 days regardless of the level of water stress (Fig. 1A). In some instances, a necrotic response accompanied by collapsing of the stem was observed at the site of inoculation by 10 days (Fig. 1B). In these situations, the mycelium expanded very rapidly on the necrotic areas, often resulting in the death of the plantlets within 14 days (Fig. 1C).

Mycelial growth increased with higher levels of water stress on all clones tested (Fig. 2). The average responses on all clones (based on the 1–5 rating scale) were 1.7, 2.1. 2.6, 3.0, and 3.8 for osmotic potentials of −0.45 (controls), −0.6, −0.8, −1.0, and −1.2 MPa, respectively. All these values were significantly different among each other (*k*-ratio = 100, minimum significant difference [MSD] is 0.38). Under high water stress (−1.2 MPa), clonal responses were

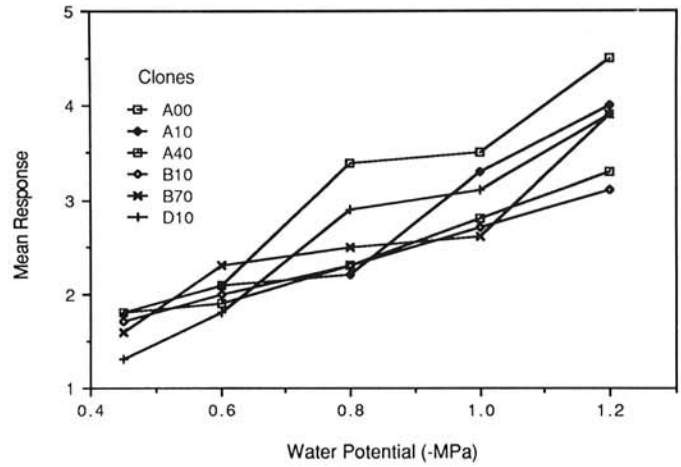

Fig. 2. Mean response to ascospore infection of *Hypoxylon mammatum* (based on 1–5 rating scale) of plantlets from six clones of *Populus tremuloides* cultured under five different water potentials.

rated as 3.1. 3.3, 3.9., 3.9, 4.0, and 4.5 for clones B10, A40, B70, D10, A10, and A00, respectively. Clone A00 had a significantly greater response than clones B10 and A40 (*k*-ratio = 100, MSD is 1.1).

H. mammatum was cultured and identified from all samples of infected plantlets or mycelial filaments from infected plantlets. No other fungi were recovered from the samples. Black cherry plantlets inoculated with *H. mammatum* did not support fungal growth at any level of water stress.

DISCUSSION

The use of mannitol was preferable for inducing a gradient of water stress on plantlets grown in solid media (9,16,23), although concerns have been raised about its potential uptake by plants (25). However, this uptake was observed in cases where mannitol was the only supply of carbohydrates; the presence of sucrose in the medium is known to be used preferentially over mannitol (9) which makes it unlikely that mannitol directly affects growth of *H. mammatum* in these tests. Moreover, no detectable amounts of mannitol were found in plantlets grown in media containing

mannitol, and the absence of development of *H. mammatum* on black cherry plantlets further suggests that mannitol did not affect the results by acting as a nutrient source for the fungus.

Ascospores of *H. mammatum* can germinate and induce necrosis of aspen (Fig. 1), and the lack of signs and symptoms on plantlets of *P. serotina* confirms the host specificity of the fungus. These results present the first reproducible ascospore inoculation assay for *H. mammatum* on aspen since 1945 when Gruenhagen (15) reported successful induction of symptoms on aspen plants following ascospore inoculations; several authors failed to reproduce his results (7,11,22,26).

Our observations show that rapid fungal invasion is associated with intense necrosis in the infected areas (Fig. 1B and C). Schipper (20) attributed the development of necrosis to a toxin released by the fungus. He hypothesized that necrotic tissues were necessary for infection of *H. mammatum*.

Our results indicate that fungal invasion is stimulated when plantlets are exposed to water stress (Fig. 2). Based on the mean response of the controls (1.7), consistent infection was not achieved when the plantlets were not stressed. This could explain the high rate of failure at infecting aspen with ascospores in previous experiments (22,26); most likely, trees tested were not under water stress. The role of water stress as a predisposing factor in the development of plant diseases, and specifically hypoxylon canker, has been suggested by several authors (4,5,8,10,14,21); our results support this hypothesis.

The response to infection increased with water stress for all clones tested (Fig. 2), but clonal differences were observed. These clonal differences suggest a range of genetic responses that could be used in basic host-pathogen interaction studies or could be applied directly in breeding aspen for hypoxylon canker resistance.

This paper presents the first reproducible ascospore inoculation assay of aspen with *H. mammatum*. The assay used micropropagated aspen plantlets exposed to different levels of water stress ranging from −0.45 to −1.2 MPa. The success of the inoculation was enhanced when plantlets were exposed to a high level of stress. This supports the hypothesis that water stress is a key factor in the development of hypoxylon canker. Clonal differences observed could be applied in basic physiological studies or in poplar breeding programs for hypoxylon canker resistance.

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