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Histochemical and microspectrophotometric analyses of early wound responses of resistant and susceptible *Populus tremuloides* inoculated with *Entoleuca mammata* (=Hypoxylon *mammatum*)

B. Bucciarelli, M.E. Ostry, R.G. Fulcher, N.A. Anderson, and C.P. Vance

Abstract: Stem tissue of resistant and susceptible genotypes of *Populus tremuloides* Michx. wounded or wound-inoculated with *Entoleuca mammata* (Wahlenberg: Fr.) J.D. Rogers & Y.-M. Ju was prepared for histochemical and microspectrophotometric analysis. Samples were collected over a 96-h period. Parenchyma cell walls associated with the response zone of infected resistant and susceptible genotypes accumulated phenolic substances having lignin-like properties. Features of the lignified zone distinguished resistant from susceptible genotypes. This zone in the resistant genotype was uniformly lignified, while in the susceptible genotype, it was discontinuous. Wound callus developed in the infected resistant but not in the susceptible genotype. In the former, callus developed internal to the lignified zone, contained phenolic substances, and was visible 48 h after inoculation. In the susceptible, callus failed to develop. Wounded tissue of both genotypes displayed no distinguishing response characteristics. Both produced equivalent amounts of callus, accumulated similar levels of lignin-like substances, and deposited it in identical locations. It is concluded that resistant *P. tremuloides* limits infection by *E. mammata* by two distinct mechanisms: (i) by the development of an intact and localized lignified barrier zone and (ii) by the development of wound callus rich in phenolic substances. The susceptible is ineffective at developing either of these barriers.

Key words: aspen, Hypoxylon canker, disease resistance.

Résumé : Les auteurs ont préparé les tissus caulinaires de génotypes résistant et susceptible du *Populus tremuloides* Michx. blessés ou blessés et inoculés avec l'*Entoleuca mammata* (Wahlenberg: Fr.) J.D. Rogers & Y.-M. Ju, afin d'en faire l'analyse histochimique et microspectrophotométrique. Les échantillons ont été récoltés pendant une période de 96 h. Les parois des cellules de parenchyme associées avec la zone de réaction des génotypes inoculés, résistants et susceptibles, accumulent des substances phénoliques ayant des propriétés ressemblant à la lignine. Les caractéristiques de la zone lignifiée permettent de distinguer les génotypes résistants de ceux qui sont susceptibles. Chez le génotype résistant, cette zone est uniformément lignifiée alors qu'elle est discontinue chez le génotype susceptible. Un callus traumatique se développe chez le génotype résistant infecté mais est absent chez le génotype susceptible. Chez le premier, le cal se développe à l'intérieur de la zone lignifiée, contient des substances phénoliques et est visible 48 h après l'inoculation. Chez le génotype susceptible, le cal ne se développe pas. Les tissus blessés des deux génotypes ne montrent pas de réactions caractéristiques distinctives. Les deux produisent des quantités équivalentes de cal, accumulent les mêmes quantités de substance ressemblant à la lignine et les déposent dans les mêmes endroits. Les auteurs concluent que le *P. tremuloides* limite l'infection par l'*E. mammata* à l'aide de deux mécanismes distincts : (i) par le développement d'une zone de barrage lignifiée intacte et localisée et (ii) en développant un callus traumatique riche en substances phénoliques. Le phénotype susceptible n'arrive pas à développer ces deux barrières.

Mots clés : peuplier faux-tremble, chancre hypoxylon, résistance à la maladie.

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Introduction

Populus tremuloides Michx. (trembling aspen), widely used in the paper industry, is the predominant forest tree in the Great Lakes States. *Entoleuca mammata* (Wahlenberg: Fr.) J.D. Rogers & Y.-M. Ju (= *Hypoxyton mammatum* (Wahlenberg: Fr.) P. Karst.) (Rogers and Ju 1996), the causal agent of Hypoxylon canker, is the most important pathogen of *P. tremuloides* in this area.

Infection by *E. mammata* occurs through aspen stem wounds that penetrate the periderm. Established hyphae cause extensive sapwood decay resulting in branch and stem canker formation. Resistant aspen genotypes are capable of walling off the cankered area and limiting penetration (Baranyay 1967; Ostry and Anderson 1983). Conversely, susceptible genotypes fail to effectively isolate and wall off the canker. Unrestricted cankers are capable of girdling the infected stem, resulting in tissue death distal to the infection site. This can be fatal to the tree if the pathogen spreads to the main trunk.

Considerable evidence indicates that lignification of cell walls in some plants is crucial to disease resistance (see reviews by Bostock and Stermer 1989; Biggs 1992a, 1992b; Vance et al. 1980; Ride 1983; Hahlbrock and Scheel 1989). Histochemical analysis of woody species show that rapid development of an intact lignified response zone near the wound site is highly correlated to resistance (Biggs 1992a, 1992b). The development of this lignified barrier is also essential to subsequent defense responses such as suberin deposition and restoration of periderm integrity (Mullick 1975; Biggs 1992a). Delayed development or the development of discontinuous barriers result in a response zone easily penetrated by pathogens (Rioux and Ouellette 1991; Biggs 1992a, 1992b).

Limited work has been done on the aspen – *E. mammata* interaction in relation to barrier zone formation and compartmentalization of infected tissue. Past research has shown that rapid development of a physical barrier, such as wound callus, in various poplar species is associated with resistance to infection (Berbee and Rogers 1964). However, in some resistant genotypes wound callus formation was not observed, suggesting that an alternate host response is involved in resistance to infection (Berbee and Rogers 1964). Our study evaluated barrier zone formation and compartmentalization in *E. mammata* resistant and susceptible aspen genotypes. The goals of this research were to (i) evaluate differences in early response zone development in resistant and susceptible aspen genotypes and (ii) determine resistance mechanisms involved in preventing infection of aspen by *E. mammata*.

Materials and methods

Plant and fungal material

Resistant (Pike Bay[®]) and susceptible (clone 422) genotypes of *P. tremuloides* were propagated from lateral root cuttings (Starr 1971) and maintained in the greenhouse as stock plants. Pike Bay[®], a mature superior aspen genotype, was located near Cass Lake, Minnesota. Clone 422 is a 30-year-old aspen originating from a cross between two cankered parents. This clone was located in the poplar research plantation at the University of Minnesota Agricultural Research Station in Rosemount, Minn. (Anderson et al. 1979). All plants used in this study were propagated from sub-

sequent lateral root cuttings excised from the above stock plants. These plants were maintained as hedges in the greenhouse under a 16 h light : 8 h dark photoperiod for 1 year prior to experimentation.

A pathogenic culture of *E. mammata* was isolated from a cankered aspen grown at the poplar research plantation at the University of Minnesota Agricultural Research Station (Rosemount, Minn.). The isolate was cultured on one layer of dialysis tubing (mwco = 6000–8000) placed over malt agar (Difco[™]). All inoculations were performed using a 3-week-old mycelial culture grown in the dark at 20°C.

Wounding and inoculation

Wounds were made on green internodal stem tissue, specifically, internode six and seven (3–4 mm stem diameter) proximal to the first unrolled leaf of the shoot apex. Wounds were made with a scalpel and extended the length of the internode. Wounds were approximately 2 mm wide and 0.5 mm deep, penetrating the cortex and phloem and exposing newly formed xylem tissue. In the wound-inoculated treatment, strips of mycelium were placed mycelium-side-down along the entire length of the wound. Nonwounded, wounded, and wound-inoculated internodes were wrapped with one layer of Parafilm[™] to prevent desiccation. Tissue was collected 12, 24, 48, 72, and 96 h after treatment. Wounding and inoculations were staggered over a 96-h period allowing tissue to be collected on the same day for all time points. Nonwounded internodes were also collected at this time. Individual trees, with four to six treated branches, were used exclusively for each specified treatment and time point. Three internodes from each treatment were evaluated.

Histochemical analysis

Samples were collected and immediately placed in formalin – acetic acid – alcohol (FAA) fixative, dehydrated in a tertiary butyl alcohol series (Johansen 1940), and embedded in TissuePrep[®] (Fisher Scientific Co., Fairlawn, N.J.). Embedded material was sectioned at 10 µm using a rotary microtome. Tissue was mounted on glass slides using Haupt's adhesive (Johansen 1940) and deparaffinized with xylene. Histochemical tests to detect lignin, suberin, and general phenolics were conducted as follows: phloroglucinol-HCl (PG-HCl) and the Maule tests (Johansen 1940; Faulkner and Kimmins 1975) were used to detect lignin; Sudan IV, Sudan Black B (Jensen 1962), and the fluorescence technique developed by Biggs (1984b) were used to localize suberin; general phenolics were detected by exposing tissue for 15 min in a solution of 0.02 M ferric chloride – 0.02 M potassium ferricyanide (Sherwood and Vance 1976); hyphae were observed with fluorescein isothiocyanate coupled to wheat germ agglutinin (FITC-WGA) (Vector Laboratories Inc., Burlingame, Calif.) by following the procedure of McManus et al. (1989) and Morrell et al. (1985). Tissue autofluorescence was examined under UV illumination (Osram HBO 100W/2 bulb) with a filter cube having the following wavelength parameters: BP365 FT395 LP397.

UV microspectrophotometry

Stems were sectioned as above and mounted in glycerine under a quartz coverslip on quartz slides. A Zeiss UMSP-80 scanning microspectrophotometer equipped with an Osram high-pressure xenon lamp (XBO 75W), an ultrafluar quartz condenser, and a 100× ultrafluar quartz objective was used to analyze the tissue. A measuring diaphragm of 0.08 mm was positioned over fluorescent areas of cell walls. An absorption spectrum, ranging from 200–400 nm, of the fluorescent area was obtained using the spectral analysis program Lambda-Scan (Carl Zeiss, Inc., Thornwood, N.J.). The procedure followed was similar to that described by Hartley et al. (1990). Statistical analysis was performed on the

spectral data using a principal component analysis. Analysis of variance was subsequently performed on the first principal component since it captured 92% of the variability within the data.

Specimen scanning at a specified wavelength was done using the MAPS (Microscopic Analysis for Photometric Scanning) software program (Carl Zeiss, Inc., Thornwood, N.J.). This program controlled a scanning stage and permitted measurement of a field consisting of up to 65 000 data points. Prior to scanning, adjustments were made at 270 nm on an area devoid of plant tissue. This analysis was similar to that described by Ames et al. (1992).

Results

Histochemical response zone development

Genotypic differences in *E. mammatata* infected resistant and susceptible aspen stem tissue was evaluated by following the PG-HCl staining pattern of phloem and cortical parenchyma cell walls near the infection site. Three separate internodes from each treatment were evaluated. The staining pattern observed was consistent among all three individual internodes for each specific time point. Results showed that by 24 h after inoculation the response zone in the resistant genotype developed directly adjacent to the wound margin in both phloem and cortical parenchyma, whereas in the susceptible genotype, it developed approximately 0.1 mm internal to the wound margin and predominately in phloem parenchyma.

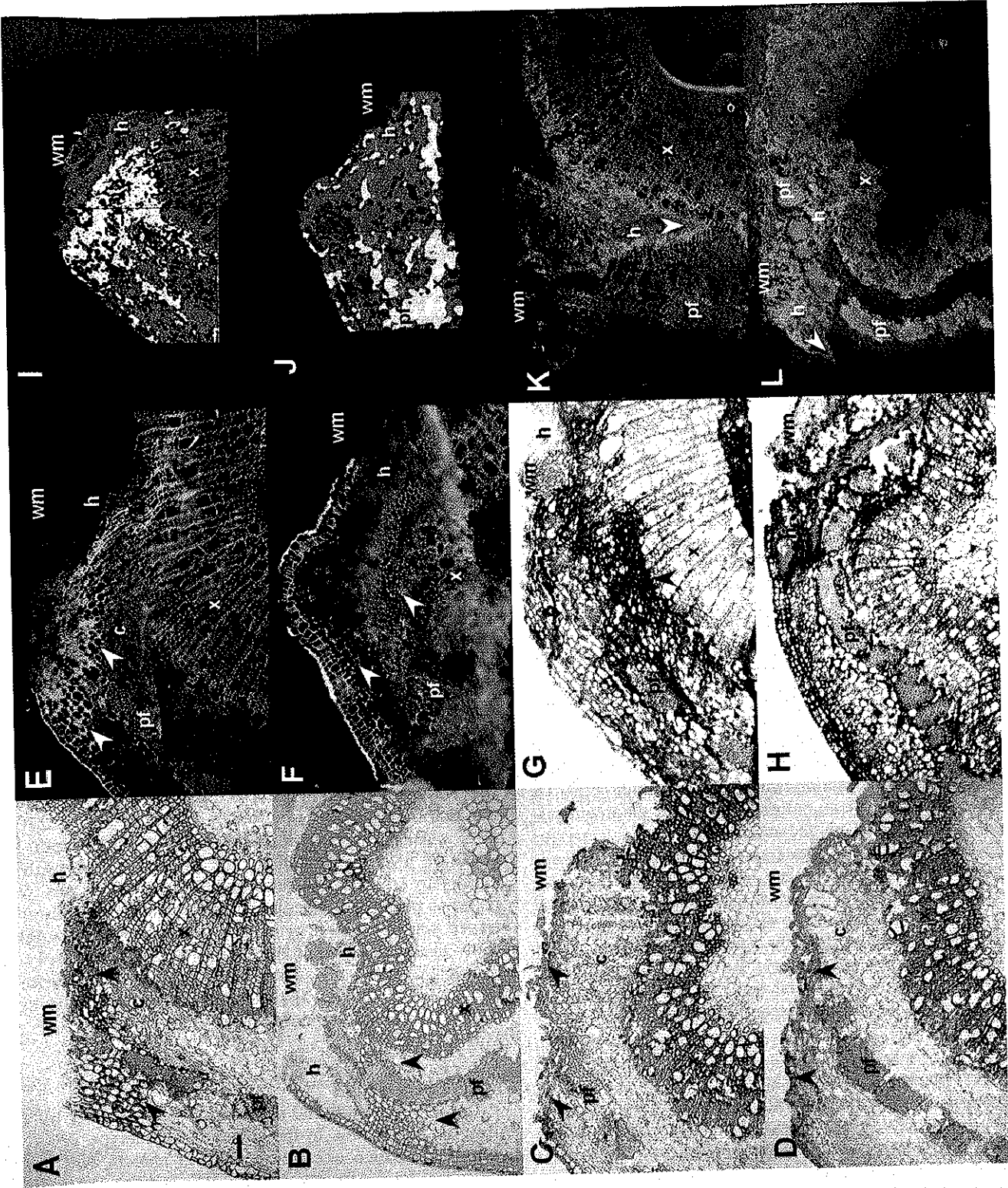
By 48 h after inoculation the response zone of the resistant genotype extended approximately 0.2 mm internal from the wound margin. In the susceptible genotype, the response zone extended 0.45–0.5 mm internally, more than twice that of the resistant genotype. Both phloem and cortical parenchyma cell walls within the response zone of the resistant genotype were uniformly stained with PG-HCl. In contrast, the PG-HCl positive-staining cell walls in the susceptible genotype continued to be predominately visible only in the phloem parenchyma, with the cell corners of these responding cell walls being the most PG-HCl reactive. Pith parenchyma cell walls closest to the wound margin in the resistant genotype stained positive for PG-HCl. Contrarily, in the susceptible genotype, pith parenchyma failed to react with PG-HCl.

Seventy-two hours after inoculation the cell walls in the response zone of the resistant genotype displayed an intense and localized PG-HCl positive-staining zone that extended 0.15–0.3 mm internal from the wound margin. In the susceptible genotype, the PG-HCl positive-staining zone appeared discontinuous and extended up to 0.5 mm internally.

Ninety-six hours after inoculation the PG-HCl response zone of the resistant genotype (Fig. 1A) had not changed in intensity. However, the area occupied by this zone decreased because of the growth of wound callus tissue developing immediately internal to the PG-HCl response zone. In the cortical cell walls of the susceptible genotype (Fig. 1B), PG-HCl staining appeared to be distributed uniformly; however, in phloem parenchyma cell walls, PG-HCl staining continued to be distributed in a discontinuous pattern.

In wounded stem tissue (Figs. 1C and 1D), the area occupied by the PG-HCl positive-staining zone was insignificant relative to that observed in *E. mammatata* infected tissue. The positive-staining cell walls developed as a single cell layer along the wound margin, and by 96 h these cells appeared

Fig. 1. Response zone development in resistant and susceptible *Populus tremuloides* wounded and wound-inoculated with *Entoleuca mammatata* (=Hypoxylon mammatum) 96 h after treatment. (A and B) Phloroglucinol-HCl positive-staining response zone along the wound margin of wound-inoculated resistant (A) and susceptible (B) aspen. Note the intactness of the response zone in the resistant relative to the susceptible genotype (red color, arrowheads) and hyphal (h) penetration beyond the initial wound margin (wm) in the susceptible. 100x. (C and D) Phloroglucinol-HCl positive-staining tissue and wound callus (c) development along the wound margin (wm) of wounded resistant (C) and susceptible (D) aspen. Note the location and size of the response zone (red color, arrow heads) and the abundance of wound callus in both genotypes. 100x. (E and F) Ultraviolet illumination of parenchyma cell walls associated with the response zone of wound-inoculated resistant (E) and susceptible (F) aspen. Note the location and abundance of the autofluorescent tissue (blue-white color, arrowheads) in the two genotypes and its location relative to the phloroglucinol-HCl staining tissue (A and B). 100x. filter set parameters: BP365 FT395 LP397. (G and H) Ferric chloride – potassium ferricyanide staining of wound-inoculated resistant (G) and susceptible (H) aspen. Note the staining of wound callus (arrowhead) in the resistant genotype. 100x. (I and J) Ultraviolet absorbance scanned images of the response zone along the wound margin of wound-inoculated resistant (I) and susceptible (J) aspen. Tissue scanned at 270 nm. Yellow and white colored areas represent high UV absorbance (arrowheads). (K and L) Fluorescein isothiocyanate – wheat germ agglutinin stain illustrating the path of fungal penetration in wound-inoculated resistant (K) and susceptible (L) aspen. Note penetration in both genotypes is by hyphal aggregation (h, arrowheads). In the susceptible genotype the leading edge of penetration is in both cortical and phloem parenchyma cells. Lignified phloem fibers (pf) are left intact in the wake of fungal penetration. In the resistant genotype the leading edge of penetration is along the vascular cambium. 100x. All images are transverse internodal stem sections. Scale bar = 0.1 mm.



compressed by the rapidly forming wound callus tissue (Figs. 1G and 1H). The development of the PG-HCl positive-staining zone in wounded tissue occurred 24 h later than observed in *E. mammata* infected tissue.

Another feature distinguishing resistant and susceptible *E. mammata* infected aspen genotypes was the development of wound callus. Wound callus developed internal to the PG-HCl response zone in the *E. mammata* infected resistant genotype but not in the susceptible genotype (compare Figs. 1A and 1B). Wound callus, in the resistant genotype, was initiated near the vascular cambium and was microscopically visible by 48 h after inoculation. By 72 h a callus ridge was visible around the wound perimeter, and by 96 h after inoculation, the growth of wound callus lead to the appearance of crushed cells in the PG-HCl positive response zone (Fig. 1A). The cell walls and cytoplasm of wound callus cells were PG-HCl negative but stained positive for both ferric chloride – potassium ferricyanide (Fig. 1G) and the Maule reaction (data not shown). In contrast, the susceptible genotype showed no visible sign of wound callus formation or reaction of the response zone to either the Maule reaction (data not shown) and ferric chloride – potassium ferricyanide staining (Fig. 1H) above background levels.

The development of wound callus in wounded stem tissue was similar for both resistant and susceptible genotypes (compare Figs. 1C and 1D) and exceeded that observed in the *E. mammata* infected resistant genotype (compare Figs. 1A and 1C). Callus tissue in wounded stem tissue was produced in such large amounts that, on occasion, the entire wound site became enclosed (data not shown).

Suberin was not detected in cell walls associated with the PG-HCl response zone or in callus tissue using either Sudan IV, Sudan black B, or the PG-HCl quenching technique developed by Biggs (1984b). However, epidermal cells reacted positive using all three methods.

Phloroglucinol-HCl positive-staining parenchyma cell walls within the response zone autofluoresced yellow-blue when viewed with epifluorescent UV illumination (Figs. 1E and 1F). Lignified xylem, phloem fibers, and suberized epidermal cell walls also emitted autofluorescent characteristics similar to those of the response zone. Parenchyma cell walls not associated with the response zone did not autofluoresce. Congruent with the PG-HCl data, the autofluorescent cell walls in the resistant genotype displayed an intact boundary zone extending the length of the wound margin from cortex to xylem (compare Figs. 1A and 1E). In the susceptible genotype, the autofluorescent cell walls of the boundary zone displayed a fragmented and discontinuous appearance (compare Figs. 1B and 1F).

UV microspectrophotometry

Reactive parenchyma cell walls within the response zone of both *E. mammata* infected and wounded stem tissue show peak absorption in two areas of the UV light spectrum: 220–240 and 260–300 nm (Figs. 2A and 2B). Absorbance values in both of these spectral zones increased progressively from 24 to 96 h after treatment (data not shown). In contrast, absorbance spectra of nonreactive parenchyma cell walls, not associated with a response zone, show peak absorbance only in the 220–240 nm range, the values remained relatively low and did not change over time (Figs. 2A and 2B).

Principal component analysis of the lambda-scan spectral data confirm that absorbance values for wavelengths between 260 and 300 nm provide the greatest variability in this data set. This indicates that differences between reactive parenchyma cell walls within the response zone and non-reactive parenchyma cell walls outside of the response zone was due predominately to the accumulation of UV absorbing substances having maximum absorbance values in the 260- to 300-nm range. Hartley et al. (1990) and Goldschmidt (1971) have demonstrated that phenylpropanoid lignin monomers such as *p*-coumaryl, guaiacyl, and syringyl units, display absorbance maxima between 270 and 300 nm.

Mean absorbance values of reactive parenchyma cell walls of resistant and susceptible *E. mammata* infected aspen stem tissue displayed distinct genotypic differences 96 h after inoculation even though their spectral pattern of absorption was similar (Fig. 2A). Higher overall absorption values were measured in the resistant relative to the susceptible genotype ($P = 0.03$). In contrast, mean absorbance values of reactive parenchyma cell walls of wounded resistant and susceptible aspen stem tissue (Fig. 2B) displayed no distinct genotypic differences 96 h after wounding ($P > 0.5$).

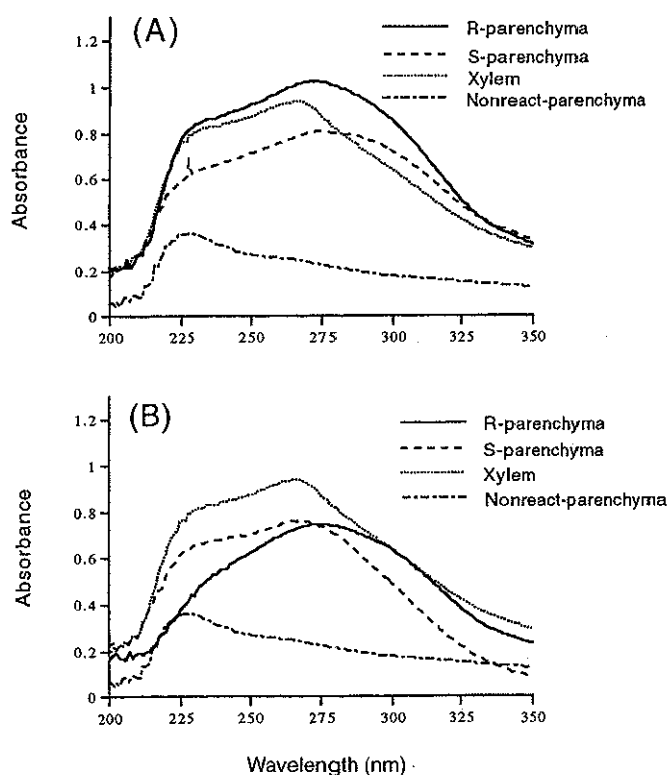
The absorbance pattern and values of reactive parenchyma cell walls located within the response zone of both resistant and susceptible *E. mammata* infected and wounded stem tissue mirrors that of lignified xylem (Figs. 2A and 2B). This strong spectral relationship is suggestive of a similar phenolic composition between developmentally lignified xylem cell walls and reactive parenchyma cell walls associated with the response zone of aspen stem wounds.

Distribution of the deposited phenolic substances within the response zone of *E. mammata* infected and wounded resistant and susceptible aspen stem tissue was examined using a UV absorption scanning microspectrophotometer controlled by the MAPS software program. This technique resulted in a visual image of the distribution pattern of phenolic substances having maximum absorbance at the specified wavelength used in the scan. Figures 1I and 1J represent the distribution patterns of substances absorbing at 270 nm in *E. mammata* infected resistant and susceptible aspen stem tissue 96 h after inoculation. The 270-nm wavelength was chosen to scan the response zone because 270 nm was a common peak absorbance wavelength identified in the spectra of reactive parenchyma cell walls. Spectral analyses show that areas of peak absorbance at 270 nm closely correspond to cell walls that are PG-HCl positive (compare Figs. 1A and 1B with Figs. 1I and 1J) and autofluorescent under UV illumination (compare Figs. 1E and 1F with Figs. 1I and 1J).

Fungal penetration

Tissue penetration and degradation were caused by large mycelial aggregates. In the susceptible genotype (Fig. 1L), fungal penetration occurred equally in phloem and cortical tissue that stained poorly with PG-HCl and showed a discontinuous autofluorescent pattern. In contrast, if penetration occurred in the resistant genotype it did so predominantly through cells near the vascular cambium (Fig. 1K). Although the rate of fungal penetration was difficult to accurately assess, degradation, as evidenced by phloem fibers encircled by the mycelial aggregate, was greater in the sus-

Fig. 2. Ultraviolet absorbance spectra of autofluorescent parenchyma cell walls associated with the response zone of *P. tremuloides* infected with *E. mammata* (A) and wounded (B) tissue 96 h after treatment. Both graphs represent a mean spectrum of 10–20 individual point absorbance scans for resistant (R-parenchyma), susceptible (S-parenchyma), nonreactive parenchyma cell walls away from the response zone (Nonreact-parenchyma), and lignified xylem (Xylem). Note parenchyma cell walls within the response zone of resistant and susceptible *P. tremuloides* for both *E. mammata* infected and wounded stem tissue show peak absorption in two areas of the UV light spectrum: 220–240 and 260–300 nm. In contrast, absorbance spectra of nonreactive parenchyma cell walls, not associated with the response zone, show peak absorbance only in the 220–240 nm range. Note also that the absorbance pattern and values of reactive parenchyma cell walls located within the response zone of both resistant and susceptible *E. mammata* infected and wounded stem tissue mirrors that of lignified xylem.



ceptible relative to the resistant genotype (compare Figs. 1K to 1L).

Discussion

Parenchyma cell walls in the response zone of young *E. mammata* infected and wounded aspen stem tissue rapidly accumulated phenolic substances having lignin-like properties. This is evidenced by PG-HCl staining and autofluorescence under UV illumination. Moreover, microspectrophotometric analysis of these parenchyma cell walls showed high absorbance in the UV spectral range between 260 and 300 nm,

indicative of phenolic lignin monomers, such as *p*-hydroxyphenyl, guaiacyl, and syringyl units (Goldschmid 1971; Hartley et al. 1990). In addition, reactive parenchyma cell walls also displayed characteristics similar to those of lignified xylem and phloem fibers. Contrarily, parenchyma cell walls outside of the response zone did not have any of the qualitative features described above and also displayed low absorbance values between 260 and 300 nm. Studies done on other woody species associated with canker-causing pathogens have demonstrated that parenchyma cell walls along the wound margin become lignified in response to wounding and infection (Biggs 1984a; Bostock and Middleton 1987; Woodward and Pearce 1988; Biggs 1992a, 1992b). The development of such a lignified response zone, followed by the accumulation of suberin in the inner lining of the responding cells, appeared to be imperative for the formation of a new periderm over wounded and infected tissue. Re-establishment of this periderm was paramount, since few organisms can penetrate such a barrier and, therefore, proved to be essential to compartmentalize the affected tissue and restore stem integrity (Mullick 1975; Pearce 1989). Interestingly, in areas of the response zone where lignin, suberin, and the new periderm failed to develop or was delayed in their development, penetration by invading pathogens was commonly observed (Hebard et al. 1984; Biggs 1986; Bostock and Middleton 1987; Rioux and Ouellette 1991). Thus, initial lignification of cell walls associated with the response zone in woody tissue is crucial to impeding pathogen penetration and promoting wound closure.

Resistant and susceptible aspen genotypes inoculated with *E. mammata* were distinguished by various characteristics associated with the development of the lignified response zone. Characteristics that distinguished the response zone of *E. mammata* infected resistant from susceptible *P. tremuloides* included (i) the initial location of the response zone relative to the wound margin; (ii) its distribution throughout the 96-h study; and (iii) the subsequent development of wound callus tissue at the wound site. In wounded aspen stem tissue there were no distinguishing wound response characteristics differentiating the resistant from the susceptible genotype. Both genotypes produced equivalent amounts of wound callus, accumulated similar levels of the lignin-like substance and deposited this substance in identical locations along the cell walls of responding parenchyma cells. This suggests that the presence of *E. mammata* in wounded aspen stem tissue greatly influences the development of the response zone as well as the formation of wound callus.

The first observable difference between *E. mammata* infected resistant and susceptible aspen genotypes was the initial location of the response zone relative to the wound margin. The response zone in the resistant genotype initially developed directly adjacent to the wound margin, whereas in the susceptible genotype it developed approximately 0.1 mm internal to the wound margin. The development of a lignified response zone directly adjacent to the wound margin appeared to be effective in slowing the advancing mycelium, whereas in the susceptible genotype, the presence of non-lignified tissue between the developing response zone and the wound margin may have provided the advancing mycelial aggregates an additional energy source enhancing the growth of the pathogen (Berbee and Rogers 1964). Our ob-

servations contrast with those of Biggs (1984a) who found barrier zones in peach (*Prunus persica* (L.) Bartsch) infected with *Cytospora leucostoma* (Sacc.) to initially develop up to 1 mm internal to the wound margin. However, Biggs (1984a) studied the wound response in mature stem tissue, whereas our study focused on juvenile stem tissue.

The second distinguishing characteristic between resistant and susceptible aspen genotypes in response to *E. mammata* infection was the distribution of PG-HCl positive-staining cell walls of the response zone. In the resistant genotype the cell walls associated with the response zone displayed a lignification pattern that was intact and localized as evidenced by PG-HCl staining and autofluorescence by UV illumination. In the susceptible genotype, these cell walls were distributed in a discontinuous pattern along the response zone. In various woody species previously studied, the distribution of the newly deposited lignin along the response zone appeared to be important in controlling pathogen penetration into noninfected tissue. Biggs (1984a) found that *C. leucostoma* penetrated boundary zones in peach through areas where cell walls were poorly lignified and suberized such as through discontinuities along the developing boundary zone or through newly initiated cells along the vascular cambium. In elm (*Ulmus americana* L.), a discontinuous response zone developing in wounded stem tissue has been associated with susceptibility to *Ophiostoma ulmi* (Buism.) Nannf., whereas intact response zones were effective in resisting ingress of this pathogen (Rioux and Ouellette 1991). Berbee and Rogers (1964) reported that *E. mammata* selectively penetrates and degrades aspen stem tissue composed of nonlignified cell walls as opposed to stem tissue composed of lignified walls. In our study, *E. mammata* penetration in the susceptible genotype occurred indiscriminately and extensively through cortical and phloem tissue where the response zone appeared discontinuous and cell walls were nonlignified. In the resistant genotype, if fungal penetration through the response zone did occur, it started and progressed along the vascular cambium where cells were newly initiated and poorly lignified. *E. mammata* penetration was not observed in cortical or phloem tissue where the response zone was intact and uniformly lignified. Therefore, congruent with studies on other woody species, in addition to earlier studies in the *E. mammata* – aspen interaction, the continuity of the response zone in *E. mammata* infected aspen stem tissue appears to be crucial in restricting penetration of the pathogen and therefore vital to resistance.

The development of wound callus is a third feature distinguishing resistant from susceptible aspen genotypes in response to *E. mammata* infection. In the resistant genotype, wound callus developed immediately internal to the lignified response zone and was first visible 48 h after inoculation. By 96 h, callus ridges were macroscopically evident along the perimeter of the wound margin. The resistant *E. mammata* infected genotype consistently produced wound callus, although less than that observed in noninfected wounded tissue. In contrast, susceptible *E. mammata* infected tissue failed to develop wound callus throughout the duration of the study despite its ability to form it in the absence of the pathogen. Berbee and Rogers (1964) reported that *Populus* spp. resistant to *E. mammata* infection rapidly developed large amounts of wound callus; however, genotypic differ-

ences in the amount of callus produced was observed (Pinon 1986; Berbee and Rogers 1964). In addition, our study also showed that callus cells developing in resistant *E. mammata* infected stem tissue displayed a unique histochemical response in contrast to cells associated with the lignified response zone. The cytoplasm and cell walls of callus cells were PG-HCl negative but stained positive with ferric chloride – potassium ferrous sulfate and the Maule reaction. Such a histochemical response of wound callus tissue suggests that the phenolic composition of this tissue is different than that observed in the lignified response zone and, therefore, may provide an additional barrier that prevents or impedes infection.

In conclusion, these results indicate that, in the presence of the pathogen, the resistant genotype is capable of developing two types of barriers that restrict infection (an intact lignified barrier and a wound callus barrier rich in phenolic substances), whereas the susceptible genotype is ineffective at developing either one of these barriers. This implicates two distinct mechanisms by which *P. tremuloides* limits infection by *E. mammata*. The extent to which these mechanisms occur throughout the population of *P. tremuloides* is not known. However, the ability to identify these mechanisms plus other defense responses would be of benefit to screen aspen genotypes resistant or tolerant to *E. mammata* infection.

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References

- Ames, N.P., Hartley, R.D., and Akin, D.E. 1992. Distribution of aromatic compounds in coastal bermudagrass cell walls using ultraviolet absorption scanning microspectrophotometry. *Food Struct.* **11**: 25–32.
- Anderson, N.A., Ostry, M.E., and Anderson, G.W. 1979. Insect wounds as infection sites for *Hypoxyylon mammatum* on trembling aspen. *Phytopathology*, **69**: 476–479.
- Baranyay, J.A. 1967. Notes on *Hypoxyylon* canker of aspen in Alberta. *For. Chron.* **43**: 372–380.
- Berbee, J.G., and Rogers, J.D. 1964. Life cycle and host range of *Hypoxyylon pruinaum* and its pathogenesis on poplars. *Phytopathology*, **54**: 257–261.
- Biggs, A.R. 1984a. Boundary-zone formation in peach bark in response to wounds and *Cytospora leucostoma* infection. *Can. J. Bot.* **62**: 2814–2821.
- Biggs, A.R. 1984b. Intracellular suberin: occurrence and detection in tree bark. *Int. Assoc. Wood Anat. Bull.* **5**: 243–248.
- Biggs A.R. 1986. Comparative anatomy and host response of two peach cultivars inoculated with *Leucostoma cincta* and *L. personii*. *Phytopathology*, **76**: 905–912.

- Biggs, A.R. 1992a. Anatomical and physiological responses of bark tissues to mechanical injury. In *Defense mechanisms of woody plants against fungi*. Edited by R.A. Blanchette and A.R. Biggs. Springer-Verlag, Berlin. pp. 13–40.
- Biggs, A.R. 1992b. Responses of angiosperm bark tissues to fungi causing cankers and canker rots. In *Defense mechanisms of woody plants against fungi*. Edited by R.A. Blanchette and A.R. Biggs. Springer-Verlag, Berlin. pp. 41–60.
- Bostock, R.M., and Middleton, G.E. 1987. Relationship of wound periderm formation to resistance to *Ceratocystis fimbriata* in almond bark. *Phytopathology*, **77**: 1174–1180.
- Bostock, R.M., and Stermer, B.A. 1989. Perspectives on wound healing in resistance to pathogens. *Annu. Rev. Phytopathol.* **27**: 343–371.
- Faulkner, G., and Kimmins, W.C. 1975. Staining reactions of the tissue bordering lesions induced by wounding, tobacco mosaic virus, and tobacco necrosis virus in bean. *Phytopathology*, **65**: 1396–1400.
- Goldschmid, O. 1971. Ultraviolet spectra. In *Lignin: occurrence, formation, structure and reactions*. Edited by K.V. Sarkanen, and C.H. Ludwig. Wiley-Interscience, New York. pp. 241–267.
- Hahlbrock, K., and Scheel, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**: 347–369.
- Hartley, R.D., Atkin, D.E., Himmelsbach, D.S., and Beach, D.C. 1990. Microspectrophotometry of bermudagrass (*Cynodon dactylon*) cell walls in relation to lignification and wall biodegradability. *J. Sci. Food Agric.* **50**: 179–189.
- Hebard, F.V., Griffin, G.J., and Elkins, J.R. 1984. Developmental histopathology of cankers incited by hypovirulent and virulent isolates of *Endothia parasitica* on susceptible and resistant chestnut trees. *Phytopathology*, **74**: 140–149.
- Jensen, W.A. 1962. Botanical histochemistry. W.H. Freeman & Co., San Francisco.
- Johansen, D.A. 1940. Plant microtechnique. McGraw-Hill Book Co., New York.
- McManus, P.S., Ewers, F.W., and Fulbright, D.W. 1989. Characterization of the chestnut blight canker and the localization and isolation of the pathogen *Cryphonectria parasitica*. *Can. J. Bot.* **67**: 3600–3607.
- Morrell, J.J., Gibson, D.G., and Krahmer, R.L. 1985. Effect of fluorescent-labeled lectins on visualization of decay fungi in wood sections. *Phytopathology*, **75**: 329–332.
- Mullick, D.B. 1975. A new tissue essential to necrophylactic periderm formation in the bark of four conifers. *Can. J. Bot.* **53**: 2443–2457.
- Ostry, M.E., and Anderson, N.A. 1983. Infection of trembling aspen by *Hypoxylon mammatum* through cicada oviposition wounds. *Phytopathology* **73**: 1092–1096.
- Pearce, R.B. 1989. Cell wall alterations and antimicrobial defense in perennial plants. In *Plant Cell Wall Polymers, Biogenesis and Biodegradation*. Proceedings of the Symposium of the 3rd Chemical Congress of North America, 5–11 June 1988, Toronto, Ont. Edited by N.G. Lewis and M.G. Paice. American Chemical Society, Washington, D.C. pp. 346–360.
- Pinon, J. 1986. Test of inhibition of cambial activity of poplar by *Hypoxylon mammatum*: development and application. *Eur. J. For. Pathol.* **16**: 230–238.
- Ride, J.P. 1983. Cell walls and other structural barriers in defence. In *Biochemical plant pathology*. Edited by J.A. Callow. John Wiley & Sons Ltd., New York. pp. 215–236.
- Rioux, D., and Ouellette, G.B. 1991. Barrier zone formation in host and nonhost trees inoculated with *Ophiostoma ulmi*. I. Anatomy and histochemistry. *Can. J. Bot.* **69**: 2055–2073.
- Rogers, J.D., and Ju, Y.-M. 1996. *Entoleuca mammatum* comb. nov. for *Hypoxylon mammatum* and the genus *Entoleuca*. *Mycotaxon*, **59**: 441–448.
- Sherwood, R.T., and Vance, C.P. 1976. Histochemistry of papillae formed in reed canarygrass leaves in response to noninfecting pathogenic fungi. *Phytopathology*, **66**: 503–510.
- Starr, G.H. 1971. Propagation of aspen trees from lateral roots. *J. For.* **69**: 866–867.
- Vance, C.P., Kirk, T.K., and Sherwood, R.T. 1980. Lignification as a mechanism of disease resistance. *Annu. Rev. Phytopathol.* **18**: 259–288.
- Woodward, S., and Pearce, R.B. 1988. Wound-associated responses in Sitka spruce root bark challenged with *Phaeolus schweinitzii*. *Physiol. Mol. Plant Pathol.* **33**: 151–162.