IMMUNOHISTOCHEMICAL INVESTIGATION OF THE NECROTROPHIC PHASE OF THE FUNGUS COLLETOTRICHUM GLOEOSPORIOIDES IN THE BIOCONTROL OF HEMP SESBANIA (SESBANIA EXALTATA; PAPILIONACEAE)\(^1\)

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- **Premise of the study:** Fungal plant pathogens exert much of their effect on plant cells through alterations in the host cell walls. However, obtaining biochemical proof for this change is difficult because of the relatively small number of cells that are affected by the pathogen relative to the bulk of host tissue. In this study, we examined the differences in host wall composition between infected and uninfected areas of seedlings of the weed hemp sesbania (*Sesbania exaltata*) that were treated with the biocontrol agent *Colletotrichum gloeosporioides*.

- **Methods:** To determine the changes in cell wall composition, we used semi-thin sections and a battery of antibody probes that recognize components of the cell wall and immunogold-silver cytochemistry to visualize the probes.

- **Key results:** A loss of specific plant cell wall polysaccharides in the region surrounding the primary fungal infection and the creation of a defensive layer by the plant to limit the fungal invasion were the two most obvious changes noted in this study. At the invasion site, there was significant loss of rhamnogalacturon-1 (RGI) and esterified and de-esterified homogalacturonan (HG)-reactive epitopes from the cell walls. In contrast, boundary tissue between the vascular tissue and the fungal lesion reacted more strongly with antibodies that recognize arabinogalactan proteins (AGPs) and xyloglucans than in unaffected areas.

- **Conclusions:** These data strongly indicate a role of pectinases in the invasion of the biocontrol agent and the importance of extensins, AGPs, and xyloglucans as defense by the host.

**Key words:** biocontrol; bioherbicide; *Colletotrichum gloeosporioides*; immunocytochemistry; fungal infection; Papilionaceae; *Sesbania exaltata*.

Although plant pathogens are generally considered as organisms to be controlled to prevent crop loss, microbes that attack weedy species have the potential to be used to control weeds. The use of phytopathogenic fungi and bacteria as inundative biological control agents for weed control (bioherbicides) has been recognized as a technological alternative to chemical herbicides (Rosskopf et al., 1999; Charudattan 2001, 2005; Weaver et al., 2007). The need to develop such alternative weed management tools and strategies is more acute than ever due to concerns about environmental contamination, food safety, and the development of herbicide-resistant weeds. For example, in 1996 there were no reports of weed species resistant to glyphosate (Round-up™) in the United States, but now, glyphosate resistance has been confirmed for nine species in the United States and 17 weed species around the world (Heap, 2010). Bioherbicides offer alternatives to chemical weed control (Charudattan and Walker, 1982; Hoagland, 1990; TeBeest, 1991; Rosskopf et al., 1999; Charudattan, 2001, 2005; Hallett 2005; Weaver et al., 2007). There is worldwide interest in this field, with active scientific research and commercial development underway in the United States, Canada, Europe, Australia, Japan, and other countries (Charudattan 2001, 2005, Weaver et al., 2007). One of the few commercialized products for fungal biocontrol of a weed is a strain of *Colletotrichum gloeosporioides* f. sp. *Aeschynomene*, that was commercialized originally under the product name Collego (Templeton et al., 1989) and more recently under the trade name Lockdown (Cartwright et al., 2008). These products allow the control of northern jointvetch in rice plantings by inducing anthracnose lesions on the stem, leading eventually to the death of the plant. Although the specificity of this reaction is helpful for EPA registration, it does limit the usefulness of this bioherbicide due to the lack of control of other weed species in the same field situation. Recently, Boyette et al. (2010) found that by changing the formulation of the emulsion in which the fungus is applied, the species host range of the fungus can be increased. This formulation change...
allowed the related and economically important weed, hemp sesbania [Sesbania exaltata (Raf.) Rydb. ex A. W. Hill] to also be controlled by this biocontrol agent.

Hemp sesbania, an invasive leguminous weed that grows among soybean, cotton, and rice, is capable of reaching heights of 3 m at maturity (Lorenzi and Jeffery, 1987). It has been ranked as one of the 10 most troublesome weeds in several southern U. S. states (Dowler, 1992) and is a prolific seed producer, yielding up to 21 000 seeds per plant (Lovelace and Oliver, 2000). Populations of 0.8–12.9 hemp sesbania plants m⁻² emerging with soybean reduced soybean yield by 80% when allowed to interfere throughout the growing season (McWhorter and Anderson, 1979). Hemp sesbania also contains compounds that are toxic to livestock and humans (Everest et al., 2010). Thus, successful control of hemp sesbania is not only important for the success of the crop but also for food safety.

The biochemical and physiological interactions of a fungal pathogen with its host plant is complex and in many instances not fully understood. Generally, disease caused by the fungus results in the alteration of the host by weakening and breaching cell walls, in the invasion of the protoplast, and often, in the death of the cells (Cantu et al., 2008; Reignault et al., 2008). In some cases, the plant also resists the invasion by producing phytoalexins and by erecting physical barriers to the fungus, or it may increase the chance for invasion when physiological processes such as fruit ripening weaken the walls (Wattad et al., 1997; Miyara et al., 2008). In many cases, these defense actions also alter plant cell wall structure. For example, one of the plant defenses often noted after a successful fungal attack is the production of appositions (small outgrowths of the host cell wall) or more extensive corky layers, which impede or block the growth of the fungus (Cantu et al., 2008). Alternately, the plant can produce inhibitors of fungal enzymes that degrade host cell walls, such as pectin methyl esterase inhibitor, to suppress or slow down the rate of infection (Lionetti et al., 2007).

Unfortunately, studying these alterations in cell walls is difficult because these changes often occur in a very small area of tissue and within a bulk of host tissue that is unchanged, limiting the usefulness of standard biochemical studies of these walls. However, plant pathogenic processes are especially amenable to immunocytochemical and cytochemical protocols. For example, using cytochemical and immunocytochemical protocols, appositions have been shown to be composed of callose (e.g., Mueller et al., 1994; Hardham and Mitchell, 1998), a polysaccharide generally limited to cell plates and plasmodesmata in the uninfected plant cell (e.g., Vaughn, 2003). Fungi have a broad array of enzymes capable of wall degradation, including cellulases and pectinases (Wattad et al., 1997; Drori et al., 2003; Wijesundera et al., 1989). Cytochemical and immunocytochemical probes have been used to detect these enzymes and some of the cell wall modifications (e.g., Tenberge et al., 1996; Hardham and Mitchell, 1998; Ouelette et al., 1999; Herbert et al., 2004) that are induced by fungal invasions.

Many of these immunocytochemical studies were undertaken before the present variety of immunocytochemical probes became available to monitor the composition of the cell walls. Now, a complete array of probes to detect homogalacturonans of various esterification states, rhamnogalacturonans and side chains, arabinogalactan proteins, ligin, xyloglucans, xylans, mannos, and arabinins (Knox, 2008; Bowling and Vaughn, 2008a; Bowling et al., 2008) allows a rather complete analysis of the cell wall components and their position within the walls and within tissues than was previously unobtainable.

In this study, we take advantage of the increased variety of immunocytochemical probes for polysaccharide epitopes of cell walls to investigate wall modifications during the invasion of Colletotrichum gloeosporioides into stems of the pernicious weed hemp sesbania. Our results demonstrate the extent of and identify some of the polysaccharides involved in alterations to the plant cell walls and the barrier tissue produced by the host plant to inhibit or contain the fungus during pathogenesis. The high specificity of these reagents has allowed us to determine several novel facets of the invasion process and the plant response to this invasion.

**MATERIALS AND METHODS**

*Accesion, culture, and maintenance of the fungus—The Colletotrichum gloeosporioides culture used in the present studies was obtained from K. Cartwright (Agricultural Research Initiatives, Fayetteville, Arkansas, USA) and is the one used in the commercial formulation Lockdown (Agricultural Research Initiatives, Fayetteville, Arkansas, USA). The fungus was maintained on Emerson’s YPsS agar (Difco, Detroit, Michigan, USA) at 28°C on open-mesh wire shelves in an incubator. Twelve-hour photoperiods were provided by two 20W, cool-white fluorescent lamps positioned in the incubator door. Light intensity at dish level was approximately 200 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) as measured with a light meter (LI-185B, LI-COR, Lincoln, Nebraska). Cultures were maintained by transferring to Emerson’s YPsS agar every 5–7 d. Inoculum for all experiments was produced in liquid culture in modified Richards’ medium containing V-8 vegetable juice (Campbell Soup, Camden, New Jersey, USA) (Daniel et al., 1973), either in Erlenmeyer flasks on a rotary shaker at 125 RPM and 28°C or in laboratory fermenters (Models 214-E and 230-C; New Brunswick Scientific, Talmadge, New Jersey, USA) under similar temperature and agitation regimes.*

*Greenhouse experiments—Hemp sesbania seeds (Azlin Seed Co., Leland, Mississippi, USA) were surface-sterilized in 0.05% (v/v) NaOCl for 5 min, rinsed with sterile distilled water, and germinated on moistened filter paper. After the seeds germinated (~48 h), they were planted in a commercial potting mix (Jiffy-mix; Jiffy Products, Batavia, Illinois, USA) contained in peat strips. Each strip contained 12 plants. The potting mix was supplemented with a controlled-release (14:14:14, NPK) fertilizer (Osmocote; Grace Sierra Horticultural Products, Milpitas, California, USA). The plants were placed in subirrigated trays that were mounted on greenhouse benches. Greenhouse temperatures ranged from 25 to 30°C with 40–90% relative humidity (RH) and a photoperiod of approximately 14 h.

The treatments were made as a spore suspension/invert emulsion. The composition of the invert emulsion was identical to that used previously to investigate control of hemp sesbania with the bioherbicial fungus Colletotrichum truncatum (Schw.) Andrus & Moore (Boyette et al., 1993). The oil phase of the invert emulsion consisted of a paraffin oil (Orchex 797; Exxon Corp., Baytown, Texas, USA) (777.5 g L⁻¹), a monoglyceride emulsifier (Myerol 18-99; Eastman Chemical, Kingsport, Tennessee, USA) (14.5 g L⁻¹), household paraffin wax (Strohmeyer & Arpe, Short Hills, New Jersey, USA) (74.25 g L⁻¹), and lanolin (93 g L⁻¹). A stable invert emulsion was formed when equal parts of the oil phase and water phase were combined and stirred briskly by hand for 2–3 min. Spore densities for all treatments with the fungal component were adjusted to 5.0 × 10⁸ spores mL⁻¹ with the aid of a hemacytometer. Spray application rates were ~100 L ha⁻¹ and were made with a pressurized backpack sprayer (Spray doc, Model 101P; Glimour Mfg., Somerset, Pennsylvania, USA).*

*Light microscopy and immunocytochemistry—Nine days after inoculation with the biocontrol agent, small tissue pieces of sesbania stems, both with and without lesions, were fixed in 3% (v/v) glutaraldehyde in 0.05 mol L⁻¹ PIPES buffer (pH 7.4) for 2 h at room temperature and then washed twice (15 min each) in cold PIPES buffer before dehydration in an ethanol series (25% (v/v), 50%, 75%, 2 h each). After reaching 100% ethanol, the samples were transferred to −20°C overnight. LR White (Polysciences, Warrington, Pennsylvania, USA) resin was added in 25% steps for 24 h per step. After the samples had remained in 100% resin at −20°C for 24 h, they were warmed to room temperature, and then aged on a rocking shaker for 24 h. Samples were then transferred*
to flat-bottomed polyethylene capsules for polymerization at 55°C for 2 h as described by Bowling and Vaughn (2008b).

Samples were cut out of the blocks and reoriented by mounting on acrylic stubs so as to produce stem cross-sections. Sections were cut with a Delaware Histoknife on a Reichert Ultracut Ultramicrotome set at 0.35 µm and dried onto chrome-alum-coated slides on a slide-warming tray. Sections for light microscopic examination were stained for 10–30 s with 1% (w/v) toluidine blue in 1% (w/v) sodium borate on the warming tray, washed with distilled water, mounted with Permount, and observed with a Zeiss photomicroscope. Images were collected using an Olympus Q-COLOR 3 digital camera.

For immunocytochemistry using the monoclonal antibodies, small circles (1.25–2.5 cm in diameter) were drawn with a wax pencil on the cleaned and chrome-alum-coated slides, sections were collected in that circle and allowed to dry down on a slide-warming table. Slides were placed in a humid chamber and 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.2 (PBS-BSA) was added to the wax pencil circle, and the sections were incubated for 30 min. The PBS-BSA was decanted and primary antibody (Plant Probes, Leeds, UK; Complex Carbohydrates Research Center, Athens, Georgia, USA; Table 1) from neat to 1:20 dilution in PBS-BSA (depending upon the antibody) was added to each circle (a volume of ~100 µL), with controls consisting of a nonreactive serum or PBS-BSA alone. Slides were incubated in the primary antibody for 3–4 h. After the incubation, slides were washed 3–4 times with PBS-BSA. Secondary antibody (goat antirat IgG or goat antimouse IgG) coupled to 15 nm colloidal gold (EY Laboratories, San Mateo, CA) was diluted 1:20 in PBS-BSA and the slides were incubated for 1 h. After that incubation, the slides were washed twice in double-distilled water from a squirt bottle and silver-enhanced (IntenSE, Amersham or GE Biochemicals) for 15–30 min. The sections were washed with distilled water, dried with compressed air, and mounted with Permount before observation with a Zeiss photomicroscope. Images were collected using an Olympus Q-Color 3 digital camera.

Fig. 1. Disease symptoms on hemp sesbania and colonization of tissue after inoculation with Colletotrichum gloeosporioides. (A) At 6–9 d postinoculation, large necrotic lesions had formed on the surface of the stem. In many cases, the degradation of host tissue had progressed to such a degree that cracks had begun to form in the epidermis of the stem (between arrowheads). Scale bar = 1 mm. (B) Lesion similar to that shown in (A), imaged by SEM and presented as a three-dimensional (3-D) anaglyph to more accurately convey the 3-D nature of the lesion (should be viewed with red/cyan anaglyph glasses). Scale bar = 500 µm. (C) Cross section of resin-embedded stem bearing a large anthracnose lesion (region between arrowheads), which is populated with the remnants of collapsed cell walls. The lesion extends most of the way through the stem cortex to within a few cells of the peripheral fiber bundles (f). The limit of colonization of the fungal lesion is indicated by arrows. Scale bar = 100 µm.
In the case of the 2F4 antibody, Tris-buffered saline at pH 8.3 was used for all
the antibody incubation steps as this monoclonal antibody does not react at
pH 7.4 as do the other antibodies.

For the lignin antisera, a slightly different protocol was used for the incuba-
tion of the slides. The slides were incubated as follows: 3% (w/v) BSA in PBS
for 30 min; the primary antiserum diluted 1:200 in 1% (w/v) BSA in PBS, 3 h;
three exchanges of 1% PBS-BSA (w/v) over 10 min, Protein A coupled to 15 nm
gold particles (EY Laboratories, San Mateo, California, USA) diluted 1:20 in
PBS-BSA, 1 h; samples washed thoroughly with a wash bottle of double dis-
tilled water. Incubation and processing through silver intensification are as
described for the monoclonal antibodies.

Some samples were examined directly in an unfixed condition with an
Olympus stereomicroscope, and images were collected with an Olympus Q-
Color3 digital camera.

Scanning electron microscopy—for scanning electron microscopy, small
pieces were fixed in 6% (v/v) glutaraldehyde in 0.05 mol/L PIPES buffer
(pH 7.4) for 24 h at 4°C. Samples were washed in PIPES buffer and dehydrated
in ethanol as described for light microscopy samples, except that the ethanol
steps were done at 4°C. They were then critical-point dried in a Bal-tec CPD
030 critical-point drying apparatus (Leica Microsystems, Wetzlar, Germany)
and sputter coated with 15 nm of gold-palladium in a Hummer X coater (Ladd
Research, Williston, Vermont, USA). Samples were observed with a JEOL
JSM-840 scanning electron microscope operating at 15 kV. Digital images
were collected with a 4-Pi (Durham, North Carolina, USA) digital acquisition
program. For anaglyph production, stem sections were oriented parallel to the
stage tilt axis and a pair of images was collected with the stage tilted at ±5 and
±5 degrees.

RESULTS

Tissue morphology—Stems of hemp sesbania, by 6–9 d after
inoculation with the biocontrol agent [Colletotrichum gloeo-
sporioides (Penz.) Penz & Sacc.], produced large necrotic le-
sions immediately adjacent to tissue areas that had little or no
apparent damage (Fig. 1A). When these samples were viewed
with SEM with anaglyph production, the three-dimensional
aspects of the lesions are more readily obvious (Fig. 1B). The
biocontrol agent caused a deep lesion in the hemp sesbania
stem, with only remnants of collapsed cells present in the zone
of the lesion. Several cell layers are thus severely damaged after
treatment with the biocontrol agent.

The stems of hemp sesbania are typical of eudicots, with vas-
cular bundles arranged around the periphery of the stem, sepa-
rated from the epidermis by a region of 4–6 cortical cells that
contain chloroplasts. These latter cells with chloroplasts confer
the light green color to the hemp sesbania stem. Central to the
sesbania stem are the large, swollen, thin-walled cells of the pith.
Several changes to this basic anatomy of the hemp sesbania
stem occurred as the result of treatment with the biocontrol
reagent with the new formulation. At the site of the fungal inva-
sion, a zone of collapsed cells (resembling the cell wall stacking
zone observed in hypersensitive responses) in both the epider-
mis and cortical layers was noted (Fig. 1C). Immediately adja-
cent to the lesion, the tissue appeared to be morphologically
unaffected. At points beneath the site of fungal invasion, cells
in the vascular bundles and the parenchyma surrounding the
bundles appeared to have increased in number, and small cells,
resulting from recent divisions, were present. The vascular tis-
uce and the pith cells appear to be relatively unaffected at least
at this stage of infection.

Serial sections to those shown in Fig. 1C were probed with a
battery of antibodies (Table 1). The pattern of reaction of the
various antibodies with this tissue fell into three broad cate-
grories: those showing signs of cell wall degradation by the fun-
gus, those binding to a defensive cork layer formed by the plant,
and those antibodies whose location and intensity in the plant
wall appeared similar between infected and uninfected tissue.

Cell wall degradation—Degradation of specific cell wall
polysaccharides was detected in a region just flank-
ing the primary infection site, which occupied the length of ~10 epidermal
cells and the cortical cells beneath this region in thin sections.
Homogalacturonan (HG), with a wide range of methyl-esterifi-
cation states was degraded by the fungus, as evidenced by the
loss of binding of both JIM5- and JIM7-reactivity in this flank-
ing region of 10–15 epidermal cells and subtending cortical
cells (Fig. 2A, B). Two regions exist in this invasion zone: (1)
the region in the center of the lesion shows nearly total cellular
collapse but no change in the HGs (areas marked by “l”), and
(2) in the region farther away, some cells apparently intact, yet
unlabeled by some of the HG antibodies (areas marked by as-
terisks). This same pattern of HG distribution was observed in
sections probed with LM19 and LM20 antibodies as well (not
shown), as would be expected, because these antibodies have
similar reactivities to JIM5 and JIM7, respectively. Further-
more, several rhamnogalacturonan I (RGI)-reactive epitopes
were also degraded by the biocontrol fungus. Both the 1→4 galactan
and 1→5 arabinan side chains of RGI (recognized by LM5- and LM6-reactive antibodies, respectively) were ab-
sent/weaker in the same regions (Fig. 2C, D), as was the RGI
epitope recognized by CCRC-M2 (Fig. 2E).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Antibody</th>
<th>Antigen/epitope</th>
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<tbody>
<tr>
<td>Reduced in necrotrophic</td>
<td>JIM5</td>
<td>Partially de-esterified/unesterified HG</td>
</tr>
<tr>
<td>region</td>
<td>JIM7</td>
<td>Partially methyl-esterified HG</td>
</tr>
<tr>
<td>LM5</td>
<td>(1→4) β-D-galactan</td>
<td></td>
</tr>
<tr>
<td>LM6</td>
<td>(1→5) α-L-arabinan</td>
<td></td>
</tr>
<tr>
<td>LM7</td>
<td>Partially de-esterified HG (nonblockwise)</td>
<td></td>
</tr>
<tr>
<td>CCRC-M2</td>
<td>RGI</td>
<td></td>
</tr>
<tr>
<td>LM19</td>
<td>Highly de-esterified HG</td>
<td></td>
</tr>
<tr>
<td>Enriched in defense</td>
<td>JIM8</td>
<td>AGP</td>
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<tr>
<td>layer</td>
<td>JIM13</td>
<td>AGP</td>
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<td>JIM14</td>
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<td>JIM19</td>
<td>Extensin</td>
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<td>JIM20</td>
<td>Extensin</td>
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<tr>
<td>LM1</td>
<td>Extensin</td>
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<td>LM15</td>
<td>Xyloglucan</td>
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<tr>
<td>CCRC-M1</td>
<td>α-L-fucosylated xyloglucan</td>
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<tr>
<td>CCRC-M7</td>
<td>arabinosylated (1,6) β-D-galactan</td>
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<tr>
<td>CCRC-M10</td>
<td>RGI</td>
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<td>CCRC-M22</td>
<td>De-arabinosylated RGI</td>
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<tr>
<td>CCRC-M34</td>
<td>Arabidopsis seed mucilage, HGs</td>
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<tr>
<td>CCRC-M88</td>
<td>Xyloglucan</td>
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<tr>
<td>CCRC-M96</td>
<td>Xyloglucan</td>
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</tr>
<tr>
<td>CCRC-M108</td>
<td>Xyloglucan</td>
<td></td>
</tr>
<tr>
<td>Unaltered</td>
<td>LM2</td>
<td>AGP</td>
</tr>
<tr>
<td>LM10</td>
<td>(1→4) β-D-xylan</td>
<td></td>
</tr>
<tr>
<td>LM11</td>
<td>(1→5)-β-D-xylan/arabinoxylan</td>
<td></td>
</tr>
<tr>
<td>CCRC-M36</td>
<td>Debranched RGI</td>
<td></td>
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<tr>
<td>CCRC-M38</td>
<td>HG with a range of esterification patterns</td>
<td></td>
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<tr>
<td>2F4</td>
<td>Calcium-bridged HG</td>
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<tr>
<td>Lignin antisera (3)</td>
<td>Recognizes S, S-G, and G lignins</td>
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Note: Epitope information from the PlantProbes and CarboSource websites
and W. G. T. Willats, University of Copenhagen, Denmark; unpublished data.
CCRC-M2-depleted wall material appeared to be slightly more restricted than the areas that were labeled with JIM5, JIM7, LM5, and LM6 based upon comparisons of nonconsecutive serial sections. The CCRC-M2-extracted area appeared to coincide only with the area of collapsed cells and not to extend into the region of intact cells. These data might indicate that side chains of the RGI (such as those recognized by LM5, LM6 and possibly JIM7) are lost before the RGI backbone epitope, recognized by the CCRC-M2 antibody.

Labeling with the anti-xylolglucan antibodies LM15, CCRC-M1, CCRC-M88, CCRC-M96, and CCRC-M108 all increased at the same sites where the antibodies to HGs such as JIM5 and JIM7 had lowered in reactivity (compare Fig. 2 with the third and fourth rows of Fig. 3 for CCRC-M1 and LM15). Although this increase in signal could indicate that there is a higher xyloglucan content in these regions, we feel that these increases in antibody labeling represent unmasking of xyloglucan epitopes by the loss of the coating HG molecules because we can observe similar increases in xyloglucan antibody labeling by treating the sections with pectinases before treatment (not shown) and the increased labeling exactly matches in these areas with the loss of HG. For example, the stronger reactions of LM15 and CCRC-M1 at the left side of the panel in the “adjacent” zone exactly match those areas lacking JIM5 and JIM7 label in serial sections.

Cork layer formation—The hemp sesbania plant also responds to the presence of the fungus with wall modifications of its own. A layer of cells was observed to form between the primary infection site and the plant’s fiber bundles and vascular system, as indicated by their small size and greater number in comparison with unaffected areas of hemp sesbania stems (Fig. 3, “lesion” panel). The walls of these cells have a different composition than normal cells of the cortex that may be observed in areas of stem away from the fungal invasion (Fig. 3, “opposite” panel). Four classes of antibodies reacted in the area where new defense reactions were being induced: those reactive with extensin (LM1, JIM19, and JIM20; not shown), those reactive with AGPs (JIM8 and JIM13, Fig. 3; JIM14 and CCRC-M7, not shown), xyloglucan (CCRC-M1 and LM15, Fig. 3), and RGI (CCRC-M22, not shown). The only exception is the anti-AGP epitope recognized by LM2 (not shown), which does not appear to be increased in this area relative to the unaffected areas of stem.

The use of these particular epitopes by the plant in an attempt to prevent further movement of the fungus through the tissue is, perhaps, not surprising. Extensins are often deposited at wound sites, and arabinogalactan proteins (AGPs) are general markers for new cell wall biosynthesis. RGI and xyloglucans are normal constituents of primary wall and would be expected to be predominant in newly formed cells.

Epitopes with no salient changes despite infection—Despite the major alterations in cell walls of the hemp sesbania stems caused by the biocontrol agent, some of the polysaccharide epitopes were apparently unchanged through the course of the infection (Fig. 4). These include the xylan epitopes recognized by the LM10 and LM11 antibodies, LM2 as already mentioned, and the HG antibodies 2F4, CCRC-M34, and CCRC-M38. In the case of the xylan antibodies, the lack of change may reflect the lack of colonization of the fungus into areas of tissue where the xylans are more abundant. In the case of CCRC-M38, the HG epitopes that are recognized include both highly esterified and completely de-esterified (W. Willats, University of Copenhagen, Denmark; personal communication), so that if one epitope was modified it may still be recognized by the CCRC-M38 antibody. Only absence or destruction of all of the HGs would be recorded as a loss of reactivity, whereas the other antibodies are more restricted in the ranges of esterification states of HG epitopes that they recognize. The monoclonal antibody LM18 seems to react similarly to CCRC-M38. The 2F4 antibody only recognizes Cu²⁺-bridged HG epitopes, and these bridged HGs might be more resistant to fungal invasion than unbridged epitopes.

Oddly, none of the lignin antibodies during host–pathogen interactions reacted differently with the infected tissues compared to the uninfected, even though lignification is often a defense strategy of the infected host. The label with G lignin-specific antiserum (not shown) reacted mainly with the cork tissue and vascular tissue, whereas the S and G-S antisera reacted more evenly across the tissue (not shown), although there was stronger reaction in thickened cells such as those in the vascular tissue similar to the reaction to the xylans recognized by LM10 and 11.

DISCUSSION

Enhancing the range of biocontrol agents—Although narrow or restricted host specificity of a bioherbicidal fungus may be beneficial from biological and US-EPA registration perspectives, this trait may impede the use of these bioherbicidal agents from practical and commercial viewpoints (TeBeest, 1991). A chemical herbicide or bioherbicide that can control several weeds has greater economic potential than a product that can only control a single weed. Furthermore, only in rare instances does a weed control problem for a grower involve a single weed. A more complete understanding of the biochemical and ultrastructural events of the host–pathogen interactions that are related to disease promotion and infection processes may provide insight useful in the development of bioherbicides with improved traits, including an expanded host range and higher virulence for optimized weed control. Caution must be applied, however, when the host range of a bioherbicide is modified or expanded to provide weed control of multiple species because closely related desirable plant species may also become infected. These desirable plants will need to be assessed to ensure that host range expansion does not injure nontarget species. The data in this study address some of these aspects of expanding the range of a biocontrol agent that originally controlled a single weed species, but now, with modifications in application conditions, may control more.

Degradation of cell walls by necrotrophic hyphae—For the fungus to successfully enter the host cytoplasm, the wall must first be breached. Because of the relatively small pore size of the host wall, many enzymes would have no access to the wall itself, but relatively small fungal pectinases would be able to start this process of wall degeneration. Because pectins control the porosity of the cell wall (Baron-Epel et al., 1988), the loss of pectins would increase the ability of other degradative enzymes to reach other components of the wall and ultimately the protoplast of the host. Likewise, the pectins present in the middle lamellae (mostly highly de-esterified HGs that are recognized by JIM5 or LM19) would be the easiest targets, once the epidermal layers are breached.
The data in this report on the *Colletotrichum* invasion of hemp sesbania stems add a few more details to this general scheme of fungal invasion. First, there appears to be a loss of not only de-esterified HGs (JIM5-reactive) that are enriched in the middle lamellae, but also a loss of the more highly esterified HGs that are found in the wall per se (JIM7- and LM20-reactive) as well as side chains of RGI (LM5 and LM6) and the RGI molecule itself (CCRC-M2). In contrast, the xyloglucan epitopes recognized by LM15, CCRC-M1, CCRC-M88, CCRC-M96, Homogalacturonan-rich pectins seem to be a primary target of fungal attack, and their degradation releases signaling molecules that the plant senses to respond to the fungus (Cantu et al., 2008; Reignault et al., 2008). Although it is difficult to conceive that the loss of just one cell wall component can be so dramatic and cause the extensive lesions seen on the sesbania stems in this study, even just the addition of purified fungal polygalacturonases can cause necrotic lesions on plant stems (Kars et al., 2005).

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Fig. 4. Examples of immunoreactions where relatively little change was observed between the cell remnants within the lesion itself, the cells that are formed in response to the infection at the bottom of the lesion, and the normal, noninfected cells. (Left: A, C, E) Lesion site. (Right: B, D, F) Area on the stem opposite the lesion. These antibodies react to homogalacturonans of varied esterification states (CCRC-M38), xylan (LM10), or RGI (CCRC-M10). l, lesion; e, epidermis; f, fibers; x, xylem; c, cortex. Scale bar = 100 µm.
and CCRC-M108 appear to have increased in the same areas where the pectins are lost. However, treatment of sections from this and other material with polygalacturonases often results in the enhanced detection of xyloglucan epitopes by xyloglucan antibodies (Marcus et al., 2008; Hervé et al., 2009; A. J. Bowling and K. C. Vaughn, unpublished data); this process is known as demasking of epitopes. Pectins coat other molecules and then render the coated molecule inaccessible to the antibody. Thus, the apparent increase in xyloglucan is likely not the result of any xyloglucan increase, but rather an increase in the ability of the antibodies to gain access to the xyloglucan that were previously coated with pectins. This phenomenon is especially obvious in the low magnification micrographs of whole-stem cross sections, in which the label with xyloglucan antibodies is much more apparent in the infection site in regions that had reduced HG content. Fungal pectinases in vivo thus can cause the same sorts of demasking as exogenous pectinases applied to sections (Marcus et al., 2008). These results could still be interpreted as the plant increasing the amount of xyloglucan in response to the fungus, but we believe demasking to be a more likely scenario, considering the loss of the masking pectin epitopes in the very regions where enhanced xyloglucan label was noted. Further, it is less likely that the plant would produce much more of one and much less of another primary wall constituent in the same tissues, especially in tissues approaching death from an invading fungus.

Despite the loss of so much HG at the periphery of the lesion, the tissue at the center of the lesion labels strongly with these same antibodies. Although this is confusing and seemingly contradictory at first, knowledge of the growth habits of Colletotrichum species sheds some light as to how this pattern is produced. Many Colletotrichum species, including the one in this study, are described as hemibiotrophic (Perfect et al., 1999; Goodwin, 2001; Dieguez-Uribeondo et al., 2005; Munch et al., 2008). The initial stages of infection are termed biotrophic because they do not result in the immediate death of the host cell, but later “necrotrophic” secondary hyphae develop and spread within the host tissue, and eventually the host cells die. At this stage, the anthracnose lesion becomes prominent (Fig. 1A). A polygalacturonase is excreted by Colletotrichum spp. only in the necrotrophic phase (Goodwin, 2001; Herbert et al., 2004), and loss of JIM5 reactivity was noted in the host cells associated with the necrotrophic phase but not the biotrophic. Thus, the biphasic lifestyles of the fungi are clearly demarcated on the sesbania stem in the retention of pectins during the initial biotrophic stage at the center of the lesion and in their loss in the more peripheral areas of the lesions characterized by necrotrophic hyphae. This increase of polygalacturonase in the necrotrophic phase is echoed by the loss of HGs in the areas where necrotrophic hyphae have advanced but not in the zone of the initial biotrophic stages. There is notable cellular collapse in the cells at the center of the lesion, and some of the apparent lack of loss of polysaccharide epitopes could be related to the greater density of these collapsed walls as well. Even there though, there is loss of some RGI residues (recognized by CCRC-M2) but not HG residues. That data indicate that it is more likely a lack of loss of HG components in the biotrophic zone rather than an enhanced density of collapsed cells. The relatively normal invasion pattern of the biocontrol Colletotrichum indicates that the culture conditions and application technology of this biocontrol fungus have overcome its inability to attack hemp sesbania. A simplified diagram of an anthracnose lesion on a hemp sesbania stem is shown in Fig. 5.

**Plant defense**—Pectins are also critical molecules in the plant defense. As HGs are broken down by fungal polygalacturonases, pectic fragments called oligosaccharins are produced. Oligosaccharins serve as elicitors of the defense pathways in the plants (e.g., Fry et al., 1993; Cote and Hahn, 1994), and it is clear that many such oligosaccharins could be produced in the large wound site produced by Colletotrichum in the hemp sesbania stem, as evidenced by the loss of HG reactions in this area, although we have not measured them directly in this study. These molecules presumably could induce the production of new cell wall synthesis in the cork layer between the cortex and vascular tissue. A conspicuous cork layer forms between the primary lesion and the vascular tissue of the hemp sesbania plant. This layer was labeled strongly with antibodies that recognize AGP (JIM8-, JIM13-, JIM14-reactive), xyloglucan (both LM15- and CCRC-M1-reactive), and extensin (LM1-, JIM18- and JIM19-reactive). AGPs are often early indicators of new wall biosynthesis (e.g., Vaughn et al., 2007), and extensins are often found in tissue that is stiffened and ready for defense. So the presence of these epitopes at high concentration in the area of cork cell production is not surprising.

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**Fig. 5.** Diagram of cross section of hemp sesbania stem bearing an anthracnose lesion caused by Colletotrichum gloeosporiodes. The fungus enters the stem in the area labeled Biotrophic. This phase of pathogenesis can be identified by the relatively low extraction/degradation of cell wall polysaccharides seen in the second, necrotrophic phase. The region of the stem with necrotrophic-phase hyphae is clearly identifiable by the loss of certain species of HG and RGI side chains (Fig. 2; Table 1). The plant forms a defense layer between the invading hyphae and the fiber bundles and the internal vasculature of the plant stem.
We were surprised, however, that there was no increased reactivity with three different lignin antisera in the wound site because lignins are often molecules that increase during plant defense strategies (Vance et al., 1980), and lignolytic enzymes from fungi are well documented (e.g., Joselau et al., 1994). However, elicitor-induced lignin is structurally and compositionally distinct from the lignins associated with thickened cell walls (Lange et al., 1995), which are recognized by the antisera used in this study. Moreover, fungal degradation of lignins was probably not observed because the lignified tissue was well beneath the lesion site in the vascular bundles, distant from sites of fungal colonization.

Conclusions—The invasion of Colletotrichum gloeosporioides into hemp sesbania stems is accompanied by a number of changes to the polysaccharide composition of the wound site. Pectins, especially homogalacturonans, are lost with high consistency from the zone of the invasion marked by the necrotroph phase of the fungal growth cycle. No loss was noted in the biotrophic phase (center of the lesion). The loss of the pectins from the wall increases the pore size of the remaining wall, allowing for larger molecules to enter the wall space, with eventual breaching of the plant cell wall. The plant responds to the presence of the wounds by producing a cork layer that may inhibit the spread of the fungus into the vascular tissue and pith. These changes involve the production of new cell walls enriched in extensin that may inhibit the fungus’s ability to colonize tissue.

LITERATURE CITED


