Production of Plant Cell Wall Degrading Enzymes by *Phoma medicaginis* f. sp. *pinodella*

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ABSTRACT

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Infection of Austrian winter peas (*Pisum sativum* var. *arvense*) by *Phoma medicaginis* f. sp. *pinodella* resulted in necrosis and stem girdling. Extracts prepared from diseased pea stems contained enzymes that degraded xylan, CM-cellulose, pectin, galactan, and arabinogalactan, as well as exoglycosidases specific for hydrolysis of α-linked galactose and β-linked xylose and glucose. Extracts of healthy stem tissues contained lower levels, if any, of these enzymes. During culture of *P. medicaginis* f. sp. *pinodella* on isolated pea leaf and stem cell walls, enzymes similar to those detected in diseased pea tissues were secreted sequentially. Identical patterns of enzyme production were obtained with cultures grown on cell walls isolated from young or old stems. In contrast, inoculation studies with the fungus showed colonization of young stems only.

Austrian winter peas (*Pisum sativum* L. var. *arvense*) grown in the Willamette Valley of western Oregon displayed severe symptoms consisting of blackened lesions on stems and leaves. Complete stem girdling usually occurred (M. L. Powelson, *unpublished*). Inoculation of Austrian winter peas with the causal agent, *Phoma medicaginis* f. sp. *pinodella*, produced lesions on young stem tissue but only small necrotic flecks on old stem tissue.

The occurrence of stem girdling suggested that plant cell wall degrading enzymes may function in the disease process. It also seemed possible that the resistance of old stems might be a result of the pathogen's inability to degrade the plant cell walls. Indeed, resistance in aged bean stems to *Rhizoctonia solani* Kühn was previously correlated with an inability of the pathogen to degrade these plant cell walls (4). Consequently, a study of hydrolytic enzymes in diseased plant tissue and in cultures of *P. medicaginis* f. sp. *pinodella* grown on isolated pea cell walls was initiated.

MATERIALS AND METHODS

Cultures. A culture of *P. medicaginis* f. sp. *pinodella* isolated from diseased Austrian winter peas was maintained on malt agar medium and spores were obtained by exposure to near ultraviolet light for 4 days at 24 C.

To determine the production of plant cell wall degrading enzymes, the pathogen was grown in liquid culture with isolated pea cell walls as the carbon source. The cell walls were isolated from pea leaves and from the basal (old tissue) and terminal (young tissue) portions of 24–36 day old pea plants (7,10). The cell walls were added at 1% (w/v) to a basal medium as previously described (7); 10-ml quantities of this medium were inoculated with 10^4 fungal spores and incubated at 24 C. At daily intervals, one culture was filtered through a coarse sintered glass funnel and the filtrate was stored at 4 C. After 9 days the mass of material retained by the filter was collected so that its neutral sugar composition and undegraded plant cell walls could be compared. The material was washed sequentially with 100 ml of 0.5 M NaCl, 200 ml of water, and 100 ml of acetone and then allowed to air dry.

Assay of hemicellulose fractions of plant cell wall material. Neutral sugar analyses were performed on isolated old and young pea cell walls and the wall residues isolated from the *Phoma* cultures. Samples (0.1 g) of the pea cell walls were hydrolyzed in 2 N trifluoroacetic acid for 1 hr at 121 C to yield neutral sugars that were quantitatively analyzed by a gas-liquid chromatographic technique described previously (9).

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Inoculation of pea tissues. Austrian winter peas were surface sterilized in 10% (v/v) commercial bleach for 3 min, planted in vermiculite, and placed in a growth room maintained at 24 C with a 10-hr dark and 14-hr light regime. Intact 12 day old pea plants were sprayed with a suspension of 5 day old spores (10^4/ml) and incubated at 24 C to permit symptoms to develop. Stem sections, inoculated by the same procedure, were obtained from the fifth to seventh internodes (young tissue) and from the first to third internodes (old tissues) of 24–36 day old plants. The inoculated stem sections were incubated on sterile moistened perlite at 24 C to allow symptoms to develop.

Preparation of enzyme-containing extracts. Extracts of diseased and healthy plant stems were prepared by homogenizing 30 g of plant tissue in 60 ml of water for 1 min at 4 C in a Waring Blendor. The homogenate was immediately mixed with 3 g of polyvinylpolypyrrolidone (Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178), and the slurry was stirred at 4 C for 5 min before being filtered through Whatman GF/A paper (Whatman Incorporated, Clifton, NJ 07014). The filtrate was centrifuged at 15,000 *g* for 10 min and the supernatant was dialyzed at 10 C in 20 L water for 12 hr. The dialyzed extract was stored at 4 C.

Assay of plant cell wall degrading enzymes. Filtrates from plant cell wall grown cultures and extracts from diseased and healthy pea stems were assayed for exoglycosidases and endoglycanases. Exoglycosidases were assayed by measuring the release of *p*-nitrophenol from the *p*-nitrophenyl derivatives of α-linked glucose and galactose and β-linked glucose and xylose (10). All these derivatives were purchased from Sigma Chemcial Company. Endoglycanase assay involved measurement of the increase in reducing groups as the following polysaccharides were hydrolyzed (10): 0.1% solutions in 50 mM of sodium acetate pH 5.2 of CM-cellulose (Hercules Incorporated, Wilmington, DE 19899), xylan (Sigma), pectin (Sigma), mannan (Sigma), laminarin (ICN Life Science Group, Cleveland, OH 44128), arabinogalactan, and galactan. Arabinogalactan and galactan were prepared by the methods of Labavitch et al (11).

Effect of cell-free preparations containing cell wall degrading enzymes on pea tissue. Pea stem sections were treated with filtrates from the cultures grown on cell walls or extracts from diseased plant tissues to see if the symptoms characteristic of *Phoma* infection could be duplicated. The extracts were sterilized by passage through presterilized 0.2-μm pore Unipore membrane filters (Bio-Rad, Richmond, CA 94804). Five-centimeter sections of stems from 14-day-old plants were cut, surface sterilized by immersion in 5% (v/v) commercial bleach for 2 min, and extensively washed with sterile distilled water. Five stem sections

were placed in a sterile petri dish and 10 ml of the sterile pea extracts or fungal culture filtrates were added. Control pea tissues were treated with sterile distilled water. The stem sections were incubated at 24 C and visually examined for symptoms over a 3-day period. The stem sections then were removed and teased apart with forceps to judge the extent of tissue softening.

Assay for pectic enzyme inhibitor. Extracts of pea tissues were prepared by a modification of a procedure used to isolate an inhibitor of fungal polygalacturonases from bean hypocotyls (2). Thirty grams of stem tissue from the basal portions of 24 day old pea plants were homogenized in 60 ml of 0.5M NaCl at 4C for 1 min in a Waring Blendor. The resulting homogenate was filtered through a sintered glass funnel and centrifuged at 15,000 *g* for 10 min. The precipitate was discarded and the supernatant was dialyzed at 4 C against four changes of 12 L of 50 mM sodium acetate, pH 5.2. Samples of 0–200 μl of the dialyzed extract were added to the standard assay for measuring pectin-degrading activity.

Preparation of isolated mycelial walls. Mycelia of *P. medicaginis* f. sp. *pinodella* were grown at 24 C for 4 days in liquid malt medium with an inoculum of 10^6 spores per liter. The cultures were filtered through a sintered glass funnel and the mycelia retained by the funnel were washed extensively with water. The mycelia were homogenized for 1 min in a Waring Blendor with 50 ml of water for each gram of material, and the insoluble cell walls were pelletized by centrifugation at 10,000 *g* for 10 min. The cell wall material was homogenized four more times in water, 0.5 M NaCl, water, and acetone before being air dried.

TABLE 1. Exoglycosidases and polysaccharide degrading enzymes in extracts prepared from healthy pea stems and pea stems with necrotic symptoms caused by *Phoma medicaginis* f. sp. *pinodella*

Enzyme substrate	Units of enzyme activity[a] in extracts prepared from	
	Healthy tissue[b]	Diseased tissue[b]
p-Nitrophenyl glycoside of		
α-Glucose	1	48
β-Glucose	39	1,500
α-Galactose	10	400
β-Xylose	10	200
Polysaccharides		
CM-cellulose	0	40
Arabinogalactan	4	20
Galactan	1	50
Pectin	8	120
Xylan	5	35
Laminarin	16	82

[a]One unit of exoglycosidase is the activity in 200 μl of extract that hydrolyzed 15 μg of *p*-nitrophenyl glycoside at 30 C during a 2-hr incubation in a final reaction volume of 1 ml (10). One unit of polysaccharide degrading activity is the activity in 200 μl of extract that, when incubated for 2 hr at 30 C in a final reaction volume of 1 ml, produced an increase in reducing groups equivalent to 500 μg of glucose (10).
[b]Extracts were prepared from 30 g of diseased or healthy stem tissue excised from intact plants.

RESULTS

Extracts from stems of peas that showed severe blackened lesions had greater amounts of plant cell wall degrading enzymes than did extracts prepared from healthy tissues (Table 1). The diseased tissue extracts contained high levels of exoglycosidases and enzymes that degrade pectin, cellulose, and hemicellulose fractions of the plant cell wall.

Isolated pea cell walls from leaves and young or old stems suspended in a basal medium supported growth of *P. medicaginis* f. sp. *pinodella*. In all cultures fungal mycelium was visible within 3 days after inoculation, and sporulation occurred by day 7. Although considerable plant cell wall material remained after 9 days of incubation, compositional changes had occurred (Table 2). In general the levels of rhamnose, galactose, and arabinose decreased, whereas those of mannose and glucose increased. An increased level of xylose also was observed in the residue from cultures with cell walls from old pea stems. Neutral sugar analysis of isolated mycelial walls from cultured *P. medicaginis* f. sp. *pinodella* confirmed that, like other fungal species (3), these walls contained glucose (64%) and mannose (10%), as well as ribose (10%), arabinose (10%), and galactose (6%).

The array of enzymes produced during growth on isolated pea cell walls was similar to that in the diseased tissue extracts. Enzyme production varied with culture age, and the secretion pattern was similar in old or young stem cell walls (Tables 3 and 4). Pectinase was the first endoglycanase to reach maximum activity at 3 days after inoculation. Arabinogalactanase, xylanase, and galactanase, although present in 3 day old cultures, reached maximum activity at 4 and 5 days. CM-cellulase was detected after 6 days of culture, and no mannanase was produced. Laminarinase was produced throughout the growth period. Exoglycosidases for β-linked glucose and xylose and α-linked galactose, first detected in 3 day old cultures, were at a maximum after 6 days of growth.

Attempts to reproduce the intense blackening of the lesion areas by incubating young stem sections with culture filtrates or extracts from the diseased tissues were unsuccessful, although the stem tissues softened. No inhibition of the pectic degrading activities in either extracts of diseased pea tissue or the cell wall culture filtrates was observed with extracts from old pea stems.

DISCUSSION

The array of potential plant cell wall degrading enzymes detected in extracts of diseased pea tissue may function in lesion formation during infection of peas by *P. medicaginis* f. sp. *pinodella*. It is possible that some of these enzymes are pathogen-produced since a similar spectrum of activities was detected in filtrates from cultures of the fungus grown on isolated pea cell walls. One exception, the elevated level of laminarinase in diseased tissue, could be a

TABLE 2. Neutral sugar composition of the hemicellulose fraction of plant cell walls isolated from young and old stem tissues before and after 9 days of culture with *Phoma medicaginis* f. sp. *pinodella*

Cell wall source	% Neutral sugar[b]					
	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose
Young stems[a]						
Not inoculated	4	10	64	2	8	10
9 Day inoculated	2	3	62	8	3	20
Old stems[a]						
Not inoculated	8	24	47	3	6	8
9 Day inoculated	0	2	65	8	3	20

[a]Samples of the cell walls were assayed before and after 9 days of incubation with *P. medicaginis* f. sp. *pinodella*. The cell walls extracted from the 9 day old cultures contained fungal spores and mycelia.
[b]Expressed as percent of total neutral sugar. The results are an average of three analyses and the compositional variance is less than 3%.

consequence of altered plant metabolism (1,12).

Pectin and arabinogalactan appear to be the plant cell wall components that were preferentially degraded by *P. medicaginis* f. sp. *pinodella*. Hydrolysis of these components was demonstrated by the reduced levels of rhamnose, galactose, and arabinose in the wall residue after 9 days of culture. The structural integrity of these wall residues indicated that hydrolysis of the cellulose component was not significant. Indeed CM-cellulase was detected only late in wall-grown cultures, whereas enzymes that degraded pectin and arabinogalactan were produced rapidly and maintained (Tables 3 and 4). This early production of pectin-degrading enzymes and subsequent sequential secretion of other cell wall degrading enzymes, observed with *P. medicaginis* f. sp. *pinodella*, support similar data obtained with several other fungal pathogens cultured on isolated plant cell walls (8,10,14).

Our results on pectin degradation in the pea-*Phoma* interaction complement studies of pectic transeliminase activity in sugar beet tissue infected with *Phoma betae* (5,6). These studies (6) suggested that resistance or susceptibility of beets to *P. betae* was partially determined by a reduced ability of resistant beet cell walls to induce the pathogen's pectic transeliminase. In our study of *P. medicaginis* f. sp. *pinodella*, however, similar levels of all the assayed endoglycanases were produced whether old (resistant) or young (susceptible) pea cell walls were provided in the growth medium (Table 3). Equivalent formation of mycelia and spores in the young and old wall cultures was observed visually. The presence of this fungal material could explain the similar increase in the proportion of mannose and glucose in 9 day old residues from both old and young wall cultures. Because xylose, unlike glucose and mannose, was not detected in *Phoma* cell walls, the increase in the proportion of xylose in the 9 day old residue from the old wall cultures could indicate some reduced ability of *Phoma* to utilize xylan as pea walls age.

Another possiblity for the failure of the old pea stems to be colonized is that an inhibitor of the pectic enzyme in the pea tissue blocked wall degradation. Plant cell wall degradation by a complement of pathogen-produced enzymes was previously demonstrated to be totally inhibited by the addition of a protein, isolated from bean, that inhibited only the pectic-degrading activity (10). However, attempts to demonstrate an inhibitor in aqueous extracts from pea tissue for the *Phoma*-produced pectic-degrading enzyme were unsuccessful.

A characteristic symptom of the *Phoma*-produced lesions on pea is their intense blackening. Our attempts to reproduce the blackened symptoms on pea with *P. medicaginis* f. sp. *pinodella* culture filtrates or diseased tissue extracts were unsuccessful. Similarly, Paulson and Schoeneweiss (15) were unable to duplicate these symptoms by treating *Vinca minor* tissue with culture filtrates from *Phoma exigua* var. *exigua*. The softening of the pea sections treated with the *P. medicaginis* f. sp. *pinodella* culture filtrates was similar to other examples (13) that correlate plant tissue maceration with the activity of the pathogen's pectin-degrading enzymes.

TABLE 3. Endoglycanase production by *Phoma medicaginis* f. sp. *pinodella* grown on isolated young or old pea stem cell walls

Culture age (days)	Units of enzyme activity on:[a]						
	lam[b]	pectin	A Gal	gal	xyl	CM-cell	mann
Young stem cell walls							
1	10	0	0	0	0	0	0
2	10	19	9	26	0	0	0
3	10	23	42	26	16	0	0
4	10	24	44	53	46	0	0
5	10	24	46	50	40	0	0
6	10	16	40	50	35	13	0
9	10	16	67	52	62	11	0
Old stem cell walls							
1	10	0	0	0	0	0	0
2	10	35	9	25	0	0	0
3	10	40	32	25	21	0	0
4	10	30	36	35	40	0	0
5	10	26	60	44	42	0	0
6	10	20	40	42	46	14	0
9	10	15	60	50	41	11	0

[a]One unit of enzyme activity is the activity in 200 μl of culture filtrate under conditions described in Table 1.
[b]lam = laminarin, A Gal = arabinogalactan, gal = galactan, xyl = xylan, CM-cell = CM-cellulose, mann = mannose.

TABLE 4. Exoglycosidase production by *Phoma medicaginis* f. sp. *pinodella* grown on isolated young or old pea stem cell walls

Culture age (days)	Units of enzyme activity on *p*-nitrophenyl derivative of:[a]			
	α-Glucose	β-Glucose	α-Galactose	β-Xylose
Young stem cell walls				
1	0	0	0	0
2	0	0	0	0
3	3	20	23	5
4	8	60	40	10
5	8	41	52	10
6	9	84	81	20
9	6	75	35	13
Old stem cell walls				
1	0	0	0	0
2	0	0	0	0
3	3	34	15	4
4	6	48	22	7
5	9	74	85	9
6	15	150	100	16
9	12	100	75	20

[a]One unit of enzyme activity is the activity in 200 μl of culture filtrate that hydrolyzed 15 μg of *p*-nitrophenyl glycoside at 30 C in 2 hr.

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