Growth of Two Fungal Pathogens on Isolated Cell Wall and Polysaccharide Fractions from Tomato Fruit

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ABSTRACT

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Conidia of *Botrytis cinerea* and *Mucor mucedo* were germinated on petri dishes 15 × 100 mm or in wells 15 mm in diameter containing 1% Bacto agar amended with fruit cell walls or cell wall fractions from mature-green (30 days postpollination) and red-ripe (60 days postpollination) cultivar Rutgers tomato or its ripening-inhibited *rin* isoline. The three fractionated cell wall preparations were ionically associated pectin, covalently bound pectin, and a hemicellulosic fraction. Fungal colony expansion and spore germ tube elongation were influenced significantly by tomato cell wall substrates. Growth of *B. cinerea* colonies was stimulated by all unfractionated cell walls compared with the water or glucose controls. Conversely, expansion of *M. mucedo* colonies was better on glucose than on any of the unfractionated cell walls, and spores failed to germinate on water agar. Germ tube growth of *B. cinerea* was suppressed by red-ripe and *rin* II covalently bound pectic fractions and also by mature-green and *rin* II ionically associated pectic fractions in the presence, but not in the absence, of a nitrogen source. In the presence of nitrogen, growth of *M. mucedo* germ tubes was inhibited by all ionically associated pectic fractions and by mature-green, red-ripe, and *rin* II covalently bound pectic fractions. Hemicellulosic fractions stimulated growth of *B. cinerea* with or without nitrogen, but *M. mucedo* growth was only stimulated in the presence of nitrogen. When wall fraction concentrations were increased fivefold or 10-fold, the growth of both fungi was inhibited. Although factors affecting germination and growth of the two fungi on tomato cell walls differ, the presence of antifungal factors has been demonstrated in at least two cell wall fractions.

Additional key words: host-pathogen interaction, neutral sugars.

The cell wall is a primary carbon source encountered by fungal pathogens after initially penetrating the skin or cuticle of a plant host. Fungal pathogens produce hydrolytic enzymes that degrade cell wall polysaccharides and utilize constituent monosaccharides as a source of carbon for their growth and development (1,4). The fruit cell wall is in a dynamic state; certain components are constantly turned over (10), and substantial changes in carbohydrate composition occur during fruit ripening and softening (7,8,10). Components of the host cell wall may also act as elicitors or repressors of the infection process (1,4).

The *rin* (ripening inhibitor) gene is a single allele mutation on chromosome 5 that results in multiple phenotypic effects characterized by the inability of *rin* fruit to ripen or soften normally (15). *Rin* fruits are also less susceptible to certain postharvest decay microorganisms than are fruits of their parental line, cultivar Rutgers, that ripen and soften normally (2,14,15). Although the basis for this extended pathogen resistance has not been determined (3,14), it is possible that the mechanism may involve cell wall composition and structure. Substantial changes in tomato cell wall composition occur during the ripening process, many of which do not occur in the *rin* mutant (7,9). Any changes that lead to cell wall deterioration and tissue softening may influence the susceptibility of fruit to fungal colonization and subsequent decay. Because *rin* fruit do not ripen or soften normally, they provide an excellent system for studying the role of the cell wall in host-pathogen interactions.

In an earlier study, we demonstrated that the ability of spores from five pathogenic fungi to germinate and grow on cell wall-related monosaccharides varied substantially (12). The objective of the present study was to characterize the growth of two of these fungi, *Botrytis cinerea* (Pers.) Fries and *Mucor mucedo* (L.) Fresenius, on natural substrates, i.e., extracted tomato cell wall and cell wall fractions of known carbohydrate composition from normal and nonripening mutant tomatoes.

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MATERIALS AND METHODS

Cell wall fractionation and analysis. The methods for fractionation of tomato fruit cell walls into ionically associated pectin, covalently bound pectin, and a hemicellulosic fraction that were used in this study have been described in detail elsewhere (7). Briefly, walls were extracted from outer pericarp tissue sequentially with 80% ethanol, 20 mM HEPES-NaOH (pH 6.9), phenol/acetic acid/H_2O (2:1:1, w/v/v), chloroform/methanol (1:1,v/v), and acetone. After drying, walls were sequentially treated with 50 mM cyclohexanediamine tetraacetic acid/50 mM Na-acetate (pH 6.5) to yield ionically associated pectin, with cold 50 mM Na_2CO_3 to yield covalently bound pectin, and with 4 M KOH/100 mM $NaBH_4$ to yield a hemicellulosic fraction. The carbohydrate composition of these wall fractions from Rutgers and *rin* tomatoes are discussed in detail elsewhere (7).

Fungal culture and growth measurement. *B. cinerea* was cultured on a medium prepared by homogenizing frozen strawberries in a blender, adding 2% Bacto agar, and autoclaving for 20 min at 121 C. *M. mucedo* was maintained on potato-dextrose agar.

Growth of fungal colonies was determined by transferring spores and mycelium to five petri dishes 15 × 100 mm that were divided into numbered quadrants. Inoculations were made at the edge of the dish in each quadrant, allowing radial measurement of growth. Petri dishes of each treatment contained 5 ml of 1% water agar amended with 1 mg/ml of mature-green and red-ripe Rutgers and *rin* tomato cell walls of comparable maturity (*rin* I and *rin* II). Glucose (1 mg/ml) and Bacto water agar served as controls. Measurements of fungal growth were recorded daily for 12 days or until colonies covered the petri dishes. The experiment was replicated twice.

In a second experiment, conidia were harvested from vigorously growing 7- to 10-day-old *B. cinerea* or *M. mucedo* cultures and diluted in sterile distilled water to a concentration of 10^5–10^6 conidia per milliliter. A 0.1-ml aliquot was transferred to each of 10 15-mm-diameter cell wells containing 0.5 ml of solidified 1% Bacto agar, 0.3 M MES-NaOH (pH 6.0), 0.5 mg of ionically associated pectin, covalently bound pectin, or hemicellulose fraction from Rutgers or *rin* fruit of equivalent chronological age with and without 5 mM NH_4NO_3. *Rin* I and Rutgers mature-green fruit were 30 days postpollination and *rin* II and Rutgers red-ripe fruit were 60 days postpollination. Ten wells containing buffered agar only or NH_4NO_3-amended agar served as controls. Conidia were incubated for 18 hr at 20 C. Germination was observed and germ tube length was measured on a Leitz Ortholux compound microscope fitted with a Bioquant image analysis system (R & M Biometrics, Nashville, TN). No conidial branching was observed. The experiment was replicated twice.

In a third experiment, designed to determine the effect of cell wall fraction concentration on growth, spore germination and germ tube elongation of *B. cinerea* and *M. mucedo* were measured in 10 cell wells containing 1% agar, 0.3 M MES-NaOH (pH 6.0), and either 1, 5, or 10 mg/ml of the cell wall fractions. The experiment was replicated twice.

Statistical analysis was carried out with analysis of variance and Duncan's multiple range test at $P = 0.05$.

RESULTS

Colony expansion of *B. cinerea* on media containing unfractionated tomato cell walls was greater than growth on unamended water agar or water agar containing glucose as a carbon source (Fig. 1). Colonies enlarged most rapidly on *rin* II and red-ripe cell walls. Fungal colonies covered the petri dishes supplemented with *rin* II and red-ripe cell walls after 8 days, *rin* I walls after 11 days, and mature-green walls after 12 days. Little growth was observed on water agar amended with glucose or on water agar controls after 12 days.

Conversely, colonies of *M. mucedo* expanded slower on all unfractionated cell wall substrates than on water agar supplemented with glucose (Fig. 1). Cell walls from red-ripe fruit appeared to stimulate colony expansion slightly more than did *rin* I, *rin* II, or mature-green walls. However, all cell wall preparations were poor substrates for growth of *M. mucedo*; no growth was observed on unamended water agar.

Growth of *B. cinerea* hyphae on buffered water agar without a supplemental nitrogen source was more rapid than with a nitrogen source (Tables 1 and 2). Growth was not significantly inhibited by any cell wall fraction in buffered water agar. Growth was enhanced by all hemicellulosic fractions, as well as the covalently bound and ionically associated pectic fractions from red-ripe and *rin* II fruit.

In the presence of 5 mM NH_4NO_3, growth of *B. cinerea* hyphae was reduced on all substrates including the buffered water agar control (Tables 1 and 2). Hemicellulosic fractions supported the best growth (about 200% of control). Growth was further suppressed by all ionically associated pectic fractions, but only mature-green and *rin* II were significantly different (Table 2). There was also a significant reduction of growth on red-ripe and *rin* II covalently bound pectic fractions but a stimulation of growth by the *rin* I covalently bound pectic fraction (Table 2).

Spores of *M. mucedo* germinated only in the presence of a nitrogen source on buffered water agar (Tables 1 and 2). All ionically associated pectic fractions inhibited hyphal growth (Table 2). The mature-green, red-ripe, and *rin* II covalently bound pectic fractions also inhibited hyphal growth. Growth was stimulated by the hemicellulosic fraction from red-ripe, *rin* I, and *rin* II fruit and by the covalently bound pectic fraction of *rin* I fruit cell walls.

Increasing the concentration of cell wall fractions in the agar reduced fungal growth (Table 3). The greatest inhibition of *B. cinerea* growth was observed on ionically associated pectic fractions, where growth was reduced to 46 and 21% of the control at 5- and 10-mg/ml concentrations of substrate, respectively. Growth on the covalently bound pectic fraction was suppressed more at 5 mg/ml (49% of control) than at 10 mg/ml (82% of control). Growth of *M. mucedo* spores was completely inhibited at the 10-mg/ml carbon source concentration on each of the three cell wall fractions (Table 3) and was reduced significantly at the 5-mg/ml level.

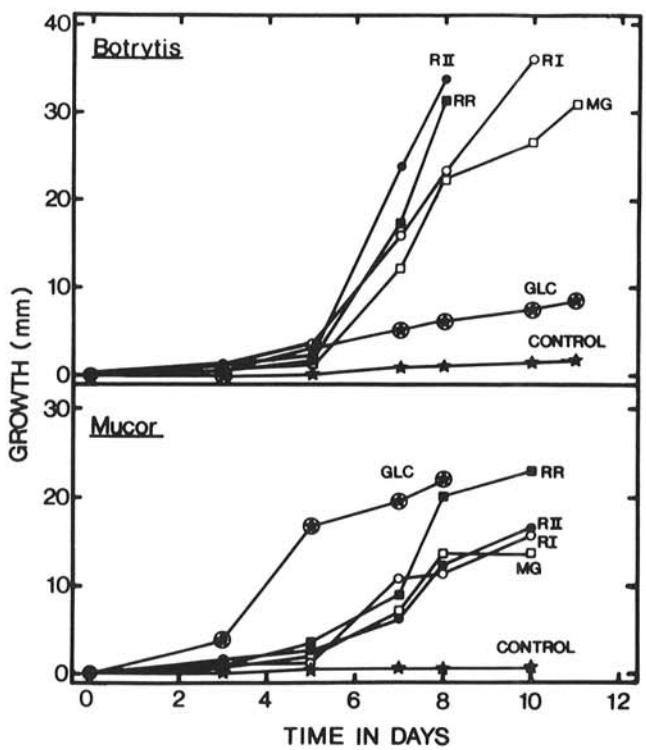

Fig. 1. Colony growth of *Botrytis cinerea* and *Mucor mucedo* on water agar containing cell walls (1 mg/ml) isolated from *rin* and cultivar Rutgers tomato fruit at two maturity stages: *rin* I and mature-green cultivar Rutgers 30 days postpollination and *rin* II and red-ripe Rutgers 60 days postpollination. Water agar and water agar amended with 1 mg of glucose (GLC) per milliliter served as controls.

TABLE 1. Growth of germinating *Botrytis cinerea* and *Mucor mucedo* conidia after 18 hr of incubation at 20 C on media containing 1 mg/ml of cell wall fractions from mature-green cultivar Rutgers, red-ripe Rutgers, *rin* I, and *rin* II tomato fruit

Substrate	Mature-green	Red-ripe	*Rin* I	*Rin* II[u]
B. cinerea				
Water agar control	2.29[v] a[w]	2.45 a	2.52 ab	2.22 a
IAP[x]	2.48 a (108%)[y]	3.22 b (131%)	2.57 ab (102%)	2.94 b (132%)
CBP	2.80 a (122%)	2.97 ab (121%)	1.99 a (79%)	2.94 b (132%)
HF	4.20 b (183%)	3.81 b (156%)	3.06 b (121%)	3.01 b (136%)
M. mucedo				
Water agar control	0.00[z]	0.00	0.00	0.00
IAP	0.46 a	0.55 ab	0.37 ab	0.41 a
CBP	0.53 a	0.35 a	0.27 a	0.48 ab
HF	0.67 a	0.88 b	0.56 b	0.68 b

[u] *Rin* I is the same chronological age as mature-green Rutgers and *rin* II is the same age as red-ripe Rutgers.

[v] Growth in millimeters, each value represents the mean of 100 germ tube measurements.

[w] Means within a single column followed by a different letter are significantly different ($P = 0.05$).

[x] Cell wall fractions: IAP = ionically associated pectin, CBP = covalently bound pectin, and HF = hemicellulosic fraction.

[y] Percent of control.

[z] Conidia of *M. mucedo* failed to germinate on water agar.

TABLE 2. Growth of germinating *Botrytis cinerea* and *Mucor mucedo* conidia after 18 hr of incubation at 20 C on buffered media (pH 6.0) containing various tomato cell wall fractions (1 mg/ml) and 5 mM NH_4NO_3 as a nitrogen source

Substrate	Mature-green	Red-ripe	*Rin* I	*Rin* II
B. cinerea				
Water agar control	0.68[w] a[x]	0.68 a	0.75 a	0.75 a
IAP[y]	0.47 b	0.56 a	0.65 a	0.42 b
	(69%)[z]	(82%)	(87%)	(56%)
CBP	0.59 a	0.37 b	1.32 b	0.32 b
	(87%)	(54%)	(176%)	(43%)
HF	1.39 c	1.49 c	1.57 c	1.40 c
	(204%)	(209%)	(209%)	(187%)
M. mucedo				
Water agar control	0.85 a	0.85 a	0.58 a	0.58 a
IAP	0.20 b	0.44 b	0.40 b	0.28 b
	(24%)	(52%)	(69%)	(48%)
CBP	0.25 b	0.00 c	0.56 a	0.34 b
	(29%)	(0%)	(97%)	(59%)
HF	0.84 a	2.45 d	1.38 c	2.16 c
	(99%)	(288%)	(238%)	(372%)

[w] Growth in millimeters, each value represents the mean of 100 germ tube measurements.
[x] Means within a single column followed by a different letter are significantly different ($P = 0.05$).
[y] Cell wall fractions: IAP = ionically associated pectin, CBP = covalently bound pectin, and HF = hemicellulosic fraction.
[z] Percent of control.

DISCUSSION

Glucose, which supported only minimal growth of *B. cinerea*, was the preferred substrate for growth of *M. mucedo* (Fig. 1). In contrast, growth of *B. cinerea* exceeded that of *M. mucedo* on all other substrates tested. *B. cinerea* was more capable of utilizing unfractionated tomato cell walls as a substrate than was *M. mucedo*; both fungi are tomato pathogens (13,16). It seems noteworthy that unfractionated cell walls provided a better carbon source for growth of *B. cinerea* than glucose, although glucose is a soluble sugar that is readily taken up and metabolized.

The greater growth of *M. mucedo* on red-ripe tomato cell walls (Fig. 1) may be related to a favorable neutral sugar balance in this substrate compared with mature-green or *rin* cell wall preparations. The carbohydrate composition of these wall fractions has been published previously (7). Lindberg (11) showed that the growth rate of *Ophiostoma multiannulatum* can be limited by the relative level of pentoses in the medium. The general growth stimulation by cell wall fractions over controls may also involve a difference in available neutral sugars, because the hemicellulosic fraction, which supports the most growth of both pathogens (Table 1), contains the highest percentage of total neutral sugar (7). The ratio of these available neutral sugars in the cell wall fractions may also partially account for the differential growth on these substrates (Table 1). Lindberg (11) observed growth-limiting effects of neutral sugars, notably the galactose/xylose ratio. The noncellulosic neutral sugar and galacturonic acid composition of certain tomato cell wall fractions changes substantially during ripening (7). For example, the galactose content of Rutgers ionically associated and covalently bound pectic fractions decreases 65% during ripening. This 65% decrease of galactose occurs while the level of free, monomeric galactose in tomato outer pericarp tissue increases fourfold to sixfold (6). In contrast, the content of galactose in *rin* pectic fractions decreased only 20% (7), and no increase in free galactose occurred (6). Because the germination and growth of *M. mucedo* and *B. cinerea* are significantly reduced in the presence of galactose (12), it is possible that either the reduced level of galactose-containing polysaccharides in the cell wall or the increased level of soluble galactose in the host tissue may influence the ability of these pathogenic fungi to become established in tomatoes. This influence would depend on the primary carbon source being used by the fungus for growth and energy production during invasion, i.e., soluble sugars, cell wall components, other extracellular polysaccharides, and other metabolites (5). Studies on the enzymes secreted by these fungi during invasion as well as a structural analysis of tomato cell wall polysaccharides should be useful in determining the nature of the host-pathogen interaction during tomato fruit decay.

TABLE 3. Growth of germinating conidia of *Botrytis cinerea* and *Mucor mucedo* on different concentrations of cell wall fractions from red-ripe Rutgers fruit

Carbon source	Substrate concentration (mg/ml) 1	5	10
B. cinerea			
Water agar control	2.45[w] a[x]	2.31 a	3.11 b
IAP[y]	3.22 a	1.06 b	0.66 c
CBP	2.97 a	1.14 b	2.56 a
HF	3.81 a	3.10 a	3.38 a
M. mucedo			
Water agar control	0.00[z]	0.00	0.00
IAP	0.55 a	0.12 b	0.00
CBP	0.35 a	0.13 b	0.00
HF	0.88 a	0.34 b	0.00

[w] Growth in millimeters, each value represents the mean of 100 germ tube measurements.
[x] Means within a single row followed by a different letter are significantly different ($P = 0.05$).
[y] Cell wall fractions: IAP = ionically associated pectin, CBP = covalently bound pectin, and HF = hemicellulosic fraction.
[z] Conidia of *M. mucedo* failed to germinate on water agar or on any of the three cell wall fractions at the 10-mg/ml level.

The ability of *B. cinerea* spores to germinate without a carbon or nitrogen source while *M. mucedo* spores require an external nutrient source to germinate indicates that the pathogens have different mechanisms controlling spore dormancy and germination. At the concentration used in this study, NH_4NO_3 reduced growth of *B. cinerea* hyphae (Table 2), whereas it stimulated the germination of *M. mucedo* spores.

The hemicellulosic cell wall fraction was a good carbon source for both fungi and did not appear to contain any inhibitory component active at the 1-mg/ml concentration. The remaining fractions were less suitable as nutrient sources and demonstrated antifungal activity in the presence of a nitrogen source (Table 2). Growth of *M. mucedo* was completely inhibited at the 10-mg/ml level of each of the three cell wall fractions and growth of *B. cinerea* was significantly reduced, indicating the presence of a growth-inhibiting factor in all three substrates (Table 3). Additional fractionation and purification of polysaccharides of tomato fruit cell walls will allow us to further characterize antifungal components.

Although we have demonstrated that carbohydrates influence germination and growth of tomato pathogens, it is difficult to correlate polysaccharide content with specific growth responses of these pathogens. The role of fruit cell wall polysaccharides in mediating host-pathogen interactions merits additional investigation.

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