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Isolation of Phenol Cells from Waterhyacinth Leaves and Possible Effects on the Growth of Foliar Pathogens[1]

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ABSTRACT

Intact phenol-storing cells were separated from waterhyacinth leaves by enzymatic digestion and filtration and assayed for phenolic acids by two-dimensional paper chromatography. Four phenolic acids were detected: chlorogenic, vanillic, p-coumaric, and protocatechuic. The growth of two potential biocontrol agents of waterhyacinth (*Cercospora rodmanii* and *Acremonium zonatum)* was monitored on media containing standard preparations of each of the phenolic acids identified at three concentrations (10, 100, and 1000 ppm). *C. rodmanii* grew as well or better on the o-dihydroxyphenols (chlorgenic and protocatechuic acids) at all three concentrations than on glucose, but was inhibited by p-coumaric and vanillic acids at 10 and 100 ppm. Growth was halted completely at 1000 ppm. In contrast, *A. zonatum* grew as well or better on all four phenolic acids at 10 and 100 ppm, but was severely inhibited or stopped completely at 1000 ppm of each. *C. rodmanii* was affected more by the type of phenolic acid than by concentration,

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while *A. zonatum* seemed to be affected more by the concentration of the acid.

Key words: Aquatic weeds, disease resistance, tannin cells, *Cercospora rodmanii, Acremonium zonatum,* histochemistry.

INTRODUCTION

Acremonium zonatum (Sawada) Gams and *C. rodmanii* Conway are leaf-spotting fungi of waterhyacinth [*Eichhornia crassipes* (Mart.) Solms] and are being tested as potential biocontrol agents (3,5,8). Each is capable of causing severe damage to the plant under optimum conditions, but rarely do either cause enough damage to stop the spread of the plants. We believe this is partially related to the phenol cells in waterhyacinth leaves which impart some disease resistance. Phenol-storing cells were first described in waterhyacinth leaves by Martyn (7) and Martyn et al. (11) in 1977. They occur in the palisade cell layer on both adaxial and abaxial leaf surfaces and near the level of the vascular tissue. Waterhyacinth phenol cells are of two distinct morphologies. Those in the palisade cell layer are long and narrow while those near the vascular bundles are much more isodiametric. Both types stain cherry-red with the nitrous acid reaction (7,11) and, therefore, presumably contain o-dihydroxyphenols (13). Martyn and Freeman (8) showed that lesions caused by *A. zonatum* increased to 40% of the leaf surface and stopped. Additionally, an ultrastructural study of the leaf colonization by *A. zonatum* revealed that hyphae which penetrated the phenol cells appeared vesiculated and nonviable, and that the phenol bodies within the phenol cells tended to accumulate around the invading hyphae (9, 12). Hence, it appeared that disease severity was limited to 40% when the fungus had penetrated a sufficient number of phenol cells to stop its progression.

Martyn (7) identified seven phenolic acids, as well as three additional unidentified phenolic acids in waterhyacinth leaf tissue, but was unable to ascribe specific compounds to the phenol cells. The purpose of this present study was to separate the phenol cells intact from surrounding tissue and identify the phenolic acids present. Secondly, the effect of the acids identified was determined on the *in vitro* growth of two waterhyacinth pathogens, *A. zonatum* and *C. rodamanii.*

MATERIALS AND METHODS

Separation of phenol cells. One thousand grams of fresh waterhyacinth leaves were chopped in a Waring blender for 5 minutes in 800 ml of cold 0.1 M citrate buffer, pH 5.0, and then filtered through four layers of cheese cloth. To macerate the tissue further and obtain single cells, the slurry was treated with a 2% solution of the pectolytic enzyme preparation "Macerase" (Calbiochem-Behring, LaJolla, CA.) for 16 hours at room temperature (22 C) with constant stirring. Optimum enzyme concentration and treatment for cell separation was determined previously (1). After enzyme treatment, the cell suspension was passed through two 7.62 cm stainless steel Tyler standard nematode screens and rinsed with 2 volumes of distilled water. The upper screen was #200 mesh with a pore size of 74 μm and the bottom was a #325 mesh with a pore diameter of 43 μm. With this method, most large debris was held by the first screen while the phenol cells collected on the second screen. Many smaller parenchyma cells passed through the second screen and were discarded.

Extraction of phenolic acids. The phenol cells were rinsed from the #325 mesh screen with distilled water and sonicated in an Artek sonic membrane disrupter for 10 minutes. After sonication, the suspension was filtered through Whatman No. 1 filter paper in a Büchner funnel and washed with enough distilled water to make a final volume of 450 ml. Fifty ml aliquots were extracted three times with equal volumes of petroleum ether to extract remaining chlorophyll. The aqueous phase of each extraction was combined and base-hydrolyzed to break the ester linkages and liberate the phenolic moiety (14). The solution was adjusted to 2 N with 32 g of NaOH pellets and boiled for 3 minutes. The hydrolyzed solution was then placed immediately into an ice bath and acidified to pH 1.0 by the dropwise addition of concentrated HCl. Fifty milliliter aliquots were extracted three times with 30 ml of anhydrous diethyl ether. The organic phase of each extraction was combined, reduced to dryness, resuspended in 5 ml of 95% ethanol, and stored at –10 C until used.

An additional 100 ml of phenol cell suspension was treated as described, except with the deletion of the alkaline hydrolysis steps to determine the presence of free phenolic acids. After extraction into diethyl ether and drying, the residue was resuspended into 1.0 ml of 95% ethanol and combined with the hydrolyzed preparation.

Identification of phenolic acids by paper chromatography. Approximately 0.5 ml of the phenolic acid extract was spotted onto Whatman No. 1 chromatography paper in 50 μl aliquots. Two-dimensional chromatograms were prepared. The sheets were run first in a benzene: acetic acid: water (41:24:1) solvent system (BZA) for 8 hours at room temperature (22 C). They were then air-dried, rotated 90 degrees and run ascending in 2% formic acid (FA). Chromatograms of authentic samples of p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, and chlorogenic acid were prepared simultaneously in the same manner. All chromatograms were dried in a hot air oven (100 C) and observed with a Woods mineral light (253 nm). Replicate chromatograms were sprayed with sulfanilic acid or p-nitroaniline locating reagents (15). Identification of the phenolic acids was based on comparisons of R_f values and color reactions to the known standards.

Effect of phenolic acids on the growth of two waterhyacinth pathogens. Four phenolic acids (chlorogenic, p-coumaric, protocatechuic and vanillic) were individually incorporated into 3% water agar at 10, 100, and 1000 ppm and poured into Petri dishes (15 x 120 mm). An additional set of Petri dishes was prepared with glucose instead of the phenolic acids at the same three concentrations. The plates were seeded with a 5mm plug of either *A. zonatum* or *C. rodmanii* and incubated at 22 C for 2 weeks. After incubation, colony diameters were recorded and analyzed statistically (4). There were five replicate plates for each concentration of each phenolic acid and glucose. The entire experiment was repeated twice for *A. zonatum* and three times for *C. rodmanii.*

RESULTS AND DISCUSSION

In waterhyacinth leaf tissue, phenol cells are arranged vertically along the palisade cell layer on both upper and lower leaf surface (Figure 1a). Tissue homogenization and Macerase treatment effectively separated the cells from one another and liberated intact phenol cells (Figure 1b). Neither the cell wall nor the cell membrane were apparently damaged since the cells still retained their characteristic cherry-red color after staining.

Length and width measurements of 135 individual phenol cells were made. The size of the cells varied considerably from a maximum of 16 x 111 μm to a minimum 24 x 55 μm. Because there was a great amount of size variation in individual cells, a length to width ratio (l/w) was taken as a more meaningful size description. The smallest cells had a l/w ratio of 2.3 while the largest cells had a l/w ratio of 7.0. Almost 60% of the separated phenol cells had a l/w ratio of 5.0 or higher.

Paper chromatography of the phenolic extracts yielded four distinct spots (Table 1). When viewed under ultraviolet light, all four spots had a blue-purple fluorescence indicative of phenolic acids (14). R_f values and color after spraying with p-nitroaniline and sulfanilic acid locating reagents were similar to those of the four authentic phenolic acid samples. Based on this, the four spots were identified as chlorogenic acid, protocatechuic acid, vanillic acid and p coumaric acid. Of these, chlorogenic and protocatechuic acids are o-dihydroxy phenols and stain cherry-red by the nitrous acid reaction (13). These two phenolic acids are those most likely responsible for the red color of the phenol cell.

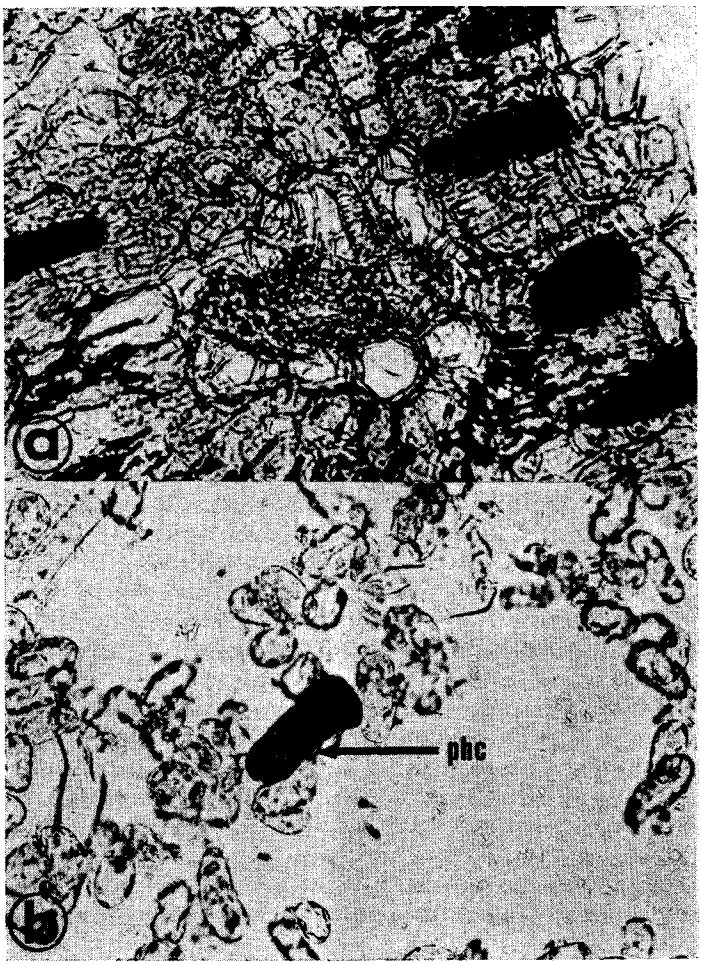

Figure 1. a) Cross-section of a waterhyacinth leaf showing the phenol-storing cells in the palisade cell layer. The tissue has been stained by the nitrous acid reaction (13) and the phenol cells appear cherry-red. (Magnification 375x). b) An isolated phenol cell (arrow) separated intact from the surrounding tissue. The surrounding cells are primarily mesophyll parenchyma. (Magnification 400x).

TABLE 1. COLOR REACTIONS AND R_f VALUES OF THE FOUR PHENOLIC ACIDS DETECTED FROM WATERHYACINTH PHENOL CELLS.

Phenolic acid	R_f values[a]		Color reactions	
	BzA[b]	FA[c]	p-NA[d]	SA[e]
Chlorogenic acid	0.10	0.57	brown	olive green
Protocatechuic acid	0.53	0.62	tan	tan
Vanillic acid	0.98	0.73	purple	orange
p-Coumaric acid	0.88	0.30	red-brown	orange

[a]R_f values calculated from authentic samples on duplicate chromatograms.
[b]Benzene:acetic acid:water solvent system (41:21:1).
[c]2% Formic acid solvent.
[d]p-Nitroaniline reagent.
[e]Sulfanilic acid reagent.

The increase in mean colony diameters of *C. rodmanii* and *A. zonatum* grown on phenolic acid media and on glucose are illustrated in Figure 2a and b. The effect that the phenolic acids had on the growth of the two fungi were quite different. Growth of *C. rodmanii* was adversely affected by the type of phenolic acid and not necessarily its concentration (Figure 2a). Growth on the o-dihydroxy-phenolic acids (chlorogenic and protocatechuic) was similar to that on glucose. In fact, it was significantly larger ($P = 0.05$) on 1000 ppm chlorogenic acid than on glucose which suggests that *C. rodmanii* is capable of utilizing these two phenolic acids as a carbon source at concentrations to at least 1000 ppm. On the other hand, p-coumaric and vanillic acids significantly reduced growth ($P = 0.05$) at each concentration tested and growth was inhibited completely by both at 1000 ppm.

In contrast to that of *C. rodmanii,* the growth of *A. zonatum* increased on all four phenolic acids at 10 and 100 ppm, but was stopped completely at 1000 ppm of each phenolic acid. *A. zonatum* was adversely affected by the concentration of the phenolic acids as opposed to the type of acid. At lower concentrations, *A. zonatum* was capable of utilizing the phenolic acids as a carbon source for growth. However, at the highest level (1000 ppm) the same compounds were fungitoxic.

The concentration of phenolic compounds within the phenol cells may be quite high, possibly reaching localized concentrations of 1000 ppm. In addition, polyphenoloxidase has been shown to be localized within plastids in the phenol cells (10). The action of this enzyme on the phenols could produce high levels of fungitoxic quinones. (6). These data support the hypothesis that phenol cells play a role

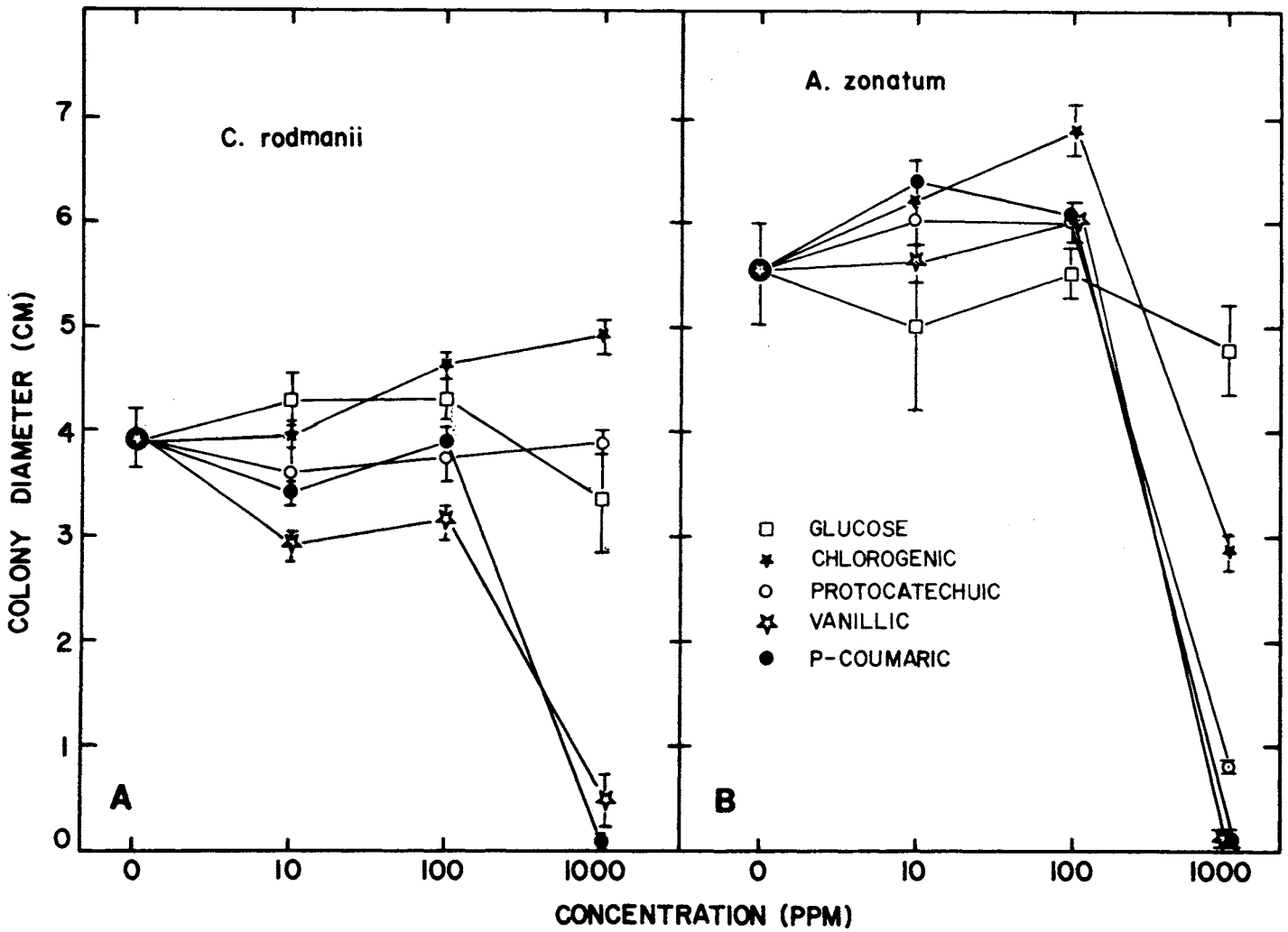

Figure 2. Growth of *C. rodmanii* (a) and *A. zonatum* (b) on water agar supplemented with 10, 100, or 1000 ppm of four phenolic acids or glucose. Statistical bars represent one standard deviation from the mean.

in the defense mechanisms of waterhyacinth against fungal pathogens.

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