Growth of and Oxalic Acid Production by *Cristulariella pyramidalis* on Selected Culture Media

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ABSTRACT

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Oxalic acid was isolated from media that had supported growth of *Cristulariella pyramidalis*. Oxalic acid was produced at all stages of growth and in all media that supported growth. Application of either isolated or authentic oxalic acid to wounded bean leaves caused lesions similar to those observed in *C. pyramidalis*-infected plants.

Few papers dealing with the leaf-spotting pathogen, *Cristulariella pyramidalis* Waterman and Marshall, were published before 1970. Since that time, however, it has become apparent that this fungus infects a wide variety of woody and herbaceous plants (15–17).

We recently reported that a toxic substance is produced in cultures of *C. pyramidalis* (6) and in a brief report (5) we identified that chemical as oxalic acid.

This paper reports the methods used to identify oxalic acid in and isolate it from culture filtrates of *C. pyramidalis*. It also presents the cultural conditions that favor growth and production of oxalic acid by this fungus.

MATERIALS AND METHODS

Oxalic acid isolation and identification. *C. pyramidalis* was grown in liquid media as previously described (6). The cultures were filtered 10 days after inoculation and were neutralized with NH_4OH. A saturated solution of $CaCl_2$ was added to the filtrates (10 ml/800 ml of filtrate), which were incubated for 24 hr at room temperature. The resulting precipitate was collected by centrifugation, washed with water, and dissolved in 2 N HCl. This solution was passed through a 2.5 × 30-cm column of AG 50W-X1 in the H^+ form and eluted with water. The eluant was concentrated by flash evaporation at 45 C until crystallization was evident. The concentrated solution was kept at room temperature for 1–2 hr and then at 5 C for several hours. Crystals formed and were collected by filtration. The filtrate was further concentrated and a second batch of crystals was similarly obtained. The crystals were combined, air-dried, washed with benzene, and stored at 5 C.

The melting point of the purified chemical was determined and it was analyzed by infrared, nuclear magnetic resonance, and mass spectrometry.

The purified chemical was dissolved in distilled water and 10-μl aliquots of various dilutions were applied to wounded pinto bean leaves as described (6). Some of the solutions were neutralized to pH 5–7 before application. The leaves were examined for necrosis after 24 hr.

Studies on growth and oxalic acid accumulation. *C. pyramidalis* was grown for 6 or 8 days in 250-ml Erlenmyer flasks containing 40 ml of unbuffered liquid medium (pH 5.7) (6). The flasks were inoculated with 0.25-cm^2 plugs from the leading edge of 8-day-old mycelium. In some experiments, glucose and fructose in the medium were replaced with equivalent amounts of other carbon sources to maintain a constant total amount of carbon in the media. When the effect of pH was studied, the media were buffered with sodium citrate or sodium phosphate.

The contents of three flasks were filtered, the mycelial mats were washed, and the dry weights were determined. The filtrates and wash solutions of each flask were combined and adjusted to 40 ml with water, and the pH values were determined. The three 40-ml solutions were combined and the oxalic acid concentration was determined in duplicate by titration with potassium permanganate (1,12).

In some experiments, concentrations of reducing sugar also were determined (4) in duplicate.

RESULTS

Oxalic acid identification. The purified toxic substance had a melting point of 101–104 C, which agrees with the reported melting point of 101–102 C for oxalic acid dihydrate (10). The nuclear magnetic resonance spectrum in D_2O had a single peak corresponding to that of water. This indicates that all of the hydrogens of the toxic substance are exchangeable. The infrared spectra of the purified chemical and oxalic acid dihydrate were identical. The mass spectrum of a dried sample of the purified chemical gave a molecular ion peak m/e 90, which would be expected from anhydrous oxalic acid.

Necrosis was obtained when 10 μl of purified isolated oxalic acid at a concentration of at least 0.01 M was applied to detached wounded bean leaves. However, no necrosis was obtained with isolated or commercial oxalic acid adjusted to pH 5–7 with dilute NaOH.

Time study. A study of growth, pH, oxalic acid production, and sugar utilization was conducted over a 14-day period (Fig. 1). Oxalic acid production nearly paralleled growth. However, the growth curve peaked on day 8 whereas the oxalic acid accumulation was maximal on day 9. This corresponded to the minimum pH. The concentration of oxalic acid then rapidly decreased for 1 day and thereafter continued to decrease at a slower rate. In contrast, the pH of the solution inceased from day 9 to 10 and then remained almost constant. Substantial amounts of reducing sugar still were present when growth and oxalic acid production ceased.

Effect of pH. *C. pyramidalis* growth and oxalic acid production both depended on the initial pH in buffered media (Table 1). Growth was positively correlated with oxalic acid production in all cases. No growth occurred in 0.05 M citrate at pH 5.8 or 6.7 and it was only slight at pH 5.0 and 2.5. In 0.05 M phosphate, growth was significant at pH 3 but was slight at pH 6. The most rapid growth was at pH 3.4 and 4.2 in 0.05 M citrate and at pH 4.5 in 0.05 M phosphate. This growth was significantly greater than that in the unbuffered medium (Fig. 1). Growth was considerably lower in 0.1 M and especially in 0.2 M phosphate than in 0.05 M phosphate at pH 4.5.

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Effect of carbon sources. Various carbohydrates, alcohols, and organic acids were used as carbon sources, and dry mycelial weights and oxalic acid levels were measured after 6 or 8 days of growth.

Table 2 summarizes the data of the carbon-source experiments that resulted in at least 10 mg of dry mycelial weight. No growth was observed when the carbon source was acetic, ascorbic, or oxalic acid. Less than 10 mg of dry mycelium was obtained when the carbon source was ethanol, erythritol, glycol, mannitol, or xylose at approximately pH 6 or glycine, citric acid, glycolic acid, or glyoxylic acid at pH 4.2. Even slight growth was accompanied by oxalic acid production in all cases.

DISCUSSION

Oxalic acid is a common metabolic product of fungi (3,7–9), but our recent report (5) and this paper are the first evidence of oxalic acid production by *C. pyramidalis*. Application of an aliquot of the culture filtrate or oxalic acid to wounded bean leaves caused identical necrotic lesions. The observation that these lesions were nearly identical to those associated with *C. pyramidalis* infection suggests that oxalic acid may function as a toxin during pathogenesis by this fungus. We have not, however, presented evidence that oxalic acid is actually produced in plants infected with this fungus. No necrosis occurred when a neutralized culture filtrate or oxalic acid was applied to leaves. This indicates that the necrosis resulting from this toxic substance may be primarily due to the hydrogen ion concentration. *C. pyramidalis* synthesized oxalic acid at all stages of growth (Fig. 1). Furthermore, the maximum rates of growth and oxalic acid production were correlated (Fig. 1) and oxalic acid accumulated in all media that permitted growth (Table 1 and 2).

The oxalic acid level decreased slightly after the maximum accumulation (Fig. 1). Similar decreases have been observed in cultures of other fungi at low pH values (11,13). Shimazono (14) and Emiliani and Bekes (2) reported the presence of an oxalate decarboxylase, but this enzyme was not detected in preliminary

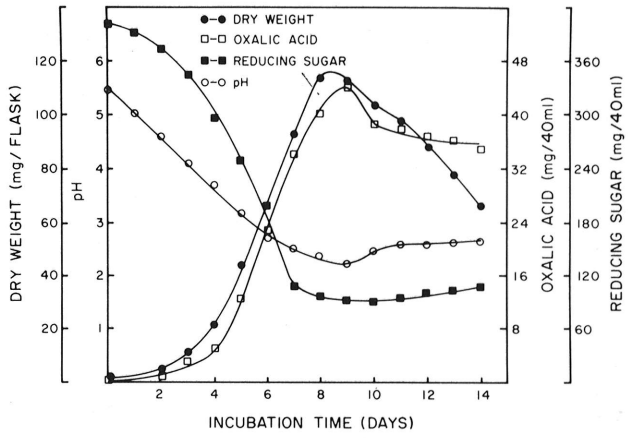

Fig. 1. Growth of *Cristulariella pyramidalis* and pH, oxalic acid, and reducing sugars in a glucose-fructose medium supporting this fungus over a period of time.

TABLE 1. Oxalic acid production and growth of *Cristulariella pyramidalis* on media containing glucose and fructose and buffered with sodium citrate or sodium phosphate.

Buffer		pH		Time (days)	Mycelial dry weight (mg/flask)	Oxalic acid (mg/40 ml)
Type	Conc.	Initial	Final			
Citrate	0.05 M	6.7	6.6	6	0	0.05
		5.8	5.8	6	0	0.05
		5.0	4.8	6	3.5	1.2
		4.2	3.3	6	161.8	82.9
		3.4	3.0	6	99.2	53.7
		2.5	2.4	6	2.5	0.7
Phosphate	0.05 M	6.0	4.6	8	13.2	5.9
		4.5	2.4	8	130.4	57.2
		3.0	2.6	8	30.1	12.2
	0.1 M	4.5	3.1	8	65.9	30.5
	0.2 M	4.5	4.0	8	11.3	5.1

TABLE 2. Oxalic acid production and growth of *Christulariella pyramidalis* on media containing various carbon sources

Carbon source	pH		Time (days)	Mycelial dry weight (mg/flask)	Oxalic acid (mg/40 ml)
	Initial	Final			
Mannose	6.2	2.4	8	95.5	34.9
Glucose	6.2	2.6	8	87.7	28.6
Starch	6.2	2.8	8	84.1	25.3
Raffinose	6.2	3.1	8	59.8	15.2
Fructose	6.2	3.0	8	42.8	14.5
Lactose	6.2	3.3	8	38.9	9.2
Arabinose	6.3	3.8	8	24.3	4.9
Cellobiose	6.2	3.9	8	20.3	4.6
Galactose	6.2	3.9	8	13.8	4.3
Glycerol	6.2	4.2	8	10.0	4.0
Glycerol	4.2	3.8	8	10.0	3.6
Succinic acid	4.2	4.2	6	49.1	20.4
Malic acid	4.2	4.2	6	20.9	9.5

studies of mycelial homogenates of *C. pyramidalis* (authors, *unpublished*).

The optimal pH for growth and oxalic acid production by *C. pyramidalis* was within the range of 3.4–4.5 (Table 1). There was slight growth in media containing phosphate adjusted to pH 6 even though no detectable growth occurred in media containing citrate adjusted to pH 5.8. This may have happened because phosphate is a poor buffer at this pH, thus synthesis of any oxalic acid would lower the pH of the solution to values that would permit some growth. The greater growth and oxalic acid production in buffered media probably occurred because the fungus was maintained at an optimal pH for a longer time. Phosphate may inhibit the fungus, as indicated by less growth and oxalic acid production in the presence of 0.1 M or 0.2 M phosphate than in the presence of 0.05 M phosphate.

There was a wide variation in growth on media prepared with different carbon sources, but the ratio of growth to oxalic acid synthesis was fairly constant on all media that permitted growth (Table 2). This and the fact that oxalic acid was synthesized at all stages of growth (Fig. 1) is consistent with the suggestion that oxalic acid is a necessary product of metabolism of *C. pyramidalis*.

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