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CARBOHYDRATE METABOLISM AND OXALIC ACID SYNTHESIS BY BOTRYTIS CINEREA [1,2]

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Most of the work on the metabolism of the fungi has dealt with the production of organic acids and other compounds formed via pathways which are, presumably, shunts from the main respiratory cycles (6, 14). Few attempts have been made to integrate these pathways into over-all metabolic systems. This study will follow the formation of oxalic acid, which appears to be a metabolic end product, from glucose through the respiratory system of *Botrytis cinerea* Pers.

Materials and Methods

In all experiments a strain of *Botrytis cinerea* (7) was grown at room temperature in 250-ml Erlenmeyer flasks containing 100 ml of the following medium: $NaNO_3$, 2.0 gm; KH_2PO_4, 1.5 gm; $MgSO_4 \cdot 7 H_2O$, 1.0 gm; Hoagland's A–Z trace element solution (9), 1.0 ml; glucose, 20.0 gm; H_2O to make 1 liter. Inoculum for flasks was provided by growing the fungus in Petri dishes on the above medium solidified with 2 % agar. Uniform discs were cut out with a cork borer 7 mm in diameter and each flask inoculated with 1 disc. Unless otherwise noted, the cultures were mechanically shaken during incubation (10 days). The pellets of mycelium which formed were then washed and shaken for 3 days in numerous changes of the above liquid medium without a carbon source. These pellets of starved mycelium were used in all respiratory and biosynthesis experiments (tables II to IV).

Oxalic acid was removed from culture filtrates essentially according to Pucher, Vickery, and Wakeman (12). The filtrates were acidified with H_2SO_4 and the oxalic acid precipitated with saturated $Ca(NO_3)_2$. One ml of the oxalic acid solution obtained was placed in a Warburg vessel and, at zero time, 0.5 ml of acid permanganate solution (8 % $KMnO_4$ in 7 *N* H_2SO_4) was tipped in. Under these conditions 2 moles of CO_2 were formed from each mole of oxalic acid. In numerous tests with known concentrations of oxalic acid, it was found possible to obtain an accuracy of about 5 % with this method. Of all the organic acids discussed in this paper, only citric interfered in this method. However, no trace of citric acid was found in any filtrates of this organism when they were analyzed chromatographically. Glucose was determined by the procedure of Folin and Malmros (5), nitrate by a phenoldisulfonic acid method (13), and organic acids, qualitatively, by the method of Lugg and Overell (8). All manometric studies were conducted at 26°C, pH 4.0, using standard Warburg techniques. The vessels contained 3.0 ml of the medium previously described, with added substrate, where indicated, and KOH with filter paper wicks were used in the center well.

Results

A 6-week time-course study was undertaken to investigate the pattern of oxalic acid formation and the physiological changes accompanying its synthesis. Each value in figure 1 represents the results of duplicate analyses for oxalic acid of combined filtrates and in addition the average mycelial weights of 6 standing cultures. The pH of the cultures rose slowly from 4.2 at the time of inoculation to 6.5 at the end of 6 weeks. Oxalic acid formation does not appear to accompany the growth of the fungus, since most of the oxalic acid was produced before the log phase of growth. There was only a slight increase in total oxalic acid during the second week, and it began to fall off slightly after this time. Oxalic acid synthesis apears to be concomitant with the rapid utilization of glucose. At the end of the 6 week period, the growth curves began to level off although only about 50 % of the carbohydrate and 30 % of the nitrate available in the medium had been utilized. Cessation of growth may have been due to production of "staling products" rather than to any limitation of available nutrient.

Figure 2 shows the rates of O_2 uptake of mats of mycelium of various ages, each value obtained from a random sample of segments of mats taken from several standing cultures. The segments were cut into small squares and shaken in Warburg vessels, each vessel containing about 20 mg dry weight of mycelium suspended in 3.0 ml of medium containing glucose (5×10^{-3} M). The Q_{O_2} reached a peak at 2 weeks and subsequently fell off rapidly, leveling off by

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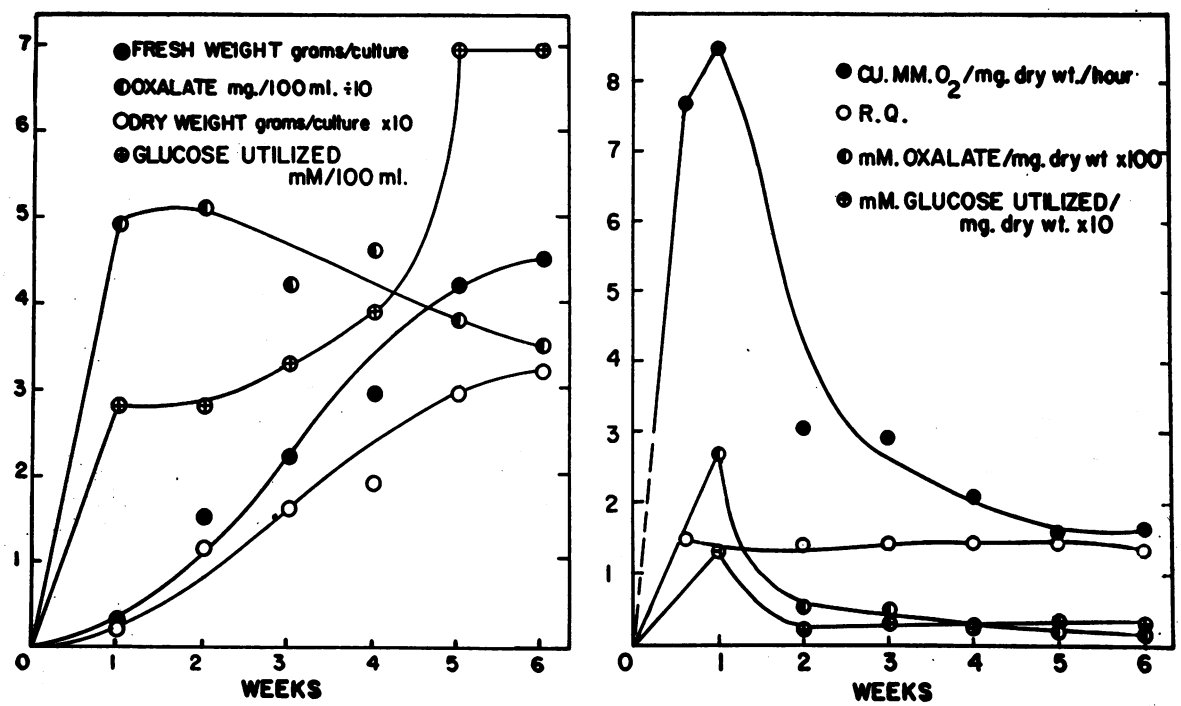
5 weeks. The respiratory quotient (R.Q.) remained above unity throughout the 6-week period. The curves for glucose utilization and oxalic acid formation per unit dry weight parallel each other, indicating a relationship between the amount of glucose utilized and the amount of oxalic acid formed. The amount of oxalic acid produced by this organism was independent of the pH of the medium (table I).

To investigate the pathways of carbohydrate oxidation by *Botrytis*, hexoses, pentoses, trioses, and selected phosphorylated intermediates were used as respiratory substrates (table II). The figures presented here and in table III were calculated from the mean rates of O_2 uptake obtained from at least 2 separate experiments. Both endogenous and substrate respiration were measured in duplicate vessels in each experiment, each vessel containing 8 to 12 mg dry wt. of mycelium. Under the conditions of growth used here the Q_{O_2} of starved pellets of mycelium originating from different cultures varied from about 2.0 to 4.0. However, in any one experiment the tissue used was all obtained from the same culture and the variation about the mean Q_{O_2} was $\pm$ 0.5. Under these conditions, the percentages of stimulation of O_2 uptake obtained in duplicate experiments were in close agreement (10 %). In every case where stimulation of O_2 uptake is reported, the lower substrate Q_{O_2}, was significantly higher than the upper endogenous Q_{O_2}.

Stimulation of O_2 uptake with glucose, gluconic acid, phosphogluconic acid, and ribose-5-phosphate suggests that glucose oxidation proceeds via direct oxidation through phosphorylated intermediates to pyruvic acid. Increased O_2 uptake was obtained with xylose and arabinose, but ribose was not oxidized. All tested phosphorylated hexose sugars as well as 5-keto-gluconic acid failed to increase the rate of respiration. Increased O_2 uptake was obtained with 3-phosphoglyceraldehyde but not with α- or β-glycerophosphate. In all instances where addition of substrate failed to enhance O_2 uptake, the possibility that the substrate failed to enter the cells cannot be eliminated. An acetone powder extract of mycelium prepared according to Arnon (1) contained glyceraldehyde phosphate dehydrogenase, which was completely DPN dependent, but did not appear to contain aldolase. Over 80 % inhibition of both endogenous and glucose respiration was obtained with sodium fluoride (10^{-3} M, pH 4.0). These results indicate that the oxidation of triose to pyruvic acid proceeds via the Meyerhof-Embden pathway. Fermentation was lacking, since neither CO_2 nor lactic acid was produced anaerobically. However, lactic acid was found in some aerobic cultures after several weeks of growth.

Paper chromatography of the filtrates from the cultures showed the presence of pyruvic, succinic, fumaric, and malic acids—all intermediates of the Krebs cycle. In addition, glycolic and lactic acids were found. Various Krebs cycle acids, pyruvic, citric, α-ketoglutaric, and succinic (table III), stimulated O_2 uptake of starved pellets of mycelium.



FIGS. 1 and 2. Fig. 1 (*left*). Growth, glucose utilized, and oxalic acid produced by *Botrytis cinerea* over a 6-week period. Fig. 2 (*right*). Oxygen uptake, respiratory quotient, oxalic acid formed, and glucose utilized per unit dry weight of *Botrytis cinerea* over a 6-week period.

TABLE I

OXALIC ACID FORMATION BY BOTRYTIS CINEREA GROWN FOR 4 WEEKS AT VARIOUS HYDROGEN ION CONCENTRATIONS

INITIAL pH	FINAL pH	OXALIC ACID (MG/100 ML MEDIUM)
2.0	3.0	54
2.6	3.6	38
3.9	3.8	58
4.8	3.2	68
6.7	5.8	50

Malic acid did not significantly increase O_2 uptake. Malonic acid (10^{-2} M, pH 4.0) inhibited O_2 uptake 37 %, this inhibition being completely relieved by succinic acid (5×10^{-2} M). Stimulation of the respiration of starved mycelium with most Krebs cycle intermediates, as well as the inhibition of succinic dehydrogenase with malonic acid and reversal of this inhibition with succinic acid, suggest that a Krebs cycle is functioning in this microorganism. There was no significant difference in O_2 uptake with oxalic acid as compared with the endogenous respiration. Since long-term growth experiments also demonstrated that oxalic acid could not be utilized as a carbon source, it appears that oxalic acid is an end product of the metabolism of *Botrytis cinerea*.

In order to obtain information on the terminal oxidase system of this organism, experiments on the effect of sodium azide on oxygen uptake were performed. Sodium azide (10^{-3} M, pH 4.0) showed 85 % inhibition of both glucose and endogenous respiration, indicating that the terminal oxidase system is probably mediated by heavy-metal enzymes.

Experiments to determine the pathway of oxalic acid synthesis were conducted. Approximately equal weights of pellets of starved mycelium were incubated at 30°C for 3 hours in 10 ml of standard medium with various organic acids. As a control the organism was incubated in this medium without substrate. All experiments were done in duplicate. At the end of the incubation period, trichloroacetic acid was added to each flask to a final concentration of 15 %. The flasks were allowed to stand for 30 minutes; the media were filtered, and then analyzed for oxalic acid. High yields of oxalic acid were obtained with malic acid as the substrate. Considerable oxalic acid was also formed from oxaloacetic acid. No significant amounts of oxalic acid were produced when acetic, glyoxylic, glycolic, or formic acids were used as precursors.

TABLE II

OXYGEN UPTAKE OF BOTRYTIS CINEREA WITH VARIOUS SUGARS AND SUGAR DERIVATIVES AS SUBSTRATES *

SUBSTRATE	O_2 UPTAKE AS PERCENT OF ENDOGENOUS
Control	100
Glucose	170
Fructose	160
Glucose-1-phosphate	97
Glucose-6-phosphate	103
Fructose-1,6-diphosphate	104
Gluconic acid	210
5-Ketogluconic acid	95
Phosphogluconic acid	190
Ribose **	107
Xylose **	190
Arabinose **	138
Ribose-5-phosphate **	160
3-Phosphoglyceraldehyde **	140
α-Glycerophosphate **	87
β-Glycerophosphate **	90

* 5×10^{-3} M concentration; pH 4.0.
** 1×10^{-3} M concentration.

TABLE III

OXYGEN UPTAKE OF BOTRYTIS CINEREA WITH VARIOUS ORGANIC ACIDS AS SUBSTRATES *

SUBSTRATE	O_2 UPTAKE AS PERCENT OF ENDOGENOUS
Control	100
Pyruvic	224
Acetic	134
Citric	272
α-Ketoglutaric	156
Succinic	195
Malic	110
Glycolic	121
Oxalic	96

* 5×10^{-3} M concentration; pH 4.0.

Since malic and oxalacetic acids appeared to be precursors of oxalic acid, experiments were conducted using these acids as well as oxalosuccinic and succinic acids. These experiments were conducted essentially in the manner described above. Controls for these experiments consisted of flasks without mycelium and flasks containing boiled mycelium. The boiled mycelium was used to estimate the oxalic acid in the tissues at zero time. At the end of a 2 hour incubation period, the dry weights of the mycelia were obtained and the filtrates analyzed for oxalic acid (table IV). Here, as in preliminary experiments, the highest yields of oxalic acid were obtained from malic acid. Considerable yields of oxalic acid were obtained from oxaloacetic and oxalosuccinic acids and relatively small yields from succinic acid.

In experiments with oxaloacetic and oxalosuccinic acids, the spontaneous decarboxylation of these com-

TABLE IV

OXALIC ACID FORMATION BY BOTRYTIS CINEREA IN 2 HOURS ON VARIOUS SUBSTRATES, pH 3.0

SUBSTRATE	μM SUBSTRATE PER 25 ML MEDIUM	μM OXALIC ACID FORMED PER 25 ML MEDIUM	μM OXALIC ACID FORMED PER MG DRY WEIGHT	MOLAR PERCENTAGE CONVERSION
Malic	125	16.6	0.17	13.3
Oxalosuccinic	25	2.2	0.03	8.8
Oxaloacetic	125	9.0	0.11	7.2
Succinic	125	4.0	0.03	3.2

pounds in aqueous solution to pyruvic and α-ketoglutaric acids, respectively (11) must be taken into account. Neither of these breakdown products proved to be precursors of oxalic acid. Thus the molar percentage conversion figures presented in table IV are probably low, if we consider the lowering concentrations of oxaloacetic and oxalosuccinic acids available for enzymatic action as the experiment progressed.

Discussion

The findings presented here suggest that glucose is being oxidized directly to gluconic acid which is phosphorylated (2) to phosphogluconic acid and then proceeds via phosphorylated intermediates to a triose. The phosphorylated pentose in this pathway could be derived from xylose or arabinose. Although ribose was not oxidized, ribose-5-phosphate may be the phosphorylated pentose formed by pathways other than direct phosphorylation (2, 3). Increased O_2 uptake with phosphoglyceraldehyde, the presence of glyceraldehyde phosphate dehydrogenase, and the inhibition of respiration with fluoride suggest that the pathway from triose to pyruvate proceeds according to the Meyerhof scheme. The failure of phosphorylated hexoses, 5-ketogluconate, and phosphoglycerate to enhance O_2 uptake cannot be interpreted since it is probable that these compounds failed to enter the cells.

The stimulation of respiration with acids of the Krebs cycle, the detection of Krebs cycle acids in culture filtrates, plus the inhibition of respiration with malonic acid and the reversal of this inhibition with succinic acid indicate that a Krebs cycle is operating. Enhanced O_2 uptake with acetic and glycolic acids suggests an additional pathway, possibly from succinic to acetic, then to glycolic, glyoxylic, and formic acids (10). Evidence presented here suggests that the main route of oxalic acid formation proceeds via direct oxidation of malic acid. Nord and Vitucci (10) reached this conclusion on the basis of long-term growth experiments. This pathway has now been demonstrated in short-term biosynthesis studies. Failure to obtain appreciable oxalic acid synthesis from glycolic, glyoxylic, and formic acids eliminates the possibility that the pathway from malic to oxalic acid via these intermediates (10) is operating to any apreciable extent in this organism. Some oxalic acid also can be formed by dismutation of oxalosuccinic and oxaloacetic acids. The oxalic acid obtained from succinic acid was probably formed via oxidation of the succinic to malic acid.

A long-term study of oxalic acid formation has shown that most of the acid is formed during the first week of growth. During this period malic acid could not be found in the culture filtrates, although it was the acid most readily found in older cultures. Oxalic acid formation does not take place during the period of maximum growth of the organism, but only during the period when the organism is highly oxidative. In view of the low rate of oxidation of malic acid even in vigorous cultures and the failure of malic acid to accumulate during the early period of growth, it would seem that the malic-to-oxalic route is operating. The formation of oxalic acid then serves as a mechanism for the removal of excess malic acid from the system and permits the Krebs cycle to operate at a rapid rate. The formation of oxalic acid by this organism virtually ceases after 1 week in culture, possibly owing to inactivation of the enzyme system involved or to the accumulation of the end product of the reaction. The pathway from malic to oxalic acid appears to be irreversible since oxalic acid could not be used for growth or as a respiratory substrate. The termination of oxalic acid formation coincides with a sharp decline in O_2 uptake. Presumably all the malic acid being oxidized after this time is going through the Krebs cycle.

The pH value of the medium does not appear to affect oxalic acid formation. It has been reported that alkalinity of the medium is the chief factor in the accumulation of oxalic acid (4). This situation may apply to organisms which utilize the free acid and not the salts, in which cases the alkali serves to trap the acid and make it unavailable to the organism.

Summary

Experimental evidence suggests that glucose is oxidized directly to gluconic acid, and then, via phosphogluconate, pentose phosphate, and triose phosphate, to pyruvate. The organism does not produce CO_2 or lactic acid anaerobically. A Krebs cycle appears to be functioning. Inhibition of respiration with azide suggests that the terminal oxidase system may be mediated entirely by heavy-metal enzymes.

Botrytis cinerea is capable of synthesizing oxalic acid, most of which is formed during the first week of growth. Oxalic acid synthesis does not parallel growth of the fungus but occurs when the organism is in a phase of high oxidation. A decline in O_2 uptake and in glucose utilization is coincident with the cessation of oxalic acid formation. The main pathway of oxalic acid synthesis is via direct oxidation of malic acid, although some oxalic acid may be formed by the hydrolytic dismutation of oxaloacetic and oxalosuccinic acids. Oxalic acid cannot be utilized as a respiratory substrate or as a carbon source for growth.

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NATURE AND DISTRIBUTION OF GLYCOLIC ACID OXIDASE IN PLANTS[1,2]

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Clagett et al (3, 4) reported the presence of a soluble enzyme in the green parts of higher plants which rapidly oxidizes glycolic and L-lactic acids, and Kolesnikov (6) also observed a rapid oxidation of glycolic acid added to ground barley leaves. Glycolic acid was oxidized more rapidly than L-lactic acid, and, among many other compounds tested, only DL-*a*-hydroxy-n-butyric acid was oxidized and this at a very slow rate (3). Tolbert et al (12) further purified the enzyme from tobacco leaves and identified the product of L-lactic acid oxidation as pyruvic acid. With both crude and purified enzyme preparations, the first product of glycolic acid oxidation was glyoxylic acid, which also was oxidized slowly. Oxidation of glycolic acid by purified preparations yielded formic acid and carbon dioxide, but the end products formed by crude preparations were not identified. Tolbert and Burris (11) later reported that the enzyme was absent from etiolated plants but was activated by light; the activation did not follow chlorophyll formation.

Zelitch and Ochoa (19, 20) have obtained a catalase-free enzyme preparation from spinach leaves which was 74 to 80 % pure by electrophoretic and ultracentrifugal criteria and which oxidized both glycolic and L-lactic acids. Apparently only one enzyme was involved in the oxidation of the two acids. They showed that riboflavin monophosphate is the prosthetic group, and that hydrogen peroxide is produced in the oxidation of glycolic acid to glyoxylic acid and in the oxidation of L-lactic acid to pyruvic acid. In the absence of catalase, glyoxylic and pyruvic acids are oxidized non-enzymatically by hydrogen peroxide to formic acid plus carbon dioxide and to acetic acid plus carbon dioxide, respectively. Kenten and Mann (5) also had demonstrated the production of hydrogen peroxide from the enzymatic oxidation of lactic and glycolic acids, and had shown that glyoxylic acid is enzymatically oxidized to oxalic acid in the presence of catalase.

Glycolic and L-lactic acids are present in a wide variety of plants. Reports of their occurrence have been summarized by Rabinowitch (8) and Thimann and Bonner (10). Moreover, Benson and Calvin (2) have found that glycolic acid is one of the early products of photosynthesis. Tolbert and Cohan (13) demonstrated the incorporation of considerable C^{14} into glycine, polyglycine, and serine from C^{14}-labeled glycolic acid added to crude barley juice. Schou et al (9) supplied C^{14}-labeled glycolic acid to Scenedesmus and observed particularly high aerobic incorporation of the C^{14} into phosphoglyceric acid, serine, glutamic acid, and hexosephosphates in the dark, and into phosphoglyceric acid, sucrose, serine, glutamic acid, and malic acid in the light. Anaerobically in the dark, much C^{14} accumulated in glycine.

This paper reports the occurrence of glycolic acid

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