Endogenous and Exogenous Respiration of Conidia of Verticillium albo-atrum

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ABSTRACT

Conidia of *Verticillium albo-atrum* harvested from shake cultures maintained a consistent respiratory response to glucose after up to 6 hr starvation, with RQ's near 1.2. Endogenous respiration decreased consistently with starvation time with a concomitant decrease in RQ from 0.9 to 0.7, indicating a transition to lipid utilization. Cells treated to induce germination responded similarly to glucose, but had slightly lower RQ's. Their unstarved endogenous respiration, expressed as a percentage of comparable exogenous respiration, was less than for unstarved noninduced cells. With increasing starvation time, however, the decrease in endogenous relative to exogenous respiration was less than with equally starved noninduced cells. Furthermore, there was no evident decrease in RQ.

Respiratory response to glycerol was initially similar to that of glucose, but was greater than with glucose after 3-hr exposure to these substrates.

On a dry wt basis, respiratory activity of cells from progressively older cultures decreased through 14 days, with the most evident decreases at 2 to 3 days. On a per cell basis, comparable decreases occurred through 6 days, but respiratory rates then increased with further culture age. Correspondingly, dry wt per cell was relatively constant through 6 days, then increased markedly.

Responses to inhibitors and other added materials varied with the endogenous or exogenous nature of the substrate. Endogenous respiration generally showed a greater response to stimulatory materials, whereas the effect of inhibitory materials was usually more evident with glucose present. Membrane permeability effects and endogenous-exogenous respiration differences are considered as possible influencing factors, though the data preclude strong support of either case. Phytopathology 60:143-147.

Endogenous respiratory metabolism of various microorganisms, particularly bacteria and yeast, has received considerable attention (9). Though fungi other than yeast have in general been less studied in this regard, reports have been noted on such species as *Neurospora crassa* (3), *Fusarium solani* (6), *Myrothecium verrucaria* (12), *Penicillium chrysogenum* (4), and *Aspergillus sojae* (13). Nothing is known about endogenous respiratory characteristics of *Verticillium albo-atrum* other than the observation that respiratory response to certain additives varied with the endogenous or exogenous (glucose) substrate conditions (1). The lack of detailed information on endogenous respiration of this important plant pathogen, and the need for a fuller understanding of its physiology, prompted the present investigation.

MATERIALS AND METHODS.—Details of the origin and maintenance of the *Verticillium albo-atrum* Reinke & Berth. culture used were described previously (1). Cultures for harvest were grown in 250-ml DeLong flasks containing 50 ml Czapek-Dox broth at room temperature on a platform shaker. Flasks were inoculated with plugs from cultures grown on potato-carrot-dextrose agar plates.

At harvest, contents of 8 to 16 flasks were combined and agitated vigorously on a magnetic stirrer for about 3 min. Most of the mycelial growth, generally sparse in comparison with conidia production under these growth conditions, was filtered off on four layers of cheesecloth; the remainder was centrifuged out at 300 *g* for 3 min. Conidia were pelleted from the supernatant fraction and from subsequent wash suspensions by centrifugation at 1,000 *g* for 10 min at near freezing temperature. Cells were washed twice in, and finally suspended in, 0.1 M phosphate buffer (Na_2-K), usually at pH 6. Other pH's used are noted in each case.

As appropriate, cells were subjected to starvation at this point. Most experiments involved starving an aliquot of the total cell suspension from a single harvest; others involved starvation of all harvested cells. Conidia to be compared with starved cells from the same harvest were either suspended and used immediately or stored cold as a pellet and suspended just prior to use. Starvation was imposed at room temperature (24-26°C) on a shaker for the designated time in about 100 volumes of buffer. Starved conidia were washed twice in buffer, as in the initial harvest. Direct comparisons within an experiment necessitated that equal aliquots of cell suspensions be taken, and that final suspensions for rate determinations be equivalent.

O_2 uptake and CO_2 evolution (indirect method) were measured at 30°C by standard manometric technique, using air as the gas phase. Liquid volumes, including 0.2 ml of 20% KOH in the center well for O_2 uptake or 0.2 ml of 6 N HCl in the sidearm for initial and final bound CO_2 measurements, were maintained at 3 ml. Each flask received 1.4 ml of cell suspension in 0.1 M phosphate buffer at the designated pH, resulting in a 0.05 M phosphate reaction concentration. When glucose was added, the final concentration was 0.1 M. Rates were measured at 10-min intervals over a 70-min period, and plotted to provide a calculated hourly rate.

All experiments were done at least three times, with duplicate flasks for each treatment involved. Variation was usually slight between the results of repeated experiments; where obvious variability was encountered, additional experiments were performed for further verification.

RESULTS AND DISCUSSION.—The effect of pH on gas exchange was determined on cells starved 3 hr with the starvation, wash, and reaction media at pH 4, 5, 6, and 7. Except at pH 4, where pthalate was used, all were

buffered with phosphate. Exogenous (hereafter indicated for added glucose) respiration was about 50% of that at the higher pH's, but endogenous respiration was only about 15% less. The fact that pthalate, rather than phosphate, was used at the low pH does not permit a strict comparison between pH 4 and the others. Malca et al. (11) observed no difference in respiratory response between pH 4 and 6, both buffered with phosphate, with glucose added to nonstarved cells of the same organism. There were no differences in rates at pH 5, 6, and 7 in the present study, except that at pH 7 there was an apparent greater CO_2 evolution, and a resulting slightly higher respiratory quotient (RQ). This difference is likely associated with the determination of CO_2 production involving initial and final bound CO_2, although permeability response to pH or some intracellular response cannot be discounted as possible contributing factors. The difference between initial and final bound CO_2 was negligible at pH 6 and lower. Most of the subsequent measurements were made at pH 6, particularly where CO_2 evolution was determined.

Endogenous and exogenous respiratory rates were compared, using nonstarved cells and comparable cells starved for varying times, and the RQ's determined to assess, to the extent possible with such values, the nature of endogenous reserves utilized. RQ's for nonstarved cells were 1.17 with glucose and 0.89 without added substrate (Table 1). Nonstarved cells had endogenous O_2 uptake and CO_2 evolution rates that were about 30 and 50% less, respectively, than when glucose was added. Starvation caused no marked change in the gas exchange or RQ response to glucose. Endogenous rates, however, consistently decreased with increasing length of starvation. O_2 uptake dropped with 6 hr of starvation from a rate that was about 70% of the exogenous rate at the nonstarved stage to about 35% of comparable glucose respiration. This was a decrease of about 60% from the initial endogenous rate, indicating depletion of readily available reserves in this time. RQ's with glucose added were consistently above unity, superficial evidence of lipid synthesis and deposition concurrent with oxidation of glucose. The initial endogenous RQ of 0.89 suggests mixed carbohydrate and lipid utilization. Starvation resulted in a decreasing RQ, indicative of increasing lipid utilization concomitant with rapid depletion of an already low carbohydrate reserve. Though no quantitative estimation of lipids was made, a pronounced fatty-material layer is evident in cell-free preparations from these cells (11, 17). Thus, these data, though subject to the usual dangers of interpretation (2, 6), do suggest a reliance on endogenous lipids in the absence of an exogenous substrate. This is not unexpected, since other fungi have yielded endogenous RQ's evidencing lipid metabolism (6, 10, 13, 14, 18).

Germ-tube formation at the normal 7-day harvest was microscopically evident in about 10% of the cells. Conidia showing more germination, for experiments equivalent to the above, were obtained as follows. Cells were harvested by routine procedure at 6 days and placed in 2.5 times the original volume of fresh growth medium for the remaining 24 hr. This increased the germination percentage 3- to 4-fold. Fresh growth medium for the last 2 days of the 7-day growth period had little more effect.

As with the cells having a lower germination percentage (Table 1), starvation had no marked effect on respiratory response to glucose by cells showing a greater germination percentage (Table 2). RQ's were only slightly lower. Endogenous O_2 uptake, adjusted to a proportion of exogenous respiration of comparably treated cells, reacted to starvation in a different manner to that of cells of lower germination percentage. Relative uptake was lower with no starvation, comparable after 3-hr starvation, and higher after 6-hr starvation. Furthermore, starvation did not cause decreases in the RQ under these conditions as it did for the lower germination condition (Table 1). Although the data are not presented, absolute endogenous O_2 uptake (dry wt basis) was considerably higher for germination-induced cells than for regularly harvested cells. This effect agrees in principle with that reported by Cochrane et al. (6) for *Fusarium solani* germinated spores. The lower endogenous to exogenous respiratory ratio found here for nonstarved, germinated cells as compared to comparable cells of low germination can perhaps be explained by a greater response to glucose by germinated cells. Cochrane et al. (6) also reported a higher RQ for germinated than for ungerminated *F. solani* spores. There may be a relationship between this obser-

TABLE 1. Effect of starvation on exogenous (glucose) and endogenous respiration of conidia of *Verticillium albo-atrum*[a]

Starvation (hr)	Glucose		Endogenous	
	O_2	RQ[b]	O_2	RQ[b]
0	100[c]	1.17	71	0.89
2	101	1.19	57	0.79
3	101	1.20	51	0.76
4	99	1.20	46	0.74
6	94	1.18	30	0.70

[a] All conditions at pH 6 following harvest, including 0.05 M phosphate buffer in respiratory vessels; glucose at 0.1 M when present.
[b] RQ = respiratory quotient.
[c] Respiratory rates are relative, based upon 100 for unstarved cells with glucose present.

TABLE 2. Effect of starvation on exogenous (glucose) and endogenous respiration of conidia of *Verticillium albo-atrum* after induction of germination,[a,b]

Starvation (hr)	Germination %	Glucose		Endogenous	
		O_2	RQ[c]	O_2	RQ[c]
0	33	100[d]	1.13	56	0.91
3	42	95	1.11	47	0.94
6	42	92	1.12	43	0.91

[a] All conditions at pH 6 following harvest, including 0.05 M phosphate buffer in respiratory vessels; glucose at 0.1 M when present.
[b] Germination induced by fresh culture medium during last 24 hr of growth period.
[c] RQ = respiratory quotient.
[d] Respiratory rates are relative, based upon 100 for unstarved cells with glucose present.

vation and the present data with regard to maintenance in germinated cells of a higher RQ even upon starvation of germinated cells, and the consistent rather than decreasing RQ may reflect differences in the nature of and mobilization of reserves important to the germination process. The results suggest that germinated or germination-oriented *V. albo-atrum* conidia do not shift to the lipid utilization pattern suspected at the lower germination level.

Endogenous O_2 uptake was compared with glucose- or glycerol-added respiration, and the effects of 2,4-dinitrophenol (DNP) and arsenite were determined for each condition (Table 3). Conidia were not starved, but respiration was measured directly after harvest and then again after 3 hr in the same reaction medium. The initial respiratory response to glycerol was similar to that of glucose, but respiration was higher with glycerol after 3 hr, probably due to greater uptake with time. DNP stimulation of O_2 uptake for the three substrate conditions was about the same during the 1st hour. With glucose or glycerol present, the DNP-influenced level of respiration was maintained through the later period. However, there was less actual DNP stimulation with added glycerol at the later time as compared to the rate for glycerol alone. DNP-stimulated endogenous respiration was less for the second measurement period than the first; however, relative stimulation compared to the untreated endogenous control was greater than with either glucose or glycerol. Arsenite, at the concentration used, inhibited under all conditions. It is interesting that rates with arsenite for the second measurement period were similar in magnitude, as opposed to the varied untreated rates. These results suggest that metabolic reactions occurring in the presence of an exogenous substrate are more sensitive to this inhibitor than those occurring with endogenous substrate only. There may be differences in metabolic reactions and pathways of utilization of endogenous and exogenous materials; such differences could result in varied responses to added inhibitors. It is realized, of course, that magnitude of cellular uptake may vary with the metabolic activity of the cell, and that this factor cannot be discounted as a major possibility.

The relationship between the age of culture at harvest, dry wt of cells, and endogenous and exogenous O_2 uptake per unit dry wt for cells with and without prior starvation was examined (Fig. 1, left). Harvest was at the times indicated and as described earlier, with half the cell harvest starved 3 hr in each case. Exogenous respiration of starved cells was consistently slightly less than that for nonstarved cells; rates for both cases decreased with increasing culture age. The greatest decreases were evident at the earlier ages, as might be expected. Endogenous rates of nonstarved cells showed a similar pattern, but rates were lower at each interval. The difference between respiration of these cells and exogenous respiration was greatest at the 2-day stage, probably due to a low level of endogenous substrate relative to the potential for respiration at this point. This difference became less with increasing age. No day-to-day marked decrease was evident for endogenous rates of starved cells, but the rate at the 2-day harvest was considerably less than for nonstarved cells. Again, this large initial difference probably reflects a rapid depletion, during starvation, of an already low endogenous substrate supply. After what appears to be a small initial increase in O_2 uptake per unit dry wt, there was a subsequent gradual decrease with age. All respiratory rates were lower for cells from 14-day cultures; the relative position of rates for the four conditions remained the same; but the absolute differences between them were much smaller. Dry wt per cell was constant, or nearly so, through 6 days. At 7 days, however, dry weight was about 70% greater; at 14 days, the increase was about 3-fold.

For comparison, respiratory determinations from the above experiments were also based on cell number (Fig. 1, right). Rate trends on this basis were very similar to those on a dry wt basis through the sixth day. The respiratory rate per cell subsequently increased with age, though not in proportion to dry wt increase through the 14th day. Thus, some but not all of the increased cell wt corresponded to greater respiration per cell. Also, the differences between rates for exogenous as opposed to endogenous respiration again were greater at the 14-day age. While some of the increased cell wt was associated with respiratory potential evident by the response to added glucose, less was readily utilized as endogenous substrate. There was no marked change in the RQ with age for each of the conditions, indicating no major change in the oxidizable substrate in each case.

The general pattern of decreasing respiratory activity per unit dry wt with age basically agrees with that reported for some other fungi (5, 7, 12, 18). The eventual sudden increase in cell dry wt accompanied by an increase in respiration per cell may correspond to certain stages of development designated by Taber (16) to be involved in sequential formation of shunt products.

The foregoing data provide some information on the nature of endogenous and exogenous respiration of *V. albo-atrum*. Additional data are presented in Tables 4 and 5 which further indicate, by the effect on these cells of various additives, that basic differences exist between the reactions or systems involved. Table 4 includes results from both starved and nonstarved cells

TABLE 3. Effect of 2,4-dinitrophenol (DNP) and arsenite on exogenous (glucose, glycerol) and endogenous respiration of conidia of *Verticillium albo-atrum*[a]

Condition	O_2, Hr 0-1	O_2, Hr 3-4
Glucose	100[b]	95
Glycerol	97	147
Endogenous	86	51
Glucose, 0.1 mM DNP	170	166
Glycerol, 0.1 mM DNP	168	177
Endogenous, 0.1 mM	160	113
Glucose, 10 mM arsenite	75	50
Glycerol, 10 mM arsenite	56	42
Endogenous, 10 mM arsenite	53	41

[a] All conditions at pH 6 after harvest, including 0.05 M phosphate buffer in respiratory vessels; glucose and glycerol at 0.1 M when present.

[b] Respiratory rates are relative, based upon 100 for unstarved cells with glucose present.

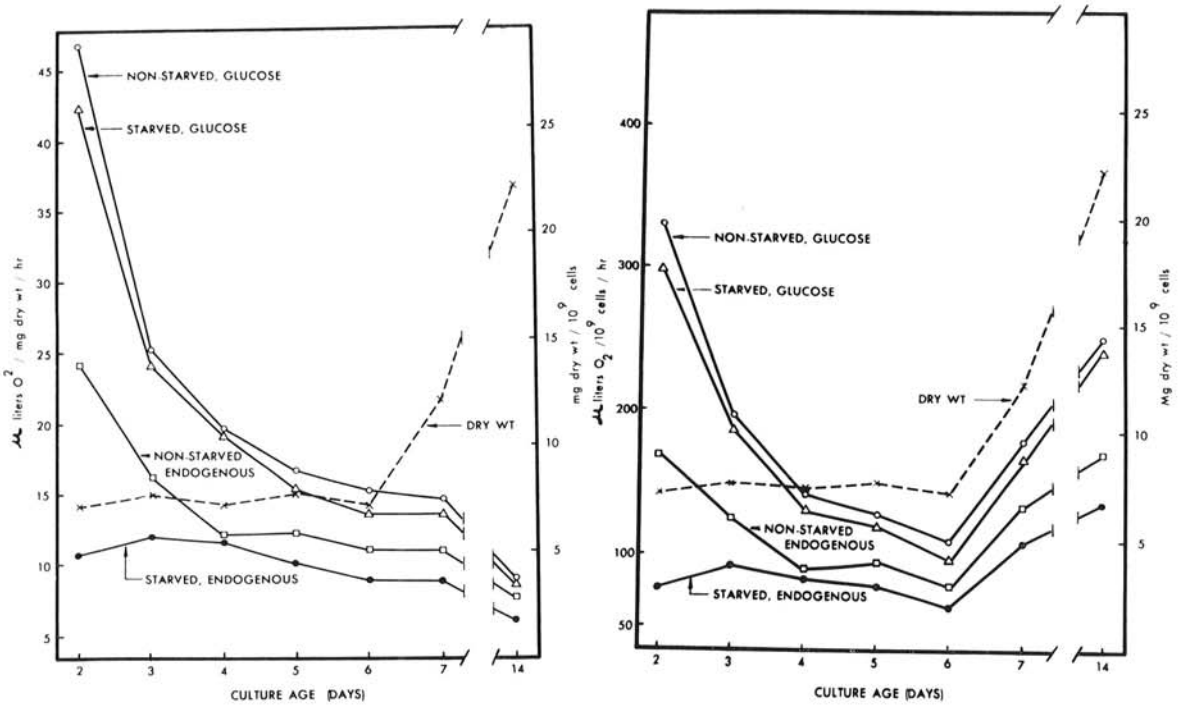

Fig. 1. (Left) Effect of age of culture on respiration (dry wt basis) and cell dry wt of conidia of *Verticillium albo-atrum*. (Right) Effect of age of culture on respiration (cell basis) and cell dry wt of conidia of *Verticillium albo-atrum*.

with all conditions at pH 6, while the results from Table 5 pertain only to nonstarved cells at a pH 7 environment. The responses of starved and nonstarved cells to the additives were generally similar in the presence of glucose. Under endogenous conditions, however, there were differences in the reactions of starved and nonstarved cells to some of the materials, notably azide, methanol, and perhaps *p*-nitrophenol. The differential response was generally greater between endogenous as opposed to exogenous respiration, evident from the effect of DNP, azide, N-ethylmaleimide, arsenate, and dimethylsulfoxide (DMSO) in Table 4, and all the materials in Table 5. With few exceptions, stimulation was more pronounced with endogenous respiration than when glucose was added. Inhibition, on the other hand, was more pronounced where glucose was present. Materials having a similar effect, regardless of substrate condition, were hydroxylamine, antimycin A, ethanol, and acetone.

These data, considered individually, do not indicate any radical departure in metabolic behavior of conidia of *V. albo-atrum*. Azide stimulation of endogenous respiration, noted here, and observed in other fungi (6, 8, 12, 15), has been ascribed to uncoupling action. Certainly, the effect of DNP, and probably several of the other materials which also stimulated respiration, can be related to an uncoupling effect. The general significance of the data seems to lie in the varied responses

TABLE 4. Percentage inhibition or stimulation by certain materials on exogenous (glucose) and endogenous O_2 uptake of conidia of *Verticillium albo-atrum*[a]

Material	Glucose		Endogenous	
	Unstarved	Starved[b]	Unstarved	Starved[b]
0.1 mM 2,4-dinitrophenol	+56	+66	+131	+145
0.1 mM azide	−63	−49	0	+27
1 mM hydroxylamine	−22	−18	−34	−16
1 μM antimycin A	−33	−31	−64	−33
1 mM N-ethylmaleimide	−33	−43	−14	0
10 mM arsenate	−16	−13	+20	+33
10 mM *p*-nitrophenol	−84	−85	−70	−47
7% Ethanol (v/v)	−60	−63	−52	−35
7% Acetone (v/v)	−82	−82	−81	−72
7% Methanol (v/v)	−29	−32	0	0
1 M dimethylsulfoxide	−22	−34	0	0

[a] All conditions at pH 6 after harvest, including 0.05 M phosphate in respiratory vessels; glucose at 0.1 M when present.
[b] Starved 3 hr in 0.1M phosphate buffer.
[c] All figures based on untreated control.

TABLE 5. Percentage inhibition or stimulation by certain materials on exogenous (glucose) and endogenous O_2 uptake of starved conidia of *Verticillium albo-atrum*[a,b]

Material	Glucose	Endogenous
0.1 M EDTA	−30[c]	0
0.1 M fluoride	−47	0
10 mM glutamine	+23	+78
10 mM hydrocinnamic acid	+45	+100
10 mM cinnamic acid	+28	+106
1 mM *p*-nitrophenol	+42	+194
1 mM 2,4-dinitrophenol	0	+52
0.3 mM quinacrine	0	+61
10 mM resorcinol	0	+28

[a] All conditions at pH 7 after harvest, including 0.05 M phosphate buffer in respiratory vessels; glucose at 0.1 M when present.
[b] All cells starved 3 hr in 0.1 M phosphate buffer.
[c] All figures based on untreated controls.

evident with endogenous as compared to exogenous respiration. It is tempting to interpret this as evidence of differential effects on metabolic systems, and that such systems are therefore preferentially operative under these conditions. It is highly possible, however, that active uptake is an associated factor responsible for some of the differential responses noted. Certainly, greater inhibition in the presence of glucose would have to be considered in this light. Cases in which stimulation is greater without an exogenous substrate, or where there is stimulation as opposed to inhibition, present a more complex situation. Even such effects might result from different rates of permeation, causing subsequent concentration effects within the cell that would inhibit or stimulate accordingly. Yet, methanol and DMSO, to which there should be little restriction to entry, each had differential effects. Perhaps also relevant, 1 mM NaCl and 10 mM KCl, $MnCl_2$, Na_2SO_4, and thiourea were among some compounds that did not affect respiration under any conditions used. Therefore, there was no apparent "salt-respiration" effect. The data do not permit a definitive explanation of the factor or factors involved, but they do show for the organism studied that the metabolic status of the cell markedly influences its response to external materials.

The results of this study of endogenous and exogenous respiration of conidia of *V. albo-atrum* expands the understanding of the physiology of this plant pathogen. The need is obvious, however, for verification of the reserve lipid role in endogenous respiration. Furthermore, the interrelationships of metabolism, cell mass increase, nature of cellular accumulation of reserves and other materials, and growth dynamics need to be examined intensively. The endogenous-exogenous respiratory balance, and the possibility of basic differences between the systems involved, seem worthy of further investigation in regard to the relation of the fungus cell to its microenvironment. Such information can only enhance an eventual unraveling of the complexities of the host-pathogen interaction as a part of the study of the incited disease.

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