Some physiological responses of *Verticillium albo-atrum* to zinc

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Zinc markedly stimulated growth of *Verticillium albo-atrum* R. & B. in Czapek-Dox broth shake cultures. Minimum zinc concentration producing optimum growth response was 0.15 to 0.2 µg/ml. On a dry weight basis added zinc resulted in increased total nitrogen content and oxygen uptake. Oxygen uptake per unit total nitrogen was essentially unaffected. Cells from 5- and 7-day-old zinc-free cultures showed less response to zinc than did those from 3- and 10-day-old cultures. Zinc response was greatest with L-alanine as the nitrogen source as compared with nitrate, urea, and ammonium nitrate sources.


En culture agitée, dans le bouillon de Czapek-Dox, le zinc a sensiblement stimulé la croissance de *Verticillium albo-atrum* R. & B. La concentration minimale de zinc capable d'entrainer une croissance optimale était de 0.15 à 0.2 µg/ml. Sur la base du poids sec, le zinc ajouté a provoqué une augmentation dans le contenu total d'azote et dans la consommation d'oxygène. La quantité d'oxygène consommée par quantité unitaire de l'azote total n'a pas été sensiblement affectée. Les cellules cultivées pendant 5 et particulièrement 7 jours en absence de zinc ont moins réagi à une addition de zinc que celles cultivées dans les mêmes conditions pendant 3 et 10 jours. La réponse au zinc a été plus forte avec la L-alanine comme source d'azote qu'avec le nitrate, l'urée et le nitrate d'ammonium.

**Introduction**

Enhancement of growth of many fungi in culture by zinc is well known (2, 3, 4, 7). This has been attributed in general to catalytic effects on carbohydrate metabolism, enabling the organism to more efficiently use the carbon source available. It has been suggested that zinc functions in some regulatory manner in the metabolic interrelationships of carbon and nitrogen (6). Specific roles of zinc relate to its being a component or activator of certain enzymes and to its requirement for normal RNA and ribosome production (8, 9).

Initial tests in the present investigation showed that zinc added to commercial Czapek-Dox broth markedly stimulated growth of *Verticillium albo-atrum* R. & B., resulting in a several-fold increase in dry weight production. As the zinc effect on growth was so pronounced, more definitive data were sought.

**Materials and Methods**

Stock cultures of *V. albo-atrum*, originally isolated from diseased cotton, were maintained on potato-carrot-dextrose-agar plates under fluorescent light at 25°C. The basal liquid medium used for fungal growth was Czapek-Dox broth (Difco), made with glass-distilled water. Where indicated, zinc as ZnCl₂ was added to the medium before autoclaving.

Growth of inoculum for subsequent zinc experiments was in the basal medium without zinc. This growth period was normally 7 days, but ranged from 3 to 10 days for the culture age study. Metal-capped flasks (250 ml), each containing 50 ml medium seeded with one plug from plate cultures, were used. Flasks were agitated on a platform shaker at about 100 cycles/min under fluorescent light at 25°C. At harvest, the contents of 8 to 16 flasks were pooled, strained through four-layered cheesecloth to remove hyphal growth, and then centrifuged at about 1000 × g for 10 min to concentrate the conidia (unicellular, yeast-like structures produced profusely by this organism in liquid shake culture (1, 12)). Conidia were then washed by suspension in sterile distilled water and recentrifuged, and homogenous inoculum suspensions in water were prepared.

Growth for experimental determinations was in either the 250-ml flasks mentioned above, four per treatment containing 50 ml medium each, or 2000-ml Erlenmeyer flasks, one per treatment containing 400 ml medium each. The latter were used in the culture age experiments. The smaller flasks were each inoculated with 0.5 ml conidial suspension, equivalent to conidial production in about 4 ml inoculum growth medium. The large flasks were each inoculated with 10 ml suspension, equivalent to the conidial production in about 150 ml inoculum growth medium. Greater inoculum density was used in this case because of the shorter growth period to be used. No further attempt was made to rigidly regulate inoculum concentration, as all flasks of a single experiment received equal volumes of the same homogenous suspension.

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At the end of the indicated time period, total growth (hyphae and conidia) was collected by centrifugation (1000 \( \times g \) for 10 min) and washed as described above. Harvest from the smaller flasks, where only dry weight production was measured, was then transferred to tared foil cups, one for each flask, for drying. Growth from the larger flasks, in the age study where nitrogen and respiratory measurements were also made, was blended 15 s in water to obtain an even suspension, then brought to a known volume. Duplicate samples from this suspension were used for dry weight, total nitrogen, and respiratory determinations. Total nitrogen was determined by micro-Kjeldahl analysis. Oxygen uptake was measured at 30°C by standard manometric technique (11), with KOH in the center well and cells suspended in 0.05 M phosphate buffer (Na\(_2\)-K) at pH 6.

All experiments were repeated at least twice, with zinc-free controls in each case. The results presented represent means obtained from the various replications of treatments and experiments.

Results and Discussion

Initial trials showed that zinc added at 5 \( \mu \text{g/ml} \) to the basal Czapek-Dox broth caused a marked increase in dry weight production by \( V. \) albo-atrur. Additional tests were then made to determine the response to various concentrations, ranging up to 16 \( \mu \text{g/ml} \) actual zinc. No growth response was evident at 0.001 and 0.005 \( \mu \text{g/ml} \). Increasing stimulation resulted from increasing concentration from 0.01 to 0.1 \( \mu \text{g/ml} \), and maximum response was attained at 0.15 \( \mu \text{g/ml} \) through the highest concentration examined. No evidence of toxicity was apparent over this range. The concentration range for maximum response in this organism thus corresponds well with that quoted by Cochrane (3) for fungal growth response to zinc. The magnitude of stimulation varied from time to time in the present work, perhaps associated with inoculum variation. While the average response was about a five-fold increase, the range was from about three- to seven-fold. Such variability may be related to genetic instability of this organism, apparent from periodic fluctuations in its morphological appearance.

Effects of zinc on dry weight accumulation, total nitrogen content, and respiration were determined on cells harvested from 3-, 5-, 7-, and 10-day-old zinc-free cultures. Determinations were made after 72 h exposure to zinc (Table 1). The expected stimulation of dry weight production was evident; it was, however, noticeably less for the 7-day-old cultures and some less for the 5-day-old cultures compared to the other ages. Essentially the same pattern occurred in total nitrogen accumulation and total respiratory potential. Nitrogen content (dry weight basis) was consistently higher, about half again as much, for all culture ages examined. Net respiratory activity was higher on a dry weight basis but not on a nitrogen basis. It is possible that the increased nitrogen content reflects increased protein production, part of which might be enzymes involved in the respiratory process. This interpretation would agree in principle with the projected role of zinc being essential to nitrogen-carbohydrate relationships (6).

The reduced response to zinc by 7-day and, to a lesser extent, 5-day culture cells is puzzling. This characteristic is particularly evident in that cells from 10-day cultures again responded essentially like those from 3-day cultures. As no observations for lysis were made, it cannot be discounted that 10-day cultures had lysed sufficiently to allow production of enough young cells to resemble the 3-day cultures. It nevertheless is apparent that cells from the

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Culture age in days as inocula spores</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Total dry wt. produced</td>
<td>+326(^a)</td>
</tr>
<tr>
<td>Total N accumulated</td>
<td>+453</td>
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<tr>
<td>N content</td>
<td>+39</td>
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<tr>
<td>Total ( \text{O}_2 ) uptake</td>
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<tr>
<td>( \text{O}_2 ) uptake per unit dry wt.</td>
<td>+32</td>
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<tr>
<td>( \text{O}_2 ) uptake per unit N</td>
<td>+2</td>
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</tbody>
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\(^a\)Growth in Czapek-Dox broth; Zn at 5 \( \mu \text{g/ml} \) added as ZnCl\(_2\).

\(^b\)Data represent percentage changes from controls of equivalent cells grown without Zn.
intermediate culture age exhibited an associated ebb in their response to zinc in comparison with younger and perhaps older cultures. That this effect was not evident in total nitrogen accumulation is additionally puzzling. A straightforward explanation of these differences is not obvious. Possibly some sort of altered metabolism at this age or some aspect of culture growth dynamics influences the response to zinc. Prior work has shown that at the 6- to 7-day culture age cells of this organism start to show a definite increase in dry weight per cell (10). Of the ages examined, the 7-day growth period produced cells that varied most in their response to zinc. If this general age is truly one of altered response to zinc, the variability encountered could be related to attempting to associate a given chronological culture age with a definite morphological and physiological cellular condition.

As zinc seemed to function in some manner to promote nitrogen utilization, or perhaps in nitrogen–carbohydrate interrelationships, the effect of zinc in the presence of different nitrogen sources was examined. Verticillium albo-atrum has been reported to be able to use a variety of nitrogen sources (5). Sources used here were sodium nitrate (as in Czapek-Dox broth), urea, ammonium nitrate, and L-alanine, at equivalent actual nitrogen concentrations. Inocula from 7-day cultures were used, and response was determined after a 7-day growth period. A stable pH was maintained in this experiment with phosphate at 0.2 M, pH 7.3, added to the medium. The four nitrogen sources were essentially equivalent in their effect on dry weight production with no zinc present. The greatest response to zinc at 5 µg/ml was with L-alanine, less with nitrate, and still less with both urea and ammonium nitrate. The greater response with the amino acid nitrogen source than with the other sources may be presumptive evidence that the zinc effect is most closely allied with carbohydrate and organic nitrogen relationships. The lesser response with urea present, however, precludes more than mere speculation on this point. The present data are not of the type to allow any serious conjecture on the possible role of zinc on RNA-ribosome metabolism (8, 9).

It is clear from the present work that zinc is essential to V. albo-atrum to the extent that it markedly enhances growth, as is the case with many other fungi, and at comparable concentrations. The increase in nitrogen content and respiratory activity per unit dry weight indicates some sort of involvement with nitrogen utilization and interconversions with carbohydrates, perhaps in the formation of proteins, including enzymes. Specific roles of zinc in this organism remain to be established.