Growth Response of Sclerotium rolfsii to the Herbicide EPTC in Liquid Culture and Soil

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Supported in part by Research Grant 12-14-100-8015 from the Crops Protection Research Branch, ARS, USDA, and by Public Health Service Grant UI-00152 from the National Center for Urban and Industrial Health.

Accepted for publication 1 October 1969.

ABSTRACT

Effect of the herbicide EPTC (ethyl-*N*,*N*-dipropylthiolcarbamate) on growth of *Sclerotium rolfsii* was determined in liquid and soil cultures. In modified Czapek's solution, mycelial production was inhibited by all concentrations of the herbicide (10, 25, 50, and 100 μg/ml), inhibition being directly proportional to concentration. A corresponding decline in utilization of glucose, NO_3-N and inorganic phosphate (Pi) was observed. Increase in titratable acidity of the culture medium at the two highest herbicide concentrations, and an increase in the ratio relating glucose consumed in Pi uptake, suggested a possible action of the herbicide in the respiratory cycle of the fungus. In soil cultures containing 0, 1.0, 2.5, 5, and 10 μg of EPTC/g, growth of *S. rolfsii* was measured in terms of saccharase activity. Enzyme activity increased at herbicide concentrations of 5 and 10 μg/g. The ratio of saccharase activity to glucose that was removed increased with increasing herbicide concentration. Phytopathology 60: 431-436.

Literature reviews by Audus (2), Bollen (4), and Fletcher (8, 9) indicate that certain herbicidal compounds in soil may cause an increase or decrease in total microbial populations, but long-term effects seldom occur. Herbicides may vary greatly in their effects on various groups of soil microorganisms, being either toxic or stimulatory (27). Kaufman (14) found that *s*-triazine and phenylurea herbicides significantly affected populations of *Fusarium* spp. in corn- and soybean-cropped soil; the effect varied with the herbicide and cropping system.

Inhibition of growth or respiratory processes of several plant-pathogenic fungi by herbicides has been demonstrated in laboratory cultures (3, 5, 17, 29). Previous investigations in our laboratory have revealed inhibitory or stimulatory effects of herbicides on growth of *Sclerotium rolfsii* (7, 23, 24, 25, 26), *Rhizoctonia solani* (22), *Fusarium oxysporum* f. *vasinfectum* (6), and *Trichoderma viride* (7, 24, 26).

Richardson found that some herbicides and insecticides either increased or suppressed disease development in barley (19) or tomato (20). Huber et al. (12) observed a reduction in severity of foot rot of winter wheat following fall applications of diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. Altman & Ross (1) demonstrated increased disease severity in sugarbeets when soil was treated with *R. solani* and either pebulate (*s*-propyl butylethylthiocarbamate) or pyrazon [5-amino-4-chloro-phenyl-3 (2H) pyridazinone] before planting.

EPTC (ethyl-*N*,*N*-dipropylthiolcarbamate) is used for control of certain broad-leaf weeds and grasses in a variety of field crops, usually at rates of 1-4 lb./acre. Its action apparently is one of contact toxicity, and it probably acts as an antimetabolite blocking essential reactions in root cells and causing rapid local injury (2). The action of EPTC on soil-borne plant-pathogenic fungi is not known. Since *S. rolfsii* commonly inhabits the organic matter in soils of warm regions, it may frequently come in contact with this herbicide. The following study represents part of a series of investigations to determine the influence of herbicides on growth-related activities of soil fungi.

MATERIALS AND METHODS.—Technical grade EPTC (Eptam) was supplied by Stauffer Chemical Co., and the isolate of *S. rolfsii* Sacc. used was obtained from *Trifolium repens* L. and maintained on potato-dextrose agar (PDA).

Liquid culture experiment.—The basal medium was filter-sterilized Czapek's solution modified to contain 30 g of glucose/liter, and dispensed in 25-ml quantities in 125-ml flasks. The medium was adjusted to pH 3.0 with 0.0625 M citric acid buffer to prevent wide fluctuations in pH caused by oxalic acid production by the pathogen (15). From alcoholic stock solutions of the herbicide, serially diluted in 95% alcohol, 0.1 ml was added to each flask to provide herbicide concentrations of 0, 10, 25, 50, and 100 μg/ml. The flasks were inoculated with single mycelial discs (1-cm diam) from 3-day-old PDA cultures of *S. rolfsii*, and incubated at 27 C. Samplings were made from four flasks/treatment at 0, 3, 5, and 11 days after inoculation. The mycelium from each flask was washed, oven-dried, and weighed. Filtrates from two flasks were pooled, restored to initial volume, and analyses for residual nutrients were performed.

Glucose was determined by Nelson's method (18), inorganic phosphate (Pi) by the vanadomolybdophosphoric procedure (13), and nitrate nitrogen by the disulphonic acid method (10). In each case, serial dilutions of the culture medium were performed to bring the nutrient concentration within the range of the methods. Hydrogen ion concentration was measured with a Beckman H-2 pH meter, and conductivity of the medium was determined by means of an Industrial Instrument Model RC 16B2 conductivity bridge equipped with a conductivity cell (K = 2). For titratable acidity, 10 ml of filtrate was titrated with 0.1 N NaOH to a pH of 9.0.

Soil experiment.—Norfolk sandy loam soil was col-

lected from a field plot that had not been fertilized for 10 years. The soil was air-dried, screened (2 mm), and lightly moistened for autoclaving. One hundred g (oven-dry wt) were delivered into 250-ml flasks and sterilized by autoclaving.

Inoculum of *S. rolfsii* was prepared by chopping 48 mycelial discs (7-mm diam), from 7-day-old PDA cultures for 30 sec in 40 ml of sterile demineralized water in a sterile Monel semimicro blender. The suspension was aseptically diluted in 680 ml of sterile water. Two ml of inoculum were pipetted in a straight line across the soil surface of each flask. Twenty-four hr was allowed for the fungus to become established before EPTC in a nutrient solution (10 ml) was added. The nutrient solution was prepared so that 10 ml provided per flask: 300 mg of glucose; 2.80 mg of NO_3-N as KNO_3; and 1.78 mg of P as K_2HPO_4. The solution was adjusted to pH 5.5 with 50% lactic acid and divided into six equal parts. A stock solution of EPTC was prepared in 95% ethyl alcohol, and a series of dilutions from this stock was prepared. Aliquots of these solutions were added to portions of the nutrient solution to provide, when added to soil, concentrations of 1.0, 2.5, 5.0, and 10 μg EPTC/g oven-dry soil. The final alcohol content of the nutrient solution was 0.5% (v/v). Controls with alcohol alone and nutrient solution alone were included. The final moisture content of soil following treatment was 60% of the moisture-holding capacity.

The flasks of inoculated soil were incubated at 27 C, and samplings were made after 3, 4, 7, and 10 days. Eight flasks/treatment (or control) were sampled each time; the flask contents were pooled in pairs providing four replicates. The soil was air-dried at room temperature (26 ± 1 C) for 24 hr, then lightly ground to break the crumbs and stored until analyses were performed. Methods used for the determination of NO_3-N, titratable acidity, and pH have been described previously (23). For glucose determination, 10 g of soil were shaken with 20 ml of water for 30 min, and the suspension was centrifuged at 3,000 rpm for 20 min. Aliquots of the supernatant, or dilutions of them, were then analyzed for reducing sugars by Nelson's procedure (18).

The procedure for determining saccharase activity of *S. rolfsii* in soil has been described previously (21). Mycelium from Czapek's liquid cultures was chopped in 25 ml of cold demineralized water in a sterile Monel semimicro blender. The suspension was diluted with sterile water, and increasing amounts were added to 20 g of autoclaved soil in a series of flasks. The soil was treated with toluene to suppress growth, and saccharase activity was determined according to the method of Hofmann (11). The results were used to construct a line relating enzymatic activity to increasing mycelial dry wt in the soil. To test whether EPTC affected saccharase activity, this procedure was repeated in presence of 10 μg of the herbicide/ml of reaction mixture. Since saccharase activity in soil cultures of *S. rolfsii* maintains linear relationships with mycelial dry wt and CO_2 production (21), this served as a guide for determining effect of EPTC treatments.

Data from both liquid and soil experiments were statistically analyzed by procedures for simple factorial experiments, and differences between means were compared by the sequential method described by Snedecor (28). Mean differences referred to in this paper were significant at the 5% level of probability unless otherwise stated. Correlation coefficients and regression equations were calculated where pertinent.

RESULTS.—*Liquid culture.*—Growth of the fungus (Fig. 1) was significantly lower in all EPTC treatments than in the herbicide-free control. Reduction in growth was directly related to increasing concentrations of the herbicide. Production of mycelium for the control at the last sampling (11 days) was five times that for the 100-μg treatment. The rate of growth of the fungus ($\Delta M/\Delta T$), measured as the average growth increment/day, was highest for the control during the first 3 days of incubation. Growth rates in the EPTC treatments were highest between 3 and 5 days after inoculation, except for the 100-μg treatment, which showed a maximum rate of growth in the interval between the last two samplings.

The amount of glucose utilized in different treatments was inversely related to the amount of EPTC added; this is reflected in Fig. 2 by residual glucose in the medium. The daily rate of glucose uptake remained essentially constant for the control during the first 5 days after inoculation. For the herbicide treatments, rates of glucose uptake were highest between 3 and 5 days after inoculation. Rates for the last 6 days of the experiment were higher for the herbicide treatments than for the control.

Economic coefficients (EC) relating the amount of dry mycelial matter produced to the amount of glucose consumed are shown for two sampling dates in Fig. 3. Five days after inoculation, the value declined constantly with increasing amounts of the herbicide. The lowest value for the 11th day was in the 50-μg treatment.

Uptake of Pi (represented in terms of residual P, Fig. 4) revealed a pattern similar to that for glucose, being inversely related to the concentration of herbicide in the medium; however, the difference between the control and the 10-μg treatment was pronounced only at the 5th-day sampling. The daily rate of Pi uptake was generally higher in the control than in the herbicide treatments, as indicated by the first two samplings; the rate then decreased for the remaining period. The EC values (Fig. 5), representing the ratio of mycelium produced to the amount of Pi removed, showed a constant decline for the 5th-day sampling as herbicide concentration was increased. For the last sampling (11 days), the lowest EC value was in the 50-μg treatment, followed by a rise in the 100-μg treatment. Thus, this pattern also was similar to that for glucose. The ratio relating the amount of glucose consumed to Pi removed (Fig. 6), calculated at the 5th-day sampling, revealed an increase in values for herbicide treatments of 25 μg and above. Values for the 11th-day sampling are not shown, since the pattern was similar to that for the 5th day.

The amount of NO_3-N (Fig. 7) in the medium

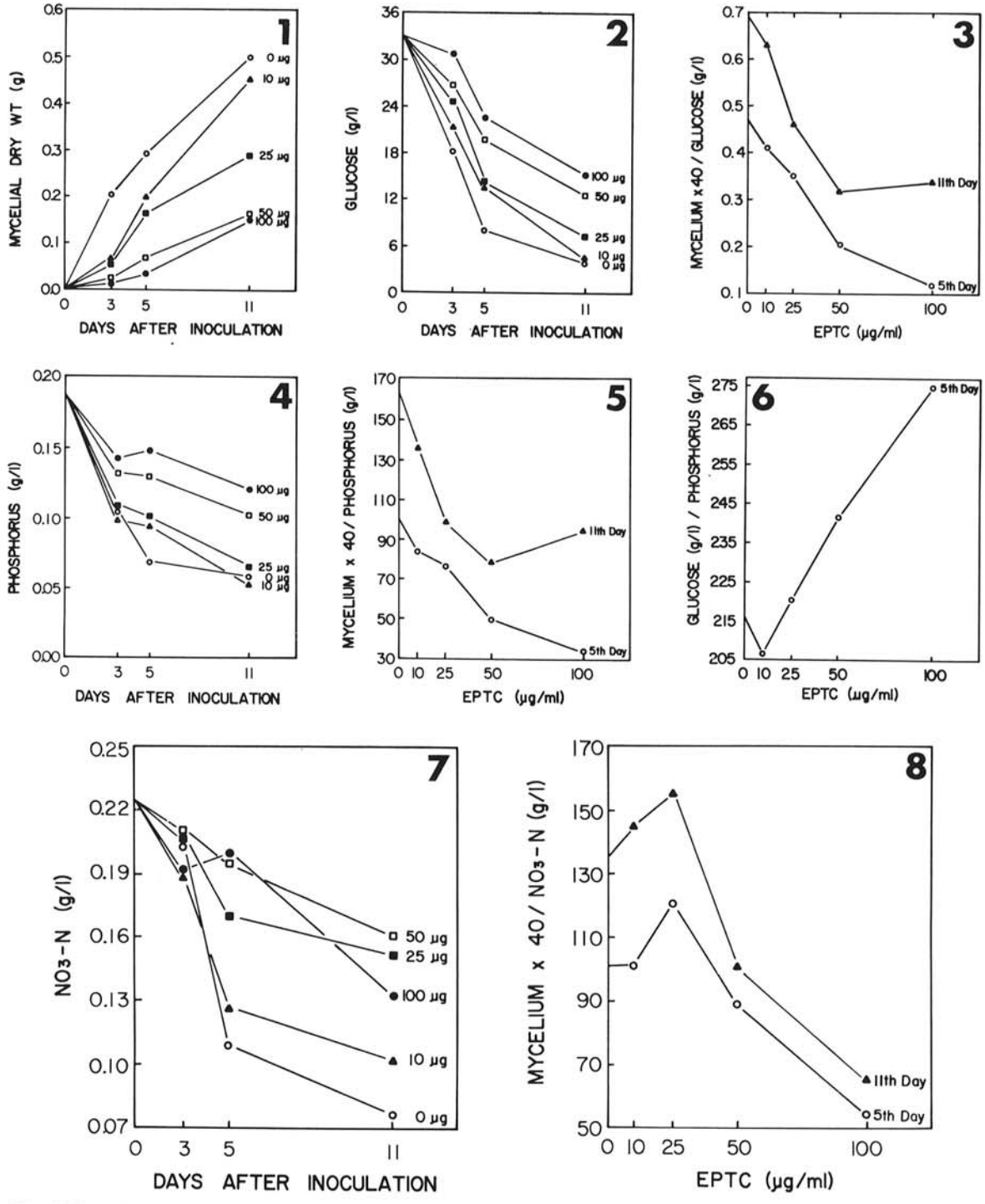

Fig. 1-8. Effect of EPTC on mycelial production and related growth responses of *Sclerotium rolfsii* in modified Czapek's liquid medium. **1)** Mycelial dry wt. **2)** Residual glucose in the medium, indicating quantity consumed. **3)** Ratio of mycelium produced/glucose consumed. **4)** Inorganic phosphorus in the medium. **5)** Ratio of mycelium produced/P consumed. **6)** Ratio of glucose/P consumed. **7)** Nitrate-N in the medium. **8)** Ratio of mycelium produced/ NO_3-N consumed.

generally decreased with time, but appreciable differences in the rate of uptake between treatments did not become apparent until the 5th-day sampling. Analysis at this time showed that the amount of nitrate removed from the medium was related to the amount of EPTC present. Rate of NO_3-N uptake between the 3rd and 5th days of the experiment was higher for the control than for any of the herbicide treatments. The rates subsequently decreased in all treatments except 100 μg. For the 100-μg treatment, the rate of uptake was reduced between 3 and 5 days, then increased sharply; the final residual NO_3-N value was less than in the 25- and 50-μg treatments. Economic coefficients (Fig. 8) calculated on the 5th and 11th days demonstrated a pattern different from that of glucose and Pi. Thus, herbicide concentrations of 10 and 25 μg/ml resulted in an increase in the EC value, followed by a sharp decrease for the two highest EPTC concentrations.

Titratable acidity (Fig. 9) of the culture medium after 3, 5, and 11 days of incubation varied considerably with herbicide treatment. Acid values for the control and two lowest herbicide concentrations had increased at 3 days, then declined to low values at 11 days. The lowest value for the control, however, was found at 5 days. Acid production in the 50-μg treatment increased sharply for 5 days, then dropped to a value that was not significantly different from the initial content. For the 100-μg treatment, the acid value had decreased slightly at 3 days, then increased to a maximum at 11 days. The daily rate of acid production for this treatment, however, became progressively less with time.

Conductivity of the medium (Fig. 10) showed generally a direct relationship to changes in titratable acidity. Thus, values for the control and two lowest levels of EPTC decreased sharply to a minimum at 11 days. The value for the 50-μg treatment increased to a maximum at 5 days after inoculation, and then declined. All of these values at the final sampling were far below the initial conductivity of the culture medium. The value for the 100-μg treatment increased consistently after the 3rd day, but at a declining daily rate.

Values for pH of the medium (Fig. 11) varied inversely with titratable acidity, as one would expect. Very low values were evidenced for the three highest EPTC levels at the 3rd- and 5th-day determinations. The pH for the 100-μg treatment remained low for the duration of the experiment.

Soil culture.—Effect of EPTC on the relationship

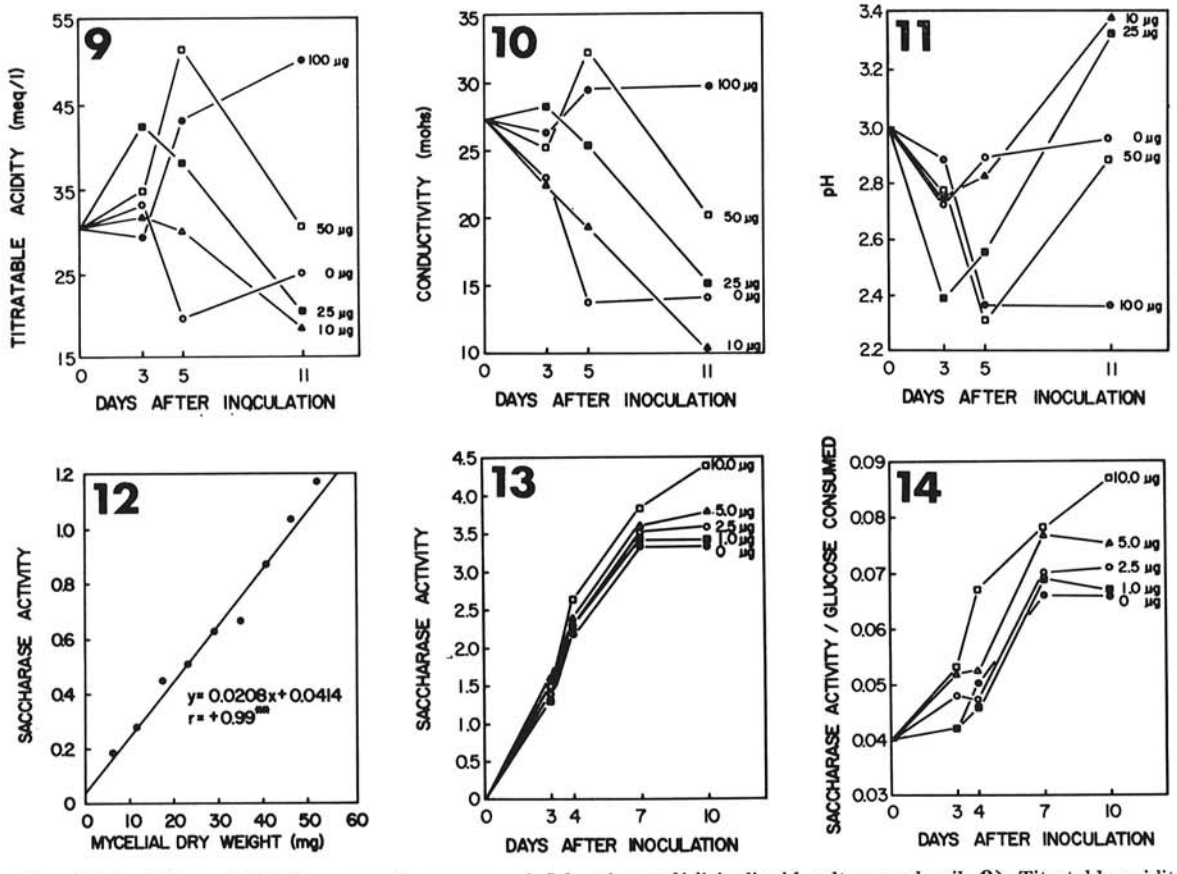

Fig. 9-14. Effect of EPTC on growth response of *Sclerotium rolfsii* in liquid culture and soil. **9)** Titratable acidity of the liquid culture medium during incubation. **10)** Conductivity of the liquid medium. **11)** pH of the liquid medium. **12)** Relationship between saccharase activity and mycelium added to autoclaved soil. **13)** Saccharase activity of the fungus in soil culture during 10 days' incubation. **14)** Ratio of saccharase activity/glucose consumed.

TABLE 1. Glucose uptake by *Sclerotium rolfsii* in herbicide-treated soil, as reflected by the amount of residual glucose (mg/100 g soil) present after intervals of incubation[a]

EPTC (μg/g)	3 days	4 days	7 days	10 days
0	108.25	40.84	14.64	5.56
1.0	107.28	19.17	17.84	6.44
2.5	113.68	20.19	17.60	8.34
5.0	115.13	31.62	18.57	7.48
10.0	123.95	61.58	16.85	8.69

[a] Initial value = 300 mg.

between saccharase activity and mycelium (dry wt) added to autoclaved soil is illustrated in Fig. 12. No significant difference was observed between the linear equation obtained in the presence of EPTC and the corresponding equation for the control. In both cases the correlation coefficient was $r = 0.99^{**}$.

Saccharase activity (Fig. 13) in all treatments increased with time during the 10-day incubation period. Whereas no significant differences were detected between treatments in the first sampling at 3 days, saccharase values for the 5.0- and 10-μg treatments were significantly higher than values for any other treatment in the last three samplings of the experiment. After 4 days of incubation, saccharase activity for the 10-μg treatment was significantly higher than for any other treatment. The difference between mean saccharase activity at the two highest herbicide concentrations did not become significant until the 7th-day sampling; this difference was even more pronounced by the 10th day.

Rate of glucose uptake by the fungus was maximal for all treatments in the first 4 days after inoculation (Table 1). The 10-μg treatment had significantly higher amounts of residual glucose at the first two samplings, but this effect disappeared with time. Thus, no significant differences in residual glucose were detected at the last two samplings. Calculation of ratios (Fig. 14) relating the amount of saccharase activity in the soil to the amount of glucose removed showed increased values with increased herbicide 7 and 10 days after inoculation.

Significant differences in pH values (Table 2) were detected only at the first two samplings and for the two highest herbicide concentrations; values for these two treatments were higher than all others in both cases and titratable acidity values were correspondingly smaller. These differences vanished with time.

TABLE 2. Changes in soil pH with growth of *Sclerotium rolfsii* in herbicide-treated soil determined at intervals after inoculation[a]

EPTC (μg/g)	3 days	4 days	7 days	10 days
0	4.28	3.92	4.13	4.24
1.0	4.28	3.93	4.10	4.23
2.5	4.35	3.91	4.12	4.23
5.0	4.47	3.99	4.10	4.26
10.0	4.53	4.04	4.07	4.27

[a] Initial soil pH value = 5.8.

DISCUSSION.—In liquid culture, the proportional decrease in uptake of nutrients by *S. rolfsii* with increasing concentration of EPTC indicated a general antimetabolic action of the herbicide similar to its effect on higher plants (2). This action was reflected in the difference obtained for daily rates of growth and glucose utilization between the control and heribicide treatments. These rates were maximal in the first 3 days for the control and between the 3rd and 5th days for the herbicide treatments. Economic coefficients furnish additional evidence for a general antimetabolic effect of this compound. These coefficients indicate that the "efficiency" of nutrient utilization declined with increasing amount of the herbicide. The only exception to this was the NO_3-N coefficient, which showed an increase at the 25-μg level of EPTC.

The increase in titratable acidity with high concentrations of EPTC (25-100 μg/ml) is of particular significance with regard to mode of action against the pathogen. Acidity produced by this fungus under the conditions of the experiment was largely due to oxalic acid (15). The acid is formed by the action of glyoxylate dehydrogenase coupled to the glyoxylate bypass in the TCA cycle (16). Accumulation of acid with no increase in mycelial production could result from a blockage by EPTC in the TCA cycle, which would shuttle glucose into increased production of oxalic acid. This is suggested by the increase in value of the glucose/phosphorus ratios (Fig. 6) in response to added herbicide. Although the exact nature of the inhibition cannot be shown with the data available, the results suggest that the action of the herbicide at the concentrations used in the liquid culture experiment could be related to abnormalities in the respiratory pathways of the fungus.

The progressive increase in production of organic acid with increased concentration of the herbicide was also reflected in the close relation of changes in conductivity to changes in titratable acidity. Thus, conductivity was more affected by the uptake of Pi, nitrate, and other ions in the control and the 10-μg treatments than by the production of acids; the reverse was true for treatments containing higher concentrations of the herbicide (Fig. 10).

Interpretation of data from the soil experiment is based on the known direct relationship between saccharase activity and amount of mycelium in soil (21). Since the presence of the herbicide did not affect enzymatic activity with the method of assay used, saccharase activity may be considered as equivalent to mycelial production, although the exact correspondence is not known. The results then indicate that presence of the herbicide in the range of 5-10 μg/g promoted growth by the fungus in soil culture. This explains the increase in the saccharase/glucose ratios (Fig. 14). The soil experiment represents a range of herbicide concentrations that includes recommended field rates; these rates were not included in the liquid culture study. Since no significant accumulation of titratable acidity was detected at the concentration used in the soil experiment, an explanation of the effect of the herbicide on the fungus cannot be advanced at present.

It should be pointed out that figures for concentrations of the herbicide used represent the amounts of the compound added to the soil initially, but no accurate measure of the active concentrations can be given. The soil culture represents a system which, unlike the liquid culture, possesses sorptive properties.

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