Cellular Basis of Growth Rate Differences in Isolates of Rhizoctonia solani: Choice of Isolates, Properties, and Content of DNA, RNA, Protein, and ATP

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ABSTRACT

Investigations were made on the biochemical bases for differences in the growth rate among some isolates of *Rhizoctonia solani*. Isolates of this fungus were sought whose growth rates relative to each other were different, but which remained in the same order relative to each other under a wide range of nutritional conditions, in response to plant hormones and other growth-promoting metabolites, and under different temp. Three out of the eleven isolates examined possessed these characteristics and were used in these studies. The concns of several important metabolites were determined for these isolates. DNA varied between 0.4 and 0.6%; RNA was constant about 11.5%; and protein ranged between 27.5 and 36.4%. The different isolates contained between 2,870 and 4,900 picomoles of ATP per mg dry weight tissue. The amounts of DNA, RNA, and protein did not correlate with the growth rates of the isolates, nor did the amount of ATP, on a dry weight basis. Indoleacetic acid was deleterious to growth at concns between 1.0 and 40 μg per ml, kinetin increased growth at various concentrations between 10 and 60 μg per ml but there was no clear response to a concentration gradient with either hormone. Gibberellic acid was not stimulatory to any of the three isolates between 50 and 250 μg per ml, but small reductions in growth occurred at all the concentrations that were used. Differences in the growth rates of these isolates could not be attributed to deficiencies in nutrients or other growth-promoting substances or to different temperatures.

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One of the most readily observed and measured variables among individuals of a species is the difference in rate of growth. This characteristic is important in plant pathogenic fungi because competition between various organisms in soil can be affected by differential growth rates, and so may determine which strain of the pathogen predominates. More directly it could be responsible, among other factors, for the virulence of any one strain of the pathogen compared to other strains.

The axenic rate of growth of an organism as ordinarily seen is dependent on genetic determinants, nutrient availability, metabolic activity, and environmental influences. Growth is the result of many individual anabolic and catabolic reactions in the cells, and the observed differences in growth can be a function of one or more of these biochemical reactions. The important question usually is whether or not a specific decrease in a biochemical reaction is the proximal cause of diminished growth or whether it is a secondary or corollary effect. Furthermore, most studies that have been carried out with bacteria have involved regulating the growth rate by varying the nutritional and environmental parameters and, except for mutants, not the genetic determinants. Surprisingly little research has been published on the factors responsible for differences in growth rate among individuals in a species. Growth limitation has been attributed in different animals, plants, and microbes to such factors as a balance of growth-retarding and growth-promoting substances (24), rate of protein synthesis (22), rate of nuclear replication (14), cell size (20), content of RNA (1, 12, 13, 15, 16, 18, 19, 21), base composition of RNA (5), and oxygen consumption (26).

The study on the growth of *Rhizoctonia solani* Kühn reported here is an attempt to determine changes in cellular biochemical reactions that might be responsible for differences in growth among the different isolates of the species. Optimum nutritive and environmental conditions were used so that we were dealing with growth differences that were probably genetically determined factors, and were innate to the different isolates. However, genetic studies were not done to determine the gene systems that were involved. Three isolates of the fungus were found whose relative growth rates were generally constant, different from each other, and independent of temperature and the composition of the media.

MATERIALS AND METHODS. – Eleven isolates of *R. solani* were obtained for this study. Their growth rates were measured on several media to determine which of the isolates had stable but different growth rates, and had relative rates of growth that were independent of nutritional conditions. Most of the isolates were discarded because of their inability to meet these criteria. The three isolates meeting these criteria were: a fast-growing isolate, MSUR-51, and a medium-growing one, MSUR-42, both from D. J. De Zeeuw of Michigan State University; and a slow-growing isolate, R221, from J. R. Parmeter, the University of California at Berkeley.

Growth rates were measured on nine media sterilized at 121 C for 15 min. Five media were obtained from Difco Laboratories: Potato-Dextrose Agar; Corn Meal Agar: Sabouraud Dextrose Agar; Malt Extraction Broth to which agar was added to 1.5%; and Czapek Agar. In addition, two chemically defined media were used (without and with agar to 1.7%). Glucose-Asparagine Medium had the following composition: glucose, 25g; DL-asparagine monohydrate, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 4.4 mg; $MnSO_4 \cdot H_2O$, 2.75 mg; $CuSO_4 \cdot 5H_2O$, 0.4 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.8 mg; $CaCl_2$, 4.5 mg; NaCl, 2.6 mg; and distilled water to one liter. Glucose-casamino acid-vitamin medium (GCV) had the same basic composition as the glucose-asparagine medium except that asparagine was omitted, vitamin-free casamino acids were substituted at 2 g/l, and the following vitamins were added at the indicated final concentrations: thiamine hydrochloride, pyridoxine, calcium pantothenate, *p*-aminobenzoic acid, and nicotinic acid, each 0.5 mg/l; inositol, 5 mg/l; riboflavin, 0.25 mg/l; and biotin, 5 μg/l.

All stock cultures were maintained on GCV agar but were transferred to the medium on which specific experiments were to be performed so that the inocula were not traumatized by a change in nutrients. Plugs of inocula were cut by cork borers from the periphery of young colonies. The plugs were 0.6 cm in diam for the fast isolate and 1.8 cm for the medium and slow ones for growth experiments in liquid culture, and 0.6 cm for all isolates in growth experiments on agar media. For biochemical experiments, the cultures were always grown in the GCV liquid medium in specially designed flasks as previously described (10). The cultures were grown for 4 days at 25 C under fluorescent light unless otherwise specified.

The influences of plant growth hormones and other possible growth-promoting substances were determined on GCV agar. Aqueous solutions of the various compounds were first filtered through Millipore filters of 0.45-μm pore diam; the aliquots were transferred aseptically to the medium after it had cooled to about 50 C. The hormones were gibberellic acid, 0-250 μg/ml; 3-indoleacetic acid, 0-40 μg/ml; kinetin, 0-60 μg/ml; and 6-(γ,γ-dimethylallylamino)purine, 0-660 μg/ml. The other compounds added as a mixture were: xanthine, hypoxanthine, thymine, cytosine, guanine hydrochloride, adenine sulfate, and uracil, each at 5.0 mg/ml; inosine-5′-monophosphate, 0.5 mg/ml; and uridine-5′-monophosphate, 1.0 mg/ml.

Two types of growth measurements were made on the isolates: (i) linear growth, by measuring the diameter of the eleven isolates on all the agar media, and (ii) gravimetric, by washing the fungus mat that had grown on liquid culture with distilled water in a Büchner funnel with a piece of filter paper and then drying the mycelium in preweighed weighing bottles under vacuum at 80 C until constant weight was

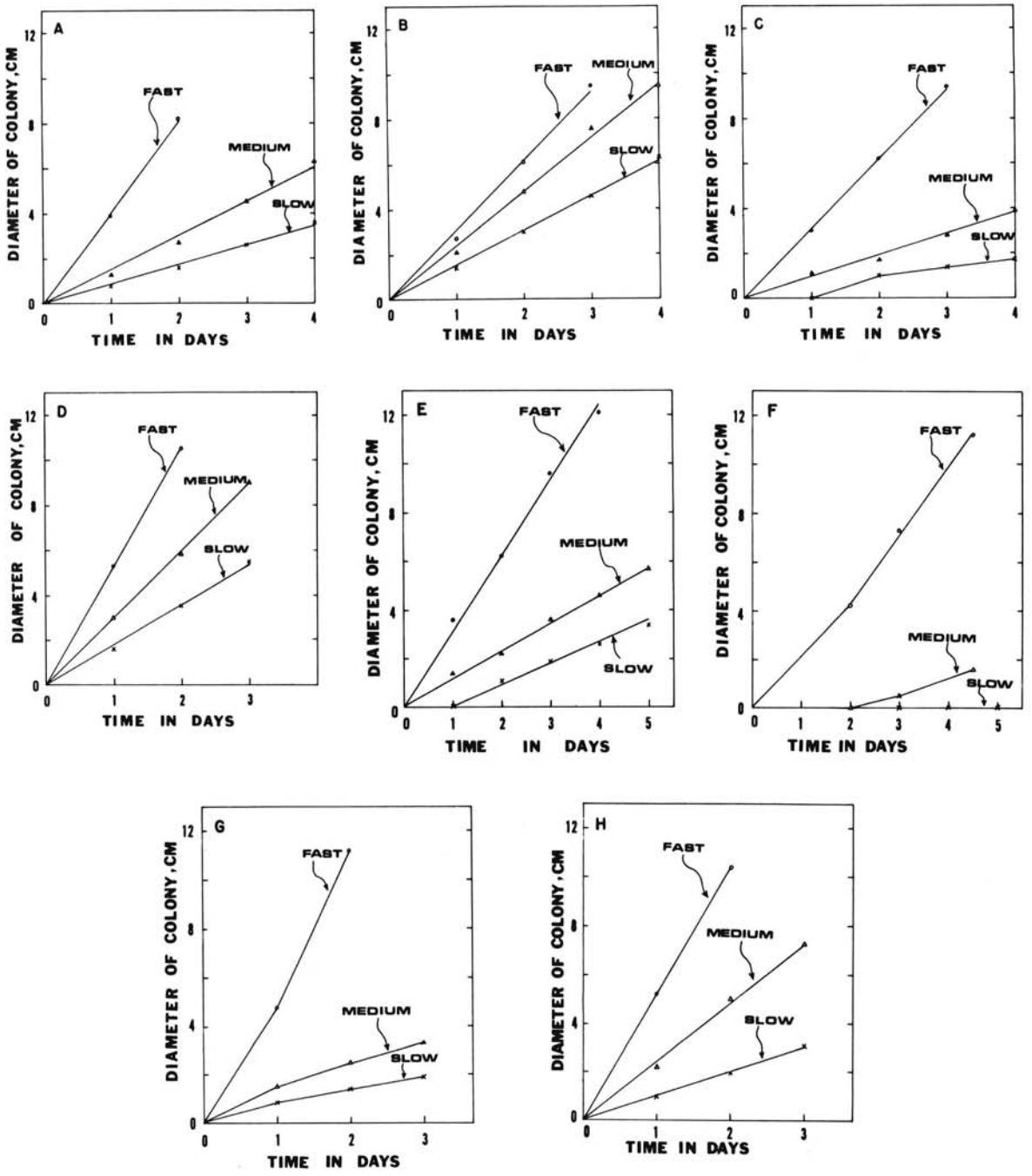

Fig. 1-A to H. Growth of fast medium and slow-growing isolates of *Rhizoctonia solani* on various media: **A)** glucose-casamino acid-vitamin agar, **B)** malt extract agar, **C)** Sabouraud dextrose agar, **D)** cornmeal agar, **E)** glucose-asparagine agar, **F)** glucose-asparagine liquid medium, **G)** Czapek agar, **H)** potato-dextrose agar.

attained. Triplicate flasks were run in all cases and the entire experiments repeated at least once.

RNA, DNA, and protein fractions of the cells were obtained by the procedure of Gottlieb and Van Etten (10). RNA was estimated by the orcinol procedure (3), DNA by diphenylamine (23), and protein by the method of Lowry et al. (17). ATP was determined in the neutralized acid-soluble fraction by the procedure of Addanki et al. (2), using a luciferin-luciferase ATP-dependent luminescence reaction as determined in a scintillation spectrometer. The neutralized acid-soluble fraction was prepared as follows. Fresh mycelium was frozen in liquid nitrogen in a mortar, ground to a fine powder, then allowed to

thaw in cold 4% perchloric acid (PCA). For every 0.5 gm of mycelium, 4 ml of PCA were used. The suspension was centrifuged at 20,000 × *g* for 10 min and the pellet was resuspended in cold PCA followed by another centrifugation. The process was repeated twice more and the three supernatant fractions were combined and neutralized with potassium hydroxide. The potassium perchlorate precipitate was removed by centrifugation and the resultant supernatant was used for ATP determination.

RESULTS. – Eight of the eleven isolates were discarded because they did not meet the criteria of constant, clear measurable differences in growth rate and the independence of relative growth rate from difference in the available nutrients in media. The remaining three isolates, fast, medium, and slow, were further tested for relative growth rates on nine different media including natural substrates, chemically defined constituents, and with and without agar. Using colony diameter as a measure, the three fungi grew essentially linearly in all media for the period of the experiments (Fig. 1, 2). The differences in growth were always similarly reflected in differences in dry weight of the fungus. An example of the congruence of measurements of diam and dry wt of the mycelium is shown in the experiment for growth on the GCV liquid medium (Fig. 2B). At the end of 4 days, the dry wt were: fast, 316 mg; medium, 131 mg; and slow, 21 mg. The diam of these mycelia in the same order of growth were: fast, 12.0 cm; medium, 7.4 cm; and slow, 4.3 cm. On GCV agar, the ratio of the relative growth rates of the fast, medium, and slow isolates at the end of 48 hr was 5.1: 1.7: 1.0 (Fig. 1A). Similar trends of the ratios were always in the same direction for the three isolates on the other media.

None of the plant hormones that were added to the media markedly stimulated the growth of the three isolates, and the small increases that occurred could not be correlated with the relative growth of the different isolates. Indoleacetic acid had no noticeable effect between 0.001 and 0.1 μg/ml, but it reduced the growth of all isolates at concns of 1.0 to 40 μg/ml. However, the inhibition did not follow a dosage-response gradient (Table 1). Gibberellic acid was not stimulatory to any of the three siolates at levels of 50 to 250 μg/ml, but it slightly reduced the growth of the medium and slow isolates at concentrations of 100 to 250 μg/ml. Kinetin and 6-(γ,γ-dimethylallylamino)purine each caused slight increases in growth at different concns between 10

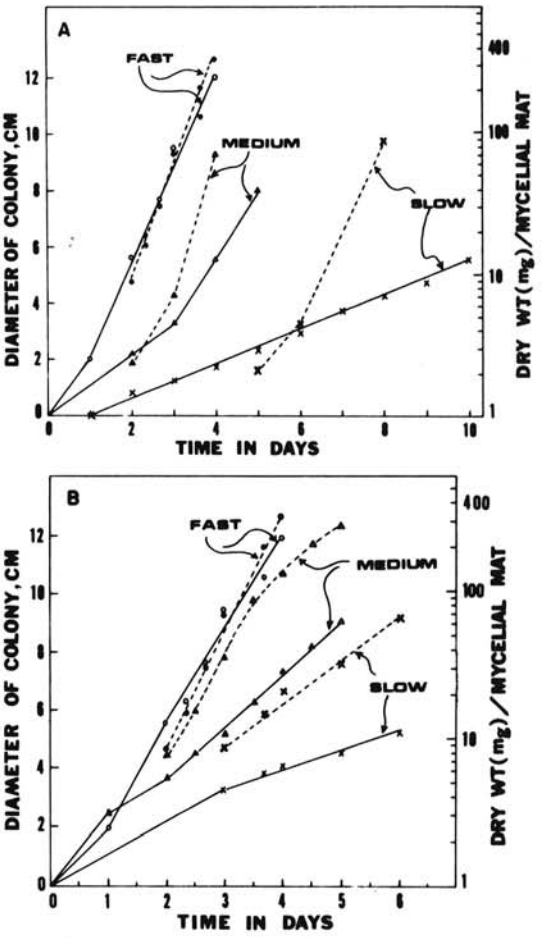

Fig. 2-A, B. A comparison of gravimetric and colony diameter growth measurements on three isolates of *Rhizoctonia solani* on glucose-casamino acid -vitamin liquid medium with different size inocula. **A)** Inoculum plugs 0.6 cm in diam; **B)** Inoculum plugs 0.6 cm for the fast isolate and 1.8 cm for the medium and slow ones. Solid lines represent colony diam and the dashed lines the colony dry weight.

TABLE 1. Effect of indoleacetic acid on the growth of three isolates of *Rhizoctonia solani*

Isolate	Growth time (hr)	Colony diam (cm) in cultures with three ranges of concn of indoleacetic acid (μg/ml)[a]											
		Low				Medium				High			
		0[b]	0.001	0.01	0.1	0	1.0	5.0	10	0	20	30	40
Fast	24	3.8	3.7	3.6	3.9	5.0	2.9	1.8	1.8	4.4	1.6	2.1	2.3
	48	> 9.0	> 9.0	> 9.0	9.0	> 9.0	6.0	3.5	2.9	8.9	2.5	2.5	2.6
Medium	96	6.7	6.2	6.5	6.8	6.8	5.6	4.9	5.1	6.1	4.1	3.8	4.0
Slow	96	3.8	4.2	4.3	4.1	4.8	4.2	3.1	2.7	5.3	2.5	2.2	2.3

[a] Indoleacetic acid was dissolved in 0.025 N NaOH and was prepared fresh each time it was used.

[b] 0 represents the solvent alone; the numbers in these columns represent the growth in the controls.

TABLE 2. Effect of different temp on the linear growth rates of three isolates of *Rhizoctonia solani* on glucose-casamino acid-vitamin agar

Temperature (C)	Diam of colony at 48 hr (cm)		
	Fast	Medium	Slow
35	0.0	0.0	0.0
32	6.0	2.5	3.1
25	9.0	3.0	1.7
20	7.9	2.6	1.5
15	8.1	1.8	1.2
10	5.7	1.4	1.0

TABLE 3. Percentage of DNA, RNA, and protein in 4-day-old colonies of three isolates of *Rhizoctonia solani* having different growth rates

Isolate	Composition (% of dry weight)		
	DNA	RNA	Protein
Fast	0.54	11.7	34.9
Medium	0.60	11.7	36.4
Slow	0.42	11.5	27.5

TABLE 4. ATP content in colonies of various ages of *Rhizoctonia solani* isolates having different growth rates

Growth time (hr)	ATP (pmoles)/mycelial dry wt (mg)		
	Fast	Medium	Slow
48	4550	–	–
72	4900	3090	4330
96	3220	3180	3270
120	–	3980	2870

and 60 μg/ml, but again without any clear response to a concn gradient.

The following potential growth-promoting metabolites at concns described in the methods section did not stimulate the growth of any of the three isolates: xanthine, hypoxanthine, thymine, cytosine, guanine, adenine, uracil, inosine-5′-monophosphate and uridine-5′-monophosphate.

Differences in growth rates of the three isolates might be caused by their growth-temp relationship. If the three isolates had different temp optima for growth, then it is possible that if only one temp were used in these studies, the rate of growth would reflect the different growth responses of the different isolates at this one temp. At another temp another isolate might grow fastest. The ratios of growth would then depend on temp and would not be an innate independent function of the isolate, no matter what the environmental conditions. The data in Table 2 indicate that the relationship between temperature and growth was almost the same for all three isolates. They grew at temp from 10 to 32 C but not at 35 C. The optimum for the fast and medium isolates was approximately 25 C, and for the slow one approximately 32 C (Table 2).

The DNA content on a dry weight basis varied with the isolate, 0.54% for the fast, 0.60% for the medium, and 0.42% for the slow one (Table 3). On the same basis, the percent RNA of the isolates was more uniform, 11.7% for fast and medium isolates and 11.5% for the slow one. The protein content in the same order of isolates was 34.9%, 36.4%, and 27.5%. Calculating RNA content on the basis of protein and/or DNA content showed no correlation with rate of growth of the three isolates. Protein content on the basis of DNA gave relative values that were different from those obtained on the basis of dry wt of the fungus.

TABLE 5. Comparison of increments of ATP content and dry weight in cultures of various ages of *Rhizoctonia solani* isolates having different growth rates

Isolate	Growth period (hr)	Dry wt of mycelial mat (mg)	Daily dry wt increment/ mycelial mat (mg)	ATP/ mycelial mat (pmoles)	Daily ATP increment mycelial mat (pmoles)	Daily ATP increment (pmoles)/ mycelial dry wt increment (mg)
Fast	48	6.30		28,650		
			51.30		253,780	4,950
	72	57.60		282,430		
			241.50		681,950	2,820
	96	299.10		964,380		
Medium	72	12.00		37,050		
			66.40		211,940	3,190
	96	78.40		248,990		
			39.50		220,220	5,580
	120	117.90		469,210		
Slow	72	5.65		24,540		
			7.65		19,010	2,490
	96	13.30		43,550		
			8.00		17,570	2,190
	120	21.30		61,120		

R. solani contained between 2,870 and 4,900 picomoles (pmoles) ATP per mg dry wt of mycelium, depending on the particular isolate and the age of the colony (Table 4). For all isolates the total amount of ATP increased with time as the colony grew (Table 5). The increment in pmoles of ATP per mycelial mat per unit time increased in the three isolates in the order of their growth rates. Thus, in the 72- to 96- hr period the increments of slow, medium and fast isolates were 19,010, 211,940, and 681, 950 pmoles, respectively. The daily increment on a mg dry wt basis showed no such trend, and there was no constant correlation between the ATP content and the growth rate of the isolates.

DISCUSSION AND CONCLUSION. – From the data in this study it is evident that differences in the growth rates of various isolates of *R. solani* need not be dependent on the nutritional and temp parameters that usually influence the rates of growth of fungi. Nutritional deficiences are known (8, 9) and could be responsible for reduced growth of some strains of a fungus compared to others. Hormonal factors such as indoleacetic acid and gibberellic acid are also produced by some fungi, and their absence or the inability to synthesize them might explain the limitations on the growth of certain isolates in a species (25, 27). Such deficiencies might be brought about by mutation, by segregation and recombination of genes, or by parasexual means to become fixed in certain strains of *R. solani.* Adding exogenous sources of such vital compounds then allows an organism to approximate its highest growth potential on a given medium. Such isolates were discarded since, for the purpose of these studies on the cellular basis of growth rate, we were seeking isolates which did not vary in relative growth rate with the availability of nutrients. The criterion for choosing isolates was always the constant growth rate of one isolate relative to that of another despite the variation in media. Similarly, one could not use isolates whose ratios of growth rates to each other were dependent on the temp at which they are grown. Three isolates that met the conditions were found; their relative rates of growth seemed independent of such external parameters and would depend on the internal biochemical machinery of the cell. The absolute growth rates for each of the three isolates did vary with the medium, but their relative rankings nevertheless remained the same despite the nutritional and temp stress under which they were grown.

The content of DNA, RNA, and protein in the three isolates were in the ranges that have previously been reported for fungi, but the differences in growth could not be correlated with their concns. Nor could differences in the following ratios of substances account for the various growths of the isolates: RNA/protein, RNA/DNA, and protein/DNA. The absence of any correlations of RNA concn with rates of growth of the three isolates is similar to the results obtained from other studies on yeast (1), *Polytomella coeca* (12, 13), and *Proteus vulgaris* (16).

Most of the energy that is available in the cell for synthetic activities is stored as ATP, and growth has been related to the synthesis of this energy-rich compound in bacteria (4, 6, 7, 11). However, such measurements were often made by varying the amount of the nutrients that were supplied to the organism; thus their oxidation would determine the amount of energy stored in ATP. Our experimental design was different, in that nutrients available to the fungal isolates were always the same, and any differences in the ATP would be dependent on differences in the activities of the synthesizing apparatus. Such differences were not indicated by the data; there was no correlation between growth and ATP content on a dry wt basis.

To ascertain the nature of the differences in the growth of the various isolates of *R. solani*, it now appears that studies on the catabolic and synthetic activities themselves will be needed. These are not reflected in the steady state metabolism but can best be followed by the use of radioactive precursors.

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