Influence of Riboflavin on the Effects of 3-Amino-1, 2, 4-Triazole on Schizosaccharomyces octosporus

ANN COSGROVE, Senior, Norman High School, Norman

(Lois C. Brandt, Teacher)

INTRODUCTION

The growth-regulating effects of the herbicide 3-amino-1,2,4-triazole (3-AT) have attracted considerable interest in recent years.[5,15,16] This hetero-cyclic compound, which is an elongated white crystal with the empirical formula $C_2H_4N_4$,[23] was first reported in 1954 (Hall, Johnson, and Leinweber) to cause leaf abscission, chlorosis, and growth inhibition in cotton.[16] Subsequent studies have shown that green plants treated with sublethal doses of this chemical manifest toxic effects in three ways: growth of meristematic tissues is inhibited;[10,21] leaves developed after treatment are devoid of chlorophyll;[4,5,22,29,31] and carbohydrate metabolism and respiration are affected.[12,19,30,31]

In 1955 Heim, Appleman, and Pyfrom found that the catalase activity in the liver and kidneys of rats injected with 3-AT was reduced, but the blood catalase and hemoglobin of the treated animals remained normal.[12,14,26] Both of these effects on catalase activity are similar to those caused by a malignancy anywhere in the animal's body.[12] Then in 1959 data obtained from further experimentation indicated that 3-AT caused thyroid cancer in rats when it was added to their diets at levels as low as 10 ppm.[7] This finding resulted in the removal from the market of the 1959 cranberry crops of Oregon and Washington. These crops had been contaminated by the careless use of 3-AT to kill weeds in the growing area.[6,7]

The foregoing information concerning 3-AT prompted a study to determine through experimentation whether or not the herbicide 3-amino-1,2,4-triazole affects the growth of a one-celled organism, and then on the basis of any findings to conduct further experimentation that might seem warranted.

To determine the effects of 3-AT on cell growth, the one-celled homothallic organism *Schizosaccharomyces octosporus* was chosen. *S. octosporus* is a haploid yeast that reproduces by fission and produces eight ascospores.[1,8,17,27] It grows best at 30 C and under slight acidic conditions (pH 6).[8]

EXPERIMENTAL PROCEDURE—PART 1

The *Schizosaccharomyces octosporus* culture was obtained from the mycology stock cultures, University of Oklahoma. For the growing of this yeast throughout the experimental study, two media based on the work of Northam and Norris (1951) were used. These media contained the following ingredients:[24]

Medium A (for *S. octosporus* growth in liquid medium)

Dextrose	10.00 g	Aneurin HCl	250.00 µg
L-asparagine	1.00 g	Pyridoxin HCl	250.00 µg
Casein hydrolyzate	1.00 g	Ca D-pantothenate	250.00 µg
$(NH_4)_2SO_4$	1.00 g	Nicotinic acid	250.00 µg
KH_2PO_4	0.75 g	KI	50.00 µg
$CaCl_2 \cdot 6\ H_2O$	0.25 g	Adenine	16.70 µg
$MgSO_4 \cdot 7\ H_2O$	0.25 g	Inositol	6.70 µg
		D-biotin	0.10 µg

Distilled water, made up to a volume of 500 ml.

Medium B (for *S. octosporus* growth on a solid medium)

2× concentration of Medium A

Equal amount of 30% water-agar

Both Medium A and the 2× concentration of Medium A were sterilized with a Type HA 0.45 μ-millipore filter under vacuum. The water-agar was autoclaved at 19 lb pressure for 20 min. The media was checked at pH 6 with a Beckman Zeromatic pH meter.

To determine the effects of 3-amino-1,2,4-triazole on the growth of *S. octosporus*, the following steps were taken:

1. One loop of cells from a 6-day-old slant was added to 10 ml of sterile distilled water.
2. One ml of *S. octosporus* and water suspension was added to a 250 ml flask containing 50 ml of Medium A.
3. The culture was incubated for 6 days at 30 C.
4. This culture (colorimetric reading, 31% transmittance) was used to inoculate 24 125-ml flasks. (Per cent transmittance was measured by a Bausch and Lomb Spectronic-20 Colorimeter at 525 $M\mu$). Inoculum consisting of 0.5 ml of the culture was pipetted into each sterile flask containing 20 ml of Medium A.
5. The 24 cultures were incubated at 30 C for 2 days to establish active growth. At the end of the 2-day period, each of the 24 flasks had a colorimetric reading of 54% transmittance.
6. Solutions of 3-AT ranging from 10^{1} M through 10^{-6} M, were prepared and filter-sterilized.
7. The 24 flasks were divided into eight treatment groups of three each and treated as shown in Table I.

TABLE I. PART 1, TREATMENT GROUPS OF *Schizosaccharomyces octosporus*.

Treatment Group No.	Amount of 3-amino-1,2,4-triazole added per flask	Concentration
1 (Control)	None	None
2	0.5 ml of 10^{1} M solution	0.25×10^{-1} M
3	0.5 ml of 10^{-1} M solution	0.25×10^{-3} M
4	0.5 ml of 10^{-2} M solution	0.25×10^{-3} M
5	0.5 ml of 10^{-3} M solution	0.25×10^{-4} M
6	0.5 ml of 10^{-4} M solution	0.25×10^{-5} M
7	0.5 ml of 10^{-5} M solution	0.25×10^{-6} M
8	0.5 ml of 10^{-6} M solution	0.25×10^{-7} M

8. The cultures were incubated for 6 days at 30 C.
9. Colorimetric determinations of each culture were made for data.
10. From each culture 0.5 ml was removed and diluted with 10 ml of distilled water; 1 ml of this dilution was used to inoculate a sterile petri dish containing 20 ml of Medium B.
11. The 24 petri dishes were incubated for 6 days at 30 C.
12. Macroscopic examinations of the cultures were made for data.

The complete experiment for this part of the study was run in triplicate.

EXPERIMENTAL RESULTS—PART 1 (See Table II)

EXPERIMENTAL PROCEDURE—PART 2

The findings from Part 1 concerning the growth-inhibiting effects of 3-AT on *Schizosaccharomyces octosporus* brought out the need for further study. An experiment to learn whether or not the growth-inhibiting effects of 3-AT on this yeast could be reversed seemed warranted and was undertaken.

For the further experimentation, a hypothesis was devised based on the 1960 findings of Sund and Little. They found that corn and pea leaf tissues which were albinistic as a consequence of treatment with 3-AT had a greatly lowered riboflavin content.[20] The hypothesis, consequently, was worded as follows: 3-amino-1,2,4-triazole affects the synthesis of riboflavin; thus through the use of riboflavin, reversal of the growth-inhibiting effects of 3-AT on *S. octosporus* might be possible. On the basis of this hypothesis, riboflavin was selected as the experimental compound for this part of the study. Riboflavin ($C_{17}H_{20}N_4O_6$) is an orange-yellow crystalline powder commonly known as Vitamin B_2.[9,11,26,28,33] This compound is involved in a cell's release of energy as an essential part of the coenzymes in the respiratory process.[18,33]

In order to test the hypothesis, a concentration of 3-AT which does not completely inhibit the growth of *S. octosporus* was necessary. In Part 1, it was found that at a 0.25×10^{-4} M concentration of 3-AT there was little growth of the yeast; at higher concentrations there was no growth, but at lower concentrations there was good growth. Consequently, a 0.25×10^{-4} M concentration of 3-AT was chosen for the reversal experiments with riboflavin.

To determine whether or not the growth-inhibiting effects of 3-AT on *S. octosporus* can be reversed by the addition of riboflavin, the following steps were taken:

1. The first five steps of the experimental procedure in Part 1 were followed to prepare 33 125-ml flasks.
2. A 10^{-4} M solution of 3-AT was prepared and filter-sterilized.
3. Solutions of riboflavin, ranging from 10^{-3} M through 10^{-10} M, were prepared and filter-sterilized.
4. The 33 flasks were divided into 11 treatment groups of three each and treated as shown in Table III.
5. The cultures were incubated for 6 days at 30 C.
6. Colorimetric determinations of each culture were made for data.
7. From each culture 0.5 ml was removed and diluted with 10 ml of distilled water; 1 ml of this dilution was used to inoculate a sterile petri dish containing 20 ml of Medium B.
8. The 33 petri dishes were incubated for 6 days at 30 C.
9. Macroscopic examinations of the cultures were made for data.

The complete experiment for this part of the study was run in triplicate.

TABLE II. EFFECTS OF 3-AMINO-1,2,4-TRIAZOLE ON THE GROWTH OF *Schizosaccharomyces octosporus*, WITH PH 6 AFTER 6-DAY INCUBATION PERIOD AT 30 C

Treatment Group No.	Concentration of 3-amino-1,2,4-triazole, used (M)	Mean optical density* In Liquid Medium			Amount of growth at end of 6-day incubation period** on Solid Medium
		6 days of incubation	Change	Change, %	
1 (Control)	none	16.3	37.7	69.8	++++
2	0.25×10^{-1}	54.0	0.0	0.0	0
3	0.25×10^{-2}	54.0	0.0	0.0	0
4	0.25×10^{-3}	54.0	0.0	0.0	0
5	0.25×10^{-4}	54.0	0.0	0.0	0
6	0.25×10^{-5}	54.0	0.0	0.0	+
7	0.25×10^{-6}	42.0	12.0	22.2	++
8	0.25×10^{-7}	31.0	23.0	42.6	+++

*Initial value 54

**The results are based on macroscopic examination; ++++ = excellent, +++ = good, ++ = fair, + = poor, and 0 = no growth.

TABLE III. PART 2, TREATMENT GROUPS OF *Schizosaccharomyces octosporus*.

Treatment Group	Concentration of 3-amino-1,2,4-triazole used (M)	Conc. of riboflavin used (M)
A (Control)	none	none
B (Control)	none	2×10^{-5}
C (Control)	0.25×10^{-5}	none
D	0.25×10^{-5}	2×10^{-5}
E	0.25×10^{-5}	2×10^{-6}
F	0.25×10^{-5}	2×10^{-7}
G	0.25×10^{-5}	2×10^{-8}
H	0.25×10^{-5}	2×10^{-9}
I	0.25×10^{-5}	2×10^{-10}
J	0.25×10^{-5}	2×10^{-11}
K	0.25×10^{-5}	2×10^{-12}

EXPERIMENTAL RESULTS—PART 2 (See Table IV)

DISCUSSION

The data obtained in Part 1 (Table II) indicate that 3-amino-1,2,4-triazole affects the growth of *S. octosporus*. In high concentrations ranging from 0.25×10^{-1} M to 0.25×10^{-4} M, this herbicide kills the yeast; at lower concentrations, the yeast growth is inhibited. The 0.25×10^{-5} M concentration seems to be the highest concentration of 3-AT which inhibits the growth of *S. octosporus* without killing the yeast cells. The degree of yeast-growth inhibition tends to decrease progressively as the concentration of 3-AT administered to the yeast decreases.

Consideration of the data from Part 2 (Table IV) indicates that riboflavin can reverse the growth inhibition caused by the addition of 3-amino-1,2,4-triazole. The reversal occurred only with concentrations of riboflavin between 2×10^{-5} M and 2×10^{-11} M. The concentrations of riboflavin ranging from 2×10^{-4} M to 2×10^{-7} M failed to reverse the growth inhibition. Moreover, reversal did not occur with a concentration lower than 2×10^{-11} M. It would appear that the low concentration of riboflavin (2×10^{-12} M) was insufficient to cause reversal. However, the failure to cause reversal with high concentrations (2×10^{-4} M to 2×10^{-7} M) may have resulted from an interaction between the vitamin and the herbicide. The results seem to confirm the hypothesis that the synthesis of riboflavin in the yeast cell is being affected by 3-amino-1,2,4-triazole.

CONCLUSIONS

1. Concentrations of 3-amino-1,2,4-triazole between 0.25×10^{-1} M to 0.25×10^{-4} kill *S. octosporus*.
2. A 0.25×10^{-5} M concentration of 3-amino-1,2,4-triazole is the highest concentration which inhibits the growth of *S. octosporus* without killing the yeast.
3. Concentrations of riboflavin ranging from 2×10^{-5} M to 2×10^{-11} M reversed the growth-inhibitory effects of a 0.25×10^{-5} M concentration of 3-amino-1,2,4-triazole on *S. octosporus*.
4. Riboflavin alone seems to have no inhibitory activity on *S. octosporus*.

TABLE IV. REVERSAL INFLUENCE OF RIBOFLAVIN ON THE GROWTH INHIBITION OF 3-AMINO-1,2,4-TRIAZOLE ON *Schizosaccharomyces octosporus* WITH pH 6 AFTER 6-DAY INCUBATION PERIOD AT 30 C

Treatment Group	Concentration of 3-amino-1,2,4-triazole used (M)	Concentration of riboflavin used (M)	Mean optical density* in liquid medium			Amount of growth at end of 6-day incubation period** on solid medium
			6 days of incubation	Change	Change, %	
A (Control)	none	none	16.3	37.7	69.8	++++
B (Control)	none	2×10^{-4}	16.4	37.6	69.6	++++
C (Control)	0.25×10^{-3}	none	54.0	0.0	0.0	+
D	0.25×10^{-3}	2×10^{-5}	54.0	0.0	0.0	+
E	0.25×10^{-3}	2×10^{-6}	54.0	0.0	0.0	+
F	0.25×10^{-3}	2×10^{-7}	54.0	0.0	0.0	+
G	0.25×10^{-3}	2×10^{-8}	50.0	4.0	7.4	++
H	0.25×10^{-3}	2×10^{-9}	41.0	13.0	24.1	+++
I	0.25×10^{-3}	2×10^{-10}	23.0	31.0	57.4	++++
J	0.25×10^{-3}	2×10^{-11}	37.0	17.0	31.5	+++
K	0.25×10^{-3}	2×10^{-12}	54.0	0.0	0.0	+

*Initial value 54

**The results are based on macroscopic examination; ++++ = excellent, +++ = good, ++ = fair, and + = poor growth.

FURTHER STUDY

Experimental studies should be conducted to determine whether or not there is a difference in the cellular structure of 3-amino-1,2,4-triazole-inhibited cells before and after the addition of riboflavin; to determine whether or not the optimum concentration of 3-AT for the inhibition of *Schizosaccharomyces octosporus* would also be the optimum concentration for the inhibition of the growth of other fungi; and to determine whether or not the optimum concentration of riboflavin for reversal is caused by metabolism of *S. octosporus*, by chemical reactions between 3-amino-1,2,4-triazole and high concentrations of riboflavin or by other factors.

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