PROLIFERATION OF ASPERGILLUS NIGER (v.T.) IN BIO-ASSAY INVESTIGATIONS WITH SULFUR

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Sulfur has been established as an essential element for all plant and animal growth. This element is supplied to soil organisms and higher plants principally from organic and inorganic soil components. Responses to sulfur fertilization have been reported within soil regions of the United States and other countries.

Increased use of high analysis fertilizers, containing little or no sulfur, may increase the need for this element with many crops and soils not recognized at present.

Diagnosis of soil characteristics contributing to deficiencies of available soil sulfur is necessary for sulfur fertilization recommendations. There is need for chemical and biological methods for determining the forms and amounts of available sulfur in soils.

Little is known of the soil organic fractions and biological reactions of sulfur that determine the availability of soil organic sulfur compounds to higher plants.

The soil micro organism, Aspergillus niger (v.T.), has been employed for bio-assay studies with soil for availability of many essential plant nutrients including phosphorus, potassium, magnesium, copper, zinc

and molybdenum.

The objective of this study was to determine the response of Aspergillus niger to sulfur sources and rates. Suitability of this organism for assessing the availability of sulfur in soils was investigated.

CHAPTER II

LITERATURE REVIEW

The essentiality of sulfur for plant growth was recognized in the early years of agricultural chemistry. Theodore de Saussaure, according to Powers (36), showed sulfur to be essential for plant growth in 1804.

Sulfur occurs in plants chiefly in components of proteins, volatile compounds and sulfates (16). It is a consitiuent of some plant hormones, the mustard-oil glucosides and glutathione (19). Although not a constituent of plant chlorophyll, sulfur is known to affect the chlorophyll formation in plants indirectly. It is essential in many metabolic processes, especially carbohydrate metabolism. Sulfur has been found to influence the up-take of other plant nutrients (19).

Sulfur deficient plants develop a peculiar chlorosis with a disrupted, abnormal growth and undesirable fruit qualities, particularly in citrus (12,19).

Many investigators have proposed that sulfur deficiency is a principal cause of the damaging diseases of tea in Nyasaland and tobacco in Kentucky, USA (15, 46, 50, 51).

Sulfur fertilization has been reported beneficial to crop plants especially those of high protein content growing in sulfur deficient

soils (9, 27, 32).

The elemental and sulfide forms of sulfur are rare in normal agricultural soils. Sulfates and organic sulfur compounds make up the major portion of known sulfur components in soils (14, 26, 36, 39). Sulfates may be released from the weathered parent material and may even accumulate in sub-humid areas. Amounts of sulfur, principally sulfate, are added to the soil with rainfall especially in heavy industrial areas. Some sulfur may be added incidently with the use of low analysis fertilizers containing sulfur. Application of fungicides, insecticides and animal manures also supply some sulfur in comparatively smaller amounts (16, 19, 49).

Loss of sulfur from soils occur through leaching by drainage water, through crop removal, by the burning of crop residues and volatilization when released from organic matter through microbial decomposition (16).

In the United States, need for sulfur fertilization was first reported in 1911 by Hart and Peterson in Wisconson (18). Other studies include the sulfur surveys for Oregon (37, 38), Kentucky (43), Iowa (10), Kansas (47), Indiana (7), and Vermont (20), with a need for sulfur fertilization indicated.

In southern Oregon, sulfur was shown to be a first limiting factor in the production of alfalfa on some soils (38). Sulfur ranks a close third to N and P as a major deficiency in California and must be supplied to maintain crop production (28). There is an increasing number of reports where soils do not respond to applications

of lime, phosphorus or potassium but where excellent results are obtained by applying sulfur or gypsum in California (13).

Sulfur deficient areas have been reported from other states including Washington (34), Idaho (31), Montana (11), North Dakota (35), Florida (17), Arkansas (53,54) and Minnesota (1,2).

Eaton in 1922 (14) reported that certain soils from Alabama, Maryland and Oklahoma were deficient in sulfur. Reports indicating sulfur deficiencies in other states as well as some other countries especially in Canada are also found in the literature.

As higher analysis fertilizers are used, particularly the ammonium phosphate materials, the amount of "incidental sulfur" in these fertilizers will be less. This may result in the need for means of supplying sulfur in fertilizers in areas where a sulfur deciciency problem is not recognized at present (49).

Many areas of sulfur deficiency are now known through results with field plots studies. These have shown the need to determine the sulfur status of soils used for crop production in order that proper fertilization practices can be used (28). Bardsley and Kilmer (6) indicated that a need for chemical methods to assess the sulfur status of the soils has become more apparent as new areas of acute sulfur or potential sulfur deficiency are found.

Generally, the methods used for this purpose are either chemical or field techniques or a combination of both. In 1960, Kilmer and Nearpass (21) explained that few laboratory procedures have been developed for determining available sulfur in soils. It was also explained that efforts

along this line have generally been confined to the extraction and determination of inorganic sulfates. However, the concentration of soluble sulfates at any given time does not appear to be a reliable reflection of the amount of sulfur which is likely to become available to a crop during the growing season, being in this respect analogous to soil nitrogen. They concluded that there is a real need for a laboratory procedure which will adequately characterize soils with respect to their available sulfur level. As a result of their work they developed a laboratory method which was thought to determine available sulfur in soils. Many investigators have used plants as indicators in methods for available sulfur determination (2). Although laboratory procedures for assessing ion status can be carried out under much more rigidly controlled conditions, crop yields in the field are influenced by many factors other than the ion in question (6). Several workers (5, 21, 24, 30, 41, 44, 52) have used greenhouse experiments to evaluate various chemical methods for determining available sulfur in soils. Values obtained from some of these methods have been highly correlated with plant growth and sulfur up-take in greenhouse experiments. However, correlation studies with field response have received less attention (6). An important limitation in sulfur studies is the fact that very little is known about the organic sulfur forms and transformations in the soil (40), while soil organic matter is the sole known reservoir of sulfur in many soils of the humid region (19).

Molds under the name of Aspergillus niger have been used in

literally hundreds of biochemical investigations (48). According to Martens (23) as early as 1909, Butkewitsch reported that the weight of Aspergillus niger increased with an increase in phosphorus or potassium from deficiency to sufficiency levels. He suggested the use of this microorganism as a biological indicator of the amounts of the available potassium and phosphorus in soils. Also according to Martens (23), Pantanelli in 1924 reported the use of molds for determining phosphorus deficiencies in soil. He cultured Aspergillus niger, Aspergillus oryzae and Aspergillus flavis on a sterilized mixture of soil in a nutrient solution containing 10% sucrose and 2% $(NH_4)_2SO_4$. The phosphorus which accumulated in the tissue was taken as a measure of the available phosphorus. A method of routine determination of available phosphorus and potassium has been developed by Niklas et al. in 1930. According to Martens,(23) their method consisted of addition of 2.50 g. of soil and 30 ml. of a nutrient solution to 75 ml. Erlenmeyer flask, inoculation with a suspension of Aspergillus niger spores and incubation at 35°C. for four days. The resulting growth was then harvested, dried and weighed. Requirement of phosphorus or potassium fertilization was indicated if the soil fell below a certain minimum. The nutrient solution used by Niklas, et al. differed from that of other workers in that it contained experimentally determined optimum levels of carbon, nitrogen, magnesium, iron, zinc, copper and potassium or phosphorus. Possibly other nutrients were present in the nutrient solution as contaminations. In 1933, Mehlich et al. (25), refined Niklas's method so that it permitted quantitative determination of available potassium fertilization of soil. As a quantitative determination

they compared the dry weight of tissue grown on culture medium containing the soil under test with that grown on a series of cultures to which increasing increments of potassium had been added. It was found that lower levels of available potassium indicated by the bio-assay method corresponded to the soils which responded to potassium fertilization. The refined method by Mehlich et al. was used by Mulder in 1939 (29) to determine quantitatively available magnesium. He tested 24 sandy soils, which ranged in pH from 4.20 to 5.00,and found that soils bearing magnesium - deficient plants contained less than 33 ppm. of available magnesium.

Assessing micro elements by such methods was developed when purification of the nutrient in question was satisfactory. Mulder (29) was the first to report a procedure for the determination of a micronutrient element using Aspergillus niger. He estimated available copper in soil by comparing the spore color of Aspergillus niger growing on soil cultures containing known amounts of copper. He tested 14 soils and found that severe deficiency of oats occurred when soil contained less than 0.60 ppm. of available copper. An Aspergillus niger bio-assay was also used by Bould et al. in 1949 (8) in order to confirm zinc deficiency of fruit trees. They used a procedure similar to that of Mehlich et al. with compounds that could be readily purified of zinc contamination. In 1950 Nicholas et al. (33) found that the method of Mehlich et al. was useful for the differentation of available molybdenum levels in soils. Martens, D. C. in 1962 (23) obtained satisfactory results for determining the available zinc in some Wisconsin soils using Aspergillus niger.

Little information has been reported on the evaluation of soil sulfur by the use of Aspergillus niger. However, some extensive studies have been conducted on the nutrition of the organism including sulfur. In 1941, Steinberg (45) surveyed the effects of some organic and inorganic sulfur compounds on the growth of this organism. He found that the sulfur of inorganic sulfur compounds is reduced to sulfoxylate prior to its conversion to organic sulfur. Sulfides and disulfides were not assimilated. Assimilability of organic sulfur compounds varied with molecular configuration and was also correlated with the presence of attached or adjacent oxygen in the molecule. Alkylmercaptans, sulfides and disulfides could not be used as source of sulfur whereas alkylsulforate and alkylsulfinate were readily available due to free sulfinic acid and an unsaturated residue. Steinberg reported that anabolites, particularly cystine and its derivatives, homocystine and methionine, were readily available as sole sources of sulfur supply irrespective of the state of oxidation of their contained sulfur. These were assumed to follow the normal channels for their metabolism. He also found that catabolites and other miscellaneous synthetic organic sulfur compounds require a process of digestion before assimilation.

Armstrong (4) found that the compounds $MgSO_4$, Na_2SO_4, $MnSO_4$, KSH, $KHSO_3$, $K_2S_2O_8$, KSCN and NH_4CNS to be favorable sources of sulfur for Aspergillus niger and some other fungi in the order named. He also found that little growth was obtained with K_2S and that $K_2S_2O_8$, while usable by Aspergillus niger inhibited the growth of the fungi Penicillum glaucum.

CHAPTER III

MATERIALS AND METHODS

Two strains of Aspergillus niger (v.T.) were used in this study. Most of the experiments were made with a strain obtained from General Biological Supply House Inc., Chicago, Illinois. This strain was termed "Turtox" for the purpose of this study. It originated from the Kraal Collection of Vienna, Austria (22) and proved to be highly responsive to sulfate additions in growth media. A strain from the American Type Culture Collection, (ATCC) 6275, was used in the studies for comparative purposes in establishing the magnitude of response to sulfate by the "Turtox" strain.

The ATCC 6275 strain is also designed as the C. Thom, strain 4247, USDA, 1938; as the Steinberg strain used in mineral nutrition studies; also QMRDC 458 (NRRL 334) for fungus resistance tests (3).

Fifty ml. of the basic sulfur - free media in 500 ml. Erlenmeyer flasks were used per culture. Cotton stoppers were used to provide aeration and to prevent contamination. Increments of the various sulfur compounds or soils extracts investigated were added to the media before or after sterilization depending on the treatment. The prepared culture media in stoppered flasks were steam sterilized at 15 psi for 15 to 20 minutes. Approximately 2 ml. of heavy spore suspensions per culture of the respective strain used in each experiment were added to the cooled sterilized media and incubated in the dark at 34° C. for the designated

time for each treatment. The mycelia pad from each culture was carefully removed, washed to remove occluded media and dried in tared aluminum weighing dishes for 24 hours at 99 - 100°C. Weights were recorded and net weights (treatment - check) were calculated. All investigations included three replications for each treatment.

Soil extractions for these studies were obtained by mechanically shaking 40 grams of soil in 80 ml. of distilled water for 20 minutes and filtering the mixture.

The basic sulfur-free media used had the following constitutents in the amounts indicated:

Sucrose (commercial), $C_{12}H_{22}O_{11}$	200.00 gms
Citric acid, $C_6H_8O_7$	20.00 "
Potassium chloride, KCl	2.00 "
Ammonium nitrate, NH_4NO_3	8.00 "
Calcium phosphate (mono basic), $CaH_4(PO_4)_2 \cdot H_2O$	1.00 "
Ammonium phosphate (mono basic), $NH_4H_2PO_4$	1.60 "
Magnesium chloride, $MgCl_2 \cdot 8H_2O$	4.00 "
Copper acetate, $Cu(C_2H_3O_2)_2 \cdot H_2O$	13.3 ml. from a 0.9420 g./liter solution.
Zinc chloride, $ZnCl_2$	13.3 ml. from a 0.6300 g./liter solution
Ferrous chloride, $FeCl_2$	13.3 ml. from a 6.2300 g./liter solution
Distilled water	to 2000.00 ml.

Sulfur containing compounds used in this study included:

Sulfur	S
Sodium Sulfate	Na_2SO_4
Sodium thiosulfate	$Na_2S_2O_3 \cdot 5H_2O$
Sodium sulfite	Na_2SO_3
Sodium Sulfide	$Na_2S \cdot 9H_2O$

Potassium thiocyanate	$KSCN$
L-cystine	$(SCH_2CH(NH_2)COOH)_2$
L-cysteine	$HSCH_2CH(NH_2)COOH$
L-methionine	$CH_3SCH_2CH_2CH(NH_2)COOH$
Sulfanilamide	$NH_2C_6H_4SO_2NH_2$
Para-amino benzoic acid	$NH_2C_6H_4COOH$
Thiourea	NH_2CSNH_2

Three soil treatments were used in the soil extraction studies: untreated soil, pH6.3; Ca-treated with 5m.e.of $CaCO_3$ added per 100 grams of soil, pH7.5; and S-treated soil with 5 m.e. sulfur added per 100 grams of soil, pH3.4. The Ca and S treated soils were incubated at approximately field capacity for three months at room temperature before drying for storage and later experimental use. Characteristics of the soil used, Norge fine sandy loam, are in Appendix, Table XXII.

CHAPTER IV

RESULTS AND DISCUSSION

Experimental results are summarized in Tables I to X. Detailed Tables containing yields of individual cultures are found in Appendix Tables XI to XXI.

Results in Table I and II show the high response of Aspergillus niger (v.T.) Turtox strain, to sulfur supplied as sodium sulfate. The range in mg. of S from 0.30 to 1.20 per culture (Table I) showed an increase in both the total growth obtained and the growth per mg. S. Results in Table II showed that with expanded sulfur rates from 0.0 to 8.0 mg. of sulfur per culture, an apparent maximum growth is obtained between 1.5 and 2.0 mg.. S/culture. From these data the assumption was made that an optimum rate for maximum growth was 2.0 mg. S/culture as Na_2SO_4. Details of Table I and II are reported in Tables XI and XII, respectively. They show replications to be rather close with good agreement of growth magnitude with similar rates in the two experiments.

The growth curve of the organism under the experimental conditions used was first determined for 96 hour period as shown in Table III. There was rapid growth increase during this period with no apparent leveling off. The study was repeated with the time expanded to 117 hours. Results are reported in Table IV indicating that a leveling

off of growth occurred between 93 and 101 hours. These data indicated that a 96 hour period was near optimum for these studies. Detailed culture yields at the various sampling periods are shown in Tables XIII and XIV.

Sulfur availability to the organism from some organic and inorganic sources was determined and results are shown in Table V. Detailed culture yields are presented in Table XV. Results indicated that a ranking of the sulfur compounds investigated supplying 2 mg. S/ culture were as follows in descending order:

sodium sulfate) potassium thiocyanate > sodium sulfide > sulfur
sodium thiosulfate) thiourea > sulfanilamide.

Sodium sulfite was also investigated but no growth was obtained in two of the three cultures used. Sodium sulfite sulfur was considered unavailable or toxic to the organism. Slight growth in one of the cultures was thought to be due to experimental error.

It is not known whether organic sulfur was used as such or was first transformed to another form. A general observation in the study with rates and sources reported in Table VI and XVI was that cultures with addition of L-cystine showed very rapid proliferation compared with inorganic sulfur sources including sodium sulfate and thiosulfate. The magnitude of response to different sulfur rates varied with the nature of the compound in question. Sodium sulfate, sodium thiosulfate and L-cystine gave increasing yields with increasing increments of sulfur. Potassium thiocyanate gave growth increase at 1 and 2 mg. sulfur per culture then decreased at the 3 mg. rate. Only two of the three cultures could be measured at the 3 mg. rate, however, growth per mg. sulfur supplied per culture did not follow a consistent pattern but in most cases was highest at the one mg. sulfur rate.

TABLE I

RESPONSE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, TO 0.03-1.20 MG. RATES OF SULFUR FROM SODIUM SULFATE

Mg. S/ culture	Net ave. dry pad wt. in g.	Dry pad wt. in g./mg.S
0.030	0.0455	1.5160
0.075	0.1851	2.4680
0.150	0.1992	1.3280
0.225	0.2288	1.0160
0.300	0.3388	1.1290
0.450	0.5066	1.1250
0.600	0.6879	1.1460
0.900	1.1200	1.2440
1.200	1.1560	1.3000

Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur = 0.016 g./culture) from the dry weight of the respective sulfur rate treated cultures.

See Table XI for individual culture yields after incubation for 96 hrs. at 34°C. Sulfate additions were made before sterilization.

TABLE II

RESPONSE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, TO 0.5-8.0 MG. RATES OF SULFUR FROM SODIUM SULFATE.

Mg. S/ culture	Net ave. dry pad wt. in g.	Dry pad wt. in g./mg. S
0.5	0.6128	0.3064
1.0	1.3499	1.3499
1.5	1.8906	1.2604
2.0	1.9245	0.9623
3.0	1.9073	0.6358
4.0	1.8996	0.4749
5.0	1.9097	0.3819
6.0	1.9583	0.3264
7.0	1.9246	0.2749
8.0	1.9322	0.2415

Figures are net ave. figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur = 0.0266 g./culture) from the dry pad weight of the respective sulfur rate treated cultures.

See Table XII for individual culture yields after incubation for 96 hrs. at 34°C. Sulfate additions were made before sterilization.

TABLE III

GROWTH CURVE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, IN RESPONSE TO SULFUR FROM SODIUM SULFATE AT THE RATE OF TWO MG. SULFUR PER CULTURE DURING 96 HOURS INCUBATION

Sampling intervals in hours	Net ave. dry pad wt. in g.	Dry pad wt. in g./mg. S
48	0.2421	0.1210
54	0.4760	0.2380
60	0.7895	0.3947
66	1.0968	0.5489
72	1.7178	0.8589
78	1.5009	0.7504
84	1.8740	0.9370
90	1.8552	0.9276
96	2.1184	1.0592

First sampling was after 48 hrs. then at 6 hr. intervals. Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur) of each interval sample from the dry pad weight of treated samples determined at the same time interval.

See Table XIII for individual culture yields incubated at 34°C. Additions of sulfur were made before sterilization.

TABLE IV

GROWTH CURVE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, IN RESPONSE TO SULFUR FROM SODIUM SULFATE AT THE RATE OF TWO MG. SULFUR PER CULTURE DURING 117 HOURS INCUBATION.

Sampling intervals in hours	Net ave. dry pad wt. in g.	Dry pad wt. in g./mg. S
48	0.2222	0.1111
61	0.7158	0.3579
69	1.2975	0.6487
77	1.5772	0.7886
85	1.8847	0.9423
93	2.1246	1.0623
101	2.1617	1.0303
109	2.1365	1.0682
117	2.0650	1.0325

First sampling was after 48 hrs., the second at 61 hrs., then at 8 hr. intervals. Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur) of each interval sample from the dry pad weight of treated samples determined at the same time interval.

See Table XIV for individual culture yields incubated at 34°C. Additions of sulfur were made before sterilization.

TABLE V

RESPONSE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, TO DIFFERENT SOURCES OF SULFUR AT THE RATE OF TWO MG. PER CULTURE.

Sulfur compound	Net ave. dry pad wt. in g.	Dry pad wt. in g./mg. S
Sodium sulfate	2.1426	1.0713
Sodium thiosulfate	2.1122	1.0561
Sulfur	0.0872	0.0436
Sodium sulfide	0.3820	0.1910
Sodium sulfite	———	———
Potassium thiocyanate	1.2092	0.6046
Sulfanilamide	-0.0148	-0.0074
Thiourea	0.0424	0.0212

Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur = 0.0251 g./culture) from the dry pad weight of each sample.

See Table XV for individual culture yields incubated for 96 hours at 34°C. Sulfur compound additions were made before sterilization. No growth was obtained in two cultures of the sodium sulfite treatment. Slight growth in the third replication culture was thought to be due to experimental error or contamination.

TABLE VI

RESPONSE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, TO DIFFERENT RATES OF SULFUR FROM DIFFERENT SOURCES.

Sulfur compound	Mg.S/ culture	Net ave. dry pad wt. in g.	Dry pad wt. g./mg. S.
Sodium Sulfate	1	1.2811	1.2811
	2	2.1345	1.0672
	3	2.2094	0.7364
Sodium thiosulfate	1	1.0332	1.0332
	2	2.1638	1.0812
	3	2.3326	0.7775
Potassium thiocyanate	1	0.7886	0.7886
	2	1.1215	0.5607
	3	0.8581	0.2860
L-cystine	1	1.1119	1.1119
	2	2.0201	1.0100
	3	2.2145	0.7381

Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur = 0.0276 g./culture) from the dry pad weight of the respective sulfur rate treated cultures.

See Table XVI for individual culture yields incubated for 96 hrs. at 34°C. Sulfur compound additions were made to cool sterilized media before incubation..

TABLE VII

RESPONSE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, TO RATES OF SULFUR FROM THREE SULFUR CONTAINING AMINO ACIDS.

Sulfur containing amino-acid	Mg. S/ culture	Net ave. dry pad wt. in g.	Dry pad wt. in g./mg. S.
L-cystine	1	1.1723	1.1723
	2	2.1433	1.0716
	3	2.3934	0.7978
L-cysteine	1	0.9943	0.9943
	2	1.8094	0.9047
	3	2.2198	0.0733
L-methionine	1	1.1572	1.1572
	2	2.0176	1.0088
	3	2.2086	0.0695

Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur = 0.0160 g./culture) from the dry pad weight of the respective amino acid treated culture.

See Table XVII for individual culture yields incubated at 34°C, for 96 hrs. Amino acid additions were made to cool sterilized media prior to incubation.

Response of Aspergillus niger (v.T.), Turtox strain, to three sulfur containing amino acids was studied with results shown in Table VII and Table XVII. L-cystine and L-methionine gave approximately the same yield response. L-cysteine gave slightly lower total yields at the one and two mg. sulfur per culture rates. The overall magnitude of response was somewhat similar to that of sodium sulfate and thiosulfate. Fungus growth from the three sulfur containing amino acids per mg. sulfur supplied decreased with increasing rates of sulfur addition.

A comparative response study was conducted with the Turtox strain and the ATCC 6275 strain. Both strains were found to be highly responsive to sulfur but the Turtox strain was slightly higher in response as shown in Table VIII with more details in Table XVIII. The relatively high response of the ATCC 6275 strain to sulfur as sodium sulfate gives sound basis for using this established strain in future bio-assay studies with sulfur compounds.

Sulfanilamide could apparently not supply sulfur in significant amounts in an available form to Aspergillus niger in these studies. Results with rates and combinations of sulfanilamide, para-aminobenzoic acid and sodium sulfate are shown in Table IX and XIX. Little difference could be observed from combinations of p-aminobenzoic acid and sulfanilamide.

Response obtained to sulfur as sodium sulfate with sulfanilamide and p-aminobenzoic acid was in normal nagnitude at the 2 mg. sulfur per culture rate obtained in the other studies.

Aspergillus niger (v.T.), Turtox strain, was investigated as an indicator of available sulfur in soil.

Extracts of untreated, $CaCO_3$-treated and elemental sulfur-treated Norge fine sandy loam soil were added at different rates to the basic sulfur free media cultures. The growth response is shown in Table X with details in Table XX. The water extractable sulfates from the different soil treatments used are presented in Table XXI. The $CaCO_3$-treated soil contained over five times more soluble sulfur than the untreated soil. This can account for its higher growth yields in the rates used. A possible explanation is based on favorable calcium and pH effects for microbial activity on soil organic matter thus releasing more available forms of sulfur. The soil reaction was raised to 7.50 from an original of 6.30. The sulfur-treated soil, pH 3.40, contained about sixty times more soluble sulfur than the untreated soil. Magnitudes of response with these soil extract treated cultures was somewhat similar to those obtained from rates of sulfur applied as sodium sulfate. The growth per mg. sulfur supplied per culture was also similar to those using sodium sulfate as the sulfate source.

TABLE VIII

RESPONSE OF TWO STRAINS OF ASPERGILLUS NIGER (v.T.), TURTOX AND ATCC 6275, TO DIFFERENT RATES OF SULFUR FROM SODIUM SULFATE.

Mg. S/ culture	Turtox strain Net ave. dry pad wt.in g.	Turtox strain Dry pad wt. in g./mg.S	ATCC 6275 strain Net ave. dry pad wt.in g.	ATCC 6275 strain Dry pad wt. in g./mg.S
0.15	0.1548	1.0320	0.1390	0.9262
0.30	0.2868	0.9560	0.2856	0.9520
0.60	0.6502	1.0833	0.6393	1.0655
0.90	1.1775	1.3083	0.9982	1.1090
1.20	1.6062	1.3385	1.5282	1.2735

Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no sulfur = 0.176 g./culture for Turtox and 0.0282 g./culture for ATCC 6275) from the dry pad weight of the respective sulfur rate treated cultures.

See Table XVII for individual culture yields incubated for 130 hrs. at 34°C. Sulfur additions were made before sterilization.

TABLE IX

RESPONSE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, TO SULFANILAMIDE AS AFFECTED BY p-AMINOBENZOIC ACID AND SODIUM SULFATE ADDITIONS

Sulfanilamide mg. S	P-aminobenzoic acid mg.	Sodium sulfate mg. S	Ave. dry pad wt. in g.
0	0.0	0	0.0746
0	0.1	0	0.0948
0	0.2	0	0.0738
1	0.0	0	0.1100
1	0.1	0	0.0879
1	0.2	0	0.0998
2	0.0	0	0.0826
2	0.1	0	0.1012
2	0.2	0	0.0965
1	0.2	2	2.0746

The basic sulfur free media was used with the above additions made before sterilization. Cultures were incubated at 34°C. for 96 hours. Dry pad weights are average figure from three replications.

TABLE X

EVALUATION OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, FOR DETERMINING AVAILABLE SULFUR IN SOILS.

Soil treatment	G.soil/ culture	Ext. S in soil in mg.	Net ave. dry pad wt. in g.	Dry pad wt. in g./mg.S
Untreated	1	0.01	0.0028	0.2800
	2	0.02	0.0055	0.2750
	4	0.04	0.0417	1.0175
5m.e.$CaCO_3$/ 100 g. soil	1	0.05	0.0604	1.2080
	2	0.10	0.1260	1.2600
	4	0.20	0.2811	1.4055
5 m.e. elemental S/ 100 g. soil	1	0.60	0.7053	1.1755
	2	1.20	1.6911	1.4092
	4	2.40	1.8493	0.7705

Figures are net average figures from three replications. The net figure is determined by subtracting the average dry pad weight of the check (no soil extract added = 0.1042 g./culture) from the dry pad weight of the respective extraction treated cultures.

Soil extractions were made with distilled water in 2:1, water: soil, ratio.

See Table XX for individual culture yields, incubated for 96 hrs. at 34°C. Soil extraction additions were made before sterilization. Extractable sulfate levels from soils are shown in Table XXI.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objectives of this study were to investigate the response of Aspergillus niger (v.T.) to various sulfur sources and rates and to evaluate the organism as an indicator of available sulfur in soil.

Reported data were obtained using a basic sulfur-free media with various sulfur containing compounds and extracts of untreated, $CaCO_3$-treated and sulfur-treated Norge fine sandy loam soil. Two strains of A. niger (v.T.) were investigated with incubation temperature of 34°C. Dry weights of mycelia pads were obtained for each culture with three replications used for all treatments.

Results may be summarized as follows:

1) The organism was highly responsive to available forms of sulfur added to a basic sulfur-free media.
2) Two mg. sulfur per culture supplied as sodium sulfate with incubation at 34°C. for 96 hours gave a relatively maximum growth.
3) High availability of sulfur to the organism was found using sodium sulfate, sodium thiosulfate and sulfur containing amino acids. Lower availability was associated with sodium sulfide, potassium thiocyanate and elemental sulfur.
4) Though both Turtox and ATCC 6275 strains showed high response to sulfur, Turtox strain was of a relatively greater magnitude of

response than the ATCC 6275 strain.

5) Investigating response to sulfanilamide, p-aminobenzoic acid and sodium sulfate rates and combinations indicated that this organism apparently could not utilize sulfur from sulfanilamide even with p-aminobenzoic acid additions.

6) Addition of extract from untreated, $CaCO_3$-treated and sulfur-treated Norge fine sandy loam soil showed response of A. niger (v. T.), Turtox strain, to soil sulfur that was in proportion to soluble sulfur content extracted from each soil.

7) These studies indicate promise of employing strains of this organism, A. niger (v.T.), for bio-assay studies concerning available forms of soil sulfur.

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APPENDIX

TABLE XI

EFFECT OF SULFUR RATES OF 0.00 TO 1.20 MG. PER CULTURE AS SODIUM SULFATE ON GROWTH OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN.

Mg. S per/ culture	Dry pad wt./culture in g. Rep. I	Rep. II	Rep. III	Ave. dry pad wt. in g.
Blank	0.0314	0.0250	0.0383	0.0315
0.030	0.0747	0.0622	0.0943	0.0770
0.075	0.1520	0.1527	0.3453	0.2166
0.150	0.2410	0.1935	0.2578	0.2307
0.225	0.2715	0.2829	0.2265	0.2603
0.300	0.3886	0.3672	0.3553	0.3703
0.450	0.5741	0.4916	0.5486	0.5381
0.600	0.7541	0.6784	0.7259	0.7194
0.900	1.1703	1.1510	1.1333	1.1515
1.200	1.6135	1.5936	1.5682	1.5917

The basic sulfur-free media was used with sulfur addition before sterilization. Incubation was at 34°C. for 96 hrs.

Treatment $F = 423.6^{**}$ C. V. = 8.23% $\hat{y} = .0068 + .926x + .289x^2$.

TABLE XII

EFFECT OF SULFUR RATES OF 0.00 TO 8.00 MG. PER CULTURE AS SODIUM SULFATE ON GROWTH OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN.

Mg. of S/ culture	Dry pad wt./culture in g. Rep. I	Rep II	Rep III	Ave. dry pad wt. in g.
Blank	0.0285	0.0216	0.0298	0.0266
0.5	0.6389	0.6343	0.6450	0.6394
1.0	1.3987	1.3849	1.3459	1.3765
1.5	1.9004	1.9370	1.9143	1.9172
2.0	1.9580	1.9092	1.9862	1.9511
3.0	1.9160	1.9246	1.9610	1.9339
4.0	1.9322	1.9535	1.8930	1.9262
5.0	1.9200	1.9436	1.9455	1.9363
6.0	1.9681	1.9925	1.9940	1.9849
7.0	1.9705	1.9494	1.9338	1.9512
8.0	2.0044	1.8813	1.9819	1.9558

The basic sulfur-free media was used with sulfur addition before sterilization. Incubation was at 34°C. for 96 hrs.

Treatment F = 1494.8** C.V. = 1.86% $\hat{y} = .509 + .674x - .066x^2$

TABLE XIII

GROWTH RATE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN IN RESPONSE TO SULFUR AS SODIUM SULFATE AT THE RATE OF TWO MG. OF SULFUR PER CULTURE DURING 96 HOURS INCUBATION

Sampling intervals in hours	Dry pad weight per culture in g. Blank	Rep I	Rep II	Rep III	Ave.	Net ave. g.
48	0.0260	0.2497	0.3021	0.2526	0.2681	0.2421
54	0.0282	0.4900	0.5115	0.5112	0.5042	0.4760
60	0.0355	0.7649	0.8183	0.8920	0.8250	0.7895
66	0.0402	1.1803	1.1069	1.1264	1.1370	1.0968
72	0.0235	1.4443	1.3735	1.5960	1.4713	1.4478
78	0.0517	1.5563	1.5396	1.5619	1.5526	1.5009
84	0.0552	2.0531	1.7722	1.9624	1.9292	1.8740
90	0.0357	1.8180	2.0046	1.8503	1.8909	1.8552
96	0.0526	2.1962	2.1801	2.1378	2.1710	2.1184

The basic sulfur-free media was used with sulfur addition before sterilization and incubation at 34°C.

Treatment F = 218.0^{**} C.V. = 5.98% $\hat{y} = 3.375 + .092x - .0004x^2$

TABLE XIV

GROWTH RATE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, IN RESPONSE TO SULFUR AS SODIUM SULFATE AT THE RATE OF TWO MG. OF SULFUR PER CULTURE DURING 117 HOURS INCUBATION

Sampling intervals in hours	Dry pad wt. per culture in g. Blank	Rep I	Rep II	Rep III	Ave.	Net ave. g.
48	0.0503	0.2538	0.3115	0.2522	0.2725	0.2222
61	0.0413	0.6407	0.8721	0.7586	0.7571	0.7158
69	0.0398	1.3590	1.3090	1.3500	1.3393	1.2975
77	0.0568	1.4230	1.6600	1.8192	1.6340	1.5772
85	0.0525	1.9117	1.9186	1.9813	1.9372	1.8847
93	0.0603	2.1789	2.1816	2.1942	2.1849	2.1246
101	0.0509	2.1854	2.2828	2.1697	2.2126	2.1617
109	0.0630	2.1596	2.2091	2.2298	2.1995	2.1365
117	0.0712	2.1682	2.1464	2.0941	2.1362	2.0650

The basic sulfur-free media was used with sulfur addition before sterilization and incubation at 34°C.

Treatment $F = 240.3^{***}$ C. V. = 4.8% $\hat{y} = 4.381 + 0.121x - .0006x^2$

TABLE XV

EFFECT OF SULFUR FROM DIFFERENT SOURCES AT THE RATE OF TWO MG. OF SULFUR PER CULTURE ON THE GROWTH OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN.

Sulfur compound	Dry pad wt. / culture in g. Rep I	Rep II	Rep III	Ave. g.
Blank	0.0297	0.0263	0.0192	0.0251
Sodium sulfate	2.2397	2.1779	2.0655	2.1677
Sodium thiosulfate	2.2060	2.1306	2.0608	2.1373
Sulfur	0.1259	0.1065	0.1044	0.1123
Sodium sulfide	0.3910	0.4432	0.3871	0.4071
Sodium sulfite	0.0592	———	———	———
Potassium thiocyanate	0.9934	1.4843	1.2254	1.2343
Sulfanilamide	0.0140	0.0070	0.0100	0.0103
Thiourea	0.0906	0.0900	0.0221	0.0675

The basic sulfur media was used with sulfur compound additions before sterilization. Incubation for 96 hours at 34°C. Slight growth was obtained in only one of the three replicated cultures with sodium sulfite and this was thought to be the result of contamination or experimental error.

Treatment F = 283.3*** C. V. = 12.59%

TABLE XVI

EFFECT OF SULFUR RATES FROM DIFFERENT SOURCES ON THE GROWTH OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN.

Sulfur compound	mg. S/ culture	Dry pad wt. per culture Rep I	Rep II	Rep III	ave. g.
Blank	0	0.0235	0.0274	0.0321	0.0276
Sodium sulfate	1	1.3650	1.1978	1.3633	1.3087
	2	2.1143	2.2035	2.1686	2.1621
	3	2.2311	2.1918	2.2887	2.2370
Sodium thio-sulfate	1	1.0761	1.0523	1.0539	1.0608
	2	2.2210	2.1810	2.1723	2.1914
	3	2.4163	2.3064	2.3580	2.3602
Potassiumthio-cyanate	1	0.8314	0.8088	0.8085	0.8162
	2	1.1452	1.1956	1.1066	1.1491
	3	0.8092	0.9023	lost	0.8857
L-cystine	1	1.0635	1.2038	1.1514	1.1395
	2	2.2479	1.8631	2.0322	2.0477
	3	2.2129	2.2864	2.2271	2.2421

The basic sulfur free media was used with sulfur compound additions to cool sterilized media and incubation at 34°C. for 96 hours.

Treatment F = 515.7** C. V. = 4.82%

TABLE XVII

EFFECT OF DIFFERENT LEVELS OF SULFUR FROM THREE SULFUR-CONTAINING AMINO ACIDS ON THE GROWTH OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN.

Amino acid containing sulfur	mg. S/ culture	Dry pad wt. in g. Rep I	Rep II	Rep III	ave. g.
Blank	0	0.0147	0.0148	0.0185	0.0160
L-cystine	1	1.1428	1.2420	1.1803	1.1883
	2	2.1306	2.1484	2.1990	2.1593
	3	2.4719	2.3771	2.3792	2.4094
L-cysteine	1	0.9867	0.9993	1.0450	1.0103
	2	1.6929	1.7003	2.0797	2.8254
	3	2.2310	2.2760	2.2005	2.2358
L-methionine	1	1.1500	1.2528	2.1770	1.1732
	2	2.1660	1.8982	2.0450	2.0336
	3	2.1520	2.2345	2.2873	2.2246

The basic sulur-free media was used with amino acid additions made to cool sterilized media prior to inoculation and incubation at 34°C. for 96 hours.

F values: source = 4.5* Rate = 7.2**

source x rate = 2.2 (m. s.)

C. V. = 11.48%

TABLE XVIII

A COMPARATIVE STUDY ON THE RESPONSE OF TWO ASPERGILLUS NIGER (v.T.) STRAINS: TURTOX AND ATCC 6275, TO RATES OF SULFUR AS SODIUM SULFATE.

Mg. S/ culture	TURTOX Dry pad wt. per culture in g. Rep I	Rep II	Rep III	Ave.	ATCC 6275 Dry pad wt. per culture in g. Rep I	Rep II	Rep III	Ave.
Blank	0.0163	0.0190	lost	0.0176	0.0275	0.0168	0.0205	0.0282
0.15	0.1929	0.1820	0.1423	0.1724	0.1835	0.1563	0.1620	0.1672
0.30	0.2902	0.3086	0.3146	0.3044	0.3215	0.3144	0.3062	0.3140
0.60	0.6878	0.6632	0.6526	0.6678	0.6600	0.7071	0.6355	0.6675
0.90	1.1611	1.1330	1.2912	1.1951	1.0325	0.9769	1.0700	1.0264
1.20	1.6248	1.6368	1.6098	1.6238	1.6705	1.5004	1.4985	1.5564

The basic sulfur free media was used with sodium sulfate additions before sterilization then incubated at 34°C. for 130 hours.

F values: cultures = 8.5** Levels = 1094.4** C. V. = 6.73%

TABLE XIX

AVAILABILITY OF SULFUR FROM SULFANILAMIDE WITH ADDITIONS OF p-AMINOBENZOIC ACID AND SODIUM SULFATE FOR TURTOX STRAIN.

Sulfanilamide. mg. S	P-amino benzoic acid mg.	Sodium sulfate mg. S	Dry pad wt./culture in g. Rep I	Rep II	Rep III	Ave. g.
0	0.0	0	0.0778	0.0780	0.0682	0.0746
0	0.1	0	0.1006	0.0943	0.0896	0.0948
0	0.2	0	0.0708	0.0721	0.0785	0.0738
1	0.0	0	0.1546	0.0816	0.0893	0.1100
1	0.1	0	0.0768	0.1027	0.0842	0.0879
1	0.2	0	0.1127	0.1020	0.0847	0.0998
2	0.0	0	0.0923	0.0888	0.0667	0.0826
2	0.1	0	0.1259	0.0935	0.0844	0.1012
2	0.2	0	0.0968	0.0899	0.1028	0.0965
1	0.2	2	2.0986	2.0456	2.0797	2.0746

The basic sulfur free media was used with the above additions made before sterilization. Cultures were incubated at 34°C. for 96 hours.

Treatment F = 4216.9*** C.V. = 5.8%

TABLE XX

RESPONSE OF ASPERGILLUS NIGER (v.T.), TURTOX STRAIN, TO SULFUR FROM WATER EXTRACTS OF A NORGE FINE SANDY LOAM.

Soil treatment	g. soil/ culture	Dry pad wt. in g. Rep I	Rep II	Rep III	Ave.
Check	0	0.1084	0.0930	0.1114	0.1042
Untreated	1	0.1100	0.1092	0.1019	0.1070
	2	0.1135	0.1040	0.1116	0.1097
	4	0.1512	0.1460	0.1460	0.1459
5 m. e. $CaCO_3$/ 100 g. soil	1	0.1677	0.1625	0.1639	0.1646
	2	0.2469	0.2444	0.2005	0.2302
	4	0.3914	0.3867	0.3780	0.3853
5 m. e. elemental S/100 g. soil	1	0.8850	0.7431	0.8005	0.8095
	2	1.5938	1.6335	1.5588	1.5953
	4	1.8900	1.9690	2.0017	1.9535

Soil samples were extracted with distilled water in a 2:1 water : soil ratio.

Additions of soil extracts were made before sterilization with incubation at 34°C. for 96 hours.

F values: Soil treatment = 4141.7** Rates = 43.8** C.V. = 6.08%

TABLE XXI

EFFECT OF CALCIUM CARBONATE AND SULFUR TREATMENT ON EXTRACTABLE SULFUR FROM A NORGE FINE SANDY LOAM SOIL.

Soil treatment	Mg. sulfate in 25 g. of soil Rep I	Rep II	Ave.	Ave. mg. S/ 100 soil g.
Untreated	0.62	0.62	0.62	1.00
5 m. e. $CaCO_3$/ 100 g. soil	3.34	3.17	3.25	5.35
5 m. e. elemental S/100 g. soil	36.61	35.18	35.89	59.50

Extractable sulfates were determined by extracting the soil with distilled water in a 2:1 water:soil ratio and precipitating the soluble sulfate as $BaSO_4$.

TABLE XXII

SOME PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE NORGE FINE SANDY LOAM USED IN THE SOIL SULFUR STUDY.

Texture:	
Percent sand	75.0
Percent silt	21.5
Percent clay	3.5
Cation exchange capacity	
(Meq./100 g.)	4.12
pH	6.3
Percent organic matter	0.89
Percent nitrogen	0.036
Easily soluble phosphorus	
(lbs./acre)	28.16
Exchangeable potassium	
(lbs./acre)	94.00

Norge Fine Sandy Loam Soil

This soil was obtained at the Paradise Station, approximately ten miles south and seven miles west of Stillwater, Oklahoma on the NW$\frac{1}{4}$ SE$\frac{1}{4}$ Section 34, T 18 N, R 1 E.

The Norge fine sandy loam soil was developed from old alluvium and is a well developed rich praire soil.

The above chemical and physical characteristics were determined by Shultz (42).

VITA

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Candidate for the Degree of

Master of Science

Thesis: PROLIFERATION OF ASPERGILLUS NIGER (v. T.) IN BIO-ASSAY INVESTIGATIONS WITH SULFUR.

Major Field: Soils

Biographical:

Personal Data: Born in Benha, Egypt, UAR, August 28th, 1933. Son of Ibrahim Mohamed Katta and Na-eima Al-Sayed Ahmad.

Education: Attended elementary and high school in Cairo and Benha, Egypt. Graduated from high school in June, 1952. Received a Bachelor of Science degree with honors in soils from Ain-Shams University, Cairo, Egypt, UAR in 1956. In junior and senior years of college received distinction awards. Received a UAR scholarship for graduate studies in 1960. Studied in the University of California, Berkeley, in 1960-1962. Graduate study at Oklahoma State University from 1962 to present.

Professional Experience: Employed as Agricultural Officer in Ministry of Agriculture for high quality cotton seed production in July 1956. In November 1956, was appointed research worker at the Soil Department of the Desert Instition of Mattareia, Cairo, Egypt. In December 1957, was appointed teacher-assistant in the Soil Dept. of the College of Agriculture, Ain-Shams University.