Sulfide Inhibition of Oxidases in Rice Roots

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ABSTRACT

Levels of H_2S prevalent in Louisiana (USA) rice fields during the heading-flowering stage were toxic to rice seedlings in vitro. Rice seedling biological assays showed significant inhibition of the respiration of rice roots pretreated with H_2S at levels as low as 0.07-0.1 μg/ml. Drastic inhibition of the activities of the metallo enzymes (cytochrome oxidase, catalase, peroxidase, ascorbic acid oxidase, and polyphenol oxidase) in roots of H_2S-pretreated rice seedlings was also shown.

These empirical data suggest that reduction of rice root oxidative capacity, terminal oxidation, and other physiological functions such as nutrient uptake by hydrogen sulfide levels occurring in Louisiana rice fields may cause hitherto unrecognized toxicant diseases of rice, manifested as reduced grain yields.

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Extensive literature shows that anaerobic metabolism of bacteria in submerged rice soils can produce an array of organic substances whose composition, concentration, and stability are governed by the nature and content of organic matter, environmental factors, microbial activity, and duration of submergence (1, 22). One of the toxic metabolites produced by anaerobic bacteria is hydrogen sulfide (H_2S). Under the anaerobic conditions prevailing in rice fields, attention has been focused principally on ferrous iron (Fe^{++}), the soluble sulfide species H_2S, HS^-, $S^=$, and their possible toxicity to rice roots (9, 21).

The possible presence of hydrogen sulfide in rice soils raises the question of how its known toxicity as an enzyme inhibitor might influence events in a rice field (14, 17, 18). The formation of free H_2S in soil has been demonstrated conclusively and linked to the so-called Akiochi (Autumn decline) disease of rice in Japan (19, 20). Postulated consequences of the possible presence of this gas in rice fields have been outlined with respect to the mentek disease of rice in Java and the straighthead disease in the USA (3, 19, 20).

H_2S is a well-known inhibitor of the enzymes of aerobic respiration (11), and iron-containing enzymes also have been shown to be inhibited by H_2S (10, 16). Furthermore, H_2S is known as one of the principal inhibitors of nutrient uptake by rice plants (4). Mitsui (15) showed evidence linking phosphorus uptake with oxidative phosphorylation; potassium accumulation, with nucleic acid metabolism; and nitrogen absorption, with the tricarboxylic acid (TCA) cycle.

Hollis (9) advanced an hypothesis that symptomless toxicant diseases caused by soluble

sulfides (sulfide disease of rice) occur in Gulf Coast (USA) rice areas. These diseases are not detectable until late season effects on rice plants appear. These effects include nutrient deficiencies, fungus diseases associated with weakened plants, or simply differences in grain yields. They are truly symptomless in a specific sense if the only observable differences are in grain yield.

In light of these findings and the principles underlying sulfate reduction in flooded soils, we attempted to study the effects of H_2S, at its prevailing levels (0.00005 to 0.64128 μg/ml) in Louisiana (USA) rice fields (2), on the activities of some oxidative enzymes in rice seedling roots, in order to further test the hypothesis that H_2S is a yield-reducing factor in Louisiana (USA) rice fields.

MATERIALS AND METHODS.—*Pretreatment of plants with H_2S.*—The Saturn cultivar of rice (*Oryza sativa* L.) was used in all experiments. Plants were grown in vermiculite in 1-quart polyethylene containers at 24 ± 2 C for 12 hr in fluorescent light. Seedlings 15 to 21 days old were selected for root uniformity. Rice plants were grouped into 30-40 seedlings/group, and roots of each group were fully immersed in solutions of known concentration of H_2S in long test tubes for 3, 5, or 6 hr as specified in each experiment.

Following pretreatment with H_2S, the seedling roots were washed 3 times in distilled water and cut from plants under the water surface before use in studies of respiration.

Changes in H_2S concentration in culture solution with time.—Potentiometric titrations were used in this study to determine the changes in H_2S concentrations in culture solutions at 1, 3, 5, 6, 7, 8, and 18 hr in which no plants were included. Changes in H_2S concentrations were also determined at 1, 2, 3, 5, and 7 hr for culture solution in which rice roots were immersed. Concentrations of H_2S used ranged from 0.17 to 8.81 μg/ml. Analyses of variance were run for all treatments with and without plants and for combined treatments, and the experimental results are presented for four of the lower H_2S levels and for four time periods.

Preparation of rice root homogenates.—Samples weighing 0.6 g from either nontreated or pretreated roots at lower levels of H_2S (3.2-0.1 μg/ml) were homogenized for 2 min at top speed in a Sorvall Omni-Mixer in 10 ml potassium phosphate buffer at an ionic strength and pH specified for each experiment. The homogenizing cup was immersed in an ice bath during grinding. The homogenates were strained through four layers of cheesecloth and kept in an ice bath until used. For enzyme extracts, the homogenates were centrifuged at 1,000 *g* at 4 C for 20 min, and the supernatant fluids were kept refrigerated prior to spectrophotometric studies.

The optimum concentration of substrate and tissue homogenate or enzyme extract used in each assay was determined by preliminary work. The substrates and tissue homogenates were prepared on the same day as the assays.

Determination of respiratory activity of the whole root.—Oxygen consumption was determined manometrically with a Gilson differential respirometer at 21 C. Duplicate flasks were run for all treatments, with air as the gas phase. Excised roots were floated in 3.0 ml of 0.02 M potassium phosphate buffer at pH 6.0. The ionic concentration maintained with this buffer prevented plasmolysis of root tissue and resulted in a high level of respiratory activity. The center well contained 0.3 ml of 10% KOH and filter paper wicks.

The first manometer reading was recorded after a 15- to 20-min equilibration period. Oxygen consumption by root tissue is presented as μliters/g dry wt per hr. Results are reported as average per cent inhibition. The individual comparisons were made according to Duncan (8). The same experiment was repeated using KCN at initial concentration of 0.01 M and 0.001 M at 0.3 ml tipped from the side arm after 30 min.

Determination of enzymatic activity.—Enzymatic activities were estimated with a Perkin-Elmer recording spectrophotometer at 24 ± 2 C. Distilled water or a boiled enzyme extract replaced the substrate solution in the reference cuvette. The enzymatic activities were estimated as the slopes of the linear portions of the curves obtained by plotting absorbance versus time and data were recorded for representative experiments.

1) *Iron-containing oxidases.*—Peroxidase activity was determined by measuring the oxidation of pyrogallol to purpurogallin in the presence of H_2O_2 at 425 mμ. The sample cuvette contained 0.5 ml 0.1 M potassium phosphate buffer at pH 7.0, 0.3 ml enzyme extract, 0.3 ml 0.05 M pyrogallol, 0.1 ml 1.0% H_2O_2, and distilled water to bring cuvette contents to 3.0 ml.

Catalase activity was determined manometrically with a Gilson respirometer by measuring the evolution of oxygen in the presence of H_2O_2. One-half ml of homogenate was pipetted into the side arm. After equilibration, the contents of the side arm were tipped into the main compartment, which contained 3.0 ml of 0.01 M potassium phosphate buffer at pH 5.8 and 0.2 ml of 0.1% H_2O_2. Readings were taken at 1-min intervals for 5 min. Results were based on averages of two flasks as μliter O_2/g fresh wt per hr and presented as per cent inhibition.

Cytochrome oxidase activity was determined spectrophotometrically at 550 mμ by the change in optical density accompanying the oxidation of reduced cytochrome *c* by the enzyme extract. The reaction mixture contained 2.8 ml reduced cytochrome *c* solution (0.6 mg/ml) at pH 7.7 and 0.2 ml enzyme extract. Cytochrome *c* was reduced by adding sufficient sodium hydrosulfite (0.004 M) to obtain an absorbance ratio (550 nm):565 nm greater than 6.0 (13).

2) *Copper-containing oxidase.*—Ascorbic acid oxidase activity was measured by following the disappearance of ascorbic acid at 265 nm (13). The sample cuvette contained 1.0 ml potassium phosphate buffer at pH 7.0, 0.3 ml 0.01 M ascorbic acid, and 1.2 ml enzyme extract brought to a final volume of

3.0 ml with distilled water. The results were expressed as the changes in absorbance for the first 5 min of the reaction per 1.0 ml of extract.

Polyphenol oxidase activity was measured by following the oxidation of catechol at 546 nm (12). The reaction mixture contained 1.0 ml 0.1 M potassium phosphate buffer at pH 7.0, 0.1 ml of (10.0 mg/ml) catechol, 0.5 ml (5 mg/ml) L-proline, and 1.0 ml enzyme extract. The reaction mixture was incubated at room temperature for 15 min prior to reading. The enzyme activity was expressed as the change in absorption versus time (hr).

RESULTS.—*Effect of H_2S concentration and contact period on rice root respiration.*—The per cent inhibition of respiration in H_2S-pretreated rice roots increased in a cumulative manner in relation to the duration of the contact period (Table 1). This inhibition, however, was linear over the first 4 hr of exposure, after which it decreased with time.

Per cent inhibition of respiration was significant at all exposures except at the first-hour period. Critical inhibition of respiration was apparent at 1, 3, 4, and 5 hr after contact.

Inhibition of respiration of rice roots pretreated with low levels of H_2S for 5 hr in water culture is shown in Table 2. The per cent inhibition of oxygen consumption was about 40, 25, and 14% at initial concentrations of 16.0, 3.2, and 0.1 μg/ml H_2S, respectively. Inhibition by low levels of H_2S was significant at almost all concentrations when compared with the control. Mean differences between the two highest concentrations at each level were not significantly different; i.e., between 16.0-10.0, 4.8-3.0, or 3.2-0.5 μg/ml.

TABLE 1. Effect of contact period on respiration of rice roots pretreated with H_2S at 3 μg/ml H_2S

Contact period (hr)	% Inhibition[a] (at 3.0 μg/ml H_2S)
0.0	0.0 a[b]
1.0	9.6 a
2.0	12.3 ab
3.0	20.8 bc
4.0	29.5 c
5.0	26.8 bc

[a] Mean inhibition of O_2 uptake/g dry wt per hr.

[b] All means which are followed by a letter in common do not differ significantly from each other at the .05 level of probability.

TABLE 2. Effect of H_2S concentration on respiration of rice roots pretreated with H_2S for 5 hr

H_2S concentration (μg/ml)	Mean oxygen uptake μliter/hr/g dry weight	Inhibition, % of control[a]
3.2	2,828.80	25.6 a
0.5	3,183.33	16.7 ab
0.1	3,288.09	14.0 b
0.0	3,820.24	00.0 c

[a] All means which are followed by a letter in common do not differ significantly from each other at the .05 level of probability.

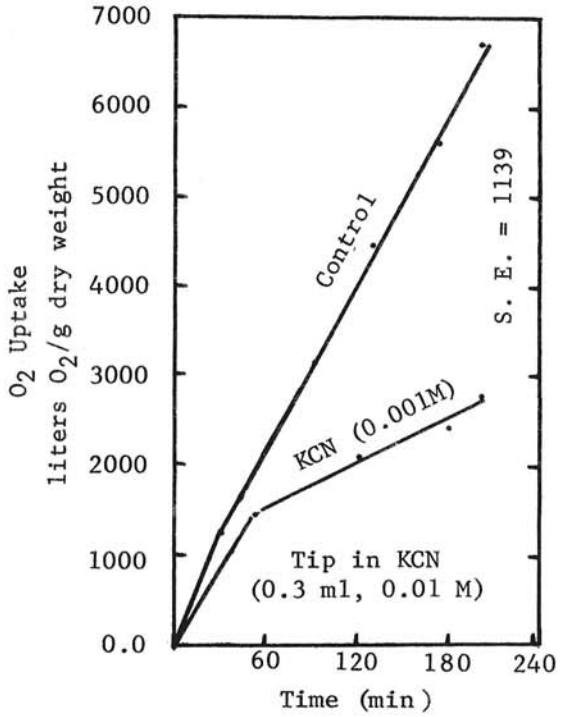

Fig. 1. Response of rice root respiration to the addition of KCN at a final concentration of 0.001 M.

Rice root respiration was 50 to 60% inhibited when treated with potassium cyanide at a final concentration of 0.001 M (26.017 μg/ml CN) (Fig. 1). This inhibition was statistically significant and indicative of the presence of the metal-containing terminal oxidases.

Changes in H_2S concentrations in culture solution with time.—The H_2S concentrations in culture solutions (in which plants or no plants were included) were measured and found to decrease with time (Table 3). There was no significant difference in the rate of H_2S loss between cultures containing plants and cultures in which no plants were included. However, a highly significant difference was apparent for H_2S concentrations, period of exposure, and all first and second order interactions, except for the plant times concentration interaction (Table 4).

H_2S concentration reached 43 and 64.7% for initial concentrations of 0.71 and 0.17 μg/ml, respectively, after 5 hr of exposure in culture solutions with rice seedlings (Table 3). H_2S concentration after 6 hr of exposure was almost half the 0.17 μg/ml H_2S initial concentration. The H_2S effect is a product of time and concentration; the use

TABLE 3. H_2S changes in culture solution with or without rice plants as a function of exposure time

Elapsed time (hr)	H_2S concentration[a] (μg/ml)			
	Without plants		With plants	
0	0.71	0.17	0.71	0.17
1	0.51	0.12	0.54	0.13
3	0.32	0.11	0.33	0.12
5	0.29	0.10	0.31	0.11
6	0.28	0.09	0.28	0.09

[a] Average of two readings.

TABLE 4. Analysis of variance of average change in H_2S concentration[a] in culture solution, with and without plants[b], as a function of exposure time

Source of variation[a]	Degrees of freedom	Mean square
Treatment (Plant)	1	2.849
Time	5	12.592**
Concentration	9	116.234**
Plant × Time	5	1.268**
Plant × Concentration	9	0.401
Time × Concentration	45	1.196**
Plant × Time × Concentration	45	0.209**
Error	120	0.00000019

[a] These data include higher concentrations of H_2S not shown in Table 3.

[b] Plants were considered as fixed effects.

[c] ** = Significant at the .01 level of probability.

of high concentration permits a short exposure period.

Effect of H_2S on enzymatic activities.—We made an attempt to investigate the effect of H_2S on root oxidases, especially catalase and peroxidase activities in H_2S-pretreated rice seedlings, since our previous findings showed inhibition of oxygen consumption (2).

1) *Catalase.*—Per cent catalase activity, in roots at a root concentration of 15 mg tissue/ml homogenate, was inhibited 20.7 ± 1.8, 26.4 ± 2.1, 31.1 ± 2.35, and 34.6 ± 2.40, by 0.1, 0.7, 1.2, and 2.4 μg/ml of H_2S, respectively, after an initial 5-hr pretreatment. In further experiments, the per cent inhibition of catalase activity was relatively the same.

2) *Cytochrome oxidase.*—Cytochrome oxidase activity was inhibited 40, 60, and 90% at concentrations of 0.1, 0.5, and 3.0 μg/ml H_2S, respectively, after an initial 6-hr pretreatment (Fig. 2).

3) *Peroxidase.*—Substantial inhibition of peroxidase activity occurred as a function of H_2S concentration and contact period (Fig. 3-A, B). Per cent inhibition of peroxidase activity at a 3-hr pretreatment was, on the average, 64% of that at a 6-hr pretreatment.

4) *Copper-containing enzymes.*—Ascorbic acid oxidase activity was inhibited 29.0 and 40.0% at concentrations of 0.1 and 3.2 μg/ml H_2S, respectively, after an initial 6-hr pretreatment (Fig. 3-C). Per cent inhibition of polyphenol oxidase

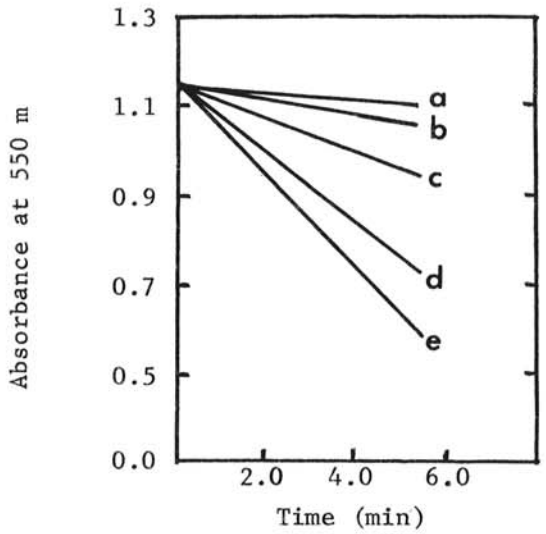

Fig. 2. Effect of H_2S concentrations (b = 3.0, c = 0.7, d = 0.1, and e = 0.0 μg/ml) on cytochrome oxidase activity; a = boiled enzyme served as control.

activity was 7.8 and 37.7% at H_2S concentrations of 0.1 and 3.2 μg/ml, respectively, after 6-hr pretreatment (Fig. 3-D).

DISCUSSION.—The inhibition of cytochrome oxidase by rice field concentrations of H_2S suggests the possibility of deleterious effects or damage to rice plants in the field and the occurrence of reduced grain yields even in the absence of seasonal symptoms of disease. There is abundant evidence that the terminal oxidase system of living organisms, including plants, is cytochrome oxidase (4, 5, 6, 7, 11). The significance of inhibition of the other oxidase enzymes lies in: (i) the number of enzymes inhibited; (ii) their generic relations to cytochrome oxidase; and (iii) the possibility that vital plant functions may be interrupted (7, 11). For example, Mitsui (14, 15) postulated that the oxidative power of the rice root is due to the decomposition of H_2O_2 produced in the glycolic acid pathway; thus, the inhibitions or alterations of peroxidase activity demonstrated here suggests that the rice root oxidative capacity may be affected.

Inhibition of in vitro root respiration at all levels of H_2S confirms the linear nature of inhibition (14) and extends the inhibitory effect to lower concentrations of H_2S under controlled conditions. The loss of H_2S in root exposure treatments is caused by seedling uptake of H_2S and by its oxidation and contribution to carbonate formation, in addition to its loss to the atmosphere. Thus, the reported minimum toxic range of H_2S, 0.7-0.1 μg/ml, is undoubtedly higher than the actual levels in vitro. The use of high H_2S concentrations in vitro was necessary for achieving a full range of time-concentration exposures of rice roots.

The average inhibition of rice roots pretreated by 0.1 μg/ml H_2S was 22% for both respiration of rice roots and for activity of all oxidase enzymes. Mitsui

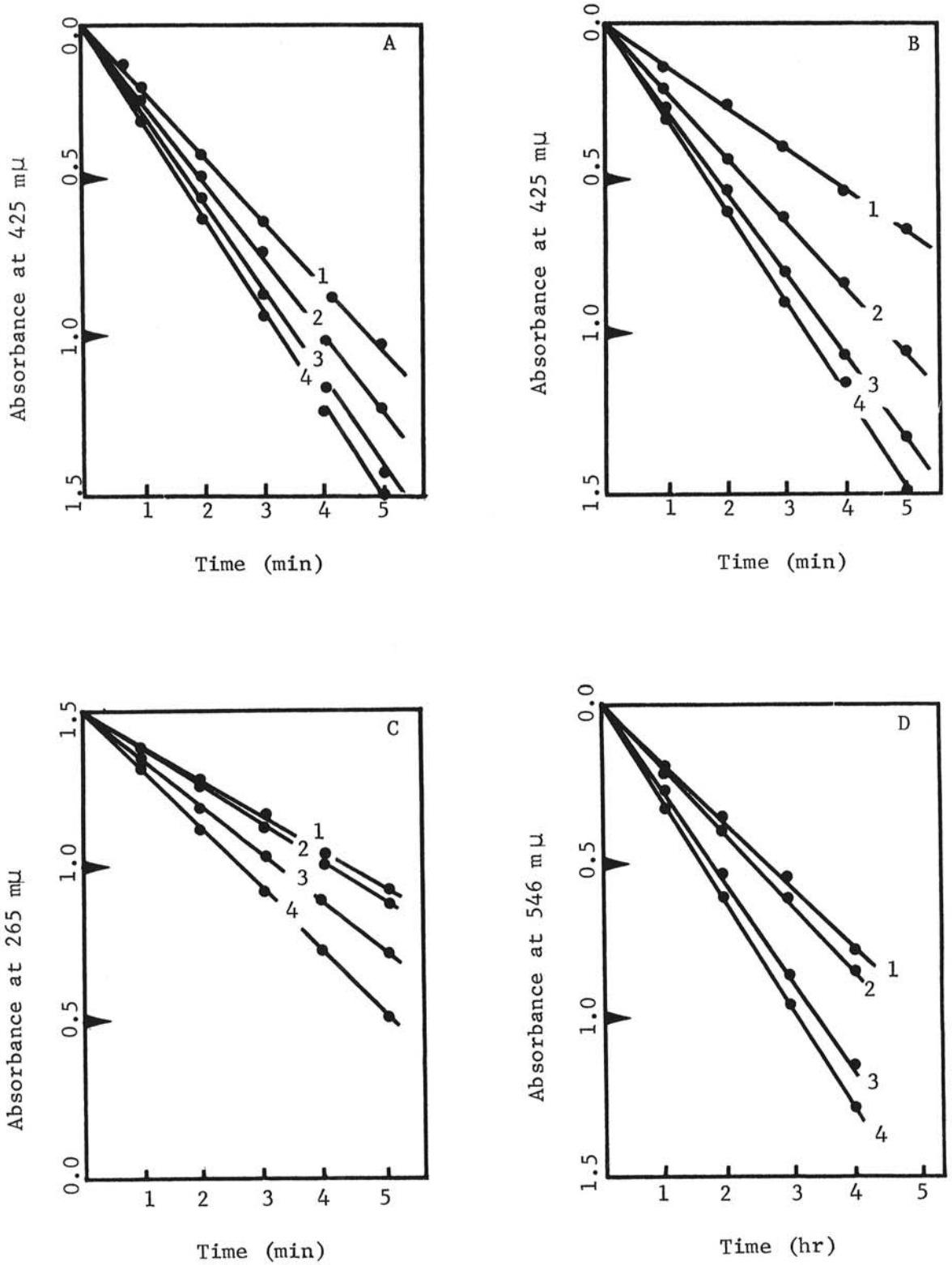

Fig. 3. Effect of H_2S concentrations (1 = 3.2, 2 = 0.7, 3 = 0.1, and 4 = 0.0 μg/ml) on peroxidase, ascorbic acid oxidase, and polyphenol oxidase activities. **A)** Peroxidase: 3-hr pretreatment. **B)** Peroxidase: 6-hr pretreatment. **C)** Ascorbic Acid Oxidase: 6-hr pretreatment. **D)** Polyphenol Oxidase: 6-hr pretreatment.

(15) demonstrated that inhibition of respiration affects nutrients uptake by the rice plant; we have shown that enzyme activities mediating oxygen uptake and transfer are inhibited by H_2S levels occurring in Louisiana rice fields. It would be of interest to make quantitative tests of the apparent relationships between these variables and rice plant vigor and grain yield.

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