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IN VITRO STUDY OF TOXICITY OF SOLUBLE SULPHIDES TO THREE NEMATODES PARASITIC ON RICE IN SENEGAL

BY

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Toxicity of sulphides to three species of nematodes parasitic on rice from Senegal (*Hirschmanniella oryzae, H. spinicaudata* and *Tylenchorhynchus mashhoodi*) was studied *in vitro* using three different methods.

A Sulphide Time Unit (STU) was defined related to the average concentrations of sulphides and their variations during the tests.

With hydrogen sulphide dissolved in water, the very low pH increased nematode mortality. When hydrogen sulphide was dissolved in a buffered medium used for bacterial culture, mortality was lower but a shock effect appeared due to sudden contact with high concentrations of sulphides and toxicity of some of the components of the medium. When hydrogen sulphide was produced by sulphate reducing bacteria a similar shock effect was still observed, but only when high concentrations were reached within 1 or 2 days: in such experiments nematodes were killed by low STU, and differences were observed in resistance of the three species. However, when sulphide accumulated more slowly, i.e., when lethal concentrations were reached in 3 or more days, the sensitivity of the three species was similar: 50% mortality was obtained at about 120-130 STU.

The possibility of using sulphate reducing bacteria for biological control of parasitic nematodes of rice is discussed.

A survey of root parasitic nematodes of rice in Senegal has shown that in some fields, particularly in the Casamance region, few or no nematodes were found (Fortuner & Merny, 1973). In four of these fields surveyed for sulphate reducing bacteria (Jacq, 1972) their activity was found to be very high. This suggested that the very low nematode populations were possibly due to the nematicidal properties of hydrogen sulphide. This phenomenon was first reported from Louisiana rice fields by Rodriguez-Kabana *et al.* (1965) who also established *in vitro* the relation between H_2S concentration and the time required to kill *Tylenchorhynchus martini.*

The present study was undertaken to determine *in vitro* the lethal dose of sulphides against the three most common species of rice parasitic nematodes in Senegal: *Hirschmanniella spinicaudata* (Schuurmans Stekhoven), *Hirschmanniella oryzae* (van Breda de Haan) and *Tylenchorhynchus mashhoodi* Siddiqi & Basir. They were subjected for 1 to 6 days to various concentrations of sulphides produced in three different ways:

— hydrogen sulphide dissolved in deaerated water;
— hydrogen sulphide dissolved in a sterile buffered medium used for sulphate reducing bacteria culture;
— hydrogen sulphide produced by sulphate reducing bacteria in the same buffered medium.

MATERIALS AND METHODS

Nematodes were obtained from stock cultures maintained at the O.R.S.T.O.M. laboratory at Dakar, originating from specimens from the Casamance and the Senegal River regions in Senegal. Two to four lots extracted at different times were used for each trial.

Nematodes were extracted from soil by elutriation (Seinhorst, 1962) or from rice roots by a mistifier apparatus (Seinhorst, 1950) and put in deaerated water. About one hundred nematodes in 1 ml of water were injected with a syringe into each test flask. A total of 650 flasks (excluding controls) were subjected to various concentrations and for various times to sulphide produced by the following methods.

Hydrogen sulphide dissolved in deaerated water

4N-hydrochloric acid (HCl) was reacted with iron monosulphide (FeS) at 60° C; the gaseous hydrogen sulphide produced was conducted by a nitrogen stream into vessels containing 100 ml of deaerated water through which it bubbled and dissolved. By varying the durations of bubbling and reagent contents hydrogen sulphide concentrations between 0.1 and 60 ppm $S^{=}$ were produced.

Soluble concentrations of sulphides were measured at 22° (Chaudhry & Cornfield, 1966). Aliquots of less than 2.2 ml were pipetted into 2.7 ml plastic test tubes containing 0.5 to 1 ml of a nematode suspension. The tubes were then filled with deaerated water, avoiding air bubbles under the caps to prevent oxidation of sulphide. The tubes were incubated at 32°. Sulphide and pH were measured again at the end of the test.

Hydrogen sulphide dissolved in a sterile buffered medium used for bacterial culture

0.5 to 5 ml of hydrogen sulphide solution obtained as described above were syringed through the rubber membrane of the screw cap into 11 ml flasks containing about 0.5 ml of nematode suspension. Sterilised Starkey's medium modified by Pichinoty (1966), used for cultivation of sulphate reducing bacteria, was added to fill the flasks in a manner to avoid air bubbles. The flasks were incubated at 32°. Sulphide concentrations were measured every day withdrawing 1 ml samples with a syringe without opening the flasks. Each flask was then refilled with 1 ml of newly prepared hydrogen sulphide solution. The pH was measured at the end of the tests.

Hydrogen sulphide produced by sulphate reducing bacteria in a buffered medium

Flasks containing the nematode suspension and Starkey medium were prepared as described above, then inoculated with 0.5 to 2 ml of 2-3 days old pure culture of a *Desulfovibrio* strain (S6) isolated from a rice field at Savoigne, Senegal. The flasks were filled completely to ensure anaerobiosis and maintained at 32°. The bacteria produced hydrogen sulphide which dissolved in the medium. Concen-

trations of soluble sulphide were measured daily or twice a day as previously described, after which the flasks were refilled with either Starkey's medium or sterile deaerated water. The pH was measured at the end of the tests.

Controls

Several lots of nematodes were put in 11 ml flasks with the same water as used during the tests, then similarly processed to estimate the number of healthy nematodes lost in the extraction procedure and so correct the results.

Other check lots were kept in the flasks up to 75 days without aeration.

Hydrogen sulphide in water greatly lowers the pH, therefore the effect of low pH values on the nematodes was tested during 12 days. Three buffers were used (cf. Brunel, 1948):

— lactate buffers of Michaelis (pH 2.65 to 5.9)
— phosphate buffers of Michaelis (pH 5.3 to 8)
— phosphate-borax buffers of Kolthoff (pH 6 to 9).

At the end of each trial, the total number of nematodes, dead, alive, or inactive, present in the test tube was determined under a dissecting microscope. The nematode suspension was then poured onto two layers of tissue paper in a holder with a screened bottom. Excess water flowed immediately through this filter and was thus lost. The filter was then put in a Petri dish with distilled water.

Nematodes in the Petri dish were counted after 24 hrs to calculate the percentage of active nematodes, based on the assumption that all living nematodes recovered and wriggled through the tissue filter in less than 24 hours, but that the dead or permanently incapacitated ones were retained.

RESULTS

Controls

The number of nematodes lost during the described procedure ranged from 7 to 12% depending on the various lots used.

When the nematodes were left in pure water up to 13 days for *H. spinicaudata*, 40 days for *T. mashhoodi* and 50 days for *H. oryzae*, the number of lost nematodes was found to be independent of the duration of the tests. When the nematodes were left longer, there was a highly significant correlation between the duration of the tests and the percentage (transformed in degrees) of lost nematodes. (coefficient of correlation $r = 0.7158$ with 20 degrees of freedom for *H. spinicaudata* and $r = 0.6614$ with 12 d.f. for *T. mashhoodi*). Tests with *H. oryzae* did not last long enough for differences to appear (see figure 1).

With the phosphate-borax buffers, nematode mortality was very high for all the pH values tested (6 to 9).

No mortality was observed for any of the three species with the phosphate buffers at pH 5.3 to 8. With the lactate buffers mortality occurred at the lower

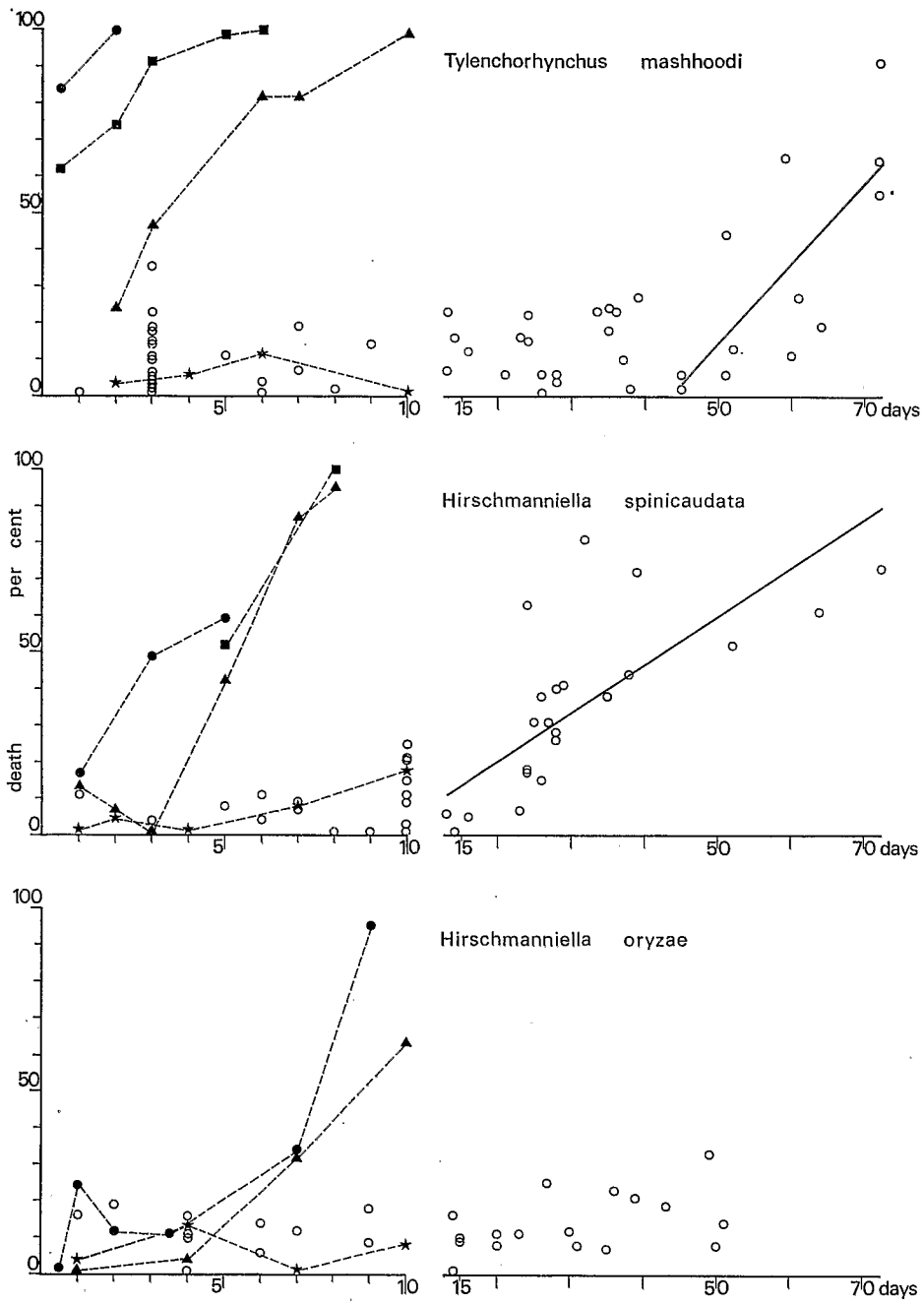

Fig. 1. Percentage mortality of the tested nematode species: — when left in water for a number of days: ○ — when left in a lactate buffer at pH 2.6: ●; 3.5: ■; 4: ▲ and 5.3: ✱.

pH values (2.6 to 4) as shown in fig. 1. The three species reacted differently, *T. mashhoodi* being the most susceptible to low pH, *H. spinicaudata* being less so, while *H. oryzae* was the most resistant. No mortality occurred for any of the three species at pH 5.3. However mortality occurred for *T. mashhoodi* at pH 5.5 and 6.9 probably because of the high percentage of Na lactate used with this buffer (9.9 g/l). This percentage is only 6 g/l in Starkey's medium.

Definition of a toxicity unit combining sulphide concentrations and duration of tests

Concentrations of sulphide in the flasks are not constant: chemically produced sulphide disappears by oxidation while biological sulphide increases with the growth of sulphate reducing bacteria.

We defined a Sulphide-Time Unit (STU) which is the toxic action of 1 ppm of soluble sulphide during 24 hours.

In every flask, the concentrations of soluble sulphide: S_0, S_1, S_2 ... were measured after: t_0, t_1, t_2 ... hours as previously described. We calculated the average concentration $\frac{S_0 + S_1}{2}$ during time $t_1 - t_0$. The number of STU was then:

$$\text{STU} = \frac{S_0 + S_1}{2} \times \frac{t_1 - t_0}{24}$$

The different STU calculated from successive measurements were added to obtain the total STU to which the nematodes had been subjected during the test.

When calculating the average concentration $\frac{S_0 + S_1}{2}$, we assumed that decrease or increase of concentration of sulphide was linear. Actually, the variations are not linear but parabolic. A systematic error was made which we attempted to lessen by shortening the time between the two measurements of sulphide.

Effects of hydrogen sulphide dissolved in deaerated water

The death of nematodes occurred at very low STU: 100% death was observed with as low as 1.4 STU for *T. mashhoodi*, 2.3 STU for *H. spinicaudata* and 7.4 STU for *H. oryzae*. In other test tubes, some individuals were found still alive at higher STU but no living nematode was observed in any tube at more than 5.3 STU for *T. mashhoodi*, 9.6 for *H. spinicaudata* and 20.1 for *H. oryzae*.

We considered that the low pH values (2.7 to 5.0 depending on the concentration of hydrogen sulphide) were responsible for the death of the nematodes more than the sulphide itself.

The results of the other experiments are shown in fig. 2. Every curve groups the results obtained with two or three lots of nematodes.

Effects of hydrogen sulphide dissolved in a sterile buffered medium used for bacterial culture

Due to the Na lactate concentration of Starkey's medium, the pH was higher than during the previous tests (5.2 — 7.2). For *T. mashhoodi* 100% mortality

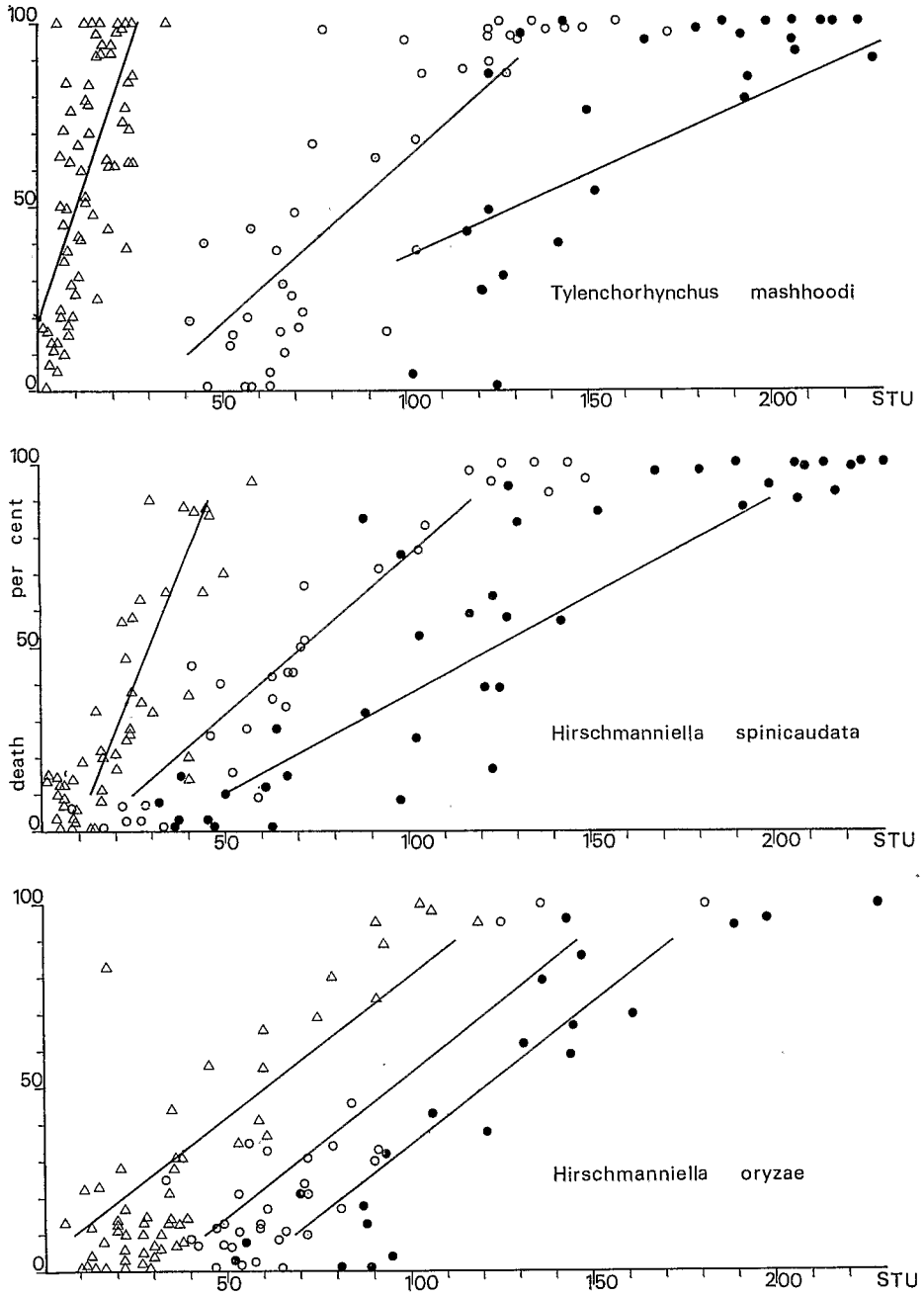

Fig. 2. Lethal dose of soluble sulphide for three rice nematodes — chemically produced sulphide: △ — microbiologically produced sulphide; tests: 1 — 2 days long: ○; tests: 3 — 6 days long: ●.

was observed with an STU as low as 5.8 and no living nematode was observed in any flask at an STU greater than 26.3. For the two species of *Hirschmanniella* 100% mortality was not obtained with the STU tested but some nematodes were still living at 57.5 STU for *H. spinicaudata* and 124.2 STU for *H. oryzae*. The coefficient of correlation between the number of STU and the percentage (transformed to degrees) of dead nematodes was calculated for the tests resulting in 10 to 90% mortality. The coefficients were $r = 0.5941$ with 50 d.f. for *T. mashhoodi*, $r = 0.5987$ with 35 d.f. for *H. spinicaudata* and $r = 0.8792$ with 30 d.f. for *H. oryzae*, showing the percentage mortality to be closely related to the number of STU. The regression lines were drawn on the graphs of figure 2. They cross the 50% mortality line at 10 STU for *T. mashhoodi*, 29 STU for *H. spinicaudata* and 60 STU for *H. oryzae*.

Effects of hydrogen sulphide produced by bacteria in a buffered medium

At the end of these tests, the pH was as high as 8.5 because the growth of bacteria was using up the lactate. It appeared that for the tests which lasted only one or two days, the regression line was different from that of longer tests (3 — 6 days). For the three species the regression coefficients r (1 — 2 day tests) and r′ (3 — 6 day tests) were calculated for the tests resulting in 10 to 90% death. All the results ($r = 0.7936$ with 20 d.f. and $r' = 0.8092$ with 10 d.f. for *T. mashhoodi*; $r = 0.8509$ with 14 d.f. and $r' = 0.5897$ with 16 d.f. for *H. spinicaudata*; $r = 0.7340$ with 19 d.f. and $r' = 0.8977$ with 10 d.f. for *H. oryzae*) showed the percentage of death to be strongly correlated with the number of STU. The regression lines were drawn on the graphs of figure 2.

The 50% mortality for 1 — 2 day tests and 3 — 6 day tests respectively occurred at 86 and 130 STU for *T. mashhoodi*, 71 and 124 STU for *H. spinicaudata* and 94 and 120 STU for *H. oryzae*.

DISCUSSION

Sensivity of nematodes to sulphide varied with pH, and the great sensitivity to hydrogen sulphide dissolved in water may be explained by the low pH values to which the three nematode species tested were found highly susceptible, *T. mashhoodi*, in particular.

During experiments in buffered media, higher pH values (5.2 to 8.5) were harmless, but sensitivity was greater during short term tests. If nematode mortality was always highly correlated with STU, it is to be noticed that the same STU can be attained by two means: first, by using high sulphide concentrations in one or two days, in experiments with chemically produced sulphide or very active sulphate reducing bacteria; or, secondly, by using lower concentrations but for a period of three or more days. Sensitivity was greater in the former. Such results can be explained by a "shock effect" produced when nematodes were quickly transferred from demineralized water to a highly concentrated medium. Thus, tests of sul-

phide toxicity should last at least three days to avoid such a side effect: it occurred in other experiments on rice soil that accumulation of soluble sulphides was low.

When different lots of nematodes were tested in the same conditions, the results were found to be the same. This means that the experimental procedure and the calculation methods for STU and percentage of incapacitated nematodes are reproducible with other populations of the species tested or with other species of nematodes.

Our results are difficult to compare with those obtained by Rodriguez-Kabana *et al.* (1965) because these authors used another experimental method, insufficiently described. It seems that they dissolved hydrogen sulphide in pure water or in an undescribed medium, added the nematodes and sealed the vials. They did not describe the method they used to measure the concentration of sulphide and they did not seem to consider: 1 — the decrease of sulphide during the tests and 2 — the low value of pH. If, however, we accept the data reported by these authors, we can calculate the STU for 100% mortality of *Tylenchorhynchus martini* as about 120 units in 2 days, 160 units in 4 days and 100 units in 6 days.

During our experiments with microbiologically produced sulphide tested for a duration of 3 days or longer, the 50% mortality of the three species occurred approximatively at the same STU: 130 for *T. mashhoodi*, 124 for *H. spinicaudata* and 120 for *H. oryzae*. The 100% mortality occurred at 190-250 STU for the three species.

In vitro experiments show that sulphate reducing bacteria could be used for biological control of rice root nematodes. But *in situ* conditions are different.

The classic determination method for sulphide concentrations of Chaudhry & Cornfield (1966) gives the total concentration of sulphides in the soil, i.e. the insoluble sulphides such as FeS solubilized by HCl 4N at 60° C, as well as the soluble sulphides, whereas we measured only the latter in these tests. A new method is needed to measure in the field only the soluble sulphides in water-extracts of samples.

The distribution of sulphides in a rice field is very irregular. Jacq (1972, 1976) has shown that sulphate reducing bacterial activity is higher in the rhizosphere and, perhaps, around decomposing organic material. On the other hand, aeration prevents accumulation of hydrogen sulphide by stopping the activity of the bacteria, which are anaerobic. Micro-sites of low concentration of sulphides are thus created, "protecting" the nematodes.

An artificial increase of sulphate reduction is not to be attempted during rice cultivation. Many authors have pointed out the toxicity of hydrogen sulphide to rice, producing physiological decay known as "bruzone" in Hungary (Vámos, 1958), "akiochi" in Japan (Park & Tanaka, 1968) and "straight head" in the USA (Atkins, 1958). In Senegal, Jacq (1976) has shown that rice seedlings decay when total concentration of sulphides around the roots reach 10 ppm and die at 30 ppm.

An attempt to use such a biological control method is however possible after

the harvest, as long as the bacterial population is lowered again before initiation of the next culture.

RÉSUMÉ

Etude in vitro *de la toxicité des sulfures solubles envers trois nématodes du riz au Sénégal*

La toxicité des sulfures solubles envers *Tylenchorhynchus mashhoodi, Hirschmanniella oryzae* et *H. spinicaudata* a été étudiée *in vitro* en utilisant trois méthodes différentes.

Une unité de toxicité (STU) a été définie: elle est fonction des teneurs moyennes en sulfures solubles et des temps de contact avec les nématodes.

Quand on utilise de l'hydrogène sulfuré dissout dans de l'eau désaérée, le pH de la solution obtenue est très bas, ce qui augmente beaucoup la mortalité des trois espèces. Quand on utilise de l'hydrogène sulfuré dissout dans un milieu tamponné, qui est le milieu de culture habituel des bactéries sulfato-réductrices, la mortalité est plus faible, mais l'application brutale de fortes concentrations en sulfures et la toxicité de certains constituants du milieu provoquent un effet de choc augmentant cette mortalité. Quand l'hydrogène sulfuré est produit *in situ* par les bactéries sulfato-réductrices, cet effet de choc est encore sensible dans le cas où les concentrations léthales de sulfures sont atteintes rapidement, en moins de deux jours. Mais dans le cas où les nématodes ne sont tués qu'en trois jours ou plus, l'effet de choc ne se manifeste pas et la sensibilité des trois espèces testées est comparable: une mortalité de 50% est obtenue pour des STU de l'ordre de 120 à 130 unités.

La possibilité d'utilisation de l'activité des bactéries sulfato-réductrices comme moyen de lutte biologique contre les nématodes parasites des racines du riz inondé est discutée.

REFERENCES

ATKINS, J. C. (1958). Rice diseases. *Fmr's Bull. U. S. Dep. Agric.* **2120**, 14 pp.

BRUNEL, A. (1948). *Traité pratique de Chimie Végétale.* Tome 1. Georges Frère, Tourcoing, 568 pp.

CHAUDHRY, I. A. & CORNFIELD, A. H. (1966). Determination of sulphide in waterlogged soils. *Pl. Soil* **25**, 474-479.

FORTUNER, R. & MERNY, G. (1973). Les nématodes parasites des racines associés au riz en Basse-Casamance (Sénégal) et en Gambie. *Cah. ORSTOM, sér. Biol.* **21**, 3-20.

JACQ, V. A. (1972). Biological sulphate-reduction in the spermosphere and the rhizosphere of rice in some acid sulphate soils of Senegal. In: *Proc. Intern. Symp. Acid Sulphate Soils.* 13-20 August 1972, Wageningen, The Netherlands, edited by I.L.R.I., Wageningen **18**, vol. II, 82-98.

JACQ, V. (1976). La sulfato-réduction en relation avec l'excrétion racinaire. (In press) in a special issue of *Ann. Soc. Botanique de France,* Paris.

PARK, Y. D. & TANAKA, A. (1968). Studies of the rice plant in a "Akiochi" soil in Korea. *Soil Sci. Pl. Nutr.* **14**, 27-34.

PICHINOTY, F. (1966). Mesure de l'activité de quelques réductases de microorganismes. *Information Exchange Group N° I. Oxidative phosphorylation and terminal electron transport, scientific memo* N° **555**, 1-13.

RODRIGUEZ-KABANA, R., JORDAN, J. W. & HOLLIS, J. P. (1965). Nematodes: biological control in rice fields: role of hydrogen sulfide. *Science N.Y.* **148**, (3669), 524-526.

SEINHORST, J. W. (1950). De betekenis van de toestand van de grond voor het optreden van aantasting door het stengelaaltje, (*Ditylenchus dipsaci* (Kühn) Filipjev). *Tijdschr. PlZiekt.* **56**, 291-349.

—— (1962). Modifications of the elutriation method for extracting nematodes from soils. *Nematologica* **8**, 117-128.

VÁMOS, R. (1958). H_2S, the cause of the "bruzone" (Akiochi) disease of rice. *Soil Pl. Food.* **4**, 37-40.

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