Techniques

Factors Affecting Staining of *Sclerospora graminicola* Oospores with Triphenyl Tetrazolium Chloride

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ABSTRACT

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Oospores of the pearl millet downy mildew pathogen, *Sclerospora graminicola,* at various concentrations, were treated with 2,3,5-triphenyltetrazolium chloride (TTC) at various temperatures, TTC concentrations, and for various time periods following presoaking in distilled water at two temperatures for several time periods. TTC-treated oospores remained unstained or developed colored cytoplasm varying from light pink to deep red. The proportion of oospores stained varied greatly with presoaking time, incubation time, incubation temperature, TTC concentration, and oospore concentration. In some instances, oospores stained at one examination had lost the stain by the next examination (destaining). The most staining occurred when an aqueous oospore suspension containing $\sim 48 \times 10^5$ oospores per milliliter that had been presoaked at 40 C for 48 hr was incubated in 1% TTC solution at 40 C for up to 9 wk. The results indicate that the technique previously reported for routine screening of *S. graminicola* oospores for viability using TTC is unreliable and emphasize that a technique useful for one fungal species may not be directly applicable to another. More work is needed to determine whether TTC can be used to give a reliable quantitative measure of viability of *S. graminicola* oospores, and the problems of differential response to environmental factors, oospore wall permeability, mycoparasitism, concentration effects, and destaining all must be recognized and dealt with.

Additional key words: Pennisetum americanum.

Downy mildew (DM), caused by *Sclerospora graminicola* (Sacc.) Schroet., is the most important disease of pearl millet (*Pennisetum americanum* [L.] Leeke) in Asia and Africa, and is also serious on foxtail millet (*Setaria italica* [L.] Beauv.) in Asia and North America (11,14,16). In the tropics the pathogen survives the hot dry season as thick-walled oospores, which are reported to remain viable in soil for periods ranging from 8 mo to 10 yr (1–3,5,6,15,21).

Despite the importance of the oospores of *S. graminicola* and the several reports of techniques to induce their germination in vitro (2,7,12,19,20), there is still no reliable repeatable method to quantitatively determine the viability of the oospores of this pathogen isolated from pearl millet. Our many attempts to induce in vitro germination by the reported techniques have been unsuccessful. Recent reports of the use of 2,3,5-triphenyl-

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tetrazolium chloride (TTC) for routine quantitative evaluation of viability of oospores of *S. graminicola* (17,18) appeared to provide the solution to the problem. However, preliminary studies with TTC in our laboratory indicated problems in repeatability of results and interactions with environmental factors (I. Huibers-Govaert, *unpublished*). The results of more detailed studies reported in this paper indicate that the technique previously reported (17,18) is not reliable for evaluation of viability or non-viability of oospores of *S. graminicola* from pearl millet.

MATERIALS AND METHODS

The experiments were conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Center, from November 1978 through October 1979.

Two samples of *S. graminicola* oospores were used; one was harvested from DM-infected pearl millet plants in November 1977 (1977 oospores), and the second was harvested in October 1978 (1978 oospores). They were stored as dry powder in the laboratory following pulverization of sun-dried infected leaves and sieving the resulting powder through a series of brass sieves (to 45 μm) to remove most of the plant debris.

We used the general methodology of Shetty et al (17) of soaking the oospores in distilled water prior to TTC treatment (presoaking) and of examining oospores for color changes following the TTC treatment. We examined the effect of variation in time and temperature during presoaking and during TTC treatment, and the effects of variation of TTC and oospore concentrations.

Unless otherwise stated the presoaking was for 48 hr at 30 C; the oospore powder concentration in the presoaking suspension was 25 mg/ml (1 mg oospore powder contained ca. 1.9×10^5 oospores); 1 ml of 2% TTC solution was added to 1 ml oospore suspension to give 1% TTC treatment.

The oospores were presoaked in distilled water adjusted to pH 7.0 with NaOH, and the TTC solution was prepared in distilled water at pH 7.0 (adjusted by the use of NaOH). In two experiments a sodium phosphate buffer was used during the TTC preparation as described by Pathak et al (13), and the oospore reactions were compared in both types of TTC solution.

The temperature treatments were established with Percival I35LL incubators in which the oospore suspensions were maintained in small, glass, screw-capped vials inside light-proof cardboard boxes.

At each examination at least 1,000 oospores from each treatment were examined for color changes by the use of a Zeiss Standard-18 compound microscope.

RESULTS

Oospore color changes. The cytoplasm of TTC-treated oospores either remained unstained or developed color which ranged from light pink to deep red. To facilitate recording we established a one to five color scale, in which 1 was not stained, 2 was light pink, and 3, 4, and 5 represented increasing intensities of red coloration. A standard color chart was prepared and used throughout the series of experiments to enable standardization of classification.

In a few instances, in the experiments on TTC and oospore concentrations, and on the effects of presoaking treatments, oospores in one of the four replicate vials showed a massive reduction in proportion of oospores stained (destaining) from one scoring time to the next (generally from 4 days after initiation of TTC treatment). In these cases the experiments were repeated, or the data from the single destained replication were eliminated from the analysis. In other instances a more gradual reduction in stained oospores with time was found in all four replicate vials, and these results were maintained as a representative reaction to the particular treatment.

Effect of time and temperature during TTC treatment. In the first experiment, the 1977 and 1978 oospores were kept at 30, 35, and 40 C during the TTC treatment and were examined 1,2,4,8,15,23, 30,37,44,51,58, and 65 days after the initiation of the TTC treatment. The proportion of stained oospores varied greatly with time and temperature (Fig. 1). After 48 hr at 40 C, 41% of 1978 oospores were stained, whereas only 9% were stained in the same time period at 30 C. A similar difference was seen with the 1977 oospores but at a lower level. After 65 days more than 60% of 1977 and 1978 oospores were stained, and temperature effects were still apparent although they were not so marked as during the first 20 – 30 days. At the final scoring, 65 days after initiation of the TTC treatment, the proportion of oospores stained was still increasing but at a slow rate. The 1978 oospores had more oospores stained than the 1977 oospores at all examination times at all temperatures but the degree of difference steadily decreased over time to a plateau of about 5 – 10% (Table 1). The plateau was reached most rapidly at 40 C and least rapidly at 30 C.

In addition to the differences in numbers of spores stained, time and temperature had major effects on the intensity of staining. Oospores first developed a light pink coloration which became richer and darker red with time. The progression from light stain to dark stain occurred most rapidly at 40 C (Fig. 2). At 30 C no oospores attained the deepest staining (category 5) until 15 days after initiation of the TTC treatment, whereas at 35 and 40 C category 5 stain was detected 4 days after initiation of the TTC treatment. Sixty-five days after initiation of the TTC treatment, the proportions of stained 1978 oospores with category 4 and 5 stain intensity were 81, 87, and 93%, respectively, for 30, 35, and 40 C, and for 1977 oospores the proportions were 47, 65, and 92%, respectively (Fig. 2).

In a second experiment, the 1978 oospores were incubated at 30 C and 40 C in TTC prepared in distilled water adjusted to pH 7.0 with NaOH and in TTC prepared with sodium phosphate buffer. The

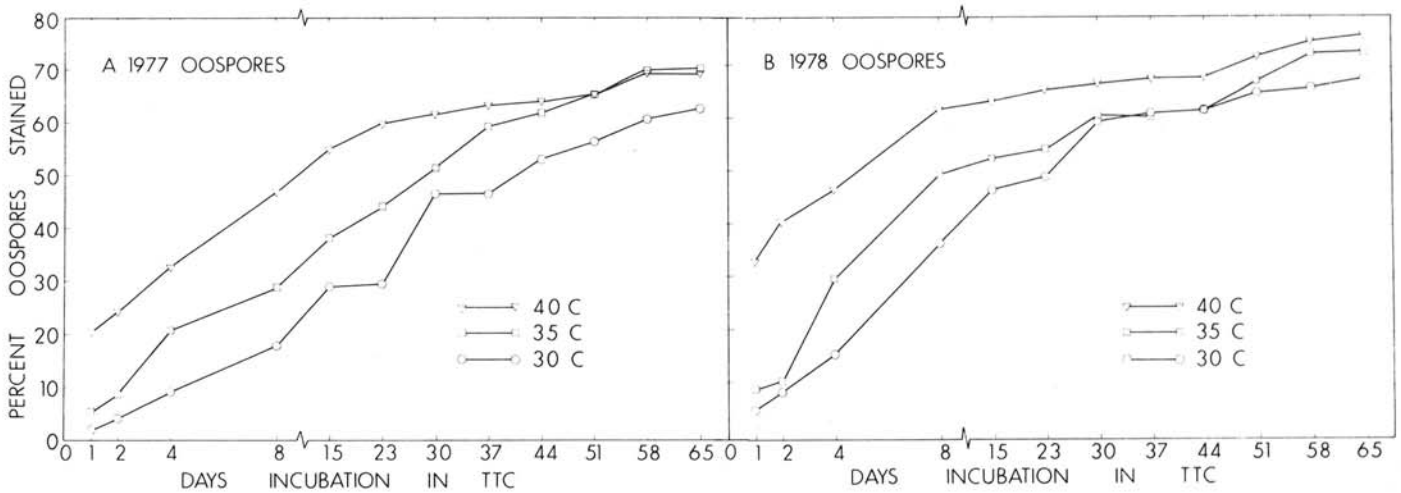

Fig. 1. Percentage of 1977 and 1978 oospores of *Sclerospora graminicola* stained when incubated for up to 65 days in 1% 2,3,5-triphenyl-tetrazolium chloride solution at 30, 35, and 40 C, following presoaking in distilled water for 48 hr at 30 C.

responses to time and temperature were similar to those obtained in the first experiment and there were no significant differences in reactions between the two TTC solutions (Fig. 3).

Effect of presoaking time and temperature. The 1978 oospores were presoaked for 96, 72, 48, and 24 hr at 30 and 40 C, or were not presoaked, and were then incubated in 1% TTC at 40 C for 8 days. Four replicate vials were maintained for each treatment and at each scoring 250 oospores were examined from each vial. Presoaking time had significant effects on oospore staining but there were no significant effects of presoaking temperature (Fig. 4). Maximum staining occurred following 48 hr of presoaking and the least followed no presoaking.

TABLE 1. The proportion of 1978 oospores of *Sclerospora graminicola*[a] stained expressed as a percentage of the 1977 oospores stained[b] following treatment in 1% TTC[c] at 30, 35, and 40 C for 1 day to several weeks

Treatment time (days)	30 C	35 C	40 C
1	311	185	166
2	228	131	172
4	181	148	144
8	208	174	134
15	163	139	117
23	170	124	110
30	129	119	109
37	133	102	107
44	117	101	107
51	116	103	111
58	109	104	108
65	108	104	110

[a] *Sclerospora graminicola* oospores from pearl millet.
[b] For each oospore age group, 1,000 oospores were examined at each temperature and examination time.
[c] TTC = 2,3,5-triphenyl-tetrazolium chloride.

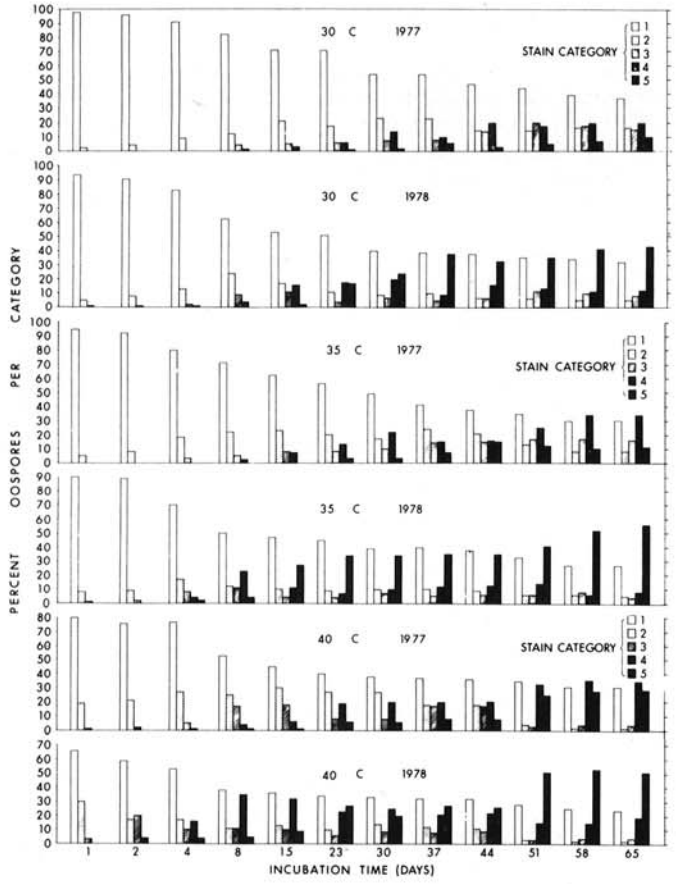

Fig. 2. Percentage of 1977 and 1978 oospores of *Sclerospora graminicola* in five staining intensity categories following presoaking in distilled water for 48 hr at 30 C and incubation for up to 65 days in 1% 2,3,5-triphenyl-tetrazolium chloride solution at 30, 35, and 40 C. (Rating scale: 1 = no stain; 2 = light pink; 3 = deep pink; 4 = mid-red; 5 = deep red).

Effect of TTC concentration. The 1978 oospores were incubated at 40 C for 9 days in six concentrations of TTC solution (0.25, 0.5, 1, 2, 4, and 8%). At all scoring dates the 1% treatment gave the greatest proportion of stained oospores. In the 0.25, 0.5, and 1% treatment the proportion increased steadily throughout the incubation period, whereas in the 2, 4, and 8% treatments it was greatest after 2 days of incubation and declined at each subsequent scoring time (Fig. 5).

Effect of oospore concentration. In the first experiment on oospore concentration, suspensions of 1978 oospores were prepared containing 50, 25, 10, and 2.5 mg oospore powder per milliliter of water, and were adjusted to pH 7.0 with NaOH. Following the normal presoaking treatment, the oospores were incubated in TTC (1%) for 8 days at 40 C, and were examined for staining 1, 2, 5, and 8 days after the start of incubation. Oospore concentration had a major effect on degree of staining (Fig. 6). At 2.5 mg/ml, less than 0.5% of the oospores stained throughout the incubation period; at 10 mg/ml about 5% of the oospores were stained with no response to time; at 25 mg/ml staining increased steadily with time from 32 to 57%; and at 50 mg/ml the staining increased from 33% to 40% and then decreased to 30%.

In order to re-check these responses a second experiment was conducted with 25, 10, and 2.5 mg oospore powder per milliliter using TTC solutions made with water and NaOH, and with sodium phosphate buffer, with four replicate vials per treatment. The results were similar to those of the first experiment (Fig. 7).

DISCUSSION

The application of the principle of reduction of colorless TTC to red, stable, nondiffusible triphenyl formazan by reduction processes in living cells has proved valuable in studies of viability of dormant seeds of higher plants (8–10). However, it was realized that standardization of techniques was essential to avoid misinter-

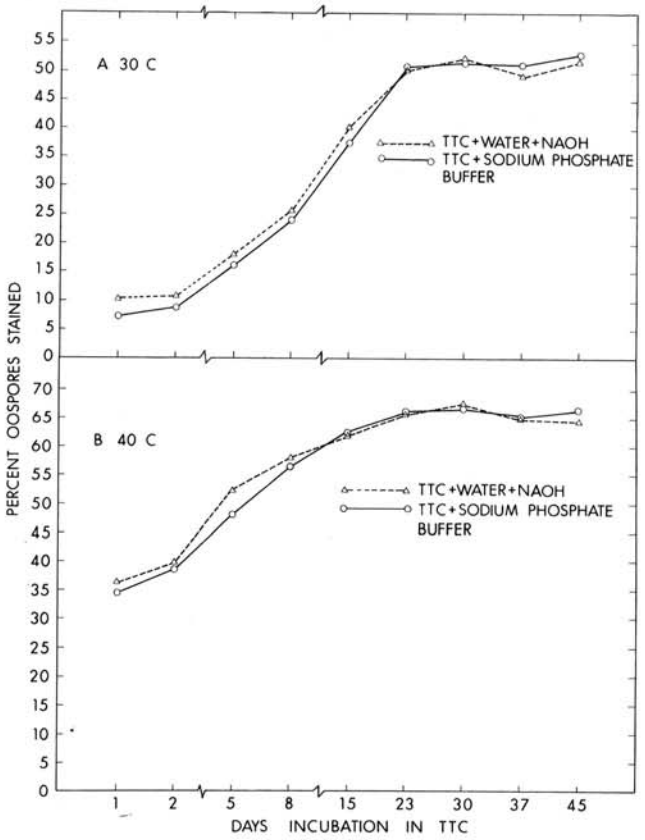

Fig. 3. Percentage of 1978 oospores of *Sclerospora graminicola* stained when incubated for up to 45 days in 1% 2,3,5-triphenyl-tetrazolium chloride solution, made up to pH 7.0 with water and NaOH, and with sodium phosphate buffer, following presoaking at 30 C for 48 hr.

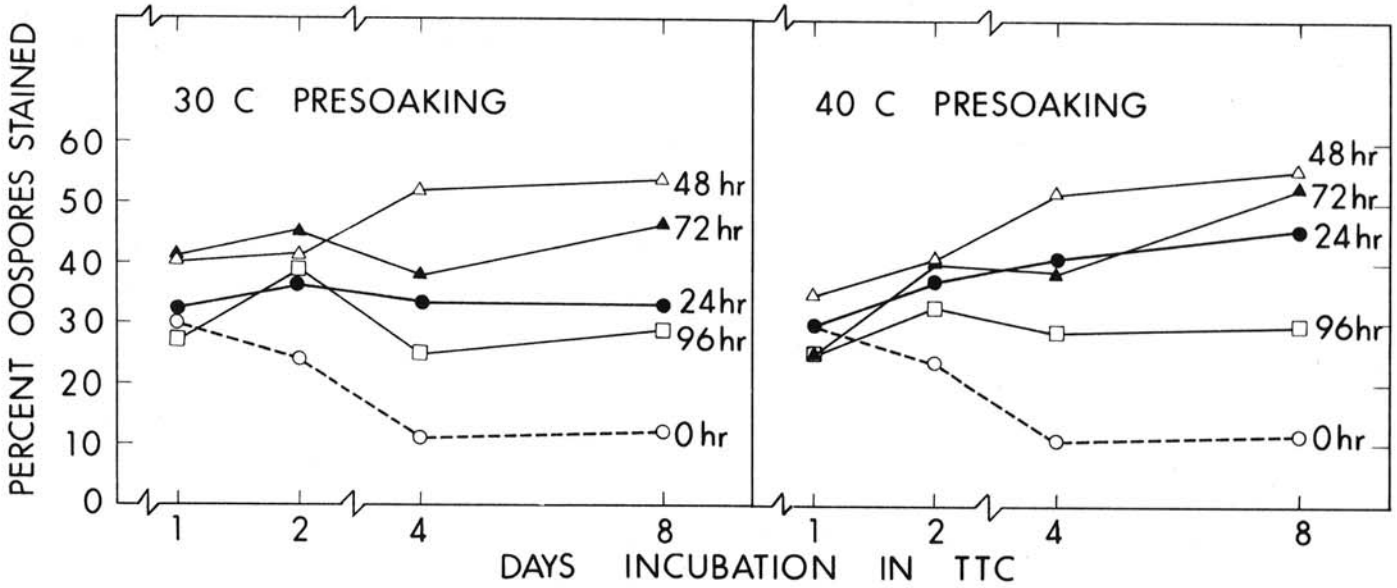

Fig. 4. Percentage of 1978 oospores of *Sclerospora graminicola* stained when incubated for up to 8 days in 1% 2,3,5-triphenyl-tetrazolium chloride solution at 40 C, following presoaking in distilled water for up to 96 hr at 30 C and 40 C.

pretations, and that the technique suitable for one species could be unsuitable for another (4,9). More rapid staining of embryo tissue at higher temperatures, and the need in certain species to treat for longer periods, or to remove the impermeable pericarp, have been reported (8,9).

The application of the TTC test to determine viability of oospores of plant pathogenic fungi appeared to offer great promise based on the results of Pathak et al (13) who found a "good correlation" between germination and staining for oospores of *Peronospora manshurica* (Naum.) Syd. However, in their tests with 48 hr presoaking and 48 hr TTC treatment at 30 C, the percentage of oospores stained was always greater than the actual percent germination. Shetty et al (17), working with *S. graminicola* oospores, used the same technique as Pathak et al (13), and, without data on actual viability, stated that the TTC test can be used routinely for testing the viability of oospores of *S. graminicola*. Our results show that treatment of *S. graminicola* oospores of unknown concentration with 0.5% TTC for 48 hr at 30 C following 48 hr of presoaking cannot be regarded as a reliable treatment for determining their stainability. In our tests the most staining occurred when and aqueous oospore suspension containing ~48×10^5 oospores per milliliter that had been presoaked at 40 C for 48 hr was incubated in 1% TTC solution at 40 C for up to 9 wk. It is possible that at higher temperatures more rapid and higher levels of staining would occur. There is no information for *S. graminicola* on the relationship between stainability and actual viability, for the absence of a technique to induce in vitro germination of oospores has precluded the examination of this vital aspect.

Shetty et al (18), in their study on seed transmission of pearl millet DM, used the negative results of the TTC test as the evidence precluding the possibility that the 0.3% infected plants obtained could have been infected by oospores detected on the seeds. As the oospores would have been present in low concentration, and as they were treated with TTC for only 48 hr at 30 C, their nonstaining does not necessarily indicate their nonviability. The fact that the DM symptoms did not occur at the seedling stage, but appeared at the 6th- to 8th-leaf stage, is an indication that the infection may have been caused by oospores carried on and with the seed, and not by internal mycelium.

The effects of presoaking time, and TTC incubation time and

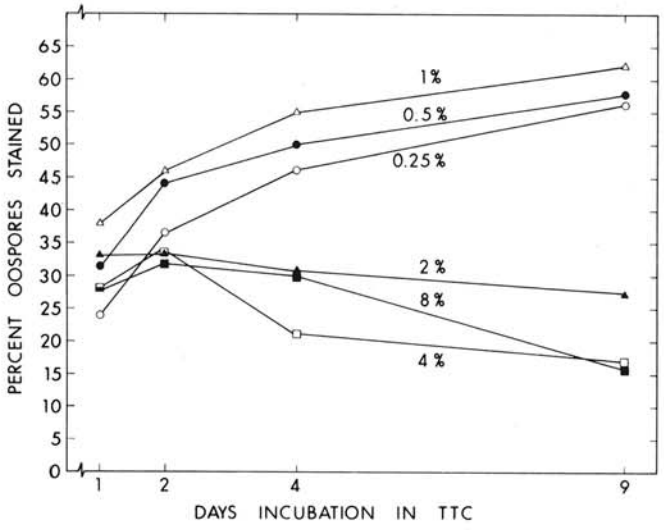

Fig. 5. Percentage of 1978 oospores of *Sclerospora graminicola* stained when incubated for up to 9 days in six concentrations of 2,3,5-triphenyl-tetrazolium chloride at 40 C, following presoaking in distilled water for 48 hr at 30 C.

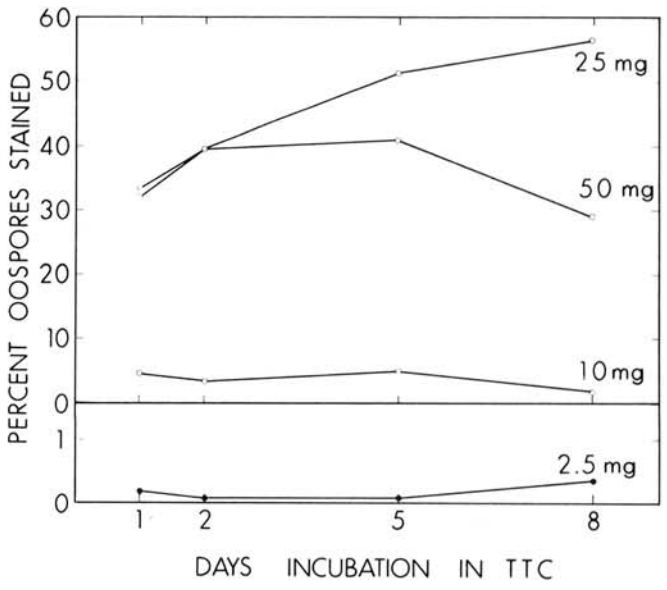

Fig. 6. Percentage of 1978 oospores of *Sclerospora graminicola* stained when incubated at four concentrations for up to 8 days in 1% 2,3,5-triphenyl-tetrazolium chloride solution at 40 C following presoaking in distilled water at 30 C for 48 hr.

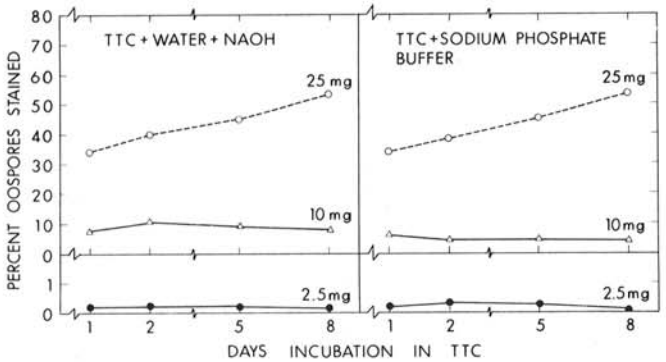

Fig. 7. Percentage of 1978 oospores of *Sclerospora graminicola* stained when incubated at three concentrations (x mg/ml) for up to 8 days in 1% 2,3,5-triphenyl-tetrazolium chloride (TTC), solution made up to pH 7.0 with water and NaOH, and with sodium phosphate buffer, at 40 C, following presoaking in distilled water for 48 hr at 30 C.

temperature, are probably related to permeability of the oospore wall, for unless the TTC can reach the living cell, staining will not occur. We have no explanation for the effects of oospore concentration or TTC concentration, and we do not understand why rapid destaining occurred in some instances, because triphenyl formazan is reported to be stable and nondiffusible (8).

We believe that the use of vital stains such as TTC are potentially a useful means for quantitative determination of viability of oospores of plant pathogens, but the specific techniques will have to be worked out for each species. The problems posed by differential response to environmental factors, oospore wall permeability, mycoparasitism, destaining, concentration effects, and the lack of a reliable in vitro germination technique for oospores of some species, will all have to be recognized and dealt with.

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