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Measurement of Fungitoxicity using the Filter Paper Disk Method

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Abstract

The main objective in using this method was to determine the concentration of each of some six important fungicides used in African Agricultural and Horticultural practices, but which lacked information on their action against isolates of *Sarocladium* species that was necessary to prevent the radial growth (mm) of these pathogenic fungi. In this study experiments involving the quantitative measurement of organism response to the toxic compounds were conducted. The method of bioassay used in this study did not distinguish between merely inhibiting the fungus growth, a fungistatic action, and the outright killing of it, a true fungicidal action. From studies on the effect of fungicide concentration on radial growth of two isolates of *Sarocladium* species, Difolatex, Ortho Difolatan, Benlate, Dithane M-45 and Calixin M (Tridemorph), in that order, were found effective at different concentrations in preventing the radial growth of S. *attenuatum* (I_3) The actions of Calixin M and Bordeaux mixture were, however, generally poor. In a similar test against S. *oryzae* (I_4), Ortho Difolatan, Difolatex and Benlate, in that order, were the best three fungicides against this isolate. *S. oryzae* (I_4) was found to be more resistant to the fungicides tested than *S. attenuatum* (I_3). The results showed that the most economical and effective concentration of a given fungicide depended on (1) the fungus, (2) the fungicide, and (3) the fungus-fungicide combination. The two isolates did not rate the fungicides in the same order, suggesting inherent variation in the chemical behaviour, the two isolates, or both groups. From the filter paper disk method used to measure the fungitoxicity of these fungicides it was found that (1) Difolatex and Ortho Difolatan were the best two fungicides against the two isolates of *Sarocladium* species tested. (2) Considering the six fungicides tested, the isolates did not rate these chemicals in like order, suggesting that (a) the recommendation for the best chemical depends on the causal organism of the disease, and (b) careful identification of the causal pathogen is very important in a control programme that seriously considers the use of chemicals. The implications of these studies are two-fold: (1) farmers like those of the European Development Fund financed Upper Noun Valley Development Project headquartered at Ndop, Cameroon, who changed from cocoa and coffee to rice farming will already be familiar with these chemicals from their previous use for protection against the cocoa pod and coffee berry diseases, (2) the fungicides Difolatex and Ortho

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Difolatan may therfore be included in an integrated pest management (IPM) programme in the African rice farming systems for the control of important diseases like the grain discoloration and sheath rot caused by *Sarocladium* species.

1 Introduction

Quantitative bioassay has been defined as a determination of the potency of a physical, chemical or biological agent by means of a biological indicator. The biological indicators in bioassay are the reactions of living organisms or tissues. As such they are subject to variation, an area which is peculiarly the domain of the biometrician and statistician. Quantitative bioassay has been developed by scientists from different fields (Bliss, 1957). The principles involved in laboratory toxicity experiments on fungicides are closely related to those involved in the determination of the potency of drugs by the methods of bioassay. Toxicity experiments may be divided into two general types.

In experiments of the first type the response of the organisms to the toxic agent is measured quantitatively, as for example where the diameter or radius of fungal colonies, or the weight of mycelial mats is measured. In experiments of the second type the organisms being tested are divided into two groups depending upon whether a given individual has or has not exhibited a definite observable response to the toxic agent. Such a division might be into germinated or non-germinated spores in the case of the usual spore germination tests. This type of experiment is said to be one involving a "quantal response" (Wilcoxon & McCallan, 1939). In this study experiments involving the quantitative measurements of organism response to toxic agents were conducted. A chemical may exert its protectant properties either by merely inhibiting the fungus, a fungistatic action, or by the outright killing of it, a true fungicidal action. Most methods of bioassay do not distinguish between these two actions. (McCallan, 1947; Wilcoxon & McCallan, 1939).

2 Materials and methods

Preparation of antibiotic solution

Streptomycin sulphate crystal (40mg) and penicillin crystal (60mg) were carefully weighed out using the Mettler HIOT analytical balance, and dissolved in 20 ml of sterile distilled water in a 50 ml beaker previously thoroughly washed in Omo detergent and sterilized. Twenty millilitres of this solution were used per litre of medium to suppress bacteria. This antibiotic solution was added to cooled agar media just before dispensing into petri dishes.

Preparation of stock isolate conidial suspensions

Stock conidial suspensions of isolates of *Sarocladium attenuatum* and of *S. oryzae* all previously identified by the Commonwealth Mycological Institute (CMI), UK, and Centralbureau Voor Schimmelcultures (CBS), The Netherlands during some earlier studies (NGALA, 1982, 1983) were used. Pure cultures of the isolates were grown on PDA plates at the room temperature of 25 ± 2°C for 3-5 days. The surfaces of the replicate colonies of each isolate were scraped, put in sterile distilled water in sterilized volumetric flasks, shaken, and filtered through three layers of cheesecloth. Tween 20 was added to ensure a uniform dispersion of the conidia.

Standardization of conidia concentrations

Aliquots from the stock conidial suspensions of different isolates of the species were counted in replicates using the Improved Neubauer (B. S. 748) Haemocytometer mounted under the high power of model KHS Olympus microscope. Calculations of the conidia concentration per millilitre for each isolate stock were made and these concentrations standardized at 1×10^6 conidia per ml.

Preparation of the orange juice stimulant

The orange juice stimulant was prepared by cutting a good quality orange and squeezing its juice into a beaker, filtering through three layers of cheesecloth and then centrifuging twice, each time at a speed of one for five minutes. The resulting clear filtrate was used in various experiments to test its effect, and that of its concentration on the per cent germination of conidia of isolates of *Sarocladium* species.

Preparation of fungicide stock solutions

The test fungicides were available in powder form. Based on the per cent active ingredients and the necessary correction factor for each fungicide, calculations were made using the methods described by Sharvelle (1979) to determine the number of grams of each chemical needed to make a standard concentration in parts per million (ppm) in 100 ml of sterile distilled water. The fungicides used were Bordeaux mixture, prepared in the laboratory according to the methods of APS (1943b), and considered to be 100% active ingredient (a.i.), Benlate (50% a.i.), Dithane M-45 (57% a.i.), Difolatex (80% a.i.), Ortho Difolatan (80% a.i.), and Calixin M (Tridemorph, 75% a.i.). The rationale in selecting these fungicides was that they are important chemical agents already in use in African Agricultural and horticultural practices but without any information available on their action against *Sarocladium* species. The chemicals also have wide ranging uses and protective properties (WORTLING, 1979), and already commonly used by farmers in tropical Africa (Cameroon) to control cocoa pod and coffee berry diseases (Difolatex, Ortho Difolatan, and Calixin M). Desiring to prepare a stock solution of 10,000 ppm of each test chemical, 1g, 2g, 1.33g, 1.25g. 1.25g and 1.33g respectively of each were there-

fore weighed out carefully using the Mettler analytical balance. Each sample was dissolved in 100 ml sterile distilled water in a separate 250 ml volumetric flask and shaken thoroughly. The content of each flask was diluted serially using the serial dilution technique (APS,1947) to give an array of concentrations from 10^0 to 10^9. Sterile distilled water was used as the check.

Preparation of filter paper disks

Several filter paper disks each of 12.7 mm diameter were cut out from ordinary laboratory Whatman No. 1 filter paper, using an iron borer. They were packed in 9.0 cm sterile petri dishes and sterilized in the Gallenkamp Hotbox Oven at 160°c for 24 hours.

Application of stimulant, fungicide and fungus to bioassay disk

Using a different 5 ml dropping pipette for each fungicide, fungus isolate, the check, and the stimulant, and mixing the content of each tube thoroughly before taking a sample, one drop of the stimulant (orange juice) was centrally placed on each of the eleven bioassay disks. This was followed by one drop of the fungicide from a numbered tube to its corresponding numbered bioassay disk, working from the most dilute to the most concentrated. Each disk, including the check, was then seeded with one drop of the 1 x 10^6 conidia/ml of the test fungus. In the case of the checks, one drop of sterile distilled water was added again, so that this brought the final concentrations of the active material of the fungicides to 2500, 250, 25, 2.5, *0.25*, 0.025, 0.0025 0.00025, 0.000025, 0.0000025 ppm respectively. For each fungicide - fungus test, the check bioassay disks were set up first to avoid contamination. The methods used were based on the test tube dilution technique of the APS (1947) and Sharvelle (1979). Three replicate disks from each dilution of each fungicide were placed on PDA medium and the plates incubated under laboratory conditions of 25 ± 2C for ten days. Observations over the concentration series of the test fungicides at 48 hours intervals for ten days permitted the determination, in each case, of the minimum inhibitory concentration (MIC) and the growth measurements from the edge of the treated and untreated (control) disks, which provided the dosage response data.

3 Results and discussion

Observations over the concentration series of the test fungicides at 48 hours intervals for 10 days permitted the determination of the growth measurements from the edge of the treated and untreated (check) disks. The results (Table 1) show that when conidia suspension samples (1 x 10^6 conidia/ml) of *S attenuatum* (I_3) were incubated with the different concentrations of each of the fungicides, their responses were variable. Difolatex at concentrations down to 10^{-6} prevented the growth of this isolate, and growth was only possible from concentration 10^{-7} (2.8 mm) increasing to 8.8 mm in the check. Prevention of growth by Ortho Difolatan was between concentrations 10^0 and 10^{-5} and growth

started from concentration 10^{-6} (3.5 mm) and increased to 7 mm in the check. Benlate prevented growth of the isolate between concentrations 10^{0} and 10^{-4} and growth started from 10^{-5} (6.3 mm) and increased to 9.8 mm in the check. Dithane M -45 prevented growth between 10^{0} and 10^{-1} and growth started from 10^{-2} (5.3 mm) and increased to 7.8 mm in the checks. Calixin M (Tridemorph) prevented growth of the isolate only at the highest concentration of 10^{0} where there was no dilution, while Bordeaux mixture had little effect on the isolate since it did not completely prevent growth of the isolate even at the highest concentration of 10^{-0}. When these fungicides were similarly tested against *S. oryzae* (I_4) the results show that only concentrations between 10^{0} and 10^{-4} of Ortho Difolatan, Difolatex and Benlate could prevent the growth of this fungus isolate. Dithane M-45 only stopped the fungus growth effectively at 10^{0}, while Bordeaux mixture and Calixin M (Tridemorph) could not control the fungus at any of the concentrations used.

From the studies on the effect of fungicide concentration on the radial growth of two isolates of *Sarocladium* species, of the fungicides tested, Difolatex, Ortho Difolatan, Benlate, Dithane M - 45 and Calixin M (Tridemorph), in that order, were effective at different concentrations in preventing the radial growth of *S. attenuatum* (I_3). Taking

Table 1: Effect of fungicide concentration on the radial growth (mm) of two isolates of *Sarocladium* species (paper disk method).

Isolate	Fungicide	Fungcide concentration and mean^{x} radial growth (mm)										
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Check
S. attenuatum (I_3)	Difolatex	-	-	-	-	-	-	-	2.8	5.3	5.5	8.8
	Ortho Difolatan	-	-	-	-	-	-	3.5	4.5	5.8	6.3	7
	Benlate	-	-	-	-	-	6.3	6.3	6.8	6.8	8.3	9.8
	Dithane M-45	-	-	5.3	5.3	6.	6.	6.5	7.3	7.5	7.8	7.8
	Calixan M (tridemorph)	-	14.5	14.5	15.8	16.5	17.8	17.8	18.3	18.3	19.3	20.3
	Bordeaux Mixture	5.3	5.5	5.8	6.3	7.3	7.8	8.8	9.5	10.3	14.5	15.5
S. oryzae (I_4)	Difolatex	-	-	-	-	-	6.6	7	7.3	11.5	16	20
	Ortho Difolatan	-	-	-	-	-	3.8	4.8	7.8	11.8	14.3	14.8
	Benlate	-	-	-	-	-	14.5	14.8	15.8	17	17.8	18
	Dithane M-45	-	3.5	6	6.8	7	12	12	13	14	15	15.5
	Calixan M (tridemorph)	11.8	12	13	13	13.5	13.5	14	14.5	14.8	15.5	15.5
	Bordeaux Mixture	9.8	9.8	12.5	12.8	13.3	13.5	13.8	17.5	18.5	21	21

Each value is the mean of 4 replicates. - = No growth observered

Each at 1 x 10^{6} conidia/ml. Concentration 100 = 10,000ppm

the best fungicide to be one whose smallest possible concentration gives effective control of the fungus growth, the results therefore showed that amongst these effective fungicides tested, Difolatex, Ortho Difolatan, and Benlate, in that order were the best three for controlling the growth of *S. attenuatum* (I_3). Although Calixin M (Tridemorph) and Bordeaux mixture showed evidence of some action against this isolate, for instance there was a general increase in the amount of fungus growth from the highest concentration of each of these fungicides to the checks, this fungus completely resisted being controlled, in this method, by Bordeaux mixture at all concentrations tested and was only controlled by the highest concentration of Calixin M (Tridemorph). This therefore suggests that in this method of bioassay these two fungicides were poor in their action against *S. attenuatum* (I_3). When similarly tested against *S. oryzae* (I_4) the results indicated that Ortho Difolatan, Difolatex, and Benlate, in that order, were the best three fungicides which acted against this isolate. In comparison, it is found that *S. oryzae* (I_4) was more resistant to these fungicides than *S. attenuatum*(I_3). Dithane M -45 required very high concentrations to be effective against *S. oryzae* (I_4) while Bordeaux mixture and Calixin M (Tridemorph) were ineffective at all concentrations tested by this method. The results therefore suggest that the most economical and effective concentration of a given funguicide depended on (1) the fungus (2) the fungicide, and (3) the fungus - fungicide combination referred to. In general, Difolatex, Ortho Difolatan and Benlate were the best chemical agent, while *S. oryzae* was the more resistant isolate tested.

In general, considering the nature of this method, it is suggested that other factors in the culture medium such as dissolved nutrients and pH may have affected the response of the whole fungus culture. However, since Difolatex and Ortho Difolatan still proved to be the best two chemicals acting against both isolates of *Sarocladium* species tested, the results therefore suggest that if there was any interference from the environmental factors of the culture medium, it was insignificant in this study. (2) Although Difolatex and Ortho Difolatan were found to be the best two fungicides against the two isolates of *Sarocladium* species tested, and considering all the six compounds used, the isolates did not rate the chemical agents in like order. This suggests that the recommendation for the best chemical depends on the causal organism of the disease, and that careful identification of the causal pathogen is therefore very important in a control programme that seriously considers the use of chemicals. The results from this study using the above mentioned method confirm the work of Ragunathan et al., (1976), Purkayastha et al., (1977), Kannaiyan (1979), Chinnaswamy et al., (1981), and Singh et al., (1981).

Since the rationale in selecting the fungicides used in this study was that (1) they are important chemical agents already in use in African Agricultural and horticultural practices but without any information on their action against *Sarocladium* species, (2) they have protective and wide range use (WORTLING, 1979), and (3) they are already being used in tropical Africa (Cameroon) to control cocoa pod and coffee berry anthracnose diseases (Difolatex, Ortho Difolatan, and Calixin M (Tridemorph), and considering their effectiveness against isolates of *Sarocladium* species as hereby reported, their active spectrum may therefore be expanded to include protection against fungus diseases of

cereals (rice) as well. The implications of these findings are that (1) Farmers who change from coffee to rice farming will already be familiar with these chemicals from their previous use for protection against the coffee berry disease. Rice farmers of the European Development Fund financed Upper Noun Valley Development Project headquartered at Ndop, Cameroon, may be living examples of this situation because of their recent shift from coffee to rice farming where both crops are grown mainly for cash. (2) These fungicides (Difolatex and Ortho Difolatan) may therefore be included in an integrated pest management (IPM) programme in any African rice farming system for the control of grain discoloration and sheath rot caused by *Sarocladium* species.

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