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SEMI-SELECTIVE MEDIUM FOR ISOLATION OF *COLLETOTRICHUM GLOEOSPORIOIDES* FROM SOIL

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SUMMARY

Isolation of *Colletotrichum gloeosporioides* (causal agent of yam anthracnose) from soil is difficult because of the presence of fast growing micro-organisms like *Rhizoctonia* spp., *Rhizopus* spp., *Penicillium* spp. and bacteria. Therefore, a semi-selective medium consisting of antimicrobial agents such as pencycuron 50 mg L^{-1}, tolclofos-methyl 10 mg L^{-1}, streptomycin sulphate 100 mg L^{-1}, chloramphenicol 100 mg L^{-1} and chlortetracycline 100 mg L^{-1} was developed using potato dextrose agar as the basal medium. The procedure involved screening of candidate fungicides, developing a suitable combination of antimicrobial agents, assessing the proposed semi-selective medium and adjustment of pH of the medium to 5. The effectiveness of the medium is due to selective inhibition of micro-organisms by the antimicrobial agents and the development of distinct salmon-pink colonies of *C. gloeosporioides*.

INTRODUCTION

Yam anthracnose caused by the fungus *Colletotrichum gloeosporioides* is one of the most important limiting factors in the production of yam, particularly *Dioscorea alata*, all over the world (Nwankiti and Okpala, 1981; Leach, 1988; Green, 1994). The inoculum of *C. gloeosporioides* initiating primary infections on yams (at the onset of the rainy season) could originate from tubers (Adebanjo and Onesirosan, 1986; Green, 1994), yam debris (Singh and Prasad, 1967), or alternate hosts (Simons, 1990; 1991; Green, 1994) on which the pathogen is believed to perennate. There has been some controversy on the ability of the pathogen to survive in soil (Nwankiti and Okpala, 1981; Sweetmore, 1990; Green, 1994). The question of whether or not the pathogen can survive in soil is important, as this could assist in developing control strategies for managing the disease. There is therefore a need to establish the survivability or not of *C. gloeosporioides* in soil. Initial trials to recover the pathogen from artificially inoculated soil using potato dextrose agar amended with streptomycin sulphate (Sweetmore, 1990; Green and Simons, 1991; Green, 1994) proved extremely difficult because the pathogen was often overgrown by fast growing micro-organisms such as *Rhizoctonia* spp., *Rhizopus*

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spp., *Penicillium* spp. and bacteria which interfered with detection of the pathogen. It was therefore, considered that a selective or semi-selective medium would be helpful in the isolation of *C. gloeosporioides* from soil.

The principle of media for selective isolation of a specific fungus has been described by Tsao (1970). Such media were developed for the detection of other *Colletotrichum* spp. such as *C. coccodes* and *C. acutatum* (Farley, 1972; Nair and Corbin, 1979; Barker and Pitt, 1987). In addition, Agostini and Timmer (1992) developed a selective medium for differentiating strains of *C. gloeosporioides* on citrus. This paper describes four successive experiments conducted to develop a semi-selective medium suitable for isolation of *C. gloeosporioides* from soil. The first experiment was primarily to screen fungicides for toxicity to *C. gloeosporioides*, the second experiment was to determine suitable combinations of antimicrobial agents, while the third and fourth experiments were conducted to assess the efficacy of a proposed semi-selective medium and to adjust the pH level of the medium.

MATERIALS AND METHODS

Screening of candidate fungicides for growth activity and sporulation of C. gloeosporioides

Four fungicides: benomyl (wettable powder containing 50% a.i, Benlate, DuPont), tolclofos-methyl (wettable powder containing 50% a.i, Basilex, Sumitomo Chemical Co., Ltd), pencycuron (wettable powder containing 12.5% a.i, Monceren DS, Bayer) and iprodione (wettable powder containing 50% a.i, Rovral, Rhône-Poulenc), were selected on the basis of their broad spectrum activity and tested as possible anti-microbial agents, using potato dextrose agar (PDA) at quarter strength as the basal medium. The effect of varying concentrations of each of these fungicides 0 (control), 10, 20, 30, 40 and 50 mg product L^{-1} (hereafter referred to as mg L^{-1}), on the radial growth and sporulation of an isolate of *C. gloeosporioides* (DaN_1) was studied in order to determine which of the fungicide concentrations would be least toxic to the fungus. Three antibiotics, streptomycin sulphate (100 mg L^{-1}), chlortetracycline (100 mg L^{-1}), and chloramphenicol (100 mg L^{-1}) were added to each of the treatments to reduce bacterial contamination (Barker and Pitt, 1987). Stock solutions of the fungicides and antibiotics were prepared in sterile distilled water and the required concentrations were added to the basal medium after autoclaving and cooling to a temperature of about 30–40 °C. Six replicate plates for each treatment per fungicide were inoculated with the selected isolate of *C. gloeosporioides* (DaN_1) taken from the centre of 7- to 8-d-old cultures. The plates were incubated at a temperature of 30 $\pm$ 3 °C, under 12 h light and 12 h darkness (Manandhar *et al.*, 1995). The radial growth of the fungus was recorded as the mean of measurements along two diametrical axes on the reverse of each plate after 8 d incubation (similar procedures were used in subsequent experiments). The amount of conidia produced (sporulation) was estimated by washing conidia from three replicate plates per treatment with 10 ml sterile distilled water. The conidial suspension was

filtered through one layer of sterile cheese cloth and the conidial concentration was counted using a haemocytometer at ×400 magnification. Data for radial growth and sporulation were analysed by ANOVA with the exception of data for the effect of benomyl on sporulation of the isolate as there was no conidia production on most of the plates.

Developing a suitable combination of anti-microbial agents

On the basis of Experiment 1, pencycuron, tolclofos-methyl and iprodione were selected and tested further in various combinations after which three treatments were developed: (i) basal medium + antibiotics (control), (ii) basal medium + pencycuron (50 mg L^{-1}) + tolclofos-methyl (10 mg L^{-1}) + antibiotics and (iii) basal medium + pencycuron (50 mg L^{-1}) + tolclofos-methyl (40 mg L^{-1}) + iprodione (10 mg L^{-1}) + antibiotics. The effect of each of the treatments was tested on the radial growth of three isolates of *C. gloeosporioides* (DaN_1, DaN_2, DaN_3) in order to determine which of the combinations could be selected as a potential semi-selective medium. Each treatment was replicated three times.

Assessing selective ability of the proposed semi-selective medium

The proposed semi-selective medium obtained from Experiment 2 was used for the following assessment. Five fungal isolates consisting of (i) *C. gloeosporioides* (DaN_1), (ii) Rhizoctonia spp., (iii) *Rhizopus* spp., (iv) *Penicillium* spp. and (v) *Trichoderma* spp., were used for this trial. Isolates (ii) to (v) (hereafter referred to as contaminant fungi) were selected because they are fast growing fungi, commonly isolated from field soil and yam foliage and which constitute a major problem in isolating *C. gloeosporioides* from soil and leaf material. Inoculation of the fungal isolates was carried out on (i) basal medium + antibiotics (control) or (ii) basal medium + pencycuron (50 mg L^{-1}) + tolclofos-methyl (10 mg L^{-1}) + antibiotics. Radial growth of the fungal isolates was measured after 6 d incubation at 30 ± 2 °C. Inhibition of mycelial growth on the fungicide-amended media (%) was calculated using the formula:

$$\frac{\text{Radial growth in control} - \text{Radial growth in fungicide amended medium}}{\text{Radial growth in control}} \times 100$$

(Manandhar *et al.*, 1995).

Effects of pH on efficacy of the proposed semi-selective medium

The fourth experiment was finally set up using the proposed semi-selective medium to determine the best pH for the recovery of *C. gloeosporioides* from soil. The medium (consisting of pencycuron (50 mg L^{-1}) + tolclofos-methyl (10 mg L^{-1}) + antibiotics in PDA basal medium) was prepared and the pH adjusted to give the following treatments: unadjusted pH (control), pH adjusted to 5.5, 5, and 4.5 (Barker and Pitt, 1987). The pH was measured using a Griffin

(Model 50) pH meter, and adjusted to the required levels with 0.01M hydrochloric acid and 0.01M NaOH. In order to determine the effects of pH on the recovery of *C. gloeosporioides* from artificially inoculated soil, 3 g top soil was taken from a representative sample of soil collected from the field experimental site at the University of Agriculture, Makurdi, Nigeria. The subsample was inoculated with 1 mL conidial suspension of an isolate of *C. gloeosporioides* (DaN_1) containing $1 \pm 0.3 \times 10^6$ conidia mL^{-1}. The soil was mixed thoroughly and 5 mL distilled water was added. The suspension was shaken using a magnetic stirrer for 3 min then 1 mL was taken and serially diluted with sterile distilled water to 10^{-3} (Ritchie, 1991). An aliquot of 0.5 mL was transferred to a 9-cm Petri dish containing the fungicide-amended medium and spread on the solidified medium using a sterile glass rod. For each pH level, six replicate plates were prepared. The plates were arranged in a completely randomized design and incubated at 12 h light and 12 h darkness and at a temperature of $30 \pm 3\,^{\circ}C$. The number of colony-forming units (cfu) of both *C. gloeosporioides* and contaminant fungi were recorded after 7 d incubation. The effect of pH on colony count was analysed by ANOVA.

RESULTS

Screening of candidate fungicides for growth activity and sporulation of C. gloeosporioides

Figures 1 and 2 show the effects of benomyl, tolclofos-methyl, iprodione and pencycuron on the radial growth and sporulation of an isolate of *C. gloeosporioides* (DaN_1). Analysis of variance revealed that pencycuron had no effect on the radial growth of *C. gloeosporioides*, but increased conidial production by 9% at 50 mg L^{-1} ($p < 0.001$) compared with the non-amended medium. Radial growth and sporulation were significantly ($p < 0.001$) lower in all concentrations of tolclofos-methyl and iprodione compared with the control. However, the effects were moderate at the lowest concentration (10 mg L^{-1}) reducing radial growth by 18% for tolclofos-methyl and 22% for iprodione. Reductions in sporulation caused by incorporating tolclofos-methyl and iprodione at 10 mg L^{-1} were also moderate but iprodione changed the colour of the conidial masses from salmon-pink to black. Benomyl inhibited the radial growth and sporulation of *C. gloeosporioides* at all concentrations. For example, at the lowest concentration (10 mg L^{-1}) the radial growth of *C. gloeosporioides* was 9.6 mm compared with 78 mm in the control. Given this high inhibition level, benomyl was excluded from further trials.

Developing a suitable combination of anti-microbial agents

Table 1 shows the effects of treatments tested on the radial growth of three isolates of *C. gloeosporioides*. Radial growth was significantly ($p < 0.001$) higher in isolate DaN_1 compared with isolates DaN_2 and DaN_3, but all of the isolates sporulated. With respect to fungicidal amendments, radial growth of *C. gloeosporioides* was significantly ($p < 0.001$) higher on the basal medium compared with the fungicide-amended media. Amongst the fungicide-amended media, colony

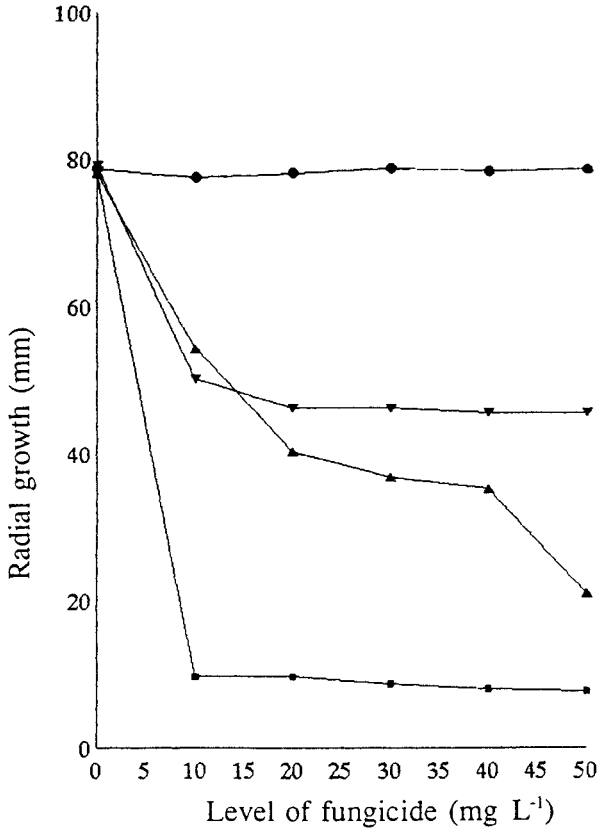

Fig. 1. Effects of benomyl (■), tolclofos-methyl (▲), pencycuron (●) and iprodione (▼) on the radial growth of *C. gloeosporioides* after 8 d at 30 ± 3 °C.

Table 1. Effects of different combinations of fungicides on the radial growth of three isolates of *C. gloeosporioides* after 8 d incubation at 30 ± 3 °C (mm).

Fungicide combinations	Radial growth of isolates			Mean
	DaN_1	DaN_2	DaN_3	
PDA† + antibiotics (control)	80.3	44.3	45.3	56.7
PDA + antibiotics + pencycuron (50 mg L^{-1}) + tolclofos-methyl (10 mg L^{-1})	54.3	22.3	20.0	32.2
PDA + antibiotics + pencycuron (50 mg L^{-1}) + tolclofos-methyl (40 mg L^{-1}) + iprodione (10 mg L^{-1})	39.0	21.7	21.0	27.2
Mean	57.9	29.4	28.8	
s.e.d. fungicide combinations = 1.286 isolates = 1.286 fungicide × isolate interactions = 2.228				

†PDA = potato dextrose agar.

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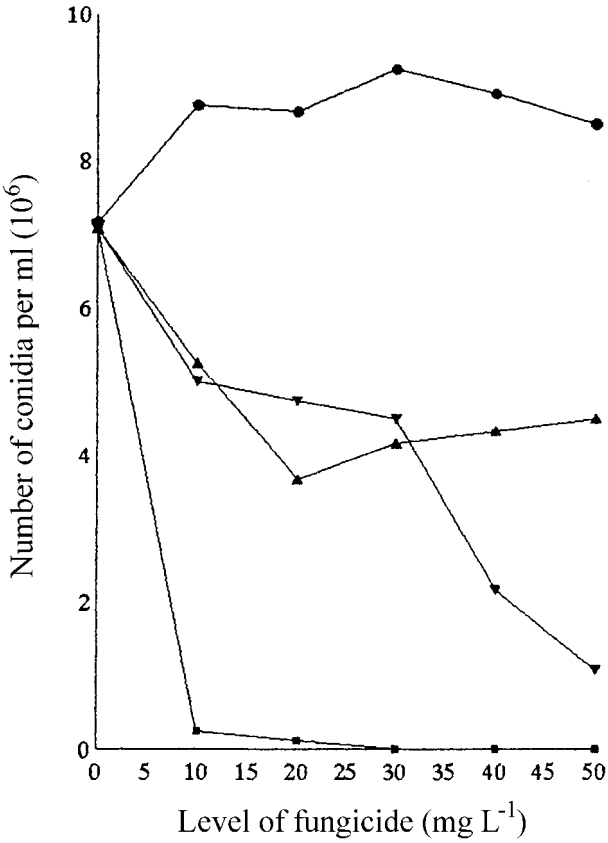

Fig. 2. Effects of benomyl (■), tolclofos-methyl (▲), pencycuron (●) and iprodione (▼) on the sporulation of *C. gloeosporioides* after 8 d incubation at 30 ± 3 °C.

diameter of *C. gloeosporioides* isolates grown on the basal medium amended with pencycuron (50 mg L^{-1}) + tolclofos-methyl (10 mg L^{-1}) + antibiotics were not significantly higher than those grown on basal medium amended with pencycuron (50 mg L^{-1}) + tolclofos-methyl (40 mg L^{-1}) + iprodione (10 mg L^{-1}) + antibiotics. However, the incorporation of iprodione in the latter treatment, changed the colour of spore masses from salmon-pink typical of *C. gloeosporioides* to black. The treatment without iprodione was therefore selected as a potential semi-selective medium for further trials.

Assessing selective ability of the proposed semi-selective medium

Inhibition of the mycelial growth of five fungal species when grown on the proposed semi-selective medium (basal medium amended with pencycuron (50 mg L^{-1}) + tolclofos-methyl (10 mg L^{-1}) + antibiotics) was as follows: *Rhizoctonia* spp. 82%, *Rhizopus* spp. 76%, *Penicillium* spp. 70%, *Trichoderma* spp. 60% and *C. gloeosporioides* 46%.

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Table 2. Recovery of *C. gloeosporioides* and contaminant fungi on semi-selective medium adjusted to three pH levels.

pH	Number of replications	Colony-forming units (cfus)†	
		C. gloeosporioides	Contaminant fungi
Control	6	104.6	18.8
5.5	6	103.0	21.2
5.0	6	147.4	6.4
4.5	6	114.2	8.6
s.e.d.		26.9	7.5

†cfus = mean colony-forming units counted after 7 d incubation at 30 ± 3 °C.

Effects of pH on efficacy of the proposed semi-selective medium

The recovery of *C. gloeosporioides* and contaminant fungi from artificially inoculated soil using the proposed semi-selective media adjusted to three different pH levels are shown in Table 2. The addition of antibiotics inhibited bacterial growth. An ANOVA on the data showed that there were no significant differences in the colony-forming units of *C. gloeosporioides* and contaminant fungi when the medium was prepared without pH adjustment and when the pH was adjusted to 4.5, 5 and 5.5. However, the critical values showed that the incidence of contaminant fungi was lowest at pH 5.

DISCUSSION

Selectivity in a medium designed for isolating a fungal species, is usually achieved by adding chemical anti-microbial agents (Tsao, 1970). The chemical agents (fungicides and/or antibiotics) are usually added to obtain a selective inhibition of the undesirable micro-organisms or a selective enhancement of the fungal species desired (Tsao, 1970). In this investigation, four fungicides (benomyl, iprodione, tolclofos-methyl and pencycuron) were tested singly and in combinations in order to determine their influence on the growth and sporulation of *C. gloeosporioides*. In addition, three antibiotics (streptomycin sulphate, chloramphenicol and chlortetracycline) were added to inhibit bacterial growth. The ability of pencycuron to enhance sporulation of *C. gloeosporioides* (Fig. 2), was considered to be a desirable attribute because sporulation is the most important diagnostic feature of *C. gloeosporioides* in the presence of other contaminant micro-organisms. Although tolclofos-methyl moderately reduced the growth and sporulation of *C. gloeosporioides*, this fungicide did not have an adverse effect on the cultural appearance of the pathogen. It was, therefore, retained as an integral part of the medium being developed for the selective isolation of *C. gloeosporioides*.

Iprodione had been used previously in a semi-selective medium for isolation of *C. gloeosporioides* from pepper (*Capsicum annuum*) (Manandhar *et al*., 1995), while benomyl was successfully used in a selective medium for the isolation of *C. coccodes* and *C. acutatum* from artificially inoculated soil (Farley, 1972; Nair and Corbin,

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1979). In this investigation, iprodione moderately inhibited the radial growth of *C. gloeosporioides* but changed the colour of the conidial masses of the pathogen from salmon-pink typical of the fungus to black. This change in colour of conidial masses was considered undesirable because it prevented a positive diagnosis of *C. gloeosporioides* on the strength of cultural morphology. Benomyl inhibited the growth and sporulation of *C. gloeosporioides*; this would not improve the recovery of the pathogen in the presence of other fungi routinely isolated from soil (Fig. 1 and 2). Benomyl and iprodione were therefore excluded as anti-microbial agents for selective isolation of *C. gloeosporioides* from soil.

In order to develop a suitable fungicide combination for selective isolation of *C. gloeosporioides*, it was necessary to test the influence of the fungicide combination on a range of isolates with different cultural characteristics. Isolate DaN_1 had white mycelia while isolates DaN_2 and DaN_3 were darkly pigmented. Isolates with white mycelia tended to be fast growing while the darkly pigmented isolates were usually slow growing in culture (Winch *et al.*, 1984). This inherent characteristic may have been responsible for the significant differences observed in the radial growth of the isolates. In a fungicide combination where iprodione was excluded, sporulation was not altered or inhibited in any of the isolates tested.

By amending PDA basal medium with pencycuron (50 mg L^{-1}) + tolclofos-methyl (10 mg L^{-1}) + antibiotics, the conidial production in *C. gloeosporioides* was enhanced. This characteristic was considered important in facilitating the differentiation of *C. gloeosporioides* from the contaminant fungi. Further to the enhancement of sporulation of *C. gloeosporioides*, the medium greatly inhibited the radial growth of contaminant fungi making it an extremely valuable tool for the selective identification of *C. gloeosporioides*. This medium was therefore proposed as a semi-selective medium for the isolation of *C. gloeosporioides* from soil. Similarly, when the medium was adjusted to pH 5, there was a lower incidence of contaminant fungi. Although the difference was not significant, pH 5 was adopted for the semi-selective medium. The medium has been successfully used to study the survival of *C. gloeosporioides* in soil, and this will form the subject of another report.

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