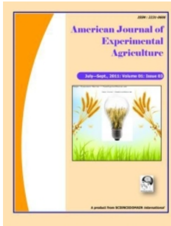

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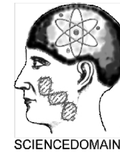

The Influence of Benomyl on Penicillin Production and Rhizosphere Organisms

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ABSTRACT

Aims: To evaluate the antimicrobial effects of benomyl (a systemic fungicide) on penicillin production and rhizosphere organisms of cowpea plant.
Study Design: 3 factor factorial experiment.
Place and Duration of Study: Department of Microbiology, Federal University of Technology, Akure, Nigeria, 2006.
Methodology: Complementary plate and pot experiments were designed to achieve these objectives. Bioassay methods such as the agar cup plate and the agar plug techniques were used to examine the metabolic fitness of *Penicillium italicum* and *Penicillium oxalicum* for penicillin production when cultured in agar medium with varying concentrations of benomyl. The pot experiment was also carried out to determine the effect of 0.8g of benomyl on the microbial load of 1.5kg of rhizosphere soil of cowpea.
Results: Biological assay predominantly shows that benomyl at different concentration has the ability to impair the metabolic and mitotic activity of *Penicillium* species mentioned above. This development resulted in the inhibition of penicillin and other allied metabolites. It was discovered that there was a reduction in the microbial load of rhizosphere soil containing benomyl and the fungicide was incriminated to be responsible for it.
Conclusion: Certain species of bacteria and fungi that predominated in the rhizosphere soil sample without benomyl were either few or absent in the sample with benomyl.

Keywords: Benomyl; penicillin; rhizosphere; mitotic activity; cowpea.

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1. INTRODUCTION

Fungicides are antibiological agents designed to control one or several types of agricultural peats as inhibitors for biological systems, the pesticides may exert simultaneously a deleterious influence upon the saprophytic soil population, the result of which may be detrimental to plant growth (Behera et al., 2006). In the past several decades, on enormous array of biocide formulations have been produced and applied in attempts to control key pests in agricultural systems (Kidd and James, 1991). These chemicals include bactericide, herbicides, insecticides, nematicides, algicides, plant growth stimulators and retarders. They have responded to the food production needs of all sufficient countries of the world and will inevitably play a major role in the tropics as this region moves from deficiency to surplus production while many insecticides such as chlorinated hydrocarbons have reportedly created major environmental pollution problem in the normal pursuit of destruction of unwanted fungal pathogens (Prescott et al., 2005). Fungicides are introduced into the environmental and had the chance of getting into the air, water, sand and plant. It will also come in contact with both target and non-target organisms. Similar findings are obtained with most insecticides. Much greater disruption of soil biota is however caused by fungicides (Tortora et al., 2002).

Fungicides are designed specifically for the control of soil/plant pathogenic fungi. Following fungicides application to the soil at an eradicant rate for the target organisms, there is commonly a depression in the rate of soil respiration for one to several weeks, but after one to several months, there is greater cumulative CO_2 evolution from treated than untreated soil (Chanway et al., 1991).

The nitrifying bacteria are the most susceptible and inhibition of nitrification is occasionally observed following a foliar application of a biocide. The rhizosphere population of non-pathogens and asymbiotic organisms on the pesticide may be increased or decreased depending on the pesticide employed and its rate of application (Brock and Madigan, 2002).

Benomyl was introduced into the U.K. market in 1971 by upon agricultural products, an American Company. It is a systemic benzimidazole fungicide that is selectively toxic to microorganisms. It has the widest spectrum of all the newer systemic including the *Sclerotinia, Botrytis* and *Rhizoctonia* sp. and the powdery mildew and Apple Scab. Its principal trade name is benlate. Benomyl is compatible with many other fungicides. Their mode of action appears to be induction of abnormalities in spore germination, cellular multiplication and growth as a result of interference in the synthesis of vital nucleic acid, materials DNA. Benomyl is strongly bound to the soil in the environment and does not dissolve in water in flooded rice fields to any significant extent (Wauchope et al., 1992).

The effects of fungicide on biological processes in the soil come to light most clearly only via a repeated multifunctional approach to the toxicant (Bethlenfalvay, 1992). Pesticides incorporated into the soil may change the composition of the soil microflora. Soil fungicides and fumigants as a rule have negative effect on the soil microflora. There is increased evidence that foliarly applied fertilizers are fungicides that can cause marked changes in soil microflora either directly through translocation and oxidation of the applied compound or indirectly by changing the chemical nature of the root exudates (Laich et al., 2002).

The role of rhizosphere microorganisms on plant species distribution and community structure is a neglected structure in plant population biology (Law, 1988). Yet, certain lines limiting factors in the distribution range of many plant species. In recent years, the

widespread use of synthetic compound for the control of insect pests, weed or plant pathogenic fungi or nematodes is a major factor determining the efficiency and yields of modern agriculture. However, the fate of these substances in the environment has become a matter of concern. Therefore in considering the contributions of chemical weed and pest control and the major role played by microorganisms in nutrient recycling, mineralization and solubilization of some complex compounds, it is imperative to examine the advantages and deleterious effects of these processes on the environment and sustainable agriculture (Pelczar et al., 2002). The present study was undertaken to determine the fitness of *P. italicum* and *P. oxalicum* for penicillin production by determining their antimicrobial activity against some bacterial pathogens before and after exposure to a systemic fungicide (benomyl) and also evaluate the effects of benomyl on the microbial load of rhizosphere microorganisms of cowpea plant.

2. MATERIALS AND METHODS

2.1 Sources of Cowpea, Soil Samples and Microorganisms Used

P. italicum was obtained from the Department of Microbiology, Federal University of Technology, Akure, Nigeria. Cowpea tagged (IT93K-452-1); stock cultures of *Fusarium solani* and *P. oxalicum* were collected from International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria. Stock cultures of pathogenic bacteria obtained from the University College Hospital (UCH) Ibadan include *Staphylococcus aureus, Shigella dysenteriae, Bacillus cereus, Salmonella typhi* and *Salmonella paratyphi. Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* were collected from Microbiology Laboratory of Neimeth Pharmaceuticals, Lagos, Nigeria. Soil samples used were obtained from the back of the green house in Biology Department, FUTA.

2.2 Agar Cup Plate Sensitivity Test

The sensitivity of bacterial pathogens to metabolite obtained from pure broth cultures of *P. oxalicum* in the absence and presence of benomyl at the following concentrations: (C_o-0.00 g mL^{-1}, C_1-0.002 g mL^{-1}, C_2-0.004 g mL^{-1} and C_3-0.008 g mL^{-1}) was determined using modified method of Tortora et al. (2002). The metabolites were obtained by adding varying qualities of benomyl to the culture plates/bottles containing SDA/SDB (Saboraud Dextrose Agar/ Saboraud Dextrose Broth) and standard inoculum of *P. italicum* was inoculated. The mixture was homogenized to ensure proper interaction of the mixture. Incubation was done at 29ºC for 7-10 days. Intermittent homogenization of culture mixture and aeration were done by shaking and opening of bottles to allow diffusion of air. Filteration of the metabolite was carried out using Whatman filter paper No.1. Centrifugation at a speed of 10,000 rpm for 5 min was undertaken to further separate the mixture. The supernatant was aseptically decanted into a sterile bottle.

2.2.1 Bioassay for penicillin production in liquid medium

Nutrient Agar (NA) plates were appropriately seeded with standard inoculum (0.1 mL) of 24 h old bacterial suspension by the pour plating method. Holes were bored on the agar with a sterile 12 mm diameter size cork borer and the metabolite was aseptically dispensed into the holes. The bioassay process was undertaken in triplicates for each test bacterial pathogen. Incubation was done at 29ºC for 24 h and zones of inhibition were measured and recorded (Pelczar et al., 2002). The procedure was also repeated for *P. oxalicum.*

2.2.2 The agar plug sensitivity test method

A 25 mL of SDA was seeded with varying qualities of benomyl (0.0, 0.05, 0.1 and 0.2 g) to make appropriate concentrations of C_o, C_1, C_2, and C_3, respectively. The plates were gently swirled to ensure proper homogenization of the contents. *P. italicum* was inoculated-on the plate and incubated at 29ºC for 7-10 days.

2.2.3 Bioassay for penicillin production in solid medium

The NA plates were seeded with 0.1 mL of 24 h old bacterial inoculums using the pour plating method. Twelve millimeter diameter agar plugs were bored out of the NA plates and a corresponding size of agar plugs of *P. italicum* on SDA cultured in the presence of varying concentrations of benomyl were inserted. Incubation of culture plates at 29ºC for 72 h was done. The test procedure was in triplicates zones of inhibition of test bacteria were measured and recorded. The procedure was repeated for *P. oxalicum* (Laich et al., 2002).

2.2.4 The pot experiment

Seventy two pots were used and 1.5 kg of sterilized soil was weighed into each of the pots arranged in rows. The experiment is a three factor; factorial experiment (2×2×6) in triplicates and the factors considered were Mycorrhizal inoculated (M) and non Mycorrhizal (MO) inoculated soil: *Fusarium* inoculated (F) and non *Fusarium* (F_0) inoculated. Soil, concentrations of benomyl used designated as C_0, C_1, C_2, C_3, C_4, C_5 are: 0.00, 0.05, 0.10, 0.20, 0.40 and 0.80 g, respectively. Ten grams of mycorrhiza *(G. mosseae)* and 5 mL of *F. solani* in solution were inoculated on the 1st day into appropriate pots at a depth of 5 cm. Benomyl were added at appropriate concentrations to centre of the pots. Cowpea seeds (*Vigna unguiculata)* were surface sterilized in 70% alcohol for 2 min, washed in sterile water and planted at a depth of 3 cm at the center of the pots. The plants were placed under a green house condition and watered to field capacity every 7 day to maintain soil moisture condition. Plants were harvested on the 30th day after germination and rhizosphere soil samples were collected from pots designated as $M_0F_0C_0$ and $M_0F_0C_5$. The condition of cowpea leaves and roots were examined and documented appropriately.

2.3 Isolation of Rhizosphere Organisms

On rhizosphere soil designed as $M_0F_0C_0$ and $M_0F_0C_5$, five-fold serial dilution was carried out for fungi while eight-fold was also done for bacteria. 0.1 mL of five-fold diluent was introduced into plating and pour plating was done with SDA for fungal isolates for bacteria isolation 0.1 mL of eight-fold diluent was inoculated into plates and pour plating was carried out with NA. Incubation was done at 29ºC for 5-6 days for fungal isolates and at 37ºC for 24 h for bacterial isolates. The culture plates were prepared in triplicates.

2.4 Identification of Microorganisms

The identification of both bacterial and fungal isolates was determined using standard methods (Tortora et al., 2002; Prescott et al., 2005). The pure cultures were identified to the species level. For bacteria, elevation, colour and shape were studied on the agar. The cells were Gram stained, tested for motility, sugar fermentation and enzymes such as catalase, urease etc. For the fungi, morphological studies such as examination of the size, shape, colour, spore formation and the number of days taken for the fungus to reach maximum diameter (9cm) of the Petri dish and the texture of fungal growth were observed. The fungi,

the spore – bearing mycelia were then carefully sectioned, teased out and stained on a slide and then observed with a light microscope. The fungi were then identified and characterized according to the method of Carpenter (1977).

3. RESULTS AND DISCUSSION

The antimicrobial activity (Tables 1-4) observed from the result of this research against -lactamase producing organism (*B. cereus, P. aeruginosa, E. coli* and *K. pneumoniae)* and even against non -lactamase producing organism by agar plugs of *P. oxalicum* cultured in the absence of benomyl suggest that penicillin was not the only antimicrobial agent that was synthesized. The observed -lactamase antibacterial activity could not be solely attributed to penicillin, (Brakhage, 1998) because -lactamase producing bacteria were also inhibited.

Table 1. Antimicrobial effects of *P. italicum* against bacterial pathogen using the agar cup plate method

Benomyl concentration	Diameter of zones of inhibition (mm)							
	A	B	C	D	E	F	G	H
C_0	0.00	0.00	0.00	0.00	0.00	0.00	16.50 ± 1.06	24.50 ± 0.35
C_1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Keys: A: K. pneumoniae. B: E. coli, C: S. typhi, D: S. aureus, E: S. dysenteriae, F: S. paratyphi, G: B. cereus, H: P. aeruginosa, C_0 0.00 g mL^{-1}, C_1: 0.002 g mL^{-1}, C_2: 0.004 g mL^{-1}, C_3: 0.008 g mL^{-1} of benomyl

Table 2. Antimicrobial effects of *P. italicum* against bacterial pathogen using the agar plug method

Benomyl concentration	Diameter of zones of inhibition (mm)							
	A	B	C	D	E	F	G	H
C_0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.010 ± 0.70
C_1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Keys: A: K. pneumoniae, B: E. coli, C: S. typhi, D: S. aureus, E: S. dysenteriae, F: S. paratyphi, G: B. cereus, H: P. aeruginosa, C_0: 0.00 g mL^{-1}, C_1 0.002 g mL^{-1}, C_2: 0.004 g mL^{-1}, C_3: 0.008 g mL^{-1} of benomyl.

Table 3. Antimicrobial effects of *P. oxalicum* against bacterial pathogens using agar cup plate method

Benomyl concentration	Diameter of zones of inhibition (mm)							
	A	B	C	D	E	F	G	H
C_0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.50±0.35
C_2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.33±0.00

A: K. pneumoniae, B: E. coli, C: S. typhi, D: S. aureus, E: S. dysenteriae, F: S. paratyphi, G: B. cereus, H: P. aeruginosa, C_0: 0.00 g mL^{-1}, C_1: 0.002 g mL^{-1}, C_2: 0.004 g mL^{-1}, C_3: 0.008 g mL^{-1} of benomyl.

Table 4. Antimicrobial effects of *P. oxalicum* against bacterial pathogens using the agar plug method

Benomyl concentration	Diameter of zones of inhibition (mm)							
	A	B	C	D	E	F	G	H
C_0	8.00 ±1.41	6.00 ±0.82	7.50 ±1.77	3.50 ±0.35	7.00 ±0.82	10.50 ±1.77	10.33 ±0.72	9.67 ±2.23
C_1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Keys: A: K. pneumoniae. B: E. coli, C: S. typhi, D: S. aureus, E: S. dysenteriae, F: S. paratyphi, G: B. cereus, H: P. aeruginosa, C_0: 0.00 g mL^{-1}, C_1: 0.002 g mL^{-1}, C_2: 0.004 g mL^{-1}, C_3: 0.008 g mL^{-1} of benomyl.

This occurrence might be credited to the presence of other mycotoxins that were produced by the fungi (Laich et al., 2002). The antibacterial activity of agar plugs from *P. oxalicum* cultured in the absence of benomyl (Table 4) had a wider spectrum of activity than that of *P. oxalicum*. However, metabolite of *P. italicum* exerted a stronger antimicrobial effect (Tables 1 and 2) against *B. cereus* and *P. aeruginosa* than metabolite and agar plugs from *P. oxalicum* when cultured in the appropriate medium without benomyl (Tables 3 and 4). *S. aureus* and *S. paratyphi* were inhibited by agar plugs of *P. oxalicum* cultured in SDA with benomyl concentration of 0.002 g mL^{-1}. *P. oxalicum* could have been genetically altered by benomyl to produce a stronger metabolite (Feng et al., 1994) that rendered *S. aureus* more susceptible to inhibition by the metabolic. (Behera et al., 2006) *P. aeruginosa* showed decreasing zone of inhibition by metabolic of *P. oxalicum* cultured in SDB with increasing concentrations of benomyl (0.002 and 0.006 g mL^{-1}), but there was no inhibition in metabolites produced in culture medium without benomyl. Benomyl at these concentrations could have modified the genes in *P. oxalicum* and stimulated them for penicillin production (Howard, 1991; Feng et al., 1994).

From the finding of this research, it was discovered that benomyl was not an utilizable substrate for both *P. oxalicum* and *P. italicum.* The fungicide has the ability to inhibit their growth by impairing their metabolic function for the production of penicillin and other allied mycotoxins (Brakhage, 1998).

The result of the pot experiment showed that benomyl does not support the rhizosphere activity of *S. cerevisiae* (yeast). The rhizosphere population of *Varicosporum elodea* was lesser when compared to that isolated from rhizosphere soil without benomyl. *Diplosporium flavum* was only isolated from rhizosphere soil the presence of benomyl. *Bacillus* sp. was the only bacterium isolated from rhizosphere soil samples in the presence and absence of benomyl. The microbial load of *Bacillus* sp in rhizosphere soil without benomyl was higher than that with benomyl. Benomyl could have inhibited the rhizosphere population and activities of this bacterium (Tortora et al., 2002).

4. CONCLUSION

The research finding indicated that the addition of the fungicide benomyl affected the ability of the two *Penicillium* species to produce antimicrobial metabolite (penicillin) and thus reduce the possible antagonistic activity of this soil flora against some phytopathogens. It equally led to lowering of the microbial load quantitatively and qualitatively suggesting a possible reduction in soil fertility because nutrient composition has been reported to be

enhanced by certain soil microbes. It is therefore necessary that complementary laboratory and field studies that focus on the ecological significance of rhizosphere organisms, their metabolites and their interaction with benomyl at varying concentration geographical soils locations. Relevant inferences should be drawn by putting these factors, soil minerals and weather conditions of the soil into consideration. Consequently, this will foster the constructive use a benomyl on agricultural soil and agricultural plant will experiences a major boost in production via positive contribution from soil microorganisms. For sustainable and productive agronomy practices, individual and stakeholders in agriculture and other related fields are advised to support productive researches in soil microbiology today.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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