# ANTIFUNGAL AND NOVEL ROOD PRESERVATIVE FOTENTIALS OF SELECTED TRIORGANOTINS

A DISSERTATION SUBMITTED BY

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OCTOBER, 1992

# DECLARATIONS

This is to certify that I carried out the work reported in this thesis in the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria under the joint supervision of Dr. P.F. Olurinola and Dr. J.J. Bonire. The work of other investigators is acknowledged and referred to accordingly. I solemnly declare that no part of this thesis has been submitted elsewhere for a degree.

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# CERTIFICATION

This thesis entitled *"Antifungal and Novel Food Preservative Potentials of Selected triorganotins"* by EHINMIDU, OLORUNFEMI OLORUNMOLA JOSEPH meets the regulations governing the award of the degree of Doctor of Philosophy (Pharmaceutical Microbiology) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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# DEDICATION

This work is dedicated to God the Father, God the Son, and God the Holy Spirit, my family members and to those that love the truth and hate ungodliness in all forms.

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## ABSTRACT

The antifungal effects of tributyltin acetate [TBTA], triphenyltin acetate [TPTA], triphenyltin salicylate [TPTS] alone and in combination with Propylene glycol [PG] under controlled experimental conditions have been evaluated in order to determine the desirability or otherwise for the use of these agents as yam anti-rot preservatives system.

Using zone of inhibition, MICs and MFCs to measure the antifungal activities of test triorganotins, their fungitoxic effects against Aspergillus flavus, Rhizopus stolonifer, Penicillium citrinum and Aspergillus niger was found to be in the order of TBTA > TPTA> TPTS. The in-vitro MICs of test triorganotins, for example TBTA against $10^5$ and $10^3$ CFU\ml of test fungi spores ranges from 2.5 to 5.0 µg/ml [0.0072 - 0.0143mM] and from 5.0 to 10.0 µg/ml [0.0143 - 0.029] respectively. An indication that the MICs of test triorganotins against test fungi spores were inoculum size dependent.

The effects of fungicidal concentrations of TBTA, TPTA and TPTS on changes in viable spores number at different time interval showed rapid lethal effects against the test fungi spores and exhibited an inverted type C death curve. Addition of 0.5% polysorbate 80 and above to triorganotin-fungi spores system reduced the killing rates of test triorganotins. It was also observed that there are significant strain differences in susceptibility responses of A. flavus and A. niger which are the least and most sensitive of the test yam rot fungi to TBTA, TPTA and TPTS respectively. The results obtained, indicate that the mechanism of fungicidal action of TBTA, TPTA and TPTS was suspected to be highly dependent upon coagulation of cytoplasmic constituents.

The effect of boiling at 100℃ and exposure to light for three days on the antifungal activity of test triorganotins was examined. A 3.3 to 5.4 and 7.5 to 13.0 reduction in biological activity of TPTA exposed to light for three days and boiling at 100℃ for 10 minutes respectively was observed. This result appears to suggest fairly rapid photo and thermal degradation of test triorganotins which may present very low toxicity threat if any, to either environment or the consumer of yam treated with test triorganotins as preservatives.

The in-vivo antifungal effects of TBTA, TPTA, TPTS alone and in admixture with PG on sliced yam at 90% relative humidity, a condition which support maximum growth of yam rot fungi spores was investigated. This study has shown that TBTA and TPTA are very effective in controlling yam rot fungi, however at a higher concentration when compared with in-vitro MIC. Furthermore, addition of PG to TBTA, TPTA or TPTS displayed enhanced antifungal effects against all the test yam rot fungi spores. This work establishes the fungicidal action of TBTA, TPTA and TPTS against spores of A. flavus, R. stolonifer, P. citrinum and A. niger and indicates that their antifungal activity, especially 400 ug/ml of test triorganotins plus PG offer potentially effective and safe fungal yam rot preservatives at reduced cost.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| CFU/ml | - | Colony forming Unit per millilitre |
| $^0C$ | - | Degree celsius |
| DM | - | Diluent medium |
| $D_{10}$-values | - | One log cycle reduction |
| ED-90 | - | Effective dose that give 90 percent Kill of test organisms |
| F | - | Analysis of variance of completely randomised design |
| FIC | - | Fractional inhibitory concentration |
| ΣFICs | - | Sum of franctional inhibitory concentrations |
| g | - | gramme |
| $gm/dm^3$ | - | gramme per cubic decimetre |
| h | - | hour |
| kg, a.i/ha | - | Kilogramme per area in hectare |
| K-values | - | The rate of Kill |
| $LD_{50}$ | - | Lethal dose at 50 percent |
| LPD | - | Lecithin - polysorbate 80 Diluent |
| M | - | Mole |
| MEC | - | Minimum effective concentration |
| MIC | - | Minimum inhibitory concentration |
| MFC | - | Minimum fungicidal concentration |
| mg | - | Milligramme |
| mg/kg | - | Milligramme per Kilogramme body weight |
| ml | - | Millilitre |
| mm | - | Millimetre |

| | | |
|---|---|---|
| mM | - | Millimole |
| nm | - | Nanometre |
| % w/v | - | percentage weight over volume |
| % v/v | - | percentage volume over volume |
| PG | - | Propylene glycol |
| pH | - | Hydrogen ions concentration |
| PVC | - | Polyvinyl chloride |
| r | - | Rat |
| RH | - | Relative Humidity |
| RLPM | - | Recovery Lecithin polysorbate 80 medium |
| SA | - | Salicylic acid |
| SDA | - | Sabouraud dextrose agar |
| TBTA | - | Tributyltin acetate |
| TPTA | - | Triphenyltin acetate |
| TPTS | - | Triphenyltin salicylate |
| ug/kg | - | Microgramme per kilogramme body weight |
| ul | - | Microlitre |
| uv | - | Ultra - violet light |
| WHO | - | World Health Organisation |
| > | - | Greater than |
| < | - | Less than |

# CHAPTER ONE

# INTRODUCTION

## 1.1 THE YAM ROT PROBLEM

The cultivated yam (Dioscorea species) is an important staple food in many tropical countries of the world. However, post harvest loss of yam tuber has been a very serious problem to yam farmers.

Yam losses during storage are due primarily to biological factors and a little to chemical and physical factors. Among the biological factors, decay by fungal pathogens account for the greatest losses (Adeniji, 1970; Agarawal and Gupta, 1973; Ogundana, et al, 1970; Okafor, 1966). Rawnsley (1969) and Bonire (1985) reported a total post-harvest loss of about 15% and 40% of yam tubers in West Africa and Nigeria respectively.

Matured root tubers are susceptible to attack by a variety of micro-organisms to which they were resistant during the period of their development in the field (Table 1.1).

TABLE 1.1: MAJOR POST-HARVEST DISEASES OF TUBERS

| CROP | DISEASE | PATHOGENS |
|---|---|---|
| Carrot | Gray mould rot | Botrytis cinera Pers ex Fr. |
| | Centrospora rot | Centrospora acerina (Hartig) Newhall |
| | Watery soft rot | Sclerotinia sclerotiorum (Lib) de Bary |
| Potato | Bacterial soft rot | Erwinia carotovora (Jones) |
| | Dry rot | Fusarium sp and Aspergillus niger van Tiegh |
| Sweet Potato | Black rot | Ceratocystis fimbriata Ellis and Halst |
| | Rhizopus soft rot | Rhizopus stolonifer (Ehr, ex Fr) Vuill |
| | Tuber rot | Corticium rolfsii (Curzi) |
| | Blue mould rot | Penicillium Sp. |
| | Foot rot | Plenodomus destruens Harter |
| | Java black rot | Diplodia tubericola (Ell and Ev.) Taub |
| | | Botryodiplodia theobromae Pat. |
| | Ring rot | Pythium ultimum Trow |

*Table 1.1 (Cont'd)*

| CROP | DISEASE | PATHOGENS |
|---|---|---|
| Cassava | Dry rot | Rhizopus sp. |
| | Soft rot | Bacillus sp. |
| | Wet rot | Fomes sp. |
| Yam | Tuber or soft rot | Aspergillus niger Van Tiegh., Botryodiplodia theobromae Pat., Penicillium sp., Rhizopus sp., Fusarium sp., Rosellinia bunodes (Berk & Br.), Glomerella cingulata, Cercoseptoria sp., Phaeoramularia sp., Pseudocercospora sp., Gibberella Fujikuroi var. subglutinans Edwards |

[Coursey and Booth, 1972; Eckert, 1977; Adeniji, 1970; Bammi, et al, 1972; Baudin, 1956; Ogundana, et al, 1970].

The dynamic innovation in the areas of mechanical harvesting, consumer packaging, bulk handling and transportation of yam tubers tend to intensify post-harvest disease problems. These occur either by increasing the opportunity for wound infection or by creating an environment favourable for disease development. Mechanical harvesting and slicing of yam tubers for planting has been reported to result to high frequency of yam rot unless urgent steps are taken within 24 hours after harvest to avoid the growth of pathogens on the open yam surface which cause decay (Okafor, 1966; Eckert, 1977).

### 1.1.1 Development in Post-Harvest disease control of Yam tubers

Many methods have been used in the attempt to control yam tuber rot caused by microbial infections. Among these methods are the use of dust and wood ashes, lime and borax as yam preservatives. None of these has proved to be exactly effective. The thiabendazole and benomyl introduced in the early 1960s failed to arrest the yam-rot problems, because of their narrow spectrum of anti-fungal activity (Bammi, et al, 1972). The tuber rot and soft rot of yam by Botryodiplodia theobromae, Aspergillus niger, Rhizopus stolonifer and Glomerella cingulata and bacterial soft rot are all examples of yam diseases which have not been reported effectively controlled by treatment with antimicrobial agents (Eckert, 1977).

An indication of effectiveness of some organotin compounds in preventing yam rot was first reported by Bonire (1985) and Olurinola and co-workers (1992). Thus, it appears that some organotins may have good potential for prevention of yam rot due to fungal pathogens.

## 1.2 REVIEW OF LITERATURE OF HIGHER TRIORGANOTIN CARBOXYLATES

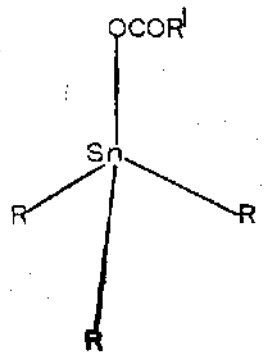
The first systematic synthesis of organotin biocidal compounds has been credited to Sir Edward Frankland (1825-1899) by Blunden and Evans (1982). Organotin carboxylates remained no more than laboratory curiosities for nearly 100 years, since the findings then, did not reveal any obvious commercial applications.

The search for novel antimicrobial organotins continued throughout the early part of twentieth century, until 1925 when the first commercial application of an organotin was reported as a moth proofing agent (Nicklin

and Robson, 1988). In the early 1950s, attention was focused on more generalised commercial applications of organotin carboxylates as pesticides most notably following the systematic biocidal investigation of Van der Kerk at Utrecht in 1954. As a consequence of Van der Kerk biocidal investigation (1954), the annual world consumption of organotins has grown rapidly from 500 tons per year in the early 1950s to about 50,000 tons in the late 1980s (Blunden and Chapman, 1986). The success recorded in triorganotins control of crops pest infection of leaves prompts investigation into effect of triorganotin carboxylates on fungal isolates - a biological causative agent of yam rot.

### 1.2.1 General Outline of Higher Triorganotin Carboxylates Chemistry

Triorganotin carboxylates are derivatives of tin (IV), an element of atomic number 50 in the Group IVA of the periodic table. These organotins often contain one tin atom per molecule. In most cases, a central tin atom is present which is basically tetrahedrally surrounded by four substituents. The basic structure of triorganotin carboxylates is as follows:




TRIORGANOTIN CARBOXYLATE.

R, $R^1$ = alkyl, arakyl or aryl substituent

In one and the same compound, the group R may be the same (symmetrical compounds) or different, referred to as unsymmetrical (Sijpesteijn, et al, 1969).

The tin-carbon bond in these compounds is generally stable against oxidation by air at room temperature. Ultra-violet irradiation (290 nm) however, causes a gradual breakdown of triorganotin carboxylates (Blunden and Chapman, 1986) by breaking the Sn-C bond.

### 1.2.2 Methods of Synthesis of Organotin Carboxylates

The two most common methods employed for the synthesis of triorganotin carboxylates utilise the corresponding oxide or chloride as starting materials. Reaction of carboxylic acids with organotin oxides (or hydroxides) in many cases, gives the organotin carboxylate.

Equation 1

$$R_3SnOSnR_3 + 2R'CO_2H \xrightarrow{PhME} 2R_3SnOCOR' + H_2O$$

Equation 2

$$R_3SnOH + R'CO_2H \xrightarrow{PhME} R_3SnOCOR' + H_2O$$

Reaction of triorganotin chlorides with carboxylates of alkali metals or heavy metals in suitable solvents also give organotic carboxylates.

Equation 3

$$R_3SnCl + MOCOR' \longrightarrow R_3SnOCOR' + MCl$$

### 1.2.3 Some Physical Properties and Solubility of Higher Triorganotin Carboxylates

Triorganotin carboxylates such as triphenyltin acetate are usually white solids. Triphenyltin acetate is thermally stable to about 122$^0$C, above which this stability break down completely. Most of these organotin carboxylates are usually oxidised slowly by air in the presence of UV light (Davies and Smith, 1982). The melting points of some common triorganotin esters are as listed in Table 1.2

TABLE 1.2: MELTING POINTS OF SOME COMMON TRIORGANOTIN CARBOXYLATES ($R_3SnOCOR^1$)

| COMPOUND | MELTING POINT $^0$C |
|---|---|
| $Bu_3SnOCHO$ | 120 - 125.7 |
| $Bu_2SnOCO$ Me | 85 |
| $Bu_2SnOCO$ Ph | 166 - 168 |
| $Ph_2SnOCO$ Me | 121 - 122 |
| $Ph_2SnOCO$ Ph | 84 - 85.5 |
| $Bu_2SnOCO\ (CH_2)_4\ CO_2SnBu_3$ | 105 |

[Davies and Smith, 1982].

The triorganostannyl esters are generally of a low solubility in organic solvents because of their polymeric associated structure. However, aliphatic organotin carboxylates are more soluble in organic solvent such as acetone than the aryl derivatives (Davies and Smith, 1982).

Dilution of the associated triorganotin carboxylates in organic solvents usually produces oligomeric and finally, monomeric species (Davies and Smith, 1982). In water, triorganotin carboxylates are known to form hydrated cations of the type $[(R_3SnCH_2O)_2]^+$, in which the anionic group is no longer directly bound to the tin atom (Blunden, et al, 1984). Thus, in both of these situations, the biological activity of triorganotin carboxylates in water should be independent of the anionic radical.

It has been reported by Ascher (1976) as cited by Blunden and co-worker (1984) that the toxicity of fentin acetate in acetone to houseflies was drastically reduced when compared with that in water. This phenomenon was attributed to the polymeric structure of triphenyltin acetate - a triorganotin carboxylate in acetone as against its monomeric half ionized structure in water. This monomeric structure is known to form biological active hydrate cation in water.

In general, the aqueous solubility of trialkyltin carboxylates decreases with increase in alkyl chain length or molecular weight as shown in Table 1.3.

TABLE 1.3: AQUEOUS SOLUBILITIES OF TRIORGANOTIN STANNYL ESTERS AT ROOM TEMPERATURE

| COMPOUNDS | SOLUBILITY ($gm/dm^3$) |
|---|---|
| $[C_2H_5]_3$ SnOCOCH$_3$ | 7500.0 |
| $[C_4H_9]_3$ SnOCOCH$_3$ | 16.0 |
| $[C_6H_5]_3$ SnOCOCH$_3$ | <3.3 + |
| $[C_4H_9]_3$ Sn $SO_3$ $C_2H_5$ | 29,000.0 + |
| $[C_6H_5]_3$ Sn $SO_3$ $C_2H_5$ | 1000.0 + |

Solubility values marked + refer to distilled water; unmarked refer to sea water.

[Blunden and Chapman, 1986].

### 1.2.4 Toxicity of the Higher Triorganotin Carboxylates

The increasing interest in the toxicological properties of triorganotins compounds is primarily due to the growing practical applications of these substances. The interest was increased by the discovery of their biocidal properties.

Toxicity of a compound is the basis of its chemotherapeutic and other biocidal applications. Generally, tin in its inorganic state is relatively non-toxic, the oxides are unreactive and the metal is essentially non-ionized at physiological pH (WHO, 1980). The addition of one or more organic groups to the tin atom however, has a profound effect in terms of overall biological activity.

In general, the biological activity within each class of the triorganotins is related to the number of carbon atoms per chain (Fig. 1.1) or its molecular weight.

Variation of the anionic radical x within any given $R_3SnX$ series is usually found to have no significant effect on the overall bioactivity of the molecule, unless, of course X is itself biologically active. In this case, the bioactivity of the compound may be enhanced. It has recently been shown, however, that in complexes where the x group results in the formation of polymeric structures or a five coordinate chelated monomer as in tributyltin (IV) dialkylamino-alkoxide, a significant reduction in activity may occur (Blunden, et al, 1984).

The literature on toxicity of triorganotins to mammals is extensive. The findings in this field of study is difficult to compare because of the great differences in the design of the experiments. These differences relate to the substances studied, their purity, form of

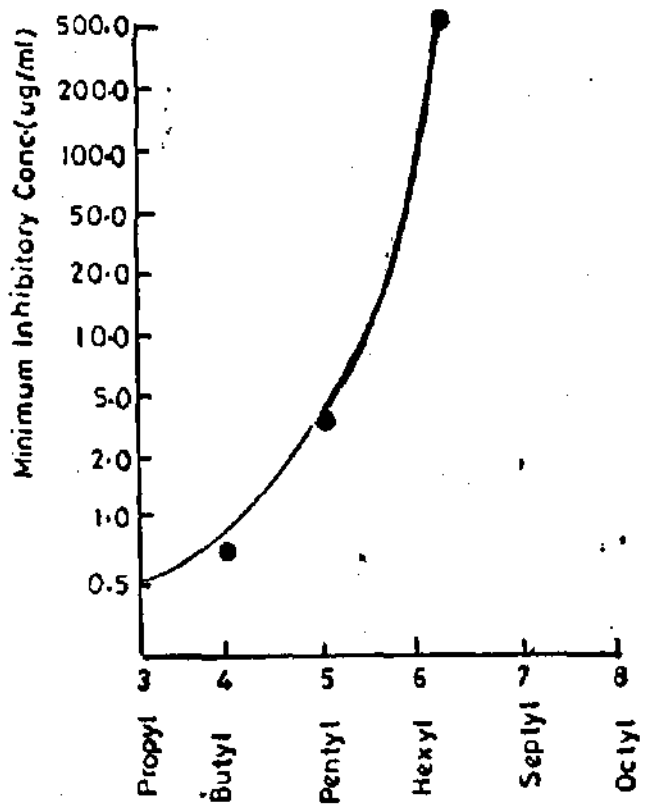



Fig. 1.1: Influence of Carbon chain length of trialkyltins on MIC to Aspergillus sp.

dosage, the mode of administration and the species used. In addition, there are differences in the number of animals employed per experiment and the period of observations (Blunden and Evans, 1982). However, the commonly used animal model is the laboratory rat, although some studies have also reported the use of rabbits, mice, guinea-pigs and dogs occasionally (Table 1.4).

The toxicity parameter commonly reported in the afore- mentioned studies is $LD_{50}$. This $LD_{50}$ values give no indication of mechanisms of action of the triorganotins. Many workers in the field of toxicological study tend to abandon the $LD_{50}$ approach in favour of more reasoned analytical method. To this end, the minimal effective and no-observed-effect doses of test compounds appear to be more reported than $LD_{50}$ (Barnes and Stoner, 1958; Blunden and Evans, 1982).

TABLE 1.4: ACUTE ORAL $LD_{50}$ VALUES FOR SOME TRIORGANOTIN COMPOUNDS

| COMPOUND | $LD_{50}$ mg/kg | TEST ANIMAL |
|---|---|---|
| Tributyltin acetate | 125-380.2 | r |
| Triphenyltin acetate | 125-491.0 | r |
| Triphenyltin chloride | 118-135.0 | r |
| 1-Tricyclohexylstannyl - 1, 2; 3 triazole | 631.0 | r |
| Trioctyltin-S, S$^{1}$-bis [1SO-octylthio glycollate] | 1200-2100.0 | r |
| Dioctyltin maleate | 4500.0 | r |

[Smith, 1978] r = rat

It has been reported that the no-observed-effect dose of triphenyltins used for two years chronic toxicities study on SPF-Wistar-K rats, dogs and guinea-pigs were 25 ug/kg, 2 mg/kg and 5 mg/kg respectively. Generally, the doses showed no adverse effect on general health, fertility or post-natal development of test animals in the generation examined (Blunden and Evans, 1982).

Although it is always possible to establish a no- observable-effect limit, more subtle changes may occur even in the absence of growth retardation or overt pathological changes. This is particularly relevant with respect to the immune system, where modern approaches can demonstrate treatment-induced changes in the level of immunological important molecules within hours of exposure.

It is important to note that liver microsomal monooxygenase enzyme has been reported to metabolise triorganotins via carbon hydroxylation of the organic groups (Kimmel, et al, 1977; Fish, et al, 1976). Furthermore, it has been reported that none of the tested higher triorganotins in the field trials display any carcinogenic effects (Blunden and Evans, 1982; Wester, et al, 1990). This finding appears to support the report of Schwienfurth (1987) that genotoxic tests of higher triorganotins in micro-organisms and in the micronucleus of mice did not indicate any mutagenic or carcinogenic potential. These observations of lack of mutagenicity or carcinogenicity in addition to transient nature of triorganotins in mammals appear to encourage the applications of these compounds as industrial and agrochemical compounds.

### 1.2.5 Industrial Uses of Higher Triorganotin Carboxylates

One of the features of organotin compounds is the diversity of applications which exist for its derivatives. There are now more organometallic compounds of tin in commercial use than for any other element, covering a spectrum of fields of which the biologically related applications represent only a part (Molloy, 1989). Indeed, non-biological applications - primarily in tonnage terms, e.g. PVC stabilizers, catalyst in polymer chemistry, biologically active agents for a wide variety of applications and precursors for forming $SnO_2$ film on glass - consume the majority of the yearly output of organotin chemicals.

With regards to the chemotherapeutic applications, there are some organotin compounds that show therapeutical potential use, such as the application of tin-protoporphyrin for the treatment of neo-natal jaudice. Neonatal jaudice is a common and sometimes severe condition which may produce neurotoxic effects. Tin protoporphyrin has been reported to inhibit heme oxidase enzyme in the liver, spleen and kidney lowering significantly the bilirubin level in the serum.

$$\text{Heme} \xrightarrow[\text{oxidase}]{\text{heme}} \text{biliverdin} \xrightarrow[\text{reductase}]{\text{biliverdin}} \text{biliverdin}$$

(Drummond and Kappas, 1981).

Increase in concentration of tryptophan and serotonin in animal brain are associated with hepatic encephalopathy and migraine headaches. These conditions have been reported to be alleviated with the administration of Sn-heme (Rideout, et al, 1987).

One of the major developments in the field of bioorganotin chemistry in the 1980s is the finding that organotins such as $Bu_2SnCl_2$.bipy,

$Bu_2SnCl_2$phen, $Bu_2$Sn-histidine play an important role in anticarcinogenesis. The observation made by Brown (1972) that prolong administration of low doses of hydrolysable triphenyltin acetate in food retarded tumour growth in rodents has stimulated research into the anticarcinogenic activity of organotin compounds. These studies have led to the suggestion that ingested organotin compounds is biochemically converted to anticarcinogenic organotin entities in animals (Cardarelli, et al, 1983; 1984a, 1984b; Cardarelli and Kanakkanatt, 1985; Cardarelli, 1985; Cardarelli and Kannakkanatt, 1987; Sherman, et al, 1987). Although the reported anti-tumour activity of bio-organotin adduct is lower than Cis-platin, organotins show less nephrotoxicity when compared with that of Cis-platin (Molloy, 1989). These potential therapeutic values of organotin derivatives have stirred up research on biological activities of these important organometallic compounds for man survival and the subsequent applications in pharmaceutical, textiles and ship industries as summarised in Tables 1.5, 1.6 and 1.7.

## 1.3 AGRICULTURAL USES OF HIGHER TRIOGRANOTINS

It is estimated that 33% of the world food production is lost to pests and bad weather conditions each year, causing damage in the region of $100 billion. The risk of damage is increased by modern farming techniques which devote large areas of agricultural land for the production of a single crop, making the control of insects and disease a necessary practice (ITRI, 1972). The current approach to this is based on application of chemical agents to the infected habitat, which are toxic to the unwanted organisms. These chemicals are important tools in

modern crop protection and the search for suitable ones has been primarily directed towards finding those which are highly efficient toxicants.

Many organotin compounds have been reported to possess effective biocidal activity amongst which are triphenyltin

TABLE 1.5: PRINCIPAL INDUSTRIAL USES OF ORGANOTIN COMPOUNDS

| APPLICATIONS | FUNCTIONS | PRINCIPAL COMPOUNDS USED |
|---|---|---|
| Heat Stabilizers | (a) Transformer, capacitor and cable stabilizers. ylthioglycollate | Tetraphenyltin. |
| | (b) Anti-oxidant and anti-cracking agents related for rigid PVC, and for= chlorinated rubber, polyvinylidene halides, chlorinated paraffins, cellulose acetate, polyamides, polycarbonates and polyolefins deterioration due to effect of heat and light. | 1. Dialkyltin di-iso-octylthioglycollate (alkyl methyl, butyl,octyl)<br>2. Dialkyltin maleate (Alkyl = methyl, butyl and octyl).<br>3. Mono-alkyltin tri-iso-octylthioglycollate (alkyl = methyl, butyl octyl and 2-butoxy-carbonylethyl).<br>4. Dibutyltin bis(Lauryl-mercaptide).<br>5.Dibutyltin-S-S-bis(iso octylmercaptoacetate).<br>6.Dibutyltin-B-mercapto-propionate.<br>7. Dioctyltin maleate.<br>8. Dioctyltin-S, S-bis (iso-octylmercapto-acetate). |
| Catalysts | 1. Curing agents for expoxy resins and RTU | Dibutyltin dioctanoate |
| | Silicone rubber meant for dental impression, encapsulating electronic parts. | Dibutyltin dialaurate |

*Table 1.5 (Cont'd)*

| APPLICATIONS | FUNCTIONS | PRINCIPAL COMPOUNDS USED |
|---|---|---|
| | 2. Homogenous catalyst which control pore size of polyurethane foam production (Urethanes-foam elastomers). | Stannaous octoate, Dibut-ltin diacetate, Diethyl-tin dilaurate, Dibutyltin bis(laurylmercaptide), Dimethyltin dichloride. |
| | 3i Esterification-trans-esterification poly-esterification. | Butanestannonic acid, Dibutyltin diacetate, Dibutyltin oxide. |
| | ii Polymerization of Olefins. | Dibutyltin dichloride. |
| Material Protection | - Wood preservations | Bis(tributyltin) oxide |
| | - Anti-oxidant for textile oils | Tributyltin naphthenate |
| | - Additive to polysiloxanes | Dimethyltin dichloride |
| | - Additive to improve water repellency of fibrous materials and to make them resistant to mildew biodegradation | Butyltin trichloride<br>Methyltin trichloride<br>Tributyltin phosphate |
| | - Corrosion inhibitors in organosilicon polymers | Tributyltin linoleate<br>Tributyltin oxide |
| | - Confer protection against biodegradation of paint, paper, textiles, leather materials, plastic, decorative stones, manila and sisal ropes, jute and jute bags. | |
| | - Precursor for tin IV oxide film on glass. | |

TABLE 1.6: PRINCIPAL INDUSTRIAL BIOCIDAL USES OF ORGANOTIN COMPOUNDS

| APPLICATIONS | FUNCTIONS | PRINCIPAL COMPOUNDS USED |
|---|---|---|
| General Biocides | a. Industrial water treatment | Bis(tributyltin) oxide |
| | i. Paper mills | Triphenyltin hydroxide |
| | ii. Cooling waters | Tributyltin benzoate |
| | iii. Secondary oil recovery | Triphenyltin chloride |
| | b. Antifoulants in ship paints and under water coatings effective against marine organisms such as barnacles, molluscs and limnoria | |

[Molloy, 1989; Nicklin and Robson, 1988; Blunden and Evans, 1982; Ross, 1965].

TABLE 1.7: POTENTIAL PHARMACEUTICAL USES OF TIN COMPOUNDS

| APPLICATIONS | FUNCTIONS | PRINCIPAL COMPOUNDS USED |
|---|---|---|
| Pharmaceuticals | - Disinfectant | Tributyltin benzoate plus QACs |
| | - anti-helmintics in poultry | Dibutyltin dilaurate, Tetra-isobutyltin |
| | - Radiopharmaceuticals | 99m Tc/Sn-dimercaptosuccinic acid |
| | - Neonatal Jaudice | Tin-protoporphyrin |
| | - Anti-carcinogenesis | Dibutyltin bipy,Dibutyltin-histidine, Dibutyltin-chloride-phen |
| | - Biocides against Leishmaniasis | Dioctyltin maleate |
| | - Ameobicides | Tributyltin(2-Fuourobenzaldehyde-s-benzyldithio carbazate) |
| | - Anti-inflamatory | Butyltin trichloride,Triphenyltin chloride |
| | - Photodynamic therapy of cancer | Dichlorotin-purpurin |
| | - Mosquito larvicides | Tributyltin fluoride, Tributyltin sucrose, Phthalate |
| | - Hypertension in rat | Tin II chloride |

[Crowe, 1987a; 1987b; Molloy, 1989; Nicklin and Robson, 1988].

acetate (Fentin acetate-brestan), triphenyltin chloride (Brestanol-Hoechst), triphenyltin hydroxide (Duter-Philips Duphar), tricyclohexyltin hydroxide (Plictran-Dow Chemical Co.), bis[tris(2-methyl-2 phenyl-propy)tin] oxide (Vendex or Torqu -Shell) and 1- tricyclohexylstannyl-1,2,4 triazole (Peropal-Bayer AG) have been developed and used as agrochemicals (Nichlins and Robson, 1988). The usefulness of these organotins in agricultural practice are shown in Tables 1.8, 1.9 and 1.10.

TABLE 1.8: PRINCIPAL USES OF ORGANOTIN COMPOUNDS AS PESTICIDES

| APPLICATIONS | INSECT PESTS | PRINCIPAL ORGANOTINS USED |
|---|---|---|
| Antifeedants | - Chrotogonus trachypterus(surface grasshopper), Diacrisio obliqua, Epilacha vigintioctopunctata, Papilio demoleus and Plusia pepanis and Tribolium confusum | Triphenyltin acetate and Triphenyltin hydroxide |
| | - Diacrisia obliqua, Leptinotarsa decemlineata (potato pest), Tribolium confusum | Triphenyltin chloride |
| | - Porthetria dispar (Gypsy moth) | Tricyclohexyltin hydroxide |
| | - Spodoptera littoralis (cotton leaf worm) | Tricyclohexyltin-1,2,4-triazole |
| Chemosterilants | - Tetranychus urticae, Musca domestica and Ceratitis capitata (Mediterranean fruit fly) | Triphenyltin acetate and Triphenyltin hydroxide |
| | - Cotton boll worm and Tetranychus urticae | Tricyclohexyltin hydroxide |
| Insecticides | - Chrotogonous trachypterus (surface grasshopper), Callosobruchus chinensis (stored product pest) Ceratitus capitata, Keiferia lycopersicella (tomato pin worm) | Triphenyltin chloride and Triphenyltin hydroxide |
| | - Aphrastacia pectinatae, Cholodovskya viridana (insect pests of Canadian spruce), Carpocapsa sp. (codling moth), Heliothis sp., Prodenia sp., Mindarus abietinus (Balsam twig aphid) | Tricyclohexyltin hydroxide |

[Blunden and Evans, 1982; Blunden, et al, 1984; Anon., 1980].

TABLE 1.9: PRINCIPAL ACARICIDAL AGRICULTURAL USES OF ORGANOTIN COMPOUNDS

| APPLICATIONS | CROP | DISEASE/CAUSATIVE ORGANISMS | PRINCIPAL COMPOUNDS USED |
|---|---|---|---|
| Acaricides | - Apple | Aculus schlectendali, Panonychus ulmi, Tetranychus urticae, Tetranychus viennensis and Brown rot disease | 1. Tricyclohexyltin hydroxide (cyhexatin) 2. Bis (trineophyltin) oxide (Fenbutan oxide 3. Tricyclohyxyltin-1,2, 4-triazole (peropal) |
| | - Egg Plant | Tetranychus gloveri | Cyhexatin, Fenbutan oxide |
| | - Straw berries | Tetranychus urticae, Tarsonemus pallidus | Cyhexatin, Fenbutan oxide |
| | - Soya bean | Tetranychus arabicus | Fenbutan oxide, peropal |
| | - Stored | Acarus siro, Glycyphagus destructor, Tyrophagus longior, Tyrophagus putrescentiae, Tetranychus urticae | Fenbutan oxide |
| | - Orange | Phyllocoptruta oleivora | Cyhexatin, Fenbutan oxide |
| | - Tea | Acaphylla theae | Cyhexatin |
| | - Vine | Tetranychus urticae | Triphenyltin, Fenbutan oxide |
| Biocides | - Black pepper | Phytophtora palmivora | Triphenyltin acetate, Triphenyltin hydroxide |
| | - Cacao | Cacao moniliasis, phytophthora palmivora | Triphenyltin acetate, Triphenlytin hydroxide |
| | - Cowpea | 1. Web blight by Rhizoctonia solani 2. Early season disease of pod rot by Choanephora sp. | Triphenyltin acetate, Triphenyltin hydroxide |
| | - Maize | 1. Sugar-can downy mildew by Sclerospora sacchari 2. Banded sclerotial disease by Rhizoctonia solani | Triphenyltin chloride Triphenyltin acetate |

[Molloy, 1989; Blunden and Evans, 1982; Blunden, et al, 1984; Anon., 1980].

TABLE 1.10: PRINCIPAL BIOCIDAL AGRICULTURAL USES OF ORGANOTIN COMPOUNDS

| APPLICATIONS | CROP | DISEASE/CAUSATIVE ORGANISMS | PRINCIPAL COMPOUNDS USED |
|---|---|---|---|
| Biocides | Carrot | Foliar disease by Cercospora carrotae and Alternaria dauci | Triphenyltins |
| | Onion | 1. Downy mildew by Peronospora destructor<br>2. Purple blotch of leafe by Cladosporium allii-cepae and Alternaria porri | Triphenyltins |
| | Rice | Sheath blight disease by Rhizoctonia solani and Sarocladium oryzae | Triphenyltins |
| | Sunflower | Leaf spot by Alternaria helianthicola, Alternaria Carthiami; Phoma macdonaldi, Phomopsis sp. | Triphenyltins |
| | Sugarbeet | 1. Damping-off disease by Rhizoctonia solani<br>2. Crown rot by Phythium aphanidermatum, Phoma betae<br>3. Leaf spot by Cercospora beticola | Triphenyltins |
| | Peanut | Early and late spot by Cercospora arachidicola, Cercosporium personatum | Tricyclohexyltin hydroxide (cyhexatin) |
| | Soyabean | 1. Pod and stem blight by Diaporthe phasedorum<br>2. Anthracnose by Collectotrichum dematium, Glomeralla glycines<br>3. Leaf blight by Cercospora kikuchi | Triphenyltins |
| | Tomato | Late blight by Phytophthora infestans | Triphenyltins |
| | Wheat | a. Leaf rust by Puccinia arachidis<br>b. Spot blotch by Helminthosporium sativum<br>c. Karnal burnt by Neovossia indica<br>d. Soil-borne fungi infection by Rhizoctonia solani, Fusarium oxysporium, Fusarium lycopersici, Pythium sp., Stemphylium solani | Triphenyltins |
| | Winged bean | a. False rust by Snychytrium psophocarpi and<br>b. Soil borne fungi Macrophoma mangifera | Triphenyltins |
| | Peach | Black spot (apple scab) and powdery mildew | Cyhexatin |
| | Groundnut | Wilt by Sclerotium rolfsii and rust by Puccinia arachidis | Triphenyltins |

[Molloy, 1989; Blunden and Evans, 1982; Blunden, et al, 1984; Anon., 1980].

Following lengthy chronic toxicity testing, the World Health organization pronounced triphenyltin compounds and trichclohexyltins as 'safe' agricultural chemicals and fixed an acceptable daily intake for man at 0.005 mg/kg and 0.007 mg/kg respectively (Blunden and Evans, 1982).

Organotins as plants protection agents offer a number of advantages over conventional ones, especially those based on mercury, copper or arsenic. These latter elements are permanently toxic, their residues remaining behind on the crops and repeated spraying is now raising concern over their accumulation within the environment and food chains. With organotin compounds, toxic residues are considered to be less of a problem, since the toxicity of the compounds resides in the $R_3Sn$ radical. This is gradually broken down by U.V. light and micro-organisms degradation to inert $SnO_2$ (WHO, 1980), as shown below.

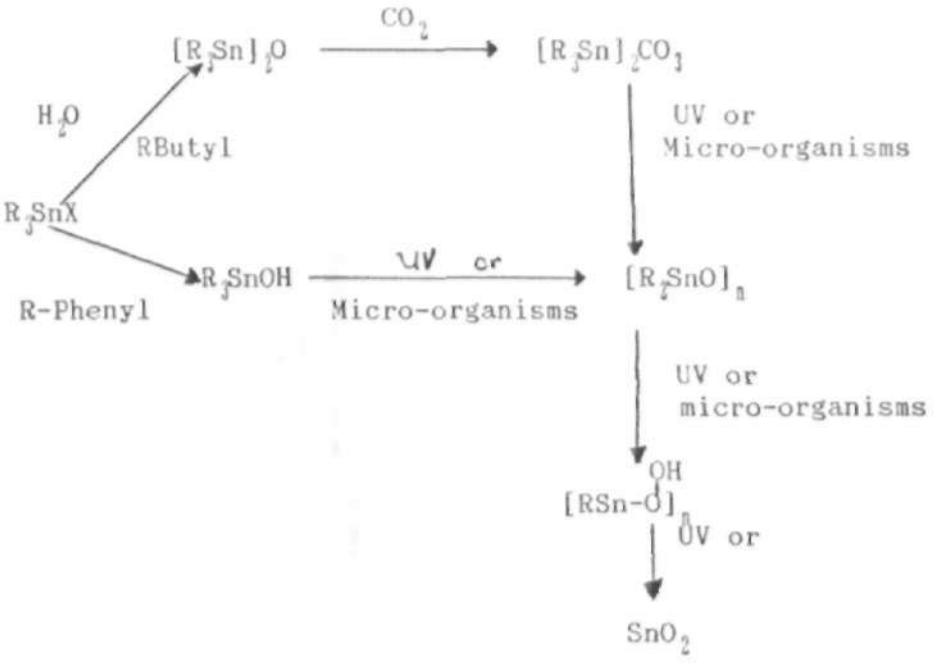



FIG. 1.2: Environmental Degradation scheme for Tributyltin and Triphenyltin Compounds (Sheldon, 1975)

For example, it has been reported that maximum residue concentration of triphenyltins in potatoes and carrots rarely exceeded 0.1 mg/kg (Blunden and Evans, 1982). Residues in food, fruit and vegetables derived from direct contact with organotin compounds could be considerably reduced by washing. Residues of tricyclohexyltin hydroxides on apples and pears decline by 50% in about three weeks due to photodegradation. A 20-50% reduction can also be achieved by washing and most of the residues can be removed by peeling the fruits after which only 0.1 mg/kg (as tin) may be expected in the fruit flesh. Thus, once the inedible sprayed portions are stripped away and the foodstuff cleaned and cooked, there is very little or no risk for human consumption of crops products treated with these higher triorganotin compounds (Table 1.11).

TABLE 1.11: WORLD HEALTH ORGANISATION RECOMMENDATION FOR USE OF TRIPHENYLTIN COMPOUNDS TO PROTECT CROPS

| CROP | DISEASE | RECOMMENDED DOSAGE (kg a.i/ha) | NO.OF APPLI-CATION | SAFETY PERIOD (DAYS AFTER APPLI-CATION | RECOMMENDED TOLERANCE (ppm) |
|---|---|---|---|---|---|
| Potatoes | Phytophtora infestans of foliage and tubers and Alternaria solani | 0.18-0.40 | 1-10 | 7 | 0.1 |
| Rice | Mycospherella corvigen and various algae | 0.75 - 1.00 (in 1000 litre water) | - | - | - |
| Sugar beet | Cercospora beticola | 0.18 - 0.40 | 1-3 | 14 | 0.2 |
| Celery and Celeriac | Septoria apii | 0.20 - 0.32 (in 600-1000 litre water) | 1-7 | 21 | Celery 1.0 Celeriac 0.1 |
| Carrot | Alternaria porri | 0.20 - 0.32 (in 600-1000 litre water) | 1-7 | 7 | 0.2 |
| Groundnut | Cercospora sp. | 0.2 - 0.6 (in >10000 litre water) | - | 7 | 0.05 |
| Coffee | Colletotriclum coffeanum | | | | |
| Cocoa | Phytophthora palmivora | 0.2 - 0.6 (in 1000 litre water) | - | - | - |

[Blunden and Evans, 1982].

## 1.4 THE RESEARCH OBJECTIVES

The cultivated yam (Dioscorea species) is a staple food in Nigeria and in many tropical countries of the world. Nevertheless, it has remained a subsistence crop largely due to biological and technical impediments to its commercialization. One of such impediments is the huge loss (up to 40% in Nigeria and 15% in West Africa) in storage, which adversely affects the availability of not only ware yams (for food) but seed yams as well (Bonire, 1985; Rawnsley, 1989).

Yam losses during storage are due to pathological factors and a little to endogenous physiological and mechanical factors. Among the pathological factors, decay by fungal pathogens such as Fusarium oxysporium, Rhizopus sp., Penicillium sp., Aspergillus niger, Botryodiplodia theobromae, Rosellina bunodes, Glomerella cingulata, Cercoseptoria sp.,Gibberella fujikuroi var. subglutinans have been found to account for the greatest losses of ware yam tubers (Adeniji, 1970; Agarawal and Gupta, 1973; Bammi, et al, 1972; Baudin, 1956; Coursey, 1961; 1967; and 1984; Eckert, 1977; Okafor, 1966; Ogundana, et al, 1970). Besides, a significant proportion of seed yam are lost to fungal decay after planting, leading to either high cost in replanting or 'gappy field'.

A number of previous efforts, including use of dust and wood ashes, lime and borax, thiabendazole and benomyl as yam preservatives have proved unsatisfactory (Bonire, 1985; Coursey, 1961; Spencer, 1977). Reports of resistance development by the pathogenic organisms to these fungicides are commonly found in literature. Consequently, in order to delay emergence of resistant fungi, combination of fungicides are commonly formulated, e.g. 75% carboxin and 80% thiram gives vitavax-200 admixtures (Eckert, 1977). Although the use of antifungal agents admixture is accepted almost unequivocally, there is a need to scientifically justify the rationale behind the formulation of any antifungal admixture.

Some higher triorganotins compounds have been reported to enjoy immense popularity in the temperate countries as disease - control agrochemicals on crops other than yam (Anon., 1980; Blunden and Evans, 1982; Blunden, et al, 1984; Crowe, 1987; Jones, et al, 1965; Molloy, 1989; Pieters, et al, 1962; Stallknecht, et al, 1968; Thayer, 1984). This study,

therefore, aims at examining the antifungal activity of some selected higher triorganotin carboxylates such as triphenyltin acetate, triphenyltin salicylate and tributyltin acetate, using acetone and polysorbate 80 as solvent systems against some causative agents of yam rot, namely Aspergillus niger, Aspergillus flavus, Rhizopus stolonifer and Penicillium citrinum, all of which were isolated from rotten yams. This study also aims at examining other alternative vehicle to acetone such as propylene glycol and to determine the effect it will have when added to test triorganotins. Furthermore, the viability of the fungal spores suspension in triorganotins alone and in admixtures with propylene glycol will be determined. It is expected that in-vivo experiment would shed more light on the potential use of these agents as seed yam preservatives.

# CHAPTER TWO

# MATERIALS AND GENERAL METHODS

## 2.1 CULTURE AND SUSPENDING MEDIA

### 2.1.1 Sabouraud Dextrose Agar: (UNIPATH LTD., Basingstoke, Hampshire, England)

Sabouraud dextrose agar (oxoid, CM 41) was prepared by dissolving 65 gm of powder in distilled water to produce a litre and heated gently to dissolve the agar completely. The medium was then distributed in smaller quantities into universal bottles with screw caps. These were then autoclaved at 121$^0$C for 15 minutes. The pH of the sterile medium was 5.4-5.8. After sterilization, some of the sabouraud dextrose agar (SDA) in bottles were slanted and allowed to set firmly to give agar slopes.

### 2.1.2 Sabouraud Liquid Medium: (UNIPATH LTD., Basingstoke, Hampshire, England)

This medium was prepared by dissolving 30 gm powder (oxoid CM 147) in one litre of distilled water, distributed into the final containers and sterilised at 121$^0$C for 15 minutes (pH = 5.7).

### 2.1.3 Recovery Lecithin-Polysorbate 80 Medium (RLPM)

This recovery medium was used for inactivating triphenyltin acetate (TPTA), triphenyltin salicylate (TPTS) and tributyltin acetate (TBTA) in order to recover surviving spores during minimum fungicidal concentration determination (Kohn, et al, 1963). The medium consists of the followings:

| | |
|---|---|
| Egg Lecithin | 0.5% w/v |
| Polysorbate 80 | 3.0% w/v |
| Yeast Extract | 0.5% w/v |
| Sabouraud Liquid Medium to | 100.0 ml |

The medium was distributed into small quantities and autoclaved at 121°C for 15 minutes.

**2.1.4 Diluent Medium (DM)**

This medium was used for ten-fold dilutions when standardizing the test-fungi spores. The medium consists the following:

| | |
|---|---|
| Sodium Chloride | 0.9% w/v |
| Polysorbate 80 | 0.05% w/v |
| Distilled water to | 100.0 ml. |

The diluent was dispensed into small quantities [9 ml] and autoclaved at 121°C for 15 minutes. These were kept until when required.

**2.1.5 Lecithin-Polysorbate 80 Diluent (LPD)**

This medium was used for ten-fold dilution in order to recover fungal spores during test spores rate of kill determination.

The recovery diluent is made up of the followings:

| | |
|---|---|
| Egg Lecithin | 0.5% w/v |
| Polysorbate 80 | 3.0% w/v |
| Sodium Chloride | 0.9% w/v |
| Distilled Water to | 100.0 ml |

This diluent was distributed as required and autoclaved at 121°C for 15 minutes.

### 2.1.6 Acetone Diluent (25% v/v)

This was of laboratory reagent (May and Baker Limited, Dagenham, England) and 25% v/v solution was prepared by adding 250 ml of acetone in distilled water to produce a litre solution. This was sterilized by membrane filteration and aseptically distributed into sterile bottles with caps.

### 2.1.7 Polysorbate 80 (Tween 80)

The polysorbate 80 - a polyoxyethylene (20) sorbitan mono- oleate diluent - was of BDH laboratory reagent grade (BDH Chemical Ltd., Poole, England). A solution of 2.5% v/v was prepared by adding 25 ml of the chemical into distilled water to produce a litre solution. This was dispensed into suitable volumes in bottles with caps and sterilised by autoclaving at 121°C for 15 minutes.

## 2.2 TEST ANTIFUNGAL AGENTS

### 2.2.1 The Selected Triorganotins

The Triphenyltin acetate (TPTA) was obtained from Alfa Product Ltd. (Danvers, M.A., USA) and purified by recrystallisation in toluene. Triphenyltin salicylate (TPTS) or triphenyltin o-hydroxy benzoate and tributyltin acetate (TBTA) were synthesised in the Department of Chemistry, Ahmadu Bello University, Zaria by Dr. J.J. Bonire. These compounds were certified pure by means of infra red, nuclear magnetic resonance and mass spectroscopy, and by elemental analysis. They were stored in vacuo in the dark throughout in brown screw cap bottles.

The solution of these chemicals were prepared by weighing separately 1 gm of each of the compounds and dissolving each in 25 ml of sterile acetone. This was made up to 100 ml volume with sterile glass-distilled water. This was then serially diluted to obtain the varying concentrations of triogranotins used in this study.

### 2.2.2 Propylene Glycol

The propylene glycol - a propan-1,2-diol - was of BDH laboratory reagent grade (BDH Chemical Ltd., Poole, England).

Stock solution of propylene glycol was prepared aseptically in sterile glass-distilled water and stored in a refrigerator. Before use, all the solutions were tested for sterility by plating.

## 2.3 TEST-ORGANISMS

The organisms were sampled from rotten portion of white yam-Dioscorea rotundata clone "Cika kwando" purchased from Samaru market in Zaria, Nigeria. These samples were isolated through cultivation and sub-culturing with sabouraud dextrose agar. The fungi isolated from the rotten yam samples were Aspergillus flavus, Aspergillus niger, Rhizopus stolonifer, Penicillium citrinum, Fusarium sp., and Nodulisporium sp (Olurinola, et al, 1992). Inoculation of healthy yam with the isolated fungi spores resulted in pronounced rottening of the yam at the portal of infection.

The first four fungal isolates were identified to species level by Prof. A.M. Emechebe and Mr. M.H. Gummel of the Department of Crop Protection, Faculty of Agriculture, Ahmadu Bello University, Zaria and

referred to as test-fungal isolates. These were sub-cultured on sabouraud dextrose agar (SDA) in MacCartney bottles and stored at $4^{0}C$ until required for use in this study.

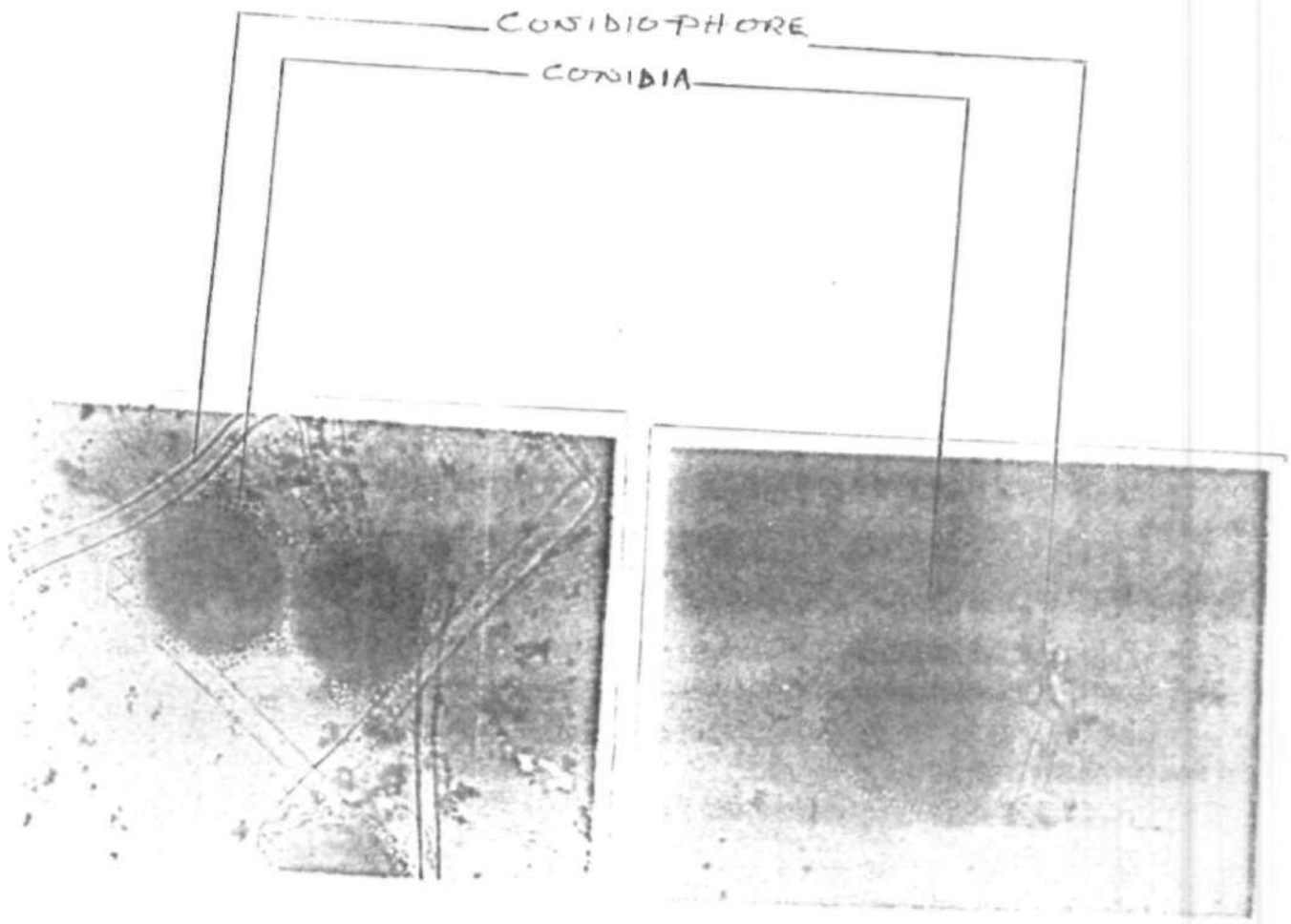
### 2.3.1 Aspergillus Species

Aspergillus is one of the best known genus in the fungus family of Eurotiaceae, class ascomycotina. Species of Aspergillus are abundant in soil, and also cause spoilage of food, crops and textiles (Webster, 1977). The Aspergillus species used in this investigation were authenticated by its colonial characteristics (Table 2.1) and microscopical morphology as shown below and also reported by Larone (1987).

TABLE 2.1: IDENTIFICATION CHARACTERISTICS OF ASPERGILLUS SPECIES

| Morphology | A. niger | A. flavus |
|---|---|---|
| Macroscopic morphology on sabouraud dextrose agar- | Wooly, at first white to yellow then turning black | Velvety, yellow to surface green |
| - Reverse side | White to yellow | Red brown |
| Microscopic morphology of condidiophores - length | long | Variable |
| - texture | smooth | Rough, pitted & spiny |
| Microscopic morphology of phialides | Biserate, cover entire vesicle, form radiate head | Uniserate & biserate, cover entire vesicle, point out in all directions. |

[Larone, 1987].




(AN)

(AF)

Figure 2.1: Microscopic morphology of Aspergillus niger [An] and Aspergillus flavus [Af]

2.3.2 Genus Penicillium

This is one of the most cosmopolitan genera of fungi, occuring whenever substrata and conditions are suitable for growth. It is abundant in soil and on all kinds of decaying materials.

In this study, P. citrinum was isolated from rotten white yam-Dioscorea rotundata with the following characteristic morphologies. This test-organism grew rapidly on sabouraud dextrose agar (SDA) with surface at first white, then becoming very powdery with bluish green pigmentation. The reverse side of the agar culture medium was white. Microscopically, the morphology was as shown below with "brush" appearance (Fig. 2.2).

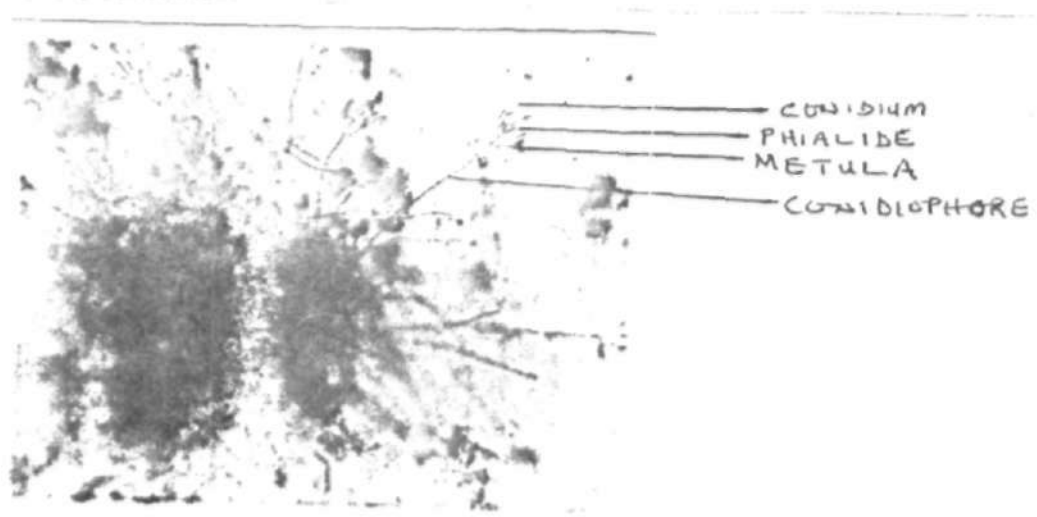



Figure 2.2: Microscopic morphology of Penicillum citrinum

2.3.3 Rhizopus stolonifer

This fungus thrive well in soil, dung and moist fresh organic matter in contact with soil. For the most part, it is a saprophyte and play an important role in the early colonisation of substrata in soil. Rhizopus stolonifer has been reported to cause rotting of sweet potatoes tuber, spoilage of bread and other food items (Webster, 1977). Rhizopus stolonifer possesses rapid growth in SDA and mature within four days. it quickly covers the agar surface with dense cotton candy-like mycelia. Initially, the mycerlia appears white which later turns gray with white reverse side. Microscopically, R. stolonifer is as shown below in Fig. 2.3.

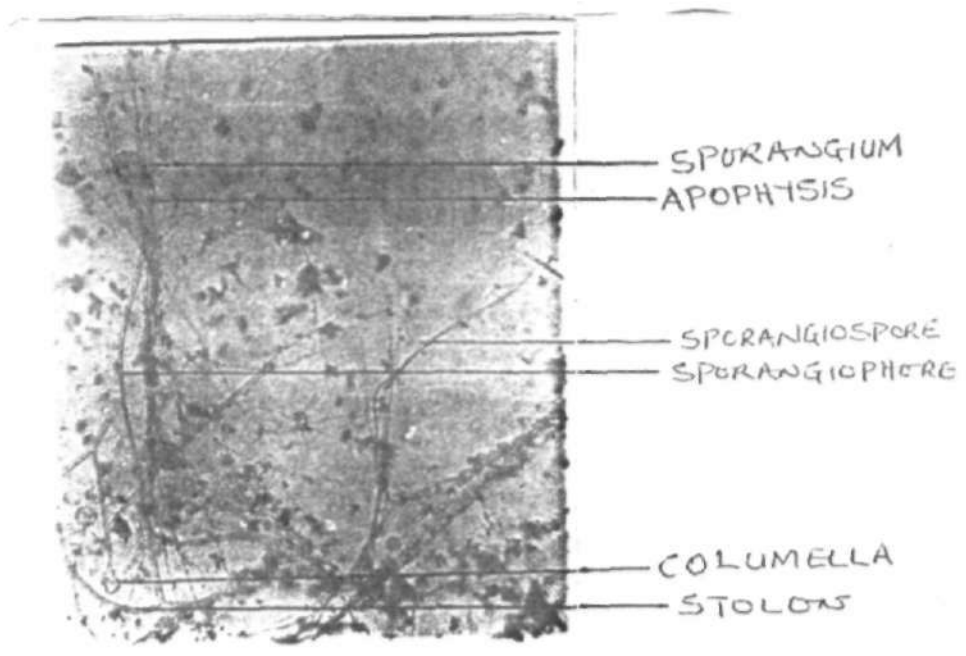



Figure 2.3: Microscopic morphology of Rhizopus stolonifer

### 2.3.4 Maintenance of Test-Fungi Stock Cultures

Four freshly prepared sabouraud dextrose agar slopes were inoculated with each test fungi spores isolated from rotten D. rotundata andincubated at 30°C for five days as described by Adeniji (1970) for maximum growth. One culture for each isolate was marked reference while the other stock cultures were stored at 4°C. These cultures were sub-cultured at three months intervals.

### 2.3.5 Preparation of Working Spores Suspension

This was prepared according to the flow chart in Fig. 2.4.

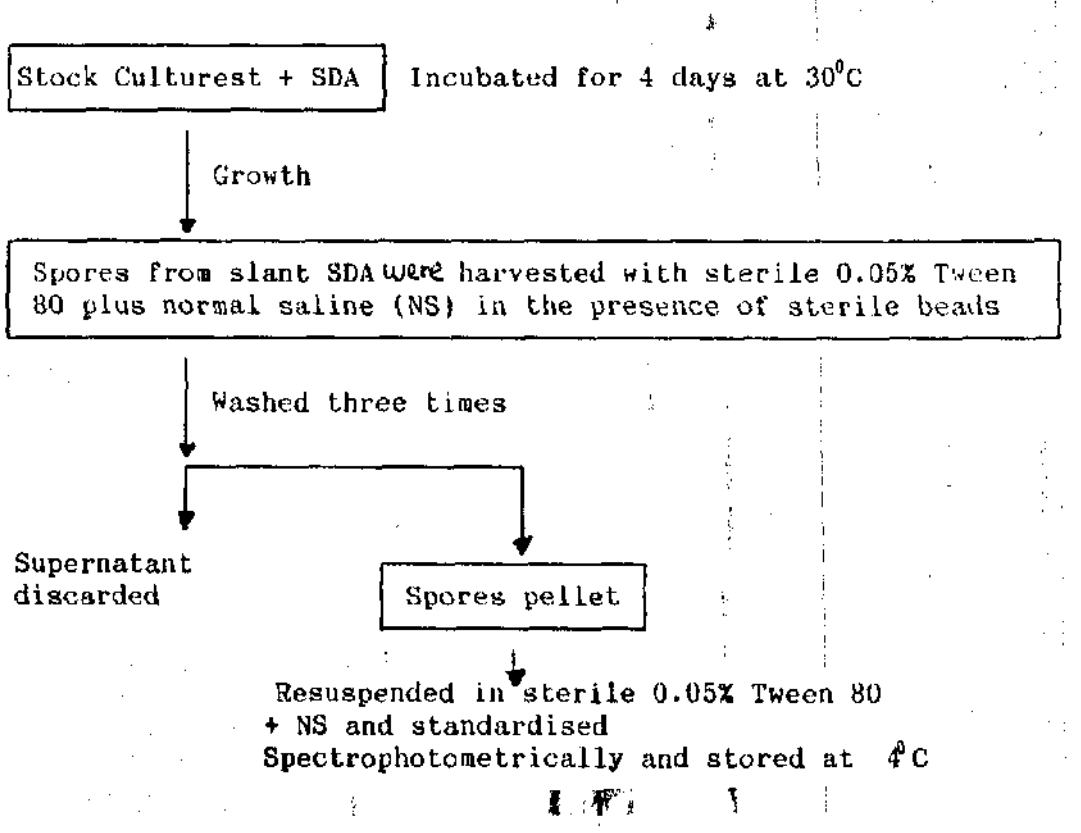



FIG. 2.4: Flow Chart for preparation of working Spores suspensions

### 2.3.6 Standardization of Working Spores Suspensions

Serial dilutions of washed spores suspension of each isolate were performed. The correspondence absorbance of these dilutions were made using the Gallenkamp spectronic 20 spectrophotometer (Bauch and Lomb, Rochester, New York). The wave- length that gave maximum absorbance in this study for A. flavus, A. niger, R. stolonifer and P. citrinum spore suspensions were 420, 565, 525 and 525 nm respectively.

Ten-fold dilutions of the different spore suspensions were carried out using sterile 0.05% Tween 80 plus normal saline with the aid of Gallenkamp whirl mixer. One millilitre of the three final dilutions of each absorbance were plated out in duplicates using pour plate technique with molten SDA kept at 45°C. The mixture was allowed to set firmly and the plates were incubated at 30°C for 48h. The colonies were counted thereafter on a Gallenkamp colony counter. A mean of the duplicate result was taken for each dilution and the viable spores count per millilitre of the spore suspension per absorbance calculated.

The logarithm of spore counts obtained was plotted against corresponding absorbance as shown in Figs. 2.5 - 2.8.

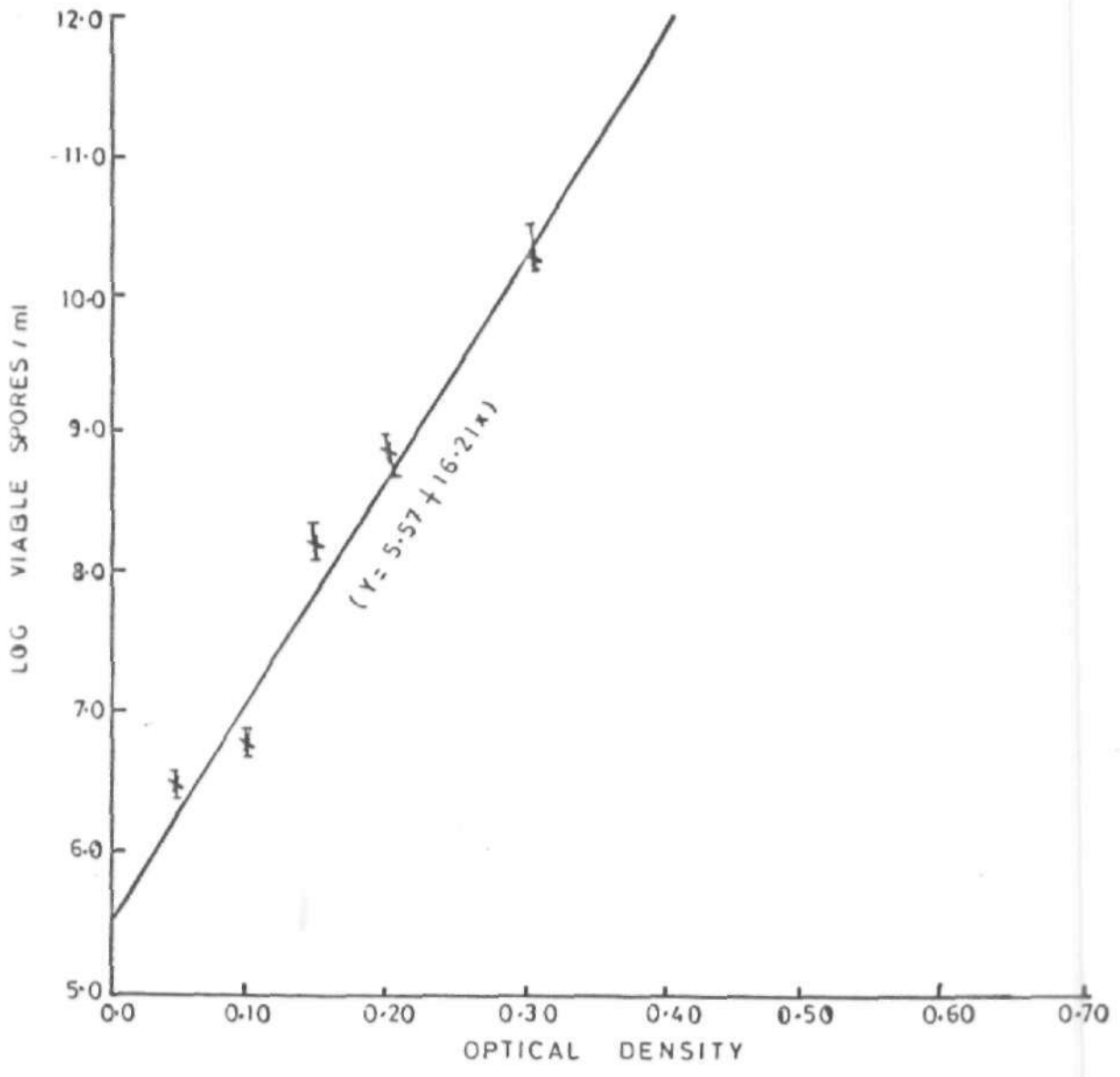



Fig.2·7 : The relationship between absorbance and viable spores of Rhizopus stolonifer

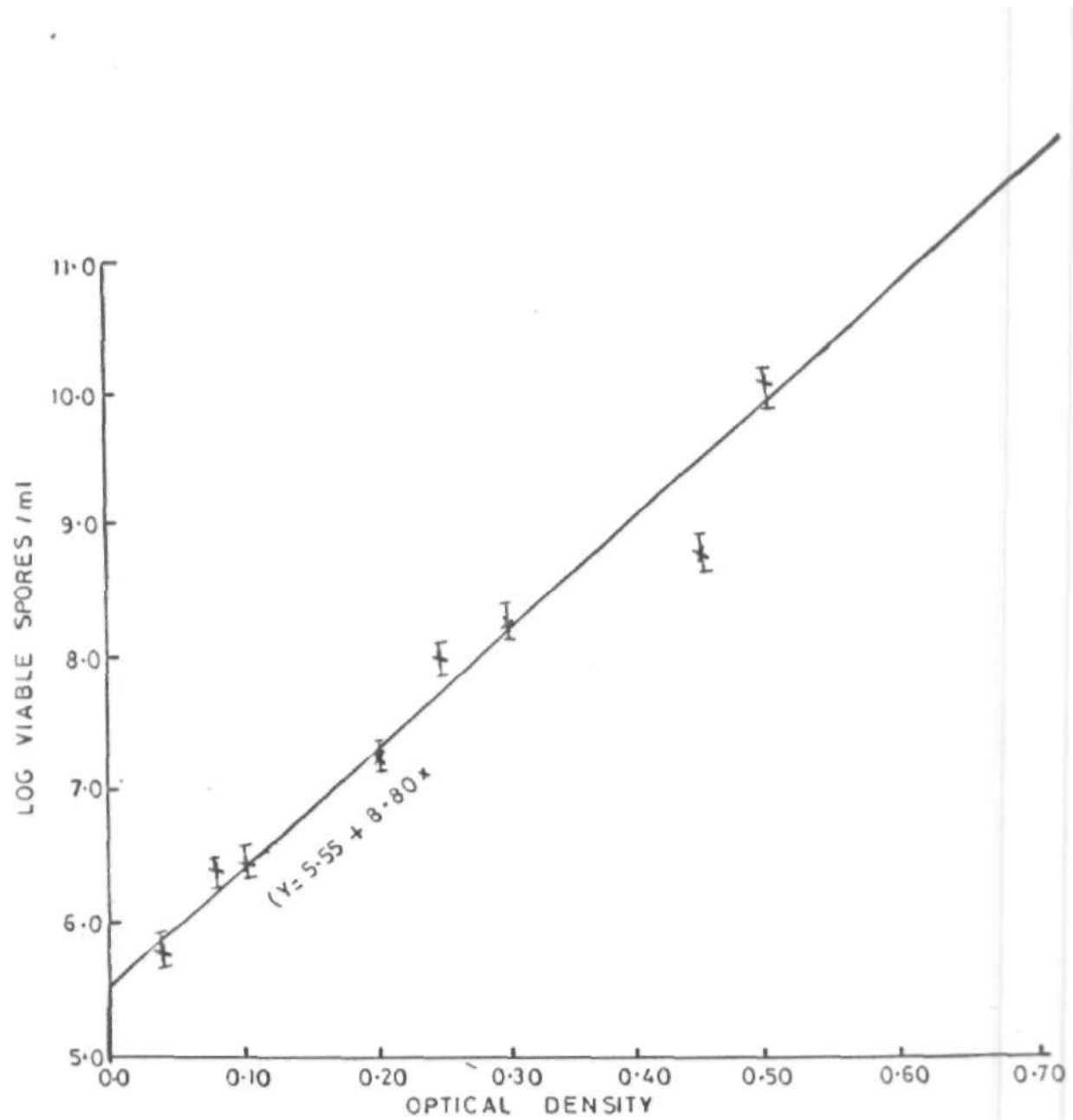



Fig. 2·6: The relationship between absorbance and viable spores of Aspergillus niger

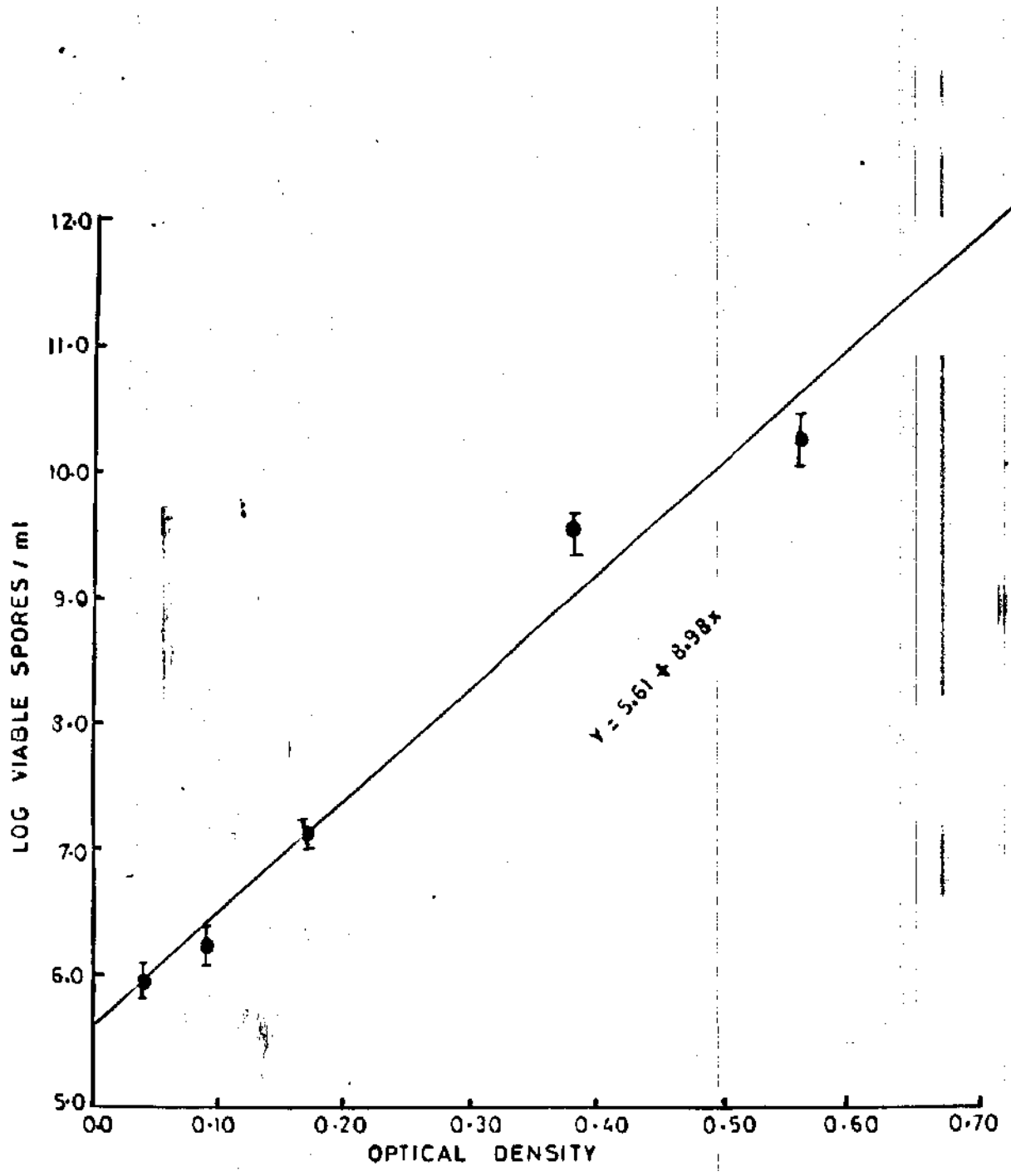



Fig.2.5: The relationship between absorbance and viable spores of Aspergillus flavus

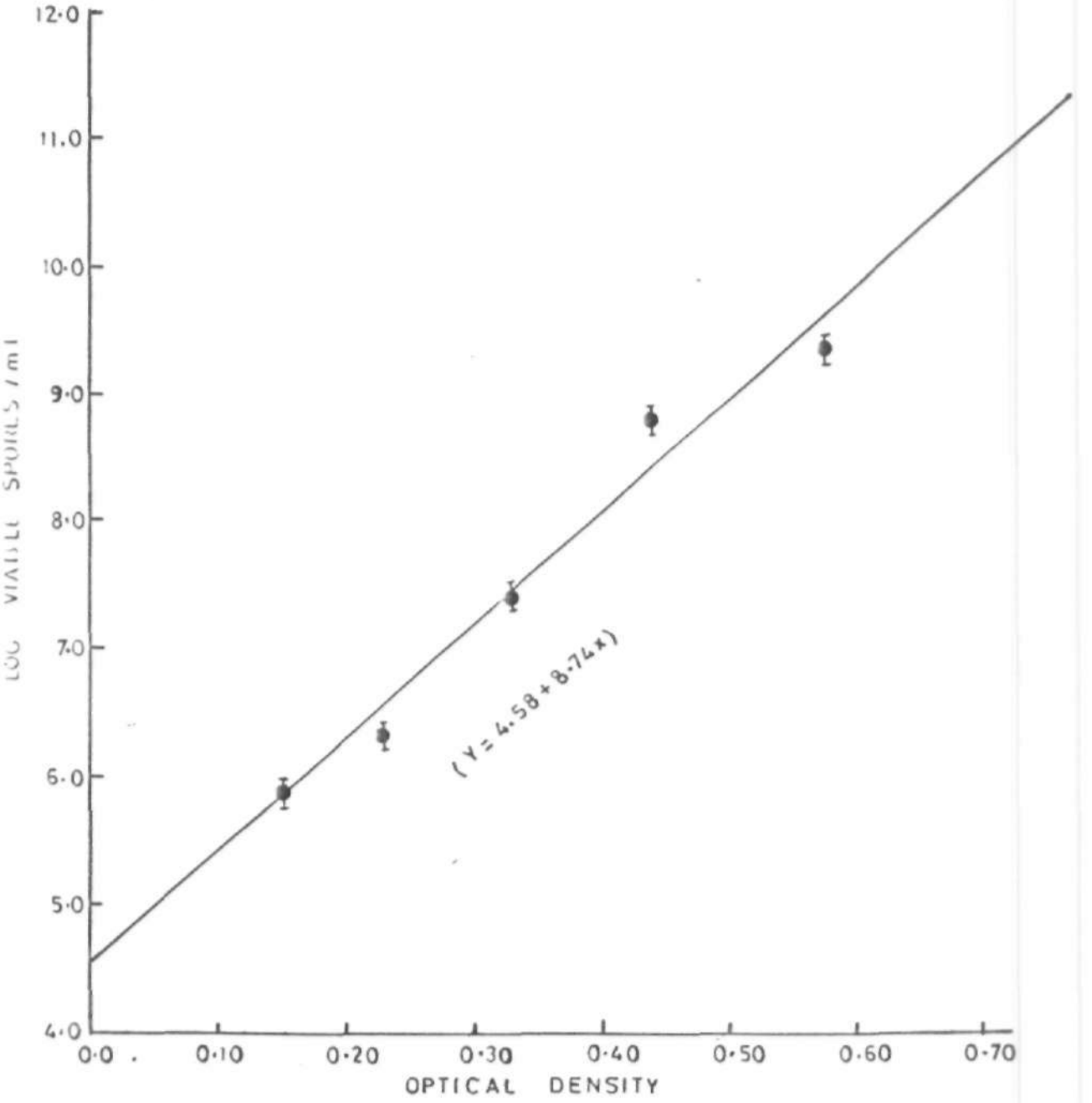



Fig.28: The relationship between absorbance and viable spores of *Penicillium citrinum*

# CHAPTER THREE
# DETERMINATION OF ANTIFUNGAL ACTIVITIES OF TPTS, TPTA, TBTA AND PROPYLENE GLYCOL

## 3.1 INTRODUCTION

The ability to measure fungitoxic effect of any chemical lies in the proper understanding of the term fungitoxicity. This term has been defined as the ability of a chemical to interfere in an adverse way with the vital functions of a fungus by physicochemical means (Anon., 1943). Fungistatic action implies continuous interferences in physiological process in the fungus as long as the organism is in the presence of the toxicant while fungicidal action denotes a persistent action on the fungi spores after the withdrawal of the toxicant (Lukens, 1971). Fungi- toxicity can be expressed in numerical terms as the dosage of toxicant that produces a unit inhibition or kill of fungal response in a prescribed period of time. This is usually measured in terms of the response of a treated culture relative to that of a control. Furthermore, growth rate, swelling spores, collapse of protoplast, sporulation, germination of spores and respiration are all responses used to determine antifungal activities of test-chemical compounds.

### 3.1.1 EXPERIMENTAL

### 3.1.2 Determination of Antifungal Activity of Acetone and Polysorbate 80

Antifungal effect of acetone and polysorbate 80 were determined by adding 10 ml of molten double strength SDA at 45$^{0}$C to 10 ml of varying concentration of these two chemicals separately in redistilled acetone 2.5% v/v to 50% v/v and polysorbate 80 0.10% v/v to 10% v/v. These were allowed to set.

The four test fungi spores, namely A. flavus, A. niger, R. stolonifer and P. citrinum were inoculated equidistantly on the acetone-SDA and polysorbate 80-SDA media with 10 ul of $10^8$ CFU/ml homogenous spores suspension. Similarly, controls, i.e. SDA without these solvents, were also inoculated. The plates were incubated at $30^0$C for five days.

### 3.1.3 Determination of Antifungal Activity of Test Compounds Using Agar Cup-plate Method

Molten SDA (19 ml) at $45^0$C was seeded with 1ml of $10^8$ CFU/ml of homogenous fungal spores suspension in petri-dish and allowed to set firmly.

Three "cups" were cut equidistantly in each of the petri- dishes, using a sterilized No. 4 cork borer (diameter - 8 mm) and the base of the hole sealed with 10 ul of molten SDA aseptically. The cups were filled with 20 ul of TPTS suspension using concentration ranging from 2.5 to 1280 ug/ml.

The plates were kept on disinfected working bench under aseptic screen cabinet for one hour at ambient temperature of 27 +_ $2^0$C to allow for diffusion and subsequently incubated at $30^0$C for 24 hr. The sensitivity of the test fungi spores to the agents was determined by the zones of inhibition using well calibrated white metre ruler to measure the diameter across the cups. The procedure was repeated for the other chemical agents, namely TPTA, TBTA, and propylene glycol.

### 3.1.4 Determination of MIC and MFC Using Agar Dilution Method

For the determinaton of minimum inhibitory concentrations (MIC), graded concentrations of each test compound were mixed with melted

double strength sabouraud dextrose agar (45°C) in sterile petri-dishes of diameter 90 mm and allowed to set. Eight sterile discs were aseptically placed equidistantly on the dried plate and inoculated with 10 ul of $10^8$ or $10^{10}$ CFU/ml of the four test fungi spores suspensions. The CFU/ml of homogeneous spores suspensions were determined from its logarithm viable CFU/ml- optical density graph as reported in this study. Control was set up, i.e. sabouraud dextrose agar plate without test chemical agents but inoculated with the test fungi spores. The plates were incubated at 30°C for five days.

The lowest concentration of the agent which inhibited visible growth of the fungal spores was taken as the MIC under this experimental conditions.

In order to determine the minimum fungicidal concentration (MFC), the membrane filter discs, showing no visible growth of test fungal spores in the MIC determination above, were removed aseptically. These were inoculated to Recovery Lecithin-Polysor- bate 80 medium. These were incubated at 30°C for seven days. The lowest concentration of the antifungal agent which indicated no growth was considered as the MFC. This procedure was repeated for the other test antifungal agents.

## 3.2 RESULTS

The antifungal effect of acetone and polysorbate 80 (probable vehicle for the test chemical agents) on the test fungal spores are as shown in Tables 3.1 and 3.2. The test fungal spores were unaffected by the presence of 1.25-25% v/v acetone and 0.05 to 5.0% v/v polysorbate 80. However, experience has shown in this study that acetone and polysorbate

80 do interfer with the setting of molten SDA at concentration 40% v/v and 5% v/v in water respectively.

The susceptibility of the test fungal spores are as given in Tables 3.3, 3.4 and Figs. 3.1 and 3.2. All the fungal spores tested were susceptible to all the test chemical agents. Generally, A. flavus appears least susceptible to all the chemical agents tested.

The MIC, that is, the minimum inhibitory concentration of an antifungal agent that prevents detectable growth in a specified inoculum of fungal spores was studied. Table 3.5 illustrates the MIC of TBTA against test fungal spores for inoculum sizes of $10^5$ and $10^3$ spores per ml. With the lower inoculum size, the growth of the test fungal spores was inhibited by the same concentration of TBTA except that against A. flavus.

TABLE 3.1: EFFECT OF VARYING CONCENTRATIONS OF ACETONE ON TEST-FUNGAL SPORES IN SDA INCUBATED AT 30°C FOR FIVE DAYS

| TEST-FUNGUS | CONCENTRATION OF ACETONE (% v/v) | | | | | |
|---|---|---|---|---|---|---|
| | 1.25 | 2.5 | 5.0 | 10.0 | 20 | 25 |
| A. flavus | ++ | ++ | ++ | ++ | ++ | ++ |
| A. niger | ++ | ++ | ++ | ++ | ++ | ++ |
| R. stolonifer | ++ | ++ | ++ | ++ | ++ | ++ |
| P. citrinum | ++ | ++ | ++ | ++ | ++ | ++ |

TABLE 3.2: EFFECT OF VARYING CONCENTRATIONS OF POLYSORBATE 80 ON TEST-FUNGAL SPORES IN SDA INCUBATED AT 30°C FOR FIVE DAYS

| TEST-FUNGUS | CONCENTRATION OF POLYSORBATE 80 (% v/v) | | | | |
|---|---|---|---|---|---|
| | 0.05 | 0.10 | 1.0 | 2.5 | 5.0 |
| A. flavus | ++ | ++ | ++ | ++ | ++ |
| A. niger | ++ | ++ | ++ | ++ | ++ |
| R. stolonifer | ++ | ++ | ++ | ++ | ++ |
| P. citrinum | ++ | ++ | ++ | ++ | ++ |

TABLE 3.3: MEAN DIAMETERS OF ZONES OF INHIBITION OF THE FOUR TEST-FUNGAL ISOTATES IN SDA WHEN TREATED WITH VARIOUS CONCENTRATIONS OF TPTS IN 25% ACETONE IN WATER

| USED CONCS. (µg/cup) | DIAMETER OF ZONE OF INHIBITION (mm) | | | |
|---|---|---|---|---|
| | A. niger | A. flavus | R. stolonifer | P. citrinum |
| 0.05 | 1.0 ± 0.1 | 1.0 ± 0.3 | 0.0 | 2.0 ± 0.2 |
| 0.10 | 1.5 ± 0.05 | 2.0 ± 0.2 | 0.0 | 3.0 ± 0.3 |
| 0.20 | 3.5 ± 0.4 | 3.0 ± 0.2 | 1.0 ± 0.2 | 4.5 ± 0.4 |
| 0.40 | 5.5 ± 0.1 | 4.5 ± 0.4 | 2.0 ± 0.2 | 6.5 ± 0.4 |
| 0.80 | 6.5 ± 0.4 | 6.0 ± 0.3 | 4.0 ± 0.3 | 8.0 ± 0.5 |
| 1.20 | 7.5 ± 0.3 | 7.0 ± 0.4 | 6.5 ± 0.3 | 9.0 ± 0.2 |
| 1.60 | 8.5 ± 0.3 | 8.0 ± 0.3 | 7.5 ± 0.3 | 9.5 ± 0.3 |
| 3.20 | 9.0 ± 0.4 | 9.0 ± 0.4 | 8.5 ± 0.4 | 10.5 ± 0.3 |
| 6.40 | 10.0 ± 0.1 | 10.0 ± 0.3 | 9.5 ± 0.3 | 12.0 ± 0.1 |
| 12.80 | 10.5 ± 0.2 | 11.0 ± 0.4 | 11.0 ± 0.4 | 16.5 ± 0.3 |

TABLE 3.4: MEAN DIAMETER OF ZONES OF INHIBITION OF THE FOUR TEST-FUNGI SPORES IN SDA WHEN TREATED WITH VARIOUS CONCENTRATIONS OF PROPYLENE GLYCOL

| USED CONCS. (µg/cup) | DIAMETER OF ZONE OF INHIBITION (mm) | | | |
|---|---|---|---|---|
| | A. niger | A. flavus | R. stolonifer | P. citrinum |
| 1.0 | 0.0 | 0.0 | 1.0 ± 0.1 | 2.0 ± 0.2 |
| 2.0 | 0.0 | 0.0 | 4.5 ± 0.4 | 4.5 ± 0.1 |
| 3.0 | 0.0 | 0.0 | 5.0 ± 0.2 | 5.0 ± 0.2 |
| 4.0 | 1.0±0.2 | 1.0±0.3 | 5.5 ± 0.3 | 5.5 ± 0.3 |
| 5.0 | 2.5±0.1 | 3.5±0.1 | 6.0 ± 0.6 | 6.0 ± 0.4 |
| 6.0 | 3.0±0.3 | 6.0±0.2 | 6.5 ± 0.4 | 6.5 ± 0.2 |
| 7.0 | 6.0±0.2 | 7.0±0.5 | 7.5 ± 0.3 | 7.5 ± 0.3 |
| 8.0 | 7.0±0.1 | 8.0±0.3 | 8.0 ± 0.3 | 8.5 ± 0.7 |
| 10.0 | 8.0±0.3 | 9.0±0.2 | 9.0 ± 0.1 | 9.0 ± 0.2 |

Increasing the inoculum size to $10^9$ spores/ml resulted in an increase in the MIC of TBTA against virtually all the fungal spores. For example, 14.3 mM and 29.0 mM of TBTA inhibited the growth of $10^6$ and $10^8$ spores/ml of the test organisms respectively.

TABLE 3.5: EFFECT OF CHANGES IN INOCULUM SIZE OF THE TEST FUNGI SPORES ON THE MIC OF TBTA IN 25% ACETONE IN WATER AFTER FIVE DAYS INCUBATION AT 30°C

| FUNGAL ISOLATES | MINIMUM INHIBITORY CONCENTRATION (mM)* $10^5$ CFU/ml | $10^3$ CFU/ml |
|---|---|---|
| A. flavus | 7.2 | 29.0 |
| A. niger | 14.3 | 29.0 |
| R. stolonifer | 14.3 | 29.0 |
| P. citrinum | 14.3 | 14.3 |

*One mM of TBTA = 349.1 ug/ml.

TABLE 3.6: MIC AND MFC VALUES OF TPTS, TPTA, TBTA, SALICYLIC ACID (SA) AND PG AGAINST $10^8$ CFU/ML OF THE TEST-FUNGAL ISOLATES AT 30°C

| ANTI-FUNGAL AGENTS | MIC [mM] | | | | MFC [mM] | | | |
|---|---|---|---|---|---|---|---|---|
| | A. flavus | A. niger | R. stolonifer | P. citrinum | A. flavus | A. niger | R. stolonifer | P. citronum |
| TPTS | 0.100 | 0.100 | 0.100 | 0.040 | 0.40 | 0.20 | 0.40 | 0.30 |
| TPTA | 0.049 | 0.037 | 0.037 | 0.024 | 0.50 | 0.20 | 0.40 | 0.20 |
| TBTA | 0.029 | 0.029 | 0.029 | 0.014 | 0.40 | 0.30 | 0.20 | 0.03 |
| PG (m) | 1.971 | 1.971 | 1.971 | 1.577 | 3.94 | 5.26 | 5.26 | 3.29 |
| SA | 1.810 | 1.810 | 1.810 | 0.905 | - | - | - | - |

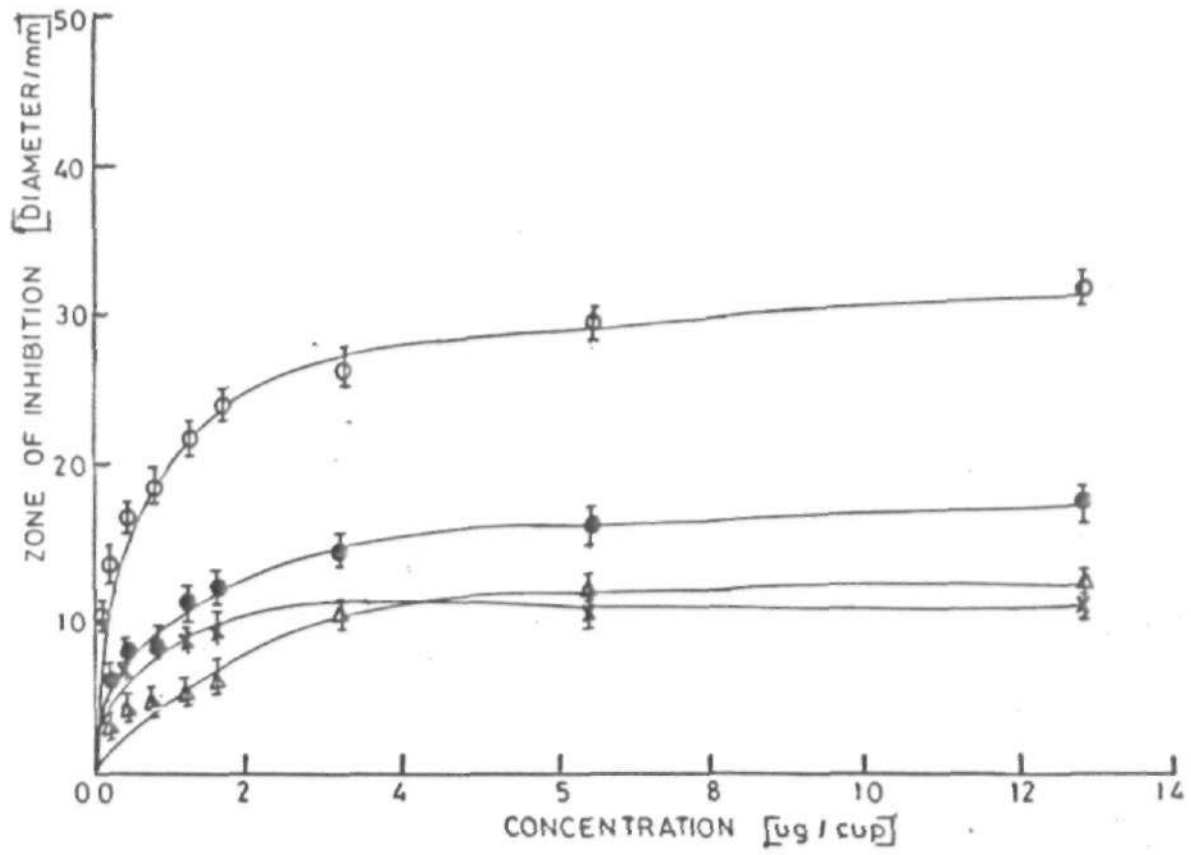
*One mM of test agents are as follows:

TBTA = 349.1 ug/ml; TPTA = 409.0 ug/ml; TPTS = 457.1 ug/ml;
PG = 76.1; SA = 138.1 ug/ml.

The MIC of TBTA, TPTA, TPTS, SA and PG against $10^3$ CFU/ml of test fungi spores, A. flavus for example were 0.029, 0.049, 0.10, 1.81 and 1.97 mM respectively (Table 3.6). It can be seen that the order of fungistatic activity of the test chemical agents are TBTA > TPTA > TPTS > SA > PG.

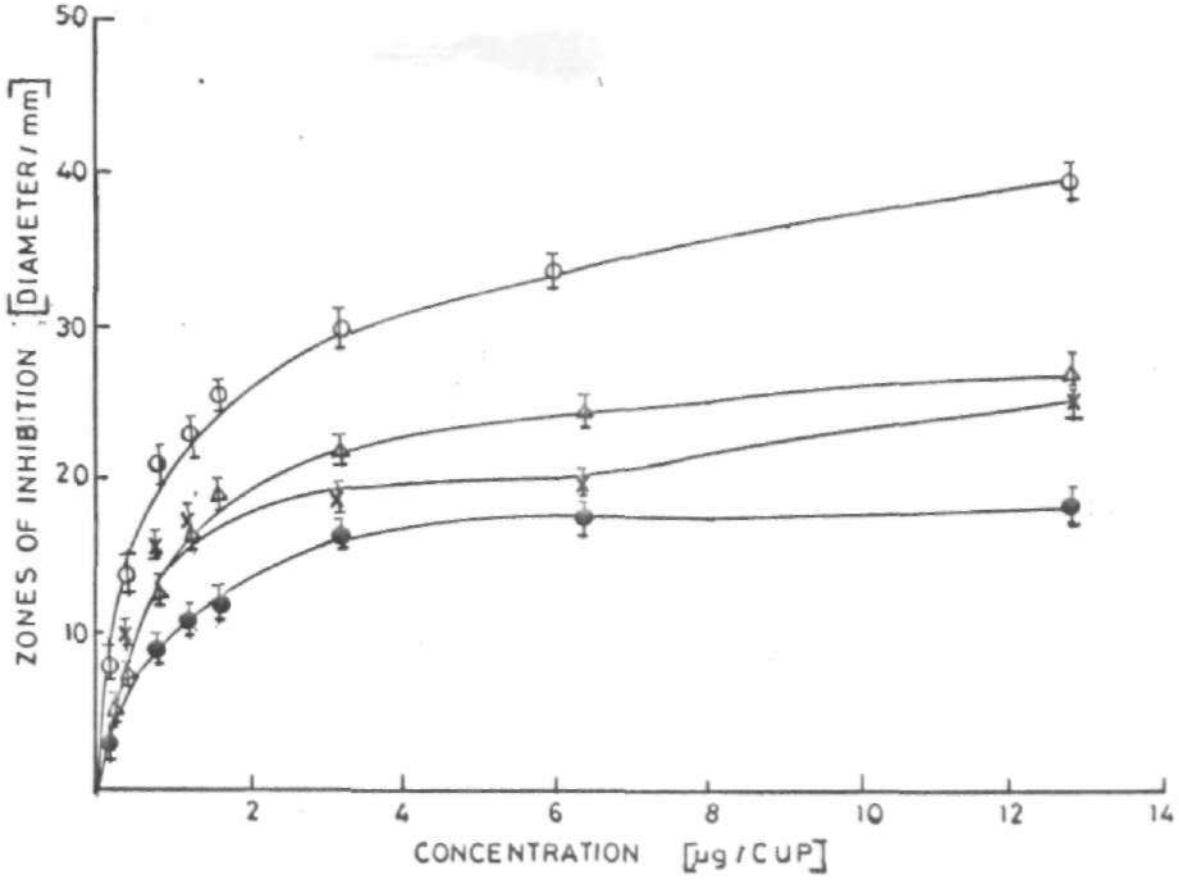
The MFC, that is, the minimum fungicidal concentration of the agents are generally greater than the MIC values as observed in this study. The increase in concentration ranged from two to twenty times greater than the MIC values (Table 3.6).

General inspection of all the tables (Tables 3.3-3.6) indicate that A. flavus was the least sensitive to the test triorganotins.




Fig·3·1: Susceptibity of the test-fungal spores grown on SDA to TPTA at varying concentrations using the cup-plate method

o—o A·niger ●—● P·citrinum ▲—▲ R·stolonifer ×—× A·flavus




Fig·3·2; Susceptibility of the test-fungal spores grown on SDA to TBTA at varying concentrations using the cup-plate method

o—o A. niger  Δ—Δ R. stoloniter  x—x A. flavus  •—• P. citrinum

## 3.3 DISCUSSION

The fungitoxic effect of any compound can be expressed in a numerical term as the effective concentration of toxicant that produces unit inhibition or kill of the fungal spores in a prescribed period of time. Other methods which have been reported for studying fungitoxicity includes determination of effect of the agent on viable spores count, the dry weight or carbon contents of the spores and respiration.

Of these methods, the measurement of zone of inhibition, MIC and MFC determinations have been shown to be reliable parameters for determining the fungitoxic effect for similar organotin chemical compounds (Olurinola, et al, 1992).

The observed lack of antifungal activity of acetone and polysorbate 80 at the tested concentrations indicate that they can be used as neutral vehicles for dissolution or suspension of the triorganotins within 1.25% to 30% for acetone and 0.05% to 2.5% for polysorbate 80.

The highest concentration used in this study for acetone has never been beyond 25% v/v, in view of the observation made in this study that concentration of acetone from about 40% v/v prevent setting of molten agar.

Polysorbate 80, a potential vehicle for triorganotin suspensions, has been reported to interfere to some extent with the antifungal activity of triorganotins (Olurinola, et al, 1992), hence, it was not used in this study as a suspending vehicle for the test agents. From the responses of fungal spores to test triorganotin compounds in acetone (Table 3.3, 3.4 and Figs. 3.1 and 3.2), it appears that there was no significant differences in the sensitivity of A. flavus, P. citrinum and R. stolonifer to the test

antifungal agents at 99% confident limit (calculated F for TPTA = 4.00 < Tabular F 4.26).

The threshhold effective concentration for the selected triorganotins used was about 3.0 ug/"cup" of 20 ul against all the test fungal spore. Thereafter, the increase in zones of inhibition was not significantly pronounced with increase in concentration of the antifungal agents (Figs. 3.1-3.2). Increase in zones of inhibition with increase in concentration was more pronounced with TPTA, TBTA, PG than with TPTS.

Minimum inhibitory concentration are usually carried out by serial dilution by using graded proportion of antimicrobial agents. However, arithmetic dilution, as recommended by Croshaw (1983) for accurate measurement of MIC was adopted in this study.

The MICs of the test triorganotins were dependent on the inoculum size of the test fungi spores. For example, with inoculum size of $10^5$ CFU/ml, the growth of the fungi spores was inhibited by 14.3 uM (or 8.9 x $10^{12}$ molecules of TBTA per spore) except A. flavus with MIC of 7.2 µM (or 4.5 x $10^{12}$ molecules/spores). Increasing the inoculum size to $10^8$ CFU/ml resulted in double increase in the MICs of TBTA to 29 µM (or 1.81 x $10^{13}$ molecules per spore) except P. citrinum with MIC of 14.3 µM (or 8.9 x $10^{12}$ molecules per spore).

Proper comparisons of two types of triorganotins compounds can only be made on molarity or molecules of the triorganotins per spore basis. This is because cellular response of test- organisms correlate better to molarity or molecules/spore than with the concentration used, expressed in ug/ml. The use of concentration in term of ug/ml is further complicated by variation in molecular weight of the different triorganotins

involved (Sijpestejin, et al, 1969).

Generally, Table 3.6 shows that there was no significant differences in the response of the A. flavus, A. niger, and R. stolonifer to all the test chemical agents in terms of MICs at 95% confident limit. However, P. citrinum was the most sensitive test-organism. The MICs of these chemical agents indicate the following order of activities TBTA (1.7) > TPTA (1.6-2.7) > TPTS (18.1-22.6) > SA (1.1-1.7) > PG [1.0].

Assay for the antifungal activity of triorganotins are somewhat difficult, because organotins have limited solubility in water and ability to diffuse through agar (Cooney, et al, 1989; Orsler and Holland, 1984). Thus, comparisons of antifungal activity which compare fungitoxicity of organotins in agar media which suggest that TPTA is three-fold more fungitoxic than TPTS are measuring the combined effects of fungitoxicity and diffusibility. Yet, fungitoxic effect of organotins determined by SDA has been reported as one of the most effective methods in view of its limited solubility and cloudy suspension it forms in water (Shadomy, et al, 1977).

The MIC and MFC values have been employed to evaluate the efficacy of agents such as antiseptics, disinfectants and indeed triorgano-tins (Croshaw, 1983; Ehinmidu and Otsapa, 1991; Olurinola, et al, 1991). The MIC is helpful in measuring the fungistatic activity of antimicrobial agent while MFC measures fungicidal activities under similar conditions.

Most chemical antimicrobial agents exhibit both inhibiting and fungicidal activities to some extent, depending on the concentration used. Other factors such as duration of treatment (Ehinmidu and Olaniyan, 1992), resistance of the organisms, inoculum level as shown in this study (Table

3.5) and an external factor like temperature, may influence the activity of the anti- microbial agents. It has been found in this work that the MFC of TBTA, TPTA, TPTS and PG increase by 2.1-13.8, 8.3-10.2, 4.0-7.5 and 2.5-2.7 times respectively more than their corresponding MIC values against the four test-organisms. Sijpestejin and co- worker (1969) had observed that the values of MIC and MFC of triorganotins acting against A. niger and Botrytis allii were not the same. Similarly, Ehinmidu and associate (1992) studied the antimycotic properties of TPTA, tioconazole, clotrimazole and chlormidazole, and found that their MIC and MFC were quite different. For example, the MIC and MFC of clotrimazole against T.rubrum was 100 ug/ml and 400 ug/ml respectively.

Comparing the response of test-fungal spores, this work has shown that A. flavus and R. stolonifer appear to be less sensitive to the selected triorganotins than A. niger and P. citrinum as indicated by their MFC values (Table 3.6). From available literatures and observations from this study, it appears that all the test fungal spores, whether they belong to phycomycetes, ascomycetes or the basidiomycetes, are susceptible to varying concentrations of TBTA, TPTA, TPTS, SA and PG examined.

# CHAPTER FOUR

# THE EFFECT OF TBTA, TPTA, AND TPTS ON THE VIABILITY OF TEST FUNGAL SPORES

## 4.1 INTRODUCTION

The fungicidal activities of an antimicrobial agent may be assessed by performing viable counts at varying time intervals on the surviving members of the fungal spore population in contact with the solution of the agent. Most of these assessment fall into one or two categories namely: (a) the all or none response as determined indirectly in spore germination tests and (b) the graded response as determined in growth and respiration tests. How much information is obtained from either, depends upon the variation in the fungal spores and the relation between dosage and response.

When a microbial population is subjected to the adverse influences of an antimicrobial agent, the number of cells decreases usually geometrically at least for some time, in a manner that the logarithmic number of the surviving cells at any time when plotted against that of time, fall on a descending straight line. This is usually referred to as the logarithmic order of death (or first order kinetics).

Linear dosage-response curves are required to evaluate the fungitoxic characteristics of chemicals. The dosage is expressed logarithmically to remove the j-shaped component of the curves, which Horsefall (1945) attributed to the law of diminishing returns. Thus, biological responses change arithmetically with logarithmic change in stimulating agent. When data of graded concentrations are plotted against logarithm of viable spores, an extended sigmoid curve is formed (Bliss, 1957). The sigmoid

curve, common to many biological phenomena, is a variation of the bell-shaped frequency-distribution curves for sensitivity of fungal spores to a toxicant. Bliss (1957) defined this symmetrical sigmoid curve as a cumulative normal distribution. That is, a few individual spores are extremely sensitive or resistant to the toxicant, but the majority fall in the middle. Thus, each increment in dosage of fungicide gives unequal increase of affected individual fungal spores.

Death curves have always been assumed to be first order kinetic, converting to a straight line on semi-log coordinates. This however, can no longer be sustained. For example, Burton (1977) reported that careful experimentation has shown deviation from linearity of thermal death curves for spores through the development of "tails" to the curves at low proportions of survivors. The presence of such tails could probably be explained as genuine occurrence of a small number of highly resistant surviving spores in a normal population, that is, the test organism was physiologically heterogeneous. Figure 4.1a shows a typical or main type of death curves in disinfection processes.



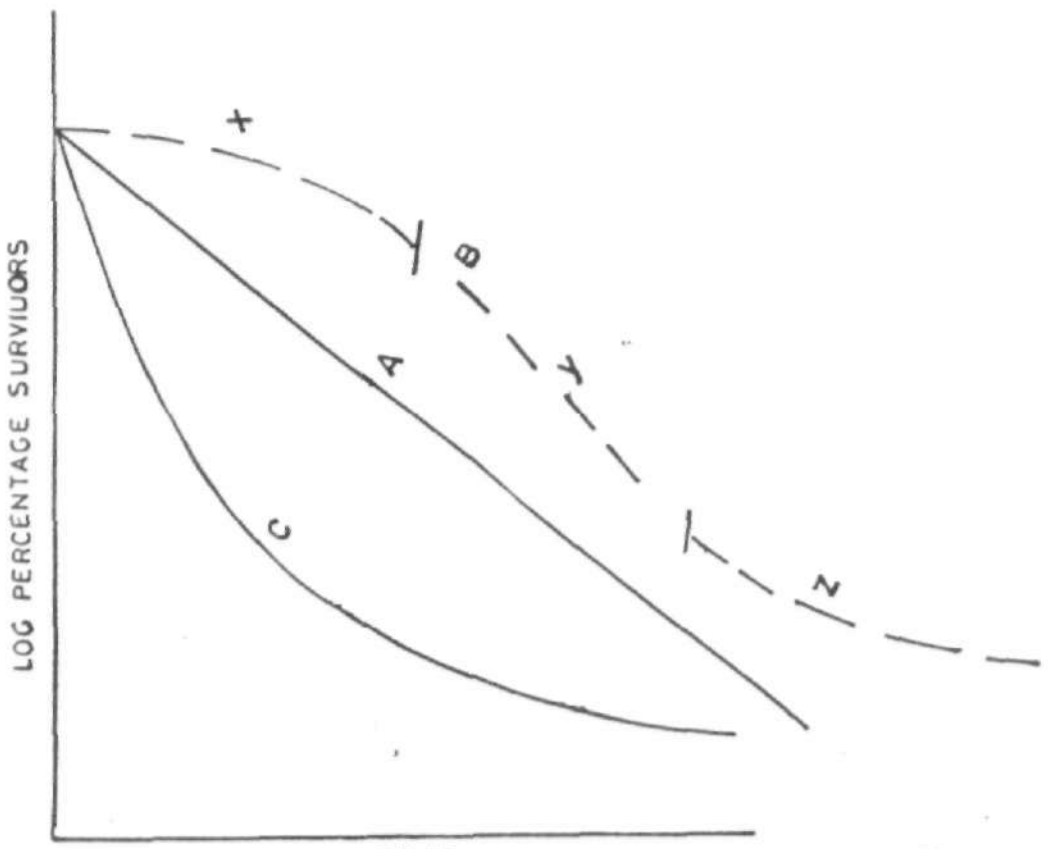



Fig. 4.1a: Death curves showing disinfection processes

A = Type of disinfection/Antimicrobial agent obeying first order kinetics.
B = Sigmoidal curves common with many biological responses to antimicrobial agent at low microbiocidal concentration with subsections.
(X) A slow initial kill mainly of subsceptible members of the population.
(Y) A faster near linear rate of kill showing a similar pattern to first order kinetics.
(Z) A slow death rate of resistants members.
C = The initial rate of kill is very fast, but subsequently decreases with time. This curve is often obtained with high concentrations of antimicrobial.

It is expected that the rate of kill curves obtained in this study will fall to any of the above curves.

## 4.2 EXPERIMENTAL

### 4.2.1 Determination of Inactivating effect of Polysorbate 80 on fungicidal activity of Test Triorganotin Ccompounds

Homogeneous spores suspension of test fungi are difficult to obtain in sterile distilled water without wetting agent. Hence, polysorbate 80 - a wetting agent without antifungal activity was used to harvest the spores of test fungi. Polysorbate 80 is known to inactivate various antimicrobial agents, including methyl - and propylparaben, quaternary ammonium compounds, chlorhexidine and sodium O - phenyl-phenate (Dimonde, 1962; Russell, et al, 1979, Hugo and Russell, 1987).

It was therefore considered appropriate to investigate the inactivating effect of polysorbate 80 on fungicidal activity of test triorganotins to provide a guide on the highest safe concentration of polysorbate 80 to be used as wetting agent without interfering with the antifungal activity of test triorganotins. Five concentrations of polysorbate 80 viz. 0.0, 0.05, 0.50, 1.0, and 5.0% were prepared in sterile distilled water with fixed 300-µg/ml of TPTA. These were inoculated with $10^3$ spores of test fungi at room temperature 27±2°C. Ten-fold dilution of 1.0ml of the reaction mixtures were made with sterile 9ml of 0.5% egg-lecithm and 3% polysorbate 80 broth diluent at specified time interval viz 5, 20, 45 & 60 minutes. After adequate dilution, 1.0ml samples were taken from the last three tubes of dilutions series and mixed with melted sabouraud dextrose agar at 45°C and plated out in duplicates. The plates were incubated at 30°C for 5 days after which the viable spores count were taken.

### 4.2.2 Determination of rate of Kill of Test Fungal spores by TBTA, TPTA and TPTS

The effect of graded fungicidal concentrations of each triorganotin carboxylate solutions on the viability of the spores of test fungi was studied. 20ml - volume solutions of the desired concentrations (e.g. 125, 175, 225, 275, 325 µg/ml) of the antifungal agents were prepared aseptically using 25% acetone in distilled water. The graded antifungal agents were prepared in amber coloured 20ml clinbritic bottles at 27±2°C. These 20-ml solution of test agents were inoculated with $10^3$ spores of test fungi. At specified time interval viz 5, 20, 45, and 60 minutes, 1.0ml sample was taken from the fungicide-spores reaction mixtures and adequately diluted with sterile inactivating diluent containing normal saline with 0.5% egg - lecithin and 3% polysorbate 80 to obtain countable spores. After adequate dilution; 1.0 samples were taken from the last three tubes of dilution series and mixed with 20 melted sabouraud dextrose agar at 45°C and plated out in duplicates. The plates were incubated at 30°C for 5 days after which the viable spores count were taken. The viability of the test fungi spores and the sterility of the SDA medium used were also determined.

A graph of log viable spores count versus time was plotted to determine the rate of Kill. In order to determine the extent of fungal spores at used concentrations, log viable spore count versus concentrations of the used chemical agents were also plotted.

Using the data obtained from this study, the rate of Kill constant (K - Values) and one log cycle or 90% reduction of viable spores population in the homogeneous suspension exposed to the test compounds were determined by calculation using the following standard equations:

$$K - value = 1/t \ (\log No/Nt)$$

$$D_{10} - value = \frac{t}{\text{Log } (No - Nt)}$$

where t = time for viable spore count to fall from No to Nt.

No = Initial number of test spores

Nt = Final number of fungal spores survivors after tune t.

K - values were calculated for all concentrations used and the values obtained were plotted against both the concentrations and the varying time of contact.

### 4.2.3 Determination of Cytoplasmic constituents leakage from Aspergillus niger spores in varying concentration of TPTA

The influence of TPTA on leakage from spores of Aspergillus niger of purine, pyrimidine and their derivatives were determined at varying concentration of the test compound.

Graded concentrations of TPTA ranging from 50 ug/ml to 475µg/ml were aseptically prepared in sterile 0.05% polysorbate 80 in normal saline. These were prepared in 20 ml volume and inoculated with $10^9$ cfu/ml spores and maintained at 27±2°C.

In order to obtain total release of 260-nm absorbing material, a similarly prepared suspension was held at 100°C for 10 minutes and referred to as "boiled spores" (Salton, 1951).

At a fixed time of 5 minutes contact time which marked the beginning of diminishing rate of Kill, the reaction mixtures were contrifuged at 3,000g for 15 minutes to precipitate the lysed spores (Hugo and Bloom-field, 1971). The amount of 260nm - absorbing materials present in the supernatant was then determined spectrophotometrically

using Milton Roy spectronic 1001 plus, two replicates being obtained for each sample. Corrections were made for the extinction of the suspending solutions by measuring the extinct using centrifuged blank corresponding test compound concentrations.

No = Initial number of test spores

Nt = Final number of Fungal spore survivors after time t

K - values were calculated for all concentrations used and the values obtained were plotted against both the used concentrations and the varying time of contact.

## 4.3 RESULT

### 4.3.1 Effect of Polysorbate 80 on Fungicidal activity of test triorganotins

Counting methods have been used to determine the number of fungal spores that survived the effects of 300 ug/ml of TPTA in admixtures with 0.05, 0.50, 1.00, and 5.00% polysorbate 80. The effect of these concentrations of polysorbate 80 on the antifungal activity of 300-ug/ml of TPTA on A. flavus are as shown in figure 4.1b.

The response of A. flavus spores to the fixed fungicidal concentration (300 ug/ml) of TPTA in 0.05% polysorbate 80 compared favourably with that obtained with TPTA in sterile distilled water. However, when polysorbate 80 concentration was increased to 0.5% and above, there was rapid decline in the fungicidal effect of TPTA. For example, whereas 300 ug/ml TPTA alone effected two log cycles of Kill within 5 minutes of contact, the same concentration of TPTA with 1% polysorbate 80 effected only one log cycle of Kill within the same period. Over five log cycles and three log cycles A. flavus spores Kill were

effected by the same concentration of TPTA alone and TPTA plus 1% polysorbate 80 respectively after 60 minutes of exposure.

A similar pattern of fungicidal activity was observed when TPTS and TBTA were test agents alone and in admixtures with varying concentrations of polysorbate 80.

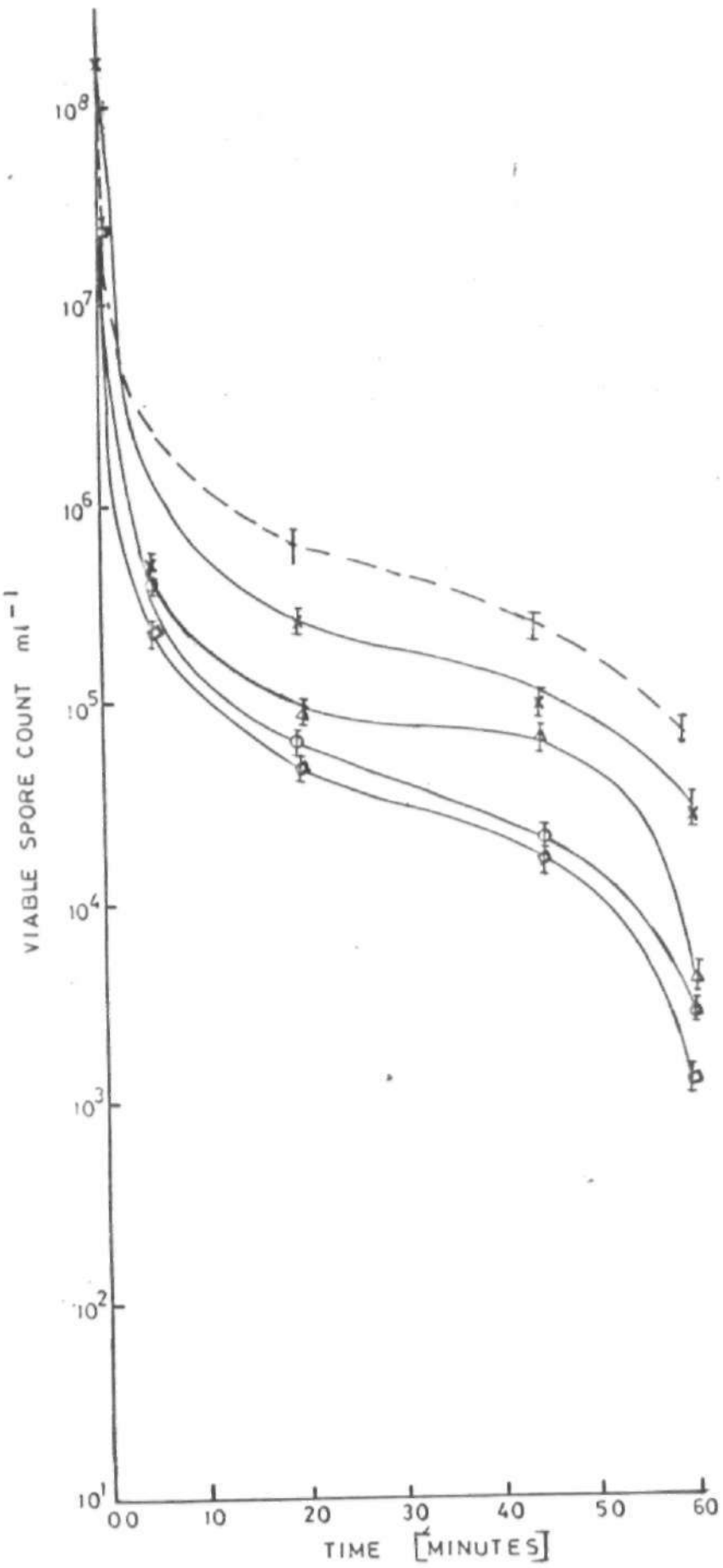



Fig.4-1b: Effect of polysorbate 80 on fungicidal activity of MFC of TPTA on A.flavus spores suspension

o—o SDW  o—o 0.05% Tw80  ▲—▲ 0.5% Tw80  x—x 1.0% Tw 80
– – – 5.0% Tw80

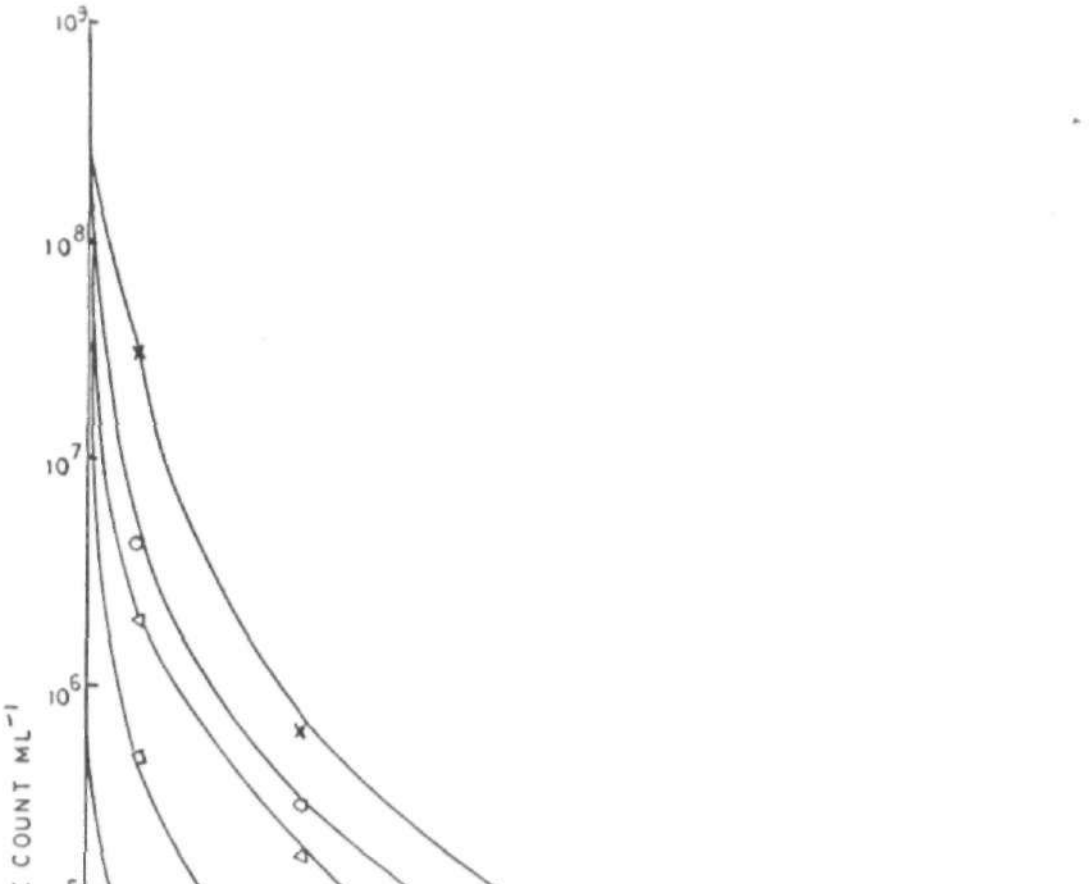



Fig-42: Effects of fixed concentration of TPTA on viability of A. niger at 25 °c

×—×125 ug/ml ○—○175 ug/ml △—△225 ug/ml □—□275 ug/ml ■—■325 ug/ml

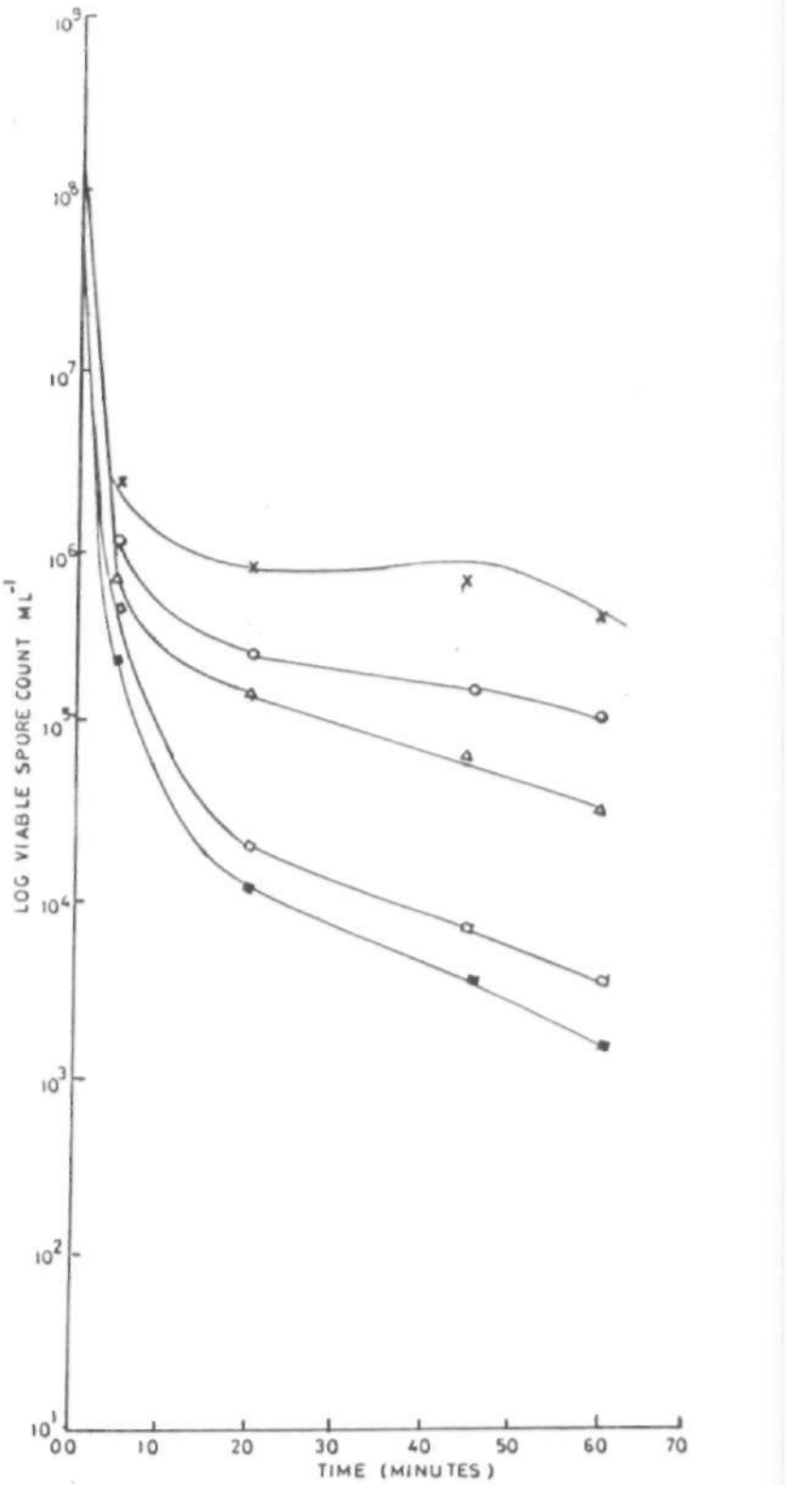



Fig.4.3: Effect of fixed concentration of TPTA on viability of Penicillium citrinum at 25°c

### 4.3.2 Rate of Kill

The rate of Kill of the test fungal spores by the test compounds are clearly demonstrated in figures 4.2 and 4.3, which depict the death curves of fixed but varying concentra-tions of TPTA on A. niger and P. citrinum at different time intervals respectively. From figure 4.2 it can be observed that the rate of Kill of A. niger by the fungicidal concentrations of TPTA progressively diminishes with time, but increases with increase in concentration. For instance, the rate of Kill by 125ug/ml, after initial 8 minutes contact was 2.38 x $10^7$ spores per minute, that of the next 7 minutes was 1.32 x $10^6$ spores per minute. Whereas, there was one log cycle (90%) reduction at 5 minutes with 125ug/ml while about 4 log cycles of Kill were effected by 325ug/ml within the same time.

Figure 4.3 illustrates a more dramatic decline of rate of Kill with time and increase rate of Kill with increased concentration. within 2.5 minutes of fungal spores treatment with 125 ug/ml, 2 log cycles Kill of fungal spores was observed. However within the next 10 minutes only about 1/5 log cycle reductions of test spores was observed. This observation gives an indication of the rapid diminishing rate of Kill of this test fungal spores with this same test compounds compared with the effect displayed when A. niger was the test fungal spores.

This pattern of rate of fungicidal activity was the same for rest test triorganotins against other test fungi spores as illustrated here. For example, figures 4.4, 4.5, and 4.6 illustrate the effect of treating various test fungal spores with 0.5mM of TBTA, TPTA and TPTS respectively for about one hour. Generally, the three triorganotins were obviously fungicidal against all the test fungal spores. Reductions in spores

population range between 2 to 4 log cycles within 20 minutes for most of the fungi spores and in some cases up to 6 log cycles within 50 minutes. Aspergillus flavus appears to be the least susceptible among the tested fungal species, while Aspergillus niger is the most susceptible. (This is rather surprising that members of the same genus would give the extremes in their response to this concentration of the test agents). The reason for this unusual behaviour species of the genus is not clear.

The shape of the death curves were all similar, presenting the 'C' type curve Fig. 4.1a in which there is an initial very rapid rate of Kill, but which diminishes with time. The concave shape is however not maintained throughout the exposure time, but eventually became an inverted or reversed of the usual type B curves Fig. 4.1a.

There were some differences in the effects of individual test triorganotins on the test fungi spores. TBTA appeared generally to be more fungicidal than all the other agents. The fungicidal rate of Kill of these test agents at equimolar

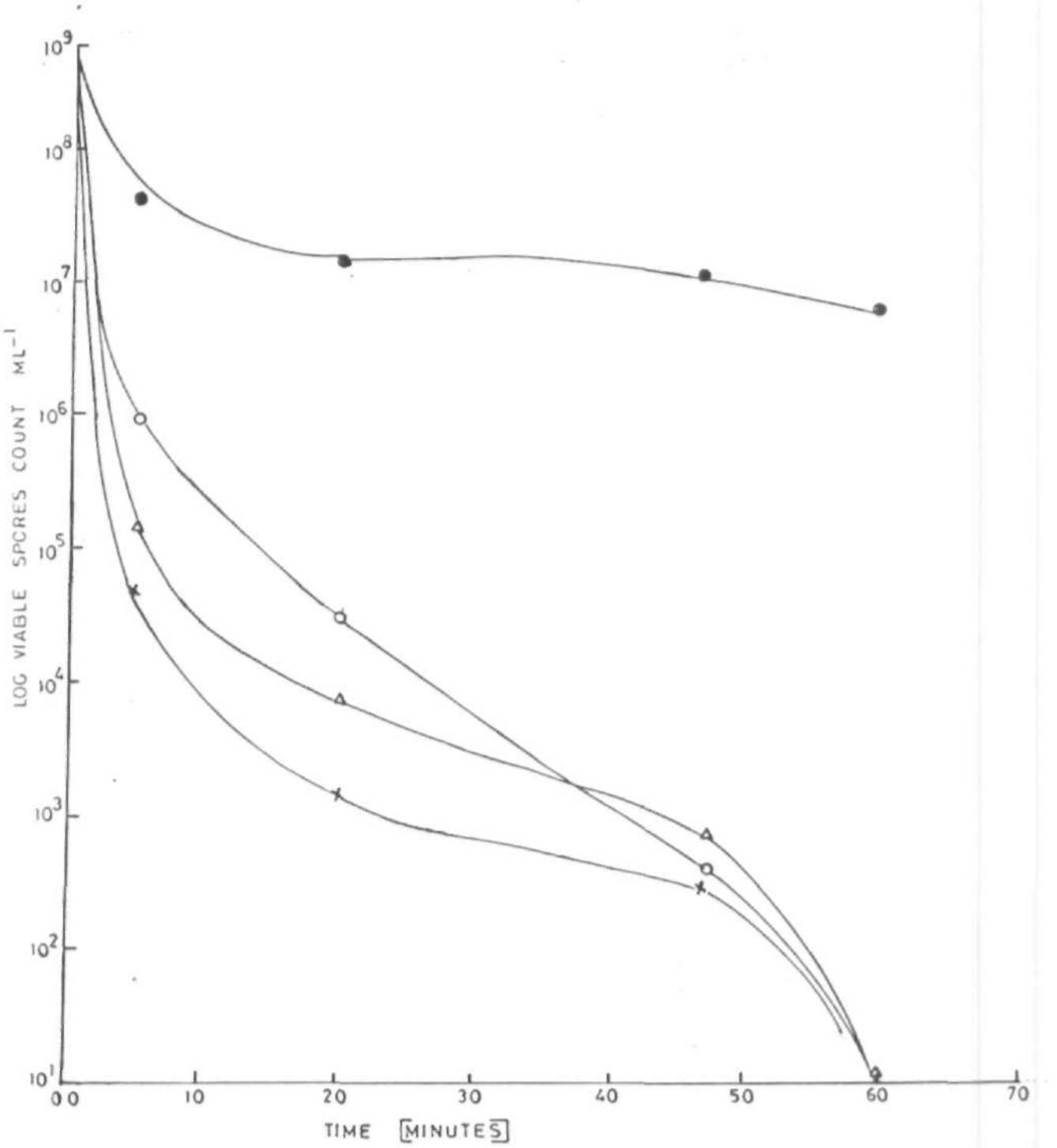



*Fig.4.4: Effect of 0·5mM of TBTA in 25% aqueous acetone solution on the viability of spores suspension of A. flavus, A. niger, R. stolonifer and P. citrinum at 27 ± 2°c*

●—● A. flavus ×—× A. niger △—△ R. stolonifer ○—○ P. citrinum

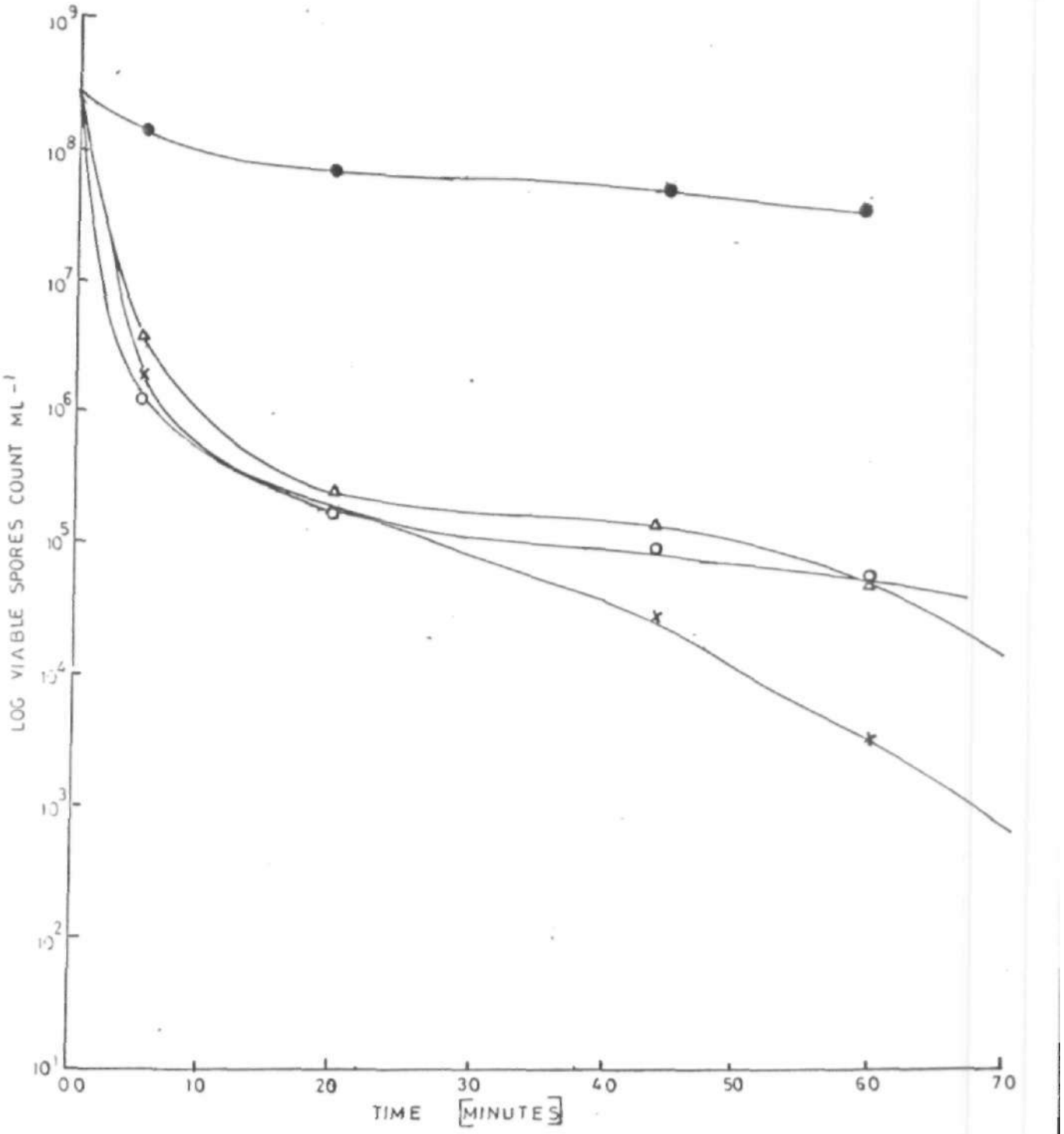
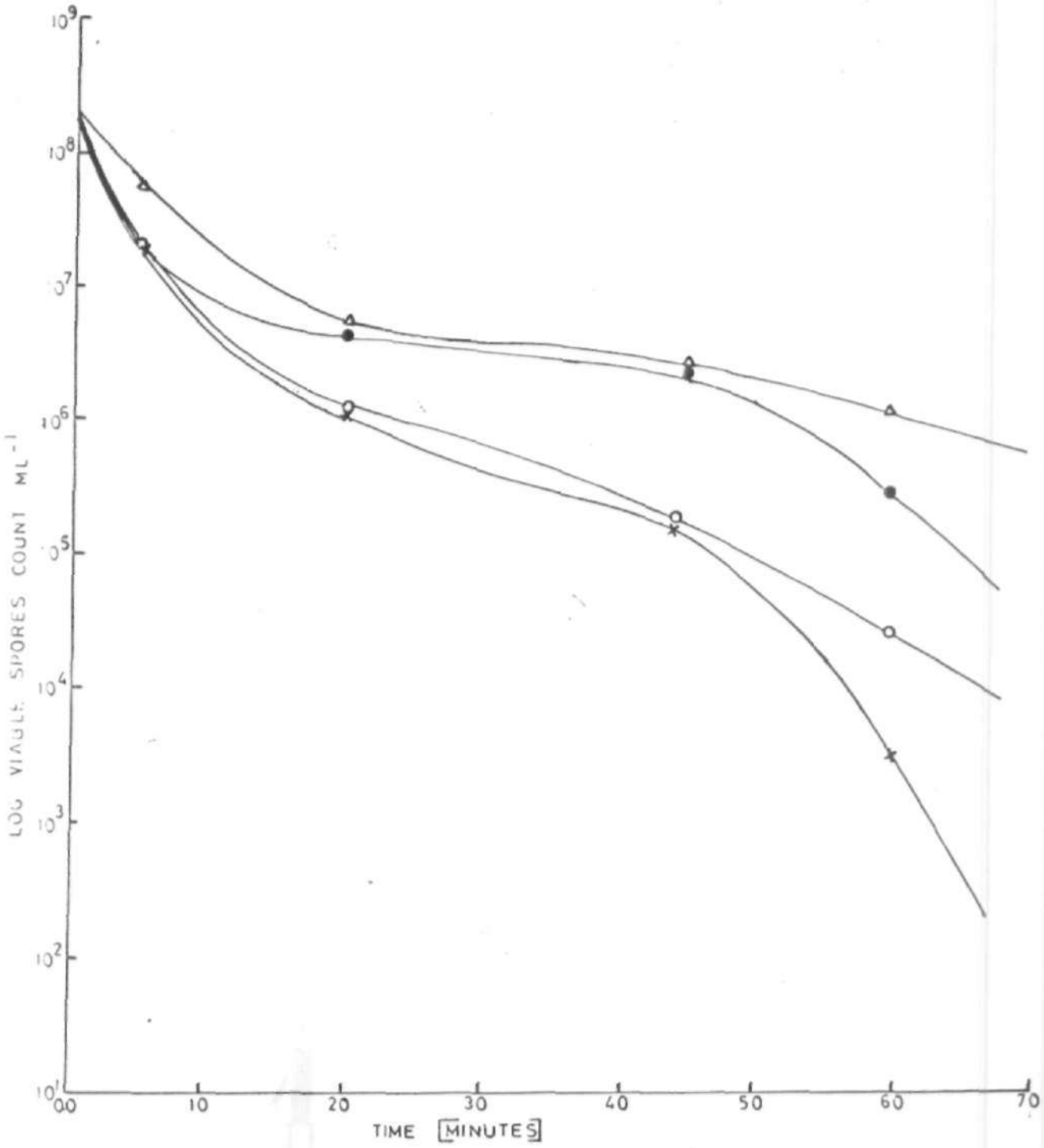



Fig.4.5: Effect of 0.5 mM of TPTA in 25% aqueous acetone solution on the viability of spores suspension of A. flavus, A. niger, R. stolonifer and P. citrinum at 27 ± 2 °c

●—● A. flavus  x—x A. niger  △—△ R. stoloniter  ○—○ P. citrinum




Fig·4·6 : Effect of 0 5mM of TPTS in 25% aqueous acetone solution on the viability of spores suspension of A·flavus, A·niger, R· stolonifer and P·citrinum at 27±2°c

●—● A·flavus ×—× A·niger △—△ R·stolonifer ○—○ P·citrinum

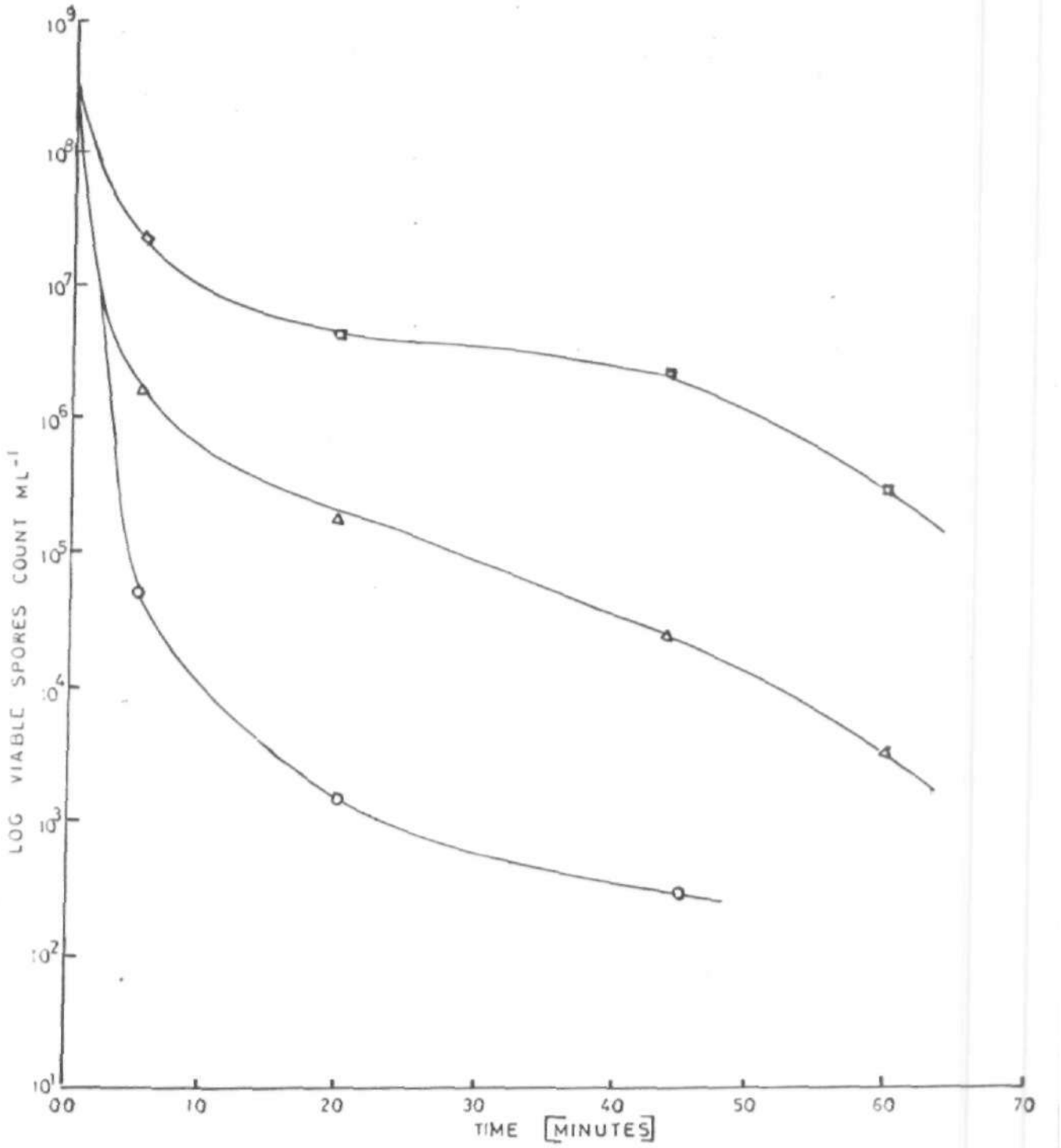



Fig-4.7: The rate of kill of A. niger spores suspension treated with 0.5mM of TBTA, TPTA and TPTS in 25% aqueous acetone solution at 27 ± 2°c

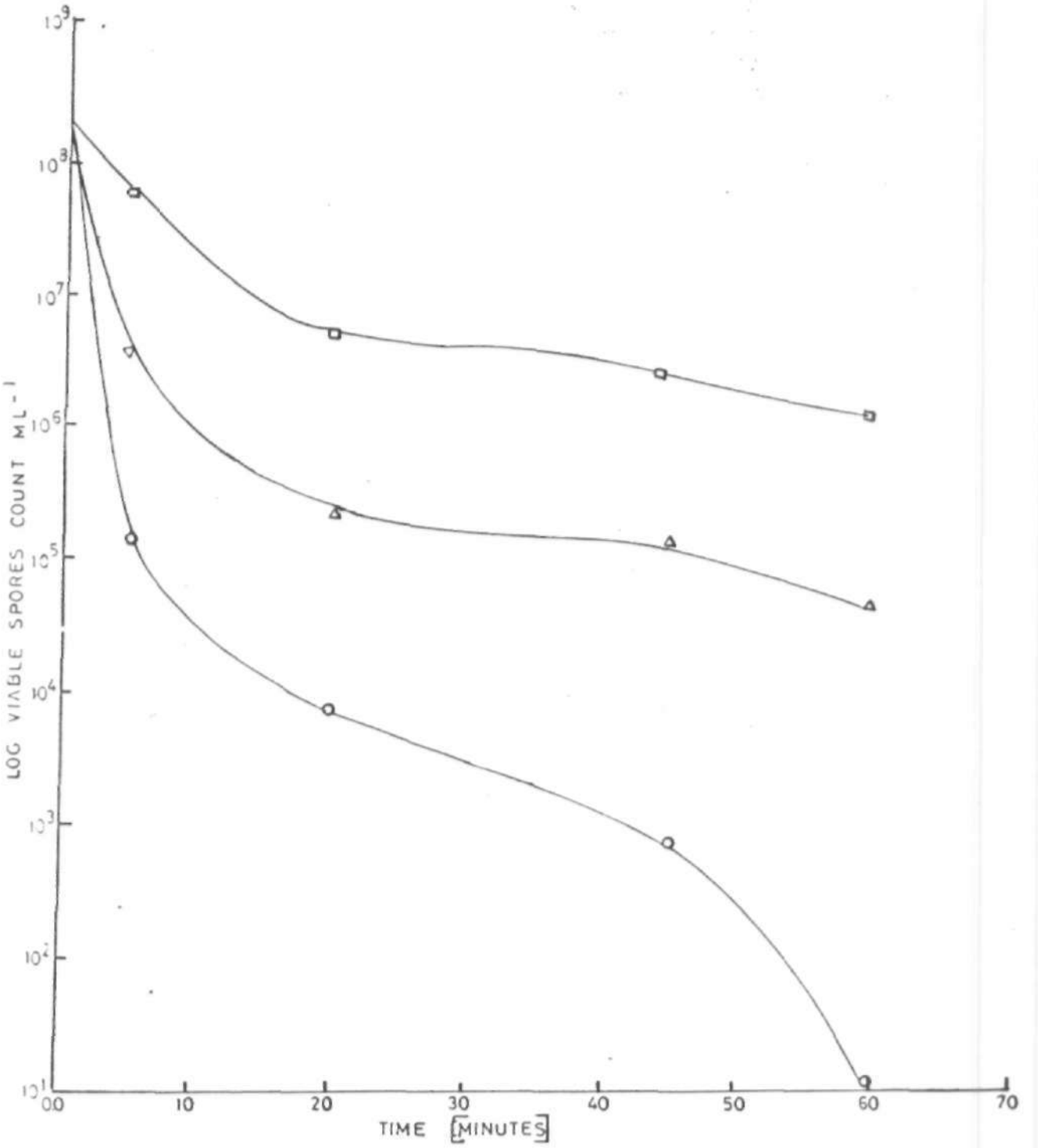



Fig.4.8 : The rate of kill of R. stolonifer spores suspension treated with 0.5 mM of TBTA, TPTA and TPTS in 25% aqueous acetone solution at 27 ± 2°c

o—o TBTA  Δ—Δ TPTA  □—□ TPTS

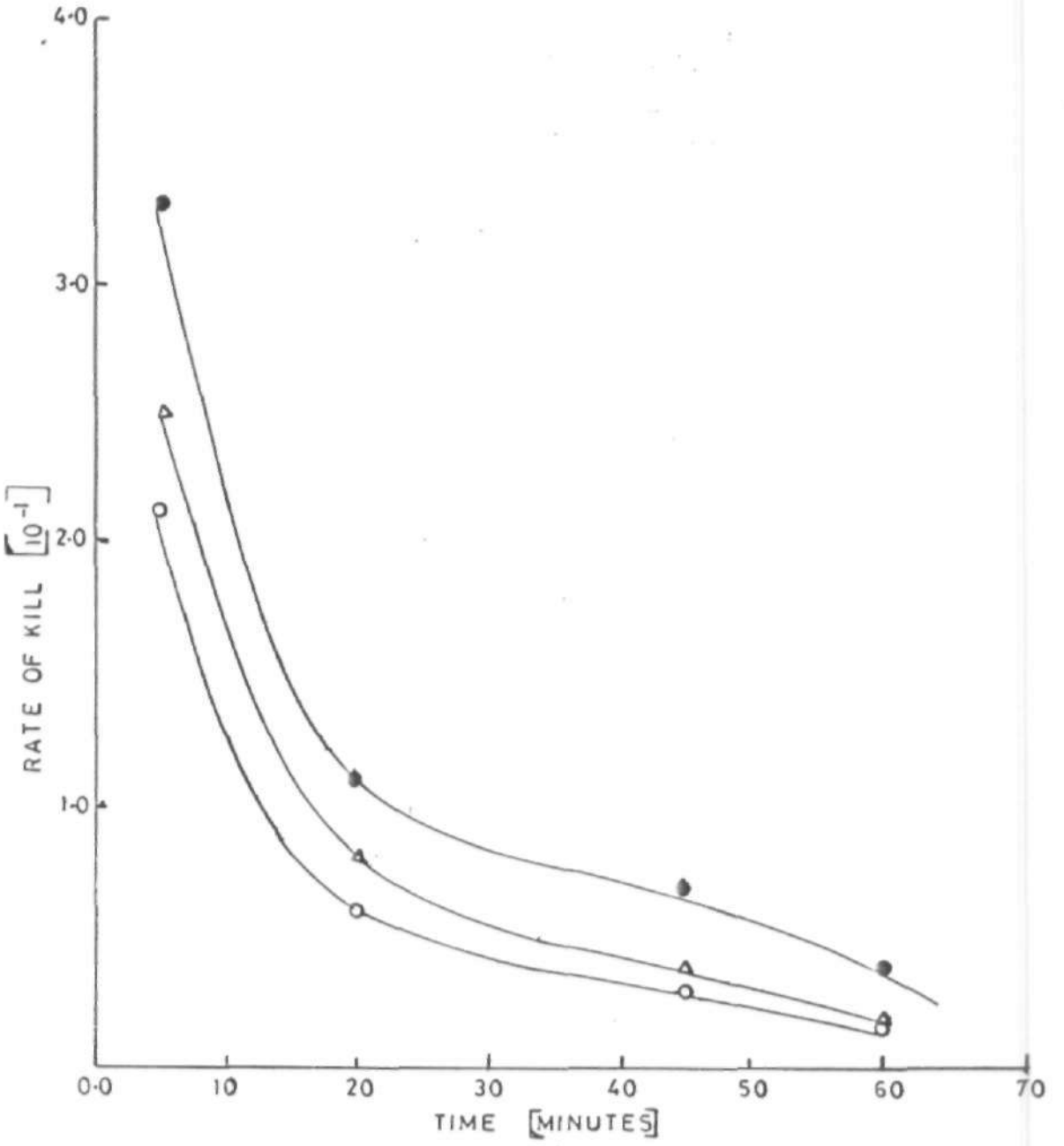



Fig. 4.9: Influence of contact time of TBTA, TPTA and TPTS at 0.5mM fungicidal concentration on K-value for R. stolonifer

●——● TBTA  △——△ TPTA  ○——○ TPTS

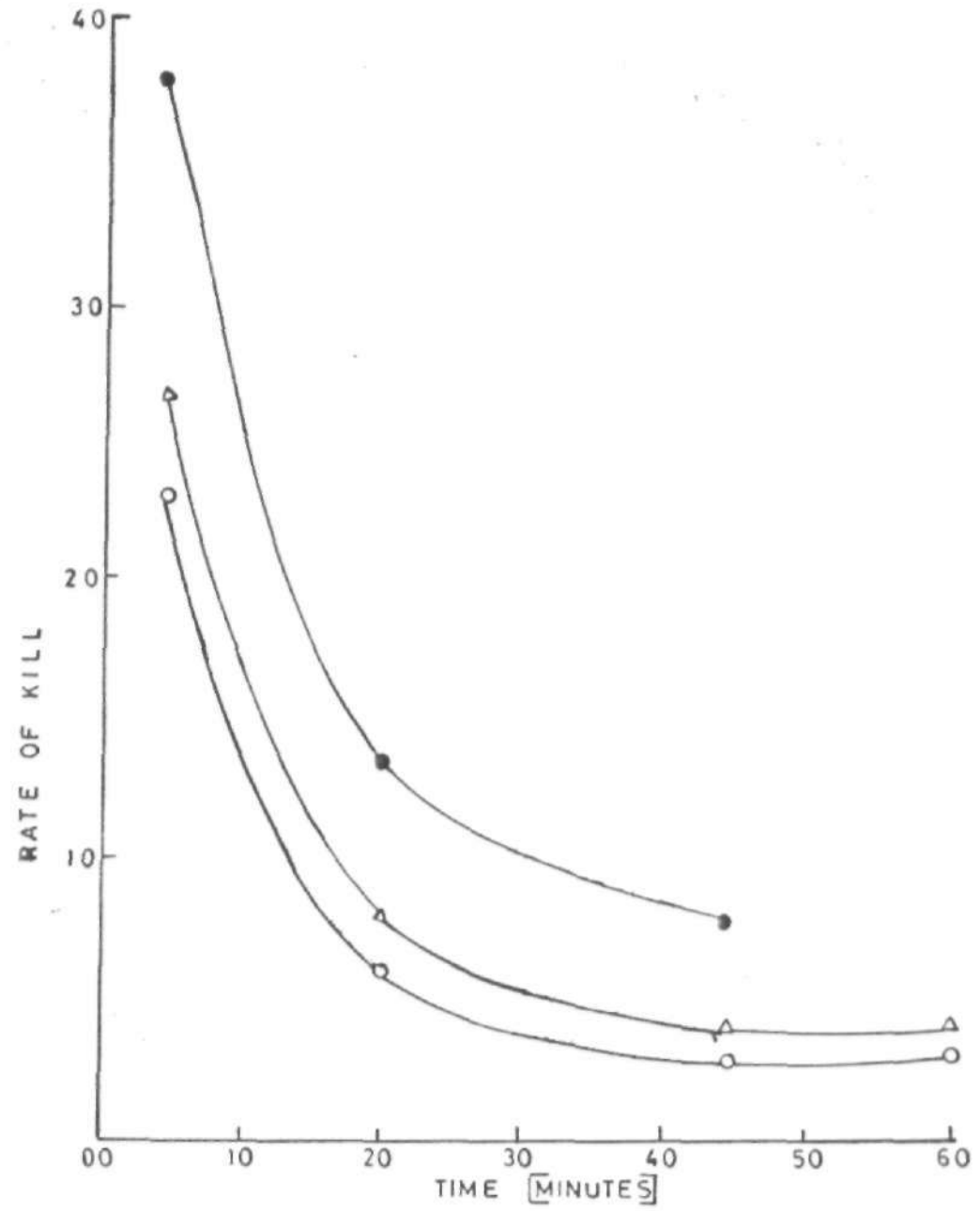



Fig.4.10: Influence of contact time of TBTA, TPTA and TPTS at 0,5mM fungicidal concentration on K-value for A. niger

concentration was in the order of TBTA > TPTA > TPTS as given in figures 4.7 and 4.8.

Table 4.1 showed the rates of Kill of the treated fungal spores after 5 minutes contact time under conditions described above. The death rates, that is, K - values of $10^3$ CFU/ml of spores of test fungi were calculated from the slope obtained from the plots of log survivors versus time according to equation in $K = 1/t[\log N_0/N_t]$ applying to unimolecular reaction.

**TABLE 4.1 RATE OF KILL OF $10^3$ CFU/ml OF TEST FUNGAL SPORES SUSPENSION AFTER 5 MINUTES CONTACT TIME WITH 0.5mM TRIORGANOTIN CARBOXYLATE.**

| CHEMICAL AGENTS | K ---- VALUES | | | |
|---|---|---|---|---|
| | A. flavus | R. stolonifer | P.citrinum | A. niger |
| TBTA | 0.23 | 0.38 | 0.33 | 0.29 |
| TPTA | 0.22 | 0.27 | 0.25 | 0.28 |
| TPTS | 0.21 | 0.23 | 0.21 | 0.26 |

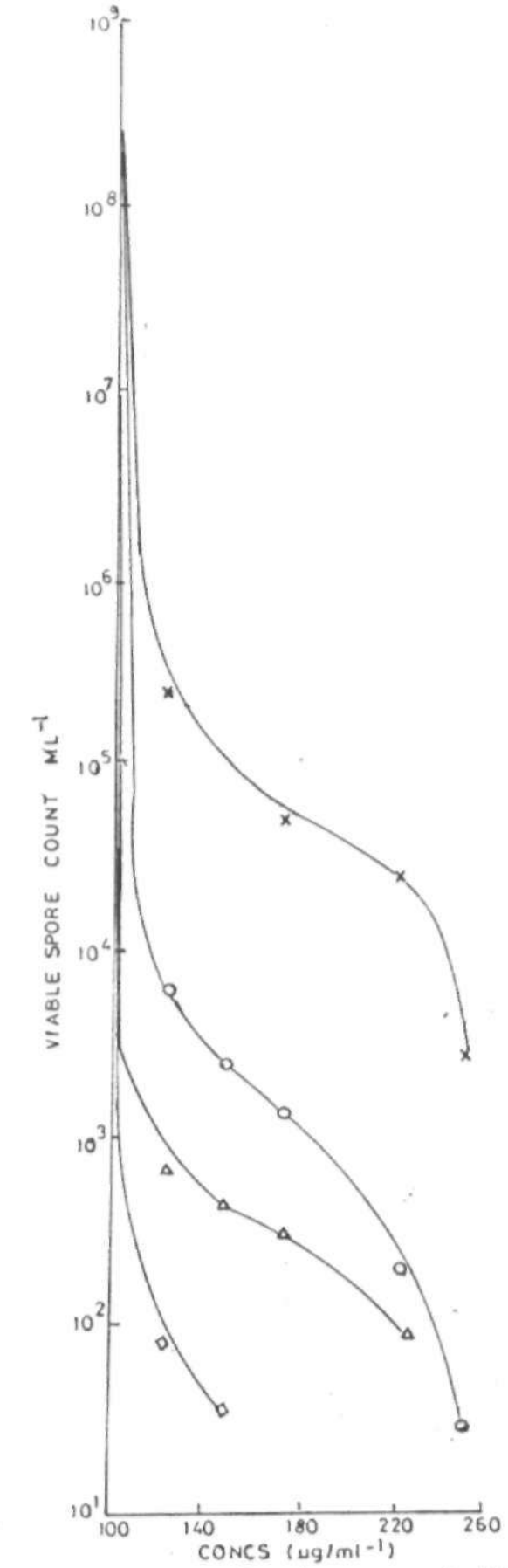
The higher the value of K, the faster the efficiency of the lethal process. The rates of Kill of the test triorganotins generally was observed to be in the order of TBTA > TPTA > TPTS. When the K - values were plotted against time as given in Figures 4.9 and 4.10, concave curves were obtained. The rate of Kill of test fungal spores decreases rapidly with time till after 20 minutes of contact time generally. This time appears to mark the beginning of a very slow rate of Kill for the test triorganotin carboxylates.

### 4.3.3 Effect of changes in concentration of test triorganotins on the viability of fungal spores

Figures 4.11, 4.12 and 4.13 illustrate the effect of variation in concentration of triorganotins on its fungicidal action against spores of the test fungi at different fixed time. A quick glance on the viability curves of the test fungi spores indicate that A. niger and P. citrinum displayed an initial concave shape curve. This shape was however not maintained, but eventually become an inverted or reversed of the usual B type curves Fig. 4.1a with increase in test antifungal agent concentration. However, the response of A. flavus and R. stolonifer to varying concentrations of the test agents showed a concave viability curve, that is, type 'C' curve as illustrated in Figures 4.14, 4.15 and 4.16. Gene-rally, there was an initial rapid Kill of the test fungi spores with increase in fungicidal concentration, which later diminishes with further increase in concentration of the test agents as given in figures 4.17, 4.18 and 4.19.

The severity of the fungicidal activity of the test agents was in the order of TBTA > TPTA > TPTS as shown in figures 4.20 and 4.21. For example, 150 ug/ml of TBTA, TPTA AND TPTS effected about 5.5, 3.0, 175 log cycles Kill of A. niger spores within 20 minutes contact time respectively.

The effect of varying fungicidal concentrations of test agents on K-values are as illustrated in figures 4.22 and 4.23. It was observed that increase in test triorganotins concentrations produced no significant increase in the rate of Kill of P. citrinum spores when compared with the response of A. flavus, A. niger and R. stolonifer. For example, increase in the rate of Kill of P. citrinum with 250 µg/ml and 316 ug/ml of TPTA




Fig·4·11: Influence of various concentration of TBTA on viability on A. niger spores at 25°c

×—×5 minutes ○—○20 minutes △—△45 minutes □—□60 minutes

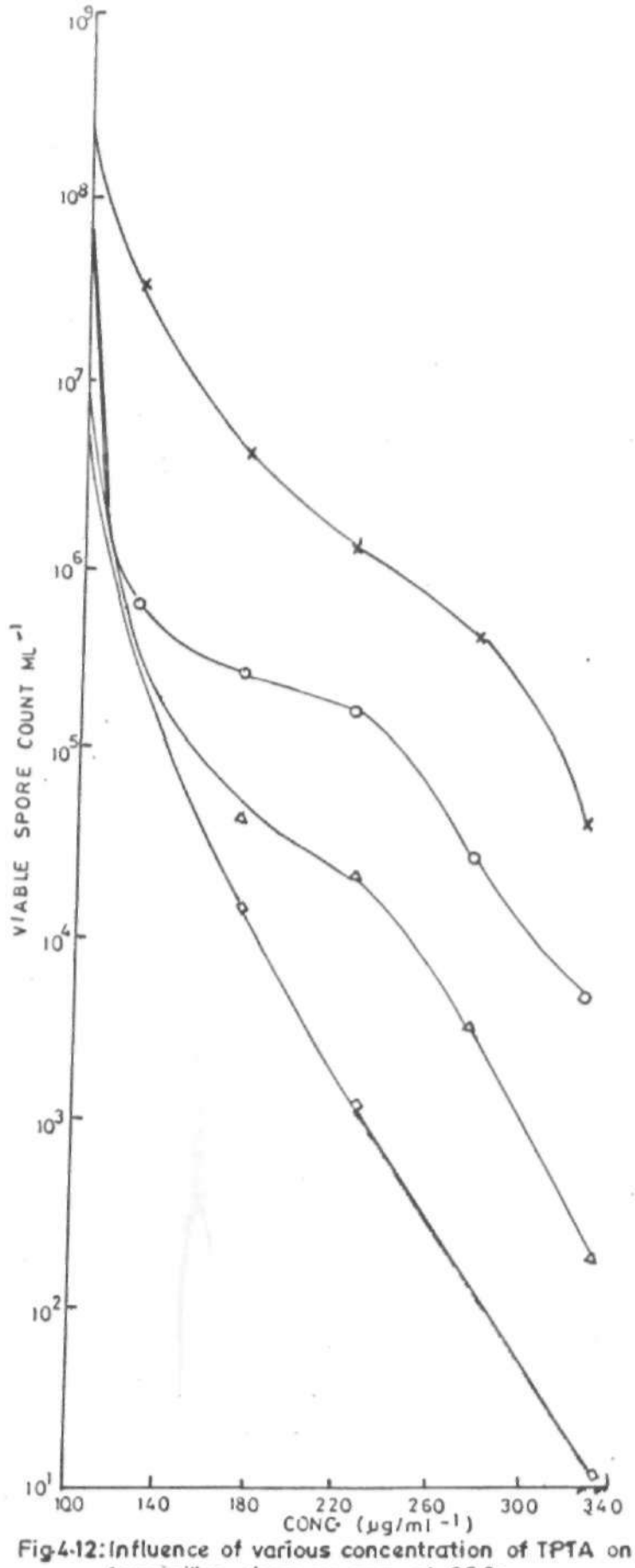
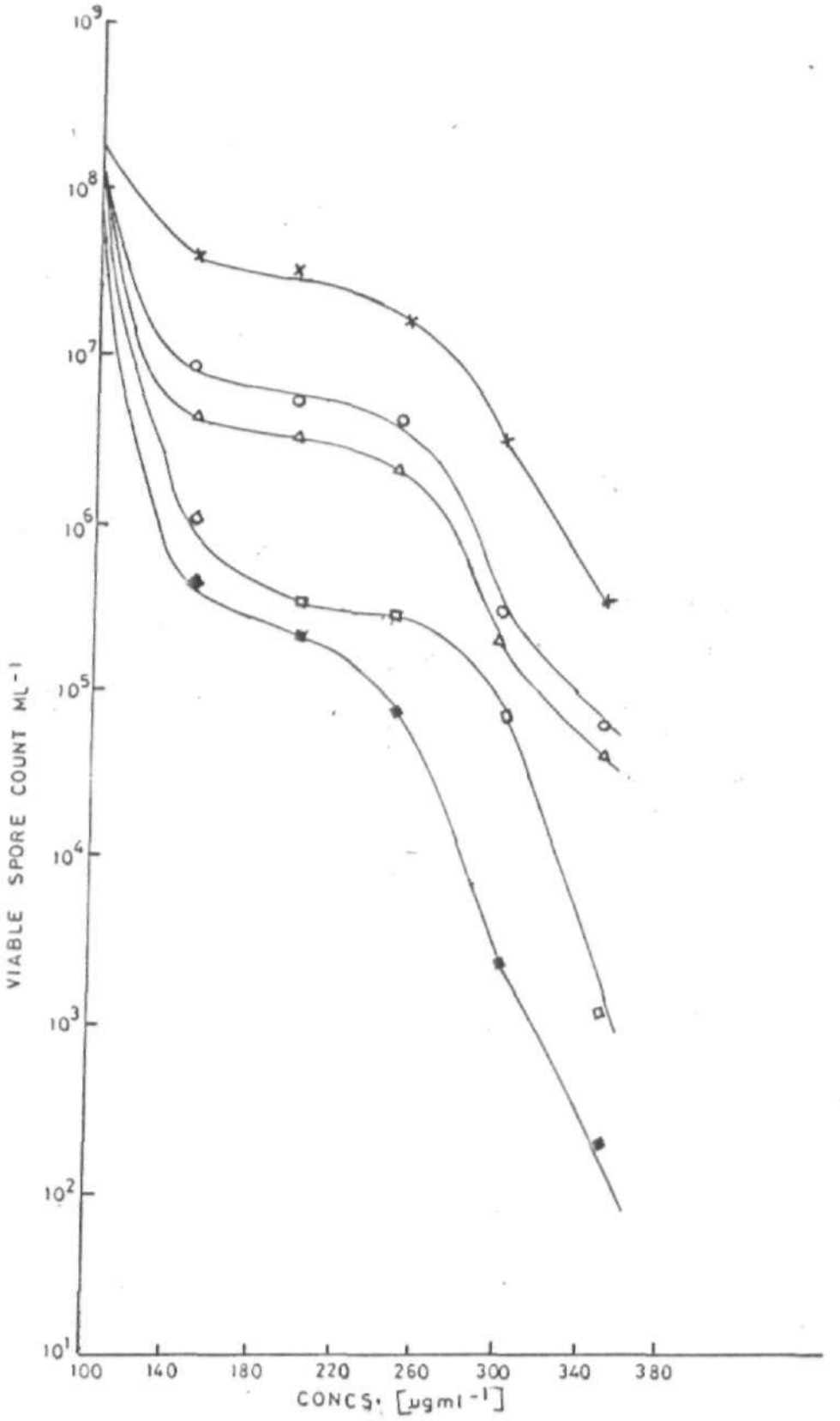
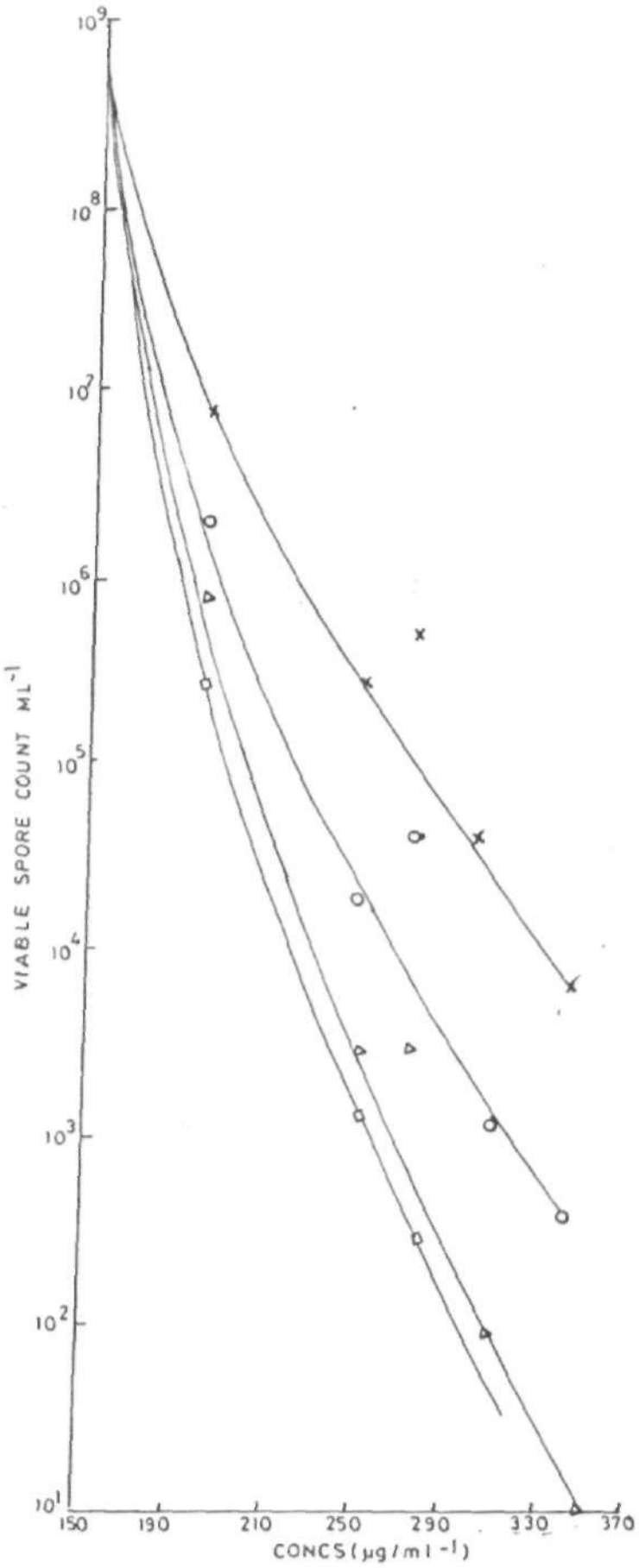



Fig.4-12: Influence of various concentration of TPTA on viability of Aspergillus niger spores at 25°c

×—× 5 minutes o—o 20 minutes ▲—▲ 45 minutes □—□ 60 minutes




*Fig·4·13: Influence of various concentration of TPTS on viability of A niger spores at 25°c*




Fig·4·14: Influence of varying concentration of TBTA on viability of A·flavus spores at 25°c

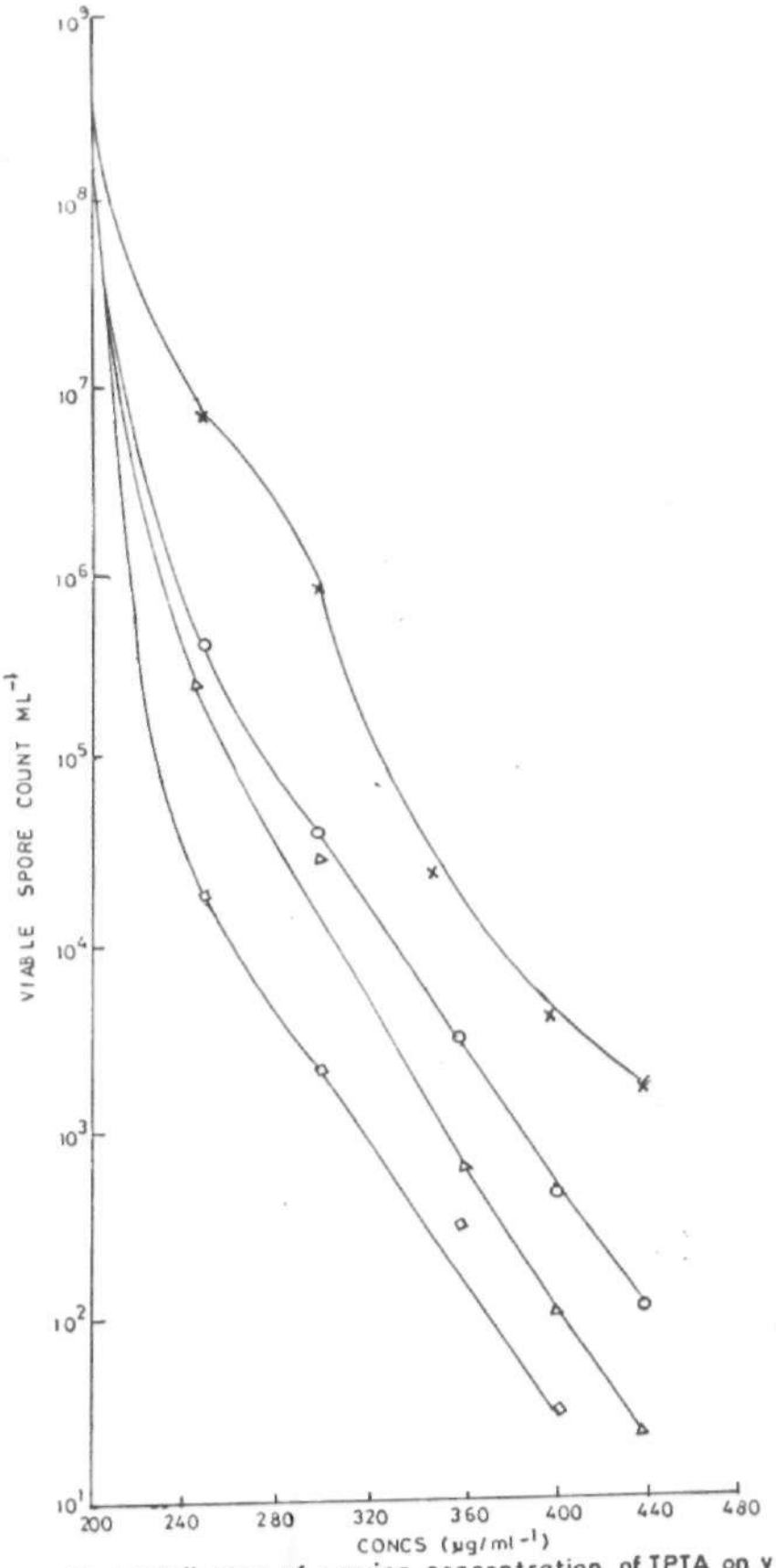



Fig-4·15: Influence of varying concentration of TPTA on viability of A. flavus spores at 25°c

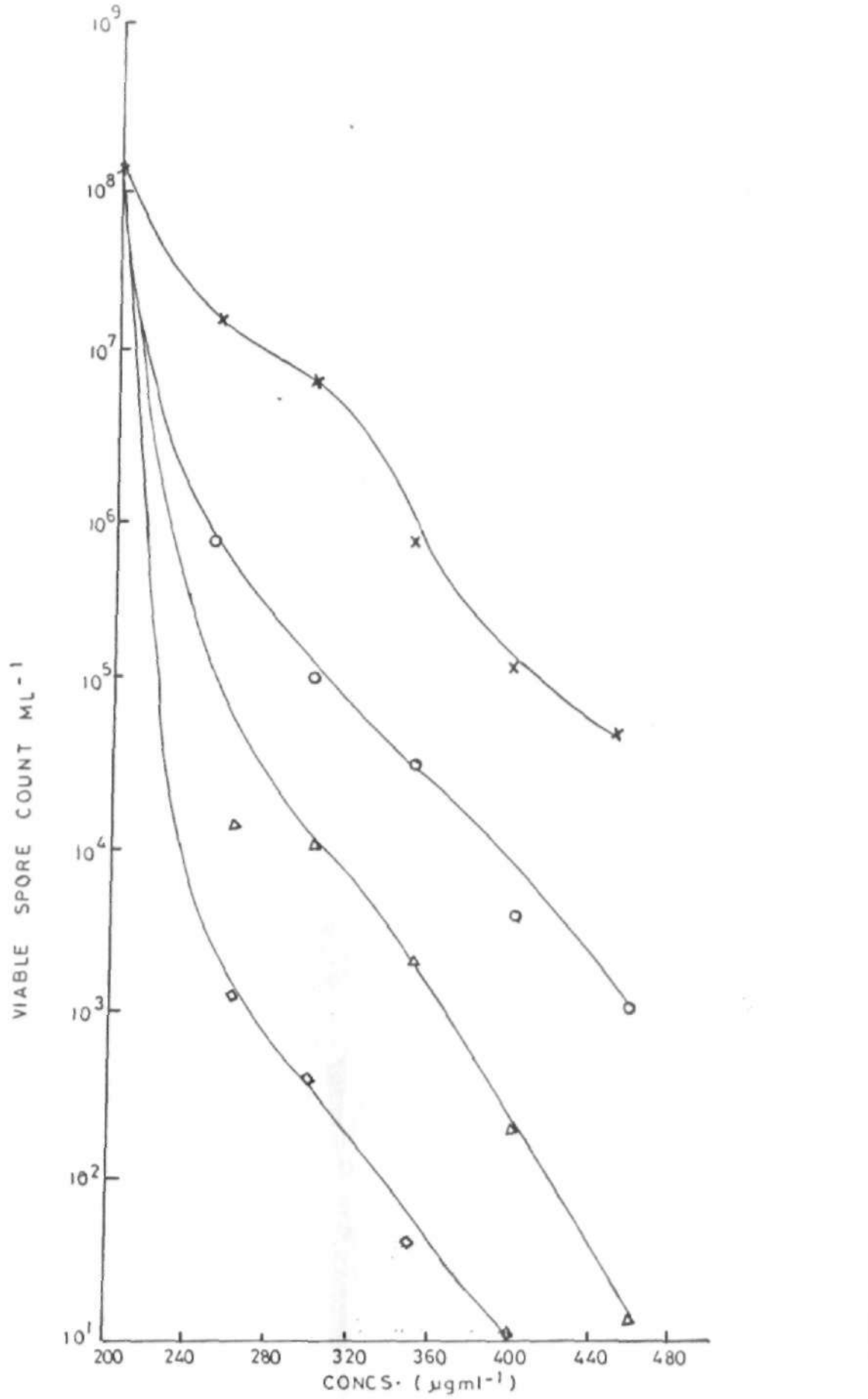



Fig.4.16: Influence of varying concentration of TPTS on viability of A. flavus spores at 25°c

×—×5minutes o—o20minutes Δ—Δ45minutes □—□60minutes

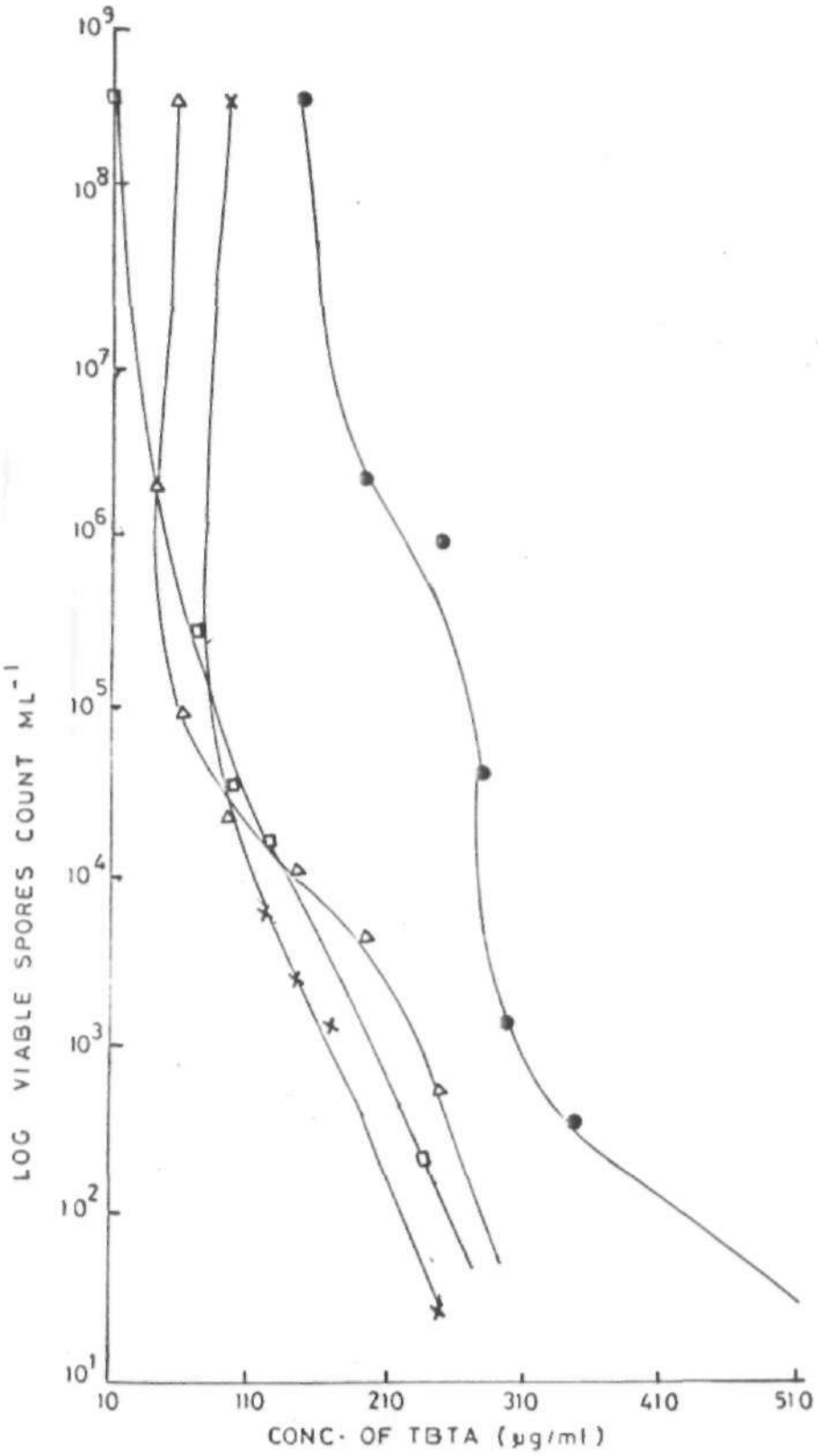
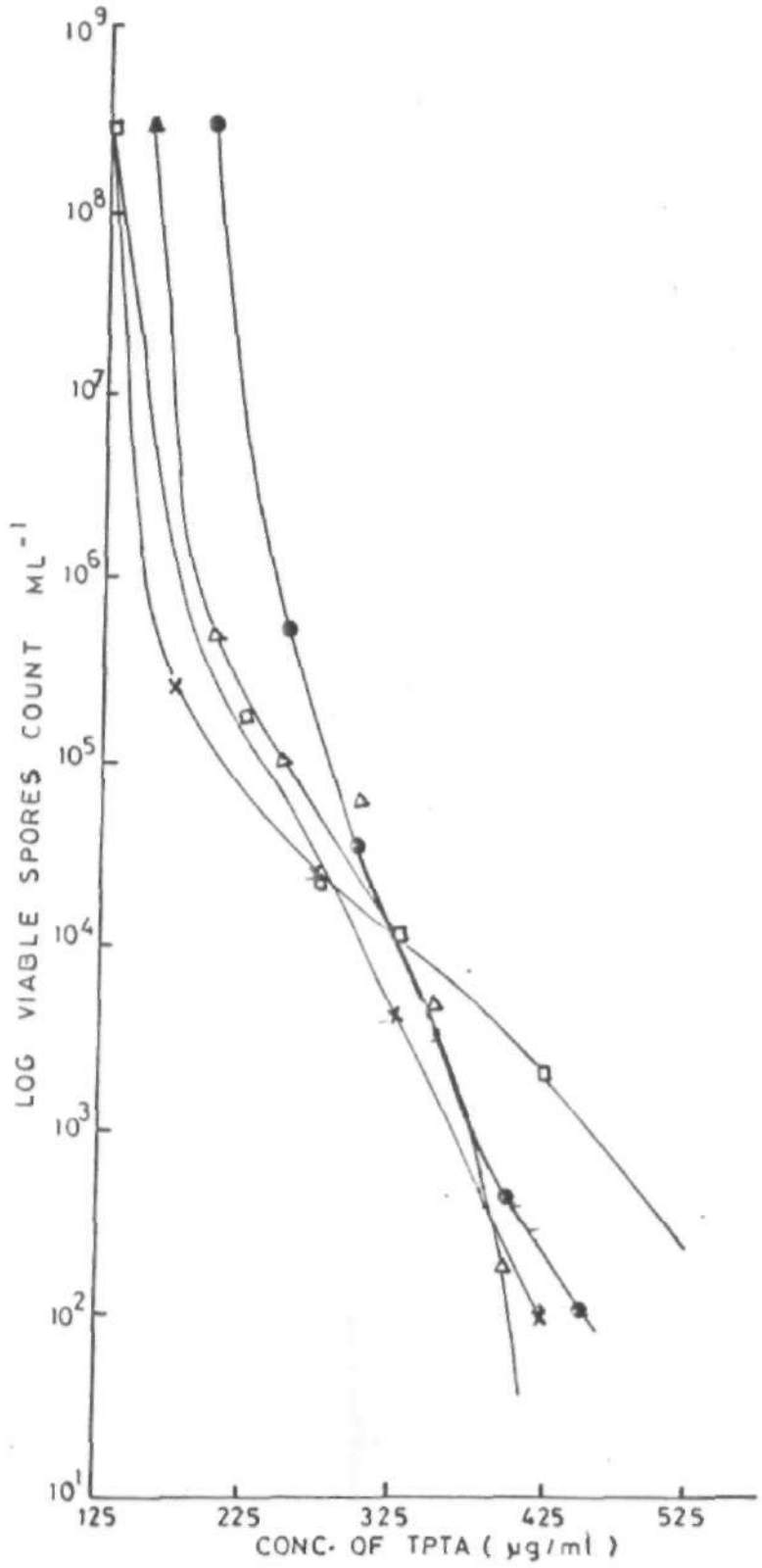



Fig-4.17: Effect of change in concentration of TBTA on viability of test fungi spores after 20 minutes contact time at 27±2°c

●—● A. flavus ×—× A. niger △—△ R. stolonifer □—□ P. citrinum




Fig·4.18 : Effect of change in concentration of TPTA on viability of test fungal spores after 20 minutes acontact time at 27±2°c

●—● A. flavus  ×—× A. niger  △—△ R. stolonifer  □—□ P. citrinum

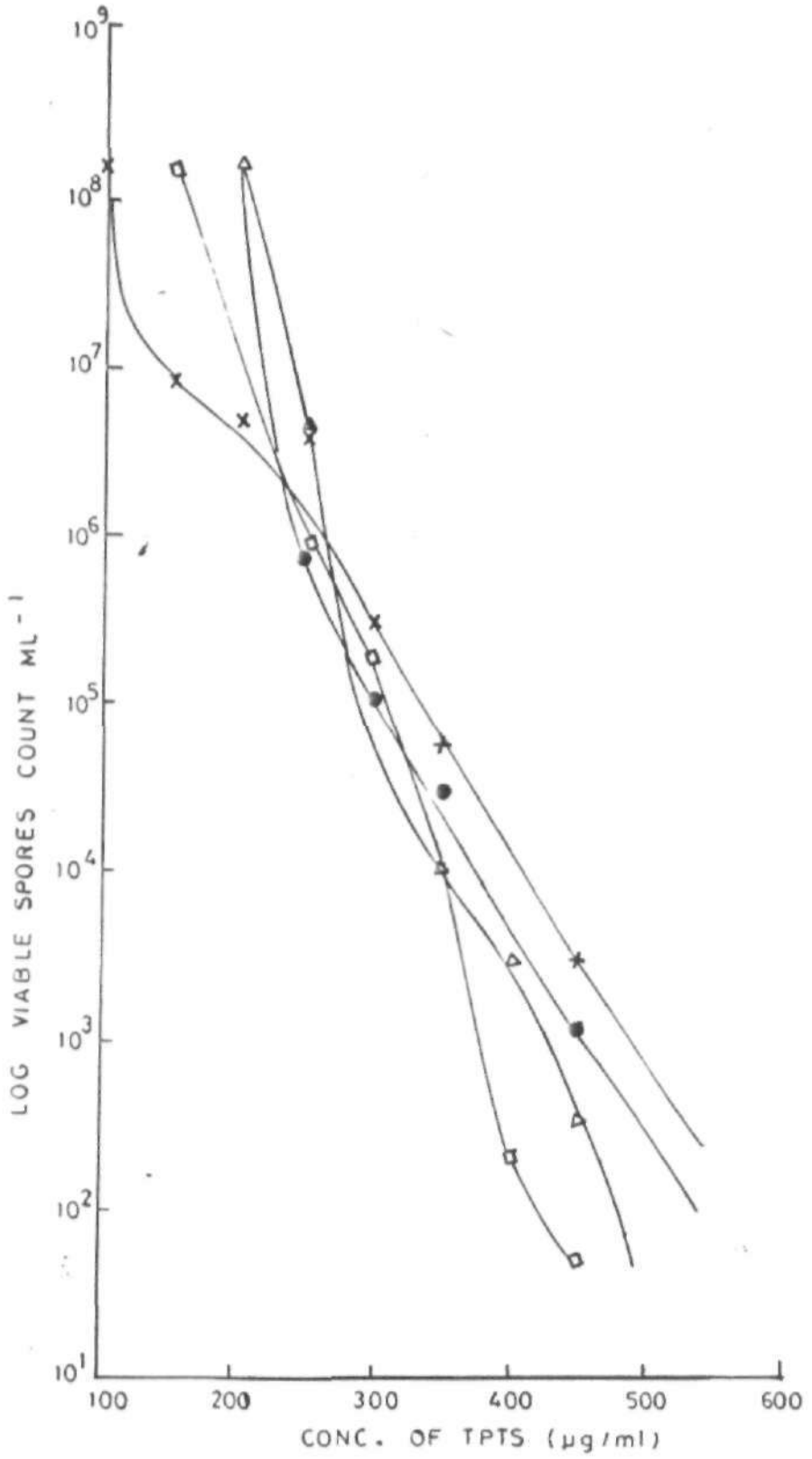
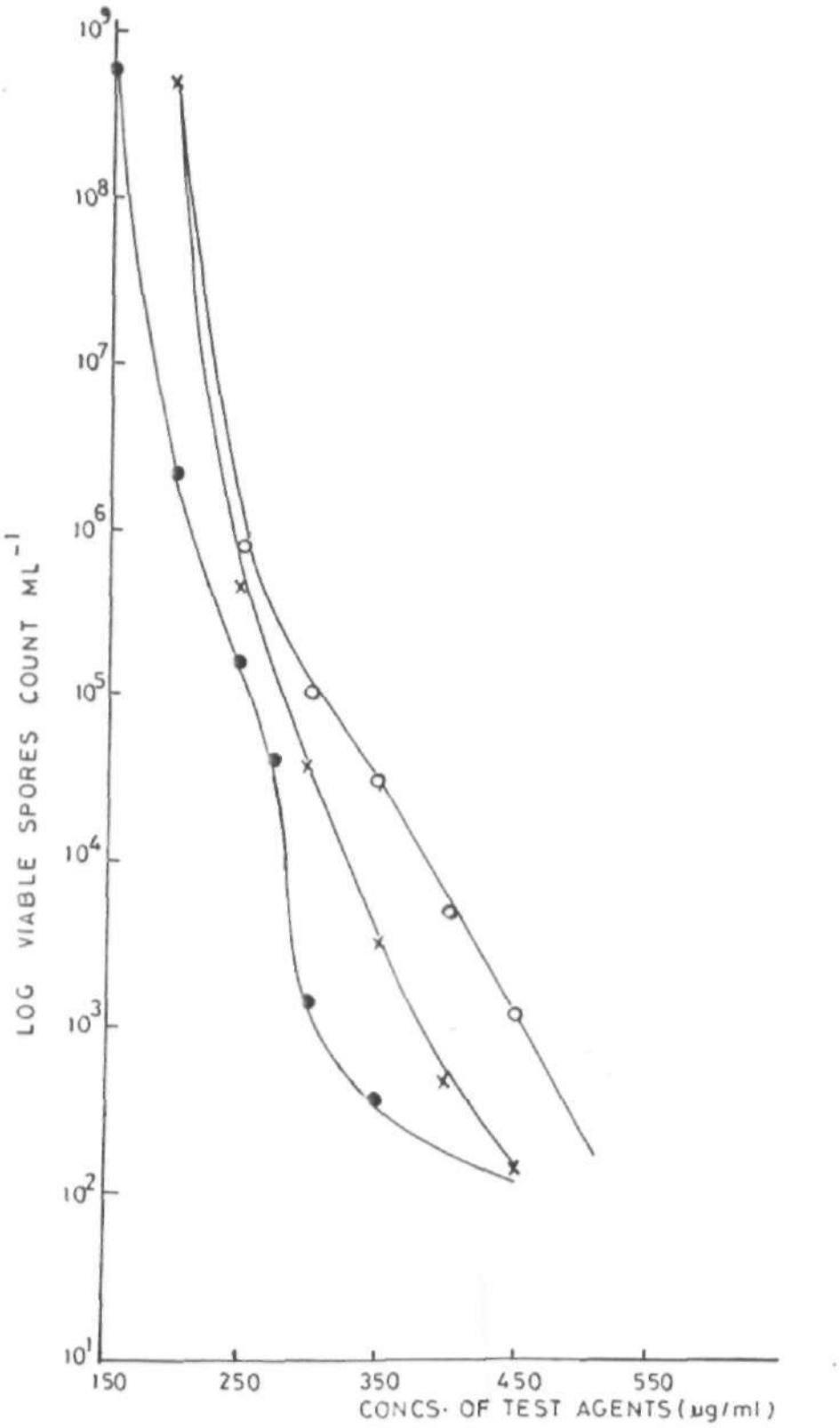



Fig.4.19: Effect of change in concentration of TPTS on viability of test fungi spores after 20 mintes contact time at 27 ± 2°c

●—● A. flavus ×—× A. niger △—△ R. stolonifer □—□ P. citrinum




Fig·4·20: Effect of changes in concentrations of TBTA, TPTA and TPTS on viability of A·flavus spores after 20minutes contact time at 27 ± 2°c

●—● TBTA ×—× TPTA o—o TPTS

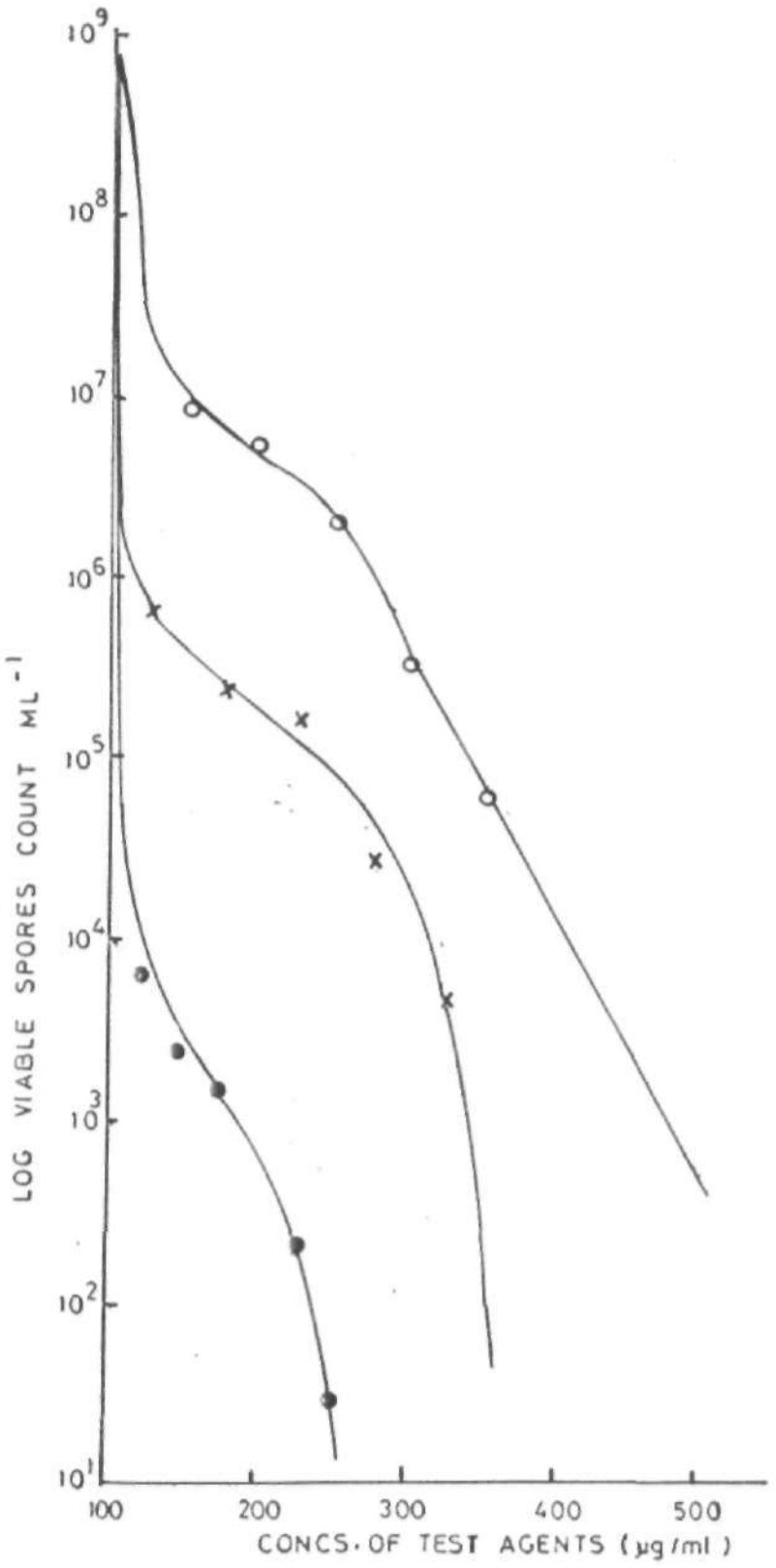



Fig. 4.21: Effect of changes in concentrations of TBTA, TPTA and TPTS on viability of A. niger spores after 20 minutes contact time at 27 ± 2°c

●—● TBTA ×—× TPTA ○—○ TPTS

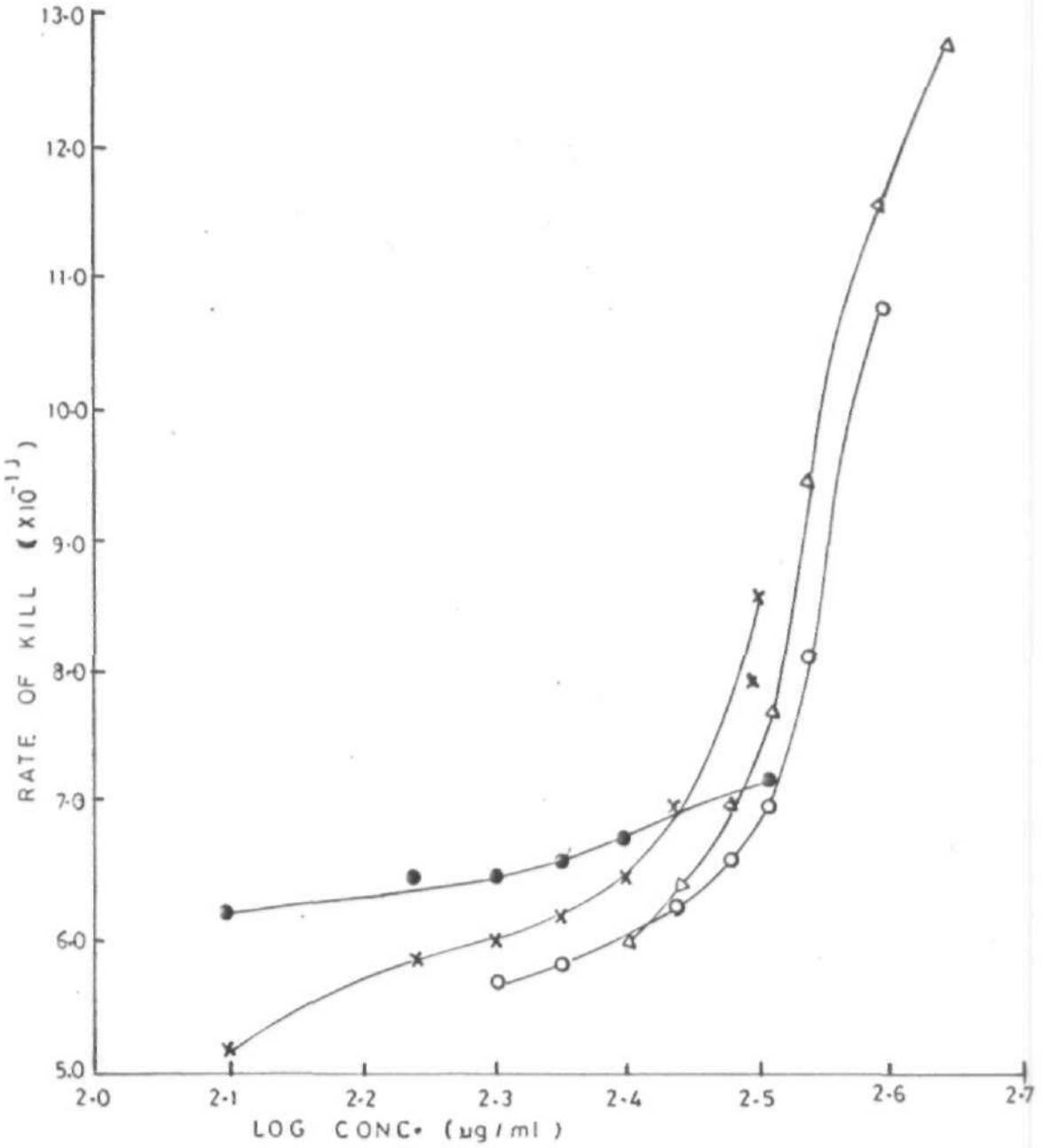



Fig.4.22: Effect of varying fungicidal concentrations of TPTA after 5minutes contact time on K-value of $10^8$ CFu/ml of test – fungal spores suspension at 27 ± 2°c

●—● P. citrinum ×—× A. niger △—△ A. flavus ○—○ R. stolonifer

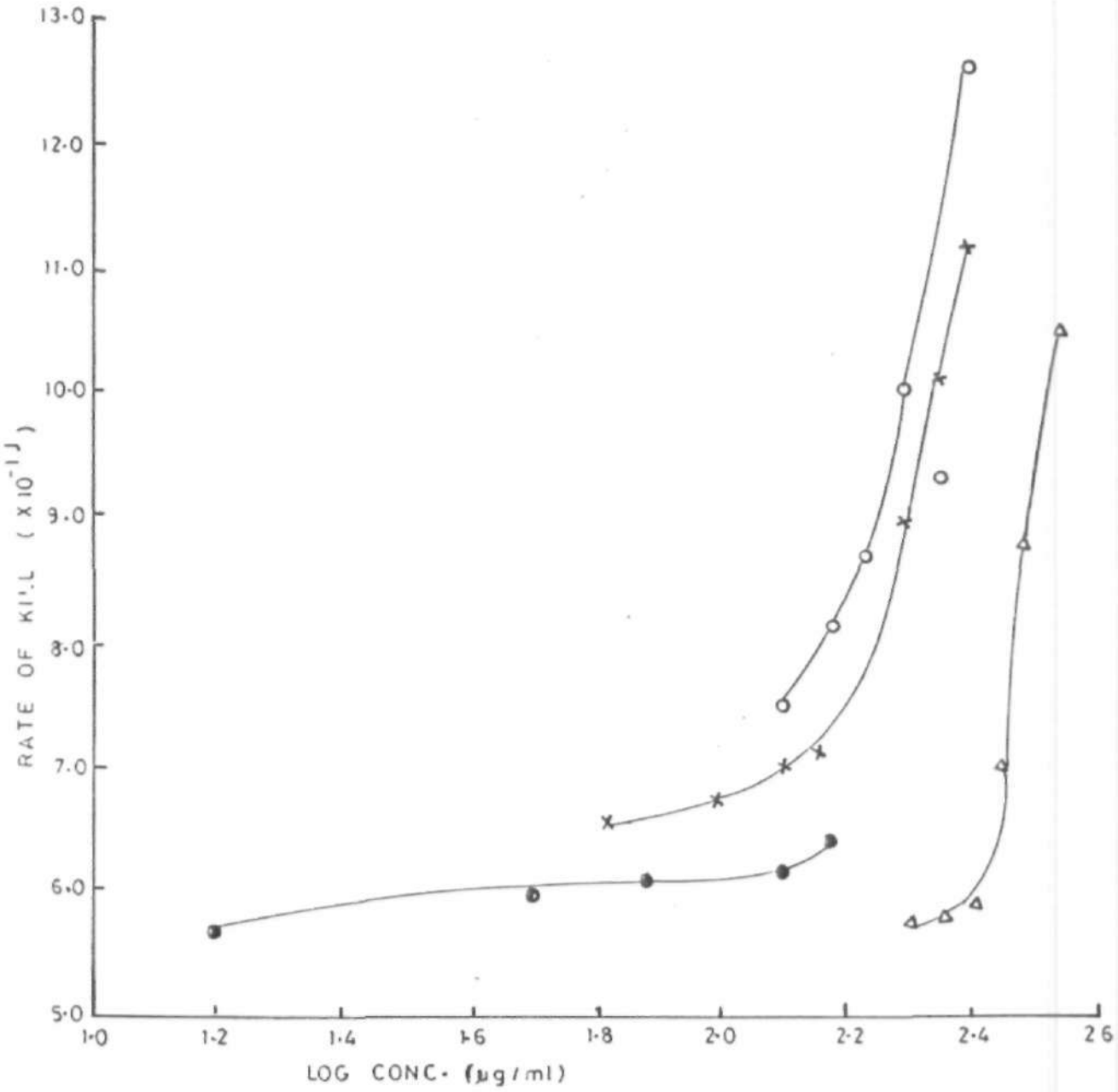



Fig.4.23: Effect of varying fungicidal concentrations of TBTA after 5 minutes contact time on K-value of $10^8$ CFu/ml of test-fungal spores suspension at 27 ± 2°c

o—o A. niger  x—x R. stolonifer  •—• P. citrinum  Δ—Δ A. flavus

### 4.3.4 Effect of Changes in concentration of TPTA on leakage of cytoplasmic constitutent of A. niger spores

The disruption of the integrity of the cell membrane and functions appears to be common features of membrane active compounds or lipophilic compounds which in turn is related to their antimicrobial action (Hugo and Bloomfield, 1971; Cooney and Wuertz, 1989).

The treatment of Aspergillus niger spores with TPTA resulted in a rapid loss of a small amount of 260nm - absorbing constituents from the metabolic pool of the cell. The maximum spore exudates in presence of TPTA was not greater than 5.2% of the total leaked 260nm absorbing materials in the spore (Figure 4.24).

It is worthy of note that seemingly diphasic leakage concentration - pattern was found. Although the maximum material leaked in the presence of TPTA was very minimal (5.2%) compared to the total material leaked from boiled spores. This probably indicates that TPTA may have less capability of disrupting spore membrane or conversely, TPTA caused protein inactivation and or coagulation at much low concentrations which may prevent further leakage. Similar result was obtained when TBTA was the test compound (Figure 4.26).

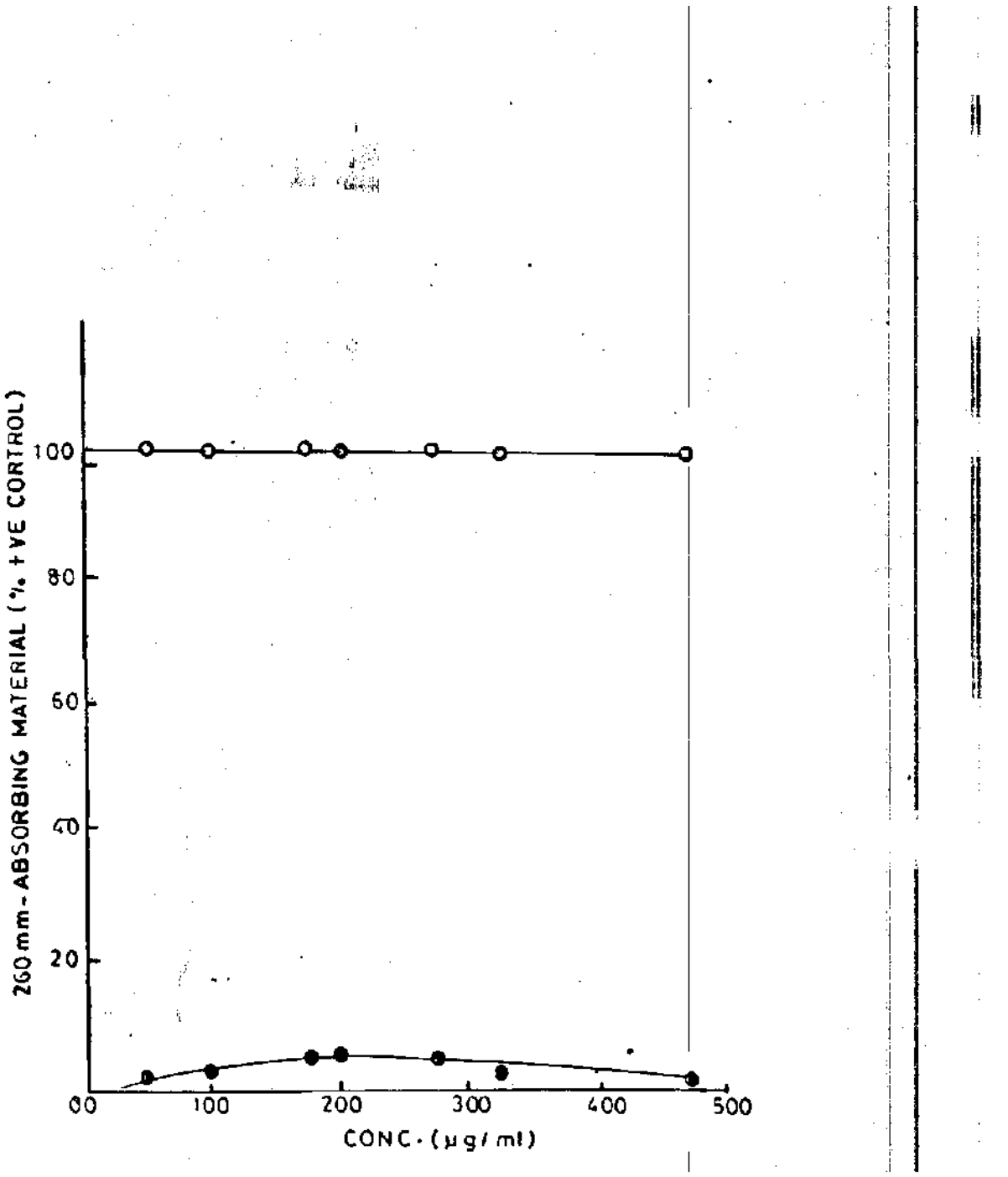
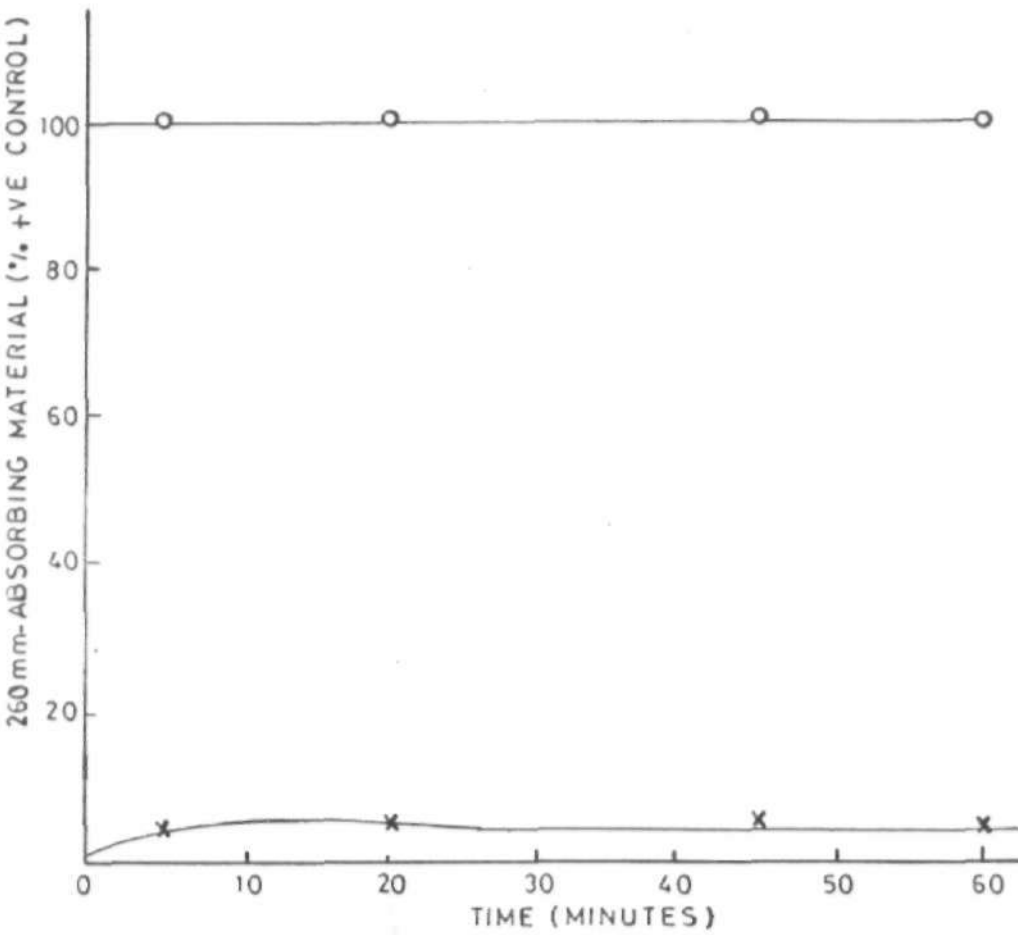



Fig.4.24: Leakage of 260 nm-obsorbing material from A. niger at different concentration of TPTA after 5 minutes contact time

o—o Total 260nm-absorbing material from boiled spores

●—● Leakage of 260nm-absorbing material from TPTA treated spores




Fig·4·25: Effect of 0·5mM of TPTA on leakage of 260mm absorbing material from A-niger spores at different time interval

o—o Total 260nm-absorbing material from boiled spores
x—x Leakage of 260nm-absorbing material from 0·5mM TPTA treated spores

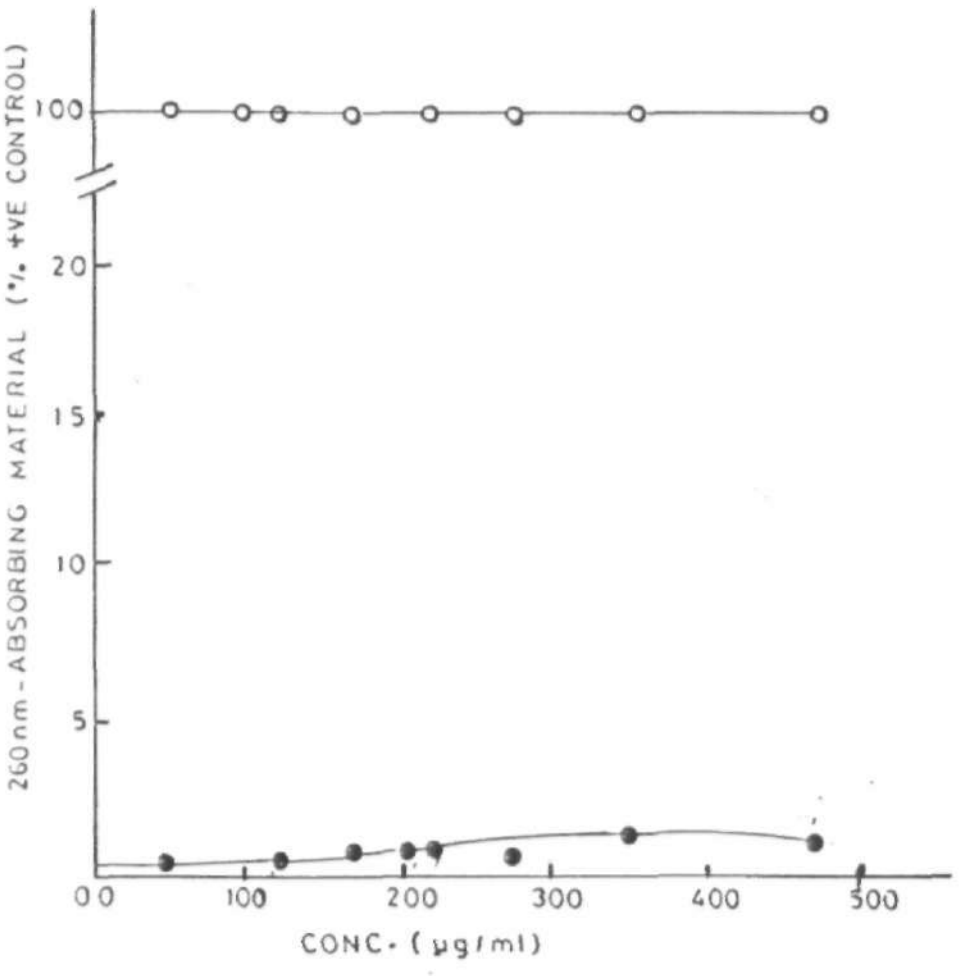
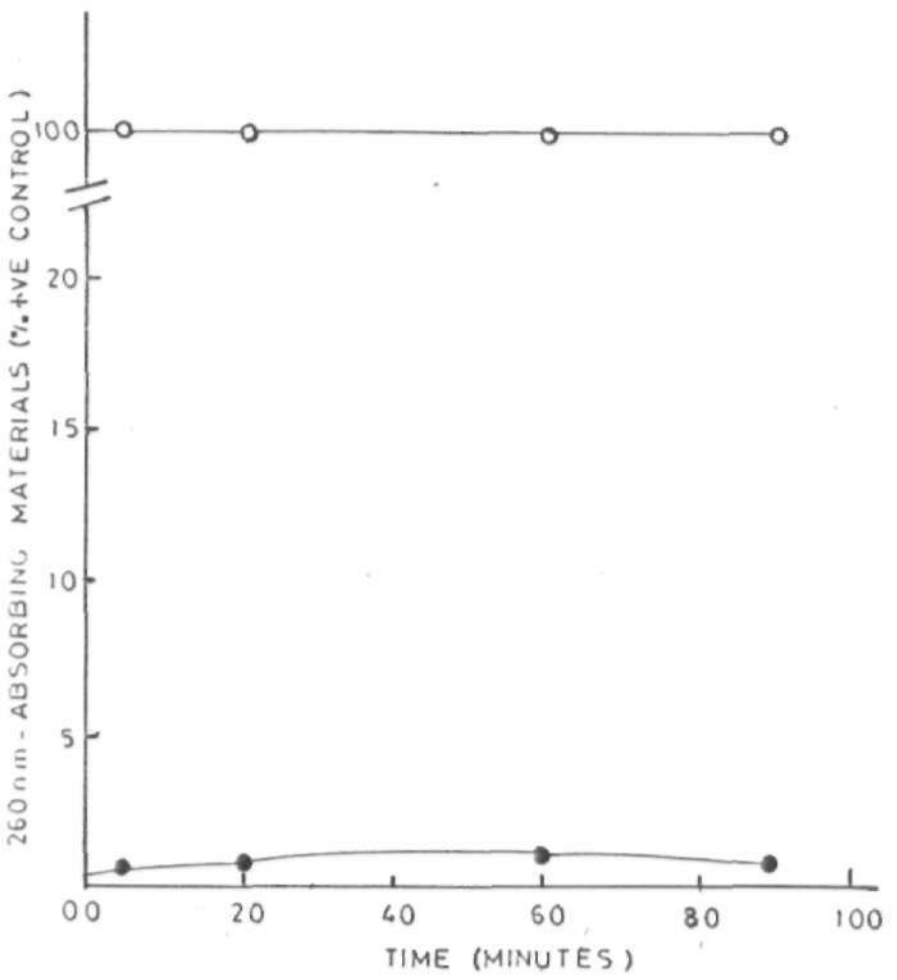



Fig.4.26: Leakage of 260nm - absorbing material from A. niger at different concentration of TBTA after 5minutes contact time

c—o Total 260 nm - absorbing material from boiled spores

●—● Leakage of 260 nm - absorbing material from TBTA treated spores




Fig·4·27: Effect of 0·5mM of TBTA on leakage of 260nm absorbing material from A. niger spores at different time interval

o—o Total 260nm-absorbing material from boiled spores

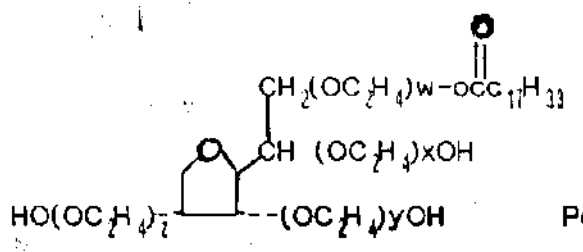
●—● Leakage of 260n m-absorbing material from 0 5mM TBTA treated spores

Although, the exact modes of action of TPTA on spore membrane and the subsequent leakage of spore constituents reported here, can not be mooted from these results alone, it is pertinent to note that TPTA and TBTA produced rapid onset of action on leakage of 260nm-absorbing materials (figures 4.25 and 4.27) which become evident in five to twenty minutes. This is probably a reflection of physical action of TBTA and TPTA on the spore membrane rather than one involving a gradual and progressive incorporation of TBTA or TPTA molecule into the 'metabolic pool' of the test fungal spores.

## 4.4 DISCUSSION

### 4.4.1 Effect of triorganotin on viability

The fungicidal activities of an antifungal agent may be assessed by performing viable spore count at specified time interval on the surviving members of a fungal population in contact with solutions of the agent. The log viability curves given in figure 4.1 established that 300 ug/ml of TPTA exerted virtually it's maximum fungicidal effect on the test fungal spores in 0.05% polysorbate 80. Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate) has been reported to be used



Polysorbate 80

as wetting agent to harvest fungal spores for enumeration (Jarvis, et al, 1983; Mossel, et al, 1983). On the other hand, it has been reported to be

widely employed in antimicrobial experiment as inactivating agents (Dimonde, 1962; Hugo and Russell, 1987, Russell, et al , 1979). Polysorbate 80 inactivating activity has been reported to be due to solubilization or partitioning of the antimicrobial agents between the micelle and water when the critical micelle concentration of polysorbate 80 is exceeded. This solubilised fraction reduced the effective concentration of the antimicrobial agent in the system. Furthermore, the ether oxygen present in polysorbate 80 has been reported to form complexes with antimicrobial agents by hydrogen bonding and dative bonding of its alicyclic oxygen to the tin centre in this case, thereby increasing steric crowding and electron density around the tin, making it less effective as an active site (Zografi, 1975). However, observation in this study has shown that antifungal activities of test triorganotin in 0.05% polysorbate 80 used as wetting agent to harvest fungal spores has no significant effect on fungicidal activity of TPTA and other test compounds (figure 4.1b).

### 4.4.2 Effect of fixed concentration of triorganotin on viability of test fungi spores at varying time

The effects of fixed concentration of test compounds on the viability of spores of test fungi were studied by determining the survivors after some contact period up to 60 minutes.

The test spores when treated with fixed concentrations of triorganotin were killed in a manner similar to the logarithmic order of death which diminishes progressively with time.

Generally, at a contact time of 5 minutes, equimolar concentrations of test triorganotins e.g. 0.5mM of the test compounds achieved one to three and half log cycles reduction of $10^3$ spores/1ml of the test fungi

(figures 4.7 and 4.8). This concentration appears to be a good index of the fungicidal activity of the test triorganotins. This is because, the dosage of a fungicide that gives a desired response such as ED-90 or ED-50 value is commonly used to index antimicrobial potency. Although, the threshold dosage i.e. 100% Kill of microbial population has practical value in chemical disinfection, this is not easily attainable in a physiologically heterogeneous microbial population. The ED-90 value is a wiser choice than the threshold value of 100%, because ED-90 value is not easily distorted by the few extremely resistant spores during fungal chemical control.

### 4.4.3 Death rate constant slope

The slope of the dosage response curve is the ratio of the change in fungi response to the corresponding change in time. Slopes measure potencies of the fungicides under the prevailing experimental conditions.

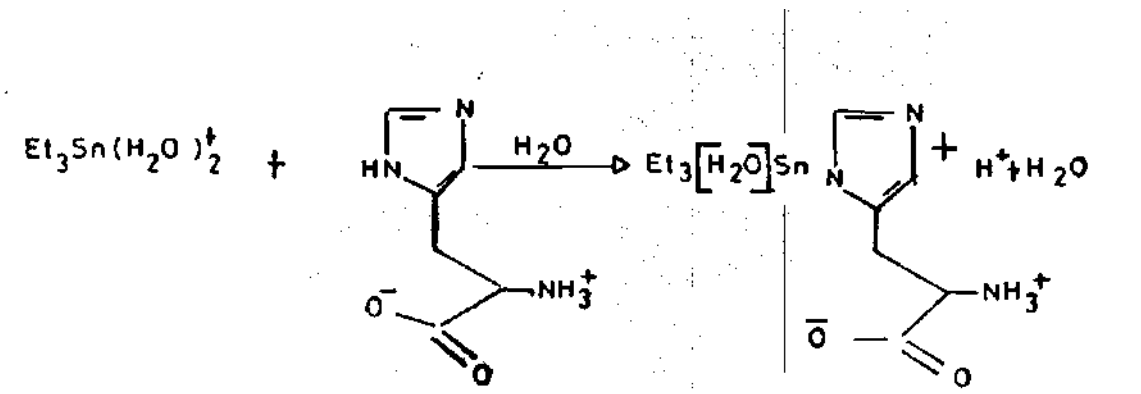
The steepness of a slope is influenced by the fungus, the conditions of the experiment and the fungicide. Slopes of dosage response curves of a fungicide vary with the test organisms. With the same organism, young spores produce steeper slopes than old spores (Dimond and Dugger, 1941). However, the level of inoculum does not affect slope. Nutrient, pH of the medium or any other factors that favour spore survival and growth tends to promote slopes in dosage response curves (Dimond and dugger, 1941., Lukens and Sisler, 1958). Apparently, slopes are steeper when fungal spores respond uniformly under conditions for maximum germination and growth.

Since slope measures potency with changing time, the observed slope will be characteristics of that fungicide which may vary with others. Furthermore, shape of spore survival data, also describe in part the mechanism of the action of the test compound (Horsefall, 1956). Thus, similarity or equal shape of survivor curves suggest that the products of fungi spores responses to the toxicants are equal. The similarity illustrated by the shape of the death curves of TBTA, TPTA and TPTS against all the test fungi spores e.g. R. stolonifer and A. niger in this study, suggest that these test triorganotin compounds have similar mechanisms of antifungal effect. It has been suggested that organotin carboxylates half ionize in aqueous media and the centre of biocidal activity is the tin. (Blunden, et al, 1984; Cooney and Wuertz, 1989, Gibson, et al, 1980). This tin centre is common to all the test compound, hence the similarity in rate of Kill curve appears to be justified.

A plot of the test fungal spores death rate constant (K - values) at 0.5mM of test-agent against time of exposure (figure 4.9 and 4.10) showed initial rapid rate of Kill for these test agents against each test fungal spores. This rapid rate of Kill diminishes with time. This diminishing K - values with time also indicate typical observation in type "C" death curve. This sharpness of death rate curves according to Hugbo (1983) indicate a physical mode of action rather than systematic enzymatic mode of Kill. That is, on contact, or adsorption, there is the possibility of the agent causing physical disruption to the structural make-up or cytoplasmic constituents of the microbial cells or spores leading to leakage or coagulation of the spores or cells constituents (Aldridge, 1976).

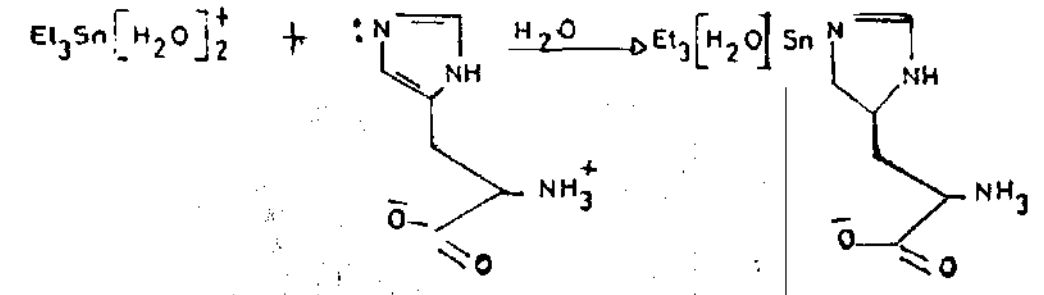
An understanding of the biochemical modus operandi of triorganotin compounds may provide an insight into the fungicidal activity of triorganotin compounds at the molecular level. On a molecular scale, a number of researchers have attempted to identify possible binding sites for triorganotins. Under judicious experimental conditions, biocidal effects of triorganotins can be classified into two broad areas namely: triorganotins effects on (A) protein and (B) cell or mitochondrial membranes.

Aldridge (1976) in his in-vitro study, reported that histidine amino acid present in cat or rat haemoglobin provide two binding sites for triethyltin chloride possibly via N-1 of the imidazole ring or lone pair of electron on the Nitrogen as shown in equation A1 and A2:




Triethyltin ions Histidine 1-triethyltin–imidazole compound

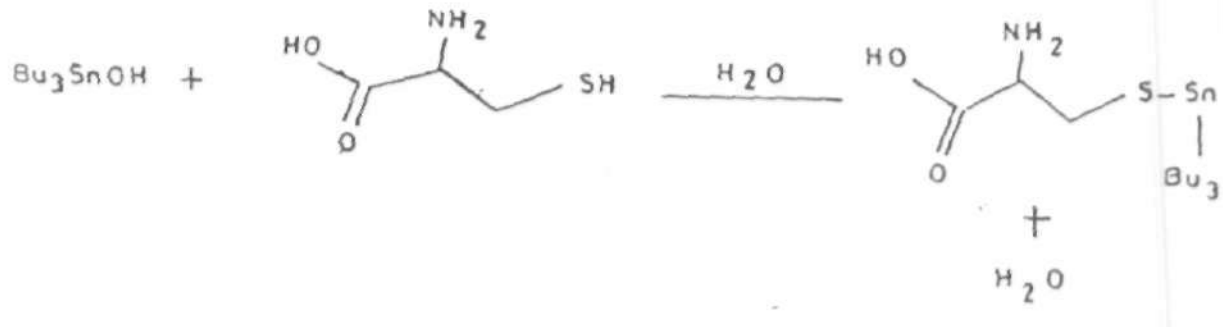
Equation A1




Triethyltin ions Histidine

Equation A2

Sulphydryl group has also been implicated as another possible protein binding site with triorganotins. For example, Tan and co-workers (1978) reported that - SH groups appeared to be responsible for the inhibitory effect of trialkyltins in the colchicine - binding activity due to organo-sulphur complexes formed which was hydrolytically stable with Sn-S-C linkages as




Equation B

Cardarelli (1977) in his in-vitro studies with TBTO and snail protein suggested that mortality of cell may arise from the direct reaction between triorganotins and cell protein. Majority of triorganotins have been reported toxic, because they combine with the specific amino acid such as histidine and cysteine in cell protein, thereby inactivate cell protein function at room temperature and physiological pH of the cell (Cardarelli, 1977).

In addition to triorganotins cell protein inactivation or coagulation, triorganotins have been reported to interfere with the functions of cell membrane and vital organelle membrane like mitochondria. Triorganotin by their chemical nature are known to be lipophilic. Their solubility in cell membrane decreases with increase in carbon chain or molecular weight. When triorganotin is in contact with the cell, it is believed to reside in the lipid bilayer and form $R_3SnOH$ and $R_3SnA$ (A=anion) which have both aqueous and lipid solubility as shown in figure 4.28.

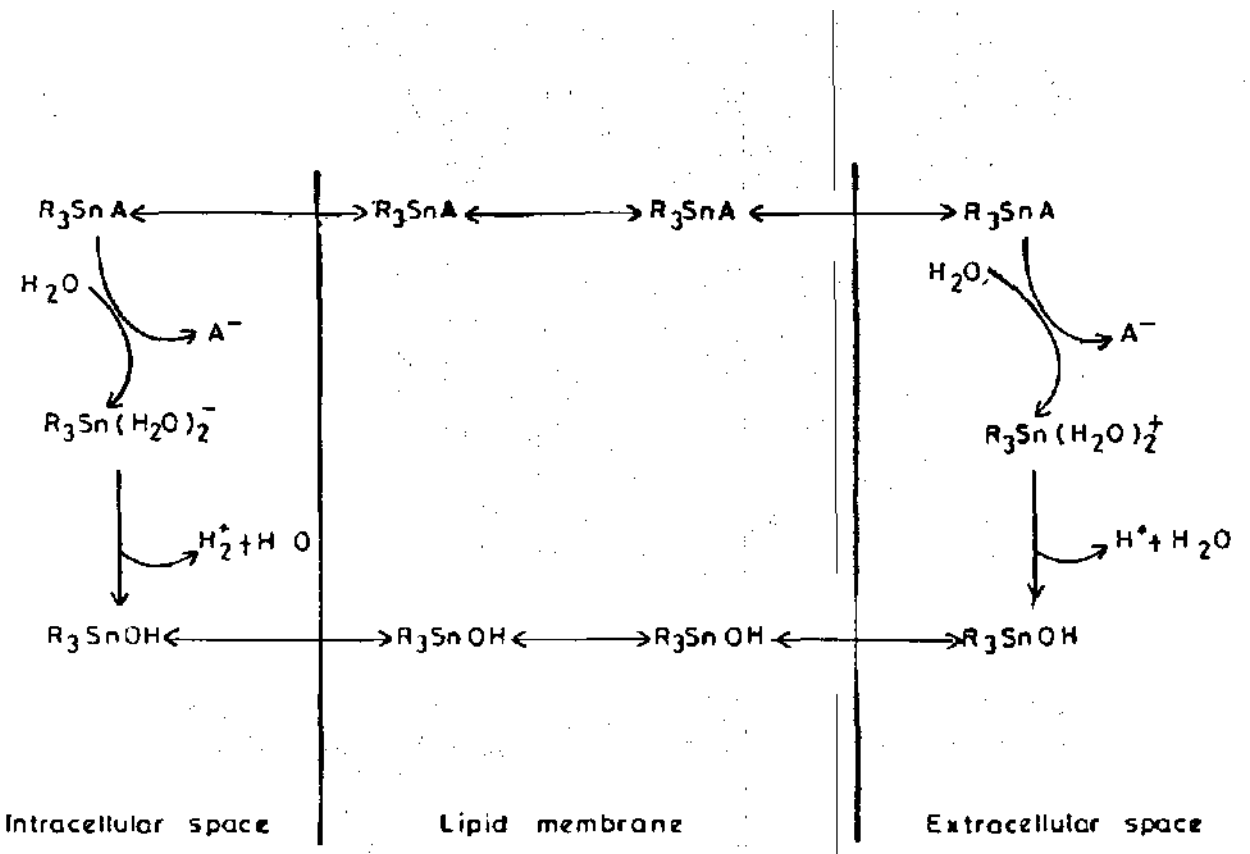



Figure 4-23 : Proposed mechanism for the trans-membrane anion-hydroxide exchange mediated by triorganotins (Molloy, 1989 )

The self-aggregation of triorganotins in the lipid bilayer encourages lipid intercalation which disrupt the lipid bilayer integrity. The intercalation of lipid bilayer results to swelling of cell and mitochondrial membranes and subsequent damaging effects to their functions. Aldridge and Street (1964) in their study on the effect of trimethyltin chloride on rat mitochondria suggest that trimethyltin chloride promote the exchange of $Cl^-$, $Br^-$, $SCN^-$, $I^-$ and $F^-$ with $OA^-$ across phospholipid membranes with resultant clamps of trans-membrane pH differences. These concomitant shift in the pH optima of the membrane enzymes result to uncoupling of electron transport phosphorylation and the enhanced movement of a variety of ions.

Furthermore, Cooney and Wuertz (1989) studied the effect of TBTO on Candida albicans and suggested that the fungicidal effect of TBTO may be due to leakages of $Na^+$, $K^+$ ions and other cytoplasmic constituents as a result of physical disruption of cell membrane.

Result from the effect of fungicidal concentrations of test triorganotins appears to be concentration dependent. The maximum amount 260-nm absorbing materials (5.2%) leaked from Aspergillus niger, the most sensitive test fungi spores, appears to be insignificant to warrant the rate of the test fungi spores death observed in this study (Figure 4.22). Although, this leakage may contribute to the test fungi spores death, the main other factors may be due to inactivation or coagulation of spores vital proteins in addition to derangement of mitochrondrial membranes functions.

### 4.4.4 Effect of change in concentration of triorganotin on spores viability

The result shown in figure 4.11, 4.12, and 4.13 illustrate the effects of varying fungicidal concentration of TBTA, TPTA and TPTA on viability of A. niger and P. citrinum which was similar to that of TPTS to A. flavus and R. stolonifer. These results appear to indicate that the spores of these fungi were not physiologically homogeneous in their behaviour toward the test triorganotins. For example in figure 4.13, there was an initial rapid Kill of A. niger spores up to 150ug/ml. Subsequent increase of TPTS concentration witnessed a decrease in rate of Kill until 260 ug/ml was attained. This pattern of spores viability response to test triorganotins seems to conform with the observation of Mouton (1977) that a non-physiologically homogeneous microbial populations tend to produce inverted sigmoid curve with increase in concentration of test antimicrobial agents. This sigmoid curve observed has been reported to be common to many biological responses to antimicrobial agents (Bliss, 1957). However, the response of A. flavus and R. stolonifer to TBTA, and TPTA was similar to that of A. niger and P. citrinum to TPTS particularly after 20 minutes exposure (figures 4.14, 4.15 and 4.16). This concave or type 'C' curve observed suggest a uniform behaviour of the test fungal spores to the test compounds. It is a rather surprising occurrence that such a wide range triorganotins concentrations produce such similar effects on spores viability.

When rate of Kill or K - values of P. citrinum was plotted against varying concentration of test agents (figure 4.22 and 4.23) increase in fungicidal concentrations examined produced no significant increase in its lethal rate. This observation may indicate inability of test-agent to reach



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the target sites within P. citrinum spores probably due to spore structure differences or other factor that may delay fungicidal effect irrespective of concentration used.

### 4.4.5 Comparative antifungal activity of TBTA, TPTA and TPTS

Tributyltin acetate (TBTA) was chosen for comparison with TPTA and TPTS not because of any structural relationship (I) but only because it is a triorganotin carboxylate with potent antimicrobial effect (Van der Kerk, et al, 1954).

The results of the fungicidal activities of TBTA, TPTA and TPTS at fixed and varying concentrations indicate that TBTA was the most active compound while TPTS was the least fungicidal agent (figures 4.7; 4.8; 4.20 and 4.21). These observations from this study appear to indicate that the order of antifungal activity of test compounds was TBTA (349.1), > TPTA (408.36) > TPTS(487.09).

It has been shown that increase in molecular weight or carbon number of triorganotin decreases it fungitoxic effect, due to steric encumbrance and solubility in lipid problem. (Sijpesteijn, et al, 1969).

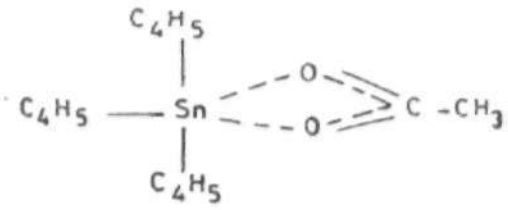
In addition to these stated reasons, it is thought that TBTA ionizes better in aqueous medium than either TPTA or TPTS. This is because tributyl has intrinsic property of donating electrons to the anion i.e. acetate thereby enhancing higher degree of ionization of TBTA unlike Triphenyl which can only withdraw electrons from its anion. Exposure of the tin centre by ionization, enhances it fungicidal activity. Thus, this variation in degree of ionization may be another possible explanation for the observed order of fungicidal activity of TBTA > TPTA > TPTS against

test fungi spores in aqueous medium.

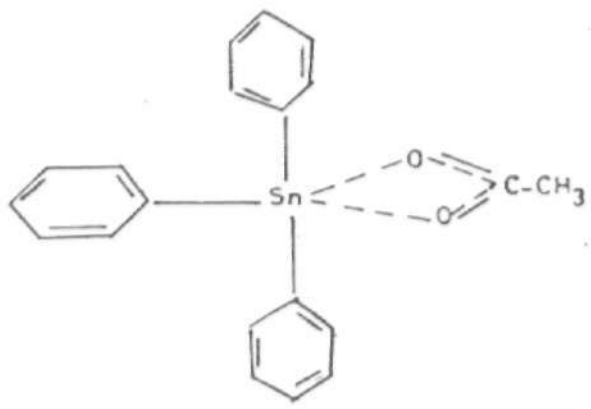
Apart from differences in molecular weight, comparative activities of TPTA (II) and TPTS (III, IV) would be interesting because of their structural similarity.

Two structures (III and IV) can be postulated for TPTS, tin being 5-coordinate in III and 6-coordinate in IV. Although the structure IV would be unlikely in highly diluted 25% acetone solution, where tin is the centre in activity, the presence of 2- OH in TPTS could make its biological activity differ from that of TPTA. The results of this study as shown in figures 4.7; 4.8; 4.20 and 4.21 and table 4.1 indicate that intra-molecular coordination enhanced by the presence of 2-OH on TPTS may have also contributed to the slight reduction of antifungal activity of TPTS as observed compared to that of TPTA.

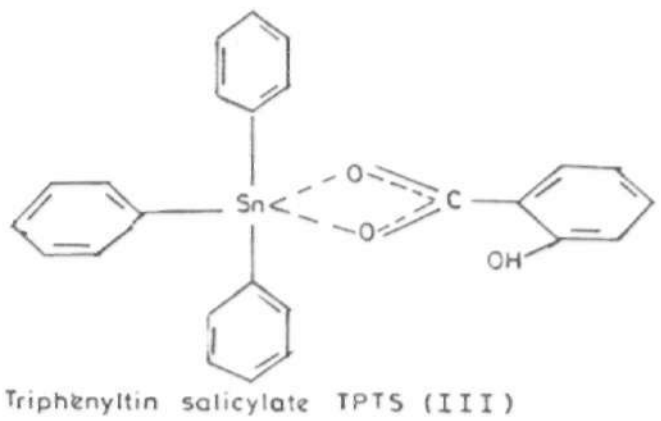
These molecular or structural differences of the test triorganotin carboxylates do not affect the pattern of the sensitivity of the test fungi spores. For example, a comparison of the sensitivity of all the test fungi spores to 0.5mM test triorganotins at varying specified time intervals shows that A. flavus was the least sensitive of the test fungi to all the test agents. This finding probably indicate the consistency in the mechanism of action of the test agents on the fungal spores irrespective of the species; the target site for each agent remain the same as discussed in the body of this thesis.



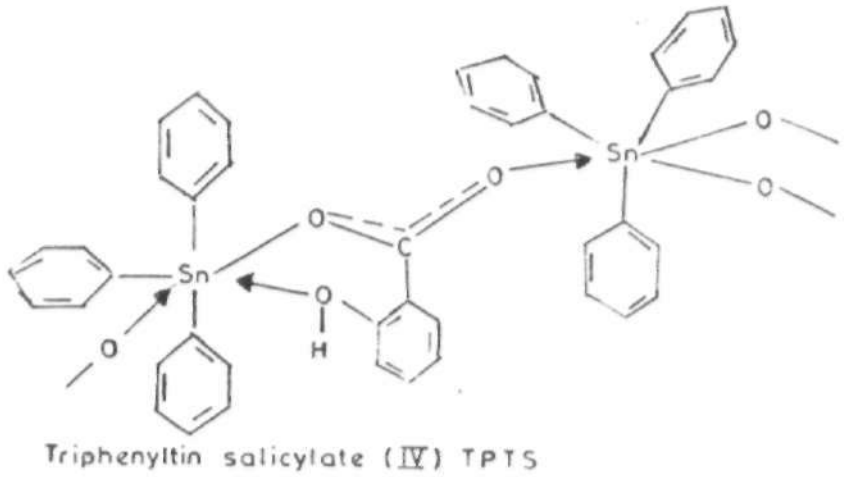

Tributyltin acetate (I) TBTA




Triphenyltin acetate TPTA (II)




Triphenyltin salicylate TPTS (III)




Triphenyltin salicylate (IV) TPTS

Generally, the test triorganotins compounds investigated, apart from being strongly fungicidal, the rate of Kill of the test fungi spores diminished progressively with time. The shape of death curves especially with survivors versus time, indicate similarity in mechanism of antifungal action. However, the rapidity of the test triorganotin compounds was observed to depend on its chemical structures. The order of biological activity of the test triorganotins was TBTA > TPTA > TPTS.

# CHAPTER FIVE

# DETERMINATION OF COMBINED ANTIFUNGAL ACTIVITIES OF TBTA, TPTA AND TPTS WITH PG

## 5.1 INTRODUCTION

When chemical antimicrobial agents are selected for any formulation where the need for effective activity or potency exists, there are often a number of physical or chemical limitations which may warrant use of less effective antimicrobial agents. In an attempt to overcome this short coming, multiple agents are usually employed. The aim of the use of multiple agents, is to obtain 'ideal' antimicrobial effect, i.e. broad spectrum of activity, rapid rate of Kill with possible synergistic effect without toxicity to the host or user and good capacity effect.

There are many reported methods for the assessment of the combination responses of antifungal agents. The use of disc on solid media and interference of two linear zones of inhibition have been advocated (Dong Heub, et. al, 1990). However, these two methods have their limitations in arriving at reliable interpretations for synergism or otherwise. Structurally different chemical antimicrobial agents may not have the same diffusion rate, hence interpretation of some combined effects on solid agar will become inconclusive (Jawetz and Gunnison, 1953).

Jawetz (1967) proposed another method in an attempt to assess and interpret combined antimicrobial responses. This method essentially is as follows: Separate antimicrobials and mixtures of antimicrobials A and B were inoculated with identical inocula of the micro-organism and counts were taken at specified time interval. Interpretation of the effects were revealed by inspection of the curves obtained (figure 5.1) which were

grouped as follows:-

**SYNERGISM:-** The antimicrobials in combination produce a greater increase in the rate of early biocidal action as compared to the rate with either antimicrobial drug alone.

**ADDITIVITY:-**The biocidal rate of the antimicrobials in combination is approximately that expected from simple arithmetic summation of single antimicrobial drug response.

**INDIFFERENCE:-** The biocidal rate of the combination is the same as that of the more active component, that is, the second antimicrobial in no way influences the activity of the first.

**ANTAGONISM:-** The biocidal rate of the combination is less than that of one or both of its components.

This assessment and interpretation of combined antimicrobials response has been criticised on the ground of being purely qualitative and lacking in precise terminology (Garret, 1958).

Other methods widely reported for quantitative interpretation of combined antimicrobial responses include the determination of single and combined minimum inhibitory concentration (MICs). This involves the determination of MIC of antimicrobial agent 'a' and MIC of antimicrobial agent 'b'. A solution is then prepared in the same medium containing multiples or ten-fold of the MIC 'a' and MIC 'b' and from this solution, serieal dilution are made, inoculated and incubated. The first dilution which inhibit growth is the MIC of the two antimicrobials. The interpretation of this experiment as cited by Hugbo (1973) is as follows:

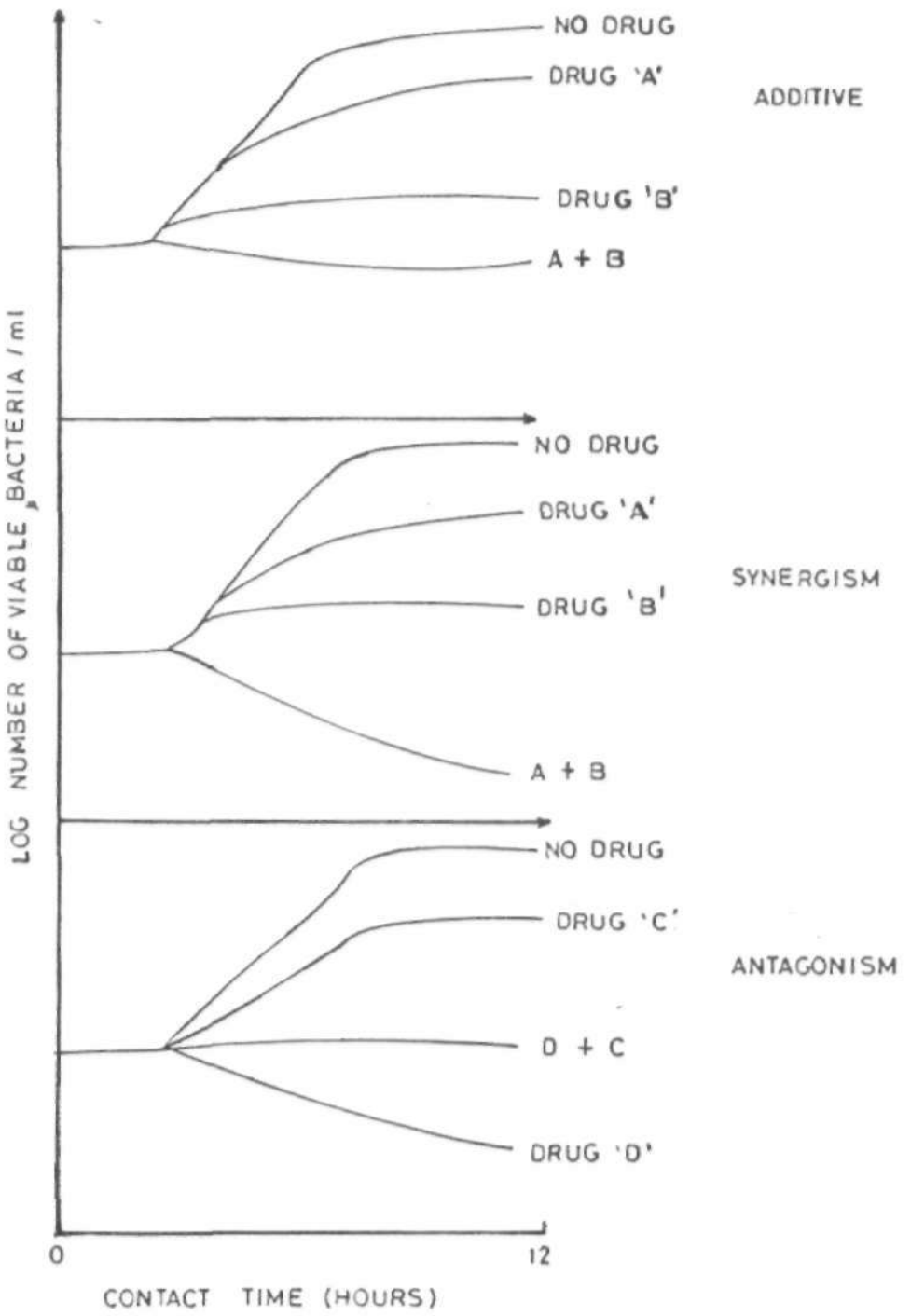



Fig.5.1: Schematic representation of biocidal action (In-vitro) showing the possible types of results when one drug or two act on a homogenous population of bacteria under conditions permitting growth (Jawetz, 1967)

If Pa + Pb represent the respective fractions of MIC 'a' and MIC 'b' present in the dilution of the mixture which corresponds to the MIC - "a + b", the Pa + Pb = 1 Additivity

Pa + Pb < 1 = Synergism

Pa + Pb > 1 = Antagonism If either Pa or Pb = 1

Pa + Pb > 1 = No interaction If neither Pa or Pb = 1

Earlier in 1954, Elion and co-workers based their interpretation on what they termed fractional inhibitory concentration. (FIC) which was defined as FIC = a/b;a = concentration of antimicrobial agent present in the combination that is inhibitory. b = concentration of each antimicrobial agent which would produce the same effect as the combination.

The FICs of the antimicrobials in combination were plotted and when the points fall on straight line, additive response was implied, but when it deviates to the left or right, synergism and antagonism were warranted respectively.

Hahn (1960) described this graphical approach as a 'clear - cut criterion' for defining additivity or synergism and believed that, it permits unequivocal conclusions to be created regarding the effects of mixture of antimicrobial agents. An example is given below to clarify the points given above.

Table 5.1 SYNERGISM IN COMBINATION OF A FIXED CONCENTRATION OF TRIPHENYLTIN ACETATE (TPTA) CONTAINING VARYING DOSES OF PROPYLENEGLYCOL (PG) AGAINST Microsporium canis.

| MIC OF TPTA (21.0mM) | | MIC OF PG (0.154mM) | | |
|---|---|---|---|---|
| TPTA strength in Admixture (mM) | FIC | PG strength in Admixture | FIC | EFICs (mM) |
| 0.01 | 0.07 | 13.67 | 0.65 | 0.72 |
| 0.02 | 0.13 | 12.30 | 0.59 | 0.72 |
| 0.03 | 0.20 | 8.88 | 0.42 | 0.62 |
| 0.04 | 0.26 | 6.83 | 0.33 | 0.59 |
| 0.07 | 0.46 | 5.47 | 0.26 | 0.72 |
| 0.13 | 0.84 | 1.64 | 0.08 | 0.92 |

Mean of sum of FICs = 0.72 [Olurinola, et. al, 1991].

In the present study, the methods of Jawetz (1967) and Elion and co-workers (1954) as described under the experimental in this work, were used to determine the combined effects of the admixtures of TBTA, TPTA and TPTS with PG against the test fungi spores.

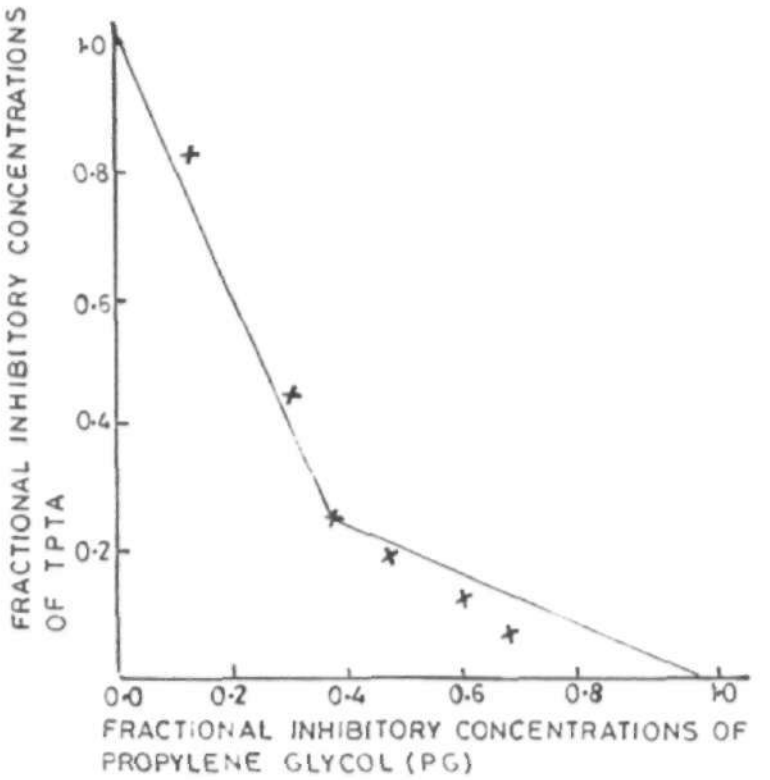



Figure 5·2: Strong synergism in combination of TPTA and propylene glycol against Microsporium canis in terms of fractional inhibitory concentrations (Fics)

## 5.2 EXPERIMENTAL

### 5.2.1 Determination of combined MIC of TBTA with TPTA, TPTS and PG

Graded concentrations of each test compound ranging from 1.2 ug/ml to 500 ug/ml were mixed with melted sabouraud dextrose agar (SDA) at 45°C in sterile petri dishes and allow to set.

For experiments involving mixtures, fixed doubling concentrations of TBTA for example were mixed with doubling dilution of TPTA beginning with the MIC or above for each drug in 10ml volume. The admixtures dilutions were mixed with 10ml melted double strength SDA at 45°C. These were allowed to set. The admixtures of TBTA and TPTS; TBTA and PG; TPTS and PG; TPTS and PG were similarly prepared aseptically. Sterile discs were aseptically placed on the dried plate and inoculated with spectrophotometrically standardized spores of test fungi to $10^3$ in triplicates. These were incubated at 30°C for 5 days. Control was also set up, i.e. SDA plate without drug, but inocubated with the test fungi spores. The susceptibilities of the test fungi spores to each drug were recorded as minimum inhibitory concentration (MIC) while the MIC for each drug-pair was calculated in terms of fractional inhibitory concentration (FIC) according to Elion and co-worker (1954).

### 5.2.2 Determination of combined fungicidal activity of test triorganotin-propylene glycol admixtures

The effect of the fungicidal concentrations of test triorganotin-propylene glycol mixtures and TBTA-TPTA suspensions on the viability of the spores of test fungi was studied. 20ml- volume solutions of the desired concentrations of the antifungal agents in 10% acetone in distilled water were prepared aseptically. These were inoculated with $10^8$

spores of test fungi.

At different specified time interval, 1.0ml was taken from the reaction mixtures and ten-fold dilutions made in an inactivating lecithin-polysorbate 80 diluent (sterilised normal saline with 0.5% w/v egg lecithin and 3.0% polysorbate 80). One millilitre of each dilution was mixed with 19ml of melted SDA at 45°C and plated in triplicates.

The viability of the test fungi spores and the sterility of the SDA medium used were also determined.

## 5.3 RESULTS

### 5.3.1 Fractional inbibitory concentrations (FICs) of test triorganotins - propylene glycol admixtures

Fractional inhibitory concentrations as described in this thesis permits a quantitative interpretation of the type of response obtained from combinations of the antifungal agents. Thus, the sum of the FICs and/or the shape of the resulting graph when the FICs of one member of a pair of drugs are plotted against those of the other member, makes it possible to determine whether the combination exhibits additive, synergistic or antagonistic response against a specified test fungal spores.

Table 5.2 and 5.3 illustrate how the FICs of triorganotin - propylene admixtures against spores of test fungi were calculated, for example in table 5.2; 2.0, 4.0, 6.0, 8.0, 13.0

Table 5.2 FRACTIONAL INHIBITORY CONCENTRATION (FIC) OF TBTA IN ADMIXTURES WITH PG THAT GIVE COMPLETE INHIBITION OF A. niger SPORES SUSPENSION.

| MIC of TBTA µg/ml | | MIC of PG 16 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (% ) | FIC | EFICs |
| 4.0 | 0.25 | 10.0 | 0.67 | 0.92 |
| 6.0 | 0.38 | 9.0 | 0.60 | 0.98 |
| 8.0 | 0.50 | 8.0 | 0.53 | 1.03 |
| 10.0 | 0.63 | 7.0 | 0.47 | 1.10 |
| 12.5 | 0.78 | 3.0 | 0.20 | 0.98 |

Mean of Sum of FICs = 1.00
Range of EFICs = 0.18

FIC = a/b a= the concentration of the antifungal agent present in the combination that is inhibitory and

b = the concentration of the antifungal agent which would produce the same effect as the combination when acting alone (Elion, et. al, 1954).

TABLE 5.3: FRACTIONAL INHIBITORY CONCENTRATION (FIC) OF TBTA IN ADMIXTURES WITH PG THAT GIVES COMPLETE INHIBITION OF A. flavus SPORES SUSPENSION.

| MIC of TBTA µg/ml | | MIC of PG 16 15 % | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (% ) | FIC | EFICs |
| 2.0 | 0.13 | 10.0 | 0.67 | 0.80 |
| 4.0 | 0.25 | 9.0 | 0.60 | 0.85 |
| 6.0 | 0.38 | 8.0 | 0.53 | 0.91 |
| 8.0 | 0.50 | 7.0 | 0.47 | 0.97 |
| 13.0 | 0.80 | 3.0 | 0.20 | 1.00 |

Means of Sum of FICs = 0.91 Range of EFICs = 0.20

TABLE 5.4: SUMMARY OF THE ACTIVITY OF SELECTED HIGHER TRIORGANOTIN CARBOXYLATES IN ADMIXTURE WITH PROPYLENE GLYCOL AND TBTA AGAINST TEST-FUNGAL SPORES BY FICs DETERMINATIONS.

| Combination of Antifungal agents | Aspergillus flavus Mean FICs | Inference | Rhizopus stolonifer FIC | Inference | Penicillium Citrinum FIC | Inference | Aspergillus niger FIC | Inference |
|---|---|---|---|---|---|---|---|---|
| TBTA + PG | 0.91 | W. Synergistic | 1.00 | Additive | 1.04 | Additive | 1.00 | Additive |
| TPTA + PG | 0.91 | W. Synergistic | 0.99 | Weakly Synergistic | 0.88 | Additive | 0.91 | W. Synergistic |
| TPTA + PG | 1.02 | Additive | 1.09 | Additive | 0.85 | W. Synergistic | 1.02 | Additive |
| TPTA + TBTA | 1.08 | Additive | 1.08 | Additive | 1.38 | Additive | 1.03 | Additive |
| TPTS + TBTA | 0.88 | W. Synergistic | 0.81 | Weakly Synergistic | 0.85 | W. Synergistic | 0.88 | W. Synergistic |

ug/ml TBTA in combinations with 10.0, 9.0, 8.0, 7.0 and 3.0% PG

respectively inhibit the growth of A. niger spores. Fractional inhibitory concentrations (FICs) of each agent were determined by calculating the ratio of concentration of the agent in combination with it's MIC against the test fungi spores when acting alone. Thus, the FICs of each of the concentration of TBTA and PG in admixture were 0.13, 0.25, 0.38, 0.50, 0.80 and 0.67, 0.60, 0.53, 0.47 and 0.20 respectively. The FICs of TBTA and PG in combination were added and the average of total sum of these FICs determined as shown in table 5.2 and 5.3. The combination responses of TBTA-PG admixture against A. niger and A. flavus displayed additive and weakly synergistic response respectively.

Generally, it can be seen from table 5.4 that propylene glycol potentiated the antifungal effect of the test triorganotins. The combination of TBTA - TPTA and TBTA - TPTS produced additive and weakly synergistic response respectively (Table 5.4).

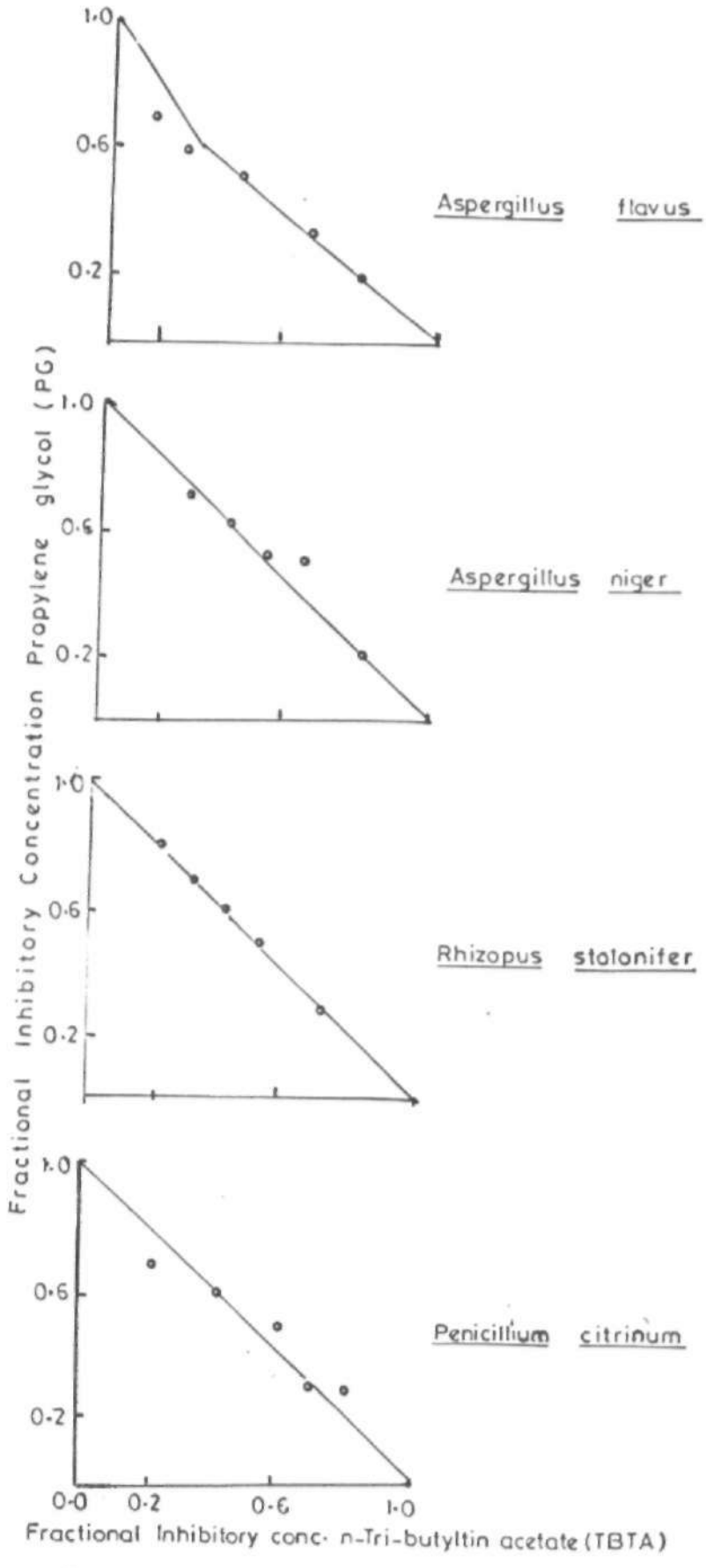
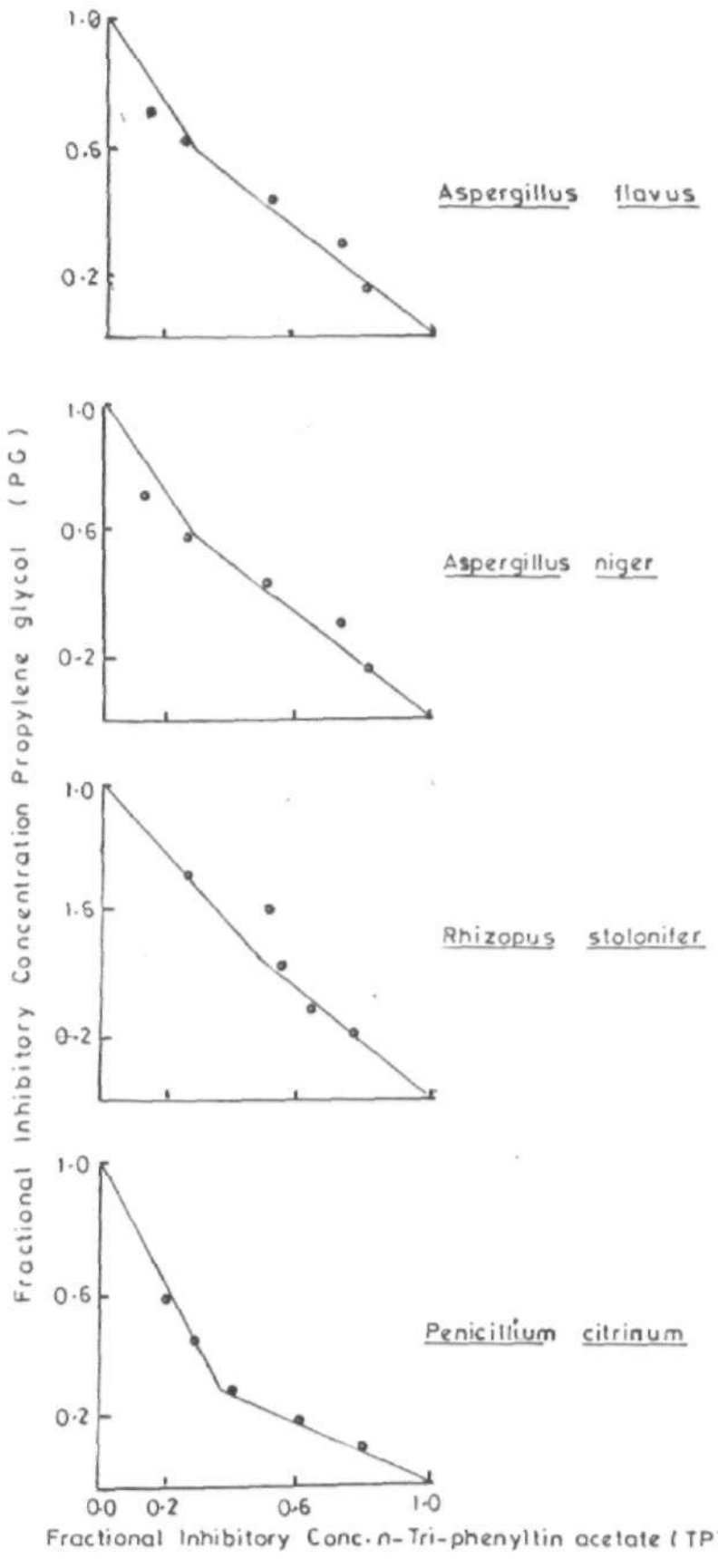



Fig. 5-3: Effects of TBTA and PG combination on test fungi spores




Fig·5·4: Effects of TPTA and PG combination on test fungi spores

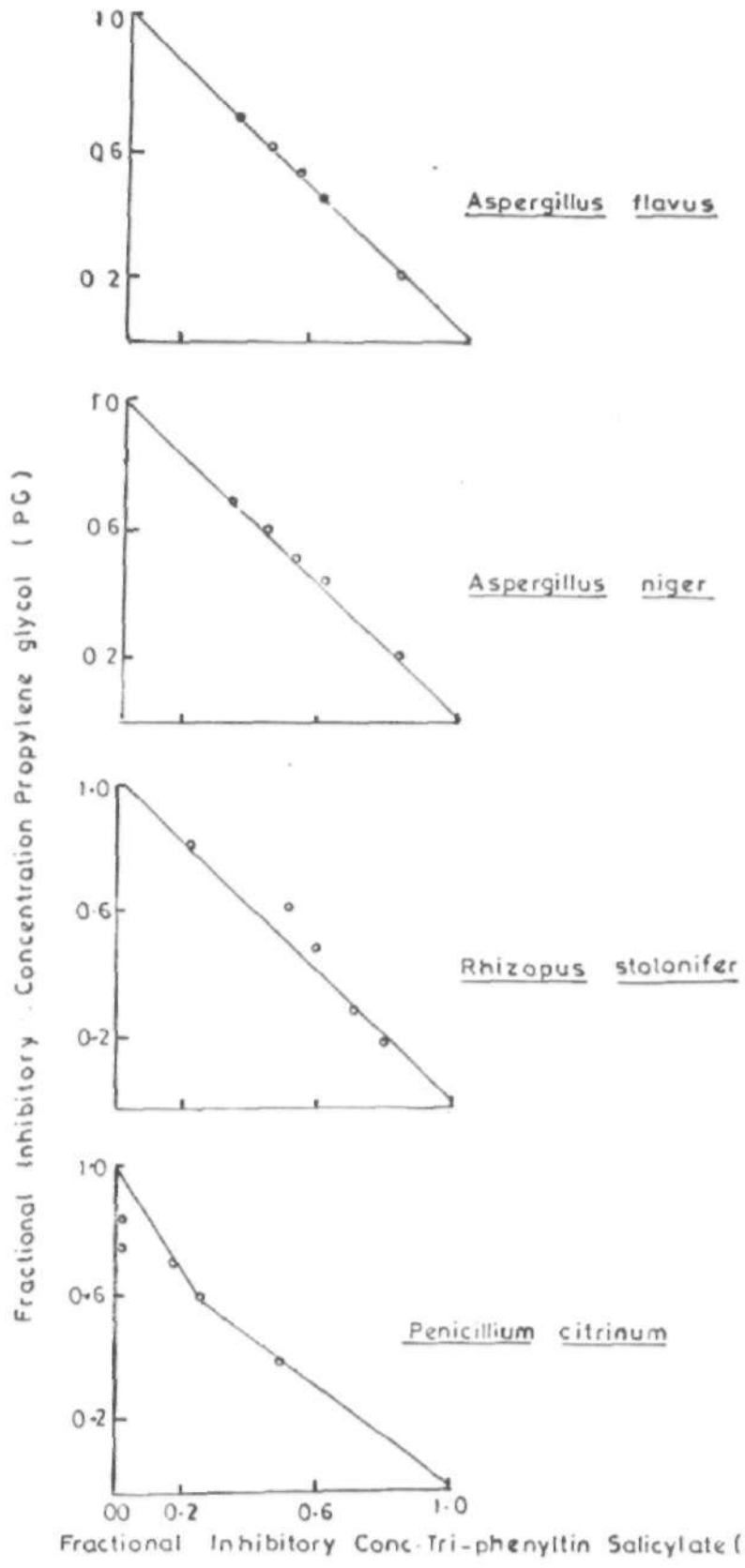



**Fig.5-5: Effects of TPTS in Admixtures with PG on test-fungi spores**

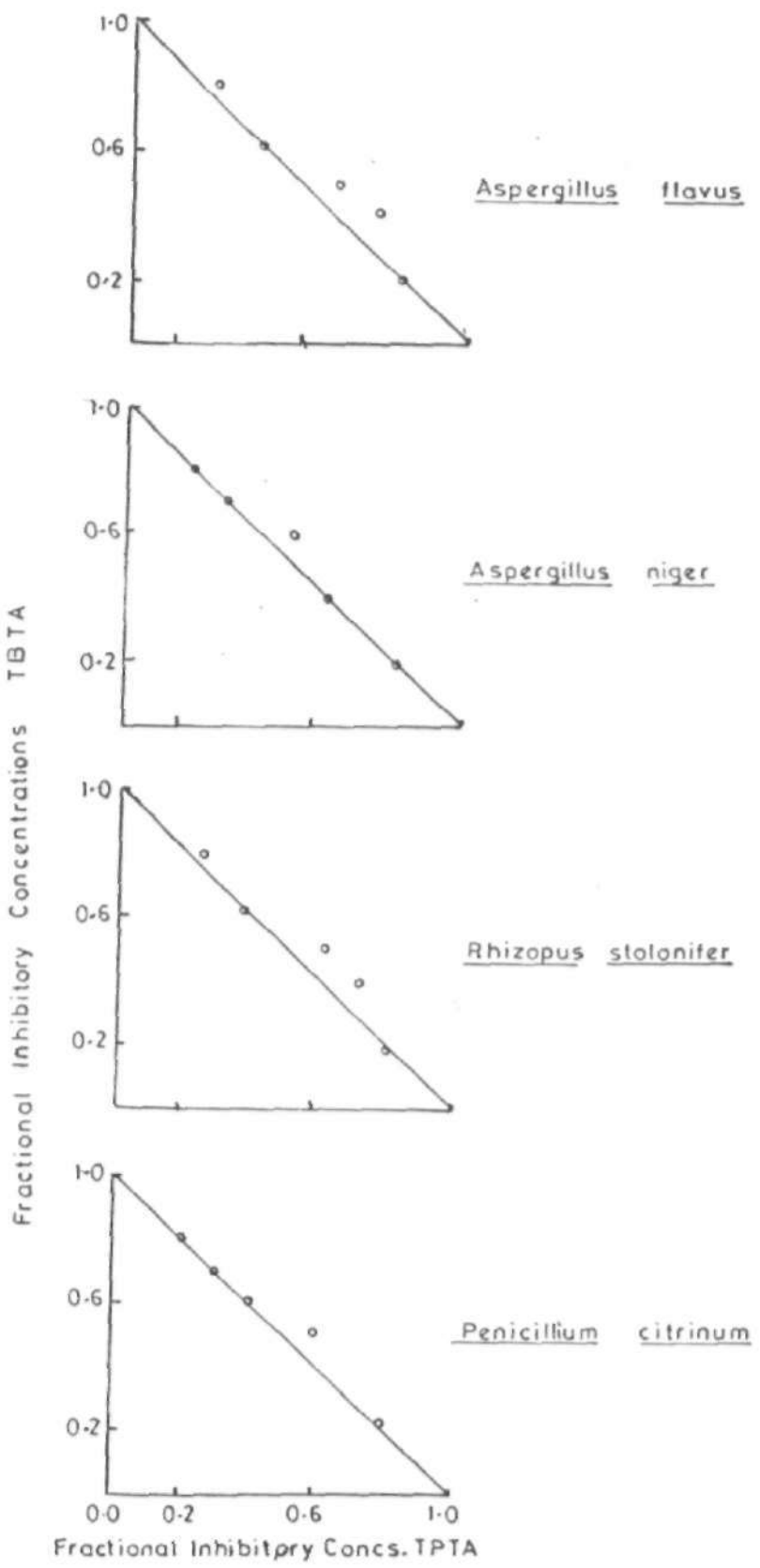



Fig. 5.6: Additivity effects of TPTA and TBTA mixtures on test-fungi spores

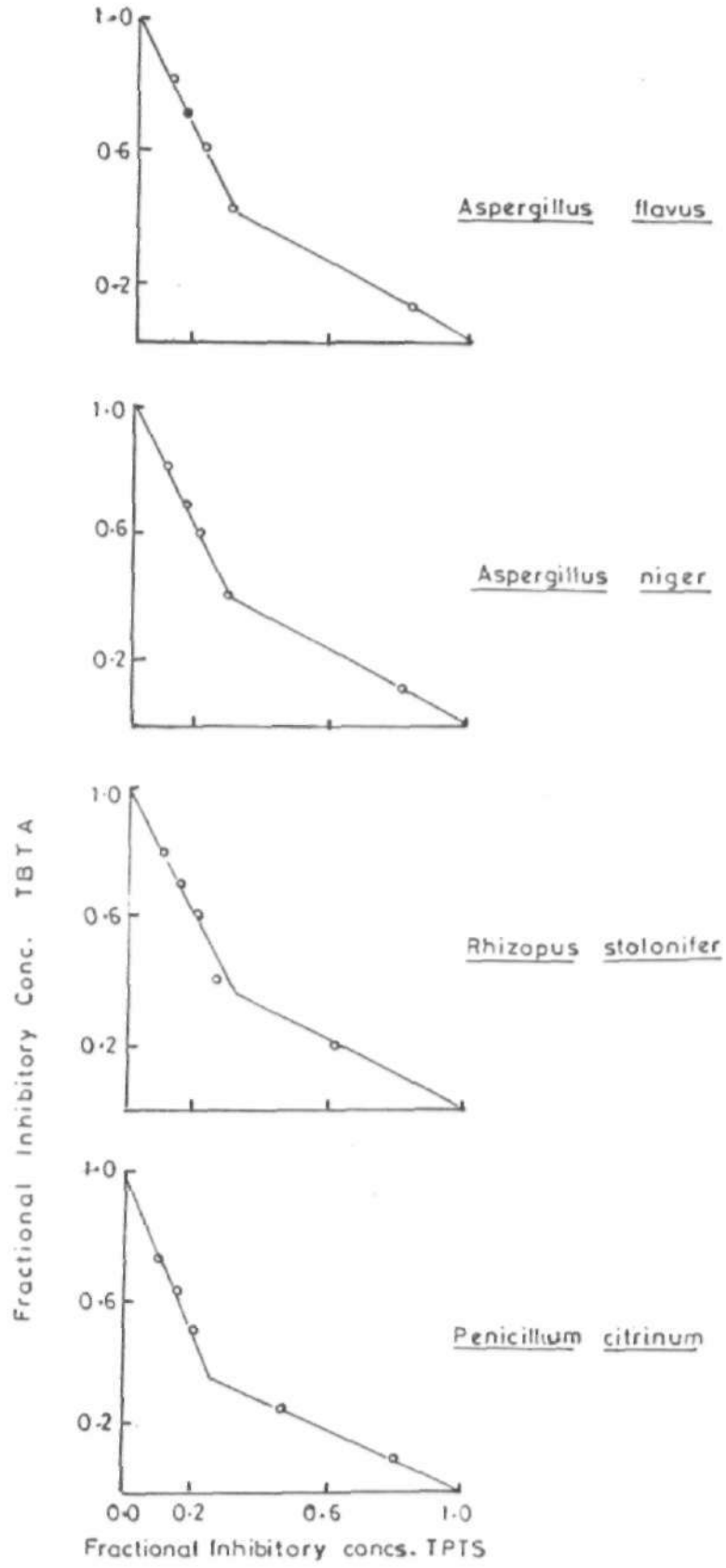


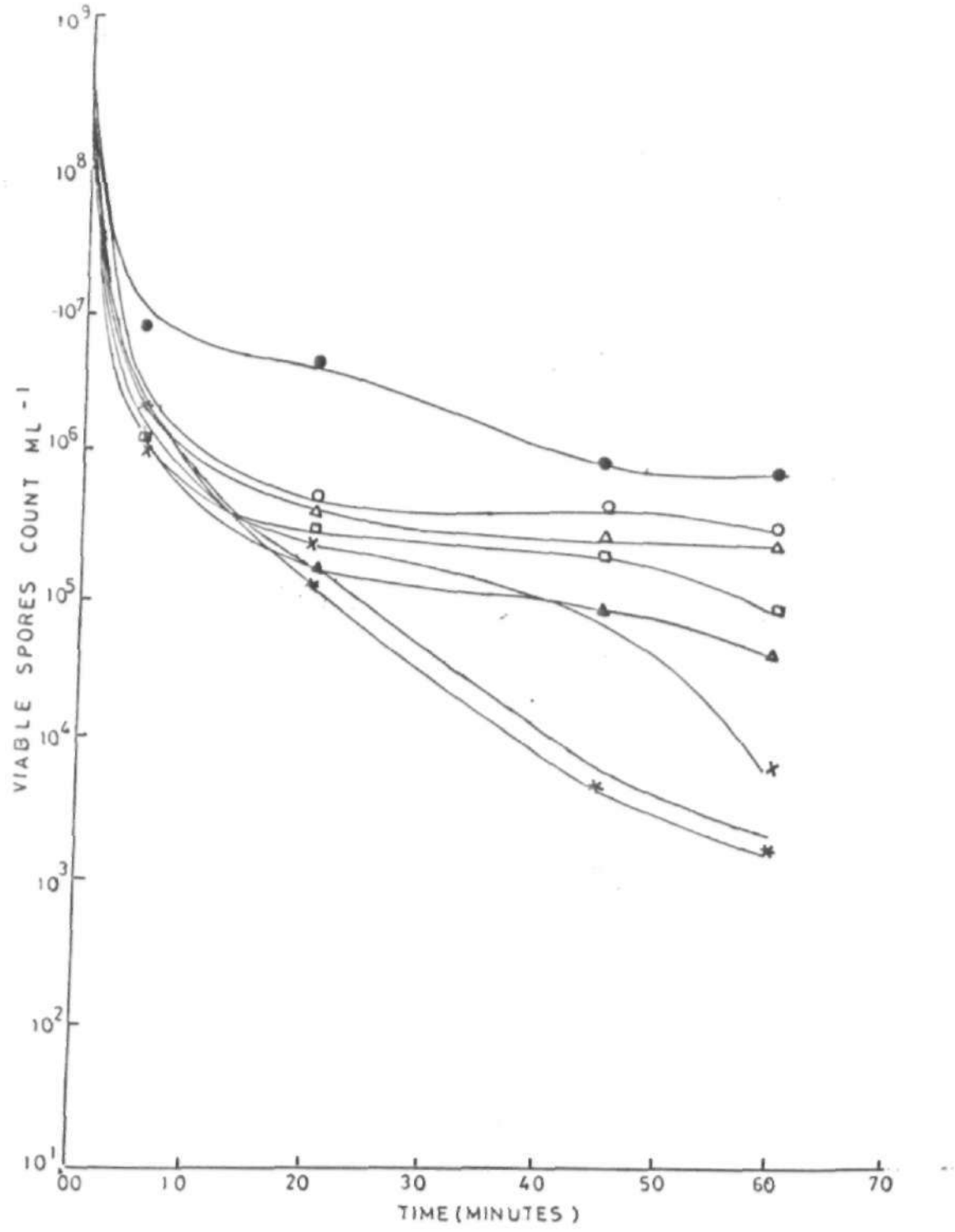

Fig.5·7: Synergistic effects of TPTS and TBTA mixtures on test-fungi spores

Hahn (1960) described graphical approach as a 'clear-cut criterion' which permits unequivocal interpretation of responses of antimicrobial combinations. For example, when the FICs of TBTA and PG against A. niger was plotted as given in figure 5.3 to 5.7, these figures clearly demonstrate the potentiating effect of propylene glycol on test triorganotins as earlier on shown in table 5.4.

### 5.3.2 Fungicidal activity of triorganotin - propylene glycol combinations

When the approach of Jawetz (1967) was used for the interpretation of the combined fungicidal responses of test triorganotin - propylene glycol admixtures, a clear indication of synergism or additive could not be reliably determined (Figure 5.8 to 5.12). However, a careful examination of these results showed that the enhanced effect produced by triorganotins - propylene glycol admixture was more than TBTA - TPTA or TBTA - TPTS admixtures.

Generally, the death curves of the test organisms by these admixtures were of 'C' type while the duration of exponential death phase for the test fungal spores was not more than 3 to 5 minutes. This was followed by drastic reduction in the death rate. For instance, 50 ug/ml TBTA effected 1.5 log cycle Kill of P. citrinum spores within 3 minutes while not more than 0.33 log cycle Kill was effected within the next 6 minutes. Similarly, 125µg/ml TPTA after 3 minutes and the next 3 minutes contact time effected 2 log cycles and 1 log cycle Kill of P. citrinum respectively (figure 5.8). An indication of decrease in death rate. It is interesting to note that 100µg/ml TBTA effected a similar rate of test spores Kill like 50/225µg/ml of TBTA/TPTA combination.




Fig·5·8: Effects of TBTA, TPTA and their combinations on viability of *P. citrinum*

●—● 50µg/ml TBTA ×—× 100µg/ml TBTA o—o 125µg/ml TPTA ▲—▲ 250µg/ml TPTA
◇—◇ 175µg/ml TPTA □—□ 50/125µg/ml of TBTA/TPTA ×—× 50/175 ug/ml of TBTA/TPTA
—— 50/225µg/ml of TBTA/TPTA

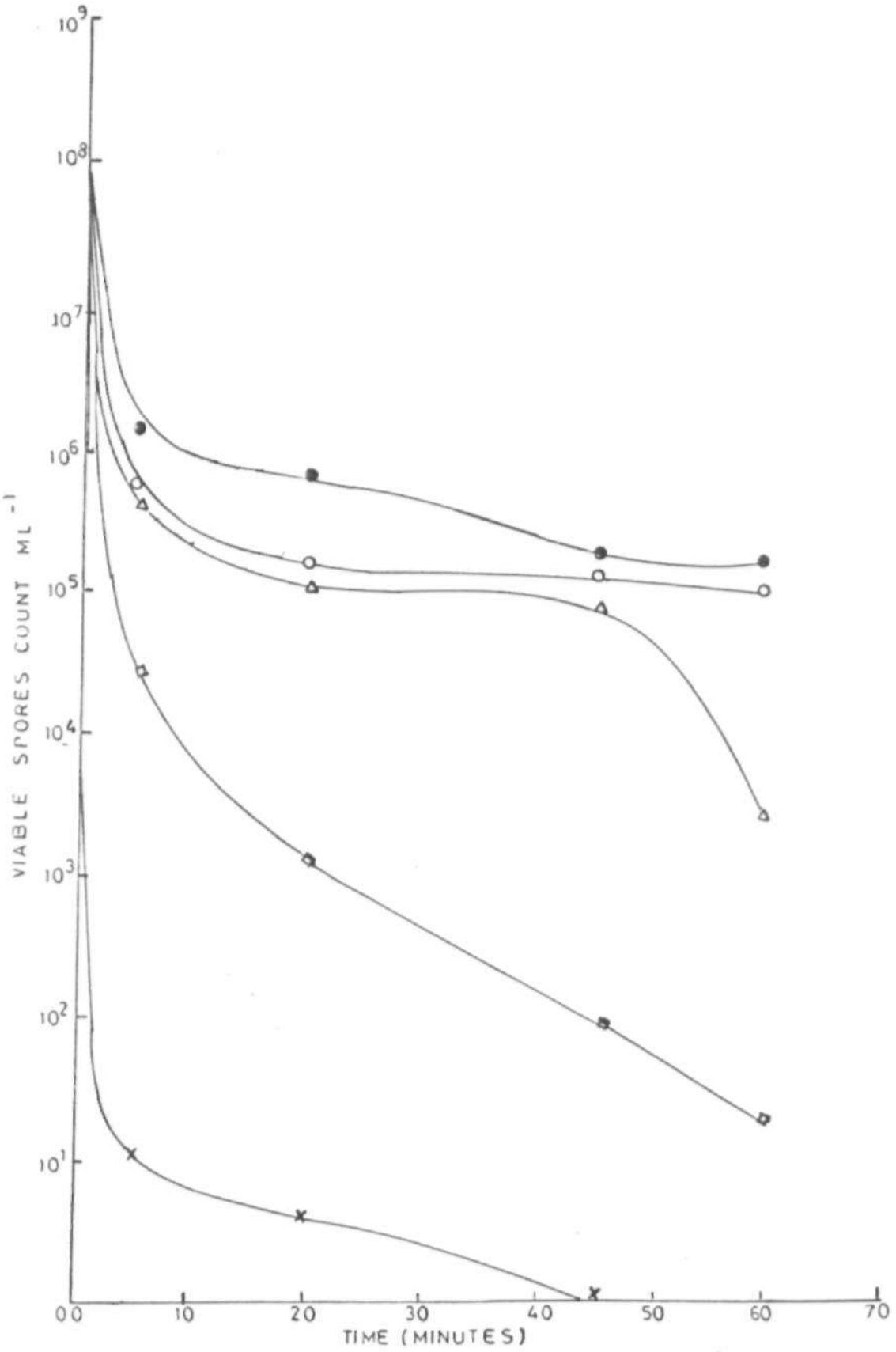



Fig.5-9: Synergistic effects of TPTA and PG admixtures against *P. citrinum*

●—● 400µg/ml TPTA  o—o 25% PG  Δ—Δ 35% PG
o—o 400µg/ml TPTA/25% PG  x—x 400µg/ml TPTA/35% PG

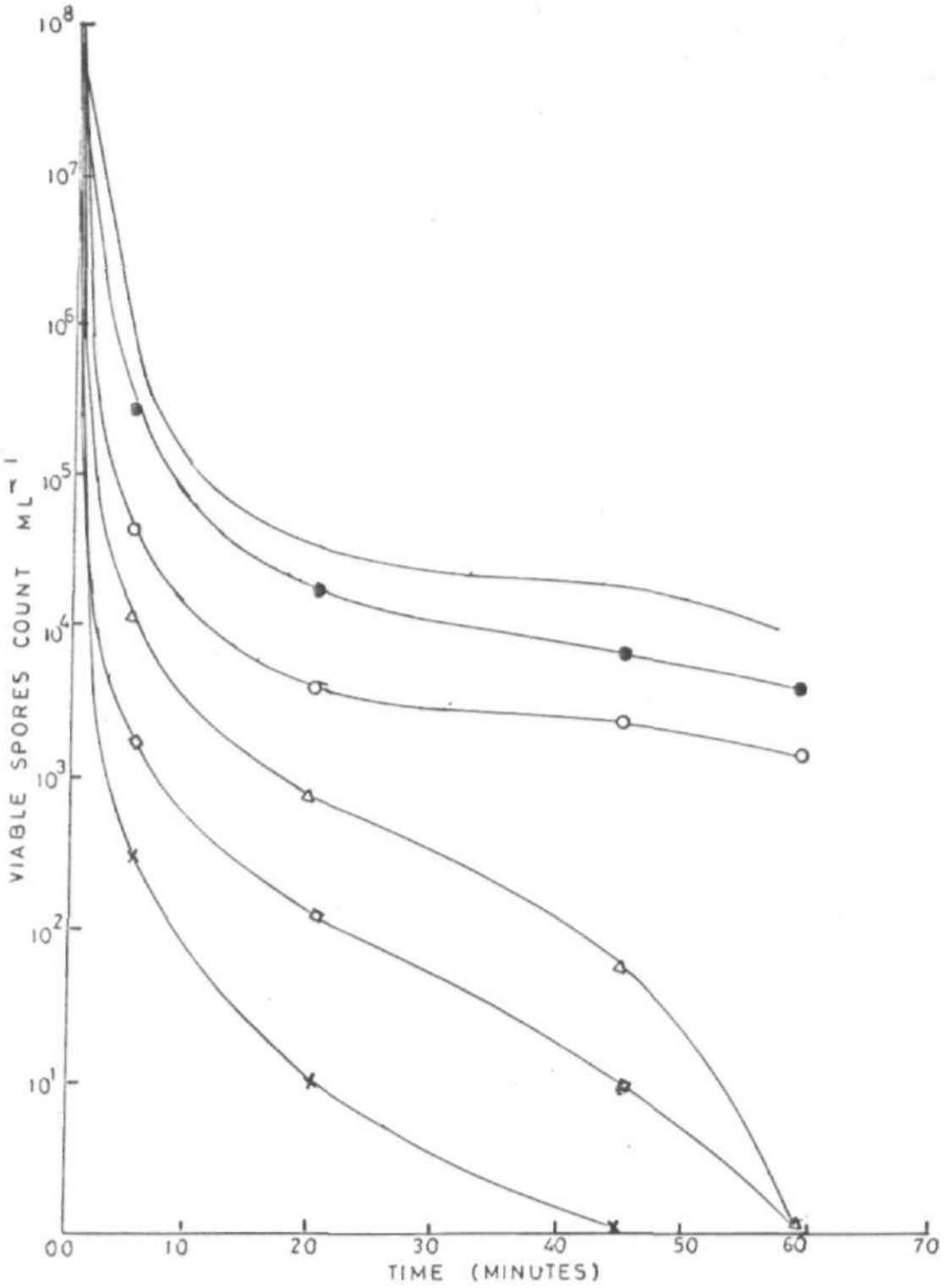



Fig.5.10: Effects of TPTA, PG and their combinations on viability of A. flavus

—— 30% PG ●—● 45% PG o—o 400µg/ml TPTA Δ—Δ 400µg/ml TPTA/30% PG
o—o 400µg/ml TPTA/35% PG x—x 400µg/ml TPTA/45% PG

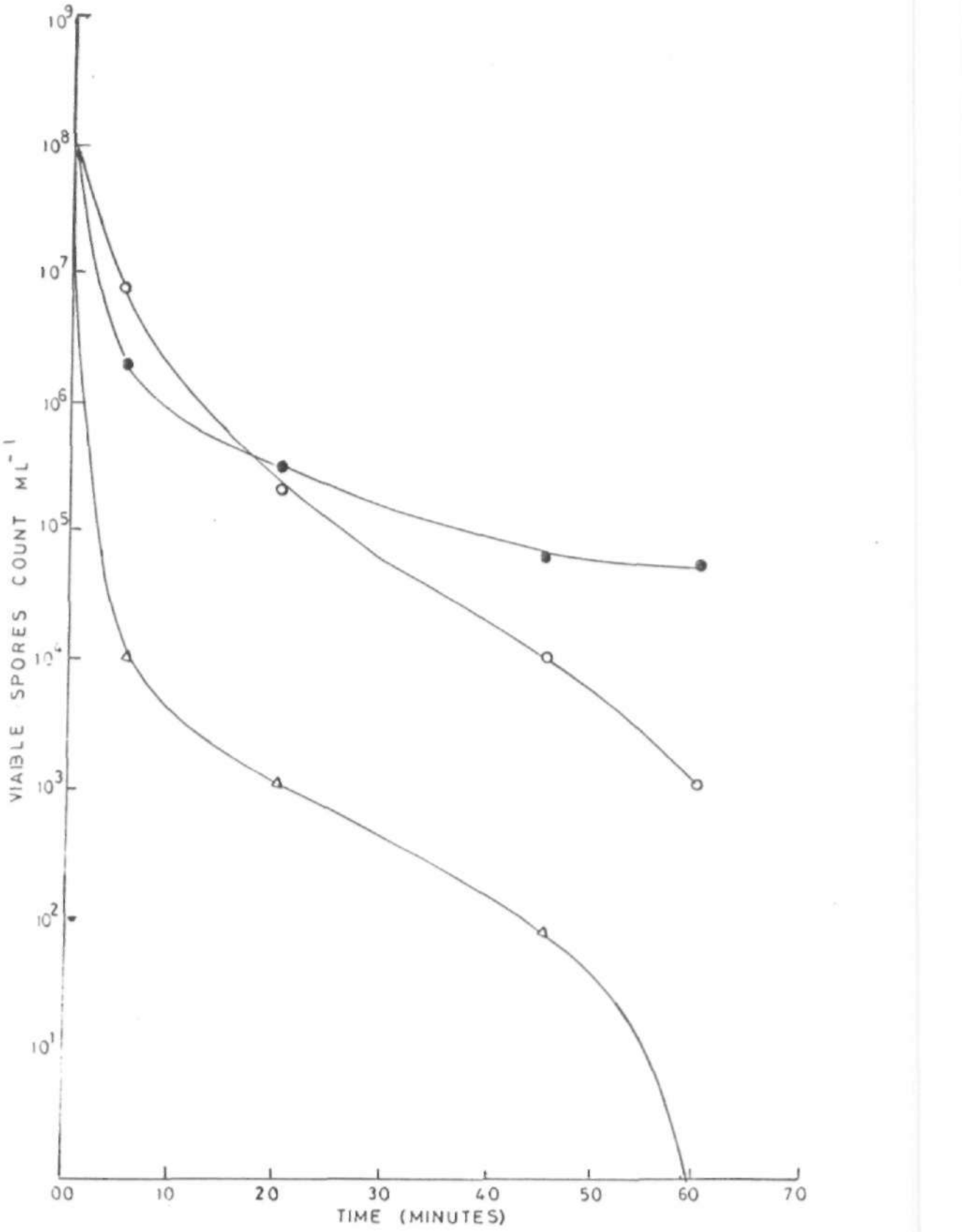



Fig.5.11: Synergistic effect of PG and TPTA admixtures against A.flavus

●—● 35% PG ○—○ 300µg/ml TPTA △—△ 35% PG + 300µg/ml TPTA

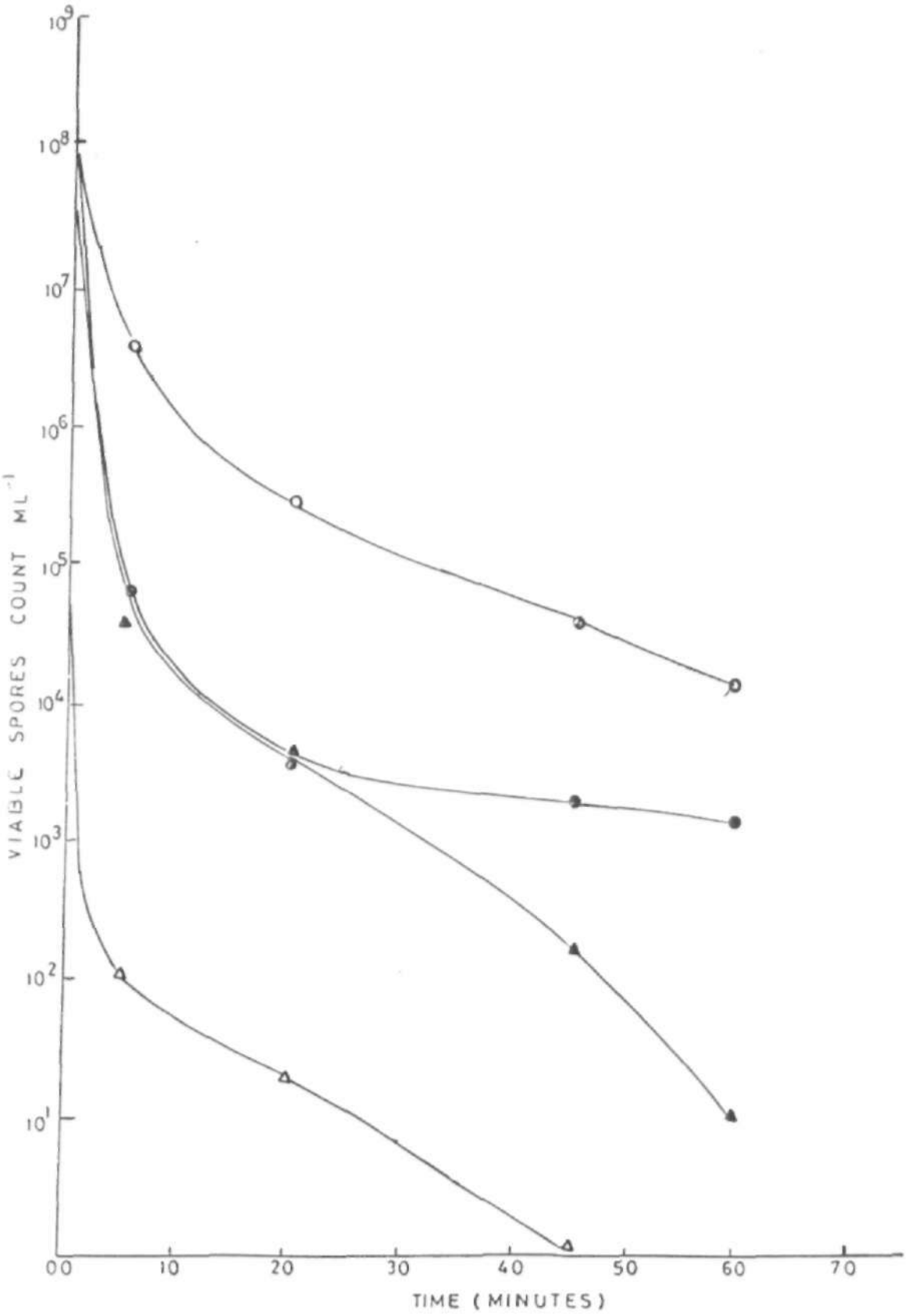



Fig.5.12: Synergistic effect of PG and TPTA admixtures against A. niger spores suspension

o—o 200 ug/ml TPTA ▲—▲ 400 µg/ml TPTA ●—● 35% PG
Δ—Δ 35% PG + 200 µg/ml TPTA

This combination response probably indicate an additive effect and possible way of reducing the toxicity of TBTA in admixtures (figure 5.8). However, 400µg/ml TPTA produced less fungicidal activity compared to that obtained with 35% PG and 200µg/ml TPTA combination as given in figure 5.12. This observation probably indicate synergism.

The ED-90 or one log cycle reduction time of the fixed concentration of TPTA e.g. 400µg/ml in doubling dilution of 15, 25, 35, and 45% PG appears to be PG concentration dependent. This is because 400µg/ml TPTA in combination with 15% PG has $D_{10}$ - values of 2.2 and 1.5 minutes when P. citrinum and A. flavus were

TABLE 5.5: TIME TAKEN TO EFFECT 1 LOG CYCLE OR 90% REDUCTION IN $10^8$ cfu/ml OF TEST FUNGAL SPORES SUSPENSION WITH TPTA AND PG ADMIXTURES.

| Fixed TPTA 400 (µg/ml) | $D_{10}$ - values (minutes) | |
|---|---|---|
| PG Strength in Admixture (%) | P. citrinum | A. flavus |
| 15 | 2.2 | 1.5 |
| 25 | 2.1 | 1.3 |
| 35 | 1.4 | 1.0 |
| 45 | 0.7 | 0.9 |

test fungi spores. However when 35% PG was added to 400 ug/ml TPTA, the $D_{10}$ - values i.e. one log cycle reduction time was drastically reduced to 1.4 and 1.0 minutes respectively against P. citrinum and A. flavus respectively. (Table 5.5). This result suggest that PG played a vital role in the rapid fungicidal effect of TPTA - PG combinations.

## 5.4 DISCUSSION

In order to advance possible explanation for the results obtained in this study, it may be necessary to first recognize the type of responses the TBTA, TPTA and TPTS in combination with propylene glycol have against the test fungi spores viz A. flavus, R. stolonifer, P. citrinum and A. niger.

Combinations containing fixed sub-inhibitory concentration of TBTA plus graded concentrations (4.0, 5.0, 7.0, 8.0, 9.0 and 10.0% ) of PG produced sums of FICs, the means of which were 0.91, 1.00, 1.04, and 1.00 against A. flavus, R. stolonifer, P. citrinum and A. niger respectively. These results clearly indicate at least an additive effect at all the levels of concentrations of PG and TBTA examined against all the test fungi except A. flavus where weak synergism was displayed. Weak synergism was also observed for TPTA/PG combination against all test fungi minus P. citrinum that indicated additive effect. However, additivity was produced by TPTS/PG admixture against A. flavus, R. stolonifer and A. niger while weakly synergistic effect was observed with P. citrinum.

Considering the mixtures of TBTA and TPTA, it was found that varying concentrations (2.0 to 48.0ug/ml) of TPTA with fixed concentrations of TBTA produced additive response while TBTA/TPTS admixture evoked weak synergism against all test fungi spores.

The results of this study showed that TBTA, TPTA or TPTS in combination with PG produced either additive or weak synergistic response against all the test fungi spores.

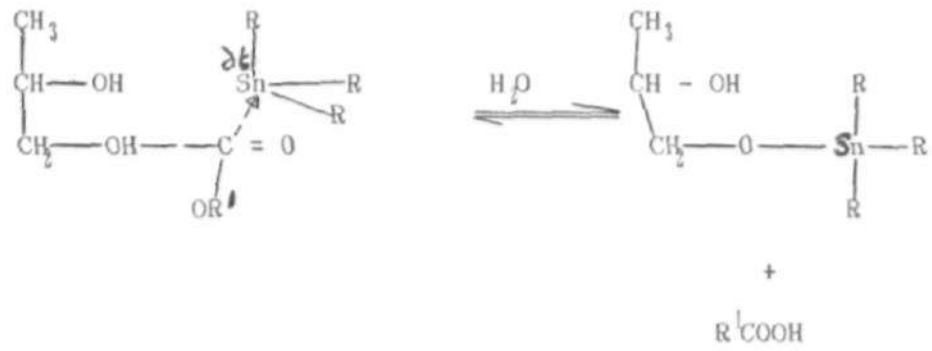
In the light of these findings, it is necessary at this stage to recognise some of the fundamental differences in the known mode of action of test antifungal agents employed in order to propose a tangible explanation for the combined effects of the antifungal agents.

The probable underlying cause of the broad spectrum of biocidal activity of triorganotins has been reported by Aldridge (1976) and Cooney and Wuertz [1989] to be due to its antimicrobial mode of action. Cooney and his co-worker (1989) in their review of antimicrobial activity of tributyltin acetate reported that the mechanism of action of this triorganotin is thought to be due to:

1. Intercalation of tributyltin with mitochrondrial membranes which cause swelling and subsequent physical disruption of mitochrondrial membrane.
2. Secondary effects due to triorganotins intercalation of lipid bilayer of mitochondrial membrane resulting to derangement of mitonchondrial function through mediation of $Cl^-/OH^-$ exchange across the lipid membrane.
3. Triorganotins ability to inhibit the fundamental energy conservation processes involved in the synthesis of ATP and ADP by reacting with histidine and sulphur - containing amino acids in enzymes involved in these processes as discussed in of chapter four.

The fungicidal activity of propylene glycol as reported by Olitzky (1965) against Microsporum canis and Trichophyton metagrophytes was suggested to be due to cell permeability effect, resulting in leakages of vital cell constituents. PG is known to be highly lipophilic like any

organic polyhydroxyl compound. It is possible that PG chemically interact with either tributyltin or triphenyltin acetate which ionize partically in aqueous state as shown in equation



Equation A.

This probable chemical interaction between triorganotin - propylene glycol may enhance cell permeability with minimal effective concentration of triorganotin at the binding sites.

The potentiated effect of triorganotin PG combinations against A. flavus, R. stolonifer, P. citrinum and A. niger in this study, thus appears to validate the concept of multiple and/or different mode of action for enhanced antifungal response of drugs combination reported by Lacey (1958) for antibiotics combinations. The differences in the degree of potentiation of antifungal responses of TBTA, TPTA or TPTS in combination with PG against test fungi spores may be due to intrinsic properties of TBTA, TPTA and TPTS such as the level of ionization in aqueous medium or their lipid solubility and species variations of the test fungi spores.

This study has also shown that the approach of Jawetz (1967) was

not the best for the quantitative differentiation between synergistic or additive effect of combination responses of test triorganotin-propylene glycol admixtures. This is because, by visual inspection of death curves produced by triorganotin - PG combinations, it is very difficult to determine which curves was synergistic or additive (figure 5.8 to 5.12). However, it is important to note that 400 ug/ml of TPTA in admixture with 15 - 45% PG achieved an ED-90 within 3 minutes. The effectiveness observed with this low concentration of TPTA/PG combination is an indication of a potential fungicide combination, that is worth in-vivo trials.

# CHAPTER SIX

# IN-VIVO ANTIFUNGAL ACTIVITIES OF TBTA, TPTA,TPTS ALONE AND IN ADMIXTURE WITH PG.

## 6.1 INTRODUCTION

In the tropics with the most rapidly expanding third world's population, post harvest losses of yam tuber can be very severe. Brody and Sacharow (1970) reported post harvest losses of yam tubers in the tropic in the third world to be about 200 million dollars annually.

Attacks by micro-organisms especially fungi and sometimes bacteria are probably the most serious agents of these post harvest losses. Among the organisms most frequently reported to cause yam rot are: Rhizopus stolonifer, Cladosporium sp., Rhizoctonia sp, Glomerella Cingulata, Penicillium sp, Phaeoramularia sp and Pseudocercospora sp (Adeniji, 1970, Bammi, et al, 1972; Baudin, 1956; Coursey, 1984; Eckert, 1977; Ogundana, et al, 1970). The 'soft rot' of tubers caused by the above mentioned fungi is a serious post harvest disease, because the fungi grow rapidly under tropical climatic and environmental conditions. These pathogens in feeding on the host, secrete extracellular enzymes which macerate the fleshy tissues of the host into an incoherent, watery mass within a few days. This 'yam rot' exemplified by Rhizopus stolonifer may become a serious problem when a substantial portion of untreated wounded or insect bite yam tuber are infected at the time of harvest.

Several previous efforts to control this post harvest fungal rot of yam tuber chemically with the use of earth dust and wood ashes, lime and borax, thiabendazole and benomyl have proved unsuccessful (Bonire, 1985; Coursey, 1961; Spencer, 1977).

In this study, the potentials of TBTA, TPTA, TPTS, alone and in admixtures with PG was examined. This is an attempt to determine the usefulness or otherwise of the in-vitro antifungal effect displayed by these test compounds against yam rot fungi.

## 6.2 EXPERIMENTAL

### 6.2.1 In-vivo test for antifungal activity of TBTA and TPTA using fresh yam slices.

Fresh white yams (Discorea rotundata "Clone Cika Kwando") were sliced. On each sliced surface four shallow pits (6.0mm diameter and 2.0mm deep) were equidistantly made using a sterile cork borer. The yam slices were then immediately dipped in various concentrations of TBTA in 25% acetone in water and drained. The bored pits on the sliced yam surfaces were then inoculated with 10ul of $10^3$ viable spores/ml in duplicates using one fungal species per pit.

The triorganotins treated and inoculated yam slices were placed in sterile plates and incubated on disinfected laboratory bench at 27±2°C for 7 days.

For experiment involving 90% relative humidity (RH), the triorganotins treated yam slices with the inoculated test fungal spores were placed in a desiccant free, 70% alcohol disinfected, cubic plastic desiccator cabinet (Aldrich). Two petri dishes, each containing 10ml of sterile 10%w/w potassium hydroxide were placed at the base of the cabinet to keep its inside environment moist at 90% RH (Solomon, 1951) which favour maximum growth of yam rot fungi (Adeniji, 1970).

These procedures were repeated with similar yam slices dipped in

various concetrations of sterile TPTA in 25% acetone in water. Controls, that is, inoculated sliced yams dipped in plain 25% acetone were also included in both cases. These yam slices were incubated at $30^0$C for 7 days.

### 6.2.2 Determination of antifungal activity of test trioganotin - propylene glycol admixtures using fresh yam slices.

The procedures used in section 6.2.1 above are repeated with triorganotin-proylene glycol combinations to determine the effects of such combinations on the antifungal potency of the member compounds. The concentrations range of TBTA, TPTA or TPTS in combination with fixed concentration of propylene glycol ranged between 5ug/ml to 2,500ug/ml. The triorganotin-PG treated yam slices were inoculated with 10ul. of $10^8$ test fungi spores. Controls were also set up i.e. viability of the test fungi spores on sliced yam tuber and the sterility of the test admixtures were determined.

These yam slices with the cabinet were incubated at $30^0$C for 7 days.

The lowest concentration of the agents alone or in combination that showed no growth of the test fungal spores was regarded as single or combined minimum effective concentration (M.E.C).

## 6.3 RESULTS

Table 6.1 to 6.2 illustrate the activity of TBTA and TPTA against heavy fungal spores inoculations on fresh sliced yam surfaces which had been pre-treated with various concentrations of TBTA or TPTA in 25% acetone in water. The minimum effective concentraion (MEC) of TBTA for

A. flavus. R. stolonifer and A. niger in the open laboratory was 0.31 mg/ml while the MEC against P. citrinum was 0.16mg/ml. The MEC of TPTA against all the test fungi spores under similar conditions doubled that of TBTA, that is, 0.63mg/ml for A. flavus,A. niger, R. stolonifer and 0.31mg/ml against P. citrinum. Concentration lower than MEC of TBTA and TPTA proved incapable of arresting the test fungal growth. Though, growth observed at these concentrations were less pronounced than those of the untreated controls. The untreated controls showed advanced tissue maceration.

Yam slices which were treated with various concentrations of TBTA or TPTA and incubated in 90% relative humidity favourable \ for most fungi causing rot of yam as reported by Adeniji (1970) were slightly less protected, as the lowest effective concentration of TBTA against

TABLE 6.1: DETERMINATION OF MINIMUM EFFECT CONCENTRATION (mg/ml) OF TBTA AND TPTA IN 25% ACETONE IN WATER TO PREVENT YAM ROT BY SELECTED YAM ROT FUNGI AFTER 7 DAYS INCUBATION IN THE OPEN LABORATORY AT 27±2°C.

| Test Fungi | MIC (mg/ml) of | |
|---|---|---|
| | TBTA | TPTA |
| A flavus | 0.31 | 0.63 |
| A niger | 0.31 | 0.36 |
| R stoloifer | 0.31 | 0.63 |
| P citrinum | 0.16 | 0.31 |

TABLE 6.2: DETERMINATION OF MEC (mg/ml) OF TBTA AND TPTA IN 25% ACETONE IN WATER TO PREVENT YAM ROT BY SELECTED YAM ROT FUNGI AFTER 7 DAYS INCUBATION IN 90% RELATIVE HUMIDITY AT 30°C.

| Test Fungi | MIC (mg/ml) of | |
|---|---|---|
| | TBTA | TPTA |
| A flavus | 0.63 | 1.25 |
| A niger | 0.63 | 1.25 |
| R stolonifer | 0.63 | 1.25 |
| P citrinum | 0.31 | 0.63 |

A. flavus, A. niger and R. stolonifer was 0.63mg/ml. The growth of P. citrinum was totally arrested at 0.31mg/ml. The pattern of MEC of TPTA obtained against test fungi spores at 90% RH was similar to the MEC observed in the open laboratory experiment. The MEC of test triorganotins in 90% RH was 2 - folds higher than that obtained during the open laboratory in-vivo experiment. For example the MEC of TBTA against A. flavus, A. niger and R. stolonifer in open laboratory and in 90% in-vivo experiments was 0.31mg/ml and 0.63 mg/ml respectively. (Table 6.1 and 6.2)

The untreated sliced yam controls displayed rot with tissue maceration and profuse growth of test fungi.

For experiments involving mixtures of TBTA-PG, TPTA-PG and TPTS-PG against test fungi spores on sliced yams surfaces, the results shown in table 6.3, 6.4 and 6.5 indicate similar responses. For instance, the MEC TBTA in combination with 15% PG against A. flavus, A. niger, R. stolonifer and P. citrinum was 0.10, 0.005, and 0.005mg/ml respectively. Similarly, the MEC of TPTA in admixture with 15% PG against the above set of test fungi spores was 0.10, 0.10, 0.01, 0.01 mg/ml respectively.

These results tend to indicate that A. flavus, A. niger were the least sensitive one to the triorganotins-PG combination while the reverse response was observed with R. stolonifer and P. citrinum.

The antifungal effect of presumably potent TBTA-TPTA

TABLE 6.3: DETERMINATION OF MEC OF TBTA IN 15%, 25% AND 30% PG TO PREVENT YAM ROT BY SELECTED YAM ROT FUNGI AFTER 7 DAYS INCUBATION IN 90% RH AT 30°C.

| Test Fungi | MIC of TBTA(mg/ml) Admixture with | | |
|---|---|---|---|
| | 15%PG | 25%PG | 30%PG. |
| A. flavus | 0.10 | 0.05 | 0.05 |
| A. niger | 0.10 | 0.05 | 0.05 |
| R. stolonifer | 0.005 | 0.0025 | 0.0025 |
| P. citrinum | 0.005 | 0.005 | 0.0025 |

TABLE 6.4: DETERMINATION OF MEC OF TPTA IN 15%, 25% AND 30% PG TO PREVENT YAM ROT BY SELECTED YAM ROT FUNGI AFTER 7DAYS INCUBATION IN 90%RH AT 30°C.

| Test Fungi | MIC of TBTA (mg/ml) in ADMIXTURE WITH | | |
|---|---|---|---|
| | 15%PG | 25%PG | 30%PG |
| A. flavus | 0.10 | 0.05 | 0.05 |
| A. niger | 0.10 | 0.05 | 0.05 |
| R. stolonifer | 0.01 | 0.005 | 0.0025 |
| P. citrinum | 0.01 | 0.005 | 0.0025 |

TABLE 6.5: DETERMINATION OF MEC OF TPTS IN 15%, 25% AND 30% PG TO PREVENT YAM ROT BY SELECTED YAM ROT FUNGI AFTER 7DAYS INCUBATION IN 90%RH AT 30°C.

| Test Fungi | MIC of TPTS (mg/ml) in ADMIXTURE WITH | | |
|---|---|---|---|
| | 15%PG | 25%PG | 30%PG |
| A. flavus | 0.25 | 0.05 | 0.05 |
| A. niger | 0.10 | 0.05 | 0.05 |
| R. stolonifer | 0.01 | 0.005 | 0.005 |
| P. citrinum | 0.01 | 0.005 | 0.0025 |

combinations like TBTA-TPTS did not appear to display any better antifungal effect compared with that of triorganotin-PG combinations (Table 6.6). For example, the MEC of TPTA in admixtures with 15% PG against A. flavus, A. niger, R. stolonifer and P. citrinum was 0.01, .10, 0.01 and 0.01 mg/ml respectively. While the MEC of TPTA in admixtures with 0.032mg/ml TBTA against A. flavus, A. niger, R. stolonifer and P. citrinum was 0.25, 025, 0.10 and 0.05 respectively. PG is known to be less toxic to mammal, hence it combination with triorganotin may achieve the same purpose like TBTA-TPTA admixtures, except that PG would evaporate rather quickly. But in situation of high humidity where evaporation would be slower, organotin/PG mixture probably will be very useful.

## 6.4 DISCUSSION.

The results of this study indicate pronounced activity of the TBTA, TPTA and TPTS against A. flavus, R. stolonifer, A. niger and P. citrinum. For example, the MEC of TBTA and TPTA against test fungi spores using sliced yam in the open laboratory ranges form 0.16 to 0.32mg/ml and 0.32 to 0.63mg/ml in 25% acetone respectively. The MEC of

TBTA was 2 times lower than TPTA MEC in both in-vivo experiments performed in the open laboratory and in cabinet under favourable environmental conditions for most yam rot fungi. It has been reported that increase in molecular weight or carbon numbers decreases the antimicrobial activity of higher triorganotins. This decrease in antimicrobial activity has been attributed to either lipid solubility problem or the increased steric hinderance at the tin atom caused by bulky hydrocarbon groups. (Sijpesteijn, et al, 1969; Kuthubutheen, et al 1989). The reports of workers in this field

TABLE 6.6: DETERMINATION OF MEC OF TPTA IN 0.032, 0.064 AND 0.128mg/ml TBTA TO PREVENT YAM ROT BY SELECTED YAM ROT FUNGI AFTER 7 DAYS INCUBATION IN 90%RH AT 30°C.

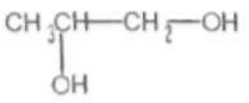
| Test Fungi | MEC OF TPTA mg/ml) IN ADMIXTURE WITH | | |
|---|---|---|---|
| | 0.032 | 0.064 | 0.128mg/ml TBTA |
| A. flavus | 0.25 | 0.10 | 0.05 |
| A. niger | 0.25 | 0.10 | 0.05 |
| R. stolonifer | 0.10 | 0.05 | 0.01 |
| P. citrinum | 0.05 | 0.005 | 0.0025 |

of study as stated above appear to support the observed high antifungal activity of TBTA compared to TPTA; although additional factors as explained in chapter 4 can be responsible.

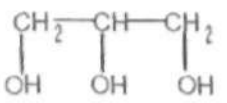
The MEC of TBTA and TPTA against test fungi spores at 30°C in 90%RH was 2 times the MEC of TBTA and TPTA obtained in the open laboratory in-vivo experiment. The relatively drier environment in an open laboratory (compared to moist environmental conditions at 90%RH) appears to contribute to the enhanced antifungal activity of the TBTA and

TPTA. This observation, thus suggest that relatively dry environment or proper ventilation of yam treated with these triorganotins may give better protection of the sliced or wounded yam from fungal spoilage. However, this observation may be due to no more than the fact that a wound on yam dries faster in dryer weather.

The protection offered by the admixtures of triorganotins-PG appears to be OF significant interest to note. For example, the MEC of TBTA acting alone against A. flavus, A. niger, R. stolonifer and P. citrinum at 30°C in 90%RH was 0.63, 0.63 and 0.31mg/ml respectively. While the MEC of TBTA in combination with 15% PG against the same set of test fungi under the same conditions was 0.10, 0.10, 0.10 and 0.005mg/ml respectively. The observed MEC of triorganotin-PG combinations showed a drastic reduction in the MEC of the individual triorganotins when acting alone. From the above example of the observed results, the MEC of TBTA in combination with 15%PG was 6.3 to 62 times lower than that obtained with TBTA alone. Similar results were obtained when TPTA or TPTS was used as illustrated in table 6.4 and 6.5. The implication of this 6.3 to 62 times reduction in minimum effective concentration of TBTA against yam rot fungi is interesting. It means reduction of production cost and toxicity, if PG is employed in combination with triorganotin as a fungicide formulation. This propylene glycol has similar hydroxyl group with glycerol:



Propylene glycol



Glycerol

which is reason to increase partial ionization of test triorganotins in the aqeous medium, thereby increasing the biologically active tin centre. Thus, it is possible that glycerol may display similar potentiated antifungal effect on triorganotins. Glycerol likely effect,is considered, because it is known to be common, less volatile and cheaper than propylene glycol, thus, it may be used as substitute for PG.

Generally, it is important to note that increasing PG concentration beyond, 25%, did not drastically increase the potency of TBTA, TPTA or TPTS-PG admixtures. This concentration provides an advisable choice in triorganotin-PG fungicide formulations.

A closer study of the antifungal activity of TBTA/TPTA or TPTS combinations (Table 6.6) indicate no better antifungal effect against test fungi than TBTA, TPTA or TPTS-PG. This is because, addition of 15%PG drastically reduced the MEC of TPTA when acting alone to 0.10, 0.10, 0.01 and 0.01mg/ml against A. flavus, A. niger, R. stolonifer, and P. citrinum respectively. Thus, triorganotin-PG combinations may provide better formulations for effective fungicide against yam rot fungi, provided ecological dis-stabilization will not be promoted with the introduction of this fungicide.

From the results obtained in this study, it is clear that triorganotins acting alone and in combination with PG provide effective fungicide in combating yam rot due to A. flavus, R. stolonifer, P. citrinum and A. niger. It is inportant to note that triorganotin/PG combinations provide the greatest potency in this study.

# CHAPTER SEVEN.

# DETERMINATION OF EFFECT OF HEAT AND LIGHT ON ANTIFUNGAL ATIFUNGAL ACTIVITY OF TBTA, TPTA AND TPTS.

## 7.1 INTRODUCTION

Owing to the widespread industrial and agricultural uses of organotins, their fate in the environment has become things of considerable interest to know. There are varieties of path ways by which triorganotins can be envisaged to enter into the environment as shown in table 7.1

TABLE 7.1: POSSIBLE DIRECT MODES OF ENTRY OF TRIORGANOTINS INTO THE ENVIRONMENT.

| Medium | | Source |
|---|---|---|
| Air | - | Agricultural spraying |
| | - | Volatilization from biological treatments |
| | - | Antifouling paint sprays |
| Soil | - | Agricultural applications |
| | - | Wood preservations |
| Water | - | Antifouling coatings |
| | - | Molluscicides |
| | - | Overspray from agricultrual applications |
| | - | Land run-off from agricultural usage |
| | - | Industrial process e.g. similicides in paper manufature. |

(Blunden and Evans, 1982).

Consequently, if these test triorganotins have to be used as anti yam rot preservative, there is the need to determine the stability of this potential fungicide in order to predict it's long-term impact on environmental ecology and the safety perspective of the treated wounded yam tubers.

## 7.2 EXPERIMENTAL.

### 7.2.1 Determination of effect of heat and 40 watt tropical day light florescence on aantifungal activity of TBTA, TPTA and TPTS.

Ten millilitreS aqueous solutions of graded double concentrations of each test triorganotin 2.5 to 2,560µg/ml were prepared in four sets in sterile white universal bottles. Each concentration of the first set was mixed at same temperature with melted 10ml of double strength SDA at 45$^0$C in sterile petri dish of diammeter 90mm. These were allowed to set. The second set of solution of graded concentrations of the test compounds were boiled at 100$^0$C in water bath for 10 minutes. After boiling, these solutions were cooled to about 45$^0$C, mixed with melted 10ml of double strength SDA at 45$^0$C in sterile petri dish and allowed to set. The third and fourth sets of solutions of graded concentrations of the test compounds were exposed to 40 watt tropical day light florescence for 24h and 72h at 27±2$^0$C respectively. After this, each 10ml solution was mixed at same temperature with melted 10ml of double strength SDA at 45$^0$C in sterile petri dish and allowed to set.

Eight sterile discs were equidistantly placed on the dried plate under aseptic condition. The four test fungi spores were inoculated on the eight sterile discs on the chemically seeded agar with 10ul of $10^3$ CFu/ml of homogeneous fungal spores suspension.

Control was set up, that is, SDA plate without any of the test compounds, but inoculated with the test fungi spores. The plates were incubated at 30$^0$C for 5days.

The lowest concentration of the agent which inhibited visible growth of the fungal spores was taken as the MIC under this experimental conditions.

## 7.3 RESULTS.

The effect of light emitted by 40watt tropical day light florescence at a height of about 3.66 metres on the antifungal activity of test triorganotins were studied. It was observed that 24h exposure of the graded concentrations of these triorganotins to this light has no significant impact on it's fungistatic activity (Table 7.2). For example, MIC of TPTA against A. flavus, A. niger, R. stolonifer and P. citrinum after 24h exposure to light was 0.049, 0.05, 0.04, and 0.024mM respectively. The corresponding control MIC of TPTA against these test fungi spores was 0.049, 0.037, 0.037 and 0.024mM respectively. This period is probably indicative of effective contact time before photo-degradation impact become pronounced. However, continous exposure of graded concentrations of TBTA, TPTA and TPTS to this light source for 72h, resulted to 42.9 - 106.9%, 233.3 - 440.5% and 75.0 - 100.0% increase in MICs of these test triorganotins respectively. An indication that antifungal activity of TBTA, TPTA and TPTS was reduced by 1.42 - 2.07, 3.33 - 5.41 and 1.75 - 2.0 folds respectively in this light source after 72h exposure. (Table 7.2).

Table 7.3 depicts the effects of boiling of the test compound solutions for 10 minutes. It is worth noting that

TABLE 7.2: MIC OF TBTA, TPTA AND TPTS EXPOSED TO 40 WATT TROPICAL DAY LIGHT FLORESCENCE FOR 0h, 24h AND 72h AGAINST $10^3$ cfu/ml OF TEST FUNGI SPORES SUSPENSIONS.

| Test Fungi spores | Minimum Inhibitory Concentration (MIC) in mM[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TBTA | | | TPTA | | | TPTS | | |
| | 0h | 24h | 72 | 0h | 24h | 72h | 0h | 24h | 72h |
| Aspergillus flavus | 0.029 | 0.03 | 0.06 | 0.049 | 0.049 | 0.20 | 0.10 | 0.12 | 0.24 |
| Aspergillus niger | 0.029 | 0.03 | 0.06 | 0.037 | 0.050 | 0.20 | 0.10 | 0.12 | 0.24 |
| Rhizopus stolonifer | 0.029 | 0.03 | 0.05 | 0.037 | 0.040 | 0.16 | 0.10 | 0.12 | 0.24 |
| Penicillium citrinum | 0.01 | 0.014 | 0.02 | 0.024 | 0.024 | 0.08 | 0.04 | 0.04 | 0.07 |

a. 1.0mM of TBTA, TPTA and TPTS = 349.1, 409.0 and 487.1ug/ml respectively.

TABLE 7.3: MIC OF TBTA, TPTA AND TPTS AT 28°C AND BOILED FOR 10 MINUTES AT 100°C AGAINST $10^8$ cfu/ml OF TEST FUNGI SPORES SUSPENSIONS.

| Test fungi spores | Minimum inhibitory Concentration (MIC) in mM[b] | | | | | |
|---|---|---|---|---|---|---|
| | TBTA | | TPTA | | TPTS | |
| | 28°C | 100°C | 28°C | 100°C | 28°C | 100°C |
| Aspergillus flavus | 0.023 | 0.03 | 0.049 | 0.63 | 0.10 | 0.75 |
| Aspergillus niger | 0.023 | 0.03 | 0.037 | 0.48 | 0.10 | 0.75 |
| Rhizopus stolonifer | 0.023 | 0.03 | 0.037 | 0.48 | 0.10 | 0.75 |
| Penicillium citrinum | 0.014 | 0.02 | 0.024 | 0.31 | 0.04 | 0.30 |

b 1.0mM of TBTA, TPTA and TPTS = 249.1, 409.0 and 487.1 ug/ml respectively.

boiling of tested concentrations of TBTA has no significant effect on it's fungistatic activity. However, the antifungal activity of TPTA and TPTS was drastically reduced by 12.9 and 7.5 folds respectively, that is, about 1,191.7% and 650.0% increase in TPTA and TPTS controls MIC will be required after boiling to display inhibitory effect. These results suggest that heating to boiling of triphenyltin acetate and triphenyltin salicylate has a direct effect on its biological activities contrary to the observation with TBTA that have almost the same MIC at 28°C and 100°C.

## 7.4 DISCUSSION

The widespread biocidal applications of the organotins necessitate the study of its biological stability under

simulated environmental conditions. This is an attempt to assess the impact of these compounds on the environment.

Some organotins are known to be degraded by stepwise loss of organic groups through biological and chemical cleavage i.e. $R_1Sn$ -- $R_2SnX_2$ -- $RSnX_3$ and by ultra- violet irradiation. Thus, once an organotin has exerted its biocidal function, it apparently does not pose any long-term hazard to its surrounding environment.

The results from this study has shown that MIC of TBTA exposed to light for up to 72h was about two times higher than the observed MIC when exposed to light for up to 24h. This observation tends to suggest that the antimicrobial effect of TBTA when used as a fungicide system for yams may still be significant even after 3 days. TBTA has been reported to have strong affinity with organic matter in loam, clay and sandy soils, hence, its suspected persistency in the soil (Blunden, et al, 1984). However, Blunden and co-workers (1984b) have reported that TBTO at 10 to 100ug/ml had no deleterious biological impact on soil ammonification, nitrification, sulphur oxidation, soil respiration or microbial population. The presence of 10 to 100ug/ml of this compound in soil appear not to be a serious threat to valuable microbial ecology of the soil provided this value is not exceeded. The MIC of TBTA after exposure to light as reported above for 72h, was 5.0 to 21.0ug/ml (0.014 - 0.06mM), this is clearly within the maximum values tolerated by useful micro-organism

in the soil as proposed by Bollen and Tu (1972).

The exposure of TPTA or TPTS to light for 72h suggest an appreciable reduction on its biological activity. For example, the MIC of TPTA after 72h exposure to 40 watt tropical day light florescence against A. flavus, A. niger, R. stolonifer and P. citrinum were 0.20, 0.20, .016 and 0.08mM respectively. The corresponding control MIC of TPTA against these fungi spores were 0.049, 0.037, 0.037 and 0.024mM respectively. This 3.33 to 5.41 reduction in antifungal activity of TPTA when exposed to light for 72h may indicate fast photo-degradation of TPTA, compared with TBTA. Blunden et al (1984) reported that there was a gradual disappearance of triphenyltin compounds when exposed to light in watch glass. Thus, the seemingly increase in the effective concentration of TPTA or TPTS might be due to disappearance of the potent TPTA or TPTS in the reaction mixture.

Similarly, there was 7.50 to 12.97 reduction in the fungistatic effect of TPTS or TPTA when heated to boiling in water bath at 100°C. Thus, if this reduction in biological activity was due to disappearance of potent triphenyltin compounds, light and heating processes may confer a kind of safety to any consumer of wounded yam tuber treated with TPTA or TPTS.

# CHAPTER EIGHT.

# GENERAL DISCUSSION AND CONCLUSION.

## 8.1 GENERAL DISCUSSION.

This project was designed to study the efficacy of TBTA, TPTA and TPTS alone and in admixtures in combating yam rot. The methods used have been discussed in chapters two to seven. However, the following points of interest on methodology may be noted. The organisms isolated and identified as fungi yam rot pathogens are Aspergillus niger, Rhizopus stolonifer, Penicillium citrinum and Aspergillus flavus. It is the first time that the last two identified fungi, are being identified as yam rot fungi pathogens.

In addition to the common methods known for determination of minimum fungicidal concentrations, the use of sterile paper discs, has not been widely reported and it appears to suggest that the method can be used for testing antifungal compounds in suspension. It appears also that the use of acetone to solubilize or disperse the test compound had not been widely known for triorganotin compounds. It is thus fortunate, because acetone was found to be capable of either solubilizing or forming very fine suspensions of the test compounds. Unlike, other known suspending agent like polysorbate80, acetone as observed in this study does not interfere with the potency as highlighted in chapters three and four.

The methods used for the determination of rates of kill, were commonly known microbiological methods. However, the methods used in the determination of test compounds effectiveness on yam-rot, had to be

designed almost totally with the aim of meeting the natural conditions which favour maximum growth of yam rot fungi spores in the tropics as much as possible. Study had to be done at 30°C and 90% relative humidity to simulate the best conditions for growth of yam rot fungi spores. This approach should give an insight into what to expect in any field test where yam rot is very rampant as far as the physical conditions are concerned.

Studies of this nature, using yam tuber with triorganotins had not been much reported before now. This method may serve as a useful guide for subsequent working on root crops.

The test of the compounds under light and heat to boiling conditions were carried out to pre-empt effects of the physical conditions under which subsequent users of the compounds may work in. Studies under these varying conditions of yam treatment with TBTA, TPTA and TPTS as the ultimate goal has not been much reported.

One of the contributions of this work is that the test concentration of polysorbate 80 for harvesting spores such that would not inactivate test triorganotin was studied. Hitherto, polysorbate 80 has been recommended for harvesting fungi spores without much thought of non-inactivating concentration of polysorbate 80 to be used to get the most accurate result on the antimicrobial potency of the test compounds.

The results reported in this thesis have shown that TBTA and TPTA are effective against mycelial fungi spores including A. flavus, R. stolonifer, P. citrinum, and A. niger TPTS synthesised in Department of Chemistry, A.B.U Zaria, Nigeria, is similarly fungicidal. Besides, the results have shown that their fungicidal activity increase with increase in concentration.

The results of MICs and MFCs of TBTA, TPTA and TPTS against test fungi spores have shown that the order of antifungal activity of test triorganotins was TBTA > TPTA > TPTS (corresponding molecular weight being 349, 409 and 487 respectively). This observation is in conformity with the knowledge that antimicrobial activity of higher triorganotins decreases with increase in molecular weight.

Triphenyltin salicylate differ chemically from triphenyltin acetate by replacement of the acetate group with a salicylate. Salicylic acid itself in un-ionised form is known to have an antifungal activity as indicated in this study. However, the replacement of acetate with salicylate does not confer significant activity on this compound. These results also appear to confirm with the suggestion that the biocidal activity of the triorganotin compounds is in the cation. Furthermore, the slight decrease in antifungal activity of TPTS compared to TPTA might be due to the intra-molecular co-ordination enhanced by the presence of 2-OH on TPTS which reduces ionisation of the compound thereby masking the fungicidal active stannyl centre.

The rate of kill of TBTA, TPTA and TPTS studied are probably being reported for the first time for yam rot fungi spores or other fungi spores. This is a useful information and probably open a new field of study as to how fast triorganotins act after applications for combating fungal attack of yam. The results obtained from the rate of kill experiments using TBTA, TPTA and TPTS indicate that these compounds possess significant fungicidal activity against spores of the four yam rot fungi viz A. flavus, R. stolonifer, P. citrinum and A. niger. Their fungicidal activity was rapid and generally concentration dependent.

Garret and Brown (1964) reported that there was no single concentration of an antimicrobial agent at which all cells in a suspension would be killed instantaneously. Killing of cells occur chiefly as a function of time within a range of concentration and this probably explains the increased lethal activity of higher concentrations of these agents above the minimum fungicidal concentration.

It was also observed from the results obtained that the four test yam rot fungi were not equally susceptible to the triorganotins as one was more sensitive than the others. The determination of death rates as reported in this thesis has made it possible to compare the sensitivities of different test fungi species at the same concentration of the test compounds as well as the sensitivity of any of the test fungi spores at different concentrations of the agent. Aspergillus flavus and A. niger are the least and most sensitive of the four test yam rot fungi respectively. Although this observation was not expected, it is not an unusual response by microbes of the same species. While one strain of the same species may be very susceptible to a concentration of an antibiotic such as ampicillin, another strain of the same species may be resistant to a concentration hundred times higher than the former concentration. The reduced susceptibility may be due to atypical structural differences within the cells or spores. Each of the test compounds studied showed similar pattern of antifungal activities against the four test fungi spores within the range of concentration examined. For any particular agent, as the concentration increases, the susceptibility becomes more pronounced as indicated in the death curves of A. niger and A. flavus.

This general pattern of the death curves was also similar to the death curves displayed by P. citrinum and R. stolonifer spores with increase in fungicidal concentration of test triorganotins. This similarity in pattern of antifungal activity may suggest similar mode of action against the yam rot fungi spores. It is known that the active centre for biological activity of the triorganotins is the tin ion radical in solution. This tin centre is common to all the test drugs, hence the similarity in the antifungal activity.

One of the aims of this investigation is to establish probable combination advantage of TBTA, TPTA or TPTS with PG as fungicide against yam-rot fungi, in view of the inherently potent activity of these test triorganotins when acting alone. Results from these studies have shown that TPTA-PG and TPTS-TBTA combinations produced synergistic, while TBTA-PG and TPTA-TPTS generally displayed additive effects. The most desirable effects resulting from simultaneous application of two antimicrobial responses as observed in this study. Furthermore, the combination of 400ug/ml of TPTA with 15 - 45% PG for example achieved an ED-90 within three minutes. This considerably lower concentrations of triorganotins - propylene glycol achieved one log cycle reduction of the test fungi spores within three minutes.

The in-vivo results have shown that more triorganotin is needed to prevent the growth of pathogen fungi spores compared with in-vitro MIC values of the test compounds. Such concentration increase with in-vivo experiments is not an un- usual observation. Reasons for this are not very clear, but it is possible that the tissue and chemical component of the host can adsorb some of the test compounds. These concentrations,

i.e. 0.63mg/ml and 1.25mg/ml of TBTA and TPTA respectively were found very effective in arresting the growth of yam rot fungi spores even at high relative humidity of 90% that favour maximum growth of these fungi on yam tuber. This concentration of TPTA, that is, 1.25mg/ml is far less than 2-10%, that is, 20.0 to 100.0mg/ml of triphenyltin derivatives in Duter and Brestan currently in use for spraying leaves of sugar beet, potatoes, black pepper, cacao, cowpea, maize, carrot, onion, winged beans, apples, ground nut and rice against various fungal diseases. The findings from this study has shown that much lower concentration of test triorganotins has been effective alone and in admixtures with 25% PG in combating yam rot fungi spores.

It is therefore expected that this considerably lower concentration of the test triorganotin in the admixtures, will minimise the toxicity and solubility problems associated with high concentrations of these biocidal compounds. This is because, the in-vivo minimum effective concentration i.e. 100ug/ml of these triorganotins in combination with 25% PG was about 200-1000 folds less than concentration in-use when acting alone. Thus, these compounds in combination with PG may not after all pose any significant toxicity threat when used as yam preservative. Furthermore, the 3.3 to 5.4 and 7.5 to 13.0 reduction in biological activity of triphenyltin acetate exposed to three days light and 10 minutes boiling at $100^0$C respectively appear to support the report of fairly rapid degradation of the triorganotins which may present no toxicity threat to either the environment or the consumer of yam treated with these triorganotins as preservatives.

In view of this observed photo-and thermo-labile nature of TBTA,TPTA and TPTS as reported in this study and the unlikelihood of their posing any toxicity problem at the reported very low effective concentrations, tributyltin acetate (TBTA), triphenyltin acetate (TPTA), or triphenyltin salicylate (TPTS) alone or in combination especially with propylene glycol can be recommended for formulation as preservatives against fungal yam rot. The important finding of potentiated effects of PG on the antifungal activity of TBTA, TPTA and TPTS is worth noting. This is because, Aldridge (1976) cited a patent report in U.S.A that sucrose has been found to confer synergistic antifungal effects on TBTO. He postulated that, this could be due to the fact that, the organisms utilised the sucrose as nutrient which have easy permeability across the cell membrane with resultant absorption of the triorganotins. But, it is not likely that PG is as good a nutrient as sucrose. The submission of Aldridge (1976) therefore probably might not be all the explanations for the potentiated effects of sucrose with triorganotins. Alternative explanation have been given in this study. In any case, the observation that PG has similar potentiating effects on the antifungal properties of triorganotin is because much lower concentration of triorganotin will be needed for potent antifungal effects in practical use. PG is a common additive in a number of drugs formulations and therefore have been proved over time not to pose any health hazard. It is possible from this finding that glycerol could also have a similar potentiated effects on triorganotin carboxylates. This aspect is left for future study.

Furthermore, it may be helpful to suggest more work on stability of TBTA, TPTA and TPTS alone and in combination with PG in the tropical soils and waters and their impact on useful micro-organisms in tropical soils and waters. The knowledge gained will be helpful in producing effective preservatives for yam setts for planting or stored ware yams.

## 8.2 CONCLUSION.

The main contributions to scientific knowledge from this study can be summarised as follows:

1. These studies most likely represent the first report that implicated and proved Aspergillus flavus and Penicillium citrinum as soft yam rot causative agents in the tropic.
2. This is the first reported comprehensive investigations into fungicidal activity of TBTA, TPTA and TPTS against some yam rot fungi spores namely, A. flavus, A. niger, R. stolonifer and P. citrinum in Nigeria.
3. The antifungal activities of TBTA, TPTA and TPTS are drastically reduced by polysorbate80 with concentrations of 0.5% and above
4. TBTA, TPTA and TPTS can now be described as fungicidal for A. flavus, A. niger, R. stolonifer, P. citrinum spores and this effect vary significantly with variations in concentrations above the threshold for minimum fungicidal activity.
5. A strong case is presented mainly in favour of chemical interaction with probable coagulation of vital components of test fungi spores by the rapid rate of kill of the test spores.

6. There is a strong indication that the order of fungicidal activity against the test fungi spores by test triorganotins was TBTA > TPTA > TPTS.

7. This study showed that there are differences in fungicidal activity of TPTA and TPTS inspite of their structural similarity.

8. There are significant species differences in the susceptibility response of A. flavus and A. niger which are the least and most sensitive of the test fungi respectively to TBTA, TPTA and TPTS.

9. The in-vitro and in-vivo experiments indicate that combinations of TBTA, TPTA or TPTS with PG elicited potentiation response against all test fungi spores and offer potentially more effective and safer antifungal yam rot preservatives at reduced cost.

10. Exposure of effective concentration of TPTA or TPTS to either 40 watt tropical day light fluorescent or boiling at 100°C for 10 minutes strongly indicate degradation of these compounds.

11. Formulations of TBTA, TPTA and TPTS alone or in combination with one another and/or propylene glycol are being proposed for development as yam anti-rot agent or preservatives especially for yam sett meant for planting.

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## APPENDICES

TABLE A.1: FIC OF TBTA IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF P. citrinum

| MIC of TBTA 5µg/ml | | MIC of PG 12% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 1.0 | 0.2 | 8.0 | 0.67 | 0.87 |
| 2.0 | 0.4 | 7.0 | 0.58 | 0.98 |
| 3.0 | 0.6 | 6.0 | 0.50 | 1.10 |
| 3.5 | 0.7 | 5.0 | 0.42 | 1.12 |
| 4.0 | 0.8 | 3.5 | 0.30 | 1.10 |
| | | | | 5.17 |

Mean {FICs = 1.03 Range 0.35

FIC = a/b

a = the concentration of antifungal agent present in the combination that is inhibitory and

b = the concentration of the antifungal agent which would produce the same effect as the combination when acting alone (Elion, et al, 954)

FIC = Fractional inhibitory concentrations of the combined drugs.

TABLE A.2: FIC OF TBTA IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Rhizopus stolonifer

| MIC of TBTA 15µg/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 3.0 | 0.02 | 12 | 0.80 | 1.00 |
| 4.0 | 0.27 | 10 | 0.67 | 0.94 |
| 6.0 | 0.40 | 9 | 0.60 | 1.00 |
| 8.5 | 0.53 | 8 | 0.53 | 1.06 |
| 10.0 | 0.67 | 7 | 0.47 | 1.14 |
| | | | | 5.0 |

Means of FICs = 1.00

Range = 0.20

TABLE A.3: FIC OF TBTA IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Aspergillus flavus

| MIC of TBTA 16µ/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 2.0 | 0.13 | 10.0 | 0.67 | 0.80 |
| 4.0 | 0.25 | 9.0 | 0.60 | 0.85 |
| 6.0 | 0.38 | 8.0 | 0.53 | 0.91 |
| 8.0 | 0.50 | 7.0 | 0.47 | 0.97 |
| 13.0 | 0.80 | 3.0 | 0.20 | 1.00 |
| | | | | 4.53 |

Mean of FICs = 0.91

Range = 0.17

TABLE A.4: FIC of TBTA IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Aspergillus niger

| MIC of TBTA 16µ/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 4.0 | 0.25 | 10.0 | 0.67 | 0.92 |
| 6.0 | 0.38 | 9.0 | 0.60 | 0.98 |
| 8.0 | 0.50 | 8.0 | 0.53 | 1.03 |
| 10.0 | 0.63 | 7.0 | 0.47 | 1.10 |
| 12.5 | 0.78 | 3.0 | 0.20 | 0.98 |
| | | | | 5.10 |

Means of FICs = 1.00
Range = 0.18

TABLE A.5: FIC OF TPTA IN ADMIXTURE WITH PG THAT GIIVE COMPLETE INHIBITION OF Penicillium citrinum

| MIC of TPTA 16ug/ml | | MIC of PG 12% | | |
|---|---|---|---|---|
| Conc. in Admixture (ug/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 3.5 | 0.20 | 7.0 | 0.58 | 0.78 |
| 5.0 | 0.30 | 5.0 | 0.43 | 0.73 |
| 6.5 | 0.40 | 3.5 | 0.28 | 0.68 |
| 9.5 | 0.60 | 2.5 | 0.20 | 0.80 |
| 13.0 | 0.88 | 1.5 | 0.10 | 0.90 |
| | | | 3.89 | |

Means of FICs = 78
Range 0.22

TABLE A.6: FIC OF TPTA IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Rhizopus stolonifer

| MIC of TPTA 16µg/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (ug/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 4.0 | 0.25 | 10.0 | 0.67 | 0.92 |
| 8.0 | 0.50 | 9.0 | 0.60 | 1.10 |
| 9.0 | 0.56 | 6.5 | 0.43 | 0.99 |
| 10.0 | 0.63 | 5.0 | 0.33 | 0.96 |
| 12.5 | 0.78 | 3.0 | 0.20 | 0.98 |
| – | | | | 4.95 |

Means of FICs = 0.99

Range = 0.18

TABLE A.7: FIC OF TPTA IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Aspergillus flavus

| MIC of TPTA 16µg/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 2.0 | 0.13 | 10.0 | 0.67 | 0.80 |
| 4.0 | 0.25 | 8.5 | 0.57 | 0.82 |
| 8.0 | 0.50 | 6.5 | 0.43 | 0.93 |
| 12.0 | 0.75 | 5.0 | 0.33 | 1.08 |
| 13.0 | 0.80 | 2.5 | 0.17 | 0.97 |
| | | | | 4.60 |

Means of FICs = 0.92

Range = 0.28

TABLE A.8: FIC OF TPTA IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Aspergillus niger

| MIC of TPTA 16ug/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 2.0 | 0.13 | 10.0 | 0.67 | 0.80 |
| 4.0 | 0.25 | 8.5 | 0.60 | 0.82 |
| 8.0 | 0.50 | 6.5 | 0.43 | 0.93 |
| 12.0 | 0.75 | 5.0 | 0.33 | 1.08 |
| 13.0 | 0.80 | 2.5 | 0.18 | 0.98 |
| | | | | 4.61 |

Means of FICs = 0.92

Range = 0.28

TABLE A.9 FIC OF TPTS IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Penicillium citrinum

| MIC of TPTS 60µ/ml | | MIC of PG 12% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 3.0 | 0.05 | 10.0 | 0.83 | 0.88 |
| 5.0 | 0.08 | 9.0 | 0.75 | 0.83 |
| 10.0 | 0.17 | 8.0 | 0.67 | 0.84 |
| 15.0 | 0.25 | 7.0 | 0.58 | 0.83 |
| 30.0 | 0.50 | 4.5 | 0.40 | 0.90 |
| | | | | 4.28 |

Means of FICs = 0.86

Range = 0.05

TABLE A.10: FIC OF TPTS IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Rhizopus stolonifer

| MIC of TPTS 60µg/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 30.0 | 0.50 | 10.0 | 0.67 | 1.17 |
| 35.0 | 0.58 | 8.0 | 0.53 | 1.11 |
| 40.0 | 0.67 | 6.0 | 0.38 | 1.05 |
| 45.0 | 0.75 | 4.0 | 0.27 | 1.02 |
| 48.0 | 0.80 | 3.0 | 0.20 | 1.00 |
| | | | | 5.35 |

Means of FICs = 1.07

Range = 0.15

TABLE A.11: FIC OF TPTS IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Aspergillus flavus

| MIC of TPTS 60µg/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 20 | 0.33 | 10.0 | 0.67 | 1.00 |
| 25 | 0.42 | 9.0 | 0.60 | 1.20 |
| 30 | 0.50 | 8.0 | 0.53 | 1.03 |
| 35 | 0.58 | 7.0 | 0.47 | 1.05 |
| 48 | 0.80 | 3.0 | 0.20 | 1.00 |
| | | | | 5.10 |

Means of FICs = 1.02

Range = 0.05

TABLE A.12: FIC OF TPTS IN ADMIXTURE WITH PG THAT GIVES COMPLETE INHIBITION OF Aspergillus niger

| MIC of TPTS 60µg/ml | | MIC of PG 15% | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (%) | FIC | FICS |
| 20.0 | 0.33 | 10.0 | 0.67 | 1.00 |
| 25.0 | 0.42 | 9.0 | 0.60 | 1.02 |
| 30.0 | 0.50 | 8.0 | 0.53 | 1.03 |
| 35.0 | 0.58 | 7.0 | 0.47 | 1.05 |
| 48.0 | 0.80 | 3.0 | 0.20 | 1.00 |
| | | | | 5.10 |

Means of FICs = 1.02

Range = 0.05

TABLE A.13: FIC OF TPTA IN ADMIXTURE WITH TBTA THAT GIVES COMPLETE INHIBITION OF P. citrinum

| MIC of TPTA 10µg/ml | | MIC of TBTA 4µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 2.0 | 0.20 | 3.2 | 0.80 | 1.10 |
| 3.0 | 0.30 | 2.8 | 0.70 | 1.00 |
| 4.0 | 0.40 | 2.4 | 0.60 | 0.90 |
| 6.0 | 0.60 | 2.0 | 0.50 | 1.10 |
| 8.0 | 0.80 | 1.0 | 0.25 | 1.05 |
| | | | | 5.15 |

Means of FICs = 1.03

Range = 0.20

TABLE A.14: FIC OF TPTA IN ADMIXTURE WITH TBTA THAT GIVES COMPLETE INHIBITION OF Rhizopus stolonife

| MIC of TPTA 16µg/ml | | MIC of TBTA 10µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 13.0 | 0.80 | 2.0 | 0.20 | 1.00 |
| 12.0 | 0.75 | 4.0 | 0.40 | 1.15 |
| 10.0 | 0.63 | 5.0 | 0.50 | 1.13 |
| 6.0 | 0.38 | 6.0 | 0.60 | 0.98 |
| 4.0 | 0.25 | 8.0 | 0.80 | 1.05 |
| | | | | 5.31 |

Means of FICs = 1.06

Range = 0.17

TABLE A.15: FIC OF TPTA IN ADMIXTURES WITH TBTA THAT GIVES COMPLETE INHIBITION OF Aspergillus flavus

| MIC of TBTA 16µg/ml | | MIC of TBTA 10µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 13.0 | 0.80 | 2.0 | 0.2 | 1.00 |
| 12.0 | 0.75 | 4.0 | 0.4 | 1.15 |
| 10.0 | 0.63 | 5.0 | 0.5 | 1.13 |
| 6.0 | 0.38 | 6.0 | 0.6 | 0.98 |
| 4.0 | 0.25 | 8.0 | 0.8 | 1.05 |
| | | | | 5.31 |

Means of FICs = 1.06

Range = 0.17

TABLE A.16: FIC OF TBTA IN ADMIXTURE WITH TBTA THAT GIVES COMPLETE INHIBITION OF Aspergillus niger

| MIC of TPTA 20µ0/ml | | MIC of TBTA 10µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 48.0 | 0.80 | 2.0 | 0.20 | 1.00 |
| 12.0 | 0.60 | 4.0 | 0.40 | 1.00 |
| 10.0 | 0.50 | 6.0 | 0.60 | 1.10 |
| 6.0 | 0.30 | 7.0 | 0.70 | 1.00 |
| 4.0 | 0.20 | 8.0 | 0.80 | 1.00 |

5.7

Means of FICs = 1.02

Range = 0.10

TABLE A.17: FIC OF TPTS IN ADMIXTURE WITH TBTA THAT GIVES COMPLETE INHIBITION OF P. citrinum

| MIC of TPTS 60µ/ml | | MIC of TBTA 4µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 48.0 | 0.80 | 0.5 | 0.10 | 0.90 |
| 25.0 | 0.45 | 1.0 | 0.25 | 0.70 |
| 12.0 | 0.20 | 2.0 | 0.50 | 0.70 |
| 10.0 | 0.17 | 2.5 | 0.63 | 0.80 |
| 6.0 | 0.10 | 3.0 | 0.75 | 0.85 |

4.32

Means of FIC = 0.86

Range = 0.20

TABLE A.18: FIC OF TPTS IN ADMIXTURE WITH TBTA THAT GIVES COMPLETE INHIBITION OF Rhizopus stolonifer

| MIC of TPTS 60µg/ml | | MIC of TBTA 10µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 36.0 | 0.60 | 2.0 | 0.20 | 0.80 |
| 15.0 | 0.25 | 4.0 | 0.40 | 0.65 |
| 12.0 | 0.20 | 6.0 | 0.60 | 0.80 |
| 10.0 | 0.17 | 7.0 | 0.70 | 0.87 |
| 6.0 | 0.10 | 8.0 | 0.80 | 0.90 |
| | | | | 4.02 |

Means of FICs = 0.80

Range = 0.25

TABLE A.19: FIC OF TPTS IN ADMIXTURE WITH TBTA THAT GIVES COMPLETE INHIBITION OF Aspergillus flavus

| MIC of TPTS 60µg/ml | | MIC of TBTA 10µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 48.0 | 0.80 | 1.0 | 0.10 | 0.90 |
| 15.0 | 0.25 | 4.0 | 0.40 | 0.65 |
| 12.0 | 0.20 | 6.0 | 0.60 | 0.80 |
| 10.0 | 0.17 | 7.0 | 0.70 | 0.87 |
| 6.0 | 0.10 | 8.0 | 0.80 | 0.90 |
| | | | | 4.12 |

Means of FICs = 0.82

Range = 0.25

TABLE A.20: FIC OF TPTS IN ADMIXTURE WITH TBTA THAT GIVES COMPLETE INHIBITION OF Aspergillus niger

| MIC of TPTS 60µg/ml | | MIC of TBTA 10µg/ml | | |
|---|---|---|---|---|
| Conc. in Admixture (µg/ml) | FIC | Conc. in Admixture (µg/ml) | FIC | FICS |
| 48.0 | 0.80 | 1.0 | 0.10 | 0.90 |
| 15.0 | 0.25 | 4.0 | 0.40 | 0.65 |
| 12.0 | 0.20 | 6.0 | 0.60 | 0.80 |
| 10.0 | 0.17 | 7.0 | 0.70 | 0.87 |
| 6.0 | 0.10 | 8.0 | 0.80 | 0.90 |
| | | | | 4.12 |

Means of FICs = 0.82

Range = 0.25