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# SENSITIVITY OF SOME PATHOGEN ISOLATES TO FUNGICIDES COMMONLY USED IN VEGETABLE GREENHOUSES IN ZIMBABWE

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## Abstract

The adoption of greenhouse technology for the production of high value vegetables is on the increase in Zimbabwe. This production system is chemical intensive in pest, disease and mineral nutrition management. This study sought to survey the prevalence of fungal diseases in greenhouse grown tomatoes, cucumber and green pepper in and around Bulawayo and to investigate the sensitivity of the isolated fungal pathogens to commonly used fungicides. Infected plant parts were collected for laboratory analyses from clearly diseased plants. Six fungal pathogens, namely *Phytophthora infestans*, *Fusarium oxysporum f. Sp lycopersici*, *Peronospora*, *Botrytis cinerea*, *Leveillula taurica* and *Spaerotheca fuliginea*, were isolated from these crops in the laboratory. These fungal isolates were evaluated for their susceptibility to the following commonly used fungicides: Copper Oxychloride, Chlorothalonil, Dithane M-45, Saaf and Didecyl Dimethyl Ammonium Chloride (DDAC – Spore Kill), using the broth macro-dilution method. The fungal isolates showed varied sensitivity to the test fungicides. All fungal isolates were completely inhibited by all the concentrations of Copper Oxychloride and Chlorothaloni used, except for *Phytophthora infestans*, which showed resistance at all the concentrations of these fungicides used, and *Fusarium oxysporium* which was resistant to 0.075 % Chlorothalonil. Both Dithane and Saaf were able to inhibit fungal growth at the recommended concentration, i.e. 0.2 %. Spore Kill (DDAC) completely inhibited all the fungal isolates at all the concentrations used. The best fungicidal activity was obtained with Spore kill, followed by Copper Oxychloride and then Chlorothalonil. *Phytophthora infestans* displayed resistance to all the fungicides except to Spore Kill, followed by *Fusarium oxysporium*. *Botrytis cinerea* was the most susceptible isolate to all the fungicides tested. All fungicides were effective at, or just below, their recommended concentration levels, except for Spore Kill which was effective across the board. Even though Spore Kill is a recent addition to the fungicides currently in use in greenhouses in Zimbabwe, the results of this study show that it can be adopted as a preventative fungicide in greenhouses with high levels of success.

**Key words**: Greenhouse technology, fungal pathogens, fungicide, blight, wilt, mildew

## 1. INTRODUCTION

An ever-increasing number of Zimbabwean farmers are embracing greenhouse technology, particularly those in horticulture. This technology enables farmers to realize higher yields due to the generally optimum and stable conditions inside a greenhouse for crop production. However despite these apparent benefits, the conditions inside a greenhouse also promote the growth and proliferation of pathogens, particularly those of a fungal nature. Fungal diseases are a major cause of yield and financial loss in greenhouses (Agrios, 2005). Crop damage and loss varies from mild to as much as 100 % (Jardine, 2006; Moses *et al.*, 2005). The fungus *Magnoporthe grisea* which causes rice blast disease destroys 157 million tonnes of rice annually (Rai, 2009). Losses due to fungal pathogens may be incurred pre- or post-harvest, depending on the crop, the pathogen, agronomic methods being employed and post-harvesting management. Financial losses are incurred as farmers switch to disease resistant, but often less productive varieties, or through having to invest heavily in chemicals, spraying technology and labour in order to try and manage fungal diseases (Agrios, 2005). Yield losses include reduced yields and low quality of the yield due to fungal diseases.

In Zimbabwe, several crops are commonly grown in greenhouses. These include tomatoes, strawberries, bell pepper, cucumber and other cucurbits. These are all susceptible to fungal attack. One common fungal disease in greenhouses is Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *Lycopersici*. Fusarium wilt is a vascular wilt disease that affects many crops, including those grown in greenhouses. The mycelia of *Fusarium oxysporum* f. sp. *lycopersici* are normally white to pink, and sometimes with a purple hue (Jones and Woltz, 2001).

Late blight, a disease that affects mainly tomatoes and potatoes, is caused by the fungus *Phytophthora infestans*. It can devastate tomato plants during periods of cool, rainy weather. The mycelium of *P.infestans* produces branched sporangiophores bearing lemon-shaped, papillate sporangia at the tips (Agrios, 2005). Another common greenhouse fungal disease is Powdery mildew, caused by the fungi *Spaerotheca fuliginea* and *Erisyphe cichoracearum*. *S. fuliginea* is more prevalent and more aggressive (McGrath, 2005). Conidia (asexual spores) are produced on plant surfaces during the growing season, and these develop either singly or in chains on conidiophores (Janisiewicz and Korsten, 2002). Powdery mildew fungi also reproduce sexually through sexual spores called ascospores carried in a sac-like structure called an ascus (Janisiewicz and Korsten, 2002).

Perhaps one of the most important fungal diseases of greenhouse crops is gray-mold rot or Botrytis blight, caused by *Botrytis cinerea*. This fungus affects almost all greenhouse vegetables, and a wide array of other non-greenhouse crops and even trees. This disease is characterized by a light-gray fuzzy growth that appears on stems, leaves and even fruits. Soft rot of the stem and of the fruit can also occur.

One of the biggest investments in the greenhouse production of crops is in the prevention and/ or management of fungal diseases. The most common management strategy employed by greenhouse farmers is the use of an intensive chemical management regime using fungicides like Copper Oxychloride (0.5% a.i.), Saaf (Mancozeb, 63% and Carbendazim, 12%), Azoxystrobin (22.9%, Flowable concentrate) and Chlorothalonil (54%). The advantages of using fungicides are decreased disease intensity leading to higher production, and consequently more profits (Batta, 2004).

Fungicides are generally classified as systemic, translaminar and contact. Contact fungicides are not taken up by the plant, but instead protect only that part of the plant on which they are deposited during spraying. Translaminar fungicides are similar to contact fungicides in that they are not taken up by the plant, but they have a special ability to spread from the upper, and usually commonly sprayed, surface of plant leaves to the unsprayed underside, ensuring a more complete application of the fungicide. Lastly, systemic fungicides are taken up by the plant and are distributed through the plant's vascular system.

It is vital to monitor the use of fungicides in the control and management of fungal diseases in greenhouses. Firstly, this is to ensure that the fungicide is not abused as this may lead to the development of resistance. Early types of fungicides targeted many vital biological processes at one goal (multi-site activity), thus it was highly unlikely for any fungal pathogen to develop resistance against such fungicides (Brent and Holloman, 2007). However, many modern fungicides have single-site activity, targeting only one metabolic pathway. When such a fungicide is sprayed to a population of fungal pathogens, a few resistant strains will survive the chemical attack, and spread the resistance. This spread of resistance is much faster if the fungicide is abused. Resistance to fungicides may be due to several reasons, including the possession of alternative metabolic pathways to the one targeted by the fungicide, or the ability to degrade and inactivate the active compound in the fungicide (Brent and Holloman, 2007).

Secondly, monitoring of fungicide use is important in the prevention of environmental contamination by fungicides. Repeated use of fungicides results in the accumulation of these chemicals in the soil, and/ or their escape into water ways and into water bodies through spray drift (negligible in greenhouses) and runoff (Wightwick *et al.*, 2010). Some fungicides persist in the soil for long, for example copper-based fungicides, thus they will have a negative effect on soil microbiology and potentially on soil fertility (Wightwick *et al.*, 2010). The escape of fungicides from greenhouses into water bodies comes with a number of environmental consequences. These fungicides, some of which are toxic, find their way into food chains and end up in both

terrestrial and aquatic ecosystems, resulting in oxidative stress and sometimes death of both aquatic and terrestrial biota (Aktar *et al.*, 2009).

Therefore, it is important to monitor fungicide use particularly by greenhouse farmers as these use more of these chemicals per area under crop than other farming systems. To reduce or prevent the development of resistance to fungicides by common pathogenic fungi, and the escape of fungicides into the environment, it is important to ensure that farmers are applying these chemicals at recommended rates. It is important to determine the minimum fungicide dosage that should be applied to achieve desired results. All fungicides come with recommended concentration levels but these may differ with the strain of pathogen being targeted, among other factors. In this study, fungal pathogens were isolated from some infected greenhouse grown vegetables and tested for their sensitivity to commonly used fungicides.

## 2. MATERIALS AND METHODS

### 2.1. Sample collection and preparation

Diseased plant parts were aseptically collected from visibly infected plants growing in greenhouses. Sampling was conducted from greenhouses in Esigodini (near Bulawayo) and Rosebank (Bubi district). In the lab, the samples were placed in a moisture chamber for 20 hours to allow for sufficient spore discharge in the leaves.

### 2.2. Isolation of fungi from infected leaves

The leaves were first surface sterilized in 10 % bleach for 10 minutes, and then discs cut off using sterile blades. A minimum of 3 discs were transferred into malt extract agar (MEA) plates and incubated at 25-28°C for 5 days. Any fungal growth on the MEA plates was followed by repeated sub-culturing on this growth medium until pure fungal cultures were obtained.

### 2.3. Isolation of fungi from infected stems

Infected stems had their leaves and secondary roots trimmed; leaving only the main stem and main root. The stems were surface sterilised by soaking in 10% bleach solution for 5 minutes, and then dried on paper towels. Using a sterile blade, thin (2-4 mm thick) discs were cut out of one side of the stem near the root/stem junction making sure to include xylem tissue during each cut. Five to six discs from each infected stem were placed on MEA plates and incubated at 37° C. After sufficient growth, the fungal isolates were transferred onto fresh MEA plates, followed by several rounds of sub-culturing on the same medium until pure cultures were obtained.

### 2.4. Microscopic examination of fungal mycelia and spores

The wet mount technique was used in the microscopic examination of mycelia and spores of the fungal isolates. In the case of spores, a sterile inoculating loop was used to collect as many spores as possible from the surfaces of 14-day old MEA plates. These were transferred to a drop of sterile water placed on a microscope slide and evenly spread on the slide using the inoculating loop. The spores were then viewed under the microscope. For mycelia, a sterile inoculating loop was used to collect mycelia from the edges of the colonies of 5-day old fungal MEA cultures. The mycelia were transferred to a drop of sterile water on a microscope slide and viewed under the microscope as described for the spores.

### 2.5. Fungal isolate sensitivity testing

The fungal isolates were tested for their sensitivity to the following fungicides commonly used to control fungal pathogens in greenhouses: Copper Oxychloride, Saaf (Carnendazim 12% and Mancozeb 63%), Chlorothalonil (54%), Dithane M-45 (Mancozeb 80% w.p.) and Didecyl Dimethyl Ammonium Chloride (Spore Kill).

#### 2.5.1 Preparation of fungicide solutions

For each fungicide a stock solution was first made in Sabouraud broth, and then this was used to make a serial dilution of the fungicide. The stock concentration for each fungicide was equivalent to the recommended application rate for each fungicide as specified by individual manufacturers. Each initial stock solution was serially diluted 4 times to give a serial range of fungicide concentrations as shown in Table 1.

**Table 1: Fungicides used and the different concentrations tested.**

| Fungicide | Concentration range used (% a.i.) |
|---|---|
| Copper Oxychloride | 0.50 – 0.0313 |
| Saaf | 0.20 – 0.0125 |
| Dithane | 0.20 – 0.0125 |
| Spore Kill | 0.10 - 0.0625 |
| Chlorothalonil | 0.15 – 0.0094 |

Each test tube contained 10 ml of fungicide diluted in Sabouraud broth. The six fungal isolates were tested for their sensitivity to these serial fungicide dilutions.

### 2.5.2 Test Tube Inoculation and Growth Assessment

For inoculation into the fungicide serial dilutions, agar plugs of fungal colonies were cut off from the edges of 14-day old MEA cultures of each isolate using a 2mm sterile cork borer and transferred into the fungicide-amended media. A control tube, without fungicide, was included in each set-up. The test tubes were incubated at 28°C for 72 hrs before assessing them for any fungal growth. Each setup was done in triplicate.

### 2.5.3 Growth Assessment

For each fungicide concentration used, fungal growth was determined. A scale that stretches from 0 - 5 was used to represent growth, with 0 representing no growth and 5 representing maximum growth (Table 2). Maximum growth was obtained when the hyphae covered the entire diameter of the test tube (15mm) containing the growth amended media.

**Table 2: Fungal Growth Assessment Criteria in Fungicide Solutions.**

| | | | | | | |
|---|---|---|---|---|---|---|
| **Hyphae diameter (mm)** | 0 | 5 | 7.5 | 10 | 12.5 | 15 |
| **Growth score** | 0 | 1 | 2 | 3 | 4 | 5 |

These fungicide sensitivity results were used to determine the minimum inhibitory concentration (MIC) of each fungicide to the fungal isolates.

## 3. RESULTS AND DISCUSSION

### 3.1 Pathogen Identification from Various Plant Hosts

Determining the presence of a specific fungal pathogen on a crop can easily be done by visual inspection. This, however, requires a great deal of experience. This task was made a lot easier in this study by combining the pathological symptoms presented on the different diseased plants with the appearance of the fungal pathogens, or their components, on culture media and under the microscope. As a consequence, the following fungal pathogens were identified among the isolates: *Phytophthora infestans, Fusarium oxysporum f. Sp lycopersici, Pseudoperonospora cubensis, Botrytis cinerea, Leveillula taurica,* and *Spaerotheca fuliginea* (Figure 1).

Fungi differ in both their vegetative and reproductive morphology. In this study, the microscopic appearance of both vegetative and reproductive structures of the fungal isolates was used in identification (figure 1). Some of the reproductive structures viewed under the microscope include microconidia, macroconidia and chlamydospores of *Fusarium oxysporum*¸ different shaped sporangia of *Phytophthora infestans* (lemon-shaped) and *Pseudoperonospora infestans* (elongate), conidiophores of *Botrytis cinerea* and chasmothecia of *Leveillula taurica* and *Spaerotheca fuliginea*. This study was limited to a few select greenhouse crops and fungal isolates, but there are way more fungal pathogens that are a cause for concern in greenhouses.

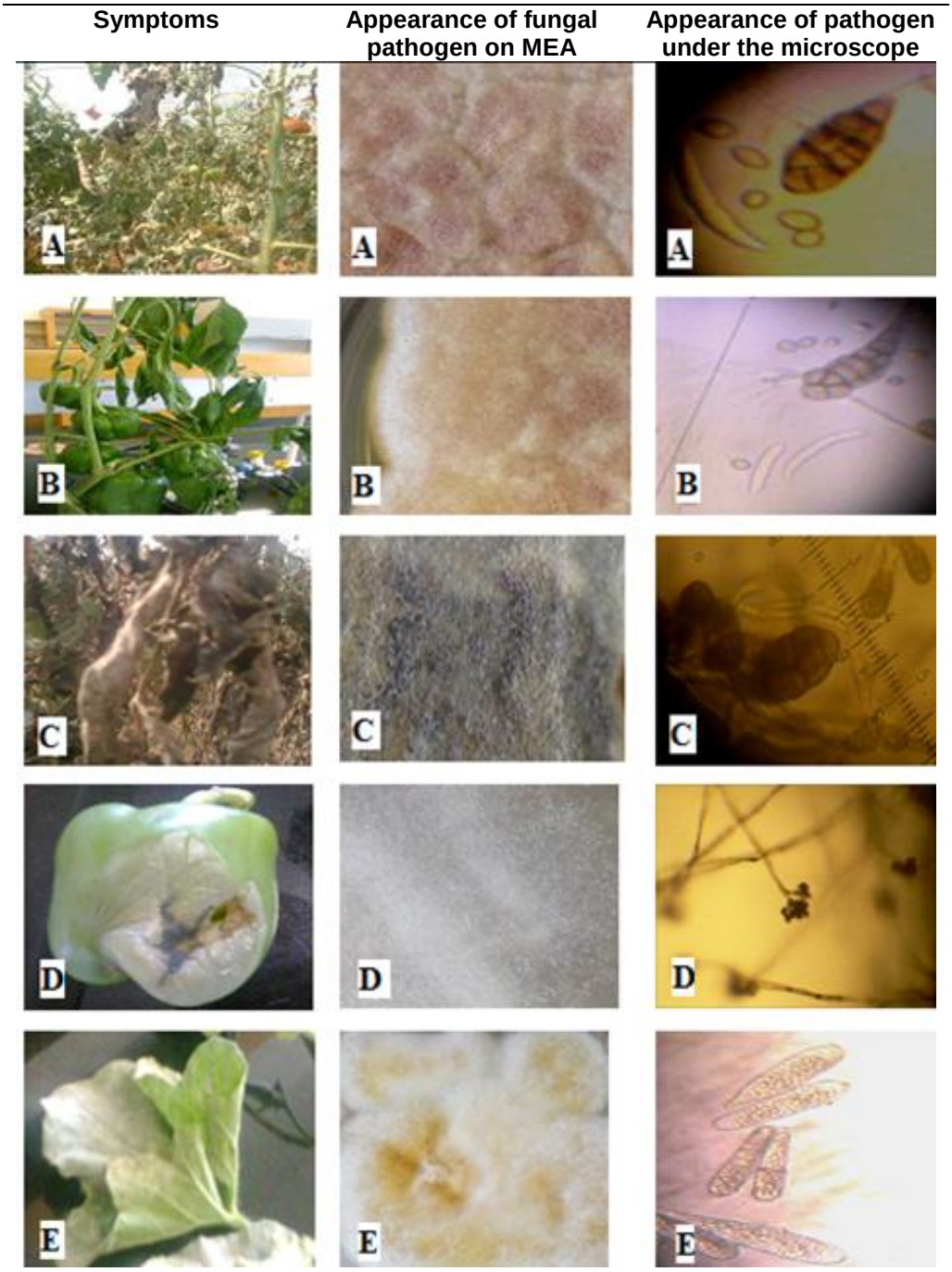


**Figure 1:** Selected fungal diseases identified in greenhouse crops, their causative agents and what these appear like in on MEA agar and under the microscope. Each picture series shows firstly an image of the field symptoms of the disease, followed by the appearance of the fungal pathogen growing on malt extract agar (MEA) and lastly the appearance of different vegetative and reproductive stages of growth of the pathogen under the microscope. The diseases identified are: **A** – Fusarium wilt damage on tomatoes, caused by *Fusarium oxysporum f. sp. neolycopersici*, **B** - Fusarium wilt damage on green pepper plant, caused by *Fusarium oxysporum f. sp. Neolycopersici*, **C** - Late blight damage on tomato leaves, caused by *Phytophthora infestans*, **D** - Grey mold damage on green pepper, caused by *Botrytis cinerea*; **E** – Downy mildew damage on cucumber leaves, caused by *Pseudoperonospora infestans*.

### 3.2. Fungicide Susceptibility Tests

The method of choice in the management and control of fungal diseases in greenhouses in the Bulawayo area of Zimbabwe is to spray using preventative fungicides. This involves the use of a weekly alternate spray comprising of copper sulphate and dithane. Thus, even though spraying is done weekly, each fungicide is sprayed every fortnight. These are preventive fungicides, effective in the prevention of fungal colonization but highly ineffective when used post-infection. As a consequence, part of this study was devoted to the determination of sensitivity of common fungal pathogens causing diseases in greenhouse crops to these, and other locally available, fungicides.

The fungal isolates were tested for their sensitivity against 6 fungicides at a wide range of concentrations, including the recommended application rate. The fungal isolates showed varied sensitivity to the test fungicides (Figure 2). Apart from Phytophthora infestans, all the other fungal isolates showed susceptibility to Copper oxychloride at all the concentrations tested. P.infestans was resistant to Copper Oxychloride at all the concentrations tested. This trend clearly shows resistance of Phytophthora infestans at all the concentrations used. Even though Botrytis cinerea showed resistance to copper oxychloride at 0.03125 %, this is far from worrying because this concentration is 16 times lower than the recommended application rate of 0.5 %, meaning Copper Oxychloride can still be used to control B. cinerea in greenhouses.

With Saaf and Dithane, the recommended application rates, i.e. 0.2 %, were sufficient to completely inhibit all fungal pathogens. However, lower concentrations of these compounds were insufficient as fungal growth was observed (Figure 2). With Chlorothalonil, the recommended fungicide concentration was effective against all fungal isolates except Phytophthora infestans. Of note on figure 2 is the effect of Spore Kill on the fungal isolates. This fungicide completely inhibited the growth of all the isolates at all the concentrations tested. Spore Kill is a fairly new addition to the fungicide collection used by greenhouse farmers in Zimbabwe. The results of this study shows that this is perhaps the most effective of the fungicides tested against the test fungal isolates. However, the recommended concentration (0.1 %) appears to be too high since even concentrations 16-times lower managed to inhibit all fungal growth. It is recommended that more sensitivity tests are conducted on a wider spectrum of fungal isolates with a view to revising the recommended application rate down.

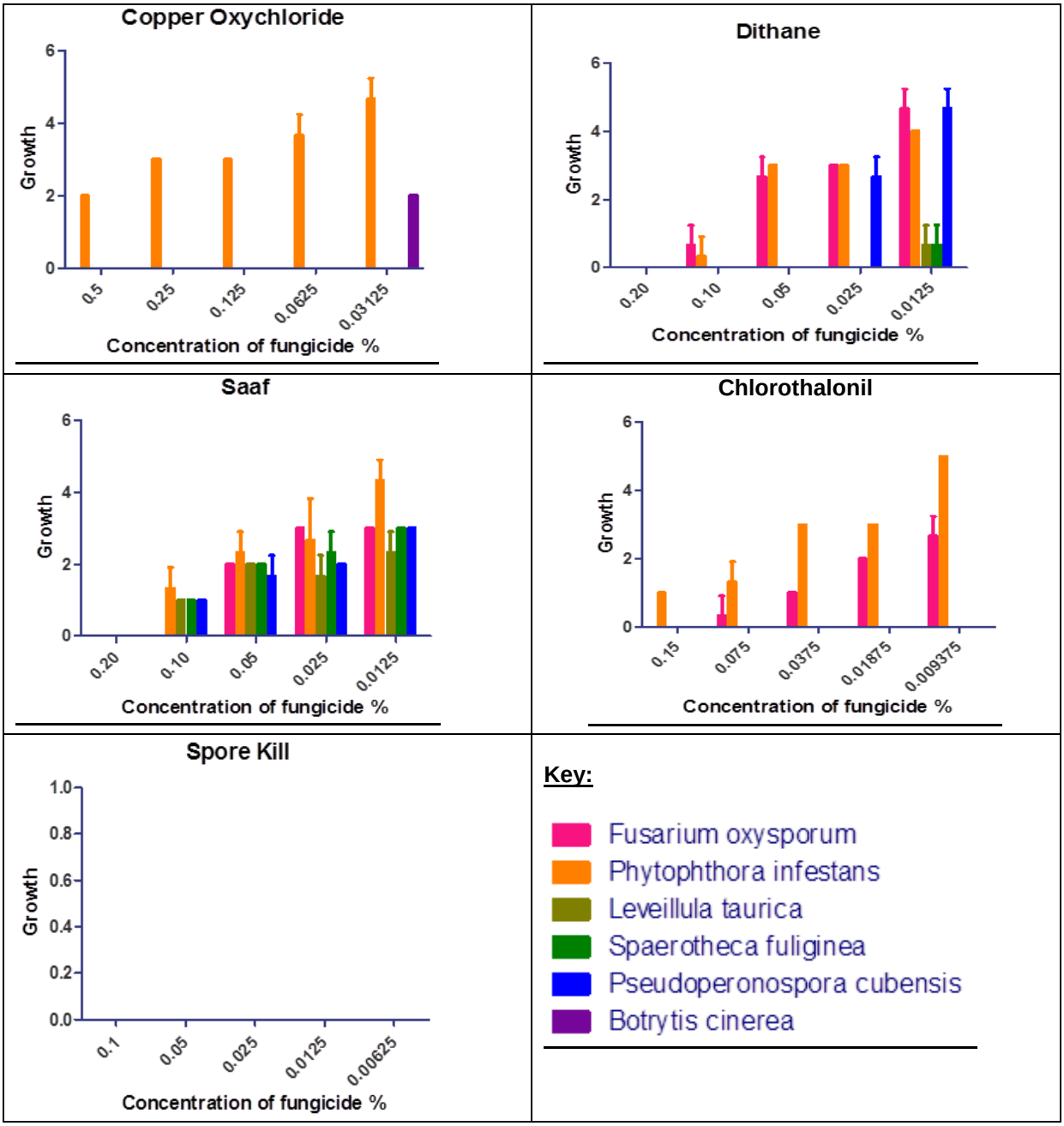


**Figure 2**: Effect of selected fungicides on the growth of the fungal isolates. The figure shows susceptibility of the fungal isolates against Copper Oxychloride, Dithane, Saaf, Chlorothalonil 1 and Chlorothalonil 2. The fungal isolates were tested for their sensitivity to different concentrations of the fungicides

### 3.3. Determination of minimum inhibitory concentrations (MICs)

The fungicide sensitivity results were used to determine the MIC for each fungicide against the fungal isolates (Table 3). The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that completely inhibits the visible growth of a microorganism after a defined period of incubation under standard culture conditions (Yilmaz, 2012). In the context of this study, it is the lowest fungicide concentration that completely inhibits mycelial growth or spore germination.

The MIC is used to determine the concentration of fungicide that should be applied to completely inhibit a given fungal pathogen. During curative application, where normally a few known fungi are being targeted, information as contained in table 3 will be valuable in coming up with the effective fungicide application rates. However, with preventative application, one will be targeting a wide range of fungal pathogens, both known and unknown. This means for the operation to be successful, the concentration applied should be the highest required inhibiting the growth of the most resistant pathogen present, even though this concentration may be several times higher than what is required to kill other members of the fungal community.

Several methods are available for the evaluation of the sensitivity of fungal pathogens to different fungicides (Rex *et al.*, 2001). In this study we used the broth macro dilution assay. This method is easy and straight forward. However one of its major limitations is its laborious nature, involving a huge volume of glassware. Another problem associated with this method is that sometimes it is difficult to measure and score mycelial growth from a test tube. This problem can be solved by the use of the solid agar method where fungicide solutions are prepared in a solid agar like potato dextrose agar (PDA) and test fungi are applied on the surface of the petri dishes as mycelial plugs of a standard size, and growth is scored by measuring radial mycelial growth about the inoculation point. This method was used successfully to evaluate the sensitivity of some important rice fungal pathogens to some of the commonly used fungicides to control these fungi (Lore *et al*., 2007).

**Table 3: Minimum Inhibitory Concentrations (MICs) of the test fungicides against the fungal isolates.**

| | Minimum Inhibitory Concentration (% a.i) | | | | | |
|---|---|---|---|---|---|---|
| **Fungicide** | ***F. oxysporum*** | ***P. infestans*** | ***L. taurica*** | ***S. fuliginea*** | ***P. cubensis*** | ***B. cinerea*** |
| **Copper oxychloride** | 0.03125 | ** | 0.03125 | 0.03125 | 0.03125 | 0.06250 |
| **Dithane** | 0.20000 | 0.20000 | 0.02500 | 0.02500 | 0.05000 | 0.01250 |
| **Saaf** | 0.10000 | 0.20000 | 0.20000 | 0.20000 | 0.20000 | 0.12500 |
| **Chorothalonil** | 0.15000 | ** | 0.00938 | 0.00938 | 0.00938 | 0.00938 |
| **Spore Kill** | *** | *** | *** | *** | *** | *** |

**Key:**

**** - Resistance at all concentrations tested**

***** - Complete inhibition at all concentrations tested.**

In overall, the oomycete *Phytophthora infestans* was the most resistant isolate to the test fungicides, being especially resistant to all the concentrations of copper oxychloride and chlorothalonil used. The ascomycete *Fusarium oxysporium* appeared to have moderate resistance to low fungicide concentrations, even though it was generally inhibited by recommended concentrations, while *Botrytis cinerea* showed the most sensitivity. Spore Kill

was the most effective fungicide, completely inhibiting all the fungal isolates tested even at the lowest concentration tested (0.0625 %), followed by copper oxychloride, which was effective against all the fungal isolates save for *Phytophthora infestans*. Thus, where resistant strains of the various pathogens are prevalent the adoption of the newly introduced fungicide Spore Kill should be considered.

## REFERENCES

Agrios, G. (2005). Plant Pathology. 5th Ed.Academic press. New York. USA. p703.

Aktar, M.W., Sengupta, D. and Chowdhury, A. (2009). Impact of pesticides in Agriculture: their benefits and hazards. Interdisciplinary Toxicology 2(1): 1–12.

Batta, Y. A. (2004). Effect of treatment with *Trichoderma harzinaum Rifai* formulated in invert emulsion on postharvest decay of apple blue mold. International Journal of Food Microbiology 96: 281- 288.

Brent, K.J. and Holloman, W. (2007). Fungicide Resistance in Crop Pathogens: How Can It be Managed. 2nd Ed. Crop International. Brussels. Beigium.

Jardine, D. (2006). Stalk Rots of Corn and Sorghum, Kansas State University.

Janisiewicz, J. and Korsten, L. (2002). Biological Control of Post-harvest Disease of Fruit. Annual Review of Phytopathology 40:411-441.

Jones, J.P. and Woltz, S.S. (2001). *Fusarium*-incited diseases of tomato and potato and their control; in *Fusarium*: Diseases, Biology, and Taxonomy. Nelson, P.E., Toussoun, T.A. and Cook, R.J. Pennsylvania State University Press, University Park, p157-168.

Lore, J.S., Thino, T.S., Hunjan, M.S. and Gael, R.K. (2007). Performance of Different Fungicides Against Multiple Diseases of Rice. Indian Phytopathology 60 (3) : 296-301.

McGrath, M.T. (2005). Guidelines for Managing Cucurbit Powdery Mildew with Fungicides. Department of Plant Pathology, Cornell University Long Island Horticultural Rsearch and Extension Centre, NY, USA.

Moses, E., Asafu-Agyei, J.N. and Ayuoboteng, F. (2005). Identification and Control of Root Rot Diseases of Cassava. CSIR-Crops Research Institute. International Society for Plant Pathology.

Rai, M. (2009). Advances in Fungal Biotechnology. I.K.International. New Delhi. India. p60.

Rex, J.H., Pfaller, M.A., Walsh, T.J., Chatuverdi, V., Espinel-Ingroff, A., Ghannoum, M.A., Gosey, L.L., Odds, F.C., Rinaldi, M.G., Sheehan, D.J. and Warnock, D.W. (2001). Antifungal Susceptibility Testing: Practical Aspects and Current Challenges14 (4): 643–658.

Wightwick, A., Walters, R., Allinson, G., Reichman, S. and Menzies, S. (2010). Environmental Risks of Fungicides Used in Horticultural Production Systems, Fungicides, Odile Carisse (Ed.), ISBN: 978-953-307-266-1.

Yilmaz, M.T. (2012). Minimum inhibitory and minimum bactericidal concentrations of boron compounds against several bacterial strains. Turkish Journal of Medical Science 42 92: 1423-1429.