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# *In vitro* growth inhibition of Kenyan *Phytophthora cinnamomi* isolates by different fungicide formulations

Mbaka[1*] J.N., Wamocho[2] L.S., Turoop[2] L. and Waiganjo[1] M.M.

[1]Kenya Agricultural Research Institute, Thika, Kenya; [2]Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

*Corresponding author e-mail: jnmbaka@yahoo.com

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ABSTRACT

*Objective*: To determine the inhibitory effects of locally available fungicides on local strains of *Phytophthora cinnamomi* causing root rot and stem canker of macadamia in Kenya.

*Methodology and results*: Pathogen isolates were obtained from soil and diseased macadamia plant parts collected during a survey, carried out from December 2005 to April 2006 in all macadamia growing areas (Central, Eastern, Western, Rift Valley and Coast provinces) of Kenya. Six commercial fungicide formulations commonly used for management of Phytophthora diseases in the horticulture industry in Kenya were evaluated. These were: Melody Duo (Iprovalicarb 55g/kg, Propineb 613 g/kg), Equation Pro (Famoxadone 225 g/kg, Cymoxanil 300 g/ kg) , Nordox 75 WP (Copper oxide 86 % w/w, 75 % metallic copper 14 % w/w), Victory 72 WP (Metalaxyl 80 g/kg, Mancozeb 640 g/kg), Topsin (Thiophanate methyl 50% w/w) and Benovarp 50 WP (Benomyl 50% w/w). Fungicide stocks were prepared in sterile de-ionized water and added to corn meal agar (CMA) at the recommended dosages. Agar discs (5mm diameter) from actively growing CMA cultures of each of the seven isolates of *P.cinnamomi* were plated on non-amended and fungicide amended CMA media in 90 mm diameter Petri dishes. The Petri dishes were wrapped with parafilm, inverted and incubated at 25 ±2 $^0$C in the dark. Mycelial growth inhibition at the recommended fungicide dose was calculated as the difference between the control and the treatment after 7 days of growth. The growth inhibition data was subjected to General Linear Models (GLM) analysis of variance using SAS version 9.1 for windows. Benovarp, Topsin and Melody duo were lethal to all the *P. cinnamomi* isolates. Nordox caused a growth inhibition of 95-100 % on all the isolates.

*Conclusion and application of findings*: The results show that some fungicides recommended for other Phytophthora diseases have an inhibitory effect on *P. cinnamomi*. These can therefore be incorporated in an integrated management strategy for macadamia root rot. Benovarp, Topsin and Melody Duo are systemic, curative and protective and can be used as soil drench, foliar sprays or trunk injections. Nordox, which is a contact protective fungicide, can be used as paint on stem cankers or wounds caused by mechanical injury, to stop entry of the fungus. However the efficacy of these formulations should be assessed further under field conditions.

Key words: Chemical control, macadamia, root rot

## INTRODUCTION

In the year 2003, Kenya was the fifth largest producer of macadamia nuts after Australia, Hawaii, South Africa and Guatemala (Onsongo, 2003). The area under macadamia cultivation in

2007 was estimated at 3,276 hectares that produced 18,161 metric tons of in-shell nuts valued at Kenya shillings 822,002,292 (USD 11,742,890) (MoA, 2007). The main export markets are in Japan, United States of America and China that purchase 80 % of nuts exported from Kenya (Onsongo, 2003). However root rot caused by the soil borne fungus *Phytophthora cinnamomi* Rands was reported as a major disease with 60 % reduction of yields in Kenya by 2005 (Muthoka *et al.,* 2005).

*P. cinnamomi* has a wide distribution with over 3,000 host species including crops such as avocado, peach, pineapple, chestnut and macadamia (Adrienne, 2005). Affected trees exhibit poor growth and display general decline, such as spurge, tufted foliage which is light green to yellow, branches' die back and substantial reduction in incremental growth rate. Disease progression may result in decline over several seasons or death within one or two seasons (Downer, 1998). *P. cinnamomi* is known to survive for as long as six years in moist soil (Zentmyer & Mircetic, 1966), and it is clear that moisture is a key factor in the establishment, spread and longevity of *P. cinnamomi* diseases.

There is no one simple method for controlling *P. cinnamomi*. Cultural control measures include reduction of soil moisture levels and improving aeration by increasing drainage and attention to mineral nutrition (Downer, 1998). Various instances of biological control of *P.cinnamomi* have been reported. Some natural soils are reported to suppress growth and reproduction of *P. cinnamomi* (Broadbent & Baker, 1974; Messenger-Routh, 1996). Several factors that influence control include the organic matter content, calcium, phosphorous and magnesium content and the population of micro-organisms (Baker & Cook, 1974). Mycorrhizal fungi exude volatile monoterpenes and sesquiterpenes which suppress *P. cinnamomi* (Kupa & Nyland, 1972; Marx, 1975; Menge *et al*., 2001).

Chemical control is achieved using systemic fungicides such as Fosetyl-Alumimium (Alliete) (Guest, 1984), phosphoric acid and metalaxyl applied as soil drench, foliar spray or trunk injection (Nartavaranat *et al.,* 2006). Phosphanates have also been shown to enhance plant defense responses, including lignification, phytoalexin accumulation and hypersensitive cell death (Guest, 1984; Saindrenan, 1990). Coffey and Joseph (1985) found that phosphoricid (4.1 to 6.2 μg $ml^{-1}$) inhibited the mycelial growth of *P. cinnamomi* in *in vitro* studies. Fenn and Coffey (1985) suggested that phosphanate metabolism may be one target of phosphanate toxicity in oomycetes. Mycelial growth of 80 isolates of *P. cinnamomi* were inhibited at an average of 89.3% at 1 or 1.25 μg active ingredient (a.i) metalaxyl in one milliliter of water with another 86 completely inhibited (Benson & Grand, 2000).

A combination of sanitation measures, good horticultural management, and selective use of fungicides and addition of organic matter to soil can be used in an integrated pest management strategy to retard the activity of *P. cinnamomi* in macadamia cultivation (Aryantha, *et al*., 2000). The objective of this study therefore, was to determine the effect of selected locally available fungicides on the *in vitro* growth of local strains of *P. cinnamomi*. The effective ones would henceforth be incorporated in the development of integrated pest management options for macadamia root rot and stem canker.

## MATERIALS AND METHODS

**Source of isolates:** Soil samples from rhizospheres of symptomatic and asymptomatic macadamia trees were collected from Central, Eastern, Western, Rift Valley and Coast provinces of Kenya. This was done during macadamia disease surveys between December 2005 and April 2006. The samples were labeled accordingly and stored at 4 ˚C. Bark and root samples from symptomatic trees were also collected and stored in a similar manner.

Isolation of *P. cinnamomi* from soil was done by using host (avocado) bait method as previously described by Zentmyer *et al*. (1960). A sub-sample (250 g) of soil was put in a plastic container and saturated with 250 ml of distilled water. Green

avocadoes (variety Fuerte) were carefully harvested to avoid injury; surface sterilized by wiping with cotton wool soaked in 70% ethanol and then rinsed in sterile distilled water. One avocado was pushed into each of the wet soil samples and incubated at 25±2 ˚C for seven days in the dark.. After 7days , the fruits were cleaned under running tap water, observed for development of a hard brown rot at the soil line and surface sterilized by dipping in 5 % sodium hypochlorite (Jik®) for 2 min then rinsed in sterile distilled water and blotter dried. A 1mm long piece was cut from the edge of the rot lesion (between healthy and necrotic area), using a sterile scapel (No. 11 fitted to a No. 3 scapel blade holder), plated on corn meal agar (CMA) in 90 mm disposable Petri dishes and incubated at 25±2 °C in the dark. The isolates were characterized on the basis of colony morphology under high power microscope (× 400) and pathogenicity to green apple fruits.

Pathogenicity tests:The use of green apple fruits was based on the principle that *P.cinnamomi* strains pathogenic to macadamia cause a hard brown rot on green apples within 2-3 days after inoculation (Zentmyer *et al*., 1960). Green apple fruits were obtained from the local market. These were surface sterilized by wiping with cotton wool dipped in 70% alcohol. Triangular slits were made on each apple by use of a sterile scapel blade inside a laminar flow cabinet to avoid contamination. The same scapel was used to cut 3mm$^2$ agar plugs from the edges of actively growing *P.cinnamomi* cultures. These were placed on the slits on the apples and sealed with Vaseline® petroleum jelly. The inoculated apples were incubated at 25±2˚C. Observations were done starting 3 days after inoculation. Development of a hard brown rot was indicative of virulence of the *P.cinnamomi* isolate (Fig. 1).

Figure1: Brown rot on apple fruits caused by *Phytophthora cinnamomi* isolates from Macdamia trees in Kenya.

Roots and bark from an individual tree were washed under running tap water to dislodge soil particles and cut into 5-10mm long segments. These were then surface sterilized by dipping in 5 % sodium hypochlorite for (Jik®) 2 min, rinsed in sterile distilled water thrice and dried with a blotter paper (Whatman No.2) before plating on potato dextrose agar (PDA). After 48 hours, mycelial growth on the agar was examined microscopically for presence of *Phytophthora* spp. hyphae. Axenic cultures were established on CMA and characterized in the same way as those from the soil samples. In both cases, the isolates were grouped on the basis of their growth on media and virulence to the green apple fruits (based on presence and size of hard brown rot).

Fungicide assays: Six commercially formulated fungicides commonly used for management of *Phytophthora* diseases in the horticulture industry in Kenya were evaluated in this study. The commercial formulations and recommended dosage rather than the active ingredient alone were evaluated to make realistic representation of compounds available for and used by growers in the field (Table 1).

Table 1: Fungicides evaluated for effect in vitro on Kenyan *Phytophthora cinnamomi* isolates.

| Trade name | Active ingredients | *Application dosage | Disease controlled | Manufact-urer | Cost (per kg or litre-Kshs) |
|---|---|---|---|---|---|
| Melody Duo | (Iprovalicarb 55g/kg, Propineb 613 g/kg), | 100g in 20 liters of water | Phytophthora blight of Tomato | Bayer crop Science | 3,500** |
| Equation Pro | Famoxadone 225 g/kg, oxide 86 % w/w | 10g in 20 liters of water | Early &late blights in tomato and potato | Dupont Crop Science | 4,500 |
| Nordox 75 WP | Copper oxide 86 % w/w, 75 % metallic copper 14 % w/w | 70g in 20 liters of water | Coffee berry disease, Phytopthora blights in potatoes | Nordox As Sweden | 800 |
| Victory 72 WP | Metalaxyl 80 g/kg, Mancozeb 640 g/kg | 50g in 20 litres of water | Late blight of tomatoes | Topsen Biotech Co., Ltd, China | 3,800 |
| Topsin | Thiophanate methyl 50% w/w | 1 ml in 1 liter of water | Broad spectrum | Dow , Newzealand | 5,600 |
| Benovarp 50 WP | Benomyl 50% w/w | 1g in 1 liter of water | Broad spectrum | VAPCO, Ltd. Jordan | 2,500 |

*Preparations recommended for foliar foliar spray. **1 USD = 75 Kenya shillings

Fungicide stocks were prepared in sterile de-ionized water and added to corn meal agar (CMA) at the recommended dosage. Four agar discs (5mm diameter) from cultures of each of the seven *P. cinnamomi* isolates actively growing on CMA were each plated on non-amended and fungicide amended CMA in 90 mm diameter Petri dishes. The Petri dishes were wrapped with parafilm, inverted and incubated at 25±2 $^0$C in the dark. The experimental design was CRD replicated 3 times (12 plates for each fungicide). The experiment was repeated three times. Measurements of colony diameter (in millimeters) were done after one week of incubation.

Inhibition of mycelial growth at the recommended fungicide dose was calculated as the difference between the control and the treatment. The difference in colony diameter between the fungicide amended and non-amended CMA cultures was calculated as the percentage growth inhibition The growth inhibition data was subjected to analysis of variance using SAS version 9.1 for windows program, and means were separated by the LSD test.

## RESULTS

Avocado fruits were effective as baits for recovery of *P.cinnamomi* from the soil samples. After surface sterilization with 5 % sodium hypochlorite, pure cultures were obtained on CMA. Based on their virulence on green apples, seven isolates coded JPc1, JPc2, JPc3, JPc4, JPc5, JPc6 and JPc7 were selected for this study.

All the seven isolates grew well on the non amended CMA and had an average diameter of 88.5-90 mm after one week. Means of percentage growth inhibition were significantly different at P=0.0496. The highest sensitivity was obtained from Benovarp 50 WP (Benomyl 50 % w/w), Topsin (Thiophanate methyl 50% w/w), and Nordox 75 WP(Copper oxide 86 % w/w, 75 % metallic copper 14 % w/w )that were lethal to six, five and three out of the seven *P. cinnamomi* isolates, respectively (Table 2).

In this study, Victory 72 WP, (Metalaxyl 80g/kg) caused the least growth inhibition (51.85-60.74 %) on six of the seven *P. cinnamomi* isolates. This could be due to resistance or the dosage of the active ingredient (metalaxyl (0.2 μg $l^{-1}$) used in this study. In *in vitro* growth inhibition studies done by Benson and Grand (2000), 1 or 1.25 μg active ingredient (a.i.) metalaxyl/ ml gave an 89.3% growth inhibition of 80 *P. cinnamomi* isolates with another 86 isolates being completely inhibited. Repeated use of metalaxyl for the control of tomato late blight (*P .infestans*) led to resistance (Mukalazi *et al*., 2001; Pappas, 2007). Phosphite was found to be a more ecologically friendly

alternative to the use of metalaxyl fungicide for the treatment of *P.cinnamomi* infestation (Guest & Grant, 1991).

Table 2: Percentage growth inhibition of isolates of *Phytophthora cinnamomi* after 2-week incubation at 27 ºC in the dark on fungicide amended Corn Meal Agar.

| | *P. cinnamomi* isolates | | | | | | |
|---|---|---|---|---|---|---|---|
| Fungicide | JPc1 | JPc2 | JPc3 | JPc4 | JPc5 | JPc6 | JPc7 |
| Melody Duo | 97.04a | 93.98a | 97.78a | 95.95a | 92.59a | 95.95a | 44.44c |
| Equation Pro | 65.19b | 77.78b | 74.81b | 29.63d | 77.78b | 22.96d | 15.15d |
| Nordox 75 WP | 97.78a | 100a | 94.04a | 100a | 98.52a | 100a | 95.95a |
| Victory 72 WP | 94.81a | 60.74bc | 55.56bc | 59.26bc | 54.81bc | 54.81bc | 51.85bc |
| Topsin | 100.00a | 100.00a | 100.00a | 90.37a | 100.00a | 95.95a | 100.00a |
| Benovarp 50 WP | 100.00a | 100.00a | 100.00a | 100.00a | 100.00a | 92.02a | 100.00a |
| LSD P=0.05 | 10.01 | 14.03 | 11.1 | 10.5 | 9.6 | 10.6 | 12.6 |

*Means in the same column followed by the same letter are not significantly different according to SNK test at P=0.0496

Benovarp 50 WP (Benomyl 50 % w/w) is a broad spectrum systemic protective and curative fungicide. Its proven toxicity to *P.cinnamomi* isolates in this study implies that it can be subjected to further experimentation for consideration in the management of *P. cinnamomi* diseases.

Five out of the six fungicides tested in this study are systemic, with an eradicative and protective mode of action. They have potential for use as foliar sprays or as soil drenches. Nordox 75 WP is a contact fungicide with a protective mode of action and can be used for painting wounds on the trunks to stop entry of the pathogen. Growth inhibition by Victory 72 WP was low (below 65 % in all the isolates except Jpc 1.

## CONCLUSION AND RECOMMENDATION

This study established that the fungicides Benovarp 50 WP, Topsin, Nordox 75 WP and Melody Duo were highly toxic to Kenyan isolates of *P.cinnamomi*. Any of the four fungicide formulations or their combination could be incorporated in an integrated management strategy for root rot of macadamia. Benovarp, Topsin and Melody Duo are systemic, curative and protective and can be used as soil drench or foliar sprays.

However, to establish the optimum rates of application for *P. cinnamomi* growth inhibition, there is need to carry out sensitivity tests with different active ingredient concentrations. Nordox, which is a contact protective fungicide, can be used as paint on stem wounds that may arise during farm operations or stem cankers caused by *P.cinnamomi* infection. This would stop entry of the fungus. However the efficacy of these formulations should be assessed further under field conditions. The selection of fungicides to be included in an integrated management strategy for macadamia root rot should also put into consideration the cost effectiveness and the effect of the fungicide to the soil micro flora.

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