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# *In vitro* inhibitory effect of selected fungicides on mycelial growth of ambrosia fungus associated with the black coffee twig borer, *Xylosandrus compactus* Eichhoff (Coleoptera: Curculionidae) in Uganda

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**Black coffee twig borer is a new but rapidly spreading insect pest of coffee in Uganda. Female beetles bore into primary branches/twigs and cultivate an ambrosia fungus for feeding their larvae. Thus, controlling the fungus means depriving the brood a source of food. Three fungicides, chlorothalonil (Glider), tebuconazole (Orius 25EW) and dimethomorph + mancozeb (Volar) were evaluated *in vitro* for their effectiveness in inhibiting mycelial growth of ambrosia fungus associated with the beetle. The pathogen was exposed to four concentrations (1.5x, 1.25x, 1.0x and 0.5x times the manufacturer recommended rate) incorporated into potato dextrose agar using inhibition and food poisoning techniques. The three fungicides inhibited fungal growth to some extent, even at the lowest concentration (0.5x) and percentage inhibition was significantly different (P≤0.05) from each other. Tebucozanole caused 100% growth inhibition irrespective of concentration and technique used while chlorothalonil and dimethomorph + mancozeb caused less than 40% inhibition for both techniques. Therefore, research should determine effectiveness of tebucozanole for suppressing fungal growth under field conditions for diminishing beetle incidence and fungal pathogenic effects in infested branches. This will pave way for integration of use of tebucozanole into overall Integrated Pest Management package (IPM) for the beetle in Uganda.**

**Key words:**Ambrosia-fungus, black-coffee-twig-borer, chlorothalonil, dimethomorph + mancozeb, fungicides, tebuconazole, *Xylosandrus-compactus*.

## INTRODUCTION

Coffee plays a vital role in the economy of Uganda, being the main export crop and a major source of

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foreign currency. It is estimated that the coffee sub-sector in Uganda provides livelihood to over 1.3 million households and about 5 million people in various businesses along the coffee value chain (UCDA, 2010). Despite its importance, coffee production has been declining for over a decade due to a number of constraints, in particular, the Coffee Wilt Disease (CWD) (Adipala-*Ekwamu et al.,* 2001). Just as the sustained effort to manage CWD led to recent release of 7 CWD resistant Robusta coffee varieties (Musoli et al., unpublished), the threat of the black coffee twig borer (hereafter abbreviated as BCTB), *Xylosandrus compactus* Eichhoff (Coleoptera: Curculionidae) has emerged (Egonyu et al., 2009). BCTB is a highly invasive and damaging pest that spreads far and wide over a short period of time. A survey carried out in 2011/2012 shows that the pest is rapidly spreading countrywide causing severe damage on coffee particularly in central, Busoga and southwestern regions (Kagezi et al., unpublished data). These regions produce the bulk of the country's Robusta coffee (Musoli et al., 2001).

The female beetle bores into the primary branches (twigs), causing them to wilt and die within a few weeks (Egonyu et al., 2009). The beetle cultivates an ambrosia fungus in the bored coffee galleries for feeding its larvae (Hara and Beardsley, 1976; Ngoan et al., 1976). The ambrosia fungus found in the BCTB is *Fusarium solani* (Martius) Saccardo (Egonyu et al., 2009). However, little is known about the actual cause of the wilting and eventually death of the attacked twigs exists. This could be due to either disruption of water and nutrient movement across the galleries made by the pest or pathogenicity of the ambrosia fungus to coffee. *Fusarium* spp. are among the most important plant pathogens in the world (Nelson et al., 1983). *F. solani* has been isolated from coffee plant tissues (Serani et al., 2007; Tshilenge et al., 2010) and has been reported to cause cankers, root rot, wilt and dieback symptoms on coffee (Baker, 1972; Venkatasubbaiah et al., 1984; Dudley et al., 2008). Thus, in addition to being a source of food for the young beetles (Hara and Beardsley, 1976; Ngoan et al., 1976), it is most probable that the ambrosia fungus might be pathogenic to the coffee. Fungal pathogens are normally controlled by use of fungicides (Murdoch and Wood, 1972) and a number of *in vitro* studies have demonstrated that some fungicides may restrict or prevent mycelial growth of many Fusaria. For example, Mancozeb gave 100% inhibition of mycelia growth of *F. solani* at 0.2 and 0.3% concentrations *in vitro* (Chavan et al., 2009). However, sensitivity of the ambrosia fungus associated with *X*. *compactus* in Uganda to fungicides has not yet been determined. This information will provide a baseline for evaluating the potentiality of these fungicides for field use to suppress the ambrosia fungus, hence control the twig borer. Consequently, successful candidate fungicides shall be incorporated into the overall IPM strategy for *X*. *compactus* in Uganda, since controlling the fungus means depriving the young beetles their exclusive source of food. Pursuant to the above therefore, we conducted *in vitro* experiments to evaluate the effect of three fungicides *viz* chlorothalonil (Glider), tebuconazole (Orius) and dimethomorph + mancozeb (Volar) on the mycelial growth of ambrosia fungus isolated from female beetle mycangium, and from its associated coffee galleries.

## MATERIALS AND METHODS

### Study site

The study was conducted at the Coffee Research Center (COREC), Kituza located about 40 km from Kampala in Mukono district, south-central Uganda. Kituza lies on the longitude 32°45'0" E and latitude 0°22'0" N. Mukono district experiences two rainy seasons (March-May and September-December) with a mean annual rainfall of 1400 to 1600 mm. The mean annual maximum temperature is 25 to 27.5°C and the mean annual minimum is 15 to 17.5°C. The predominant soils types are mainly ferralic with sandy clay- loams as the main constituents (DSER, 1997).

### Isolation of the ambrosia fungus, media preparation and fungicides used

The ambrosia fungus used in this study were isolated from the mycangium of the female beetles and the debris in the associated coffee galleries collected from coffee fields at COREC in 2011. To isolate the ambrosia fungus, the beetles were chopped into small pieces and plated on tap water agar (2% Agar technical-Oxoid in 1000 ml tap water). The debris from the galleries was also scrapped with surgical blades and sprinkled on solidified tap water agar (TWA). Both cultures (from the beetle and the galleries) were incubated separately at 25°C for three days and then sub-cultured on synthetic nutrient agar (SNA) (Nirenberg, 1976) and potato dextrose agar to reveal the characteristic conidia shapes and pigmentation respectively. The cultures were incubated under 12 h fluorescence light and dark cycles at room temperature for 10 days. Evaluation of fungicide against the fungus was done by two methods, namely: inhibition techniques (Meah et al., 2002) and food poisoning (Borum and Sinclair, 1968) as described below.

Three fungicides, chlorothalonil (Glider), 720 g/L with a dosage of 2 to 2.5 ml/L, tebuconazole (Orius 25 EW) 250 g/L with a dosage of 70 ml/L and dimethomorph + mancozeb (Volar), 690 g/kg with a dosage of 2.7 g/L at four concentrations (1.5x, 1.25x, 1.0x and 0.5x: where x is the field rate recommended by the manufacturer) were used in this study. The fungicides were mixed into 10 ml of sterile water before application and allowed to set to obtain concentrations of 3.6, 7 and 2.6 mg/L for chlorothalonil, tebuconazole and dimethomorph + mancozeb respectively.

### The inhibition technique

A plug of the ambrosia fungus (4 x 4 mm) from a 10 day-old culture was placed in the middle of a Petri dish containing potato dextrose agar (PDA). Three sterile pieces of filter paper squares (5 x 5 mm) were placed on the surface of solidified agar at a distance of about 30 mm from the inoculum in a radial pattern in each Petri dish (Plates 1a, 2a, 3a). The fungicides were then applied on the filter paper in order to defuse into the agar. The experiment was laid in completely randomized design (CRD) with the fungicide concentration as the treatment. Each treatment was replicated 4 times. The radial growth of the fungus was measured after 14 days

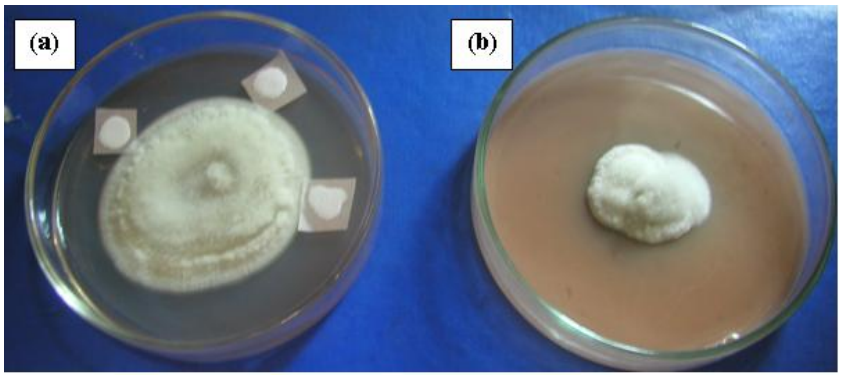


**Plate 1.** *In vitro* effect of fungicide chlorothalonil (Glider) on the mycelial growth of ambrosia fungus isolated from mycangium of female beetles and debris in the associated coffee galleries using the inhibition technique (a) and food poisoning technique (b).

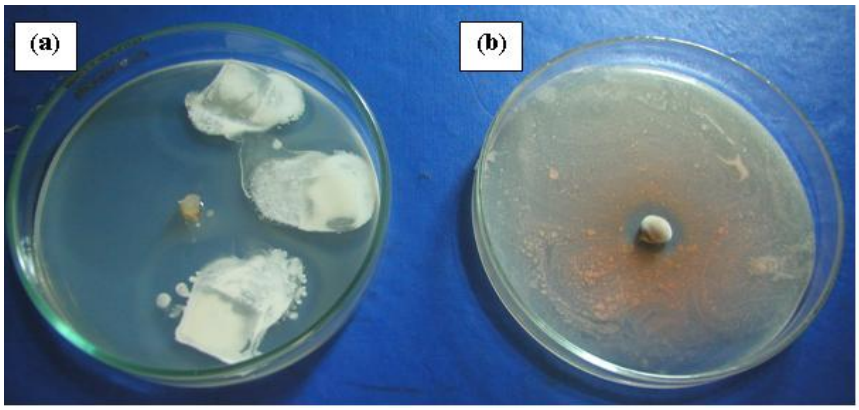


**Plate 2.** *In vitro* effect of fungicide tebuconazole (Orius 25 EW) on the mycelial growth of ambrosia fungus isolated from mycangium of female beetles and debris in the associated coffee galleries using the inhibition technique (a) and food poisoning technique (b).

of growth (when the fungus completely covered the plates in the control). The percentage growth inhibition (I) for the different fungicides was assessed as follows (Fokkema, 1973):

$$I = \frac{r1 - r2x100}{r1}$$

Where: I=percentage growth inhibition, *r1*=radius of the pathogen away from the fungicide (mm/cm) and *r2*=radius of the pathogen towards the fungicide (mm/cm).

**Food poisoning technique**

This was done by incorporating the fungicides into the medium before setting in Petri dishes. A 5 mm diameter agar disk of the test fungus was extracted from a 10 day-old PDA culture plate using a sterile surgical blade and placed in the centre of Petri plates that contained the fungicides incorporated into the PDA at the various concentrations levels (Plates 1b, 2b, 3b). The plate without fungicides served as the control (Plate 4). The plate without fungicides served as the control (Plate 4). The experiment was laid in a completely randomized design (CRD) with fungicide type and concentration as the treatments. These were replicated four times. The radial fungal growth was recorded after 45 to 50 days (when the fungus completely covered the plates in the control). The percentage inhibition (PI) of the fungus over control was calculated using the following formula:

$$PI = \frac{A - B}{A} x100$$

Where: A=colony growth of the fungus in control plate and B=colony growth of the fungus in treated plate.

**Data analysis**

Before the analysis, the percentage inhibition data were arcsine-transformed to reduce non-normality and heterogeneity of variances. The analysis of variance (ANOVA) was performed with the general linear model (GLM) procedure of the Statistical Analysis System (SAS) software (SAS Institute, 2008). Means were

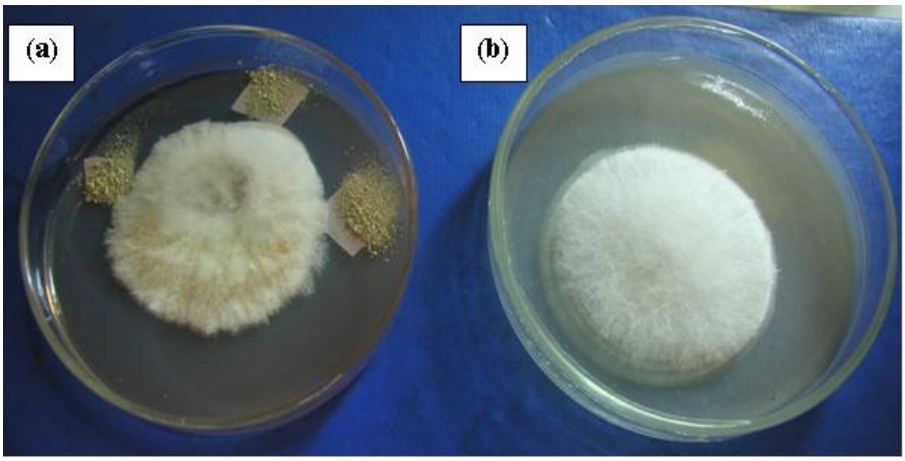


**Plate 3.** *In vitro* effect of fungicide dimethomorph + mancozeb (Volar) on the mycelial growth of ambrosia fungus isolated from mycangium of female beetles and debris in the associated coffee galleries using the inhibition technique (a) and food poisoning technique (b).

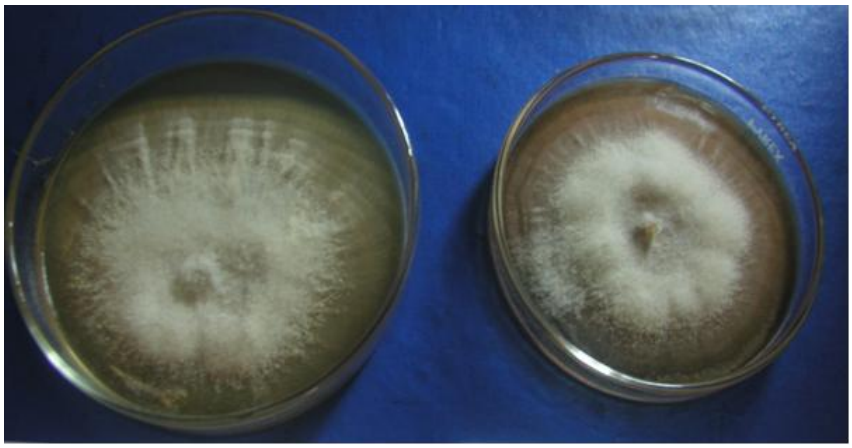

**Plate 4.** *In vitro* effect of the control (without fungicide) on the mycelial growth of ambrosia fungus isolated from mycangium of female beetles and debris in the associated coffee galleries using the food poisoning technique.

separated by the Tukey test at 5%. Note that the data for fungicide tebucozanole (Orius) were omitted when analysing for the effect of fungicidal concentration on mycelial growth of the fungus because 100% inhibition was attained for all the concentrations irrespective of the technique used.

## RESULTS

Our results clearly show that irrespective of the concentration level and technique used, all the three fungicides tested in the study were able to inhibit growth of the ambrosia fungus to some extent (at least >20%; Tables 1 and 2). The percentage fungal growth inhibition varied across the fungicides and they were highly significantly different (P≤0.05) from each other for both techniques (food poisoning and inhibition). Of the three fungicides, tebucozanole (Orius) was the most effective in inhibiting the mycelial fungal growth, causing 100% inhibition in both techniques (Table 1). Overall, for both techniques, fungicides chlorothalonil (Glider) and dimethomorph + mancozeb (Volar) caused less than 40% inhibition of mycelial fungal growth (Table 1). Further, the inhibitory effect of fungicides, chlorothalonil (Glider) and dimethomorph + mancozeb (Volar) increased significantly with increasing fungicidal concentrations in both techniques. However, the inhibitory effect of chlorothalonil (Glider) did not differ significantly for concentrations 1.5x and 1.25x, and also for 1.25x and 1.0x. Similarly, the inhibitory effect of dimethomorph + mancozeb (Volar) did not differ significantly for concentrations 1.5x and 1.25x, and, 1.0x and 0.5x (Table 2).

**Table 1.** Percentage inhibition of radial mycelial growth of ambrosia fungus isolated from mycangium of female beetles and debris in the associated coffee galleries by fungicides, tebucozanole (Orius), chlorothalonil (Glider) and dimethomorph + mancozeb (Volar) using food poisoning and inhibition techniques. Same letters within a column indicate means (after arcsine transformation) are not significantly different by Tukey's test (*P≤0.05). Values in parenthesis are the untransformed means.

| Fungicide | Food poisoning technique | Inhibition technique |
|---|---|---|
| Orius | 7.9 (100.0)[a] | 7.9 (100.0)[a] |
| Glider | 4.4 (31.8)[b] | 4.8 (37.4)[b] |
| Volar | 3.7 (23.3)[c] | 4.3 (29.6)[c] |
| F value | 361.74** | 234.53** |
| CV | 8.78 | 9.0 |

**Table 2.** Percentage inhibition of radial mycelial growth of ambrosia fungus isolated from mycangium of female beetles and debris in the associated coffee galleries using food poisoning and inhibition techniques by fungicides, chlorothalonil (Glider) and dimethomorph + mancozeb (Volar) at varying concentration using food poisoning and inhibition techniques. Same letters within a column indicate means (after arcsine transformation) are not significantly different by Tukey's test (*P≤0.05). Values in parenthesis are the untransformed means.

| Concentration | Food poisoning technique | | Inhibition technique | |
|---|---|---|---|---|
| | Glider | Volar | Glider | Volar |
| 0.5X | 3.6 (22.5)[d] | 2.8 (12.5)[d] | 3.8 (23.6)[c] | 3.7 (21.4)[b] |
| 1.0X | 4.2 (28.4)[c] | 3.5 (19.7)[c] | 4.7 (36.2)[b] | 3.8 (22.5)[b] |
| 1.25X | 4.6 (34.4)[b] | 4.0 (25.9)[b] | 5.2 (42.6)[ab] | 4.8 (36.7)[a] |
| 1.5X | 5.1 (41.9)[a] | 4.4 (31.3)[a] | 5.5 (48.3)a | 4.9 (37.6)[a] |
| F value | 156.51** | 208.10** | 34.88** | 597.56** |
| CV | 2.07 | 2.58 | 5.05 | 1.28 |

## DISCUSSION

This study evaluated the efficacy of three fungicides, chlorothalonil (Glider), tebuconazole (Orius 25 EW) and dimethomorph + mancozeb (Volar) *in vitro* for inhibiting radial mycelial growth of ambrosia fungus using the inhibition and food poisoning techniques. Our data show that all the three fungicides tested were able to inhibit mycelial growth of the fungus, even at the lowest concentration (0.5x) irrespective of the technique used. These results are in agreement with a number of earlier *in vitro* studies which have demonstrated that various fungicides may restrict or prevent growth of *F. solani* and other Fusaria (Tepper et al., 1983; Chavan et al., 2009; Sultana and Ghaffar, 2010). Tebucozanole (Orius) had the greatest inhibitory effect, causing 100% inhibition irrespective of the fungicide concentration or techniques employed. These results are in line with earlier research studies which reported very strong *in vitro* inhibition effects of tebuconazole-based fungicides on fungal mycelial growth of several *Fusarium* species. For example, *F. avenaceum* (Simpson et al., 2001; Ivić et al., 2011), *F. culmorum* (Simpson et al., 2001), *F. graminearum* (Ramirez et al., 2004 ; Ivić et al., 2011) and *F. verticillioides* (Ivić et al., 2011) among others. The high effectiveness of tebucozanole (Orius) is of great importance in the management of the ambrosia fungal gardens and thus the twig borer. First of all, tebucozanole-based products are is highly systemic with protective, curative, and eradicant action (Shtienberg and Dreishpoun, 1991; Labrinos and Nutter, I993). The fungicide is absorbed rapidly into the vegetative parts of the plants by the leaves and stems and is translocated acropetally upward in the plants. Thus, the fungicide can easily reach the ambrosia fungal gardens located deep inside the galleries/tunnels in the coffee twigs (Hara and Beardsley, 1976; Ngoan et al., 1976). Secondly, the fact that tebucozanole is equally effective even at half the manufacturer's recommended rate implies that future research can explore the possibilities of using even lower dosages that would minimize costs. One of the major limitations of using tebucozanole in disease management is its cost. Currently, a liter of tebucozanole costs 150,000 Uganda shillings (approximately US$ 60) on the market which is definitely too high for the small scale coffee farmers who produce more than 80% of the coffee in the country (Musoli et al., 2001).

Our data further show that chlorothalonil (Glider) and dimethomorph + mancozeb (Volar) caused far less fungal growth inhibitory effect (<40%) compared to tebucozanole

(Orius) irrespective of the inhibition technique used. These results are in agreement with earlier *in vitro* studies which reported low fungal mycelial inhibitory effect (>25%) by chlorothalonil-based fungicides on several *Fusarium* species including *F. oxysporum* f. sp. *cumini* (Bardia and Rai, 2007) and *Fusarium avenaceum* (Kopacki and Wagner, 2006). Similarly, Kim et al. (2005), Cha et al. (2007) and Mamza et al. (2008) reported low inhibitory effect of dimethomorph- and mancozeb-based fungicides on mycelial growth of *F. pallidoroseum* and *F. oxysporum* respectively. However, our results contradict studies by Tepper et al. (1983) who reported complete (100%) *in vitro* inhibition of mycelial growth of *F. solani* by chlorothalonil-based fungicides at 1000 mg/L. Similarly, Tepper et al. (1983), Chavan et al. (2009) and Sultana and Ghaffar (2010) reported that mancozeb-based fungicides caused complete (100%) *in vitro* inhibition of mycelial growth of *F. solani* at 100 mg/L, 0.2 and 0.3% concentrations respectively. The contradiction in the results could have been due to the difference in the content of the fungicides in final commercial products and also the source of the fungicide. Incidentally, counterfeit or adulterated chemicals including fungicides are very common on Ugandan market (MAAIF, 2009).

Further, the inhibitory effect of chlorothalonil (Glider) and dimethomorph + mancozeb (Volar) increased significantly with increasing fungicidal concentrations in both techniques. These results are in agreement with earlier studies which reported that the fungal inhibitory effect of the chlorothalonil-, mancozeb- and dimethomorph-based fungicides increased with increasing concentration of the fungicide (Kopacki and Wagner, 2006; Bardia and Rai, 2007; Cha et al., 2007; Mamza et al., 2008).

In conclusion, our results clearly show that fungicide tebucozanole (Orius25 EW) caused 100% inhibition of the mycelial growth of the ambrosia fungus. Thus, further research should concentrate on determining the effectiveness of this fungicide for controlling ambrosia under field conditions. Secondly, this fungicide should be combined with candidate insecticides (tank mixture) and integrated it into the chemical control option and the overall Integrated Pest Management (IPM) strategies for managing the ambrosia fungus and thus its associated beetle.

## Conflict of Interest

The authors have not declared any conflict of interest.

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