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Evaluation of several fungicides on mycelial growth and conidial germination of *Alternaria* species causing leaf spots in sunflowers under in vitro conditions

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Abstract. *Muljowati J, Hikam AR. 2023. Evaluation of several fungicides on mycelial growth and conidial germination of* Alternaria *species causing leaf spots in sunflowers under in vitro conditions. Asian J Agric 7: 47-51. Alternaria* leaf spot caused by *Alternaria* species is the most destructive disease of sunflowers. Fungicides, such as mancozeb, carbendazim, benomyl, propiconazole, and iprodione, are commonly used to control diseases. However, the continuous use of synthetic fungicides can cause pathogen resistance to these fungicides. Therefore, the aim of this study was to conduct an in vitro test on the effect of fungicides, such as benomyl, carbendazim, mancozeb, iprodione, and propiconazole, on the mycelial growth and germination of conidia of *Alternaria* species causing leaf spot on sunflower. The experiment was performed in a completely randomized design with factorial patterns. The first factor was the type of fungicide, namely benomyl, mancozeb, iprodione, carbendazim, and propiconazole. The second factor was the concentration level of the (0%, 25%, 50%, 75%, 100%, and 125%) recommended dose. Data were Analyzed of Variance (ANOVA) using SPSS 18 version. The results showed that *Alternaria* species were resistant to carbendazim (32.98%) and benomyl (40.32%). It also shows an intermediate level of resistance to mancozeb (62.59%), iprodione (65.38%), and sensitivity to propiconazole (78.38%). Based on the research results, the authors suggest sunflower farmers use propiconazole to control *Alternaria* species. However, such fungicides may trigger the use of fungicides with higher doses than the recommended dose. That led to the emergence of Alternaria species resistant to the fungicides benomyl, carbendazim, mancozeb, and iprodione.

Keywords: *Alternaria*, fungicide effectivity, leaf spot, sensitivity, sunflower

INTRODUCTION

Nowadays, sunflowers have many benefits, including medicine, cosmetics, textile dyes, and food (Purwati and Herwati 2016; Adeleke and Babalola 2020). However, the limiting factor in sunflower cultivation is foliar diseases such as leaf spots caused by *Alternaria* species (Zhang et al. 2021). Symptoms of the disease are dark brown, oval to circular spots with pale margins and a yellow halo. Spots are found on leaves, stems, petioles, sepals, and petals. In severe infections, lesions become irregular by coalescing, leading to blight, defoliation, and plant death. The disease is severe in wet weather and under moist conditions (Bianchini and Bullerman 2014).

Alternaria leaf spot threatens sunflowers production causing yield losses in all production areas that may reach 60-80% (Viriyasuthee et al. 2019). The high destructive potential of leaf spots makes fungicide control an option and requires repeated application of fungicides to prevent yield loss (Aalum et al. 2016). However, the continuous of synthetic fungicides use or not using recommended doses can lead to pathogen resistance to these fungicides (Ons et al. 2020). Benomyl and carbendazim belong to the benzimidazole group of fungicides with a heterocyclic nitrogen component. The benzimidazole's effect on the plant could inhibit the mitochondrial fumarate reductase enzyme, reduces glucose transport, and eliminates oxidative phosphorylation (Allen and Gottlieb 1970). Benomyl attaches to the microtubule, disrupting processes in the cell, such as cell division and intracellular transport (Rouabhi 2010). Furthermore, benomyl inhibits the growth of *Cercospora beticola* (Utlwang et al. 2017). Carbendazim controls pathogens that cause leaf blight in sunflowers (Devi et al. 2015) and *Alternariaster helianthi* (Jena et al. 2020).

Mancozeb is a dithiocarbamate fungicide that acts on the plant surface where it is applied. This fungicide cannot inhibit the development of fungi in plant tissues but is capable of multi-site inhibitors, which can inhibit the growth of fungi through several places in fungal cells (Rouabhi 2010). Furthermore, mancozeb can hinder the development of several phytopathogenic fungi, including *Choanephora cucurbitarum* (Chandrakala et al. 2018) and *A. helianthi* (Jena et al. 2020).

Iprodione is a dicarboximide fungicide widely used worldwide in agriculture (Bitar et al. 2019). It belongs to the dicarboximide group, which inhibits steroidogenesis (Blystone et al. 2007). Iprodione inhibits several pathogens because it has a broad spectrum (Wei et al. 2020) and, among others, can hinder the growth of *Monilinia fructicola* (Thomidis et al. 2009).

Based on its constituent compounds, propiconazole belongs to the triazole fungicide, which inhibits fungal growth by inhibiting sterol biosynthesis, an essential

component in fungal cell defense (Rouabhi 2010). Propiconazole can inhibit the growth of *C. beticola* (Hudec et al. 2020) and *A. helianthi* (Usha et al. 2019). Evaluated for mycelial growth and conidial germination can determine the sensitivity of pathogenic fungi to fungicides. Sensitivity is a condition where the fungus is sensitive to a fungicide and reacts by inhibiting its growth. The growth of colony diameter indicates the sensitivity of the fungus to fungicides. The aim of this study was to conduct an in vitro test on the effect of fungicides, such as benomyl, carbendazim, mancozeb, iprodione, and propiconazole, on the mycelial growth and germination of conidia of *Alternaria* species causing leaf spot on sunflower.

MATERIALS AND METHODS

Procedures

Fungicides and fungal material

The fungicide bioassay experiment was conducted at the Laboratory of Mycology of the Faculty of Biology, Universitas Jenderal Sudirman, Indonesia. Five commercial fungicides, including: Masalgin 50WP (benomyl), Bendas 50WP (carbendazim), Dithane M-45 80WP (mancozeb), Rovral 50WP (iprodione), and Remazole 490EC (propiconazole) were obtained from pesticide distributor used in the experiment. Isolates of *Alternaria* sp. were isolated from sunflowers, and pure cultures were maintained on a PDA medium. As per the manufacturer's specifications, a stock solution of the fungicides was prepared by dissolving them in distilled water. The first factor was the type of fungicide, namely benomyl, mancozeb, iprodione, carbendazim, and propiconazole. The second factor was the concentration level of the (0%, 25%, 50%, 75%, 100%, and 125%) recommended dose. Furthermore, from the stock solution, different concentrations (0%, 25%, 50%, 75%, 100%, and 125%) of each fungicide were prepared, in which fungicide at 0% served as a negative control.

Mycelial growth sensitivity test

The prepared fungicide solution was mixed with 20 mL of PDA media and poured into a 9 cm diameter Petri dish (Ogolla et al. 2021). A fungal disc of 5 mm diameter was placed in the center of the petri dish and incubated at room temperature for 12 days. The control treatment was made without using fungicides. The entire procedure was done under aseptic conditions in Laminar Air Flow. Measurement of the diameter (in millimeters) of mycelial growth was taken 12 days after incubation. The Relative Inhibition Level (RIL) of the colony diameter was calculated using the following formula (Catao et al. 2013):

$$\%\mathbf{RIL} = \frac{\mathbf{d1}-\mathbf{d2}}{\mathbf{d1}} \times \mathbf{100\%})$$

Where:
d1: colony diametre of control
d2: colony diametre of treatment

Conidial germination sensitivity test

A drop of conidial suspension of each fungicide concentration was kept on a glass slide for conidial germination. Conidial germination was observed under a microscope after 24 hours of incubation at room temperature. One hundred conidia were observed per fungicide concentration. The treatment sensitivity level calculation was based on the relative resistance level of the fungicide to conidia germination in a medium mixed with fungicides. The percentage of Relative Inhibition Level (RIL) of conidial germination was calculated using the following formula (Catao et al. 2013):

$$\mathbf{RIL} = \frac{\sum \mathbf{control} - \sum \mathbf{treatment}}{\sum \mathbf{control}} \times \mathbf{100\%}$$

Where:
RIL: Relative Inhibition Level (%)
∑Control: number of germinated conidia in the control
∑Treatment: number of conidia grew in each treatment

Data analysis

The data obtained were analyzed by analysis of variance using the software SPSS at a 95% confidence level, followed by Duncan's test at a 95% confidence level (Steel and Torie 1995).

RESULTS AND DISCUSSION

The propiconazole and iprodione fungicides showed the highest (94.05% each) inhibition of mycelial growth, followed by mancozeb (82.14%), carbendazim (70.24%), and benomyl (59.76%). Meanwhile, the lowest mycelia growth inhibition was observed by the fungicide benomyl and carbendazim (29.28% each) (Table 1). This finding is aligned with Mahapatra and Das (2016), who reported iprodione and propiconazole could control *Alternaria* sp. in India. Furthermore, our findings differ from the findings of Desmukh et al. (2020) that the fungicide mancozeb is most effective in inhibiting *Alternaria* sp.. Prathuangwong et al. (1991) found that benomyl had the lowest ability to control *Alternaria* sp.. Vighe et al. (2018) reported that propiconazole is most effective in inhibiting the mycelium growth of *Alternaria* sp.. In comparison, mancozeb can inhibit the sporulation and germination of conidia *Alternaria* sp.. Ogolla et al. (2021) also reported that the fungicide carbendazim had the lowest inhibitory ability.

The highest (100%) inhibition of spore germination was recorded by fungicides propiconazole, iprodione, mancozeb, and carbendazim. Fungicide benomyl showed 97.0% inhibition of conidia germination (Table 2). Results showed that tested fungicides could inhibit the conidial germination of *Alternaria* sp. Our findings are in line with the findings of Koka et al. (2021) reported that the fungicide carbendazim was able to inhibit mycelium growth and conidia germination *Alternaria* sp..

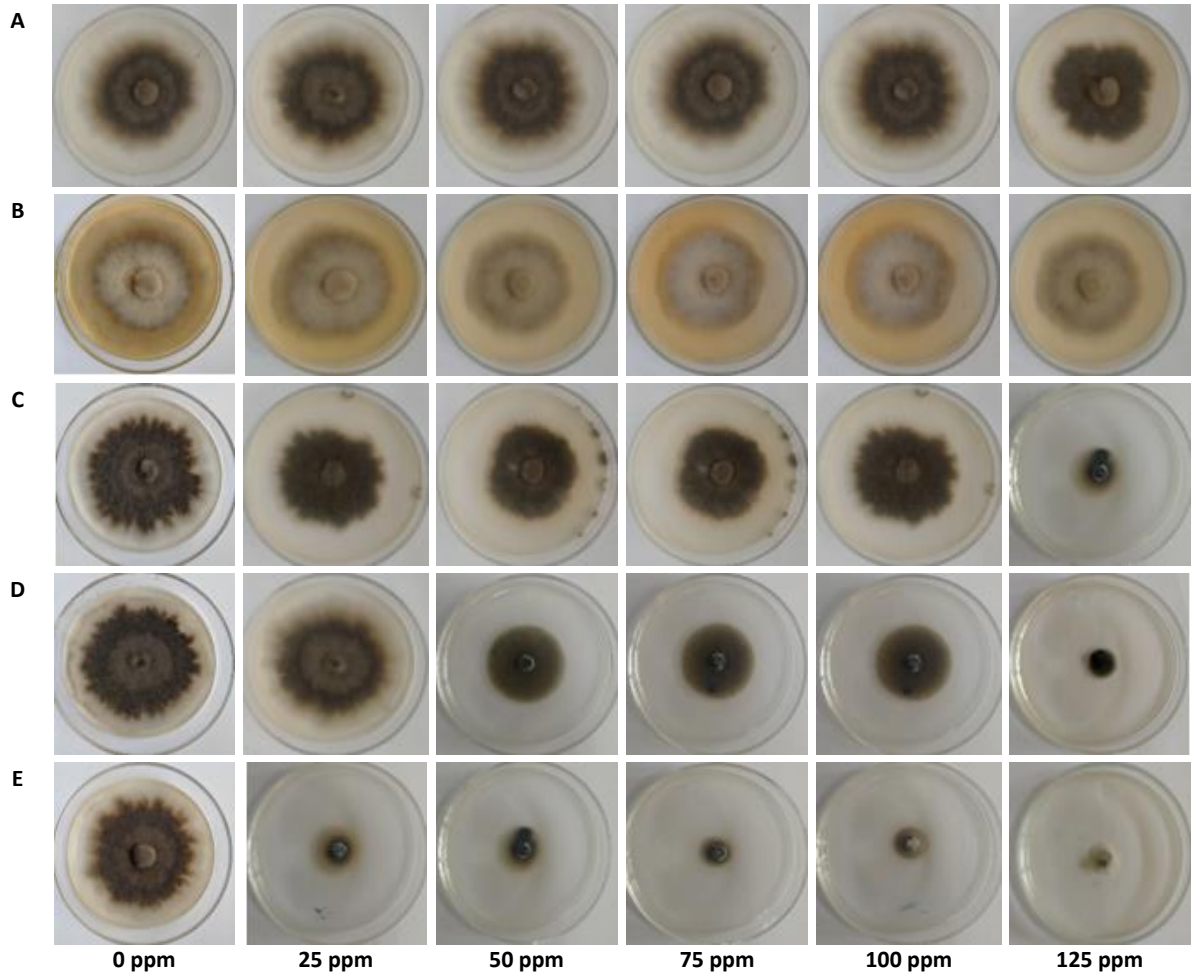

Figure 1. Mycelial growth of *Alternaria* sp. on several fungicides with various concentrations. A. Benomyl, B. Carbendazim, C. Mancozeb, D. Iprodione, and E. Propiconazole

Table 1. Percentage of inhibition of several synthetic fungicides on the mycelium growth of *Alternaria* sp.

Fungicides Dosage (%)	Benomyl	Carben-dazim	Mancozeb	Iprodi-one	Propicona-zole
0	0 VR	0 VR	0 VR	0 VR	0 VR
25	38.56 VR	29.28 VR	76.19 S	70 RS	94.05 VS
50	42.24 R	36.9VSR	76.92 S	71.43 MR	94.05 VS
75	52.38 R	44.05 R	77.62 S	73.81 MR	94.05 VS
100	54.28 R	61.9 MR	77.86 S	82.85 S	94.05 VS
125	59.76 R	70.24MR	82.14 S	94,05 VS	94,05 VS

Note: VR: Very Resistant, IR: Intermediate level of Resistance, R: Resistant, S: Sensitive, VS: Very Sensitive

Table 2. Percentage of inhibition of several fungicides on spore germination of *Alternaria* sp.

Fungicides Dosage (%)	Benomyl	Carben-dazim	Mancozeb	Iprodi-one	Propicona-zole
0	0 VR	0 VR	0 VR	0 VR	0 VR
25	89.60 VS	97.60 VS	98.20 VS	97.60 VS	95.40 VS
50	91.20 VS	100 VS	98.60 VS	98.40 VS	97.80 VS
75	92.40 VS	100 VS	100 VS	100 VS	100 VS
100	96.20 VS	100 VS	100 VS	100 VS	100 VS
125	97.00 VS	100 VS	100 VS	100 VS	100 VS

Note: VR: Very Resistant, IR: Intermediate level of Resistance, R: Resistant, S: Sensitive, VS: Very Sensitive

Table 3. Relative Inhibition Level (RIL) of fungicides on mycelium growth and spore germination of *Alternaria* sp.

Fungicides	Mycelial growth inhibition (%)	Spore germination inhibition (%)
Benomyl	40.32 b	77.73 a
Carbendazim	32,98 a	82.93 a
Mancozeb	62.59 c	82.80 a
Iprodione	65.38 c	82.67a
Propiconazole	78.38 d	82.20 a

Note: The number followed by the same letter in the same column is not significantly different at the 5% level (P>0.5)

The application of fungicides to the culture medium of *Alternaria* sp affected the growth of the mycelium of the fungus. The difference in the fungicide concentration also affects the mycelium's growth. The highest inhibition of mycelium growth *Alternaria* sp. was by propiconazole fungicide. While the lowest inhibition was by the fungicides benomyl and carbendazim (Figure 1). The relative-inhibition fungicides on mycelium growth was propiconazole (78.38%), followed by iprodione and mancozeb at 65.38% and 62.59%, respectively. The lowest (40.32%) mycelium growth inhibition was recorded by benomyl fungicide.

There is no difference in the inhibition ranges of fungicides against spore germination, indicating that they were all in the very sensitive range, which means that administration of all types of fungicides had a very high ability to inhibit spore germination of *Alternaria* sp. (Table 3). This finding is in line with Vighe et al. (2018), which stated that propiconazole was most effective in controlling *Alternaria cassia*. In addition, van den Berg et al. (2002) stated that carbendazim and propiconazole had the same ability to inhibit the mycelium growth of *Alternaria* sp.. This finding is also in line with Yang et al. (2011), who stated that fungicides could inhibit sporulation, spore germination, and germination of germ tubes. Those effects are due to interference in the osmoregulatory signal transmission pathway, thereby inhibiting hyphae formation.

Differences in the ability of fungicides to inhibit mycelium growth and germination of *Alternaria* sp. spores were due to the different activities of each fungicide. Benomyl and carbendazim affect mitosis and cell division, namely tubulin polymerization into microtubules. Rouabhi (2010) stated microtubules are cytoskeletal polymers in eukaryotic cells and play important roles in many cellular functions. Mancozeb has multi-site activities. Multisite-activity fungicides are widely used in agronomic activities because of their broad spectrum of disease control activity. Still, they may have side effects on other microorganisms due to the impact of their various biochemical sites. Mancozeb affects the metabolism of target microorganisms. Iprodione has the activity of inhibiting glycerol synthesis and hyphae development by cutting signal transduction. In addition, propiconazole inhibits Demethylation (DMI) and sterol biosynthesis in fungal cells. Sterols are another important component of cell membranes in fungi.

In addition to differences in activity, differences in the ability of fungicides to inhibit mycelium growth and spore germination may be influenced by the genetic conditions of pathogenic fungi. Genetic differences possessed by pathogenic fungi can cause differences in sensitivity because genetic diversity affects the ability of fungi to survive and adapt to active fungicide applications. The emergence and development of resistant pathogens can be caused by repeated use of fungicides over a long period in a season, systemic fungicides, and continuous use at doses above lethal concentrations (van Tuyl 1977). Each fungicide has a different way of working in inhibiting pathogenic fungi. Systemic fungicides work simultaneously with plant metabolic processes and only act on one site of fungal cells, so they are said to have a single site or specific mechanism of action. Fungicides with a single-site mechanism of action generally have a high risk of developing fungal resistance to this active ingredient more rapidly than fungicides with a multi-site mechanism of action (Secor and Rivera 2012).

The application of fungicides to control hinders the growth of pathogenic fungi, and in some instances, the pathogen tries to adapt continuously, causing resistance to the given fungicide (Yang et al. 2019). The mechanism of the strains resistant to several fungicides occurs due to a decrease in the permeability of pathogenic cells to absorb chemical compounds. The mechanism for the emergence of strains resistant to several fungicides is a decrease in the permeability of pathogenic cells to absorb chemical compounds, detoxification of chemical compounds by pathogenic cells, decreased conversion to more toxic metabolites, decreased affinity for pathogenic cells, and by-passing. In addition, the sequence of reactions in metabolic processes and the production of replacement enzymes is inhibited by treatment with chemical compounds (Agrios 2005). The differences in the sensitivity level of *Alternaria* sp. to all fungicides were caused by the mode of action of each active ingredient of the fungicide, the level of fungicide toxicity to pathogens, the method of use carried out by farmers, and the genetic diversity of the two species of pathogenic fungi.

Application of fungicides following the recommended dose on the fungal growth medium affected the sensitivity level of tested fungi. The level of sensitivity of fungi to fungicides can be known from differences in the growth of colony diameters. Based on test results of inhibition of mycelium growth in each treatment, there were differences in the size of the colony diameter. Therefore, a more effective fungicide inhibits the growth of fungal mycelium. Vighe et al. (2018) stated that propiconazole effectively inhibits mycelium growth, sporulation, and conidial germination of *A. cassiae*. This study revealed that *Alternaria* species were sensitive to mancozeb and propiconazole but resistant to benomyl, carbendazim, and iprodione. In addition, it has also been observed that fungicides mancozeb and propiconazole effectively controlled the pathogens.

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