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Research Article
Determination of Median Effective Inhibitory Concentration of Three Fungicides Widely Used for Treatment of Wheat on the Target Pest *Fusarium* sp.

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Abstract
The pathogenic *Fusarium* species attack plants of major economic importance including wheat. Infection of cereal plants causes significant losses in crops but also the deterioration of the quality of grains and economic losses resulting are often very heavy. In the present study, the efficacy of three fungicides (thiram, tebuconazole and fludioxonil+difenoconazole) tested *in vitro* for their inhibitory activities against the target pest *Fusarium* sp., isolated from infected seed of wheat. Fungi toxicity was expressed as a percentage of inhibition of mycelial growth. The median lethal dose was calculated for each tested active ingredient. Among the chemical fungicides which were used, thiram was the most effective with EC_{50} value of 0.15 mg L^{-1} followed by the mixture fludioxonil-difenoconazole with a value equal to 0.27 mg L^{-1} and finally tebuconazole with 3.79 mg L^{-1}.

Key words: Thiram, tebuconazole, fludioxonil, difenoconazole, *Fusarium* sp., EC_{50}

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fusarium wilt is the most devastating disease affecting cereal crops with small grains; it can devastate a crop in a few weeks before harvest and cause significant yield losses. The causative agents include two genera of pathogenic fungi: Microdochium and Fusarium (Atanasov, 1920; Bottalico, 1998; Arseniuk *et al*., 1999; Xu *et al*., 2005). They lead to a series of symptoms that are characterized by damping and necrosis of plant tissues and ears (Bottalico and Perrone, 2002; Xu and Nicholson, 2009). Furthermore, some *Fusarium* sp., produce mycotoxins which are toxic to animals and humans (Leonard and Bushnell, 2003). In addition, some highly toxic mycotoxins have recently been reclassified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic in humans (Dalie, 2010). Smith (1884) described the disease in the UK. It may be associated with both high yield losses (abortion and low grain weight), reduction in their germination quality and a decrease in their quality by the presence of toxins in grains.

Devastating epidemics have been reported in Europe, USA, China and South America and caused yield losses of up to 30% (Mesterhazy and Rowaished, 1977; McMullen, 1997). In 2000, a 100% rate of loss has been recorded in certain French plots (Carlier, 2001).

To control this disease, several strategies have been developed. Chemical control remains one of the most important interventions and the efficiency of culture is directly related. Several families of fungicides are used including the triazoles (Maufras *et al*., 1994; Caron, 1995; Mielke and Weinert, 1996; Mesterhazy *et al*., 2003; Mateo *et al*., 2011). They inhibit the biosynthesis of ergosterol and are considered the most active against Fusarium wilt. Other fungicides classes are also used such as dithiocarbamates (Fravel *et al*., 2005).

Therefore, the objective of the present study was (1) To assess the sensitivity, *in vitro*, of the pathogenic *Fusarium* sp., isolated from seeds of wheat, towards three fungicides including tebuconazole, thiram and the mixture fludioxonil-difenoconazole which have been introduced recently against *Fusarium* spp. and (2) to determine the median effective inhibitory concentration (EC_{50}) values for each fungicide.

MATERIALS AND METHODS

Biological material: The seed of wheat (*Triticum aestivum* L. subsp. *Aestivum*) was supplied to us by the National Institute of Plant Protection (INPV), Annaba, Algeria.

Fungal strains: The strains of *Fusarium* sp. were obtained from seed of wheat. The seeds were previously disinfected by soaking in a solution of sodium hypochlorite 2% followed by thorough rinsing with sterile distilled water. They are then dried with sterile filter paper.

Surface disinfected seeds were plated on the PDA medium (Potato Dextrose Agar) and were usually incubated for 7-10 days at 25°C (ISTA., 1996; Mathur and Kongsdal, 2003). The identification of the pathogen was done based on some morphological characteristics of colonies (growth rate, colony appearance, pigmentation) and microscopic observation of spores (shape and size).

Fungicides: The active ingredients used in the *in vitro* tests were: Tebuconazole (Rival), fludioxonil+difenoconazole (syngenta) and thiram (Sipcam Inagra) (Table 1). Preliminary tests were conducted for each fungicide to choose the range of concentrations to obtain a dose-effect response.

The experiments were performed by testing five concentrations for each active ingredient: 0.06, 1.39, 2.79, 5.58 and 11.16 mg L^{-1} for tebuconazole, 0.025, 0.05, 0.1, 0.21 and 0.42 mg L^{-1} for Thiram and finally 4 concentrations were selected for the mixture fludioxonil-difenoconazole (0.05, 0.1, 0.5 and 1 mg L^{-1}).

***In vitro* toxicity tests:** The objective of this experiment is to assess inhibitory activity on the growth of Fusarium cultures. Synthetic fungicides were incorporated aseptically to get desired concentration in the culture medium kept supercooled (40-45°C). The mixture is then poured equally into three petri dishes. Mycelial disks of the pathogen (6 mm in diameter) will be cut from young cultures of *Fusarium* sp. (7 days old culture) and were then deposited in the center of petri dishes with a PDA media amended with the fungicides. Meanwhile, untreated control will be made in the same way where the same amount of fungicide is replaced by sterile distilled water. The dishes are then incubated at 25°C. After 7 days of incubation, the mean diameter of the colonies was estimated from two perpendicular diameters. The experiment was repeated 4 times. Fungi toxicity was recorded in terms of percentage colony inhibition and calculated according to Pandey *et al*. (1982). Percentage growth inhibition was determined using the Eq. 1:

$$I(\%) = \frac{D_0 - D_f}{D_0} \times 100 \quad (1)$$

Where:

D_0 = Average diameter of fungal colony with control
D_f = Average diameter of colonies in the presence of the fungicide

Table 1: Fungicides tested on *Fusarium* strains

Fungicides	Products name	Concentrations	Groups
Fludioxonil+difenoconazole	Celest extra	25 g L^{-1}	Phenylpyrroles+triazole
Tebuconazole	Acil	60 g L^{-1}	Triazole
Thiram	Thiramchim	80%	Dithiocarbamates

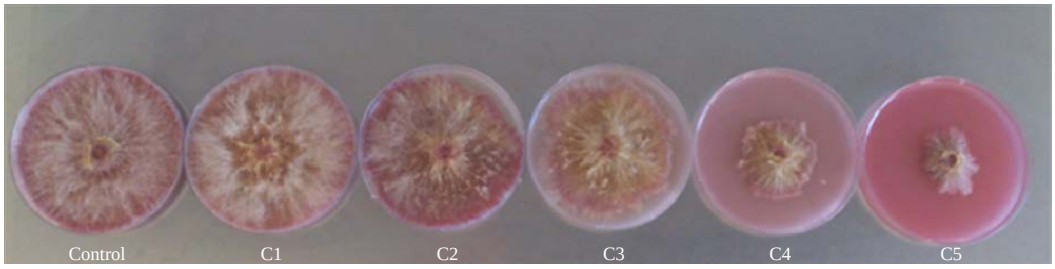

Fig. 1: Evolution of *Fusarium* sp., colonies treated with increasing concentration (Case of tebuconazole)

EC_{50} determination: The median effective inhibitory concentration (The concentration of fungicides which reduced the mycelial growth of *Fusarium* sp. by 50%) is calculated according to the method of Finny (1971).

The percentages of inhibition are transformed into probit values. The regression lines are drawn according to the Eq. 2:

$$y = ax+b \quad (2)$$

Where:

a = Regression coefficient
b = Constant
y = The probit
x = log10 of the concentrations

Statistical analysis: The obtained data was analyzed using Minitab16 student t-test and analysis of variance (ANOVA) to detect significant differences reported for the studied parameters.

RESULTS

Isolation and identification of the pathogen: On PDA medium, strain growth results in the production of aerial and dense mycelium (cottony) of a white color on the front and yellow on the back. The color of the back turns crimson when culture is older.

Under an optical microscope (Olympus CH20), we observe macroconidia with 4-6 septate, curved in shape and may have a pointed terminal as described by Champion (1997).

Effect of fungicides on mycelium growth: The results of studying the influence of the fungicides upon the growth of the phytopathogenic strain of *Fusarium* sp. show that the 3 molecules affected significantly the growth of paramecia ($p \leq 0.001$). Indeed, for Fusarium strains treated with the highest concentration of thiram (0.42 mg L^{-1}), colonies displayed a diameter of about 1.60 cm compared to control colonies (8.29 cm). As to the strains treated by fludioxonil-difenoconazole and tebuconazole, the diameter of colonies is about 1.70 for the respective concentrations of 1 and 11.16 mg L^{-1}. Figure 1 shows the evolution of Fusarium colonies treated with increasing concentration (Case of tebuconazole).

Our results gave clear and gradual decrease of diameter of colonies in dependent dose manner (Fig. 2).

Response inhibition: Based on the acute toxicity results, a percentage of inhibition of mycelial growth was calculated. The highest percentage of inhibition was obtained with Thiram where inhibition reached 81% for the concentration of 0.42 mg L^{-1}. Fludioxonil-difenoconazole mixture and tebuconazole also showed high percentages of inhibition of about 79% for the respective concentrations of 1 and 11.16 mg L^{-1} (Fig. 3). It should be noted that statistical analysis revealed a significative inhibition ($p \leq 0.001$) of mycelial growth of *Fusarium* sp., treated with fludioxonil-difenoconazole mixture dice the lowest concentration (0.05 mg L^{-1}).

EC_{50} determination: The EC_{50} of the fungicides was calculated according to the linear relation between inhibitory probit and concentration logarithm. Table 2 shows the EC_{50} values of the three fungicides: thiram, tebuconazole and fludioxonil-difenoconazole (0.15, 3.79 and 0.27 mg L^{-1}). Of these, thiram and the mixture fludioxonil-difenoconazole proved to be the most effective in inhibiting mycelial growth.

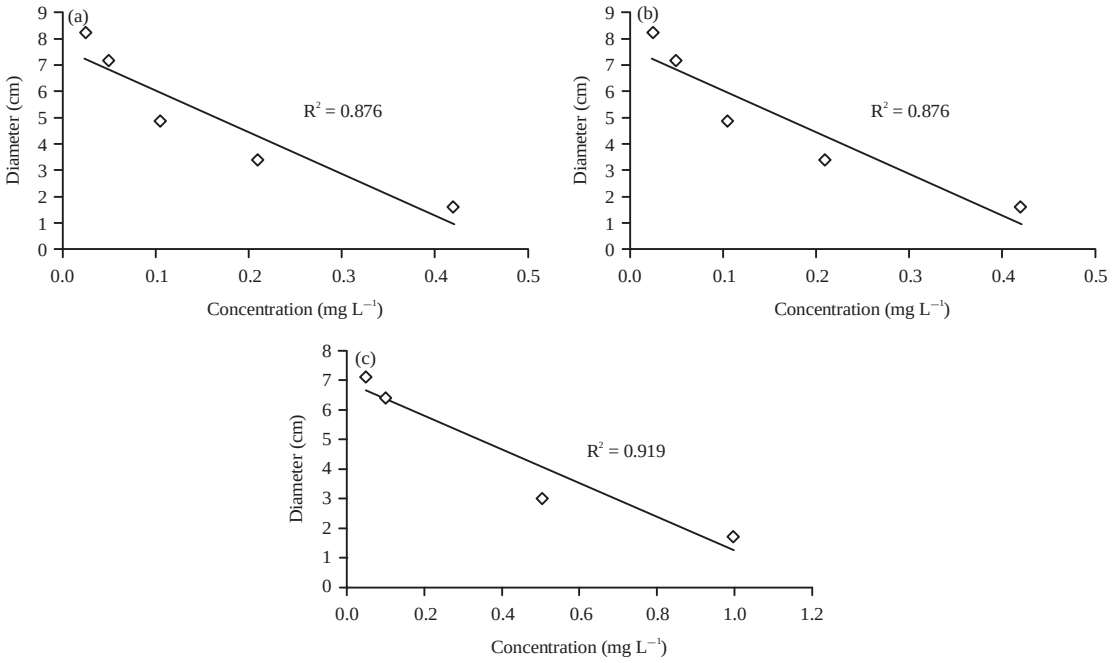

Fig. 2(a-c): Effects of selected fungicides on mycelium growth of *Fusarium* sp. treated by, (a) Thirame, (b) Tebuconazole and (c) Fludioxonil-difenoconazole after 7 days of incubation at 25°C ($p \leq 0.001$)

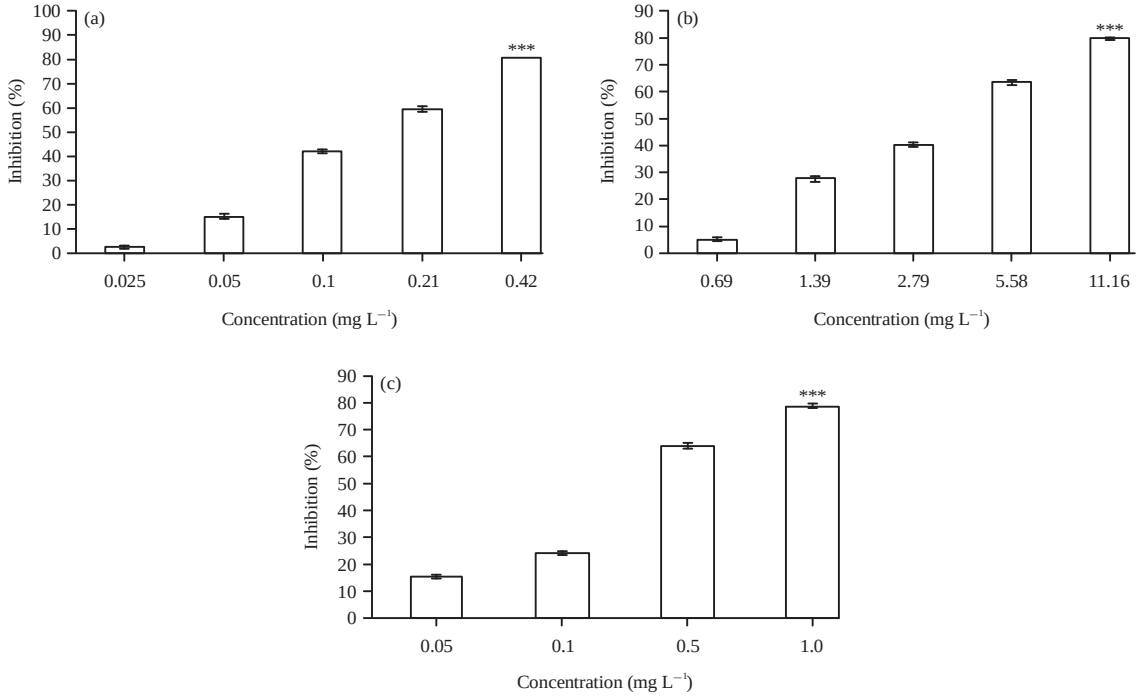

Fig. 3(a-c): Inhibitory effect of (a) Thiram, (b) Tebuconazole and (c) Fludioxonil-difenoconazole, on *Fusarium* sp. Results expressed in response percentage and each value is average±standard error of four replicates (***$p<0.001$)

DISCUSSION

Fusarium wilt is regarded as one of the most important diseases of wheat. The development of this disease may have serious consequences on both crop yields and grain quality (Pirgozliev *et al*., 2003). Several synthetic antifungal agents have been developed (Prasad *et al*., 2010) but fungicide application still the key factor in the control of fungal diseases.

Table 2: Values of EC_{50} for the three fungicides screened *in vitro* on *Fusarium* sp.

Fungicides	Percentage of inhibition 1 week after treatment (%)	EC_{50} (mg L^{-1})
Thiram	81.05	0.15
Tebuconazole	79.76	3.79
Fludioxonil-difenoconazole	79.41	0.27

In this study we focused first on evaluating the efficiency of three fungicides from different chemical classes. We noticed that the selected fungicides reduced the mycelial growth of Fusarium in dose-dependent manner. At the concentrations tested in the *in vitro* experiments, thiram reduced significantly the mycelium growth and final colony size of *Fusarium* sp. at the concentration of 0.42 mg L^{-1} compared to growth on unamended thiram medium. Similar observation was done by Fravel *et al*. (2005) when studying the effects of thiram on mycelial growth of *Fusarium oxysporum* strain CS-20.

As shown reported in literature, triazoles proved to be the most active molecules against *Fusarium* species (Edwards *et al*., 2001; Matthies and Buchenauer, 2000; Menniti *et al*., 2003; Blandino *et al*., 2006, 2012). These types of fungicides interfere with the metabolism of fungal pathogens, mainly by inhibition of ergosterol biosynthesis (Ragsdale, 1977; Hewitt, 1998) and often cause striking morphological malformations of cell wall. This confirms the strong decrease of colony sizes observed in strains exposed to tebuconazole and difenoconazole+fludioxonil. Indeed, the diameter of the colonies decreases significantly until it reaches a value of about 1.7 cm compared to the control (8 cm).

On the other hand, the response percentages confirm the toxic effects of the increasing concentrations tested. Indeed, for the higher concentrations of tebuconazole, the inhibition rate has reached a value of 79.76%. These results correlated well with the recent data on the effects of tebuconazole on growth and production of toxins by *Fusarium langsthiae* (Mateo *et al*., 2011). These results also agree with previous reports (Ramirez *et al*., 2004). These authors emphasize the great efficiency of triazoles, against the pathogenic species of *Fusarium graminearum* and report that none of the isolates of *F. graminearum* were able to grow in the presence of any active triazole substance at concentrations >15 mg mL^{-1}.

The strain treated with a mixture of triazoles and phenylpyrroles (Fludioxonil) led to a significant inhibition (79.41%), as great as that obtained with triazole alone. However, concerning the median effective inhibitory concentration, the EC_{50} value for tebuconazole was lower than fludioxonil+difenoconazole suggesting better efficacy overall of the mixture in controlling growth of *Fusarium* sp. The results obtained are in agreement with those of loos *et al*. (2005), who observed the most efficient control of *F. graminearum* by mixing the triazoles and strobilurins. Mesterhazy *et al*. (2011) demonstrated that the mixture of two triazoles provide a significative higher reduction of FHB disease.

CONCLUSION

The *in vitro* exposure showed that the three selected fungicides inhibit mycelial growth of *Fusarium* sp. till 80%. Additionally, the efficiency of the combination of two or more active ingredients has been demonstrated. The EC_{50} values vary from 0.27 mg L^{-1} for the mixture fludioxonil-difenoconazole and 3.79 mg L^{-1} for tebuconazole and thiram provide to be the most effective with a value of 0.15 mg L^{-1}.

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