

## Growth inhibition of *Fusarium oxysporum* f. sp. *nicotianae* by two *in vitro* poisoning methods with fungicides of different toxicological groups

Inhibición del crecimiento de *Fusarium oxysporum* f. sp. *nicotianae* por dos métodos de envenenamiento *in vitro* con fungicidas de diferentes grupos toxicológicos

Inibição de *Fusarium oxysporum* f. sp. *nicotianae* por dois métodos de envenenamento *in vitro* com fungicidas de diferentes grupos toxicológicos

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### Crop Production

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### Abstract

Wilt caused by *Fusarium oxysporum* f. sp. *nicotianae* is one of the most important fungal diseases in tobacco cultivation, being also one of the most difficult to control. The *in vitro* effectiveness of two poisoning methods was evaluated; sensi-disc (SD) and medium dilution (DMC) to determine the inhibitory effect of four fungicides of different toxicological groups on *F. oxysporum* isolated from tobacco in different regions of the province of Granma, Cuba. A differential response was observed in the susceptibility levels of all strains tested, regardless of the method of poisoning. The DMC method was more efficient than the SD, observing increases from 4.37 % (S) to 45.57 % (Ip+Pr) with respect to the SD. The highest inhibition values were observed in DMC with mancozeb (100 %), Tz+Az (79.74 %), and Ip+Pr (96.9 %). The greatest effectiveness in sporulation inhibition was with mancozeb by the SD method (0 %). The *in vitro* inhibitory effect of the fungicides evaluated (alone or in combination) is indicative of the fungicidal effect on the fungus under study and establishes the importance of inhibition methods for the study of fungicides used in management programs of diseases caused by *Fusarium* spp., in the tobacco growing areas of Cuba and other parts of the world.

## Resumen

La marchitez causada por *Fusarium oxysporum* f. sp. *nicotianae* es una de las enfermedades fúngicas más importantes en el cultivo del tabaco siendo también una de las difíciles de control. Se evaluó la efectividad *in vitro* de dos métodos de envenenamiento; sensi-disco (SD) y dilución en el medio (DMC) para conocer el efecto inhibitorio de cuatro fungicidas de diferentes grupos toxicológicos sobre *F. oxysporum* aislado de tabaco en diferentes regiones de la provincia de Granma, Cuba. Se observó una respuesta diferencial en los niveles de sensibilidad de todas las cepas evaluadas, independientemente del método de envenenamiento. El método DMC fue más eficiente que el SD, observándose incrementos del 4,37 % (S) al 45,57 % (Ip+Pr) con respecto al SD. Los valores de inhibición más altos observados fueron en DMC con el mancozeb (100 %), Tz+Az (79,74 %) y el Ip+Pr (96,9 %). La mayor efectividad en la inhibición de la esporulación fue con mancozeb por el método SD (0 %). El efecto inhibitorio *in vitro* de los ingredientes activos evaluados (solos o en combinación) es un indicativo del efecto fungicida sobre el hongo y establece la importancia de estos dos métodos de inhibición para el estudio de los productos que mayormente se utilizan en los programas de manejo de las enfermedades causadas por *Fusarium* spp., en las zonas tabacaleras de Cuba y otras partes del mundo.

**Palabras clave:** Combate químico, fusariosis, tabaco, sensibilidad, fungitoxicidad, marchitez.

## Resumo

Murcha causada por *Fusarium oxysporum* f. sp. *nicotianae* é uma das doenças fúngicas mais importantes na cultura do tabaco, sendo também uma das mais difíceis de controlar. A eficácia *in vitro* de dois métodos de envenenamento foi avaliada; sensi-disco (SD) e diluição média (DMC) para determinar o efeito inibitório de quatro fungicidas de diferentes grupos toxicológicos sobre *F. oxysporum* isolados de tabaco em diferentes regiões da província de Granma, Cuba. Uma resposta diferencial foi observada nos níveis de suscetibilidade de todas as cepas testadas, independentemente do método de intoxicação. O método DMC foi mais eficiente que o SD, com aumento da inibição de 4,37 % (S) para 45,57 % (Ip+Pr) em relação ao SD. Os maiores valores de inibição observados foram no DMC com mancozeb (100 %), Tz+Az (79,74 %) e Ip+Pr (96,9 %). A maior eficácia na inibição de esporos foi mancozeb pelo método SD (0 %). O efeito inibitório *in vitro* dos fungicidas avaliados (sozinhos ou em combinação) é indicativo do efeito fungicida sobre o fungo em estudo e estabelece a importância dos métodos de inibição para o estudo de fungicidas utilizados em programas de manejo de doenças causadas por *Fusarium* spp., nas áreas de cultivo de tabaco de Cuba e outras partes do mundo.

**Palabras-chave:** Combate químico, fusarium, tabaco, sensibilidade, fungitoxicidade, murcha.

## Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the main crops in Cuba, being the essential raw material for the tobacco company, one of the most iconic in the country. Of the total surface available for annual crops, tobacco represents around 6 % with an average sown area of ~25,000 ha (Wikle, 2015), which has decreased by ~3,000 by 2020

(CubaNews, 2022). However, the estimated annual production is 26,000 t.leaf<sup>-1</sup>. Cuba is the main exporter of cigars in the world, being the tobacco industry with an estimated income of 267 million dollars, the fourth most important sector in the generation of the country's gross domestic product (GDP) (Cosner, 2015).

One of the most important limitations in production and quality is the presence of vascular diseases, mainly those caused by various species of *Fusarium oxysporum* (Olivares *et al.*, 2021; Martín *et al.*, 2021). In Cuba, 31 species of *Fusarium* associated with different commercial crops have been reported (López, 2004), although *F. oxysporum* f. sp. *nicotinae* is the one that has had the greatest importance due to its high affectation in the Burley-type varieties, which are the most productive and with highest commercial value (García *et al.*, 2015). These peculiarities combined with the climatic conditions and the type of soils prevalent in the region (fluvisols) have been particularly difficult in the tobacco-growing regions of Bayamo, province of Granma (López, 2004; Mariña de la Huerta *et al.*, 2005; Villa *et al.*, 2015; Ceiro *et al.*, 2021). In fact, the biological complexity described for *Fusarium* spp. (Edel-Hermann and Lecomte, 2019; Lombard *et al.*, 2019) is one of the main characteristics that has made it difficult to control the disease in the field, in addition to the low availability of varieties tolerant to the fungus (García *et al.*, 2015). On the other hand, the range of fungicides for the control of *Fusarium* spp. in Cuba, including broad-spectrum (Cu, S) and specific (benzimidazoles, triazoles, strobirulins, and carbamates) have shown some type of resistance in other fungi for which the product was designed, thereby causing unwanted effects on agricultural ecosystems (Silva-Marrufo and Marin-Tinoco, 2021). In this way, the various methods of poisoning the culture medium are an important tool to evaluate the biological effectiveness on the growth and/or sporulation of the fungus, especially where it is necessary to evaluate fungicides with native phytopathogenic strains (Ceiro *et al.*, 2021).

The objective of the present work was to evaluate the *in vitro* inhibitory effect of fungicides of different toxicological groups by two poisoning methods on mycelial growth and sporulation of *F. oxysporum* f. sp. *nicotinae* isolated from tobacco in Granma, Cuba.

## Materials and methods

### Collection of *Fusarium* spp. strains

The fungal strains were collected from infected stems of tobacco crops (*Nicotiana tabacum* L.) with symptomatology according to the reported for *Fusarium* spp. (Nelson *et al.*, 1990; Blancard, 1998). The regions of collection were Buey Arriba (20°14'20.5"N 76°46'28.8"O), Monjará, Guisa (20°18'07.3"N 76°36'42.6"O) and El Dorado, Bayamo (20°15'52.3"N 76°44'43.8"O) in the province of Granma, Cuba. The nomenclatures of the strains under study were assigned according to the region and the variety of tobacco where the isolation was carried out, where; Fus= *Fusarium*, BA= Buey Arriba, G= Guisa, B= Bayamo, Hab = CV of 92 Havana tobacco, Cor (CV Corojo-2006). The other strains were isolated from SS-96 variety. The strains were prepared as monosporic culture in the Agricultural Microbiology Laboratory of the Faculty of Agricultural Sciences, University of Granma for subsequent identification and evaluation.

### Identification of *Fusarium* spp.

The identification was carried out according to the morphological characters for the determination of genus and specie. The morphometric tests for the characterization of the fruiting bodies were according to the color, pigmentation, and mycelial growth of the colonies. The characterization of conidia and microconidia was carried out according to size, shape, and the number of septa, the shape of apical cells, shape, and size of microconidia, presence

and arrangement of chlamydo spores, the color of sporodochia, and observation of phialides according to Summerell *et al.* (2007). Molecular identification was performed by PCR amplified with primers Bik1 (5'-TTCAAGAGAGCTAAAGGTCC-3') and Bik4 (5'-TTTTGACCAAGATAGATGCC-3'). The PCR conditions were according to the species (Holguín-Peña, 2007). All *F. oxysporum* strains were compared with those of reference from the Phytopathology Laboratory of the Northwest Research Center. For identification, each strain from each locality was morphologically characterized.

#### Sensitivity studies

The sensitivity study of strains to fungicides of different taxonomic groups was performed by two poisoning methods; 1) by diffusion of the active ingredient (i.a) on a paper disc (sensi-disc) (Clinical Laboratory and Standards Institute [CLSI], 2008) and 2) by the method of serial dilutions in culture medium (DMC) (Webster *et al.*, 2008). The sensi-disc method was performed by immersing (5 minutes) a paper disc in a solution with the fungicide and depositing four sensi-discs in the Petri dish with PDA medium. A 5 mm diameter disc of PDA with 3-day-old fungal mycelium was placed in the center of each one. After incubation time (28 °C/7 d), mycelial growth was determined for the calculation of inhibition using the equation [%inhibition = (CMC-CMT)/CMC\*100], where: CMC is the mycelial growth of the control (mm) and CMT is the mycelial growth of the treatment (mm). For the DMC method, PDA medium (39 g.L<sup>-1</sup>) plus streptomycin (50 mg.L<sup>-1</sup>) was prepared according to the methodology described by Ceiro *et al.* (2015).

#### Fungicide selection

Fungicide treatments were assigned according to table 1, considering six toxicological groups (alone or in combination). For *in vitro* doses, they were calculated according to each method and as reported in the literature (Nisa *et al.*, 2011; González-Merino *et al.*, 2021).

**Table 1. Fungicides of different toxicological groups used for *in vitro* control of *Fusarium oxysporum* in tobacco.**

Fung <sup>1</sup>	Active Ingredient (IA)	Concentration of IA (%) <sup>2</sup>	Chemical group	Trade name
Mz	Mancozeb	0.8	Dithiocarbamate	Mancozeb PH 80 (Limin Chemical Co. LTD)
Tz+Az	Tetraconazole+ Azoxystrobin	0.08/0.01	Triazole+ strobilurin	Galileo SC18 (Isagro S. P. A.)
Ip+Pr	Iprovalicarp+ Propineb	0.09/0.6	Isopropyl carbamate+ dithiocarbamate	Positron Duo 69 WP, Bayer CropScience
S	Sulfur	0.8	Inorganic element	Thiovit Jet 80 WG, Sygenta

<sup>1</sup>Fung = abbreviation according to the formulation of the active ingredient (IA).

<sup>2</sup>Percentage (%) of active ingredient (i.a) in the commercial formulation. The concentration of each fungicide is according to those reported on the trade name label. The selection of fungicides was according to prior consultation of the inhibition of the active ingredient; Mz (Gullino *et al.*, 2010; Runkle *et al.*, 2017), Az (Wang *et al.*, 2016), Tz, Ip, Pr and Az (Ceiro *et al.* 2015; Lucas *et al.*, 2015; Nisa *et al.*, 2011).

#### Sporulation inhibition

For the quantification of macroconidia concentration, 10 mL of distilled sterile water was added over the fungal growth on each plate and homogenized with a Drigalsky spatula. The spore suspension was collected in a 20 ml test tube. Dilutions up to 10<sup>-2</sup> were prepared from these. A capillary tube was used to extract small portions of the last dilution and a drop was deposited in a Neubauer chamber to determine the concentration of macroconidia with the help of a Novel brand optical microscope at 400X. The methodology for quantification was according to Savin-Molina (2021) and González-Merino *et al.*, (2021).

#### Statistical analysis

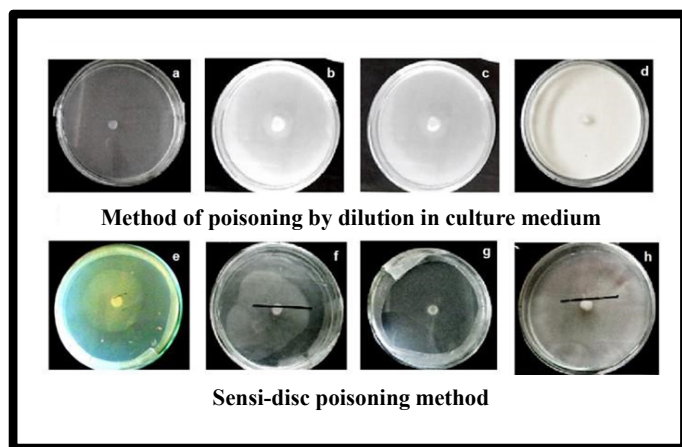
Statistical processing was performed using a 5x2x4 factorial ANOVA where; factor I = *Fusarium* isolates (two from Buey Arriba, two from Guisa, and one from Bayamo), factor II = poisoning methods (two poisoning methods DMC, SD), and factor III = fungicides (four fungicides; Mz, Tz+Az, Ip+Pr, S). From the combinations made, 40 treatments resulted in four replicates, under a completely randomized design. The control consisted of purified water. When detecting significant differences, Tukey's multiple comparisons of means test was used (P≤0.05). The values of macroconidia concentration were previously transformed to ANOVA by LOG (x+1) as well as the percentage of inhibition by  $ACOS\sqrt{X+1}$ . Processing was performed with InfoStat statistical package, 2008.  $ACOS\sqrt{X+1}$

## Results and discussion

The fungus associated with tobacco stem wilt disease was identified as *Fusarium oxysporum* f. sp. *nicotianae*. The morphological characteristics were in accordance with what was reported (Blancard, 1998; Summerell *et al.*, 2007; Rentería-Martínez *et al.*, 2019), observing a colonial growth (72 h) of salmon/purple-colored hairy appearance at the bottom of the Petri dish. The observed macroconidia were ≈18x140 μm, crescent-shaped in appearance, and hyaline with three to four septa. The presence of microconidia of ≈2-3 x 5-12 μm with oval to reniform appearance without obvious septa, was observed. Globose chlamydo spores (single and double) with short monophialides were observed. Sporodochium formation was not observed. The specie was confirmed by molecular techniques according to the amplification of the expected fragment 943 bp with primers Bik 1 and Bik 4, which corresponds to that reported for *F. oxysporum* (Holguín-Peña, 2007; Watanabe, 2013).

In sensitivity studies according to the inhibition of mycelial growth (%) significant differences (Tukey P≤0.05) were found between the poisoning methods and the fungicides evaluated. A higher inhibition was observed by the dilution method in culture medium (DMC), compared to the sensi-discs method (SD) (figure 1).

The highest inhibition values were obtained with the DMC method with mancozeb (100 %), followed by tetraconazole + azoxystrobin (79.74 %) and iprovalicarp + propineb (51.7 %). The lowest values with DMC were observed with sulfur (37.12 %). With the SD method, inhibition ranges were from 6.13 % (Ip+Pr) to 85.55 % (Mz) (table 2). It has been documented that methods with serial dilutions such as DMC are more sensitive for assessing biological effectiveness and the homogenization of the medium with the active ingredient is more consistent (Zgoda and Porter, 2001). In addition, the log dilution series (2.5 log) can be interpreted in a probabilistic trend curve (probit) for the calculation of the minimum inhibitory concentration (CMI) and the effective concentration at 50 % (EC50)



**Figure 1. Inhibition at 168 h of mycelial growth of *F. oxysporum* (Fus-By) isolated from tobacco (*Nicotiana tabacum* L.) by two different poisoning methods. DMC method. a) mancozeb, b) tetraconazole + azoxystrobin, c) iprovalicarp + propineb, d) sulfur. SD method; e) mancozeb, f) tetraconazole + azoxystrobin, g) iprovalicarp + propineb, h) sulfur.**

or 90 % (EC90) (Förster *et al.*, 2004; Webster *et al.*, 2008). In the SD method, the fungicide only interacts with the phytopathogen on the poisoned seed disc, causing a fungistatic effect in the first hours of interaction. However, if the mycelium of the fungus exceeds the area of interaction with the fungicide, it can grow and multiply on the non-poisoned growing medium.

Mancozeb is one of the most used active ingredients for the control of this type of fungi, due to the fact that its mechanism of action is aimed at inhibiting enzymatic processes and respiration; as well as, it inactivates the sulfhydryl groups (-SH), affects the Krebs cycle, prevents the formation of ATP and denatures the lipids of the cytoplasmic membrane (Runkle *et al.*, 2017). This implies that the different activation sites increase the spectrum of action and decrease the selection pressure associated with the appearance of resistant strains (Gullino *et al.*, 2010).

In the present research, mancozeb proved the most effective fungicide in both poisoning methods, coinciding with the reported inhibition range of the  $CI_{50}$  between 500 and 1000 ppm (Sultana and Ghaffar, 2013). Regarding the effect of azoxystrobin, in our study, the inhibition percentages at 168 h, were from 56.06 % with the strain (Fus-BA1) to 87.74 % (Fus-BA2), both strains from Bayamo.

**Table 2. Mycelial inhibition (%) at 168 h of fungicides of different toxicological groups on *Fusarium oxysporum* f. sp. *nicotinae* isolated from tobacco in the province of Granma, Cuba.**

Strain <sup>1</sup>	Method <sup>2</sup>	Fungicides (% inhibition) <sup>3</sup>			
		Mancozeb	Tetraconazole+ azoxystrobin (Tz + Az)	Iprovalicarp + propineb (Ip + Pr)	Sulfur (S)
<i>Dilution in culture medium</i>					
Fus-BA1	DMC	100 a	56 de	26.9 h	36.8 fg
Fus-BA2	DMC	100 a	87.7 b	80.1 bc	34.8 g
Fus-G(Hab)	DMC	100 a	84.5 b	35.4 fg	34.2 g
Fus-G(Cor)	DMC	100 a	86.1 b	42.1 fg	34.7 g
Fus-By	DMC	100 a	81.2 bc	73.9 c	45.1 f
<i>Sensi-disc</i>					
Fus-BA1	SD	100 a	62.2 d	2.9 j	24.2 h
Fus-BA2	SD	45.4 f	60.7 d	9.7 i	26.4 h
Fus-G(Hab)	SD	97.9 ab	39.7 fg	2.7 j	20.6 h
Fus-G(Cor)	SD	84.5 b	53.6 e	8.2 i	51.1 e
Fus-By	SD	100 a	61.9 d	7.2 ij	41.3f g
<i>Averages</i>					
DMC		100	79.74	51.7	37.12
SD		85.55	55.66	6.13	32.75

<sup>1</sup>Strain abbreviations; Fus (*Fusarium*), BA (Bayamo), G (Guisa), Hab (tobacco C.V. Havana 92), Cor (tobacco C.V. Corojo-2006), By (Bayamo). The BA and By isolates were isolated from tobacco C.V. SS-96.

<sup>2</sup>Poisoning method; SD = sensi-disc, DMC = dilution in culture medium.

<sup>3</sup>Different lowercase letters in columns differ significantly according to Tukey's test ( $P \leq 0.05$ ).

Azoxystrobin belongs to the methoxy-acrylates chemical group with a translimiting action within leaves and petals and it is an effective inhibitor of respiratory processes in mitochondria and also affects the proper electron transfer between cytochromes, thereby affecting the proper flow of ATPs and the adequate cellular energy transfer (Wang *et al.*, 2016).

In the study of the effects of fungicides on sporulation, significant differences were observed (Tukey  $P \leq 0.05$ ) between all the treatments. The highest inhibition values were observed with the DMC method with 96.7 % ( $0.2 \times 10^5$  spores.mL<sup>-1</sup>), compared to the sensi-discs (SD) where the highest concentrations ( $5.18 \times 10^5$  spores.mL<sup>-1</sup>) (S and Tr+Az) were observed (table 3). The lowest sporulation was recorded with Bayamo strains (Fus-BA2 and Fus-G/Hab) with mancozeb ( $0.15 \times 10^5$  mL<sup>-1</sup>). The highest concentration of spores ( $5.9 \times 10^5$  mL<sup>-1</sup>) was observed in sulfur with the SD method with the Fus-BA2 and Fus-G(Cor)/Tr+Az strains. Castellanos-González *et al.* (2011), showed that doses higher than 100 mg.L<sup>-1</sup> of mancozeb could totally inhibit the sporulation of the entomopathogenic *Beauveria bassiana*, and in other fungi such as *Pochonia chlamydosporia* var. *catenulate* can also inhibit the production of chlamydospores (Ceiro *et al.*, 2015). On the other hand, in addition to the inhibition effect, it has been observed that some mixtures such as fludioxonil + metalaxyl could affect the morphology of the hyphae and cause cell wall disorders (Miguel *et al.*, 2015).

According to the results obtained, all the fungicides evaluated can efficiently inhibit the mycelial growth of the fungus. The evaluated methods DMC and SD showed to be a good criterion for the evaluation of the *in vitro* activity of *F. oxysporum*. The widespread use of fungicides with single-site mechanisms of action requires a diverse set of chemical management tools to slow the evolution of fungicide-resistant pathogens (Avenot and Michailides, 2010; Lucas *et al.*, 2015). In this sense, both methods (DMC and SD) can be complementary and an excellent tool to establish phytosanitary management programs where fungicides with different modes of

action are involved. However, it is important to consider that the most effective *in vitro* fungicides will only be a reflection of the sensitivity of the active ingredient in each strain evaluated, and the efficacy in open-field conditions will not necessarily coincide.

## Conclusions

The results of the *in vitro* studies indicate that the greatest inhibitory effect on mycelial growth in *F. oxysporum* f. sp. *nicotinae* was with mancozeb and tetraconazole + azoxystrobin, regardless of the poisoning method, although the highest efficiency was observed with the dilution method in the culture medium, compared to the sensi-disc method.

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**Table 3. Inhibition of sporulation of *Fusarium oxysporum* f. sp. *nicotinae* due to the effect of fungicides of different toxicological groups.**

		Macroconidia concentration (x10 <sup>5</sup> mL <sup>-1</sup> )			
		Mancozeb	Tetraconazole+azoxystrobin	Iprovalicarp+propineb	Sulfur
		Xt <sup>2,3</sup>	Xt	Xt	Xt
Fus-BA1	DMC	0.15 a	4.0 defgh	3.0 cd	5.2 fghi
Fus-BA2	DMC	0.15 a	4.7 fgh	3.7 def	5.3 ghi
Fus-G(Hab)	DMC	0.2 a	3.3 cde	4.2 defgh	3.7 def
Fus-G(Cor)	DMC	0.2 a	4.8 fgh	4.2 defgh	4.9 fghi
Fus-By	DMC	0.3 a	3.7 def	3.0 cd	4.7 fgh
Fus-BA1	SD	0.2 a	5.3 ghi	4.2 defgh	5.5 ghi
Fus-BA2	SD	4.6 fgh	5.0 ghi	5.4 ghi	5.9 hi
Fus-G (Hab)	SD	1.2 ab	5.3 ghi	4.0 defgh	4.7 fgh
Fus-G(Cor)	SD	2.2 bc	5.9 hi	4.4 efghi	5.92 hi
Fus-By	SD	0.1 a	4.4 fgh	4.2 defgh	3.9 defgh

<sup>1</sup>MET = method; DMC (dilution in culture medium), SD (sensi-disc). 2Xt = transformed value LOG (x+1) of the average for each treatment. Control concentration (without fungicide) = 6 x10<sup>5</sup> mL<sup>-1</sup>. 3 Different letters in the columns differ significantly according to Tukey's test. (P≤0.05).

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