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Chemical control of *Septoria lycopersici in vitro* as first screening for fungicide efficacy studies

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Abstract

The disease septoriose causes severe defoliation in tomato plants that can reach 100% leaf fall. Consequently, the losses are significant due to the decrease in photoassimilate production and sun scald on tomato fruits. To apply a fungicide the dose used must follow the fungicide label instructions to keep the resistance risk low and comply with current legislation. This work presents *in vitro* studies of 16 active ingredients (17 commercial products) at the recommended doses to control the tomato septoriose and the molecular identification of 26 *Septoria lycopersici* isolates performed with the ITS gene. *In vitro* test was performed at Petri dishes filled with 20 mL of malt-extract medium (20 g/L malt extract and 20 g/L agar). 100 µL of spore suspension (10^5 spores/mL) were spread onto Petri dishes with the Drigalski loop. In their label dose, 10 µL of each fungicide was added at the centre of the Petri dishes to measure the inhibition halo formed. Petri dishes were incubated for 14 days at 25 ºC and 12h of photoperiod. Among the fungicides tested, 14 commercial products formed inhibition halo showing the dose used was able to control the spore germination which indicates efficacy of the fungicide and also an adequate dose to control the pathogen. Only cuprous oxide (1344 mg a.i./L), azoxystrobin (80 mg a.i./L) and thiophanate methyl (490 mg a.i. /L) were unable to prevent spore germination and did not form an inhibition halo. The *Septoria* isolates were identified as *Septoria lycopersici* and sequences were deposited at the Genbank. No variation was observed for the species when using ITS region.

Keywords – Chemical control – *Septoria* leaf spot – *Septoria lycopersici* – *Solanum lycopersicum*

Introduction

The septoriose was reported for the first time in Argentina in 1882 (Sutton & Waterston 1966) and nowadays occur anywhere in tomato crops (Stevenson 1991). *S. lycopersici* infect tomato leaves by both stomata and direct penetration (Martin-Hernandez et al. 2000). Its importance depends on the favourable weather conditions, which occur when the relative humidity is above 85%, temperature is between 20 to 25ºC (Kurozawa & Pavan 2005) and wetting periods greater than 20h (Elmer & Ferrandino 1995).

Symptoms appear one week after inoculation, and after six weeks, defoliation is close to 100%, and losses are significant due to the sunscald on tomato fruits (Sohi & Sokhi 1974), when in humid conditions and no control measures are used (Parker et al. 1997). In each cultivation cycle, the disease begins in the leaves of the shallows due to the raindrops that fall on fragments of plants

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with *Septoria* spores and cause splashes spreading the spores to the surrounding tomato leaves (Douglas 2008).

A study to determine resistance to septoriosis with more than 500 plants, including strains, accessions and cultivars revealed that all tested cultivars demonstrated susceptibility to this disease (Poysa et al. 1993). Despite attempts to include septoriose resistance, no commercial cultivar has it. Since there is no genetic resistance, the recommended control tactics include crop rotation with a non-host species, removal of alternative hosts (*Datura stramonium*, *Physalis* sp., *Solanum carolinense*) and cultural remains (Seymour & Ridings 1980, Zitter 1987, Stevenson 1991, Malnati 1993, Bhardwaj et al. 1995), and the application of the recommended fungicides.

Fungicides, natural or synthetic, protect plants against invasion by fungi or eradicate established fungal infection to ensure the yield potential, measure as the quantity or quality of production (Oliver & Hewitt 2014). The effectiveness of some fungicides may vary from time to time because pathogens can develop resistance to it. The risk of fungicides resistance varies depending on the number of action sites of the active ingredients and pathogen propensity to develop resistance (Oliver & Hewitt 2014).

Scientific data on the efficiency of fungicides in controlling tomato septoriosis were published more than 20 years ago highlighting the use of chlorothalonil (Poysa & Tu 1993) and mancozeb (Dillard et al. 1997). A more comprehensive work and more current studied the efficiency of 29 active ingredients in the control of *S. lycopersici* showing which were the most efficient in controlling the disease (Monteiro et al. 2021).

The objective of this work was to determine the effect of 16 active ingredients (17 commercial products) at the label recommended doses to control *S. lycopersici in vitro* including the pidiflumetofen, a molecule recently raised on the market, and perform the molecular identification of 26 *S. lycopersici* isolates via ITS gene.

Materials & Methods

Molecular identification

Twenty-six isolates of *S. lycopersici* were identified by sequencing the Internal Transcribed Spacer (ITS gene), and the sequences were deposited at the GenBank (Fig. 1). The DNA was extracted via PureLink Genomic DNA Mini Kit and PCR was performed in thermocycler TC-9639 (Loccus) with the following program: 94 ºC for 4 min followed by 30 cycles of 94 ºC for 30 sec, 55 ºC for 1 min, 72 ºC for 1:30 min, and a final extension at 72 ºC for 10 min. PCR products were analyzed by agarose gel (0.7%) electrophoresis in 0.5x TBE buffer conducted at 90V for 1h and scanned using the imaging system L-PIX EX (Loccus). The evolutionary history was inferred using the UPGMA method using ITS gene. The percentage of replicate trees in which the associated taxa clustered together was performed with the bootstrap test (10,000 replicates). The evolutionary distances were computed using the Kimura 2-parameter method.

Germination of *Septoria* spores when in contact with the registered fungicides at the label dose

The experiment was conducted in Petri dishes of 9 cm and three replicates per treatment. After pouring the culture media malt extract (malt extract 20 g/L and agar 20 g/L), 100 µL of a spore suspension at 10^5 spores per ml was spread over the culture media surface using a Drigalski handle. In the center of the Petri dish was placed 10 µL of the fungicides solution at the recommended dose. Fungicides doses were employed as describe in their labels (Table 1). The inhibition halo was measured after 14 days of incubation at 25ºC and a photoperiod of 12h.

Results

Molecular identification

No genetic variability was found among the isolates via ITS gene sequencing (Fig. 1).

Table 1 Label doses of fungicides.

Fungicides	Label dose (a.i.)
Difenoconazole + Pidiflumetofem	200 + 120 mg/L
Pyraclostrobin + Fluxapiroxade	58.5 + 116.6 mg/L
Difenoconazole	125 mg/L
Mancozeb	4000 mg/L
Captan	1250 mg/L
Chlorothalonil	1500 mg/L
Chlorothalonil + Difenoconazole	1000 + 100 mg/L
Thiophanate methyl + Fluazinam	375 + 375 mg/L
Thiophanate methyl + Chlorothalonil	350 + 875 mg/L
Metiram + Pyraclostrobin	1100 + 100 mg/L
Pyraclostrobin	100 mg/L
Mancozeb + Copper oxichloride	880 + 600 mg/L (340mg/L metallic eq.)
Benzalkonium chloride	250 mg/L
Propineb	2100 mg/L
Thiophanate methyl	490 mg/L
Azoxystrobin	80 mg/L
Cuprous oxide	1344 mg/L (50% metallic copper)

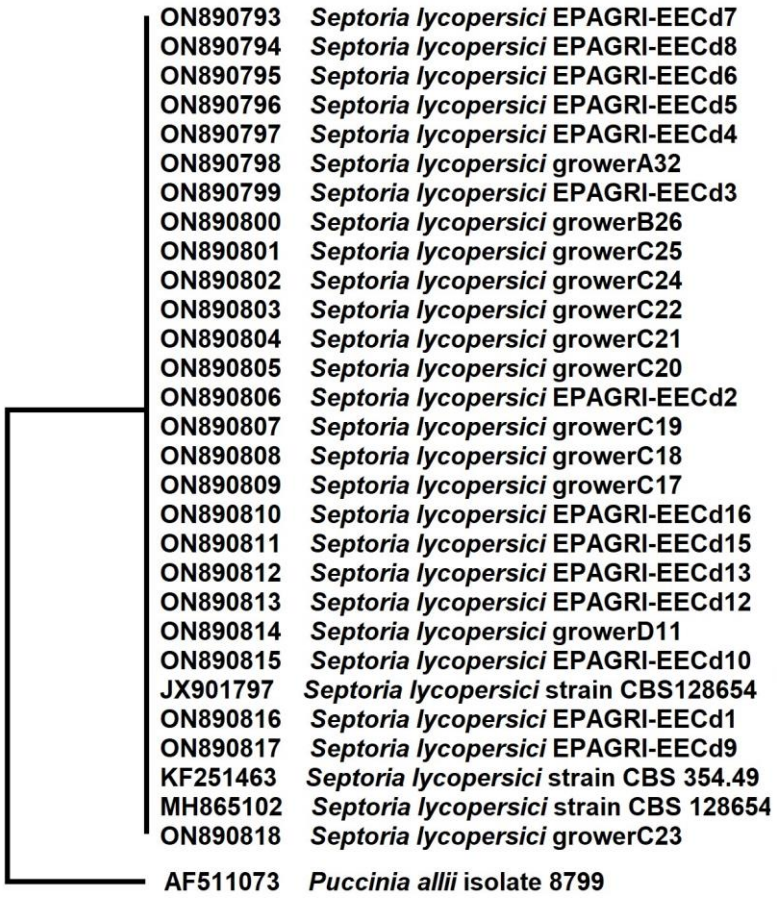

Fig. 1 – Phylogenetic tree inferred using the UPGMA method using ITS gene.

Inhibition halo formed by fungicides application

Among the fungicides tested, 14 commercial products formed inhibition halo showing the dose used was able to control the spore germination which indicates efficacy of the fungicide and also an adequate dose to control the pathogen (Figs 2, 3). Only cuprous oxide (1344 mg a.i. /L),

azoxystrobin (80 mg a.i./L) and thiophanate methyl (490 mg a.i. /L) were unable to prevent spore germination and form an inhibition halo (Figs 2, 3).

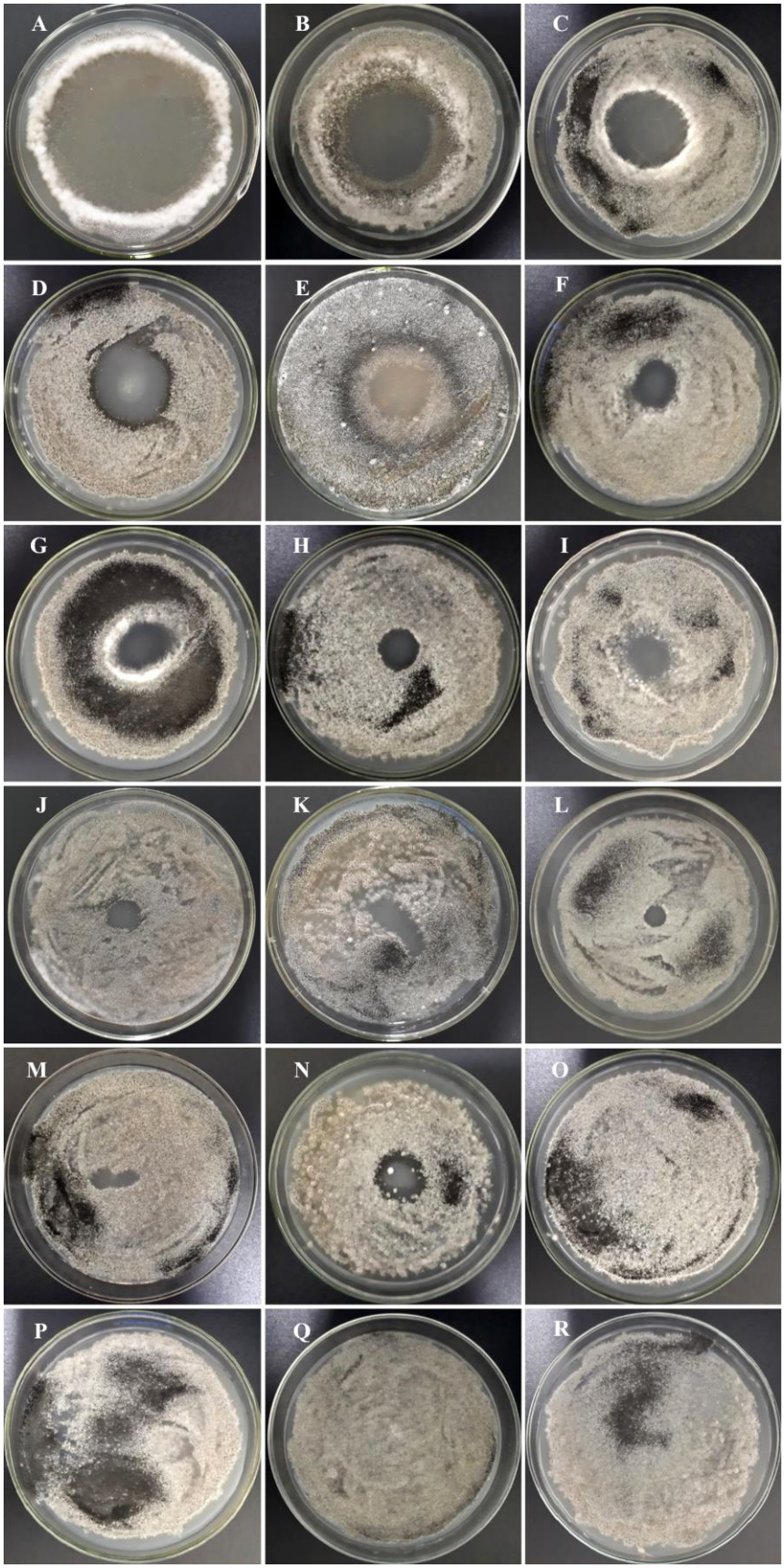

Fig. 2 – Effect of fungicides in their label doses against *S. lycopersici*. A Difenoconazole + Pidiflumetofem (200 + 120 mg/L). B Pyraclostrobin + Fluxapiroxade (58.5 + 116.6 mg/L).

C Difenoconazol (125 mg/L). D Mancozeb (4000 mg/L). E Captan (1250 mg/L). F Chlorothalonil (1500 mg/L). G Chlorothalonil + Difenoconazol (1000 + 100 mg/L). H Thiophanate methyl + Fluazinam (375 + 375 mg/L). I Thiophanate methyl + Chlorothalonil (350 + 875 mg/L). J Metiram + Pyraclostrobin (1100 + 100 mg/L). K Pyraclostrobin (100 mg/L). L Mancozeb + Copper oxichloride (880 + 600 mg/L [340 mg/L metallic eq.]). M Benzalkonium chloride (250 mg/L). N Propineb (2100 mg/L). O Thiophanate methyl (490 mg/L). P Azoxystrobin (80 mg/L). Q Cuprous oxide (1344 mg/L 50% metallic copper). R Check.

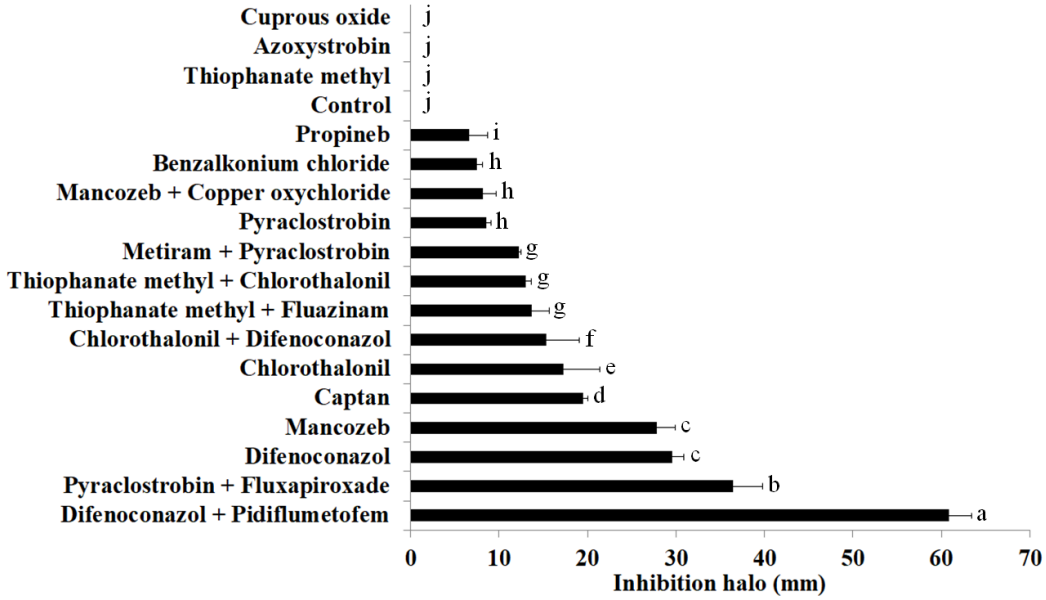

Fig. 3 – Inhibition halo promoted by the fungicides at their label doses against *S. lycopersici*.

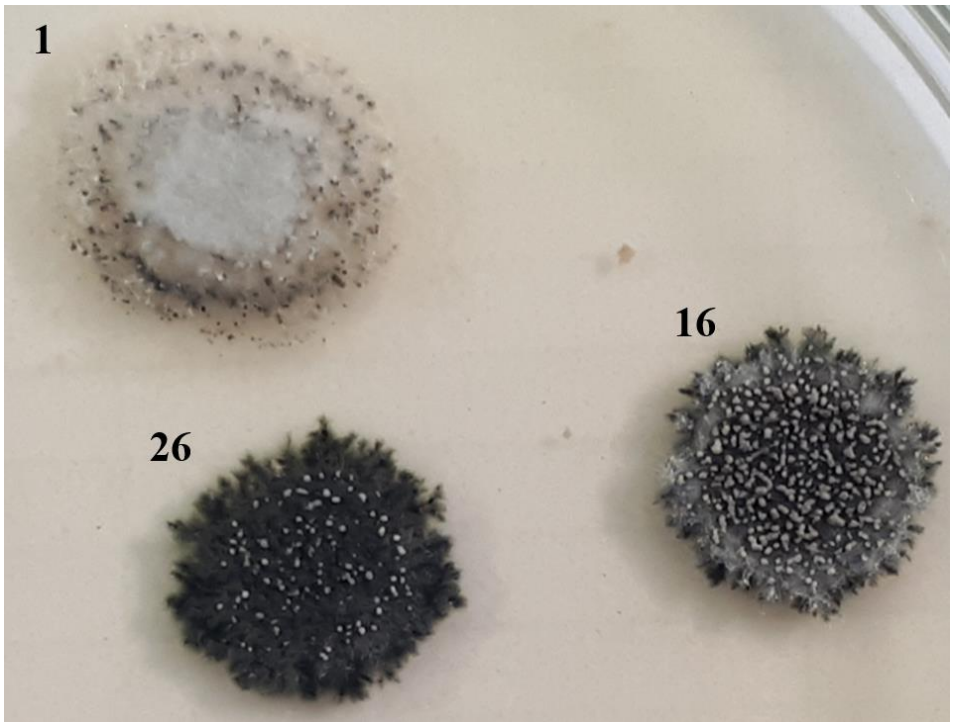

Fig. 4 – Morphological variation among isolates (Isolates nº 1, 16 and 26).

Discussion

The *S. lycopersici* is the unique etiologic agent of the septoriose in Brazil, and genetic diversity was not found for isolates from Brazil using Tub, Cal and EF1-α sequencing region

(Costa 2019). Using the ITS region in this work, the same conclusion was reached. Costa (2019) reported that despite the lack of genetic diversity based on Tub, Cal and EF1-α sequencing region, the isolates of *S. lycopersici* have some morphology variability and variation in aggressiveness to tomato plants. In fact, morphological differences among isolates were also observed in this work (Fig. 4).

Since all fungicide herein is recommended to control *S. lycopersici* seems to be weird some of them not forming any inhibition halo and allow fungus grow normally as the check (Fig. 2O, P, Q, R). The low sensibility of some *S. lycopersici* isolates to azoxystrobin was reported at the dose of 40mg/L (Costa 2019). Two possible reasons for this inefficiency is the development of resistance to the active ingredient or the use of a very low dose to a particular isolate unable to prevent spore germination and mycelial growth. Differences among *S. lycopersici* isolates in terms of sensibility to the fungicides exists (Monteiro et al. 2021). By adding 10 µL of each fungicide at the recommended dose in the center of the Petri dishes, it was possible to observe different sizes of inhibition halos, indicating that some fungicides not only act in the place where they were deposited, but also influence the surrounding environment via diffusion or vapor phase (Figs 2, 3).

The method of inhibiting spore germination used herein seems to demonstrate the reality of what happens *in vivo* (Monteiro et al. 2021), when the fungicide is applied to tomato plants, aiming to control *S. lycopersici*, and with the advantage of not needing a microscope to evaluate the result. In this context, we believe that the proposed method can be a fast and effective method for determining fungicide efficiency, aiming at field control. The disadvantage of *in vitro* methods is that it is impossible to know any deleterious effects to plants caused by applying some fungicides in some doses.

According to Oliver & Hewitt (2014) *in vitro* test tends to generate many false-positive results, which occur when a compound that inhibits growth in the Petri dishes assay fails to inhibit fungal growth in plant. In general, the greater the halo of inhibition caused by the fungicide, the more efficient the fungicide would be in controlling the *S. lycopersici* in tomato plants. On the other hand, the fungicide dose is unable to promote any inhibition halo has little chance of controlling the disease in tomato crop in field.

Monteiro et al. (2021) demonstrated that the preventive treatment with fluxapyroxad + pyraclostrobin (58.5+116.6 mg/L of the active ingredient, respectively), mancozeb (4000 mg/L), difenoconazole (125 mg/L), chlorothalonil (1500 mg/L), propineb (2100 mg/L), fluazinam + thiophanate-methyl (375+375 mg/L) or metiram + pyraclostrobin (1100+100 mg/L) controlled the tomato septoriose efficiently. Among the fungicides recommended for other tomato diseases than septoriose, those with mancozeb or chlorothalonil in doses higher than 1920 and 1200 mg/L, respectively, as part of the active ingredients and boscalide (75 mg/L) also controlled the disease efficiently (Monteiro et al. 2021).

This approach allows establishing efficient doses of products in test and also makes it possible to study new fungicides before setting up field trials so that they have better performance in controlling the disease.

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