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In vitro susceptibility test of *Cladosporium cladosporioides* isolates from Argentinian tomato crops against commercial fungicides

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ABSTRACT

Tomato cultivation is an important agricultural activity in northeast of Argentina, and disease control is indispensable for its production. The purpose of the current study was to identify two fungi isolated from tomato plants cultivated in greenhouses, with symptoms of leaf mould disease and a strain of the genus *Cladosporium* from a culture collection, and evaluate their *in vitro* susceptibility to four commercial fungicides. Macro and microscopic examination, molecular characterisation and sequence analysis were applied for identification. Broth dilution and spread plate methods were used to determine the minimal inhibitory concentration (MIC) and minimal fungicide concentration (MFC). The active ingredients of the products were azoxystrobin+difenoconazole, trifloxystrobin+tebuconazole, chlorothalonil and metalaxyl-M+mancozeb. The results were processed using the Kruskal-Wallis method. The isolates were identified as *Cladosporium cladosporioides*$^{a-c}$; consequently, lesions found on tomato plants did not correspond to *Cladosporium fulvum*. There was a significant statistical difference between the obtained values. Qualitatively, the three strains had a similar behaviour for chlorothalonil (MIC values: 0.25 - 0.5 µg/ml, MFC values: 4 µg/ml). In all cases, tests with metalaxyl-M+mancozeb yielded higher values than those achieved for chlorothalonil (MIC values: 8 µg/ml, MFC values: 8- 32 µg/ml). trobilurin-formulated fungicides were less effective against *C. cladosporioides*$^{a-b}$ (MIC values: 16-256 µg/ml, MFC values: >64 µg/ml). *C. cladosporioides*^{c} was the most sensitive isolate. The information about the presence of a non-frequent fungus and its fungicide susceptibility, would be useful for establishing control strategies and enhance production.

Keywords: Antifungals, characterisation, *Cladosporium cladosporioides*, greenhouse, *Solanum lycopersicum*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an annual vegetable crop with a wide geographic distribution and consumption throughout the world. It is one of the most important vegetables in Argentina. In some regions, it is produced in greenhouses, which account for almost a half part of the area used for vegetables cultivation. This area is principally located in the green belt of La Plata, followed by Corrientes and other important provinces (Sinavimo, 2018; Sosa, 2013).

Producers adopt integrated disease management (IDM) to maintain healthy crops during a long production period (March-December). IDM congregates several practices maintaining low incidence levels (Obregón, 2018). However, diseases continue to cause serious economic losses (Lucas et al., 2015). Phytopathogenic fungi can cause numerous diseases in plants, such as leaf mould by *Passalora fulva*. In relation to the last microorganism mentioned, it is one of the species that commonly attacks tomato crops (Thomma et al., 2005). The province of Corrientes does not escape this reality and producers must pay special attention to this pathogen.

Initially, leaf mould infects older leaves. Yellowish spots appear on the upper leaf surface and an olive-

green mould on the lower leaf surface. As the disease develops, the spots become darker, leaves roll up, and defoliation may occur (Fig. 1). Hence, the photosynthetic area is diminished, which affects the yield and quality of fruits. *P. fulva* grows best at high temperature and relative humidity. Its primary dissemination method is wind, and it lives as a saprophyte on crop debris. Currently, disease control methods involve the application of fungicides (preventives and curatives), and among other agricultural practices, this guarantees crops protection (Obregón, 2018).

Fig. 1 : Tomato plants with symptoms of leaf mould disease

Consequently, when climate conditions are favourable for disease development, this pathogen plays an important role in agricultural production causing big economical damage. The purpose of the current study was on identify two fungi isolates from different tomato plants cultivated in greenhouse conditions, with typical leaf mould symptoms, and one isolate from a culture collection, applying conventional and molecular methods; and to determine their *in vitro* susceptibility against commercial fungicides by broth dilution and spread plate methods.

MATERIALS AND METHODS

The identification of the three phytopathogens was made by morphological and molecular characterisation. Two of them were from the Laboratorio de Fitopatología Hortícola, INTA EEA, Bella Vista, Argentina and the other was from a culture collection of Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina. Isolates were denominated Ia, Ib and Ic, respectively. Experiments were done at Instituto de Investigaciones Científicas, Argentina, during the year 2021.

Fungicides used for *in vitro* susceptibility tests were azoxystrobin 20 g + difenoconazole 12.5 g (Syngenta®), trifloxystrobin 25 g + tebuconazole 50 g (Bayer Crop Science®), chlorothalonil 72 g (Syngenta®) and metalaxyl-M4 g + mancozeb 64 g (Syngenta®). The first three products were selected taking into account the recommendations established by Obregón (2018) for the treatment of tomato plants with leaf mould. The latter one was included due to its broad antifungal spectrum, in order to examine its efficacy for strains under study (Emara et al., 2021). The amount of active ingredients is presented per 100 g/100 ml.

Morphological characterisation

To verify the presence of a monosporic culture, moulds were sub cultured on Fungi and Yeast Agar (Laboratorios Britania S.A.). Later, each isolate was inoculated in a Petri dish with Malt Extract Agar for 14 days in dark (Piontelli-Laforet, 2011; Pitt & Hocking, 2009). For macroscopic examination, colonies description, the size, appearance, colour, presence of pigments in culture medium, border, elevation, exudate and zoning were registered. These colonies were assessed visually and with stereomicroscope. Microscopic examination was made applying the microculture technique to observe the complete mycelium structure. Microcultures were incubated for 5-7 days (Pitt & Hocking, 2009). A detailed observation and a schematised description of the fungal structures were done (Piontelli-Laforet, 2011; Winn et al., 2006).

Molecular characterisation

After 7 days of incubation, the mycelium of each isolate was used for deoxyribonucleic acid (DNA) extraction, with minor modifications. The extracted

DNA was used for polymerase chain reaction (PCR) amplification. PCR was carried out using primers which a code for a segment of the internal transcribed spacer (ITS) region of ribosomal nuclear: ITS-1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS-4 (5' TCCTCCGCTTATTGATATGC 3'). The obtained products were observed on a 1.5% agarose gel in 1x TAE buffer by electrophoresis and after running, the gel was visualised under ultraviolet light (UV) using a UV transilluminator (MLB - 21, MAESTROGEN) (Ibañez et al., 2022).

Products from amplification were sequenced with the same primers in Macrogen Inc., Korea. Sequences were aligned using MEGA 7 software (Kumar et al., 2016). Identification was performed by comparing the sequences in GenBank Nucleotide Database of the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST).

Susceptibility tests

Broth macro dilution test was used to determine *in vitro* fungicides' minimal inhibitory concentration (MIC). M38-A standard of the Clinical Laboratory Standards Institute (CLSI) methodology was taken as basis (Balouiri et al., 2016; CLSI, 2008). Antifungal stock solutions should be prepared at least ten times the highest concentration to be tested. Stock solutions of water-soluble or water-dispersible antifungal agent were prepared in a concentration of 5120 µg/ml. For the non-soluble or non-dispersible in water fungicide the concentration was 6400 µg/ml using DMSO as solvent. Dilutions were done with Sabouraud Dextrose Broth (SDB) (Laboratorios Britania S.A.) at pH 7.0 ± 0.1, an affordable and useful medium for the cultivation of fungi. Dilutions concentration range varied from 0.03125 to 64 µg/ml for chlorothalonil, metalaxyl-M+mancozeb and trifloxystrobin+ tebuconazole and from 0.03125 to 512 µg/ml for azoxystrobin+difenoconazole.

Seven-day old cultures of Ia, Ib and Ic, were used to prepare inoculums in a concentration between 0.4 x 10^4 and 5 x 10^4 colony forming unit per ml (CFU/ ml), aid by a Neubauer chamber. Inoculum concentration was verified by spread plate method. All macrodilution tests were incubated for 48-72 h to determine MIC (Machuca & Murguía, 2020; Machuca et al., 2015). All the tests included drug-free and fungi-free controls. Tests with non-soluble in water products also included 1% DMSO as a dilution control.

For the determination of *in vitro* minimal fungicide concentration (MFC), dilution tubes that showed a complete inhibition, the last tube with visible growth and positive control tube, were selected. Subsequently, without shaking the content of the tubes, 20 µL of each one was transferred to Sabouraud Dextrose Agar Petri dishes. Plates were incubated until positive control showed visible colonies. The MFC (µg/ml) was determined by the lower fungicide concentration that did not show any growth or when it showed less than 3 CFU (Díaz Dellavalle, 2011; Espinel-Ingroff et al., 2002). Assays were carried out in duplicate.

Statistical analysis

Statistical analysis of MIC and MFC values were done separately by Kruskal-Wallis non-parametric test, to determine the differences between them. This method was chosen because of the abnormal distribution of values and the small sample (n=3) and hypothesis are the following: (Hernández Sampieri, 2010; Spiegel & Stephens, 2009).

H_o: There is not a significant difference in the antifungal activity of the commercial fungicides against the three isolates.

H_1: There is a significant difference in the antifungal activity the commercial fungicides against the three fungi isolates.

RESULTS AND DISCUSSION

Isolates presented colonies diameters between 30 and 40 mm, being dense, flat, slightly velvety, olive-green at obverse and grey-blue at reverse. Microscopely, ramoconidia was about 11.4-45.6 µm long, not geniculate nor nodose conidiophore, oblong to limoniform conidia. For bicellular conidia, measurements of 11.4-15.2 x 3-3.8 µm were recorded; meanwhile for unicellular conidia measures were of 6.8-7 x 3.5-3.8 µm. Characteristics coincided with the key related for *Cladosporium cladosporioides* (Piontelli-Laforet, 2011; Pitt et al., 2009) (Fig. 2 & Fig. 3).

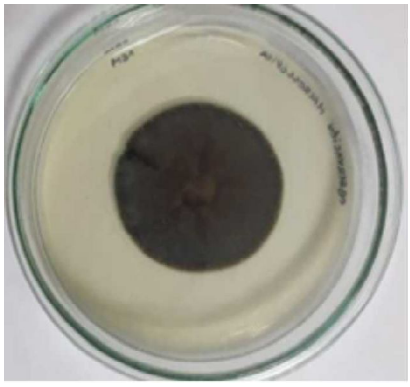

Fig. 2 : Cladosporium cladosporioides culture (malt extract agar): obverse (a) and reverse (b)

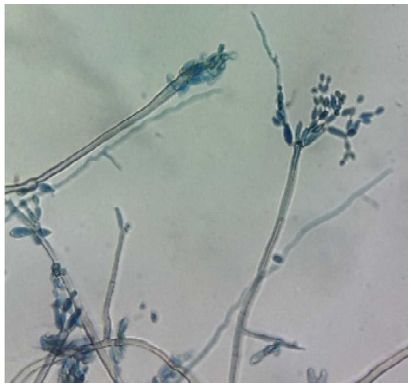

Fig. 3 : Cladosporium cladosporioides microscopic examination (400x)

Sequences of isolates Ia, Ib and Ic were identical to those recorded for *C. cladosporioides* in GenBank and were finally identified as *C. cladosporioides*. From this section on, isolates will be mentioned as *C. cladosporioides*[a], *C. cladosporioides*[b] and *C. cladosporioides*[c], respectively, with NCBI accession numbers ON585709, ON585710 and ON585708. The results for MIC and MFC are exposed in Table 1.

For *C. cladosporioides*[a] and *C. cladosporioides*[b], MIC readings were done at 48 h of incubation and *C. cladosporioides*[c] MIC reading was performed at 72 h. MFC was determined after MIC readings and were read with the same criteria.

Statistical processing was done by the application of the Kruskal-Wallis test to MIC and MFC values. To carry out MFC data processing, values that were higher than the upper limit concentration ranges were configured as lost values. The quantitative analysis of *in vitro* susceptibility of *C. cladosporioides* isolates against fungicides, showed that values for MIC (P = 0,159) and MFC (P = 0,740) were both $P > 0.05$. This means there is significant statistical difference between values.

Cladosporium cladosporioides[a-b] exhibited higher MIC values with azoxystrobin+difenoconazole and trifloxystrobin+tebuconazole. Coincidentally, these fungicides have an active ingredient of strobilurins chemical group in their composition. Chlorothalonil and metalaxyl-M+mancozeb showed lower MIC values, so *C. cladosporioides*[a-b] were more susceptible to these fungicides, being chlorothalonil the most effective and recommended. For *C. cladosporioides*[c], trifloxystrobin+tebuconazole exhibited the lowest MIC. For Metalaxyl-M+mancozeb showed the highest MIC value and was more susceptible to formulates with strobilurins. The three isolates had a similar MIC behaviour for chlorothalonil.

Regarding MFC values, the isolates showed the same behaviour for MFC values for metalaxyl-M+mancozeb were found into the concentration ranges, but they were higher than those for

Table 1 : *In vitro* susceptibility of *Cladosporium cladosporioides* isolates against fungicides, expressed in minimal inhibitory concentration (MIC) and minimal fungicide concentration (MFC) in µg/ml

Fungicide	*C. cladosporioides*[a]		*C. cladosporioides*[b]		*C. cladosporioides*[c]	
(Active ingredients per 100 g - 100 ml)	MIC	MFC	MIC	MFC	MIC	MFC
Azoxystrobin 20 g + difeconazole 12,5 g*	64	> 512	256	> 512	0.125	4
Trifloxystrobin 25 g + Tebuconazole 50 g**	32	> 64	16	> 64	0.0625	0.125
Chlorothalonil 72 g**	0.25	4	0.25	4	0.5	4
Metalaxyl-M 4 g + mancozeb 64 g**	8	8	8	16	8	32

*Concentration range tested: 0.03125-512 µg/ml, at higher concentrations the formulate precipitated

**Concentration range tested: 0.03125-64 µg/ml, at higher concentrations the formulate precipitated

chlorothalonil. Chlorothalonil was the most potent formulate. The results showed that *C. cladosporioides*[c] was the most sensitive isolate.

MFC values that were higher than the upper limit concentration ranges in *C. cladosporioides*[a-b], could be due to resistance caused by constant exposure to strobilurins, as these fungicides are frequently used to control tomato diseases (Longone & Escoriaza, 2017; Veloukas et al., 2007; Watanabe et al., 2017) found an azoxystrobin resistance of 80% or more, in *P. fulva* isolates collected from greenhouses where crops were treated with this ingredient at least two times per harvest period, for six years.

The development of resistance to strobilurins seems to be facilitated due to the high specificity of its mode of action, which results in a high risk for the appearance of resistance genotypes among populations of plant pathogens (Bartlett et al., 2002; Del Puerto Rodríguez et al., 2014; FAO, 2012). In this way, resistance generated by various pathogens was concreted faster than expected. However, the alternate or combined use of strobilurins with other fungicides proved to be a good strategy to reduce risk of resistance (Veloukas et al., 2007).

Therefore, considering the origin of the isolates used in this study, strobirulins resistance could be a factor to investigate since *C. cladosporioides*[a-b] was recently collected from greenhouses crops, where disease management is based primarily on fungicides. Whereas *C. cladosporioides*[c] was reactivated from colony fragments conserved in sterile distilled water dated from year 2007, and has not been exposed to strobilurins for fourteen years, it has shown a better *in vitro* susceptibility. In order to verify this assumption, more studies should be carried out.

CONCLUSION

As the three plant pathogens were identified as *Cladosporium cladosporioides*, it was shown that symptoms found on tomato leaves do not correspond to *P. fulva*. Since it is a different species, it would be important to study if *C. cladosporioides* accompanies *P. fulva* on tomato leaves. Finally, the current discovery provides information about the presence of an infrequent fungus in tomato crops in the region and its fungicide susceptibility, which would be useful for establishing control strategies and enhance production.

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