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Fungicides and alternative products in the mycelial growth and germination control of *Alternaria tomatophila*

Fungicidas y productos alternativos en el crecimiento micelial y en control de la germinación de Alternaria tomatophila

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ABSTRACT

This study aimed to characterize the *in vitro* effect of different products on the mycelial growth and conidial germination of *Alternaria tomatophila*, the etiologic agent of tomato early blight. The experiment was installed using a completely randomized design, in factorial scheme 5x4, with five levels of concentration (0%, 25%, 50%, 75% and 100% of the commercial recommended doses) x 4 products (cymoxanil + mancozeb, copper oxychloride, potassium phosphite 0-28-26 and biofertilizer) in four replicates. The products were added and homogenized to the PDA mean, to evaluate the inhibition of mycelial growth and incorporated in agar-water mean to test inhibition of conidial germination. The fungicide cymoxanil + mancozeb and the potassium phosphite 0-28-26 were the best evaluated products totally inhibiting the mycelial growth; the copper oxychloride was shown to be intermediary while the biofertilizer was less effective. The products behaved in a similar way in the conidial germination. The fungicide cymoxanil + mancozeb and the phosphite stood out, followed by copper oxychloride and the biofertilizer, however, the products did not completely inhibited the conidial germination of *A. tomatophila*.

Key words: early blight, *in vitro* control, mycelial growth, conidia, tomato.

RESUMEN

Este estudio tuvo como objetivo caracterizar el efecto in vitro *de diferentes productos en el crecimiento micelial y en la germinación de conidios de* Alternaria tomatophila*, agente etiológico del tizón temprano del tomate. El experimento se estableció mediante un experimento completamente aleatorizado en un esquema factorial 5x4, con cinco concentraciones (0%, 25%, 50%, 75% y 100% de la dosis comercial recomendada) x 4 productos (cimoxanil + mancozeb, oxicloruro cobre, fosfito de potasio 0-28-26 y biofertilizante) con cuatro repeticiones. Los productos se homogeneizaron y añadieron al medio PDA para evaluar la inhibición del crecimiento micelial y se incorporaron en medio agar-agua para probar la inhibición de la germinación de los conidios. El fungicida mancozeb + cimoxanil y el fosfito de potasio 0-28-26 fueron los mejores productos evaluados inhibiendo totalmente el crecimiento micelial, el oxicloruro de cobre demostró tener un efecto intermedio, mientras el biofertilizante fue menos eficaz. La germinación de conidios tuvo un comportamiento similar frente a los productos evaluados. El fungicida mancozeb + cycomaxil y el fosfito sobresalieron, seguido de oxicloruro de cobre y del biofertilizante; sin embargo, los productos no inhibieron completamente la germinación de esporas de* A. tomatophila*.

Palabras clave: *tizón temprano, control* in vitro*, conidios, tomate.*

Introduction

The tomato (*Solanum lycopersicum* L.) has been highlighted as one of the most widely cultivated vegetable crops in Brazil and worldwide (Marim *et al.*, 2005). Brazil is the biggest producer of this vegetable in Latin America and the state of São Paulo the largest consumer market in MERCOSUR and the Southeast, Midwest and Northeast the main centers of production and productivity (Camargo Filho and Mazzei, 2002; Silva and Giordano, 2000). Even though obtaining an expressive production a

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lot of regions have many difficulties in producing due to the occurrence of pests and diseases affecting the crop creating high.

Among the fungal diseases that affect tomato early blight is one of the most important in that crop and it can attack the plant anywhere and at any age if it find favorable conditions. Although the cause of the disease has been traditionally attributed to the fungus *Alternaria solani* Sorauer (Rotem, 1994), in 2000 was described a new specie, *Alternaria tomatophila* Simmons, whose individuals were commonly associated with early blight of tomato (Simmons, 2000). The symptoms manifest with greater intensity in the leaves, reducing plant vigor due to the high rate of defoliation and on the fruits due to depreciation inflicted by the injury of the pathogen. Therefore causes indirect and direct losses (Vale *et al.*, 2000).

The high destructive potential of the disease allowed the use of fungicides to be a key measure for an effective disease control. Alternatives to chemical control are being studied in order to minimize the problems caused by pesticides. The constant search for alternatives in controlling diseases have proposed the development of substances capable of inducing the plant defense system (Kessmann *et al.*, 1995; Leroux, 1996), or have a direct effect on the structures of the pathogens. The use of organic matter both by soil incorporation as by processing for later use has been made viable (Hoitink and Fahy, 1986; Boehm and Hoitink, 1992). The transformation of organic matter via anaerobic fermentation of manure produces an effluent known as biofertilizer. This product is used in foliar spray or applied to the soil, both for nutritional purposes and for the control of diseases and pests. The control of *Botrytis cinerea*, with aqueous extract from horse cattle and poultry manure's originated compounds, were reported by several authors in beans, lettuce, tomato and pepper (Stindt and Weltzien, 1988; Elad and Shtienberg, 1994; McQuilken *et al.*, 1994).

Another employed control measure has been the use of phosphites in an attempt to practice agriculture with lower pesticide contamination. The phosphites are compounds derived from the neutralization of phosphorous acid (H_3PO_3), by a base that can be sodium hydroxide, potassium hydroxide, ammonium hydroxide, among others, being the potassium hydroxide the most widely used to form potassium phosphite (Reuveni, 1997). These products are being marketed as fertilizers and they have an effect on the control of various diseases, especially fungal. Despite this technology is presented as emergent, using phosphites have been quite effective in controlling pathogens (Araújo *et al.*, 2010; Nojosa *et al.*, 2009).

The aim of this study was to evaluate the *in vitro* effect of fungicides (cymoxail + mancozeb and copper oxychloride) and products considered alternatives in controlling diseases (potassium phosphite 0-28-26 and biofertilizer) on mycelial growth and conidial germination of *Alternaria tomatophila*.

Materials and Methods

The experiments were performed at the Laboratory of Plant Pathology of the Instituto de Ciências Agrárias of the Universidade Federal de Minas Gerais, in Montes Claros, Minas Gerais State, Brazil. The fungicides were cymoxanil + mancozeb, copper oxychloride, potassium phosphite (0-28-26) and liquid manure biofertilizer. The antifungal activity was evaluated by the fungistatic and fungitoxic effect of the products, by the action on mycelial growth and by the action on the conidia germination of *A. tomatophila*. The monosporic's isolated and the pure cultures of *A. tomatophila* were obtained from characteristic lesions of early blight on tomato fruits purchased in the region (Alfenas and Mafia, 2007).

For the mycelial growth an evaluation of the experiment was performed using a completely randomized in a 5x4 factorial scheme, with five concentrations (0%, 25%, 50%, 75% and 100% of the comercial dose of each product) versus 4 products (cymoxanil + mancozeb, copper oxychloride, potassium phosphite 0-28-26 and liquid manure biofertilizer), with 4 replicates, being each petri dish considered a repeat.

The biofertilizer was prepared from the fermentation of fresh manure in an anaerobic system for 40 days. The manure was mixed in equal parts with not chlorinated pure water and placed in a plastic container with a capacity of 200 L, containing a hose attached to the lid to release the methane produced. The dose of biofertilizer was established according to the recommendation proposed by Penteado (2007). This was autoclaved at 121 °C and 1 atm for 20 min prior to addition to the culture medium.

The products were homogenized and added to 100 ml of PDA melting medium, according to

the concentration to be tested. Then was poured an amount of 20 mL of each prepared medium in a petri dish with 9 cm diameter. All plates were inoculated at the center with a mycelial disc of 5 mm diameter with a 7 days old monosporic culture. Funguses grown on PDA without any additions were considered as the only control. The whole procedure was performed under aseptic conditions in a laminar flow hood. The Petri dishes were incubated at 25 °C under a photoperiod of 12 hours.

To evaluate the effect of the product`s concentrations the diameter of the colonies in two orthogonal axes was measured daily (average of two measurements diametrically opposed), starting 24 hours after the inoculation of fungi and for a period of 7 days obtaining the mycelial mean growth per plate (Benício *et al.*, 2003).

The percentage of growth inhibition (PIC) of fungal colonies was determined for each product compared to the control, where:

$$PIC = \left(\frac{\text{øcontrol - øtreatment}}{\text{øcontrol}} \right) x100$$

PIC = percentage of growth inhibition, Øcontrol = diameter control, Øtreatment = diameter treatment.

To evaluate the effect of the products on spore germination, conidia suspension of *A. tomatophila* was placed on water-agar medium containing different products. For that was obtained a pure colony grown for 7 days on V8-agar medium a spore suspension of *A. tomatophila* at a concentration of 2 x 10^5 conidia/mL.

To the doses of each respective product were added 100 mL of water-agar (20%). After solidification culture medium blocks were made with a surface area of 2 cm^2 and a thickness of 1 cm. Then the blocks were deposited onto a sterilized microscope slides. After that aliquots of 30 µL of conidial concentration were placed on the medium and covered with a coverslip. After mounting, the slides were kept in 14 cm diameter glass plates containing two sheets moistened filter paper with sterile distilled water. The set was stored in incubation chambers BOD at 25 °C with a photoperiod of 12 hours. The study also was conducted in a completely randomized 5x4 factorial scheme, similar to the experimental evaluation of mycelial growth, with four replications, each repetition being considered microscope slide with an agar block.

After 18 hour's incubation, 20 µL of lactophenol and cotton blue were added to inhibit the germination of conidia and facilitate viewing by optical microscopy. Conidial germination was assessed in two viewing fields of (40X objective) and randomly selected by analyzing 100 conidia per field. Those showing any emission germ tube were considered germinated conidia, regardless of its length (Silva *et al.*, 2009; Tavares and Souza, 2005). The percent inhibition of conidial germination (PIG) was then calculated, where:

$$PIG = \left(\frac{\text{nº control conidia - nº treatment conidia}}{\text{nº control conidia}} \right) x100$$

PIG =Percent inhibition of conidial germination, nº control conidia= control number of conidia, nº control treatment= treatment number of conidia.

The data from both trials were subjected to variance analysis, and the means of qualitative data were compared by the Scott-Knott test ($p \leq 0.01$) and quantitative data submitted to polynomial regression ($p \leq 0.01$).

Results and Discussion

The products showed differentiated behavior, regarding the following criteria: percentage of mycelial growth inhibition (PIC) and percentage inhibition of conidia germination (PIG). There was significant interaction between the products and the tested concentrations and the polynomial models best suited to describe the behavior of PIC and PIG variables. The fungicides mancozeb + cymoxanil and potassium phosphite were those that promoted the highest percentage of inhibition. Copper oxychloride was shown to be intermediate, while the biofertilizer was the less effective in the in vitro control of *A. tomatophila* (Tables 1 and 2, Figures 1 and 2).

A significant increase in PIC is observed with increasing concentrations of the tested products, except for the biofertilizer (Figure 1). The fungicide mancozeb + cymoxanil was the product that most inhibited mycelial growth of *Alternaria tomatophila* and already at a dose of 25% of the active ingredient ceased in vitro development of the pathogen, followed by phosphite and copper oxychloride. The cymoxanil + mancozeb were recommended only for pathogens such as *Phytophthora infestans* and mildews. In the followed doses the phosphite was

Table 1. Mycelial growth inhibition (%) of *Alternaria tomatophila* grown on PDA medium for different products and concentrations. Montes Claros, 2010.

Product	Concentration (%)				
	0	25	50	75	100
Cymoxanil+mancozeb	0.0 A	93.0 A	100.0 A	100.0 A	95.0 A
Phosphite	0.0 A	84.7 B	99.0 A	100.0 A	99.0 A
Copper oxychloride	0.0 A	67.7 C	80.0 B	83.5 B	83.0 B
Biofertilizer	0.0 A	2.0 D	3.2 D	5.2 C	8.3 C
CV (%)			5.55		

* Means followed by the same letter in the column do not differ by Scott-Knott test at 1% probability.

statistically equal to cymoxanil + mancozeb and these were superior and different from the other products. Copper oxychloride at a dosage of 100% of the active ingredient inhibited 83% growth, while the biofertilizer was less efficient inhibiting only 8% of the mycelial growth of *A. tomatophila* at its higher concentration.

Products with systemic and protective action such as the fungicide mancozeb + cymoxanil have a higher fungistatic, fungitoxic action and high specificity instead to copper oxychloride. Systemic fungicides action as tebuconazole, difenoconazole, iprodione and fluazinam had superior efficiency in vitro control of *A. solani*, inhibiting more than 80% from 1 µg/mL and a 100% of mycelial growth at a dose of 100µg/mL when compared to products with contact action (Tofoli *et al.*, 2003). That author also states that the fungicides mancozeb and chlorothalonil with a contact action, similar to the action of copper oxychloride, had an intermediate inhibition level, mainly due to its inherent low fungitoxicity.

The action of phosphites has also been observed in other pathosystems. Araújo *et al.* (2010), demonstrated their direct action on mycelial growth of *Colletotrichum gloeosporioides*, where a dose of 1.5 µL/mL inhibited significantly the diameter of the colony and reduced the rate of mycelial growth rates.

Similar results have been observed with the use of systemic fungicides and potassium phosphite 0-28-26 in control of plant pathogens in vitro (Araújo *et al.*, 2010; Batista *et al.*, 2002; Tofoli *et al.*, 2003). The use of fungicides and phosphites was also highlighted in the control of postharvest rottenness caused by *Penicillium* spp., not allowing the growth of mycelium in apple fruits (Brackmann *et al.*, 2005). The potassium phosphite also had a direct effect on mycelial growth of *Venturia inaequalis*, promoting, using the commercial dose, a reduction of 68.4% (Boneti and Katsurayama, 2005).

Copper oxychloride was less effective in inhibiting the mycelial growth of early blight,

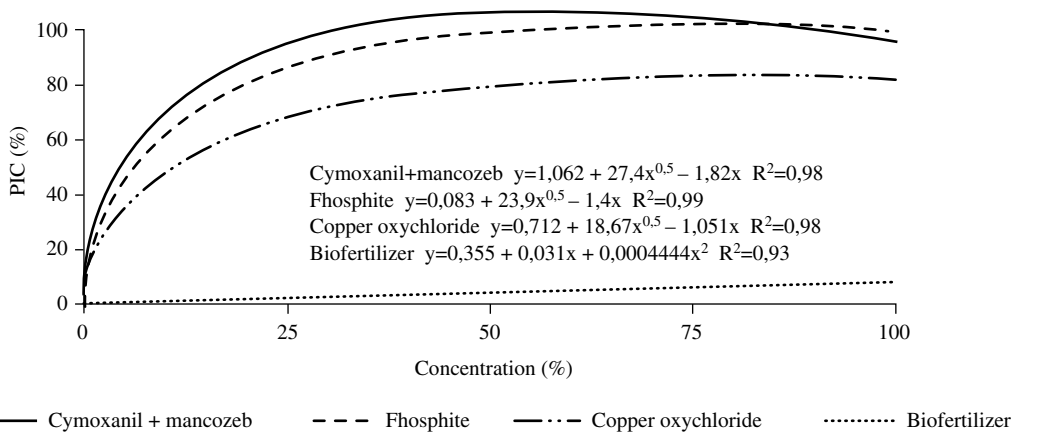

Figure 1. Mycelial growth inhibition (PIC) for different products and concentrations in *in vitro* control of *Alternaria tomatophila*. Montes Claros, 2010.

diverging from the results presented by Brignani Neto and Oliveira (1980), which indicate the high potential of this product in the inhibitory control of *A. solani*. Tavares and Souza (2005), evidenced the low efficacy of copper oxychloride on the mycelial growth of *Colletotrichum gloeosporioides*.

The biofertilizer used in this review did not inhibit mycelial growth of early blight, but there are several examples in the literature demonstrating its effect on pathogenic fungi. Tratch and Bettiol (1997), evidenced that doses above 10% of biofertilizer supermagro inhibited completely the mycelial growth of various pathogens, including *A. solani.* The action of biofertilizer originated from anaerobic fermentation of cattle manure was also reported by Carmo and Côrrea (2006) on *Colletotrichum gloeosporioides* and *Uromyces appendiculatus*. McQuilken *et al.* (1994), using an aqueous extract of compost's horse manure and poultry verified the inhibition of mycelial growth and conidial germination of *B. cinerea*, at all ages of extract's extraction.

The aqueous extract of pig manure provided greater inhibition of mycelial growth of *Pythium*, *Phytophthora*, *Fusarium* and *Sclerotium* (Nakasone *et al.*, 1999). At doses above 30%, the extract inhibited the growth of *Pythium* on a 100%; On the concentration of 40% inhibited the development of *Phytophthora* by 50%, at all tested doses, there was inhibition of *Sclerotium,* around 50% and *Fusarium* less than 50% (Nakasone *et al.*, 1999).

In this study, the inhibitory effect of the biofertilizer may have declined with autoclaving. Some researches claim that the loss of inhibitory capacity may occur in some cases (Elad and Shtienberg, 1994). In accordance with Hoitink and Fahy (1986) and Hoitink *et al.* (1997), at temperatures above 60 °C, there is loss of suppressiveness of organic compounds to *Pythium* spp. and *Fusarium* spp., besides the elimination of beneficial microorganisms. Visconti *et al.* (2010), found that aqueous extract of autoclaved organic matter (aqueous extract of bovine manure) did not control the mycelial growth of *Cylindrocladium sphathiphylli* but an opposite effect occurred stimulating its development. Similar data were observed in this study showing that the biofertilizer of bovine manure was not able to control the *in vitro* growth of *Alternaria tomatophila* and also contributed to its development.

The percentage of inhibition of conidial germination (PIG) of *A. tomatophila* is shown in Table 2 and Figure 2. The effects of the different products on the germination of conidia were similar to those seen in PIC. The cymoxanil + mancozeb and potassium phosphite were the ones which most inhibited germination of conidia, however were statistically different at all doses above 25%. Copper oxychloride had an intermediate action, while the biofertilizer was the least effective.

The fungicide mancozeb + cymoxanil at a concentration of 25% inhibited the PIG at 88,5% and in the concentrations above 25% more than 92% of the germination. The phosphite, when used at its highest concentration (100%), inhibited at 76% the germination of conidia. Copper oxychloride at a concentration of 100% inhibited 32% of the conidia. The biofertilizer had the highest inhibition of conidia germination at concentration of 25%.

The cymoxanil + mancozeb showed high levels of inhibition of germination of conidia of *A. tomatophila*. Tofoli *et al.* (2003), evaluating the fungicide tebuconazole, difenoconazole, fluazinam, iprodione, among others, found that concentrations above 1µg/mL inhibited completely the germination of conidia of that fungus. Copper oxychloride showed intermediate levels of inhibition, and increased as the concentration was increased. Contact fungicides, chlorothalonil and mancozeb, evaluated by Tofoli

Table 2. Inhibition of conidial germination (%) of *Alternaria tomatophila* for different products and concentrations. Montes Claros, 2010.

Product	Concentration (%)				
	0	25	50	75	100
Cymoxanil+mancozeb	3.50 A	88.50 A	99.50 A	98.90 A	92.10 A
Phosphite	3.50 A	54.84 B	67.25 B	73.35 B	76.15 B
Copper oxychloride	3.50 A	9.10 C	16.75 C	24.10 C	32.00 C
Biofertilizer	3.50 A	9.30 C	8.50 D	6.60 D	4.10 D
CV (%)			11.83		

* Means followed by the same letter in the column do not differ by Scott-Knott test at 1% probability.

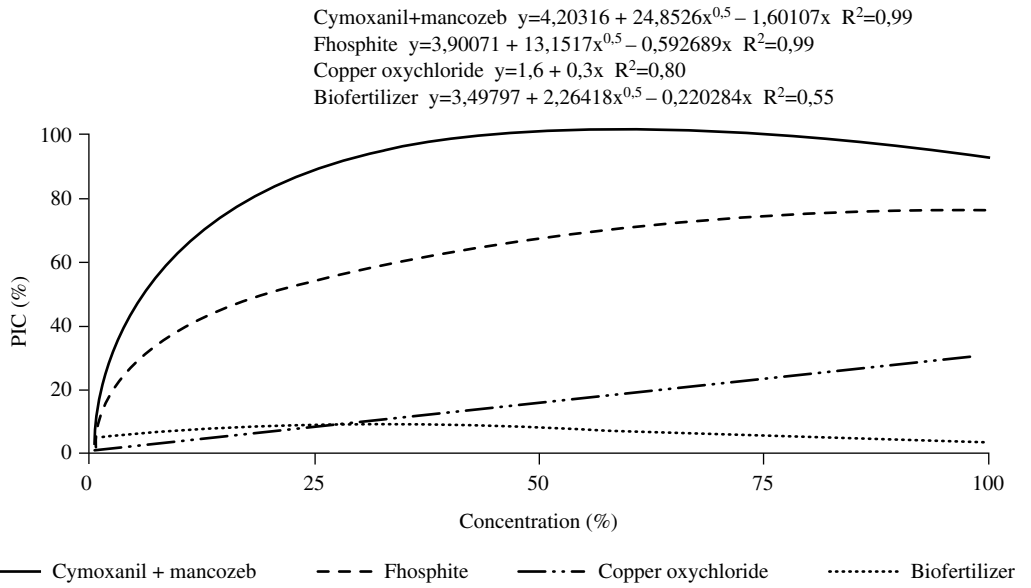

Figure 2. Inhibition percentage of conidial germination (PIG) due to different products in different concentrations in the *in vitro* control of *Alternaria tomatophila*. Montes Claros, 2010.

et al. (2003) were intermediate, showing percentages of inhibition of 45-56% of conidial germination.

When the copper oxychloride was evaluated on the conidia germination effect of *Colletotrichum gloeosporioides* that fungicide performed better in concentration of 1 ppm with only 10% of germinated conidia, while systemic action fungicide as azoxystrobin, chlorothalonil, imazalil, propiconazil, tebuconazole, among others, were effective at concentrations above 50 ppm (Tavares and Souza, 2005).

Ribeiro Júnior *et al.* (2006), testing the direct effect of doses (0.62; 1.25; 2.5 and 5 mL/L) of potassium phosphite 0-27-27 on conidial germination of *Verticillium dahliae*, found that all doses showed some toxic effect on conidial germination of *Verticillium*. A work done by Nojosa *et al.* (2009) with *Phoma costarricensis* showed that potassium phosphite reduced the germ tube length by 32% at doses from 1.5 to 10 mL/L.

Tratch and Bettiol (1997) found a reduction in the percentage of conidia of *A. solani*, with an increasing concentration of the biofertilizer supermagro. At the concentration of 2.5%, reduced by approximately 50% the germination of the conidia in relation to the control and, from 10% the inhibition reached almost 100% of the germination.

It was verified a higher germination of conidia of *Botrytis cinerea* when using the biofertilizer supermagro at concentrations of 2.5 and 5%, however, at higher concentrations its total inhibition occurred. In the same work, the autoclaved biofertilizer supermagro proved to be less effective at concentrations of 0.01 to 0.5% and had effective control over the germination of urediniospores of *Hemileia vastatrix* and *Coelosporium plumierae* in doses of 1 and 5% (Tratch and Bettiol, 1997). In this research, the autoclaved biofertilizer had no effect on the inhibition of conidia of *Alternaria tomatophila.*

Conclusions

The active ingredients mancozeb + cymoxanil and potassium phosphite 0-28-26 were effective in inhibiting the mycelial growth and conidial germination of *Alternaria tomatophila*. The lowest concentrations of these products, 25 and 50%, allowed total inhibition of the growth of *A. tomatophila*, while for conidial inhibition higher concentrations were required.

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