

# The *in vitro* evaluation of the antifungal potential of natural *Capsicum annuum* L. extract and synthetic compounds against the pathogens *Alternaria spp.* and *Fusarium oxysporum*.

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## ABSTRACT

In the last decade, interest in organic agriculture has been steadily gaining ground, leading to studies on the control and management of phytopathogens using plant extracts and essential oils. This study aimed to evaluate the *in vitro* antifungal potential of the natural extract obtained from the *Capsicum annuum* L. species, as well as synthetic compounds, as a control method against the pathogenic fungi *Alternaria spp.* and *Fusarium oxysporum*. The natural extract was used in four different concentrations (0.5%, 2%, 9%, and 12%) and was characterized in terms of total polyphenol content (TPC) using the Folin-Ciocalteu spectrophotometric method, as well as its antioxidant activity using the DPPH radical scavenging method. The results of this study, in which *Capsicum annuum* L. extract was used, demonstrated high efficacy, showing an inhibitory percentage of 33.33% against the pathogen *Alternaria spp.*, and 21.90% against the pathogen *Fusarium oxysporum*. Regarding the efficacy of synthetic products, it can be said that the mancozeb-based product at a concentration of 0.2% exhibited a strong antifungal effect on both pathogens, with an inhibition percentage of 100%.

**Keywords:** bioactive compounds, capsaicin, phytopathogens, *Capsicum annuum* L, *in vitro*

## INTRODUCTION

Currently, synthetic pesticides are the most effective method for controlling and combating fungal pathogens (Amoako Ofori *et al.*, 2022); however, repeated use of these substances can lead to resistance, as well as the accumulation of their residues in soil and water (Gevao *et al.*, 2000; Garcia *et al.*, 2018).

Organic farming has become increasingly popular, generating a growing interest in studies related to the control and management of phytoparasites using natural extracts and essential plant oils (Tomazoni *et al.*, 2018). However, the current progress of organic

agriculture is insufficient due to two main aspects: the lack of research and evaluation in the various functions of agro-ecosystem services, as well as the difficulty in achieving significant economic benefits due to the lack of market-oriented approaches, large-scale management, specialized production, and marketing strategies (Li *et al.*, 2011). Research on plant compounds with bioactive properties against phytopathogens has garnered increasing interest, as biopesticides represent eco-friendly, sustainable, and potentially less toxic alternatives to synthetic fungicides (Vuerich *et al.*, 2023).

The *Capsicum* L. genus (family *Solanaceae*, subfamily *Solanoideae*) originates from South America (Barboza and Bianchetti, 2004), but both Mexico and Central America are also considered diversity centers for this taxon (Barboza, 2011). *Capsicum annum* L. is one of the cultivated species that exhibits a wide range of cultivars with economic, medical, and industrial importance, being used for food, capsaicinoids, carotenoids, and antioxidants (Olatunji and Afolayan, 2018). This genus includes around 32 species native to the Americas, five of which have been domesticated for consumption: *C. annum* L., *C. frutescens* L., *C. baccatum* L., *C. pubescens* Ruiz & Pav., and *C. chinense* Jacq. (Martínez-Ávalos *et al.*, 2018).

All plants in the *Capsicum* genus produce varying amounts of capsaicin. Additionally, this compound provides a unique characteristic and spicy flavor to each cultivar (Morrine *et al.*, 2018).

*In vitro* tests have shown that capsaicin extract obtained from *Capsicum spp.* can be used as a preventive method against the pathogenic fungus *Penicillium expansum*, preventing colony development during the 14-day monitoring period (Fieira *et al.*, 2013). According to a study by Xing *et al.* (2006), capsaicin (8-methyl-vanillyl-6-nonenamide) demonstrated antifungal efficacy in tests against the pathogenic fungus *Botrytis cinerea*. Additionally, Buitimea-Cantua *et al.* (2018) demonstrated that *Capsicum* extract exhibited a 50% inhibitory effect on the phytopathogen *Aspergillus parasiticus*. Previous research has demonstrated that natural extracts obtained from *Capsicum spp.*, in addition to being used as biopesticides against insects (Koleva-Gudeva *et al.*, 2013), have also been effective against plant pathogens, showing post-harvest antifungal action against the pathogenic fungi *Alternaria alternata*, *Fusarium oxysporum*, *Verticillium dahliae*, *Rhizoctonia solani*, *Sclerotinia minor*, and *Phytophthora capsici* (Tewksbury *et al.*, 2008; Koleva-Gudeva *et al.*, 2013; Veloso *et al.*, 2014; Rodriguez-Maturino *et al.*, 2015; Pane *et al.*, 2015).

The aim of this study was to test the efficacy of the natural extract obtained from *Capsicum annum* L., as well as synthetic fungicides, in evaluating antifungal activity against the hyphal growth of two pathogens, namely *Alternaria spp.* and *Fusarium oxysporum*.

## **MATERIALS AND METHODS**

The conventional extraction technique was used to obtain the natural extract from *Capsicum annum* L.. The pericarp and seeds were dried and then ground for 3 minutes. The solvent used was made from a binary mixture of distilled water and ethyl alcohol in a 50:50 v/v ratio. The extraction was carried out by immersing 20g of plant powder in 200 ml of solvent and extracting for 180 minutes at a temperature of 70°C. After cooling, the extract was filtered to remove solid particles and stored at +4°C until further analysis and testing. The natural extract was used in four different concentrations (0.5%, 2%, 9%, and 12%)

### ***Antifungal Activity***

The pathogens *Fusarium oxysporum* and *Alternaria spp.* were isolated by taking mycelial fragments and asexual fruiting bodies from the collar and infected tomato fruits, then cultivated on Potato-dextrose agar (PDA) medium. Inoculation was carried out using a seeding needle, which was sterilized beforehand by “red heat.” Observations regarding the

growth and development of the pathogen colonies were made at regular intervals: 3, 5, 7, and 10 days post-inoculation. After the colonies developed, about 7 days after seeding, they were replated to evaluate the antifungal potential of the natural extract obtained from *Capsicum annuum* L., as well as two synthetic products based on mancozeb (Dithane M45) and azoxystrobin (Amistar).

Thus, sterilized PDA medium was distributed in each Petri dish, to which synthetic pesticides were added at a concentration of 0.2%, along with the natural extract in four different concentrations: 0.5%, 2%, 9%, and 12%. For an accurate interpretation of the results, three repetitions were performed for each experimental variant. The control variant for the two phytopathogens consisted of PDA medium without the addition of natural extract or fungicides.

The colony diameter was measured in two directions, and the average value was recorded at 3, 5, 7, and 10 days. The percentage of mycelial growth inhibition was calculated using Vincent's formula (Vincent, 1947), where  $I$  = Percent inhibition,  $C$  = Radial growth in control,  $C$  = Radial growth in control, and  $T$  = Radial growth in treatment (fungicide).

$$I = \frac{C - T}{C} \times 100$$

#### **Total Polyphenol Content (TPC)**

The total polyphenol content (TPC) was measured using the Folin-Ciocalteu spectrophotometric method (ISO 14502-1). In this method, the Folin-Ciocalteu reagent is reduced by the phenolic compounds in the extract, forming a blue-colored complex. The experimental variants were prepared by taking different volumes of the extract, diluting them, and then adding 1 mL of the Folin-Ciocalteu reagent (diluted 10 times) to 200  $\mu$ L of each dilution. After 5-6 minutes, 800  $\mu$ L of a 7.5% Na<sub>2</sub>CO<sub>3</sub> solution was added.

The samples were kept in the dark at room temperature for 60 minutes and analyzed spectrophotometrically at 765 nm. The results were reported as mg gallic acid/g plant (mg GA/g) based on a calibration curve created using concentrations of the standard, with 7 concentration points ranging from 10 to 70  $\mu$ g/mL of gallic acid.

#### **Determination of Antioxidant Capacity (DPPH)**

The antioxidant capacity of the natural extract was evaluated using the DPPH radical scavenging method (2,2-diphenyl-1-picrylhydrazyl), according to the protocol developed by Shimamura *et al.* (2014), with subsequent adjustments. To highlight the antioxidant reaction of the control (Trolox in this case) and the experimental variants, a DPPH solution and the standard/extract were added to a test tube in a volumetric ratio of 1:7. The absorbance of each sample was measured at a wavelength of 517 nm, after a stabilization period of 5 minutes under UV light, followed by plotting the calibration curve for Trolox. The Trolox calibration curve was generated for 6 different concentrations of the reagent, and the antioxidant capacity of each sample was expressed as TEAC (Trolox Equivalent Antioxidant Capacity), Where:  $IC_{50}$  Trolox = half the maximum concentration of Trolox inhibitors and  $IC_{50}$  Extract = half of the maximum inhibitory concentration of Extract using the formula:

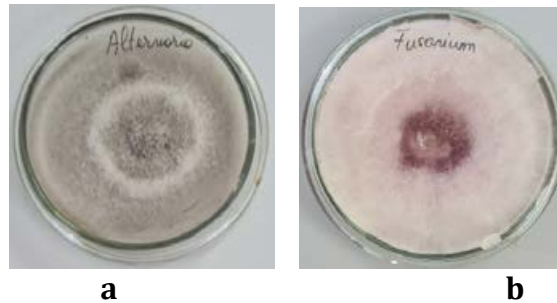
$$TEAC = \frac{IC_{50} Trolox}{IC_{50} Extract}$$

All analyzed data were processed using one-way ANOVA, followed by Šídák's multiple comparisons test. Processed data were expressed as mean  $\pm$  standard error (SE). This analysis was performed using GraphPad Prism 9.0.0.0 software.

## RESULTS AND DISCUSSIONS

### ***Dynamics of mycelial hyphae growth on culture medium***

*Alternaria* spp. and *Fusarium oxysporum* were isolated on PDA medium (Figure 1) being suitable for the cultivation and replication of the two pathogens (Din *et al.*, 2022). The dynamics of mycelial growth was fast, with a radial expansion from the point of inoculation observed. At 3 days after inoculation, the diameter of the colonies for *Alternaria* spp. was on average 1.6/1.7 cm, reaching 7/7 cm at 10 days. In contrast, *Fusarium oxysporum* showed a more accelerated growth, with an average colony diameter of 3/3.1 cm at 3 days and 7/7 cm at 5 days after inoculation.

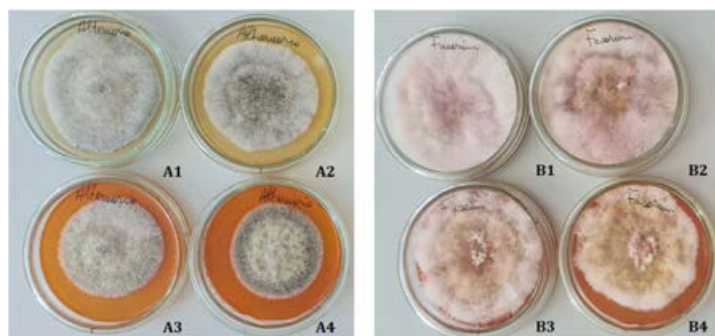


**Figure 1.** *Alternaria* spp. (a) and *Fusarium oxysporum* (b) on PDA plate

### ***Antifungal Activity of the Natural Extract of Capsicum annuum L. and Synthetic Products***

Unlike synthetic products, it can be stated that the natural extract obtained from the *Capsicum annuum* L. species exhibited fungistatic effects directly proportional to the concentration used for both pathogens. To establish a notable inhibitory percentage, four different concentrations of the natural extract were set: 0.5%, 2%, 9%, and 12%.

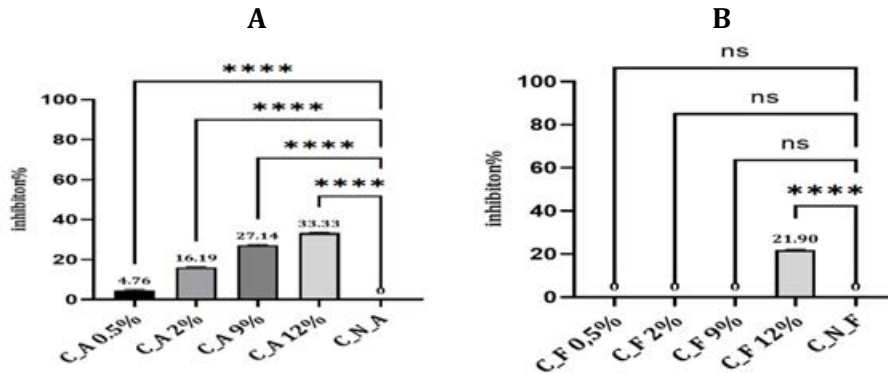
Figure 2 shows the fungistatic action of the natural extract obtained from *Capsicum annuum* L. on the mycelial hyphae of the pathogens *Alternaria* spp. and *Fusarium oxysporum*.



**Figure 2.** Growth of *Alternaria* spp. and *Fusarium oxysporum* isolate on PDA amended with ethyl extract of *Capsicum annuum* L. (*Alternaria* spp.: A1=0,5%; A2=2%; A3=9%; A4=12% and *Fusarium oxysporum*: B1=0,5%; B2=2%; B3=9%; B4=12%).

The statistical analysis of the results after 10 days is illustrated in Figure 3, where a 33.33% inhibition rate of the mycelial growth of the pathogen *Alternaria* spp. is observed at the maximum concentration used in our experiments (12%), compared to the pathogen *Fusarium oxysporum*, which showed an inhibition rate of 21.90% at the same concentration of the natural extract.

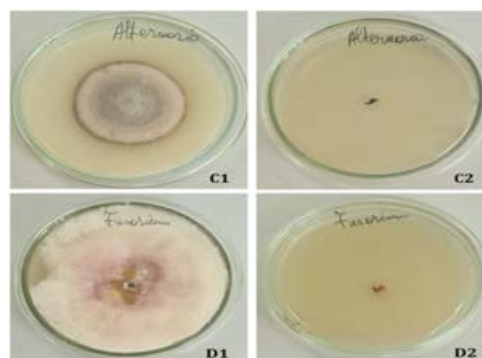
At the concentration of 0.5% for the growth and development of *Alternaria spp.*, the inhibition percentage was 4.76%; at 2%, it was 16.19%; and at 9%, the inhibition percentage was 21.14%. Regarding the growth and development of the pathogen *Fusarium oxysporum* at concentrations of 0.5%, 2%, and 9%, the inhibition percentage after 10 days of monitoring was 0%, which was equal to that of the control variant.



**Figure 3.** Effect of concentrations of the three types of extracts on the pathogenic fungus *Alternaria spp.* (A) and *Fusarium oxysporum* (B). The data are expressed as the mean  $\pm$  SD values of three independent experiments performed in triplicate, and the values of p were calculated by the one-way ANOVA method followed by Šídák's multiple comparison test \*\*\*\*p < 0.0001; ns p > 0.9999 (C... - *Capsicum annuum* L extracts A... - *Alternaria spp.*; F... - *Fusarium oxysporum*, C\_N - negative control).

Prasad *et al.* (2018) studied the effect of the extract obtained from the *Capsicum annuum* L. species against the pathogen *Fusarium oxysporum*, and the results showed a maximum inhibitory effect of 75.7%. Additionally, López-Muñoz *et al.* (2019) demonstrated that the habanero pepper extract (*Capsicum chinense* var. *Habanero*) is effective for controlling *Cochliobolus spp.*, with an inhibition percentage of 82.29%, as well as against the pathogen *Fusarium andiyazi*, with an inhibition percentage of 55.61%.

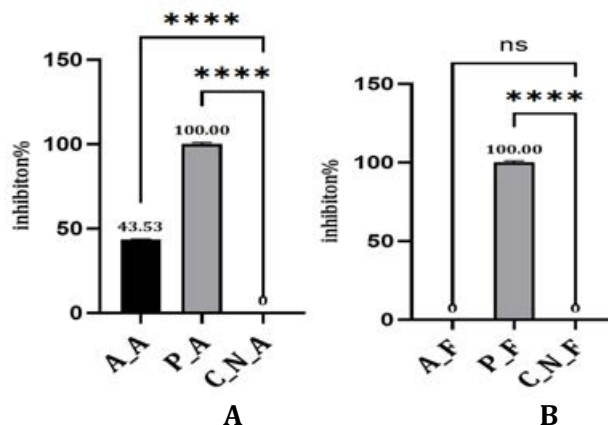
The sensitivity of these two pathogens to the natural extract of *Capsicum annuum* L. was compared to their sensitivity to synthetic pesticides, using active substances azoxystrobin and mancozeb (Figure 4). The concentrations used for the two synthetic pesticides were 0.2%, according to the manufacturer's instructions.



**Figure 4.** Antifungal effect of fungicides *in vitro* *Alternaria spp.*=series C (C1=azoxistrobin 0,2%; C2=mancozeb 0,2%) and *Fusarium oxysporum* series D (D1=azoxistrobin 0,2%; D2=mancozeb 0,2%).

*In vitro* experiments using synthetic products showed that the mancozeb-based product demonstrated an inhibition capacity of 100% for both pathogens, proving very effective in combating and controlling these pests in tomato crops. Regarding the azoxystrobin-based synthetic product, the results were different: for the pathogen *Alternaria spp.*, the inhibition percentage was 43.53%, while for the pathogen *Fusarium oxysporum*, the effects

were not visible, with the inhibition percentage of the fungal colony equal to that of the control variant (0%) (Figure 5).



**Figure 5.** The effect of synthetic pesticides on the pathogenic fungus *Alternaria spp.*(A) and *Fusarium oxysporum* (F). The data are expressed as the mean  $\pm$  SD values of three independent experiments performed in triplicate, and the values of p were calculated by the one-way ANOVA method followed by Šídák's multiple comparison test \*\*\*\*p < 0.0001 (A- azoxistrobin, P - mancozeb, A-*Alternaria spp.*, F-*Fusarium oxysporum*, C\_N- negative control)

Therefore, it was highlighted that the natural extract obtained from the *Capsicum annuum* L. species possesses authentic antifungal qualities that can be exploited industrially in the agricultural sector, representing a sustainable alternative to synthetic pesticides, as the raw material acquisition and production process do not involve high costs.

### Analysis of Polyphenols and Antioxidant Capacity

The total polyphenol content (TPC) of the natural extract obtained from *Capsicum annuum* L. showed a value of  $70.58 \pm 0.46$  mg/ml GAE, while the antioxidant capacity (DPPH) was  $0.0134 \pm 0.0040$ , which was correlated with the lowest value of the half-maximal inhibitory concentration (IC<sub>50</sub>), specifically 2834.6  $\mu$ g/mL (Table 1).

**Table 1.** The total polyphenol content and antioxidant capacity of the *Capsicum annuum* L. extract.

Plant extract	TPC (mg/ml GAE)	IC <sub>50</sub> ( $\mu$ g/mL) (DPPH)	TEAC (DPPH)
<i>Capsicum annuum</i> L.	$70.58 \pm 0.46$	2834.6	$0.0134 \pm 0.0040$

Salamatullah *et al.* (2022) used different cultivars of the *Capsicum annuum* L. species (green, yellow, and red peppers) to obtain natural extracts. The analyses showed that the total polyphenol content varied, with values ranging from 30.15 mg GAE/g for the green pepper cultivar, followed by 28.73 mg GAE/g for the red pepper, and 27.68 mg GAE/g for the yellow pepper.

The same study also determined the antioxidant capacity, which ranged from 72.76% (green pepper) to 70.26% (red and yellow peppers). Additionally, Kavuncuoğlu *et al.* (2021) reported a high polyphenol content of 515.85 mg GAE/g for the yellow pepper extract and 362.48 mg GAE/g for the *Charleston pepper* extract.

## CONCLUSIONS

The overall effectiveness assessment of a plant protection product involves analyzing several aspects, including the duration of its biological activity (persistence of action), compatibility with various protection strategies or cultural practices, and ease of application. To evaluate the *in vitro* antifungal activity of the natural extract obtained from *Capsicum annuum* L. against the pathogens *Alternaria spp.* and *Fusarium oxysporum*, four concentrations were used: 0.5%, 2%, 9%, and 12%, which were incorporated into PDA culture media. The highest inhibition rate for the pathogen *Alternaria spp.* was observed at a concentration of 12%, where the inhibition percentage was 33.33%. For the pathogen *Fusarium oxysporum*, the inhibition percentage was 21.90% at the maximum concentration used. Synthetic fungicides demonstrated good antifungal activity against the studied pathogens. The mancozeb-based synthetic product achieved a 100% inhibition rate for both studied pathogens.

Thus, our experimental results suggest that the extract from *Capsicum annuum* L. could be utilized as a control method in managing the phytopathogenic fungus *Alternaria spp.*

## ACKNOWLEDGEMENTS

This research paper would not have been possible without the exceptional support of the Regional Center of Research & Development for Materials, Processes and Innovative Products Dedicated to the Automotive Industry (CRC&D-AUTO), (National University of Science and Technology POLITEHNICA Bucharest, Pitesti University Centre).

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