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Influence of Environmental Factors on the Antagonistic Effects of *Aspergillus* Species on Phytopathogenic *Alternaria alternata*

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ABSTRACT

The control of phytopathogens such as *Alternaria alternata* in plants, including tomato by chemical method has sometimes yielded satisfactory results. However, the excessive use of chemical fungicides has contributed to the development of resistant strains. Biocontrol is considered a promising alternative by the integration of microbiological agents. Environmental factors have been observed to influence the antagonistic efficacy of biocontrol microorganisms. This study was aimed at determining the influence of different environmental conditions on the antagonistic efficacy of *Aspergillus* sp. against *Alternaria alternata*. From infected tomato fruits, *A. alternata* was isolated and identified using morphological characterization. A total of seventy-three fungal isolates from a collection of 254 isolates were screened for antagonistic activity against *A. alternata* using the dual culture technique. Antifungal activity of *Aspergillus* sp. against *A. alternata* was evaluated using a dual culture technique. Also, the effects of temperature, culture media and pH on the cultures of the antifungal activity assay were investigated. Data on percentage and zone of inhibition, as well as radial mycelial growth, were collected. The results showed a difference in the mycelial growth of *A. alternata* during *in vitro* interaction with *Aspergillus* sp. The antifungal potential was significantly higher in malt extract agar medium (66.6%) after seven days of incubation. *A. alternata* was inhibited with a large zone of inhibition at 28°C (63%). The optimum antifungal activity of *Aspergillus* sp. was achieved at pH 5. This study demonstrated that *Aspergillus* sp. is an effective and promising biocontrol agent against *A. alternata* under different environmental conditions.

Keywords: *Alternaria alternate*, Antagonism, *Aspergillus* sp., Biocontrol, Environmental factors.

Introduction

Natural products have been a prolific source and an inspiration for numerous medicinal agents with widely divergent chemical structures and biological activities.[1] The resistance of fungi to fungicides is one of the topical issues and is increasingly of research interest.[2] Microbial biocontrol strategy of plant diseases has been considered as a potential tool in recent years because chemical control by the use of synthetic fungicides results in the accumulation of harmful chemical residues, which may lead to serious ecological problems.[3] Biocontrol is environmentally safe and in some cases is the only option available to protect plants against pathogens.[4] The biological control agents with antifungal activity include oils, phytohormones, antifungal proteins, and antifungal peptides.[5] In the biocontrol of plant diseases, the focus is usually on the augmented introduction of antagonists to control diseases.[6] Sources of biocontrol agents (BCAs) are microorganisms such as fungal and bacterial strains.[7-10] There are a variety of mechanisms of antagonistic microorganisms; nutritional and spatial competition, parasitism as well as secretion of bioactive substances, which is the most important mechanism.[11]

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Fruits and vegetables represent a crucial part of the resources contributing to human nutrition, with a relevant impact on human health.[12] Tomato (*Solanum lycopersicum* Mill) is one of the most cultivated culinary vegetables in the world.[13] It has curative properties and high nutritive value.[14,15] However, it is affected by fungal diseases such as downy mildew and gray mold which causes serious problems on the yield and quality of the tomato crop.[16-18] The colonization of undesired microorganisms in pre-harvest can affect the postharvest quality, influencing crop production, yield, and storage.[19,12] In the concept of researching new antifungal molecules, and production optimization for filamentous fungi, the most common antifungal detection methods include the confrontation "dual cultures" in solid media of two filamentous fungal strains,[20] or the use of fungal extracts to be tested for antibiosis against other filamentous strains of some microorganisms, are mostly used for testing fungal strains.[21] To the best of our knowledge, there are no studies or reports of the use of *Aspergillus* species as a biological antagonist agent in controlling tomato fungal pathogens.

This research was therefore conducted to investigate the influence of some environmental factors (incubation conditions, media pH, and media composition) on the antagonistic effect of *Aspergillus* species on *Alternaria alternata*.

Materials and Methods

Sources of fungal species

Alternaria alternata was isolated according to the Rapilly technique.[22] A small segment was obtained from infected tomato fruits, surface sterilized, and inoculated in Petri dishes containing malt extract agar (MEA) medium. The cultures were incubated at 28 ± 2°C for five days in dark conditions. In addition to *A. alternata*, six fungal species;

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Aspergillus, Fusarium, Penicillium, Cladosporium, Paecilomyces, and *C. albicans* ATCC 10231 were also isolated and used to evaluate their sensibility to the selected strains. Also, the antagonist agent, *Aspergillus* species were isolated from green residue that has decomposed for 45 days in red agricultural soil by the method of serial dilutions.

Morphological characterization of fungal isolates
Mold isolates were purified by successive subcultures until stabilization of their macro and microscopic morphological characters was achieved.[23] Morphological identification of the filamentous fungi (conidia production, color, shape, size, hyphae, and diffusible pigments) was carried out by the lactophenol cotton blue staining technique. In addition, the species were identified according to the Barnett and Hunter key.[24-26] A confirmation of the morphological identification of the fungi isolates was made by Prof Iraqi Housseini Abdelilah at the Department of Biology, Faculty of Sciences, Sidi Mohammed Ben Abdellah University, Fez, Morocco.

In vitro screening of antagonistic agents
In vitro screening for antagonistic agents was performed by the dual culture technique on MEA medium using pure isolates. A total of seventy-three fungal isolates from a collection of 254 isolates were screened for antagonistic effect against *A. alternate*.

Antifungal activity assay
The antifungal activity was determined by taking an inoculum of 6 mm from an actively growing border culture of *A. alternata* and was placed in the center of sterile Petri dishes. The antagonist and the phytopathogenic fungus were inoculated simultaneously on the opposite sides of a Petri dish at the same distance (1 cm) from the center of the Petri dish. The test dishes were incubated in dark conditions.[27] Index of antagonism as percentage inhibition of radial mycelial growth in Petri dishes after seven days of incubation was calculated as:

$I\% = [(C - T) / C)] \times 100$.[23,28,29,30]
Where,
I = Inhibition percent
C = Colony diameter (mm) in control
T = Colony diameter (mm) in treatment

Determination of the effect of temperature on fungal growth
The effect of temperature on fungal growth was determined by incubating cultures of the antifungal activity assay at different temperature (25, 28, 30, 37, and 42°C).[31] Petri dishes were covered with parafilm and incubated for seven days until the fungal mycelia reached the edge of the control experiment.

Evaluation of the effect of culture media on fungal growth
The antagonistic assay was optimized by a dual culture technique on different culture media containing different carbon sources. MEA, Sabouraud dextrose agar (SDA), Potato dextrose agar (PDA), and Czapek dox agar (CDA), were prepared and used for culturing.[32] The *in vitro* cultures were incubated at 28 ± 1°C and observed for the growth of the test and antagonistic fungi for seven days.

Assessment of the effect of pH on fungal growth
To determine the optimum pH of *Aspergillus* sp. antagonism towards *A. alternata*, different pH levels of the MEA medium were prepared. The pH value of the MEA medium was adjusted by the addition of Citrate-Phosphate Buffer (Tampon McIlvaine) at different pH levels using the pH meter. In addition to the normal pH value (pH 5.5) of the MEA medium, pH values of 4, 5, 6, 8, and 10 were tested.[33,34] The radial mycelial growth was measured after seven days of incubation at 28°C.

Determination of inhibition spectrum
Aspergillus sp. was tested against 16 pathogen isolates, including fifteen filamentous fungi of different genera (*Aspergillus, Fusarium, Penicillium, Cladosporium*, and *Paecilomyces*), and a yeast *C. albicans* ATCC 10231. The experiment was conducted by the confrontation method on MEA medium for seven days.

Statistical analysis
The experiments were performed in three replicates for all the *in vitro* tests. Statistical analysis of the data obtained was performed using SPSS software (IBM SPSS Statistics 21, United Kingdom). Data were expressed as a mean ± standard deviation (SD) of the mean and analyzed using a two-way ANOVA test at a P-value of less than 0.01.

Results and Discussion

Morphological characterization of fungal species
The selection and isolation of the antagonist constitute the most important phases when a biological control agent is sought.[35] In this study, a collection of fungi isolates was characterized by cultural and colony morphology characteristics and microscopic observation. *A. alternate* was defined by a white grey thallus at the beginning, which becomes dark (olive black) and downy to cottony appearance. Under the microscope, it has large spores and is characterized by a sinuous and long conidiophore: 75-100 µm, and a spherical vesicle (8-10 µm). The phialides are carried by metulae inserted on the upper part of the vesicle, with round conidia of 3.0-3.4 µm. Filamentous fungi, including *Aspergillus*, are widely used for the industrial production of enzymes.[36] Each test was compared with the diameter of each *A. alternata* control. Out of the seventy-three fungal isolates from a collection of 254 isolates that were screened, *Aspergillus* sp. was the most effective antagonist for controlling *A. alternata in vitro*.
Based on the cultural characterization and identification system of Barnett and Hunter,[25] the mold was suspected to be *Aspergillus* sp. It was the fungus that was isolated from the green decomposing residue that showed the strongest antagonistic activity against *A. alternata*. The inhibition of fungal mycelial growth at a distance, as observed in this study is commonly ascribed to antibiotic production by *Aspergillus* sp. The percentage of growth inhibition of mycelial growth after one week of incubation was approximately 65%. This result is very comparable to the *in vitro* antagonistic activity test by Samiya *et al*..[37] They found that isolates SQUCC-33Y (yeast), SQUCC-LB11 (bacterium), and SQUCC-BY1 (bacterium) alone showed inhibitory activities against *A. alternata* and recorded mycelial growth inhibitions of 29.7, 35.8, and 50.1%, respectively.

Effect of environmental factors on the pathogenic and antagonistic fungi
In the current study, the use of full conditions demonstrated the complex factors affecting the antifungal effect of *Aspergillus* sp. against *A. alternata*. The optimal temperature was determined by incubating the cultures of the antifungal activity assay at 25, 28, 30, 37, and 42°C. Temperature affected both mycelial growth and fungal activity. According to the data obtained, 28°C was observed to be the optimal assay temperature value due to the significant inhibition of the vegetative hyphal growth. At 28°C, the co-culture assay showed that the growth of the fungal pathogen was significantly ($p < 0.0001$) inhibited by approximately 62.5% (Figure 1). However, there was no fungal growth recorded at temperature values of 37 and 42°C, for both the pathogen and antagonist. The results obtained are in agreement with the findings of Sempere *et al*., where they showed that the antifungal activity of *T. harzianum* isolate against *A. alternata* varies due to temperature variation.[38]
Different methods have been used to determine fungal growth in different substrates and conditions.[39] To investigate the influence of culture media on *Aspergillus* sp. antagonism against *A. alternata*, a dual culture assay was conducted. Different media which included MEA, CSA, and PDA were tested to select a suitable assay medium and evaluate antifungal activity. The assay displayed a significant ($p < 0.0001$) inhibitory mycelial growth in MEA (3 mm) than in the PDA (4.8 mm) compared to the control (9 mm). Czapeck medium showed no fungal growth of confronted strains (Figure 2).
The effect of various physiological parameters like nutrients, pH, and temperatures on the production of antifungal compounds by biocontrol agents has been reported.[40-42] Figure 3 revealed the effects of different media pH on the antibiosis of *Aspergillus* sp. after seven days of

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incubation. Different ranges of antifungal activity against *A. alternata* between pH 4, 5, and 6 were observed, with the maximum activity of *Aspergillus* sp. at the acidic pH. This observation provided a significant inhibition ($p < 0.01$) of the vegetative hyphal growth of *A. alternata* of 52% at pH 5.

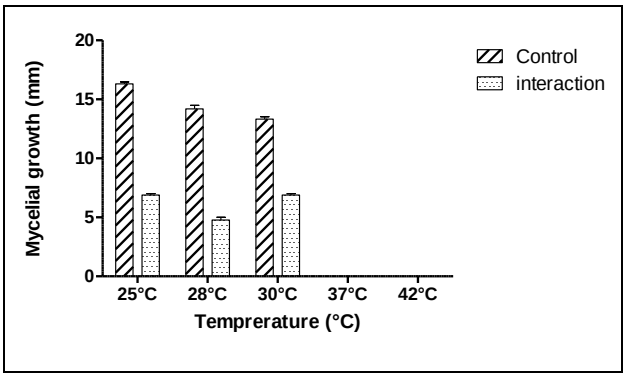

Figure 1: Effect of test temperature on the antagonistic interactions between *Aspergillus* sp. *and Alternaria alternata*. Following fungal growth (mm) at different incubation temperatures (25, 28, 30, and 42 °C) for 8 days. The results were expressed as mean ± SD of 3 independent experiments.

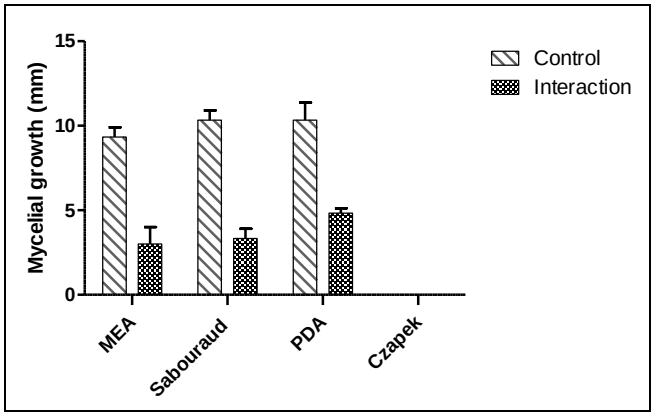

Figure 2: Effect of culture media on the antifungal activity of *Aspergillus* sp. against *Alternaria alternata*.
Following fungal growth (mm) in different media: MEA: Malt extract agar; CDA: Czapek dox agar; SDA: Sabouraud dextrose agar; and PDA: Potato dextrose agar for 8 days. The results were expressed as mean ± SD of 3 independent experiments.

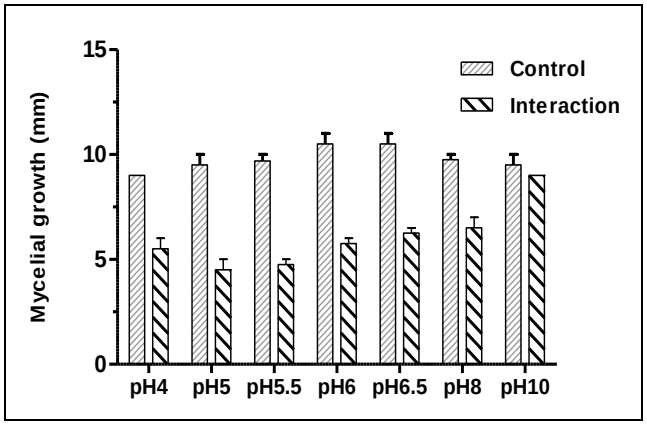

Figure 3: Effect of pH on the antifungal activity of *Aspergillus* sp. against *Alternaria alternata*.
Following fungal mycelial growth (mm) for 7 days. The results were expressed as mean ± SD of 3 independent experiments.

Inhibition spectrum of Aspergillus species
The biological characterization study of *Aspergillus* sp. showed the presence of a very strong inhibition spectrum. On the collection of 16 isolates, the fungal interaction between *Aspergillus* sp. and the test isolates showed that *Aspergillus* sp. had a significant inhibitory effect against the four filamentous fungi; P19 *Fusarium oxysporum, Aspergillus terrus, P11 Fusarium oxysporum,* and *Aspergillus restrictus* by inhibition of mycelial growth of the test isolates. The inhibition rates vary depending on the sensitivity of the test isolates with values of 49.2 ± 1.4%, 49.9 ± 0.2%, 55.6 ± 1.7%, and 61.7 ± 1.5% for *Aspergillus terrus, Aspergillus restrictus, P11 Fusarium oxysporum,* and P19 *Fusarium oxysporum,* respectively (Table 1).

Table 1: Inhibition rate of *Alternaria alternata* co-cultured with *Aspergillus* sp

Test fungal strains	% inhibition
Fu I25: *Fusarium Solani*	-
P19: *Fusarium oxysporum*	61.7 ± 1.5
Fu I26: *Aspergillus terrus*	49.2 ± 1.4
Fi I22: *Aspergillus ustus*	-
P11: *Fusarium oxysporum*	55.6 ± 1.7
Fi I20: *Penicillium simplicissum*	-
Fi I4: *Aspergillus restrictus*	49.9 ± 0.2
Fi I1: *Paecilomyces variotii*	-
C. albicans ATCC	-
FU I17: *Aspergillus corudidus*	-
FU I30: *Penicillium Italicum*	-
ASP37: *Aspergillus fumigatus 42*	-
ASP42: *Aspergillus fumigatus 37*	-
FI I12: *Aspergillus versicolor*	-
FiI27: *Cladosporium cladosporoid*	-
A3: *Penicillium sp*	-

Conclusion

The findings in the study of the effects of environmental factors on the antagonism of *Aspergillus* sp. against *A. alternata* reveal useful information for the applicability of biocontrol agents in plant diseases. This research provides important new knowledge supporting the optimum conditions for the antagonistic activity of *Aspergillus* sp. Therefore, *Aspergillus* sp. may be useful as a biocontrol agent under diverse physiological and certain temperatures, media composition, and pH of the culture media.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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