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Full Length Research Paper

Spices, fungi mycelial development and ochratoxin A production

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The effect of ten powdered spice plants was evaluated (at 1, 2, 3 and 4% concentration) to observe the micelial growth and esporulation of *Penicillium roqueforti* Thompson and *Rhizopus stolonifer* (Ehrenberg: Fries) Vuillemin. The spices were added to the culture media PDA and CYA20S. Clove completely inhibited the mycelial growth of the tested fungi. The other spices; cinnamon, garlic, thyme, mint, anis, oregano and basil also have promising antifungal properties. Laurel and basil did not show a pronounced fungicide effect. In agreement with the first stage of work four spices were selected for the extraction of the essential oil by utilizing the vapor "draging" technique. The antitoxigenic potential of the species was tested against one ochratoxin A-producing strain of *Aspergillus ochraceus* Wilhelm. Clove completely inhibited the micelial growth of the fungi *A. ochraceus*. Garlic and laurel completely inhibited the ochratoxin A production. Cinnamon and anis inhibited the synthesis of ochratoxin A starting from the concentration of 3%, and mint starting from 4%. The other spices did not have a pronounced antiochratoxigenic action.

Key words: Spices, ochratoxin A, fungi.

INTRODUCTION

By definition, condiments and spices are aromatic compounds of vegetal origin, mainly employed to offer taste to foods. According several authors, besides this utility spices also have antioxidant, medicinal, antifungal and antitoxigenic activites (Bullerman, 1974; Bullerman et al., 1977; Arun Sharma et al., 1979; Dewit et al., 1979; Hitokoto et al., 1980; Azzous and Bullerman, 1982; Farag et al.,1989; Shelef 1983; 1989; Chalfoun et al., 2004). The antimicrobial properties of spices and their essential oils have been studied mainly in relation to their inhibition effect on the pathogenic microorganisms present in foods.

Azzous and Bullerman (1982) evaluated the inhibition activity of spices and commercial chemical compounds on toxigenic fungi and reported that clove, cinnamon, mustard, allspice, garlic and oregano were, in decreasing order, the most efficient antifungals; the combination of different concentrations of clove and potassium sorbat showed a possible synergic effect on the fungi development. The inhibition activity of oregano was confirmed by a research developed by Akgul and Kivanç (1988), in which they observed a synergic effect resulted of the combination between oregano and sodium chloride. Beuchat (1976) observed that oregano and thyme were bactericidal to *Vibrio parahaemoliticus* in the concentration of 0,15% while their essential oils demonstrated this activity in the concentration of 100 µg/mL.

The inhibition effect of powdered spices; rosemary, garlic, cinnamon, onion, cumin, clove, turmeric, ginger, laurel, mustard, nutmeg, oregano, paprika, allspice, black pepper on the development of *Yersinia enterocolitica* was verified by Bara (1992) emphasizing the pronounced effect of clove. Bullerman et al. (1977) reported that cinnamon had inhibited the development of *Aspergillus parasiticus* and also the production of aflatoxin in an study with breads.

According to Krogh et al. (1997), the ochratoxin A is a

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Table 1. Botanical classification and active principles of the spices.

Common name	Scientific name	Family	Active principle
Garlic	*Allium sativum* L.	*Liliaceae*	Allicin
Cinnamon	*Cinnamomum burmannil* Meissn	*Lauraceae*	Aldehyde cinnamic
Onion	*Allium cepa* L.	*Liliaceae*	Thipropanal-S-oxide
Clove	*Caryophillus aromaticus* L.	*Myrtaceae*	Eugenol
Anis	*Pimpinella anisum* L.	*Umbelliferae*	Anetol
Mint	*Mentha piperita* L.	*Labiatae*	Carvone
Bay leaf	*Laurus nobilis* L.	*Lauraceae*	Cineol
Basil	*Ocimum basilicum* L.	*Lauraceae*	Linalool
Oregano	*Origanum vulgare* L.	*Lamiaceae*	Carvacrol
Thyme	*Thymus vulgaris* L.	*Labiatae*	Thymol

hepatocarcinogenic, mutagenic, teratogenic and toxic metabolite produced by fungi of the genera *Aspergillus* and *Penicillium* which can develop themselves in a variety of foods that are naturally contaminated, such as: cereals, nuts, dry fruits, milk, coffee and fruit juice.

This study was carried out to determine the effect of the addition of powdered spices in culture media on mycelial growth and sporulation of *Penicillium roqueforti* Thompson and *Rhizopus stolonifer* (Ehrenberg: Fries) Vuillemin and also on the production of ochratoxin A by one strain of *Aspergillus ochraceus* Wilhelm.

MATERIAL AND METHODS

The present work was developed at the Laboratory of Phytopatology at Eco/Centro/EPAMIG, located in the Federal University of Lavras, in the city of Lavras, South region of the State of Minas Gerais.

Isolation of the fungi

Tests were performed on the following kinds of fungi; *P. roqueforti* Thompson and *R. stolonifer* (Ehrenberg: Fries) Vuillemin, which are kept at the mycoteca of the Ecological Manegement Research Center of Pests and Diseases/ EPAMIG. A strain of *A. ochraceus* Wilhelm number EcoCentro 1161T01-01 producer of aflotoxin which is already available in pure culture at the mycoteca of EcoCenter of CTSM/EPAMIG – Lavras, MG was also used for this work. The choice of this fungus was based in the food deterioration effect of the *P. roqueforti* and *R. stolonifer* and the mycotoxigenic potential of *A. ochraceus*.

Botanical material

The inhibiting effect of ten powdered spices in the concentrations of 1, 2, 3 and 4%, was tested. The spices offered by the SantosFlora Medicinal Herbs and Aromatics Enterprise, São Paulo-SP were added to the culture as a powedery. The spices listed in Table 1 were tested according to the inhibiting effect on the development of fungi and the ochratoxin A production, through *in vitro* tests. Evaluation of the effect of the powdered spices on the development and sporulation of the isolated fungi. The *in vitro* studies of the antifungal activities of powdered spices were conducted by using a culture with the addition of a bactericide (chloranfenicol), PDA for *R. stolonifer* and CYA+20S for *Penicillium roqueforti*. (Samson et al., 1995), using Petri dishes with a 9 cm diameter. The powdered spices were added to the media at the concentrations of 0 to 4%. They were sterilizeded by irradiation during six hours and revolved at each two hours, with the exception of basil, mint and laurel that were autoclaved together with the culture media (Bara, 1992), because they were with contamination levels above to the others spices.

After seven days of incubation, ortogonals measurements were performed on the colonies diameter using the development of the check plate as reference. Newbauer's chamber was used for counting spores.

Evaluation of the powdered spices on the production of ochratoxin A

The effect of powdered spices was evaluated on the production of ochratoxin by *A. ochraceus* in a culture YES, incubated for ten days at 25-26°C (Filtenborg and Frisvad, 1980). With the help of an automatic pipe pointer a circular cut of about 25 mm was made on the mycelial fungus with agar containing spices and these mycelia fragments were tranfered to plate of CCD (Merk-Sílica Gel 60, 20X20) previously actived, side by side with a distance at 1.5 cm between them, and after that, a elution phase was developed in a glass cube containing as variable phase TEF-toluene, ethyl acetate and 90% formic acid (50:40:10).

After the eluition, the plates were dried in a flow chapel. Confirmation was acknowledged in ultraviolet light with 366 nm CAMAG (UV- BETRACHTER). The intensity of the fluorescence was expressed by the symbols (+) adopted subjectively, giving that which showed an intenser fluorescence the symbol (+++); medium fluorescence (++); low fluorescence (+) and not detected (ND), which did not show the fluorescence characteristic of the production of ochratoxin A.

Experimental design

A completly randomized experimental design was used with three replicates, with the factors arranged in a 9 x 2 (spice x fungi) factorial scheme. Data analyses were carried out using the statistics package "Analyses System Modification for Balanced Data – Sisvar, according to Ferreira (2000). Spices which totally inhibited the mycelial growth were not included in the statistics analysis.

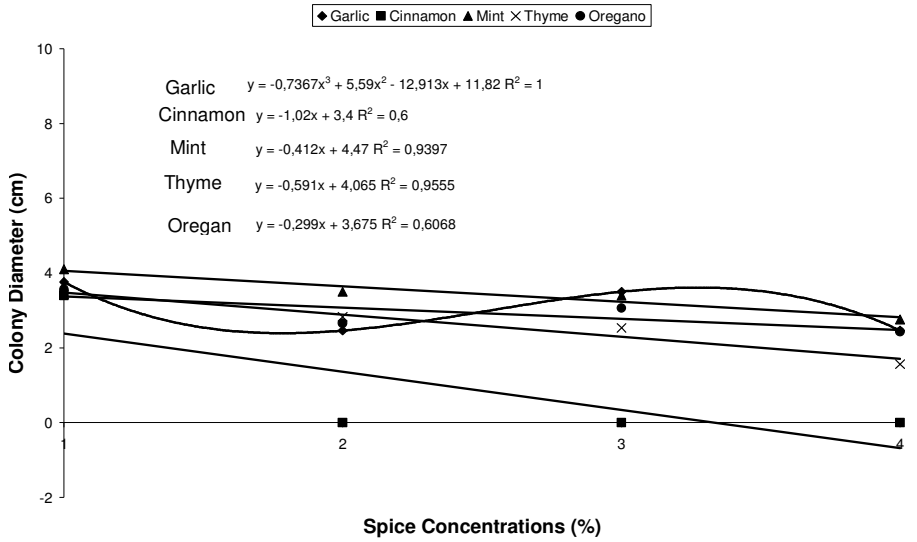

Figure 1. Effect of the powdered spices concentrations on mycelial growth of *P. roqueforti*..

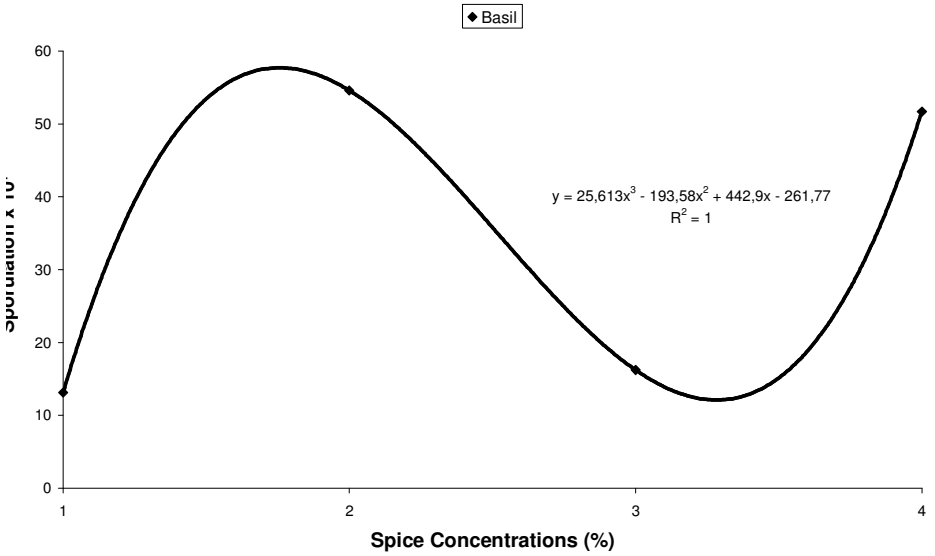

Figure 2. Effect of powdered basil spice concentration on sporulation of *P. roqueforti.*

RESULTS AND DISCUSSION

The spices which were tested in this study, showed in general, a high index of mycelial development and sporulation control of the *P. roqueforti* and *R. stolinefer*. There was high positive correlation between the spices and the different tested concentrations. It was observed that clove promoted total inhibition of *P. roqueforti* micelyal growth in all concentrations tested, which is in agreement with previous works (Hitokoto, 1980; Azzous and Bullerman, 1982; Shelef, 1983; Karapinar and Aktug, 1987; Farag et al., 1989; Bara, 1992). According to the representation in the Figure 1, there is a trend of progressive increase in the inhibition of mycelial growth with increase in concentrations of the spices especially cinnamon and thyme, followed by mint and oregano. Garlic affected the mycelial growth of the fungus *P. roqueforti* in a bimodal way in concentrations at about 2 and 4% with decrease in mycelial growth, while at 1 and 3% mycelial growth increased. This kind of behavior (bimodal) requires further study, because it involves several factors like: kind of matrix, fungi reaction to matrix and other kinds of compounds that can interfere in the fungi behavior. The spices; onion, laurel, anis, basil, were unable to significantly inhibit the mycelial growth of the fungus at the tested concentrations.

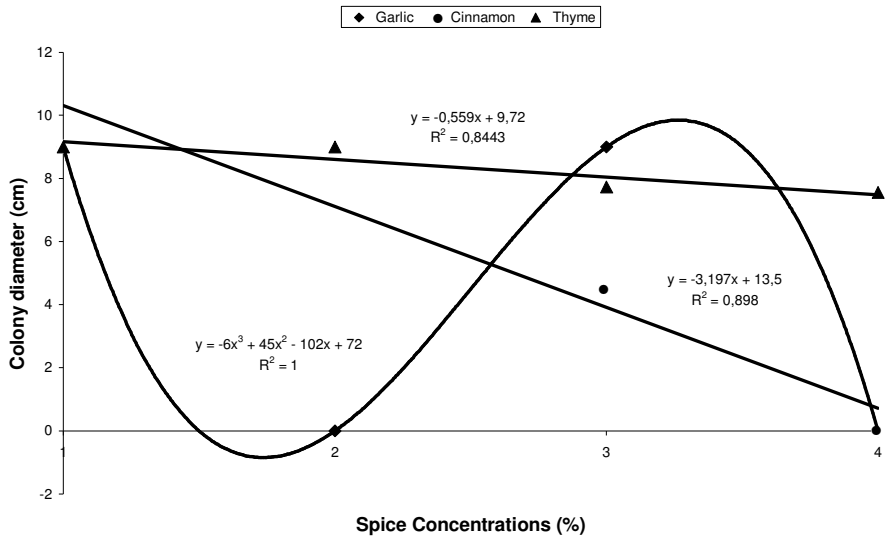

Figure 3. Effect of the powdered spices concentrations on mycelial growth of *R. stolonifer*.

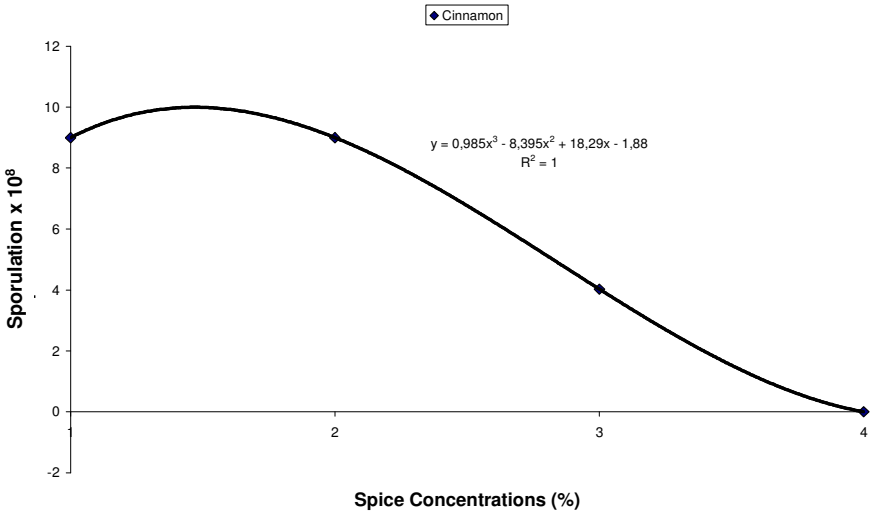

Figure 4. Effect of powdered basil spice concentration on sporulation of *R. stolonifer*.

Regarded the effect of spices on *P. roqueforti* sporulation (Figure 2), it was observed that only basil presented a significant effect, although in a bimodal way, observing that at the concentrations of 1 and 3% the sporulation decreased while at 2 and 4% the sporulation tended to increase. It was observed that clove powder promoted total inhibition of the fungus *R.* stolonifer, which is the reason for not being included in the graphic representation.

Cinnamon and thyme (Figure 3) presented a progressive increase in the inhibition of fungi mycelial growth with the increase of the tested concentrations. The spice garlic presented the highest inhibition indexes at the concentrations of 2 and 4%, indicating a bimodal effect on the fungi mycelial development. The spices; onion, anis, mint, oregano, basil and laurel, were unable to significant inhibit the fungi mycelial growth.

Regarding the effect of spices on fungi sporulation (Figure 4), it was verified that only cinnamon presented a significant correlation with the different tested concentrations, while the other spices that have been tested were unable of any effect on the sporulation decrease.

This work confirmed the potential of some spices in the inhibition of mycelial growth of the fungi *P. roqueforti* and *R. stolonifer*. Clove promoted total inhibition of the mycelial growth in all tested concentrations and cinnamon, thyme and garlic presented a trend of progressive increase in the inhibition of mycelial growth with the incre-

Table 3. Effect of the addition of powdered spices in relation to the production of ochratoxin A.

Concentation (%)	Thyme	Garlic	Cinnamon	Oregano	Laurel leaf	Basil	Mint	Anis	Onion
1	ND	ND	+	+	ND	+++	ND	+++	ND
2	+++	ND	+	++	ND	+	+++	+	+++
3	+	ND	ND	+++	ND	+	++	ND	++
4	+++	ND	ND	++	ND	+++	ND	ND	+

ND – Not detected
+ : Low Fluorescence
++ : Medium Fluorescence
+++ : Intenser Fluorescence

Table 2. Mycelial development and fungi sporulation without spice addition after seven days.

Fungi	Diameter of colony*	Sporulation
Penicillium roqueforti	6.8	$8.875x10^7$
Rhizopus stolonifer	9.0	$1.3625x10^8$

*Average of three experiments.

ase of spices concentrations. Mint inhibited the mycelial growth of *P. roqueforti*. Although basil did not inhibit the fungi mycelial growth, it inhibited the sporulation of the fungi. Oregano inhibited the mycelial growth. Laurel and anis did not show any significant inhibition of the mycelial growth and sporulation of the fungi (Table 2).

The development of the *A. ochraceus* fungus was totally inhibited by clove, in agreement to the previous results obtained by other workers (Hitokoto et al., 1980; Azzous and Bullerman, 1982). According to the results shown in Table 3, garlic and laurel presented a total inhibition of ochratoxin A synthesis, while cinnamon and anis presented an inhibition proportional to the tested concentrations. Thyme presented a total inhibition only in the lowest concentration, while promoting the highest degrees of toxin synthesis in at 2 and 4%. Treatments with oregano and mint, basil and onion, did not correlate with the tested concentrations. The analysis of the effects of spices on the ochratoxin A synthesis, showed a greater inhibition effect by cinnamon, anis and laurel. The results of this work confirmed that besides the antifungal activity, some spices present an antitoxigenic activity.

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