

Origanum vulgare L. essential oil as inhibitor of potentially toxigenic *Aspergilli*

Efetividade do óleo essencial de orégano (*Origanum vulgare* L., *Lamiaceae*) como inibidor do crescimento de espécies de *Aspergillus* potencialmente toxigênicas

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Abstract

Origanum vulgare L. essential oil has been known as an interesting source of antimicrobial compounds to be applied in food conservation. In this study, the effect of *O. vulgare* essential on the growth of *A. flavus*, *A. parasiticus*, *A. fumigatus*, *A. terreus* and *A. ochraceus* was assessed. The essential oil had a significant inhibitory effect on all assayed fungi. MIC was 0.6 $\mu\text{L}\cdot\text{mL}^{-1}$ for all fungi, while MFC was in the range of 1.25-2.5 $\mu\text{L}\cdot\text{mL}^{-1}$. The radial mycelial growth of *A. flavus* and *A. parasiticus* was strongly inhibited over 14 days at 0.6, 1.25 and 2.5 $\mu\text{L}\cdot\text{mL}^{-1}$ of oil in solid medium. The mycelial mass of all fungi was inhibited over 90% at 0.6 and 0.3 $\mu\text{L}\cdot\text{mL}^{-1}$ in liquid medium, while it was 100% at 1.25 $\mu\text{L}\cdot\text{mL}^{-1}$. The oil in a range of concentrations (0.6 to 2.5 $\mu\text{L}\cdot\text{mL}^{-1}$) was effective in inhibiting the viability and spores germination in a short time of exposure. The main morphological changes caused by the essential oil in *A. parasiticus* observed under light microscopy were absence of conidiation, leakage of cytoplasm, loss of pigmentation, and disrupted cell structure. These results demonstrated that *O. vulgare* essential oil produced a significant fungitoxic effect supporting its possible rational use as anti-mould compound in food conservation.

Keywords: *Origanum vulgare* L.; anti-mould activity; *Aspergillus*; biocontrol.

Resumo

O óleo essencial de *Origanum vulgare* L. tem sido reconhecido como uma interessante fonte de compostos antimicrobianos para ser aplicado na conservação de alimentos. Neste estudo, avaliou-se o efeito do óleo essencial de *O. vulgare* sobre o crescimento de *A. flavus*, *A. parasiticus*, *A. fumigatus*, *A. terreus* e *A. ochraceus*. O óleo essencial apresentou um significativo efeito inibitório sobre todos os fungos ensaiados. A CIM foi 0,6 $\mu\text{L}\cdot\text{mL}^{-1}$ para todos os fungos, enquanto a CFM variou entre 1,25-2,5 $\mu\text{L}\cdot\text{mL}^{-1}$. O crescimento micelial radial de *A. flavus* e *A. parasiticus* foi fortemente inibido ao longo de 14 dias pelo óleo essencial nas concentrações de 0,6; 1,25; e 2,5 $\mu\text{L}\cdot\text{mL}^{-1}$ em meio sólido. O óleo essencial a 0,6 e 0,3 $\mu\text{L}\cdot\text{mL}^{-1}$ inibiu o aumento da massa micelial de todos os fungos em valores superiores a 90% em meio líquido, enquanto a 1,25 $\mu\text{L}\cdot\text{mL}^{-1}$, a inibição foi de 100%. O óleo essencial em variadas concentrações (0,6-2,5 $\mu\text{L}\cdot\text{mL}^{-1}$) inibiu a viabilidade e germinação dos esporos fúngicos em um curto tempo de exposição. As principais alterações morfológicas provocadas pelo óleo essencial em *A. parasiticus*, observadas sob microscopia óptica, foram ausência de conidiação, perda de citoplasma, perda de pigmentação e ruptura de estrutura celular. Estes resultados demonstraram que o óleo essencial de *O. vulgare* exerce um significativo efeito fungitóxico, suportando seu uso racional como agente antifúngico na conservação de alimentos.

Palavras-chave: *Origanum vulgare* L.; atividade antifúngica; *Aspergillus*; biocontrole.

1 Introduction

Fungi are significant destroyers of foodstuffs during storage resulting in a reduction in quality and quantity, as well as rendering them unfit for human consumption (ATANDA; AKPAN; OLUWAFEMI, 2007). Some *Aspergillus* species are xerophilic fungi, which are responsible for many cases of food and feed contamination all over the world (KUMAR et al., 2007).

The presence of *Aspergilli* in foods is toxicologically significant since some of them produce mycotoxins known as potential hazard for human and animal health (RASOOLI; OWLIA, 2005). Mycotoxin-producing *Aspergilli* are widely distributed in nature and frequently contaminate human food resources (SAMAPUNDO et al., 2007).

Aflatoxins-B₁, B₂, G₁, G₂ (produced by *A. flavus* and *A. parasiticus*), aspergillic and hidroxiaspergillic acid (produced by *A. flavus*), ascladiol (produced by *A. clavatus*), gliotoxin (produced by *A. fumigatus*, *A. chavalieri* and *A. terreus*), austamide (produced by *A. usutus*), ochratoxins (produced by *A. ochraceus*), oxalic acid (produced by *A. flavus* and *A. glaucus*), and terreic acid (produced by *A. terreus*) are some mycotoxins produced by *Aspergillus* species in foods when exposed to suitable conditions (OVERY et al., 2003; SALEEMULLAH; KHALIL; SHAH, 2006).

Chemicals are used to inhibit the fungal growth in/on foods, but the negative consumer perception of chemical preservatives

Recebido para publicação em 29/7/2008

Aceito para publicação em 14/6/2009 (003730)

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drives attention toward natural alternatives (RASOOLI; OWLIA, 2005). Recently, the antimicrobial potential of essential oils has been of great interest in both academia and food industry since their possible use as natural additives emerged from a growing tendency to replace synthetic additives (SAMAPUNDO et al., 2007; TZORTZAKIS; ECONOMAKIS, 2007). Many studies worldwide have showed the efficacy of essential oils and extracts of various plants in inhibiting the growth of spoiling and/or toxigenic food-related fungi (VELLUTI et al., 2003; FENG; ZENG, 2007; OMIDBEYGI et al., 2007). Phyto-compounds are expected to be far more advantageous than synthetic antifungals for sheer magnitudes of complexity, diversity and novelty of chemicals and reactions since they are bio-degradable in nature, non-pollutant, and possess no residual properties (SHARMA; TRIPATHI, 2008).

The genus *Origanum*, Labiateae, is an annual, perennial, and shrubby herb that is native to the Mediterranean, Euro-Siberian, and Irano-Siberian regions. *Origanum* species grow abundantly on stony slopes and rocky mountain areas at a wide range of altitudes (0-400 m) (SAHIN et al., 2004). *Origanum vulgare* L. is widely known as a very versatile plant with many therapeutic properties (diaphoretic, carminative, antispasmodic, antiseptic, tonic) being applied in traditional medicine systems in many countries (SAGDIÇ et al., 2002; CHUN et al., 2005). It has been widely used in agricultural, and perfumery for its spicy fragrance (SOUZA; STAMFORD; LIMA, 2006; SOUZA et al., 2007).

O. vulgare has presented interesting results in inhibiting the growth of bacteria, fungi, and the synthesis of microbial metabolites (MARINO; BERSANI; COMI, 2001; BAYDAR et al., 2004). *O. vulgare* is rich in essential oil characterized for high amount of phenolic compounds which are believed to be responsible for their antimicrobial property (SKANDAMIS; TSIGARIDA; NICHAS, 2002; FERRARA; MONTESANTO; CHIANTESE, 2003).

This study aimed to evaluate the effect of *O. vulgare* essential oil on various aspects related to the growth/survival and morphogenesis of some food related potentially toxigenic *Aspergillus* species. To our knowledge, the effect of *O. vulgare* essential oil on the morphology of *Aspergillus* genus has not been reported to date.

2 Materials and methods

2.1 Essential oil

The essential oil (batch 209, November/2006) was obtained from Ferquima Ind. e Com. Ltda. (Vargem Grande Paulista, São Paulo, Brazil), and its quality parameters (appearance, color, purity, odor, density -20 °C, refraction index -20 °C) were described in a accompanying technical report. The essential oil was assayed under concentrations ranging from 80 to 0.3 $\mu\text{L}\cdot\text{mL}^{-1}$. Essential oil solutions were prepared in Sabouraud broth using bacteriological agar (0.15 g.100, mL^{-1}) as stabilizing agent according to Mann and Markam (1998) and Bennis et al. (2004).

2.2 Moulds strains

Aspergillus flavus ATCC 6013, *A. flavus* ATCC 40640, *A. ochraceus* ATCC 22947, *A. parasiticus* NRRL 2999,

A. fumigatus ATCC 40640, and *A. terreus* ATCC 7860 were used as test microorganisms. These strains were obtained through the courtesy of the Laboratory of Mycology, Federal University of Paraíba, João Pessoa, Paraíba, Brazil. The stock cultures were kept on sterile Sabouraud Agar (SA) slants under 8 °C (± 1 °C). For anti-mould assays, 7 day-old culture grown on sterile SA at 25-28 °C were used. Mould spores were taken by adding sterile NaCl (0.85 g.100 mL^{-1}) to the medium followed for gentle shaking for 30 seconds. Each suspension was filtered through a sterile triple layer cheesecloth to remove mycelial fragments. The mould spores were counted using a hemocytometer. The spore suspension was adjusted using sterile NaCl (0.85 g.100 mL^{-1}) to contain approximately 10^6 spores. mL^{-1} (RASOOLI; ABYANEH, 2004; RASOOLI; OWLIA, 2005).

2.3 Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

MIC and MFC were determined by macrodilution in broth. 5 mL of double strength Sabouraud Broth (SB) was inoculated with 1 mL of the mould suspension (approximately 10^6 spores. mL^{-1}). Next, 4 mL of the essential oil solution with different concentrations was added to the system and followed by shaking for 30 seconds using a Vortex. The system was incubated for 48-72 hours at 25-28 °C without shaking. The MIC was defined as the lowest concentration of the essential oil required to completely prevent visible fungal growth. An aliquot (1 mL) of the tubes showing no visible mould growth was subcultured on sterile SA Petri dishes at 25-28 °C for 72 hours to determine if the inhibition was reversible or permanent. The MFC was defined as the lowest concentration of the essential oil at which no growth was noted on SA. The control flasks without essential oil were tested in the same way (RASOOLI; ABYANEH, 2004; SHARMA; TRIPATHI, 2008).

2.4 Measure of radial mycelial growth

The inhibition of the essential oil on the radial mycelial growth was determined using the poisoned substrate technique (dilution in solid medium). For this, a 2 mm plug taken from a 10 day-old mould culture grown on SA slants at 25-28 °C was placed on the center of sterile SA Petri dishes added with 2.5, 1.25 and 0.6 $\mu\text{L}\cdot\text{mL}^{-1}$ of the oil at 25-28 °C. At different intervals (0, 2, 4, 6, 8, 10, 12 and 14 days) after incubation, the radial mycelial growth was measured (mm) using calipers. The control flasks without the essential oil were tested in the same way (ADAM et al., 1998; DAFERERA; ZIOGAS; POLISSIOU, 2003).

2.5 Determination of mycelial dry weight

The inhibition of the essential oil on the mycelial dry weight was determined using the poisoned substrate technique (dilution in liquid medium). Hence, 10^6 spores. mL^{-1} of the assayed fungi was inoculated in sterile SB Erlenmeyer flasks added with the essential oil at concentrations of 1.25, 0.6 and 0.3 $\mu\text{L}\cdot\text{mL}^{-1}$. After 15 days of incubation, the dry weight of the mycelium was determined. Flasks containing mycelia were autoclaved (121 °C for 30 seconds) in order to inactivate the spores. Thereafter, the content of the flasks were filtered by

Whartman filter n°1 and washed twice with sterile distilled water. Mycelia were allowed to dry at 60 °C for 6 hours and then at 40 °C overnight. The filter paper containing dry mycelia was weighed. The control flasks without essential oil were tested in the same way. The percentage of mycelia growth inhibition in terms of dry weight was calculated in comparison with the control assay (RASOOLI; REZAEI; ALLAMEH, 2006).

2.6 Mould spores viability

5 mL of sterile double strength SB were inoculated with 1 mL of the mould suspension (approximately 10^6 spores.mL⁻¹). Next, 4 mL of the essential oil solution (1.25 and 0.25 $\mu\text{L.mL}^{-1}$) were added to the system and followed for shaking using a Vortex for 30 seconds. The system was incubated at 28-30 °C. At different time intervals (0, 15, 30, 45, 60, and 120 minutes) of post-incubation, 1 mL of the suspension was serially diluted in PBS (10^{-1} – 10^{-4}), spread on sterile SA Petri dishes, and incubated for 28-30 °C/48–72 hours. The control flasks without the essential oil were tested in the same way. After the incubation time, the mean number of Colonies Forming Units (cfu.mL⁻¹) was counted. The percentage of inhibition of spore viability was calculated in comparison with the control assay (RASOOLI; REZAEI; ALLAMEH, 2006; TZORTZAKIS; ECONOMAKIS, 2007).

2.7 Spore germination assay

Aliquots of 0.1 mL of the essential oil at concentrations of 10, 5, 2.5, and 1.25 $\mu\text{L.mL}^{-1}$ were mixed with 0.1 mL of mould spore suspension (approximately 10^6 spores.mL⁻¹). The mixture was placed on separated glass slides which were incubated in a moist chamber at 25-28 °C for 24 hours. At the end of the incubation period, each slide was fixed with lacto-phenol-cotton blue stain and observed under a light microscope for spore germination. Control flasks without essential oil were tested in the same way. About 200 spores were counted and the percentage of spore germination was calculated in comparison with the control assay (FENG; ZENG, 2007).

2.8 Fungal morphogenesis study

For the evaluation of morphological alterations caused by the essential oil (2.5 $\mu\text{L.mL}^{-1}$) in *A. parasiticus* NRRL 2999, a sample of mycelium was taken from the periphery of a 10-day-old fungal colony grown on SA at 25-28 °C containing the essential oil. The samples were fixed in lacto-phenol-cotton blue stain and observed under a microscope at 400 \times to examine morphological abnormalities. The samples from the control SA plates without the essential oil were also stained and their morphological aspects were observed (SHARMA; TRIPAPHI, 2008).

3 Results and discussion

O. vulgare L. essential oil showed broad anti-mould spectrum against all investigated fungi. The MIC and MFC values of *O. vulgare* essential oil on potentially toxigenic food-related *Aspergillus* species in liquid medium are shown in Table 1. The MIC was 0.6 $\mu\text{L.mL}^{-1}$ for all fungi. The MFC was in the range of 1.25 – 2.5 $\mu\text{L.mL}^{-1}$. The MFC of 2.5 $\mu\text{L.mL}^{-1}$ was found to *A. parasiticus* and *A. terreus*.

Velluti et al. (2003) found significant inhibitory effect of *O. vulgare* essential oil at 500 and 1000 $\mu\text{g.g}^{-1}$ against the growth of *Fusarium proliferatum* and production of fumonisin B₁ in maize grains. Sahin et al. (2004) found MIC values of *O. vulgare* ssp. *vulgare* essential oil of 125 and 31.25 $\mu\text{L.mL}^{-1}$ toward *A. flavus* and *A. versicolor*, respectively.

The effect of *O. vulgare* essential oil on the radial mycelial growth of *A. flavus* and *A. parasiticus* in solid medium is shown in Figures 1 and 2. The essential oil at 0.6, 1.25, and 2.5 $\mu\text{L.mL}^{-1}$ exhibited a fumigant effect against both fungi. *O. vulgare* provided a 100% of lethal effect against *A. flavus* after 2 days. *A. parasiticus* presented a light increase in the radial growth up to 8 days of exposure to 1.25 and 0.6 $\mu\text{L.mL}^{-1}$ of the oil. However, the growth was always smaller than that of the control assay. These findings show a cidal property of the assayed essential oil providing a fast and steady rate of mycelial growth inhibition.

The effect of *O. vulgare* essential oil on the dry micelyal mass weight of *Aspergillus* species in liquid medium is shown in Table 2. The oil at all tested concentrations (1.25, 0.6 and 0.3 $\mu\text{L.mL}^{-1}$) caused a 100% inhibition of the biomass of *A. flavus* and *A. terreus*. For *A. fumigatus*, *A. parasiticus*, and *A. ochraceus* the biomass inhibition was between 91 and 100% showing dependence of the assayed concentration. The inhibition percentage in terms of radial growth and dry weight showed similar efficacy of the oil in solid and liquid medium.

Table 1. MIC and MFC of the *O. vulgare* L. on potentially toxigenic *Aspergillus* species.

Moulds	MIC ($\mu\text{L.mL}^{-1}$)	MFC ($\mu\text{L.mL}^{-1}$)	Control	Mould viability*
<i>A. flavus</i> ATCC 6013	0.6	1.25	+	+
<i>A. fumigatus</i> ATCC 40640	0.6	1.25	+	+
<i>A. parasiticus</i> NRRL 2999	0.6	2.5	+	+
<i>A. ochraceus</i> ATCC 22947	0.6	1.25	+	+
<i>A. terreus</i> ATCC 7860	0.6	2.5	+	+

*Mould growth in Sabouraud agar without adding the essential oil.

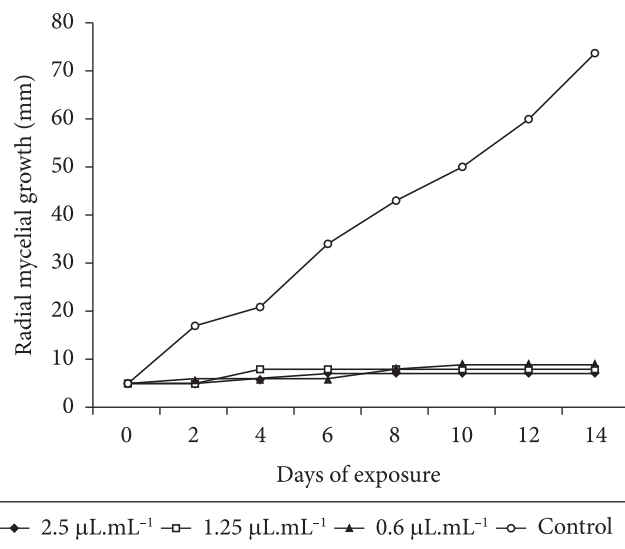


Figure 1. Effect of *O. vulgare* L. essential oil on the radial mycelial growth of *A. flavus* ATCC 6013.

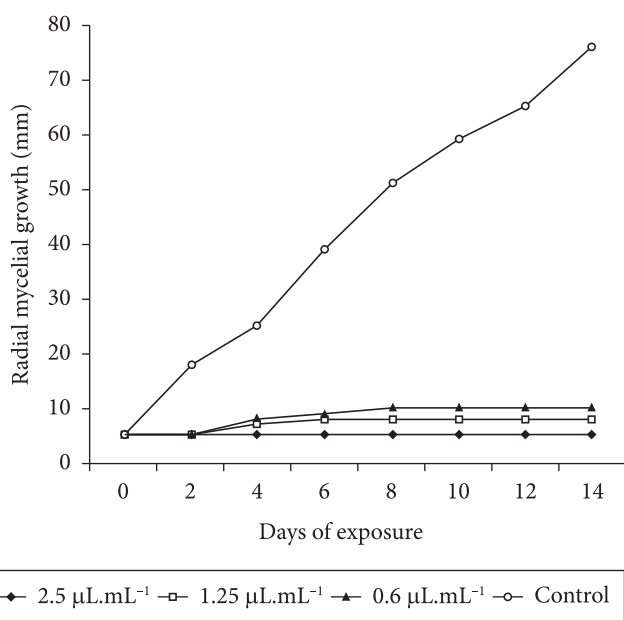


Figure 2. Effect of *O. vulgare* L. essential oil on the radial mycelial growth of *A. parasiticus* NRRL 2999.

The effect of *O. vulgare* L. essential oil (0.6 and 1.25 $\mu\text{L.mL}^{-1}$) on the spore viability of *A. flavus* ATCC 6013 and *A. parasiticus* NRRL 2999 in liquid medium revealed over 80% inhibition after 120 minutes of exposure in comparison with the control assay. It was found that the oil at 1.25 $\mu\text{L.mL}^{-1}$ caused a 100% inhibition of the spore viability of both fungi in a maximum time of 30 minutes (data not showed).

A few studies have focused on the effect of essential oils on the viability of mould spores. Rasooli and Abyaneh (2004) noted no viability of spores of *A. parasiticus* after treating with thyme (*Thyme eriocalyx* and *Thyme x-porlock*) essential oils (1/4 v/v) for 120 minutes. Rasooli, Rezaei and Allameh (2006) found a decrease in the viability of *A. niger* spores exposed to 250 and 500 ppm of *Thyme eriocalyx* and *Thyme x-porlock* essential oils.

The results of the effect of *O. vulgare* essential oil at concentrations of 10, 5, 2.5, and 1.25 $\mu\text{L.mL}^{-1}$ on the spore germination of *A. parasiticus* NRRL 2999 in liquid medium are shown in Table 3. As can be noted, the essential oil exhibited a 96% inhibition of spore germination at 10 $\mu\text{L.mL}^{-1}$ concentration, while it was in the range of 25 – 46% at 5, 2.5 and 1.25 $\mu\text{L.mL}^{-1}$.

In agreement with previous studies, the inhibition of spore germination caused by *O. vulgare* essential oil was in a dosage response manner (RANA; SINGH; TANEJA, 1997; TZORTZAKIS; ECONOMAKIS, 2007). Sharma and Tripathi (2008) reported a 100 and 50% inhibition of germination of *A. niger* spores exposed to *Citrus sinensis* essential oil at 1.5 and 0.3 $\mu\text{g.mL}^{-1}$, respectively.

Observations of *A. parasiticus* under a light microscope at 400 \times magnification after exposure to 2.5 $\mu\text{L.mL}^{-1}$ of *O. vulgare* essential oil showed some morphological abnormalities (Figure 3). Microscopic examination of the control mycelium (untreated cell) showed a regular cell structure with homogenous

Table 2. Inhibitory effect of *O. vulgare* L. essential oil on the micelyal mass of potentially toxigenic *Aspergillus* species (results expressed as percent inhibition of dry mycelial mass in comparison with the control assay).

Moulds	Essential oil concentration ($\mu\text{L.mL}^{-1}$)		
	1.25 (%)	0.6 (%)	0.3 (%)
<i>A. flavus</i> ATCC 6013	100	100	100
<i>A. fumigatus</i> ATCC 40640	100	94	91
<i>A. parasiticus</i> NRRL 2999	100	100	96
<i>A. ochraceus</i> ATCC 22947	100	100	95
<i>A. terreus</i> ATCC 7860	100	100	100

Table 3. Inhibitory effect of *O. vulgare* L. essential oil on the spores germination of *A. parasiticus* NRRL 2999 (results expressed as percent inhibition of spore germination in comparison with the control assay).

Essential oil ($\mu\text{L.mL}^{-1}$)	Inhibition of spores germination (%)
10	96
5	46
2.5	37
1.25	25

cytoplasm, clearly visible sterigmata bearing conidia and profuse conidiation on a large and radiated conidial head. The mycelia cultivated in the medium added with the essential oil appeared to present morphological changes with a heterogeneous mycelial structure. The alterations included lack of sporulation, loss of cytoplasm content (empty hyphae), loss of pigmentation, distorted development hyphae, and empty hyphae. The hyphae became distorted with swelling along its structure and budded apical tips. Still, the oil caused a clear absence of conidiation. Abnormalities in hyphae and spores coloring were not evaluated.

Previous studies reported that essential oils are able to cause morphological changes in *Aspergillus* species including lack of sporulation, loss of pigmentation, aberrant development of conidiophores (flattened and squashed), and distortion of hyphae (DE BILLERBERK et al., 2001; RASOOLI; ABYANEH, 2004; SHARMA; TRIPATHI, 2008). These findings suggested that the mode of antifungal activity of essential oils could include an attack on the cell wall and retraction of the cytoplasm in the hyphae ultimately resulting in the death of the mycelium. In addition, it was also related to the interference of the essential oil components in enzymatic reactions of cell wall synthesis, which affects the fungal growth and morphogenesis.

Velluti et al. (2003) suggest that the antimicrobial activity of the essential oil depends on the chemical structure of their components. Carvacrol and thymol, phenolic compounds known as major constituents of *O. vulgare* essential oil, have their antimicrobial property attributed to the presence of an aromatic group that is known to be reactive and to form hydrogen bonds with active sites of target enzymes (DIMITRIJEVIĆ et al., 2007; SOUZA et al., 2007). However, it is also suggested that the effectiveness of complete essential oils is higher than the activity of each separated compound (MILOS; MASTELIC; JERKOVIC, 2000).

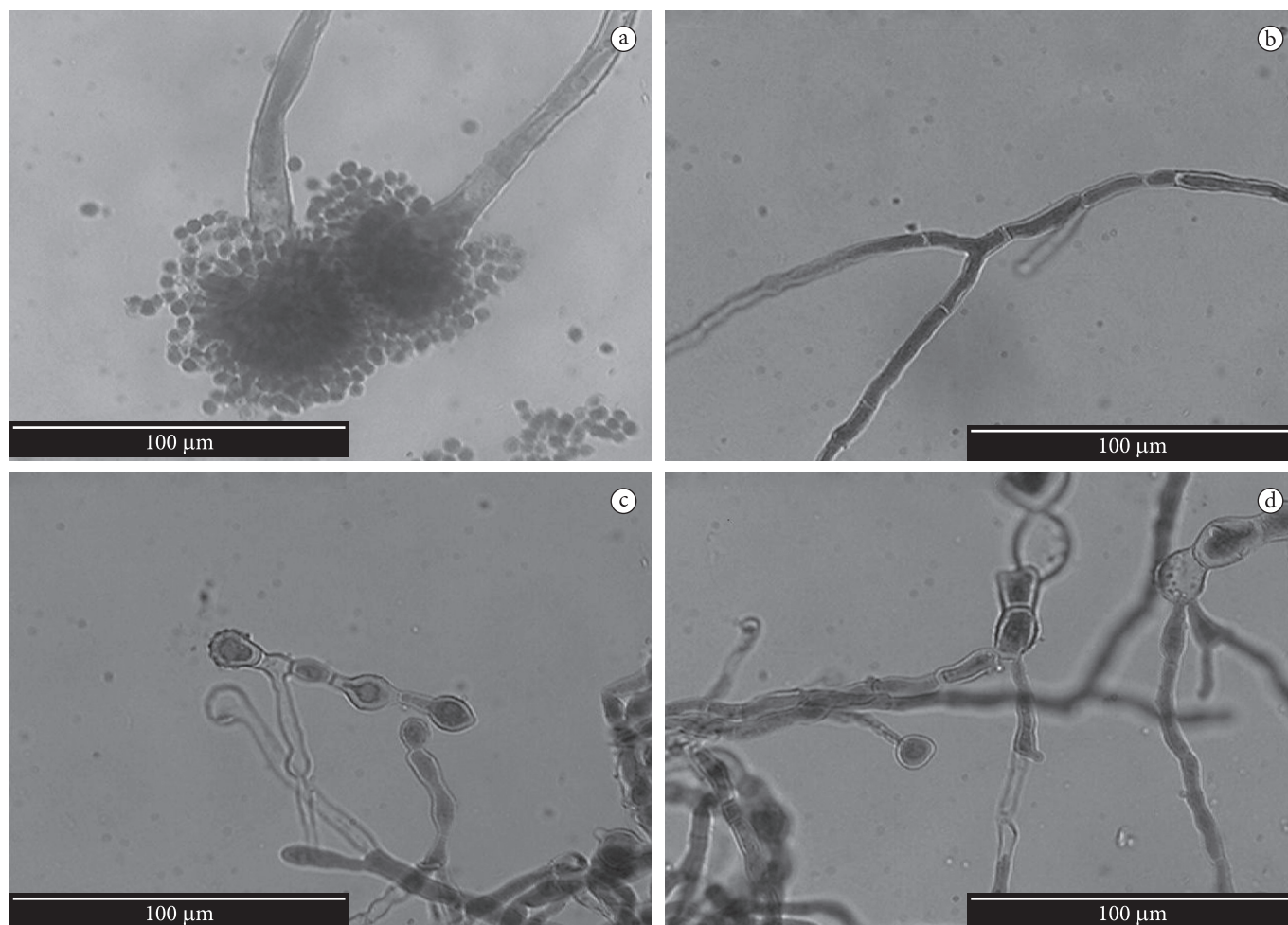


Figure 3. Light microphotographs of *A. parasiticus* NRRL 2999 mycelium growing on AS without or with *O. vulgare* essential oil ($2.5 \mu\text{L.mL}^{-1}$) during 7 days of incubation at 25-28 °C. a) Control conidial head of *A. parasiticus*, large and radiated, development of vesicle on conidiophore, conidia clearly visible, Bar 100 μm ; b) *A. parasiticus* control mycelium, homogeneous structure and regular growing hyphae, control magnification of single hyphae, Bar 100 μm ; c-d) Hyphae modification induced by $2.5 \mu\text{L.mL}^{-1}$ of *O. vulgare* essential oil showing anomalous structure and loss of pigmentation; clear leakage of cytoplasm content and destruction of cell structure noted by disrupted hyphae integrity, Bar 100 μm ; and d) conidial head with clear absence of conidiation, Bar 100 μm .

The present study confirmed the anti-*Aspergillus* activity of *O. vulgare* essential oil based on MIC, MBC, mycelia growth, spore germination/viability, and morphological changes. The assayed essential oil presented a fast and steady fungitoxic property supporting its rational use as an alternative to be applied in the biocontrol of *Aspergillus* species in foodstuffs.

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