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Antimicrobial activities of various essential oils against foodborne pathogenic or spoilage moulds

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Abstract - The use of essential oils in the food industry, as natural sanitizing agents, requires the definition of optimal conditions. The aim of the present work was to evaluate some antimicrobial activity parameters as mycelial growth inhibition, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of six essential oils against *Aspergillus niger, Aspergillus terreus, Chaetomium globosum, Penicillium chrysogenum, Penicillium pinophilum, Trichoderma harzianum* and *Trichoderma viride*. The antimicrobial activity of essential oils was monitored by the macrodiluition technique. The mycelial growth inhibition, fungistatic and fungicidal concentrations were recorded for each strain that showed sensitivity to the essential oils. The essential oils of catnip, cinnamon, tea tree and thyme essential oils exhibited a large spectrum antimicrobial activities; those of clary sage and laurel inhibited the mycelial growth in a few fungal strains. The essential oils of cinnamon and thyme had the lowest MIC and MFC values against all the fungi assayed, followed by catnip, tea tree, clary sage and laurel. The use of these natural products rather than, the currently used antifungal chemicals, may be of interest given that: i) essential oils are of natural origin which means they are safer for human health and the environment and ii) there is less chance that the pathogenic microorganisms will develop resistance.

Key words: essential oils, antimicrobial activity, foodborne pathogens, spoilage moulds.

INTRODUCTION

Public concern for food safety is increasing despite the progress that has been made in food production technology (WHO, 2002a).

New methods that reduce or eliminate food pathogenic microbes are still needed to improve current technology (Leistner, 1978). Concurrently, modern society is looking for natural products that have less impact on the environment and that contain less synthetic antimicrobial food additives. This is the so-called "green" consumerism (Tuley de Silva, 1996; Smid and Gorris, 1999). A lower food salt content is also being promoted by the World Health Organization, in an attempt to reduce the incidence of cardiovascular disease (WHO, 2002b). Alternative natural additives are therefore needed in order, to guarantee food safety in preserved products. The antimicrobial properties of some essential oils is well known. The scientific interest in these natural additives has flourished alongside "green" consumerism and the food preservative action of some essential oils has been investigated (Burt, 2004).

Food-spoiling organisms include some filamentous fungi that grow on all kinds of foods: cereals, meat, milk, fruit, vegetables, commercial mushrooms, nuts, fats and their products. The fungal growth may result in several kinds of food-spoilage: off-flavours, toxins, discolouration, mycolytic enzymes, rotting and formation of pathogenic or allergenic propagules. The deterioration of sensorial properties is often due to the production of exoenzymes during growth. Filamentous fungi can produce a vast number of enzymes: lipases, proteases and carbohydrase. Once inside the food, these enzymes may continue their activities independent of the destruction or removal of the mycelium (Filtenborg *et al*., 1996). The production of mycotoxins, in particular, has a major negative impact of fungal growth in foods. According to experts, five kinds of mycotoxins are important world-wide for human health: aflatoxins, ochratoxin A, funonisins, certain trichothecenes and zeazalenone (Pitt *et al*., 2000). Long-term ingestion of these toxins as a result of eating contaminated foods has been associated with liver and kidney tumors in animals and humans (Park, 1995).

Some of the most dangerous and well-known fungal species, that are related to food spoilage, belong to the genera *Aspergillus* and *Penicillium*. *Chaetomium* and *Trichoderma* also have species that produce mycotoxins that are nocivous to human and animal health and cause perilous food losses (Hou *et al*., 1972; Udagawa *et al*., 1979; Corley *et al*., 1994; Pitt and Hocking, 1999; Sautour *et al.*, 2002; Dantigny *et al*., 2005; Mandeel, 2005). *Trichoderma harzianum* and *Trichoderma viride,* for instance, can cause severe losses in mushroom production because, their presence in the mushroom compost or casing layer, cause the emerging mushrooms to be badly spotted and often distorted

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(due to the production of chitinases and glucanases). The mushroom are therefore unmarketable (Mamoun *et al*., 2000; Williams *et al*., 2003).

The aim of the present work was to evaluate some antifungal activity parameters (mycelial growth inhibition, minimum inhibitory concentration and minimum fungicidal concentration) of the essential oils of catnip (*Nepeta cataria* L.), cinnamon (*Cinnamomum zeylanicum* Breyne), clary sage (*Salvia sclarea* L.), laurel (*Laurus nobilis* L.), tea tree (*Melaleuca alternifolia* Cheel) and thyme (*Thymus vulgaris* L.), against different foodborne pathogenic or spoilage moulds.

MATERIAL AND METHODS

Essential oils. The essential oils used in this study were of *Cinnamomum zeylanicum* Breyne, *Laurus nobilis* L., *Melaleuca alternifolia* Cheel, *Nepeta cataria* L., *Salvia sclarea* L. and *Thymus vulgaris* L. They were all supplied by Aboca Erbe, S. Sepolcro, AR, Italy.

Strains of filamentous fungi and growth conditions. The fungi used in this study were: *Aspergillus niger* Tieghem (ATCC 9642, American Type Culture Collection), *Aspergillus terreus* Thom (ATCC 1069), *Chaetomium globosum* Kunze (ATCC 6205), *Penicillium chrysogenum* Thom (ATCC 6205), *Penicillium pinophilum* Hedgcoock (ATCC 9644), *Trichoderma viride* Pers ex Fries (CECT 2423, Colección Española de Cultivos Tipo) and *Trichoderma harzianum* Rifai (CECT 2424). The fungi were maintained on Sabouraud dextrose agar (SDA medium, BioLife). Cultures were stored at 4 °C and subcultured once a month.

Antimicrobial assay. The disc diffusion method (Wistriech, 1997) was used to investigate the antimicrobial activity of essential oils. SDA medium was prepared and poured into 90 mm diameter Petri plates. The paper disc size was 6 mm (Whatman no. 1). The fungus was streaked on the surface of the SDA medium using a sterile cotton swab in order to get a uniform microbial growth on both the control and test plates. The essential oils were diluted 1:5 (v/v) with acetone. Under aseptic conditions, discs were placed on the agar plates and soaked with 10 µl of essential oil. Solvent controls were prepared with 10 µl of acetone.

Determination of minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and mycelial growth inhibition (MGI). MIC and MFC techniques were used to assess the fungistatic and fungicidal oil properties. Fungistatic and fungicidal essential oil concentrations were only determined against the filamentous fungi that exhibited sensitivity during the previous assay.

The minimum inhibitory concentrations (MICs) were determined by the macrodilution technique (Ishii, 1995; Behravan *et al*., 2004). Five concentrations of essential oils were prepared within the concentration range of 0.0625 to 1 µl/ml (0.0625, 0.125, 0.25, 0.5, 1.0 µl/ml). The concentration of 1 µl/ml was chosen as the maximum value because anything higher would probably not be acceptable in food (Smith-Palmer *et al*., 1998). Oil samples were first dissolved in acetone; 30 µl of the specific oil concentration and acetone mix were then added to Petri dishes containing 15 ml of SDA medium. Positive controls were water (in place of oil) and acetone added to Petri dishes containing 15 ml of SDA medium. The growth medium was inoculated the next day at the centre of the plates, using 5 mm cores taken from mycelial stock culture plates and incubated at 25 °C for 7 days.

The MIC values of the tested essential oils were the lowest concentrations that did not exhibit any visible growth of the fungal mycelium, but which remained viable and grew when plated on SDA medium.

The MFC values were determined by the method of Garber and Huston (1959). This was done by subculturing the inhibited fungal discs at MICs on SDA medium. Observations were recorded after 7 days of incubation at 25 °C. Fungal growth on the seventh day was indicative of a fungistatic nature, while the absence of fungal growth denoted a fungicidal action of the oil.

The MGI percentage was calculated according to the equation:

$$MGI = (dc\text{-}dt)/dc \times 100$$

where dc is the fungal colony diameter measured in control sets, dt is the fungal colony diameter measured in treatment sets after 7 day incubation.

Every experiment was performed in triplicate.

Statistical analysis. The relative standard error was determined. Analyses of variance (Anova), followed by LSD post hoc determinations, were performed. All computations were done using the statistical software SuperAnova for Mac Plus (1989-90, Abacus Concepts, Inc).

RESULTS

The MGI, fungistatic and fungicidal activity values of the essential oils against the tested fungi are reported in Table 1. Of the oils analysed in this work, the essential oils of cinnamon and thyme were the most effective as antifungal agents, followed by catnip and tea tree. Clary sage and laurel oils had the least antifungal activity. Furthermore, the mycelial growth inhibition activity of clary sage and laurel oils was only observed against certain fungal species. Cinnamon and thyme oils severely inhibited fungal growth at the lowest experimental concentration levels (0.125 and 0.0625 µl/ml), while catnip and tea tree showed extensive and mycelial growth inhibition at concentrations of 0.25 and 0.5 µl/ml, respectively.

The MIC and MFC values of cinnamon and thyme oil against all the moulds assayed were 0.25 and 0.5 µl/ml, respectively.

Trichoderma harzianum was the least sensitive to thyme oil, with MIC and MFC values of 0.5 and 1 µl/ml, respectively.

Catnip essential oil showed both fungistatic and fungicidal properties at the highest concentrations used: 0.5 µl/ml MIC and 1 µl/ml MFC. Tea tree oil had fungistatic activity (not significantly different) against *A. terreus*, *C. globosum*, *P. chrysogenum* and *P. pinophilum* at concentration of 1 µl/ml (MIC). Moreover, the strains of *T. harzianum* and *T. viride* showed the least sensitivity to the tea tree oil; while the strains of *T. harzianum* and *A. terreus* showed the least sensitivity to the catnip oil.

Clary sage essential oil exhibited antimicrobial activity mainly against *P. chrysogenum*, *P. pinophilum* and *T. viride*, with MGI values of 50%, 40.9% and 40.7%, respectively.

Laurel essential oil showed the weakest activity, with

TABLE 1 – Mycelial growth inhibition, fungistatic and fungicidal activity of various essential oils on tested fungi

Essential oils (µl/ml)	Mycelial growth inhibition (%)						
	Aspergillus niger	*Aspergillus terreus*	*Chaetomium globosum*	*Penicillium chrysogenum*	*Penicillium pinophilum*	*Trichoderma harzianum*	*Trichoderma viride*
Catnip							
1	100 (a) Fc	100 (a) Fs	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fs	100 (a) Fc
0.5	100 (c) Fs	91.3 ± 2.8 (b)	100 (c) Fs	100 (c) Fs	100 (c) Fs	80.8 ± 2.9 (a)	100 (c) Fs
0.25	65.5 ± 1.9 (ab)	57.1 ± 0.6 (a)	78.1 ± 6.9 (c)	72.1 ± 5.7 (bc)	66.6 ± 3.5 (abc)	66.2 ± 3.1 (abc)	100 (d)
0.125	60.7 ± 4.2 (d)	20 ± 1.8 (a)	47.6 ± 3.4 (c)	62.1 ± 2.3 (d)	41.1 ± 1.4 (c)	31.8 ± 1.0 (b)	67.6 ± 1.2 (d)
0.0625	10.3 ± 0.4 (b)	18.2 ± 0.1 (e)	11.4 ± 0.2 (c)	17.6 ± 0.2 (e)	27.2 ± 0.6 (f)	2.2 ± 0.1 (a)	14.2 ± 0.1 (d)
Cinnamon							
1	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc
0.5	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc
0.25	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs
0.125	60.7 ± 4.2 (b)	50 ± 2.8 (a)	57.1 ± 2.8 (ab)	72.9 ± 2.3 (c)	61.1 ± 1.4 (b)	87.5 ± 2.8 (d)	70.9 ± 1.2 (c)
0.0625	55.1 ± 1.7 (c)	36.3 ± 3.5 (a)	60 ± 4.6 (c)	52.9 ± 3.4 (bc)	45.4 ± 2.4 (ab)	75 ± 2.3 (d)	73.4 ± 1.9 (d)
Clary sage							
1	27.6 ± 0.5 (b)	18.1 ± 0.4 (a)	–	50 ± 1.2 (d)	40.9 ± 1.2 (c)	–	40.7 ± 1.3 (c)
0.5	22.5 ± 0.6 (b)	12.1 ± 0.3 (a)	–	32.7 ± 1.6 (d)	29.4 ± 1.3 (c)	–	31.3 ± 0.8 (cd)
0.25	18 ± 1.1 (c)	9.5 ± 0.3 (a)	–	13.7 ± 0.2 (b)	14.2 ± 0.3 (b)	–	22.2 ± 0.2 (d)
0.125	15.7 ± 0.4 (d)	7.5 ± 0.1 (b)	–	0 (a)	9.5 ± 0.6 (c)	–	0 (a)
0.0625	10.3 ± 0.5 (c)	4.1 ± 0.3 (b)	–	0 (a)	0 (a)	–	0 (a)
Laurel							
1	28.2 ± 0.6 (d)	26.8 ± 0.5 (c)	–	10.8 ± 0.3 (b)	–	–	9.2 ± 0.2 (a)
0.5	15 ± 0.8 (c)	18.5 ± 0.2 (d)	–	6.7 ± 0.2 (b)	–	–	3.5 ± 0.3 (a)
0.25	11.3 ± 0.2 (c)	13.8 ± 0.5 (d)	–	4.2 ± 0.3 (b)	–	–	0 (a)
0.125	7.1 ± 0.6 (c)	5 ± 0.1 (b)	–	0 (a)	–	–	0 (a)
0.0625	0 (a)	0 (a)	–	0 (a)	–	–	0 (a)
Tea tree							
1	79.4 ± 0.7 (b)	100 (d) Fs	100 (d) Fs	100 (d) Fs	100 (d) Fs	63.6 ± 1.2 (a)	83.3 ± 0.3 (c)
0.5	55 ± 2.8 (b)	56.5 ± 1.7 (b)	70.3 ± 0.6 (d)	72.9 ± 0.8 (d)	57.1 ± 0.3 (b)	35.9 ± 0.5 (a)	64.9 ± 0.5 (c)
0.25	10.7 ± 0.3 (b)	32.4 ± 0.8 (d)	28.6 ± 0.3 (c)	52.9 ± 0.6 (f)	28.5 ± 0.3 (c)	2.2 ± 0.2 (a)	38.8 ± 1.5 (e)
0.125	6.3 ± 0.2 (b)	10 ± 0.4 (c)	13.4 ± 0.3 (d)	44.8 ± 0.7 (e)	5.8 ± 0.2 (b)	0 (a)	0 (a)
0.0625	0 (a)	0 (a)	8.5 ± 0.2 (b)	37.8 ± 1.1 (c)	0 (a)	0 (a)	0 (a)
Thyme							
1	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc
0.5	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fc	100 (a) Fs	100 (a) Fc
0.25	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs	100 (a) Fs
0.125	60.7 ± 0.6 (d)	55 ± 0.8 (c)	52.3 ± 0.6 (b)	67.6 ± 0.8 (e)	36.4 ± 0.3 (a)	86.3 ± 0.7 (g)	77.4 ± 0.9 (f)
0.0625	37.9 ± 0.2 (b)	40.9 ± (c)	37.1 ± 0.7 (b)	55.8 ± 0.6 (d)	29.3 ± 0.1 (a)	56.1 ± 0.6 (de)	57.5 ± 0.3 (e)

Fs: fungistatic activity; Fc: fungicidal activity; –: no antifungal activity was shown by agar diffusion assay.
Data in the line followed by different letters in the parentheses are significantly different in LSD post hoc test ($p \leq 0.05$).
The values are means of three repetitions ± standard error.

only moderate antimicrobial activity against *A. niger, A. terreus, P. chrysogenum* and *T. viride.*

DISCUSSION

Six essential oils were tested to determine the ability to antagonize foodborne pathogens and spoilage moulds. Traditionally, the mycoflora of foods has received considerably less attention than the bacterial flora. The general antifungal activity of essential oils has been well documented but there have been fewer studies on the effects of essential oils on food spoilage and pathogenic moulds (Burt, 2004). The antimicrobial activities of tested essential oils have only been investigated on a limited number of fungal species (Sinha *et al*., 1993; Hammer *et al*., 2002; Ranasinghe *et al*., 2002; Soliman and Badeaa, 2002; Velluti *et al*., 2003; Simic *et al.*, 2004).

Among the essential oils tested, those of cinnamon and thyme were the most effective as antifungal agents, followed by catnip, tea tree and clary sage; laurel oil showed the lowest antifungal activity. In all cases the inhibitory effects were dose-dependent.

The essential oils of cinnamon, tea tree, catnip and thyme exhibited broad-spectrum antimicrobial activities; while those clary sage and laurel only showed antimicrobial activity against a few strains, and only at concentrations above 1%. The oils extracted from cinnamom, thyme, and catnip showed natural sanitizing properties which could have an important technological application in the food industry. However, the other essential oils tea tree, clary sage and laurel, which had lower antimicrobial activities, could be of used for sequential washing steps. Singh *et al*. (2002) reported that a sequential washing might be an important component to reduce contamination in freshly cut vegetables.

The use of synthetic fungicides to control food spoilage moulds has been discouraged due to their effects on food, carcinogenicity, teratogenicity, high and acute residual toxicity, long-term degradation, and other side-effects in humans (Roller, 2003). One of the major problems related to the use of these chemicals is that the fungi develop resistance. The use of higher concentrations of chemicals, to overcome the microbial resistance further enhances the risk of high level toxic residues in the products. In contrast the use of natural products to control food spoilage moulds does not seem to favour the development of resistance by the pathogens. This is due to the presence of a mixture of oil components which, apparently, have different mechanisms of antimicrobial activity (Tyler, 1992). Another important factor is that, biological compounds are comparatively biodegradable and most of them are nearly non-residual in nature. In addition, the essential oils extracted from several plants are the non-toxic, at least at the oral level. This safety feature is very important because it reduces the time and costs involved in the development and registration of a new formulation for commercial purposes. The development period and registration time frame, for most chemical fungicides, is long and registration costs are high mainly due to concern regarding the possible high toxicity of such materials; long-term toxicological testing on experimental animals is therefore required. In contrast, biological products usually only require short-term toxicological tests because of their target specificity (Roller, 2003).

However, further studies are needed to determine the acceptability of foods that have been treated with essential oils to reduce and control pathogen contamination or native microflora.

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