Scientific Bulletin, Series F, Biotechnologies, Vol. XVI, 2012
ISSN Online 2285-5521, ISSN-L 2285-1364

PRELIMINARY RESEARCH TO DEVELOP ACTIVE PACKAGING FOR BAKERY PRODUCTS USING ESSENTIAL OILS

Alina A. DOBRE[1], Petru NICULIȚĂ[2]

[1]University of Agricultural Sciences and Veterinary Medicine, Faculty of Agriculture, 59 Marasti Blv, 011464, Bucharest, Romania;
[2]University of Agricultural Sciences and Veterinary Medicine, Biotechnology Faculty, 59 Marasti Blv, 011464, Bucharest, Romania;

Corresponding author email: dobrealinaa@yahoo.com

Abstract

In this research the aim was to evaluate the antifungal properties of aromatic plant essential oils in vapour phase, against various fungal strains that causes bakery products alteration. The vapours of clove oil, oregano and white thyme oil were tested against five fungal strains (Aspergillus flavus, Aspergillus oryzae, Aspergillus brasiliensis, Fusarium culmorum, Fusarium graminearum) in a closed system using disc volatilization method which identifies the antifungal potential of essential oil vapours. The results obtained were expressed as minimum inhibitory concentration (MIC) in ppm in a 365.64 cm^3 volume Petri dish. Were evaluated nine different concentration of these essential oils (50, 100, 200, 400, 600, 800, 1000, 1500, 2000 ppm) obtained by dilution in a 10% DMSO solution. The best result after seven days of exposure, were shown by oregano oil against all tested fungi with a MIC of 1000 ppm, especially against Fusarium spp., which presented no growth in the area above the disc with oil and no sporulation activity in rest of the Petri dish. Aspergillus ssp. was more resistant to the action of oregano vapours and gives a MIC of 1500 ppm. The vapour phase of clove and white thyme oils were more active at a MIC of 1500 ppm against all fungal strains while at 1000 ppm induced low mycelia growth, influenced the germination of spores which caused immature spores. Vapours of clove oil affected mainly Fusarium spp. by total inhibition of growth in the area of action and in rest affected mycelia development. White thyme oil had a low impact against Aspergillus spp. under study and the most efficient vapours concentration was of 2000 ppm. Main morphological changes observed under light microscopy on fungal strains grown in atmosphere of essential oils were disrupted cell structure, considerable alterations in hyphae, reduced number of conidiospores and loss of pigmentation. In conclusion, tested essential oils were effective in vapour phase leading to important alterations in fungal structure and sporulation process. This work gave us the opportunity to put the base of a protective active atmosphere using essential oils like natural antifungal agents, which could extend the shelf life of packaged bakery products.

Key words: *antifungal activity, essential oils, light microscopy, vapour phase.*

INTRODUCTION

In bakery industry, bread occupies a unique position both in production and utilization as compared to other bakery products. Bread is the most important commercial product of wheat flour consumed as staple food by most of the wheat-eating people. In Romania annual bread consumption is in the region of 97 kg per person, a third more than the European average, twice than Danes and almost seven times over the consumption in UK [8]. Consumption of bread in Romania is so high because bread is considered a basic food and also because of the national tradition, the people budget and living standards.

Since, bread is an important part of our daily diet ways and means should be explored to improve the quality and shelf life. Mould spoilage is the most common form of deterioration of bread. At the present time mould spoilage of bread is prevented by addition of chemical preservatives such as propionic, sorbis and acetic acids and their salts [11]. Since the 1980's the bread industry has been working to reduce the number of additives and so called synthetic preservatives in a genuine effort to make bread as natural and fresh as possible [7]. In the last few decades a great interest has emerged for natural preservation of food products using plant extract and essentials oils. Essential oils are volatile oily liquids obtained from

different plant parts and widely used as food flavours [4]. The antimicrobial activity of essential oils has been studied and demonstrated for years against a number of microorganisms, by different test methods such as direct contact antimicrobial assays, diffusion assays or dilution methods [4] [10]. The most interesting area of application for essential oils consist of their incorporation directly into the packaging material, coated onto polymer surface, or immobilized to polymers [3], resulting an antimicrobial packaging which preserves, in a natural manner, different food products. The use of essential oils and their chemical compounds, categorized as flavourings by the European Union [1] [2] and as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration, in food preservation is a attractive opinion for "green" food products.

This research paper has two aims. First, to evaluate the antifungal activity of essential oils in vapour phase at different concentration against food spoilage and toxigenic fungi. This aim is important in identification of minimum inhibitory concentration (MIC) of volatile vapours in order to be used in future researches in active packaging of bread. Second, to identify the changes and abnormalities caused by essential oils against fungal cell by microscopic methods.

MATERIAL AND METHOD

Fungal strains and growth media

In this study were used five toxigenic fungal strains involved in grain contamination and spoilage and decay of food products especially bakery products. The fungal strains selected for the assessment of antifungal activity of volatile vapours of essential oils were as fallows: *Fusarium culmorum* 46 and *Fusarium graminearum* 96 from I.C.D.A Fundulea, *Aspergillus flavus* and *Aspergillus oryzae* from C.B.A.B Biotehnol, *Aspergillus brasiliensis* (niger) ATCC 16404 from MicroBioLogics, SUA.

Fungal strains were maintained on Potato Dextrose Agar (Biokar Diagnostics) at 4^0C and used as stock cultures. Spore suspensions were prepared in a 10 ml solution of NaCl 1% (w/v) containing 5% Tween 80 (w/v) from fresh stock cultures grown on PDA Petri dish for 7 days at 25^0C. Spore suspension obtained was diluted in sterile Peptone Physiological Serum to a concentration of 10^5spores/ml. Spore suspension was counted using plate agar method on PDA culture medium.

Essential oils and work concentrations

Based on preliminary researches on the antifungal activity of seven essential oils, three essential oils were chosen due to their active inhibition against presented fungal strains.

The essential oils of white thyme oil (*Thymus vulgaris),* oregano oil (*Thymus capitatus),* and clove bud oil (*Eugenia caryophyllata*) obtained by steam distilation, purchased from Sigma Aldrich, Germany, were the oils selected for this research. Essential oils quality parameters (Chemical Abstract Service, CAS, number, appearance, color, purity, odor, and density at -20^0C and refraction index at -20^0C) were described in an accompanying technical report.

In order to obtain work concentrations of 200, 400, 600, 800, 1000, 1500 and 2000 ppm, the essential oils were diluted in a 10% DMSO (Dimethyl sulfoxide, Sigma Aldrich, Germany) sterile medium solution based on their density.

The work concentration prepared were mixed for total solubilization at 180 rpm for 10 minutes and kept at room temperature until subsequent use.

Antimicrobial assay [12]

In vitro antifungal activity of volatile vapours of essential oils was evaluated by micro atmosphere method, a modified disc diffusion method at seven different concentrations (200 – 2000 ppm).

This method allowed us to determine the MIC (minimum inhibitory concentration) of essential oils vapour phase. The MIC is defined as the lowest concentration of the compound that inhibits growth of a microorganism after a specified incubation period [13].

In brief, 15 ml of warm PDA medium was poured into a 90 mm sterile plastic Petri dish (volume recorded with the medium poured into the dish of 365.64 cm^3) and after

solidification the medium in the dish was inoculated with 100 µl of spore suspension (10^5spores/ml) of the microorganism under study. In the cover of the Petri dish was cast a thin layer sterile medium on which was placed a 6 mm diameter paper disc (Whatman, no 1) with 10 µl of different essential oils concentration. The dishes were then sealed using sterile laboratory Parafilm to avoid evaporation of the essential oils, fallowed by incubation at 25^0C for seven days.

After incubation, the minimum inhibitory concentrations (MICs) were recorded based on the inhibitory activity against fungal growth. The MIC was defined as the lowest concentration witch made clearly visible inhibition zones. Blank discs with and without 10 % DMSO solution served as negative control.

Microscopic observation of morphological changes

Fungal strains grown on nutritive medium (PDA) treated with essential oils vapours were evaluated for any morphological changes using a light microscope (Olympus U-CMAD 3). Representative samples were taken from the surface of the fungal colony, from the margin next to the inhibition zone due to essential oils activity. The sample was placed on a clean microscopic slide and viewed on the microscope for any abnormalities. The important changes in the fungal microscopic appearance were noted and commented.

RESULTS AND DISCUSSIONS

Results obtained from the antimicrobial assay of volatile vapours of essential oils after seven days of exposure are summarized in Table 1. As was expected, all the essential oils tested expressed antifungal activity in vapour phase but with a highest concentration than in direct contact method (results are not shown in this article). Notable inhibition areas due to volatile vapours of essential oils were presented for higher concentration, the main concentrations selected were 1000, 1500 and 2000 ppm.

Table 1. MIC (ppm) of essential oils in vapour phase against toxigenic fungal strains

Essential oils	Fungal strains under study				
	F. c.	*F. g.*	*A. b.*	*A. f*	*A. o.*
Clove bud	1500	1500	1500	1500	1500
White thyme	1500	1500	2000	2000	2000
Oregano	1000	1000	1500	1500	1500

MIC – minimum inhibitory concentration; F.c. – *Fusarium culmorum*; F.g. – *Fusarium graminearum*; A. b. – *Aspergillus brasiliensis*; A. f. – *Aspergillus flavus*; A. o. – *Aspergillus oryzae*

The vapour of essential oil of oregano has showed an inhibition activity for all the fungal strains tested at 1000 ppm, especially for Fusarium spp. In this case, Fusarium spp. presented no growth in the area above the disc with oregano oil and no sporulation in the rest of the plate (Photo 1.).

In terms of susceptibility expressed by Aspergillus spp. at oregano oil vapours, the MIC who gave better result of inhibition was 1500 ppm, which limited the growth of the mycelium and development of fungal spores. *Aspergillus oryzae* and *Aspergillus flavus* growth were inhibited by vapour phase of oregano oil at 1500 ppm concentration.

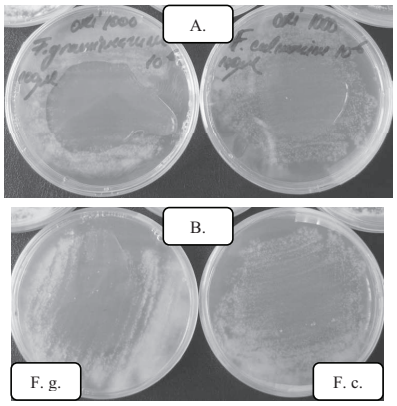

Photo 1. Vapour phase activity of oregano oil at 1000 ppm against Fusarium ssp.
F. g. – *Fusarium graminearum*; F. c. – *Fusarium culmorum*; A – front side of the plates; B - reverse side of the plates.

Growth of these strains was observed only on the margins of the Petri dish were the vapour did not have a direct diffusion, just mycelium development and no sporulation action. *Aspergillus brasiliensis* presented a low

development in the area above the oregano essential oil, but a good sporulation in the rest of the Petri dish. Clove bud essential oil expressed the same MIC against fungal strains tested as oregano oil, but Fusarium ssp. were more sensitive to 1500 ppm concentration of clove oil that 1000 ppm by oregano oil. At 1500 ppm concentration, clove bud oil inhibited fungal growth in the area above the disc and in the rest of the Petri dish there were visible affected mycelia, no sporulation and discoloration of the fungal mass.

The less effective in vapour phase against tested fungal strains was white thyme essential oil. MIC of thyme oil was of 2000 ppm in case of Aspergillus spp., from which *Aspergillus oryzae* was the most susceptible to the activity of the vapours, presenting no growth in the area above the disc and no sporulation in the rest of the Petri dish. Spore germinations of Fusarium *culmorum* and *Fusarium graminearum* were completely inhibited by the volatile vapours of thyme essential oil at 1500 ppm concentration (Photo. 2).

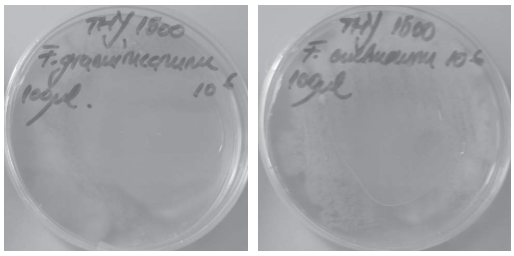

Photo 2. Vapour phase activity of 1500 ppm concentration of thyme oil against Fusarium spp.

Fusarium spp. were more sensible to the action of essential oils vapours, situation presented in the case of all tested essential oils but more predominant to oregano and thyme oil. *Aspergillus brasiliensis* showed more resistance to essential oils activity, and have grown in all the Petri dish after 10 days of exposure (data not showed).

Morphological alteration of fungal strains

Observation of fungal strains under the light microscope at 200, 400 and 500 X magnification after exposure to essential oils vapours showed some morphological abnormalities. Fungal cells exposed to clove oil vapour underwent considerable morphological abnormalities in comparison with the control. Control cells of *Aspergillus brasiliensis* showed regular growing hyphae with homogenous cytoplasm and visible conidiation on a large conidial head (Photo no. 2 – A) while the mycelia trated with clove oil vapours presented lack of sporulation, loss of cytoplasm content and distorted development of hyphae (Photo 3 – B).

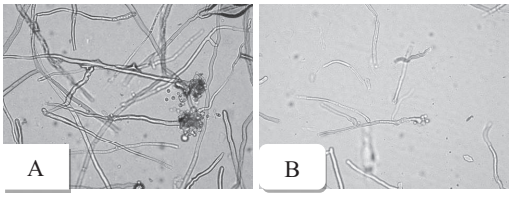

Photo 3. Light microscopy (100 X) of *A. brasiliensis* mycelium growing on PDA in clove bud oil atmosphere during 7 days of incubation at 25 – 28 °C. A – Normal conidial head of *A. brasiliensis*, development of vesicle on conidiophores, conidia clearly visible; B – treated with clove oil vapours, showing clear reduction in conidiation, disrupted hyphae integrity.

The same changes, caused by clove bud oil and oregano oil, in fungal cellular structure was observed also in microscopic preparations of *Aspergillus oryzae* and *Aspergillus flavus* (figure no. 4). The fungal cells were affected by the active vapours of clove oil by presenting reduction in conidiation, unspecific development of conidiophores and shorter hyphae.

The essential oils clearly caused reduction in conidial heads of *Aspergillus flavus*, with distorted presence of conidiophores (Photo 4 - B), decreased conidiation (lack of sporulation), visible loss of cytoplasm content, abnormal development of hyphae (Fig. 4. - C). Thyme essential oils caused the same abnormalities to Aspergillus spp., mainly reduction in conidial head.

In the case of Fusarium spp., vapour of essential oils tested act against macroconidia formulation, reduce chalmydosopores development, lack of pigmentation of hyphae and loss of structure (Photo 5).

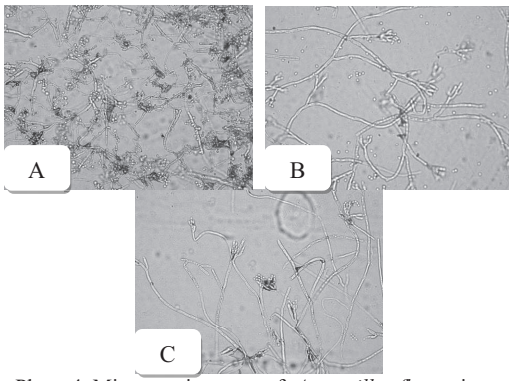

Photo 4. Microscopic aspect of *Aspergillus flavus* in control (A) and after exposure to clove (B) and oregano (C) essential oils

It is clear from these results that the vapour treatement not only alters the cell dimension and overall morphology, but has an important impact also on the surface of the cells.

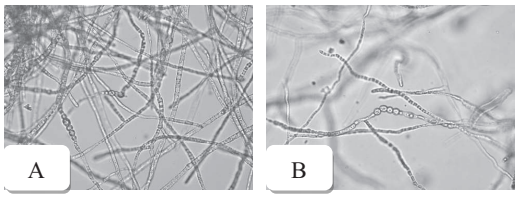

Photo. 5. Morphological changes induced by oregano oil vapours against *Fusarium graminearum*. A – Control sample present normal mycelia and chalmydospores. B – Treated mycelia present a significant reduction in hyphae and chalmydospores formation.

CONCLUSIONS

The investigations on antifungal activity of vapour phase of three essential oils against toxigenic fungi showed that by the micro atmosphere method is necessary a higher concentration of essential oils than in direct contact method, in order to express a good antifungal activity. All the tested essential oils inhibited the growth of fungal strains but at a higher concentration as presented in Table no. 1 in which are given the values of MIC's for each fungal strain. Although the MIC of essential oils was between 1000 – 2000 ppm, this concentration has a sensorial print that is not unpleasant. Fusarium spp. were more sensitive to the action of esssential oils vapours than Aspergillus spp, presenting low development of mycelia and lack of sporulation in all the Petri dish not only in the impregnated essential oil disc. The essential oils tested expressed a suppressing effect on the fungal spore development, fact presented by light microscopy.

The observations of light microscopy showed that the main morphological changes caused by tested essential oils on Aspergillus spp. were associated with the degeneration of fungal hyphae and in the sporulation process. In terms of morphological structure of Fusarium spp, the treatment with essential oils presented a reduction in chlamydospores formation and loss of structure of hyphae. Different behavior of the fungal strains in the essential oil atmosphere may be determined by the different chemical composition and concentration of phenol compounds in each type of essential oil utilized in this study.

In conclusion, our results indicate that essential oils could find a practical and applicable use in the inhibition of mould growth. Therefore, using volatile vapour of essential oils as an antifungal agent may provide a useful application in active packaging of different food products especially bakery products.

ACKNOWLEDGEMENTS

This research was supported by the Sectorial Operational Programme Human Resources Development 2007-2013 under the project number POSDRU/88/1.5/S/52614 „PhD scholarships to increase the quality of training young researchers in the field of agronomy and veterinary medicine".

REFERENCES

[1] 2002/113/EC, 2002, *Commission Decision of 23 January 2002 amending Commission Decision 1999/217/EC as regards the register of flavouring substances used in or on food stuffs*. Official Journal of the Europe Community, L49, 1 – 160.

[2] 89/107/EEC, 1989, *Council Directive of 21 December 1988 on the approximation of the laws of the Member States concerning foods additives authorized for use in foodstuffs intended for human consumtion.* Official Journal of the European Community, L40, 27 – 36.

[3] Appendini, P., and Hotchkiss, JH. 2002. *Review of antimicrobial food packaging. Innovative Food Science & Emerging Technologies*, 3, 113–126.
[4] Burt, S. 2004. *Essential oils: their antibacterial proprieties and potential application in food. Journal of Applied Microbiology,* 94, 223-253.
[5] Cavanagh, H.M.A., 2007, *Antifungal activity of the volatile phase of essential oils: a brief review. Natural* Product Communications, 2, 1-3.
[6] Chee Hee, Y., and Lee Eun, H. 2004. *Antifungal activity and inhibitory modes of volatile vapours of essential oils. Mycrobiology,* 32(2), 102-104.
[7] Casdagli, T., 2000, *Bakery industry 2000+: the challenges ahead*, Food Sci Techn Today, 14, 87- 90;
[8] *General Bakary Market Statistics – American Institute of Baking*, https://www.aibonline.org/resources/statistics/generalmarket.html.
[9] Guynot, M.E., Ramos, A.J., Setó, L., Purroy, P., Sanchis, V., and Marín, S. 2003. *Antifungal activity of volatile compounds generated by essential oils against fungi commonly causing deterioration of bakery products.* Journal of Applied Microbiology, 94, 893-899.
[10] Holley R.A., Patel D. 2005. *Improvement of shelflife and safety of perishable foods by plant essential oils and smoke antimicrobials*. Food Microbiology, 22: 273–292.
[11] Karin I. Suhr, Per V., Nielsen, 2005, *Inhibition of fungal growth on wheat and rye bread by modified atmosphere packaging and active packaging using volatile mustard essential oil*, vol 70, nr. 1, Journal of Food Science.
[12] Lopez, P., Sanchez, C., Batlle, R., and Nerin, C. 2005. *Solid- and vapor-phase antimicrobial activities of six essential oils: Susceptibility of selected foodborne bacterial and fungal strains*. *Journal of Agricultural and Food Chemistry,* 53(17), 6939-6946.
[13] Parish, M.E., Davidson, P.M., 1993, *Method for evaluation. In Antimicrobials in Food*, 2 nd ed, ,pp 597 – 615.
[14] Pitt, J. I., Hocking, A. D., 2009, *Fungi and Food Spoilage*, Ed Springer, Third edition, p.15.
[15] Stanley P. Cauvain, 2003, *Bread Making: Improving Quality, Woodhead Publishing,* 2003, pag 505 – 511.
[16] Suhr, K.I., and Nielsen, P.V. 2003. *Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi.* Journal of Applied Microbiology, 94, 665–674.
[17] Tyagi Amit, K., and Malik, A. 2010. *Liquid and vapour-phase antifungal activities of selected essential oils against Candida albicans: microscopic observations and chemical characterization of cymbopogon citrates.* BMC Complementary and Alternative Medicine, 10, 65.
[18] Velutti, A., Sanchis, V., Ramos, AJ., Agido, J., and Marin, S. 2003. *Inhibitory effect of cinnamon, clove, lemongrass, oregano and palmarose essential oils on growth and fumonisin B1 production by Fusarium proliferatum in maize grain*. International Journal of Food Microbiology, 89, 145 – 154.