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Environmental Factors Affecting Efficacy of Some Essential Oils and Potassium Sorbate to Control Growth of *Aspergillus flavus*, *Aspergillus parasiticus* on Wheat and Maize Grains

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ABSTRACT

The antifungal potential of essential oils of thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.), and laurel (*Laurus nobilis* L.) was determined. To establish this antifungal potential, two molds related to feed spoilage, namely, *Aspergillus flavus* and *Aspergillus parasiticus,* were selected. The agar dilution method was employed for the determination of antifungal activities. The investigated essential oils exhibited inhibitory effects on both molds tested. Thyme oil showed the highest inhibition of mold growth, followed by rosemary and laurel. Thyme essential oil was a stronger inhibitor against *A. parasiticus* than against *A. flavus.*The finding of the present study suggests that thyme essential oil inhibits the growth of fungi attacking stored feed and strengthens the possibility of using it as the alternative to potassium sorbate as effective inhibitor of biodegrading and storage contaminating fungi.

Keywords: Antifungal potential, Laurel, Rosemary, Thyme.

INTRODUCTION

The presence and growth of fungi in food may cause spoilage and result in a reduction in quality and quantity. Some *Aspergillus* species are responsible for many cases of food and feed deterioration (Abarc *et al*., 1994). *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxins in food and feedstuffs. Aflatoxins are known to be potent hepatocarcinogens in animals and humans (Bennet and Klich, 2003; Galvano, 2005). Therefore, the presence of toxigenic fungi and mycotoxins in foods and grains stored for long periods of time presents a potential hazard to human and animal health. Considerable interest has developed in the preservation of feeds by the use of essential oils to effectively suppress growth of such fungi and mycotoxin production.

Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. For these reasons, consumers tend to be suspicious of chemical additives and, thus, the demand for natural and socially more acceptable preservatives has been intensified (Leite *et al.,* 2006). The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Schuenzel and Harrison, 2002). Antimicrobial properties of herbs and spices have been recognized and used since ancient times for food preservation and in medicine. Conner (1993) reported on natural antimicrobial agents dating back more than a century. A renewed interest in natural preservation appears to be stimulated by the present food safety concerns, growing problems with microbial resistance, and a

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rise in production of minimally processed food, together with green image policies of food industries. Numerous studies have documented the antifungal (Cairns and Magan, 2002; Bluma and Etchevery, 2008) and antibacterial (Canillac and Mourey, 2001; Dorman and Deans, 2000) effects of plant essential oils. Screening experiments with 13–52 essential oils and their major active constituents against 5–25 microorganisms, (Conner and Beuchat, 1984; Deans and Ritchie, 1987; Dorman and Deans, 2000) have reported thyme, clove, cinnamon, rosemary, oregano, laurel, and lemongrass to be some of the best broad spectrum candidates for inhibition of food-borne pathogens and spoilage organisms (Burt, 2004).

This study was undertaken to investigate the antifungal activities of essential oils of three spices (thyme, laurel, and rosemary) on maize and wheat grains under different conditions of water activity and temperatures.

MATERIALS AND METHODS

Fungal Strains and Culture Medium

For inoculation purposes, *A. flavus* and *A. parasiticus* were obtained from a culture at the Laboratory of Food Microbiology, TUBITAK Turkey. The cultures were grown on yeast extract sucrose (YES) basal medium (20 g yeast extract, 20 g agar, 150 g sucrose, 1 L distilled water were autoclaved for 20 minutes at 121°C, 1 atm^{-1}).

Preparation of Essential Oil Extract

Thyme, rosemary, and laurel were collected from Istanbul Province in Turkey. The fresh leaves of thyme, laurel, and rosemary were placed in a round-bottom flask of a Clevenger-type apparatus with water and the oil was hydrodistilled for 3h in this apparatus (Hussain *et al*., 2008). After distillation, the essential oils were stored in sealed glass containers and refrigerated in the dark at 4°C until use.

Essential Oil Analyses

GC/MS analyses of the main components of each essential oil extract (Table 1) were done on a Perkin-Elmer Q700 chromatograph equipped with SE-30 capillary column 30 m×0.25 mm×0.25 µm film thickness. Operating conditions were as follows: Oven temperature was held at 60°C (3 minutes) and then linearly programmed to 240°C at a rate of 5 °C min^{-1}. Injector and detector were heated to 250°C as well as ion source (EI, 70eV). Helium was used as a carrier gas at constant flow of 0.90 ml min^{-1}. The oil components were identified using retention indices as a preselection routine and comparison of acquired mass spectra to

Table 1. Content of major constituents in tested essential oils (%).

Thymus vulgaris		*Rosamarinus officinalis*		*Laurus nobilis*	
Thymol	25.13	Borneol	7.40	α-Pinene	3.21
Carvacrol methyl ether	5.30	Camphor	8.80	Sabinene	6.35
Camphene	2.58	1,8-Cineole	2.20	β-Pinene	2.69
α-Humulene	2.20	γ-Terpinene	13.20	1,8-Cineole	37.7
Carvacrol	15.93	β-Pinene	8.69	Linalool	9.10
α-Pinene	2.05	Caryophyllene oxide	3.30	α-Terpineol	2.80
Camphor	2.80	α-Pinene	3.21	α-Terpinyl acetate	11.30
p-Cymene	12.18	Sabinene	6.35	Eugenol	3.80
γ-Terpinene	8.75	p-Cymene	12.18	Methyl eugenol	6.80
Borneol	8.85				

those from available literature (Craveiro *et al*., 1984; Adams, 2007).

Antimicrobial Assay (Disk Diffusion Aassay)

The essential oils were screened for antimicrobial activity using the agar diffusion technique (Turkusay and Onogur, 1998) against two microorganisms of significant importance.

Filter paper disks (Whatman No. 1, 6 mm diameter) containing 15 µL of each essential oil were applied to the surface of agar plates that were previously seeded by spreading of 0.1 ml overnight culture. The plates were incubated overnight at the appropriate temperature and the diameter of the resulting zone of inhibition was measured in millimetres. The results indicated in Figure (1a-b) and in the text represent the net zone of inhibition including the diameter (6 mm) of the paper disk. The scale of measurement was the following (disk diameter included): ≥ 20 mm, strongly inhibitory zone of inhibition ; < 20–12 mm, moderately/mildly inhibitory zone of inhibition , and < 12 mm, not inhibitory.

Substrate

Stored maize and wheat grains were irradiated with 14.5 kGy of gamma irradiation and stored aseptically at 4°C. In this way, the grains had retained germinative ability. The maize grains were checked for sterility and absence of AFB_1. Initial water activity (a_w) of the grains was 0.60. For all experiments, irradiated maize grains were weighed into sterile flasks and hydrated to the desired a_w levels (0.60, 0.77, and 0.92) by addition of sterile distilled water. After that, flasks were vigorously shaken to homogenously distribute water and essential oils were added to the grains and stored at 4°C for 48 hours in order to allow balanced conditions.

Inoculation, Incubation and Growth Assessment

Rehydrated maize and wheat were placed in sterile Petri dishes (20 g per plate, approximately) forming a single layer of grains covering the whole plate. A 5-mm diameter agar disk was taken from the margin of a 3–day–old growing colony on MMEA at 25°C of each isolate and transferred to the grain placed in the centre of each plate. Plates containing grain at the same a_w level and the same essential oil were placed in containers along with beakers containing glycerol water solutions of the same a_w as the grains in order to create an atmosphere with the same equilibrium relative humidity. Containers were kept at 21 and 34°C. All treatments were repeated twice. Diameters of growing colonies were

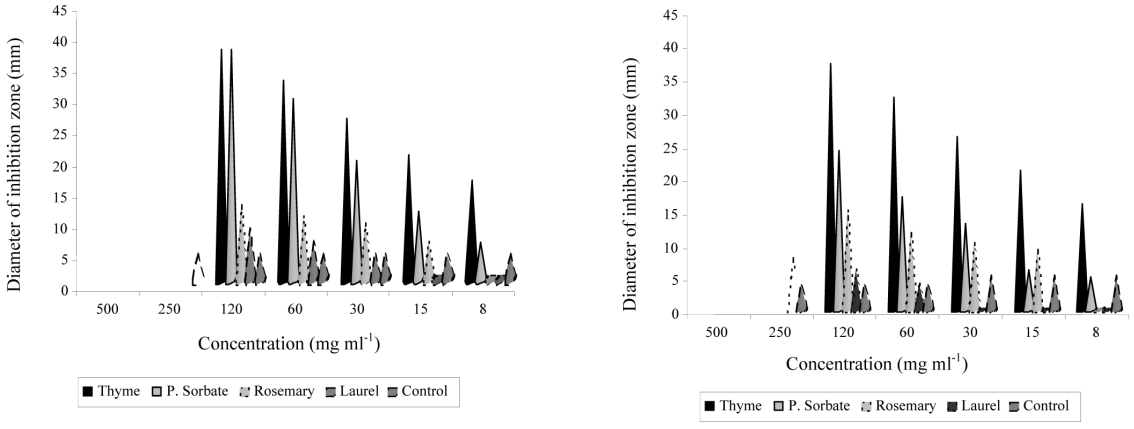

Figure 1. Effects of treatments on growth of (a) *Aspergillus parasiticus*, (b) *Aspergillus flavus*.

measured every day with the aid of a binocular magnifier. Two diameters were obtained from each colony and growth rates expressed as mm day^{-1} were calculated by linear regression of colony radius against time for each set of conditions tested. After 28 days, the grains were frozen at -20°C for later AFB_1 analysis.

Aflatoxin B_1 Analyses

After 28 incubation days, AFB_1 was extracted from maize grains and quantitatively determined by HPLC following the methodology proposed by Trucksess *et al.* (1994). Fifty grams of milled maize grains samples were homogenized with acetonitrile/water (90:10) by shaking in an orbital shaker and the extracts were filtered through Whatman No. 4 filter paper. A 3 ml aliquot of each extract was applied to a clean up column (Mycosep 2224 MFC, Romer). A 200 µL aliquot was derivatized with 700 µL of trifluoratic acid/acetic acid/water (20:10:70). The derivatized aflatoxins (50 µL solution) were analyzed using a reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of an HP1100 pump (Hewlett Packard, Palo Alto, CA, USA) connected to an HP10464A progrmmable flurescence detector, interfaced to an HP ChemStation. Chromatographic separations were performed on stainless steel, C18 reverse-phase column (150×4.6 mm ID, 5 µm particle size; Luna-Phenomenex, Torrance, CA, USA). Water/methanol/acetonitirle (4:1:1) was used as the mobile phase, at a flow rate of 1.5 ml min^{-1}. The fluorescence of AFB_1 was detected at excitation and emission wavelengths of 360 and 440 nm, respectively. Calibration curves were constructed with different levels of AFB_1. This toxin was quantified by correlating the peak height of sample extracts to that of the calibration curve. The mean recovery percentage for AFB_1 was 94.5±3.2%. The limit of detection of the analytical method was 1 ng g^{-1}.

Statistical Analyses

A full factorial design was used. The factors were a_w, temperature, concentration of essential oil, and the response were diameters of growing colonies and AFB_1 concentration. Analysis of variance was performed for colony diameters and AFB_1 concentration using SAS version 16.0 (SAS Institute, Cary, NC, USA). Statistical significance was judged at the 5% level.

RESULTS

Composition of Essential Oils

The main components of thyme, laurel, and rosemary essential oils were identified by GC-MS analyses and listed in Table 1.

Antifungal Activity

Antifungal Activity *In vitro*

Each essential oil showed notable antifungal activities against *A. parasiticus* and *A. flavus,,* Figure (1a-b). Statistical results showed that kind and amount of essential oils had a significant effect. But, storage time had no significant influence on the antifungal activity (P> 0.05).

Antifungal Activity *In vivo*

The effects of different temperatures (22 and 34°C) and different concentrations of essential oils on *A. parasiticus* and *A. flavus* in sterile maize grain are given in Table 2. During 28 days period, no significant difference was shown between the mould values of the control and the essential oils treatments. In the case of maize grain samples, the results showed a significant

Table 2. Analysis of variance of the effect of different temperature (22 and 34°C) on the control of *A. parasiticus* and *A. flavus* in sterile maize grain by the essential oils and potassium sorbate.[a]

Mould	Temperature	Essential oil	Maize				
			Moisture	pH	AW	Mould	AFB_1
A. parasiticus	22°C	Control	16.56a	6.01ab	0.66ab	731.67a	13.17a
		Thyme	12.89ab	6.12ab	0.66ab	157.33b	2.80b
		Rosemary	12.50b	6.35ab	0.54b	168.33b	3.00b
		Laurel	12.64b	6.26ab	0.70ab	154.67b	2.67b
		P. sorbate	13.09ab	6.33ab	0.72ab	171.67b	3.04b
A. flavus		Control	16.41a	6.08ab	0.70ab	645.67a	11.87a
		Thyme	13.27ab	6.13ab	0.65ab	170.67b	3.01b
		Rosemary	12.85ab	6.20ab	0.67ab	67.67b	0.00b
		Laurel	14.47ab	6.35ab	0.60ab	127.67b	1.95b
		P. sorbate	13.01ab	6.42a	0.68ab	142.33b	2.17b
A. parasiticus	34°C	Control	12.72ab	6.69a	0.71ab	7.33b	0.00b
		Thyme	10.51b	6.65a	0.71ab	19.17b	0.08b
		Rosemary	10.33c	6.98a	0.63ab	17.00b	0.00b
		Laurel	9.92c	6.52a	0.66ab	7.33b	0.00b
		P. sorbate	6.25d	6.57a	0.77a	4.00b	0.00b
A. flavus		Control	12.34b	6.73a	0.63ab	3.00b	0.00b
		Thyme	10.83b	6.96a	0.70ab	6.33b	0.00b
		Rosemary	10.6 b	6.73a	0.63ab	3.67b	0.00b
		Laurel	11.43b	6.83a	0.68ab	14.67b	0.00b
		P. sorbate	9.00cd	5.42 b	0.60ab	3.50b	0.00b
Standard error of mean			0.314	0.068	0.012	21.13	0.391
Source of variation					*P* level		
Mould			0.193	0.618	0.393	0.288	0.192
Temperature			<0.001	<0.003	0.594	<0.001	<0.001
Essential oil			<0.001	0.394	0.324	<0.001	<0.001
Mould×Temperature			0.676	0.519	0.254	0.387	0.204
Mould×Essential oil			0.764	0.354	0.292	0.929	0.842
Temperature×Essential oil			0.288	0.057	0.903	<0.001	<0.001
Mould ×Temperature×Essential oil			0.866	0.341	0.360	0.942	0.823

[a] Values with different letters in the same column are statistically significantly different (P< 0.05).

effect of temperature (P< 0.001), except for pH (P< 0.003) and, a_w (P= 0.594). In a similar way, the essential oil additive was also significant (P< 0.001), except for pH (P= 0.394) and, a_w (0.324), but the interaction (mould×essential oil×temperature) were not significant in any case.

The effect of different temperatures (22 and 34°C) and different concentrations of essential oils on *A. parasiticus* and *A. flavus* in sterile wheat grain are given in Table 3. During 28 days period, no significant difference was shown between the mould values of the control and the essential oil treatments. In the case of wheat grain samples, the results showed a significant effect of temperature (P< 0.001), except for pH (P< 0.004). In a similar way, the essential oil additive was also significant (P< 0.001), except for moisture content (P= 0.012) and, a_w (0.968), but the interaction (mould×essential oil×temperature) were not significant in any case.

DISCUSSION

In the experiment, thyme essential oil showed the highest inhibition of mold

Table 3. Analysis of variance of the effect of different temperature (22 and 34°C) on the control of *A. parasiticus* and *A. flavus* in sterile wheat grain by the essential oils and potassium sorbate.[a]

Mould	Temperature	Essential oil	Wheat				
			Moisture	pH	AW	Mould	AFB_1
A. parasiticus	22°C	Control	16.02ab	6.72abc	0.70	734.67a	13.19a
		Thyme	11.88b	6.16d	0.73	192.83b	3.44b
		Rosemary	13.42abc	6.56bc	0.66	152.67bc	2.73bcd
		Laurel	13.35abc	6.51bc	0.71	167.50bc	3.19bc
		P. sorbate	14.40ab	6.84ab	0.71	149.33bc	2.85bcd
A. flavus		Control	16.99a	6.62abc	0.68	709.00a	12.07a
		Thyme	13.29abc	6.44c	0.70	132.00bc	2.56bcd
		Rosemary	13.78ab	6.39cd	0.70	121.17bc	2.32bcd
		Laurel	14.38ab	6.66abc	0.72	144.00bc	2.70bcd
		P. sorbate	15.22ab	6.75abc	0.69	158bc	3.01bcd
A. parasiticus	34°C	Control	13.71abc	6.80ab	0.80	7.00c	0.11cd
		Thyme	12.18b	6.48c	0.82	1.33c	0.00cd
		Rosemary	11.41c	6.71abc	0.79	2.67c	0.00cd
		Laurel	11.36c	6.67abc	0.80	12.00c	0.11bcd
		P. sorbate	12.21b	6.56bc	0.82	9.00c	0.14bcd
A. flavus		Control	13.72abc	6.74abc	0.81	2.00c	0.00cd
		Thyme	10.59c	6.48c	0.76	2.00c	0.00cd
		Rosemary	11.60b	6.76abc	0.80	6.33c	0.00cd
		Laurel	12.80abc	6.72abc	0.82	5.67c	0.08cd
		P. sorbate	12.27b	6.89a	0.82	0.83c	0.00d
Standard error of mean			0.3039	0.0241	0.0115	22.11	0.3877
Source of variation					*P* level		
Mould			0.4202	0.259	0.877	0.546	0.476
Temperature			<0.001	<0.004	<0.001	<0.001	<0.001
Essential oil			0.012	<0.001	0.9688	<0.001	<0.001
Mould ×Temperature			0.441	0.457	0.941	0.631	0.562
Mould×Essential oil			0.967	0.218	0.848	0.997	0.993
Temperature×Essential oil			0.916	0.112	0.952	<0.001	<0.001
Mould ×Temperature×Essential oil			0.910	0.056	0.991	0.991	0.990

[a] Values with different letters in the same column are statistically significantly different (P< 0.05).

growth, followed by rosemary and laurel. Thyme oils possess useful antimicrobial and antioxidant properties that may be utilized in the food industry and as a dietary supplement. Various species of thyme have been reported to possess antifungal properties (Lambert *et al.,* 2001; Soliman and Badeaa, 2002; Rasooli and Razzaghi, 2004; Pillai and Ramaswany, 2012). The oil showed a strong inhibitory effect against all fungi investigated (Couladis *et al*., 2004). At present, the essential oils of many *Thymus* species are widely used as flavoring agents in food processing and many pharmacological preparations, and particularly thyme oil is still among the world's top 10 essential oils (Stahl-Biskup, 1991; Chia-Wen *et al*., 2009; Chrpova, 2010).

Maize and wheat are used in many feedstuffs. Today, considerable interest has developed in the preservation of foods and feeds by the use of essential oils to effectively control growth of fungi and mycotoxin production. The fungal growth and survival of these genera are markedly affected by water availability, which is one of the limiting factors in the functioning

ecosystems (Aldred *et al*., 2008). Water activity did not seem to be influenced in any of the investigated feed components. Harvested grains that contain aflatoxigenic fungi can significantly decrease the quality and economic value of the harvested grain. The moisture content of harvested grains is often 18–20% (a_w of 0.90–0.93) and they must subsequently be dried. Sometimes, this process is inefficient and environmental conditions often result in rapid aflatoxin production (Nesci and Etchevery, 2006). When the water activity of the grain decreases to the range of 0.68 to 0.80, the *Aspergillus* and *Penicillium spp*. predominate, with minor contributions from *Absidia* and *Mucor* spp. (Ono *et al*., 1999; Raid and Kucharek, 2005; Samapundo *et al*., 2005). *A. parasiticus* was more sensitive to thyme essential oil than *A. flavus., A. flavus,* and *A. parasiticus* grow best and produce aflatoxin at temperatures greater than 21^{o}C (Thompson and Henke, 2000; Rahimifard *et al*., 2008; Sumalan *et al.,* 2013). Fungal infection is enhanced when the crops are stressed, such as during drought or infestation. Field fungi are characterized by high moisture content requirements (greater than 200 g kg^{-1}), and thus are vulnerable to drying post-harvest. Though some mycelium may remain dormant in feeds after harvesting, most die during storage or international transport (Sauer *et al*., 1992).

There are complex interactions of environmental factors, like water availability, which influence the efficacy of essential oils. It is possible to use a combination of them to reduce growth of *A. flavus* and *A. parasiticus* and aflatoxin production (Centeno *et al*., 2010). It can be concluded that food and feedstuffs industry, such as maize and wheat grain were influenced by several factors that could affect the effectiveness of the essential oils: pH of the environment, lipids that decrease activity of hydrophobic compounds, and proteins that may cause binding of some compounds and reduce activity.

Feed is a rich environment for most bacteria. Food-borne illnesses caused by consumption of food contaminated with pathogenic bacteria and/or their toxins are a great problem in animal health. Essential oils represent a source of natural antimicrobial substances and have the potential to be used in the feed industry as a preservative to prevent spoilage and to increase the shelf life of products. The essential oils could also reduce side effects by their replacement of chemical preservatives. A variety of molecules derived from essential oils also possess bioactive properties with antibacterial activity that could be used directly in feed products or in products for cleaning feed. Natural antimicrobials could be used alone or in combination with other preservation technologies (Tiwari *et al*., 2009; Sharafi *et al.,* 2010).

The finding of the present study suggests that thyme essential oil extract can be used against fungi attacking stored feed and strengthen the possibility of using it as an alternative to potassium sorbate as effective inhibitor of biodegrading and storage contaminating fungi.

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عوامل محیطی موثر بر توان بعضی اسانس ها و سوربات پتاسیم در کنترل رشد قارچ های *Aspergillus flavus* و *Aspergillus parasiticus* روی دانه گندم و ذرت

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چکیده

در این پژوهش، توان ضد قارچی اسانس آویشن (*Thymus vulgaris* L.) ، اکلیل کوهی (رزماری) (*Rosmarinus officinalis* L.) و برگ بو (*Laurus nobilis* L.) بررسی شد. برای بررسی این توان، دو کپک عامل ضایع شدن و پوسیدگی علوفه شامل *Aspergillus flavus* و *Aspergillus parasiticus* انتخاب شدند. برای تعیین فعالیتهای ضد قارچی مواد مورد نظر از روش رقیق کردن آگار استفاده شد. اسانس های مطالعه شده روی هر دو کپک اثرات بازدارندگی داشتند. اسانس آویشن روی *A. parasiticus* قدرت بازدارندگی بیشتری در مقایسه با *A. flavus* داشت.اسانس آویشن بیشترین بازدارندگی رشد قارچ را داشت و بعد از آن اکلیل کوهی و برگ بو قرار داشتند. بر پایه نتایج این بررسی می توان گفت که اسانس آویشن بازدارنده رشد قارچ های انبار علوفه است و نتایج به این گمان قوت می بخشد که می توان آن را به عنوان جایگزین سوربات پتاسیم به عنوان بازدارنده موثر ضد قارچ های زیست-تجزیه گر وآلوده کننده علوفه انباری در نظر گرفت.