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Temperature-dependent Changes in Susceptibility of *Stenotrophomonas maltophilia* to the Essential Oils of Sweet Basil (*Ocimum basilicum*) and Black Pepper (*Piper nigrum*)

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Abstract

The aims of this study was to determine the activities of essential oils from sweet basil and black pepper against *Stenotrophomonas maltophilia* and, as temperature is known to affect the *in vitro* susceptibility of *Sten. maltophilia* to aminoglycosides and quinolones, to identify temperature-dependent susceptibility. Minimum inhibitory concentrations (MICs) of the oils were determined using broth microdilution at 30°C and 37°C. Linalool was further studied using an agar-incorporation method and gradient plates using tryptone soya agar containing lecithin and Tween 80 (TSALT agar) at 30°C and 37°C. Linalool was more active (MIC 0.125% v/v) than black pepper oil (MIC 8% v/v) at 37°C using broth microdilution. MICs for linalool were two- to fourfold higher in the agar-incorporation assay compared with the broth microdilution. At 30°C, MICs for linalool and black pepper oil were twofold higher than those obtained at 37°C. Temperature-dependent susceptibility to linalool was demonstrated using TSALT gradient plates. In conclusions, linalool is more active than black pepper oil against *Sten. maltophilia*. Susceptibility to both oils manifests temperature dependence. Susceptibility to linalool is affected by the nature of the assay (i.e., agar vs broth techniques). These results highlight the need for standardization of methods used to assess susceptibility of microorganisms to plant essential oils.

Keywords: Black pepper, *Stenotrophomonas*, susceptibility testing, sweet basil, temperature dependence.

Introduction

Essential plant oils have been used for centuries as culinary ingredients and fragrant aromatics, as well as for their medicinal properties. With increasing reports of the emergence of multiple antibiotic-resistant bacteria (Alonso & Martinez, 1997; Cookson, 1998), there has been much research into the use of alternative antimicrobials like essential plant oils, including popular herbs and spices such as sweet basil (order, Lamiales; family, lamiaceae; species, *Ocimum basilicum*) and black pepper (order, Piperales; family, Piperaceae; species, *Piper nigrum*).

Sweet basil is a popular culinary herb that traditionally has been used in high-acidity foods that are prone to spoilage by acid-tolerant bacteria and has been demonstrated to be an effective antimicrobial against this group of bacteria (Lachowicz et al., 1998). Sweet basil is also an effective antimicrobial against a variety of Gram-positive bacteria, Gram-negative bacteria, yeasts, and molds (Wan et al., 1998). The constituents associated with the antimicrobial and antifungal activities of sweet basil are linalool and methyl chavicol (Sinha & Gulati, 1990). Lachowicz et al. (1998) demonstrated that pure linalool has a broad antimicrobial spectrum similar to sweet basil oil, whereas pure methyl chavicol has a much narrower antimicrobial spectrum in comparison. The activity of black pepper oil against 25 different genera of bacteria was demonstrated by Dorman and Deans (2000), who suggested its potential use as a chemotherapeutic agent, food-preserving agent, and disinfectant.

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Unlike susceptibility testing of conventional antimicrobials that is rigorously standardized, a plethora of essentially nonstandardized techniques have been used to study the activity of plant essential oils against medically important bacteria. This inevitably raises issues of reproducibility and hampers comparison of data reported in published studies. In particular, the effect of variables known to influence observed results in conventional antimicrobial testing remains underinvestigated. Knowledge of these is, therefore, of importance in the development of standardized susceptibility testing protocols for products such as plant essential oils.

The antimicrobial properties of linalool from sweet basil oil and black pepper oil were investigated using *Stenotrophomonas maltophilia* an emerging nosocomial pathogen. Infections with this bacterium are particularly difficult to manage as *Sten. maltophilia* manifests resistance to most commercially available antimicrobials including the carbapenems, and new therapeutic strategies are needed (Denton & Kerr, 1998). *In vitro* testing of susceptibility of the bacteria to antimicrobials of the aminoglycoside and quinolone class is known to be affected by temperature of incubation (Denton & Kerr, 1998). Accordingly, we assessed the activity of essential oils of sweet basil and black pepper and investigated whether temperature-dependent susceptibility could occur during in *in vitro* testing.

Materials and Methods

Bacterial Isolates and Essential Oils

Three strains of *Sten. maltophilia* (CXC 1, CXU 1, 44606) isolated from blood culture were obtained from The Leeds General Infirmary, Leeds; and two strains of *Sten. maltophilia* (M33, M99) isolated from sputum cultures were obtained from St. James' Teaching Hospital, Leeds. All bacterial isolates were maintained on horse blood agar and subcultured as required from 40% glycerol in phosphate-buffered saline (PBS) pH 7.3 (Oxoid BR149, Basingstoke, UK) at −70°C. Black pepper oil and linalool from sweet basil, produced by steam distillation, were obtained from Graham Page Ltd. (Loughton, Essex, UK) and British Natural Products Ltd. company out of business, respectively. Nutrient broth was supplemented with 0.15% w/v agar and autoclaved at 121°C for 15 min. Prior to use, the test media was melted by steaming and cooled to 37°C to maintain a homogenous mix. Tryptone soya agar (TSA) was supplemented with 0.07% w/v lecithin and 0.5% v/v Tween 80 (TSALT agar) and autoclaved at 121°C for 15 min.

Minimum Inhibitory Concentration Assays

Minimum inhibitory concentrations (MICs) of the oils were determined using a modified resazurin broth microdilution technique at 30°C and 37°C (Mann & Markham, 1998). Doubling dilutions of each essential oils were prepared in 96-well microtiter plates (100 µl total volume per well) as described by Carson et al. (1995). Nutrient broth, as described above, was used as a diluent for the essential oil dilutions. The range of dilutions used was 32–0.5% (v/v) for black pepper oil and 1–0.015625% (v/v) for linalool. Optical densities of inocula were measured at 600 nm and adjusted to 5×10^4 cfu/ml of which 100 µl was used to inoculate each well. The concentration of each inoculum was confirmed with viable counts. Plates were incubated at both 37°C and 30°C for 24 h.

After incubation, 10 µl resazurin (0.01% w/v) (Sigma R2127) was added to each well. After two further hours of incubation, bacterial growth was indicated by the color change from blue to pink (Mann & Markham, 1998). The MIC for each organism was determined in duplicate by reading the last concentration of essential oil at which no bacterial growth had occurred.

Linalool was selected for further study using an agar-incorporation method (Dube et al., 1989). Linalool was added to TSALT agar poured into sterile Petri dishes. Broth cultures with a cell density of 1×10^8 cfu/ml were prepared and inoculated onto the agar's surface using a multipoint inoculator (Brown et al., 2004) and incubated at 37°C for 24 h. The MIC for each organism was determined in triplicate by reading the last concentration of essential oil at which no bacterial growth the occurred.

Gradient Plates

A slope of TSALT agar was prepared in a 10×10 cm square sterile Petri dish as described by M'Zali et al. (2000). Linalool was emulsified in ethanol and Tween 80 in a 2:3:1 ratio and added to TSALT agar supplemented with 0.005% resazurin, giving a final concentration of 0.5% linalool oil. The plates were then placed horizontally and the agar containing the oil poured to create a proportional increase in oil along the horizontal axis.

Broth cultures of *Sten. Maltophilia* CXC 1, CXU 1, M33, M99, and 44606 with a cell density of 1×10^8 cfu/ml were prepared: 500 µl of inoculum was spread-plated onto the agar surface. The concentration of each inoculum was confirmed with viable counts. The plates were incubated for 48 h at 30°C and 37°C. The plates were then examined for zones of inhibition.

Results

MICs for the broth and agar dilution techniques are shown in Table 1. Temperature-dependent susceptibility to linalool oil was also shown in experiments using the gradient plate. Resistant colonies beyond the MIC were seen at both temperatures. After subculture onto essential oil-free

Table 1. Minimum inhibitory concentration of five isolates of *Sten. Maltophilia* to pepper and linalool (% v/v) at 30°C and 37°C.

	Broth microdilution				Agar dilution
Isolate	Pepper (30°C)	Pepper (37°C)	Linalool (30°C)	Linalool (37°C)	Linalool (37°C)
CXC 1	16	8	0.25	0.125	0.5
CXU 1	16	8	0.25	0.125	0.5
M33	16	8	0.25	0.125	0.25
M99	16	8	0.25	0.125	0.25
44606	16	8	0.25	0.125	0.25

agar, MICs of these isolates were determined again using the resazurin broth microdilution method. MICs reverted to the values of the parent strains (data not shown).

Discussion

Both linalool and black pepper oil were active *in vitro*, and temperature-dependent activity was noted. Although the increase in MIC was only one doubling dilution, it was seen consistently with all isolates and in repeated experiments. Temperature-dependent resistance to aminoglycosides in *Sten. maltophilia* is well recognized, probably as a result of changes in membrane fluidity and conformational changes of the bacterial outer membrane, which occur upon growth at lower temperature and which may affect binding and/or uptake of these agents (Rahmati-Bahram et al., 1995, 1996). In addition to the implications for *in vitro* testing of essential oils against these bacteria, this phenomenon may well have clinical significance as essential oils would be most likely to find use as topical agents at body sites where the prevailing temperature may be < 37°C (Wilcox et al., 1994).

Colonies growing beyond the MIC for both oils were identified using the gradient plate technique. MICs of these colonies after subculture onto oil-free agar were the same as the parent strains, and the significance of these observations for the development of stable resistance to the oils is uncertain.

This study emphasizes that differences in methodology, including temperature of incubation, may affect results of *in vitro* susceptibility testing of these products and underscores the need for the introduction of standardized protocols similar to those that already pertain to conventional antibiotics. This is particularly important given the wide range of other variables—method of oil extraction, use of commercially available products (which may be adulterated with synthetic compound), and so forth—that may also affect the results obtained in susceptibility testing.

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