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Effect of cold atmospheric plasma on antifungal activities of clove oil and eugenol against molds on areca palm (*Areca catechu*) leaf sheath

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ABSTRACT

An attempt to control the growth of *Aspergillus niger*, *Penicillium* sp., and *Rhizopus* sp., commonly found on the surface of the areca palm (*Areca catechu*) leaf sheath, by using clove oil and its main components (eugenol, linalool, and caryophyllene) at concentrations of 5–100 μl ml^{-1} both in vitro (agar dilution method) and in vivo (dip method) was enhanced by discharging cold atmospheric plasma at 40 W for 10 min. The results showed that the minimum inhibitory concentration (MIC) of clove oil and eugenol was reduced tenfold against all molds at concentrations of 10 μl ml^{-1} and 5 μl ml^{-1}, respectively, after cold atmospheric plasma treatment. For the in-vivo assays, clove oil at 30 μl ml^{-1} and eugenol at 10 μl $ml^{-1}$with cold atmospheric plasma could extend the protection against the growth of all molds on the surface of an areca palm leaf sheath from 14 days (control) to at least 12 wk at 25 °C. In addition, it was observed that eugenol was the main agent responsible for the antifungal activity of clove oil after plasma treatment. This study has demonstrated the good potential of using atmospheric plasma treatment to enhance antifungal activity of clove oil and to control molds on an areca palm leaf sheath.

1. Introduction

Although plastic and foam have both been widely used as food packaging, they pollute the environment and are difficult to decompose (Marsh and Bugusu, 2007). Therefore, alternative natural packaging might be the answer to these problems (Davis and Song, 2006). In the past few years, cellulose-based food packaging has attracted a lot of attention (Gemili et al., 2009; Barbiroli et al., 2012; De Moura et al., 2012). In this study, the areca palm leaf sheaths (*Areca catechu*) used in Thailand as food packaging material for wrapping various fruit pastes such as the one with durian (Matan et al., 2011), were selected as a model for cellulose-based food packaging.

Mold growth contributing to spoilage of food and toxin production on the cellulose-based food packaging is a major problem when using this type of packaging (Matan et al., 2011). Unfortunately, microorganisms found on the surface of the packaging could also be found on and contaminate the surface of the food inside the packaging (Magrex-Debar et al., 2000). During food storage and transportation, various mold spores could be found and growth could occur on the surface. In 2011, Matan et al. reported that *Mucor dimorphosporus*, *Penicillium* sp., *Aspergillus niger*, and *Rhizopus* sp. were the major molds identified from durian paste wrapped with an areca palm leaf sheath.

Although for many years chemical control has been the main way to inhibit mold growth on the surface of food (Brul and Coote, 1999; Marín et al., 2002), concerns over the use of chemicals have been growing (Gangolli, 1983). Therefore, alternative methods to inhibit mold should be explored. Applying natural preservatives extracted from herbs or plants has been one of the most interesting research topics. Several reports have shown that the antifungal activity of essential oils can preserve cellulose materials such as the areca palm leaf sheath and wood (Matan et al., 2006, 2009, 2011, 2012; Matan and Matan, 2007, 2008). One of them, clove oil, has been found to be a great mold inhibitor. Antifungal activities of clove oil or its main component, eugenol, against molds have also been reported by Matan et al. (2006), Omidbeygi et al. (2007), Cheng et al. (2008), and Komala et al. (2012). However, high concentrations of natural compounds are required for long-term storage, and this can alter the taste of food or even exceed acceptable

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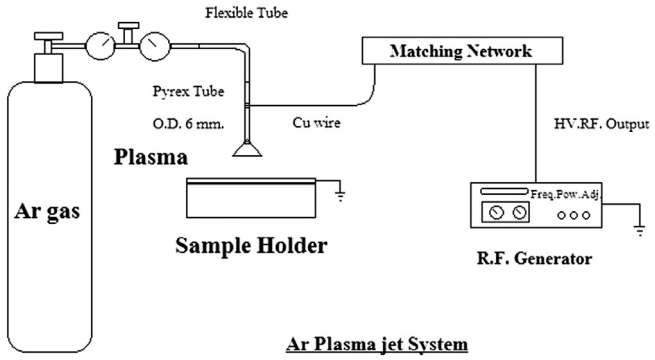

Fig. 1. Schematic view of a cold atmospheric plasma jet.

flavor thresholds. To avoid the flavor of the essential oil, enhancing its antifungal activity at a relatively low concentration using cold atmospheric plasma is an alternative. Cold atmospheric plasma is produced by excited gas. Plasma, also known as the highly energized fourth state of matter, is defined as an ionized gas with an approximately equal amount of positively and negatively charged particles, containing neutral species (radicals, excited atoms, and molecules), ions, and electrons (Lerouge et al., 2001). The effect of plasma on various mold contaminant microorganisms such as *Aspergillus flavus* (Suhem et al., 2013) and *Penicillium expansum* (Liang et al., 2012) has been studied. However, studies on the combined effect of cold atmospheric plasma and essential oil are very limited. Therefore, the objective of this research was to observe the effect of cold atmospheric plasma on the efficacy of clove oil at a relatively low concentration against major molds found on the areca palm leaf sheath.

2. Materials and methods

2.1. Chemicals, essential oils, and areca palm leaf sheath

Food-grade clove oil containing 75% eugenol, 11% linalool, and 5% caryophyllene, derived from dried flower buds of clove (*Eugenia caryophyllata*) by steam distillation, was provided by the Thai China Flavours & Fragrances Industry Co., Ltd., of Bangkok, Thailand. Pure substances of eugenol, linalool, and caryophyllene were purchased from Sigma–Aldrich of Singapore.

Areca palm leaf sheaths were obtained from Khiriwong Village in the Lansaka district of the Nakhon Si Thammarat province in southern Thailand. The average moisture content of the raw leaf sheaths prior to testing was $15 \pm 1\%$ ($n = 10$).

2.2. Cultures

Three strains of mold (*A. niger* WU 0903, *Penicillium* sp. WU 0902, and *Rhizopus* sp. WU 0904) were identified from the areca palm leaf sheath surface. The codes refer to strains held in the culture collection of the Wood Science and Engineering Research Unit of Walailak University in Nakhon Si Thammarat, Thailand. Colonies were plated on malt extract agar (MEA; Merck Ltd, Thailand), and incubated for 7 days at 25 °C. Isolates were identified by their morphological characteristics. *A. niger* isolates was identified according to Pitt and Hocking (1997). *Penicillium* sp. and *Rhizopus* sp. morphologies were characterized by using an optical microscope (Carl Zeiss, Oberkochen, Germany). All strains were again identified and confirmed by the Microbiological Resources Centre (MIRCEN), Thailand Institute of Scientific and Technological Research (TISTR), Thailand.

2.3. Preparation of inocula

Spores of test mold were obtained from mycelia grown on malt extract agar (MEA; Merck Ltd, Thailand) at 25 °C for 14 days, and were collected by flooding the surface of the plates with ~5 ml sterile saline solution (NaCl, 8.5 g l^{-1} water) containing Tween 80 (0.1% v/v). After the spores were counted using a haemocytometer, the suspension was standardized to concentrations of 10^7 spores ml^{-1} by dilution with sterile water before use. The viability of all strains was checked using quantitative colony counts at 10^7 CFU ml^{-1}.

2.4. Cold atmospheric plasma setup

The cold atmospheric plasma jet system developed by the Plasma Technology for Agricultural Application Laboratory of Walailak University, as shown in Fig. 1, was used in this study. Argon as a working gas was introduced to a 2-mm-thick Pyrex glass tube with a 6-mm inner diameter. An RF power source was provided to generate high voltage and high frequency for the plasma jet discharge. The power levels required to sustain the discharges ranged from 0 W to 40 W and 50 kHz to 600 kHz. The maximum voltage was 10 kV and the power supply could be adjusted for various plasma discharge conditions. The plasma jet was produced at atmospheric pressure using argon at a gas flow rate of 10 L/min.

2.5. Minimum inhibitory concentration (MIC) of clove oil and its main components on agar

The antifungal analysis and determination of the minimal inhibitory concentration (MIC) of clove oil and its main components (eugenol, linalool, and caryophyllene) were performed by the agar dilution method. Clove oil and its main components (5–100 µl ml^{-1}) were prepared in malt extract agar (MEA; Merck Ltd, Thailand). Then, the lids of petri dishes were removed and the agar surface was exposed to the cold atmospheric plasma jet at 40 W for 10 min with a distance of 1 cm from the tip of the Pyrex tube. Temperature on the agar surface both during and after cold plasma treatment was monitored using an infrared thermometer (Fluke Corporation, USA). The treated agar plate was next inoculated with 0.1 ml each of *A. niger*, *Penicillium* sp., and *Rhizopus* sp. inocula. Inoculated plates were left at room temperature for approximately 30 min to allow the inocula to be fully absorbed by the agar. Five replicates were performed for each treatment. Vegetable oil (soybean oil; Morakot Industries, Thailand) with plasma and non-plasma treatment at the same concentration was used as the control. The highest dilution with the lowest concentration of mold and showing no visible growth was regarded as the MIC after incubation at 25 °C for 72 h.

2.6. Mold test on areca palm leaf sheath

Sets of five random replicate specimens of the leaf sheath plates measuring 10 mm wide and 70 mm long were dip-treated with clove oil and eugenol in the range of 5–100 µl ml^{-1}. Clove oil and eugenol were diluted with methanol. Dip-treated specimens were held in a closed container overnight at room temperature before being treated with cold atmospheric plasma at 40 W for 10 min. Then, the specimens were inoculated with 1 ml of the test molds (*A. niger*, *Penicillium* sp., and *Rhizopus* sp.). The vegetable oil, diluted with methanol to the same concentration, with plasma and non-plasma was used as control.

The treated specimens were next incubated at 25 °C with 100% RH in an environmental chamber (Binder, Germany) for 12 wk. The specimens were then individually rated for mold growth cover on a