Original paper

Antimicrobial Paper with a Coating Containing Emulsified AITC and Lemongrass Oil for Protecting Japanese Pear against *Alternaria*

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Received February 5, 2014 ; Accepted November 8, 2014

***Alternaria* sp. causes discoloration and black spot disease in *Pyrus pyrifolia* (Japanese or Nashi pear). We investigated the antimicrobial properties of an emulsion oil of natural antimicrobial substances, such as allyl isothiocyanate (AITC) and lemongrass oil. The effect of oil-droplet size in the emulsified oil containing AITC and lemongrass oil with modified starch was assessed. Furthermore, the effect of different oil ratios on the antimicrobial properties was also investigated. When the emulsion oil weight ratio of AITC to lemongrass oil was 0.15, *Alternaria* sp. growth was not observed after 16 days. In paper bags coated with the emulsified oil, the amount of lemongrass oil retained on the paper increased with a decrease in the average oil-droplet size.**

Keywords: emulsion, essential oil, Japanese pear, antimicrobial activity

Introduction

Japanese pears are one of the most popular fruits in Japan, and are exported to other countries. The commercial value of Japanese pear is evaluated by taste (sugar content), size (weight), and beauty (external appearance). Recently, the appearance of brown discoloration on harvest fruits has resulted in reduced pear quality. In extreme cases, Japanese pear has been found to have a moldy smell, dull appearance, loss of color, and loss of external beauty. These features are caused by pathogens such as filamentous fungi and basidiomycetous yeasts. Although the pathogenic mechanism is unknown, several of these pathogens, including *Alternaria* sp., have been isolated from diseased pears (Yasuda *et al.*, 2006; Cowan, 1999). To protect pears from fungal diseases and injurious insects, developing pears are covered by a paper bag containing antifungal agents, thereby preventing fungal or mold growth on the fruit. An antifungal agent typically used is 2,4,5,6-tetrachloroisoph

thalonitrile (TPN). The amount of TPN used to treat pears is restricted to limit the chlorine and benzene residues, increasing food safety. However, the development of natural antimicrobial agents with low toxicity and high antimicrobial activity is desirable.

Essential oils are known to possess antimicrobial activity and have attracted attention because of increased interest in the use of natural antimicrobial agents (Nychas, 1999; Silva, 1995). Essential oils are extracts of plant origin. They are organic compounds containing volatile fragrant substances, such as monoterpenes and sesquiterpenes. These oils have shown broad-spectrum antimicrobial activity against viruses and fungi. Monoterpenes have been reported as one of the most abundant compounds present in essential oils, and a number of monoterpenes exhibit antimicrobial activity against various bacteria (Moleyar and Narasimham, 1986; Gocho, 1992; Pattnaik *et al.*, 1997). Okabe *et*

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al. (1990) evaluated the antimicrobial activity of β -tsuyopusen, and reported strong antimicrobial activity against a broad spectrum of microorganisms. Ward *et al.* (1998) reported the antibacterial and antifungal activity of allyl isothiocyanate (AITC). Furthermore, essential oils showed high activity against fungi, particularly filamentous fungi, for which other antimicrobial agents were not effective (Inouye *et al.*, 1996, 1998, 2000).

When preparing the antifungal paper, it is essential to embed the antifungal agents in a manner allowing for their controlled release in a timely manner. There are several methods used to immobilize the antifungal agents in the paper. Rehmann *et al.* (2003) and Furuta *et al.* (2006) demonstrated the potential use of surface-modified Japanese washi paper by monochlorotriazinyl- β -cyclodextrin. The modified cyclodextrins were covalently bonded to the paper, and the presence and release of hinokitiol (β -tsuyopusen) was observed. The antimicrobial activity of the fixed hinokitiol against airborne microorganisms was demonstrated. They also reported that the release rate was strongly influenced by the relative humidity. Emulsion coating of the antifungal agent onto the paper is another popular method to fix the antifungal agent to the paper. Since the coating process includes thermal processing (drying), the key point is to prevent the antifungal agent from dissipating during processing. Starches modified with octenyl succinic anhydride (OSA) are used as emulsifiers, encapsulating agents and stabilizers in many food oils and essential oils (Partanen *et al.*, 2002, 2005; Tesch *et al.*, 2002; Drusch *et al.*, 2006; Baranauskiene *et al.*, 2007; Reiner *et al.*, 2010; Paramita *et al.*, 2012; Rodríguez-Rojo *et al.*, 2012). Ben Arfa *et al.* (2007) employed OSA starch and soy protein isolates as paper coatings and inclusion matrices for two antimicrobial compounds containing cinnamaldehyde and carvacrol. They reported that soy protein isolates showed better retention of antimicrobial compounds, and that OSA enabled better release of antimicrobial compounds as well as good adhesion to the paper.

In this study, the antifungal activities of several essential oils against *Alternaria* sp. were investigated. Two of the selected agents, lemongrass oil and AITC, were mixed and the antifungal activities of the mixture and emulsion, formulated with the OSA starch CAPSUL[®], were investigated. Paper was coated with the emulsified lemongrass oil-AITC mixture at different oil-droplet sizes using CAPSUL[®]. The antifungal activity of the coated paper bags was evaluated and compared with that of the conventional TPN-coated bags.

Materials and Methods

Materials Neem oil, hiba oil, linalool, L-menthol, citral, lemongrass oil, and allyl isothiocyanate (AITC) were used as the antimicrobial oils in this study. Neem oil was purchased from M&K Laboratories Inc. (Nagano, Japan). AITC, citral, lemongrass oil, and linalool were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were of analytical reagent

grade. Hiba oil was purchased from Osaka Organic Industries, Ltd. (Osaka, Japan). L-Menthol was purchased from Nagaoka & Co., Ltd. (Hyogo, Japan). Starch sodium octenyl succinate (CAPSUL[®] National Starch and Chemical Co., Ltd., Bridgewater, New Jersey, USA), was used as an emulsifying agent. *Alternaria* sp. 0407, obtained from Tottori Prefecture Horticultural Experiment Station (Tottori, Japan), was used as the causative agent of black spot disease in Japanese pear.

Preparation of emulsions Dehydrated CAPSUL[®] (25 g) was dissolved by stirring in 70 mL of distilled water at 90°C for 1 h and then cooled to room temperature. Five grams of AITC, lemongrass, or the mixture of both oils was added to the solution. The weight ratio of lemongrass to AITC in the mixed oils was 0.65, 0.75, 0.85, 0.95, or 1.00. The mixture was homogenized using a POLYTRON homogenizer (PT 6100; Kinematica AG, Schweiz, Switzerland) at 3000, 4000, and 8000 rpm for 3 min, at 1-min intervals.

Oil-droplet size analysis The oil-droplet size in emulsions was determined using a laser light-scattering particle analyzer instrument (SALD-7100; Shimadzu Corporation, Kyoto, Japan). The volume-averaged diameter, D_{43} , was used as the mean diameter for all subsequent measurements. Each sample was analyzed in duplicate. The reflective index was set to $n = 1.70 - 0.2i$.

Antifungal activity of bulk and emulsified oil mixtures Potato dextrose agar (PDA, DAIGO for JP general general test; Nihon Pharmaceutical Co., Ltd, Tokyo, Japan) was used as the agar medium. PDA (11.7 g) was dissolved in 300 mL distilled water, and was then sterilized in an autoclave, poured onto a sterilized plate, and allowed to cool. The *Alternaria* sp. strain was incubated on PDA plates at 30°C for approximately 2 weeks. About 10 mg of cultured *Alternaria* sp. was added to 10 mL of sterile water, and the solution was agitated using a vortex mixer (Vortex GENIUS3; IKA-Werke GmbH & Co. KG, Staufen, Germany) for 2 min to produce a 1 mg/mL fungal suspension. The suspension was inoculated (25 μ L) onto a PDA plate, and 5 or 10 μ L of bulk essential oil, or the emulsified oil mixture, was poured onto a 25-mm diameter filter paper (ADVANTEC; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and placed on the center of the PDA plate. Growth of *Alternaria* sp. at 30°C was observed using a digital camera (COOLPIX5200; Nikon Corporation., Tokyo, Japan).

Preparation of paper coated with the mixed oil emulsion In addition to the antimicrobial property of fruit protection bags, other necessary functions include water resistance, air permeability, and optical transparency. In general, paraffin processing is applied to the fruit protection bag. A schematic diagram of a coater (NE Seisakusyo Co., Ltd., Tottori, Japan) used for the preparation of the coated paper is shown in Fig. 1. This coater consists of two coating rollers (width 540 mm and diameter ϕ 180 mm) to coat the antimicrobial agent and paraffin. Emulsion oil was applied to one side of the paper, and then the paper was air-dried. Subsequently, paraffin heated to 110°C was coated on the same side of the paper. The running speed of the paper was selected at 8.6 m/s. Since the

Fig. 1. Processing method for pear-specific fruit protection bags
The coating speed was fixed at 8.6 m/s. The coating machine delivered two-step emulsion and paraffin coating. The roller diameter was 180 mm.

Fig. 2. Petri dish set-up of antifungal paper and fungal cultures using the vapor-action method
The petri dishes are used to evaluate the antifungal activity of treatments.

coating process involved two thermal operations, drying and paraffin coating, the antimicrobial oil was applied in an emulsion form to prevent the loss of the antimicrobial agent during thermal processing. Two types of coated paper, with and without paraffin, were prepared to evaluate the amount of antimicrobial compounds in paper coated with emulsion oil at each step.

Quantification of antimicrobial compounds in coated paper
The coated paper (0.4 g, 10 cm × 10 cm) was added to 40 mL of water in a glass bottle (ϕ 13 mm × 100 mm), 10 mL of chloroform with 10 μ L of cyclohexanone was added, and then vigorously mixed using a vortex mixer for 1 min. To extract the emulsified antimicrobial oil from the organic solvent, the mixture was heated on a heating block at 90°C for 30 min with shaking every 10 min. After cooling to room temperature, a 1- μ L sample from the organic phase was injected into a gas chromatograph (GC-2010; Shimadzu Corporation, Kyoto, Japan) equipped with a 2.1 m × 0.32 mm capillary column (DB-WAX; Agilent Technologies, Inc., Santa Clara, California, USA) using a flame ionization detector at 200°C. The column temperature was increased at 10°C/min from 50°C to 230°C, and nitrogen was used as the carrier gas (2.36 mL/min). The analysis was repeated twice for each sample and the amount of antimicrobial compound was expressed as the mean value. The amount of antimicrobial compound was defined as the concentration per paper weight.

Antimicrobial activity of paper coated by the vapor-action method The antimicrobial activity of the coated paper was

examined by the vapor-action method as shown in Fig. 2. The 2-week-old fungal culture was hollowed out using a heat-sterilized cork borer (ϕ 4 mm). The fungal disks obtained were placed upside-down in three locations on the PDA medium, and the petri dish was covered with the coated paper (10 × 10 cm) and covered with a lid. For comparison, TPN-coated paper (containing 1,000 to 1,500 ppm) obtained from Nippon Nogyo Shizai KK (Tottori, Japan) was used. Growth of the *Alternaria* sp. incubated at 30°C was observed. Images of the PDA plates were taken with a digital camera (COOLPIX5200; Nikon Corporation., Japan) for image processing using Scion Image software (Scion Corp. Frederick, MD, USA) (Neoh *et al.*, 2008).

Results and Discussion

Antifungal activity of essential oils Seven essential oils (neem oil, hiba oil, linalool, L-menthol, citral, lemongrass oil, and AITC) were screened for antifungal activity toward *Alternaria* sp. Figure 3 illustrates the inhibitory effect of the seven essential oils (10 μ L) on the mycelial growth of *Alternaria* sp. Figure 3 (a) presents the negative control, containing no essential oils. Under this treatment, the fungus was visible and covered the entire surface of the PDA medium by day 4. A similar pattern of growth was observed following the addition of neem oil. For hiba oil, although the fungus was visible from day 4, growth was limited to the outer edge of the plate, and a clear inhibition ring was observed (Fig. 3 (c)). Fig. 3 (g) and (h) indicated that lemongrass oil and AITC exhibited the highest antifungal activity out of the seven essential oils studied. Thus, in subsequent experiments, a mixture of lemongrass oil and AITC was used as the antifungal agent toward *Alternaria* sp.

Antifungal activity of different mixing ratios of lemongrass-AITC The effect of lemongrass oil and AITC mixing ratio on antifungal activity was investigated. The difference in antifungal activity was negligible between lemongrass oil and AITC applied at 10 μ L. To clarify the differences in antimicrobial activity, the amount of antifungal agent added was reduced to 5 μ L. The antifungal activity of lemongrass, AITC, and the mixture was assessed. The growth behavior of *Alternaria* sp. is shown in Fig. 4 at different lemongrass oil and AITC ratios. Figure 4 (a) is the

Fig. 3. Antifungal activity of essential oils

(a) No additives (control), (b) neem oil, (c) hiba oil, (d) linalool, (e) L-menthol, (f) citral, (g) lemongrass oil, (h) AITC. *Alternaria* sp. (1 mg/mL) was inoculated (25 μ L) on a PDA plate and antifungal substances were added (10 μ L).

Fig. 4. Antifungal activity of the lemongrass oil (L) and AITC (A) oil mixtures

(a) No additives (control); (b) L = 1.0; (c) A = 1.0; (d) L = 0.95, A = 0.05; (e) L = 0.85, A = 0.15; (f) L = 0.75, A = 0.25; (g) L = 0.65, A = 0.35. *Alternaria* sp. (1 mg/mL) was inoculated (25 μ L) on a PDA plate and antifungal substances were added (5 μ L).

negative control, and (b) and (c) are the positive controls for lemongrass oil and AITC, respectively. The other columns are those treated with the mixture of lemongrass oil and AITC. L and A in columns (d) and (g) denote the weight fraction of lemongrass oil and AITC in the mixture, respectively. Following addition of lemongrass oil, the growth of *Alternaria* sp. was observed on day 6, which was 2 days earlier than that observed in the case shown in Fig. 3. This is likely due to the use of half the amount of antifungal agent used in the previous test. However, following treatment with AITC, *Alternaria* sp. growth was not observed by day 8. Following treatment with the lemongrass-AITC mixture, growth of *Alternaria* sp. was not observed over 8-days incubation at an AITC fraction

greater than 0.15 in the mixture, as shown in Fig. 4 (e–g). These results suggest that AITC was an effective antifungal agent towards *Alternaria* sp. Since AITC is highly volatile and difficult to handle, its incorporation with lemongrass oil is a practical method to prevent loss during processing.

Effect of emulsion oil-droplet size on antimicrobial activity

The effect of emulsion oil-droplet size on antifungal activity was investigated. Following homogenization at 3,000, 4,000, and 8,000 rpm with a POLYTRON homogenizer, the average oil-droplet sizes in the emulsions were approximately 1.3, 0.9, and 0.3 μ m, respectively (Fig. 5). The emulsion oil-droplet size decreased with an increase in the homogenizer rotation speed. The

Fig. 5. Oil-droplet size distribution of the emulsion solutions

(a) Lemongrass (L) = 1.0; (b) L = 0.90, AITC (A) = 0.10; (c) L = 0.85, A = 0.15. The solid line, broken line, and long dashed line indicate emulsions mixed with rotating speeds of 3000 rpm, 4000 rpm, and 8000 rpm, respectively.

Fig. 6. Antifungal activity of emulsion oils

(a) No additives (control), (b) oil (L = 1.0), (c) emulsion oil (L = 1.0, 1.3 μm), (d) emulsion oil (L = 1.0, 0.3 μm), (e) oil (L = 0.9), (f) emulsion oil (L = 0.9, 1.3 μm), (g) emulsion oil (L = 0.9, 0.3 μm), (h) oil (L = 0.9), (i) emulsion oil (L = 0.85, 1.3 μm), (j) emulsion oil (L = 0.85, 0.9 μm), (k) emulsion oil (L = 0.85, 0.3 μm). *Alternaria* sp. (1 mg/mL) was inoculated (25 μL) onto a PDA plate and antifungal substances were added (5 μL).

ratio of AITC and lemongrass did not affect the average oil-droplet size in the emulsions (Fig. 5).

The effect of oil-droplet size on the antimicrobial activity of the emulsions was investigated. Antimicrobial activity was measured following the addition of emulsions containing 5 μL of antimicrobial oil. For comparison, antimicrobial oil (5 μL) alone was evaluated. For lemongrass oil (L = 1.0), the addition of oil alone resulted in proliferation of *Alternaria* sp. on day 4, as shown in Fig. 6 (a). In contrast, for emulsion droplet sizes of 0.9 μm and 1.3 μm , *Alternaria* sp. growth was observed on day 8 (Fig. 6 (b) and (c)). Moreover, for the 0.3 μm emulsion droplet size, fungal growth was not observed by day 16 (Fig. 6 (d)). Antimicrobial activity increased with decreasing emulsion oil-droplet size. At L = 0.90, growth of *Alternaria* sp. was observed in the case of oil

alone, but was not observed in the emulsion treatments (Fig. 6 (e–g)). Moreover, at an oil mixture ratio of L = 0.85, growth of *Alternaria* sp. was not observed by day 16 in any case (Fig. 6 (h–k)). We predict that a similar trend would be observed for L = 0.90 and L = 0.85. The surface area of the oil droplets increased as oil-droplet size decreased, and thus the quantity of oil adsorbed onto *Alternaria* sp. increased. This suggests that as the quantity of oil adsorbed onto *Alternaria* sp. increases, the antimicrobial activity increases.

Quantification of antimicrobial substances on the coated paper

The effects of oil-droplet size and the emulsion mixing ratio of antimicrobial substances on the amount of antimicrobial substances on the paper were investigated. Table 1 shows the amount of AITC and lemongrass oil retained on the paper before paraffin treatment.

Table 1. Content of antifungal substances in fruit protection bags coated with emulsion oil

	Fruit protection bags coated with emulsion oil before paraffin treatment				
	L = 1.0, (0.3 μm)	L = 0.9, (0.3 μm)	L = 0.85, (0.3 μm)	L = 0.85, (0.9 μm)	L = 0.85, (1.3 μm)
Lemongrass oil (ppm)	6,140	5,101	5,337	3,231	1,891
AITC (ppm)	0	817	817	591	725
Total oil (ppm)	6,140	5,918	6,154	3,822	2,616

Table 2. Content of antifungal substances in fruit protection bags coated with emulsion oil and paraffin wax

	Fruit protection bags coated with emulsion oil after paraffin treatment				
	L = 1.0, (0.3 μm)	L = 0.9, (0.3 μm)	L = 0.85, (0.3 μm)	L = 0.85, (0.9 μm)	L = 0.85, (1.3 μm)
Lemongrass oil (ppm)	4,837	3,572	3,556	2,175	1,226
AITC (ppm)	0	0	750	0	0
Total oil (ppm)	4,837	3,572	4,306	2,175	1,226

For the ratios $L = 1.0, 0.9$, and 0.85 with an average oil-droplet size of $0.3 \mu\text{m}$, the total amounts of oil on the paper were 6,140, 5,918, and 6,154 ppm, respectively. The total amount of oil retained on the paper was nearly equal in each condition, and thus was hardly affected by the lemongrass oil and AITC ratio. Moreover, for oil-droplet sizes of $0.3, 0.9$, and $1.3 \mu\text{m}$ at the ratio $L = 0.85$, the total amounts of oil on the paper were 6,154 (AITC, 817 ppm; lemongrass, 5,337 ppm), 3,822 (AITC, 591 ppm; lemongrass, 3,231 ppm), and 2,616 ppm (AITC 725 ppm, lemongrass 1,891 ppm), respectively. The total amount of oil on the paper increased as the oil-droplet size decreased. In particular, the lemongrass oil content of the emulsions with a droplet size of $0.3 \mu\text{m}$ was approximately triple that of the emulsions with a droplet size of $1.3 \mu\text{m}$.

The effect of high-temperature paraffin treatment on the amount of antimicrobial substance on the paper was investigated. Table 2 shows the amount of AITC and lemongrass oil retained on the paper after paraffin treatment. The total amount of oil in the paper was reduced in all cases, and a decrease in the amount of AITC was particularly notable. Paper coated with emulsion oil ($L = 0.85, 0.3 \mu\text{m}$), in which the content of AITC was 750 ppm, only retained AITC after paraffin treatment.

Antifungal activity of paper coated by the vapor-action method
To evaluate the antifungal activity of paper coated with emulsified AITC and lemongrass oil, the vapor-action method was used. Figure 7 shows images of petri dishes used to evaluate the antifungal activity of fruit protection bags coated with emulsion oil using the vapor-action method. The images in Fig. 7 show areas of growth of *Alternaria* sp. on the paper coated with the mixed AITC and lemongrass oil emulsion as well as on TPN-coated paper. Growth of *Alternaria* sp. was suppressed compared to that in the control (no additive). In particular, the delay in *Alternaria* sp. growth was significant with the paper coated with emulsion at a

ratio of 0.85 lemongrass oil to 0.15 AITC, at an average oil-droplet diameter of $0.3 \mu\text{m}$. Suppression of *Alternaria* sp. growth was observed as the average droplet diameter of the emulsion oil decreased, a finding consistent with the result of the previous antifungal experiments on mixed lemongrass oil and AITC emulsions.

In addition to its antifungal property, fruit protection bags are also required to be water resistant, air permeable, and optically transparent. In general, paraffin processing is applied to the bags. Figure 8 shows the effect of using bags coated with emulsion oil and paraffin wax on the antifungal activity against *Alternaria* sp. The observed growth inhibition using the developed emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) was as good or better compared to the TPN-coated paper. Images of petri dishes were converted to binary images for detailed analysis. The medium area of the petri dish was assumed as S_0 and the area of fungal growth was designated as S . Therefore, S/S_0 indicates the relative fungal coverage area. The growth behavior of *Alternaria* sp. was determined based on the following equation (1):

$$S/S_0 = 1 - \exp(-(kt)^n) \quad \dots \text{Eq. 1}$$

where k , t , and n are growth rate constants [day^{-1}], culture period [day], and growth mechanism parameter [-], respectively. Growth mechanism parameter n was fixed at 2.00 , considering the delayed fungal growth in the initial period. The time course of the S/S_0 of control, TPN-coated paper, and emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) is shown in Fig. 9. Symbols and curved lines indicate experimental values and correlation values based on the Weibull equation, respectively. The S/S_0 of emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) was lower, regardless of whether it had been coated with paraffin, compared to TPN-coated paper, until day 4. The S/S_0 of emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) before paraffin treatment was equivalent to that of TPN-coated paper until day 8.

Fig. 7. Antifungal activity of fruit protection bags coated with emulsion oil
(a) No additives (control), (b) TPN, (c) emulsion oil ($L = 1, 0.3 \mu\text{m}$), (d) emulsion oil ($L = 0.9, 0.3 \mu\text{m}$), (e) emulsion oil ($L = 0.85, 1.3 \mu\text{m}$), (f) emulsion oil ($L = 0.85, 0.9 \mu\text{m}$), (g) emulsion oil ($L = 0.85, 0.3 \mu\text{m}$).

Fig. 8. Antifungal activity of fruit protection bags coated with emulsion oil and paraffin wax
(a) No additives (control), (b) TPN, (c) lemongrass oil, (d) emulsion oil ($L = 1, 0.3 \mu\text{m}$), (e) emulsion oil ($L = 0.9, 0.3 \mu\text{m}$), (f) emulsion oil ($L = 0.85, 1.3 \mu\text{m}$), (g) emulsion oil ($L = 0.85, 0.9 \mu\text{m}$), (h) emulsion oil ($L = 0.85, 0.3 \mu\text{m}$).

In contrast, the S/S_0 of emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) after paraffin treatment was higher than that of TPN-coated paper on day 8. The growth rate constant k obtained by the Weibull equation is shown in Table 3. The growth rate constant k of emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) was 0.115 day^{-1} (coated without paraffin) and 0.131 day^{-1} (coated with paraffin). Compared to 0.129 day^{-1} of the TPN-coated paper, the growth rate constant k was almost equal before being paraffin coated, but was lower than that after coating. Therefore, the S/S_0 of emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) after paraffin treatment was higher

than that of TPN-coated paper on day 8. In comparison with TPN-coated paper, the growth-inhibition effect of emulsion oil-coated paper ($L = 0.85, 0.3 \mu\text{m}$) was equivalent before paraffin treatment, and lower afterwards.

It was inferred that the decreased oil concentration in the paper following paraffin treatment reduced the volatility of the oil, and subsequently decreased the antimicrobial effect. Therefore, increasing the oil concentration on the paper, by extending the induction period, is expected to enhance the antimicrobial effect.

Table 3. Growth kinetic parameters of *Alternaria* sp. in fruit protection bags coated with and without TPN and/or emulsion oil as determined by the Weibull equation

	Fruit protection bags			
	Control	TPN	Emulsion oil (paraffin uncoated)	Emulsion oil (paraffin coated)
k (day ⁻¹)	0.183	0.129	0.115	0.131
standard error	0.01439	0.00282	0.00528	0.00672

Control: Fruit protection bags without any treatment; **TPN:** TPN-coated fruit protection bag; **Emulsion oil (paraffin uncoated):** fruit protection bag coated with emulsion oil ($L = 0.85$, $0.3 \mu\text{m}$); **Emulsion oil (paraffin coated):** fruit protection bag coated with emulsion oil ($L = 0.85$, $0.3 \mu\text{m}$) and paraffin wax.

Fig. 9. Temporal changes in relative mycelium-covered area of fruit protection bags

Non-coated (control) (●), TPN-coated bag (▲), emulsion oil ($L = 0.85$, $0.3 \mu\text{m}$, paraffin uncoated) (■), and emulsion oil ($L = 0.85$, $0.3 \mu\text{m}$, paraffin coated) (□). Solid lines indicate the correlations as determined using the Weibull equation.

Conclusion

Neem oil, hiba oil, linalool, L-menthol, citral, lemongrass oil, and AITC were used to investigate the antimicrobial activity of essential oils toward *Alternaria* sp. The antimicrobial activities of lemongrass oil and AITC were higher than the other oils. The influence of droplet size and oil mixture ratio on antimicrobial properties and oil retention following paper coating were examined. *Alternaria* sp. growth was suppressed with decreasing average droplet size of the emulsion oil. The application of emulsion oil with an average droplet diameter of $0.3 \mu\text{m}$ suppressed *Alternaria* sp. growth. At a lemongrass ratio of 0.85 in the emulsion oil, *Alternaria* sp. growth was not observed proximal to the oil and emulsion solution for 16 days. The amount of lemongrass oil retained on the paper increased with decreasing oil-droplet size. However, the amount of AITC on the paper coated with the emulsion oil was not influenced by the average oil-droplet size.

Acknowledgements This work was supported by the Regional Resource Utilization Research and Development Programs of the Ministry of Economy, Trade, and Industry.

References

- Baranauskienė, R., Bylaite, E., Zukauskaitė, J., and Venskutonis, R. P. (2007). Flavor retention of peppermint (*Mentha piperita* L.) essential oil spray-dried in modified starches during encapsulation and storage. *J. Agri. Food Chem.*, **55**, 3027-3036.
- Ben Arfa, A., Preziosi-Belloy, L., Chalier, P., and Gontard, N. (2007). Antimicrobial paper based on a soy protein isolate or modified starch coating including carvacrol and cinnamaldehyde. *J. Agri. Food Chem.*, **55**, 2155-2162.
- Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, **12**, 564-582.
- Drusch, S., Serfert, Y., and Schwarz, K. (2006). Microencapsulation of fish oil with n-octenylsuccinate-derivatised starch: Flow properties and oxidative stability. *Eur. J. Pharm. Biopharm.*, **108**, 501-512.
- Gocho, S. (1992). Antibacterial action of aroma compounds in vapour state. *J. Antibact. Antifung. Agents.*, **19**, 329-334.
- Inouye, S. (1996). *Annual Report of the Research Center for Pathogenic Fungi and Microbial Toxicoses*, Chiba University, **10**, 49.
- Inouye, S., Watanabe, M., Nishiyama, M., Takeo, K., Akao, M., Nishiyama, Y., and Yamaguchi, H. (1998). Antisporulating and respiration-inhibitory effects of essential oils on filamentous fungi. *Mycoses*, **41**, 403-410.
- Inouye, S., Watanabe, M., Takeo, K., Akao, M., Nishiyama, Y., and Yamaguchi, H. (2000). Inhibitory effect of essential oils on apical growth of *Aspergillus fumigatus* by vapour contact. *Mycoses*, **43**, 17-23.
- Moleyar, V. and Narasumham, P. (1986). Antifungal activity of some essential oil components. *Food Microbiol.*, **3**, 331-336.
- Nychas, G. J. E. (1999). Natural antimicrobials from plants. In "New methods of food preservation" ed. by Gould, G. W., Blackie Academic and Professional, London, pp. 58-89.
- Okabe, T., Saito, K., Otomo, Y., and Kudo, Y. (1990). "Aomori Hiba no fusigi.", Aomori Hiba Research Press, Tokyo.
- Paramita, V., Furuta, T., and Yoshii, H. (2012). High-oil-load encapsulation of medium chain triglycerides and D-limonene mixture in modified starch by spray drying. *J. Food Sci.*, **77**, 38-44.
- Partanen, R., Hakala, P., Sjövall, O., Kallio, H., and Forssell, P. (2005). Effect of relative humidity on the oxidative stability of microencapsulated sea buckthorn seed oil. *J. Food Sci.*, **70**, 37-43.
- Partanen, R., Yoshii, H., Kallio, H., Yang, B., and Forssell, P. (2002). Encapsulation of sea buckthorn kernel oil in modified starches. *J. Am.*

- Oil Chem. Soc.*, **79**, 219-223.
- Pattnaik, S., Subramanyam, V. R., Bapaji, M., and Kole, C. R. (1997). Antibacterial and antifungal activity of aromatic constituents of essential oils. *Microbios*, **89**, 39-46.
- Reiner, S. J., Reineccius, G. A., and Peppard, T. L. (2010). A comparison of the stability of beverage cloud emulsions formulated with different gum Acacia- and starch based emulsifiers. *J. Food Sci.*, **75**, 236-246.
- Rodríguez-Rojo, S., Varona, S., Núñez, M., and Cocero, M. J. (2012). Characterization of rosemary essential oil for biodegradable emulsions. *Ind. Crops Prod.*, **37**, 137-140.
- Tesch, S., Gerhards, C., and Schubert, H. (2002). Stabilization of emulsions by OSA starches. *J. Food Eng.*, **54**, 167-174.
- Ward, S. M., Delaquis, P. J., Holley, R. A., and Mazza, G. (1998). Inhibition of spoilage and pathogenic bacteria on agar and pre-cooked roast beef by volatile horseradish distillates. *Food Res. Int.*, **31**, 19-26.
- Yasuda, F., Yamagishi, D., Akamatu, H., Izawa, H., Kodama, M., and Otani, H. (2006). *Meira nashicola* sp. nov., a novel basidiomycetous, anamorphic yeast-like fungus isolated from Japanese pear with reddish stain. *Mycoscience*, **47**, 36-40.