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Antifungal Activity of Fatty Acid Salts Against *Penicillium pinophilum*

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The antifungal activity of nine fatty acid salts (butyrate, caproate, caprylate, caprate, laurate, myristate, oleate, linoleate, and linolenate) was tested on the spores of *Penicillium pinophilum* NBRC 6345 and *Penicillium digitatum* NBRC 9651. Potassium caprate showed the strongest antifungal activity at 4 log–units. At incubation times of 180 min, potassium caprylate and potassium laurate showed antifungal activities of 2 log–units against *P. pinophilum* NBRC 6345. These results suggest medium–chain fatty acid salts showed the highest antifungal activity. The minimum inhibitory concentration of potassium caprate against *P. pinophilum* NBRC 6345 was 175 mM, and >175 mM for other fatty acid salts. When mixed with short–chain fatty acid salts (potassium butyrate, potassium caproate) or medium–chain fatty acid salts (potassium caprylate or potassium laurate), potassium caprate caused a 4 log–unit reduction in fungal growth; however, when mixed with long–chain fatty acid salts (potassium myristate, potassium oleate, potassium linoleate, or potassium linolenate) it had no antifungal effect. Thus, long–chain fatty acid salts inhibited antifungal activity of C10K. We also evaluated the ability of C10K to inhibit fungal growth on orange rind. C10K effectively inhibited *P. pinophilum* NBRC 6345 growth on orange rind. Thus, C10K shows promise as an antifungal agent.
Keywords: *Penicillium pinophilum*, Fatty acid salts, Antifungal activity, antifungal agent

1. Introduction

Fungi are present in a wide range of natural environments, and breed in the foods such as vegetables and fruit, causing corruption and deterioration of these foods in some cases. Furthermore, some species of fungi are known to cause food intoxication or allergic reactions in some individuals. *Penicillium* spp., commonly known as '*P. italicum*' found in food items such as cheeses, dried foods, citrus fruits, herbs, spices, and cereals. These molds produce spores and easily propagate through the air.

To prevent fungal contamination, various fungicides have been developed that inhibit fungal growth. Fungicides must show high antifungal activity, sustainable activity, and a high degree of safety. The most common fungicides are orthophenyl phenol (OPP), thiabendazole (TBZ), and imazalil (IMZ). These are used by soaking fruits in the fungicides or spraying them after mixed with wax. These are approved food additives, and they show high antifungal activity. However, the safety of fungicides is greatly debated, and the acceptable daily intake ADI of OPP, TBZ, and IMZ has been determined to be 0–0.2 mg/kg/day, 0–0.1 mg/kg/day, 0–0.1 mg/kg/day, respectively. Therefore, creation of additional safe antifungal agents with high performance is required.

In this study, we focused on fatty acid salts, which are the main component of soap. Fatty acids, the raw material for the production of fatty acid salts, have been reported to show some antibacterial and antifungal activity. Fatty acids vary in length and degree of saturation, and naturally occurring fatty acids have a chain length of 4 to 28 carbons, which may be saturated or unsaturated [1]. Saturated fatty acids are straight chains and consist of a carbon chain with single bonds, while unsaturated fatty acids contain one or more carbon–carbon double bonds (C = C), which introduce fixed bends into the carbon chain [2]. The antimicrobial properties of several fatty acids have been well documented. For example, lauric acid and myristoleic acid, which are saturated fatty acids, have strong activity against oral bacteria, including *Porphyromonas gingivalis, Selenomonas artemidis*, and *Streptococcus sobrinus* [3]. Unsaturated fatty acids have

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been shown to have antibacterial activity against *Helicobacter pylori* and *Staphylococcus aureus* [4,5]. Siyun Liu *et al.* reported that some fatty acids show an inhibitory effect against spore germination of the phytopathogenic fungi *Alternaria solani, Colletotrichum lagenarium,* and *Fusarium oxysporum* f. sp. *cucumerinum* [6]. Thus, although the antifungal properties of fatty acids have been well-studied, and several reports have demonstrated the inhibitory effects of fatty acid salts on bacteria [7,8,9], little is known about the antifungal properties of fatty acid salts.

In this report, we have demonstrated that fatty acid salts are effective inhibitors of *Penicillium* spp.

2. Materials and methods

2.1 Source and preparation of fatty acid salts

Nine fatty acids were tested. Butyric acid (C4 : 0), caproic acid (C6 : 0), and linoleic acid (C18 : 2) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Caprylic acid (C8 : 0), capric acid (C10 : 0), lauric acid (C12 : 0), myristic acid (C14 : 0), oleic acid (C18 : 1), and linolenic acid (C18 : 3) were obtained from Tokyo Chemical Industry Co., Ltd.

Hydrated mixtures of fatty acid salts in solution were prepared by gravimetric determination, using fatty acids, KOH: potassium hydroxide pellets (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and water. Samples were prepared at 350 mM concentrations. The samples were then stirred for 2 h at 75℃. KOH aq was added to yield theoretical neutralization at pH 10.5 of fatty acid salts. Potassium butyrate (C4K), potassium caproate (C6K), potassium caprylate (C8K), potassium caprate (C10K), potassium laurate (C12K), potassium myristate (C14K), potassium oleate (C18:1K), potassium linoleate (C18:2K), potassium linolenate (C18:3K), and the blank were all adjusted using a KOH pH-adjusted solution (pH 10.5). All fatty acid salts and the KOH pH- adjusted solution were filter-sterilized at low temperature (4-6℃) using a 0.20-μm Millipore filter (Toyo Roshi Kaisya, Ltd., Tokyo, Japan).

2.2 Other reagents tested

o-Hydroxybiphenyl (OPP) and 2-hydroxybiphenyl sodium salt tetrahydrate (OPP-Na) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Appropriate amounts of OPP and OPP-Na were dissolved in ethanol or water, respectively, to produce concentrations of 175 mM.

Linear alkylbenzene sulfonate (LAS) is an anionic surfactant similar to fatty acid salts, and was used in this experiment for comparison (pH 10.5). LAS was obtained from Shabondama Soap Co., Ltd., Fukuoka, Japan. LAS composed a sodium salt consisting of linear alkyl benzene sulfonic acid, a number of compounds as with soap. 98% of compound elaborated LAS are linear alkyl benzene sulfonic acid sodium of 12 carbon chain, the rest is included other carbon chain length.

2.3 Fungal strains and growth conditions

P. pinophilum NBRC 6345 and *P. digitatum* NBRC 9651 were obtained from the NBRC (Biological Resource Center, NITE, Tokyo, Japan).

The fungi were initially grown on Potato Dextrose Agar (PDA: Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in the dark and the culture stocks were stored at 4℃. Plant-pathogenic fungi were routinely sub-cultured on PDA in slants in the dark at 30℃ so that the fresh cultures were available for later use.

2.4 Preparation of spore suspensions

Fungi were grown on PDA slants at 30℃ until well sporulated, approximately 10-14 d. Spores were harvested in sterile distilled water using a sterile inoculation loop and gentle agitation; a drop of 0.005% sulfosuccinic acid bis (2-ethylhexyl) ester sodium salt and 0.9% NaCl were added to aid wetting of the spores. The spore concentration was determined by counting using a hemacytometer (Thoma, Sunlead Glass Corp., Saitama, Japan). The initial spore concentration was adjusted to 3.0×10^4 spores/mL.

2.5 Effect of fatty acid salts on fungal spores

Solutions of 400 μL of fatty acid salts (final concentration of 175 mM in the tubes) and 400 μL of the spore suspension (3.0×10^4 spores/mL) were prepared in 1.5 mL plastic tubes. Spores mixed with the KOH pH-adjusted solution were used as controls. Final pH of all samples were a range of pH 9.2-10.8. The mixtures were incubated at 25℃. Samples were counted at 0, 10, 60, and 180 min by plating (100 μL) on PDA. Fungal colonies were counted after incubation for 2 d or 10 d at 30℃. Viable counts (log10 CFU) of spore was subtracted from the viable count of the control (log10 CFU), and the difference was used as a measure of the antifungal activity. All experiments were performed at least thrice.

2.6 Determination of minimum inhibitory concentrations (MICs)

The MIC is defined as the lowest concentration of drug sufficient for inhibiting visible growth of spores after 10 min of incubation. MICs against fungi were determined using the two-fold dilution method [7,10]. Each fatty acid salt was separately inoculated with 400 μL of *P. pinophilum* NBRC 6345 or *P. digitatum* NBRC 9651 at 3.0×10^4 spores/mL. 1.5 mL plastic tubes containing 400 μL of each of fatty acid salts were inoculated separately with 400 μL of the fungi. The tubes, each containing a total volume of 800 μL, were incubated at 25℃ for each organism for 10 min. After incubation, samples were plating on PDA, incubated at 30℃ for 7 d, and then examined for the growth of spores. Following incubation, the end point was visually assessed and expressed in mM. The lowest concentration of the antifungal treatment that inhibited visible growth of the fingi after incubation was taken as the MIC of the treatment [10].

2.7 Stability of fatty acid salts under autoclave condition

The thermal stability of the fatty acid salts was also evaluated. Autoclaved samples (121℃, 20 min) were counted 10, 60, 180 min by plating (100 μL) on PDA. Fungal colonies were counted using the same method used for determining the effect of fatty acid salts on fungal spores.

2.8 Effect of C10K combined with other fatty acid salts

C10K was mixed with short-chain fatty acid salts (C4K, C6K), medium-chain fatty acid salts (C8K, C12K) or long-chain fatty acid salts (C14K, C18 : 1K, C18 : 2K, C18 : 3K); final concentrations of each fatty acid salt of 1.75, 17.5, 35, 87.5 mM. Final concentration of C10K was 175 mM. These samples were used to measure the antifungal activity of C10K mixed with other fatty acid salts.

2.9 Demonstration experiment of C10K on the citrus

The testing method described in JIS Z 2911 (Japan Testing Center for Construction Materials) was the reference of this experiment [11].

Spore concentrations were determined using a hemocytometer. Spore solutions were diluted in sterile distilled water to a concentration of 10^4 spores/mL. Spore suspensions were produced immediately prior to each experiment.

Orange specimens were washed and sections of the orange rind (3.0 cm×3.0 cm) were cut and untreated (control) or sousing them in C10K (final concentration: 350 mM), and placed on PDA medium. All samples were inoculated with spore suspension (10^4 spores/mL), were cultured for 7 d at 30℃. Samples were observed visually and evaluated for the area of fungal colonization measured.

3. Results and discussion

3.1 Antifungal activity of fatty acid salts

Figure 1A and B show the antifungal activities of fatty acid salts against *P. pinophilum* NBRC 6345 and *P. digitatum* NBRC 9651. The average initial population of fungi at 0 min in all samples was approximately 3.0×10^4 spores/mL. Fungi were incubated for 2 d. Final concentration of fatty acid salts were 175 mM. C10K produced a 4 log-units reduction in the growth of *P. pinophilum* NBRC 6345 after incubation for 10 min. Thus, C10K suppressed 99.99% of fungal growth. The effect of C10K on *P. digitatum* NBRC 9651 was similar (Fig. 1B).

Against *P. pinophilum* NBRC 6345, C8K and C12K produced an antifungal effect of 2 log-units (suppressing 99% of growth) following incubation for 180 min. However, C4K, C6K, C14K, C18:1K, C18:2K, C18:3K and pH-adjusted solution (control) were ineffective after 180 min.

These results show that the compound with the 10-C chain produced the highest antifungal effect. Fatty acid salts exerted an antifungal effect, and no effect was produced by the pH-adjusted solution alone.

Hoagland *et al.* reported that incubation for 1 h with sodium caprylate produced an antifungal activity of 5 log-units against the bacterial fish pathogens *Edwardsiella ictaluri* C 91-152, *Edwardsiella tarda* 296, *Streptococcus iniae*, and *Yersinia ruckeri* 29473 [7]. Ababouch *et al.* also reported that sodium laurate and sodium linolenic inhibited spore growth of the bacteria *Clostridium botulinum* and *Bacillus cereus* [9]. Thus, fatty acid salts have been shown to inhibit bacterial growth. In the present study, we found that fatty acid salts also inhibited against fungal growth (*Penicillium* spp.).

The antifungal activity of C8K, C10K, and C12K observed in the present study may be partly attributed to the length of their carbon chains. The antimicrobial effect of fatty acids, which are the raw material for the production of fatty acid salts, decrease with increasing chain length, and medium-chain fatty acids exhibit stron-

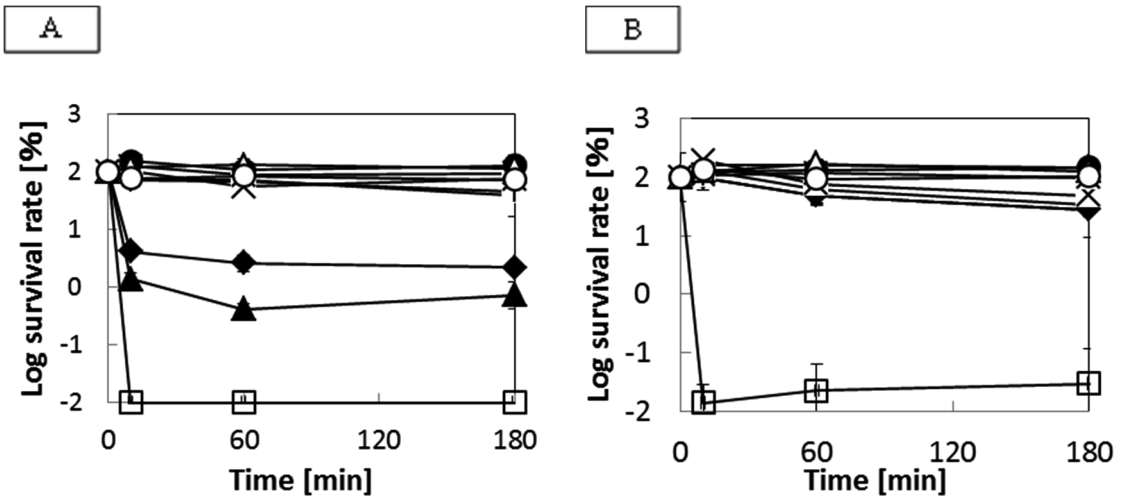

Fig. 1 Antifungal activity of 175 mM fatty acid salts against A; *Penicillium pinophilum* NBRC 6345 and B; *Penicillium digitatum* NBRC 9651. Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 μL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30℃ for 2 d were enumerated at the specified time points on PDA. Symbols: −, C4K; +, C6K; ◆, C8K; □, C10K; ▲, C12K; ×, C14K; ∗, C18 : 1K; ●, C18 : 2K; △, C18 : 3K; ○, Control (KOH pH−adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

ger activity than longer chain fatty acids [12]. The decrease in the effectiveness observed as chain length increases may be related to increased hydrophobicity and decreased solubility. Isaacs *et al*. reported that fatty acids and monoglycerides containing 8−12 carbons showed higher antimicrobial activity than their longer chain counterparts [13]. These reports fatty acid salts show antifungal similar to their antibacterial activity. In addition, we suggest that the antifungal activity of the fatty acid salts tested depended on the fungal species and the acid salts used.

A quantitative determination of the activity of fatty acid salts against *P. pinophilum* NBRC 6345 is depicted in Fig. 2. Among the 9 samples, C8K, C10K, and C12K showed the highest antifungal activity and were selected for further experimentation. These samples were inoculated with spore suspension (3.0×10^4 spores/mL), and cultured for 10 d at 30℃. The antifungal activity of C8K and C12K decreased as incubation time increased, the number of fungal colonies was observed to increase. Thus, C8K and C12K appear to have fungistatic activity. However, fungi incubated with C10K showed no growth, even after 10 d. This suggests that C10K shows sustained antifungal activity, but it remains unclear whether the activity is fungistatic or fungicidal. However, the mechanism of action of C8K and C12K appears to differ from that of C10K.

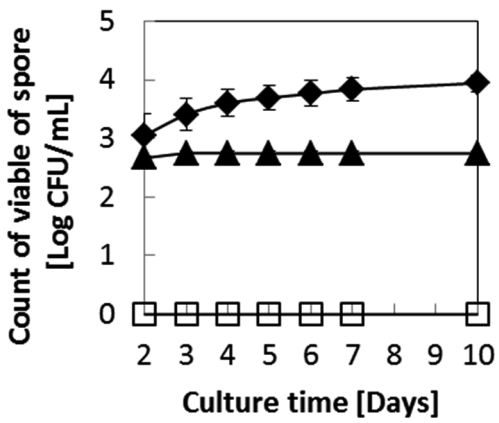

Fig. 2 Sustainability of activity of fatty acid salts against *P. pinophilum* NBRC 6345. Samples inoculated with spore suspension (3.0×10^4 spores/mL) at 10 min were cultured for 10 d at 30℃. Symbols: ◆, C8K; □, C10K; ▲, C12K. Experiments were performed in triplicate, and the error bars represent the standard deviation.

3.2 MICs of fatty acid salts and other reagents tested

Two−fold dilution samples of the 175 mM solution inoculated with fungi were incubated for 10 min, and then applied to the agar medium, and MICs were determined after 7 d of culture. The results are shown in Table. 1. C10K at 175 mM inhibited the growth of *P. pinophilum* NBRC 6345 for 7 d. However, concentrations >175 mM of the other samples were required to inhibit fungal growth. The same MIC of all samples was

Table 1 MICs of fatty acid salts and other reagents tested against *P. pinophilum* NBRC 6345 and *P. digitatum* NBRC 9651 (expressed in mM±SE).

Treatment	MIC [mM]	
	P. pinophilum NBRC 6345	*P. digitatum* NBRC 9651
C 4K	>175±0.0	>175±0.0
C 6K	>175±0.0	>175±0.0
C 8K	>175±0.0	>175±0.0
C 10K	175±0.0	>175±0.0
C 12K	>175±0.0	>175±0.0
C 14K	>175±0.0	>175±0.0
C 18 : 1K	>175±0.0	>175±0.0
C 18 : 2K	>175±0.0	>175±0.0
C 18 : 3K	>175±0.0	>175±0.0
OPP	5.4±0.0	10.9±0.0
OPP-Na	87.5±0.0	43.8±0.0
LAS	>175±0.0	175±0.0

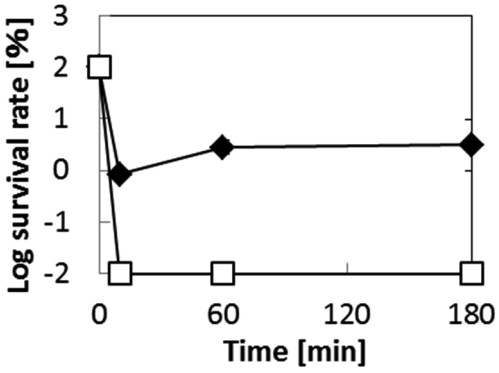

Fig. 3 Antifungal activity of 175 mM LAS against *P. pinophilum* NBRC 6345 and *P. digitatum* NBRC 9651. Spores were counted at the time of inoculation (0 min) and after 10, 60, and 180 min of incubation by means of plating 100 μL portions of the same samples. Log survival rate (expressed log CFU/ mL) in the samples stored at 30℃ for 2 d were enumerated at the specified time points on PDA. Symbols: ◆, *P. pinophilum* NBRC 6345; □, *P. digitatum* NBRC 9651. Experiments were performed in triplicate, and the error bars represent the standard deviation.

required against *P. digitatum* NBRC 9651.

Against fungi, the MICs of OPP and OPP-Na were lower than that of fatty acid salts tested. Thus, OPP and OPP-Na showed high antifungal activity. The MICs of OPP were particularly low, at 5.4 mM against *P. pinophilum* NBRC 6345 and 10.9 mM against *P. digitatum* NBRC 9651 (Table 1). The MICs of OPP-Na were 87.5 mM against *P. pinophilum* NBRC 6345, and 43.8 mM against *P. digitatum* NBRC 9651. Thus, OPP showed higher antifungal activity than OPP-Na. These results showed that OPP and OPP-Na were high antifungal activity than C10K. However, the costs of samples are higher than C10K. Thus, we suggest that C10K shows promise as an antifungal agent.

The MIC is the lowest concentration of a drug inhibiting visible growth of spores after 10 min of incubation. MIC, minimum inhibitory concentration; SE, standard error.

OPP, *o*-Hydroxybiphenyl; OPP-Na, 2-hydroxybiphenyl sodium salt tetrahydrate; LAS, linear alkylbenzene sulfonate.

LAS at a concentration of 175 mM significantly inhibited fungal growth (Fig. 3). At a concentration of 175 mM, fungal growth was reduced by approximately 2 log-units (suppressing 99% of growth) in *P. pinophilum* NBRC 6345, and by 4 log-units (suppressing 99.99% of growth) in *P. digitatum* NBRC 9651. LAS showed stronger antifungal activity against *P. digitatum* NBRC 9651 than *P. pinophilum* NBRC 6345. Thus, the MIC of LAS against *P. digitatum* NBRC 9651 was 175 mM (Table 1). These results suggest that C10K was more effective than LAS against *P. pinophilum* NBRC 6345. However, LAS showed the strongest antifungal activity against *P. digitatum* NBRC 9651.

In this study, we have described the antifungal activity of fatty acid salts and LAS, an anionic surfactant. The antifungal activities of fatty acids and monoglycerides have various mechanisms, which have yet to be clarified. Their antimicrobial activity observed has been attributed to their complex composition [13]. Monoglycerides are surfactants, similar to fatty acid salts. Glassman reported that proteins lower the bactericidal activity of surface-active agents such as monoglycerides due to the formation of lipid-protein complexes [14]. Thus, we hypothesized that the mechanisms of fatty acid salts may be analogous. Additionally, we suggest that the antifungal activity of the various fatty acid salts observed in this study could be partly attributed to their carbon numbers. Wang *et al.* reported that the antibacterial activity of fatty acids against *Listeria monocytogenes* decreases with increasing chain length, with medium-chain fatty acids exhibiting stronger activity than long-chain fatty acids [12]. Similarly, Isaacs *et al.* reported that fatty acids and monoglycerides containing 8-12 carbons showed stronger antiviral and antibacterial activities than their long-chain counterparts [13]. Our results are concordant, as we observed that medium-chain fatty acid salts (C8K, C10K, and C12K) showed higher antifungal activity. However, further experiments are required to determine their precise antifungal mechanism.

3.4 Stability of antifungal activity of fatty acid salts

The antifungal activities of the autoclaved samples (final concentration 175 mM) were comparable with those shown in Fig. 1. Therefore, the fatty acid salts showed thermotolerance (data not shown). These results suggest that fatty acid salts are resistant to heat.

3.5 Effects of alkali metal salts

Fatty acid salts can be constituted of potassium, sodium, lithium, and similar alkali metal salts. Fatty acids were neutralized with sodium carbonate, and those with 8, 10, and 12 carbon atoms, which showed higher antifungal activity, were selected. Figure 4 shows that antifungal activity of fatty acid sodium and the pH-adjustment solution (control). Ababouch *et al*. reported that sodium salts exert antimicrobial activity [7-9]. In this study, sodium fatty acids showed high antifungal activity. The results shown in C8K, C10K, and C12K of Fig. 1 (A) are in relative agreement with those shown in C8Na, C10Na, and C12Na of Fig. 4. Against *P. pinophilum* NBRC 6345, C10Na showed an antifungal effect of 4 log-units when incubated for 10 min, and C8Na and C12Na showed an antifungal effect of 2 log-units when incubated for 180 min. This result is identical to that observed with potassium salts. In addition, the MIC of C10Na was 175 mM (data not shown).

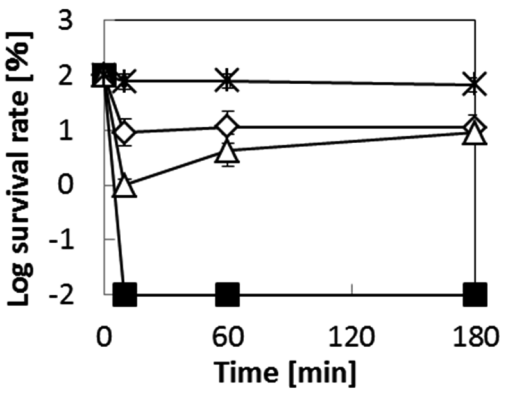

Fig. 4 Effect of antifungal activity of fatty acid sodium (C8Na, C10Na, C12Na) (final concentration 175 mM). C8K, C10K, and C12K are compared with C8Na, C10Na, and C12Na, and the pH adjusted solution. Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 μL portions of the same samples. Log survival rate (expressed log CFU/mL) of samples stored at 30℃ for 2 d were enumerated at the specified time points on PDA. Symbols: ◆, C8K; □, C10K; ▲, C12K; ×, Control (KOH pH-adjusted); ◇, C8Na; ■, C10Na; △, C12Na; ∗, Control (NaOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

This result suggests that the antifungal activity of fatty acid salts is not affected by the use of different alkali metal salts. Fatty acid potassium dissociates fatty acid ionistion and potassium ion by hydrolyze in solution, fatty acid and potassium hydrate are produced. In solution, fatty acid potassium and fatty acid are existed, these rate is one-for-one [15]. This rate is the same as fatty acid sodium. However, acid dissociation constant (pK_a) of fatty acid produced is 4.85 [16], we considered that fatty acid ionistion is heavier than fatty acid in solution because final pH of all samples were a range of pH 9.2-10.8 [17]. Thus, we suggested that fatty acid salt produced an antifungal activity by form of fatty acid ionistion. With the object of pH, C10K produced an antifungal effect of 4 log-units following incubation for 180 min when changed pH 8.5 and pH 9.5 (data not shown).

3.6 Effect of C10K mixed with other fatty acid salts

Soap can be produced using coconut, palm, or olive oils, various fatty acids. Carbon number of fatty acids are generally used the range of 8 to 22 [18]. The main component of soap is fatty acid salts bearing the carbon number and the salts formed from them. The fatty acid salts are characterized by micelle formation at high concentration. The micelle formation depends on the concentration of them, the critical micelle concentration (CMC) of long-chain fatty acid salt is low. Thus, we investigated the effect on the antifungal activity of C10K further by studying the antifungal effect of C10K, which showed the highest antifungal activity, against *P. pinophilum* NBRC 6345 when mixed with other salts. Figure 5 shows the effect of the antifungal activity with mixing C10K and other fatty acid salts (C4K, C6K, C8K, C12K, C14K, C18:1K, C18:2K, or C18:3K).

C10K mixed with C4K showed the same antifungal activity (4 log-units) as C10K alone (Fig. 5A). Thus, addition of C4K did not affect the activity of C10K. In addition, the effect of mixing C10K with C4K did not change, regardless of concentration. Similar results were obtained when C10K was mixed with short (C6K) and medium-chain fatty acid salts (C8K or C12K).

However addition of long-chain fatty acid salts (C14K, C18 : 1K, C18 : 2K, or C18 : 3K) inhibited the antifungal activity of C10K (Fig. 5E, F, G, and H). The antifungal activity of C10K decreased when mixed with long-chain fatty acid salts, and decreased more strongly as the con-

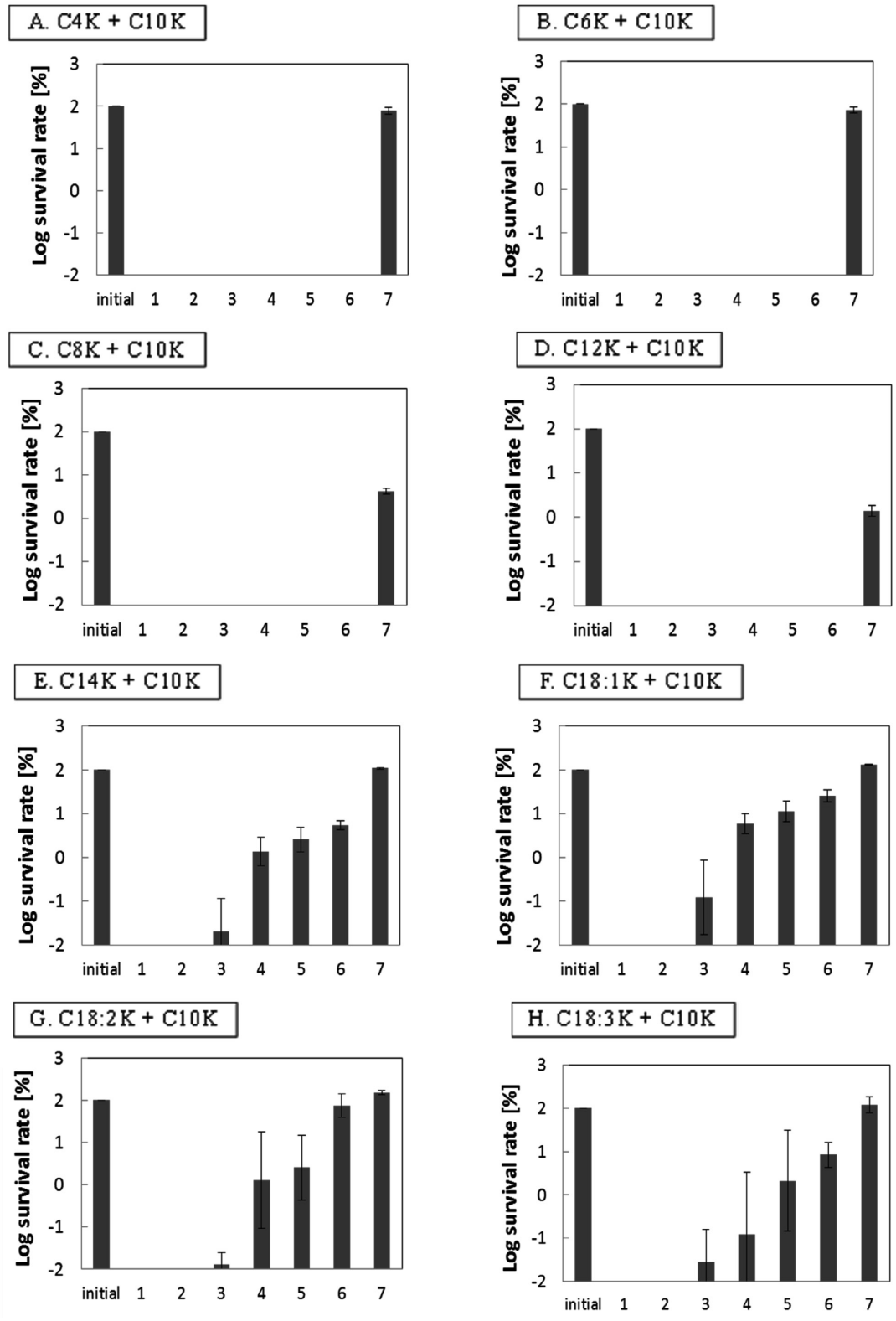

Fig. 5 The effect of mixing with other fatty acid salts (C4K, C6K, C8K, C12K, C14K, C18:1K, C18:2K or C18:3K) on the antifungal activity of C10K. A C10K with C4K, B C10K with C6K, C C10K with C8K, D C10K with C12K, E C10K with 14K, F C10K with 18:1K, G C10K with 18:2K, H C10K with 18:3K. Samples: initial, fungal suspension; sample 1, 175mM C10K alone; sample 2, 175 mM C10K and 0.175 mM other fatty acid salts; sample 3, 175 mM C10K and 1.75 mM other fatty acid salts; sample 4, 175 mM C10K and 17.5 mM other fatty acid salts; sample 5, 175 mM C10K and 35.0 mM other fatty acid salts; sample 6, 175mM C10K and 87.5 mM other fatty acid salts; sample 7, 175 mM other fatty acid salts alone. Spores were counted at the time of inoculation (0 min) and after 10 min of incubation by means of plating 100 μL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 30℃ for 2 d were enumerated at the specified time points on PDA. Experiments were performed in triplicate, and the error bars represent the standard deviation.

centration of long-chain fatty acids increased. In fact, increased numbers of mold spores were observed (sample 3-6 of Fig. 5E, F, G, and H). However, addition of 0.175 mM of long-chain fatty acid salts did not affect the antifungal activity of C10K. This may have been due to the low concentration; addition of 0.175 mM of any compound did not inhibit C10K.

In this study, we entertained that arithmetic-geometric effect of C10K and other fatty acid salts. C10K (final concentration: 87.5 mM) mixed with C8K or C12K (final concentration: 87.5 mM) did not show high antifungal activity (data not shown). Thus, antifungal activity of C10K alone was highest by this experiment.

We showed that short- and medium-chain fatty acid salts did not affect the antifungal activity of C10K. Lee *et al*. reported that combination of linoleic acid and monolaurate or monomyristin produced higher antibacterial activity than any of the compounds alone [19]. Addition of monoglycerides as emulsifiers may have increased the solubility of linoleic acid, which is insoluble on its own. Moreover, Ingram hypothesized that the antimicrobial activity of linoleic acid is due to its ability to disrupt bacterial cell membranes, causing lysis [20]. Previous studies have shown that linoleic acid shows higher antibacterial activity when mixed with monolaurin than when mixed with monomyristin [20]. These results were effect against bacteria, and different from this study. The antifungal activity of C10K was higher when mixed with short- and medium-chain fatty acid salts than when mixed with long-chain fatty acid salts. The surface tension of fatty acid salts, as anionic surfactants, decreases as concentration increases, and reaches a constant value at a certain concentration [21]. Surface tension is not altered, even when concentration increased, above the CMC. Micelles are thought to form at concentrations greater than the CMC. In this study, the antifungal activity of C10K was inhibited when mixed with high concentration of long-chain fatty acid salts (C14K, C18 : 1K, C18 : 2K, and C18 : 3K). We hypothesize that this was caused by differences in the micelle concentration of the fatty acid salts. The CMC decreases as the carbon number of a compound increases. For example, the CMCs of C14K and C18 : 1K are 6.0 mM and 0.6 mM, respectively [22].

The antifungal mechanisms underlying the antifungal activity of fatty acid salts are unknown. Walters *et al*. [23, 24] and Rihakova *et al.* [25, 26] studied the antifungal activity of fatty acids and their derivatives against highly pathogenic plant fungi, such as *Aspergillus niger*; however, the antifungal activities of fatty acids and their derivatives in general are not well known. Monoglycerides are proposed to act as nonionic surfactants that penetrate and become incorporated into bacterial plasma membranes, thereby altering membrane permeability [27, 28].

Fatty acid salts are considered to inhibit fungal growth by penetrating the cell membrane. However, because the molar weight of fatty acid salts has been suggested to become large through micellar aggregation, mixtures of long-chain fatty acid salts may decrease their activity by preventing cell membrane penetration. Mixture of C10K with C18 : 1K produced lower antifungal activity than mixture with C14K, likely due to the difference in the CMC, suggesting that C18 : 1K easily forms micelles. Among unsaturated fatty acid salts (C18 : 1K. C18 : 2K, and C18 : 3K), no differences were observed in the antifungal activity of unsaturated fatty acid salts alone. However, C10K with mixed C18 : 1K was observed the lowest inhibitory effect on antifungal activity. We found that inhibitory effect was high as increased degree of unsaturation (Fig. 5F, G, and H). Ababouch *et al.* [9] and Petrone *et al.* [29] investigated the antimicrobial activity of unsaturated fatty acids and their salts of 18-carbon unsaturated fatty acids and their salts, and showed that their antimicrobial activity increased as unsaturation increased. We observed the spore of *P. pinophilum* NBRC 6345 treated C10K by confocal laser scanning microscope, however the spore-coat appeared uniform (data not shown).

Lee *et al.* determined that leakage of adenosine triphosphate by bactericidal activity against *Bacillus cereus* and *Staphylococcus aureus* [19]. Thus, we considered the antifungal activity of C10K is same as the bactericidal activity.

3.7 Effect of C10K on citrus fruit

Figure 6 illustrates the results of application of C10K to the rind of an orange inoculated with *P. pinophilum* NBRC 6345. An orange was inoculated with the fungus alone, or with fungus and C10K. The final concentration of C10K was 350 mM. After inoculation with spore suspension (10^4 spores/mL), the samples were cultured for 7 d at 30℃.

Fungal growth was observed on the orange inoculated with the fungus alone (Fig. 6A), but was not observed on the rind of the orange also inoculated with C10K (Fig. 6B).

This result shows that C10K prevented fungal growth, and that C10K is an effective antifungal agent for citrus fruit.

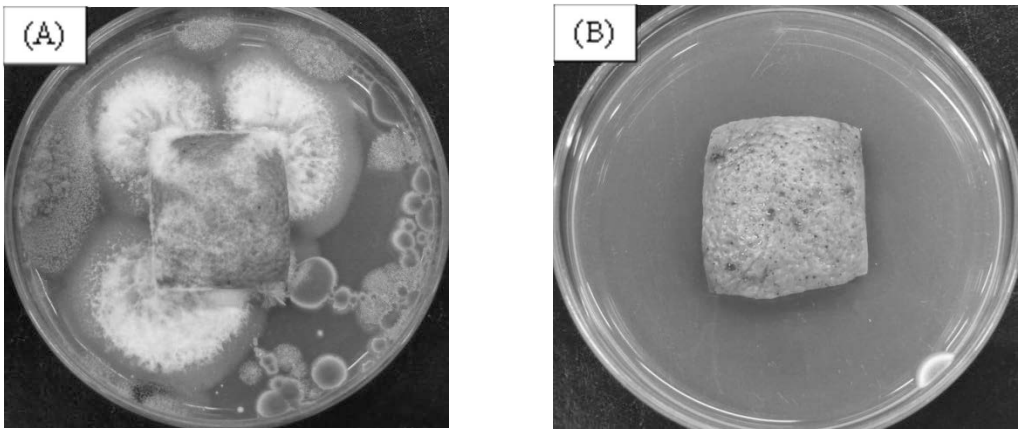

Fig. 6 Pictures of C10K-coated orange rind inoculated with (A); fungus only, (B); fungus and C10K. The samples were cultured for 7 d at 30℃.

4. Conclusions

In this study, C10K showed the highest antifungal activity (4 log-units) against *P. pinophilum* NBRC 6345 and *P. digitatum* NBRC 6345, suppressing 99.99% of fungal growth (Fig. 1A and B). A control solution at the same pH as the fatty acid salt solutions did not affect fungal growth, and we concluded that the antifungal activity was due to the fatty acid salts themselves, not pH. In addition, the antifungal activity of fatty acid salts is not affected by the use of different alkali metal salts (Fig, 4A and B). Fatty acid salts dissociate in solution, and change fatty acid ionistion and fatty acid. The pK_a of fatty acid produced is 4.85, we considered that fatty acid ionistion is heavier than fatty acid in solution because final pH of all samples were a range of pH 9.2–10.8. Thus, we suggested that fatty acid salt produced an antifungal activity by form of fatty acid ionistion. In conclusion, C10K at 175 mM showed antifungal activity against two species of *Penicillium*. This study suggests that fatty acid salts show potential as heat-stable antifungal agents. It is necessary to conduct further research on the mechanism by which the fatty acid salts inhibit fungal growth. These results provide justification for evaluation of C10K as an alternative antifungal agent.

References

1) S. L. Sado-Kamdem, L. Vannini, M. E. Guerzoni; Effect of α-linolenic, capric and lauric acid on the fatty acid biosynthesis in *Staphylococcus aureus*. Int. J. Food Microbiol., **129**, 288–294 (2009).
2) C. H. Pohl, J. L. F. Kock, V. S. Thibane; *Science against microbial pathogens: Commmunicating current Research and technological advances*. Formatex Reseach Center., 61–71 (2011).
3) S. Shapiro; The inhibitory action of fatty acids on oral bacteria. Oral Microbial Immunol., **5**, 350–355 (1996).
4) S. Khulusi, H. A. Ahmed, P. Patel, M. A. Mendall, T. C. Northfield; The effects of unsaturated fatty acids on *Helicobacter pylori* in vitro. J. Med. Microbiol., **42**, 276–282 (1995).
5) C. J. Zheng, J. S. Yoo, T. G. Lee, H. Y. Cho, Y. H. Kim, W. G. Kim; Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. FEBS Lett., **579**, 5157–5162 (2005).
6) S. Liu, W. Ruan, J. Li, H. Xu, J. Wang, Y. Gao, J. Wang; Biological control of phytopathogenic fungi by fatty acids. Mycopathologia, **166**, 93–102 (2008).
7) A. Kollanoor, P. Vasudevan, M. K. M. Nair, T. Hoagland, K. Venkitanarayanan; Inactivation of bacterial fish pathogens by medium chain lipid molecules (caprylic acid, monocaprylin and sodium caprylate). Aquaculture Res., **38**, 1293–1300 (2007).
8) H. Galbraith, T. B. Miller, A. M. Paton, J. K. Thompson; Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol and cholesterol. J. Appl. Bacteriol., **34**, 803–813 (1971).
9) L. Ababouch, A. Chaibi, F.F. Busta; Inhibition of bacterial spore growth by fatty acids and their sodium salts. J. Food Prot., **55**, 980–984 (1992).
10) K. Takashima; Mold inspection manual color atlas (Kabi Kensa Manuaru Kara Zufu). Techno System Co., Ltd., 214–215 (2009)
11) H. Otaka; Fungus resistance test method of construction for interior and exterior material (Kenchikuyo Naigaisozairyo No Kabiteikouseisiken Houho). Japan Testing Center for Construction Materials, 1–5 (2003).
12) L. L. Wang, E. A. Johnson; Inhibition of *Listeria monocytogenes* by fatty acids and monoglycerides. App. Env. Microbiol., **58**, 624–629 (1992).

13) C. E. Isaacs, R. E. Litov, H. Thormar; Antimicrobial activity of lipids added to human milk, infant formula, and bovine milk. J. Nut. Biochem., **6**, 362–366 (1995).

14) H. N. Glassman; Surface active agents and their application in bacteriology. Bacteriological Rev., 12, 105–148 (1949).

15) K. Inaba, Z. Hirano; New fatty acid chemistry (Shinban Sibousan Kagaku). Saiwai Shobo Co., Ltd., 117–118 (1997)

16) S. Hara; Oleo science (Oreo saiensu). Japan oil chemists' society, **1**, 75–82 (2001)

17) C. Q. Sun, C. J. Connor, S. J. Turner, G. D. Lewis, R. A. Stanley, A. M. Roberton; The effect of pH on the inhibition of bacterial growth by physiological concentrations of butyric acid: Iplications for neonates fed on suckled milk. Chemico–Biological Interactions., **113**, 117–131 (1998)

18) K. Inaba, Z. Hirano; New fatty acid chemistry (Shinban Sibousan Kagaku). Saiwai Shobo Co., Ltd., 106 (1997)

19) J. Y. Lee, Y. S. Kim, D. H. Shin; Antimicrobial synergistic effect of linolenic acid and monoglyceride against *Bacillus cereus* and *Staphylococcus aureus*. J. Agric. Food Chem., **50**, 2193–2199 (2002).

20) A. Hinton, D. K. Ingram; Use of oleic acid to reduce the population of the bacterial flora of poultry skin. J. Food Prot., **9**, 1282–1286 (2000).

21) S. Yamamoto, T. Enomoto, H. Mizutani, T. Hamaoka; Development of diluted working lubricant for honing utilizing micelle formation and polymer compound. Japan Soc. Mech. Eng., **77**, 1535–1543 (2011).

22) T. Yoshida, T. Ogaki, S. Shindo, K. Yamanaka; New edition Handbook of Surface Acting Agents (Shinban Kaimenkasseizai Handbook). Kougakutosho Co., Ltd., 118–119 (1987).

23) D. Walters, L. Raynor, A. Mitchell, R. Walker, K. Walker; Antifungal activities of four fatty acids against plant pathogenic fungi. Mycopathologia, **157**, 87–90 (2004).

24) R. D. Walters, R. L. Walker, K. C. Walker; Lauric acid exhibits its antifungal activity against plant pathogenic fungi. J. Phytopathol., **151**, 228–230 (2003).

25) Z. Rihakova, V. Filip, M. Plockova, J. Smidrkal, R. Cervenkova; Inhibition of *Aspergillus niger* DMF 0801 by monoacylglycerols prepared from coconut oil. Czech. J. Food Sci., **20**, 48–52 (2002).

26) Z. Rihakova, M. Plockova, V. Filip, J. Smidrkal; Antifungal activity of lauric acid derivatives against *Aspergillus niger*. Eur. Food Res. Technol., **213**, 488–490 (2001).

27) G. Bergsson, J. Arnfinnsson, S. M. Karlsson, O. Steingrimsson, H. Thormar; In vitro inactivation of *Chlamydia trachomatis* by fatty acids and monoglycerides. Antimicrob. Agents Chemother., **42**, 2290–2294 (1998).

28) G. Bergsson, J. Arnfinnsson, O. Steingrimsson, H. Thormar; Killing of gram–positive cocci by fatty acids and monoglycerides APMIS, **109**, 670–678 (2001).

29) G. Petrone, M. P. Conte, C. Longhi, S. D. Santo, F. Superti, M. G. Ammendolia, P. Valenti, L. Seganti; Natural milk fatty acids affect survival and invasiveness of *Listeria monocytogenes*. Lett. App. Microbiol., **27**, 362–368 (1998).

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◇◇◇ 和文要約 ◇◇◇

Penicillium pinophilum に対する脂肪酸塩の抗カビ効果

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カビは土壌や空気中，生物体表面など様々な場所に生育しており，食品や住環境を汚染することから，その制御が問題となっている．*Penicillium* 属菌は，自然界に広く生息し，野菜や果実などにおいて繁殖し，劣化や腐敗を引き起こす．さらに本菌は胞子の吸引によるアレルギー性疾患などの様々な健康被害を引き起こす．カビ対策としては，オルトフェニルフェノールやチアベンダゾール，イマザリルなどの防かび剤による手法が挙げられるが，安全性や持続性の低さが問題となっている．このような背景から，抗カビ効果，安全性，持続性の高い防かび剤の創出が求められている．

本研究では，界面活性剤の一種で石けんの主成分である脂肪酸塩に着目した．脂肪酸塩は鎖状炭化水素のカルボン酸塩であり，*Escrichia coli* や *Staphylococcus aureus* などの細菌に対して抗菌効果が報告されている．しかし，カビに対する抗カビ効果の知見は少ないのが現状である．そこで，本研究では JIS かび抵抗性試験法で用いられる *Penicliium pinohphilum* NBRC 6345 株および *Penicillium digitatum* NBRC 9651 株に対する脂肪酸塩の抗カビ効果について評価した．

炭素数が異なる 9 種類の脂肪酸塩の抗カビ試験の結果，*Penicillium* 属菌に対してカプリン酸カリウム；C10K が最も高い抗カビ効果を発揮することが明らかとなった．C10K は 10 分の接触で 4 オーダーの抗カビ効果（カビ胞子を 99.99％減少）を発揮した（Fig. 1）．最小発育阻止濃度（MIC）測定結果では，*P. pinophilum* に対して C10K が 175 mM で発育を抑制することが明らかとなった（Table 1）．これらの結果から，*P. pinophilum* に対して C10K が最も高い抗カビ効果を発揮することが明らかとなったため，本菌株に対して C10K とその他脂肪酸塩の共存試験を検討した．その結果，C10K の抗カビ効果に対して短鎖・中鎖脂肪酸塩は影響を与えず，長鎖脂肪酸塩は阻害作用を示すことが明らかとなった（Fig. 5A–H）．

P. pinophilum に対して C10K が最も高い抗カビ効果を発揮することが明らかとなったため，柑橘類における C10K の抗カビ効果を検討した．その結果，Control と比較して C10K は柑橘類における *P. pinophilum* の発育を抑制した（Fig. 6）．本研究より，C10K の防かび剤としての有用性が示唆された．

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