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Effect of Essential Oils on *In Vitro* Activities of 1-Aminocyclopropane-1-carboxylate (ACC) Synthase and ACC Oxidase from Winter Squash Mesocarp

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Abstract

In vitro experiments were carried out to investigate the effect of essential oils on 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase by using crude enzyme extracts from wounded mesocarp tissues of winter squash (*Cucurbita maxima* Duch. cv. Ebisu). Enzyme activities were assayed in a closed vessel including essential oils in its head space. All of the essential oils tested inhibited ACC synthase activity. A 50% inhibition was obtained with limonene and linalool at 28 and 25μl/l (liquid volume in the head space), respectively. Greater inhibition was found with aldehyde group essential oils. Citral, 1-octanal, 1-nonanal and 1-decanal inhibited ACC synthase activity by 50 to 78% and citronellal by 78 to 93% at their applied amounts ranging 3.6 to 36μl/l. On the other hand, the essential oils did not inhibit ACC oxidase activity. However, aliphatic aldehydes such as 1-octanal, 1-nonanal and 1-decanal caused a non-enzymatic breakdown of ACC into ethylene.

Key words: ACC synthase, ACC oxidase, aliphatic aldehydes, non-enzymatic breakdown of ACC

Introduction

Essential oils are volatile substances that evolve from plant tissues as secondary metabolic products. These secondary plant products are of importance in biological and ecological aspects in plants (Croteau, 1987; Tigney et al., 1991). We revealed that some essential oils inhibited ethylene biosynthesis in apple fruit and peach seed tissues (Rabbany and Mizutani, 1996). The enzymes involved in ethylene biosynthesis are ACC synthase and oxidase, which catalyze the conversion of S-adenosylmethionine (SAM) to ACC and ACC to ethylene, respectively. Here, we report effects of essential oils on *in vitro* activities of ACC synthase and ACC oxidase obtained from wounded winter squash mesocarp tissues.

Materials and Methods

Plant materials

Winter squash fruit (*Cucurbita maxima* Duch. cv. Ebisu) were obtained from a local market and stored at 12 ℃ until use. Plugs (10mm in diameter) were prepared from the mesocarp of fruit and cut into 2 mm thick slices. The slices were incubated in a plastic chamber on a moist paper towel for 16 hr at 24℃ under 90% relative humidity.

Essential oils

Three groups of essential oils were used: limonene as a hydrocarbon, linalool as an alcohol and citral, citronellal, 1-octanal, 1-nonanal and 1-decanal as aldehydes. All of the essential oils were purchased from Wako Pure Chemical Industries Ltd.

ACC synthase extraction and assay

The mesocarp tissues were homogenized with a chilled pestle and mortar in 2ml/g tissue of extraction buffer (A) containing 0.1M 3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acid (EPPS) buffer (pH8.5), 5mM 2-mercaptoethanol, 5μM pyridoxal phosphate (PLP) and 2% (w/w) insoluble polyvinylpyrrolidone (Tokyo Chemical Industry Co. Ltd.). The homogenate was centrifuged at 8000 x g for 20min at 0℃. The supernatant was passed through a column of Sephadex G-25 (PD-10; Pharmacia, Uppsala, Sweden) that had been equilibrated with the extraction buffer (A). The macromolecular fraction was collected and used as a crude enzyme extract. All procedures were carried out at 2℃. The enzyme activity was assayed in a sealed 70ml test tube, which contained 20mM EPPS, 200μM SAM, 1.6μM PLP and 0.6ml of the enzyme extract. A given amount (0.25, 0.5, 1.0, 2.5μl) of oils was applied with micro syringes (1.0 and 10μl full scale) to the interior wall of the test tube and the outer wall was heated with a hair dryer for vaporizing the essential oils and then cooled. After adding the enzyme

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solution with a hypodermic syringe, the test tube was incubated at 30℃ for 30min with reciprocal shaking (40 strokes/min). The reaction was stopped by addition of 0.1 ml of 20mM $HgCl_2$. ACC formed from SAM by ACC synthase was assayed by the method of Lizada and Yang (1979).

ACC oxidase extraction and assay

The mesocarp tissues of winter squash fruit were homogenized with a pestle and mortar in 2ml/g tissue of of extraction buffer (B) containing 0.1M Tris-HCl (pH 7.4), 10% (v/v) glycerol and 30mM Na-ascorbate. The homogenate was centrifuged at 10,000 x g for 15min at 0 ℃. The supernatant was used as a crude extract. All procedures were carried out at 2℃. The enzyme activity was assayed in a sealed 55-ml plastic syringe, which contained 0.2M 3-morpholinopropane-1-sulfonic acid (MOPS) (pH6.7), 10% (v/v) glycerol, 30mM Na-ascorbate, 50μM $FeSO_4$, 1mM ACC, 0.15% Triton X-100, 14mM $NaHCO_3$, and 0.2ml of the crude extract in a total volume of 1.5ml and 20% CO_2 in the head space. Essential oils (0.2, 0.4, 0.8, 2.0μl) were injected into the head space and vaporized as mentioned above. The syringe was incubated at 30℃ with reciprocal shaking (60 strokes/min). After 30min incubation, ethylene content in the syringe was determined by using a gas chromatograph equipped with an activated alumina column.

Results

Effects of various essential oils on ACC synthase activities

ACC synthase activities as affected by the essential oils are shouwn in Fig. 1. All of the essential oils inhibited ACC synthase activity. This inhibition occurred at a very low amount (3.6μl/l) and increased with their increasing amounts. Limonene, a hydrocarbon essential oil, inhibited ACC synthase activity by 50% at 28μl/l, whereas linalool, an alcohol essential oil, inhibited the activity by 42 to 52% over the range from 3.6 to 36μl/l. Aldehyde essential oils such as citral, citronellal, 1-octanal, 1-nonanal and 1-decanal greatly inhibited ACC synthase activity. Citronellal was most effective in the inhibition of ACC synthase activity among the essential oils tested. Citral inhibited ACC synthase activity by 50 to 78% over 3.6 to 36μ/l, whereas citronellal inhibited 78 to 93% with the same dosages. 1-Octanal, 1-nonanal and 1-decanal were similar in the inhibition of ACC synthase activity. Among these three aliphatic aldehydes, 1-octanal gave the most pronounced inhibition.

Effects of various essential oils on ACC oxidase activities

Essential oils had no effect on ACC oxidase activity, except for three aliphatic aldehyde essential oils (Fig. 2). The incubation of the enzyme with different amounts of 1 -octanal, 1-nonanal and 1-decanal caused a significantly higher rate of ethylene production with their increasing amounts. At 36μl/l of C_8, C_9 and C_{10} aldehyde essential oils, ethylene production rates were 2.8-, 18.2- and 12.9-fold over the control, respectively (Fig. 2).

Non-enzymatic breakdown of ACC by aliphatic aldehydes

The stimulation of ethylene production might be associated with non-enzymatic breakdown of ACC in the presence of these aliphatic aldehydes or the breakdown of themselves. This speculation was tested by monitoring ethylene evolution in enzyme-free and ACC-free reaction mixture with 1-nonanal. As shown in Fig. 3, a high ethylene production occurred in the assay mixture even without the enzyme extract, and there was no ethylene evolution in ACC-free mixture.

Discussion

Rabbany and Mizutani (1996) previously reported that essential oils, especially aldehyde types, suppressed the increase of ACC content and ethylene production in the assay by using excised young peach seeds. The young peach seed exhibits a rapid increase in ACC content and ethylene production just after taken from the pit (Jerie and Chalmers, 1976). The present results confirmed that the suppression is due to the inhibition of ACC synthase activity. ACC synthase is a PLP-requiring enzyme (Boller et al., 1979; Hyodo et al., 1983; Yoshii and Imaseki, 1981; Yu et al., 1979). Most of the PLP-linked enzymes have a lysine residue in their active site, where the ε-amino group of lysine makes a Schiff base with aldehyde group of PLP coenzyme. In our study the aldehyde essential oils were found to be most inhibitory to ACC synthase. If they form a Schiff base with the ε-amino group of lysine residue, the formation of the aldemine linkage between PLP and the ε-amino group of lysine residue of the enzyme might be hampered, which consequently results in the inhibition of ACC synthase activity. Therefore, further kinetic studies will reveal whether such a competition between the aldehyde essential oils and PLP operates during ACC synthase action. On the other hand, concerning other types of essential oils such as limonene and linalool, the mechanism in which they inhibit ACC synthase activity remains to be elucidated.

We also observed that the rate of non-enzymatic conversion of ACC to ethylene increased with increasing numbers of carbon in aliphatic aldehydes, whereas aromatic aldehydes showed no effect (data not shown). Earlier reports described that simple aldehydes such as methional (Beauchamp and Fridovich, 1970) and propanal (Baur and Yang, 1969) produce ethylene non-enzymatically. However, these aldehydes themselves convert to ethylene. Non-enzymatic conversion of ACC to ethylene has been reported previously under various conditions. Divalent metal ions, free radicals and lipoperoxides (Yang and Hopffman, 1984; McRae et al., 1983; Kaaceperska and Kubacka-Zehalska, 1993) facilitate the non-enzymatic conversion of ACC to ethylene. There is no information on the non-enzymatic conversion of ACC to ethylene caused by aliphatic aldehydes.

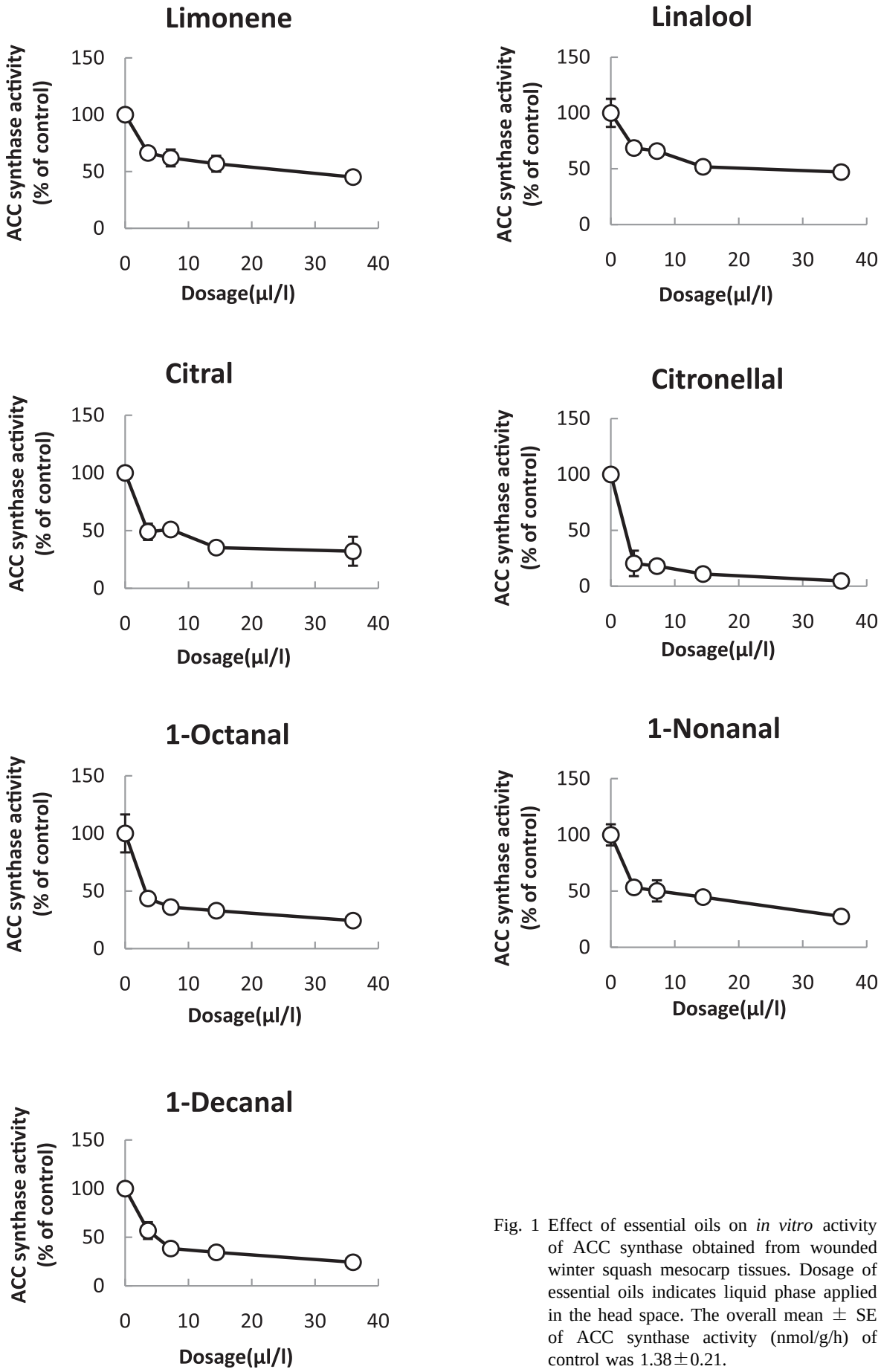

Fig. 1 Effect of essential oils on *in vitro* activity of ACC synthase obtained from wounded winter squash mesocarp tissues. Dosage of essential oils indicates liquid phase applied in the head space. The overall mean ± SE of ACC synthase activity (nmol/g/h) of control was 1.38±0.21.

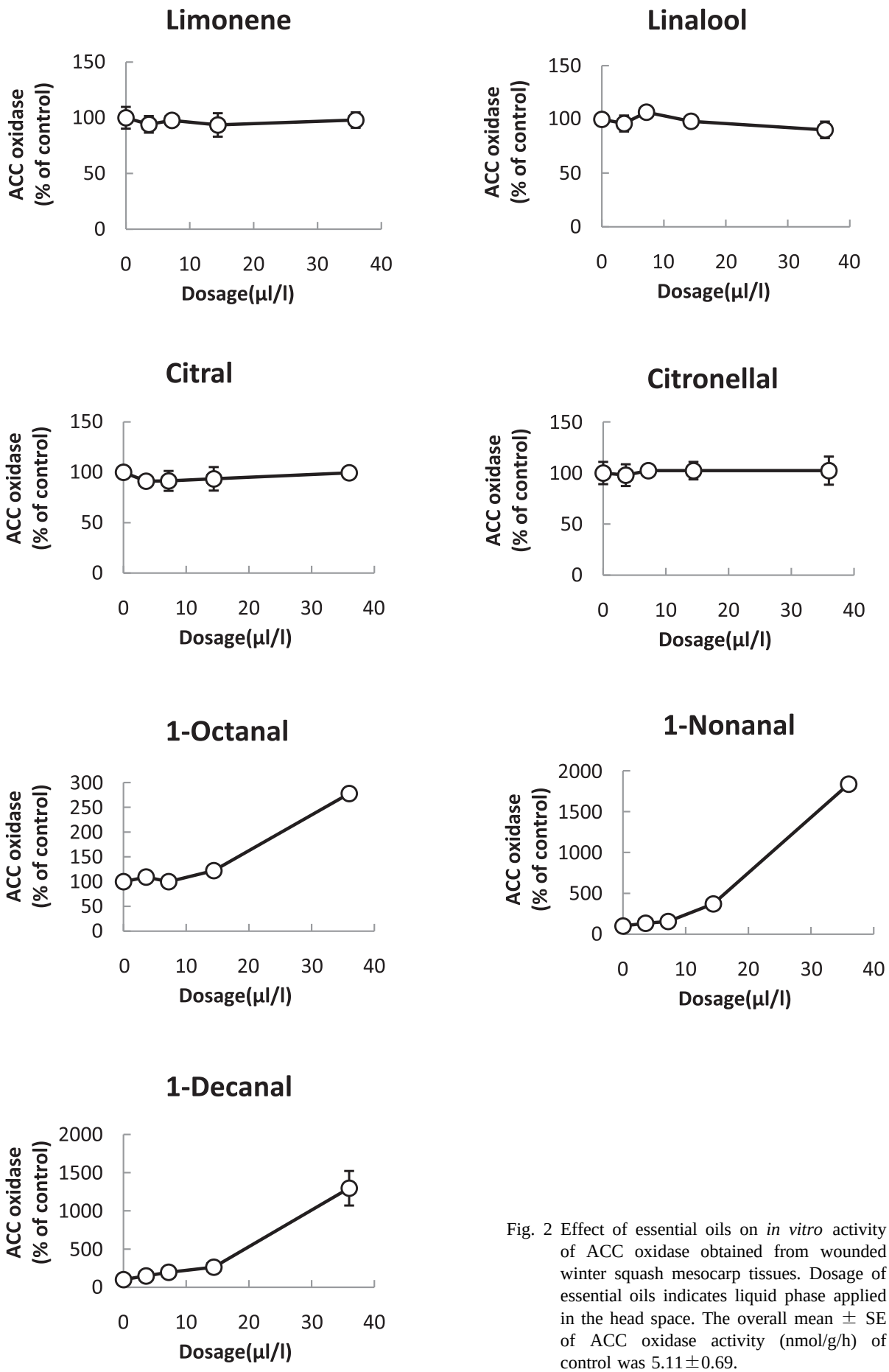

Fig. 2 Effect of essential oils on *in vitro* activity of ACC oxidase obtained from wounded winter squash mesocarp tissues. Dosage of essential oils indicates liquid phase applied in the head space. The overall mean ± SE of ACC oxidase activity (nmol/g/h) of control was 5.11±0.69.

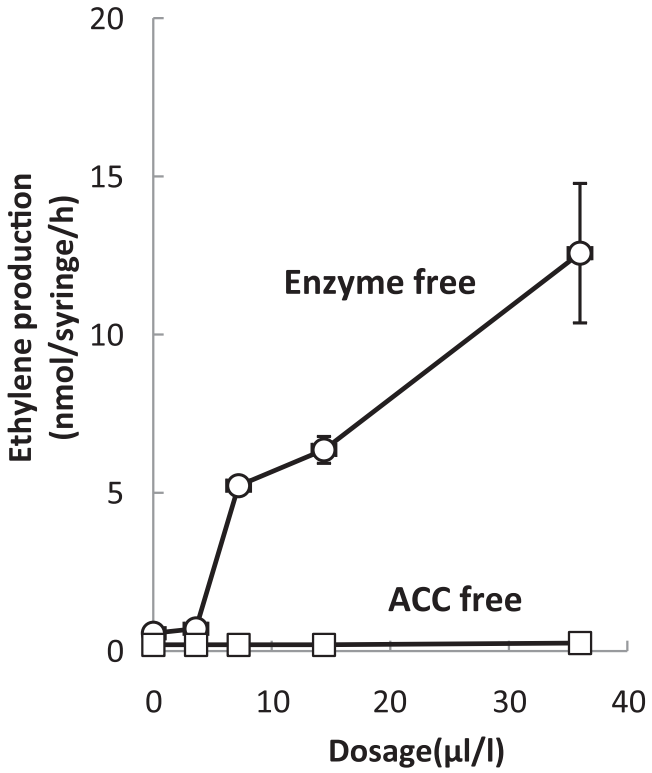

Fig. 3 Stimulation of non-enzymatic conversion of ACC to ethylene by aliphatic aldehyde 1-nonanal. Dosage of essential oils indicates liquid phase applied in the head space.

It is well known that plants emit carbonyl compounds including pentanal, hexanal, nonanal, and decanal (Kesselmeier and Staudt, 1999). Matsui and Hatanaka (2005) reported biological activities of green leaf volatiles in which C_6 aldehydes and alcohols are main constituents. Among them 1-hexanal is also involved. If these aliphatic aldehydes are produced in the plant tissues by some factors such as wounding or senescence, non-enzymatic breakdown of ACC occurs leading to ethylene production. The ethylene thus produced seems to trigger the subsequent physiological cascades like autocatalytic ethylene production.

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摘　　要

A. B. M. ゴーラム　ラバニー・水谷房雄・近泉惣次郎：カボチャから得た ACC 合成酵素と酸化酵素の試験管内での活性に及ぼす精油の影響

カボチャの果肉から調製した粗酵素液を用いて，試験管内で精油が ACC 合成酵素と酸化酵素活性に及ぼす効果を調査した．閉じた試験管内のヘッドスペースに気化した精油を含む状態で酵素活性を測定した．テストした全ての精油が ACC 合成酵素活性を抑制した．リモネンとリナロールではそれぞれ28，25µl/l の処理濃度で50％の活性抑制が見られた．アルデヒド型の精油ではさらに強い抑制活性が見られた．処理をした濃度範囲で（3.6～36 µl/l）シトラール，1-オクタナール，1-ノナナール，1-デ

カナールでは50～78%の活性抑制が，シトロネラールでは78～93%の活性抑制が見られた．一方，精油は ACC 酸化酵素活性には抑制効果を示さなかった．しかしながら，1-オクタナール，1-ノナナール，1-デカナールのような脂肪族アルデヒド型の精油は非酵素的に ACC を分解し，エチレンを生成した．