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INHIBITION OF ETHYLENE PRODUCTION AND 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE ACTIVITY BY TROPOLONES

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Key Word Index—*Malus domestica*; Rosaceae; apple; tropolone; hinokitiol; C_2H_4; ACC oxidase; Fe^{2+}; chelating.

Abstract—The effect of tropolone and hinokitiol on C_2H_4 production and *in vitro* ACC oxidase activity was examined by using apple fruit plugs. Application of aqueous tropolone and hinokitiol solution inhibited C_2H_4 production, but the addition of Fe^{2+} in the solution alleviated the inhibition. Gaseous tropolone and hinokitiol were also effective in suppressing C_2H_4 production, but the suppression was annulled when apple plugs were pretreated with Fe^{2+}. Tropolone and hinokitiol inhibited *in vitro* ACC oxidase activity, which was recovered by adding Fe^{2+} in the assay medium. Therefore part of inhibition of C_2H_4 production by these tropolones was due to their chelating action of Fe^{2+}, which is essential for the activation of ACC oxidase. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Hinokitiol (β-thujaplicin), a tropolone-type compound, was first isolated from the wood of *Chamaecyparis taiwanensis* [1]. Since then similar compounds such as α-thujaplicin and γ-thujaplicin have been isolated from the heart wood of *Thuja plicata* [2]. These compounds inhibit the growth of wood-rotting fungi and therefore their heart wood shows resistance towards decay. Among these compounds, there have been numerous reports on the biological activity of hinokitiol because of its high yield [3, 4]. Other tropolone derivatives have also been isolated from *Penicillum* sp. (stipitatic acid), gall of *Quercus* sp. (purpurogallin) and *Colchicum* sp. (colchicine). *Pseudomonas plantarii* is the causal agent of seedling blight in rice. Under iron-limiting conditions, the bacterium produces tropolone, which suppresses the growth of microorganisms [5–7] and is toxic to rice seedlings [8]. In relation to its antimicrobial activity, hinokitiol has been used as the agent that controls postharvest disease in peach [9], eggplant and pepper fruit [10].

Recently it was reported that tropolone and hinokitiol have strong inhibitory activity on the growth of plants [11]. Furthermore we reported that tropolone compounds including tropolone, hinokitiol, purpurogallin and colchicine inhibited C_2H_4 production and suppressed increase in ACC content in excised young peach seeds [12].

Tropolones are known as chelating agents [3, 5, 13, 14] and ACC oxidase requires Fe^{2+} for its activity [15]. Therefore in this study we have tried to establish if tropolone and hinokitiol inhibit ACC oxidase activity via their chelating action with Fe^{2+}, resulting in the inhibition of C_2H_4 production.

RESULTS AND DISCUSSION

Figure 1 shows the effect of application of aqueous tropolone and hinokitiol solution on C_2H_4 production of apple plugs. In tropolone-treated plugs, with higher concentrations, greater inhibition of C_2H_4 production was observed during the first 30 min incubation. But during the 60–90 min and 120–150 min incubation, there was no difference in the inhibition over the range from 0.1 mM to 1.0 mM although it seemed that the inhibition became greater as time elapsed. In hinokitiol-treated plugs, there was no difference in the inhibition of C_2H_4 production among concentrations from 0.1 mM to 1.0 mM with any incubation period. However, inhibition by hinokitiol also seemed to increase with time.

The effect of aqueous tropolone and hinokitiol solution at 0.1 mM with or without addition of Fe^{2+} at 0.25 mM on C_2H_4 production is shown in Fig. 2. During the first 30 min and 60–90 min incubation periods, the addition of Fe^{2+} to tropolone and hino-

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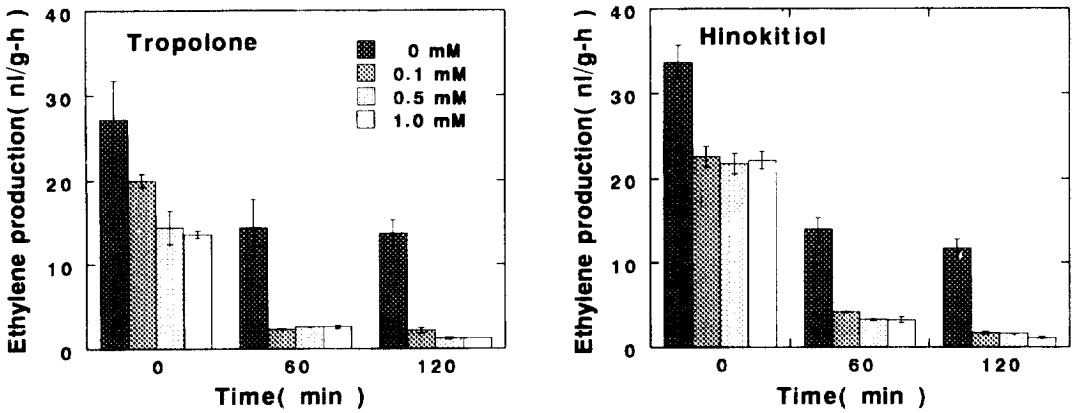

Fig. 1. Effect of application of aqueous tropolone and hinokitiol solution on C_2H_4 production in apple fruit plugs.

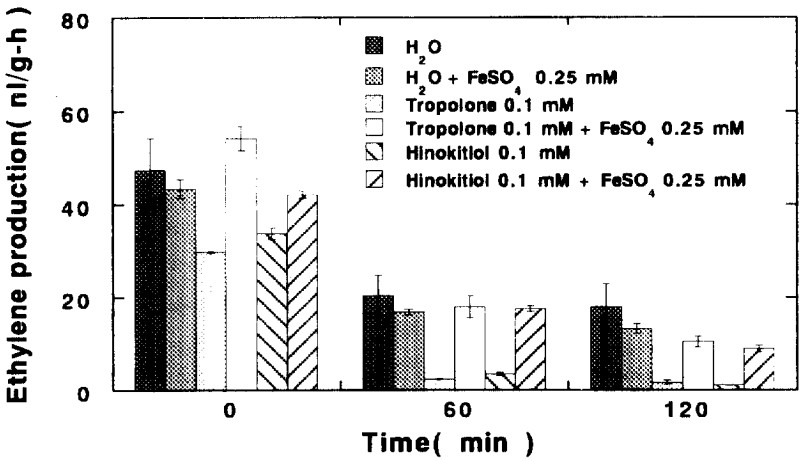

Fig. 2. Effect of application of aqueous tropolone and hinokitiol solution with or without addition of Fe^{2+} on C_2H_4 production in apple fruit plugs.

kitiol solution caused no inhibition in C_2H_4 production compared with control although tropolone and hinokitiol alone inhibited C_2H_4 production.

Tropolone and hinokitiol, with melting points 50–51° and 51–52°, respectively, readily volatilize at room temperature. Their gaseous phase was also effective in inhibition of C_2H_4 production (Fig. 3). However, apple plugs pretreated with Fe^{2+} at 0.1, 0.25 and 0.5 mM alleviated the inhibition of C_2H_4 production by these gaseous tropolone compounds. The complete recovery from inhibition was observed at 0.5 mM Fe^{2+}. In our previous work, gaseous tropolone and hinokitiol inhibited C_2H_4 production in young peach seeds excised from pits [12]. Reciprocal transfer of peach seeds from air to gaseous hinokitiol and vice versa revealed that suppression of C_2H_4 production immediately was caused by hinokitiol and the C_2H_4 production was recovered upon transfer from hinokitiol to air when the gaseous concentration was low but irreversible when high [12].

It is well documented that Fe^{2+} is an essential cofactor in the activation of ACC oxidase [15]. Within the range from 0.01–0.05 mM Fe^{2+}, gradual decreases in ACC oxidase activity were found with increasing concentrations of tropolone and hinokitiol (Fig. 4). However, at 0.1 and 0.25 mM Fe^{2+} there was little inhibition in the enzyme activity even when high concentrations of tropolone and hinokitiol were employed. Because tropolones are known as chelating agents [3, 8, 13, 14], it is most likely that *in vivo* ACC oxidase activity is also inhibited by their chelating function with Fe^{2+}. In relation to their affinity to metal ions, it was reported that the antifungal action of hinokitiol was completely eliminated by iron, and reduced to one-quarter by zinc, but not affected by copper [3]. This specificity of the copper–hinokitiol complex was assumed to be due to its substitution with iron [3]. Furthermore, in our previous work, tropolone compounds including tropolone, hinokitiol purpurogallin and colchicine suppressed not only ethylene production but also increase in ACC content in excised young peach seeds [12]. In a separate experiment, tropolone and hinokitiol inhibited *in vitro* activity of ACC synthase from winter squash mesocarps (unpublished data). When these facts and the present results are taken together, tropolone com-

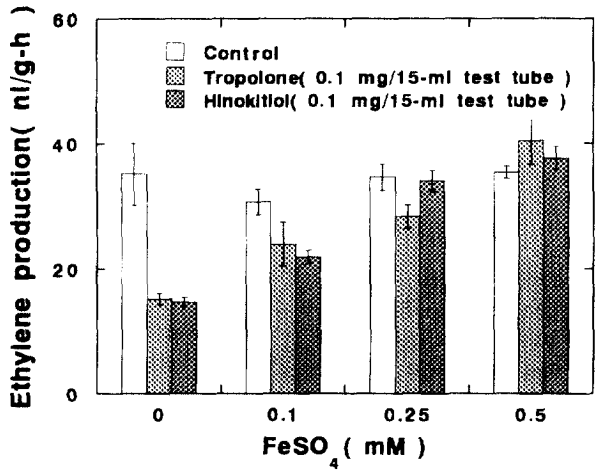

Fig. 3. Effect of gaseous tropolone and hinokitiol on C_2H_4 production in apple fruit plugs pretreated with various concentrations of Fe^{2+}. 0.1 mg (solid) of tropolone and hinokitiol was volatilized in a 15-ml test tube by slightly heating and then cooled. An apple plug pretreated with Fe^{2+} was placed in the test tube, sealed with a rubber septa and incubated at 30° for 30 min.

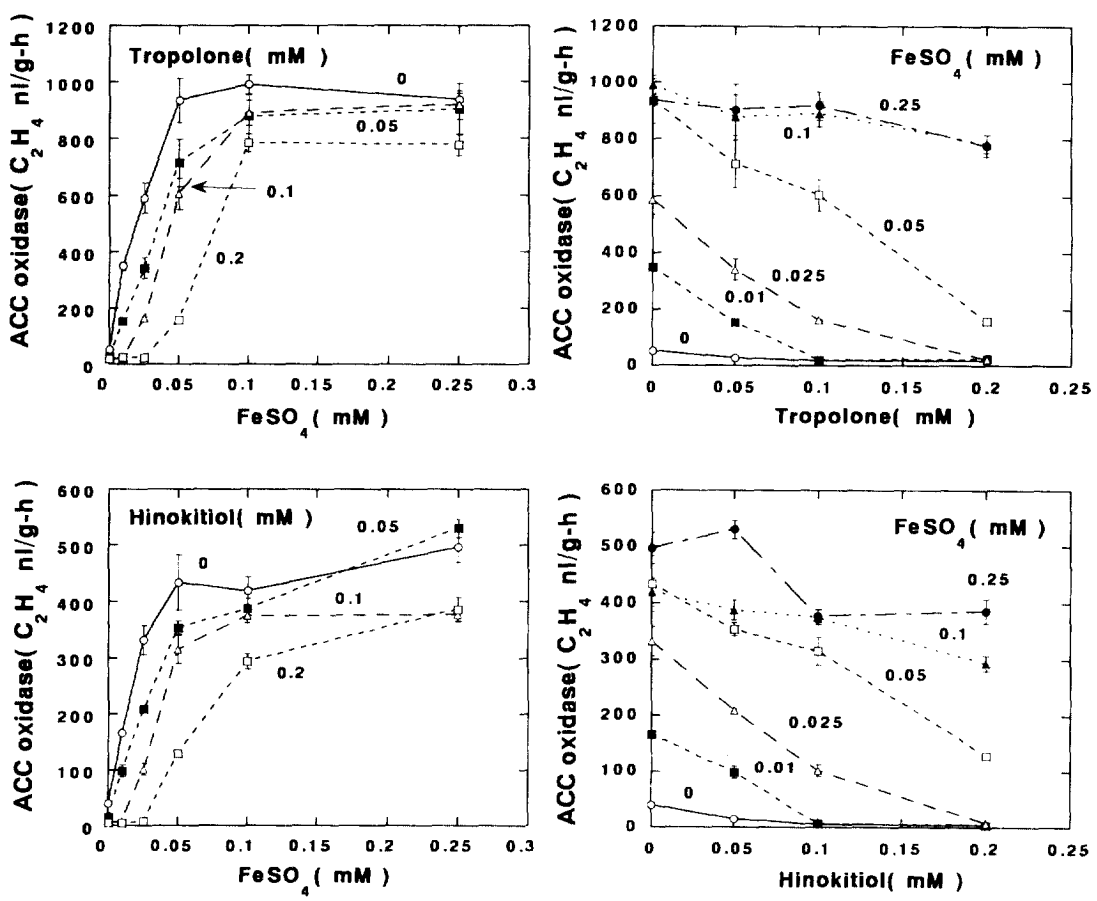

Fig. 4. Effect of aqueous tropolone and hinokitiol solution with addition of various Fe^{2+} concentrations on *in vitro* ACC oxidase activity from apple fruit.

pounds seem to inhibit C_2H_4 biosynthesis by interfering with both ACC synthase and ACC oxidase activity.

EXPERIMENTAL

Plant materials

Apple (*Malus domestica* Borkh. cv. Golden Delicious) fruit at climacteric stage as confirmed by C_2H_4 production rate were used. Plugs were made from the cortical tissues by using a cork borer (10 mm diam. × ca 7 mm, 500 mg fresh wt).

Effect of tropolone and hinokitiol on C_2H_4 production of apple tissues

Aq. soln of tropolone, hinokitiol at 0, 0.1, 0.5 and 1 mM was prepared by dissolving them in H_2O. Apple plugs were immersed in the soln in glass vials and the chemicals were impregnated into tissues for 5 min under red. pres. The plugs were taken out and blotted with filter paper. Each plug was placed in a 15-ml test tube, sealed with a rubber stopper and incubated at 30°. C_2H_4 accumulation in the head space of the glass vials during 0–30, 60–90 and 120–150 min from the beginning of incubation was determined by GC.

Effect of addition of Fe^{2+} on recovery of C_2H_4 production in tropolone- and hinokitiol-treated apple tissues

The effect of aq. soln of tropolone and hinokitiol at 0.1 mM with or without addition of 0.25 mM $FeSO_4$ on C_2H_4 production of apple plugs was investigated. Aq. soln of 0.25 mM $FeSO_4$, 0.1 mM tropolone, 0.1 mM tropolone +0.25 mM $FeSO_4$, 0.1 mM hinokitiol and 0.1 mM hinokitiol +0.25 mM $FeSO_4$ were prepared. Treatment and assay for C_2H_4 determination were similar to the method in the above section.

Effect of gaseous tropolone and hinokitiol on C_2H_4 production of apple tissues pretreated with Fe^{2+}

Apple plugs were immersed in $FeSO_4$ soln at 0, 0.1, 0.25 and 0.5 mM in glass vials and treated as described above. The plugs were taken out and blotted with filter paper. A 0.1 mg of hinokitiol and tropolone was put into a 15-ml test tube, sealed with a rubber septum and volatilized by slightly heating, then cooled. Each apple plug pretreated with various concns of Fe^{2+} was placed in the 15-ml test tube, sealed with a rubber stopper and incubated for 30 min at 30°. A gas sample was withdrawn from the test tube and C_2H_4 concn was determined by GC.

Effect of aqueous tropolone and hinokitiol with or without Fe^{2+} on in vitro *ACC oxidase activity*

Cortical tissue was ground with a pestle and mortar in 2 ml g^{-1} tissue of extraction medium of 0.1 M Tris-HCl (pH 7.4) containing 10% (v/v) glycerol and 30 mM Na ascorbate. The homogenate was filtered through four layers of cheesecloth. The filtrate was used as the enzyme source. All procedures were carried out at 4°. The enzyme activity was assayed in 12-ml plastic syringes capped with rubber septa. The reaction mixture was 0.2 M MOPS (pH 6.7) containing 10% (v/v) glycerol, 30 mM Na ascorbate, 1 mM ACC, 0.1% Triton X-100, 20% gaseous CO_2, 14 mM $NaHCO_3$, tropolone or hinokitiol (0, 0.05, 0.1, 0.2 mM), $FeSO_4$ (0, 0.01, 0.025, 0.05, 0.1, 0.25 mM) and 0.2 ml crude enzyme extract, in a total vol. of 1.56 ml. After the final addition of crude extract and rapid shaking, the syringes were transferred to a reciprocating shaker (90 cycles min^{-1}) at 30°. C_2H_4 produced in the head space of the syringe after 30 min incubation was determined by GC [15]. All the experiments were conducted in triplicate.

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