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Inhibition of Auxin-Induced Ethylene Production by Salicylic Acid in Mung Bean Hypocotyls

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Salicylic acid (SA), a common plant phenolic compound, influences diverse physiological and biochemical processes in plants. To gain insight into the mode of interaction between auxin, ethylene, and SA, the effect of SA on auxin-induced ethylene production in mung bean hypocotyls was investigated. Auxin markedly induced ethylene production, while SA inhibited the auxin-induced ethylene synthesis in a dose-dependent manner. At 1 mM of SA, auxin-induced ethylene production decreased more than 60% in hypocotyls. Results showed that the accumulation of ACC was not affected by SA during the entire period of auxin treatment, indicating that the inhibition of auxin-induced ethylene production by SA was not due to the decrease in ACC synthase activity, the rate-limiting step for ethylene biosynthesis. By contrast, SA effectively reduced not only the basal level of ACC oxidase activity but also the wound- and ethylene-induced ACC oxidase activity, the last step of ethylene production, in a dose-dependent manner. Northern and immuno blot analyses indicate that SA does not exert any inhibitory effect on the ACC oxidase gene expression, whereas it effectively inhibits both the in vivo and in vitro ACC oxidase enzyme activity, thereby abolishing auxin-induced ethylene production in mung bean hypocotyl tissue. It appears that SA inhibits ACC oxidase enzyme activity through the reversible interaction with Fe^{2+}, an essential cofactor of this enzyme. These results are consistent with the notion that ethylene production is controlled by an intimate regulatory interaction between auxin and SA in mung bean hypocotyl tissue.

keywords: ACC oxidase, auxin, ethylene, mung bean hypocotyls, salicylic acid, *Vigna radiata*

The gaseous plant hormone ethylene controls many physiological processes during plant growth and development. The rate of ethylene production is usually low in most plant tissues, but increases dramatically at certain developmental stages, including seed germination, leaf senescence and abscission, and fruit ripening (Yang and Hoffman, 1984). Ethylene production also increases in response to diverse biotic and abiotic stresses, including wounding, drought, flooding, anaerobiosis, pathogen attack or auxin treatment (Yang and Hoffman, 1984; Theologis, 1992). In higher plants, ethylene is produced from methionine via S-adenosyl-L-methionine and ACC (Met → AdoMet → ACC → C_2H_4) (Yang and Hoffman, 1984; Theologis, 1992; Kende, 1993). The last two steps of this biosynthetic pathway are catalyzed by ACC synthase and ACC oxidase, respectively. In fruit tissue, these two unique enzymes are induced during ripening and contribute to the regulatory steps for ethylene production. In vegetative tissues, however, ACC oxidase is constitutively expressed so that ACC synthase is regarded as the rate-limiting step for ethylene biosynthesis (Yang and Hoffman, 1984; Theologis, 1992). Auxin stimulates ethylene production in a wide variety of plant tissues by promoting the synthesis of ACC synthase (Yang and Hoffman, 1984; Kim et al., 1992; Theologis, 1992; Kende, 1993). IAA-induced ethylene production in mung bean hypocotyls can be inhibited both by cycloheximide (CHI), a protein synthesis inhibitor, and by aminoethoxyvinylglycine (AVG) or aminooxyacetic acid (AOA), inhibitors of the pyridoxal 5'-phosphate (PLP)-mediated reaction catalyzed by ACC synthase (Yang and Hoffman, 1984).

Salicylic acid (SA), a common plant phenolic compound, influences numerous physiological and biochemical processes in plants. These include flower stimulation (Khurana and Maheshwari, 1983), vegetative bud formation (Fries, 1984), inhibition of phosphate and potassium uptake (Glass and Dunlop, 1974), adventitious root initiation (Kling and Meyer, 1983) and thermogenesis (Raskin et al., 1987). Recently, SA has been shown to be a key signaling component involved in the plant defense responses (Durner et al., 1997) and was suggested to be a signal involved in the induction and maintenance of systemic acquired resistance (SAR) in tobacco plants (Ryals et al., 1994).

In order to gain insight into the mode of interaction between auxin, ethylene and SA, we examined the

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effect of SA on auxin-induced ethylene production in mung bean hypocotyls. Here, we report that SA inhibits auxin-induced ethylene production by inhibiting the ACC oxidase enzyme activity in mung bean hypocotyls. The possible mode of action of SA on the inhibition of ACC oxidase activity is also discussed.

MATERIALS AND METHODS

Plant Material

Dry seeds of mung bean (*Vigna radiata* L.) were imbibed overnight in aerated tap water. Seedlings were grown for 3 days in a dark room at 25°C. For various treatments, batches (2 g each) of hypocotyl segments were incubated in 10 mL of a medium consisting of 2% (w/v) sucrose, 1 mM $CaCl_2$, and 50 mM Mes buffer (pH 6.2) in Erlenmeyer flasks for various time periods with continuous shaking. Where indicated, addenda were 100 μM IAA + 50 μM BA, 100 μM IAA + 50 μM BA + various concentrations (0.1-5 mM) of SA (Figs. 1, 2, 3, and 5), 1 mM SA or 1 mM SA + 10 μL/L ethylene (Fig. 4).

Determination of Ethylene Levels

A 1 mL gas sample was withdrawn from the flask with a hypodermic syringe, and ethylene was assayed on a gas chromatograph equipped with an aluminum column and flame ionization detector.

Determination of ACC

Approximately 1 g of tissue was extracted twice at 80°C in 5 mL 80% ethanol for 2 h. The extracts were then concentrated in vacuo at 40°C and adjusted with water to 1 mL. ACC was then determined by the method of Lizada and Yang (1979).

Extraction and Assay of ACC Oxidase

For assay of ACC oxidase in vivo, 1 g of hypocotyl tissue was incubated for 1 h in 3 mL of medium containing 2% (w/v) sucrose, 1 mM $CaCl_2$, 50 mM Mes buffer (pH 6.2) and 2 mM ACC, and the ethylene produced was measured by gas chromatography (Fernandez-Maculet and Yang, 1992). For the in vitro enzyme assay, mung bean hypocotyl tissues were pulverized in liquid nitrogen and homogenized in 0.5 mL/g of extraction buffer consisting of 100 mM Mops (pH 7.2), 10% glycerol, and 1 mM PMSF. After centrifuging at 28,000g for 25 min at 4°C, the supernatant was used for enzyme assays. Enzyme activity was assayed at 30°C for 1 h in a sealed 12-mL tube which contained 2 mL of reaction mixture consisting of 100 mM Mops (pH 7.2), 10% glycerol, 1 mM ACC, 25 μM $FeSO_4$, 3 mM sodium ascorbate, 10 mM sodium bicarbonate and 200 μL crude extract. Ethylene accumulation in the head space was determined by gas chromatography (Fernandez-Maculet and Yang, 1992). Protein concentration was determined using a Bio-Rad protein assay kit with BSA as a standard.

RNA Isolation and Northern Blot Analysis

Total RNAs of mung bean hypocotyls were obtained by a method as described previously (Kim et al., 1997a). The total RNA was precipitated overnight at 4°C by the addition of 0.3 volume of 10 M LiCl and then precipitated in ethanol. Total RNA (20 μg) was separated by electrophoresis in a 1% formaldehyde-agarose gel and blotted to a nylon membrane (MSI, Westboro, MA, USA). To ensure equal loading of RNA, the gel was stained with ethidium bromide after electrophoresis. In order to confirm complete transfer of RNA to the membrane filter, both gel and membrane were viewed under UV light at the end of transferring. The filter was hybridized to ^{32}P-labeled pVR-ACO1 (Kim and Yang, 1994). The blot was washed as described previously (Kim et al., 1997a) and visualized by autoradiography at −80°C using Kodak XAR-5 film and an intensifying screen (Cronex, DuPont).

Protein Gel Blot Analysis

After separation using an 8% SDS-PAGE, proteins were transferred from the gel onto a nitrocellulose membrane filter (MSI, Westboro, MA, USA) using an electroblotting apparatus (Novex, San Diego, CA, USA). After blocking with 5% (w/v) non-fat dried milk in TTBS (20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20) for 1 h at room temperature, the membrane was incubated for 1 h with anti-ACO1 antiserum (1:2,000 dilution) (Jin et al., 1999). The blot was then washed five times with TTBS and incubated for 1 h with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5,000 dilution) (Amersham, Buckinghamshire, UK). After washing with the same buffer, antibody binding was then detected with the chemiluminescence western blot detection kit from Amersham, according to the manufacturer's protocol.

RESULTS AND DISCUSSION

In various vegetative tissues, auxin stimulates ethylene synthesis by promoting the conversion of AdoMet to ACC (Yu et al., 1979; Yoshii and Imaseki, 1982; Kim et al., 1992). Figure 1 shows the time course of the ethylene production rate of mung bean hypocotyls in the presence or absence of IAA (100 μM) and with various concentrations of SA (0.1-1 mM). BA (50 μM) was added to promote the action of auxin (Lau and Yang, 1973; Kim et al., 1992). In the absence of IAA, ethylene production was not detectable throughout the entire incubation period (Fig. 1A). In the presence of 100 μM IAA + 50 μM BA, the ethylene synthesis rate in mung bean hypocotyls was low (6 nL/g·h) initially but continuously increased, reaching a maximum (45 nL/g·h) at 10 h incubation (Fig. 1A). Subsequently, the ethylene synthesis rate declined. Figure 1 also demonstrates that SA significantly inhibits auxin-induced ethylene production in a dose-dependent manner. In the presence of 0.1 mM SA, auxin-induced ethylene production was inhibited about 15% and 23% at 7 h and 10 h incubation, respectively (Figs. 1A and B). At 1 mM of SA, the auxin-induced ethylene production rate decreased more than 60% (Figs. 1A and B).

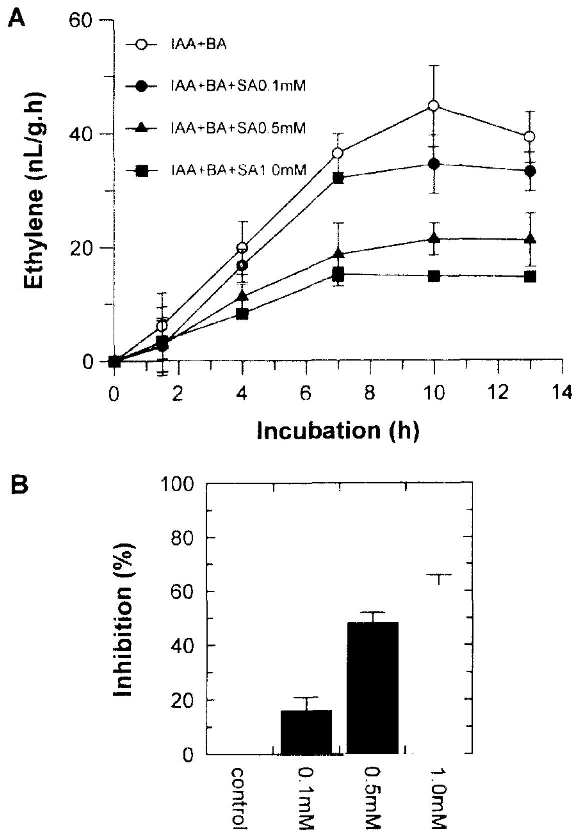

Figure 1. A. Dose-dependent inhibitory effect of SA on the auxin-induced ethylene production. Three-day-old etiolated mung bean hypocotyls were segmented into 1-cm lengths and incubated in Mes buffered medium with or without 100 μM IAA, 50 μM BA and various concentrations (0.1-1 mM) of SA for 13 h. **B.** Percent inhibition of the ethylene production rate of auxin-treated samples incubated with 100 μM IAA + 50 μM BA + various concentrations (0.1-1 mM) of SA for 7 h. Each set was duplicated. These experiments were performed three times and all presented the same pattern.

In order to examine whether the inhibition of auxin-induced ethylene production by SA was due to the decrease in ACC synthase activity, the rate-limiting step for ethylene biosynthesis, the time courses of ethylene production and ACC accumulation were compared in the presence or absence of 100 μM IAA and 1 mM SA in mung bean hypocotyls. Before the auxin treatment, both the level of ACC and ethylene production were barely detectable (Fig. 2). With auxin treatment, the ACC level increased to 25 nmol/g in 5 h, and reached 35 nmol/g after 7 h. This induction pattern of ACC level was in parallel with that of ethylene production during the incubation with auxin (Fig. 2). As shown in Figure 1, the ethylene production rate was very low (less than 5 nL/g·h) throughout the incubation with auxin + SA treatment. By contrast, the amount of ACC was not affected by SA, and the induction of ACC accumulation with auxin + SA treatment exhibited a similar pattern to that with auxin treatment (Fig. 2), indicating that SA did not exert any inhibitory effect on ACC synthase. These results demonstrate that the inhibition of auxin-

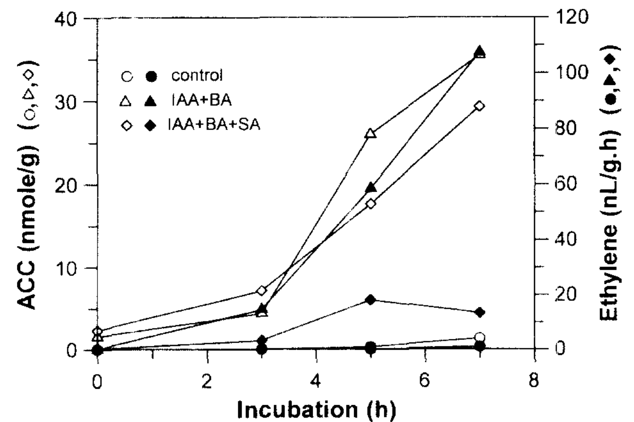

Figure 2. Time courses of differential changes in ACC content and ethylene production by SA in mung bean hypocotyls at various time points. Samples were incubated with 100 μM IAA, 50 μM BA, and with or without 1 mM SA.

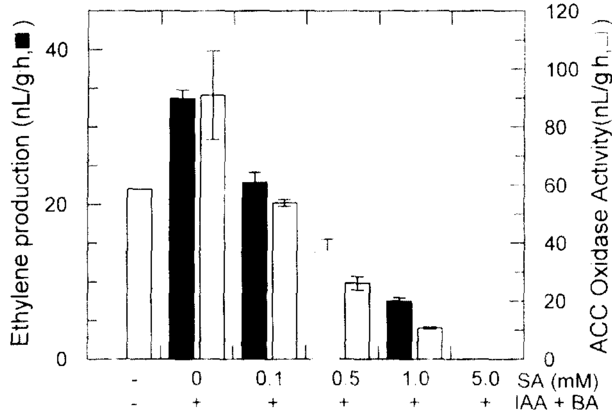

Figure 3. Similar pattern of inhibition of ethylene prouction and in vivo ACC oxidase activity by SA in mung bean hypocotyls. Samples were incubated with 100 μM IAA + 50 μM BA ± 0.1-5 mM SA for 7 h.

induced ethylene production by SA is not due to the decrease in ACC synthase enzyme activity.

Results as seen in Figure 1 and Figure 2 raise the possibility that SA inhibits the auxin-induced ethylene production through the inhibition of ACC oxidase, the last step of ethylene biosynthesis. To investigate this possibility, mung bean hypocotyls were incubated with 100 μM IAA + 50 μM BA or 100 μM IAA + 50 μM BA + various concentrations of SA for 7 h, and the ethylene production rate and in vivo ACC oxidase enzyme activity were monitored. Previous studies have shown that ACC oxidase is present constitutively, and low basal levels of ACC oxidase mRNA and enzyme activity are detected without any treatment in mung bean hypocotyls (Kim and Yang, 1994; Kim et al., 1997b; Jin et al., 1999). Before the auxin treatment, ACC oxidase enzyme activity was 24 nL/g·h., and slightly increased to 35 nL/g·h after incubation with 100 μM IAA + 50 μM BA for 7 h (Fig. 3). In the presence of 100 μM IAA + 50 μM BA + 0.1 mM SA, however, ACC oxidase activity was reduced to 20 nL/g·h, while 1.0 mM SA markedly decreased enzyme activity to 4 nL/g.h (Fig. 3). The ACC oxidase activity was not detectable when hypocotyl tissue was treated with IAA + BA + 5.0 mM SA. Thus, SA reduces not only auxin-induced ACC oxidase enzyme activity but also the basal level of activity in a dose-dependent manner. In addition, the decrease in the ACC oxidase activity by SA was in parallel with that of ethylene production (Fig. 3). These results strongly suggest that SA inhibits auxin-induced ethylene production through the decrease in ACC oxidase activity in mung bean hypocotyls.

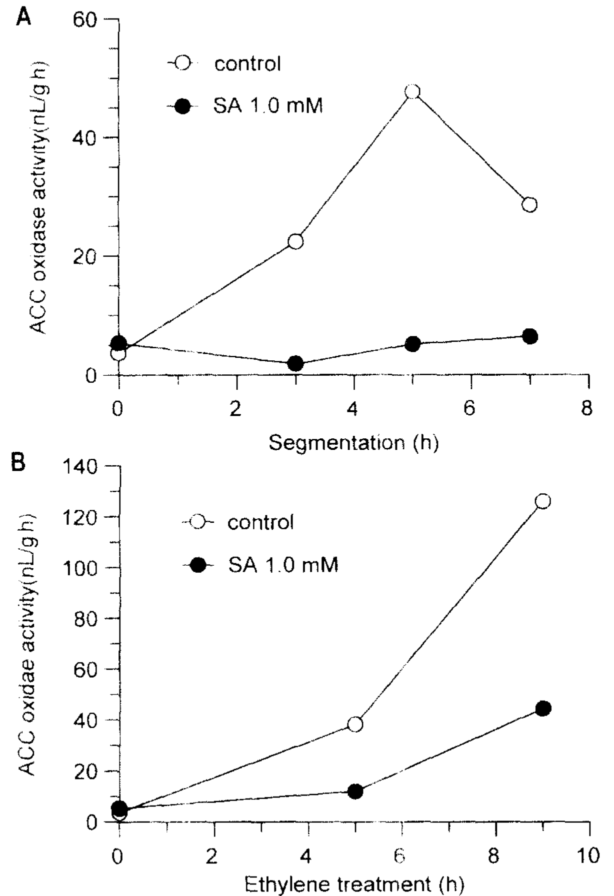

Figure 4. Inhibitory effect of SA on the wound- and ethylene induced ACC oxidase activity in mung bean hypocotyls. For wounding, hypocotyl tissue was excised into 1 cm-long sections and incubated in Mes buffered medium for several different time periods. For ethylene treatment, intact hypocotyls were enclosed in 4-L jars containing air or air with 10 μL/L ethylene for various periods. After incubation, in vivo ACC oxidase enzyme activity was immediately measured as described in Materials and Methods.

We have previously shown that wounding and ethylene markedly increase the expression of ACC oxidase, the last step of its own biosynthetic pathway, in mung bean hypocotyls by an auto-catalysis mechanism (Kim and Yang, 1994; Kim et al., 1997b; Jin et al., 1999). To assess the effect of SA on the wound- and ethylene-induced ACC oxidase activity, the excised mung bean hypocotyl segments were incubated with or without 1 mM SA or 1 mM SA + 10 μL/L ethylene for different time periods, and then in vivo ACC oxidase activities were determined. As shown in Figure 4A, the ACC oxidase enzyme activity increased from 10 nL/g·h at time zero to a maximum activity of 49 nL/g·h at 6 h wounding, and then subsequently declined. This wound-induced ACC oxidase activity,

however, was almost completely abolished by 1 mM SA (Fig. 4A). Similarly, the basal level of enzyme activity was up-regulated by ethylene treatment and reached 130 nL/g·h at 9 h ethylene treatment (Fig. 4B). With 1 mM SA treatment, the ethylene-induced increase in ACC oxidase activity was effectively blocked (Fig. 4B). These results indicate that SA decreases not only the basal level of ACC oxidase activity but also the wound- and hormone-induced enzyme activity in mung bean hypocotyls.

The results described above indicate that SA decreases the ACC oxidase activity, and hence effectively inhibits auxin-induced ethylene production in mung bean hypocotyls. Decrease in the ACC oxidase activity by SA could be due to the reduction of mRNA and protein levels of ACC oxidase. Alternatively, SA may directly inhibit the ACC oxidase enzyme activity. In order to study the mode of action of SA in decreasing the ACC oxidase activity, Northern blot analysis was carried out using total RNA isolated from mung bean hypocotyls which had been incubated for 7 h with 100 μM IAA + 50 μM BA, 100 μM IAA + 50 μM BA + 1 mM SA or 1 mM SA (Figs. 5A). Although 1 mM SA effectively reduces ACC oxidase activity (Figs. 3 and 4), the results of Figure 5A show that the level of ACC oxidase transcript is not affected by SA. These results suggest that SA does not exert its inhibitory effect on the level of ACC oxidase mRNA. This interpretation is further supported by the results of immuno blot analysis that ACC oxidase protein levels were not changed by SA during the incubation with 100 μM IAA + 50 μM BA, 100 μM IAA + 50 μM BA + 1 mM SA or 1 mM SA (Fig. 5B). Taken together, these results are consistent with the notion that SA does not reduce the expression of ACC oxidase gene, but directly inhibits the ACC oxidase enzyme activity, thereby blocking the auxin-induced ethylene production in mung bean hypocotyls.

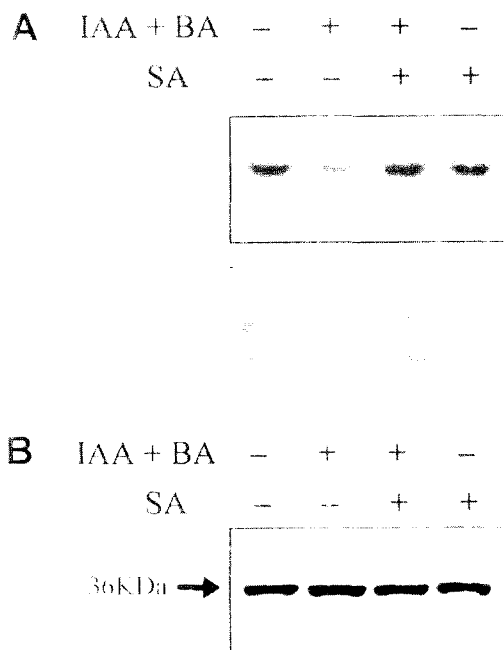

Figure 5. Effect of SA on the levels of ACC oxidase mRNA and protein in mung bean hypocotyls. **A.** Northern blot analysis of mung bean hypocotyl RNA. Total RNA (20 μg) isolated from mung bean hypocotyls treated with 100 μM IAA + 50 μM BA ± 1 mM SA was resolved and blotted as described in Materials and Methods, and hybridized to ^{32}P-labeled pVR-ACO1. **B.** Immuno blot analysis of mung bean hypocotyl protein. Total protein (12 μg) obtained from hypocotyls identically treated as in A was resolved using an 8% SDS-PAGE, transferred onto a nitrocellulose membrane filter and then incubated with anti-ACC oxidase antiserum as described in Materials and Methods. Antibody binding was visualized by the chemiluminescence detection method.

SA has been investigated at the view of the elicitor effect in plants (Raskin, 1992). A study by Sanchez-Casas and Klessig (1994) revealed that the mode of action of SA in plant defense is to bind catalase and inhibits its activity which results in an increase in the concentration of H_2O_2. They further suggested that catalase was the only SA receptor or binding component in the plant cell, playing a central role in the plant defense mechanism. This speculation, however, was reinvestigated by Rulfer et al. (1995) who showed that SA was able to bind to heme and non-heme iron enzymes so that the bound SA impaired

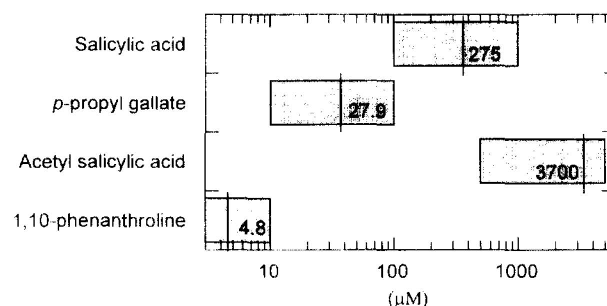

Figure 6. Effective concentration range and 50% inhibition concentration (IC_{50}) of chemicals which have an inhibitory effect on the in vitro ACC oxidase enzyme activity. *n*-Propyl-gallate is a free radical scavenger and acetyl SA is an active SA analog. 1,10-Phenanthroline is a chelator of divalent cations. In vitro ACC oxidase activity of each samples incubated with these reagents was compared with the enzyme activity of mock-treated sample. Percent inhibition and $\log_{10}$ [reagents] were processed by linear regression functions and 50% inhibition concentrations were calculated.

known to be a non-heme iron protein (Pirrung et al., 1993; Jin et al., 1998). Indeed, inhibition by SA in ethylene biosynthesis on the conversion from ACC to ethylene was reported in pear cell suspension culture (Leslie and Romani, 1988), embryonic axes of chick-pea seeds (Munoz De Rueda et al., 1995) and apple fruits (Fan et al., 1996). In addition, it has been recently shown that inhibition of ACC oxidase activity by SA could be reversed by increasing the concentration of Fe^{2+} (Jin et al., 1998). Thus, SA may inhibit the ACC oxidase enzyme activity through the direct chelation of Fe^{2+}, or it may bind and modify the site that may provide the ligand for Fe^{2+} in the ACC oxidase enzyme. In order to assess the relationship between SA and Fe^{2+} in the ACC oxidase enzyme activity in more detail, we examined the effect of cation chelating agent and radical scavenger on the ACC oxidase activity. *n*-Propyl-gallate, a radical scavenger, effectively inhibits the in vitro ACC oxdase enzyme activity and its 50% inhibition concentration (IC_{50}) was 27.9 μM (Fig. 6). Thus, ACC oxidase is about 10 times more sensitive to *n*-Propyl-gallate than to SA. Aspirin, an acetylsalicylic acid, was also able to reduce the ACC oxidase activity to a lesser extent with an IC_{50} of 3,700 μM (Fig. 6). The cation chelater 1,10-phenanthroline was the most effective inhibitor and its IC_{50} was 4.8 μM. All these results support the notion that SA inhibits ACC oxidase enzyme activity through the reversible interaction with Fe^{2+}, an essential cofactor of this enzyme.

In conclusion, our present results indicate that SA inhibits the ACC oxidase enzyme activity through the reversible interaction with Fe^{2+}, thereby abolishing the ethylene production induced by auxin-treatment in mung bean hypocotyls. Thus, it is possible that ethylene production in mung bean hypocotyl tissue would be modulated by an intimate regulatory interaction between auxin and SA. This regulatory pathway would permit the plant to fine-tune its response to diverse developmental and environmental signals. At this time, the detail molecular mechanism by which SA inhibits ACC oxidase activity is not known. In addition, the exact molecular association of ACC oxidase and Fe^{2+} remains to be elucidated. Binding assay with labeled SA and purified ACC oxidase would be helpful to answer these questions.

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