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SALICYLIC ACID INHIBITION OF GERMINATION, ETHYLENE PRODUCTION AND RESPIRATION IN COCKLEBUR SEEDS

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Abstract

Salicylic acid (SA) inhibited germination of cocklebur (Xanthium pennsylvanicum Wallr), lower seeds, which are completely after-ripened and nondormant. SA also inhibited ethylene production during a pre-germination period of the seeds. Exogenous ethylene overcame the inhibtion of the seed germination by SA. Moreover, SA reduced respiration in pre-germinating whole seeds as well as excised axial tissues. SA was suggested to inhibit more severely cyanide-sensitive respiration than cyanide-resistant respiration. The inhibition of both ethylene production and respiration by SA was thought to be the cause of prevention of cocklebur seed germination. The mechanism of the action of SA is discussed in the inhibition of cockelbur seed germination.

Introduction

Salicylic acid (SA) distributes in a wide variety of plants and is regarded as an important plant growth regulator (22). SA is known to have various kinds of physiological effects on plant growth and development (20). Exogenous SA reduces transpiration (1, 17), closes stomata (18), lowers water potential of leaves (1), inhibits phosphate and potassium uptake in barley roots (9), increases flower longevity by inhibiting ethylene production, and induces flowering in Xanthium (2). SA is present at higher concentrations in special organs, for example, necrotizing leaves infected by pathogens and inflorescences of thermogenic plants (22). Endogenous SA is found to trigger thermogenesis by stimulating cyanide-resistant respiration in aroid plants (23). Moreover, a large body of evidence suggests that SA plays a key role in both systematic acquired resistance and disease resistance, in addition to, jasmonates and ethylene (5, 26).

SA, one of derivatives of benzoic acid, is known as a strong inhibitor of seed germination (8, 31). However, the mechanism of actions of SA on seed germination is unclear. We found that germination of cocklebur (Xanthium pensylvanicum Wallr). seeds is also inhibited by SA. Recently we reported that jasmonates inhibit germination of cocklebur seeds probably by preventing ethylene production, which is essential to start germination (21). Moreover, Esashi (6) proposed that cyanide-resistant respiration plays an important role to induce germination of cocklebur seeds. Thus, it is important to study the actions of SA on cocklebur seed germination.

Keywords: Cocklebur (Xanthium pennsylvanicum); Ethylene; Respiration; Salicylic acid; Seed germination

Abbreviations: BHAM, benzohydroxamic acid; SA, salicylic acid; SHAM, salicylhydroxamic acid

In this study, we investigated effects of SA on ethylene production and respiration in relation to cocklebur seed germination.

Materials and Methods

Plant Materials

Lower seeds (large seeds) of cocklebur (Xanthium pennsylvanicum Wallr.) cultivated at the field of our department of Tohoku University were used in this study. The seeds were gathered in 1994, after-ripened and kept at 8°C in the dark until used (11).

Germination Experiments

The seeds were washed several times with tap water and once rinsed with deionized water. After blotting with tissue paper, 10 seeds were put on two layers of filter paper in a 125-ml flask or a 9-cm petri dish. The filter paper in the flask and the petri dish was wet with 3.5 and 8 ml, respecitvely, of a test solution. SA, KCN, and benzohydroxamic acid (BHAM) solution were prepared by dissolving a small amount of 1N NaOH and by adjusting the pH to around 7 with 1N HCl. A necessary amount of ethylene was injected with a hypodermic syringe (13) into a flask through a skirted rubber stopper, the hole of which was sealed with a piece of adhesive vinyl tape after ethylene injection. A small tube containing 0.5 ml of 0.2M mercuric perchloride, an ethylene absorbent, was put in the control flask. Percentages of seed germination were measured after the incubation in the dark at 25°C.

Ethylene Production

Cocklebur seeds were put on two layer of filter paper wetted with deionized water in a 15-cm petri dish and pre-incubated for 6 hours in the dark at 25°C. A 25-ml vial, which was lined with a layer of 4×7cm filter paper wetted with a test solution of 1.5 ml, was used to measure ethylene production. Pre-incubated, 30 seeds were arranged on wet filter paper in the vial. The vials were sealed with a skirted rubber stopper covered with vinyl tape to prevent any gas leakage. After a constant interval of incubation in the dark at 25°C, a gas sample of 1ml was withdrawn from the head space of each vial with a hypodermic syring and injected to assay ethylene by gas chromatography (Shimazu GC-8A equipped with an active alumina column and a flame ionization detector, supplied with N2 as a carrier gas). Finally, the fresh weight of 30 seeds was measured.

Measurement of Respiration

Cocklebur seeds were pre-incubated by the same method as used for measurement of ethylene production. Then, 15 seeds were placed on a layer of filter paper in a 10-ml vial. The filter paper was wet with 1 ml of the same test solution as used for the pre-incubation. The vials were sealed with skirted rubber stoppers and incubated in the dark at 25°C. After a constant interval, a gas sample of 1 ml was withdrawn from the head space of each vial and analyzed by gas chromatography (GL Science KOR-70 equipped with a molecular sieve column and a thermal conductivity detector, and supplied with He as a carrier gas) to determine the oxygen concentration.

Statistics

A completely randomized design was used in all experiments. Three replicates were included in each treatment. Each experiment was repeated at least twice. Data are shown as the mean with a standard error.

Results

Inhibition of Germination of Cocklebur Seeds Treated with SA

Lower cocklebur seeds start germinating around 20 hours after the incubation at 25°C in the dark (21, 27). Figure 1 shows effects of different concentrations of SA on germination of cocklebur seeds after 24, 48 and 72 hours after incubation. The rate of germination reached maximum (more than 80%) after 72 hours in the absence of SA. SA at 1 mM or above significantly decreased the rate of germination 48 hours after the treatment. The

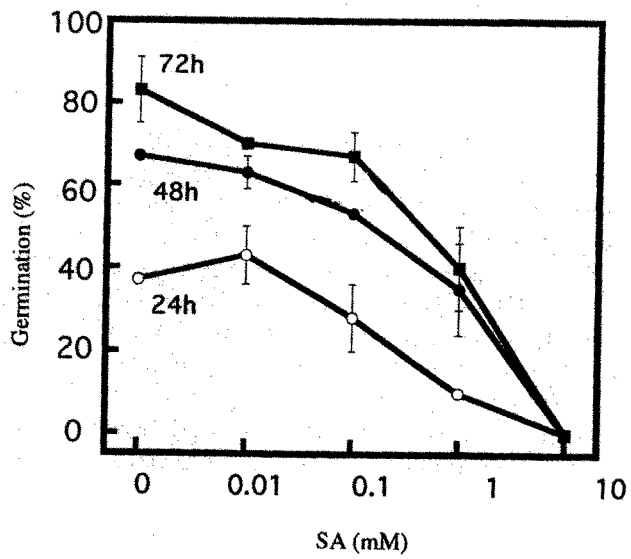

Figure 1. Effects of various concentrations of SA on cocklebur seed germination after 24, 48, and 72 hours of incubation at 25°C in the dark. Vertical bars show ±SE of the means of three replicates

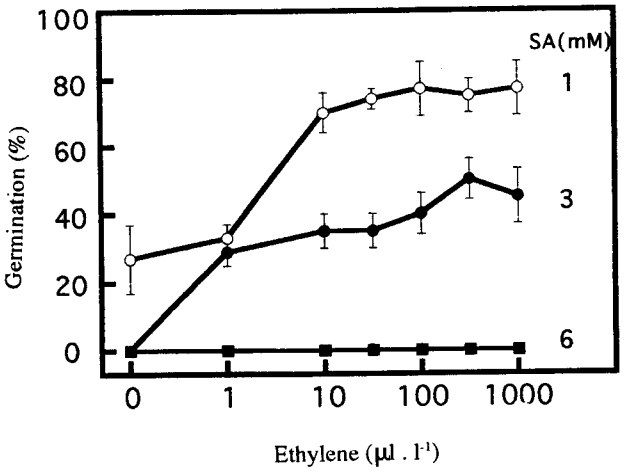

Figure 2. Alleviative effects of ethylene on the inhibition of germination in cocklebur seeds treated with 1,3, and 6 mM SA. Germination rates were counted 24 hours after the incubation at 25°C in the dark. Data show the means±SE of three replicates

seeds treated with 10 mM SA were not able to germinate at all even after 72 hours.

Ethylene is an important promoter for germination of cocklebur seeds (13). We examined whether ethylene overcomes inhibitory effects of SA on cocklebur seed germination (Fig. 2). The inhibition of germination by SA was prevented by ethylene depending on the concentrations. The inhibition by 1 mM SA was completely recovered by 10 ml.l^{-1} ethylene, and the inhibition by 3 mM SA was partially overcome by 300 µl l^{-1} ethylene. However, the inhibition induced by 6 mM SA was not overcome even by 1000 µl l^{-1} ethylene. These results showed that there is an interaction between physiological actions of SA and ethylene.

Next, we examined whether SA inhibits the ethylene production of cocklebur seeds (Fig. 3). SA at 1 mM or above inhibited the ethylene production, showing its similar dose-response curve to that for the inhibition of the seed germination shown in Figure 1. Since ethylene can overcome the SA inhibition of seed germination (Fig 2), it is plausible that the inhibition of ethylene production induced the inhibition of seed germination by SA.

Inhibitory Effects of SA on Respiration of Cocklebur Seeds

Esashi *et al.* (6,7) proposed that the germination of cocklebur seeds is regulated by a balance between cyanide-sensitive and cyanide-resistant respiration. It is well-established, on the other hand, that SA activates the alternative oxidase (22). Thus, we examined how SA affects respiration of cocklebur seeds. At first, a dose response curve of SA for the respiration was examined in axial tissues excised from cocklebur seeds (Fig. 4). SA at 0.1 mM hardly inhibited the respiration but more than 1 mM significantly inhibited it, showing again a similar dose-response curve to those for the inhibition of seed germination (Fig. 1) and ethylene production (Fig. 3). Next, we examined effects of SA on the respiration of cocklebur seeds during the germination period (Fig. 5). In the absence of SA, oxygen consumption started increasing 14 hours after the incubation and then drastically increased at the 22th hour just when some cocklebur seeds started germinating. SA completely inhibited these increases in respiration of cocklebur seeds, suggesting that this inhibition of respiration is another cause of SA inhibition of seed germination.

Next, we examined effects of KCN, a specific inhibitor of cytochrome oxidase; and BHAM, an inhibitor of alternative oxidase, in order to reveal which respiration is inhibited by SA (Table 1). Three mM KCN and 20 mM BHAM were used because Esashi *et al.* (6,7) reported that such concentrations of the inhibitors are necessary for inhbiting sufficiently the respiration of cocklebur seed tissues. The observed rate of respiration in the axial tissues of cocklebur seeds was 23.8±1.1 µmol gFW^{-1} h^{-1}. Three mM KCN and 20mM BHAM inhibited 86%

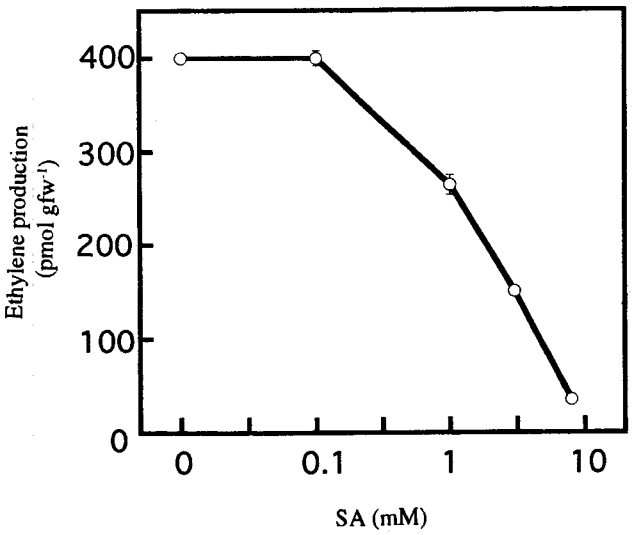

Figure 3. Dose response curve of SA for the inhibition of ethylene production in cocklebur seeds. The seeds were incubated in a sealed vial at 25°C in the dark. Amounts of ehtylene accmulated in the vials were determined by gas chromatography. Data show the means±SE of three replicates

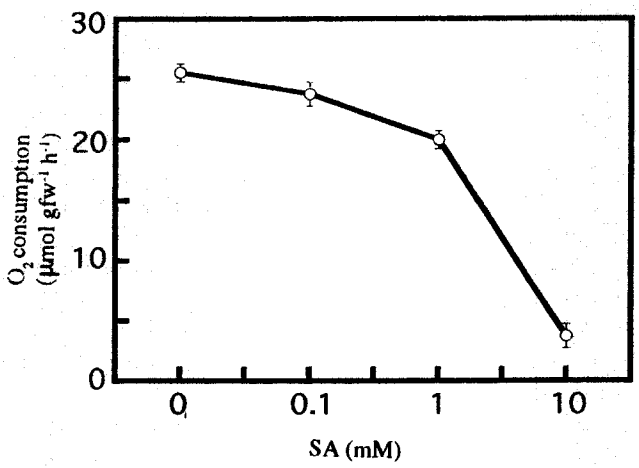

Figure 4. Dose response curve of SA for the inhibition of O_2 consumption in axial tissues excised from cocklebur seeds. The tissues were incubated in a sealed vial for 4 hours at 25°C in the dark. Decreases in oxygen concentrations in the vials were determined by gas chromatography. Data show the mean±SE of three replicates

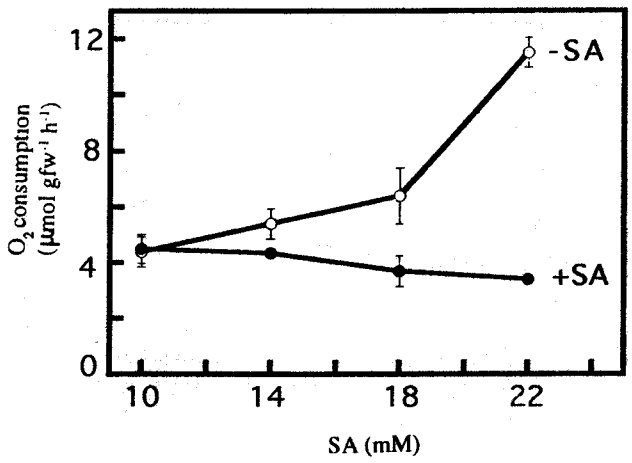

Figure 5. Effects of SA on respiration of cocklebur seeds during the germination period. Fifteen seeds, which had been incubated with or without 6 mM SA in the dark at 25°C, were placed in a sealed vial containing the same solution and further incubated for 4 hours. Initial and final oxygen concentrations in the head space of vials were determined by gas chromatography to calculate the rate of respiration. Data show the mean±SE of three replicates

and 16% of the respiration, respectively. The combined treatment with KCN and BHAM could not completely stop oxygen consumption, showing a residual oxygen consumption (16). In the presence of 1 mM SA, the total oxygen consumption was 19.1±0.9 μmol gFW^{-1} h^{-1}, showing 20% inhibition in comparison with that in the absence of SA. In the presence of SA, KCN inhibited 93% of oxygen consumption, whereas BHAM hardly reduced oxygen consumption. The combined treatment with KCN and BHAM prevented 96% of the oxygen consumption. The capacity of alternative respiration was conveniently defined as a salicylhydroxamic acid (SHAM)-sensitive respiration in the presence of KCN by Vanlerberghe and McIntosh (29). SHAM is a similar inhibitor of alternative respiration to BHAM. We calculated the capacity of alternative respiration in cocklebur seed tissues by subtracting oxygen consumption in the presence of KCN and BHAM from one in the presence of only KCN from the data shown in Table 1. The capacity of alternative respiration in the absence or presence of SA was 1.1 or 0.6 μmol.g FW^{-1} h^{-1}, respectively. These results suggested that SA inhibited more severely cytochrome electron transport than alternative electron transport.

Discussion

SA has been reported to be a strong inhibitor of seed germination (8, 20, 31). In contrast, SA is capable of breaking dormancy in dehulled red rice seeds (3) and enhancing growth of germinated embryos of maize kernels by increasing the expression of catalase genes (10). We showed that germination of cocklebur nondormant seeds is strongly inhibited by SA (Fig. 1). The most interesting result in this study was finding SA inhibition of cocklebur seed germination was alleviated by ethylene when concentrations of SA were lower than 3 mM (Fig. 2). Khan and Ungar (14) suggested that endogenous phenolic compounds, catechol, chlorogenic, salicylic, and syringic acids in *Atriplex triangularis* seeds play a key role in keeping the seeds dormant, and that the inhibition of seed germination by such phenols, except for salicylic acid, could be reversed by the application of gibberellic acid and kinetin. They did not referred to effects of ethylene, and they used only a high concentration of SA (10 mM). Also, in the case of cocklebur seeds, ethylene did not alleviate the inhibition by 10 mM SA (Fig. 2).

Ethylene production of cocklebur seeds was inhibited by SA (Fig. 3). Ethylene produced by cocklebur seeds during a pre-germination period has been through to play an important role to initiate germination (27). The inhibition of nondormant cocklebur seed germination by jasmonates is thought to come from the inhibition of ethylene production (21). In the case of SA, the inhibition

Table 1. Effects of SA on cyanide-sensitive and cyanide-resistant respiration in axial tissues of cocklebur seeds. The axial tissues were treated with both 1 mM SA, 3 mM KCN and 20 mM BHAM. Data show the mean of three replicates±SE.

SA	KCN	BHAM	O_2 consumption	
			μmol. $gFW^{-1}.h^{-1}$	Inhibition(%)
-	-	-	23.8±1.1	-
-	+	-	3.4±0.6	86[a]
-	-	+	20.1±0.4	16[a]
-	+	+	2.3±0.2	90[a]
+	-	-	19.1±0.9	20[a]
+	+	-	1.4±0.3	93[b]
+	-	+	17.6±1.0	8[b]
+	+	+	0.8±0.1	96[b]

[a]: Inhibition expressed as a percent of the value in the absence of SA, KCN, and BHAM.
[b]: Inhibition expressed as a percent of the value in the absence of KCN and BHAM.

of ethylene production is again suggested to be one of the causes inhibiting seed germination. SA has been known to be a strong inhibitor of ethylene biosynthesis in various kinds of plant tissues (20). In suspension cultured pear cells, SA inhibits ethylene production from exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) and is more effective than cobalt ions (19), suggesting that SA inhibits ACC oxidase. But it remains unsolved which step of ethylene biosynthesis in cocklebur seeds is inhibited by SA.

In response of plants to wounding and pathogen attack, SA acts as a stimulator of alternative oxidase gene expression (20). Moreover, SA is involved in the induction of alternative oxidase to produce heat in flowering *Arum* species (23) and in non-thermogenic plants (12, 25, 28). Figure 4 and 5 show that SA severely inhibited respiration during a germinating period of cocklebur seeds. In this study, we tried measuring the capacity of alternative respiration (Table 1), as defined by Vanlerberghe and McIntosh (29): BHAM-sensitive respiration in the presence of KCN. This measurement of the capacity does not reflect the actual alternative respiration activity under conditions without inhibitors because of some modification of the partitioning of electron flow between two pathways (4,30). SA hardly changed the capacity of alternative respiration, but SA inhibition of total respiration of cocklebur seeds seemed to come mainly from its inhibition of cytochrome respiration. Probably, the inhibition of respiration by SA is another cause of the inhibition of cocklebur seed germination.

The mechanism of inhibitory actions of SA remains unclear. Evenari (8) discussed effects of various organic acids including SA on seed germination and pointed out that the inhibition by such organic acids does not seem to manifest only through lowering the pH. Reynolds (24) reported that SA and catechol, ortho-substituted phenols, show abnormally strong activity to inhibit seed germination among effective aromatic acids on lettuce seed germination, and pointed out that SA may change permeability of cell membranes. On the other hand, SA is suggested to induce flowering in duckweed by acting as a chelating agent of metallic ions (15, 32). Further investigation is necessary to explore the mechanism of action of SA on the inhibition of seed germination.

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