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# The Mechanism of Stomatal Closing by Salicylic Acid in *Commelina communis* L.

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**The mechanism of stomatal closing by salicylic acid (SA) has been investigated. The addition of 1 mM SA to fully opened stomata resulted in a significant reduction of 75% in stomatal aperture. Stomata in the treatment of SA with EGTA closed as observed in the treatment of SA. However, the addition of catalase with SA completely inhibited stomatal closing. Stomatal closing induced by SA was also reduced by $Ca^{2+}$. To understand the relation bewteen stomatal closing by SA and catalase activity, the effect of SA on catalse activity and the effect of AT (catalase inhibitor) on stomatal closing was investigated. SA inhibited 32% of catalase activity. Stomata in isolated epidermis floated on an incubation solution containing 0.1 mM AT closed from 9.6 μm to 3.2 μm after 1 hour. SA stimulated $K^+$ efflux as much as the twice of the control in isolated strips. SA inhibited 53% of photosynthetic activity at the light intensity of 1000 μmole $m^{-2}$ $s^{-1}$ on SA infiltrated leaves. A similar result was found on stomatal conductance in SA infiltrated leaves. These results indicate that SA inhibit catalase activity and increase the concentration of $H_2O_2$ in guard cell cytoplasm. $H_2O_2$ oxidize the plasma membrane and increase the membrane permeability of $K^+$. The mass efflux of $K^+$ induce the loss of turgor pressure and lead to stomatal closing. The inhibition of photosynthetic activity by SA suggests that stomatal closing by SA is also related with the decrease of photosynthetic activity.**

*Keywords*: $Ca^{2+}$, catalase, photosynthesis, stomata, salicylic acid

## INTRODUCTION

Salicylic acid (SA) is a naturally occuring plant phenolic compound which has been shown to affect such diverse processes as flowering, seed germination and stomatal behaviour (Malamy and Klessig, 1992). There have been a number of reports which have suggested that SA can inhibit stomatal opening (Bhatia *et al.*, 1986; Larque-Saavedra, 1978, 1979; Manthe *et al.*, 1992: Lee, 1995). Manthe *et al.* (1992) reported that 1 μM SA inhibited stomatal opening by 67% at pH 5.0 in detached epidermis of *Commelina*. Larque-Saavedra (1979) reported that 1 mM SA took 75 min. to close totally the stomata in detached epidermis of *Commelina* which were previously open. They used the low pH of the incubation medium for the detached epidermis. However, Ral *et al.* (1986) found stomatal opening by SA as they used pH 7.0 of the incubation medium.

This contradictory report could be due to the properties of SA as it is known that SA is metabolized rapidly and its effect is pH dependent (Raskin, 1992). Lee (1995) also found that SA effect on stomatal closing was greatly pH dependent. The reduction of stomatal aperture caused by 1 mM SA was most effective at lower pH (pH 7.2, 5%; pH 6.2, 40%; pH 5.2, 78%). Therefore, if SA was properly treated to the epidermal strips in the medium, the effects of SA on stomatal closing were similar with those of abscisic acid (ABA).

The magnitude of SA and ABA induced stomatal closing was similar. When plant is exposed to water stress, stomata close. It is well known that stomatal closing induced by water stress is mediated by ABA. The role of ABA in inhibition of stomatal opening is thought to involve an increase in the permeability of the plasma membrane of guard eclls to calcium ions. $Ca^{2+}$ entering the guard eclls then acts as a second messenger to regulate the ion fluxes that lead to the loss of guard cell turgor and therefore stomata close (MaAinsh *et al.*, 1990). SA caused the col-

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lapse of the transmembrane electrochemical potential of mitochondria and ATP dependent proton gradient of the tonoplast-enriched vesicles in pea (Macri *et al.*, 1986). This report suggests that SA can induce stomatal closing by affecting ion fluxes and membrane permeability.

Surprising signals by SA in plant cells had been reported by Jones (1994). He reported that SA inhibited catalase activity. This report gives us great clue on the understanding of SA-induced stomatal closing. The inhibition of catalase activity by SA could elevate the concentration of $H_2O_2$ in guard cell cytoplasm and the increased concentration of $H_2O_2$ could lead to stomatal closing. Lee and Jun (1997) reported that the effect of $H_2O_2$ on stomatal closing was dependent on the concentration of $H_2O_2$. They found that 10 ppm $H_2O_2$ showed a clear effect on stomatal closing and 1000 ppm $H_2O_2$ induced complete stomatal closing after treatment of 3 hours. This report implys that stomatal closing by SA could be the result of the single action of $H_2O_2$ or the cooperation of SA itself and $H_2O_2$.

Therefore, in order to study the mechanism of stomatal closing by SA, a series of experiments in which the effect of $Ca^{2+}$, catalase, catalase inhibitor on stomatal closing induced by SA have been carried out. The effects of SA on ion effluxes, photosynthetic activity and stomatal conductance on SA infiltrated stems have also been investigated.

## MATERIALS AND METHODS

The experiments were carried out on the abaxial surface of leaves of *Commelina communis* L. The plants were grown from seeds in mixture of vermiculite and Peat & Loam potting compost in Growth Chamber ($22\pm2$°C) with supplementary lighting from natrum lamps (400 μmole $m^{-2}$ $s^{-1}$) to give a photoperiod of 16 h. At all stages of development the plants were kept free from water stress by periodic watering.

Epidermal strips from the fully expanded leaves were obtained by the method of Lee and Bowling (1992). The strips were cut into $5\times10$ mm pieces and placed in 5 cm petri dishes containg 10 mM MES buffer (2-[N-morpholonol ethan sulfonic acid) adjusted to pH 5.2 with KOH in which 50 mM KCl was dissolved. The dishes were incubated in a water bath for 2 h at $25\pm2$°C under photon flux density of 200 μmole $m^{-2}$ $s^{-1}$. Carbon dioxide free air was obtained by passing room air through a cylinder of soda lime and 2 M KOH solution. When stomata were fully open, SA, $Ca^{2+}$, Aminotriazole, catalase were added to the medium and the epidermal strips were incubated for a further 1.5 h.

To measure stomatal aperture, a microscope (Olympus) with a camera lucida ($\times400$ magnification) was conneted to a monitor video and monitor screen calibrated by an ocular micrometer disc. After incubation, epidermal strips were mounted under the microscope. Stomata screened in the video monitor could be measured directly with calibrated ruler. This system was more convenient and accurate for measuring the stomatal apertures than direct microscopic measurement.

### Assay of Catalase Activity

Plants were selected in the early stages of maturation (3~6 weeks) for leaf material. Strips of lamina between the major parallel veins on either side of the midrib were removed by cutting with a razor blade on a glass slide. Around 5 grams (fresh weight) of young leaves were throughly homogenized with 4 ml of 100 mM K-$PO_4$ buffer (pH 7.0; 2 mM $Na_2$-EDTA, 5 mM ascorbate) for 1 min. using a Waring blender.

Then leaf slurry was filtered through four layers of cheesecloth. Filtered solution was centrifuged for 20 min. at $15,000\times g$. The supernatant was used as a enzyme extracts.

To measure catalase activity, 0.05 ml of the pellet was resuspended in 2.9 ml of 50 mM K-$PO_4$ (pH 7.0), 0.06 ml of SA, 0.05 ml of $H_2O_2$. And then after 20 sec. a decrease of optical density was measured at 240 nm for 80 sec. Catalase activity (unit) was calculated through the degradation of the quantity of $H_2O_2$ (extinction coefficient, $\varepsilon=39.4$ $M^{-1}$ $cm^{-1}$). 1 unit was defined as the degraded concentration of 10 mM $H_2O_2$ for 80 sec.

### The Measurements of $K^+$ and Cl

The epidermis was peeled away from the mesophyll by pulling gently. The strips cut into segments and incubated for 2 hours in 5 cm diameter plastic petri dishes containing 10 mM MES-KOH buffer (100 mM KCl, pH 5.2), into which air was bubbled through hyperdermic needles fitted in the lids. Samples were placed under a light (200 μmole $m^{-2}$ $s^{-1}$ a) at $25\pm2$°C.

After incubation of 2 hours, aminotriazole and SA were treated into the medium to induce ion leakages and incubated another 3 hours in darkness. And then

the medium was analysed for $K^+$ and Cl and the strips were weighed. The concentratiojn of $K^+$ and Cl was measured by ion chromatgraphy (DX-120).

### Infiltration of Mesophyll Cells with SA and the Measurements of Photosynthetic Acitivity and Stomatal Conductance

The mature leaves (second bifoliate leaf) were excised, and the petiole was immediately dipped into a solution containing 0.5 mol $m^{-3}$ $CaCl_2$, 0.25 mol $m^{-3}$ $MgSO_4$, buffered at pH 5.9 with 5 mol $m^{-3}$ sodium citrate/10 mol $m^{-3}$ sodium phosphate with SA or without SA. The leaves were kept in a hood to stimulate transpiration under light regime of 16 h. This infiltration lasted 48 hours, and its efficiency was checked by measuring the rates of uptake of medium. The volume of uptake of the medium was around 0.9 ml for 24 hours.

Stomatal conductance and photosynthetic activity was measured using by L1-6400 Portable photosynthesis System.

## RESULTS

Figure 1 shows the effects of $Ca^{2+}$ and catalase on SA-induced stomatal closing. The addition of 1 mM SA to fully opened stomata resulted in a significant reduction of 75% on stomatal aperture. Stomata in the treatment of SA with EGTA closed similary as observed in single treatment of SA. However, the addition of catalase with SA completely inhibited stomatal closing. Lee (1995) and Manthe *et al.* (1992) also found similar results with SA on stomatal closing.

Figure 2 shows the response to $Ca^{2+}$ on stomatal closing induced by SA. The effect of $Ca^{2+}$ on stomatal closing was linear to the concentration of $Ca^{2+}$. 1 mM $CaCl_2$ inhibited 49% and 10 mM $CaCl_2$ inhibited 74% of stomatal aperture when compared with the control. De silva *et al.* (1985) and Lee and Jun (1997) also showed similar results with $Ca^{2+}$ on stomatal closing.

When SA was treated with $Ca^{2+}$ to the medium for the epidermal strips, the effect of SA on stomatal closing was significantly reduced. These results indicate that stomatal closing by SA was not mediated by $Ca^{2+}$, reversely $Ca^{2+}$ seemed to repair or protect the damage of membrane induced by SA metabolites.

To understand the relation bewteen stomatal closing by SA and catalase activity, the effect of SA on

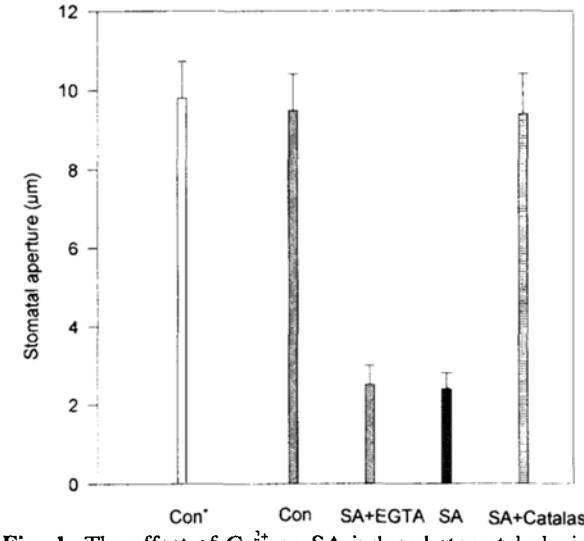


**Fig. 1.** The effect of $Ca^{2+}$ on SA-induced stomatal closing. Epidermal strips were incubated in 10 mM MES-KOH (pH 5.2) for 2 hours to induce stomatal opening. When stomata were fully open, chemicals and enzyme were added to the medium and the epidermal strips were incubated for a further 1.5 h. Con* indicates the stomatal aperture before the chemicals were treated and Con indicates the result after the chemicals were treated. Each point is the mean (±s.e.m.) of 80 stomatal apertures.

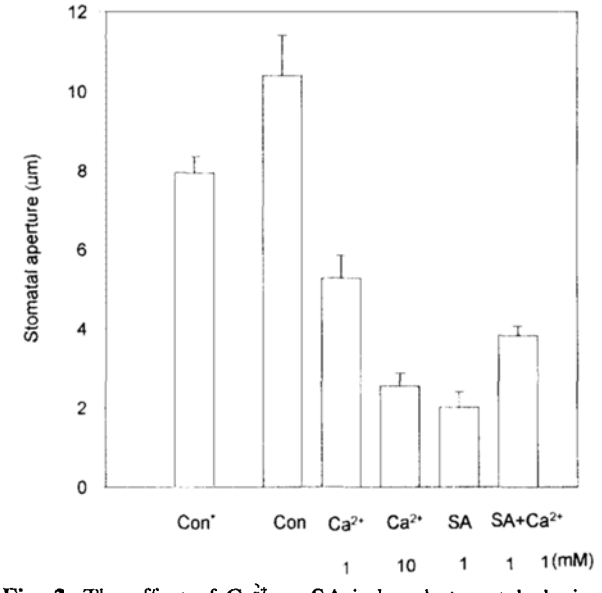


**Fig. 2.** The effect of $Ca^{2+}$ on SA-induced stomatal closing. Epidermal strips were incubated in the same buffer used in Fig. 1 for 2 hours to induce stomatal opening. When stomata were fully open, chemicals and enzyme were added to the medium and the epidermal strips were incubated for a further 1.5 h. Con* indicates the stomatal aperture before the chemicals were treated and Con indicates the result after the chemicals were treated.. Each point is the mean (±s.e.m.) of 80 stomatal apertures.

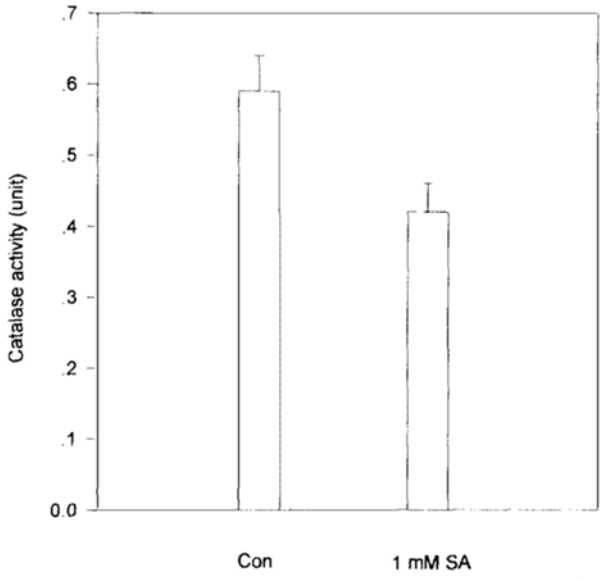


**Fig. 3.** The effect of SA on catalase activity. Each point is the mean (±s.e.m.) of 4 measurements in two separate experiments.

catalse activity and the effect of AT (catalase inhibitor) on stomatal closing was investigated (Fig. 3, 4). SA inhibited 32% of catalase activity. Stomatal

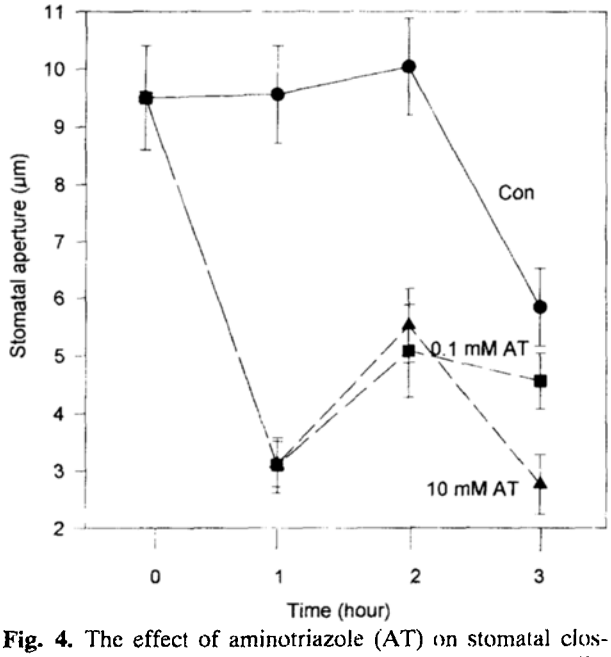


**Fig. 4.** The effect of aminotriazole (AT) on stomatal closing. Epidermal strips were incubated in the same buffer used in Fig. 1 for 2 hours to induce stomatal opening. When stomata were fully open, AT was added to the medium and the epidermal strips were incubated for a further 3 h. Each point is the mean (±s.e.m.) of 80 stomatal apertures.

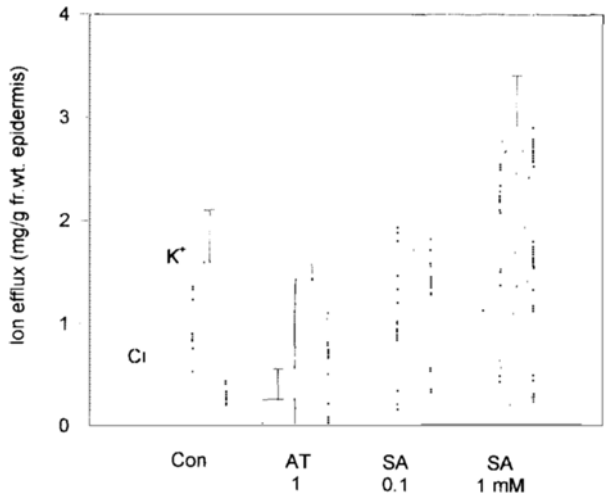


**Fig. 5.** The effect of SA on ion efflux in isolated epidermal strips. Each point is the mean (±s.e.m.) of 4 measurements in two separate experiments.

closing by AT was very clear. Stomata in isolated epidermis floated on an incubation solution containing 0.1 mM AT closed from 9.6 μm to 3.2 μm after 1 hour. The effect of 0.1 mM AT on stomatal closing was reduced according to the incubation time. In case of the treatment of 10 mM AT to the isolated epidermis stomata was almost closed after 3 hour.

Figure 5 shows the effects of SA and AT on ion

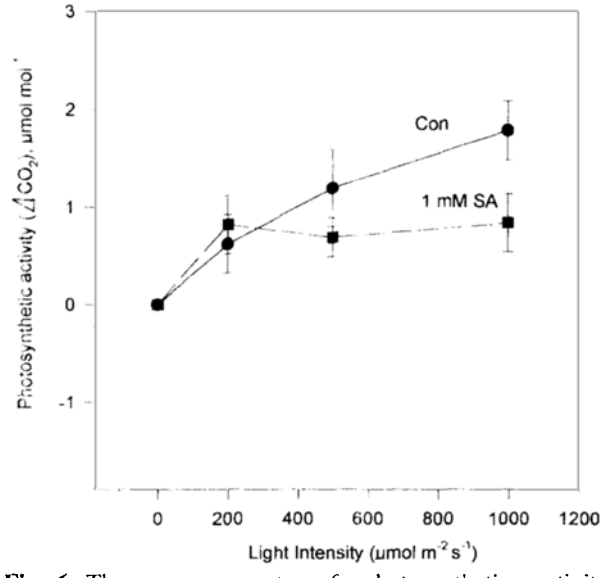


**Fig. 6.** The measurements of photosynthetic activity according to the light intensity on SA infiltrated leaves. Each point is the mean (±s.e.m.) of 4 measurements in two separate experiments.

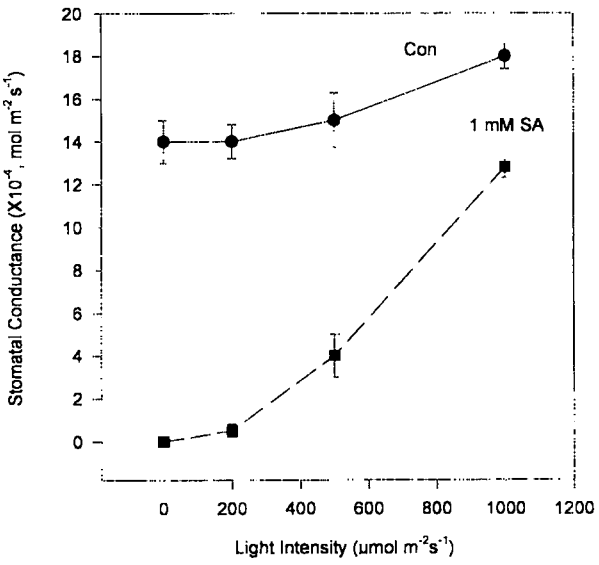


**Fig. 7.** The measurements of stomatal conductance according to the light intensity on SA infiltrated leaves. Each point is the mean (±s.e.m.) of 4 measurements in two separate experiments.

efflux in isolated epidermal strips. When isolated epidermal strips were incubated in the medium, a significant leakage of $K^+$ and Cl was observed. In the case of the treatment of 1 mM AT there was no significant difference on ion efflux when compared with the control. In the treatment of 1 mM SA $K^+$ was leaked almost the twice of the control, but Cl was leaked as same as the control.

Figures 6 and 7 show the measurements of photosynthetic activity and stomatal conductance according to light intensity on SA infiltrated leaves. SA inhibited 53% of photosynthetic activity at the light intensity of 1000 μmole m $^{-2}$ s $^{-1}$ on SA infiltrated leaves. Stomatal conductance was slightly increased according to light intensity on the control. In the treatment of SA stomatal conductance was sharply increased according to light intensity, but it was much lower than that of the control.

## DISCUSSION

Environmental factors such as darkness, water stress and high $CO_2$ concentration triggers events which may result in stomatal closing. How these signals are sensed and how they are transduced into driving the ion fluxes which control stomatal movements is not still fully understood. An important consideration in water stress studies is how the plant perceives changes in the water status of the soil and how the signal is transduced to aerial portions of plant. The nature of the proposed signal is unknown, but Zhang and Davies (1987) favor abscisic acid (ABA) delivered to leaves via the transpiration stream, which affect the stomatal aperture. Stomatal closing by ABA appeared to be dependent on the availbility of $Ca^{2+}$. Several studies have shown that SA induces stomatal closing. The mechanism by which SA induces stomatal closing is not unknown.

This study focused whether the mechanism of stomatal closing by SA, $H_2O_2$ and ABA was similar or not. If they were different, this study was approached to understand how SA close stomata.

The results in Fig. 1 suggested that stomatal closing by SA was not mediated by $Ca^{2+}$, not likely as observed in abscisic acid induced stomatal closing. Reversely $Ca^{2+}$ inhibited the SA-induced stomatal closing. This unexpected result can be explained that $Ca^{2+}$ seemed to repair or protect the damage of membrane induced by SA metabolites. Lee and Jun (1997) also found similar results.

Catalase completely inhibited the SA-induced stomatal closing (Fig. 1). SA block catalase activity (Jones, 1994). In this study SA inhibited 32% of catalase activity (Fig. 3). The effect of AT on stomatal closing was clear (Fig. 4). From these results, it can be explained that stomatal closing by SA was related with the activity of catalase. It was suggested that artificial addition of catalase reduced the concentration of $H_2O_2$ in guard cell cytoplasm and stomatal closing by SA was inhibited. Lee and Jun (1997) reported that the effect of $H_2O_2$ on stomatal closing was dependent on the concentration of $H_2O_2$. 10 ppm $H_2O_2$ showed a clear effect on stomatal closing and 1000 ppm $H_2O_2$ induced complete stomatal closing after the treatment of 3 hours. Accordingly, stomatal closing by SA follow same processes as observed on stomatal closing by $H_2O_2$.

It needs further investigation how exogenously applied catalase inhibited stomatal closing. Catalase is a macromolecule and it is not known whether it can enter guard cell cytoplasm or not. It was possible that catalase reduced $H_2O_2$ in the medium and cell wall. The low concentration of $H_2O_2$ in the medium and cell wall stimulated the diffusion of $H_2O_2$ from the cytoplasm to medium and resulted in the low concentration of $H_2O_2$ in the guard cell cytoplasm.

Lee and Jun (1997) also showed that stomatal closing by $H_2O_2$ was not mediated by $Ca^{2+}$. Reversely, $Ca^{2+}$ showed a great inhibition on stomatal closing induced by $H_2O_2$. They found that the leak-

age of $K^+$ in epidermal strips was doubled in response to $H_2O_2$ when it was compared with the control. $H_2O_2$ decreased photosynthetic activity. Fv/Fm representing the activity of photosystem II was reduced about 4% in 10 ppm $H_2O_2$ and 8% in 100 ppm $H_2O_2$ in the treatment of 1.5h.

Similary, SA stimulated $K^+$ efflux as much as the twice of the control in isolated epidermal strips (Fig. 5). SA inhibited 53% of photosynthetic activity at the light intensity of 1000 μmole $m^{-2}$ $s^{-1}$ on SA infiltrated leaves (Fig. 6). A similar result was found on stomatal conductance in SA infiltrated leaves (Fig. 7). The decrease of stomatal conductance in SA-infiltrated leaves indicated that SA-induced stomatal closing could be found in both system of in vivo and in vitro.

Stomatal opening and closing is related with the photosynthetic activity (Lee and Kim, 1997). The inhibition of the photosynthetic activity by SA indicates that SA may indirectly alter guard cell responses as a result of secondary effects from damage to the photosynthetic machinery in the leaf mesophyll. The secondary effect of SA on stomatal closing is through the effect of $CO_2$ elevation as a result of damage of the photosynthetic machinery. Morison (1987) suggested that the stomatal response to $CO_2$ is a general phenomenon. Since the intercellular $CO_2$ concentration ($C_i$) declines as assimilation increases, and since stomatal conductance in many cases increases with decreasing ci, it has been supposed that assimilation controls conductance by affecting changes in $C_i$ (Raschke, 1976).

Therefore, SA-induced stomatal closing could be explained in two ways. Firstly, SA inhibit catalase activity and increase the concentration of $H_2O_2$ in guard cell cytoplasm. $H_2O_2$ oxidize the plasma membrane and increase the membrane permeability of $K^+$. The mass efflux of $K^+$ induce the loss of turgor pressure and lead to the stomatal closing. Secondary, the inhibition of photosynthetic activity by SA suggests that stomatal closing by SA is the result of the cooperation of the mass efflux of $K^+$ and the elevated $CO_2$ concentration in $C_i$.

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