

Effect of *p*-Coumaric Acid, Benzoic Acid, and Salicylic Acid on the Activity of Glutathione Reductase and Catalase in *in vitro* Grown Tobacco Plants

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Effects of *p*-coumaric acid (*p*-CA), benzoic acid (BA), and salicylic acid (SA) on the activities of glutathione reductase and catalase were studied in *in vitro* grown tobacco plants. After culturing the tobacco plants in MS medium containing 10^{-5} mM of *p*-CA, BA, and SA, the increase in the activities of two enzymes, glutathione reductase and catalase, were compared from day 20 to day 50 day, with an interval of 10 days. The growth of the tobacco plants treated with *p*-CA, BA, and SA was the highest on day 50. Analysis of the effect of the three substances on the activity of glutathione reductase showed that BA and *p*-CA decreased the activity of the enzyme compared with a control, and SA increased the activity of the enzyme. All of them showed the highest activity on day 40. SA increased the activity of catalase, but BA and *p*-CA reduced the activity of this enzyme. In all the experimental groups, the activity was the highest on day 40. In conclusion, *p*-CA and BA appear to promote the growth of tobacco plants. The growth was the best on day 50, but the activity of the antioxidative enzyme was inhibited. On the contrary, SA seemed to inhibit the growth of the tobacco plants but to promote the activity of glutathione reductase and catalase. The growth of the plants treated with SA was best on day 40.

Key words : Benzoic acid, catalase, glutathione reductase, *p*-coumaric acid, salicylic acid

Introduction

Plants exposed to various environmental stresses produce reactive oxygen species, which gives damages to protein, lipid, and DNA, promotes aging and various diseases and makes cells to die [4]. In order to protect themselves from oxidative stress by the reactive oxygen species, plants produce antioxidative enzymes such as glutathione reductase, catalase, and peroxidase and various types of low molecular antioxidative materials such as ascorbic acid and glutathione to counteract it [20].

Glutathione composed as a tripeptide of γ -glutamate, cysteine and glycine [25], synthesized in the plant, discharged out of cells, put into and synthesized again in cells [39]. It is known that most glutathione exist as GSH activated by NADPH and glutathione reductase, but also exists as oxidation type GSSG produced by disulfide bond of 2 GSH [4]. GSH has very diverse functions and plays as a coenzyme

for metabolism and material delivery in plants as well as antioxidant, antidote of herbicide, and antidote of heavy metal ions [10]. Glutathione reductase acted to reduce GSSG to GSH [2].

Catalase is a major enzyme involved in the decomposition of H_2O_2 [7]. Catalase exists in all living organisms regardless of animals, plants, and microorganisms. In plant cells, it exists in peroxisome and plays a role in degrading H_2O_2 generated in photorespiration and oxidation process of fatty acids H_2O and O_2 [27]. Catalase is also known as an index-gene for various physiological stresses [42].

All of *p*-coumaric acid (*p*-CA), benzoic acid (BA), and salicylic acid (SA) are substances derived from phenylalanine. Phenylalanine is converted to *p*-CA and BA through cinnamic acid by two different pathways, and BA combined with OH group is produced SA. *p*-CA is involved in the system hydroxycinnamic acid [41] and has various physiological activities such as antimicrobial activity [28], cancer chemoprevention [19], antioxidant activity [12], and anti-melanogenesis [3]. BA is a precursor of SA and produced by β -oxidation of cinnamic acid synthesized through phenyl propanoid metabolic pathway in plants [31]. SA is one of the phenolic compounds consisting of aromatic ring structure containing hydroxyl and carboxyl group [33], synthesized in most plants, plays a role in reactive oxygen species

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associated signal transduction as well as stimulating plant growth through promotion of cell division and expansion of cells [22]. In addition, it is reported as an important signal material in pathogen resistance and systemic acquired resistance [11]. Thus, it is known that SA changes in plant body plays a role in signal transduction of biological stress [15].

Studies on glutathione and glutathione reductase have been progressed in diverse angles including glutathione metabolism and biological functions [30], biosynthesis and anti-oxidation function of glutathione [2] in high plants. Inhibition of ascorbate peroxidase by SA [10], participation of SA in glutathione metabolism of garden pea seedlings [36] and various studies on glutathione metabolism and antioxidative enzymes have been reported. However, there is no research on *p*-CA, BA and SA in tobacco, relating them to antioxidative enzymes.

In spite of the considerable literature on this subject, the effects of *p*-CA, BA, and SA on antioxidant enzymes in tobacco plants grown *in vitro* has not been studied. Therefore, in this study, we studied the effects of *p*-CA, BA, and SA on the growth and activity of glutathione reductase and catalase concerned with the antioxidation in tobacco plant.

Materials and Methods

Plant material culture and growth determination

Tobacco (*Nicotiana tabacum* L.) seeds were germinated and grown aseptically in a cell culture vessel containing MS agar medium [26]. The shoots were cut into 3 cm segments and used as explants. The explants were placed on an induction MS medium supplemented with 10^{-5} mM *p*-CA, BA, and SA, respectively. These explants were maintained at $27 \pm 2^\circ\text{C}$ under light for 16 hr ($800 \mu\text{M}/\text{m}^2/\text{s}$ PFD) and dark photoperiod for 8 hr [23]. Fully expanded leaves from mature plants at 20 day to 50 day with an interval of 10 day were used for growth and enzymes experiments. All experiments were independently triplicated.

Isolation of enzyme extracts

Enzyme extracts were isolated according to Srivastava and Dwivedi [36]. Tobacco leaves were ground in extraction buffer [0.1 M Tris-HCl buffer (pH 7.5), 0.05 M EDTA, 10 mM ascorbate, 0.002% polyvinylpyrrolidone], and then centrifuged for 30 min at $15,000 \times g$. The supernatant obtained were kept at 4°C until use. The activity of glutathione reductase and catalase were determined in these prepared extracts.

Determination of glutathione reductase activity

The activity of glutathione reductase was determined by the method of Foyer and Halliwell [13]. The assay mixtures consisted of 25 mM Tris-HCl (pH 7.5), 10 mM oxidized glutathione, 3 mM MgCl_2 , 0.15 mM NADPH, and 0.3 ml enzyme extracts in a final volume of 2 ml. The oxidation of NADPH was monitored by measuring the decrease in absorbance at 340 nm using a UV-visible spectrophotometer (Bio-Rad 680, USA).

Determination of catalase activity

The enzyme activity was determined according to Aebi [1]. Briefly, 2 ml of enzyme extracts was added in a cuvette and the reaction was initiated by adding 1 ml 30 mM H_2O_2 , and the change in absorbance at 240 nm was monitored at 25°C for 1 min.

Results

Effects of *p*-CA, BA, and SA on growth of tobacco plant

As results of studying effects of *p*-CA, BA and SA on *in vitro* cultured tobacco plants, it was found that the growth from *p*-CA and BA was better than control group, but the growth from SA was lower than the control group. In addition, it was found that all of the three materials showed the lowest growth on 20 day, the increase of their growth until 50 day, and the highest growth on 50 day (Fig. 1).

In order to identify these, total fresh weight of tobacco plants cultured for 20-50 days after treating with *p*-CA, BA and SA was measured. While in the control group, it was shown that the total fresh weight was 4.5638 g, 8.4264 g, 17.0015 g, and 20.3557 g on 20, 30, 40, and 50 day respectively, in the *p*-CA treatment group, it was shown as 3.7507 g, 12.5063 g, 15.1563 g, and 22.3037 g on 20, 30, 40, and 50 day respectively. Therefore, it was suggested that the growth was increased from 20 day to 50 day gradually and the highest on 50 day. Fresh weight of *p*-CA group with that of the control group, it was found that it was high on 50 day (Fig. 2).

In BA group, it was found that the fresh weight was 5.2773 g, 15.1056 g, 17.9762 g, and 25.7441 g on 20, 30, 40, and 50 day respectively, which was increased gradually from 20 day to 50 day and the highest on 50 day. Comparing them with those of the control group, the values from 20 day to 50 day were higher (Fig. 3).

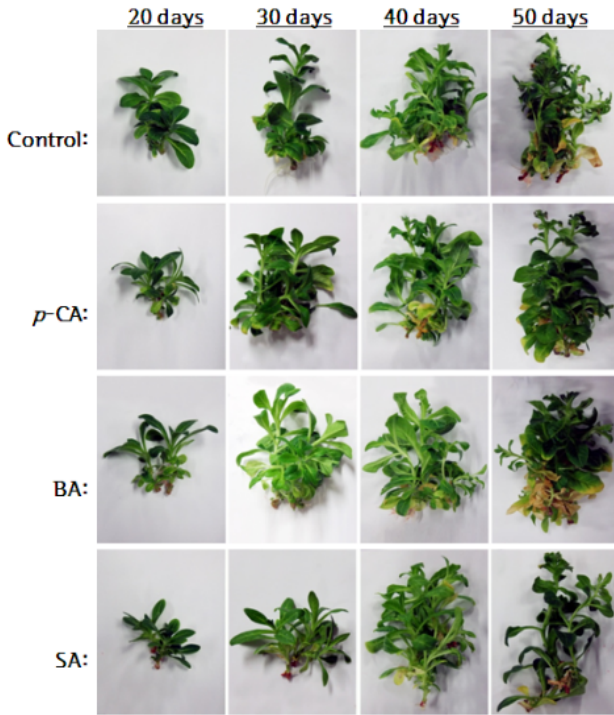


Fig. 1. *in vitro* induction of tobacco plant on MS medium containing *p*-coumaric acid, benzoic acid, and salicylic acid, respectively. These explants were maintained at 27°C ± 2°C under light for 16 hr (800 μM/m²/s PFD) and dark photoperiod for 8 hr.

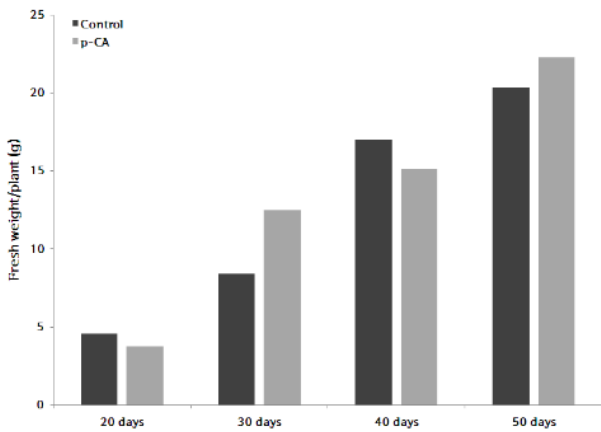


Fig. 2. Total fresh weights of tobacco plant grown on MS medium containing *p*-CA. Mature plants at 20 day to 50 day with an interval of 10 day were used for growth.

In SA group, it was found that it was 2.6066 g, 7.224 g, 14.8528 g, and 16.8081 g on 20, 30, 40, and 50 day respectively, which was also increased gradually from 20 day to 50 day and the highest on 50 day (Fig. 4). When comparing these with results of the control group, it was found that they were lower than those and showed a trend contrary to the results of *p*-CA and BA group.

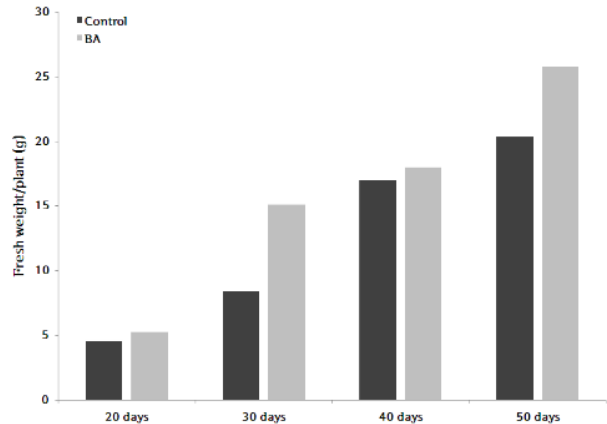


Fig. 3. Total fresh weights of tobacco plant grown on MS medium containing BA. Mature plants at 20 day to 50 day with an interval of 10 day were used for growth.

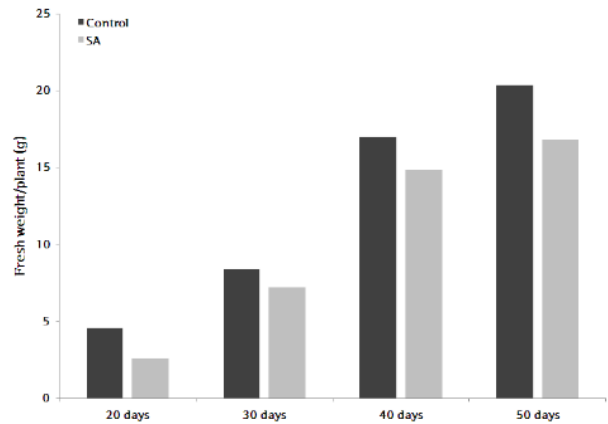


Fig. 4. Total fresh weights of tobacco plant grown on MS medium containing SA. Mature plants at 20 day to 50 day with an interval of 10 day were used for growth.

Effects of *p*-CA, BA, and SA on glutathione reductase activity

Effects of *p*-CA, BA and SA were studied on activity of glutathione reductase. In the control group, it was found that the activities were 0.2628, 0.3398, 0.4993 and 0.3398 nmoles/min/ml on 20, 30, 40 and 50 day, respectively, and in the *p*-CA group, it was found that it was 0.161, 0.176, 0.2367, and 0.172 nmoles/min/ml on 20, 30, 40, and 50 day, respectively (Fig. 5). It was suggested that the activities increased gradually from 20 day to 40 day, the highest on 40 day, and reduced on 50 day. While comparing the activity of glutathione reductase in the *p*-CA group with that of the control group, it was found that all of their values from 20 day to 50 day were lower (Fig. 5).

In the BA group, it was found that it was 0.1366, 0.162, 0.294, and 0.1163 nmoles/min/ml on 20, 30, 40, and 50 day,

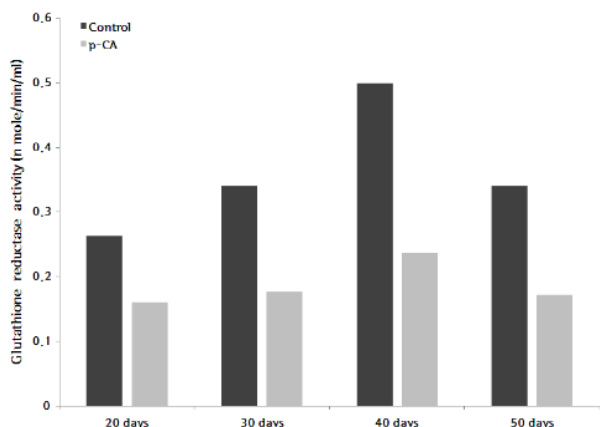


Fig. 5. Effects of *p*-CA on activity of glutathione reductase in tobacco plant. The activity of glutathione reductase was determined in extracts prepared from tobacco leaves.

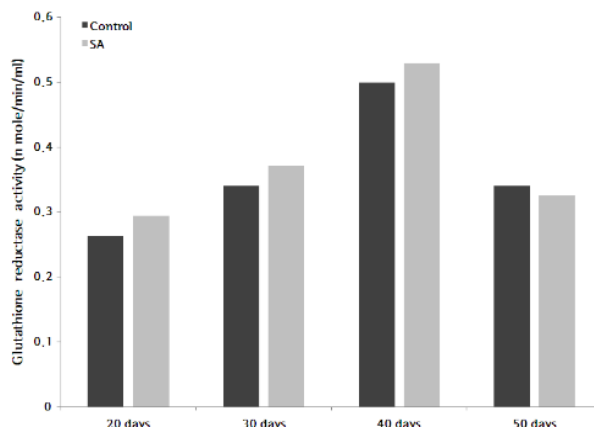


Fig. 7. Effects of SA on activity of glutathione reductase in tobacco plant. The activity of glutathione reductase was determined in extracts prepared from tobacco leaves.

respectively (Fig. 6). It was suggested that it was increased gradually from 20 day to 40 day, the highest on 40 day, and reduced on 50 day. As results of comparing them with those of the control group, it was shown that all of their values from 20 day to 40 day were higher, but it was lower on 50 day.

In the SA group, their weights were measured as 0.2944 on 20 day, 0.3709 on 30 day, 0.5289 on 40 day, and 0.325 nmol/min/ml on 50 day, which were increased from 20 day to 40 day, the highest on 40 day, and reduced on 50 day (Fig. 7). It was found that they were higher than those of the control group and showed a trend contrary to the results of *p*-CA and BA.

Effects of *p*-CA, BA, and SA on catalase activity

Effects of *p*-CA, BA, and SA on activity of catalase in the

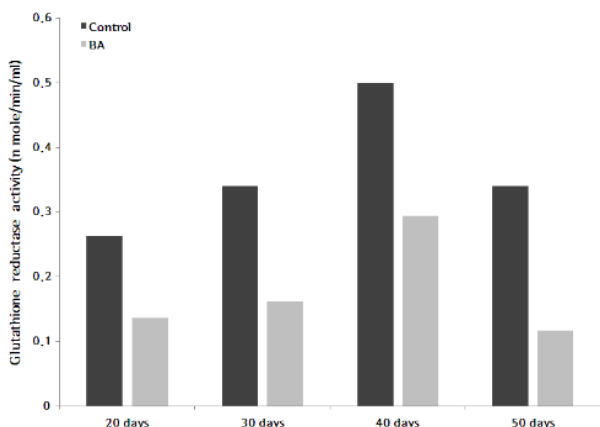


Fig. 6. Effects of BA on activity of glutathione reductase in tobacco plant. The activity of glutathione reductase was determined in extracts prepared from tobacco leaves.

tobacco plant were studied. In the control group, it was found that the activity of catalase was 1.4, 1.75, 1.84, and 1.34 μ moles/min/ml on 20, 30, 40, and 50 day, respectively and in the *p*-CA group, it was found that it was 1.04, 1.25, 1.33 and 0.98 μ moles/min/ml on 20, 30, 40 and 50 day, respectively (Fig. 8). It was suggested that it was increased gradually from 20 day to 40 day, the highest on 40 day, and reduced on 50 day.

The values of catalase activity in the *p*-CA group with those of the control group were composed and it was found that all of them were lower than those of the control group from 20 day to 50 day. In the BA group, it was found that the activity was 0.46, 0.62, 0.85 and 0.39 μ moles/min/ml 20, 30, 40, and 50 day, respectively (Fig. 9). It was suggested that it was increased gradually from 20 day to 40 day, the highest on 40 day, and reduced on 50 day. By comparing

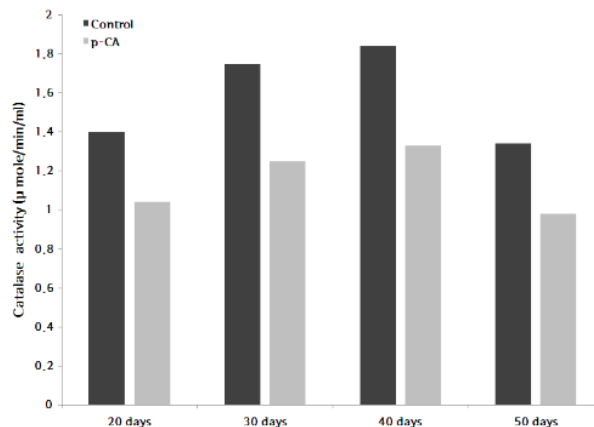


Fig. 8. Effects of *p*-CA on catalase activity in tobacco plant. The activity of catalase was determined in extracts prepared from tobacco leaves.

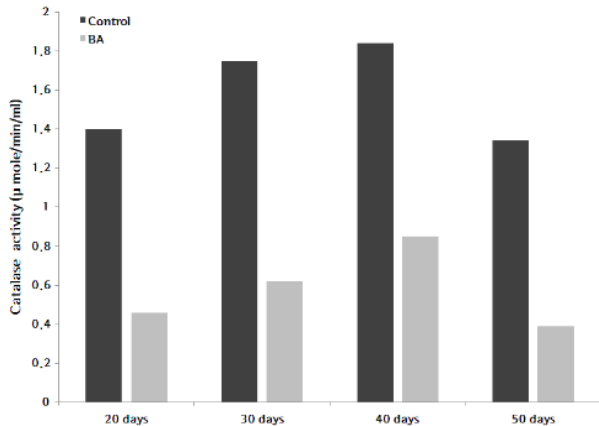


Fig. 9. Effects of BA on catalase activity in tobacco plant. The activity of catalase was determined in extracts prepared from tobacco leaves.

the values of catalase activity in the SA group with those of the control group, it was found that all of them were higher than those of the control group on contrary to the results of *p*-CA and BA group (Fig. 10).

Discussion

It had been reported that when SA was treated in advance to plant leaves, oxidative stress damages induced by toxicity of paraquat might be reduced [37]. In addition, it was also reported that activity of H_2O_2 degrading antioxidative enzymes such as catalase, glutathione reductase, and guaiacol peroxidase might be a very important role in aging process of leaves including oxidizing process, where reactive oxygen species to cause a fatal oxidative damage to cells was produced and accumulated [8].

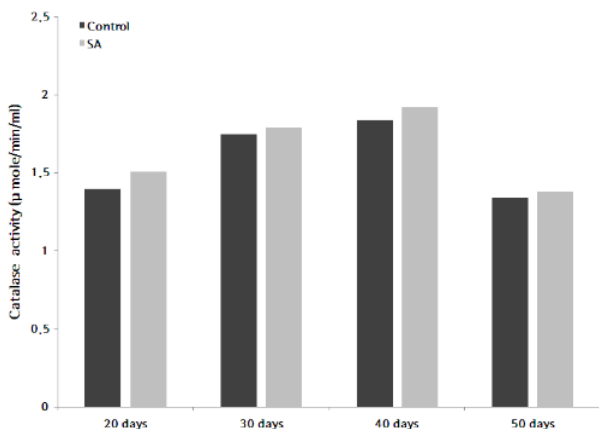


Fig. 10. Effects of SA on catalase activity in tobacco plant. The activity of catalase was determined in extracts prepared from tobacco leaves.

From the results on the effects of *p*-CA, BA and SA on the growth of tobacco plant, it was identified that while all the growth of BA group were increased from 20 day to 50 day, the growth of SA group was reduced and the growth of *p*-CA was increased from 30 day to 50 day compared with that of the control group. Therefore, it was identified that while *p*-CA and BA promoted the growth of tobacco plant and showed the best growth on 50 day, SA inhibited its growth.

Concentration of SA makes a little difference in growth of plant [40]. Gutierrez-Coronado et al [18] reported that when treating 10^{-8} M SA, growth of shoot and root of soybean increased dramatically and Shakirova et al [33] reported that 0.05 mM SA treatment promoted germination of wheat seeds and growth of their seedlings. However, different from the above results, it was identified that growth of tobacco plant was inhibited by SA. Shin et al [34] reported that grape suspension culture cells received no effect at below 1 μ M SA, their growth was inhibited at over 5 μ M and stopped at 20 μ M. Although there were some differences with plant materials, culture condition and concentration used in this study, the growth was inhibited similarly. BA and *p*-CA, the precursor and derivatives of SA, promoted the growth of tobacco plant, so showed different results.

In this study, effects of *p*-CA, BA and SA on the activity of glutathione reductase were studied. It was identified that *p*-CA and BA inhibited its activity and inhibition degree of BA was higher than that of *p*-CA. As like *p*-CA and BA, Cd reduced the activity of glutathione reductase [35]. It agreed with results of the report that SA promoted the activity of glutathione reductase in garden pea seedlings [36], and the report by Janda et al [21] that SA increased the activity of glutathione reductase in maize. There was a report that ozone increased the activity of glutathione reductase in tobacco plants [29]. It was considered that as *p*-CA and BA have lower activity of glutathione reductase than that of SA, the precursor and the analogue of SA have no effect in increasing the activity of glutathione reductase.

It was found that during the culture period, the activity was the highest by all the *p*-CA, BA and SA. Although the growth of whole tobacco plant was the best on 50 day, the growth of leaves was the best on 40 day. As like the growth of leaves, the activity of glutathione reductase was the best on 40 day. It is considered that it is because the activity of enzyme was measured using tobacco leaves, not whole tobacco plant.

Superoxide produced in various intracellular redox reaction is converted to H₂O₂ by SOD in antioxidative process. The antioxidative process is completed by degradation of H₂O₂ by catalase or peroxidase. However, when the anti-oxidation is inhibited and H₂O₂ is not eliminated and accumulated in cells, highly reactive hydroxyl radical is produced. Through this, the cell membranous lipid is oxidized or cell membrane itself loses its function, so cell death occurs [16].

Relating to anti-oxidation, this study identified effects of *p*-CA, BA and SA on the activity of catalase. It was reported that *p*-CA and BA inhibited the activity of catalase and inhibition degree of BA was stronger than that of *p*-CA. There was another report that BA reduced the activity of peroxidase in the cucumber [6]. It was identified that SA increased the activity of catalase. Although there were some differences with concentration of this study, SA increased the activity of catalase in tobacco [5], maize [17] and Arabidopsis [38], so the results agreed to the results of this study. The results were different from the report that SA inhibited the activity of catalase in seed of pea, cucumber and tomato plant [32] and pea seedlings [36]. As activation degree of *p*-CA and BA are lower than that of SA, it is considered that the precursor and the analogue of SA have no effect on increase of catalase activity. Phenylalanine and cinnamic acid as well as BA and *o*-CA, an isomer of *p*-CA, increases activity of BA2H [24].

In conclusion, it was identified that although *p*-CA and BA promoted the growth of tobacco plant, they inhibited activity of glutathione reductase and catalase. SA inhibited growth of tobacco plant but promoted activity of glutathione reductase and catalase.

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초록 : 기내배양 담배 식물에서 *p*-coumaric acid, benzoic acid 및 salicylic acid에 의해 유도되는 성장 및 glutathione reductase와 catalase의 활성

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본 연구에서는 담배식물의 성장과 glutathione reductase와 catalase의 활성에 미치는 *p*-CA, BA 및 SA의 영향을 연구하였다. 담배식물의 성장에 미치는 *p*-CA, BA 및 SA의 영향을 알아보기 위하여, 10^{-5} mM *p*-CA, BA 및 SA가 각각 함유된 MS배지에서 배양한 후 20일부터 50일까지 10일 간격으로 담배의 성장을 비교하였다. 3가지 물질 모두에 의해 50일에서 생장이 가장 잘 되었다. Glutathione reductase의 활성에 미치는 효과를 측정할 결과, BA의 활성은 대조구보다 감소하였으며, *p*-CA 역시 활성이 감소하였고, SA의 활성은 증가하였다. 또한 모두 40일에서 가장 활성이 높았다. Catalase의 활성에 미치는 효과를 측정할 결과, SA의 활성은 증가하였으며, BA와 *p*-CA의 활성은 감소하였다. 또한 모든 실험구에서 40일에 활성이 가장 양호하였다. 결론적으로 *p*-CA와 BA는 담배식물의 성장을 촉진시켰으며, 50일에서 생장이 가장 양호하였으나 항산화 효소의 활성을 억제하였다. SA는 담배식물의 성장을 억제시켰으나 항산화 효소의 활성은 촉진시켰으며, 40일에서 가장 활성이 양호하였다.