

## Influence of Benzyladenine on *in vitro* Growth, Chlorophyll and Photosynthetic Enzymes in Tobacco

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Received March 9, 2006 / Accepted May 17, 2006

The influence of N<sup>6</sup>-benzyladenine (BA) on *in vitro* growth, chlorophyll, rubisco and rubisco activase were studied in tobacco. After 11 weeks of treatment of various concentrations of BA, the most pronounced effect on *in vitro* growth and chlorophyll was found at 2  $\mu$ M BA. Rubisco content increased with increasing concentrations of BA, but a point at 2  $\mu$ M BA was reached beyond which increasing concentrations of BA cause inhibition of this enzyme. Rubisco activity showed patterns of change similar to rubisco content. These data suggest that rubisco activity was associated with an amount of rubisco protein. Under the assumption that effects of BA on rubisco may be related to by rubisco activase, in addition to, its content and activity were determined. The rubisco activase content at 2  $\mu$ M BA was more increased than other treatments. A similar change pattern was also observed in activity of rubisco activase. These results suggest that the effects of the activation of rubisco by BA seem to be related with rubisco activase.

**Key words** – Benzyladenine, *in vitro* growth, photosynthetic enzymes, tobacco

Auxin and cytokinin are used in plant tissue culture to elicit morphogenic events. A balance between auxin and cytokinin determines the morphogenic development of an explant in culture[26]. A high cytokinin to auxin ratio would favour the formation of shoots, a low cytokinin to auxin ratio root formation, and a balance between the two factors would promote callus formation.

Cytokinins are involved in plant growth and development associated with the control of cell division, chloroplast development, bud differentiation, shoot initiation, seed dormancy and the delay of leaf senescence[2]. At the cell level, cytokinins act by stimulating the synthesis of secondary metabolites like betacyanins and indolic alkaloids[14]. Cytokinin plays a significant role in seed development, tobacco increases, plerome cell division, and embryo enlargement to increase dry weight of the mature seed[11].

N<sup>6</sup>-benzyladenine (BA) as a synthetic compound with cytokinin activity has been used frequently in plant biochemical, physiological and tissue culture studies. BA can directly affect gene expression[27], mineral uptake and distribution patterns[13], and water relations[18]. BA can also inhibit PEPCase transcript induction during water stress, both in excised leaves and in intact plants[25].

In spite of the considerable literature on this subject,

however, little is known about the effect of BA on the photosynthesis at the rubisco activation level by rubisco activase *in vitro*. In order to investigate the effect of BA, we studied the influence of BA on growth and photosynthesis base on chlorophyll and rubisco/rubisco activase level in tobacco induced from the shoot of seedling *in vitro*.

### Materials and methods

#### *In vitro* growth of tobacco

Tobacco (*Nicotiana tabacum* L.) seeds sterilized in 70% (v/v) ethanol and 3% (v/v) sodium hypochlorite solution were germinated and grown aseptically in cell culture vessel containing MS[15] agar (0.8%) medium in the dark at 26 $\pm$ 1 $^{\circ}$ C. Four week-old shoots were cut into 2 cm segments and used as explants. The explants were placed on an induction MS medium supplemented with 0.2  $\mu$ M NAA[8] and various concentrations of BA (0, 0.5, 1, 2, 3, 4, 5  $\mu$ M). These explants were maintained for 11 weeks on these media at 26 $\pm$ 1 $^{\circ}$ C under a 16-h light (800  $\mu$ M/m<sup>2</sup>/s PFD) and 8-h dark photoperiod as described previously[22]. Fully expanded leaves of mature plants were used for experiments. All experiments were independently duplicated.

#### Measurement of chlorophyll

Fully expanded leaves from mature plants were frozen, then transferred to DMF and stored at 5 $^{\circ}$ C in darkness.

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Extracts were centrifuged for 5 min at 8000xg. The following equations were used to estimate the concentrations of chlorophylls from the supernatants[6].

$$\text{Chlorophyll a (mg/g fw)} = 12.70 A_{664.5} - 2.79 A_{647}$$

$$\text{Chlorophyll b (mg/g fw)} = 20.70 A_{647} - 4.62 A_{664.5}$$

$$\text{Total chlorophyll (mg/g fw)} = 17.90 A_{647} + 8.08 A_{664.5}$$

#### Purification of rubisco and rubisco activase

Two enzymes were purified according to the method by Wang et al.[29]. Frozen leaf was grinded in a mortar under liquid nitrogen, then extracted in buffer containing 50 mM BTP (pH 7.0), 10 mM NaHCO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.01 mM leupeptin, 1.5% PVPP, and 3 mM MBT. The solution filtered by four layers of cheesecloth and one layer of Miracloth was then centrifuged at 30,000 xg for 40 min. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> powder was slowly added to the supernatant to 35% saturation, and stirred for 30 min. Supernatant and pellet were both collected by centrifugation at 8000xg for 10 min. The supernatant was then brought to 55% saturation by adding (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> powder. Following centrifugation, the pellet was re-suspended in 20 mM BTP (pH 7.0) containing 0.2 mM ATP, 10 mM MgCl<sub>2</sub>, and 2 mM MBT (Buffer A). Afterward, 50% PEG-10K was added to a final concentration of 18%. The resulting precipitate was collected by centrifugation at 8000xg for 10 min and re-suspended in Buffer A. This solution was loaded onto a Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, and 10 mM NaHCO<sub>3</sub>. Elution was started with a linear gradient of 0.1 to 0.5 M NaCl, and a flow rate of 1 ml/min. The 3-ml fractions were pooled and assayed for rubisco activity and content.

To quantify rubisco activase, 50% (w/v) PEG-10K was added to pellet obtained above to a final concentration of 18%, and centrifuged at 8000xg for 10 min. The pellet was dissolved in Buffer A. This solution was cleared by spinning at 20,000xg for 10 min. This process of dissolving and spinning was performed one more time. The collected supernatants were loaded onto Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0). Elution proceeded with 20 mM BTP (pH 7.0), at a flow rate of 1 ml/min, before continuing with a linear gradient of 0 to 0.5 M NaCl in 20 mM BTP (pH 7.0). The 3-ml fractions were pooled and assayed for activity and content of rubisco activase.

#### Activity assays

Rubisco activity was determined by monitoring NADH

oxidation at 340 nm[19]. Assay medium contained 1 M Tris buffer (pH 7.8), 0.006 M NADH, 0.1 M GSH, 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.025 M 3-phosphoglycerate kinase, 0.05%  $\alpha$ -glycerophosphate dehydrogenase-triose phosphate isomerase, 0.025 M RuBP, 0.2 M ATP, 0.5 M MgCl<sub>2</sub>, 0.5 M KaHCO<sub>3</sub>, and purified rubisco solution. One unit of enzyme was defined as the amount of enzyme producing 1  $\mu$ M of RuBP per min.

Rubisco activase activity was defined as the ability to produce ADP in an ATP-dependent reaction at 340 nm[21]. The purified rubisco activase solution was added to 0.4 ml of a reaction mixture containing 50 mM tricine (pH 8.0), 20 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 40 units/ml pyruvate kinase, and 40 units/ml lactate dehydrogenase. One unit was defined as the amount that catalyzed the cleavage of 1  $\mu$ M ATP per min.

#### ELISA assays for contents

100  $\mu$ L of different dilutions of two enzymes in 0.1 M sodium carbonate-bicarbonate coating buffer (pH 9.5) was added to each well for coating of the antigen. After incubating overnight, the plate was washed with 0.01 M PBS (pH 7.4) containing 0.05% Tween 20. And then 0.1% BSA in 0.01 M PBS (pH 7.4) was added to each well and incubated for 1 hr at 37°C. After washing, 50  $\mu$ L of various dilutions of two enzymes in 0.01 M PBS (pH 7.4) was added. Approximately 50  $\mu$ L of different dilutions of a rabbit anti-rubisco and anti-rubisco activase antiserum as the primary antibody[24] was added and incubated for 30 min at 37°C. After washing, 100  $\mu$ L of peroxidase-conjugated goat anti-rabbit IgG [as diluted to 1:20,000 in 0.01 M PBS (pH 7.4)] that contained 0.1% BSA was added and incubated for 30 min at 37°C. The plate was again washed, and 100  $\mu$ L of peroxidase substrate [OPD tablets in 0.05 M citrate/0.1 M sodium phosphate buffer (pH 5.0) containing 30% of H<sub>2</sub>O<sub>2</sub>] was added. After incubation in the dark for 20 min, the reaction was terminated by adding 1 N HCl. Absorbance at 490 nm was determined by an ELISA reader (Bio-Rad Model 3550-UV).

## Results and Discussion

Cytokinins and auxins interactions control numerous developmental processes[4]. Auxin produced by the shoot apex prevents the development of axillary buds, while the application of cytokinins on these buds induced their de-

velopment in kidney bean. Also auxin induces the development and growth of secondary roots, and cytokinins have an antagonistic effect in roots[28].

In our study, the growth of leaves and stems was much decreased in plants treated by 0.2  $\mu\text{M}$  NAA alone in comparison with NAA + BA treatment after 11 weeks of culture. The growth of root is poor, when NAA was applied alone and when plants were treated with NAA + 0.5  $\mu\text{M}$  BA. The presence of BA in the culture medium stimulated shoot growth, whereas root growth was less affected by BA. These results suggest that root morphology was affected by BA treatment; at low BA (0.5  $\mu\text{M}$ ), lower biomass production was offset by increased root length, reducing the magnitude of the significant increase in total root length at 1-2  $\mu\text{M}$  BA.

In contrast to our results, however, Wei et al.[30] reported that after elongated shoots were transferred to TE medium containing 0.05  $\mu\text{M}$  NAA for 6 weeks, adventitious roots were formed. The most pronounced effect on growth of leaves, stems, and roots was found to be at 2  $\mu\text{M}$  BA (Fig. 1). These results suggest that BA had a marked effect on growth of tobacco *in vitro*. The growth was not significantly different at 1 and 2  $\mu\text{M}$  BA treatments. The plants grown in the medium with 5  $\mu\text{M}$  BA had less shoot and root growth than those with 1, 2, 3, and 4  $\mu\text{M}$  BA. In contrast to our results, however, Ramage and Williams[20] reported that high frequency shoot formation could be induced

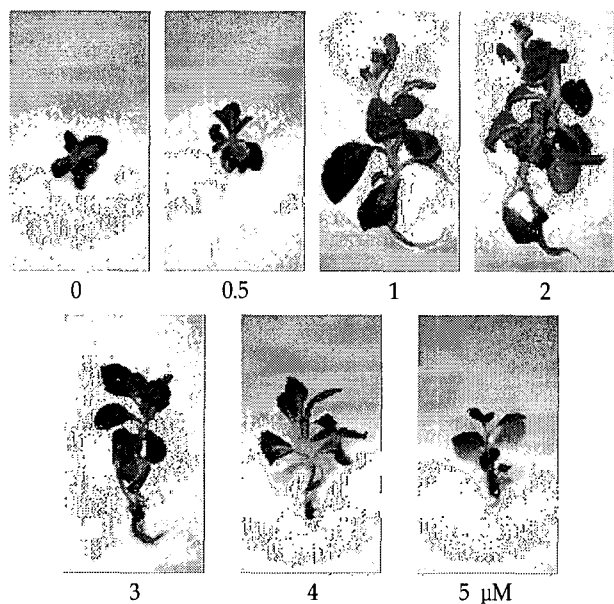


Fig. 1. *In vitro* growth of tobacco on MS medium containing 0.2  $\mu\text{M}$  NAA and various concentrations of BA for 11 weeks.

with 5  $\mu\text{M}$  of BA in tobacco leaf discs, and that increasing the exogenous BA concentration to greater than 20  $\mu\text{M}$  resulted in stunted explants with abnormal shoot morphology.

To verify the effects of BA to the growth, fresh weight was determined. Fresh weight of all plant treated with BA was significantly increased in comparison to the control. The greatest increase was obtained when BA was applied with 2  $\mu\text{M}$  (Fig. 2). As shown in Fig. 1, although there were significantly differences in growth of tobacco at BA treatments, these differences were accounted for by the weight of the shoot system. Auer et al.[1] reported that the exposure to 2.2 mM BA for 10 days induced shoot formation on 100% of the explants with a concomitant increase in the number of shoots per explant. However, Eymar et al.[5] reported that the presence of 50  $\mu\text{M}$  BA in the culture medium significantly decreased the fresh weight.

Chlorophylls are pigments capable of absorbing visible radiation that will initiate the photochemical reactions of photosynthesis in higher plants[10]. Chlorophyll a is located in the reaction center of the photosystem, and chlorophyll b is located in the light-chlorophyll-harvesting complex, rather than the reaction center[9].

To verify the contribution of BA to the regulation of photosynthesis, the changes of the chlorophyll a and b content in the leaves of tobacco treated with 0-5  $\mu\text{M}$  BA were measured (Table 1). Levels of chlorophyll a and b content were greater in explants cultured with BA compared to without BA. However, increasing the concentration of BA to 2  $\mu\text{M}$  resulted in levels of chlorophyll a and b content comparable to culture with other concentration of BA. Similarly, the change pattern was observed in total chlorophyll. But no differences were observed in

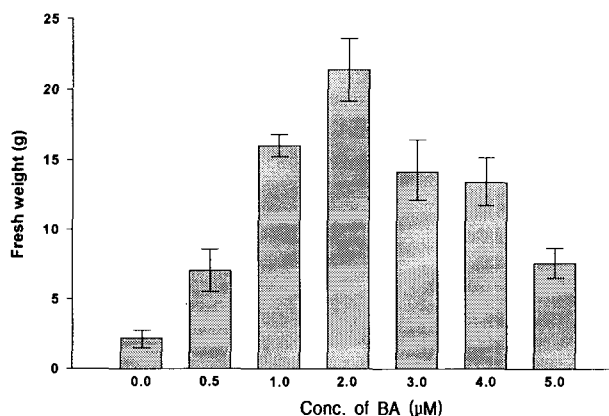


Fig. 2. Effect of BA on growth of tobacco. Plants were grown on MS media containing 0.2  $\mu\text{M}$  NAA and various concentrations of BA, respectively.

Table 1. Effect of BA on chlorophyll content in tobacco leaves. Plants were grown on MS media containing 0.2  $\mu\text{M}$  NAA and various concentrations of BA, respectively

NAA ( $\mu\text{M}$ )	BA ( $\mu\text{M}$ )	Chl. a	Chl. b	Chl. a/b	Total chl.
		(mg/g fr. wt.)			
0.2	0.0	13.13	21.35	0.61	34.46
	0.5	13.28	21.47	0.62	34.73
	1.0	13.81	21.98	0.63	35.78
	2.0	14.24	22.45	0.63	36.69
	3.0	13.60	21.76	0.63	35.35
	4.0	13.42	21.64	0.62	35.06
	5.0	13.36	21.53	0.62	34.86

the chlorophyll a/b. These results indicate that chlorophyll was regulated by BA. The changes in chlorophyll content closely parallel the changes in photosynthesis of wheat leaves[3].

The elution profiles of rubisco by anion exchange chromatography through a Q-Sepharose column are seen in Fig. 3. The fraction with the highest activity was used for measuring content and activity of rubisco.

The content of rubisco was detected by immunological method using an antibody. The effect of increasing BA concentration from 0 to 5  $\mu\text{M}$  on rubisco content is strictly dependent on concentration of BA tested. Rubisco content was enhanced by increasing BA concentration to 2  $\mu\text{M}$  BA, but it was significantly lower when it was above 3  $\mu\text{M}$  (Fig. 4). Rubisco activity also showed patterns of change similar to its content (Fig. 5). These results suggest that rubisco content was connected with an activity of rubisco protein, and induction and activation of rubisco were directly correlated with BA concentration. The behaviour in response to BA observed in this paper is in agreement with that reported by Roh et al.[23], who observed that the activation and reduction of rubisco in soybean leaves is caused by  $\text{GA}_3$ . In contrast to our results, however, Medford and Sussex[12] observed that ABA depressed the accumulation of rubisco in embryonic cotyledons of the kidney bean. In addition, BA promoted rapid accumulation of mRNAs that encoded the small subunit of rubisco. However BA hardly affected the level of mRNA for the large subunit of rubisco in etiolated cucumber cotyledons[16].

Rubisco activase acts on rubisco and allows release of the bound RuBP so that the site can bind the activator  $\text{CO}_2$  and  $\text{Mg}^{2+}$ [7]. Rubisco activase itself requires ATP, and its activity is related to the energy charge of the chloroplast [17]. Also the activity of rubisco activase can be regulated

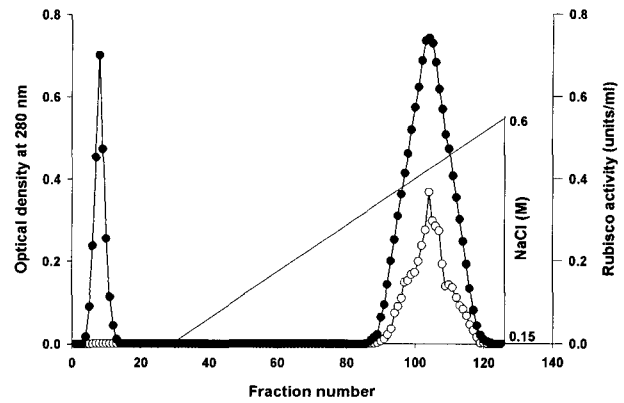


Fig. 3. Elution profile for protein (●) and activity (○) of rubisco from anion exchange chromatography on Q-Sepharose column. Rubisco was purified from leaves of tobacco treated with 2.0  $\mu\text{M}$ . Its activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0.15-0.6 M NaCl gradient in 20 mM BTP (pH 7.2).

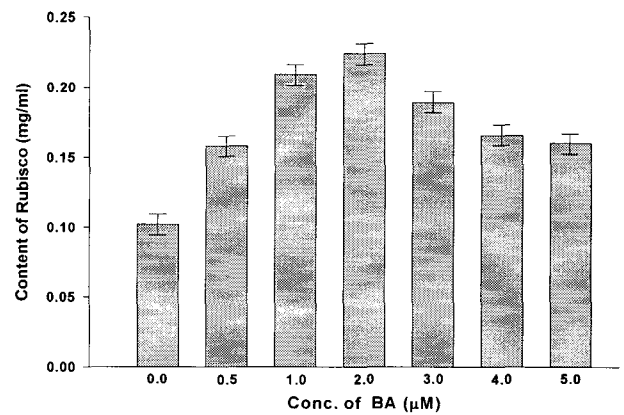


Fig. 4. Effect of BA on content of rubisco in tobacco leaves. Plants were grown on MS medium containing 0.2  $\mu\text{M}$  NAA and various concentrations of BA, respectively.

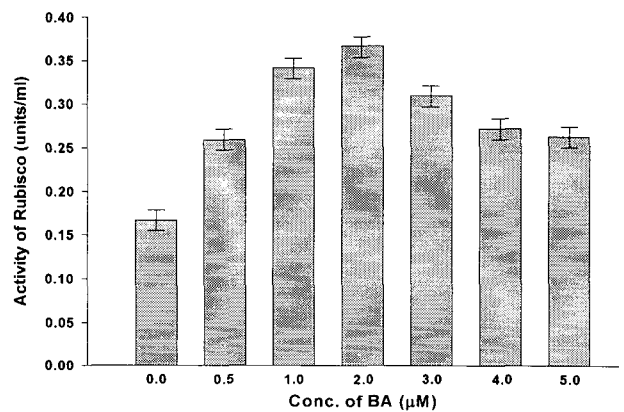


Fig. 5. Effect of BA on activity of rubisco in tobacco leaves. Plants were grown on MS medium containing 0.2  $\mu\text{M}$  NAA and various concentrations of BA, respectively.

by the ADP/ATP ratio in stroma because ADP inhibits the ATP hydrolysis reaction of the activase, which is required to remove the inhibitors[31]. Because of these reasons, we postulated that the changes of rubisco activity and content induced by BA might be related to rubisco activase. Therefore, rubisco activase was purified on a Q-Sepharose column after PEG-10K fractionation (Fig. 6), and then its content and activity were determined. The assay of rubisco activase is based on its ability to produce ADP in the presence of ATP[21].

The rubisco activase content at 2  $\mu\text{M}$  BA was more increased than other treatments (Fig. 7). A similar change pattern was also observed in activity of rubisco activase (Fig. 8). These results suggest that the effects of the activation of rubisco by BA seem to be related with rubisco activase.

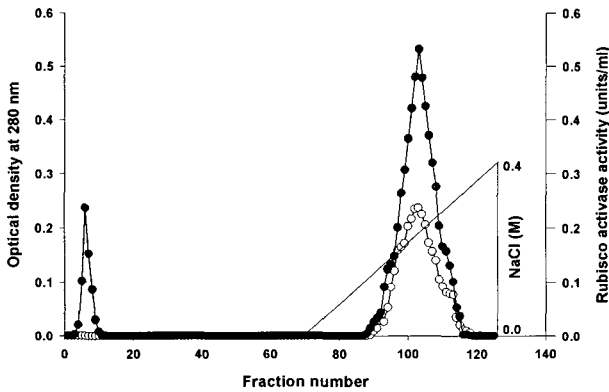


Fig. 6. Elution profile for protein (●) and activity (○) of rubisco activase from anion exchange chromatography on Q-Sepharose column. Rubisco activase was purified from leaves of tobacco treated with 2.0  $\mu\text{M}$  BA. Its activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0-0.4 M NaCl gradient in 20 mM BTP (pH 7.2).

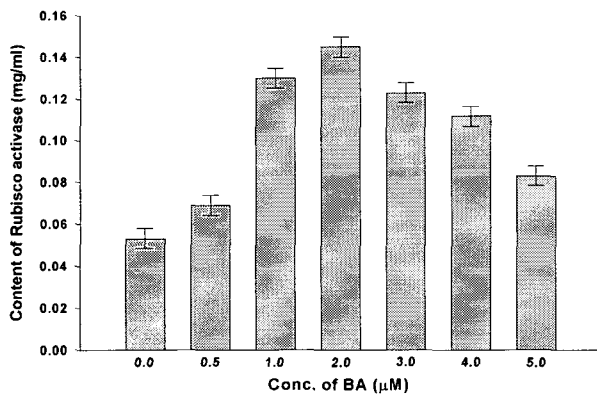


Fig. 7. Effect of BA on content of rubisco activase in tobacco leaves. Plants were grown on MS medium containing 0.2  $\mu\text{M}$  NAA and various concentrations of BA, respectively.

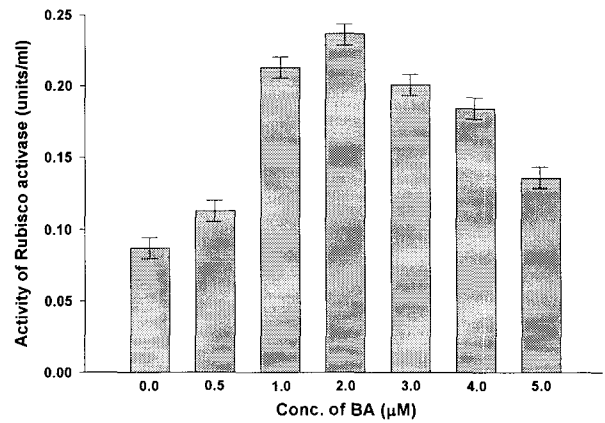


Fig. 8. Effect of BA on activity of rubisco activase in tobacco leaves. Plants were grown on MS medium containing 0.2  $\mu\text{M}$  NAA and various concentrations of BA, respectively.

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## 초록 : 담배의 기내 생장, 엽록소 및 광합성 효소에 미치는 벤질 아데닌의 영향

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담배에서 기내 생장, 엽록소 및 광합성 효소인 rubisco와 rubisco activase에 미치는 벤질 아데닌(BA)의 영향을 연구하였다. 여러 농도의 BA 처리 후 11주에 2  $\mu$ M의 농도에서 기내 생장과 엽록소의 함량이 가장 현저하였다. Rubisco의 함량은 BA의 농도가 증가함에 따라 같이 증가하여 2  $\mu$ M에서 가장 높았으나, 이후 농도의 증가에 따라 감소함으로써 억제 현상을 보였다. Rubisco의 활성 또한 함량과 같은 현상을 나타냈다. 이 결과들은 rubisco의 활성이 함량과 연관되어 있음을 의미한다. Rubisco에 대한 이러한 효과가 rubisco activase와 관련되어 있을 것이라는 가정하에 이 효소의 함량과 활성을 측정된 결과, 2  $\mu$ M BA에서 가장 증가하였다. 이러한 결과들을 종합하면 BA에 의한 rubisco의 활성화 효과는 rubisco activase와 관련되어 있음을 보여준다.