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The Mechanism of Polyamines on Ethylene Biosynthesis in Tobacco Suspension Cultures

Lee, Sun Hi and Ky Young Park
(Department of Biology, Yonsei University, Seoul)

담배 현탁 배양세포에서 Ethylene 生合成에 미치는 Polyamine의 作用機作

李 舜 熙 · 朴 基 榮
(연세대학교 이과대학 생물학과)

ABSTRACT

Effects of polyamines on ethylene biosynthesis were studied in synchronized suspension cultured cells from leaf segments of *Nicotiana tabacum* L. Putrescine, spermidine and spermine inhibited the endogenous production of both ACC and ethylene. Those production was more remarkably inhibited by spermidine and spermine than putrescine. These results were the same tendency with those obtained from exogenous application of SAM and ACC. Polyamines had more inhibitory effect on the conversion of ACC to ethyene than that of SAM to ACC, but ACC was not accumulated. The inhibition rate of exogenously applied ACC conversion to ethylene was well coincident with that of exogenously applied SAM conversion to ethyene via ACC by polyamines. However, polyamines inhibited more the activity of ACC synthase than that of EFE. From these results we can suggest that polyamines inhibit both steps of SAM to ACC and ACC to ethylene, and more effectively the latter than the former.

INTRODUCTION

Polyamines, putrescine, spermidine and spermine, are synthesized ubiquitously in plant tissues and the contents of polyamines are greatly increased at the growth stage of plant (Ramarkrishna *et al.*, 1979; Kaur–Sawhney *et al.*, 1982). Polyamines are also essential to growth regulators in metabolism of plant cell (Bagni, 1982; Smith, 1982). Polyamines prevent senescence by delaying chlorophyll breakdown in the thylakoid membrane of chloroplast (Cohen *et al.*, 1979; Popovic *et al.*, 1979; Kaur–Sawhney *et al.*, 1981) and stabilize protoplast by inhibiting the activities of RNase and protease (Galston *et al.*, 1978; Kaur–Sawhney *et al.*, 1977,

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1980). While polyamines react antagonistically on plant senescence with ethylene, which promotes senescence (Apelbaum *et al.*, 1981; Ben–Arie *et al.*, 1982; Even–Chen *et al.*, 1982), polyamines and ethylene synthesize from the same precursor: S–adenosyl-methionine (Adams and Yang, 1977; Boller *et al.*, 1979; Smith, 1985). Thus, biosynthesis of ethylene and polyamines are interdependently related. Exogenously applied putrescine and spermidine in orange peel discs stimulated the incorporation of 3,4–[C^{14}] methionine into spermidine and inhibited its incorporation into ACC, thereby polyamine inhibition of ethylene biosynthesis occured at the conversion of SAM to ACC (Even–Chen *et al.*, 1982).

Polyamines inhibited ethylene production in apple discs and protoplasts and in tobacco leaf tissue (Apelbaum *et al.*, 1981). However, the inhibitory site(s) on ethylene production is still obscure. It is generally explained that the actions of polyamines on ethylene biosynthesis are the direct effects on the activity of ACC synthase (Fuhrer *et al.*, 1982) and the indirect effect on the activity of microsomal membrane–associated ethylene–forming enzyme (Apelbaum *et al.*, 1981). SAM binds to the pyridoxal phosphate group by Schiff's base, which convert to ethylene via ACC (Boller *et al.*, 1979). On the other hand, polyamines form a Schiff's base with pyridoxal phosphate (Adams and Yang, 1979; Goodwin and Mercer, 1983). Accordingly, it is supposed to be possibilities that polyamines may cause an irreversible inhibition of ACC synthase and amino groups of amines may prevent SAM from reacting with ACC synthase by interfering with the prosthetic group (Shih *et al.*, 1982). Also, polyamines inhibit a membrane–associated ACC conversion system (Apelbaum *et al.*, 1981; Legge *et al.*, 1982), because polyamines, polyvalent cations, bind to microsomal membranes and alter the microviscosity of them, which affects indirectly the activity of EFE (Ben–Arie *et al.*, 1982). It is fact that indirect influence of polyamines inhibited ethylene biosynthesis by inhibiting ACC synthase activity and more effectively, the conversion of ACC to ethylene.

To explain the inhibitory site(s) of polyamines on ethylene biosynthetic pathway: SAM→ ACC→ethylene, we investigated the effects of polyamines on the inhibition rate at the exogenously applied SAM conversion to ethylene via ACC and the exogenously applied ACC conversion to ethylene, and also investigated activities of ACC synthase and EFE.

MATERIALS AND METHODS

Cell cultures. Callus was induced on B5 agar media at 26℃ from the leaf segments of *Nicotiana tabacum* L. The callus was subcultured every 3 weeks on B5 agar media at 26℃ and then subcultured again every 2 weeks on liquid media by shaking at 120 rpm. Synchronous cell division was induced by the double phosphate starvation method (Amino *et al.*, 1983). Cells on the 6th day of subculture were transferred to the phosphate–free media and cultured for 3 days, and then cultured again for 24 hours on the media added with 0.2 mM phosphate of final concentration. After washed with B5 phosphate free liquid media, the cells were cultured on the phosphate–free media for 4 days. The cell cycle was allowed to start by a second phosphate addition. After mitotic index (MI) was determined, the synchronized cells were used for experimental samples. The synchronized cells were incubated in 50–ml Erlenmeyer flask with

10 ml of B5 hormone free medium containing 10 mM putrescine, 10 mM spermidine, 10 mM spermine, 200 μM SAM or 1 mM ACC, which were added either alone or in combination.

Determination of ethylene production. Synchronized suspension cells (about 1 g fresh weight) were cultured in flasks which were sealed with rubber stoppers. The flasks were gently shaken at 26℃ in dark room. At indicated time intervals, 1 ml gas samples were taken from the flasks with a syringe and assayed with a gas chromatograph (Shimazu GC-3BF) equipped with a frame ionization detector.

Determination of ACC content. ACC content was assayed by the methods described by Lizada and Yang (1979), which is based on the liberation of ethylene from ACC. 0.5 g of the cells were extracted with 3% sulfosalicylic acid, and the extract was fractionated by a column of Dowex 50 (H^+ form) (bed volume, 1 ml). The amino acid fraction was eluted from the column with 2N NH_4OH, and the elute was evaporated with nitrogen gas at 40℃. The residue was dissolved in 2 ml of H_2O for ACC, and then the amount of ACC in the solution was calculated from the quotient of ethylene relased, which was determined by gas chloromatograph.

Assays of ACC synthase. Extraction and measurement of ACC synthase were assayed by the modified method of Boller *et al.* (1979) and Kang *et al.* (1984). The cultured cells were homogenized with 2 ml of 100 mM Tris-HCl (pH 8.0), containing 10 mM EDTA, 4 mM DTT, 2m M $MgCl_2$, 10 μM pyridoxal-5-phosphate and 30% glycerol. The homogenate was centrifuged for 15 min at 15,000×g, and the supernatant was passed through a column of ultrogel ACA 44 (bed volume, 30 ml), which had previously been equilibrated with 10 mM Tris-HCl (pH 8.0), containing 1 mM EDTA, 1 mM DTT, 10 μM pyridoxal-5-phosphate, 2 mM $MgCl_2$ and 15% glycerol, and followed by elution with the same buffer solution. The macromolecular fraction was collected and used for the assay of ACC synthase. The reaction mixture for ACC synthase assay contained 0.4 ml of the enzyme preparation, 50 μM SAM, 10 μM pyridoxal-5-phosphate, 100 mM HEPES (pH 8.5) in a total volume of 0.6 ml. After the reaction was stopped by adding 0.3 ml of 50 mM $HgCl_2$, the activity of ACC synthase was assayed by the determination of ethylene formed from ACC through the above reaction. Protein content in the enzyme preparation was determined by the method of Lowry *et al.* (1951).

Assay of ethylene forming enzyme (EFE). The activity of EFE was assayed by the modified method of Hoffman and Yang (1982) as following. The cultured cells were incubated in B5 hormone free media added with 1 mM aminooxyacetic acid (AOA) for 2 hours, and then 1 mM ACC was infiltrated into the culture cells at the vacuum condition. The cultured cells were collected on filter paper and washed with distilled water. After the collected cells were incubated for 1 hour in the flasks which were sealed with silicon stopper, ethylene produced from ACC was assayed by gas chromatograph.

RESULTS AND DISCUSSION

In the present study, polyamines progressively inhibited ethylene production with the

increase in concentration. Especially, the inhibiting effect was maximum at 10 mM spermidine and spermine (Fig. 1). The inhibition began to appear at 2 hours of incubation, and after 12 hours of incubation, the ethylene production was kept plateau (Fig. 2). 10 mM polyamines inhibited remarkably both ACC formation and ethylene production, but more effectively on ethylene production than ACC formation (Table 1). In the presence of exogenously applied 200 μM SAM, polyamines was shown to have a similar inhibitory effect with above results (Table 2): the conversion of SAM to ACC was inhibited about 29% of the control by putrescine, 39% by spermidine or spermine, whereas that of ACC to ethylene was inhibited about 77% of the control by putrescine, 87% by spermidine and 88% by spermine. When ACC was applied exogenously at the concentration of 1 mM, these inhibitory rates was similar to those of the conversion of ACC to ethylene (Table 3). These results suggest that polyamines inhibit both the conversion of SAM to ACC and that of ACC to ethylene. If polyamines inhibited only at the step of SAM conversion to ACC, the inhibition rate of ACC formation from SAM were similar to that of ethylene production from SAM via ACC. On the other hand, if polyamines inhibited only at the step of ACC conversion to ethylene, it led to a result of ACC accumulation. Fuhrer *et al.* (1982) reported that ACC content in oat leaves was increased,

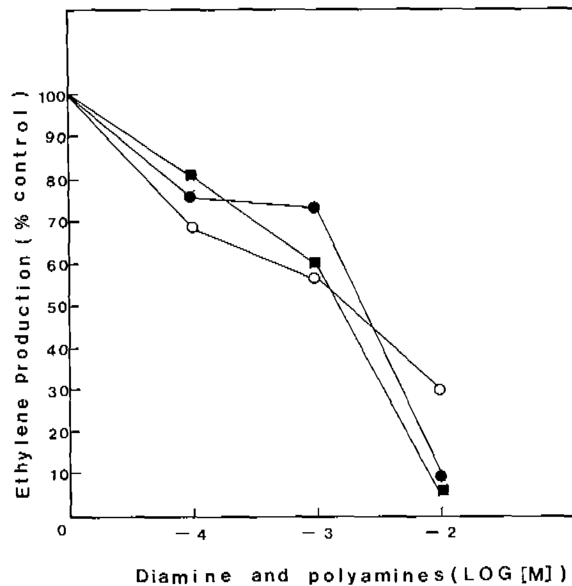

Fig. 1. Effect of polyamines at various concentrations on ethylene production in tobacco synchronized-suspension culture cells. The culture cells were incubated in 50-ml flasks containing 10 ml B5 hormone free medium. Rates of ethylene production in darkness at 26℃ were recorded after 24 h of incubation when rate in control sample was 13.47 nl/g. fr. wt. Put., ○……○; Spd, ●……●; Spm., ■……■

because spermidine inhibited more effectively the ACC conversion to ethylene, despite ACC synthase activity was decreased by it. But our results showed that polyamines reduced the level of ACC, and other experiments reported similar results (Suttle, 1981; Apelbaum *et al.,* 1982;

Table 1. Effect of polyamines on ethylene production, and ACC content in tobacco synchronized-suspension culture cells in darkness at 26℃ after 24 h incubation

Treatment	ACC Content		Ethylene Production	
	n mole/g.fr.wt.24 h.	% inhibition	n mole/g.fr.wt.24 h.	% inhibition
Control	3.17 ± 0.49	0	7.74 ± 0.06	0
Put.	2.02 ± 0.60	36	4.83 ± 0.39	38
Spd.	1.50 ± 0.23	53	1.18 ± 0.16	85
Spm.	1.25 ± 0.11	61	1.28 ± 0.12	83

Suspension cultured cells were incubated 50 ml flasks containing amines (putrescine, spermidine and spermine at 10 mM).

Table 2. Effect of polyamines on SAM-based ethylene production in tobacco synchronized-suspension culture cells in darkness at 26℃ after 24 h of incubation

Treatment	ACC Content		Ethylene Production	
	n mole/g.fr.wt.24 h.	% inhibition	n mole/g.fr.wt.24 h.	% inhibition
SAM	0.66 ± 0.04	0	10.48 ± 0.66	0
SAM + Put.	0.47 ± 0.06	29	2.43 ± 0.03	77
SAM + Spd.	0.40 ± 0.04	39	1.38 ± 0.01	87
SAM + Spm.	0.40 ± 0.03	39	1.23 ± 0.12	88

SAM, 200 μM; diamine and polyamines, 10 mM, respectively.

Table 3. Effect of polyamines on ACC-based ethylene production in tobacco synchronized-suspension culture cells in darkness at 26℃ after 24 h of incubation

Treatment	ACC content	
	n mole/g.fr.wt.24 h.	% inhibition
ACC	14.02 ± 0.52	0
ACC + Put.	6.10 ± 0.93	56
ACC + Spd.	1.97 ± 0.09	86
ACC + Spm.	2.36 ± 0.15	83

ACC, 1 mM; diamine and polyamines, 10 mM, respectively.

Table 4. Effect of polyamines on ACC synthase activity and EFE activity in tobacco synchronized-suspension culture cells

Treatment	ACC synthase activity		EFE activity	
	n mole ACC/mg protein	% inhibition	n mole ethylene/g.fr.wt.	% inhibition
Control	5.44 ± 0.51	0	2.43 ± 0.29	0
Put.	4.20 ± 0.24	23	1.77 ± 0.09	27
Spd.	2.04 ± 0.18	62	1.34 ± 0.03	45
Spm.	2.02 ± 0.13	63	1.26 ± 0.05	48

Cultured cells were pretreated by diamine and polyamines at 10 mM, respectively.

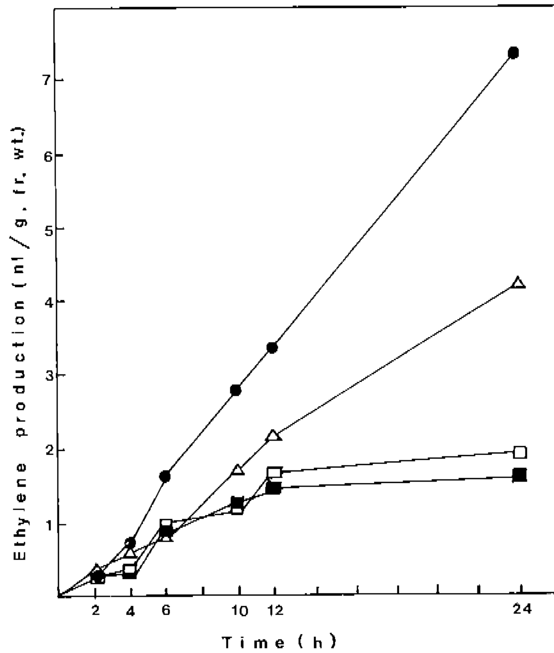

Fig. 2. Effect of polyamines on the time course of ethylene production in tobacco synchronized-suspension culture cells in darkness at 26℃. Suspension cultured cells were incubated in 50-ml flasks containing 10 ml B5 hormone free medium with Put. (△……△), Spd. (□……□), Spm. (■……■) and control (●……●).

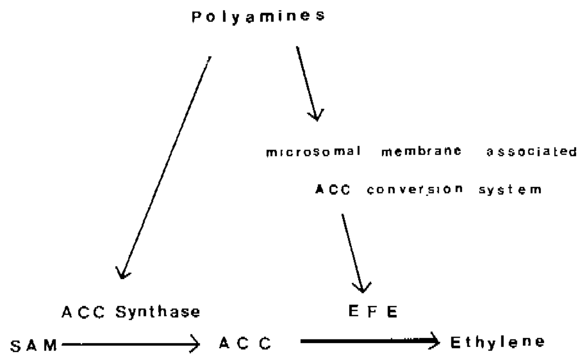

Fig. 3. Action mode of polyamines on the inhibition of ethylene biosynthesis.

Even-Chen *et al.*, 1982). Because the effect of polyamines was greater on the inhibition of ACC synthase activity than on that of EFE activity (Table 4), it was regarded that ACC accumulation did not occur. Consequently, it is fact that polyamines more effectively inhibited the conversion of ACC to ethylene than that of SAM to ACC. The inhibitory effect of polyamines on the activities of ACC synthase and EFE is not likely to result from the decrease of de novo synthesis of the enzymes, because polyamines mesurably increase DNA synthesis (Kaur–Sawhney *et al.*, 1980), as well as decrease the degradation of RNA and protein (Galston, 1983). Therefore, it is suggested that polyamines themselves inhibit the activities of ACC synthase and EFE by interfering the enzyme–substrate reaction and membrane–integrity.

It is suggested that polyamines inhibited more effectively the conversion step of ACC to ethylene, indicrectly interfering with microsomal membrane associated ACC conversion system, rather than that of SAM to ACC, directly by means of interfering with ACC synthase activity (Fig. 3).

摘 要

담배 잎에서 유도한 동시성 현탁배양세포를 재료로 ethylene생합성에 미치는 polyamine의 영향을 연구하였다. putrescine, spermidine 및 spermine은 내생적으로 생성되는 ACC와 ethylene을 모두 억제하였다. 이들의 생성은 putrescine보다는 spermidine과 spermine을 처리하였을때 보다 현저하게 억제되었다. 이러한 결과는 외부에서 SAM 혹은 ACC를 처리하였을 때의 결과와도 일치하였다. Polyamine은 SAM에서 ACC로의 전환보다는 ACC에서 ethylene으로의 전환을 더 억제하였지만 ACC는 축적되지 않았다. Polyamine이 ACC에서 ethylene으로의 전환을 억제하는 율은 ACC를 경유하여 SAM에서 ethylene으로의 전환을 억제하는 율과 일치하였다. 그러나, polyamine은 EFE활성보다는 ACC synthase활성을 더욱 억제하였다.

이상의 결과로부터 polyamine은 SAM에서 ACC로의 전환과정과 ACC에서 ethylene으로 전환과정을 모두 억제하며 후자에 더 효과적으로 작용한다고 사료된다.

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