Glyphosate Toxicity: Ⅱ. EPSP-synthase Activity in Cell Suspension Culture of *Corydalis Sempervirens* and *Lycopersicon Esculentum.*

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Glyphosate 毒性: Ⅱ. *Corydalis Sempervirens*와 토마토의 細胞培養體에서 EPSP-synthase의 活性에 미치는 影響

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ABSTRACT

Glyphosate (N-[phosphonomethyl]glycine) applied to the assimilate-exporting leaves or sprayed to the whole plants of tomato(*Lycopersicon esculentum* Mil var. Moneymaker) induced the rapid inhibition of 5-enolpyruvyl skimic acid 3-phosphate synthase(EPSP-synthase). It shows that EPSP-synthase activity precedes chlorophyll loss. There is no difference in EPSP-synthase activity between *in vivo* tomato meristem and cell suspension culture if glyphosate is not applied. The EPSP-synthase activity is in a range of 4 to 6 nkat per mg protein. The inhibition of EPSP-synthase action is induced within 36 h after glyphosate application while the Chl contents were reduced 48 h after the application. In cell suspension culture of tomato and Corydalis (Corydalis sempervirens), a sublethal concentration of glyphosate retards the fresh weight increase and prolonged lag phase. The fresh weight is reached maximal about 14 days after the subculture in the presence of glyphosate. The inhibitory effect of glyphosate on EPSP-synthase is remarkably induced in lag phase.

Key words: Cell culture, Chlorophyll, Glyphosate, Shikimic acid, EPSP-synthase, *C. sempervirens*, *L. esculentum.*

INTRODUCTION

Glyphosate is a potent nonselective postemergence herbicide which has low toxicity on mammalian cells and is easily degraded in soil[9,12,25] Glyphosate has been shown to inhibit many metabolic processes in plants including protein synthesis, nucleic acid synthesis, photosynthesis and respiration[5,7,11,20,21,23] It has been observed

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through ultrastructural and biological studies[18,28] that chloroplast swelling and shikimate accumulation are induced within 16 to 20 h after glyphosate treatment. During chloroplast swelling, it might be necessary to occur in protein turnover and lipid synthesis or redistribution within chloroplast[10]. It would be interesting to investigate the action of glyphosate on Chl degradation because EPSP synthase has been shown to be a plastidial enzyme which is synthesized on cytoplasmic ribosome as a higher molecular weight precusor and which is then transported through the plastid envelope and proteolytically processed inside the chloroplast to yield mature EPSP-synthase[8,14,19]

Since EPSP-synthase seems to be target enzyme of glyphosate, the observation of the change in the EPSP-synthase activity is needed to understand the chronological action of glyphosate[26,27] In this study, the changes in EPSP-synthase activity in *in vivo* apical meristem of tomato plant was compared to it in cell culture system. Furthermore, the inhibitory action in cell suspension culture of *C. sempervirens* with or without glyphosate is discussed.

MATERIALS AND METHODS

Plant materials and growth condition

Tomato was used for the measurement of EPSP-synthase activity. The growth condition and the glyphosate application except for that glyphosate was only applicated onto one of the middle lobe of third old leaf was same as previous method by Kim and Amrhein[15] Chlorophyll contents were measured by previous Arnon's method[2]

Suspension culture of *Corydalis* and tomato

The cells(1.5 g F. W.) were cultured in 25 ml LS-medium with 1 μM 2,4-D and 1 μM 1-NAA under 100 rev./min(Table 1). The minor change in supplements is indicated in text. Temperature (23℃) and light intensity(600 lux) were constantly controlled. Subcultures were biweekly performed.

Table 1. The LS medium used for cell suspension culture of *Corydalis sempervirens* and *Lycopersicon esculentum*.

Nutrients	mg / litre
KNO_3	1900
NH_4NO_3	1650
$MgSO_4$ $7H_2O$	370
$CaCl_2$ $2H_2O$	440
KH_2PO_4	170
$MnSO_4$ $4H_2O$	22.3
KI	0.83
H_3BO_3	6.2
$ZnSO_4$ $7H_2O$	8.6
$CuSO_4$ $5H_2O$	0.025
$NaMoO_4$ $2H_2O$	0.25
$FeSO_4$ $7H_2O$	27.86
Na_2 EDTA	37.26
Myo-Inositol	100
Thiamine-HCl	0.4

Extraction and purification of EPSP-synthase

The frozen cells were mortar ground in 4× volume(v/w) of 250 mM ice cold Tris-Malate buffer(pH 7.7) with 5 mM mercaptoethanol and 0.2 g polyclar AT/g F. W. The homogenates were filtrated through 4 layers cheesecloth and centrifuged by 12,000×g for 10 min. The supernatants were saturated to 80% $(NH_4)_2SO_4$ and then centrifuged by 39,000×g for 10 min. The pellets were redissolved in a bit of extraction buffer. After dialysis against 20 mM Tris-Malate buffer (pH 7.7) with mercaptoethanol, the emzyme extracts were collected and frozen-stored at -80℃ until analysis. The measurement of enzyme activity was carried out as previous method by Smart et al[24] Briefly, the test solution included in a total volume of 100 μl 1 mM phosphoenolpyruvate, 1 mM shikimate-3-phosphate, 50 mM Tris-Malate buffer(pH 6.7) and 0.1 mM sodium molybdate solution. The reaction was started through the addition of 40 μl

enzyme extracts after preincubation at 30℃ for 5 min and stopped through the addition of 1 ml phosphate reagents. Organic phosphate was quantified by Lanzetta's method[16]. Namely, exactly 1 min after the stop of incubation, 200 μl 34% (w/v) Na_3-citrate. $2H_2O$ was added. After standing at room temperature for 60 min, Pi is measured at 660 nm. The phosphate reagent was prepared from the following stock solutions: 0.045% malachite green hydrochloride(MG); 4.2% ammonium molybdate in 4 N HCl (AM); 2% (v/v) Tergitol in water. One volume AM and 3 volume MG are mixed, and then stand for 24 h at room temperature. After filtering, 10 ml of 2% Tergitol was added to 490 ml AM-MG mixture. Protein concentration was measured as previous method by Bradford[4].

RESULTS AND DISCUSSION

Fig. 1. shows the *in vivo* inhibition of EPSP-sysnthase. EPSP-synthase activity in the basal part of apical leaves treated with 200 nmol glyphosate strongly decreased within 36 h. The chlorophyll(Chl) loss was induced after 48 h. Although it shows that the Chl content on a basis of g F. W. is slightly reduced with leaf age even in untreated control plants, it is clear that the Chl loss is considerably stimulated by glyphosate. EPSP-synthase activity on a basis of mg protein is also somewhat reduced with leaf development. It is of interesting that the decrease in EPSP-synthase activity precedes the chl loss. Thus, the chloroplast swelling[5,18,28] and chl loss may be a secondary effect of glyphosate.

In general, it is known that the plastid is the major biosynthesis site of aromatic amino acids in plants and micro-organisms.[19,24,27] As known by previous report[15,22,24], the accumulation of shikimic acid has been sured to be a lethal effect of glyphosate. However, the compartmentation of

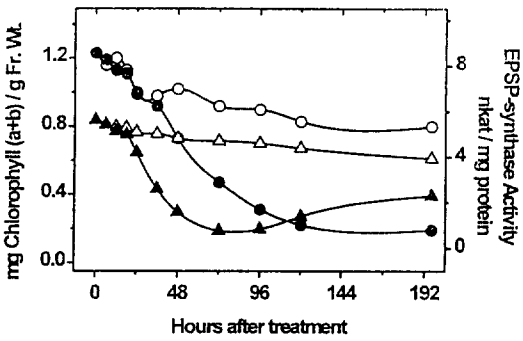

Fig. 1. The changes in chlorophyll content (left axis; -□-, -●-) and EPSP-synthase activity (right axis; -△-, -▲-). Two hundred nmol glyphosate was applied onto a middle lobe of the third old leaf of 6 week old tomato plants. The yellowing basal part of leaves was sampled. Chlorophyll contents from methanolic extracts were measured by Arnon's method (n=3). EPSP-synthase activity (n=6) was measured by the method of Smart *et al.* The empty marker and filled marker indicate control and treated plants respectively.

shikimic acid may not be limited only in plastid. Not only the chloroplast swelling but also large vacuoles are observed after glyphosate treatment [5,18,28]. The redistribution of shikimic acid and its physiological mechanism are not as yet illuminated. The EPSP-synthase activity is normally in a range of 4 to 6 nkat per mg protein if the plants were not treated with glyphosate. The *in vivo* EPSP-synthase activity in apical leaf is similar to it in tomato cell culture(Fig. 2) in which the activities are in a range of 4 to 5 nkat per mg protein for the observation. One mmol glyphosate application in the cell culture medium of *L. esculentum* retarded the increase in fresh weight(Fig. 2). This phenomenon is also observed in *C. sympervirens* cell culture in the presence of 500 nmol glyphosate(Fig. 3). The EPSP-synthase activity in *C. sempervirens* cell culture is lower than in *L. esculentum.* Interestingly, EPSP-synthase activity is again induced 96 h after glyphosate application(Fig. 2). As shown in previous report by Kim and Amrhein[15], the

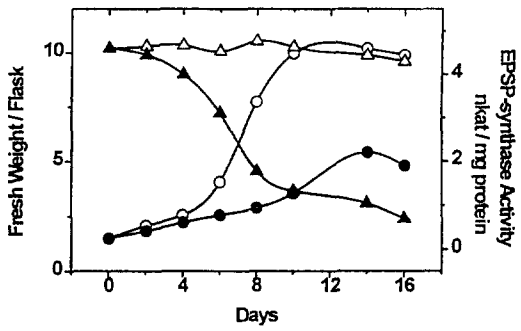

Fig. 2. The changes in fresh weight (left axis; -○-, -●-) and EPSP-synthase activity (right axis; -△-, -▲-) in *Lycopersicon esculentum*. One mmol glyphosate was included in the cell culture medium. The subcultures were performed biweekly. The empty marker and filled marker are control and treated plants respectively.

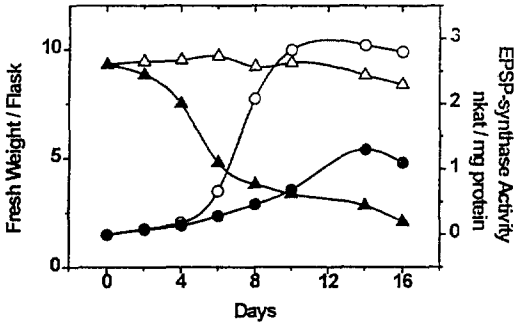

Fig. 3. The changes in fresh weight (left axis; -○-, -●-) and EPSP-synthase activity (right axis; -△-, -▲-) in *Corydalris sympervirens*. Five hundred nmol glyphosate was included in the cell culture medium. The subcultures were performed biweekly. The empty marker and filled marker are control and treated plants respectively.

decrease in the shikimic acid level accumulated after glyphosate treatment should be resulted from the increase in EPSP-synthase(Fig. 1), and the recovery effect may also be due to the sublethal concentration. The decrease of EPSP-synthase activity is induced at the lag phase in both cell cultures, *i.e.* before the increase in F. W.(Fig. 2 and 3). The lag phases in both cell culture are prolonged by glyphosate, and the maximal F. W. is not over about 50% compared to the control cell culture in abscence of gly- phosate(Fig. 2 and 3). However, we would like to conclude that the inhibition of EPSP-synthase through glyphosate is unlikely to be only res- ponsible for glyphosate's herbicidal effect since many plants naturally have very high levels of shikimic acid with no detrimental symtoms (personal communication to Prof. G. Heinrich in Univ. Graz and unpublished data). Furthermore, the synthesis of EPSP-synthase *per se* may not be inhibited by glyphosate. There are some evidences that an overproduction of enzyme may be direct responsible to be a resistance agianst inhibitors. Amrhein *et al*'s[1] indicated that an overproduction of EPSP synthase in glyphosate tolerance cell lines seemed to be a resistance mechanism. The elevation of enzyme level required for normal growth has been known to be a common mechanism of resistance to inhibitors. In the gene amplication level, it has been demonstrated in some mamalian cell lines[3]. However, since there is no difference between the properties of EPSP-synthases isolated from glyphosate-unadapted and -tolerant cell lines, gene amplication is unlikely to explain the glyphosate-resistance mechanism(data in preparing). In conclusion, the EPSP-synthase inhibition by glyphosate is not regulated by a gene amplication. Furthermore, the inhibition seems to be temporarily if the sublethal concentration is applied. Therefore, the separation of primary herbicidal effects on plastidial shikimic acid pathway from secondary or tertiary effects on whole plants is difficult and unclear in many instances. Metabolic processes are interdependent *in vivo* and thus it is difficult to extrapolate with certainty a previous *in vitro* data in micro-organisms and cultured plant cells to a living system. There is, therefore, a need to identify other factors in a level of whole plant which may be important in the

events leading to cell death.

Our next reports will show how the inhibition of EPSP-synthase and the accumulation of shikimic acid bring about herbicidal effects on the turnovering proteins of thylakoid membrane and on the lipid biosynthesis or redistribution within the chloroplast envelopes and thylakoid

摘 要

Glyphosate (N-[phosphonomethyl]glycine)를 토마토(*Lycopersicon esculentum* Mil)의 同化部位에 部分處理하거나 全 植物體에 噴霧處理하였을 때 EPSP-synthase의 活性 減少가 나타났다. EPSP-synthase의 活性은 처리된 植物體의 葉綠素의 감소보다 시기적으로 먼저 나타나는 현상이었다. EPSP-synthase의 活性은 glyphosate處理에 민감한 酵素로서 토마토의 細胞顯濁培養組織과 分裂組織간에는 活性의 차이가 없었다. EPSP-synthase의 활성은 4-6 nkat/mg protein 정도이었다. EPSP-synthase의 活性억제는 glyphosate 處理 36시간 후 부터 나타나기 시작하였고, 葉綠素의 감소는 처리 48시간 후 부터 나타나기 시작하였다. 細胞顯濁培養에서 致死濃度 以下에서 glyphosate는 生體重을 低下시켰으며 生育段階 중 lag-phase를 延長시켜 生育이 더디도록 하였다. Glyphosate 存在하에서 生體重은 繼代배양 후 14일이 지난 뒤에 生體重이 最高에 달하였다. EPSP-synthase에 대한 gly- phosate의 抑制효과는 lag-phase에서 심하게 나타났다.

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