Glyphosate Toxicity: I. Long Term Analysis of Shikimic Acid Accumulation and Chlorophyll Degradation in Tomato Plant.

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Glyphosate 毒性: I. Glyphosate 處理가 토마토의 Shikimic Acid의 蓄積과 葉綠素의 分解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). Glyphosate induced the rapid accumulation of shikimic acid within 24 h. The accumulation of shikimic acid companied with chlorophyll loss in meristematic leaves, i.e. apical leaves. The chlorosis was acropetal in apical region of young growing leaf. The degradation of chlorophyll seems to be a secondary or tertiary effect of glyphosate. However, the level of shikimic acid accumulated was reduced except for roots and apical leaves from 5 days after treatment. The accumulating levels are considerably differed through the applicated regions. The level of shikimic acid is highest at the apical meristem 4 days after the application to 3rd old leaf.

Key words: *Lycopersicon esculentum*, Chlorophyll, Chlorosis, Glyphosate, Shikimic acid.

INTRODUCTION

Glyphosate is a widely used potent nonselective postemergence herbicide which has low toxicity on mammalian cells and is easily degraded in soil.[4,6,7,12] Glyphosate has been shown to inhibit many metabolic processes in plants including protein synthesis, nucleic acid synthesis, photosynthesis and respiration.[3,5] It is known to be rapidly absorbed and transported to meristematic and young growing tissues of both the shoot and the root following the source-sink gradient within the plants. Although 5-enolpyruvylshikimic acid 3-phosphate synthase(EPSP) of the shikimic acid pathway is the primary target enzyme[15,17,19], it is not understand how glyphosate has long term toxic effect in apical meristem and young leaves after phloem mobilisation.

It has been observed through ultrastructural

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and biochemical studies[2,10,20] that chloroplast swelling and shikimate accumulation are induced within 16 to 20 h after glyphosate treatment. Glyphosate has also been shown to decrease the activities of PS I and PS II.[12,14] There is no direct evidence that PS II and 70 S ribosme are the primary inhibition site of glyphosate. The degradation of chlorophyll can directly induce the decrease in the PS I and II activity. Thus, the longterm analysis was needed to illuminate the relationship between shikimic acid accumulation and chloroplast disintegration.

MATERIALS AND METHODS

Plant materials and growth condition

Tomato, Lycopersicon esculentum Mil var. Moneymaker, was transfered from green house 3 weeks after sawing and acclimatized for the further 3 weeks until the application of glyphosate in a growth chamber(PK-10, Therma, Zürich :PGA 36, Conviron, Winnipeg, Canada). The transfered tomato seedlings were planted in flats 9 by 9 by 12cm) containing vermiculite. The flats were placed in a controlled chamber at 26 ℃ day temperature and 16℃ night temperature under 60±2% constant relative humidity. Light intensity of 4000 lux was obtained from cool white fluorescent and indecandescent lamps for 16 h every day.

Application of glyphosate

Glyphosate 200 nmol was applied as a 20㎕ drop [10 mM glyphosate in 10 mM potassium phosphate buffer, pH 6.0; 0.1% (v/v) Tween 80] onto one of the middle lobes of the forst-, the second- and the third oldest leaf or sprayed to the whole plant of 6-week-old tomato plants 4 h after the start of the photoperiod, respectively (Fig. 1). Buffer-treated and untreated plants served as controls. The tomato leaves, roots and shoots

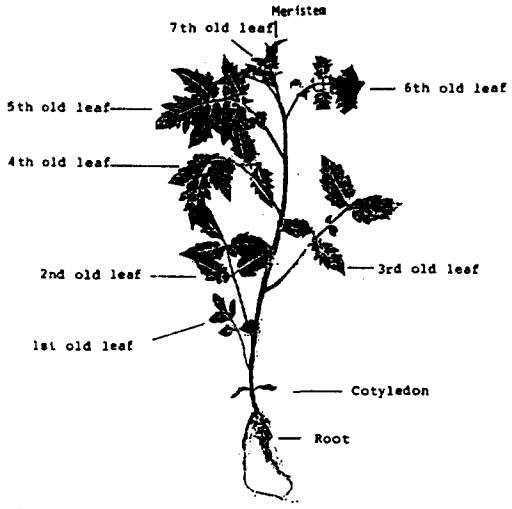

Fig. 1. Diagramatic representation of 6-week-old tomato plant. The leaves of tomato plants are numbered in a basipetal direction and cited in the text of this study. Meristem in the text is defined as apical meristem, leaf primordia, and shorter young leaf than 2cm long.

were chronologically sampled in liquid N_2 and stored at -80℃ prior to the shikimic acid analysis by high performance liquid chromatography (Bechman System Gold 126) equipped with UV detector(Detector 168). Shikimic acid is detected at 215 nm and quantified using a linear standard curve(Fig. 2).

Quantitative analysis of shikimic acid and chlorophyll

The shikimic acid content of the tissue was quantitatively determined by HPLC. The sampled parts of plant were extracted 3 times under the reflux cooling apparatus with 20 ml methanol for 20 min. The extracts were filtrated and dried in an air stream. The precipitates were liquefied with 70℃ distilled water and centrifuged for 20 min (4℃, 12500 rpm, Beckman Rotor Jar[16]. The shikimic acid was eluted using the mobile phase of 95/1/5(v/v/v/) of acetonitril(HPLC-grade, Fluka), 85% phosphoric acid and deionized water by a flowing rate of 1 ml/min with the LiChrosorb-

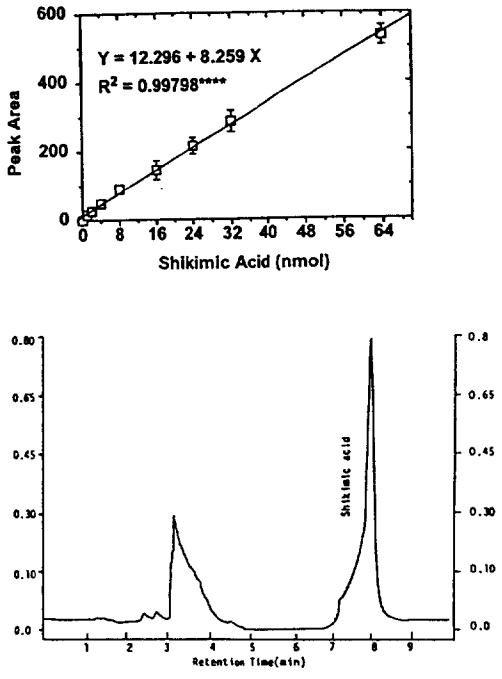

Fig. 2. The standard curve of shikimic acid quantification. The shikimic acid provieded by Monsanto Agricultural Co. was diluted to the required concentrations in 95% acetonitrile and then eluted using the mobile phase of 95/1/5(v/v/v/) of acetonitrile(HPLC-grade, Fluka), 85% phosphoric acid and deionized water by a flowing rate of 1 ml/min with the LiChrosorb- NH_2 column. Shikimic acid was eluted about 7.5 min, and the peak areas at 215 nm were counted. The elution was continued for 10 min. The vertical bars mean the standard errors(SE) of three independent experiments. Vertical bars(SE) are missing where the SE was smaller than symbol size.

NH_2 column. Shikimic acid was eluted by 7.5 min. The elution was continued for 10 min. The loading volume was 20$\mu\ell$. Acid free glyphosate was provided by Monsanto Agricultural Products Co.(St. Louis, MO).

Chlorophyll content was measured by Arnon's method.[1] The middle lobe of the leaves applied with glyphosate is removed and the rest leaves is used to measure the chlorophyll content.

RESULTS AND DISCUSSION

The first visible herbicidal effect observed after applied with 200 nmol glyphosate was an acropetal yellowing and bleaching in the emerging and maturing leaves of apical region. The chlorosis is at first limited to the proximal region of the lamina near the veins. Four days after glyphosate application to the middle lobe of third old leaf, the chlorosis proceeds in a basipetal direction until veinal chlorosis is just perceptible in 5th old leaf. The level of shikimic acid in control leaves and roots were too low for quantitative analysis. Fig. 3 shows the accumulation of shikimic acid after the glyphosate application onto middle lobe of the first or the second old leaf. It seems that the fully matured leaves are usually unaffected by glyphosate, e.g. for the first old leaf treatment, between second and fourth old leaf. Glyphosate is known to translocate via phloem into apical and young growing region of root and shoot, in which shikimic acid is accumulated[15]. Our time course analysis of shikimic acid accumulation reveals that the accumulation is concentrated in these regions within 4 days after appied with 200 nmol glyphosate, corresponding to the previous result[11]. However, the level of the shikimic acid accumulated is reduced thereafter. Albeit this phenomenon may be resulted from the thing that 200 nmol glyphosate is a sublethal concentration, there is no report what the mean of the disappearance of the shikimic acid accumulated in apical regions is physiologically. Many enzymological sutdies on shikimic acid pathway have indicated that the pathway is not controlled by a feedback system.[15,17,19] Of course, it has been noted that an unregulated carbon flow into shikimic acid pathway is involved in the herbicidal action of glyphosate.[8,14] Pinto et al.[14] have reported taht the first enzyme of this pathway, 3-deoxy

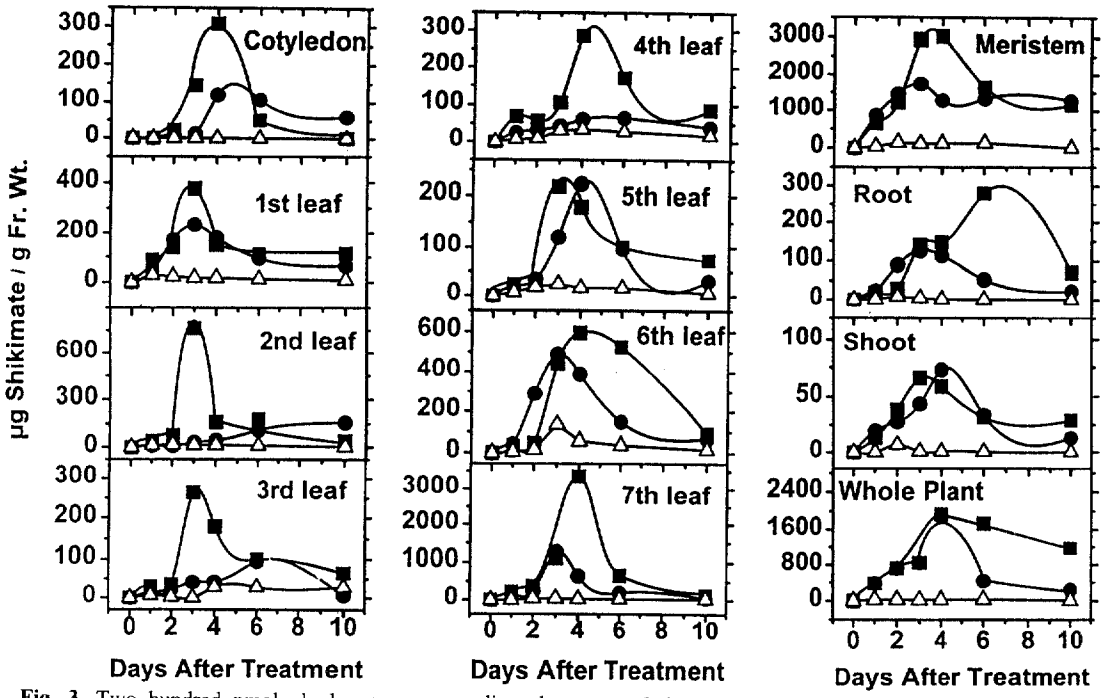

Fig. 3. Two hundred nmol glyphosate were applicated to one of the middlle lobe of the first old leaf (-■-) or the second old leaf (-●-), or sprayed to whole tomato plants (-△-). The methanolic extracts from each plant part were analyzed for shikimic acid content by HPLC. All values are the mean of three independent experiments and three repeatedly measurements (n=6).

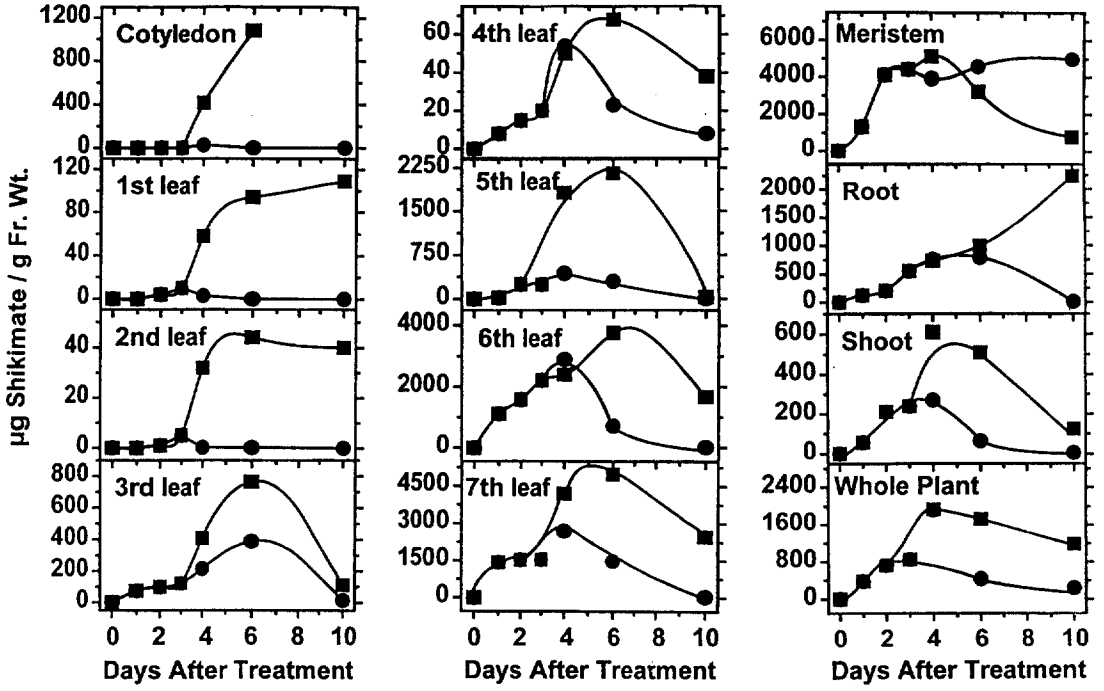

Fig. 4. Two hundred nmol glyphosate were applicated to one of the middlle lobe of the third old leaf (-■-), and after further 3days twice to the other middle lobe of same leaf (-●-). The methanolic extracts from each plant part were analyzed for shikimic acid content by HPLC. All values are the mean of three independent experiments and three repeatedly measurements (n=6).

-D-arabino-heptulosonate 7-phosphate synthase is induced by glyphosate in cultured potato cells. It may be assumed from our result and their data that the excessive flow of carbon into the shikimic acid pathway can be continuously progressed at least for 4 days after applied(Fig. 3 and 4). However, it was shown to be a difference of the accumulation rate of shikimate between the leaves sprayed in whole plant and the leaves applied to the middle lobe of the assimilate expoting leaves (Fig. 3 and 4), implying that glyphosate is absorbed more effectively in the assimilate exporting leaves in young growing region, and transported into the apical region of shoot and root as following a source-sink gradient as previously reported by Schulz et al.[15] In real, this result has an important mean in practical application to field work and weed control. Namely, the tissue-specific treatment of glyphosate (i.e. an assimilate exporting leaf, old leaf) may be more effective than the whole surface treatment if it is sprayed.

In meristematic leaves including 6th and 7th old leaves, the levels of shikimic acid are a mg range on the basis of g. Fr. Wt. The accumulating levels in apical regions are also considerably different between the applied leaves. When glyphosate is applied to the third old leaf, the level is hightest, implying that the third old leaf retains a most active assimilate exporting capacity. After applied twice to a middle lobe of third oldest leaf, the more shikimate was accumulated in the relative young growing region and meristem, suggesting that the the assimilate(glyphosate) exproting and importing(apical region) leaves had yet a viability. This may paradoxically mean that the herbicidal mode of action of glyphosate is limited only in young growing region.

Our long term quantitative analysis of shikimic acid shows also that the accumulated shikimic acid is reduced in meristematic young growing region accompanied by a chlorophyll degradation

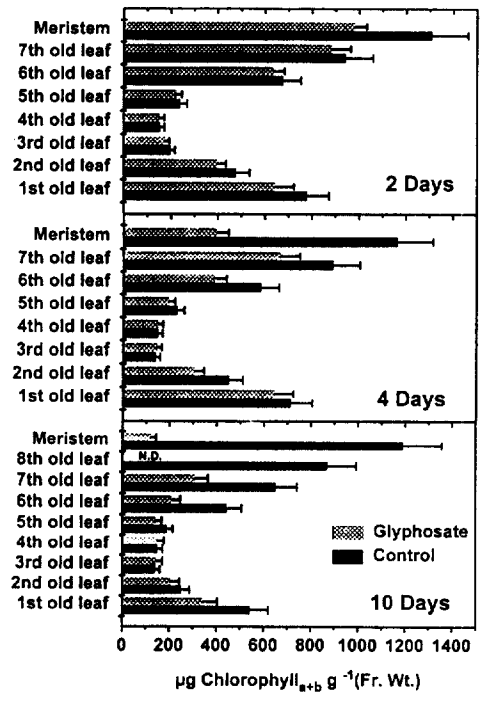

Fig. 5. The changes in chlorophyll (a+b) content after the application of 200 nmol glyphosate onto one of the middle lobe of the third old leaf. Chlorophyll is extracted and quantified by the previous method[1].

from 6 to 10 days after the application of glyphosate(Fig. 5). The leaf chlorosis occurs not within 48 h after the application of glyphosate. Four days after the application onto the middle lobe of third old leaf, the chlorophyll degradation of the apical leaves affected by glyphosate (6th, 7th, and apical leaves) is profoundly progressed. The control tomato plant developed the eighth leaf and the ninth leaf primordia while the leaf primordia of the tomato plant applied with glyphosate stopped the cell expension. Although the application time was different, such observation in ultrastructural level was precisely reported by Mollenhauer et al.[11] The total chlorophyll(a+b) content in the sixth and seventh leaves is strongly reduced below 50% to 70% compared to the control leaves. The content in

meristematic leaf decreases more than 20 fold. The degradation of chlorophyll a is more rapidly proceeded(data not shown). However, in the leaves unaffected by glyphosate in which shikimic acid is relatively not accumulated, the chlorophyll levels are not changed. Although there are no direct evidence that chlorophyll degradation is a primary effect of glyphosate, It could be confirmed from that gylphosate has a secondary or tertiary effect on the biosynthesis or degradation of chlorophyll.[9] Munoz-Rueda et al.[13] and Servaites et al.[16] indicated that the decrease of PS II activity by glyphosate can directly be related with chlorophyll degradation which is a cause of the lethal effects of glyphosate.

In base on present study, it will have to be revealed how the shikimic acid accumulated for 4 to 5 days after application of glyphosate can rapidly decraese from 5 to 10 days during which it may be a period of the last pleiotropic lethal effects of glyphosate. Furthermore, our next report will be works on the protein turnover in chloroplast and thylakoid membrane in relation to the glyphosate target enzyme EPSP synthase.

摘 要

Glyphosate (N-[phosphonomethyl]glycine)에 의한 植物體의 被害樣相을 알아보기 위하여 토마토 (*Lycopersicon esculentum* Mil)를 대상으로 하여 同化部位에 部分處理하거나 全 植物體에 噴霧處理하였다. Glyphosate는 처리 24時間 이내에 shikimic acid의 급속한 體內 蓄積을 誘導하였다. Shikimic acid의 蓄積은 頂端葉의 分裂組織에서 葉綠素의 減少를 同伴하였다. 이때 나타나는 黃化현상은 생장하는 어린잎의 頂端組織에서 向頂性인 現象이었다. 葉綠素의 減少는 glyphosate의 二次效果 내지 三次效果인 것으로 보인다. 그렇지만 蓄積된 shikimic acid의 減少는 처리 5일째부터 정단엽과 뿌리를 제외하고는 減少하였다. Shikimic acid의 蓄積 정도는 處理된 部位에 따라 매우 다르게 나타났으며, paraquat를 處理한 下位 3葉에서는 3일 後에 토마토의 頂端分裂組織에서 shikimic acid의 水準이 가장 높게 나타났다.

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