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Herbicidal Effect of 5-Aminolevulinic Acid, a Biodegradable Photodynamic Substance

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Abstract : Laboratory and greenhouse experiments were conducted to determine the herbicidal effect of two types of δ-aminolevulinic acid (ALA), microbiologically-produced ALA (Bio-ALA) and synthetically produced ALA (Synthetic-ALA), on plant growth and chlorophyll content of Chinese cabbage. ALA effect on early plant growth was greatly concentration dependant, showing significant inhibition at higher concentrations. Both pre- and post-emergence application of ALA exhibited significant degree of photodynamic phytotoxicity. Older plants with many leaves were more tolerant to ALA than younger plants, showing less injury. No significant difference in herbicidal activity of two types of ALA, Bio-ALA and Synthetic-ALA, on plant height and chlorophyll content of Chinese cabbage was observed. However, residual biological activity and physico-chemical properties of Synthetic-ALA were more stable than those of Bio-ALA. Our results suggest that ALA had herbicidal potential with both pre- and post-emergence application, and that the chemical may be a valuable mean of eco-friendly weed control based on natural microbial substance.(Received February 1, 2007; accepted March 16, 2007)

Key words : δ-aminolevulinic acid, photodynamic, herbicidal potential, residual phytotoxic effect, eco-friendly weed control.

INTRODUCTION

Porphyrin compounds play an essential role in plant metabolism. The porphyrin ring structure is derived from 5-aminolevulinic acid (ALA). In plants, algae, and a few bacteria, ALA is formed from the five-carbon skeleton of glutamate in unit of the C5 pathway (Beale, 1978; Wettstein *et al.*, 1995). This pathway utilizes glutamyl-tRNA synthetase, glutamyl-tRNA hydrogenase, and glutamate-1-semi-aldehyde aminotransferase to carry out three sequential enzymatic reactions that produce ALA from glutamate (Beal and Castelfranco, 1974). In the four-carbon (C4) pathway, which is present in animals and microorganisms, ALA is formed by the enzyme 5-aminolevulinic acid synthetase (ALAS), which catalyzes the pyridoxal phosphate-dependent condensation of succinyl-coenzyme A (succinyl-CoA) (Avissar *et al.*, 1989). A few microorganisms have both C4 and C5 pathways, as is distinct in *Euglena gracilis* (Weinstein and Beale, 1983).

ALA is very expensive because it is usually synthesized chemically via complex processes. In contrast, microbiological production of ALA involves simple reactions. Therefore, biological production using microorganisms has been suggested as an inexpensive way to produce ALA. Sasaki *et al.* (1987) observed the extracellular accumulation of ALA by *Rhodobacter sphaeroides* up to 16 mM at neutral pH with levulinic acid addition using a volatile fatty acids medium. Another approach to achieving ALA production is by metabolic pathway engineering. Extracellular accumulation of ALA by an *E. coli* overexpressing ALA synthase was achieved by inserting a *hemeA* gene from *Bradyrhizobium japonicum* and expressed under the control of T7 promoter (Choi *et al.*, 1999).

The biosynthesis of porphyrin is tightly regulated at several levels to coordinate apoprotein synthesis with

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cofactor availability and to avoid the accumulation of the intermediates, protoporphyrin IX (Proto IX) and protochlorophyllide (Pchlide), which are photosensitive to light, generating reactive oxygen species, at the stage preceding chlorophyll(Chl) biosynthesis (Papenbrock and Grimm, 2001). Plants suffer severe photodynamic damage if these control mechanisms are circumvented, e.g., by feeding early intermediates like ALA or by the action of protoporphyrinogen IX oxidase (Protox)-inhibiting herbicides, producing an accumulation of excess Proto IX (Menon *et al.*, 1989; Böger and Wakabayashi,1999; Mock *et al.*, 2002). The damage is accompanied by the destruction of photosynthetic reactions and is irreversible. When ALA-treated plants are exposed to sunlight, excess tetrapyrroles absorb the energy that is normally used for photochemical reactions and use it instead to photosensitize the production of $^1O_2$ (Hopf and Whitten, 1978; Tripathy and Chakraborty, 1991). $^1O_2$ oxidizes unsaturated membrane lipids, generating free radicals, which damage the membrane system and lead to the death of the plant. Therefore, ALA has been proposed as a selective and biodegradable herbicide and insecticide (Rebeiz *et al.*, 1984 and 1988b). However, no study on residual effects of ALA in soil or soil fractions has been reported.

In addition, ALA has agricultural applications as a growth-promoting factor (Sasaki *et al.*, 1998) and as an agent to confer salt and cold tolerance to plants (Kuramochi *et al.*, 1997; Hotta and Watanabe, 1999). ALA at low concentrations elicited 10-60% promotive effects on the growth and yield of several crops and vegetables, including radish, kidney beans, barley, potatoes, and garlic (Hotta *et al.*, 1997). Cotton seedlings treated with ALA were able to grow in soil containing 1.5% NaCl (Kuramochi *et al.*, 1997); ALA application increased the survival of rice plants at 5°C by 40-50% (Hotta and Watanabe, 1999). Further agricultural applications of ALA, including color-intensification of apples, nitrate reduction of vegetables, and green-color maintenance of grass, have been reported (Hotta and Watanabe, 1999).

ALA was named as tetrapyrrole-dependent photodynamic herbicides (TDPH) that force green plants to accumulate undesirable amount of metabolic intermediates (protoporphyrin IX) of the chlorophyll and heme metabolic pathway in darkness, namely tetrapyrrole (Rebeiz *et al.*, 1990) or as a 'laser' herbicide that is photodynamic (Rebeiz *et al.*, 1984). Under the light, the accumulated tetrapyrroles photosensitize the formation of singlet oxygen that kills the treated plants by oxidation of their cellular membranes as like diphenyl ether (DPE) herbicides. A variety of DPE herbicides such as acifluorfen-methyl, oxadiazone, and oxyfluorfen cause rapid peroxidative photobleaching and desiccation of green plant tissues (Duke *et al.*, 1991; Scalla and Matringe, 1994). The target site of action of these herbicides has been well known to be protoporphyrinogen oxidase (Protox), which catalyzes the oxidation of protoporphyrinogen IX (Protgen IX) to protoporphyrin IX (Proto IX), in the biosynthesis of hemes and chlorophylls (Duke *et al.*, 1991; Beale and Weinstein, 1990).

The present study was conducted to determine residual herbicidal activity of ALA on cabbage through pre- and post-emergence applications. The fundamental study would be useful for development of ALA as a new bioherbicide that is biodegradable, environmentally sound, and safe to human, animals and crops.

MATERIALS AND METHODS

Chemicals

ALA produced by overexpressing the *hemA* gene isolated from *Bradyrhizobium japonicum* (Choi *et al.*, 1999) was provided by Envirogen Co., Korea (Bio-ALA). ALA production by this method was around 30 mM. To compare the biological activity with Bio-ALA, synthetically-produced ALA (synthetic-ALA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Concentration Responses of Bio- and Synthetic-ALAs on Chinese Cabbage

Chinese cabbage was used for testing the difference of biological activity on between Bio-ALA and Synthetic -ALA. Stock solution of two kinds of ALA was diluted with distilled water to give final concentrations ranged from 10^{-8} to 10^{-3}M. Four milliliters of each

diluted solution was pipetted into the petri dishes with Whatman No. 2 filter paper. The distilled water was used as the control. Twenty seeds of Chinese cabbage were evenly placed on filter paper wetted with the ALA solution in each petri dish. The petri dishes were covered, sealed by wrapping with parafilm, and placed flat in a growth chamber maintained at 24°C during the14-h light period and 22°C during the 10-h dark period. Plates were illuminated with 180 μmol photons m^{-2} s^{-1} PAR provided by a mixture of incandescent and fluorescent lamps. Shoot length and chlorophyll content (Lichtenthaler, 1987) were measured on all seedlings in each petri dish at 6 days after incubation.

Response of Seedling Ages to ALA

Growth conditions were the same as those described in the previous section. Two types of ALAs, Bio-ALA and Synthetic-ALA, at 2 mM mixed with Tween 80 were foliar applied at four different growth stages; 2, 3, 4, and 5 leaf stages of Chinese cabbage grown in pot (40 × 60 × 12 cm) filled with silt-loam soil. A 15 ml of ALA solution was applied with handy sprayer at 6:00 PM. After application, post-spray dark incubation period was kept for 14 hrs, and next morning exposed to the natural sunlight ranged from 1000 to 1500 μmol photons m^{-2} s^{-1} to elicit photodynamic damage. Fresh weight was measured on all seedlings 7 days after exposure to sunlight.

Residual Effect of ALA on Seedling Growth and Chlorophyll Content

To determine the persistence of Synthetic-ALA performance when applied into soil surface, residual amount and herbicidal activity of ALA were measured. Growth conditions were the same as those described above. Aqueous solutions of 0, 4, 8, and 12 mM ALA were prepared for the experiment.

Pot (40 × 60 × 12 cm) was filled with 4 kg silt-loam soil, and added with 4 L-ALA solution of each concentration. Seeds of Chinese cabbage were planted on the pot at 5-day interval for 20 days.

Fresh weight and chlorophyll content of Chinese cabbage were measured on all seedlings 10 days after planting.

Change in Stability and Content of Bio- and Synthetic-ALA

To determine stability of 16 mM-ALA stored at room temperature for 150 days, two types of ALA were analyzed by Lermontova and Grimm method (2000) at 50-day interval. To 10 μL of supernatant, 0.5 mL of 1 M sodium acetate buffer (pH 4.7), 0.5 mL of D.W., and 50 μL of acetylacetone (2,4-petanedione) were added, and then tubes were kept in boiling water for 15 min. After cooling, 3 ml of freshly prepared modified Ehrlich's reagent (1g *p*-dimethylaminobenzaldehyde, 30 ml glacial acetic acid, 8 mL 70% (v/v) perchloric acid and 12 mL acetic acid) was added. The A553 of the mixture was measured after 15 min at room temperature. In addition, biological activity of Bio- and Synthetic-ALAs stored at room temperature was assayed on filter paper treated with 2-mM at 50-day interval for 150 days. Soil samples applied with ALA aqueous solutions of 0, 4, 8, and 12 mM were analyzed at 5-day interval for 20 days.

RESULTS AND DISCUSSION

Concentration Responses of Bio- and Synthetic-ALAs on Chinese Cabbage

ALA at 10^{-3}M treated with pre-emergence application significantly reduced shoot growth of Chinese cabbage. Bio- and Synthetic-ALAs at 10^{-3}M apparently reduced plant height of Chinese cabbage by 63 and 60%, respectively, compared with control (Fig. 1). No significant differences in herbicidal activity between two types of ALA on plant height of Chinese cabbage were observed. However, lower concentrations of ALA did not affect shoot growth of Chinese cabbage. In an earlier study, Chon (2003) reported that cotyledons of Chinese cabbage were severely bleached at 0.5 mM of ALA 24 hrs after application, and the root growth was significantly inhibited with increase of concentration when applied with pre-emergence. Kuk *et al.* (2003) also found that no significant differences in biological activity between bio-ALA and synthetic ALA on barley, wheat, rice, and weed, *Ixeris dentate* tested were observed.

Chinese cabbage cotyledones treated with ALA of 10^{-3} M were completely bleached within 24 h after light

exposure (Data not shown). Bio- and Synthetic-ALAs at 10^{-3} M reduced chlorophyll contents of Chinese cabbage by 79 and 86%, respectively, compared with control. However, lower concentrations of ALA did not affect chlorophyll content of Chinese cabbage, showing even more chlorophyll synthesis than control (Fig. 1).

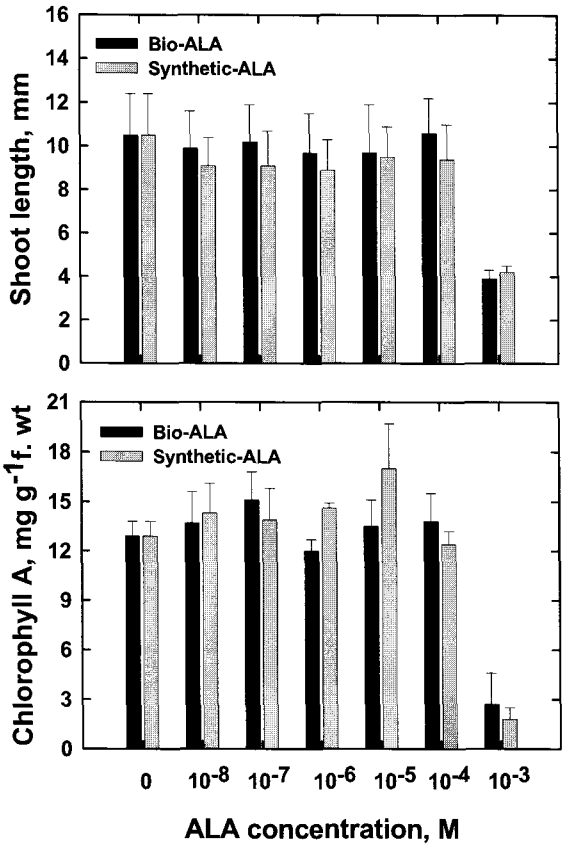

Fig. 1. Effects of Bio- and Synthetic-ALA on shoot length (upper) and chlorophyll content (bottom) of Chinese cabbage at 6 days after pre-emergence application.

ALA is not harmful to crops, animals, and humans, and used as a prodrug for photodynamic diagnosis and therapy of cancer (Schuimaker *et al.*, 1999). However, Synthetic-ALA is very expensive because it is usually synthesized chemically via complex processes. Therefore, biological production using microorganisms has been suggested as a less expensive way to produce ALA.

Response of Seedling Age to ALA

Difference in selectivity among seedling ages to ALA was examined in greenhouse experiment. ALA at 2 mM reduced shoot fresh weights of Chinese cabbage with 2-, 3-, 4-, and 5-leaf stage by 57-62, 51-52, 40-41, and 13-25%, respectively. The results showed that older plants were less affected by ALA than younger plants, showing less injury. However, no significant difference in herbicidal activity between two types of ALA on shoot fresh weight of Chinese cabbage was observed (Fig. 2). Rebeiz *et al.* (1983) suggested that photodynamic herbicides exhibit a very pronounced organ, age, and species-dependent selectivity. Chon (2003) reported that post-emergence application of ALA exhibited the greatest photodynamic activity against test plants, and that Chinese cabbage was the most susceptible to ALA. Symptoms of photodynamic injury within the first 1 hour after exposure to light after post-spray dark incubation period for 15hrs became apparent. Initial symptoms appeared on green foliage of susceptible plants as isolated bleached spots contiguous. Bleaching was accompanied by severe loss of turgidity followed by desiccation. Within 24 hrs the green plant tissue turned into a brownish desiccated mass of dead tissue (Data not shown).

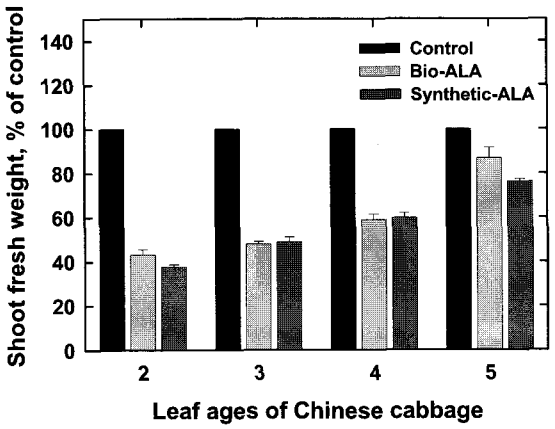

Fig. 2. Effects of Bio- and Synthetic-ALA on fresh weight of Chinese cabbage with different leaf ages 6 days after application.

Residual Effect of ALA on Seedling Growth of Chinese Cabbage

Various solutions of ALA ranged from 4 to 12 mM were soil-applied. Chinese cabbage seeds were planted on the soil at 5-day interval. At 10 days after ALA application, shoot fresh weight of Chinese cabbage investigated was significantly reduced up to 5 days after application regardless of application concentration. At 0

day after soil application, ALA of 4, 8, and 12 mM reduced shoot growth of Chinese cabbage by 49, 70, and 79%, respectively, compared with control. However, from 5 to 20 days after soil application, ALA applied did not affect shoot growth of Chinese cabbage (Fig. 3). On the other hand, chlorophyll content of Chinese cabbage was significantly affected by ALA with increasing of ALA concentration, until 10 days after application. However, at 15 days after application, chlorophyll content of Chinese cabbage was not affected by ALA (Fig. 3).

The physiological actions of ALA at high concentrations suggests that ALA increases the levels of porphyrin intermediate such as protochlorophyllide, protoporphyrin IX, and Mg-protoporphyrin IX abnormally, and the accumulated tetrapyrroles act as a photo-sensitizer for the formation of singlet oxygen triggering photodynamic damage (Askira *et al.*, 1991; Rebeiz *et al.*, 1984). Thus, in other study the selectivity among plant species would be based on tetrapyrrole accumulating capabilities and the tetrapyrrole metabolism in various plant species (Rebeiz *et al.*, 1988a).

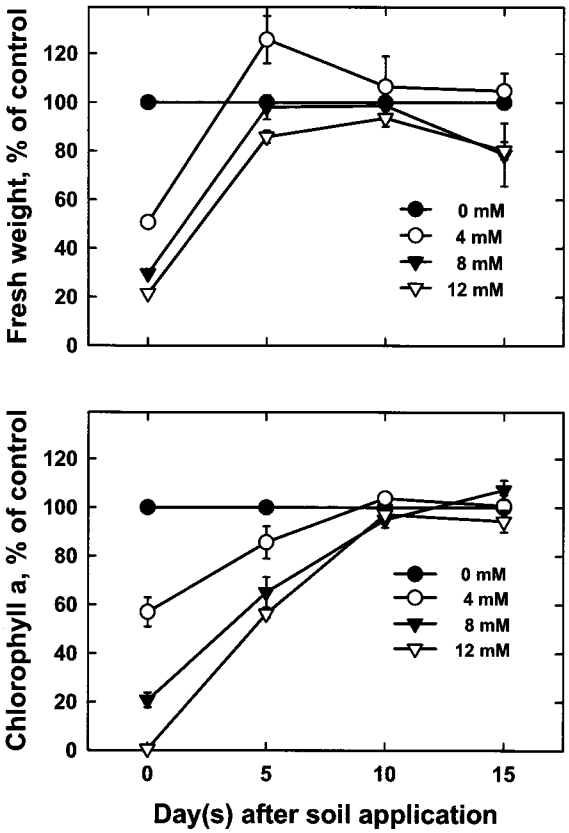

Fig. 3. Residual effects of ALA on fresh weight (upper) and chlorophyll content (bottom) of Chinese cabbage.

Change in Stability and Content of Bio- and Synthetic-ALA

Bio-ALA was less stability in chemical properties than was Synthetic-ALA. At 150 days after storing, Bio-ALA concentration was reduced by 13% comparing with original ALA concentration 16 mM. On the other hand, Synthetic-ALA concentration was not affected under the same condition, showing more stability (Fig. 4). Biological activity of Bio-ALA was gradually reduced by 18% 150 days after storing, comparing with original ALA concentration. Bio-ALA concentration was more reduced than was Synthetic-ALA, showing less persistence in biologically-produced ALA (Fig. 4). These results indicate that variations in stability and activity persistence of ALA exhibited, and that appropriate technologies could be developed for making industrial production technically feasible.

On the other hand, soil samples applied with various solutions of ALA ranged from 4 to 12 mM were collected at 5-day interval to analyze ALA content. The results showed that at 0-days after soil application, more ALA content in the soil was detected with increasing of ALA treatment concentration. However, ALA in the soil was not detected 5 days treatment, indicating that ALA applied in soil could leach into downward or adsorb to organic matters. These findings could be correlated with the results of residual biological activity of ALA (Fig. 5). However, further investigations also needed to elucidate the fate of the ALA in soil.

In conclusion, ALA has been proposed as a tetrapyrrole-dependent photodynamic herbicide and biodegradable plant growth enhancer, by the action of the proto-porphyrinogen IX oxidase (Protox IX). Effect of ALA on early plant growth was greatly concentration dependant, showing significant inhibition at higher concentrations. The present study was conducted to determine residual phytotoxic effects of ALA, a biode-gradable herbicidal substance under different conditions. No significant difference in herbicidal activity of two types of ALA, Bio-ALA and Synthetic-ALA on plant height and chlorophyll content of Chinese cabbage was observed. Both pre- and post-emergence application of

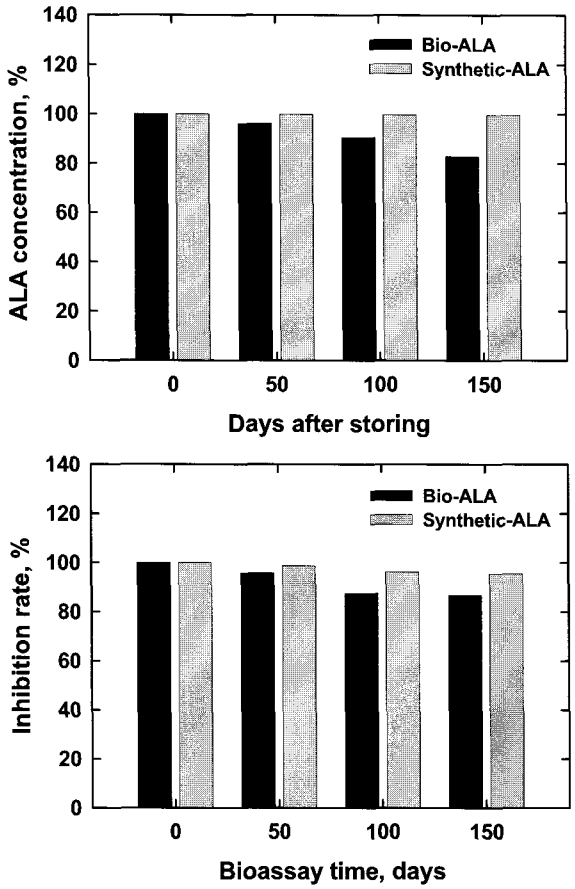

Fig. 4. Change in concentration (upper) and herbicidal activity (bottom) of Bio- and Synthetic-ALA solutions by time. Effect of herbicidal activity on alfalfa was conducted every 50 days after seeding on filter paper treated with 2-mM ALA.

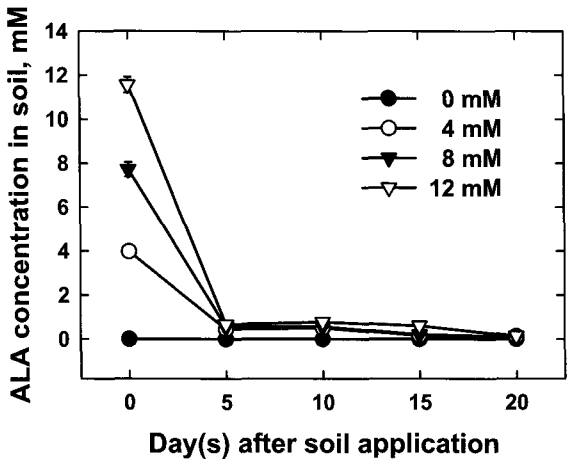

Fig. 5. Change in concentration of ALA in the soil at 0, 5, 10, 15, and 20 days after soil application.

ALA exhibited significant degree of photodynamic phytotoxicity. Older plants with many leaves were less affected by ALA than younger plants, showing less injury. However, residual biological activity and physico-chemical properties of Synthetic-ALA exhibited more stably than those of Bio-ALA. With appropriate technologies, the variations in stability and activity persistence of ALA should be improved for making industrial production technically feasible. Our results suggest that ALA had herbicidal potential with both pre- and post-emergence application, and that the chemical may be a valuable means of eco-friendly weed control based on natural substances.

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생분해성 광활성 물질 5-aminolevulinic acid의 제초활성

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요약 : 미생물학적으로 생산된 것과 합성된 2종류의 δ-aminolevulinic acid(ALA)가 배추의 생장과 엽록소 함량에 미치는 제초활성을 탐색하기 위해 실험실 및 온실시험을 수행하였다. 초기 유묘의 생장에 미치는 ALA의 효과는 농도에 비례하여 반응을 보이며 높은 농도에서 유의적인 억제효과를 보였다. 발생 전 및 발생 후 처리된 ALA는 유의적인 정도의 광활성을 나타냈다. 많은 잎을 가진 엽령의 유묘는 적은 엽령의 유묘보다 ALA에 대해 더 내성을 보였다. 미생물학적으로 생산된 것과 합성된 ALA간의 제초효과에 있어서 유의적인 차이는 인정되지 않았다. 그러나 합성된 ALA의 잔류성과 물리화학적 특성은 미생물학적으로 생산된 ALA의 그것 보다 더 안정적이었다. 따라서 잡초발생 전후 처리한 ALA의 제초활성이 인정되었으며 그 화합물은 천연 미생물 제제로 가치 있는 친환경 잡초방제 수단이 될 것으로 사료되었다.

색인어 : δ-aminolevulinic acid, 광활성, 제초활성, 잔류독성, 친환경 잡초 방제

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