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Phytotoxic Effect of 5-Aminolevulinic Acid, a Biodegradable Photodynamic Biomaterial, on Rice and Barnyardgrass

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ABSTRACT: ALA (5-aminolevulinic acid) has been proposed as a tetrapyrrole-dependent photodynamic herbicide by the action of the protoporphyrinogen IX oxidase (Protox IX). A study was conducted to determine photodynamic herbicidal effect of ALA on seedling growth of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa crus-galli* Beauv. var. *oryzicola* Ohwi) under dry and wet conditions. ALA effect on early plant growth of rice and barnyardgrass was greatly concentration dependant, suggesting that it promotes plant growth at very low concentration and inhibits at high concentration. No significant difference in herbicidal activity of biologically and synthetically produced ALAs on plant lengths of test plants was observed. ALA exhibited significant photodynamic activity regardless of PSDIP and its duration. Significant shoot growth inhibition by ALA soaking treatment exhibited apparently, indicating that ALA absorbed through root system was translocated into shoot part of plants. ALA reduced plant heights of rice and barnyardgrass seedlings by 6% and 27%, respectively, showing more tolerant to ALA in rice under wet condition. Leaf thickness was reduced markedly by ALA with increasing of ALA concentration, due to mainly membrane destruction and severe loss of turgidity in mesophyll cells, although the epidermal was little affected. It was observed that photodynamic herbicidal activity of ALA applied by pre-and post-emergence application exhibited differently on plant species, and that the activity of ALA against susceptible plants was highly correlated with growing condition.

Key Words: Eco-friendly weed management, 5-aminolevulinic acid, rice, barnyardgrass, photodynamic herbicidal potential, growing condition, leaf morphology

INTRODUCTION

Porphyrin compounds play an essential role in plant metabolism. The porphyrin ring system is derived from 5-aminolevulinic acid (ALA). δ-Aminolevulinic acid (ALA) has been well known as an intermediate for the biosynthesis of tetrapyrroles such as chlorophyll, heme, bacteriochlorophyll, and vitamin B_{12} analogues[1] in human, plants, animals and microorganisms. In plants, algae and a few bacteria, ALA is formed from the five-carbon skeleton of glutamate in unit of the C5 pathway[2,3]. 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[4]. 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)[5]. A few microorganisms have both C4 and C5 pathways, as is distinct in *Euglena gracilis*[6]. ALA is very expensive because it is usually synthesized chemically via complex processes. Therefore, biological production using microorganisms has been suggested as an inexpensive way to produce ALA. It has been well known that photosynthetic Rhodospirillaceae such as mainly *Rhodospirillum*, *Rhodobacter*, and *Rhodopseudomonas* species can extracellularly excrete ALA[7].

Bradyrhizobium japonicum is a family of rhizobia that form symbiotic root nodules on leguminous plants[8].

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In *B. japonicum*, ALA is formed by 5-aminolevulinic acid synthase (ALA-S), which catalyzes the condensation of glycine and succinyl-CoA, together with the release of carbon dioxide and CoA, and requires the essential cofactor pyridoxal 5-phosphate9). The ALA-S gene of *B. japonicum* was cloned in 1987 and identified as *hemA*, which encodes a 409-amino acid protein with a molecular weight of 44,59910). ALA-S is a rate-limiting enzyme for tetrapyrrole biosynthesis in bacteria, and synthesis of this enzyme is itself highly regulated11) via feedback regulation of the *hemA* or *hemT* gene12,13).

The biosynthesis of porphyrin is tightly regulated at several levels to coordinate apoprotein synthesis with 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) biosynthesis14). 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 IX15-17). 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$18,19). $^1O_2$ 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 herbicide20,21).

ALA was proposed as a tetrapyrrole-dependent photodynamic herbicide (TDPH) that force green plants to accumulate undesirable amount of metabolic intermediates (protoporphyrin IX) of the chlorophyll and heme metabolic pathway in darkness, namely tetrapyrrole22) or as a 'laser' herbicide that is photodynamic20). 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 tissues23). The target site of action of these herbicides has been well known to be protoporphyrinogen oxidase (Protox), which catalyzes the oxidation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX), in the biosynthesis of hemes and chlorophylls4,23).

The present study was conducted to determine photodynamic herbicidal effect of ALA on seedling growth of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa crus-galli* Beauv. var. *oryzicola* Ohwi) under different cropping conditions. The fundamental assessment would be useful for development of ALA as a new biodegradable herbicide that is environmentally sound and safe to human, animals and crops.

MATERIALS AND METHODS

Chemicals

ALA produced by overexpressing the *hemA* gene isolated from *Bradyrhizobium japonicum*24) was provided by Environgen Co., Korea (Bio-ALA). Extracellular accumulation of ALA by an *E. coli* overexpressing ALA synthase was achieved by inserting a *hemA* gene from *Bradyhizobium japonicum* and expressed under the control of T7 promoter24). ALA amount produced by this method was up to 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).

Herbicidal Activity of Bio-ALA and Synthetic-ALA

Rice and barnyardgrass was tested for the difference of biological activity on between Bio-ALA and Synthetic -ALA through pre-emergence application. Two types of ALA stock solutions were 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 a 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 the 14-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. Root lengths of two plants were measured on all seedlings in each petri dish at 6 days after ncubation. In all the present research, data collected ere analyzed using a variance (ANOVA) procedure in SAS program[25]. Treatment means were separated using Fisher's protected LSD (P=0.05).

Effect of Light on Root Soaking Treatment with ALA

Imbibed seeds of rice and barnyardgrass were seeded in small horticulture pot (10 x 10 x 5 cm) filled with silt-loam soil. The seedlings were grown for 15 days under greenhouse condition (28/21℃ day/night temperature) from July to September 2004. Fifteen-day-old seedlings of rice and barnyardgrass were soaked in various aqueous solutions of ALA at 0, 3, and 4 mM, and placed under three different light conditions, 1) light exposure after placing ALA-treated seedlings in darkness for 16 hrs at 25°C (with PSDIP; post-spray dark incubation period) to elicit photodynamic damage (DL), 2) without PSDIP, continuous light exposure (LL), and 3) placing ALA-treated seedlings in darkness for 24 hrs (DD). After light or dark treatment, all seedlings were continually placed under normal sunlight. Shoot fresh weights of rice and barnyardgrass were measured 10 days after application.

Response of Seedling Ages to ALA

Growth conditions were the same as those described in the previous section. ALA at 0, 5, 10, 15, and 20 mM mixed with Tween 80® was foliar applied to seedlings of rice and barnyardgrass at two different growth stages; 15 (1-2 leaf stage) and 35-day old seedlings (5-6 leaf stage). 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 16 hrs, and next morning exposed to the natural sunlight ranged from 1000 to 1500 μmol photons m^{-2} s^{-1} to elicit photodynamic damage. Shoot length and fresh weight were measured on all seedlings of two plant species 10 days after exposure to sunlight.

Herbicidal Activity of ALA under Dry and Flood Condition

Seeds of rice and barnyardgrass were planted in horticultural pots. The seeds for wet condition were germinated, and planted on flooded soil. ALA at 0, 5, 10, 15, and 20 mM mixed with Tween 80 was foliar applied to 10-day old seedlings of rice and barnyardgrass under dry and wet condition. Herbicide paraquat at 1 mM was used as a control chemical. A 15 ml of ALA and paraquat solutions 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 1,000 to 1,500 μmol photons m^{-2} s^{-1} to elicit photodynamic damage. Plant height and fresh weights were measured on all seedlings 20 days after exposure to sunlight.

Herbicidal Effect of ALA on Plant Morphology

The 2nd leaf blade from 10-day old seedlings of barnyardgrass applied with various ALA solutions were excised 8 mm long at 10 days after application, and fixed in FAA (Formalin : acetic acid : alcohol : water = 15 : 15 : 30 : 40, by volume) for 24 hours. The tissues were then dehydrated in a series of ethyl alcohol followed by infiltration with with xylene : paraplast (1 : 1) for 1 days, and changed into the pure paraplast for 5 days at 60℃. The tissues were embedded in paraplast for sectioning with a rotary microtome (Shandon, Southern Products Ltd., UK). The cross sections were cut 6 μm thick and the sections were stained with Fast-green. The microphotographs were enlarged along with a micrometer scale photographed under x 200 magnification cells (Olympus Optical Co. Ltd., Japan) for measuring the thickness of epidermal and mesophyll.

RESULTS AND DISCUSSION

Herbicidal Activity of Bio-and Synthetic ALA

Biological production using microorganisms has been suggested as a less expensive way to produce ALA. For this study, we used several concentrations from 30 mM of ALA accumulated by overexpressing the *hemA* gene isolated from *Bradyrhizobium japonicum*[24]. No significant difference in herbicidal activity of two types of ALA on root lengths of barnyard grass was observed (Fig. 1). Kuk et al[26]. and Chon[27], as similar reports, found that no significant difference in biological activity between bio-ALA and synthetic ALA on barley, wheat, rice, and weed, *Ixeris dentate* tested was observed. Even though the variations in stability and activity persistence of ALA were not confirmed, ALA produced by microorganisms should be developed by appropriate technologies for making industrial produc-

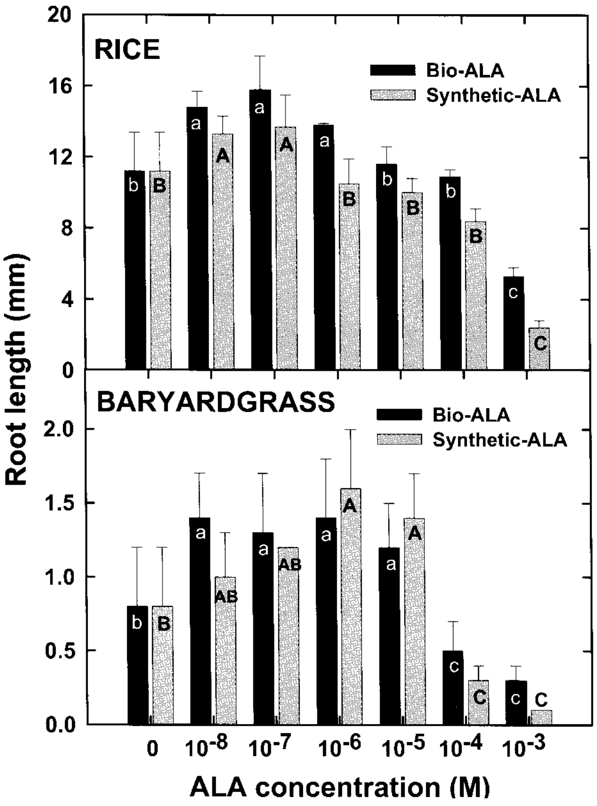

Fig. 1. Effect of bio-and synthetic-ALA on root length of rice (upper) and barnyardgrass (bottom) 6 days after pre -emergence application. Within an extract concentration, means followed by the same letter are not significantly different at $p < 0.05$. Each bar represents standard error of the mean.

tion technically feasible.

However, ALA concentrations ranged from 10^{-4} to 10^{-3}M significantly reduced root length of barnyardgrass, showing more susceptible to ALA than rice. On the other hand, root lengths of rice and barnyardgrass were greatly stimulated up to 19-41 and 26-62% over the control, respectively, by ALA ranged from 10^{-8} to 10^{-7} M. Several studies reported that ALA promotes the growth and yield of crops and vegetables at low concentrations by 10-60% over the control as observed in cases of crops of radish, kidney beans, barley, potatoes, garlic, rice, *Vigna* species, and corn [28,29]. These positive effects may be induced through the increased chlorophyll content, the enhanced photosynthetic activity, and the inhibition of respiration by ALA treatment[28].

Effect of Light on Root Soaking Treatment with ALA

To elucidate the degree of photodynamic effects of ALA on rice and barnyardgrass as affected by PSDIP (post-spray dark incubation period), ALA was applied to their roots by soaking at 0, 3, and 4 mM (Table 1). Shoot fresh weight of rice and barnyardgrass were significantly reduced at 10 days after soaking treatment with ALA solutions. ALA-treated seedlings with PSDIP to elicit photodynamic damage (DL) showed 18-25 and 51-55% inhibition in shoot growth of two

Table 1. Effect of ALA on plant height and fresh weight of rice and barnyardgrass under dry and wet conditions at 20 days after application

Treatment (mM)		Rice		Barnyardgrass	
		Dry	Wet	Dry	Wet
		Plant height (cm)			
ALA	0	64.7 (100)	55.3 (100)	61.8 (100)	66.2 (100)
	5	62.2 (96)	57.7 (104)	57.9 (94)	76.1 (115)
	10	37.8 (58)	56.9 (103)	48.7 (79)	56.5 (85)
	15	20.1 (31)	52.0 (94)	38.2 (62)	48.4 (73)
	20	0.0 (0)	43.8 (79)	0.0 (0)	47.2 (71)
Paraquat	0	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
		Fresh weight (g $plant^{-1}$)			
ALA	0	2.5 (100)	2.4 (100)	5.3 (100)	6.5 (100)
	5	1.7 (68)	2.4 (100)	3.5 (66)	6.3 (97)
	10	0.7 (28)	1.7 (71)	2.2 (42)	5.7 (88)
	15	0.3 (12)	1.6 (67)	1.6 (30)	3.7 (57)
	20	0.0 (0)	1.4 (58)	0.0 (0)	2.6 (40)
Paraquat	0	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)

* Values in parensesis represent % of untreated control.

plant species at 3 and 4 mM ALA, respectively. On the other hand, continuous light exposure (LL) to ALA-treated seedlings, without PSDIP, showed complete photodynamic damage. When ALA-treated seedlings placed in darkness for 24 hrs (DD) were exposed to light, the seedling growth was reduced by ALA. The results suggest that ALA exhibited significant photodynamic activity regardless of PSDIP, and that ALA absorbed by soaking through root system would be translocated into shoot part of plants. This contrasts with earlier reports that PSDIP could be required for photodynamic phytotoxicity[20,21].

Response of Seedling Age to ALA

Selectivity among seedling ages of rice and barnyard grass to ALA was examined in greenhouse experiment. Younger seedlings of rice and barnyardgrass with 1-2 leaf stage were more affected by ALA than older seedlings with 5-6 leaf stage (Fig. 3). ALA at 10-20 mM reduced plant heights of rice and barnyardgrass seedlings with 1-2 leaf stage by 31-41 and 53-69%, respectively, showing more tolerant rice to ALA. Especially, herbicide paraquat at 1 mM reduced shoot fresh weights of rice and barnyardgrass seedlings by 52 and 86%, respectively. However, shoot fresh weight of two plant species were more affected by ALA than plant height. On the other hand, at 5-6 leaf stage, ALA at 10 mM reduced plant heights of rice and barnyardgrass seedlings by 4 and 9%, respectively, showing less photodynamic damage than the seedlings with 1-2 leaf stage. ALA at 10 mM reduced shoot fresh weights of rice and barnyardgrass seedlings with 5-6 leaf stage by 22 and 29%, respectively. Rebeiz et al[30]. suggested that photodynamic herbicides exhibit a very pronounced organ, age, and species-dependent selectivity. 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 photosensitizer for the formation of singlet oxygen triggering photodynamic damage[20,31]. Thus, in our previous work, the selectivity among plant species would be based on tetrapyrrole accumulating capability and the tetrapyrrole metabolism in various plant species[21].

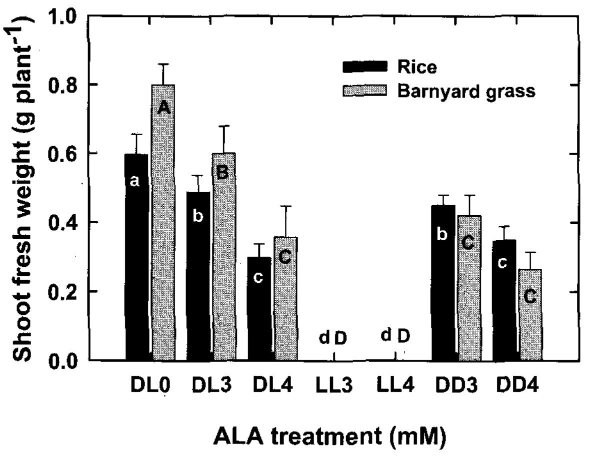

Fig. 2. Effect of root soaking application with ALA on shoot fresh weight of rice and barnyard grass 10 days after application. Within an extract concentration, means followed by the same letter are not significantly different at $p < 0.05$. Each bar represents standard error of the mean.

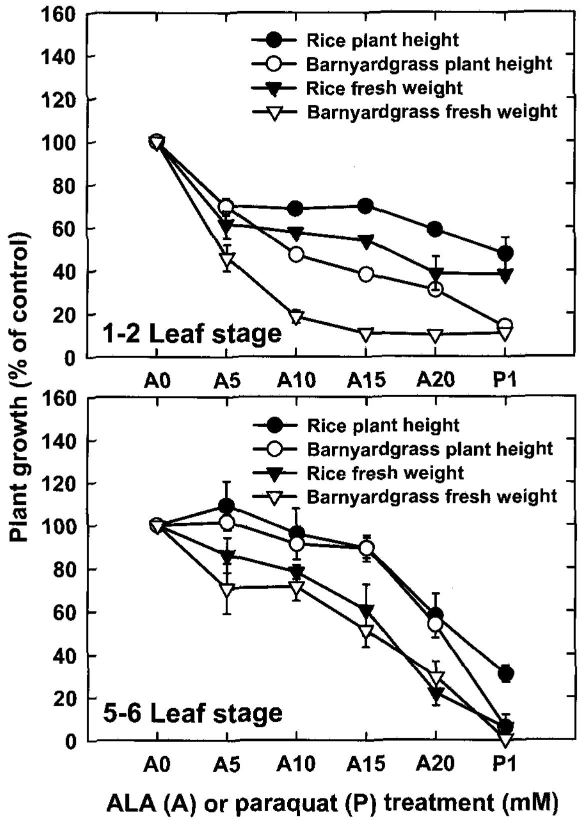

Fig. 3. Effects of post-emergence application of ALA on plant height and fresh weight of rice and barnyard grass at 1-2 leaf and 5-6 leaf stages 10 days after application. A0 to A20 represent ALA concentration at 0, 5, 10, 15 and 20 mM, and P1 paraquat at 1 mM.

Herbicidal Activity of ALA under Dry and Flood Condition

ALA significantly reduced plant height as well as shoot fresh weight of direct seeded rice and barnyardgrass seedlings under dry and wet conditions. Under dry seeding condition, rice seedlings were more sensitive to ALA than barnyardgrass. ALA at 10-15 mM reduced plant heights of rice and barnyardgrass seedlings by 42-69% and 21-38%, respectively, showing more susceptible in rice. However, under wet seeding condition, plant height of barnyardgrass seedlings was more inhibited by ALA than those of rice seedlings. ALA at 15 mM reduced plant heights of rice and barnyardgrass seedlings by 6% and 27%, respectively, showing more tolerant to ALA in rice. Fresh weights of rice and barnyardgrass seedlings were more reduced than plant heights. ALA at highest concentration 20 mM and paraquat at 1 mM reduced completely shoot growth of rice and barnyardgrass seedlings under dry growing condition.

Herbicidal Effect of ALA on Plant Morphology

Morphological symptoms of photodynamic injury within the first 1 hour after exposure to sunlight after PSDIP for 16 hrs 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 turns into a brownish desiccated mass of dead tissue. Greatly reduced chlorophyll contents from leaves of three plant species also were observed with increasing of ALA concentration (data not shown). Anatomical responses of barnyardgrass to ALA were investigated through microscopic study. Leaf thickness was reduced markedly by ALA due to destruction and severe loss of turgidity in mesophyll cells (Fig. 4), although the epidermal was little affected (Table 2). Thickness of mesophyll cells was reduced abnormally due to desiccation of cell contents. Rebeiz et al[22]. reported that breakage and nicking of the chloroplast membranes, plasmolyzed cells, and lost organells and memebrane structures also became apparent. An earlier study attributed these earlier symptoms to a loss of differential permeability of the tonoplast and seepage of vascular sap, rich in hydrolytic enzymes, into the cytoplasm[32].

In conclusion, ALA effect on early plant growth was greatly concentration dependant, suggesting that it promotes plant growth at very low concentration and inhibits at high concentration. No significant difference in herbicidal activity of two types of ALA on plant height and weight of test plants was observed. ALA exhibited significant photodynamic activity regardless of PSDIP and its duration. ALA absorbed by soaking through root system would be translocated into shoot part of plants, and significant growth inhibition and photodynamic effects were exhibited apparently. ALA reduced plant heights of rice and barnyar-

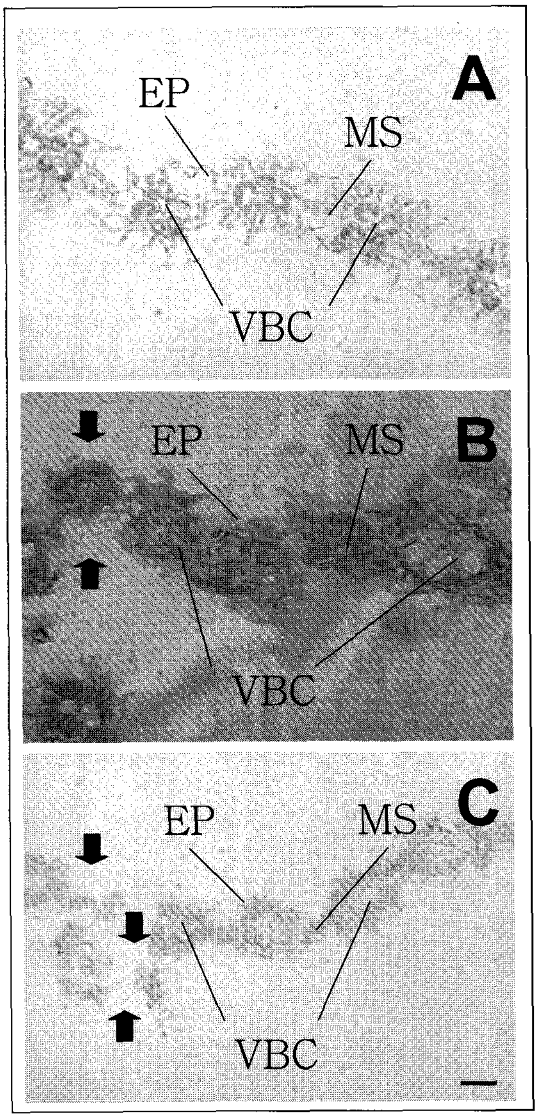

Fig. 4. Cross sections of the 2nd leaf blade of barnyardgrass treated with 0 (A), 4 (B), and 8 (C) mM of ALA 10 days after foliar application. EP: epidermal cell, MC: mesophyll cell, and VBC: vascular bundle sheath cell. Photograph is x 200 (bar size: 40 μm). Arrows represent phytotoxic damages and reduction in cell thickness due to destructing mesophyll cells and epidermal cells.

Table 2. Effect of ALA on the thickness of epidermal and mesophyll cells of barnyardgrass at 10 days after foliar application

ALA treatment (mM)	Cell thickness of barnyardgrass leaf blade (μm)		
	Epidermal	Mesophyll	Total
0	19.4 (100)	108.1 (100)	127.4 (100)
2	17.8 (92)	70.9 (66)	88.7 (70)
4	17.7 (91)	51.5 (48)	69.2 (54)
6	16.8 (87)	37.2 (34)	54.0 (42)
8	15.8 (81)	22.4 (21)	38.2 (30)

* Values in parensesis represent % of untreated control.

dgrass seedlings by 6% and 27%, respectively, showing more tolerant to ALA in rice under wet condition. Leaf thickness was reduced markedly by ALA with increasing of ALA concentration, due to mainly destruction and severe loss of turgidity in mesophyll cells, although the epidermal cells were little affected. The present study suggests 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.

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