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Ammonium accumulation is associated with senescence of rice leaves

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Abstract

The relationship between ammonium accumulation and senescence of detached rice leaves was investigated. Ammonium accumulation in detached rice leaves coincided closely with dark-induced senescence. Exogenous NH_4Cl and methionine sulfoximine, which caused an accumulation of ammonium in detached rice leaves, promoted senescence. Treatments such as light and benzyladenine, which retarded senescence, decreased ammonium level in detached rice leaves. Abscisic acid, which promoted senescence, increased ammonium level in detached rice leaves. The current results suggest that ammonium accumulation may be involved in regulating senescence. Evidence was presented to show that ammonium accumulated in detached rice leaves increases tissue sensitivity to ethylene. The accumulation of ammonium in detached rice leaves during dark-induced senescence is attributed to a decrease in glutamine synthetase activity and an increase in reduction of nitrate.

Abbreviations: ABA = abscisic acid; BA = benzyladenine; GOGAT = glutamate synthase; GS = glutamine synthetase; MDA = malondialdehyde; MSO = methionine sulfoximine; STS = silver thiosulfate

1. Introduction

Leaf senescence is an integral part of leaf development. It is, like other development processes, a well organized and regulated process rather than a passive decay of the leaves. Ammonium assimilation changes considerably with the onset of senescence [4]. Glutamine synthetase (GS, EC 6.3.1.2), the key enzyme in the generally recognized GS/GOGAT pathway, plays a crucial role in the assimilation of ammonium [14]. GS activity is known to decrease during either natural or dark-induced senescence of leaves [12, 18, 21, 24–26, 29]. Decline in GS activity in leaves during senescence may result, at least in part, in an accumulation of ammonium in leaves. In fact, various investigations have been able to show the accumulation of ammonium in leaves during senescence [21, 29]. Ammonium is considered to be toxic to plant cells [5]. Lauriere and Daussant [13] suggested that ammonium accumulation might be a factor contributing to senescence. However, no direct evidence has been provided to prove this suggestion. Ammonium has been demonstrated to accumulate after the onset of senescence of detached wheat leaves [18, 29]. Thus, emphasis in the present investigation was on the relationship between ammonium accumulation and senescence of detached rice leaves.

2. Materials and methods

Rice (*Oryza sativa* cv. Taichung Native 1) was cultured as previously described [10]. The apical 3-cm segments excised from the third leaves of 12-d-old seedlings were used. A group of 10 segments was floated in a Petri dish containing 10 mL of test solutions. Incubation was carried out at 27 °C in darkness or in the light (40 μmol m^{-2} s^{-1}).

For protein determination, leaf segments were homogenized in 50 mm sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 g for 20 min, and the supernatant liquids were used for determination of protein by the method of Bradford [3].

Protein level was expressed as mg^{-1} g^{-1} fresh weight. For ammonium determination, leaf segments were homogenized in 0.3 mM sulphuric aicd (pH 3.5). The homogenate was centrifuged for 10 min at 39,000 g and the supernatant was used for determination of ammonium by the method described previously [15]. Ammonium level was expressed as μmol g^{-1} fresh weight. For nitrate determination, leaf segments were homogenized with double distilled water. The homogenate was centrifuged for 25 min at 17,600 g. The supernatant was used for determination of nitrate by the method described by Hecht and Mohr [7]. Nitrate level was expressed as μmol g^{-1} fresh weight.

MDA was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer [6]. MDA is routinely used as an index of lipid peroxidation and was expressed as nmol g^{-1} fresh weight.

For extraction of GS, leaf segments were homogenized with 10 mM Tris-HCl buffer (pH 7.6, containing 1 mM mgCl2, 1mM EDTA and 10 mM 2-mercaptoethanol) in a chilled pestle and mortar. The homogenate was centrifuged at 15,000 g for 30 min and the resulting supernatant was used for determination of GS activity. The whole extraction procedure was carried out at 4 °C. GS was assayed by the method of Oak et al. [17]. One unit of GS activity is defined as 1 μmol L-glutamate-*r*-monohydroxamate formed per min.

A stock solution of STS was prepared by mixing equal volumes of 0.01 M $AgNO_3$ and 0.04 M $Na_2S_2O_3$ [16].

All experiments were repeated three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

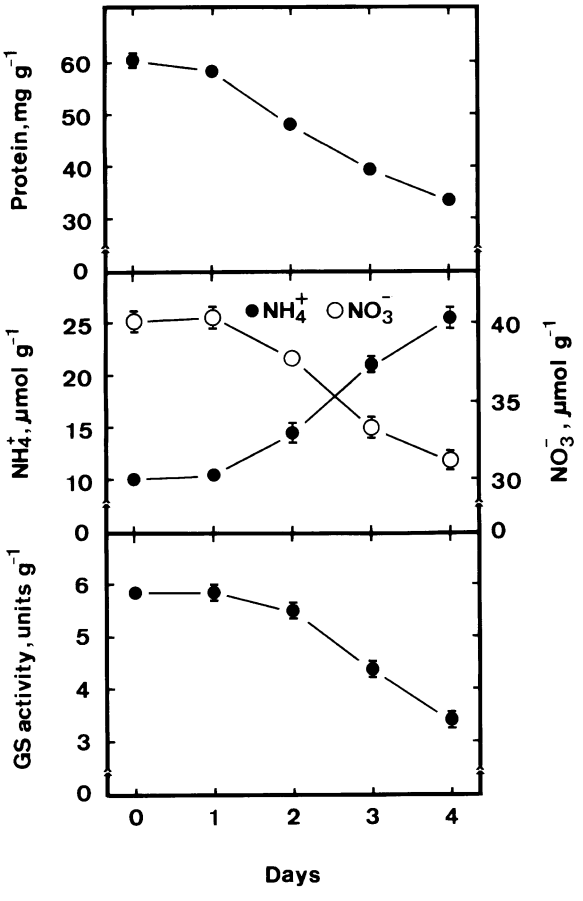

Figure 1. Protein, ammonium, and nitrate levels and GS activity in detached rice leaves during dark-induced senescence. Vertical bars represent standard errors (n=4).

3. Results

The senescence of detached rice leaves is characterized by a decrease in chlorophyll and protein levels [10]. The loss of chlorophyll and protein has been the principal criterion of leaf senescence for the largest number of workers [28]. Since protein loss in detached rice leaves is prior to chlorophyll loss [10], the senescence of detached rice leaves was monitored by the decrease of protein in the present study. Figure 1 shows the time courses of protein, ammonium, and nitrate levels and GS activity in detached rice leaves incubated in darkness. A decrease in protein level in detached leaves was evident 2 days after leaf detachment. Ammonium level in control leaves remained unchanged during the first day of dark incubation and increased subsequently. The increase in ammonium level coincided closely with the decrease in nitrate level and GS activity. Our results indicate that ammonium accumulation parallels the senescence of rice leaves under dark condition. Peeters and Van Laere [18] and Thomas [29] reported that ammonium accumulated after onset of leaf senescence.

It is clear from Figure 2 that light was effective in retarding senescence of detached rice leaves. Light treatment resulted in a lower ammonium level and a higher GS activity in detached rice leaves when compared with the dark control.

If ammonium accumulation plays a regulatory role in dark-induced senescence of detached rice leaves, it is expected that treatment of NH_4Cl would increase endogenous ammonium level and consequently promote senescence. As indicated in Figure 3, this is

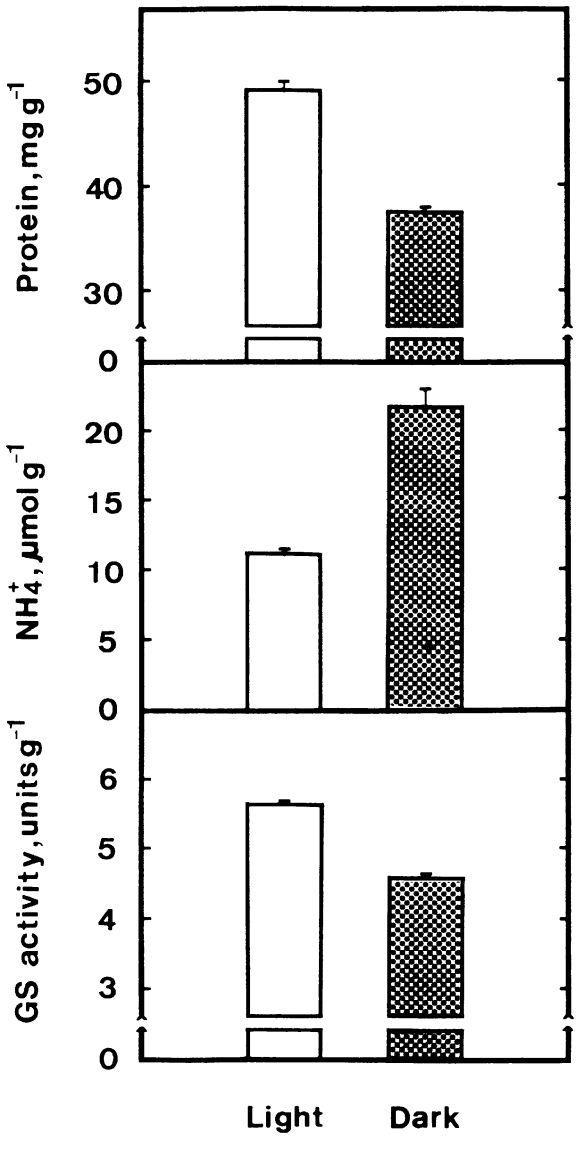

Figure 2. Influence of light on protein and ammonium levels and GS activity in detached rice leaves. All measurements were made 3 days after treatment. Vertical bars represent standard errors (n=4).

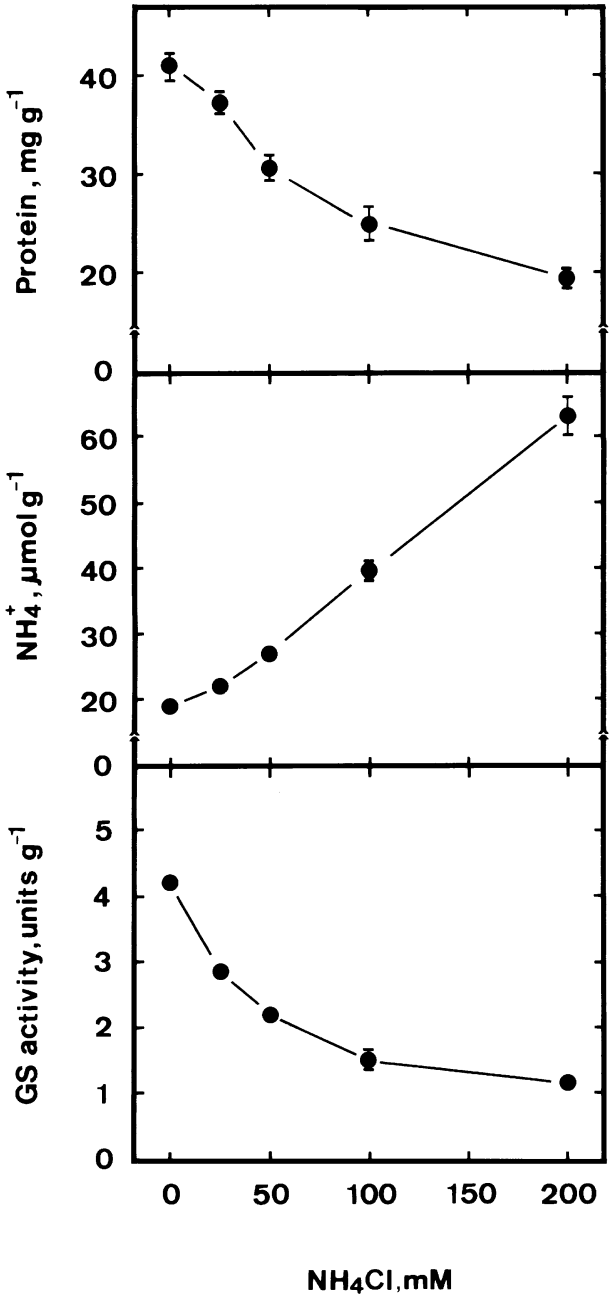

Figure 3. Influence of NH_4Cl on protein and ammonium levels and GS activity in detached rice leaves. All measurements were made 3 days after treatment in the dark. Vertical bars represent standard errors (n=4).

indeed the case. It is also clear from Figure 3 that the increase in ammonium level in NH_4Cl-treated detached rice leaves was associated with the decrease in GS activity (Figure 3).

MSO is a structural analogue of glutamate, and serves as an irreversible inhibitor of GS [22, 23]. There is evidence that addition of MSO results in an accumulation of ammonium [1, 19, 20]. To characterize further the role of ammonium accumulation in regulating dark-induced senescence, detached rice leaves were incubated in the presence of various concentrations of MSO. As indicated in Figure 4, MSO decreased GS activity, increased endogenous ammonium level, and promoted senescence.

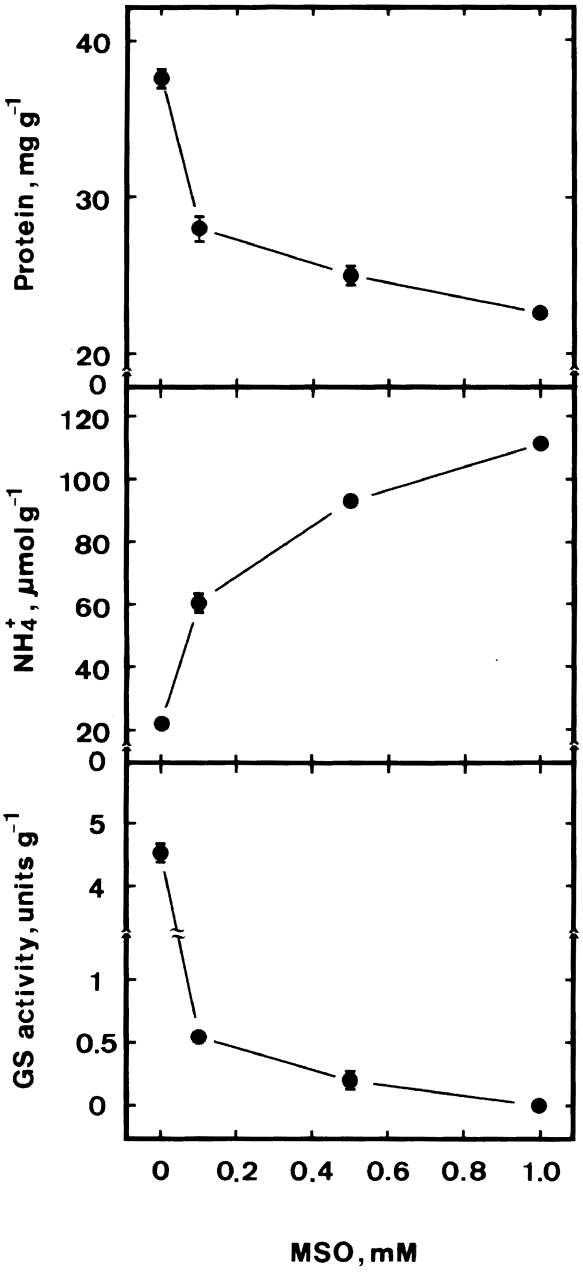

Figure 4. Influence of MSO on protein and ammonium levels and GS activity in detached rice leaves. All measurements were made 3 days after treatment in the dark. Vertical bars represent standard errors (n=4).

It has long been recognized that cytokinins are effective in retarding the senescence of most if not all leaves. The effect of cytokinins in retarding senescence is species- or variety-specific; for the rice variety used in this investigation, BA has been found to be the most active cytokinin in retarding senescence in darkness [9]. The effect of BA on leaf senescence in relation to ammonium level and GS activity is presented in Figure 5. BA effectively retarded senescence, reduced ammonium accumulation, and increased GS activity in detached rice leaves. Among the known promotors of senescence, ABA has been studied most extensively. Figure 6 shows the effects of ABA on senescence, ammonium level and GS activity in detached rice leaves. ABA promoted senescence, increased ammonium accumulation, and decreased GS activity.

Since accumulation of ammonium coincides with the decrease in nitrate level during dark-induced senescence (Figure 1), it is of interest to know whether the changes in ammonium level in BA- and ABA-treated detached rice leaves are associated with those in nitrate level. Figure 7 shows that BA treatment had higher nitrate level, whereas ABA treatment had lower nitrate level than the control leaves. These results indicate that nitrate reduction is significant as a source of ammonium.

Lipid peroxidation is considered to be an important mechanism of leaf senescence [27]. Thus we examined the changes in the level of MDA during dark incubation of detached rice leaves and the influence of NH_4Cl on MDA level in detached rice leaves (Figure 8). No increase in MDA level was observed in detached rice leaves during senescence. We also observed that the increase in MDA level did not parallel the concentrations of NH_4Cl applied. These results seem to exclude the possibility that ammonium-promoted senescence is mediated through lipid peroxidation.

Previously, we demonstrated that senescence of detached rice leaves is mediated through an increase of tissue sensitivity to ethylene [11]. It is most likely that NH_4Cl or MSO may change ethylene sensitivity of detached rice leaves. This possibility was tested by using ethylene action inhibitor, Ag^+ [2]. In the present study, STS was used as a source of Ag^+. The effect of STS on NH_4Cl- and MSO-promoted senescence of detached rice leaves is shown in Figure 9. STS was observed to inhibit NH_4Cl- and MSO-promoted senescence of detached rice leaves.

4. Discussion

The present study shows that ammonium accumulates in detached rice leaves during dark-induced senescence. In the photorespiration, the glycine decarboxylase reaction produces not only CO_2 but also an equiv-

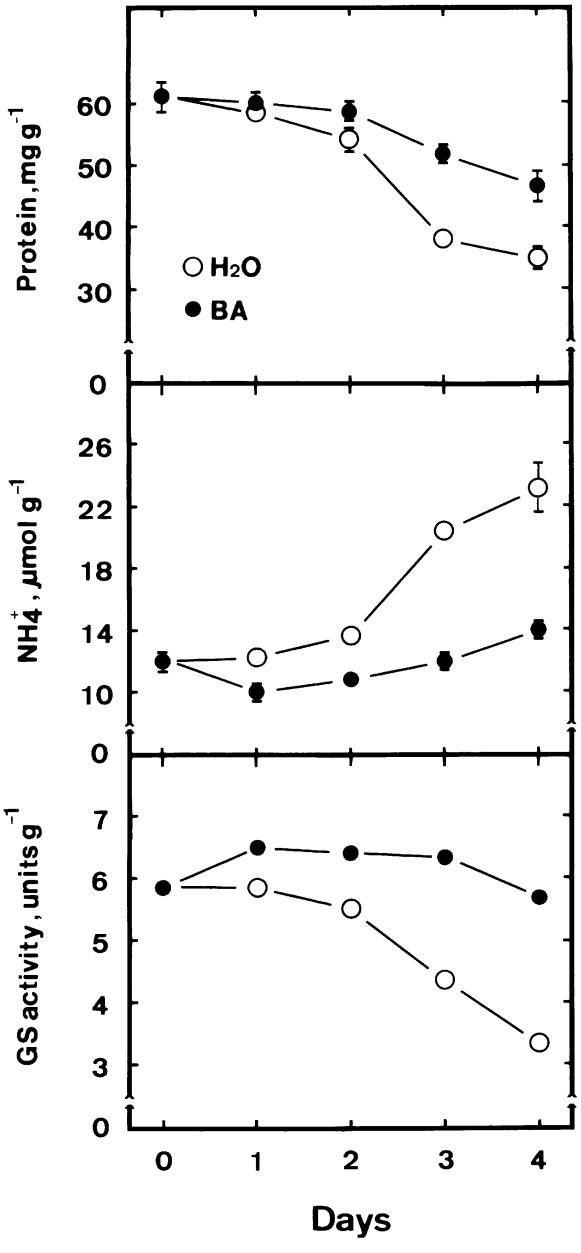

Figure 5. Changes in protein and ammonium levels and GS activity in detached rice leaves treated with BA. Detached rice leaves were treated with either water or 10 μM BA in the dark. Vertical bars represent standard errors (n=4).

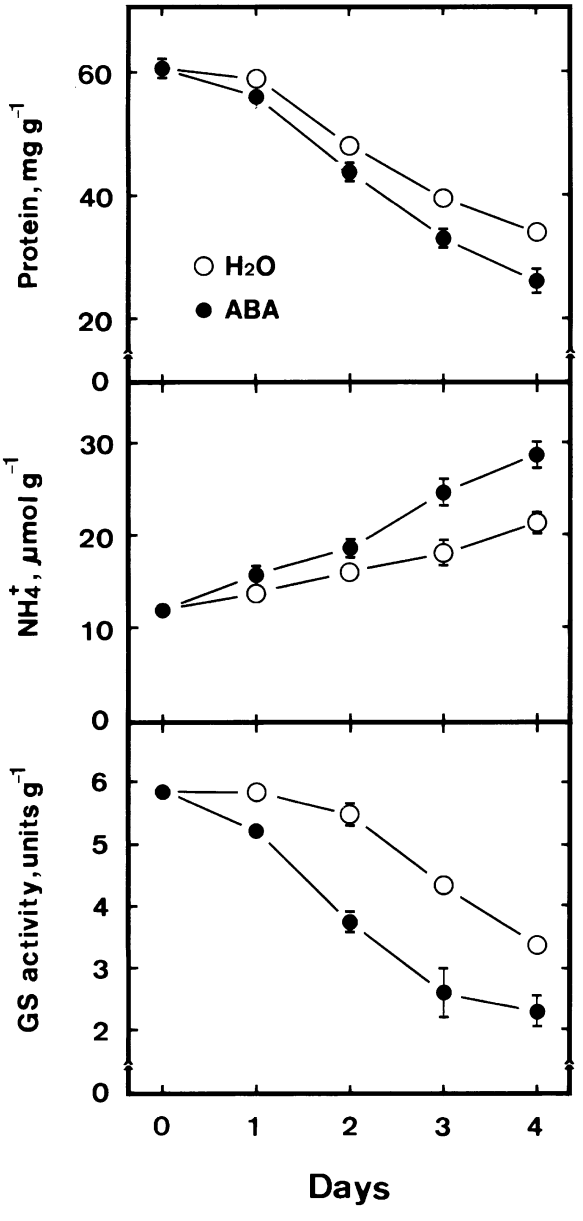

Figure 6. Changes in protein and ammonium levels and GS activity in detached rice leaves treated with ABA. Detached rice leaves were treated with either water or 45 μM ABA in the dark. Vertical bars represent standard errors (n=4).

alent quantity of ammonium. Since our experiments were conducted in darkness, ammonium is unlikely to have been produced from photorespiration. The assimilation of ammonium requires carbon skeltons, energy and GS. We have observed that GS activity in detached rice leaves decreased during dark-induced senescence. This inhibition may result in, at least in part, the accumulation of ammonium in detached rice leaves during senescence in the dark. Another metabolic process producing ammonium is the reduction of nitrate. In our experiments, we observed that ammonium level was inversely correlated with nitrate level during senescence in darkness. If nitrate is assumed to be the

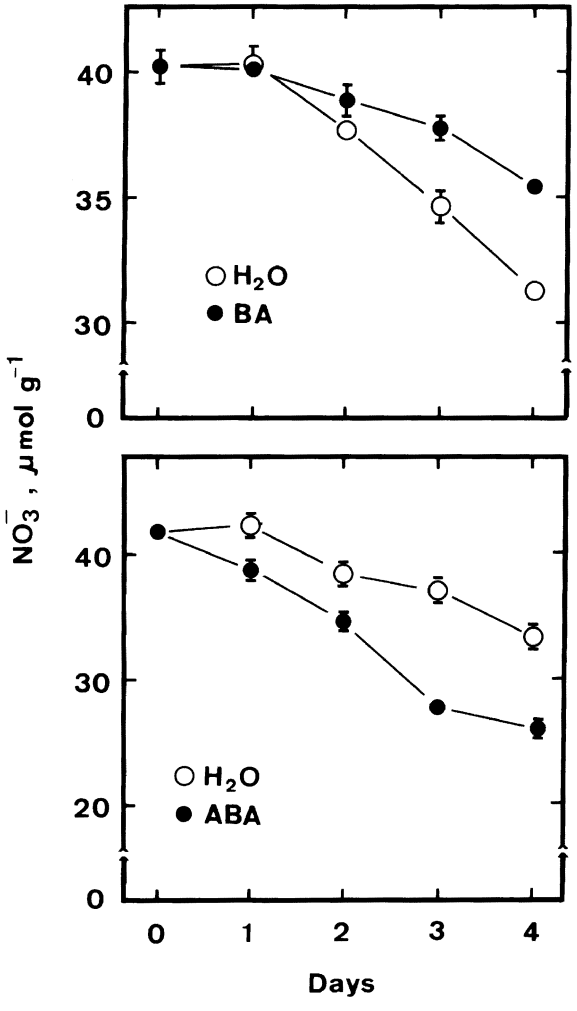

Figure 7. Changes in nitrate level in detached rice leaves treated with BA or ABA. Detached rice leaves were treated with either water, or 10 μM BA, or 45 μM ABA in the dark. Vertical bars represent standard errors (n=4).

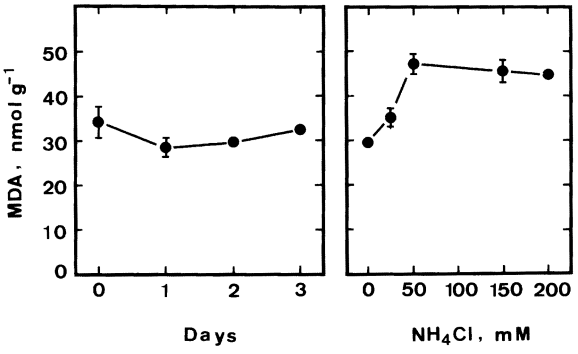

Figure 8. Changes in MDA level in detached rice leaves during senescence in darkness and influence of NH_4Cl on MDA level in detached rice leaves. For experiments of NH_4Cl, MDA level was measured 3 days after treatment in darkness. Vertical bars represent standard errors (n=4).

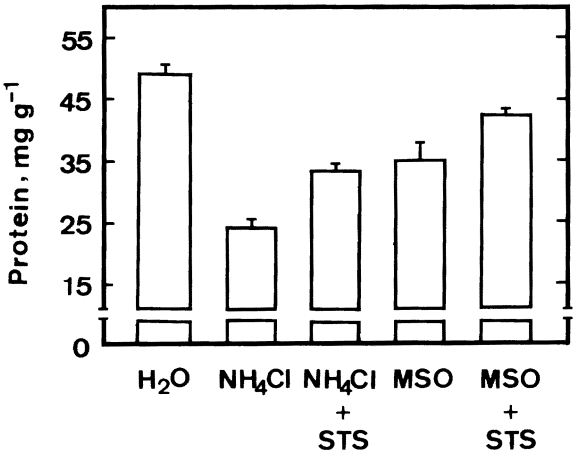

Figure 9. Influence of STS on NH_4Cl- and MSO-promoted senescence of detached rice leaves. The concentrations of NH_4Cl, MSO and STS were 100, 0.5 and 0.2 mM, respectively. Protein level was determined 3 days after treatment in the dark. Vertical bars re present standard errors (n=4).

source of ammonium, then detached rice leaves ought to accumulate more ammonium if fed with additional nitrate. In our work the exact result was observed. Detached rice leaves pretreated with 50 mM KNO_3 for 24 h, followed by treatment with water for 24 h in the dark contained about 30% more ammonium than those pretreated with 50 mM KCl. Our results seem to suggest that ammonium accumulation during dark-induced senescence in attributed to a decrease in GS activity and an increase in nitrate reduction.

Our results indicate that ammonium accumulation is likely to participate in the regulation of senescence of detached rice leaves in the dark. This conclusion is based on the observations that (a) ammonium accumulation paralleled the senescence under dark condition; (b) detached rice leaves fed with NH_4Cl or MSO, which resulted in an accumulation of ammonium in leaves, promoted senescence; (c) treatments such as light and BA, which retarded senescence, decreased ammonium level, and (d) ABA, which promoted senescence, increased ammonium level.

Inhibition of GS activity in dark-induced, MSO- and ABA-treated detached rice leaves may also result in a decrease in glutamine level. Hurst et al. [8] reported that glutamine depletion rather than ammonium accumulation could be the reason for the reduced shelf-life of asparagus treated with phosphinothricin, an

inhibitor of GS. In our work, addition of glutamine had no effect on dark-induced, MSO- and ABA-promoted senescence (data not shown), it seems unlikely that lack of glutamine is the reason for the senescence of detached rice leaves in the dark or treated with MSO and ABA.

In the present study, we show that STS inhibits NH_4Cl- and MSO-promoted senescence in detached rice leaves. It seems that ammonium accumulated in detached rice leaves increases tissue sensitivity to ethylene, which in turn results in senescence promotion.

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