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Ammonium ion, ethylene, and NaCl-induced senescence of detached rice leaves

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Abstract

The possibility that NH_4^+ accumulation is linked to the senescence of detached rice (*Oryza sativa*) leaves induced by NaCl was investigated. NaCl was effective in promoting senescence and in increasing NH_4^+ content of detached rice leaves. NaCl-promoted senescence is mainly due to the effect of both Na^+ and Cl^- ions. NaCl had no or slight effect on relative water content, suggesting that an osmotic effect is unlikely to be a major factor contributing to senescence of these leaves. NaCl-induced NH_4^+ accumulation was due to enhanced nitrate reduction and decreased glutamine synthetase activity. Exogenous NH_4Cl, which caused an accumulation of NH_4^+ in detached rice leaves, also promoted senescence. It was found that an increase in NH_4^+ content preceded the occurrence of senescence caused by NaCl. Results also show that NaCl-promoted senescence is unlikely to be due to the lack of glutamate, glutamine, aspartate, and asparagine. The current results suggest that NH_4^+ accumulation is linked to NaCl-induced rice leaf senescence. Since ethylene is known to be a potent promoter of leaf senescence, we also investigated the role of ethylene in the regulation of NH_4^+-promoted senescence of detached rice leaves. NaCl or NH_4Cl treatment resulted in a decrease of ethylene production. Evidence was presented to show that NH_4^+ accumulation in detached rice leaves does not change tissue sensitivity to ethylene. Clearly, the possible involvement of ethylene in NH_4^+-promoted senescence is excluded.

Abbreviations: Chl – Chlorophyll, DIDS – 4,4′-diisothiocyano-2,2′-disulfonic acid, FW – fresh weight, GS – glutamine synthetase, RWC – relative water content, STS – silver thiosulfate

Introduction

Leaf senescence is an integral part of leaf development. Ammonium ion (NH_4^+) assimilation changes considerably with the onset of senescence (Feller and Fischer 1994). Glutamine synthetase (GS, EC 6.3.1.2) plays a crucial role in the assimilation of NH_4^+ (Miflin and Lea 1976) and its activity is known to decrease during either natural or dark-induced senescence of leaves (Feller and Fischer 1994). Decline in GS activity in leaves during senescence may result, at least in part, in an accumulation of NH_4^+. Recently, we reported that NH_4^+ accumulation was associated with senescence of detached leaves of rice (*Oryza sativa*) induced by methyl jasmonate, under dark condition, and by water stress (Chen and Kao 1998; Chen et al. 1997; Lin and Kao 1998).

Ethylene is a potent promoter of leaf senescence. In oat and rice leaf segments an increase in ethylene production preceded the occurrence of leaf senescence (Gepstein and Thimann 1981; Kao and Yang 1983). Other studies using inhibitors of ethylene biosynthesis and action are supportive of the hypothesis that ethylene is an important plant hormone involved in leaf senescence (Gepstein and Thimann 1981; Kao and Yang 1983).

NaCl has been shown to induce leaf senescence (Kang and Titus 1989; Lutts et al. 1996a; Yeo and Flowers 1983). The accumulation of NH_4^+ due to salinity in tomato and *Pheseolus* plants has been re-

ported (Feng and Barker 1992; Lazcano-Ferrat and Lovatt 1998). Salinity has been shown to increase, decrease or have no effect on ethylene production (Chrominski et al. 1986; Helmy et al. 1994; Khan et al. 1987; Lutts et al. 1996b).

In the present investigation, the relationship between NH_4^+, ethylene and NaCl-promoted senescence of detached rice leaves was examined.

Materials and methods

Rice (*Oryza sativa* cv. Taichung native 1) was cultured as described previously (Chen et al. 1997). Briefly, rice seedlings were planted on a stainless net floating on half-strength Johnson's modified nutrient solution (pH 4.2, Johnson et al. (1957)) in a 500 mL beaker. The nutrient solution was replaced every three days. The rice plants were then grown for 12 days in a greenhouse, where natural light was provided and the temperature was controlled at 30 °C during the day and 25 °C at night. The apical 3-cm segments excised from the third leaves of 12-day-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 the light (40 μmol m^{-2} s^{-1}).

The senescence of detached rice leaves was followed by measuring the decrease in chlorophyll (Ch1) and protein. Ch1 was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein extraction, leaf segments were homogenised in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 *g* for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976).

RWC, defined as water content of leaf tissue as a percentage of that of the fully turgid tissue, was determined by the method of Weatherley (1950). For Na^+ determination, harvested leaf segments were washed three times (one minute each) with distilled water, dried at 65 °C for 2 days, extracted in 1 N HCl at room temperature (Hunt 1982) and analysed with a flame photometer (Evans, Electroselenium Ltd, England). Chloride ion was estimated in a separate extract made according to the method described by Hodson et al. (1985) and estimated using an ion meter (Mittler Delta 350, UK).

Ammonium ions were extracted by homogenising leaf segments in 0.3 mM sulphuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39,000 *g*, and the supernatant was used for determination of NH_4^+ as described by Lin and Kao (1996). For nitrate determination, leaf segments were homogenised in double distilled water. The homogenate was centrifuged for 25 mn at 17,600 *g*. The supernatant was used for determination of nitrate by the method described by Hecht and Mohr (1990).

For determination of glutamate, glutamine, aspartate, and asparagine, leaf samples were extracted with 2% sulfosalicylic acid and the homogenate centrifuged at 15,000 g for 20 min. The supernatant was used directly for amino acid analysis. Amino acid analysis was carried out by an amino acid analyser (Beckman 6300, California, USA) and contents of amino acids are expressed as nmol g^{-1} FW or μmol g^{-1} FW.

For extraction of GS, leaf segments were homogenised with 10 mM Tris-HCl buffer (pH 7.6, containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol) in a chilled pestle and mortar. The homogenate was centrifuged at 15,000 g for 30 mn 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 Oaks et al. (1980). One unit of GS activity is defined as 1 μmol L-glutamate γ-monohydroxamate formed per min.

For ethylene production, leaf segments were transferred to test tubes sealed with serum caps. After 2 h of incubation in the dark at 27 °C, a 1-ml gas sample was withdrawn from the headspace of the test tube. Ethylene was then assayed as described previously (Kao and Yang 1983). In experiments with silver thiosulfate (STS), a stock solution of STS was prepared by mixing equal volumes of 0.01 M $AgNO_3$ and 0.04 M $Na_2S_2O_3$ (Liu et al. 1990).

For all measurements, all treatments were repeated four times. All experiments described here were repeated four times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results and discussion

In the present paper, NaCl-induced senescence of detached rice leaves was assessed by the decrease in Chl and protein content. Increasing the concentration of NaCl from 50 to 200 mM progressively decreased Chl and protein contents and increased both Na^+ and

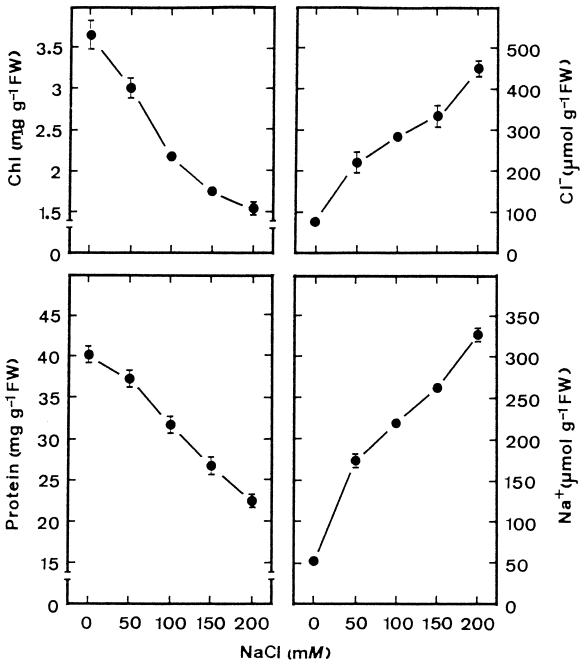

Figure 1. Effect of NaCl on Chl, protein, $Na^+$and Cl^- contents in detached rice leaves. Detached rice leaves were incubated in 5 mM sodium phosphate buffer (pH 7.0) in the presence of NaCl (0–200 mM). Measurements were made 3 days after treatment in the light. Vertical bars represent standard errors (n = 4).

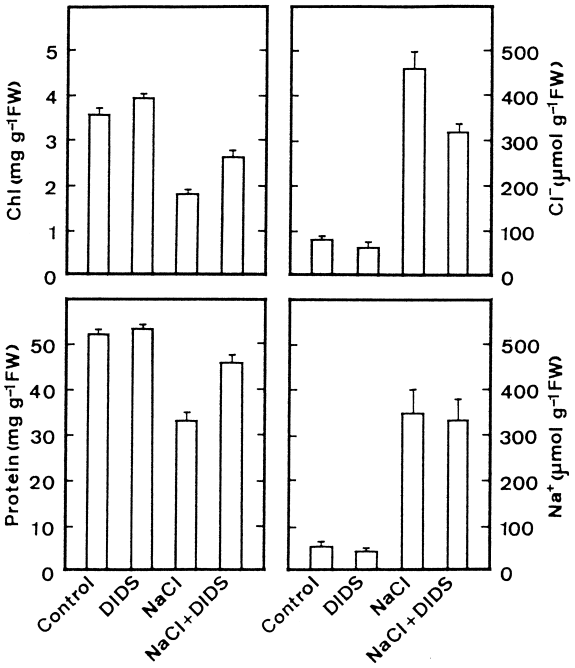

Figure 2. Effect of NaCl and 4, 4′- diisothiocyano – 2, 2′- disulfonic acid (DIDS) on Chl, protein, Na^+, and Cl^- in detached rice leaves. Detached rice leaves were incubated in 5 mM sodium phosphate buffer (pH 7.0) in the presence or absence of NaCl (200 mM) or DIDS (0.1 mM). Measurements were made 3 days after treatment in the light. Vertical bars represent standard errors (n = 4).

Cl^- contents in detached rice leaves in the light (Figure 1). Clearly, the promotion of leaf senescence by NaCl is closely correlated with the increase of Na^+ and Cl^- contents in detached leaves.

The effect of NaCl on the induction of senescence in detached rice leaves could be attributed to Na^+, Cl^-or both. Previously, we have reported that NaCl treatment results in an inhibition of root growth of rice seedlings (Lin and Kao 1999). We also observed that NaCl-inhibited root growth of rice seedlings was mainly due to Na^+, rather than Cl^- (unpublished data). Thus it is of great interest to know whether senescence induction caused by NaCl in detached rice leaves is also due to Na^+, rather than Cl^-. To test this possibility, we determined the effect of 4,4′-diisothiocyano-2,2′-disulfonic acid (DIDS), a nonpermeating amino-reactive disulfonic acid known to inhibit the uptake of Cl^- (Lin 1981), on NaCl-induced senescence in detached rice leaves. If Cl^- plays no role in promoting senescence in detached rice leaves treated with NaCl, then addition of DIDS is expected to lower Cl^- content and to have no effect on senescence promotion. Results presented in Figure 2 show that DIDS was found to decrease Cl^- content without affecting Na^+ content, and also increase Chl and protein content in NaCl-treated detached rice leaves. It appears that both Na^+ and Cl^- are involved in senescence induced by NaCl in detached rice leaves.

No or only a slight difference in RWC was observed between NaCl-Treated leaves and control leaves (Table 1), suggesting that an osmotic effect is unlikely to be a major factor contributing to senescence promotion in detached rice leaves treated with NaCl. This suggestion is supported further by the observations that detached rice leaves treated with sorbitol at a concentration iso-osmotic with 200 mM NaCl had much higher Chl and protein contents than those treated with 200 mM NaCl and significant changes in Chl and protein in leaves treated with 200 mM NaCl were due to the presence of NaCl (Table 2). The lack of effect of NaCl on RWC seems to result from a certain amount of osmotic adjustment, due to the accumulation of Na^+ and Cl^- (Figure 1).

Ammonium ion content in detached rice leaves increased with the increase of NaCl concentration (data not shown). NH_4^+ content increased about 2-fold in detached leaves treated with 200 mM NaCl for 3 days in the light (Figure 3). NH_4^+ content in control leaves remained unchanged during the first day of incuba-

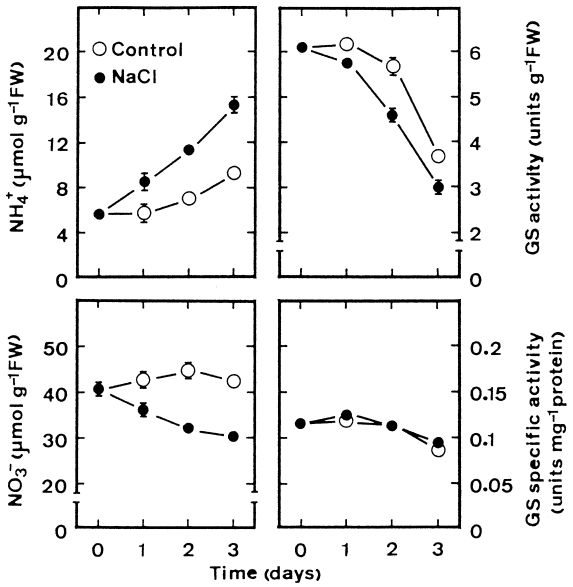

Figure 3. Time courses of NaCl effect on the contents of NH_4^+ and nitrate, and activity and specific activity of GS in detached rice leaves. Detached rice leaves were incubated in 5 mM sodium phosphate buffer (pH 7.0) with or without NaCl (200 mM) in the light. Vertical bars represent standard errors (n = 4).

Table 1. Effect of NaCl on relative water content (RWC) in detached rice leaves

Time (days)	RWC (%)	
	Control	NaCl
0	98.9 ± 0.2	
1	98.2 ± 0.4	97.0 ± 0.7
2	98.3 ± 0.7	94.3 ± 1.1
3	96.6 ± 0.9	93.5 ± 0.8

Detached rice leaves were incubated in sodium phosphate buffer (5 mM, pH 7.0) with or without NaCl (200 mM). Means ± standard errors (n = 4).

tion and increased subsequently (Figure 3). It is clear that NH_4^+ content in leaves treated with NaCl was higher than that in controls and the accumulation of NH_4^+ induced by NaCl was evident at 1 day after treatment (Figure 3).

Ammonium ion is a central intermediate in the metabolism of nitrogen in plants. NH_4^+ is produced during nitrate assimilation, deamination of amino acids and photorespiration (Miflin and Lea 1976). Figure 3 shows that NaCl treatment resulted in a decrease in nitrate content. This result suggests that NaCl-induced NH_4^+accumulation may have resulted from the promotion of nitrate reduction. If nitrate is assumed to be the source of NH_4^+, the detached rice leaves ought to accumulate more NH_4^+ when fed with additional nitrate. In our study the exact result was observed. Detached rice leaves pretreated with 50 mM KNO_3 for 12 h in the light, following by treatment with NaCl for 24 h in the light contained more NH_4^+ than those pretreated with water or KCl (Table 3).

GS is the primary enzyme responsible for NH_4^+ assimilation in plants (Miflin and Lea 1976). We observed that GS activity in control leaves remained almost unchanged during the first 2 days of incubation and subsequently decreased (Figure 3). NaCl-treated rice leaves had lower GS activity than the control leaves (Figure 3). However, no decrease in specific activity of GS was observed in detached rice leaves treated with NaCl (Figure 3). It appears that NaCl-induced NH_4^+ accumulation is attributed to the decrease in GS activity.

A high content of NH_4^+ is known to have a toxic effect on plant cells (Givan 1979). Recently, we reported that NH_4^+ accumulation is associated with water stress-, methyl jasmonate- and dark-promoted senescence of detached rice leaves (Chen and Kao 1998; Chen et al. 1997; Lin and Kao 1998). If NH_4^+ accumulation plays a regulatory role in NaCl-induced senescence of detached rice leaves, it is expected that treatment of NH_4Cl would increase endogenous NH_4^+ content and consequently promote senescence. As indicated in Figure 4, this is indeed the case. The observations that detached rice leaves treated with NH_4Cl, which resulted in a promotion of senescence and an increase in NH_4^+ content in the same way that NaCl did, further support our suggestion that NH_4^+ accumulation is likely to participate in the regulation of senescence of detached rice leaves under saline conditions.

To test the causal relationship between NH_4^+ accumulation and senescence promotion in detached leaves caused by NaCl, detached rice leaves were incubated in the presence or absence of NaCl for 4, 8, and 10 h. Changes in Chl, protein, and NH_4^+ were then monitored. As indicated in Figure 5, an increase in NH_4^+ content preceded the decrease in Chl and protein contents in detached rice leaves caused by NaCl. Thus, NH_4^+ accumulation may play a regulatory role in leaf senescence induced by NaCl.

Kylin and Quatrano (1975) suggested that a primary plant response to salinity is in amino acid metabolism, specifically key reactions involved with metabolic regulation of NH_4^+ assimilation. Pulich (1986) reported that amides decreased with increasing salinity in leaves of *Halodule wrightii* and *Thalassia testudinum*. Hurst et al. (1993) demonstrated that

Table 2. Effect of the concentration of NaCl and sorbitol on the contents of Chl and protein in detached rice leaves

Treatment	Chl (mg g^{-1} FW)	Protein (mg g^{-1} FW)
Control	3.5 ± 0.10	42.9 ± 1.3
Sorbitol, 400 mM	2.6 ± 0.04	36.8 ± 0.82
Sorbitol, 300 mM + NaCl, 50 mM	2.3 ± 0.03	34.2 ± 0.51
Sorbitol, 200 mM + NaCl, 100 mM	2.1 ± 0.03	33.0 ± 0.71
NaCl, 200 mM	1.2 ± 0.14	29.6 ± 1.0

The osmotic potential was kept equivalent to that of medium with 200 mM NaCl by replacing NaCl by sorbitol. Sorbitol and NaCl were dissolved in sodium phosphate buffer (5 mM, pH 7.0). Chl and protein were determined 3 days after treatment in the light. Means ± standard errors (n = 4).

Table 3. Effect of KNO_3 pretreatment on NaCl-induced NH_4^+ accumulation in detached rice leaves

Treatment	NH_4^+ (μmol g^{-1} FW)
H_2O → Control	8.4 ± 0.48
H_2O → NaCl	12.2 ± 0.18
KCl → Control	9.1 ± 0.46
KCl → NaCl	12.5 ± 0.31
KNO_3 → Control	9.7 ± 0.54
KNO_3 → NaCl	14.5 ± 0.48

Detached rice leaves were pretreated with either H_2O, 50 mM KCl or KNO_3 for 12 h in the light and then treated with sodium phosphate buffer (5 mM, pH 7.0) in the presence or absence of NaCl (200 mM) for 24 h in the light. Means ± standard errors (n = 4).

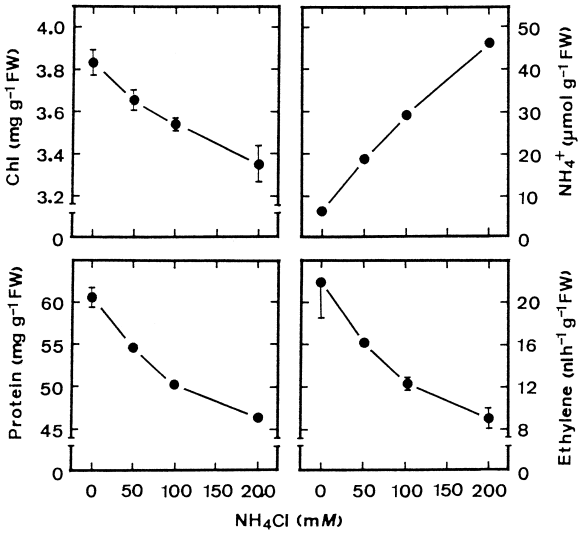

Figure 4. Effect of NH_4Cl on Chl, protein and NH_4^+ contents, and ethylene production in the detached rice leaves. Detached rice leaves were incubated in 5 mM sodium phosphate buffer (pH 7.0) in the presence of NaCl (0–200 mM). Measurements were made 3 days after treatment in the light. Vertical bars represent standard errors (n = 4).

glutamine depletion rather than NH_4^+ accumulation could be the reason for the reduced shelf-life of asparagus treated with phosphinothricin, an inhibitor of

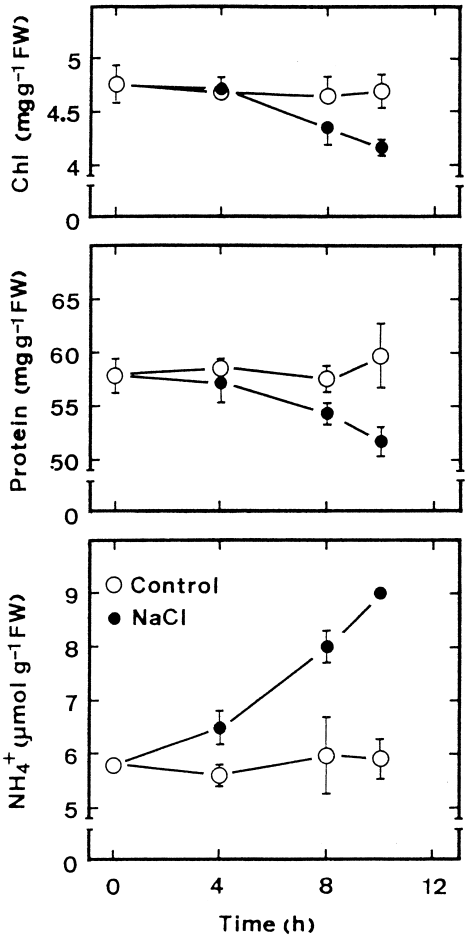

Figure 5. Time courses of NaCl effect on the contents of Chl, protein, and NH_4^+ in detached rice leaves. Detached rice leaves were incubated in 5 mM sodium phosphate buffer (pH 7.0) with or without NaCl (200 mM) in the light. Vertical bars represent standard errors (n = 4).

GS. In our work, we found that the contents of glutamate, glutamine, aspartate, and asparagine in NaCl-treated rice leaves were higher than those in control leaves (Figure 6). It appears unlikely that lack of glutamate, glutamine aspartate or asparagine is the

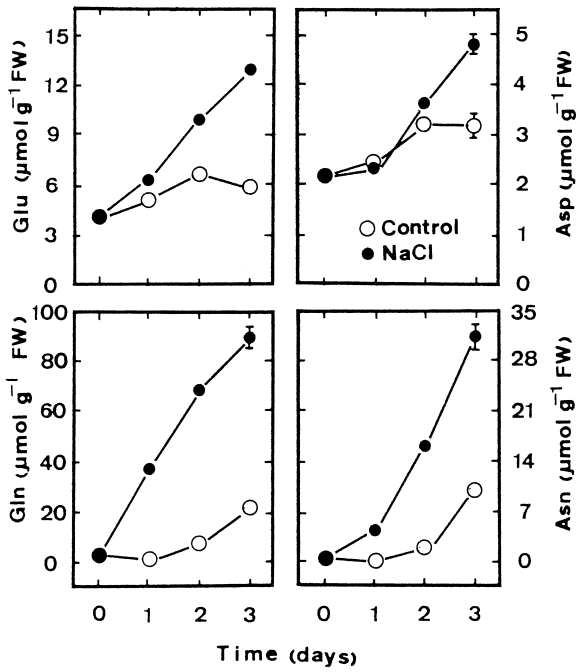

Figure 6. Time course of NaCl effect on the contents of aspartate (Asp), asparagines (Asn), glutamate (Glu), and glutamine (Gln) in detached rice leaves. Detached rice leaves were incubated in 5 mM sodium phosphate buffer (pH 7.0) with or without NaCl (200 mM) in the light. Vertical bars represent standard errors (n = 4).

reason for the senescence of detached rice leaves induced by NaCl.

Ethylene is known to be the promoter of leaf senescence (Gepstein and Thimann 1981; Kao and Yang 1983). It is of great interest to known whether senescence of detached rice leaves induced by NaCl or NH_4Cl is mediated through an increase in ethylene production. Ethylene production in control leaf segments increased significantly during the first day and decreased subsequently (Figure 7). This result is consistent with our early findings that ethylene production precedes the senescence of detached rice leaves (Kao and Yang 1983), indicating that ethylene production participates in the regulation of rice leaf senescence. If NaCl-promoted senescence of detached rice leaves is mediated though ethylene production, then ethylene production in NaCl-treated leaf segments is expected to be higher than that in control leaf segments. However, as indicated in Figure 7, this does not seem to be the case. Figure 4 also shows that increasing concentration of NH_4Cl from 50 to 200 mM progressively decreased ethylene production in rice leaf segments.

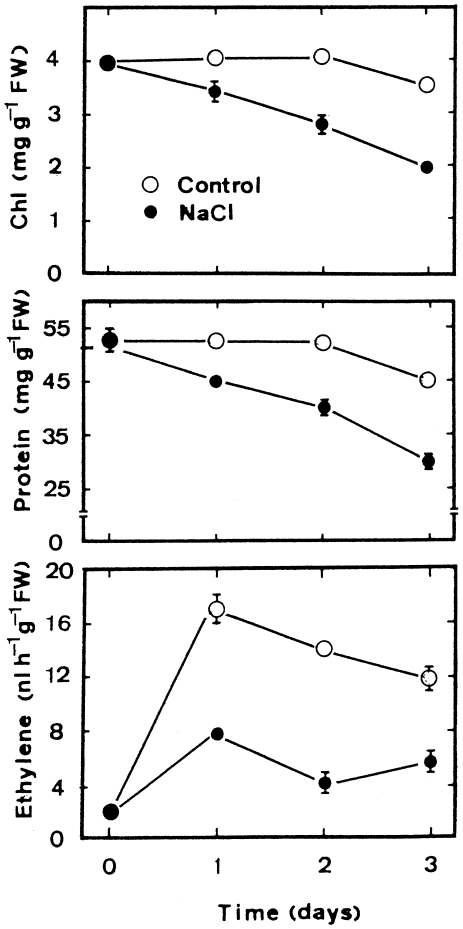

Figure 7. Time courses of NaCl effect on Chl and protein contents, and ethylene production in detached rice leaves. Detached rice leaves were incubated in 5 mM sodium phosphate buffer (pH 7.0) with or without NaCl (200 mM) in the light. Vertical bars represent standard errors (n = 4).

If a change in ethylene production is excluded as an explanation for the NaCl- or NH_4Cl-promoted senescence of detached rice leaves, a change in sensitivity to ethylene is an alternative possibility. This possibility was tested by using the inhibitor of ethylene action, STS (Liu et al. 1990). STS was ineffective in inhibiting NaCl- or NH_4Cl-promoted senescence of detached rice leaves (Table 4). It seems that ethylene is not involved in regulating senescence of detached rice leaves caused by NaCl or NH_4Cl.

Although the present investigation provides evidence to show that NH_4^+ accumulation is associated with senescence of detached rice leaves caused by NaCl stress, the actual mechanism of NH_4^+-induced senescence is still unclear. Therefore, further research

Table 4. Effect of STS on NaCl- or NH_4Cl-promoted senescence of detached rice leaves

Treatment	Protein (mg g^{-1} FW)	Chl (mg g^{-1} FW)
H_2O → Control	57.4 ± 0.9	4.7 ± 0.04
H_2O → NaCl	51.5 ± 0.5	3.7 ± 0.02
STS → NaCl	51.5 ± 1.1	3.7 ± 0.07
H_2O → Control	58.4 ± 0.8	4.1 ± 0.05
H_2O → NH_4Cl	46.3 ± 0.9	3.8 ± 0.05
STS → NH_4Cl	46.7 ± 1.1	3.7 ± 0.04

Detached rice leaves were pretreated with either H_2O or 0.4 mM STS for 12 h in the dark and then treated with sodium phosphate buffer (5 mM, pH 7.0) in the presence or absence of NaCl (200 mM) or NH_4Cl (100 mM) for 24 h in the light. Means ± standard errors (n = 4).

is necessary for a better understanding of the mechanism of NH_4^+-induced senescence.

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