Bot. Bull. Acad. Sin. (1996) **37**(4)**:** 255-259

Ammonium accumulation in relation to senescence of detached maize leaves

Shu Jiuan Chen and Ching Huei Kao[1]

Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China

(Received June 22, 1996; Accepted August 14, 1996)

Abstract. We investigated the role of ammonium in the regulation of dark-induced senescence of detached maize leaves. Ammonium levels were found to increase prior to senescence. The accumulation of ammonium was associated with a decrease in glutamine synthetase (GS). Exogenous NH_4Cl and methionine sulfoximine (MSO), an inhibitor of GS, increased ammonium level and promoted senescence. Benzyladenine, a synthetic cytokinin, retarded senescence and decreased ammonium level. Methyl jasmonate promoted senescence and increased the ammonium level. The addition of L-glutamine had no effect on dark-induced, MSO- and MJ-promoted senescence. Our results suggest that an increase in ammonium level is associated with the senescence of detached maize leaves in the dark.

Keywords: Ammonium; Benzyladenine; Leaf senescence; Methyl jasmonate; Methionine sulfoximine; *Zea mays.*

Introduction

Glutamine synthetase (GS, EC 6.3.1.2) is the primary enzyme responsible for ammonium assimilation in plants (Lea and Miflin, 1974). It has been shown that GS activity in leaves decreases during senescence (Kar and Feierabend, 1984; Peeters and Van Laere, 1992; Postius and Jacobi, 1976; Simpson and Dalling, 1981; Storey and Beevers, 1978; Streit and Feller, 1983). Decreased GS activity during leaf senescence may result, at least in part, in the accumulation of ammonium in leaves. In fact, the accumulation of ammonium in senescing leaves has already been described (Postius and Jacobi, 1976; Thomas, 1978). Ammonium is thought to be toxic to plant cells (Givan, 1979) and it has been suggested that ammonium accumulation may be a factor contributing to loss of quality of harvested aspargus spears or leaf senescence (Hurst et al., 1993). However, no clear evidence has been provided to prove this suggestion. Ammonium has been shown to accumulate after the onset of senescence in detached wheat leaves (Peeters and Van Laere, 1992; Thomas, 1978). In the present investigation, we used detached maize leaves to determine whether ammonium accumulation is associated with leaf senescence.

Materials and Methods

Plant Materials and Incubation Conditions

Seedlings of maize (*Zea mays* cv. XL 678) were grown in vermiculite in a greenhouse with natural light at 30°C day/25°C night for 7 days, by which time the primary leaves were fully expanded. The apical 2.5-cm segments were excised from the primary leaves and then floated in a glass Petri dish containing 10 ml of distilled water or test solution. Incubation was carried out at 27°C in the dark.

Determination of Protein and Ammonium

For protein determination, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 7.5). 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 (1976). Protein level was expressed as mg g^{-1} fresh weight. For ammonium determination, leaf segments were homogenized in 0.3 mM sulphuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39,000 *g,* and the supernatant liquids were used for determination of ammonium by the method described previously (Lin and Kao, 1996). Ammonium levels were expressed as μmol g^{-1} fresh weight.

GS Assay

Leaf segments were homogenized 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 mortar and pestle. 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 Oaks et al. (1980). The reaction mixture contained in a final volume of 1 ml: 80 μmol Tris-HCl buffer, 40 μmol L-glutamic acid, 8 μmol ATP, 24 μmol $MgSO_4$, and 16 μmol NH_2OH; the final pH was 8.0. The reaction was initiated by the addition of the enzyme extract and after incubation for 30 min at 30°C was stopped by adding 2 ml 2.5 % (w/v) $FeCl_3$ and 5 % (w/v) trichloroacetic acid

[1]Corresponding author. Fax: 02-3620879.

in 1.5 M HCl. After centrifugation at 3,000 *g* the absorbance of the supernatant was read at 540 nm. One unit of GS activity is defined as 1 μmol L-glutamate γ-monohydroxamate formed per min.

Results

The senescence of detached leaves is characterized by a decrease in chlorophyll and protein levels. The loss of chlorophyll and protein has been the principal criterion of senescence for the largest number of workers (Thimann, 1980). Since protein loss in detached maize leaves was observed to occur one day earlier than chlorophyll loss during senescence (Kao, 1994), the senescence of detached maize leaves was monitored along with the decrease of protein in the present study. Figure 1 shows the time courses of protein and ammonium levels and the GS activity of detached maize leaves. A decrease in protein was evident 3 days after leaf detachment. Ammonium levels remained unchanged during the first day of dark incubation but increased subsequently. The increase in ammonium was associated with a decrease in GS activity. Our results indicate that ammonium accumulation in detached maize leaves precedes the onset of senescence . It is obvious that our results are in contrast with those of Peeters and Van Laere with wheat (1992) and Thomas with *Lolium* (1978), who demonstrated that ammonium accumulates at the later stage of senescence.

If ammonium accumulation plays a regulatory role in senescence of detached maize leaves, it is expected that treatment of NH_4Cl would increase endogenous ammonium levels and consequently promote senescence. As indicated in Figure 2., this is indeed the case.

Miflin and Lea (1977) suggested that an active GS could be responsible for the efficient assimilation of ammonium, and there is evidence that addition of methionine sulfoximine (MSO), an inhibitor of GS, results in an accumulation of ammonium (Miflin and Lea, 1977). To

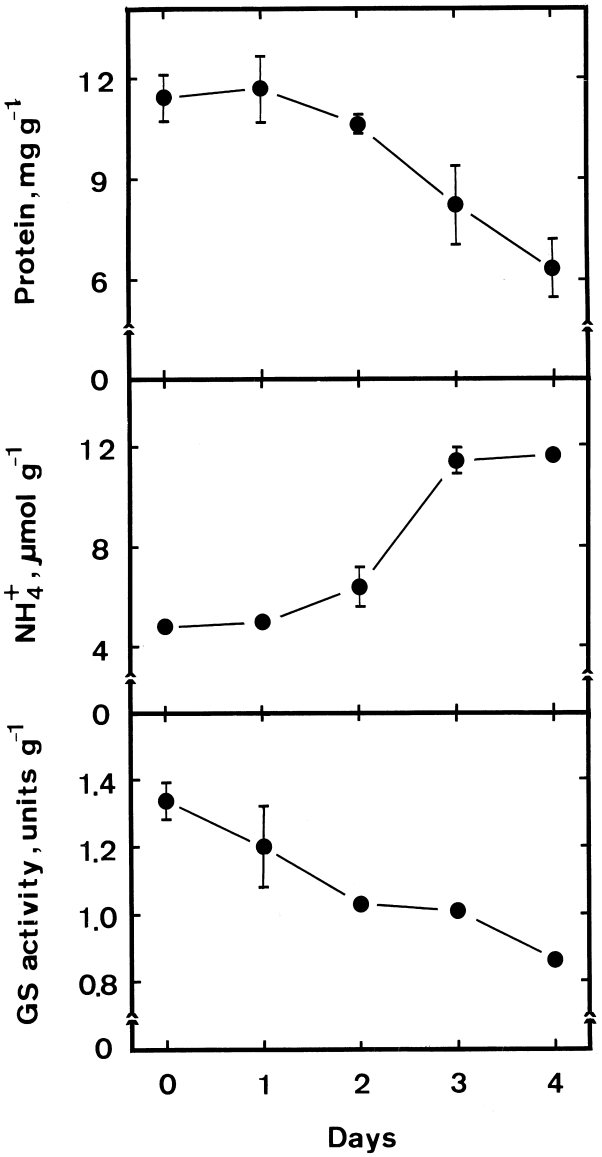

Figure 1. Protein and ammonium levels and GS activity in detached maize leaves during dark-induced senescence. Vertical bars represent standard errors (n=4).

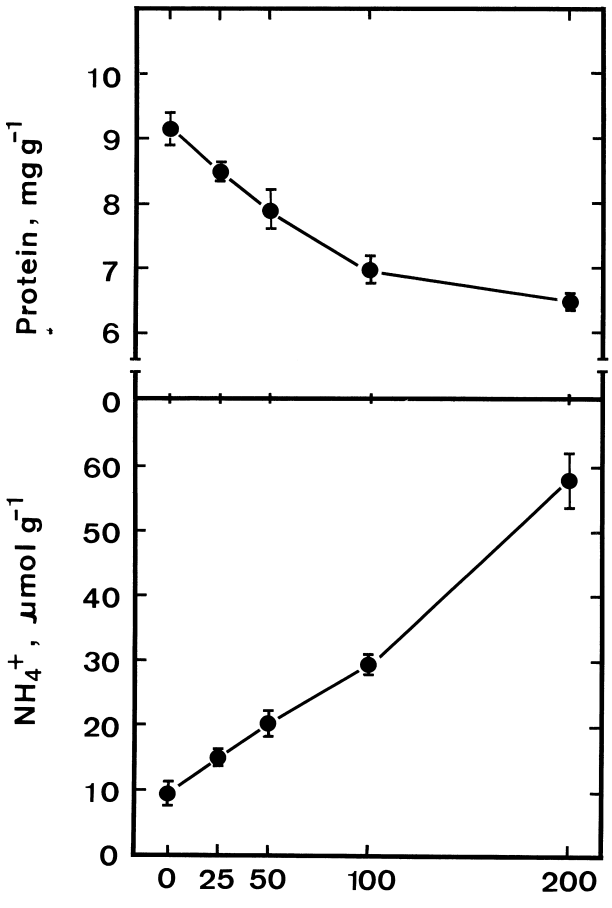

Figure 2. Influence of NH_4Cl on protein and ammonium levels in detached maize leaves. All measurements were made 3 days after treatment. Vertical bars represent standard errors (n=4).

characterize further the role of ammonium accumulation in regulating dark-induced senescence of detached maize leaves, leaf segments were incubated in the presence of various concentrations of MSO. As indicated in Figure 3, MSO decreased GS activity, increased ammonium levels in leaf segments, and promoted senescence of leaf segments. From the shape of the curves, it is obvious that ammonium levels are closely associated with senescence promotion.

The influence of benzyladenine (BA, a synthetic cytokinin) on senescence, ammonium level, and GS activity is shown in Figure 4. BA retarded senescence of detached maize leaves, decreased ammonium levels, and increased GS activity.

Figure 5 shows the influence of methyl jasmonate (MJ) on senescence, ammonium level, and GS activity of detached maize leaves in the dark. MJ promoted senescence, increased ammonium levels, and decreased GS activity.

Discussion

In the present study, we show that ammonium accumulates in leaf segments during dark-induced senescence. There are three major potential sources for the ammonium.

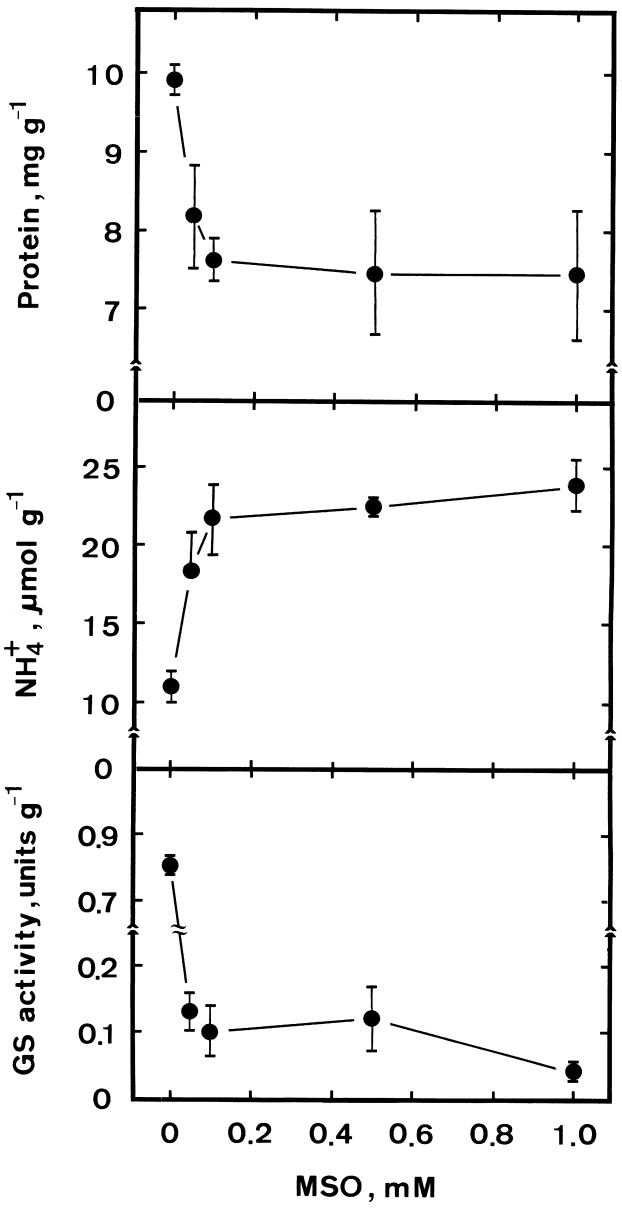

Figure 3. Influence of MSO on protein and ammonium levels and GS activity in detached maize leaves. All measurements were made 3 days after treatment. Vertical bars represent standard errors (n=4).

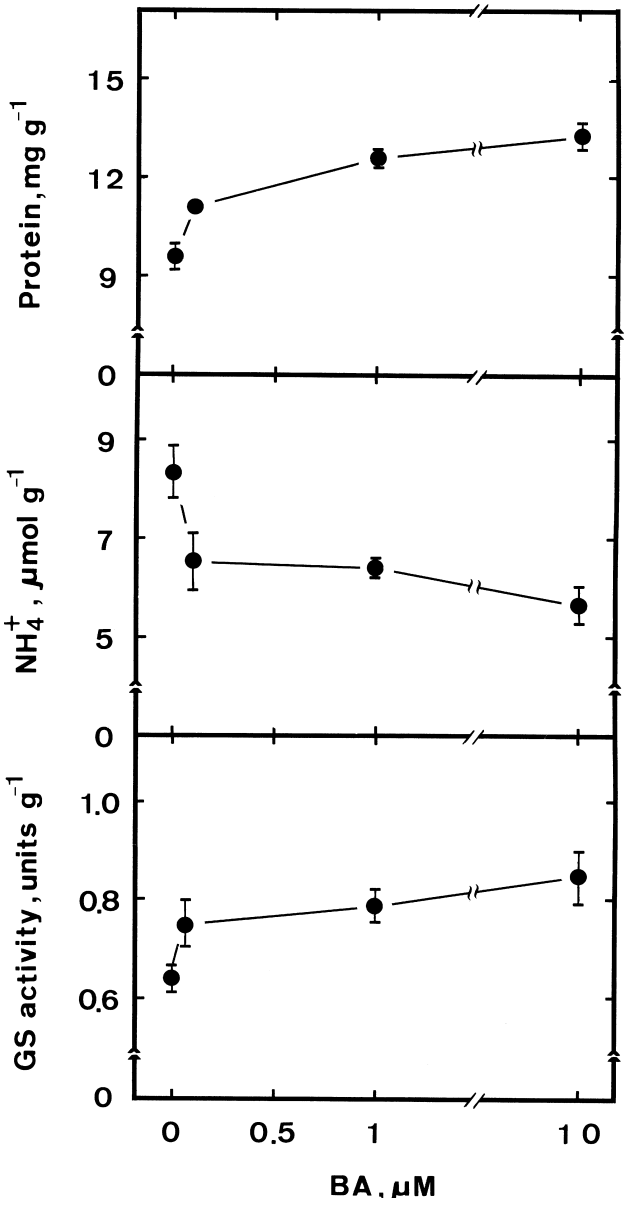

Figure 4. Influence of BA on protein and ammonium levels and GS activity in detached maize leaves. All measurements were made 3 days after treatment. Vertical bars represent standard errors (n=4).

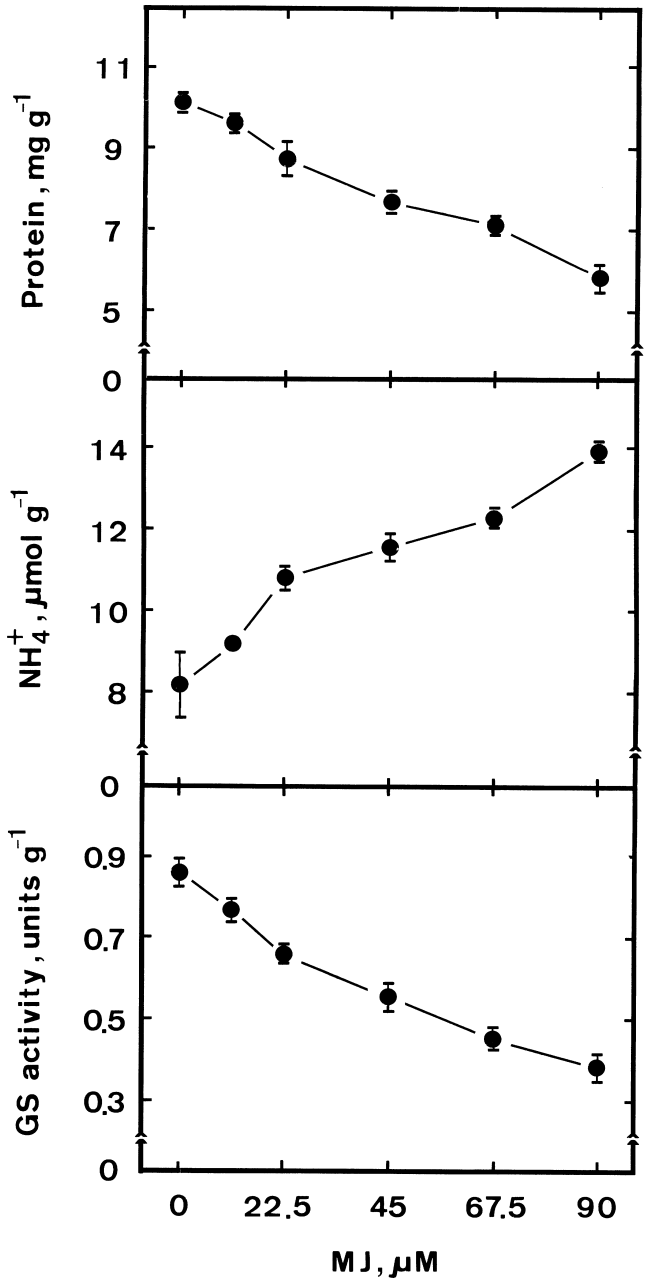

Figure 5. Influence of MJ on protein and ammonium levels and GS activity in detached maize leaves. All measurements were made 3 days after treatment. Vertical bars represent standard errors (n=4).

During photorespiration, the glycine decarboxylase reaction produces not only CO_2 but also an equivalent quantity of ammonium. Since our experiments were conducted in darkness, ammonium is unlikely to have been produced from photorespiration. Moreover maize is a C_4 species. Another metabolic process forming ammonium is the evolution of ammonium in the course of nitrate assimilation by reduction of the nitrate. This possibility was excluded because nitrate levels remained unchanged during the dark-induced senescence of detached maize leaves (data not shown). Thirdly, ammonium also occurs in catobolic and anabolic process, such as in the degradation of proteins or nucleotides and in the oxidative deamination of amino acids. In each case the ammonium liberated is reassimilated via GS. In the present investigation, we have observed that GS activity in detached maize leaves decreased during senescence. This decrease may result, at least in part, in the accumulation of ammonium in maize leaves during dark-induced senescence.

Our results indicate that ammonium accumulation is likely to participate in the regulation of senescence of detached maize leaves in the dark. This conclusion is based on the observations that (a) ammonium accumulation in leaves preceded the onset of senescence; (b) ammonium levels in leaves were associated with senescence promotion; (c) NH_4Cl or MSO treatment, which resulted in an accumulation of ammonium in leaf segments, promoted senescence; (d) BA, which retarded senescence of leaf segments, inhibited the accumulation of ammonium; and (e) MJ, which promoted senescence of leaf segments, increased the accumulation of ammonium.

Inhibition of GS activity in senescent maize leaves and the effect of MSO or MJ on maize leaves may also result in lowering the level of L-glutamine. Hurst et al. (1993) demonstrated that L-glutamine depletion rather than ammonium toxicity could be the reason for the reduced shelf-life of asparagus treated with phosphinothricin, an inhibitor of GS. In our work, the addition of L-glutamine had no effect on dark-induced, MSO- and MJ-promoted senescence (data not shown), it seems unlikely that lack of L-glutamine is the reason for the senescence of detached maize leaves in darkness or those treated with MSO and MJ. In conclusion, an increase in ammonium seems to be associated with the senescence of detached maize leaves in the dark.

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銨離子與玉米切離葉片老化關係之研究

陳淑娟　高景輝

國立台灣大學農藝學系

本研究探討玉米（XL 678 品種）切離葉片在黑暗下的老化過程中，銨離子所扮演的角色。玉米切離葉片在老化前的銨離子含量明顯增加。在老化過程中銨離子含量之增加伴隨著 glutaminesynthetase(GS) 活性的下降。氯化銨與 Methioninesulfoximine(MSO, GS 活性之抑制劑) 處理，可導致銨離子含量增加與加速老化。Benzyladenine處理，可延緩老化，同時抑制銨離子的累積。Methyl jasmonate(MJ) 處理，可加速老化，同時增加銨離子之含量。添加 L-glutamine不會影響玉米切離葉片在暗中之老化，亦不會影響 MSO 與 MJ 所加速的老化。因此，銨離子的累積似乎與玉米葉片老化有關。

關鍵詞：銨離子；Benzyladenine; 葉片老化；Methyl jasmonate; Methionine sulfoximine; 玉米。