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Research article

Is the electrolyte leakage assay an unequivocal test of membrane deterioration during leaf senescence?

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ABSTRACT

The main symptoms of leaf senescence are the degradation of chlorophyll and proteins (which may be accompanied by ammonium accumulation), and an increase of electrolyte leakage (EL), which has been traditionally attributed to disruption of cell membranes. The aim of this study was to determine if ammonium efflux contributes to the increase EL in senescing barley leaves. During senescence of detached leaves the increase of EL correlated with ammonium leakage (r^2 = 0.82) and ammonium content in tissues (r^2 = 0.73), but not with K^{1+} leakage (r^2 = 0.23). Although lower amounts of ammonium accumulated in senescing attached leaves, again changes in EL paralleled ammonium accumulation. EL increased early during senescence even though ion leakage was selective (leaves leaked proportionally more ammonium than K^{1+}), and membranes appeared intact as judged from staining with the cell impermeant stain propidium iodide. Detached leaves maintained their capacity to regreen after 3 days of senescence-acceleration in darkness, i.e., membrane integrity was not severely compromised. During the early stages of senescence, EL increases due to ammonium accumulation (possibly resulting from protein degradation) even if there is no massive disruption of cell membranes. Therefore, increased EL in senescing leaves is not an unequivocal symptom of cell membrane damage.
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1. Introduction

The measurement of electrolyte leakage from plant tissues is a classical method to estimate membrane integrity in response to environmental stresses, senescence, fruit ripening, etc. In living cells, electrolytes are contained within membrane-bound compartments. During senescence, or under stress, the proteins and lipids of these membranes are degraded, oxidized, etc, and this may lead to structural changes (e.g., phase transitions) that cause loss of integrity and increased membrane permeability [1]. Undamaged plant cells, like young cells, maintain electrolytes within the boundaries of the plasma membrane, whereas in senescing cells electrolytes may leak out into the surrounding apoplast. High solute losses by leakage have been reported for leaves during senescence [2–5]. Cell membrane damage can be estimated by comparing the conductivity of the leaked contents from injured and uninjured tissues [e.g., 1,6–9]. For example, since an increase of EL is assumed to reflect extensive disruption of the plasma membrane, it has been argued that ion leakage may be the best parameter to follow the course of leaf senescence [10].

Senescence represents the final stage of leaf development and it is characterized by the transition from nutrient assimilation to nutrient remobilization [11]. One physiological purpose of leaf senescence is the recycling of N-containing compounds, which mainly derive from the chloroplasts. Many reviews have addressed different aspects of senescence-associated N remobilization [e.g., 11–13]. In terms of nutrient redistribution, the earliest and most significant change during senescence is the breakdown of chloroplasts, which contain up to 70% of the proteins of a mesophyll cell. The chloroplasts are dismantled first, while other cellular constituents remain intact to accomplish the recycling process [14]. To allow for efficient recycling of these compounds, integrity and compartmentation of the leaf are maintained until a late stage of senescence [15]. During chloroplast breakdown, photosynthetic proteins are degraded to amino acids, amides and ammonium. Therefore, ammonium accumulation in leaves may be a symptom of senescence in some cases. Protein degradation during senescence

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EL, electrolyte leakage; PMSF, phenylmethylsulfonyl fluoride; d, day.

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coincides with NH_4^{1+} accumulation in both, attached and detached leaves [e.g., 16,17]. High levels of ammonium in tissues results in potential NH_3 emission, and the highest rates of ammonia emission are usually seen during senescence and grain filling of cereals [18]. Increased NH_3 emission during dark-induced senescence was detected in barley and ryegrass, and the increased emission paralleled protein and chlorophyll degradation, and an increase of ammonium content in the leaf. During senescence of detached leaves in light or darkness, NH_4^{1+} accumulated in the cells and apoplast, and the potential for NH_3 emission increased, while in attached leaves of different ages, senescence-induced ammonium accumulation was much less [17].

The aim of this work was to determine the possible contribution of ammonium efflux to the increased electrolyte leakage (EL) of senescing leaves. In detached spinach leaves ammonium content increased 5-fold during 6 d of incubation in darkness, and this correlated with a 5-fold increase in EL [19]. A correlation analysis on these data shows that increased EL correlated closely ($r^2 = 0.82$) with percentage increase in ammonium content. Thus, we hypothesized that ammonium accumulated in the apoplast is the main electrolyte contributing to EL during the initial stages of senescence, before significant damage to membranes occurs. To test this, we induced senescence by protracted incubation in darkness, and followed the changes in electrolyte leakage in detached or attached leaves of barley, which presumably differ in terms of ammonium accumulation during senescence.

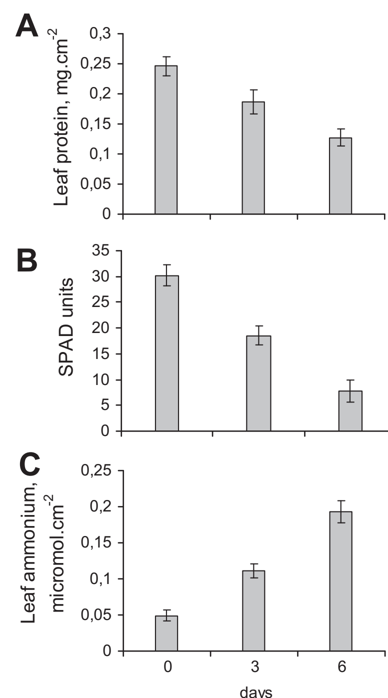

Fig. 1. Changes in the levels of total proteins (A), chlorophyll (B) and ammonium (C) during senescence of detached barley leaves. Protein and ammonium are expressed on the basis of leaf area. Eight independent extracts were made for each treatment and incubation time. Each SPAD data point represents the mean of 15 independent measurements. Bars indicate standard deviation.

2. Results

2.1. Senescence of detached leaves

2.1.1. Characterization of senescence

Primary barley leaves reached full expansion on the 10th day after sowing. Excision and dark incubation were started on the 13th day (hereafter, day 0). The senescence of detached leaves was characterized by a decrease in protein and chlorophyll levels and an increase in ammonium content. The levels of protein and chlorophyll decreased continuously during senescence, with a decrease of more than 48% and 65%, respectively, after 6 days (Fig. 1A, B). Ammonium levels increased at least 4-fold during 6 days of dark-induced senescence, concomitantly with the decrease in protein and chlorophyll levels (Fig. 1C).

2.1.2. Electrolyte leakage

Percent electrolyte leakage (EL) increased during senescence of detached leaves, from about 7% on day 0–15% after 6 days of incubation (Fig. 2A). pH in the leakage medium changed from 9.11 to 9.18 between days 0 and 6 of incubation (data not shown). There was a negative correlation between electrolyte leakage and protein content, whereas electrolyte leakage and leaf ammonium content correlated closely (Fig. 2B and C).

Electrolyte leakage measurements quantify all charged solutes in the bathing medium, but it does not provide information concerning the identity of these solutes. We measured K^{1+} and NH_4^{1+} concentrations in the bathing solution used for electrolyte leakage assays during senescence of detached leaves in darkness (Fig. 3A and B). EL increased by about 67% (from 7% EL to around 11% EL) between days 0 and 3 (Fig. 2A), and this correlated with a 71% increase of ammonium efflux into the bathing solution in the same period of time. By contrast, the final concentration of K^{1+} in the leachate was the same at 0 and 3 days after incubation. Conductivity was measured in control solutions with different concentration of NH_4^{1+} or K^{1+} (Table 1). Solution conductivity increased by 8 and 2.8 dS cm^{-2} per ppm increase in the concentration of NH_4^{1+} and K^{1+}, respectively. With this information, we calculated that 85% of the increase of EL on day 3 was accounted for by NH_4^{1+} leakage. This is confirmed by the close correlation between EL and ammonium concentration in the bathing solution ($r^2 = 0.82$), whereas the correlation between EL and K^{1+} was lower ($r^2 = 0.25$, Fig. 3C and D). The increase of electrolyte leakage showed a close correlation with K^{1+} leakage only at the end of the experiment, on day 6, when senescence was very advanced (chlorophyll and protein levels had decreased about 74% and 50%, respectively, and ammonium levels had increased approximately four fold).

2.1.3. Regreening of senescing leaves

Loss of membrane intactness, usually inferred from increased EL values [e.g., 1,8,9,20,21] represents a serious and potentially irreversible condition leading to loss of compartmentation and cell death [22]. We analysed the reversibility of senescence-associated symptoms, i.e., the regreening capacity of detached leaves of barley after 3, 6 and 8 days of senescence induction. Leaves were induced to regreen by returning them to light conditions and treatment with a standard nutrition solution added with cytokinin. To follow regreening, relative Chl content was measured non-destructively with an SPAD metre, and data were expressed as the difference between initial and final SPAD readings, after two days of regreening treatment (Fig. 4). After 3, 6 and 8 days of senescence induction, % EL increased in a time-dependent way. However, leaves were still quite capable of regreening (delta SPAD was positive, SPAD readings increased by 4 units in two days) on day 3, while this capacity seemed to be lost after 6 or 8 days in darkness (delta SPAD were negative). Regreening capacity on day 3 suggests that cells were functional and the integrity of membranes was still not severely compromised, in spite of the significant increase in EL (Fig. 2A).

2.1.4. Estimation of cell membrane integrity

Propidium iodide (PI) staining can be reliably used to estimate integrity of membranes because PI is membrane impermeant and,

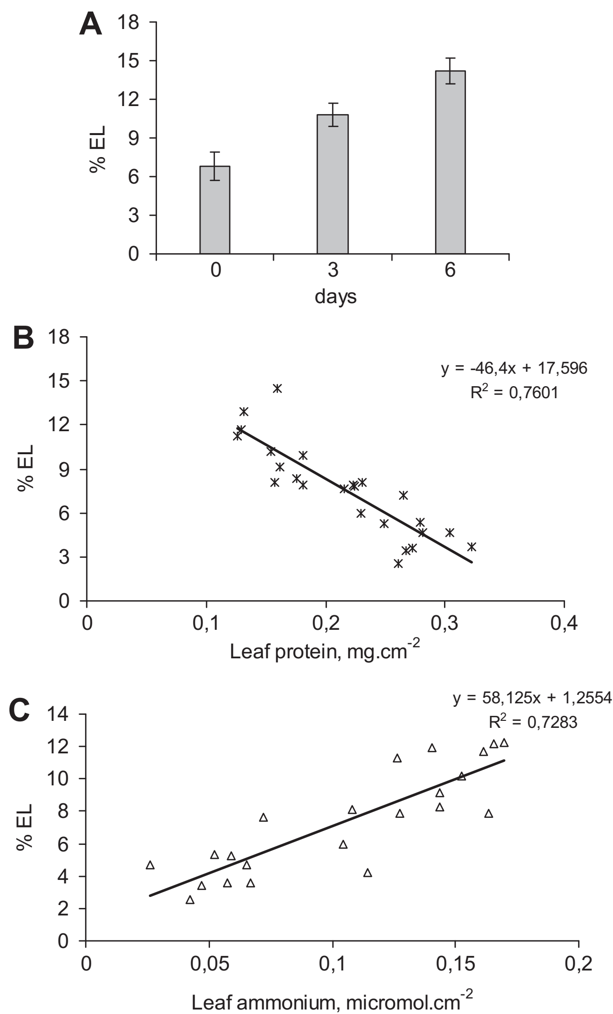

Fig. 2. Changes in electrolyte leakage (%EL) during senescence of detached barley leaves (A). Different letters indicate differences significant at $p < 0.05$. Panels B and C show the relationship between %EL and leaf protein (B) or leaf ammonium (C) concentration. Each data point represents the mean of eight independent measurements. Bars indicate standard deviation.

therefore, it is excluded from cells with an intact plasma membrane [23]. PI binds to DNA and RNA by intercalating between bases with little or no sequence preference and with a stoichiometry of one molecule of dye per 4–5 base pairs. Once the dye is bound to nucleic acids, its fluorescence is enhanced by 20- to 30-fold. We used PI to estimate cell death by fluorescence microscopy of mesophyll cells of detached leaves after 0, 3 and 6 days in darkness. There was no significant staining of cells in leaves incubated for 0 and 3 days (Fig. 5D and F), in spite of a significant decrease in SPAD readings (from 32 to 14, on days 0 and 3, respectively) and an increase in EL. On days 0 and 3, only nuclei of wounded cells adjacent to the cut end of the segment were stained. A measurement of the proportions of leaf K^{1+} and NH_4^{1+} leaked into the bathing solution on day 3 (Table 2) clearly shows that leaves leaked 70% of their NH_4^{1+} content, as compared to only 8.8% of their K^{1+}. This clearly indicates differential permeability of the cell membrane towards different ions, which implies a large degree of membrane intactness. In contrast, after 6 days in darkness, some nuclei were stained by PI in the mesophyll cells of the mid portion of leaf segments, away from the cut ends, indicating that cell death was starting to spread over the mesophyll at that time in senescing leaves (Fig. 5H and J).

2.2. Senescence of attached leaves

The senescence of attached leaves can be accelerated by darkening an individual leaf while the rest of the plant remains illuminated [24], which allows for export of degradation products, e.g., amino acids. In attached leaves of different ages, senescence-induced ammonium accumulation was much lower than in detached leaves [17], possibly because the amino acids released from protein degradation are exported out of the leaf, rather than become a source for ammonium production. The time course of senescence in attached barley leaves subjected to darkness was monitored through changes in protein and chlorophyll levels and ammonium accumulation (Fig. 6). Protein and chlorophyll levels decreased, and ammonium increased during senescence of attached leaves, as seen previously with detached leaves. However, compared to detached leaves, in attached leaves the rate of protein degradation was faster in the beginning, between 0 and 3 days, while ammonium accumulated later, between days 3 and 6 (Fig. 6A and C).

EL increased during senescence of attached leaves, but the increase occurred later than in detached leaves, between days 3 and 6 (Fig. 7A), and there was no correlation between EL and protein degradation ($r^2 = 0.05$; Fig. 7B). There was a close correlation between EL and ammonium accumulation in tissues ($r^2 = 0.69$; Fig. 7C). The increase of EL between days 3 and 6 correlated with a slight increase in the amount of ammonium leaked out of leaf discs on day 6 (Fig. 7D and E). However, we calculated that only 10% of the increase of EL on day 6 was accounted for by NH_4^{1+} leakage. There was no pH change in the bathing medium (data not shown).

3. Discussion

3.1. Electrolyte leakage during senescence with or without N remobilization

The main symptoms of senescence are the decline in chlorophyll and protein levels, a decrease in photochemical efficiency and membrane ion leakage. There are many reports where electrolyte leakage is used as the standing method for the estimation of membrane integrity in relation to environmental stresses, development, senescence and fruit ripening [e.g., 1,3,9,25], and EL has been put forward as the most accurate indicator of senescence progression [10]. Electrolyte leakage from leaves is dependent on the senescence status. Only a small leakage was detected from mature (non-senescing) leaves, whereas leakage became more important as senescence progressed [5; Figs. 2 and 7 in this paper]. During senescence, components of the photosynthetic apparatus are degraded for the remobilization and recycling of N, and frequently this is accompanied by ammonium production which may accumulate in the apoplast [16]. The leaf apoplastic NH_4^{1+} concentration increases with NH_4^{1+} concentration in the cytosol; for example, inhibition of glutamine synthetase leads to a rapid and very substantial increase in apoplastic NH_4^{1+} and barley mutants with reduced Gln synthetase activity have increased apoplastic NH_4^{1+} concentrations relative to wild-type plants [17]. If ammonium accumulates in the apoplast, diffusion into the bathing solution might lead to an overestimation of cell electrolyte leakage.

The aim of this study was to test the contribution of ammonium accumulation due to protein degradation and amino acid de-amination to increased EL of senescing barley leaves. We used two model systems in which leaf senescence is induced by protracted incubation in darkness, and which presumably differ in terms of the accumulation of the products of protein degradation. In detached leaves, in which senescence occurs without N remobilization because the leaves are severed from the sink tissues of the rest of

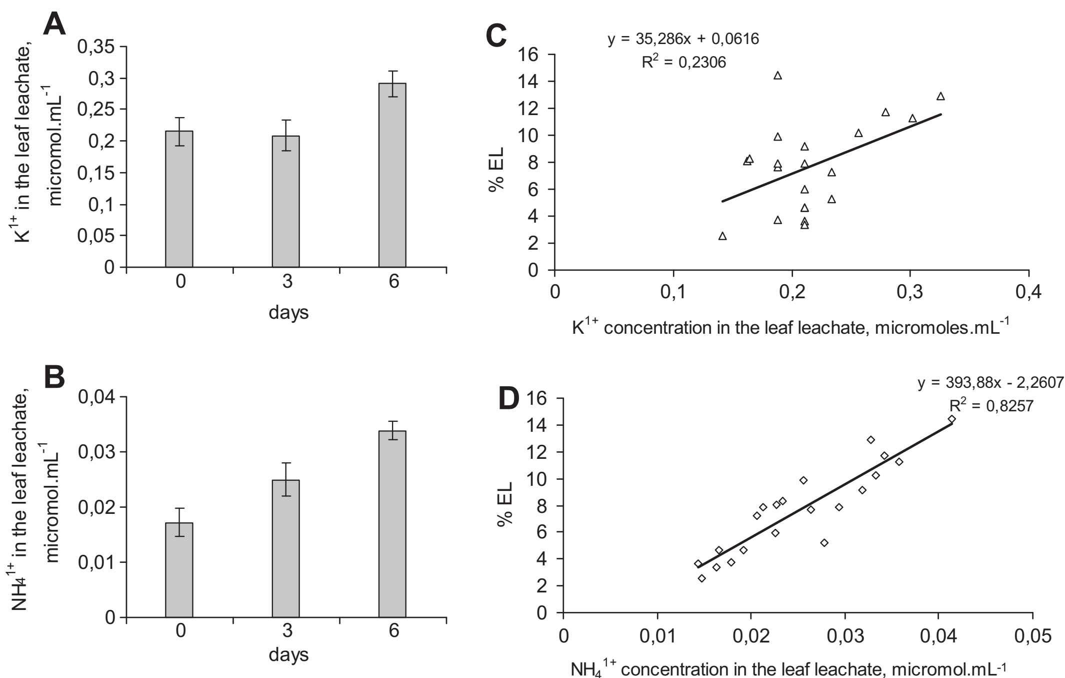

Fig. 3. Levels of K^+ (A) and NH_4^+ (B) in the bathing solution used for electrolyte leakage assays during senescence of detached leaves. Both ions were measured at the end of the 3 h leakage period. Panels C and D show the relationship between % EL and K^+ (C) or NH_4^+ leakage (D) during senescence of detached leaves. Bars indicate standard deviation.

the plant, amino acids and ammonium accumulate as a result of protein degradation. In contrast, in attached leaves senescence is coupled to N remobilization, and there is less accumulation of ammonium because amino acids are exported to other parts of the plant.

With both models, we show that there is a close correlation between accumulation of ammonium in leaves and % EL. During senescence of detached leaves (i.e., without N remobilization), leaf ammonium content and EL started to increase early (day 3), and there was a close correlation between EL and both, ammonium leaked into the bathing solution and ammonium accumulation in leaf tissues. The correlation between EL and K^{1+} leakage was lower, and only on day 6, when senescence was quite advanced, there was an increase of K^{1+} leakage (Fig. 2). We calculated that 85% of the early increase in % EL was due to the increase of NH_4^{1+} leakage (Table 1).

During senescence of attached leaves (with N remobilization), lower amounts of ammonium accumulated in leaves, and ammonium accumulation occurred later (Fig. 6C). However, as with detached leaves, changes in EL followed the same trend as leaf ammonium content and ammonium leaked into the bathing solution used for EL determination (Fig. 7A). These results show that ammonium can be the main ion contributing to increased electrolyte leakage during senescence of leaves.

Bajji et al. (2002) [26] analysed the use of electrolyte leakage to assess cell membrane stability as a water stress tolerance index in durum wheat. Although K^{1+} and Na^{1+} appeared to be the main ions contributing to the conductivity of the leachate (ammonium was not measured in this work), the increase in inorganic ion leakage could not fully account for the recorded increase in electrical conductivity (EC) in these experiments. Bajji et al. (2002) [26] hypothesized that organic ions could also be involved. Since water stress can cause protein degradation [27,28] it seems possible that amino acids and ammonium accumulation were responsible for the increase in electrolyte leakage in response to water deficit.

Given that ammonium accounts for a large part of the increase in electrical conductivity in electrolyte leakage measurements of

Table 1
Changes in electrical conductivity in standard solutions with different concentrations of NH_4^+ or K^+.

ppm	K^+ (μm mL^{-1})	NH_4^+ (μm mL^{-1})	Conductivity μS cm^{-1} (K^+)	Conductivity μS cm^{-1} (NH_4^+)
0	0	0	2.4	2.3
0.5	0.013	0.028	3.7	6.1
1	0.026	0.056	4.9	10.5
1.5	0.039	0.084	6.5	15.4
2	0.056	0.112	7.4	19.8
2.5	0.069	0.140	9.2	23.4
			$y = 3.1929x + 1.9107$ $r^2 = 0.9945$	$y = 8.1929x + 2.3821$ $r^2 = 0.9944$

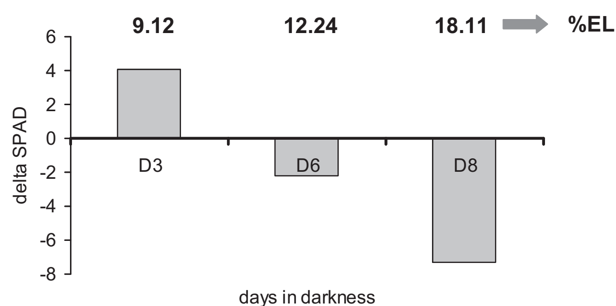

Fig. 4. Regreening capacity of detached, senescing barley leaves. Leaves senescing in darkness for 3, 6 or 8 days were transferred to light conditions and supplied with a nutrient solution added with cytokinins to test their regreening capacity. ΔSPAD is the difference between SPAD readings before and after 2 days of regreening treatment. ΔSPAD was positive if the leaf regreened upon transfer back to light conditions. % EL was measured at the start of the regreening treatment (i.e., after 3, 6 or 8 days of senescence in darkness). Each data point represents the mean value of 10 leaves measured.

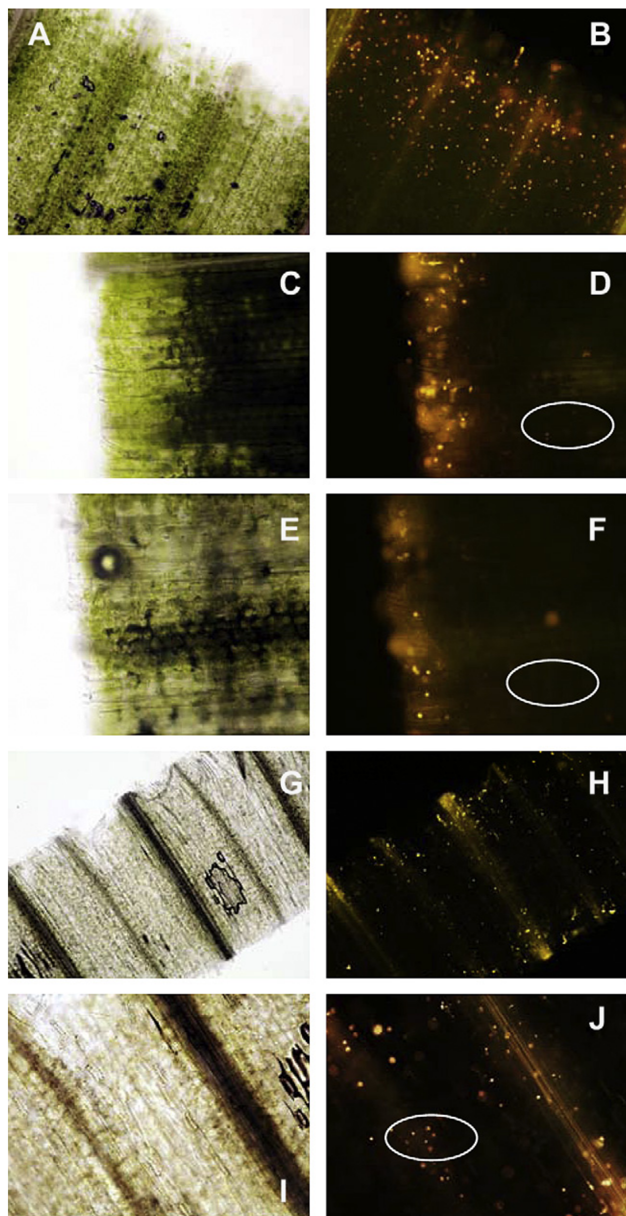

Fig. 5. Brightfield (A, C, E, G, I) and fluorescence (B, D, F, H, J) images of cross sections of barley leaves. Small segments (13 mm long) of detached barley leaves were stained with propidium iodide (stained nuclei are seen orange in fluorescence images). A and B (10×) are positive control samples, where leaf sections were boiled for 10 min before incubation with PI and observation. C and D (20×) are samples from 0 d; E and F (20×) are samples from 3 d; G and H (10×) and I and J (20×) are samples from 6 d. Ovals mark comparable areas of the mesophyll, at a similar distance from the cut edge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Proportions of ammonium and potassium leaked on day 3.

	Ammonium	Potassium
Ion content in leaf tissues	3.12 ± 0.25 μmol cm^{-2}	5.76 ± 0.62 μmol cm^{-2}
Ion content in the electrolyte leachate	2.23 ± 0.22 μmol cm^{-2}	0.51 μmol cm^{-2}
% Ion leaked	70%	8.85%

The proportions of ammonium and K^+ leaked in the bathing solution used for EL measurements in detached leaves after 3 days in darkness. The ion content in the electrolyte leachate is expressed on the basis of the leaf area of the discs used for the EL measurement. % ion leaked = ion content in the electrolyte leachate/ion content in leaf tissue x 100. Values are means ± SEM.

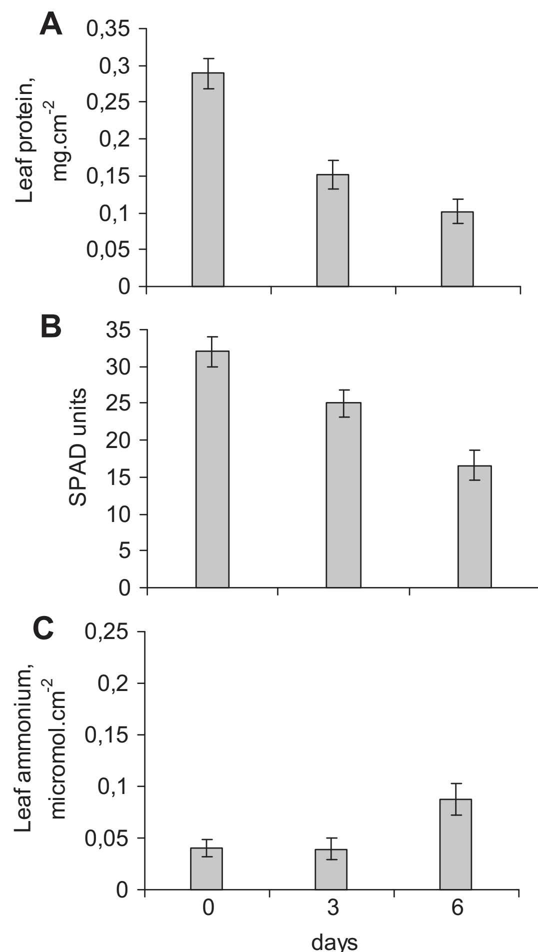

Fig. 6. Changes in the levels of leaf proteins (A), chlorophyll (B) and ammonium (C) during senescence of attached barley leaves subjected to darkness. Protein and ammonium contents are expressed on the basis of leaf area. Eight extracts were made for each treatment and incubation time. Bars indicate standard deviation.

senescing leaves, an important question is whether ammonium in the leachate is an unequivocal symptom of membrane damage. There is evidence for physiological ammonium efflux in leaves, particularly when intracellular levels of ammonium increase. For example, inhibition of glutamine synthetase in Brassica leaves impairs refixation of photorespiratory ammonium, and this might lead to an increase of intracellular ammonium concentration. In such conditions, apoplastic ammonium content increases 25-fold, and this is accompanied by ammonia emission [29]. An energy-driven, outward efflux of ammonium to keep cytosolic NH_4^{+1} concentrations outside of the toxic range has been described [30]. Several papers [e.g., 16,19,31,32] and our own data reported here (Fig. 1C) show ammonium accumulation in senescing leaves. Thus, outward movement of ammonium in senescing leaf cells may be a physiological response to increased intracellular ammonium levels associated with protein degradation and amino acid deamination. It is possible that, during senescence, ammonium might use the some transporters as K^+ to efflux across a relatively undamaged membrane. Because K^+ and NH_4^+ are univalent cations with similar hydrated atomic radius it has been suggested that they share a common transporter, and that K^+ may alleviate NH_4^+ toxicity by competing with NH_4^+ at the transport level [33]. Recent $^{13}NH_4{}^+$ work in barley has confirmed the K^+-dependent reduction of toxic NH_4^+ fluxes [34].

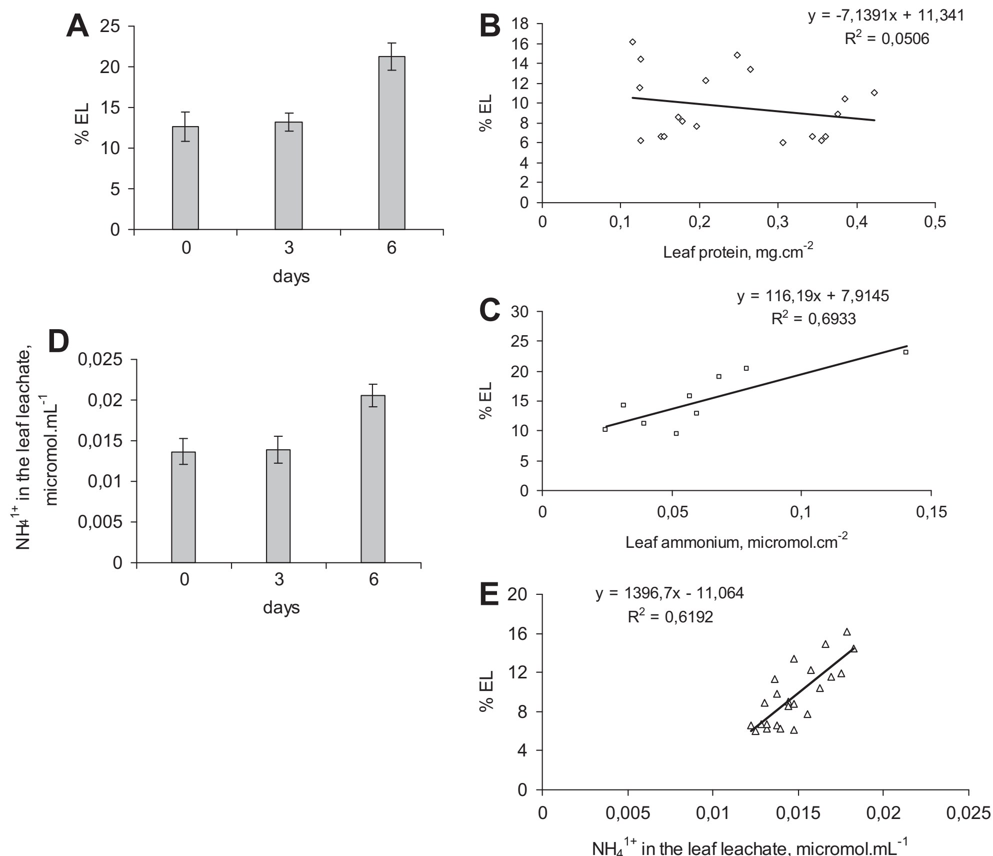

Fig. 7. Change in %EL during senescence of attached barley leaves (A). Panels B and C show the relationship between % EL and leaf protein (B) and ammonium (C) content. Panel (D) show the levels of NH_4^+ in the bathing solution used for electrolyte leakage assays during senescence of attached leaves. Ions were measured at the end of the 3 h leakage period. Panel (E) shows the relationship between %EL and ammonium in the leaf leachate. Bars indicate standard deviation.

3.2. Electrolyte leakage as an estimate of membrane integrity during senescence

An important goal of this work was to determine if increased EL is an unequivocal symptom of senescence-associated membrane damage. Three lines of evidence indicate that EL increased substantially in excised leaves senescing in darkness for 3 days without significant damage to the plasma membrane. First, the plasma membrane appeared to retain selective permeability, since the proportions of ammonium and K^{1+} leaked after 3 days were quite different (Table 2). Leaf discs leaked more ammonium than K^{1+} even though the tissue content of K^{1+} was much higher than that of ammonium. Second, we estimated membrane integrity and cell death by PI staining. PI, a cell impermeant dye, is commonly used to identify dead cells [35,36] because, if the integrity of membranes is lost, PI can diffuse into cells and bind to nucleic acids, which are otherwise inaccessible to PI. We showed that there was no difference in PI staining after 0 and 3 d in darkness (Fig. 5C, D, E, and F), in spite of a significant increase in EL. Third, there are two phases in dark-induced senescence, an initial reversible phase followed by an irreversible phase [37], and it is possible to revert chlorophyll loss and progression of dark-induced senescence during the first reversible phase by re-illuminating the leaves. Detached leaves showing different degrees of yellowing regreened slowly, but recovered their pre-senescence Chl levels more rapidly when cytokinin was also supplied [38]. Similarly, in young wheat seedlings senescence progressed through an initially reversible phase, but after some days in darkness senescence became irreversible [39]. Senescence reversion is only possible if cellular compartmentation is maintained, and the plasma membrane is not severely disrupted. We found full regreening capacity in detached leaves after 3 d in darkness (Fig. 4), in spite of a significant increase in % EL. Together, these data suggest that EL may increase even when senescence is still fully reversible (i.e., up to 3 days in darkness), and membrane disruption is probably still not significant to compromise cell viability.

4. Conclusions

Changes in EL followed the same trend as ammonium accumulation in senescing leaves, with and without N remobilization, while the correlation between EL and K^{1+} leakage, which is presumably associated with membrane damage, was much lower. In detached leaves EL increased during the first 3 days of incubation in darkness, when cell viability and membrane integrity had not changed. Thus, in the early stages of senescence, EL increases if there is a potential for ammonium accumulation associated with protein degradation, but EL does not change where senescence is not accompanied by ammonium accumulation (e.g., early senescence of attached leaves). Our data suggests that EL may be more closely related to protein degradation and ammonium accumulation than to membrane damage, and, therefore, results of EL tests should be interpreted with caution.

5. Materials and methods

5.1. Plant material and experimental design

Barley (*Hordeum vulgare* L. cv. Scarlet) seedlings were grown under controlled conditions (20 °C, 16 h light at 300 μmol m^{-2} s^{-1})

in 250 mL pots filled with soil. The pots were watered daily with deionized water. To study senescence of detached leaves, fully expanded primary leaves were detached, placed in Petri dishes on moist filter paper, and kept in the dark at 20 °C for 6 d. Samples were collected 13 (0 d in dark: D0), 16 (3 d in dark: D3) and 19 (6 d in dark: D6) days after sowing. For experiments involving dark-induced senescence of attached leaves, plants were grown under the same conditions as described above. Fully expanded, attached primary leaves were wrapped in aluminum foil, and samples were collected 13 (D0), 16 (3 d in darkness: D3) and 19 (6 d in darkness: D6) days after sowing.

5.2. Chlorophyll content

Relative chlorophyll content per unit leaf area was determined using an SPAD (Soil Plant Analysis Development) analyser (SPAD-502 Chlorophyll Meter, Konica Minolta, Tokyo, Japan).

5.3. Protein content

Three freshly cut leaf discs (0.5 cm diameter each disc) were homogenized with 500 μL of buffer (50 mM tris hydroxy-methyl aminomethane-HCl, pH 7, 2 mM EDTA and 1 mM PMSF). Extracts were centrifuged at 10,000 × g for 10 min at 4 °C and proteins were determined according to [40]. Bovine serum albumin was used as standard.

5.4. Ammonium content in leaves

Eight freshly cut leaf discs (0.5 cm diameter each) were homogenized with 1 mL of 0.3 mM H_2SO_4. Samples were centrifuged at 10,000 × g for 10 min at 4 °C and ammonium was determined with Indophenol blue according to [41]. $(NH_4)_2SO_4$ was used as standard.

5.5. Potassium content in leaves

Five freshly cut leaf discs (0.5 cm diameter each) were reduced to ash and then were solubilised with a HCl solution 0.005 M. K^{1+} content in leaves was determined by flame photometry [42].

5.6. Electrolyte leakage (EL)

Twenty five freshly cut leaf discs (0.5 cm diameter each) were floated on 15 mL of deionized water with continuous shaking. The electrolyte content in the solution was measured immediately (C0) and after 3 h (C3) of incubation at room temperature using a conductimeter (Hama Instruments Ltd., El-Hama, Israel). Total electrolyte content was determined in the same way after boiling for 10 min (TC). Results were expressed as percentage of electrolyte leakage:

$$\% \text{ EL} = 100 \times (\text{C3-C0})/\text{TC}$$

5.7. Ammonium and potassium content in the electrolyte leachate

Twenty five freshly cut leaf discs (0.5 cm diameter each) were floated on 15 mL of deionized water with continuous shaking as indicated for electrolyte leakage determinations. The bathing solution containing the leaked electrolytes was used to measure K^{1+} and NH_4^{1+} by standard methods of analysis for aqueous solutions. Flame photometry was used for K^{1+} [42], and the Nessler reagent was used for NH_4^{1+} [42]. We compared Nessler and Indophenol Blue methods of ammonium analysis in the bathing solution and we had got height correlation ($r^2 = 0.97$).

5.8. Regreening experiment

Fully expanded primary leaves were detached, placed in Petri dishes on moist filter paper, and kept in the dark at 20 °C for 3–8 d. After 3, 6 and 8 d, leaves were transferred to a light/dark regime (10 h d^{-1} light with 150 μmol m^{-2} s^{-1}) with their cut ends immersed in a standard nutrition solution added with zeatin (10 μM). During the regreening period (two days), relative chlorophyll content per unit leaf area was determined daily using an SPAD metre. Regreening capacity was expressed as δSPAD = final SPAD − initial SPAD, where final SPAD is the SPAD reading after two days of regreening treatment and initial SPAD is the corresponding value at the start of the regreening treatment, i.e., D0, D3 or D6.

5.9. Estimation of live/dead cells by fluorescence microscopy

The membrane impermeant DNA stain propidium iodide was used to estimate cell integrity in leaf segments. Propidium iodide (PI, Sigma, St Louis, MO) was dissolved in phosphate buffer (25 mM, pH 7) and used at a concentration of 2.5 μg mL^{-1}. Leaves were sectioned into small pieces (3 mm long sections) and vacuum infiltrated with PI for 10 min at room temperature. The excess dye was washed with buffer before observation. The sections were mounted on glycerin: water (1:1) and observed. The samples were collected from detached leaves placed in Petri dishes on moist filter paper, and kept in the dark at 20 °C for 3 and 6 d. At least three different samples for each time point were observed, and the experiment was repeated twice. Positive controls were boiled for 10 min to completely disrupt cell membranes. Slides were examined with a Fluorescence Microscope (Olympus BX51), and a 545/580 nm excitation/emission filter (R&B Phycoerythrin).

5.10. Statistical analysis

Data were analysed by ANOVA, and the means were compared with the Tukey's test at a significance level of 0.05. For regression analysis, r^2 values are reported if they are significant at $p < 0.05$.

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