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# Ammonium overaccumulation in senescent leaves as a novel exclusion mechanism to avoid toxicity in photosynthetically active rice leaves

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A B S T R A C T

High ammonium concentration is toxic for most plant species, but some rice cultivars are tolerant by mechanisms not understood yet. We tested the hypothesis that $NH_4^+$-tolerance could be associated with an exclusion mechanism involving ammonium deposition on the oldest leaves after triggering localized senescence. An ammonium-tolerant cultivar was exposed to increasing ammonium ($NH_4^+$) concentrations for two weeks and afterward subjected to 15 mM throughout 4 weeks, to evaluate toxicity and resistance mechanisms. Plants exposed to similar nitrate ($NO_3^-$) concentrations were taken as control. In presence of high $NH_4^+$ rice displayed similar nitrogen (N) root influx compared to nitrate-supplied roots. In parallel, these plants exhibited a strong impairment in root growth even after exposure to mild $NH_4^+$ concentration (3.75 mM). This response was related to increased activity of type III peroxidases, but no evidence for oxidative stress and tissue damages on roots were apparent. Intriguingly, high $NH_4^+$-supplied plants displayed a prominent increase in dry mass of senescent oldest leaves, which were 5-fold higher than that observed in nitrate-supplied ones. Ammonium-treatment induced strong $NH_4^+$ accumulation on the dead leaf tissues (by 2.8 mmol $g^{-1}$ DW) representing 75 % of the total-N in this part. This high deposition rate of potentially toxic $NH_4^+$ associated with a “self-destruction” strategy should indicate an exclusion mechanism. In contrast, mature leaves presented low $NH_4^+$ content, which was associated with no alterations in both $CO_2$ assimilation and photosystem II activity. Our data suggest that rice displays an unusual $NH_4^+$ exclusion mechanism by triggering fast and intense senescence on its older leaves. Afterwards, this process is followed by the death of these leaves and deposition of extremely high amounts of this toxic component but preserving younger photosynthetically active tissues.

## 1. Introduction

High ammonium concentration is toxic for the majority of plant species and this problem might affect the yield of several crops. Ammonium toxicity decreases plant growth by many direct and indirect mechanisms some of which have been well characterized in different species. The most common reported adverse constrain is the induction of stunted root growth (Araya et al., 2016; Liu et al., 2013), oxidative stress (Li et al., 2019; Podgórska et al., 2013; Xie et al., 2015), earlier leaf senescence (Wu et al., 2016), reduction in photosynthetic efficiency (Alencar et al., 2019) and intensified respiratory metabolism (Hachiya et al., 2010). However, despite plant tolerance to excess ammonium varies largely among species, the involved underlying mechanisms or genetic traits are still poorly understood (Esteban et al., 2016). In this context, the use of rice as a plant model is interesting because some cultivars are adapted to paddy soil conditions, where commonly they face high $NH_4^+$ concentrations (Wang et al., 2020).

Although several studies have attempted to investigate how plants can deal with high ammonium exposure, paradoxically, the physiological mechanisms that might confer tolerance are only partially known to date (Esteban et al., 2016; Liu and Von Wirén, 2017). Often, studies aiming to investigate such questions have adopted fragmented approaches, which are not enough to provide robust mechanistic conclusions. Some works have suggested that tolerant species could have developed an efficient enzymatic system associated with $NH_4^+$-assimilation represented by the GS/GOGAT cycle and/or

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glutamate dehydrogenase – GDH, which could be highly stimulated in different plant tissues (Jung et al., 2015; Sun et al., 2020). In parallel, these plants might also display a less intense $NH_4^+$ futile cycle at the plasmalemma level, contributing to lower respiratory drain (Balkos et al., 2010; Britto and Kronzucker, 2002).

Rice plants supplied with a high ammonium supply commonly exhibit stunted root growth and this response is frequently considered as a toxicity symptom (Di et al., 2018; Hachiya et al., 2010; Sun et al., 2020; Xie et al., 2015). However, the cogitation that such a decrease in growth also could involve an avoidance mechanism, protecting the root against excessive exposure to that hazardous molecule, is very reasonable. Indeed, some plant species when challenged by excess toxic elements around roots like high salinity can down-regulate the root expansion as a protective mechanism (Maia et al., 2013). However, it is very difficult to distinguish among generalized physiological responses from those related to specific avoidance mechanisms, such as those involved with avoiding $NH_4^+$-toxicity. Indeed, understanding these differences is complex and probably due to this, to date, is not still understood how some rice cultivars are extremely tolerant against high $NH_4^+$ concentrations (Alencar et al., 2019).

In the case of salt exclusion mechanism, tolerant plants commonly display the regulation of specific ion transporters, to avoid toxicity in the cytosol by regulating homeostasis in vacuoles and xylem (Shi et al., 2002; Volkov and Beilby, 2017; Wu and Li, 2019; Xu et al., 2012; Yang et al., 2009; Zörb et al., 2005). For instance, the expression of some genes/proteins able to regulate the $Na^+$ and $K^+$ transport through cellular membranes might also control the levels of these ions in different cell compartments and plant tissues (Shabala and Pottosin, 2014; Yaish et al., 2017). Some species are even able to drive salt deposition on leaves in specialized compartments as an effective exclusion mechanism, avoiding accumulation in younger tissues (Silveira et al., 2009; Schippers et al., 2015; Wang et al., 2012). This mechanism generally is followed by senescence, cellular death and leaf abscission (Schippers et al., 2015).

In the specific case of $NH_4^+$ toxicity avoidance, in contrast to salt ions and some heavy toxic metals, the studies are scarce. The transport of $NH_4^+$ by plasmalemma and xylem can trigger the expression of specific transporters which are tissue- and ammonium concentration-dependent (Hao et al., 2020; Yuan et al., 2007). Several works using rice and Arabidopsis plants have evidenced that $NH_4^+$ in high concentrations is capable to trigger earlier leaf senescence, possibly involving changes in ABA and ethylene pathways (Jian et al., 2018; Li et al., 2013, 2012, 2019; Sun et al., 2020). However, is not clear if these responses in rice are associated simply with toxicity or if they could represent some kind of protective strategy. If this response is part of a regulated mechanism for plant defense, it could has presupposed that the sacrificing of oldest leaf tissues via induction of localized senescence followed by cell death could allow a high deposition of ammonium on these parts.

These mechanisms, still not reported in the literature, could protect young and mature photosynthetic tissues against direct and indirect $NH_4^+$ toxicity. This type of exclusion mechanism for excess ammonium in plants, to the best of our knowledge, was never reported for $NH_4^+$ tolerance neither in rice nor in any other plant species. Possibly, under extreme conditions of high ammonium pressure via N fertilization, or in presence of favorable conditions for its accumulation, such as in paddy soils, that physiological adaption could represent an extreme response, but an effective strategy to face with that adverse environmental condition. Our group has recently demonstrated that high $NH_4^+$-supply for rice plants, in short-term exposure, especially when combined with high light, is capable to induce earlier senescence at the reproductive stage (Alencar et al., 2019). In parallel, these plants have displayed deep alterations in photosynthesis and related processes such as stomatal closure and strong photorespiration enhancement.

More recently we have empirically observed that when those plants were progressively exposed to increasing $NH_4^+$ concentrations, from seedlings to mature stages, they presented earlier, and intense senescence followed by cell death localized on the oldest leaves. To understand if this *phenomenon* could represent an effective exclusion mechanism for excess ammonium, we carried out this study. Meaningfully, our results strongly corroborate that assumption, demonstrating that rice plants can display an unusual mechanism represented by $NH_4^+$-induced senescence followed by death on the oldest leaves, accompanied by a very large deposition of this toxic substance. In parallel, mature leaves displayed low $NH_4^+$-contents and unchanged photosynthetic efficiency, strongly suggesting that this exclusion mechanism is capable to preserve the younger tissue growth from $NH_4^+$-toxicity. This mechanism is discussed from morpho-physiological and partitioning of N-forms data, associated with photosynthetic parameters.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Rice seeds (*Oryza sativa ssp. japonica cv.* Nipponbare), an ammonium tolerant cultivar (Alencar et al., 2019), were germinated in Germitest® paper under controlled conditions (25 °C, 70 % relative humidity, 100 μmol photons $m^{-2}$ $s^{-1}$ and 12 h photoperiod). Eleven days after sown, seedlings were transplanted to 3 L plastic pots and divided into two treatments: $NH_4^+$ and $NO_3^-$ as the unique N-source. Seedlings were exposed to a gradual increasing of N-source concentrations, beginning with 3.75 mM of $NH_4^+$ or $NO_3$- in a diluted ¼ modified Hoagland-Arnon's nutrient solution (Hoagland and Arnon, 1950), for a week. Afterwards, $NH_4^+$ and $NO_3^-$ concentrations were increased to 7.5 mM in a diluted $^1/_2$ nutrient solution for one week more. Next, 26-day-old plants were supplied with 15 mM of $NH_4^+$ or $NO_3^-$ in a non-diluted Hoagland and Arnon's nutrient solution, for more 4 weeks. The salts $(NH_4)_2SO_4$ and $NH_4Cl$ were utilized as $NH_4^+$-sources and Ca $(NO_3)_2$ and $KNO_3$ as $NO_3$-sources. All other nutrients were provided in an optimum concentration according to composition of the Hoagland and Arnon's solution.

Plants were cultivated in a greenhouse under natural conditions as follow: day/night average temperature of 32/25 °C, mean relative humidity of 65 %, maximum photosynthetic photon flux density (PPFD) approximately 800 μmol $m^{-2}$ $s^{-1}$ at noon, and a photoperiod of 12 h. During the growth period, the pH was adjusted every two days to 6.0 ± 0.5 and the nutrient solution was completely changed every four days to minimize oscillations in N concentrations. In order to evaluate the long-term effects of $NH_4^+$, the morphological changes were regularly analyzed by photos, visually and by measurement of the shoot and root lengths every two days. In parallel, gas exchange and chlorophyll fluorescence were also performed every two days. Periodically and after four weeks of treatment with 15 mM $NH_4^+$ or $NO_3$, Fv/Fm parameter was periodically measured, and leaf samples were taken for chlorophyll and ammonium measurements.

### 2.2. Biomass partitioning determination

To analyze the long-term effects of $NH_4^+$ supply on rice growth, the plant phenotypes were periodically assessed by taking pictures and measurements of root and shoot length. Four weeks after treatment with 15 mM $NH_4^+$ (53-day-old plants), after 42 days of $NH_4^+$ or $NO_3^-$ treatments, plants were harvested and separated into green and senescent-dead leaves, root and culm + tiller parts. Fresh mass was measured immediately and stored at −80 °C freezer for further analyses. Part of the samples were dried at low temperature via lyophilization, and dry mass of roots, green leaves, oldest senescent leaves and culms was calculated from the water content of each part.

### 2.3. Gas exchange and chlorophyll *a* fluorescence measurement

The gas exchange parameters were measured by using a portable infrared gas analyzer system (LI-6400XT, LI-COR, Lincoln, NE, USA) in mature leaves. PPFD and temperature inside the measurement chamber were kept at 1000 µmol $m^{-2}$ $s^{-1}$ and 28 °C, respectively. In all measurements, the amount of blue light was set up to 10 % of the PPFD to maximize stomatal aperture (Flexas et al., 2008), the $CO_2$ partial pressure was 40 Pa and the leaf-to-air vapor pressure difference was 1.85 ± 0.14 kPa. Measurements were recorded when the total coefficient of variation was lower than 5% and temporal stability was achieved (on average, 3 min after the beginning of each step).

in vivo chlorophyll fluorescence was measured using a fluorometer (LI-6400-40, LI-COR, Lincoln, NE, USA) coupled with the IRGA. The actinic light utilized for measuring the chlorophyll fluorescence was 1000 µmol $m^{-2}$ $s^{-1}$ of PPFD. The fluorescence parameters were measured using the saturation pulse method (Schreiber, 2004) in leaves previously exposed to natural light conditions. The intensity and duration of the saturation light pulse were 8000 µmol $m^{-2}$ $s^{-1}$ and 0.7 s, respectively. The effective quantum yield of PSII was calculated [ΦPSII = (Fm'−Fs)/Fm'], where Fm' represents the maximum fluorescence and Fs the steady-state fluorescence under actinic light conditions.

### 2.4. Measurement of potential quantum yield of PSII (Fv/Fm) and chlorophyll content

An experiment was carried out to evaluate changes in the maximum potential quantum efficiency of PSII (Fv/Fm) on the 5th mature leaf after supplying of $NH_4^+$ or $NO_3^-$ in the root medium. After exposing the plants to the highest dose of each N form (15 mM) for 16, 24, 32 and 40 days of treatment, the Fv/Fm was measured weekly with a Dual-PAM 100 fluorometer (Walz, Germany), always using the same leaf. The Fv/Fm was measured according to the saturation pulse technique (Schreiber et al., 1995), where Fv/Fm = [Fm-Fo/Fm], and Fo and Fm are the minimum and maximum fluorescence o leaves previously acclimated for 30 min in the dark. Total chlorophylls were determined in senescent/dead and green/mature leaves after 16 and 32 days of 15 mM exposure, as described by Lichtenthaler (1987). Total Chl content was calculated as mg g $MD^{-1}$ = [(7.15 $Abs_{663}$) + (18.71 $Abs_{647}$)] / g $MD^{-1}$ x D, where Abs is the absorbance and D the dilution used in the assay.

### 2.5. Oxidative stress indicators

Root membrane damage (MD) was measured by determination of the cellular electrolyte leakage as described previously by (Blum and Ebercon, 1981). Basal root samples (~500 mg FW) were transferred to tubes containing 10 mL of deionized water, incubated for 3 h, and electric conductivity in the medium (L1) was measured. Subsequently, the root samples were boiled (98 °C) for 1 h and the electric conductivity in the water medium (L2) was measured again. The relative membrane damage was estimated by MD = L1/L2 × 100. Lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) following Heath and Packer (1968). TBARS concentrations were calculated using the absorption coefficient (155 $mM^{-1}$ $cm^{-1}$), and the results were expressed as ηmol MDA-TBA $g^{-1}$ FW.

Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to measure $H_2O_2$ production (Zhou et al., 1997). For $H_2O_2$ extraction, 400 mg of fresh roots were firstly ground in liquid $N_2$ to a fine powder and then 1 mL of 100 mM potassium phosphate buffer, pH 7.5 was added for homogenization. Subsequently, 100 µL of the supernatant was incubated with 0.2 U $mL^{-1}$ of horseradish peroxidase and 100 µL Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine) at room temperature, for 30 min, under dark conditions. The absorbance was quantified spectrophotometrically at 560 nm, and the $H_2O_2$ content was calculated from an $H_2O_2$ standard curve according to the kit manufacturer's instructions and expressed as µmol $H_2O_2$ $g^{-1}$FW.

### 2.6. Nitrogen influx in roots and determination of total nitrogen, nitrate, ammonium and amino acids

Root nitrogen influx was estimated according to described by Silveira et al. (2001), through direct measurement of N-forms depletion from the nutrient solution, and the results were expressed as mmol $plant^{-1}$ $day^{-1}$. To quantify nitrate, ammonium and free amino acids contents in the different plant tissues, lyophilized samples were incubated with distilled water at 90 °C for 1 h and filtered to obtain a crude extract. Subsequently, the total-N concentration was determined using the adapted Kjeldahl method (Baethgen and Alley, 1989), the nitrate concentration was measured by the salicylic acid reaction (Cataldo et al., 1975), ammonium concentration was determined using the phenol-hypochlorite method (Felker, 1977) and the total free amino acids were measured according to Yemm et al. (1955). The N-forms content was expressed in µmol $g^{-1}$ DW and the accumulation was expressed as µmol per plant part.

### 2.7. Enzymatic activities

For glutamine synthetase measurement (GS; EC 6.3.1.2), fresh leaves, culm and roots were ground until obtaining a fine powder in presence of liquid $N_2$ and then homogenized in ice-cold 100 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 1 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The GS activity was measured according to Hirel and Gadal (1980) and the reaction medium consisted of 50 mM Tris–HCl buffer (pH 7.8) containing 5 mM ATP, 12.5 mM $MgSO_4$ and 25 mM Na-glutamate. The reaction was started with the addition of 3 mM hydroxylamine to the reaction medium and developed for 6 min at 35 °C. Subsequently, the reaction was stopped by the addition of a 10 % (w:v) ferric chloride solution in 0.2 M HCl. The concentration of the brown complex formed by γ-glutamyl-hydroxamate and $Fe^{+3}$ was determined spectrophotometrically by measuring the absorbance at 540 nm. A reference without hydroxylamine was employed to discard side products with similar absorbance. A standard curve was made with γ-glutamyl hydroxamate and the GS activity was expressed as µmol γ-glutamyl hydroxamate (GGH) $g^{-1}$ FW $h^{-1}$.

Ascorbate peroxidase (APX; 1.11.1.11) and class III peroxidase (GPOD; 1.11.1.7) activities were determined according to (Nakano and Asada, 1981) and (Amako et al., 1994), respectively. The crude extract was obtained by macerating 400 mg of fresh tissue in the presence of liquid $N_2$ and the extraction medium consisting of potassium phosphate buffer (pH 7.0), 1 mM EDTA and 1 mM ascorbic acid. APX activity was assayed in presence of 50 mM potassium phosphate buffer (pH 6.0) and 0.5 mM ascorbic acid and the crude extract. The reaction started when 0.1 mL of 30 mM $H_2O_2$ was added, and the decreasing absorbance at 290 ηm was monitored over 300 s (Nakano and Asada, 1981). APX activity was estimated by utilizing the molar extinction coefficient of ascorbate (2.8 $mM^{-1}$ $cm^{-1}$) and expressed as µmol AsA $g^{-1}$ FW $min^{-1}$. GPOD activity was assayed by measuring the rate of guaiacol oxidation. The reaction medium consisted of 50 mM K-phosphate buffer (pH 7.0), 20 mM guaiacol as an electron donor and 0.1 mM hydrogen peroxide. The intensity of the reaction product was measured in a spectrophotometer at 430 nm. GPOD activity was estimated utilizing the molar extinction coefficient of tetra-guaiacol (26.6 $mM^{-1}$1 $cm^{-1}$) and was expressed as mmol tetra-guaiacol $mg^{-1}$ protein $min^{-1}$.

### 2.8. Experimental design and statistical analysis

The experiments were arranged in a completely randomized design, using two different nitrogen sources and four biological replicates per treatment. Each biological replicate was represented by a pot containing two plants. For statistical analysis, the averages were compared by Tukey test at 5% of probability ($p \leq 0.05$) and all analyses were performed using the SigmaPlot 12.0 program (Systat Software, San Jose, USA).

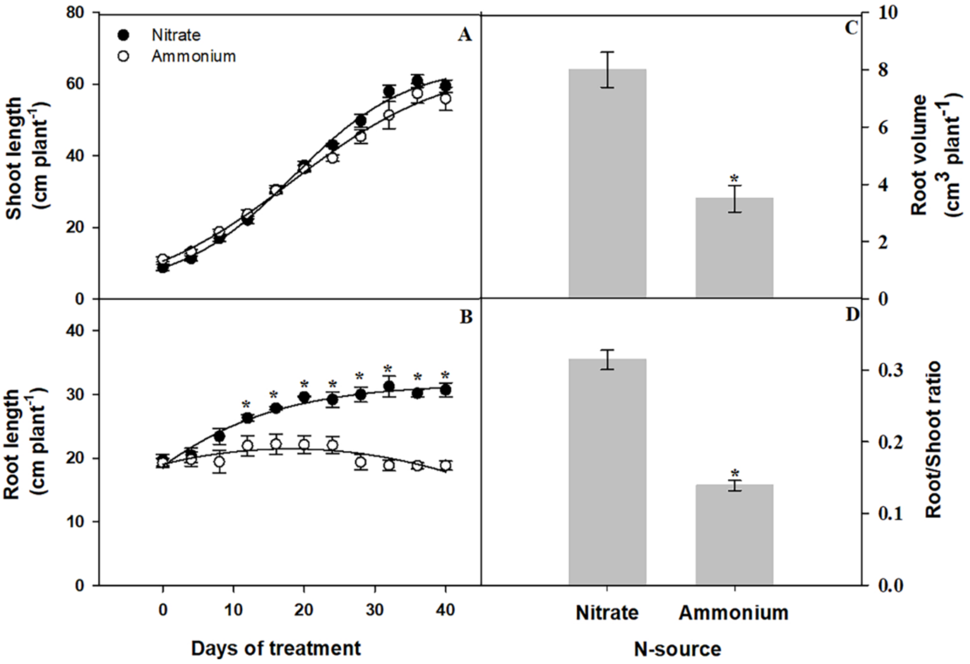

**Fig. 1.** (A) Rates of shoot growth (length) and (B) principal root elongation throughout plant development and (C) accumulated root volume and (D) shoot/root ratios in rice plants exposed to increasing $NH_4^+$ and $NO_3^-$ concentrations. Eleven-day-old seedlings were previously exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3$ for 8 days in each concentration and afterwards subjected to very high ammonium concentration (VHAC, 15 mM) in parallel to nitrate 15 mM. The plants remained under these stressful conditions for 24 consecutive days, reaching the V13 stage (51-day-old, 40 DAT). The values represent averages of four replicates ($n = 4$) and bars indicate the standard error (SE). Curves in (A) were adjusted by Gompertz regression and in (B) by quadratic polynomial adjustment whereas the values represented in (C) and (D) were obtained in 51-day-old plants (40 DAT). Asterisks represent significant differences at the 5% level according to Tukey's test.

## 3. Results

### 3.1. *High ammonium supply induces stunted root growth and increased senescence on oldest rice leaves*

Initially, aiming to understand the effects caused by long-term exposure to high $NH_4^+$ concentrations on morpho-physiological traits of rice, 11-day-old seedlings were exposed to increasing $NO_3^-$ and $NH_4^+$ levels inside the nutrient solution. Firstly, seedlings were exposed to a non-toxic ammonium concentration (3.75 mM) for 8 days to induce acclimation and not-toxic responses. Afterwards, plants were subjected to high, but still not strongly toxicity (7.5 mM) for additional 8 days in the nutrient solution. Next, 27-day-old plants were exposed to very high ammonium concentration (15 mM) for 24 consecutive days, to study toxic effects associated with plant defense mechanisms. Similar $NO_3$-concentrations were employed in parallel as a positive control since that the studied rice cultivar is facultative to $NH_4^+$ or $NO_3^-$ utilization and, this late N-source, when supplied in high concentrations, does not cause any physiological disturbances (Alencar et al., 2019). However, in this current study, exogenous $NH_4^+$, even in lower concentrations, was capable to initially induce restrictions on root growth followed by induction of senescence on the oldest leaves (Figs. 1 and 2).

After 10 days of treatment, the root elongation rates (cm/day) in plants subjected to 3.75 mM for 8 days and followed by more two days under 7.5 mM, exhibited a prominent decrease (~30 %) in comparison to nitrate-supplied roots. Indeed, whereas nitrate-supplied roots presented positive elongation rates, $NH_4^+$ treatment induced an almost complete root elongation cessation. The maximum contrast between roots from nitrate and ammonium-treated plants was achieved on the 36th day of treatment, when the elongation of the ammonium-supplied roots reached only 60 % of that nitrate-supplied plants (Fig. 1B, Fig. S2). Corroborating the data obtained of root elongation, both root volume and root/shoot height ratios exhibited a significant decrease by approximately 50 % at the 36th day, respectively, in comparison to nitrate-plants (Fig. 1C, D). In order to avoid an indirect negative effect related to acidic medium, the nutrient solution pH was adjusted to near 6.0 every two days, in both nitrate and ammonium treatments. Interestingly, high ammonium supply did not induce significant differences in the shoot elongation rates (cm/day), as compared to nitrate treatment, over the entire experimental period (Fig. 1A).

If, on the one hand, the growth data showed that the roots were much more affected than shoots in response to ammonium treatment, the visual appearance of early induced senescence on the oldest leaves was clearly observed in plants treated with ammonium on the 16th day of exposure (plants still exposed to 7.25 mM $NH_4^+$) - (Fig. 2A, Fig. S4). These visual symptoms were corroborated by the total chlorophyll content, which decreased significantly in that treatment (Fig. 2B). Indeed, the older leaves (here identified by numbers 2–6 and called senescent leaves), exhibited about a 50 % decrease in total chlorophyll content, as compared to plants supplied with nitrate. These observations are physiologically very important because, when they are taken together, they seem to indicate that rice plants can trigger the ammonium-induced senescence process localized only on the oldest leaves, preserving the youngest and mature tissues from toxic ammonium accumulation and avoiding, as a consequence, negative effects on photosynthesis capacity.

### 3.2. *Oxidative stress markers suggest that ammonium-induced root growth impairment is part of an exclusion process and not a toxicity effect*

As the root growth was strongly impaired by ammonium exposure, even under moderate concentrations, we tried to answer if that effect was due to toxicity or if it could be related to an acclimation mechanism, in order to restrict the contact of roots with excess $NH_4^+$ in the external medium (an avoidance mechanism). Firstly, we measured the activity of class III peroxidases because these enzymes are directly related to root lignification and growth impairment. Indeed, ammonium supply induced a prominent increase in GPOD activity (by 2-fold), as compared to nitrate treatment (Fig. 3A). On the other hand, total root APX activity displayed no significant differences, regardless of the N-source employed in this study (Fig. 3B). Afterwards, we measured the contents of thiobarbituric acid substances (TBARS) and $H_2O_2$ associated with electrolyte leakage to evaluate if high ammonium supply had induced oxidative stress in rice roots. The obtained data revealed that no significant changes did occur in root $H_2O_2$ and TBARS levels in that treatment, indicating neglectable disturbances on the redox homeostasis and absence of oxidative damage (Fig. 4A). Besides, these results were corroborated by the electrolyte leakage (membrane damage), an important cellular indicator of tissue integrity, which was slightly increased by high $NH_4^+$ supply in comparison to $NO_3^-$ exposure (Fig. 4B).

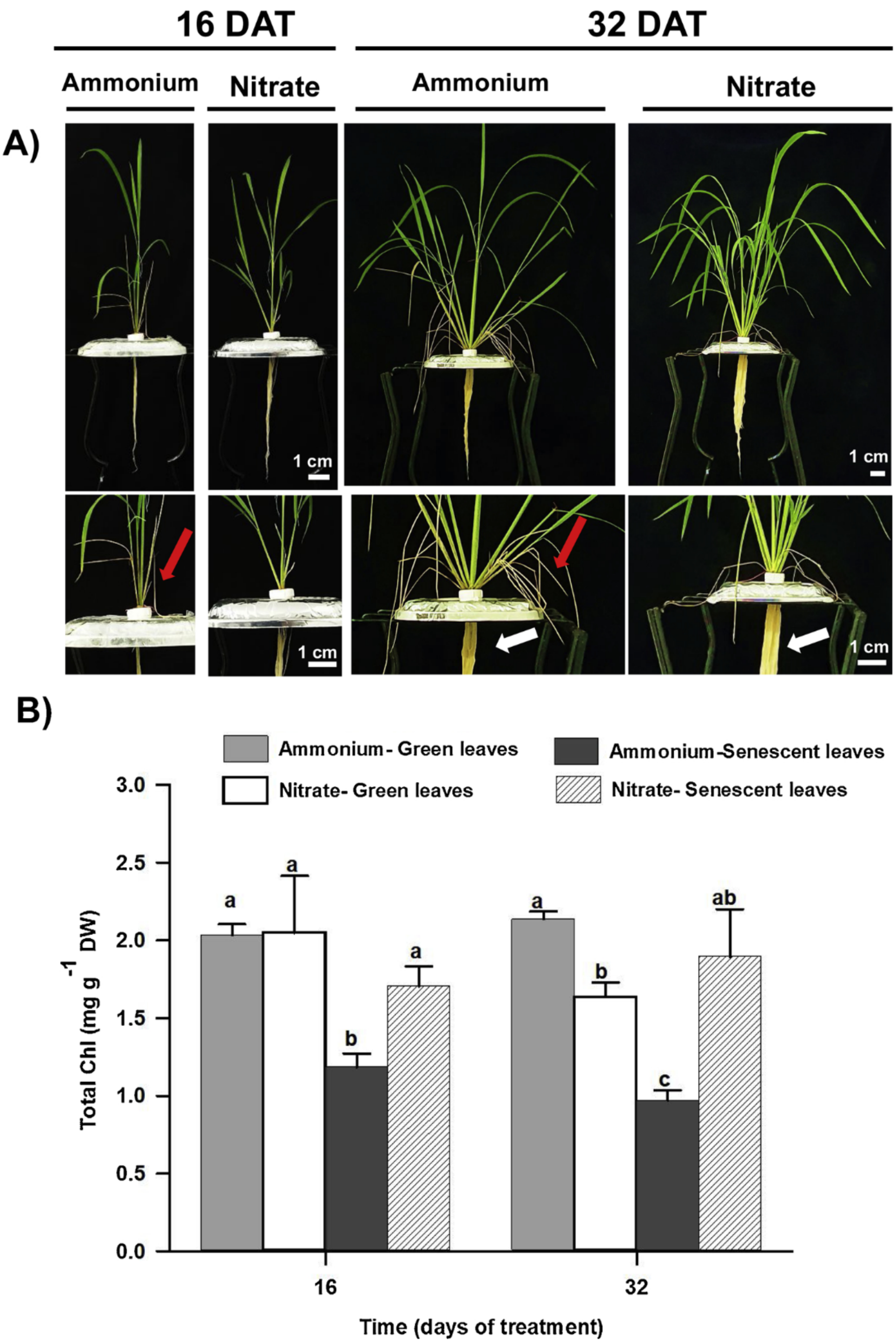

**Fig. 2.** (A) Visual aspects of rice shoot and roots and (B) total chlorophyll content in oldest and green mature leaves after exposure to increasing $NH_4^+$ and $NO_3^-$ concentrations. Eleven-day-old seedlings were initially exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ during 8 days in each concentration and afterwards subjected to 15 mM of $NH_4^+$ or $NO_3$- for more consecutive 24 days (51- day-old plants, 40 DAT). Zero-day after VHAC (0 DAT) was taken as 27-day-old plants previously supplied with 3.75 and 7.5 of $NH_4^+$ or $NO_3^-$ and 16 DAT were plants supplied with 15 mM of $NH_4^+$ or $NO_3^-$ for 16 days more. The red and white arrows highlight the effects of ammonium treatment on induced senescence on the oldest leaves and the great restriction caused by root growth, respectively, in $NH_4^+$-treated plants. To note that ammonium treatment already has induced contrasting effects on leaf senescence and root growth compared after treatment with lower concentrations, compared with nitrate supply. The average values were obtained of replicates and different letters within each experimental period and distinct N-source treatments, represent significant differences at a 5% level according to Tukey's test.

It's important to highlight that this indicator was measured as the ratio between initial electrolyte leakage (in which the apoplastic fraction is very important) and total leakage from the entire cells. Thus, any alterations on the apoplast pool and membrane permeability may induce changes in electrolyte leakage, but this will not be necessarily associated with cell integrity disturbance, especially when these alterations are minor and the reached values are low, as noticed here.

### *3.3. High $NH_4^+$ supplying induces significant changes in the partitioning of biomass and N-compounds in different rice parts probably as an acclimation mechanism*

This experiment was performed to elucidate the morpho-physiological alterations caused by high ammonium supply in terms of biomass and N-compounds partitioning as part of an acclimation mechanism. Interestingly, on the 40th day of $NH_4^+$ treatment, approximately 50 % of the green leaf dry mass was strongly decreased and converted to senescent/dead matter, while in $NO_3^-$-treatment these changes were negligible (Table 1). The dry mass of the senescent leaves represented 15 % of the plant whole biomass in ammonium-supplied

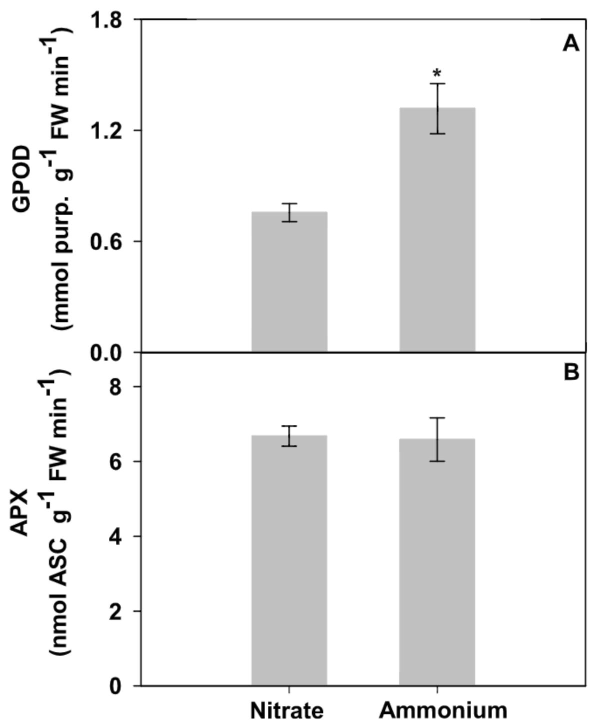

**Fig. 3.** Activities of (A) guaiacol peroxidases, (B) ascorbate peroxidases in rice roots exposed to increasing $NH_4^+$ and $NO_3^-$ concentrations. Eleven-day-old seedlings were initially exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ during 8 days in each concentration and afterwards subjected to 15 mM of $NH_4^+$ or $NO_3$- for additional 24 consecutive days (51-day-old plants). The values represent averages of four replicates and bars indicate standard error. Asterisks represent significant differences at a 5% level among treatments according to Tukey's test.

plants, whereas in nitrate-plants the senescent leaves represented only 3% of the total biomass (Table 1). The culm and whole plant biomass were similar in both groups of plants, evidencing that in response to excess $NH_4^+$ supplying rice plants were able to reallocate part of biomass from roots to shoot and part of this mass was consumed by the senescence followed by cellular death.

Based on these observations, we investigated if the increased biomass in senescent leaves, at the expense of roots and green leaves, could be related to a protective exclusion mechanism to avoid excess ammonium and preservation of photosynthetically active tissues. To test that hypothesis, we quantified the ammonium net influx from nutrient solution to roots, ammonium transport to shoots via root xylem, and accumulation of the different N-forms ammonium, nitrate, free amino acids (AA), and total-N in different plant parts (Table 2). Comparatively, the total N influx in the roots of $NH_4^+$- and $NO_3^-$ in nitrate-treated plants was very similar, i.e., 4.26 mmol $NH_4^+$ and 4.36 mmol $NO_3^-$ per plant per day, respectively. Intriguingly, the $NH_4^+$-fed plants presented an ammonium flux in root xylem 10-fold lower than nitrate flux in nitrate-supplied plants (Table 2). In parallel, the nitrate-treated plants exhibited a significantly higher (~45 %) exportation of free AA as compared to that of $NH_4^+$-supplied plants. These results evidence that high ammonium supplying does not induce high transport rates of $NH_4^+$ and amino acids from roots to shoot via xylem suggesting some downregulation mechanisms at transporter levels, or increased ammonium assimilation in root cells. Based on the observation that $NH_4^+$ supplied plants exhibited a significant decrease in the free AA exportation from roots to shoots, we verified if the GS activity in roots and leaves could be associated with this decreased flux. GS activity showed similar values in roots and green leaves in both N-treatments (Fig. 5). Thus, if ammonium-supplied rice roots presented any increase in ammonium assimilation, this was probably not related to GS activity.

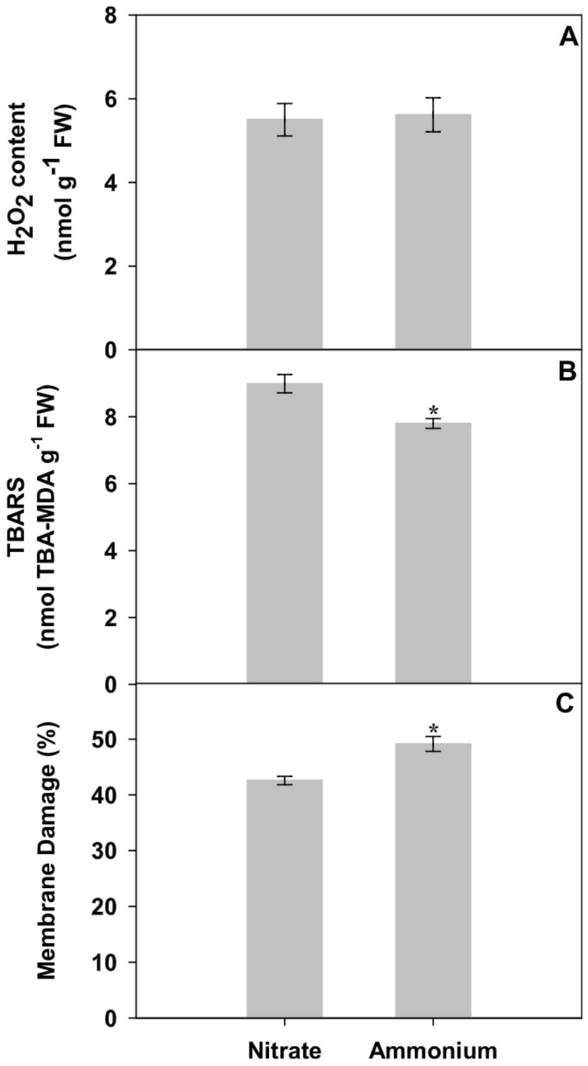

**Fig. 4.** Changes in the contents of (A) hydrogen peroxide, (B) TBARS and (C) membrane damage in rice roots exposed increasing $NH_4^+$ and $NO_3^-$ levels. Eleven-day-old seedlings were initially exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ during 8 days in each concentration and afterwards subjected to 15 mM of $NH_4^+$ or $NO_3$- for more consecutive 24 days (51- day-old plants). The values represent averages of four replicates and bars indicate standard error. Asterisks represent significant differences at a 5% level among treatments according to Tukey's test.

**Table 1**

Dry matter partitioning among parts of rice plants exposed to increasing $NH_4^+$ and $NO_3^-$ levels. Eleven-day-old seedlings were initially exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ during 8 days in each concentration and afterwards subjected to 15 mM of $NH_4^+$ or $NO_3$- for more consecutive 24 days (51- day-old plants). The values represent averages of four replicates expressed by gDM/plant and parenthesis per cent contribution of each part for the whole plant. Upper letters represent significant differences at a 5% level among treatments in the same plant part and lower-case letters represent significant differences within treatment in different plant parts according to Tukey's test. Percentages in parenthesis indicate the relative contribution of each plant part concerning the whole plant.

| | Green leaves | Senescent leaves | Culm | Roots | Total |
|---|---|---|---|---|---|
| **Nitrate** | 0.81 (22 %) Ab | 0.10 (3%) Bd | 2.33 (63 %) Aa | 0.45 (12 %) Ac | 3.71 (100 %) A |
| **Ammonium** | 0.44 (12 %) Bb | 0.53 (15 %) Ab | 2.43 (67 %) Aa | 0.20 (6%) Bc | 3.62 (100 %) A |

**Table 2**
Root-N influx and xylem flux in rice roots exposed to increasing $NH_4^+$ and $NO_3^-$ levels. Eleven-day-old seedlings were initially exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ during 8 days in each concentration and afterwards subjected to 15 mM of $NH_4^+$ or $NO_3$- for more consecutive 24 days (51- day-old plants). The values represent averages of four replicates (n = 4) and different letters represent significant differences at 5% level among treatments according to Tukey's test. The values of treatment of root N influx are expressed as mmol $plant^{-1}$ $day^{-1}$ and N xylem flux as mmol $L^{-1}$ xylem $h^{-1}$ and amino acids: μmol $L^{-1}$ xylem $h^{-1}$. ND = not detected.

| Treatment | Root-N Influx |  | Xylem Flux |  |  |
|---|---|---|---|---|---|
|  | Nitrate | Ammonium | Nitrate | Ammonium | Amino Acids |
| **15 mM Nitrate⁻** | 4.32 | ND | 2.31 | ND | 57.77 a |
| **15 mM Ammonium** | ND | 4.26 | ND | 0.24 | 40.14 b |

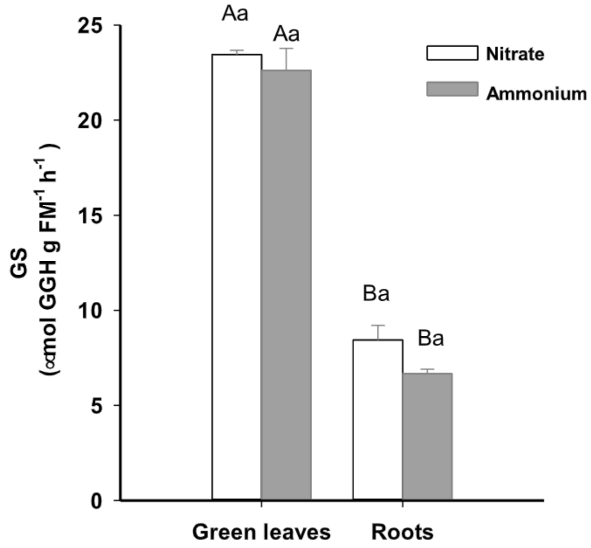

**Fig. 5.** Changes in glutamine synthetase activity in green leaves and roots in rice plants exposed to increasing $NH_4^+$ and $NO_3^-$ concentrations. Eleven-day-old seedlings were previously exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ for 8 days in each concentration and afterwards subjected to very high ammonium concentration (VHAC, 15 mM) in parallel to nitrate 15 mM. The plants remained under these stressful conditions for 24 consecutive days, reaching the V13 stage (51-day-old, 40 DAT). The values represent averages from four replicates (n = 4) and are expressed as μmolμ/g FM. Different letters represent significant differences at a 5% level among treatments according to Tukey's test.

**Table 3**
Accumulation of N-forms ($NH_4^+$, $NO_3^-$, free amino acids and total-N) among roots, senescent leaves, culm and green leaves in rice plants exposed to increasing $NH_4^+$ and $NO_3^-$ concentrations. Eleven-day-old seedlings were previously exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ for 8 days in each concentration and afterwards subjected to very high ammonium concentration (VHAC, 15 mM) in parallel to nitrate 15 mM. The plants remained under these stressful conditions for 24 consecutive days, reaching the V13 stage (51-day-old). The values are expressed as μmol/plant part from four replicates (n = 4). Upper letters represent significant differences at a 5% level among treatments in the same plant part and lower-case letters represent significant differences within treatment in different plant parts according to Tukey's test. Percentages in parenthesis indicate the relative contribution of each plant part concerning the whole plant.

| Nitrate Treatment | Green leaves | Senescent leaves | Culm | Roots | Total |
|---|---|---|---|---|---|
| Total-N | 2,498.69 (38 %) Aa | 233.48 (3%) Bc | 2,682.32 (43 %) Ba | 986.62 (16 %) Ab | 6,401.13 (100 %) B |
| Nitrate | 34.00 (5%) Ac | 153.55 (23 %) Ab | 362.47 (54 %) Aa | 123.30 (18 %) Bb | 673.3 (100 %) A |
| Ammonium | 15.63 (16 %) Ab | 3.50 (4%) Bc | 48.22 (52 %) Ba | 26.03 (28 %) Ab | 93.39 (100 %) B |
| Amino acids | 100.93 (15 %) Ab | 22.38 (3%) Bd | 473.51(71 %) Ba | 73.17 (11 %) Ac | 670.01 (100 %) B |
| **Ammonium Treatment** | **Green leaves** | **Senescent leaves** | **Culm** | **Roots** | **Total** |
| Total-N | 1,167.55 (13 %) Bc | 2,170.89 (27 %) Ab | 4,265.18 (54 %) Aa | 468.94 (6%) Bd | 8,072.58 (100 %) A |
| Nitrate | 8.19 (7%) Bc | 11.42 (11 %) Bc | 80.21 (80 %) Bb | 2.018 (2%) Aa | 101.9 (100 %) B |
| Ammonium | 13.04 (1%) Ac | 1,502.72 (75 %) Aa | 469.30 (23 %) Ab | 30.13 (1%) Ac | 2,015.21 (100 %) A |
| Amino acids | 78.25 (3%) Bc | 399.74 (20 %) Ab | 1,485.46 (75 %) Aa | 34.17 (2%) Bd | 1,997.65 (100 %) A |

Then, we attempted to answer the following question: how the different N-forms partitioning was affected in different plant parts by the two N-supply sources? In green leaves, $NH_4^+$ treatment induced a significant decrease in total-N accumulation (by 2.14-fold, expressed as μmol/total green leaves) due to a decrease in its content (by 15 %, expressed as μmol $g^{-1}$ DW), and combined with a strong reduction in dry mass accumulation (1.85-fold), compared to nitrate-plants (Table 3 and Fig. S1). In parallel, the final $NH_4^+$ content reached in green leaves (25.45 μmol $g^{-1}$ DW), which corresponded to approximately 3.23 mM as expressed in the whole tissue water basis, was similar to $NH_4^+$ content which found in leaves of nitrate-supplied plants, 19.39 μmol $g^{-1}$ DW. Remarkedly, and in strong contrast, in senescent leaves, the $NH_4^+$ treatment induced a prominent accumulation of total-N (9.31-fold) and free AA (by 18-fold), as compared to nitrate-supplied plants. Remarkably, the $NH_4^+$ content (μmol $g^{-1}$ DW) in senescent leaves of ammonium treatment was higher by approximately 100-fold in comparison to that reached in green leaves. Also, $NH_4^+$ accumulation (μmol per total senescent leaves) was approximately 10-fold higher than nitrate accumulation in senescent leaves of nitrate-supplied plants. In culms, the contents of total-N, ammonium, and free AA of $NH_4^+$ treated plants were also increased by 1.5-fold, 9.3-fold, and 3-fold, respectively, as compared to nitrate-plants. On the other hand, total-N and free AA contents in roots of $NH_4^+$ treated plants were similar to those observed in roots supplied with nitrate as the sole N source (Table 3 and S1).

Besides the evident contrasts in N partitioning, it's important to highlight the differences in the composition of N-compounds observed between green and senescent/dead leaves of ammonium treatment. Indeed, in green leaves, the $NH_4^+$ and free amino acids contents represented approximately 1% and 7%, respectively, of the total-N content, whereas in senescent/dead leaves these values reached astonishing values of 75 % and 18 %, respectively, encompassing almost 93 % of the total-N in these tissues (Table 3). Importantly, the total-N content of senescent/dead leaves was 1.5-fold higher than that observed in green leaves, suggesting that accumulation of $NH_4^+$ and free amino acids was possibly part of a deposition process rather than part of a remobilization mechanism.

Taken together these results indicate that despite the decreased root growth, rice plants were able to trigger an effective mechanism of protection in presence of excessive $NH_4^+$ exposure, which possibly involves a prominent deposition of ammonium in the senescent (followed by death) leaves. This accumulation mechanism and the subsequent abscission of this dead tissue could even represent an unknown and efficient mechanism to modulate plant systemic response to excess ammonium, avoiding accumulation in other plant tissues, which may

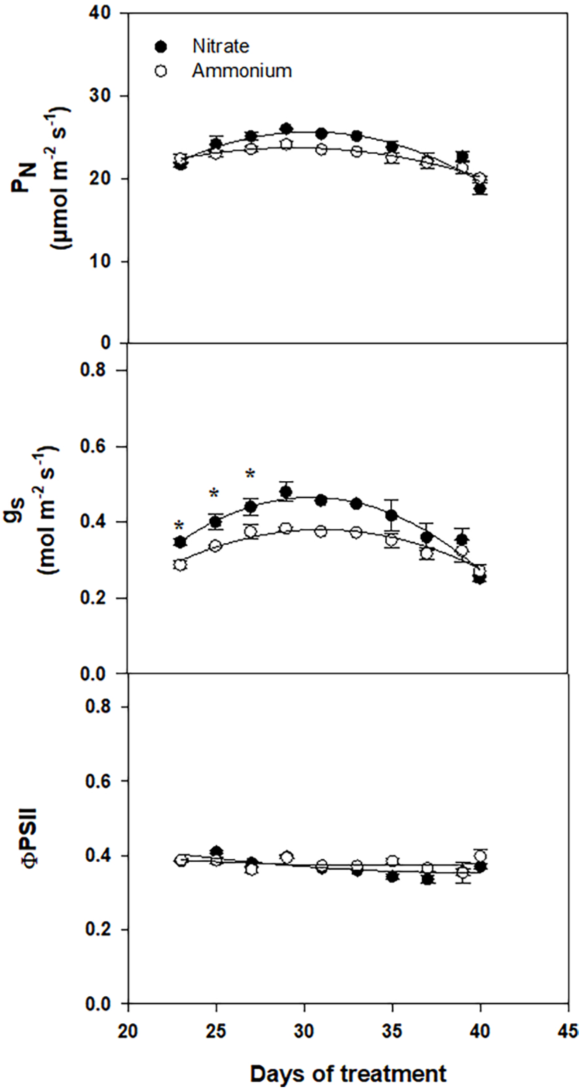

**Fig. 6.** (A) Photosynthetic $CO_2$ assimilation, (B) stomatal conductance and (C) actual quantum yield of PSII in mature leaves of rice plants exposed to increasing $NH_4^+$ and $NO_3^-$ concentrations. Eleven-day-old seedlings were previously exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ for 8 days in each concentration and afterwards subjected to very high ammonium concentration (VHAC, 15 mM) in parallel to nitrate 15 mM and this condition was defined as the zero-time treatment. The plants remained under these stressful conditions for 24 consecutive days, reaching the V13 stage (51-day-old). The values represent averages of three replicates (n = 3) and bars indicate the standard error (SE). Asterisks represent significant differences at a 5% level among treatments according to Tukey's test. Curves were adjusted by quadratic polynomial regression.

ensure appropriate levels of other N forms, such as free AA and proteins, consequently protecting shoot growth and whole plant development.

### *3.4. High $NH_4^+$ deposition in senescent leaves as a mechanism to avoid ammonium accumulation and protecting photosynthetic efficiency in green leaves*

The obtained data evidence that increased deposition/accumulation of $NH_4^+$ and free amino acids in senescent leaves could be beneficial to avoid ammonium toxicity in other plant organs with higher metabolic activity, such as green leaves, representing an effective mechanism to sustain shoot growth. To test this hypothesis, a time-course experiment was carried out where crucial variables associated with photosynthetic efficiency were measured every 2 days always in the mature fully expanded leaves, following the same procedure of gradual increase of N, that is, 8 days in 3.5 mM, 8 days in 7 mM and 24 days in 15 mM. This study revealed that no differences in the net photosynthetic rate ($P_N$) and actual quantum efficiency of PSII (ΦPSII) among plants treated with ammonium or nitrate were observed whereas stomatal conductance in $NH_4^+$-treated plants was slightly decreased in some points of the vegetative development (Fig. 6).

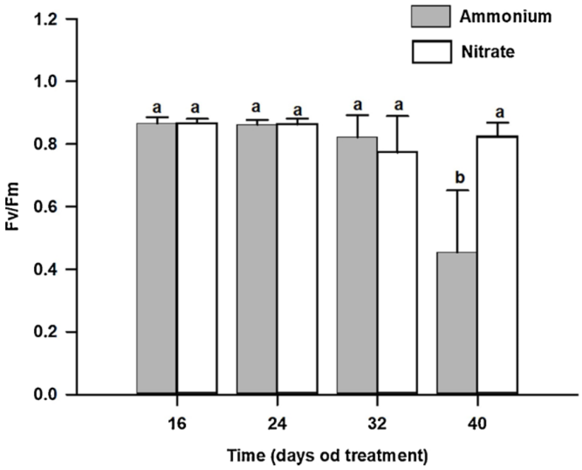

**Fig. 7.** PSII maximum quantum efficiency (Fv/Fm) in the 5th leaf of rice plants exposed to very high ammonium concentration (VHAC, 15 mM). Eleven-day-old seedlings were previously exposed to 3.75 mM and 7.5 mM of $NH_4^+$ or $NO_3^-$ for 8 days in each concentration and afterwards subjected to high ammonium concentration (15 mM) in parallel to nitrate 15 mM and this condition was defined as the zero-time treatment. The plants remained under these stressful conditions for 24 consecutive days, reaching the V13 stage (51-day-old). Letters represent significant differences at 5% between treatments according to the Tukey test. Measurements followed by the same letters within the column do not differ by the Tukey test.

To corroborate the finds concerning the photosynthetic protection in green leaves and ammonium partitioning associated with ammonium-induced early senescence, we performed a time-course assay focused on the investigating of the progress of senescence and its effects on the maximum quantum efficiency of PSII (Fv/Fm), to evaluate PSII integrity, in response to ammonium exposure. This photochemical parameter is commonly employed as an indicator of photoinhibition and damages on the PSII structure. For this, we marked physically the 5th leaf (a green leaf at the mature stage) and follow the changes in Fv/Fm, an effective photosynthetic parameter able to indicate irreversible damages on the photochemical apparatus, over time until 24 days (when it became a senescent leaf). The assay revealed that the 5th leaf of plants supplied with ammonium remained with similar levels of Fv/Fm, as compared to nitrate ones, until the 16th day, that is, after exposure to the lower ammonium levels, 3.75 mM followed by 7.25 mM. After this time, subsequently to exposure to the highest $NH_4^+$ (15 mM), coinciding with the phase when differences in chlorophyll content and senescence become noticeable, the Fv/Fm dropped prominently in the ammonium treated plants whereas in the nitrate treatment it dropped slightly (Fig. 7).

Taken together, these results reveal that the senescence process is indeed accelerated on the oldest rice leaves treated with excess ammonium; however, this feature can be advantageous whereas senescence occurs simultaneously with high deposition of ammonium and AA in these senescent and further dead leaves. Thus, this specific modular change could represent an efficient mechanism of ammonium exclusion to avoid toxic accumulation on the youngest and mature leaves of rice plants, maintaining and preserving photosynthesis and shoot growth.

## 4. Discussion

### 4.1. $NH_4^+$-suppling triggers down-regulation in root growth as an exclusion mechanism

The data set analyzed here strongly suggests the studied Nipponbare cultivar displays unusual mechanisms for coping with high $NH_4^+$ concentrations in the root medium, presenting an effective exclusion strategy. As is widely known, increased GPOD activity is highly correlated with lignification and reduction in root growth, thus, the obtained data strongly suggest that $NH_4^+$ might have induced a negative regulation on the root elongation as have been suggested by Passardi et al., (2004). As indicated by redox analysis, these effects are probably not related to ammonium toxicity *per se*, as has been widely proposed in the literature. Thus, it is reasonable to suggest here these changes might, in fact, represent a protective response for rice surviving under such severe adverse conditions as has been partially proposed (Yang and Guo, 2018). Recently, Jia et al. (2020) have shown that under moderate $NH_4^+$ concentrations (2.5 mM), very young rice roots are capable to exhibit growth restriction by an indirect mechanism related to pH lowering (acidic stress), induced after $NH_4^+$ uptake. This response is possibly specific for rice roots (but not for Arabidopsis) and it is associated with the formation of helicoidal roots that are associated with signalling by auxins.

These intriguing results reported by Jia et al. (2020) do not explain why and how some rice cultivars are tolerant to high ammonium once authors have employed mild concentrations. However, they were able to uncover a new and interesting morphological mechanism (induction of helicoidal root growth), associated with acidic stress induced by $NH_4^+$ uptake. As in this current study, the pH values of the nutrient solution were corrected to near 6.0 every two days in both nitrate and ammonium treatments, the possibility of indirect effects caused by pH changes can be ruled out. Other authors have suggested that root growth inhibition induced by excess ammonium is related to hormonal metabolism, such as the capacity to preserve auxin homeostasis (Di et al., 2018). In another recent study, has been proposed that biosynthesis of brassinosteroids is involved in $NH_4^+$-inhibited rice root elongation, suggesting a potential role of plant hormones in regulating rice root responses to N-sources (Jiao et al., 2020).

Rice, when cultivated in paddy soils, commonly faces high ammonium concentrations (Yoshida, 1981). In these conditions, restrictions in root elongation could be a good strategy to avoid excessive contact with that toxic N-source, since in these environments the availability of water and inorganic nutrients is usually non-limiting for rice growth (Alencar et al., 2019; Tabuchi et al., 2007). Plant responses displayed to high salinity can be used here as an interesting example of an effective avoidance mechanism involving the reduction of the root surface area. Indeed, salt stress induces a strong reduction in root growth associated with increased type III-peroxidase activity in cowpea plants, but this feature represents an effective strategy to avoid salt-induced oxidative stress (Maia et al., 2013). Thus, in the view of the convergent evolution of plant mechanisms to cope with potentially accumulated toxic substances, the possibility of down-regulation in root growth in response to ammonium could indeed represent a protective exclusion strategy, preserving both surviving and growth.

Exclusion mechanisms have been widely reported for other stressor types such as salt ions (Shao et al., 2021), heavy metals (Szopiński et al., 2020), and others toxic components (Chakraborty et al., 2016). Interestingly, several works have suggested that $NH_4^+$-induced root growth impairment is a toxicity symptom (Britto and Kronzucker, 2002; Li et al., 2010; Liu et al., 2013; Araya et al., 2016). However, in this current study the rice roots had not shown any stress signals, even when they were exposed to very high $NH_4^+$ concentrations, as 15 mM for long-term exposure. Indeed, discrimination between responses for plant adaption and toxicity to high $NH_4^+$ are very difficult to be established. However, our data strongly suggest that the stunted root growth of rice in response to excess $NH_4^+$ might be part of an acclimation mechanism to avoid excessive contact with that toxic nutrient. Thus, we are proposing that this exclusion strategy is part of a global adaptive response for ammonium tolerance. Nevertheless, further studies are still needed to elucidate the molecular players underlying such remarkable morpho-physiological changes.

### 4.2. High $NH_4^+$ concentration induces localized senescence on the oldest leaves as an exclusion mechanism to avoid toxicity and preserve the active photosynthetically tissues

In parallel to those responses related to root growth restriction, rice plants have displayed other and even more unusual exclusion mechanisms to minimize $NH_4^+$ toxic effects, especially to preserve the photosynthetically active leaves. Indeed, our data strongly evidence that, in this condition, a significant part of the oldest leaves suffer fast senescence followed by cellular death, but the photosynthesis of mature and young leaves is preserved. This observation is very interesting, suggesting that rice plants, which are tolerant to very high ammonium concentrations, can trigger a "localized self-destruction" process on the oldest leaves in order to avoid transference of high amounts of $NH_4^+$ to their metabolically active tissues, performing an effective exclusion mechanism. Indeed, in contrast to natural leaf senescence course, when proteins are degraded to produce amino acids to be transported for young tissues, in this study the oldest-senescent leaves progressively accumulated very high amounts of $NH_4^+$, reaching 75 % of the total-N of these tissues, accompanied by approximately 20 % of free amino acids.

Remarkedly, this $NH_4^+$-exclusion mechanism is, to the best of our knowledge, not reported in the literature yet. Despite, some metabolic mechanisms for ammonium tolerance in plants have been already widely postulated, such as high $NH_4^+$ recycling in root plasmalemma and high activities of ammonifying enzymes, especially GS/GOGAT (Cruz et al., 2006; El Omari et al., 2010) and glutamate dehydrogenase (Lasa et al., 2002). Moreover, most of the studies investigating ammonium toxicity are carried out with sensible plants exposed to relatively low-moderate $NH_4^+$ concentrations (2.5–5.0 mM) and for short-term (Frechilla et al., 2002; Podgórska et al., 2017). Interestingly, Arabidopsis plants exposed to high ammonium concentrations have evidenced that this N-form is capable to deeply alter ammonia assimilation metabolism and, in fact, regulate the leaf senescence (Jian et al., 2018).

In a recent transcriptomic study performed with rice plants subjected to high $NH_4^+$ concentrations (a range from 2 to 40 mM for 14 days), the authors found that high ammonium supplying induces strong impairment in root growth (Sun et al., 2017). The authors proposed that ammonium-induced root growth impairment may preclude an intricate adjustment of hormone metabolism, especially in ethylene and ABA pathways (Sun et al., 2020, 2017). These evidences have been obtained after investigating rice plants exposed to $NH_4^+$ 15 mM throughout a short-term time-course (hours of exposure). Moreover, it was newly published that the Arabidopsis nitrate transporters genes (AtNRT2) are regulated by three different transcription factors: TGA3, MYC1 and bHLH093 in response to N signals (Ruffel et al., 2021). Thus, in our current study, possibly the high $NH_4^+$ levels might have induced changes in several metabolic pathways, especially those involved with hormonal metabolism related to senescence on the oldest leaves.

As discussed before, it has been known for a long time that excess $NH_4^+$ is capable to induce senescence on rice leaves (Chen et al., 1997) but all studies were performed in the perception of "toxic effects" and not in an acclimation perspective. Indeed, we are proposing here that rice plants exposed to high $NH_4^+$ can trigger intense senescence localized on their oldest leaves. In our hypothesis, this initial process is possibly associated with other molecular, metabolic and physiological events such as proteolysis, cell death, apoptosis, necrosis and autophagy. After these processes, large amounts of $NH_4^+$ are accumulated in those tissues, possibly originated from protein and amino acids degradation. This assumption is reinforced by the prominent ammonium accumulation

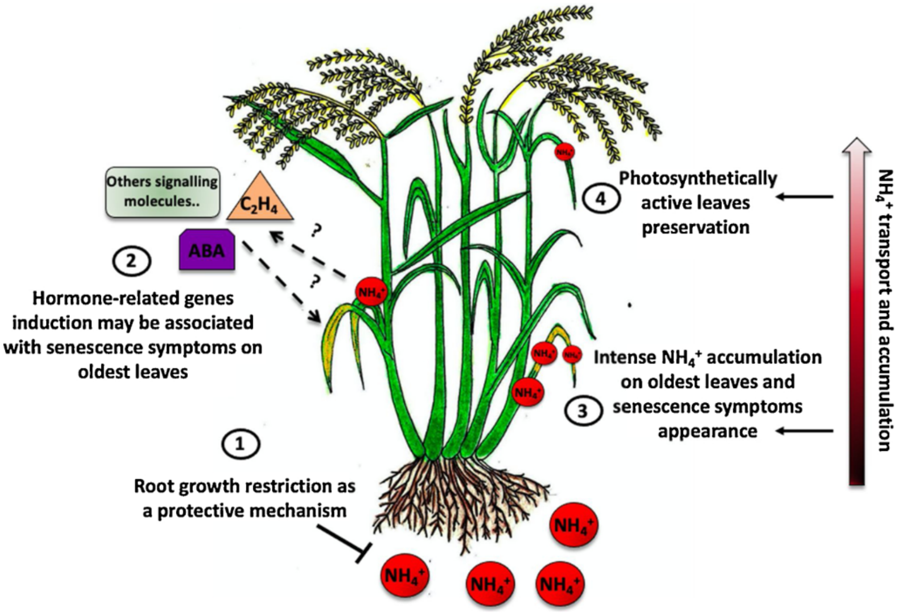

**Fig. 8.** Schematic model representing the global rice response to high ammonium exposure (15 mM) for the long-term. 1) Ammonium induced a root growth restriction as a protective mechanism to avoid contact with this toxic ion; 2) The ammonium assimilated could be act as a signalling molecule, activating complexes hormone pathways that could be related with senescence symptoms that appeared on oldest leaves; 3) The intense ammonium accumulation on oldest leaves suggests a "self-destruction" strategy to 4) preserve the photosynthetically active leaves and allow the plant survival.

throughout the oldest leaf development. In an anthropomorphic conception, it would be less plausible to assume that the oldest leaves should export ammonium and free amino acids for younger tissues in a circumstance of very high N supplying and low C availability. Indeed, this condition is very different from normal senescence when older leaves export high amounts of C and N to the youngest and reproductive tissues in the form of mainly amino acids and sucrose.

However, a question is important: how much $NH_4^+$ accumulated on the oldest/ dead leaves could have been transported from roots? In that circumstance, rice plants displayed low rates of root xylem influx suggesting that accumulated $NH_4^+$ was mainly originated from the local proteolysis. Despite that assumption, our data are not enough to clearly explain which molecular and metabolical mechanisms are involved in that complex exclusion process. Further studies involving $^{15}NH_4^+$ influx and transport to shoot, expression of genes related to senescence, cell death, apoptosis and autophagy, combined with omics approaches such as transcriptomics and proteomics are needed. We are presenting an overall schematic model (Fig. 8) suggesting some possible relationships to explain that phenome and highlighting $NH_4^+$ as a signalling ion for triggering those hormonal and molecular processes when it reaches high concentrations on the oldest leaves and roots.

In conclusion, we demonstrated that rice plants tolerant to very high $NH_4^+$ supplying can induce effective and unusual exclusion mechanisms. These features are characterized, on the one hand, by a strong non-toxic restriction on root growth, avoiding excessive contact with nutrient toxicity. Most importantly, is proposed that excess ammonium triggers initially a fast-induced senescence process on the oldest leaves (a regulated self-destruction) followed by a high $NH_4^+$ accumulation and cell death in these tissues. This mechanism allows avoiding the flux of high $NH_4^+$ amounts towards the younger leaves and other shoot tissues, thus preserving their photosynthetic capacity and growth in presence of very high $NH_4^+$ in the root medium.

## CRediT authorship contribution statement

**Rikaely T. Sousa:** Carried out all experiments and performed data analyses. **Ana L. S. Paiva:** Part of the writing of manuscript and original draft preparation. **Fabricio E. L. Carvalho:** Photosynthetic measurements and part of the manuscript writing. **Vicente T. C. B. Alencar:** Carried out some experiments and performed biochemical analyses. **Joaquim A. G. Silveira:** Manuscript conception and writing, research supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2021.104452.

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# Update

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Corrigendum

# Corrigendum to “Ammonium overaccumulation in senescent leaves as a novel exclusion mechanism to avoid toxicity in photosynthetically active rice leaves” [Environ. Exp. Bot. 186 (2021) 104452]

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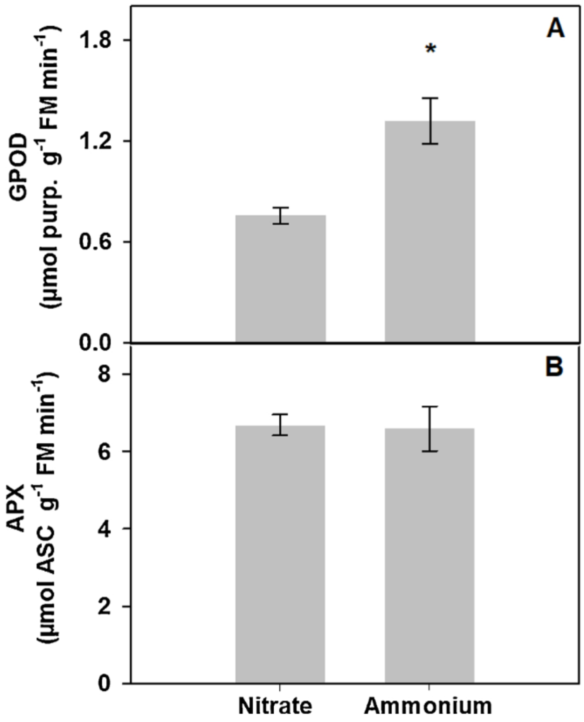
The authors apologize for the missed correction reported below:

In Fig. 3, the unit in Y axis in both figures (3A and 3B) should be micromolar, not millimolar or nanomolar.



The publisher would like to apologize for any inconvenience caused.

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