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Use of an in vitro-Assay to Investigate the Antioxidative Defence Potential of Wheat Genotypes under Drought Stress as Influenced by Nitrogen Nutrition

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Summary

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A rapid 'in vitro'-test was used to study the impact of mineral nutrition on the antioxidative defence potential of wheat genotypes under drought stress. Segments of the third leaf of hydroponically grown wheat seedlings were subjected to a 8 hour treatment by floating segments on destilled water or a solution containing PEG 6000 under high light intensity, simulating drought stress on a cellular level. After the treatment, the leaf segments were evaluated for their extent of pigment bleaching. Plasma membrane damage was estimated by measuring the efflux of K^+ into the incubation solution. Comparing six genotypes, the extent of leaf damage was significantly correlated to the activity of glutathione reductase ($r^2 = 0.909$; $P = 0.009$). Two genotypes, differing in tolerance to drought stress under field conditions, were grown at two levels (1 or 4 mM) of nitrate, ammonium or a combination of both, and were compared using the described assay. Photooxidative damage was less severe at low N-level and under NH_4^+ supply. At 4 mM N tocopherol contents decreased relatively to the chlorophyll levels, irrespectively of the form of N supply, whereas xanthophyll/chlorophyll-ratios were not affected, indicating a similar capacity for harmless dissipation of excitation energy at both N levels. At 4 mM NH_4^+ the leaf concentrations of K^+ and Mg^{2+} were markedly lower. Therefore, during desiccation harmful tissue concentrations of this ions might have been avoided. Besides, NH_4^+ grown plants contained higher amounts of reduced N compounds, especially of putrescine which could have exerted a protecting effect on the membrane integrity during the photooxidative stress treatment.

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Introduction

Under normal conditions, plants are well adapted for minimizing damage due to the inevitable formation of active oxygen species in photosynthesis (FOYER & al. 1994). Drought stress as well as many other environmental stresses, like freezing (BRIDGER & al. 1994), heat (HAVAUX & al. 1991) or high light intensity (MISHRA & al. 1995), intensify the formation of free radicals in plants (NAVARI-IZZO & al. 1993, QUARTACCI & al. 1995). Such conditions can limit the pool of $NADP^+$ available for the acceptance of electrons from PS I. Thereby the probability increases of excitation energy transfer to O_2, leading to the production of O_2^- and $^1O_2$. Although genotypical differences exist in wheat in the potential of free radical detoxification (BADIANI & al. 1990, ZHANG & KIRKHAM 1994), breeding for semi-arid regions has not systematically exploited this potential yet, but has mainly aimed at selecting genotypes with low water demand or accelerated maturity in order to escape late season drought. The study of the antioxidative defence potential under drought stress is complicated by the difficulty to exclude in long term studies the influence of alternative adaptation mechanisms. In order to achieve a rapid onset of stress, therefore, several groups have used 'in vitro'-assays (BAISAK & al. 1994, PASTORI & TRIPPI 1993) to investigate the genetic variation in photooxidative stress tolerance.

In addition, 'in vitro'-systems allow to study the antioxidative potential at different tissue levels of mineral nutrients in plants grown hydroponically. In natural environments, mineral nutrient is limited under drought conditions for several reasons, like decreased ion mobility in the soil and impaired mineralization of organic matter. Certain mineral nutrient deficiencies, especially of Mg^{2+} (CAKMAK 1994, POLLE & al. 1994) or K^+ (CAKMAK 1994), can increase the susceptibility to light-induced chlorosis. Little is known about the influence of nitrogen nutrition on the defence against free radicals in plants. Elevated chlorophyll levels at high nitrogen supply can cause excessive absorption of light energy. In addition, the concentrations of the low molecular antioxidants, ascorbate and α-tocopherol, could be diminished by N fertilization (MOZAFAR 1993). On the other hand, the accumulation of carotenoids under N-deficiency (GOODWIN 1980) relative to chlorophyll may provide an improved potential for the thermal dissipation of excitation energy (KHAMIS & al. 1990). Photooxidative stress tolerance may also be influenced by the N ion form. Nitrate can compete with O_2 as an alternative electron acceptor from PS I. In addition, in case of NO_3^--nutrition, the pH gradient of the thylakoids increases due to NO_2^- reduction. Thus, zeaxanthin formation and, thereby, the capacity for nonphotochemical dissipation of excitation energy may be enhanced (SATTELMACHER, pers. comm.). At high NH_4^+ supply NH_3 toxicity might be exacerbated by high light intensities (MAGALHAES & WILCOX 1983). On the other hand, the levels of polyamines could be elevated (GERENDAS & SATTELMACHER 1995), which can impair the activity of proteases and delay senescence (KAUR-SAWHNEY & GALSTON 1991) by stabilizing the thylakoid membranes under stress conditions (BESFORD & al. 1993).

The increased yield potential of wheat genotypes with prolonged photosynthetic activity under drought conditions due to an improved antioxidative defence capacity can be exploited only, if additional nitrogen can be applied without adverse effects on resistance. The present work, therefore, aimed at investigating the influences of different levels and forms of nitrogen nutrition on the antioxidative defence system of wheat genotypes, preselected for contrasting drought resistance.

Material and Methods

Seeds of bread wheat (*Triticum aestivum* L.) were germinated in quartz sand irrigated with saturated $CaSO_4$ solution. After 4-5-day seedlings were transplanted to nutrient solutions containing in mol*m^{-3}: 0.7 K_2SO_4, 0.1 KCl, 0.5 $MgSO_4$, 0.1 K_2HPO_4, 0.5 x 10^{-3} $MnSO_4$, 0.5 x 10^{-3} $ZnSO_4$, 0.2 x $CuSO_4$, 0.02 x 10^{-3} H_3BO_3, 0.02 x 10^{-3} $(NH_4)_6Mo_7O_{24}$, 0.1 Fe(III)-EDTA. Different levels of nitrogen (1 mol*m^{-3} or 4 mol*m^{-3}) were supplied as $Ca(NO_3)_2$ ('NT'), $(NH_4)_2SO_4$ ('AM') or NH_4NO_3 ('CB'). The 'AM'-solutions contained 1 mol*m^{-3} $CaSO_4$ and were buffered with 2 kg*m^{-3} $CaCO_3$. The plants were grown under controlled environmental conditions with a light/dark regime of 14/10 h, an air temperature of 22/18 °C, 70 % r.H. and about 400 µmol m^{-2} s^{-1} photon flux density.

After 14 d of growth segments (3 cm) from the middle part of the 3rd leaf were placed on petri dishes containing 30 % (w/w) PEG 6000 (osmotic potential 1.1 MPa), simulating drought stress on a cellular level, or destilled water as a control. The petri dishes were incubated for 8 h at 25 °C and 950 µmol m^{-2} s^{-1}. Photooxidative damage to the leaves during the stress treatment was evaluated visually, by analysis of pigment loss, and by determination of the K^+ leakage into the incubation solution via atomic absorption spectroscopy. In the first experiment, six cultivars ('Gerek', 'BDME 10', 'Dagdas', 'Kirac 66', 'Atay', 'Kunduru') were grown in high NT-solution to evaluate the assay system. The two genotypes 'Gerek' and 'BDME 10', were used to investigate the influence of form and level of nitrogen supply on the photoxidative stress tolerance.

The constitutive activities of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.7.), monodehydroascorbate radical reductase (MDAR, EC 1.1.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.8.5.1) were determined according to CAKMAK & al. 1993. Chloroplast pigments were extracted in acetone and separated by HPLC following the method of BUECH & al. 1994. Pigments were identified and quantified comparing the retention times and absorbance spectra of standards prepared by TLC. For determination of α-tocopherol, samples were homogenized in 80 % ethanol, saponified with KOH and extracted with n-hexane. Tocopherols were separated by isocratic HPLC and identified and quantified using fluorescence detection at 296 nm (excitation) and 326 nm (emission). Free polyamines were determined according to TIBURCIO & al. 1986 in lyophilized leaf material. Dansyl-polyamines were separated by TLC. Polyamine bands were visualized under long wavelength UV light, scraped into ethyl acetate and quantified with a fluorescence photometer (excitation 350 nm, emission 495 nm). Total nitrogen content of leaf material was determined with an automatic nitrogen analysator, nitrate was measured with a Technicon autoanalyser. Contents of K^+, Ca^{2+}, Mg^{2+} were analyzed by atomic absorption spectroscopy.

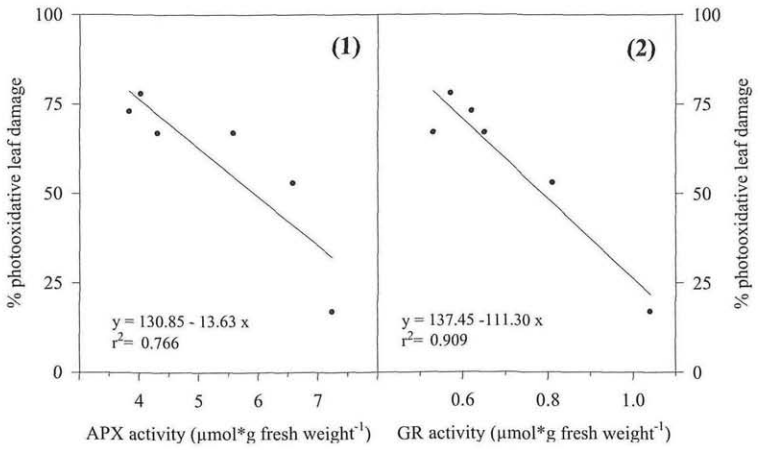

Fig. 1. Extent of visible leaf damage (as percentage of the whole leaf segment) after an 8 h stress treatment under high light intensity (950 mE $m^{-2}s^{-1}$) dependent on the genotypical activity of radical-scavenging enzymes in leaves of wheat genotypes before stress onset. (1) ascorbate peroxidase (APX); (2) glutathione reductase (GR).

Results

The 4 mM N supply lead to a 30 % increase in fresh matter production in the 'NT'- and 'CB'-, but not in the 'AM' treatment. Total nitrogen levels were elevated to 4.9-6.1 % of the dry matter compared to 2.5-3.4 % at 1 mM N. This additional nitrogen was mainly stored as nitrate in case of 'NT'- and 'CB'-supply, whereas 'AM'-grown plants accumulated higher levels of reduced N. At high 'AM'-nutrition in the leaf dry matter the content of K^+ was lowered from 5.5 % to 3.5 % and of Mg^{2+} from 0.21 to 0.12 %, but no deficiency symptoms were observed.

Visual symptoms of photooxidative pigment decomposition appeared within 6 to 8 hours in the stress treatments. Differences in symptoms between genotypes were most distinct at this time, and were levelled during prolonged incubation.. Therefore, a standard incubation period of 8 h was used. Partial shading of the segments alleviated the bleaching effect. Studying six genotypes, the extent of visual damage was, on a fresh weight basis, well correlated to the genotypical, constitutive levels of GR activity (r^2 = 0.909, P = 0.009) and of APX activity (r^2 = 0.766, P = 0.022) (Fig.1). Two genotypes were selected for the following investigation, 'Gerek', which was less, and 'BDME 10', which was more sensitive to photooxidative stress 'in vitro': This was in agreement with their different tolerance to drought stress under field conditions in Turkey and in pot experiments under controlled environmental conditions.

Visual damage to the segments was only observed in leaves grown at 4 mM N level, and was less severe in case of NH_4^+ supply. The visual symptoms resembled the effects of N levels and N forms on the extent of plasma membrane

damage, as indicated by the net K^+ efflux during the incubation (Table 1). Compared to the control treatments with negligible efflux, the efflux from 'NT' and 'CB'-segments increased about 10-fold at low, and up to 30-fold at high N supply. In contrast, the membranes of 'AM'-grown segments were markedly less affected by the osmotic stress. However, differences in stress resistance between the two varieties were only evident at high 'NT'.

Table 1. Effect of form and level of nitrogen supply during growth on the loss of K^+ from leaf segments. Data ± standard error (n=2 independent experiments) expressed in µg K^+ *(g fresh weight*8 h)$^{-1}$

N-Form	N-Level	'Gerek'		'BDME 10'	
		control	+ PEG[a]	control	+ PEG
NO_3^-	1 mM	159 ± 27	1577 ± 262	146 ± 50	1767 ± 200
NH_4^+	1 mM	306 ± 39	1409 ± 333	247 ± 48	519 ± 161
NH_4NO_3	1 mM	119 ± 24	1696 ± 276	132 ± 36	1173 ± 270
NO_3^-	4 mM	303 ± 89	3769 ± 354	140 ± 90	4203 ± 288
NH_4^+	4 mM	307 ± 119	2592 ± 64	346 ± 48	2509 ± 129
NH_4NO_3	4 mM	108 ± 10	3723 ± 109	290 ± 95	3633 ± 262

[a] PEG 30 % (w/w) polyethylene glycol 6000

At high levels of nitrogen supply, the enzyme activities of the ascorbate-glutathione cycle decreased relative to the total protein content. However, based on fresh weight, the N level had no effect (data not shown). As expected, GR activities of the drought-resistant 'Gerek' were higher. In contrast, the form of N supply showed no significant influence on the enzyme activities (Table 2).

Table 2. Effect of form and level of nitrogen supply during plant growth on the activity of glutathione reductase (GR) in leaves of wheat genotypes. Mean ± standard deviation (n = 3).

Genotype	N-Level	NO_3^-	NH_4^+	NH_4NO_3
			µmol*g FW^{-1}	
Gerek	1 mM	0.87 ± 0.10	0.81 ± 0.16	0.88 ± 0.11
	4 mM	0.77 ± 0.12	0.79 ± 0.16	0.84 ± 0.11
BDME 10	1 mM	0.55 ± 0.11	0.37 ± 0.03	0.41 ± 0.08
	4 mM	0.53 ± 0.10	0.40 ± 0.07	0.60 ± 0.11
			µmol*(g protein)$^{-1}$	
Gerek	1 mM	57 ± 22	49 ± 29	49 ± 16
	4 mM	35 ± 13	24 ± 3	23 ± 6
BDME 10	1 mM	31 ± 8	21 ± 6	29 ± 4
	4 mM	28 ± 11	13 ± 6	14 ± 3

Irrespectively of the N form, higher supply slightly lowered the total carotenoid/chlorophyll-ratios, whereas the xanthophyll/chlorophyll-ratios were not affected. At high N supply, the α-tocopherol contents decreased relative to

chlorophyll at 4 mM nitrogen. Since no distinct differences occurred between genotypes, data are presented for 'Gerek' only (Fig. 2). As indicated by the relative decrease of α-tocopherol at 4 mM in the drought-stressed treatment, this antioxidant was decomposed earlier than the chlorophylls, demonstrating its protecting effect on the thylakoid membranes (Fig. 2). The constitutive levels and the extent of tocopherol loss under stress were similar for 'NT', 'AM' and 'CB' and do not explain differences in photooxidative stress tolerance between different forms of N supply.

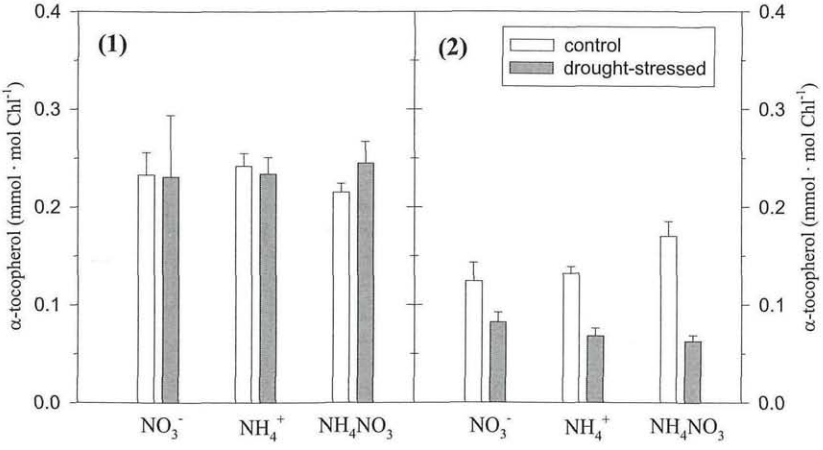

Fig. 2. α-Tocopherol content in relation to chlorophyll content in leaves of 'Gerek' before and after osmotical stress treatment as influenced form and level of nitrogen nutrition during plant growth. (1) 1 mM N; (2) 4 mM N.

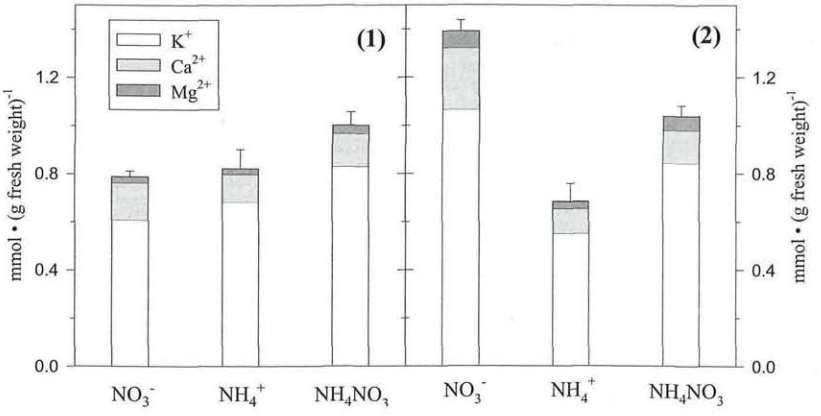

Fig. 3. Effect of form and level of nitrogen supply on the concentrations of K^+, Ca^{2+} and Mg^{2+} in leaf segments of wheat (cv. 'BDME 10') after 8 h stress treatment. (1) 1 mM N; (2) 4 mM N.

Plant culture at 4 mM N elevated the total content of free polyamines by about 80 %. Whereas the spermidine and spermine levels did not change

significantly due to different forms of N-supply, putrescine was only detected at high N-supply, the levels increasing more than twofold in case of 'AM'-nutrition (Table 3). The increased putrescine contents were accompanied by an elevated pool of free amino acids as the main storage form of reduced N.

Table 3. Effect of form and level of nitrogen supply on polyamine contents (nmol*g fresh weight^{-1}) in leaves of genotype 'Gerek' before onset of stress treatment. Mean ± standard deviation (n = 3).

N-Form	N-Level	Putrescine	Spermidine	Spermine
NO_3^-	1 mM	n.d.[a]	145 ± 18	208 ± 18
NH_4^+	1 mM	n.d.	144 ± 12	216 ± 36
NH_4NO_3	1 mM	n.d.	135 ± 25	174 ± 27
NO_3^-	4 mM	40 ± 1	200 ± 12	410 ± 66
NH_4^+	4 mM	152 ± 4	206 ± 14	338 ± 28
NH_4NO_3	4 mM	62 ± 22	282 ± 17	303 ± 1

[a] n.d. not detectable (< 10 nmol*g fresh weight^{-1})

Tissue concentrations of inorganic cations after the osmotic stress treatments were calculated, based on the concentrations of K^+, Ca^{2+}, and Mg^{2+} before onset of the stress treatment, and on the loss in fresh weight of the leaf segments during the 'in vitro'-test. Leaf segments of plants grown at 4 mM N lost about 40 % of fresh weight during the incubation period, irrespectively of the form of N-supply. At 4 mM NH_4^+ the leaves contained less K^+, Ca^{2+}, and Mg^{2+} (see above), the desiccation therefore, caused 1.5- (in 'CB') to 2-fold (in 'NT') higher concentration of this ions in the leaf tissue compared to 'AM'-grown leaves (Fig. 3).

Discussion

The experimental system was suitable to detect genotypical differences in drought resistance in wheat due to photooxidative stress tolerance. It provided some important advantages in that it allowes to impose drought stress rapidly, investigating the resistance on a cellular level and lessening the effects of alternative adaptation mechanisms. In addition, the control of mineral nutrient supply could easily be achieved by the preculture in nutrient solution. An induction of increased GR activities by drought stress has been observed previously (BAISAK & al. 1994). In contrast to the results of PASTORI & TRIPPI 1993, the constitutive levels of GR activity differed between genotypes in the present study, and were related to short term drought resistance. One explanation may be that, as suggested by OSMOND & GRACE 1995 at excessive light, the Mehler-reaction, followed by ascorbate peroxidation, may enhance the thylakoid energization, thereby inducing zeaxanthin formation and increasing the thermal dissipation of light energy. Under such conditions, the pre-set chloroplast capacity of GSH-recycling could be crucial

to prevent the depletion of the ascorbate pool due to the decomposition of the instable dehydroascorbate (SMIRNOFF 1995).

Drought resistance and susceptibility to photooxidative stress were improved by N limitation for several reasons. Additional N supply elevated the chlorophyll contents and, hence, the possibility of excessive light energy absorption. On the other hand, the radical scavenging potential of the ascorbate-glutathione cycle was not affected by the N supply and, thus, the higher probability of oxidative damage to the thylakoids could not be compensated by an increase in protective capacity. In addition, the levels of tocopherol did not increase concomitantly. According to MOZAFAR 1993 the levels in leaf α-tocopherol, and often ascorbate are even lower at high nitrogen supply. In contrast to the findings of KHAMIS & al. 1990 with N-deficient plants, in our studies the lower N level did not promote higher xanthophyll/chlorophyll- and carotenoid/chlorophyll-ratios, presumably because N deficiency was avoided even at low N-supply.

The form of N supply was crucial for drought resistance under high ligh intensities, NO_3^- exacerbating the extent of pigment bleaching at high N. Despite the accumulation of NO_3^-, this pool could not be used as an alternative sink for electrons in case of light excess, presumably, because C skeletons for N-assimilation were lacking due to the impaired photosynthetic CO_2 fixation. Consequently, no changes in thylakoid pH-gradient and de-epoxidation status were induced by NO_2^- reduction as suggested by SATTELMACHER (pers. comm.). Theoretically, photorespiration could mitigate the effects of excess light in case of stomatal closure via an internal recycling of CO_2 (STUHLFAUTH & al. 1990). NH_4^+ nutrition together with photorespiratory NH_3 formation could then exceed the capacity of N assimilation via the glutamine synthetase pathway. Indeed, NH_3 toxicity in tomato was preferentially observed under high light intensity (MAGALHAES & WILCOX 1983). However, as has been shown by BRESTIC & al. 1995, the O_2-consumption in the light under drought stress conditions is mainly linked to the Mehler O_2-reduction, but not the rubisco oxygenase reaction, suggesting that the oxygenase photorespiration does not substantially contribute to the protection from photoinhibition. A deleterious effect of NH_4^+ compared to NO_3^- nutrition due to a lower potential for photorespiratory CO_2-formation is therefore not to be expected.

In the present study, NH_4^+ supply rather alleviated photooxidative damage. One reason could be the accumulation of putrescine in the leaves. Higher levels of putrescine are induced after exposure to various types of stresses (for a review see FLORES 1991). A decrease in endogenous polyamine levels often accompanies the natural onset of senescence (KAUR-SAWHNEY & GALSTON 1991). This correlation was attributed in part to the membrane-stabilizing action (BESFORD & al. 1993) and the free radical scavenging capacity of polyamines (DROLET & al. 1986). However, spermine and spermidine were more effective than putrescine in this respect. Recently, elevated constitutive putrescine levels were detected not only in different *Conyza* species resistant to paraquat (SZIGETI & al. 1996), but also in wheat genotypes, selected by PASTORI & TRIPPI 1993 for high drought resistance

(YE & MÜLLER, unpubl.), suggesting also a role of this diamine in photooxidative stress tolerance. Presumably, in the present work the accumulation of putrescine in case of NH_4 nutrition was a response to lower K^+ levels. The assumption that putrescine levels caused the improved resistance, has to be tested under conditions, where putrescine synthesis is inhibited.

The lower contents of inorganic cations in NH_4 grown plants may have had another beneficial effect on drought stress resistance. During advancing water loss, the tissue cation concentrations can increase to, perhaps, toxic levels. As demonstrated by RAO & al. 1987, stromal Mg^{2+} concentrations doubled under drought conditions. This impaired the photosynthetic ATP synthesis and electron transport even at low photon flux density. Under high light intensities such conditions would probably enhance energy transfer to oxygen and $^1O_2$-production and would cause oxidative damage. Consequently, in NH_4^+ grown plant tissue with lower Mg^{2+} and K^+ concentrations this toxic levels would be reached later, delaying the onset of photooxidative membrane damage and, thereby, imcreasing drought resistance under high light intensities.

In conclusion, nitrogen nutrition has a strong impact on short term drought resistance in wheat due to photooxidative stress tolerance, mainly by altering the ratio of energy-collecting and thermal energy-dissipating potential. The improvement of tolerance by NH_4^+ supply may be a secondary effect of changes in polyamine and inorganic cation levels in response to NH_4^+.

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