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Research article

Nitric oxide inhibits nitrate reductase activity in wheat leaves

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Nitrate reductase (NR), a committed enzyme in nitrate assimilation, is involved in the generation of nitric oxide (NO) in plants. In wheat leaf segments exposed to sodium nitroprusside (SNP) or *S*-nitrosoglutathione (GSNO), NR activity was significantly reduced to different degrees between 3 and 21 h, whereas its activity was partially recovered when the NO scavenger cPTIO was used. At 21 h, NR activity decreased from 38% with 10 μM SNP to 91% with 500 μM SNP, respect to the C values. *S*-nitrosoglutathione reduced NR activity between 18% and 26% only at 3 h. When added directly to the incubation solution, NR activity was quickly and strongly inhibited more than 90% by 10 or 50 μM SNP, whereas 10 μM GSNO reduced the enzyme activity an average of 50%, at 30 min of incubation. L-NAME and D-arginine (nitric oxide synthase (NOS) inhibitors) increased NR activity by 14% and 52% respectively, at 21 h of exposure, leading us to suppose that endogenous NOS-dependent NO formation could also be modulating NR activity. NR protein expression was not affected by 10 or 100 μM SNP at 3 or 21 h of incubation, whereas nitration of tyrosines was not detected in the NR protein. Nitrates, which content increased along the time in the tissues, could be exerting a role in this regulation.

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1. Introduction

Higher plants acquire the majority of their nitrogen from the environment by nitrate assimilation. Nitrate reductase (NR) (EC 1.7.1.1) catalyses the transfer of two electrons from NAD(P)H to nitrate to produce nitrite, which is further reduced to NH_4^+ by nitrite reductase (NiR, EC 1.7.2.1). Studies in bacteria, fungi, and higher plants have shown that NR expression is highly regulated [1]. In plants, nitrate is the primary factor regulating NR activity [2]. In addition to nitrate, NR activity is also regulated by a number of other factors, including light, growth, hormones, and reduced nitrogen metabolites [3,4]. Moreover, in higher plants, NR is rapidly inactivated/activated by phosphorylation/dephosphorylation, respectively, in response to different environmental stimuli and treatments [5]. Sugars, cytosolic acidification and anaerobiosis are factors all known to activate NR in both leaves and roots [5,6]. One reason for the large interest in NR regulation is the high toxicity of nitrite, that when is produced in excess, could be released to the surroundings and, in the form of the undissociated HNO_2, penetrates biomembranes rather easily, particularly in roots (Botrel et al., 1996 [7]). Under most conditions, nitrite does not accumulate because, generally, the activity of NiR in plants is much higher than that of NR, which can avoid nitrite accumulation to toxic levels. However, when nitrite does accumulate, it was demonstrated that NR catalyzes one electron reduction of nitrite to form nitric oxide (NO), using NAD(P)H as an electron donor [8–10], constituting an alternative physiological function for NR in plants. This activity is different from the plastidic nitrite reducing activity catalysed by NiR, which reduces nitrite to ammonium using six electrons [10].

The production of NO by plants was described as early as 1979 by Klepper [11] in herbicide-treated soybeans. It has long been known that, in soybean, a so-called constitutive bispecific NAD(P)H:NR can catalyse the production of NO from nitrate during "*in vivo*" NR assays [12]. In roots, NO production from nitrate mediated by NR [13] and nitrite:NO reductase (Ni:NOR, [14]) has significant importance. Although NR is the only protein whose NO-producing activity has been confirmed in plants up to now [8,15,16]), several other still not completely identified pathways have been described as contributors to NO formation in plants, as the inducible NO synthase, NOS [3,17,18]. However, the only postulated plant NOS has recently been shown not to be a nitric oxide synthase, but a chloroplastic GTPase involved in proper ribosome assembly [19].

Nitric oxide may act as a gaseous signaling compound involved in communication from organ to organ or from plant to plant. In the last few years, a plethora of data have demonstrated that NO participates

Abbreviations: NR, nitrate reductase; NO, nitric oxide; SNP, sodium nitroprusside; NiR, nitrite reductase; GSNO, *S*-nitrosoglutathione.

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in plant responses to both biotic and abiotic stresses and is involved in processes such as germination, ethylene production, senescence and stomatal closure [18,20], and in cadmium-induced root growth inhibition [21]. Despite the involvement of NR in NO production is indubitable and has been extensively documented, studies regarding the role of NO in regulating NR activity and protein expression are still very scarce. This work focuses mainly on the role of NO on NR activity and expression in wheat plants. We hypothesized that NO could play an important role in regulating NR activity in plants, thus contributing largely to N assimilation and use, and providing new insights into the complex regulation of N metabolism.

2. Materials and methods

2.1. Chemicals

NADH, SNP, cPTIO, L-NAME, GSH, $NaNO_2$, casein, PVP were from Sigma Chemical Company (Saint Louis, MO). All chemicals were of analytical grade.

2.2. Plant material and treatments

Wheat seeds (*Triticum aestivum* L., provided by Buck Co.) were germinated and grown at 26/20 °C (day and night), with a 16-h photoperiod under fluorescent white light (175 μmol m^2 s) in a controlled environment growth chamber. Plants were daily watered with a nutrient solution [22]. By the end of the light period, leaf segments (8 mm length) from 12 d-old plants were placed in flasks containing as NO donors, 25 ml of either 10, 100 or 500 μM sodium nitroprusside (SNP, $Na_2[Fe(CN)_5NO]$) or 10, 100 or 500 μM of *S*-nitrosoglutathione (GSNO) in distilled water and incubated during 3 or 21 h in a rotatory shaker under continuous illumination. GSNO must be freshly synthesized right before the experiments, so it was prepared immediately before use, using equimolar amounts of GSH and $NaNO_2$ to obtain 10, 100 and 500 μM GSNO. During the preparation, the mixture was protected from light. Incubations for 6 and 9 h were also done but only for SNP. Controls were incubated in distilled water. When indicated, the NO scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide, 100 μM), L-NAME (NG–monomethyl-L-arginine, 100 μM), or D-arg (2 mM), were added to the incubation medium. Potassium cyanide was used as a control in the incubation medium at 3 or 21 h of exposure or when measuring NR activity in tubes using crude extracts, considering that cyanide is part of the SNP molecule but it has been described as NR inhibitor. The concentrations used in this study were selected after preliminary experiments.

2.3. Determination of NR activity

NR activity was measured according to Yaneva et al. [23] and Savidov et al. [24]. Wheat leaf segments were homogenized in a medium containing 5 mM EDTA, 5 mM GSH, 1% (w/v) casein, PVP and 50 mM HEPES pH 7.5 and centrifuged 15 min at 17000 *g*. The assay mixture contained: 200 μmol KNO_3, 0.2 μmol NADH and 100 μL of the homogenate. After incubation at 30 °C for 20 min, the reaction was finished by the addition of 50 μL 1 M zinc acetate. The mixture was centrifuged 5 min at 7600 *g* and the supernatant was used to determine nitrite production by reading the absorbance at 540 nm after the addition of 1% sulphanylamide in 1.5 M HCl and 0.01% N-(1-Naphthyl)-ethylenediammonium dichloride.

When NR activity was measured directly in the reaction tubes, wheat leaf segments of control treatment were homogenized to make a crude homogenate and used for the assays. SNP, at a final concentration of 10 or 50 μM, GSNO at a final concentration of 5 or 10 μM, and KCN at a final concentration of 10 or 30 μM, were added directly to the reaction tubes, and the reaction mixtures were incubated under continuous illumination. NR activity was determined at 3, 10, 20 and 30 min after SNP, GSNO or KCN addition.

Nitrites are themselves competitive inhibitor of NR, so NO_2^- content in leaves was measured immediately after SNP addition. It was verified that NO_2^- levels were not significantly different from the control without SNP or GSNO addition. After SNP was dissolved in the incubation medium, pH was measured to check that it was not lower than 4.5, which can also affect the activity of the enzyme.

2.4. Western blot analysis of NR expression and of total nitrotyrosines

Leaves were homogenized and extracted with 50 mM HEPES–KOH pH 7.5, 1 mM EDTA, 10 mM FAD, 1 mM DTT, 1% (w/v) insoluble PVP, 5 mM ascorbate and protease inhibitor cocktail (Sigma). The extract was centrifuged at 17000 *g* for 15 min at 4 °C and the protein concentration in the supernatant was determined according to Bradford [25]. Proteins were separated on a 10% SDS-PAGE in Mini PROTEAN III equipment (Bio-Rad), as described by Laemmli [26]. Following electrophoresis at 4–8 °C, proteins were transferred to PVDF membrane (GE Healthcare, Amersham Hybond P). After that, membranes were blocked with 3% (w/v) BSA dissolved in PBS, incubated overnight with the primary antibody dissolved in blocking buffer (1/5000 for anti-NR and 1/1000 for anti-nitrotyrosine), and washed several times with PBS. Immunodetection of NR was carried out using a rabbit serum antibody raised against NR from *Arabidopsis* (kindly provided by Dr. Steven Huber, Department of Plant Biology, University of Illinois). Mouse anti-nitrotyrosine IgG (Chemicon International) was used as the primary monoclonal antibody to detect nitrotyrosines. In both cases, bands were revealed using a goat anti-rabbit IgG peroxidase conjugated secondary antibody (Dako Cytomation), and 3,3′-diaminobenzidine (DAB) was used as substrate for the staining procedure.

2.5. Immunoprecipitation and Western blot analysis of nitrotyrosines in NR

The protein homogenate (100 μg) was separated by affinity chromatography. Antibodies anti-NR were linked to cyanogen bromide activated Sepharose 4% agarose matrix (100 mg) from Sigma–Aldrich (St Luis, USA). Samples were incubated overnight at 4 °C with an excess of anti-NR-agarose resin and then centrifuged for 5 min at 10.000 *g*. Resin beads were washed 3 times with Tris-buffered saline (TBS), with pellets re-suspended in 100 mM glycine–HCl (50 μl, pH 2.5). After centrifugation, the pellets were discarded, with the pH of the supernatants adjusted to 6.8 with 0.5 M Tris–HCl buffer (5 μL, pH 8.8) and used for immunodetection of the nitrotyrosine residues. NR was separated by 10% (w/v) SDS–PAGE. After electrotransfer of the proteins to PVDF membranes, the nitrotyrosine residues were detected using anti-nitrotyrosine primary antibody (Santa Cruz Biotechnology, Inc) and goat anti-mouse IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, Inc), with DAB as substrate. Membranes were photographed with a Fotodyn equipment and analyzed with GelPro software.

2.6. Measurement of nitrates content

Wheat leaf segments were dried at 85 °C until constant weight. The dried material (25 mg) was grounded to powder and incubated in 10 ml of distilled water during 2.5 h. Nitrate was measured colorimetrically after a reaction with salicylic acid [27].

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2.7. Statistics

All determinations were performed from three independent experiments. Analytical measurements were done three times for all parameters in each experiment, with a minimum of three replicates. Differences among treatments were analyzed by one-way ANOVA, taking. $P < 0.05$ as significant according to Tukey's multiple range test.

3. Results

3.1. Nitric oxide from SNP or GSNO inhibited NR activity

Considering that in plants, NR is one of the NO-former enzymes, it was possible that this important gaseous molecule was involved in the regulation of the NR activity or protein expression. To characterize the effect of NO on wheat leaves, either SNP or GSNO were selected as NO donors. SNP is a suitable compound for long-term treatments (such as 21 h) since its stability is higher than that of other known NO releasing compounds [28]. Nitrate reductase activity was measured at 3, 6, 9 and 21 h, using 10, 100 and 500 μM of SNP. As it is shown in Fig. 1 A, all SNP concentrations significantly decreased NR activity at all incubation times, and the reduction in NR activity was dependent on SNP concentration. At 10 μM, SNP-derived NO decreased NR activity by 45%, on average, at all incubation times. At 21 h, NR activity decreased from 38% to 91% of the control, with either 10 or 500 μM SNP, respectively. The enzyme activity was almost not detected between 6 and 9 h of incubation with 500 μM SNP (Fig. 1 A).

The NO scavenger cPTIO, was used to trap the SNP-released NO, only at 6 h of incubation with 10 μM SNP, to confirm that NO was involved in the inhibition of NR activity (Fig. 2). When used alone, cPTIO did not produced any effect, but used simultaneously with 10 μM SNP (which decreased NR activity to 43% of the C), both 100 and 200 μM cPTIO recovered the enzyme activity to 76% and 82% of the initial value, respectively (Fig. 2), demonstrating that the greater part of the observed effect was due to NO.

Another NO donor, GSNO, was used to verify that NO was the compound involved in the inhibition of NR enzymatic activity. The effect GSNO was assayed only at 3 and 21 h of exposure. At 3 h, this NO donor decreased NR activity 18% when was used at 10 or 100 μM

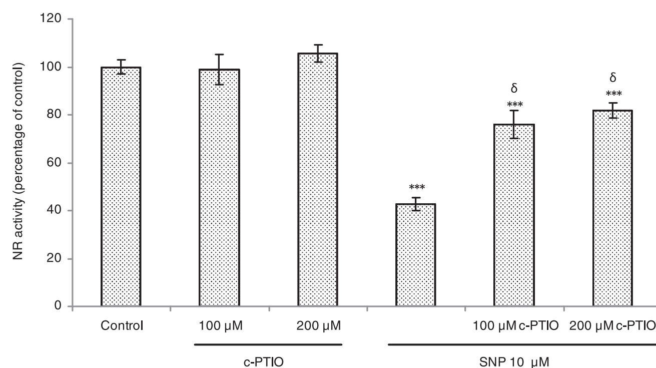

Fig. 2. NR activity of wheat leaf segments treated with SNP (10 μM) and/or the NO scavenger cPTIO (100 μM and 200 μM) during 6 h. NR activity is expressed as percentage of the control. Values are the means of three different experiments with three replicated measurements, and bars indicate SEM. *Significant differences at ($P < 0.05$) according to Tukey's multiple range test.

and 26% at 500 μM. However, at 21 h, not only no inhibition was observed but a moderate increase of 27% of the enzyme activity was detected with the highest GSNO concentration (Fig. 3).

In water solution, SNP decomposes into NO, NO_2^- and $Fe(CN)_6^{4-}$. Cyanide is a well known inhibitor of many enzymes including nitrate reductase, so we used KCN to test if CN^- modified NR activity instead of NO. Fe $(CN)_6^{4-}$ was not used because it acts as an electron acceptor that disrupt the normal electron transport in the enzymatic reaction catalyzed by NR [29]. Potassium cyanide decreased the enzyme activity 22% and 13% at 3 and 21 h, respectively, suggesting that a minor part of the inhibition exerted by SNP was due to cyanide. (Fig. 3). It was verified that NO_2^- levels were not significantly different from the control when their levels were measured immediately after SNP addition (data not shown).

3.2. "In vitro" NR activity is reduced by NO

To study the direct effect of NO on NR activity, an *in vitro* measurement of NR activity was carried out using crude wheat leaf homogenate and adding directly SNP at a final concentration of 10 or 50 μM into the reaction solution. As it is shown in Fig. 4, NR

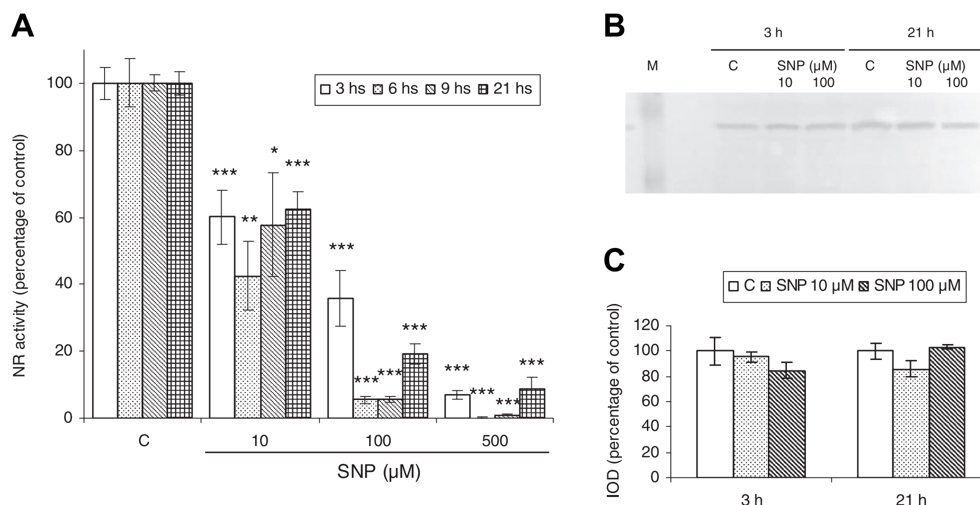

Fig. 1. A): NR activity in wheat leaf segments treated with increasing SNP concentrations at 3, 6, 9 and 16 h of exposure, as described in Materials and Methods. NR activity is expressed as percentage of the control. Values are the means of three different experiments with three replicated measurements, and bars indicate SEM. *Significant differences at ($P < 0.05$) according to Tukey's multiple range test. (B): Western blot analysis showing NR protein expression in wheat leaf segments exposed to 10 and 100 μM SNP for 3 and 21 h. The experiment was repeated three times and a representative image is presented. (C): Relative amount of proteins in B, considering control homogenates as 100 au.

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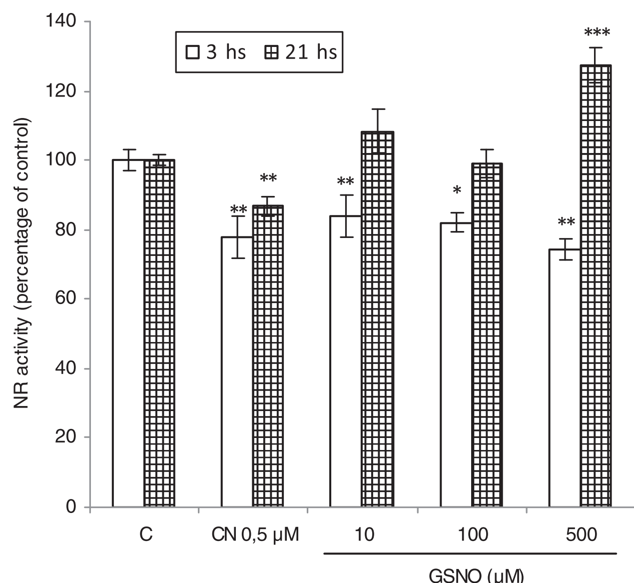

Fig. 3. NR activity in wheat leaf segments exposed to 10, 100 or 500 μM GSNO, at 3 and 21 h of exposure. GSNO was prepared as described in Material and Methods. The effect of 0.5 μM KCN is also presented. NR activity is expressed as percentage of the control. Values are the means of three different experiments with three replicated measurements, and bars indicate SEM. *Significant differences at ($P < 0.05$) according to Tukey's multiple range test.

activity was immediately and significantly reduced by the presence of SNP, reaching only 5% of the C activity at 10 min of incubation with the highest SNP concentration. The other NO donor, GSNO, significantly reduced NR activity an average of 50% when used at 10 μM (Fig. 4). No significant differences in the enzyme activity were observed respect to the controls when KCN 10 and 30 μM was used instead of SNP (Fig. 4), possibly due to the short time of the reaction. The level of NO_2^- in the tissues was almost undetectable when measured immediately after SNP addition (data not shown), thus confirming that NO released from SNP or GSNO (and not nitrites) were responsible for the observed inhibition of NR activity.

3.3. Effect of NO on the NR protein expression

Taking into account that NR activity was reduced by NO, we decided to test if this decay was accompanied by a decrease in the NR protein content. The analysis demonstrated that NR expression

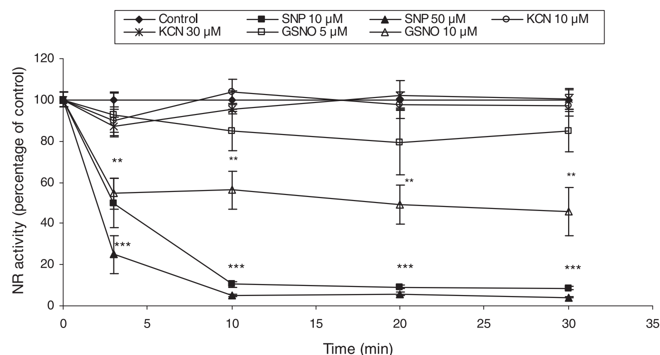

Fig. 4. NR activity measured in the reaction solution up to 30 m, after the direct addition of 10 or 50 μM SNP, 5 or 10 μM GSNO and 10 or 30 μM KCN, according to the description in Materials and methods. NR activity is expressed as percentage of the control. Values are the means of three different experiments with three replicated measurements, and bars indicate SEM. *Significant differences at ($P < 0.05$) according to Tukey's multiple range test.

was not significantly affected after 3 or 21 h of incubation, with either 10 or 100 μM SNP (Fig. 1B and C), suggesting that a post-transcriptional effect of NO on the NR protein could be responsible for the reduction in the protein activity.

3.4. NO effect on nitrative modifications in wheat leaves proteins

Another reason for the inhibition of NR activity could be the nitration of tyrosines in the protein, produced by an increased formation of peroxynitrite ($ONOO^-$) after the reaction of SNP-derived NO with O_2^-. This protein modification was evaluated by Western blot, using a mouse monoclonal anti-nitrotyrosine antibody. First, the content of nitrotyrosines was measured in total proteins, using samples from control, 10 and 100 μM SNP-treated wheat leaves. A slight increase was observed in the content of nitrotyrosines in either 10 or 100 μM SNP-exposed wheat leaves at 3 h compared to controls, whereas a more clear increase was detected at 21 h, particularly in the band corresponding to 53 kD band (Fig. 5). In order to check if NR itself was modified by tyrosine nitration, the protein was inmunopurified and inmunodetected by Western blot using the NR antibody. However, nitrotyrosines were not detected in the purified NR, either from controls or NO-treated samples (data not shown).

3.5. L-NAME and D-arg effects on NR activity

With the aim to study how NR could be affected by the endogenous NOS-dependent NO, we used 100 μM L-NAME and 2 mM D-arg (inhibitors of NOS enzyme) to measure NR activity. The enzyme activity increased between 41% and 52% over the controls, at 3 and 21 h respectively, when D-arg was used, whereas when L-NAME was used, NR activity increased by 14% only at 21 h of exposure. The results suggested that endogenous NOS-dependent NO could also be participating in the modulation NR activity (Fig. 6).

3.6. Effects of NO on NO_3^- content

As a result of the inhibitory effect displayed by NO on NR activity, it was presumed that low rates of nitrate assimilation were

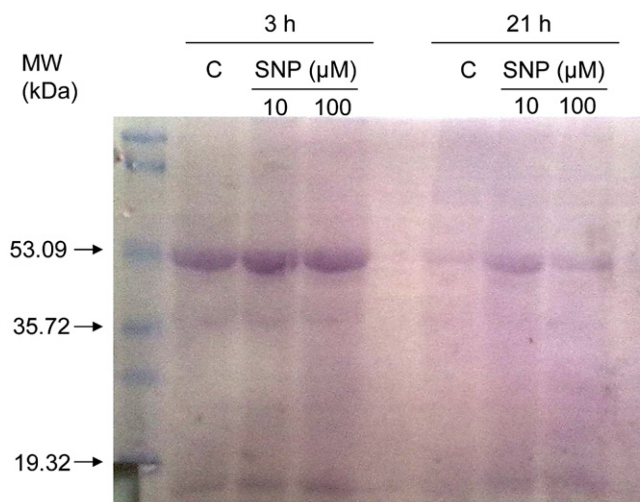

Fig. 5. Analysis of nitrotyrosines in segments of wheat leaves, incubated for 3 or 21 h in the presence of 10 or 100 μM SNP. Nitration of protein tyrosines in homogenates of wheat leaves was evaluated by semi-quantitative Western blottting, using a monoclonal anti-nitrotyrosine antibody. The experiment was repeated four times and a representative picture is shown.

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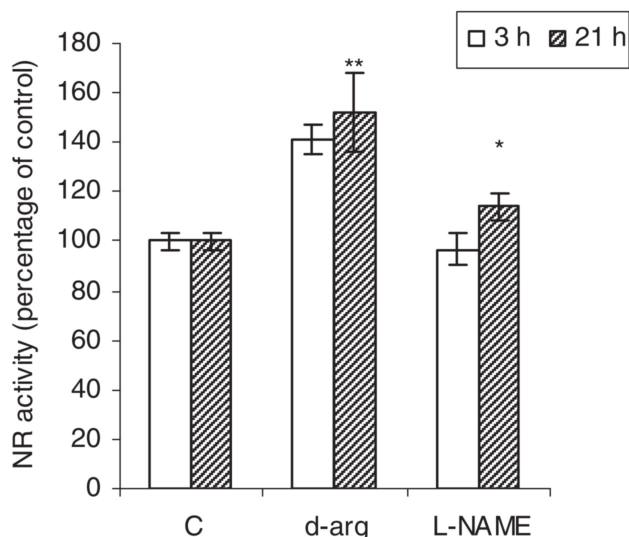

Fig. 6. NR activity of wheat leaf segments, at 3 and 21 h, after the addition of 2 mM D-arg or 100 μM L-NAME. NR activity is expressed as percentage of the control. Values are the means of three different experiments with three replicated measurements, and bars indicate SEM. *Significant differences at ($P < 0.05$) according to Tukey's multiple range test.

achieved, altering the nitrate uptake and leading to a rapid increase of the leaf nitrate pool. Nitrate content increased an average of 31% both at 16 h or 21 h of exposure with 100 μM SNP (Fig. 7). At shorter times (3–9 h), no significant differences were observed. The highest SNP concentration (500 μM) was used only at 21 h, producing an increase of 42% in the NO_3^- content of the leaf segments.

4. Discussion

It has long been recognised that NR, besides its role in NO_3^- reduction to form nitrite, catalyse the NAD(P)H-dependent reduction of nitrite to NO [13]. Nitrate reductase is an enzyme highly regulated by nitrogen availability or light and dark transitions, at many levels, like gene expression that contributes to control NR protein levels, and, reversible protein phosphorylation, that provides a more rapid regulation [2].

The results presented here demonstrate that NR activity is negatively modulated by NO released from SNP or GSNO, results that are opposite to those found by Du et al. [30] using roots from Chinese pakchoi cabbage (*Brassica chinensis* L.), who reported that

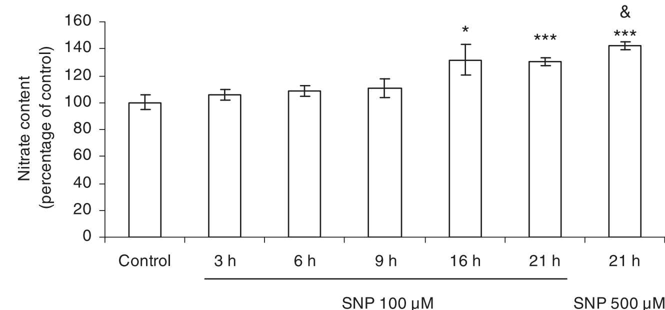

Fig. 7. Nitrate content of wheat leaf segments treated with 100 μM SNP for 6, 9, 16 and 21 h, and with 500 μM SNP only at 21 h. Results are expressed as percentage of the control. Values are the means of three different experiments with three replicated measurements, and bars indicate SEM. *Significant differences at ($P < 0.05$) according to Tukey's multiple range test.

NR activity was significantly enhanced by the addition of the NO donors SNP (up to 40 μM) and diethylamine NONOate (up to 100 μM). These authors also found that NR protein content was not affected by the SNP treatment and suggested that the stimulating effect of NO on NR activity might be due to an enhancement of electron transfer from haem to nitrate through activating the haem and molybdenum centres in the NR [30]. The same NO donors also stimulated NR activity of tomato roots fed with 0.5 mM nitrate, whereas the opposite occurred with the roots fed with 5 mM nitrate, suggesting that NO mediates the NR activity in plant roots depending on the level of nitrate supply [31].

In general, NO donors are applied as pharmacological tools in the understanding that most, if not all, of their biological effects are mediated by NO. Sodium nitroprusside and GSNO are among the NO donors commonly used, whereas phenyl-tetramethyl-imidazoline-oxyl-oxides (PTIOs) are used as NO-trapping agents [32]. In our experimental system using wheat leaf segments, the NO-donor SNP produced a quick and evident inhibition of NR activity, both in the "*in vivo*" experiments or when SNP or GSNO was added directly to the reaction solutions (Figs. 1 and 4). This rapid and massive decay should be attributed to a direct interaction of NO with a protein residue more than a post-transcriptional or post-translational regulation, considering that the NR protein content was not modified and nitration of tyrosine residues of the protein was not detected in our experimental conditions, irrespective of NO level (Fig. 1 B and C). Jin et al. [31] reported that the protein concentration of the NR enzyme in tomato roots was not affected by SNP treatment, irrespective of nitrate pre-treatment. One could suppose that NO could participates in the regulation of the phosphorylation event and/or, by this way, in the regulation of NR activity. S-nitrosylation reactions mediated by nitrosonium (NO^+, a product of NO oxidation), which reacts with a cysteine thiolate to form an S-nitrosothiol, should not be discarded among the regulatory mechanisms of NR activity [33].

GSNO is considered to represent a functionally relevant signalling molecule which might act both as NO reservoir and NO donor [34]. In physiological buffers, many S-nitrosothiols undergo relatively rapid decomposition to yield the corresponding disulfide and NO [32]. This compound produced a moderate inhibition of NR at 3 h of incubation, but this effect disappeared at 21 h, probably due to the kinetic of NO release from GSNO. Surprisingly, 500 μM GSNO increased NR activity by 27% at 21 h. This result was unexpected and could be due to the above mentioned kinetic of the reaction or to GSNO decomposition in the presence of metal ions like Cu^{2+}, GSH or ascorbic acid that could be present in the tissues [35].

The incubation of wheat leaf discs with L-NAME and D-arg produced an increase of NR activity (Fig. 6) which led us to suppose that NOS-dependent NO could also be participating in the modulation of the enzyme activity in wheat leaves. The NOS inhibitor D-arg increased significantly NR activity at both times of incubation, but L-NAME scarcely affected NR activity (only 14% at 21 h). There is abundant evidence from the plant science literature that argues for the presence of both nitrite reduction and arginine-dependent NO-formation pathways. However, the identity of the players and the importance of each biosynthetic pathway as a function of the physiological process remain unclear [36]. In PEG-treated *Arabidopsis* roots, Kolbert et al. [37] showed that neither NR nor NOS were involved in the early generation of NO, while the accumulation of NO at longer times was mediated by an NR-associated pathway. On the other hand, Zhao et al. [38] reported that cold acclimation stimulated NR activity and induced up-regulation of *NIA1* gene expression but in contrast, it reduced the quantity of *NOA1/RIF1* protein and inhibited NO synthase (NOS) activity.

Although there is ample evidence showing that nitrate is the most important factor controlling NR mRNA synthesis [4], it appears to have no direct effects on the NR phosphorylation/

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dephosphorylation state (or activation state) nor to be directly involved in the modulation of NR activity [4,39]. In wheat leaves, nitrate content significantly increased at 21 h of incubation with SNP, indicating that the substrate for NR activity was present in amounts enough to be not a limiting factor for NR activity. Moreover, nitrate could regulate NOS-dependent NO formation, as it was shown both in tomato [31] and maize roots treated with different levels of nitrate [40]. Hence, while nitrate itself appears not to be directly involved in the modulation of NR activity, an indirect effect might be expected through the alteration of endogenous NO level [2,31]. In wheat leaf segments, the cytosolic level of nitrates increased but NR activity decreased, along with SNP-derived NO increase in the incubation medium. The increase in NO_3^- in the cytosol could be due to an efflux from nitrates that previously exist in the mitochondria or chloroplasts, as occurred in *Nicotiana benthamiana* where a rapid NO_3^- efflux was shown to be essential for NO production by NR and the subsequent defense responses induced by elicitin [41]. Besides, an oxidation of SNP-derived NO by an enzymatic or non-enzymatic mechanism in an aerobic environment that could be leading to an increase in the cytosolic NO_3^- levels should not be discarded [42].

Tyrosine nitration of proteins (leading to 3-nitrotyrosine) is a widely used marker of peroxynitrite ($ONOO^-$) produced from the reaction of nitric oxide with O_2^- [43]. The content of nitrotyrosine in proteins showed an increase in soybean axes from SNP-exposed seeds in a dose-dependent manner [44]. This effect could not be detected in NR from wheat leaf segments in our experimental conditions. Graziano and Lamattina [45] speculated that there are likely other molecules, as H_2O_2, that act in the pathway upstream or downstream from the site of NO action, or in concert with NO, for what oxidative modifications of the wheat NR protein mediated by reactive oxygen species should not be discarded as a possible reason for the decreased activity.

The regulatory mechanism exerted by NO on NR activity has been scarcely described previously. The results presented in this work clearly suggest that NR activity is negatively regulated by NO released either from SNP or GSNO in wheat leaf segments, in a dose and time-dependent manner and without affecting the protein content. The fact that though nitrates were accumulated in the tissues NR activity was inhibited shows that the role of NO in the N metabolism has undoubtedly importance and needs further investigation. Therefore, it should be clue to determine whether the change in the concentration of substrate NO/NO_3^- was affecting NR activity, which component of the nitrate reductase system was affected by NO and which is the precise mechanism involved in such regulation.

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