The Nitric Oxide Production in the Moss *Physcomitrella patens* Is Mediated by Nitrate Reductase

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Abstract

During the last 20 years multiple roles of the nitric oxide gas (•NO) have been uncovered in plant growth, development and many physiological processes. In seed plants the enzymatic synthesis of •NO is mediated by a nitric oxide synthase (NOS)-like activity performed by a still unknown enzyme(s) and nitrate reductase (NR). In green algae the •NO production has been linked only to NR activity, although a *NOS* gene was reported for *Ostreococcus tauri* and *O. lucimarinus*, no other Viridiplantae species has such gene. As there is no information about •NO synthesis neither for non-vascular plants nor for non-seed vascular plants, the interesting question regarding the evolution of the enzymatic •NO production systems during land plant natural history remains open. To address this issue the endogenous •NO production by protonema was demonstrated using Electron Paramagnetic Resonance (EPR). The •NO signal was almost eliminated in plants treated with sodium tungstate, which also reduced the NR activity, demonstrating that in *P. patens* NR activity is the main source for •NO production. The analysis with confocal laser scanning microscopy (CLSM) confirmed endogenous NO production and showed that •NO signal is accumulated in the cytoplasm of protonema cells. The results presented here show for the first time the •NO production in a non-vascular plant and demonstrate that the NR-dependent enzymatic synthesis of •NO is common for embryophytes and green algae.

Introduction

The multiple roles of nitric oxide (•NO) in plant physiology have been extensively studied for almost two decades [1–3]. Nowadays, it is recognised that •NO is a fundamental signalling

molecule during plant development, from germination to floral set and senescence [4–6] as well as in the response to biotic and abiotic stress conditions [7–9]. Besides the study of •NO functions, another appealing research topic is the analysis of the mechanisms to generate •NO in plant systems. In land plants two main enzymatic systems involved in •NO production are recognized, a nitric oxide synthase (NOS-like) activity and •NO production by nitrate reductase (NR, EC 1.7.1.1–3) [10,11]. In mammals the NOS enzyme oxidizes L-arginine to •NO and citrulline [12]. Such activity has been found in a variety of plant organs such as *Lupinus albus* roots and nodules [13], *Zea mays* root tips [14] and in *Nicotiana tabacum* epidermal cells and cell cultures [15,16]. The decrease in •NO content after the application of animal NOS inhibitors reinforced the idea that plants has NOS enzyme [17–19]. However, neither a NOS protein nor a gene has been found in embyophytes [10, 20]. In the green algae, *Ostreococcus tauri* and *O. lucimarinus*, a functional NOS protein was reported, but no other algae or land plant sequence matches with the *O. tauri NOS* gene [21], leaving the identity of a plant NOS unresolved. In addition to the classical role of NR in reducing nitrate to nitrite, this enzyme reduces the nitrite to •NO in different plant species [22–25]. The *Arabidopsis thaliana* mutant *nia1/nia2* that lacks NR produces less •NO and is more susceptible to bacterial and fungal infections [26–29] demonstrating the importance of NR-derived •NO in plant physiology. In green algae, the synthesis of •NO has been reported in *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* [30–32]; the •NO production in *S. obliquus* and *C. sorokiniana* was nitrite-dependent and insensitive to NOS inhibitors. According to this, the compelling question of the evolution of enzymatic •NO sources in land plants emerges.

In land plants, all the studies regarding •NO synthesis have been performed in angiosperms and gymnosperms [19,33,34] leaving a gap of information concerning the •NO synthesis in the rest of the embryophytes (i.e. bryophytes and pteridophytes). In evolutionary terms, the bryophytes are widely accepted as the modern representatives of early land plants [35,36]. Thus the study of the enzymatic synthesis of •NO in the basal land plants will provide valuable information about the enzymatic synthesis of •NO in the Plant kingdom. The moss *Physcomitrella patens* has been used as a model system for comparative analysis between basal and higher land plants [37–40]. It has been reported that *P. patens* possesses three *NIA* genes [41] as well as the NR activity [42]. Thus, the aim of this work was to evaluate whether *P. patens* is capable of •NO production and to establish the possible role of NR in this process.

Measuring •NO in live plants is a challenge that has promoted the development of direct and indirect techniques to detect this molecule, recently reviewed in [43]. Among them, the Electron Paramagnetic Resonance (EPR) technique stands out because it accurately detects •NO in crude extracts using a specific "spin-trap" [44]. This technique has been used to detect •NO in *Pisum sativum* leaves [45], *Glycine max* chloroplasts [46] and to characterize the •NO production in the *Arabidopsis* mutant *nia1/2/noa1* [47]. Other methods, such as epi-fluorescence and confocal laser scanning microscopy (CLSM) using fluorescence probes are common in •NO research. From the family of diaminofluoresceins (DAF), 4,5-diaminofluorescein diacetate (DAF-2DA) is preferred because it enters the cell and reacts with •NO to produce the fluorescent triazole DAF-2T [48] allowing the identification and localization of •NO inside cells. This technique has been used to monitor •NO production in green algae [31], gymnosperms [49], and angiosperms [45, 50].

Here, the presence of •NO in *P. patens* protonema was detected using EPR spectroscopy and CLMS. As no mutants of *NIA* genes are available, a pharmacological strategy to inactivate NR using sodium tungstate was successfully applied. Plants with reduced NR activity had a •NO production drastically reduced, indicating that in the moss *P. patens* protonema, NR is the main source for •NO production.

Materials and Methods

Plant growth conditions and tungstate treatments

Explants from *P. patens* (Hewd) B.S.G protonema were cultivated on solid Knop medium (0.8% Agar) [51] covered with sterile cellophane discs. Plants were cultivated in a growth chamber at 21°C with 16/8h light/dark period. Light intensity was 30 μmol m^{-2} s^{-1}.

To analyse the effect of sodium tungstate on *P. patens* protonema growth, seven day-old protonema pieces were transferred from simple Knop medium to the same medium supplemented with different concentrations of sodium tungstate (Sigma-Aldrich). The plants were grown for seven days under treatment. At the day of the transfer (day 0) and seven days later when the experiment ended (day 7) the Petri dishes with the protonema cultures were photographed with a Nikon Colpix5000 camera. To analyse the effect of high nitrate concentration (8.4 mM Ca(NO$_3$)$_2$) on *P. patens* growth, the same assay was performed adding nitrate to Knop media alone and to Knop media supplemented with 30 μM sodium tungstate. The area covered by each plant at day zero and day seven were measured using ImageJ software (http://rsb.info.nih.gov/ij/). The relative growth rate was calculated using the following formula: $(ln\ a_f—ln\ a_0)t^{-1}$ [52] where a_f and a_0 *are* the areas occupied by the plant at final day of growth (day 7) and at the beginning of the experiment (day 0) and *t* is the duration of growth equal to 7 days.

Nitrate reductase activity

Nitrate reductase activity was measured following an established method [53]. The protonema was ground with four volumes (v/w) of cold extraction buffer (250 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 μM Na$_2$MoO$_4$, 5 μM FAD, 3 mM DTT, 1% BSA, 12 mM β-mercaptoethanol and 250 μM PMSF). One volume of pre-warmed (25°C) assay buffer (40 mM KNO$_3$, 8 mM Na$_2$HPO$_4$, 20 mM NaH$_2$PO$_4$ [pH7.5] and 0.2 mM NADH) was added and the mixture was incubated at 25°C. After 0, 5, 10 and 15 min, 100μl aliquots were removed from the assay mixture and the reaction was stopped by adding 25 μl of 0.6 M zinc acetate, the samples were kept for 20 min at room temperature, then 100 μl of 1% sulphanilamide dissolved in 3 N HCl supplemented with 100 μl 0.02% N-(1-naphthyl)-ethylenediamine were added. The mixture was incubated for 20 min at room temperature, centrifuged at 18,000 x g and the nitrite was measured by spectroscopy at 540 nm. Nitrite concentration expressed in μmol h^{-1} g^{-1} FW was calculated based on a nitrite standard curve. The nitrite content of control and tungstate treated plants was obtained from the samples taken at time 0. Total soluble proteins were obtained by grinding 0.1 g of protonema in 400 μl of the extraction buffer (250 mM Tris-HCl [pH 8.0], 250 μM PMSF, 12 mM ß-mercaptoethanol, 1 mM EDTA). The resulting solution was cleared by centrifugation at 13 000 x g for 5 min. Concentration of soluble proteins was quantified using Bradford reagent binding assay (Sigma-Aldrich) using bovine serum albumin as standard.

•NO detection by EPR

Plant extracts were obtained by homogenizing 2.0 g of protonema explants with 500 μl of 0.1 M phosphate buffer (pH 7.2), previously flushed with argon, then centrifuged at 13,000 x g for 10 min. The supernatants (750μl) were mixed with an equal volume of freshly made 20 mM degassed citrate buffer containing 30 mM N-methyl-D-glucamine dithiocarbamate (MGD) (Santa Cruz) and 1 mM FeSO$_4$-7H$_2$O in deionized water [54, 55]. Then the mixtures were incubated for 1h at room temperature to allow the formation of the (MGD)$_2$Fe(II)NO complex. Samples were maintained on ice and immediately measured. Since •NO reacts rapidly with O$_2$

the sample preparation was performed in an anoxic chamber. EPR spectra were measured under non-saturating conditions of microwave power on a Bruker Elexys E500 X-band spectrometer (in a quartz flat cell at 297K; microwave power, 20.0 mW; modulation frequency, 100KHz; modulation amplitude, 3G; time constant, 20.48 ms). The spectra were simulated using the Bruker software, and *g*-values were calculated by measuring the magnetic field and the microwave frequency.

•NO detection by CLMS

Protonema samples (grown as indicated before) from simple media (control) and media with 30 µM sodium tungstate were incubated for 15 min in the dark in 0.1 M phosphate buffer (pH 7.4) containing 20 µM of 4,5-diaminofluorescein diacetate (DAF-2DA) (Calbiochem). Plants treated with the •NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (Sigma-Aldrich), were pre-incubated for 45 min with 200 µM cPTIO in 0.1 M phosphate buffer (pH 7.4). Afterwards, the samples were incubated for 15 min in the same buffer supplemented with 20 µM DAF-2DA and 200 µM cPTIO. Then the plants were mounted on slides in the same solution and analysed under the microscope. All images were collected with a confocal Zeiss LSM510 inverted microscope equipped with 40x Plan Neofluar NA 0.75 objective lens. DAF-2DA fluorescence was excited with the 488 nm line of Argon laser (with HFT UV/488/543/633 DM, BP 500–530 nm settings). Images were collected using the same specific set of parameters for laser power, photomultiplier gain level, pixels (0.2197 microns) and pinhole size. The obtained data corresponded to a stack of images of 1024 x 1024 pixels, each one composed of two channels (fluorescence and bright field).

A quantitative method to compare the fluorescence intensity associated with •NO production in *P. patens* tissue was developed. From each single image, the information from bright field and fluorescence channels were combined to identify the pixels corresponding to the regions of interest (ROI) associated with fluorescent signal; this process eliminated the pixels of the background of the image. To analyze the set of pixels on the ROI's, we have developed an algorithm to calculate the fluorescence intensity histogram and to normalize its intensity distribution relative to its own area.

Applying the algorithm to the set of images associated with each experimental treatment, a set of histograms of gray intensity levels was obtained from where an average curve that describes the probability distribution of the gray intensity levels associated to the specific experimental treatment was obtained. The autofluorescence associated to the sample without DAF-2DA was calculated and subtracted from these curves. The statistical independence between the resultant distributions was determined using a Kolmogorov-Smirnov test. Finally, integrating the intensity distribution for every treatment, we obtained the total fluorescence corresponding to the different experimental conditions. The highest fluorescence corresponding to the control DAF-2DA sample was used to normalize the total intensity associated to all the experimental conditions. Two biological replicates were performed (n = 22–28 for each treatment)

Results

Nitrate reductase activity inhibition

As the interest of this work is to examine whether *P. patens* produces •NO in a NR-dependent manner and in light of the lack of null *nia* mutants, a pharmacological approach to reduce NR activity was employed. It is believed that tungsten ion substitutes for a molybdenum ion during the synthesis of the molybdate cofactor, and the addition of tungsten to growth media successfully inactivated NR in tobacco and rice leaves [45,56]. Thus, *P. patens* protonema were

cultivated in simple Knop medium or in medium supplemented with increasing concentrations of sodium tungstate.

As shown in (Fig. 1A) NR activity in the untreated (control) plants was 2.4 μmol h^{-1} g^{-1} FW, which is comparable to the range of NR activity reported for other plants as *A. thaliana*, *Nicotiana plumbaginifolia* and *Solanum lycopersicum* leaves (15, 1.9 and 0.38 μmol h^{-1} g^{-1} FW, respectively) [57–59].

As expected, sodium tungstate decreased the NR activity; plants growing with 30 μM sodium tungstate had only 32% of the NR activity from untreated control plants. Moreover, the activity was reduced to 10% of that from control plants during 150 μM sodium tungstate treatment. To confirm the NR inactivation the nitrite content from control and treated plants was measured (Fig. 1B). Nitrite is the product of NR activity, thus when the enzyme is inactive less product should be formed. Indeed, plants treated with 30 μM and 150 μM sodium tungstate had 41% and 21% of the nitrite content of control plants, respectively. These results demonstrated that NR activity was effectively blocked by the tungstate present in the growth medium. To analyse the possible effect of reduced NR activity on nitrogen metabolism the total soluble protein content from control and tungstate-treated protonema was measured. As shown in Fig. 1C, the total protein level did not change between control and treated plants, suggesting that the NR activity still present in plants treated with sodium tungstate was enough to maintain the tissue protein demands. To select the most suitable tungstate concentration for evaluating the NR role in •NO synthesis, the growth of control and treated plants was compared (Fig. 2). Tungstate treatments lasted for seven days and the relative growth was calculated from the pictures at the beginning and end of the experiment (Fig. 2A). The statistical analysis showed that tungstate treatment reduced the relative plant growth rate in a dose-dependent manner (Fig. 2B). The most severe effect was at 150 μM which reduced the growth rate to 58% of that in the control. As 30 μM sodium tungstate had a subtle effect on the relative plant growth rate but the enzyme lost 68% of its activity (Fig. 1A), this concentration was used in the treatments to analyse the NR contribution to •NO synthesis.

Detection of •NO in P. patens protonema by EPR and CLMS

The multiple roles of •NO in plant physiology have been shown in green algae and in higher plants [60, 61], but its production in non-vascular plants has not been demonstrated. Thus, the endogenous •NO in *P. patens* was detected using EPR and the spin trap (MGD)$_2$Fe(II). The EPR spectrum of (MGD)$_2$Fe(II)NO adduct at 297 K (Fig. 3) isolated from the control protonema extracts revealed a well-resolved three-line spectrum with an isotropic g value = 2.040 and an hyperfine coupling, a^N, value of 13 G typical of the EPR signal of (MGD)$_2$Fe(II)NO compounds in aqueous solution [54] demonstrating that *P. patens* produce •NO during normal protonemal growth.

Under the same concentration of extract and spin trap, the •NO signal decreased almost completely in plants that grew on 30 μM sodium tungstate indicating that the •NO production in *P. patens* protonema was dependent on NR activity. To strength this conclusion the •NO production was analysed in protonema that grew in Knop media supplemented with high nitrate concentration 8.4 mM Ca(NO$_3$)$_2$ (Knop media contains 4.2 mM Ca(NO$_3$)$_2$ [Fig. 4A]). As expected the •NO amount increased (Fig. 4B) compared to standard nitrate concentration. The •NO signal was almost lost in plants grown in standard media and media with high nitrate both supplemented with sodium tungstate (Fig. 4D and E) confirming that NR is the enzyme responsible for the •NO production. The plant growth rate and NR activity in plants growing in high nitrate did not change compared with plants growing in simple Knop media (S1 Fig.)
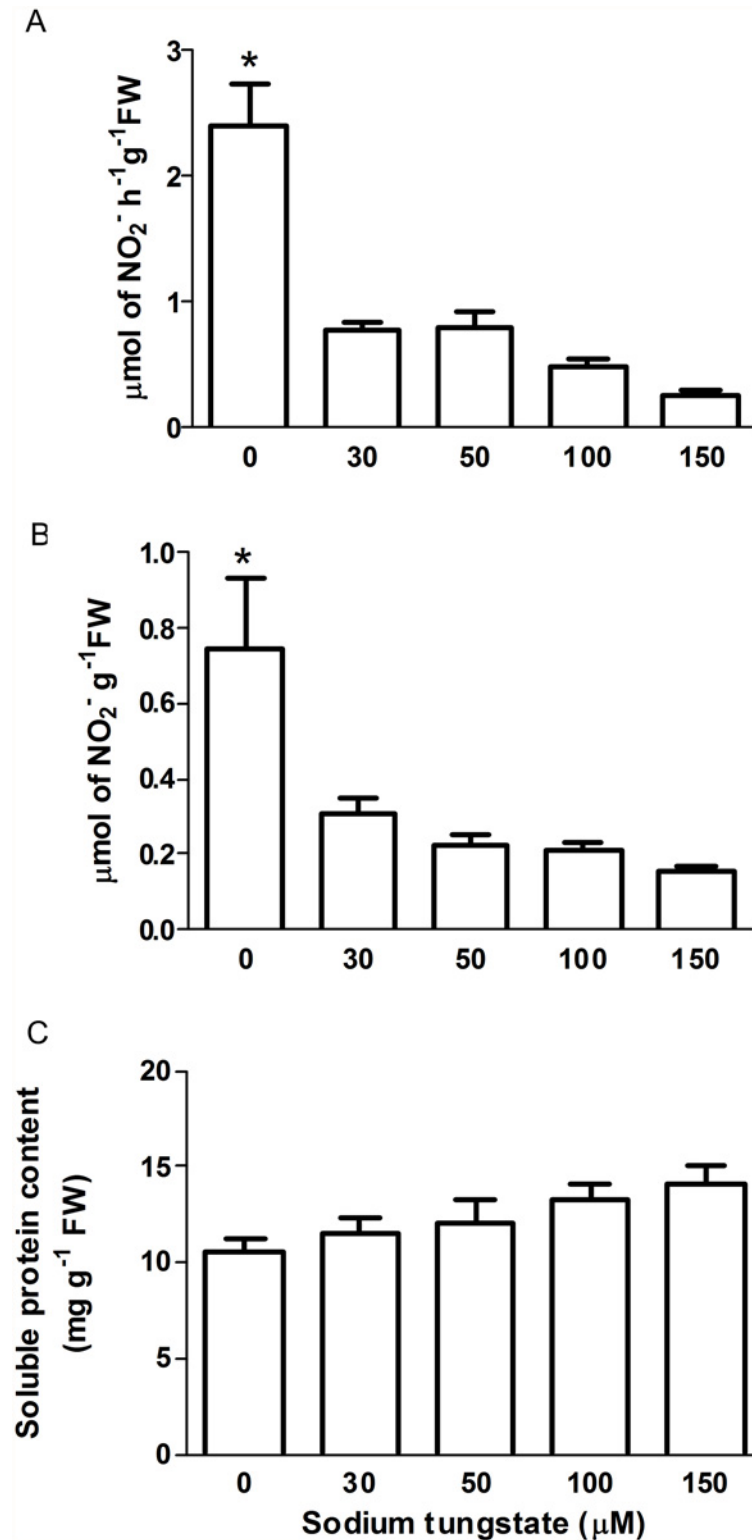
Fig 1. Analysis of the effect of sodium tungstate in *P. patens* protonema. A) Nitrate Reductase activity. B) Plant endogenous nitrite content. C) Plant total soluble proteins. Plants grew for seven days in simple Knop medium or media supplemented with increasing concentrations of sodium tungstate. Data are mean of three independent experiments. Data were analyzed by one-way ANOVA and Tukey´s multiple comparison test (n = 9 in panel A, n = 3 in panels B and C). Asterisk denotes statistically significant difference ($P < 0.05$). Error bars denote SE.
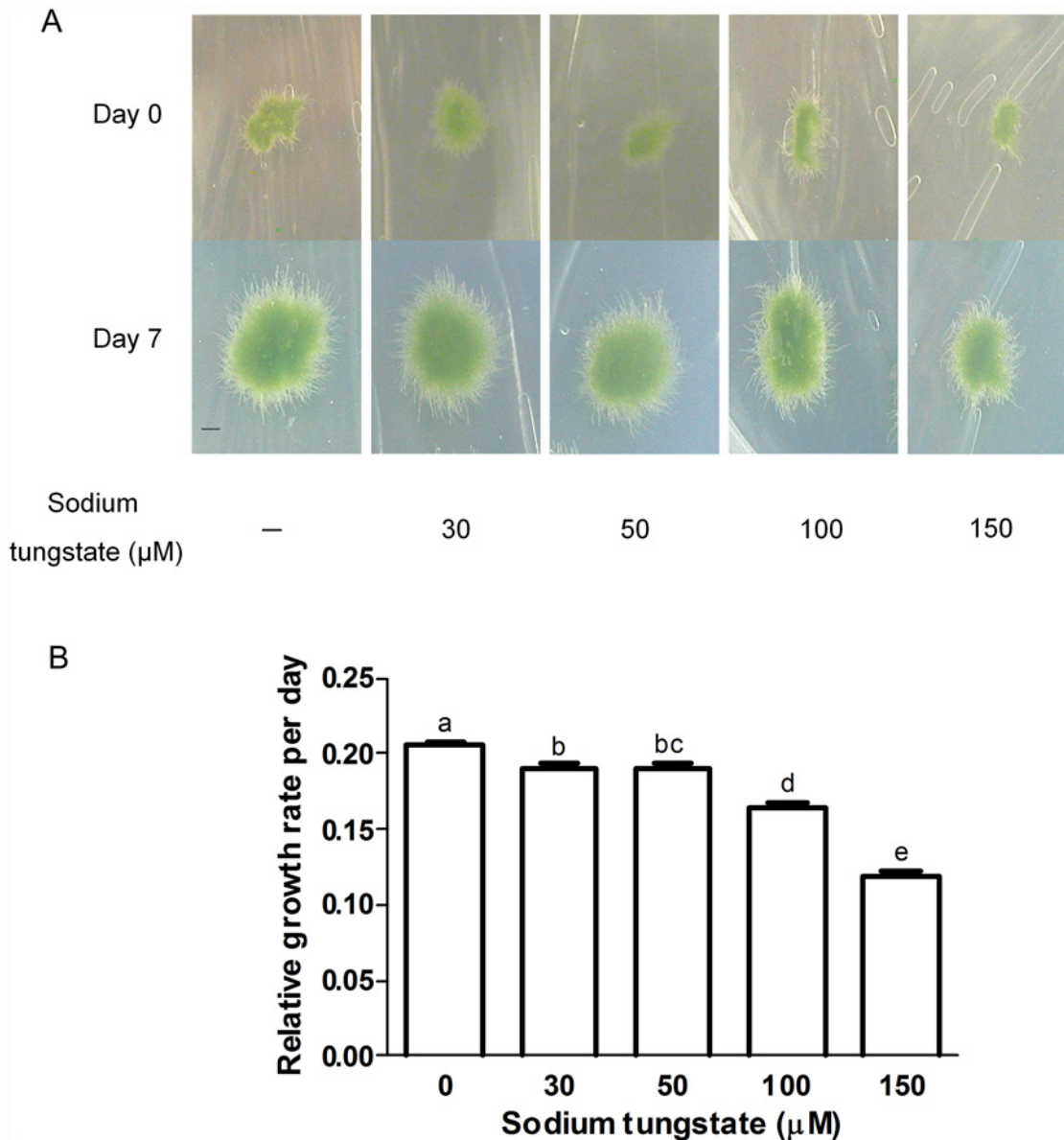
Fig 2. Effect of sodium tungstate on *P. patens* protonema growth. A) Plants grew for seven days on simple Knop medium or on media supplemented with the indicated concentrations of sodium tungstate. Pictures were taken at the beginning and end of the experiment, Day 0 and Day 7, respectively. Scale bar = 3 mm B) Plant relative growth rate was measured according to 52. ($\ln a_f - \ln a_0)t^{-1}$ where a_f and a_0 are the plant area at day 7 and at day 0, respectively. t is the time growth in days = 7. Data are mean of three independent experiments. Data were analyzed by one-way ANOVA and Tukey´s multiple comparison test ($n = 84$). Different letters indicate a statistically significant difference ($P < 0.05$). Error bars denote SE.

To further characterize the •NO production in *P. patens* protonema an analysis of •NO localisation was performed using the DAF-2DA fluorescent probe and CLSM (Fig. 5A). The •NO signal was detected in the cytoplasm of control plants, confirming the presence of •NO in protonema. The fluorescence signal was significantly decreased in protonema of control plants incubated with cPTIO as well as in plants treated with 30 μM of sodium tungstate with or without cPTIO. The quantitative analysis of the DAF-2DA emission assay presented in Fig. 5B showed that the •NO signal from control plants treated with 200 μM cPTIO was 42% of that in the control plants, indicating that the DAF-2DA green emission in untreated control was due
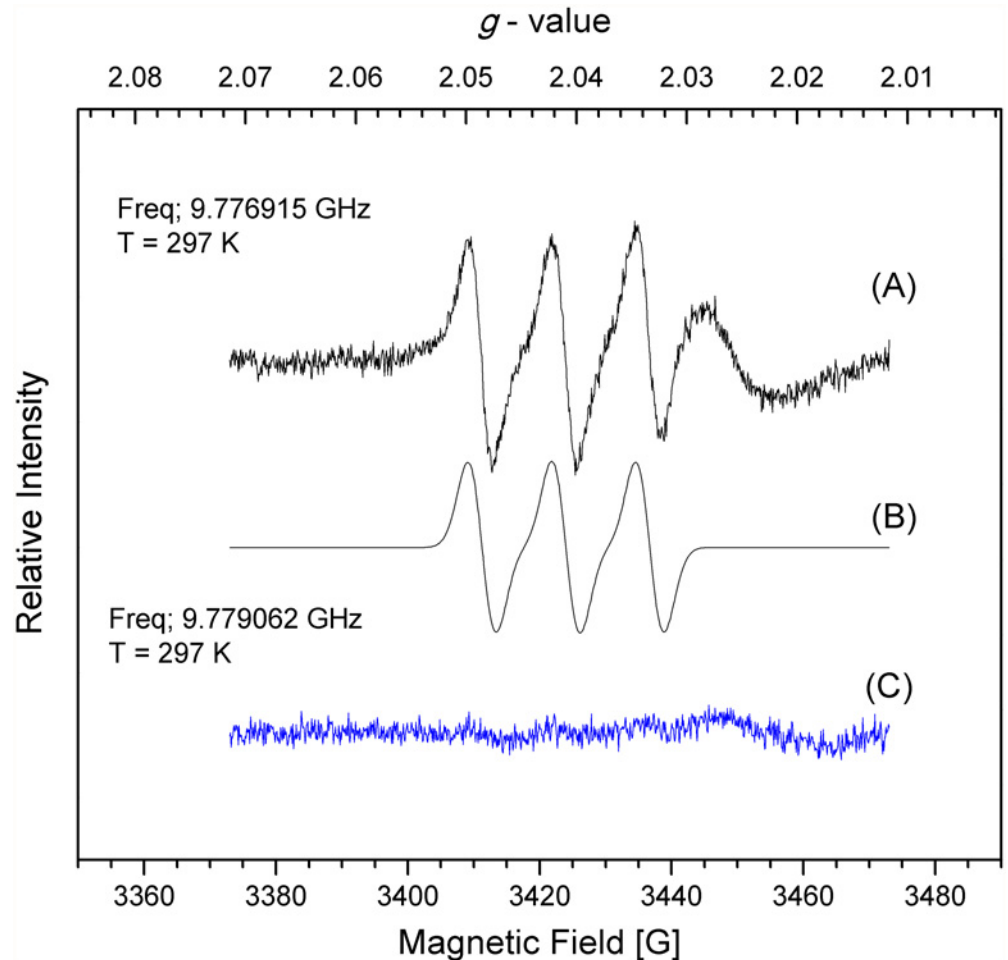
Fig 3. Nitric oxide detection by EPR in P. patens protonema. A) Plants grew for seven days on simple Knop media or C) on medium supplemented with 30 µM sodium tungstate. Homogenized tissues were mixed with $(MGD)_2Fe(II)$ and incubated for 1h. EPR spectra were recorded at approximately 9.8 GHz (X-band) and 100 kHz modulation (Microwave frequency, 9.776915 GHz for A and 9.779062 GHz for C; microwave power, 2.0 mW; modulation amplitude: 0.3 mT; T, 297 K). Each graph is a representative spectrum of four independent experiments. B represents the simulated spectrum of A.

to •NO present in cells ([Fig. 5C](#)). Moreover, the DAF-2DA signal in protonema of plants treated with sodium tungstate was reduced to the same level as that in control plants treated with cPTIO, supporting the observation that •NO is produced mainly by NR activity. The addition of cPTIO to tungstate-treated plants did not change the •NO signal intensity, confirming that in these plants the •NO synthesis or accumulation was blocked.

Discussion

The •NO synthesis mediated by NR is present in green algae and seed plants. In the latter, the NOS-like activity has been described in several species, but the protein(s) that perform such activity remains unknown. Interestingly, there is little information about •NO synthesis and function in basal land plants. Recently we described a family of three *NIA* genes in *P. patens* [41]. Here, the activity of the enzymes encoded by those genes was verified and their role in •NO synthesis examined. The NR activity of protonema growing in normal conditions
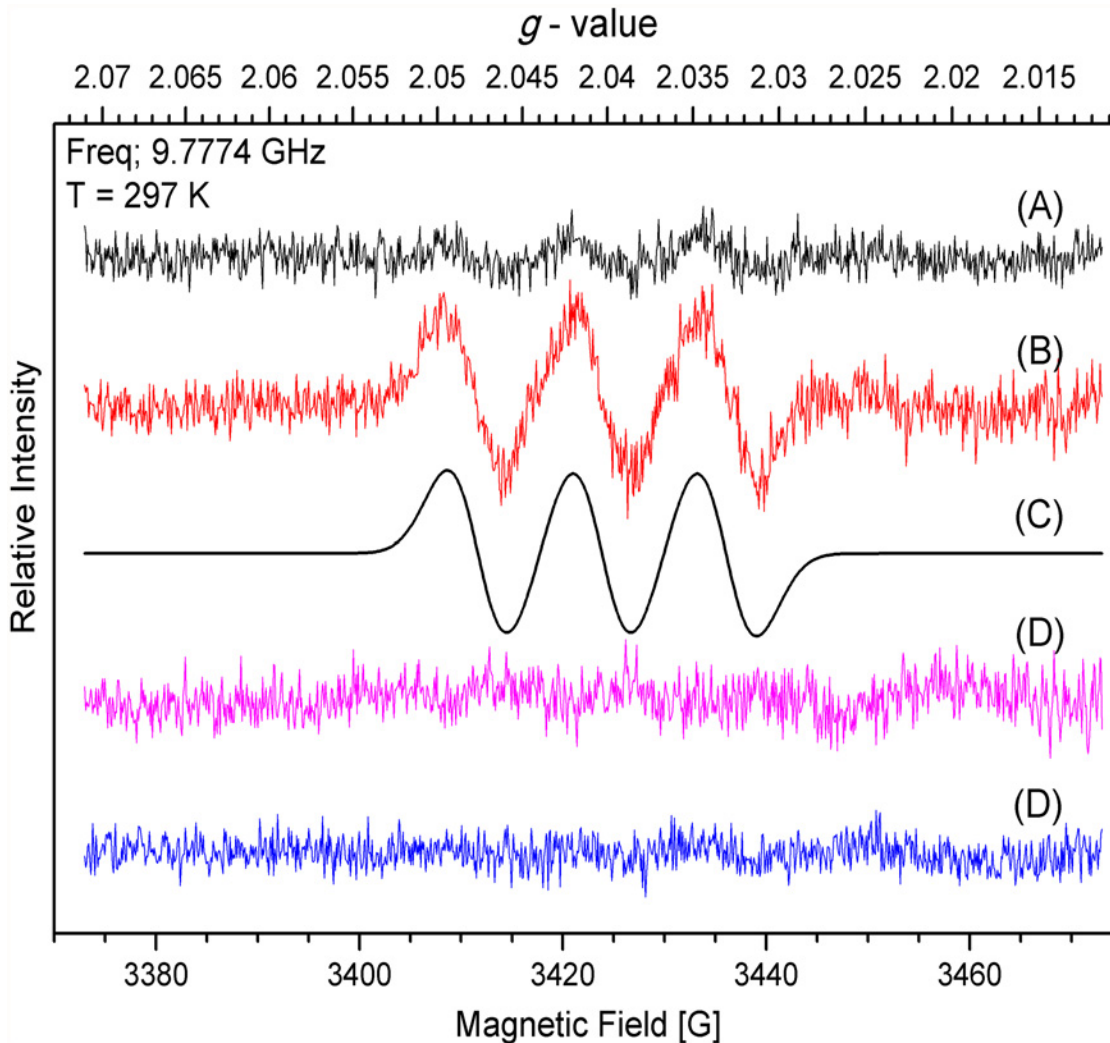
Fig 4. Nitric oxide detection by EPR in P. patens protonema growing in high nitrate. The EPR assay was performed as in Fig. 3 using plants that grew for seven days on A) Knop media, B) media added with 8.4 mM Ca(NO₃)₂, D) Knop media added with 30μM sodium tungstate and E) media added with 8.4 mM Ca(NO₃)₂ and 30μM sodium tungstate. EPR spectra were recorded at approximately 9.8 GHz (X-band) and 100 kHz modulation (Microwave frequency, 9.777400 GHz for B) and microwave power, 2.0 mW; modulation amplitude: 0.3 mT; T, 297 K). Each graph is a representative spectrum of four independent experiments. C) represents the simulated spectrum of B).

(Fig. 1A) is comparable to NR from different plant species [57– 59]. However, the result obtained here is different from a report in which the NR activity from *P. patens* is 0.24 μmol h^{-1} g^{-1} FW [42]. The age and developmental stage of the plants used in that work were not reported. Here, the NR activity corresponds to two week-old plants formed by growing protonema tissue, and thus it is likely that the nutrient demands in this developmental stage could explain the greater NR activity found in our study. The effectiveness of sodium tungstate treatment to reduce NR activity in *P. patens* provided an appropriate system to evaluate the role of NR in •NO synthesis in this plant, as an alternative to the use of *nia* mutants that are not available. The inactivation with 30 μM sodium tungstate was enough to reduce NR activity by 68% without affecting total protein content (Fig. 1C) and a decrease of only 8% on relative growth rate (Fig. 2B). The suitability of the use of tungstate to inactivate NR in studies regarding •NO synthesis has been questioned because in plants the tungsten ion substitutes the molybdenum
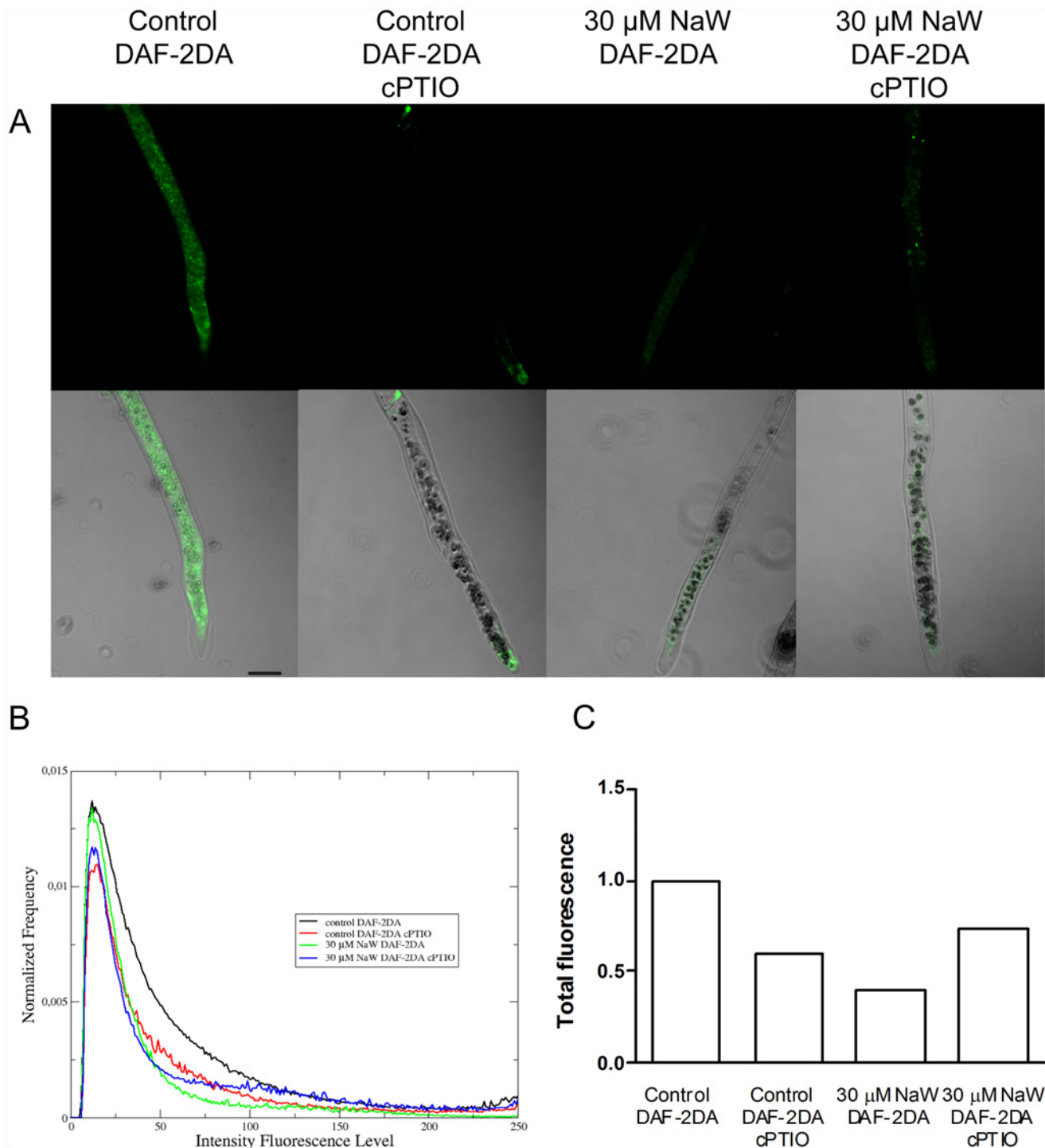
Fig 5. Nitric oxide detection in *P. patens* protonemal cells by confocal laser scanning microscopy. A) DAF-2DA green emission by protonemal cells grown for seven days on Knop medium or medium supplemented with 30 µM sodium tungstate. Protonema was incubated in growth medium supplemented with 20 µM DAF-2DA for 15 min. Some plants were preincubated with 200 µM cPTIO for 45 minutes and then with DAF-2DA. Green signal indicates •NO production. The micrographs show pairs of representative laser confocal microscopy and bright-field images of the protonemal cells. Scale bar = 50 µm. B) Normalized average histograms, each curve describes the DAF-2DA green emission intensity distribution associated to a specific experimental treatment. C) For each treatment, the total DAF-2DA green emission intensity was obtained based on the area under the curves shown in B, and normalized according to DAF-2DA green signal intensity from untreated control. Data from two independent experiments (n = 22–28). The statistical independence between the distributions (B) was calculated using the Kolmogorov-Smirnov test.

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ion in three more enzymes besides NR: sulphite oxidase, xanthine dehydrogenase and aldehyde oxidase. The inactivation of such enzymes could alter the cell metabolism and indirectly the •NO synthesis [62]. Only xanthine dehydrogenase has been linked to •NO production in mammals [62, 63] but the recombinant protein from plants is unable to support •NO synthesis [64]. On the other hand, it has been shown in *C. sorokiniana nia* mutant that treatment with 100 µM tungstate did not alter the •NO emission, detected by gas phase chemiluminescence, indicating that the potential inhibition of the other Mo enzymes do not participate in the synthesis of •NO [32]. The protein content in control and treated plants did not change (Fig. 1C) indicating that the nitrogen metabolism was not affected by the sodium tungstate treatment. Moreover, the tungstate concentration used here (30 µM) was lower than the concentration used by other authors that ranges from 1 to 0.1 mM (reviewed by [62]) ruling out the possible stress effect of tungstate in *P. patens*. The inactivation of NR by sodium tungstate treatment on *P. patens* protonema resulted in a phenotype similar to that obtained in other plant models with reduced NR activity. The *A. thaliana* single mutant *nia1*, has a NR activity of only 10% of that of wild type and presents normal shoot growth, whereas the double mutant *nia1/nia2* had NR activity that is only 0.05% of that of the wild type and the plant size is severely reduced [65]. In the case of *P. patens*, plants treated with 30 µM sodium tungstate had a NR activity representing 32% of that in control plants, a normal total protein level and almost normal growth. Accordingly, it was concluded that the best conditions to analyse the possible role of NR in •NO accumulation in *P. patens* was the treatment with 30µM sodium tungstate.

As discussed before, EPR is an excellent method to detect •NO that has been used successfully in several plants [45–47]. Here, the g value at 2.040 and a hyperfine coupling of 13 G obtained by the EPR technique was used to demonstrate beyond any doubt the existence of endogenous •NO in *P. patens* protonema. The results presented in Figs. 3 and 4 show for the first time that non-vascular plants produced •NO during normal growth, and that the •NO signal from plants with low NR activity is dramatically reduced demonstrating that NR is the main source of •NO in *P. patens*. The •NO production by NR has been demonstrated in different organs of a variety of plant species. Rockel *et al.*, [24] established the nitrate dependent •NO production in sunflower and spinach leaves. The constitutive expression of *NIA1* gene from *Eucalyptus grandis* in *A. thaliana* resulted in increased •NO levels in transgenic plants [66]. Moreover, the *A. thaliana* double mutant *nia1/nia2* produce less •NO and is more susceptible to bacterial infections [26, 27]. These results demonstrate the importance of NR in •NO synthesis in higher plants. Also in green algae the NR-dependent •NO production was proved in *C. reinhardtii* and *S. obliquus* [30, 31] and it is probably present in other species. The enhanced •NO production observed in plants growing in high nitrate (Fig. 4) indicates that *P. patens* NR, as the other NR from plants, increases the •NO production when more substrate is available.

A commonly used technique to analyse •NO location in plant cells and tissues is epifluorescence or CLMS with DAF-2DA used as a probe [18, 62, 63]. Using the DAF-2DA fluorophore and CLMS the •NO presence in *P. patens* protonema was confirmed and showed that •NO is distributed throughout the cytoplasm (Fig. 5). The DAF-2DA signal intensity was reduced when plants were treated with the •NO scavenger cPTIO, demonstrating that the signal corresponded specifically to •NO presence. The DAF-2DA signal intensity in plants with impaired NR activity was also diminished, confirming the observed effect of sodium tungstate on the •NO concentration obtained by EPR (Fig. 3). The results presented in Fig. 5A show a faint green signal in tungstate-treated protonema, but according to the quantitative analysis their comparative fluorescence is in the order of control plants treated with cPTIO (Fig. 5C), demonstrating that in tungstate-treated plants the •NO level was diminished.

Overall, the information available and the data presented here demonstrate that NR is the main enzymatic source of •NO synthesis in *P. patens*. The presence of •NO in protonema indicates that this molecule is needed for *P. patens* normal growth, which was expected, due to the known crucial roles of •NO in plant physiology. Our results are the first step in a promising new area of research that necessarily leads to the analysis of the probable cross-talk between •NO and other plant hormones that has been demonstrated in higher plants (Recently reviewed in [67]). In *P. patens*, auxin and cytokinins are important for differentiation of caulonema to chloronema and for caulonema branching, respectively [68]. It is possible that •NO is involved in these development processes. Furthermore, protonema growth occurs by tip growth and it was shown that •NO plays a crucial role in the control of tip growth in root hairs and pollen tube of angiosperms [69, 33]. Hence, the analysis of the roles of •NO in *P. patens* growth, development and physiology will open the door to understand the evolution of this gas molecule in plant biology.

Supporting Information

S1 Fig. Effect of high nitrate on *P. patens* protonema growth and NR activity. The effect of high nitrate in growth A) and NR activity B) was analyzed in plants that grew for seven days in Knop media alone or with 8.4 mM Ca(NO3)2, supplemented or not with 30μM sodium tungstate. Relative growth rate was measured according the formula: $(ln\ a_f - ln\ a_0)t^{-1}$ where a_f and a_0 are the plant area at day 7 and at day 0, respectively. t is the time growth in days = 7. Data are mean of three independent experiments. Data were analyzed by one-way ANOVA and Tukey's multiple comparison test (n = 56 in panel A and n = 7–9 in panel B). Different letters indicate a statistically significant difference ($P < 0.05$). Error bars denote SE.
(TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: JGD MEST VLR. Performed the experiments: RMA ASP JPSV SNM MEST JAPC. Analyzed the data: RMA JGD MEST SNM JAPC JPSV VLR. Contributed reagents/materials/analysis tools: VLR JGD MEST. Wrote the paper: RMA VLR.

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