Key acclimation responses to phosphorus deficiency in maize plants are influenced by exogenous nitric oxide

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ABSTRACT

Improving phosphorus (P) acquisition and utilization in crops is of great importance in order to achieve a good plant nutritional state and maximize biomass production while minimizing the addition of fertilizers, and the concomitant risk of eutrophication. This study explores to which extent key processes involved in P-acquisition, and other acclimation mechanisms to low P supply in maize (*Zea mays* L.) plants, are affected by the addition of a nitric oxide (NO) donor (S-nitrosoglutathione, GSNO).

Plants grown in a complete culture solution were exposed to four treatments performed by the combination of two P levels (0 and 0.5 mM), and two GSNO levels (0 and 0.1 mM), and responses to P-deprivation were then studied. Major plant responses related to P-deprivation were affected by the presence of the NO donor. In roots, the activity of acid phosphatases was significantly increased in P-depleted plants simultaneously exposed to GSNO. Acidification of the culture solution also increased in plants that had been grown in the presence of the NO donor. Furthermore, the potential capability displayed by roots of P-deprived plants for P-uptake, was higher in the plants that had been treated with GSNO.

These results indicate that exogenous NO addition affects fundamental acclimation responses of maize plants to P scarcity, particularly and positively those that help plants to sustain P-acquisition under low P availability.

1. Introduction

One feature that characterizes higher plants is their exquisite capacity to respond to environmental changes, displaying a wide phenotypic plasticity that helps them to avoid or reduce the deleterious effects imposed by stress factors. To a certain degree, plants possess the ability to explore their surroundings in order to get what they are missing. For this to occur, environmental conditions should be narrowly sensed to achieve the combined actions that enable sessile organisms to live in an ever fluctuating environment.

Among the mineral nutrients provided by the soil, phosphorus (P) is one of great ecological and agricultural importance. It is well known that P deficiency has a strong impact on plant performance, as plant growth restriction, increased root/shoot ratio and delayed leaf appearance are usually observed following long-term P-scarcity

(Atkinson, 1973; Rodriguez et al., 1994). P-availability in the soil solution (typically in low micromolar concentrations) is frequently limiting for plant growth, particularly when considering the high demand from tissues. In addition, as the concentration of P in the root depletion zone is very low, roots of plants grown in P-impovertised soils need to acquire and buffer cytosolic P at concentrations three orders of magnitude above that in the environment (Baker et al., 2015). In this context, the addition of P fertilizers is a common practice based on the use of mined phosphate rocks, which imposes a strong dependence on a possible finite resource as well as potential side effects on the environment, which can threaten future food security (Ashley et al., 2011; Baker et al., 2015). Therefore, the need of a more efficient use of phosphorus has been proposed, which involves maximizing P-acquisition and -utilization efficiencies (Veneklaas et al., 2012).

Plants typically respond to P deficiency by altering their root

Abbreviations: GSNO, S-nitrosoglutathione; GSNO_d, degraded GSNO; NO, nitric oxide

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morphology and whole plant physiology in a manner that tends to increase the capacity of P-acquisition and to preserve plant survival (Pang et al., 2015). The array of mechanisms displayed by plants to ensure P-acquisition includes increases in root surface area, lateral root length and root hairs, and –in a way that depends on plant species- the formation of cluster roots as well as the establishment of mycorrhizal symbioses (Lambers et al., 2006; Niu et al., 2013). Physiological responses include the production and exudation of organic acids to the rhizosphere, which help to mobilize P in soils (Jones, 1998; Pang et al., 2015), increased activity of acid phosphatases (Gaume et al., 2001) and the induction of high affinity P-transport (Cogliatti and Santa-Maria, 1990; Jia et al., 2011; Raghothama 2005).

The bioactive molecule nitric oxide (NO) participates in a wide range of functions including acclimation responses to biotic and abiotic stresses (Yu et al., 2014). Endogenous sources of NO in plants remain to be fully characterized (Jeandroz and Wendehenne, 2017), however, it is clear that NO levels vary in plant tissues according to environmental and physiological signals (Simontacchi et al., 2015). In addition, NO generated in the environment can diffuse into plant tissues and exert biological effects (Molina-Favero et al., 2008). The early observations that exogenous NO prevent iron-deficiency- induced chlorosis symptoms in maize (Graziano et al., 2002) opened a field of research concerning other aspects of mineral nutrition that may be affected by NO (Simontacchi et al., 2015).

A role for this small molecule in plant responses to conditions of P shortage slowly begins to emerge. In this regard it has been observed that, under P restriction, white lupin roots display enhanced NO accumulation in the primary and lateral root tips, which correlate with two low P-supply acclimation responses: the formation of cluster roots and increased citrate exudation (Wang et al., 2010). Moreover, the addition of NO donors stimulates the formation of lateral and cluster roots irrespective of P status (Meng et al., 2012; Wang et al., 2010). However, it is not known to what extent other crucial plant responses to P-deprivation could be mediated or influenced by NO in non-clustered roots. Interestingly, recent studies have shown enhanced levels of NO following P-deprivation in the roots of *Arabidopsis* and rice (Wu et al., 2014; Zhu et al., 2017).

In this work we report that exogenously applied NO intensifies some acclimation responses related with P deficiency in maize roots, as are the increase in the activity of acid phosphatases, the phosphate depletion capacity from a diluted P solution, and the acidification of external medium.

2. Materials and methods

2.1. Plant material, growing conditions and treatments

Maize (*Zea mays*) Ax882 Cl-MG seeds were germinated at 28 °C during 4 days in dark on wet filter paper. After this period, seedlings were placed for 4 days in 750 mL pots containing a nutritive solution with the following composition: 1 mM $\text{Ca}(\text{NO}_3)_2$; 1 mM KCl; 0.5 mM MgSO_4 ; 0.5 mM H_3PO_4 ; 200 μM FeNaEDTA; 50 μM CaCl_2 ; 25 μM H_3BO_3 ; 2 μM MnSO_4 ; 0.5 μM CuSO_4 ; 0.5 μM H_2MoO_4 ; 2 μM ZnSO_4 ; 2.5 mM 2-(N-morpholine)-ethanesulphonic acid (MES), and adjusted to pH 5.9–6.0 with $\text{Ca}(\text{OH})_2$. After that, individual homogeneous seedlings were transferred to 2 L pots (1 plant/pot), where they received any of the treatments resulting from the combination of two phosphorus (P) levels (0 and 0.5 mM) and two S-nitrosoglutathione (GSNO) levels (0 and 0.1 mM) for 6 or 21 days (Scheme 1). As pH was adjusted using $\text{Ca}(\text{OH})_2$, differences in Ca^{2+} concentration in the solutions without phosphorus addition were corrected by the addition of CaCl_2 . Pots were continuously aerated and the solution was renewed every 4 days.

S-nitrosoglutathione (GSNO) was synthesized according to (Singh et al., 1996). Equal volumes of 200 mM reduced glutathione in deionized water and 200 mM NaNO_2 prepared in HCl 0.1 N, were mixed and used without further purification. GSNO was prepared and immediately

added to the pots (final concentration 0.1 mM), every time the nutrient solution was renewed. NO release from GSNO was assessed by electrochemical measurements employing a WPI ISO NO Mark II electrode in the culture conditions.

To further evaluate the kinetic of GSNO solution, the decomposition of the 0.1 mM GSNO was followed at $\lambda = 335$ nm, using the extinction coefficient of $922 \text{ M}^{-1} \text{ cm}^{-1}$ (Broniowska et al., 2013). After one week (approximately 7 half-lives) GSNO completely decayed (first order rate constant = 0.032 h^{-1} , $t_{1/2} = 21.6 \text{ h}$) (Supp. Fig. 1), and this solution (GSNO_d) was employed as a control and applied to the pots in the same way as GSNO fresh solution. Reduced and oxidized forms of glutathione were evaluated in the GSNO_d solution according to Tietze (1969).

The experiments were performed in a greenhouse within a range of temperature between 15 and 35 °C, under sun radiation (latitude –34,93), during the end of spring and the summer seasons. Five replicates per treatment were obtained in each assay. Plants were harvested at 6 or 21 days of treatment (day 14 and at day 29 after sowing, respectively), and the fresh weight of each plant fraction was immediately measured.

2.2. Measurement of anthocyanins content in leaves

For total anthocyanins determination, leaves were ground to a powder in liquid nitrogen and extracted with 99% MeOH containing 1% HCl (100 mg FW/mL). Anthocyanin content was estimated from the absorbance at 520 nm (Suzuki, 1995).

2.3. Measurement of P in plant tissues

The amount of total P was measured in roots, leaves and shoots. After harvests, roots were briefly washed in distilled water. Tissue samples were dried at 60 °C in an oven and incinerated at 500 °C. Afterwards, samples were digested with 0.5 mL of pure HNO_3 and diluted in 3 mL of deionized water. P concentration in the digests was measured spectrophotometrically by the molybdovanadophosphate method according to Kitson and Mellon (1944).

2.4. Acid phosphatases activity

Roots were briefly washed in deionized water and, once surface water was carefully removed, placed in liquid nitrogen and frozen until biochemical measurements were performed. Tissues were homogenized in buffer solution containing 3 mM MgCl_2 , 1 mM EDTA, and 1% (w/v) polyvinylpyrrolidone in 50 mM Tris-HCl buffer (pH 7). The supernatant was obtained by centrifugation at 10.000g for 20 min at 4 °C. Acid phosphatases activity was measured through the release of p-nitrophenol from p-nitrophenyl phosphate. Supernatants were mixed with sodium acetate buffer solution pH 4.3 containing 2 mM p-nitrophenyl phosphate (Clark, 1975). The enzymatic reaction was developed at 37 °C during 3 min and stopped with 0.2 N NaOH, and the absorbance was measured at $\lambda = 400$ nm. Acid phosphatases activity was expressed as μmol of p-nitrophenol released per minute in the above described conditions, and referred to the total protein content determined according to Bradford (1976), using bovine serum albumin as standard.

2.5. Measurement of pH change in the culture solution

Plants were individually placed in 700 mL pots filled with the same solution used for plant growth but without the addition of MES (non buffered solution). The pH in each pot was recorded with a pH meter Hanna Edge® electrode from zero up to 240 min.

2.6. P-depletion measurement

Plants were transferred to pots filled with 100 mL of culture solution

Experimental design

Scheme 1. Experimental design outline employed for maize plants exposed to phosphorus restriction and/or the NO donor.

containing 50 μM phosphate, pH 5.9–6.0. Pots with and without plants were weighted prior and after the experiment to estimate the rate of evaporation and evapotranspiration. During the assay solutions were continuously aerated and aliquots of 2 mL from each pot were taken periodically from 5 to 120 min. Phosphorus concentration in the aliquots was quantified employing the method previously described by Murphy and Riley (1962).

2.7. Statistical analysis

The analysis of the data was performed using the Statistica7.0 program, StatSoft, Inc. 1984–2004. Factorial ANOVA was employed to evaluate interactions and differences between Phosphorus and GSNO treatments. Post-hoc comparisons were performed with the Tukey test ($p < 0.05$). When appropriate, Student *t*-test was used as indicated in the respective figures. Data in figures and tables represent the means of five replicates (each replicate corresponds to an independent pot) per treatment from one assay. At least two independent experiments were performed with similar results.

3. Results

We first studied, under the specific growth conditions of these assays, the effect of P-deprivation on some physiological attributes of maize plants. After 21 days of treatment P amount was severely depressed in P-deprived plants (Fig. 1A), and a significant increase in the ratio between root fresh weight and shoot fresh weight was also observed (Fig. 1B). In addition, P-starved plants exhibited a typical symptom of P-deficiency, as it was the increase of anthocyanins content in the oldest leaves (first and second) where P deficiency symptoms became evident early on (Fig. 1C).

To test whether exogenous NO affects maize plants performance under P-restriction, an NO donor (100 μM GSNO) was applied in the nutrient solution after 8 days of sowing (Scheme 1). Under these experimental conditions the NO released in the nutrient solution reached a maximum concentration of 0.5 μM after 5 h of GSNO application (Fig. 2A), in agreement with GSNO decay profile (Fig. 2B). After GSNO application, which was performed with every change of culture solution, no alterations in the pH value (5.9–6.0) were observed in the buffered nutrient solution.

Effects of NO on plant growth have been previously described (Beligni and Lamattina, 2000). A tendency to promote growth was observed only for control plants after long term exposure to GSNO (21 d) (Supp. Fig. 2), without significant effects in P-restricted plants. After 21 d of treatment root/shoot fresh weight ratio was not significantly affected by the NO donor addition in both P-deprived (0.82 ± 0.05 and 0.76 ± 0.03 for plants exposed or not to the NO donor, respectively), and P-well supplied plants (0.45 ± 0.04 and 0.42 ± 0.02 for plants exposed or not to the NO donor, respectively).

P concentration in roots and shoots declined as a consequence of the lack of P-supply in the culture solution and the subsequent dilution of the P accumulated by plants (Fig. 3). The presence of the NO donor did not affect P concentration in roots or shoots in P-restricted plants (Fig. 3). However, a significant reduction in root P concentration was

Fig. 1. Effect of long term P-deprivation in maize plants. Eight day-old plants were grown 21 days in culture solution, in the absence (–P) or the presence (+P) of 0.5 mM phosphate. **A.** P amount in the whole plant. **B.** Root:shoot fresh weight ratio. **C.** Anthocyanins content evaluated in different leaves. Asterisks denote significant differences between means for P-deprived and control maize plants based in Student's *t*-test ($p < .05$). Error bars represent SE.

Fig. 2. A. NO release from GSNO in the culture conditions. GSNO was added to the nutrient solution at a final concentration of 0.1 mM and the release of NO was measured employing the NO-selective electrode (WPI ISO NO Mark II). Solution was kept in the dark and continuously aerated. B. GSNO decay from the 100 mM stock solution was followed spectrophotometrically at $\lambda = 335$ nm, using the extinction coefficient of $922 \text{ M}^{-1} \text{ cm}^{-1}$.

observed for control plants exposed to the NO donor (Fig. 3). This finding is related with the effect of GSNO on growth promotion observed in control plants after long term exposure (Supp. Fig. 2), as total amount of P in roots remained unchanged irrespective of the presence or not of the NO donor (303 ± 28 and $327 \pm 18 \mu\text{mol phosphorus root}^{-1}$, for control plants exposed or not to GSNO for 21 days, respectively).

The pattern of P accumulation in leaves was evaluated in maize plants after 21 days of P-deprivation. A drop in P concentration -relative to that observed in P well supplied plants- was clearly observed in all leaves (blade + sheath) showing a more pronounced decrease in the oldest, first and second, than in the youngest leaves as compared to control (well supplied plants) (Table 1), suggesting the occurrence of P remobilization from the oldest to the youngest leaves accompanying plant growth. The addition of GSNO did not significantly affect the profile of P concentration in leaves (Table 1).

Changes in P accumulation, biomass distribution, and anthocyanins content clearly indicated the progress and establishment of P- deficiency in maize plants under these experimental conditions. While the data above indicated that in maize NO does not modify major growth-related responses to P-deprivation, it remained unclear whether or not NO could affect traits related with P-acquisition under P-scarcity conditions.

Cogliatti and Clarkson (1983) observed clear physiological acclimation responses to P deficiency in potato plants when growth suffered a 20% restriction in P-deprived relative to P-well supplied plants. In our experimental conditions, shoot length in maize plants showed a

Fig. 3. Effect of P-restriction and the presence of an NO donor on P concentration. Eight day-old plants were grown in culture solution, in the absence or the presence of 0.5 mM phosphate, and in the presence or the absence of 0.1 mM GSNO. P concentration was evaluated in roots (A) and shoots (B) from plants exposed to the treatments for 21 days. Values are means $\pm$ SE (standard error). The data were analyzed by Factorial analysis of variance (ANOVA), and Tukey post-hoc tests. Different letters denote significant differences between treatments ($p < .05$).

reduction of 20% after 6 days of P-restriction (data not shown). Thus, some short-term acclimation responses were examined at this time of treatment when plant growth was almost no affected by the stress condition (Supp. Fig. 2), but total P amount was significantly decreased (38 ± 5 and $13 \pm 2 \mu\text{mol P g}^{-1} \text{ FW}$ in control and P-deprived roots respectively; 40 ± 3 and $10.1 \pm 0.7 \mu\text{mol P g}^{-1} \text{ FW}$ in control and P-deprived shoots, respectively).

Among plant responses to P restriction, the increase in the activity of acid phosphatases occupies a prominent position (Zhang et al., 2014). Here we observed that in the roots of P-restricted plants the activity of acid phosphatases increased, as compared to control plants, both at 6 (Supp. Fig. 3) and 21 days of treatment (Fig. 4). Although the presence of the NO donor in the nutrient solution did not affect the activity of acid phosphatases in control (+P) plants; in roots of P-restricted plants, GSNO treatment led to a marked increase in their activities after a long-term period of P-deprivation along with the NO donor (Fig. 4). It is worth mentioning that soluble protein content was not affected among treatments (Supp. Table 1). As a consequence, a similar pattern of acid phosphatases activity response to P-scarcity and GSNO treatment was observed when expressed on protein or on fresh weight basis (Supp. Table 1).

In order to assess if GSNO decomposition led to the generation of other bioactive products responsible for the observed effect on acid phosphatases activity, GSNO stock solution was prepared and kept

Table 1Effect of exogenous GSNO on phosphorus concentration ($\mu\text{mol g}^{-1}\text{FW}$) in well supplied and P-deprived maize leaves.

P	GSNO	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5
+	–	22 ± 8a	41 ± 3a	31.1 ± 2.4a	24 ± 4a	28 ± 3a
+	+	13 ± 4a	41 ± 2a	32 ± 1a	27 ± 8a	27 ± 3a
–	–	1.4 ± 0.2b (↓93%)	2.6 ± 0.1b (↓94%)	3.6 ± 0.6b (↓88%)	5.8 ± 0.2b (↓76%)	9.0 ± 0.8b (↓68%)
–	+	1.3 ± 0.3b	3.1 ± 0.2b	3.8 ± 0.05b	6.3 ± 0.5b	9.7 ± 0.5b

Eight day-old plants were grown 21 days in the absence (–) or the presence (+) of 0.5 mM phosphate, and in the presence (+) or the absence (–) of 0.1 mM GSNO. Values in brackets express the percentage of decrease in P concentration for P-deprived leaves as compared to P-well supplied ones. Data correspond to the mean ± SE. The data were analyzed by Factorial analysis of variance (ANOVA), and Tukey post-hoc tests. Different letters indicate significant different values ($p < .05$).

Fig. 4. Effect of an NO donor on acid phosphatases activity in P-well supplied and P-deprived maize roots. Eight day-old plants were grown 21 days in the absence (–P) or the presence (+P) of 0.5 mM phosphate, and in the presence or the absence of 0.1 mM GSNO or degraded GSNO (GSNO_d). Values are means ± SE (standard error). The data were analyzed by Factorial analysis of variance (ANOVA) and Tukey post-hoc tests. Different letters indicate significant differences between treatments ($p < .05$).

under environmental light and temperature. After one week, when elapsed approximately 7 half-lives (first order rate constant = 0.032 h^{-1} , $t_{1/2} = 21.6 \text{ h}$) (Fig. 2B, Supp. Fig. 1), GSNO was considered completely decayed (GSNO_d) and this solution was employed as a control for GSNO treatment. In the GSNO_d solution the concentrations of oxidized (GSSG) and reduced (GSH) forms of glutathione were evaluated, and the ratio $[\text{GSSG}]/[\text{GSSG} + \text{GSH}]$ was

1.00 ± 0.02 suggesting that the concentration of GSH is negligible after NO release from GSNO. After 21 days of treatment with GSNO_d 0.1 mM no alterations in root acid phosphatases activity for both P-well supplied and P-restricted maize plants were observed (Fig. 4).

To further explore the nature of the NO donor effect on acid phosphatases activity, a complementary *in vitro* assay was performed. Root homogenates from 21 days control and P-deprived plants were directly exposed to GSNO $10 \mu\text{M}$ up to 45 min, and the activity of acid phosphatases was evaluated as described previously. Interestingly, no changes were observed in acid phosphatases activity from *in vitro* GSNO treated samples as compared to samples incubated for the same period in the absence of the NO donor, suggesting that the effect of NO does not involve direct activation of these enzymes, at least under the experimental conditions employed here (Supp. Fig. 4).

Another common observation in plants exposed to P-restriction is the acidification of the rhizosphere due to proton and/or organic acids extrusion (Li et al., 2012). In order to evaluate the root capacity to acidify the nutrient solution, control and P-deprived plants that had been grown in the presence or the absence of GSNO during 6 days, were transferred to a fresh non buffered nutrient solution (without MES addition), and pH values were recorded. While no major changes in pH were observed in the media containing P-well supplied plants, a clear decrease in the pH values along time was found in plants suffering from P-restriction (Fig. 5A). Interestingly, the pots containing plants previously grown in the presence of GSNO showed higher acidification of the media (Fig. 5A). Expression of ΔpH per gram of root consistently showed the strong effect of GSNO treatment observed in P-deprived plants (Fig. 5B).

In order to evaluate if the observed effects in the capacity of external medium acidification were due to NO release, controls were performed adding to the culture solution degraded GSNO (GSNO_d)

Fig. 5. Effect of an NO donor on the acidification of nutrient solution by the roots. Eight day-old plants were grown for 6 days in the absence (–P) or the presence (+P) of 0.5 mM phosphate, combined with the presence or the absence of 0.1 mM GSNO. **A.** Typical time course of pH in individual non buffered-pots from plants previously grown in the presence (circles) or the absence (squares) of phosphate combined with the presence (dark symbols) or the absence (white symbols) of GSNO. **B.** ΔpH was calculated as the difference between the initial pH and the pH value after 240 min of root immersion in the non buffered nutrient solution, expressed on fresh weight basis ($\text{pH}_t - \text{pH}_i / \text{g FW}$). Values are means ± SE (standard error). The data were analyzed by Factorial analysis of variance (ANOVA) and Tukey post-hoc tests. Different letters denote significant differences between treatments ($p < .05$).

Fig. 6. Effect of an NO donor on P uptake from a diluted P solution. Eight day-old plants were grown for 6 days in the absence (– P) or the presence (+ P) of 0.5 mM phosphate combined with the presence or the absence of 0.1 mM GSNO. **A.** Typical time course of P amount in the solution of individual 50 μ M phosphate-pots containing plants previously grown in the presence (circles) or the absence (squares) of phosphate combined with the presence (dark symbols) or the absence (white symbols) of GSNO. **B.** Rate of P uptake from plants grown in the presence (■) or absence of GSNO (□). The initial rate was calculated as the difference between initial P amount in the solution and the amount after 15 min of root immersion, and expressed on fresh weight basis. Values are means $\pm$ SE (standard error). The data were analyzed by Factorial analysis of variance (ANOVA) and Tukey post-hoc tests. Different letters denote significantly differences between treatments ($p < .05$).

following the same scheme as with freshly prepared GSNO. P-well supplied plants (+P) that had been exposed to GSNO_d for 6 days showed an almost negligible decrease from the initial pH value (0.018 ± 0.001 units g^{-1} FW after 240 min). While P-deprived plants exposed to GSNO_d exhibited a decrease in the initial pH value of 0.078 ± 0.004 units g^{-1} FW in the same period. These values compare well with those obtained for plants grown in the absence of GSNO, and were lower than those obtained in former experiments for plants treated with fresh GSNO as shown in Fig. 5B.

A key, well known, response of plants to P-deprivation is the increase in the potential capability of roots to incorporate P from diluted P-solutions (Cogliatti and Clarkson, 1983). In order to examine the possible effect of the NO donor addition on this response, we estimated P-depletion from a 50 μ M phosphate nutrient solution for plants that had been grown in the presence or the absence of GSNO. P-deprived plants exhibited a clear P depletion in the external medium, and it was observed that plants grown in the presence of GSNO displayed a more effective capacity to extract P from the medium (Fig. 6A). In turn, the initial rate of P-uptake, expressed as root weight basis, clearly shown that roots of plants formerly exposed to P-restriction combined with the presence of the NO donor exhibited an increased capability for P-uptake (Fig. 6B). The effect of GSNO on the ability of the roots to extract the P from the medium was also analyzed in well P-supplied plants (+P). In this case plants were transferred from 500 μ M phosphate solution to distilled water for 1 min, and then placed in the 50 μ M phosphate solution, employed for the uptake assay, for 5 additional minutes before to start the measuring process. This protocol helped to reduce the possible masking effect of P-efflux from root compartments involved in rapid P-elution (Cogliatti and Santa-Maria, 1990). Those plants did not show appreciable P uptake during 25 min, irrespective of the treatment or not with the NO donor (Fig. 6A and B).

To check for potential side effects of the GSNO donor, experiments were performed employing a degraded GSNO solution (GSNO_d), following the same scheme as the treatment with freshly prepared GSNO solution. The initial P uptake was 0.007 ± 0.001 μ mol P g^{-1} FW min^{-1} for P-well supplied plants treated with GSNO_d, and 0.021 ± 0.006 μ mol P g^{-1} FW min^{-1} for P-restricted plants treated with GSNO_d. These figures are similar to those obtained for plants grown in the absence of GSNO (Fig. 6B).

4. Discussion

Acquisition of P is a critical step for plants dwelling on soils affected by low P availability. Higher plants have evolved several acclimation responses that enable roots to maximize the capture of this element following P shortage (Rose et al., 2013; Vance et al., 2003). The precise signal transduction pathways linking low soil P availability with those responses remain a subject of active research, being possible that NO participates in this process (Simontacchi et al., 2015). Along this work we offered evidence indicating that, in maize roots, stimulation of external medium acidification, and two additional pivotal processes, namely the increase of the capability to extract P from diluted solutions, and enhanced acid phosphatases activity, are positively influenced by the presence of an exogenous source of NO.

The employment of an NO donor was based on previous reports showing that endogenous levels of NO increase following P-restriction in white lupin, *Arabidopsis*, and rice roots (Royo et al., 2015; Wang et al., 2010; Zhu et al., 2017). Evidence has been also obtained indicating that NO may contribute to some specific acclimation responses to P-shortage through its effects on the formation of cluster roots (Meng et al., 2012), citrate exudation (Wang et al., 2010), modification of P distribution within the plant (Zhu et al., 2017) as well as metabolic modifications (Royo et al., 2015). However, to our knowledge, no previous studies have established a link between NO and other key processes that allow plants to thrive at low P supplies. NO endogenously generated or provided from the environment have proved to exert biological effects in plants including alleviation of abiotic stress (Du et al., 2015; Foresi et al., 2015). In this study, maize plants were exposed to the NO donor GSNO, a compound that naturally occurs in plants (Barroso et al., 2006), which releases NO in a low steady state concentration (Jasid et al., 2006). In order to test to what extent side effects derived from GSNO decomposition or synthesis affected the pattern here reported, a solution of degraded GSNO was employed as a control for the key physiological responses evaluated in maize roots. According to the kinetic of GSNO decomposition, after one week GSNO was completely degraded and only oxidized glutathione could be detected in the solution.

No significant changes in biomass accumulation and biomass allocation were observed as a consequence of the NO donor treatment in plants grown under P-starvation conditions.

P is a mobile nutrient in plants and therefore, P recycling and re-distribution from source (older and senescing leaves) to sink (young

leaves and roots) is significant for plant acclimation to P-deficient conditions (Luan et al., 2017; Veneklaas et al., 2012). Under long-term P deficiency, plants could employ P sources by reallocating inorganic P to support growth and development, which results in decreased accumulation of P in old leaves and substantially increased the distribution ratio of P to young leaves (Li et al., 2015). In the experimental conditions employed here, P redistribution was not affected by the presence of the NO donor.

A fraction of P is present in soils as immobile phosphate organic esters, and acid phosphatases represent a major route for P release from organic sources (González-Muñoz et al., 2015). The exudation of acid phosphatases has a role in the extracellular medium where they hydrolyze soil organic P to release inorganic P (Gaume et al., 2001), while intracellular phosphatases are important in the internal P remobilization and recycling under P starvation conditions (Li et al., 2012) and also facilitating P remobilization during senescence (Shane et al., 2014). Both intracellular and secreted acid phosphatases tend to increase during P-limiting conditions (Tran et al., 2010). Recent studies have demonstrated that over-expression of the acid phosphatase PAP26 in rice improves plant performance under P deficient conditions (Gao et al., 2017), highlighting the relevance of this group of enzymes under P restriction. In this work, we found that the activity of acid phosphatases in maize roots increased almost 4 times after 21 days of P-restriction, and that this enzymatic activity was strongly influenced by the presence of the NO donor under P-restricted conditions (Fig. 4B). Interestingly, the effect of NO improvement on acid phosphatase activity was not observed in control plants (+P), and was only evidenced after long term treatment, when phosphatase activity was strongly increased, suggesting a time dependent role of NO on this acclimation response to P deficiency. Acid phosphatases can be regulated by means of transcriptional (Calderón-Vázquez et al., 2008) or post-transcriptional control as it was found for the principal intracellular isoenzyme up-regulated by P restriction in *Arabidopsis* (Tran et al., 2010). The fact that under the experimental *in vitro* conditions probed here no direct effects of GSNO on phosphatase activity were observed, strongly suggest that indirect signaling mechanisms could be involved. Further experiments will be required to explore this issue.

The release of organic acids and the activation of the H^+ -ATPase are mechanisms involved in external medium acidification under P-restriction which positively impact on P-bioavailability and acquisition (Baker et al., 2015; Shen et al., 2006, 2011; Yan et al., 2002). Maize plants exposed for 6 days to P restriction showed a high capacity to perform external medium acidification as compared to plants with full nutrition, and the ability to decrease the external pH was further increased in those plants that had been grown in the presence of the NO donor (Fig. 5). This effect may be related with specific NO-dependent mechanisms leading to proton extrusion and/or a rapid release of organic substances. Interestingly, a high activity of plasma membrane H^+ -ATPase was induced by nitrate in maize (Santi et al., 1995), and it has been also described that not only the amount of nitrogen (Caro and Puntarulo, 1998) but also the form of nitrogen supply (NO_3^- or NH_4^+) (Zhu et al., 2016) impacts on NO levels, thus a relationship between NO and plasma membrane H^+ -ATPase activity could be suggested. In addition, more direct evidence for the involvement of NO on the activation of plasma membrane H^+ -ATPase activity has been already offered by Zandonadi et al. (2010), showing that the NO donor sodium nitroprusside (SNP) increased plasma membrane ATPase proton pumping activity from maize plants. Therefore, under conditions of P-deficiency, a similar effect of NO on H^+ -ATPase activity may be operative.

On the other hand, the net uptake rate of P has been shown to be finely modulated by the level of P-supply (Cogliatti and Santa-Maria, 1990; Raghothama, 2005), which involves both transcriptional and post-transcriptional regulatory effects on specific transport systems (Gu et al., 2016; Yue et al., 2017). In this sense, it has been well established for maize, as well as for other plants, that the capacity of roots to take up external P increases when the supply is restored after a growing

period without the nutrient (Cogliatti and Clarkson, 1983; Lee et al., 1990; Schachtman et al., 1998). In our experimental conditions, when the P-uptake ability was evaluated in plants that had been grown simultaneously in the absence of P and in the presence of the NO donor, a higher P-depletion capacity from a 50 μ M P solution was observed (Fig. 6). Although the regulation of net P uptake by P supply involves the control of both P-influx and P-efflux in roots, at low P-supplies, the net uptake of P can be mainly attributed to the activity of the systems involved in P-influx (Cogliatti and Santa-Maria, 1990). In maize it has been early shown that the contribution of the high-affinity transport mechanism that mediates P-influx from low P-concentrations is enhanced under conditions of P-deficiency (Lee et al., 1990), which should be attributed to the enhanced activity of high-affinity P transporters (Calderón-Vázquez et al., 2011). In order to keep P homeostasis, the degradation of P transporters in P-sufficient conditions occurs through ubiquitin-mediated pathways that are reduced under P deficiency, activating P_i uptake as well as root-to-shoot translocation (Wang et al., 2017; Yue et al., 2017). Interestingly, the participation of NO regulating processes that involve ubiquitin-targeted protein degradation events has been documented in plants and animals (Arnaud et al., 2006; Peng et al., 2008) and constitutes an important issue to explore. Moreover, under P deficiency, a rise in the NO level caused an increase in soluble P, and enhanced the expression of P transporters coding genes in rice roots, facilitating the translocation of P to the shoot (Zhu et al., 2016). Further experiments will be necessary to explore specific molecular mechanisms involving NO actions on maize P transporters.

5. Conclusion

Overall, considering the effects exerted by exogenous NO addition on acid phosphatases activity, P-uptake capacity, and the acidification of the growing medium, together with those shown in previous works (formation of cluster roots, release organic acids), it can be concluded that several acclimation mechanisms involved in P-acquisition are influenced by NO, pointing out a critical role of this small molecule during the acclimation to P-deficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jplph.2018.01.001>.

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