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Nitric oxide, polyamines and Cd-induced phytotoxicity in wheat roots

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ABSTRACT

To further explore the biochemical basis of Cd toxicity in developing wheat seedlings, we studied the possible role of nitric oxide (NO) and polyamines as signaling molecules involved in metal-induced root growth inhibition. When used at 0.1 mM, sodium nitroprusside, a NO-releasing compound, inhibited root growth to a similar extent as Cd and enhanced the polyamine contents as Cd also did. Putrescine and spermidine treatments caused significant decreases in root growth with spermine giving the greatest level of inhibition (77% reduction). The simultaneous addition of Cd and inhibitors of putrescine biosynthesis (DFMA and DFMO) prevented increases in putrescine levels but did not restore normal root growth. NO content, as evidenced by the fluorescent probe DAF-FM diacetate, was found to be significantly increased in the roots of both Cd and polyamine treated plants, especially in those exposed to spermine. The effect was specific for NO since the NO scavenger cPTIO almost suppressed the fluorescent signal. Concerning the oxidative status of the root system, only Cd and spermine enhanced lipid peroxidation in roots. At the same time, all treatments led to a significant increase in levels of the non-enzymatic antioxidant defense glutathione. Our results strongly suggest that Cd and spermine treatments induce NO formation in wheat roots which, in turn, is involved in root growth inhibition.

1. Introduction

Cadmium is a non-essential element that normally occurs in soils at low concentrations (Wagner, 1993). At higher levels it becomes strongly phytotoxic due to its interference with plant uptake, transport and use of different macro and micronutrients (Hart et al., 1998). This can cause growth inhibition and even plant death (Sanitá di Toppi and Gabbrielli, 1999; Benavides et al., 2005).

Reduction of enzyme activities, particularly those enzymes rich in readily available sulfhydryl groups (Schutzendubel et al., 2001), and involved in photosynthesis (Clijtsters and Van Assche, 1985; Sanitá di Toppi and Gabbrielli, 1999), are usually observed after Cd treatment. Although the mechanism by which metals cause plant injury is not clearly understood, there is increasing evidence that, at least in part, metal toxicity is due to oxidative damage (Gallego et al., 1996; Sandalio et al., 2001; Vitoria et al., 2001; Groppa et al., 2003; Cho and Seo, 2005).

Polyamines (PAs), spermine (**1**) (Spm), spermidine (**2**) (Spd) and their precursor putrescine (**3**) (Put) are aliphatic amines found in all living cells. Put (**3**) is synthesized by decarboxylation of either arginine (**4**) or ornithine (**5**), catalyzed by arginine decarboxylase (ADC; EC 4.1.1.19) or ornithine decarboxylase (ODC; EC 4.1.1.17), respectively. The following addition of two aminopropyl groups to Put (**3**) catalyzed by Spd and Spm synthase leads to the formation of Spd (**2**) and Spm (**3**), respectively. PAs have been related to plant response under different abiotic stress conditions including drought, salinity, UV light, air pollutants and heavy metals (Bouchereau et al., 1999; Groppa and Benavides, 2008). They are multifunctional molecules that interact with polyanionic compounds such as either DNA or proteins. Despite their potential significance, the polyamine-dependent signal transduction system has not yet been established.

Nitric oxide is a highly diffusible gaseous free radical that plays a key role as an intra- and inter-cellular messenger to induce various processes in plants; these include germination, induction of cell death and pathogen response (Neill et al., 2003). Along with other plant growth regulators, such as abscisic acid or jasmonates, it may function to either mitigate or to exacerbate stressors effects in different plants (Leshem and Kuiper, 1996). Whereas some authors consider NO a stress-inducing agent (Leshem et al., 1997), others have assigned it a protective role (Beligni and Lamattina, 1999a,b; Hsu and Kao, 2004). Currently, the most confounding issue in plant NO biology is the mechanism(s) of NO production. The enzyme responsible for NO generation in animal organisms is nitric oxide synthase (NOS), catalysing the five-electron oxidation of one of the atoms in L-arginine (**4**) (N^{3-} to N^{2+}) with the participation of O_2 and NADPH. Although NOS-like activity has been detected widely in animals, plant-type NOS is still elusive. Guo et al. (2003) isolated a gene encoding NOS-like protein, AtNOS1 from the *Arabidopsis* genome, which was involved in the process of growth and hormonal signaling. However, the most recent

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studies have raised critical questions regarding the nature of AtNOS1 (Zemojtel et al., 2006; Arasimowicz and Floryszak-Wieczorek, 2007). Another enzymatic source of NO in plants is nitrate reductase (NR), capable of synthesizing NO from NO^{-2}, with the participation of NADPH (Kaiser et al., 2002).

It has been postulated that NO might function as an antioxidant by scavenging reactive oxygen species (ROS) such as superoxide radicals, thus limiting cellular damage (Laspina et al., 2005; Kopyra and Gwóźdź, 2003). Likewise, NO could function as a signaling compound in the molecular cascade leading to changes in gene expression (Delledonne et al., 1998; 2001; Lamattina et al., 2003).

Polyamines are related to NO through arginine (**4**), a common precursor in their biosynthetic routes. A recent publication by Tun and colleagues (2006) presents evidence that PAs induce production of NO in *A. thaliana* and reports that NO could be a link between polyamine-mediated stress responses and other stress mediators. Although further confirmation of PA-induced NO production is necessary, the observations reported by Tun et al. (2006) could imply the presence of an unknown enzyme responsible for direct conversion of PAs to NO.

Along previous experiments we verified that Cd treatment of developing wheat seedlings inhibited root growth and increased root PAs content (Groppa et al., 2007). In order to elucidate if increasing levels of PAs and root growth inhibition were related events, and if NO could have been involved in this deleterious effect of Cd, we carried out a set of experiments using wheat seedlings grown under different treatments and performed several determinations.

2. Results

2.1. Putrescine (*3*) is not responsible for cadmium-induced root growth inhibition

To elucidate if increases in endogenous levels of PAs (Spm (**1**), Spd (**2**) and Put (**3**)) were related to root growth inhibition and to explore if this was a direct or an indirect effect, the PAs contents and root length of wheat under different treatments were measured (Table 1). It was observed that Cd treatment significantly reduced root elongation and enhanced Spm (**1**) and Put (**3**) levels. The exogenous addition of PAs also reduced root growth and increased polyamine levels to different extents. Spm (**1**) was even more phytotoxic than Cd, reducing root length by 77%. In this case a brownish colour was also observed.

The simultaneous addition of Cd and difluoromethylarginine (**6**) (DFMA) or difluoromethylornithine (**7**) (DFMO) (inhibitors of the Put biosynthetic enzymes ADC and ODC, respectively) prevented increases in PAs levels, but did not restore normal root growth, supporting the notion that increased PAs contents (at least increased Put (**3**) levels) was not the main cause implicated in the root growth inhibition observed under Cd treatment. When tested alone, these inhibitors did not affect root growth.

In order to elucidate if NO, a molecule related to polyamines by a common precursor, arginine (**4**), was implicated in the root inhibition observed, we evaluated the effect of the exogenous addition of the NO-donor, sodium nitroprusside (**8**) (SNP). This treatment also resulted in a reduction of root elongation and an increase in polyamine levels. Although Put (**3**), Spd (**2**) and Spm (**1**) increased to similar levels after both SNP (**8**) doses assayed (0.01 and 0.1 mM), the inhibition of root growth was significantly greater for the highest SNP (**8**) concentration tested (45% vs. 29%, respectively) (Table 1).

2.2. Cadmium-induced root growth inhibition is related to NO formation

A similar degree of root growth inhibition was detected after treating wheat seedlings with 0.1 mM $CdCl_2$, with either the NO-releasing compound SNP (0.1 mM) (**8**) or with 1 mM Spd (**2**)/Put (**3**) while 1 mM Spm (**1**) produced even more inhibition. From this observation and taking into account the Tun et al (2006) findings about the induction of NO biosynthesis by PAs in Arabidopsis plants, we hypothesized that possibly NO rather than the PAs themselves could be responsible for root growth inhibition following Cd treatment. In order to explore this hypothesis, we tested the simultaneous addition of Cd, Spm (**1**) or SNP (**8**) with the NO-trapping compound 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (**9**) (cPTIO) (Table 2 and Fig. 1). It may be appreciated that under these treatments, root growth inhibition was either partially (Spm (**1**) and Cd) or completely (SNP (**8**)) reverted. To determine if NO was the compound in SNP (**8**) that inhibited root growth, control treatments using potassium ferrocyanide (**10**) (Fe(II)CN) and potassium ferricyanide (**11**) (Fe(III)CN) were carried out. These treatments produced a root inhibition similar to that observed with SNP (**8**) (Table 2 and Fig. 1) and, surprisingly, this inhibition was also reverted by cPTIO (**9**).

A direct detection of NO in root segments using the probe DAF-FM was also performed. As shown in Fig. 2A, Cd, SNP (**8**) and Spm (**1**) increased endogenous NO levels to varying extents, as evidenced by the increased green fluorescence observed under fluorescence microscopy. Potassium ferricyanide (**11**) (Fe(III)CN) and potassium ferrocyanide (**10**) (Fe(II)CN) also produced an increment of NO formation (Fig. 2B) as detected by the fluorescent probe. When the NO-trapping agent cPTIO (**9**) was included in the incuba-

Table 1
Root length and polyamine content of wheat roots

Treatment	Root length (cm)	Spermine (nmol g^{-1} PF)	Spermidine (nmol g^{-1} PF)	Putrescine (nmol g^{-1} PF)
C	7.97 ± 1.12[a]	12.50 ± 4.31[a]	143.10 ± 20.60[a]	535.03 ± 63.51[a]
Cd	4.10 ± 0.69[b]	22.93 ± 2.40[b]	171.62 ± 13.31[a]	807.24 ± 83.54[b]
SNP 0.01	5.80 ± 0.85[b]	24.62 ± 3.52[b]	242.28 ± 35.25[b]	749.09 ± 46.89[b]
SNP 0.1	4.36 ± 0.69[b]	21.91 ± 6.22[b]	280.27 ± 53.38[b]	909.50 ± 56.40[b]
Spm	1.87 ± 0.53[c]	120.89 ± 24.03[c]	167.28 ± 30.22[a]	1136.82 ± 130.02[c]
Spd	4.50 ± 0.43[b]	18.35 ± 2.44[a]	352.43 ± 45.55[b]	605.89 ± 89.97[a]
Put	5.90 ± 1.03[b]	16.93 ± 1.62[a]	156.85 ± 12.19[a]	950.39 ± 71.43[b]
DFMA	8.68 ± 1.19[a]	14.60 ± 5.68[a]	114.59 ± 28.9[a]	156.58 ± 24.39[d]
DFMA + Cd	4.50 ± 0.88[b]	13.33 ± 2.62[a]	113.63 ± 17.92[a]	152.58 ± 57.91[d]
DFMO	6.68 ± 0.64[a]	17.94 ± 3.55[a]	156.03 ± 16.27[a]	442.44 ± 61.22[a]
DFMO + Cd	4.52 ± 0.48[b]	23.77 ± 3.52[b]	150.69 ± 11.94[a]	419.11 ± 57.48[a]

Wheat seeds were germinated on wet Whatman paper for 48 h. After this time, seedlings were transferred to a hydroponic system with Hoagland solution containing: 0.1 mM $CdCl_2$, 0.01 or 0.1 mM SNP (**8**), 1 mM Spm (**1**), Spd (**2**) or Put (**3**) and 1 mM DFMA (**6**) or DFMO (**7**) with or without 0.1 mM $CdCl_2$ as described in the Section 5. Values are the means of three different experiments with three replicated measurements. Different letters within columns indicate significant differences ($P < 0.05$), according to Tukey's multiple range test.

Table 2
Effect of NO depletion on root length

Treatment	Root length (cm)	
	− cPTIO	+ cPTIO
C	7.97 ± 1.12[a]	8.90 ± 0.90[a]
Cd	4.10 ± 0.69[c]	6.33 ± 0.45[b]
Spm	1.87 ± 0.53[d]	4.50 ± 0.56[c]
SNP 0.1	4.36 ± 0.69[c]	7.81 ± 0.45[a]
Fe(II)CN	4.22 ± 0.55[c]	6.73 ± 0.86[b]
Fe(III)CN	4.03 ± 0.34[c]	6.15 ± 0.74[b]

Wheat seeds were germinated on wet Whatman paper for 48 h. After this time, seedlings were transferred to a hydroponic system with Hoagland solution containing: 0.1 mM $CdCl_2$, 0.1 mM SNP (**8**), 1 mM Spm (**1**), 0.1 mM Fe(II)CN (**10**) and 0.05 mM Fe(III)CN (**11**) with or without 300 μM cPTIO (**9**) as described in the Section 5. Values are the means of two different experiments with 15–20 plants per treatment. Different letters within and between rows indicate significant differences ($P < 0.05$), according to Tukey's multiple range test.

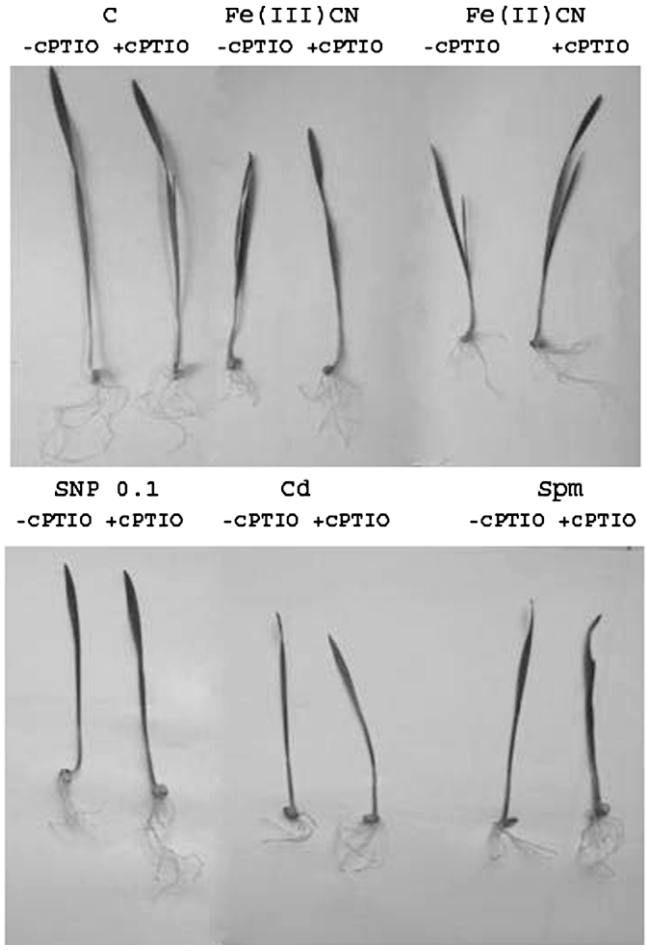

Fig. 1. Effect of endogenous NO depletion on wheat root length. Wheat seeds were germinated on wet Whatman paper over a 48 h period. After this time, seedlings were transferred to a hydroponic system with Hoagland solution containing different treatment solutions: control (C) 0.05 mM Fe(III)CN (**11**), 0.1 mM Fe(II)CN (**10**), 0.1 mM $CdCl_2$, 0.1 mM SNP (**8**) and 1 mM Spm (**1**) with or without 300 μM cPTIO (**9**) as described in the Section 5.

tion media, the signal was evidently reduced in all of these treatments, confirming that NO was the molecule involved in the response (Fig. 2). Roots treated with DFMA (**6**) and DFMO (**7**) together with Cd exhibited a similar green fluorescence than that observed with Cd alone, while Put (**3**) and Spd (**2**) treatments increased green fluorescence but less than that of either Cd or Spm (**1**) (data not shown). These results as a whole suggest that root growth inhibition under Cd or other phytotoxic treatments are coupled to NO formation.

2.3. Cd-treated wheat roots undergo lipid peroxidation despite glutathione levels increased

Thiobarbituric acid reactive substances (TBARS) were measured as an index of lipid peroxidation in wheat roots under different treatments. Cadmium and Spm (**1**) increased the amount of TBARS by 140% and 300% over the controls. However, neither the NO-releasing compound SNP (**8**) nor the other two polyamines (Spd (**2**) and Put (**3**)), which also caused root growth inhibition, modified the root TBARS levels (Fig. 3). Interestingly, glutathione (**12**), the most abundant soluble antioxidant compound in plants, was found to be significantly increased in the roots of Cd-treated wheat plants and also in all other treatments (50–300% over control), particularly after SNP (0.1 mM) (**8**) and Spm (**1**) exposure (Fig. 3). Oxidized glutathione (**13**) levels, on the contrary, remained similar to those shown by controls under all treatments (data not shown).

3. Discussion

Cadmium effects on plant polyamine metabolism and oxidative status have been previously analyzed by our group (Groppa et al., 2001, 2003, 2007); however, the precise mechanism involved in root growth inhibition caused by this metal has not been still elucidated. We noticed that 0.1 mM cadmium causes in wheat a significant inhibition of root elongation, in coincidence with previous data reported for this (Ranieri et al., 2005) an for other plant species, like barley and sunflower (Wu et al., 2004; Groppa et al., 2007).

It has been widely described that PAs accumulate in plants subjected to biotic and abiotic stresses (Bouchereau et al., 1999; Kakkar and Sawhney, 2002; Alcázar et al., 2006) including heavy metals (Groppa et al., 2001, 2003, 2007). On the last years, several reports have postulated the involvement of PAs in cell death processes in mammalian tissues (Baldini et al., 2006; Johnson and Ray, 2006), but data regarding a comparable function in plants are scarce (Kuehn and Phillips, 2005). Looking for a biochemical mechanism involved in Cd-induced root growth inhibition and taking into account that polyamine metabolism was already found to be altered under Cd stress (Groppa et al., 2007), we decided to explore if PAs were involved in root growth inhibition and the possible connection to NO, a molecule very recently linked to the biosynthesis of PAs (Tun et al., 2006; Yamasaki and Cohen, 2006).

We found that Cd increased Put (**3**) and Spm (**1**) levels in wheat roots. Shih and Kao (1996) reported that growth inhibition of cultured rice cells deprived of potassium and phosphate was associated with Put (**3**) accumulation, whereas Tun et al. (2006) observed a growth reduction in an embryogenic *Ocotea* culture treated with 0.5, 1 and 2 mM Spd (**2**) and Spm (**1**). Embryogenic suspension cultures of *Araucaria angustifolia* showed reduced cellular growth when either Spd (**2**) or Spm (1 mM) (**1**) were supplemented into the culture medium (Silveira et al., 2006). In order to see if there was a linkage between increased PAs levels and root growth inhibition, 1 mM Put (**3**), Spd (**2**) or Spm (**1**) were exogenously added. Different degrees of root growth inhibition were noticed after 5 days of treatment. These additions significantly increased root content of the corresponding polyamine, especially Spm (**1**), which caused a 10-fold Spm (**1**) increase. This polyamine elevated also Put (**3**) content (Table 1).

To evaluate if the increased levels of Put (**3**) were the main factor responsible for cadmium-root growth inhibition, the addition of Put (**3**) biosynthesis inhibitors, DFMA (**6**) and DFMO (**7**), should

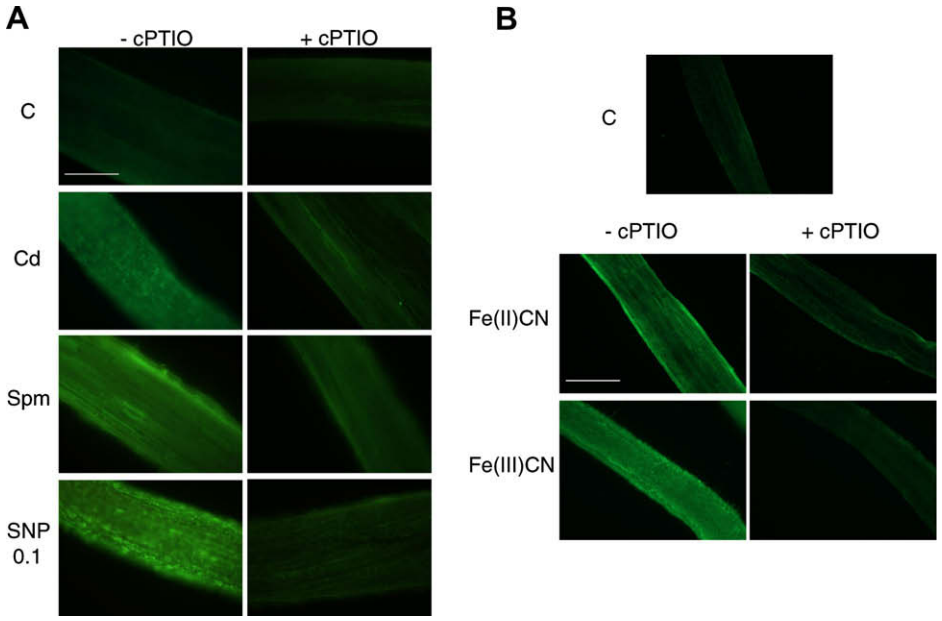

Fig. 2. NO accumulation in wheat roots. Wheat seeds were germinated on wet Whatman paper over a 48 h period. After this time, seedlings were transferred to a hydroponic system with Hoagland solution containing different treatment solutions with or without 300 μM cPTIO (**9**) as described in the Section 5. (A) Control (C), 0.1 mM $CdCl_2$, 1 mM Spm (**1**) or 0.1 mM of the NO-donor sodium nitroprusside (SNP (**8**)). Bar = 0.4 mm; and (B) control (C), 0.1 mM Fe(II)CN (**10**), 0.05 mM Fe(III)CN (**11**). Bar = 1 mm. At day 5 of treatment, the apical portions of the roots (1.5–2 cm) were incubated with 10 μM DAF-FM and were observed under fluorescence microscopy.

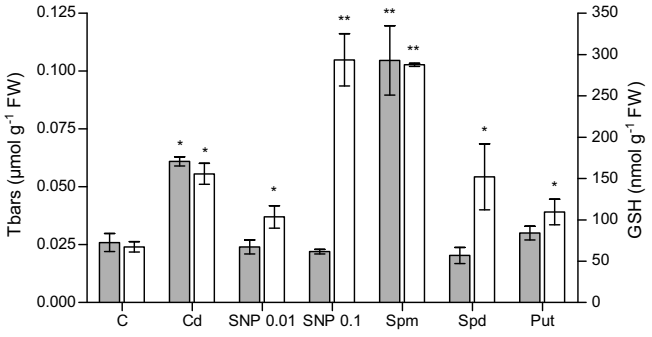

Fig. 3. Thiobarbituric acid reactive substances (filled bars) and GSH (**12**) (open bars) contents in wheat roots treated with 0.1 mM $CdCl_2$, 0.01 or 0.1 mM SNP (**8**), 1 mM Spm (**1**), Spd (**2**) or Put (**3**) as described in the Section 5. Values are the means of two different experiments with three replicated measurements, and bars indicate S.E. * Significant differences ($P < 0.05$) according to Tukey's multiple range test.

have reduced Cd-induced root growth inhibition by decreasing Put (**3**) levels. Although the diamine content significantly decreased after DFMA (**6**) treatment, both if it was added alone or together with Cd, normal root growth was not restored in the combined treatment, suggesting that other factors beyond PAs, at least beyond endogenous Put (**3**), may be involved in the reduction of wheat root length. Even so, we cannot exclude the possibility that the endogenous Put (**3**) concentration (and probably the other two PAs) could be not enough for reducing root growth by itself, because when it was exogenously added at 1 mM, it did reduce root growth by 25%.

The biosynthesis of NO in different plant tissues and its role on different physiological processes is a matter of continuous research and controversy. The participation of NO in various cell processes as growth and development, respiratory metabolism, senescence and maturation, as well as plant response to abiotic and biotic stressors is well documented (Arasimowicz and Floryszak-Wieczorek, 2007). However, it is still difficult to present a relatively comprehensive model illustrating the multiplicity of NO functions in plants. Regarding the protecting role of NO, previous results from our group showed that NO can exert a certain antioxidant role in 30 d-sunflower leaf discs incubated with NO prior to Cd treatment (Laspina et al., 2005). Kopyra and Gwóźdź (2003) have reported that root length of *Lupinus* plants were reduced only by SNP (**8**) concentrations higher than 400 μM and that exogenous applied SNP (**8**) reverted the inhibitory effect of 50 μM Cd or 1500 μM Pb in these roots. However, Correa-Aragunde et al. (2004) showed that, in tomato plants, SNP (**8**) increased lateral root proliferation but decreased principal root growth in a dose-dependent manner. In accordance with our results, Perrine-Walker et al. (2007) presented evidence that the induced growth inhibition of rice roots colonized by *Rhizobium leguminosarum* bv. *trifolii* was due to a toxic accumulation of NO, whereas Silveira et al. (2006) observed that while Put (**3**) increased NO release from the embryogenic culture of *A. angustifolia*, Spd (**2**) and Spm (**1**) inhibited the NO biosynthesis in both embryonic and suspensor cells. PAs probably affect differently NO physiology. These controversial results concerning either NO evolution or NO antioxidant effects in different plant species are due to the fact that different results have been obtained using different plants species, organs, stage of development, duration or severity of the stress period.

All treatments tested in this study elevated endogenous NO contents and reduced root growth rates. The NO-trapping agent, cPTIO (**9**), a compound routinely used to remove NO from biological tissues and from solution, considerably reversed root growth inhibition, not only in SNP (**8**) treatment but also with Cd, Spm (**1**), Fe(III)CN (**11**) or Fe(II)CN (**10**), confirming that NO was, at least in part, responsible for this inhibition and the dose-dependent relation between root growth inhibition and SNP (**8**) concentration reinforces this idea. Bartha et al. (2005) reported that NO increased in roots of *Brassica juncea* L. Czern. and *Pisum sativum* L. exposed to 100 μM Cd, Cu or Zn. Contrary to our results and those of Bartha et al. (2005), Barroso et al. (2006) and Rodríguez-Serrano et al. (2006) found a decrease of NO concentration in 50 μM Cd-treated pea roots, despite root growth was also inhibited. These opposite findings could be explained by the different cadmium concentra-

tions used, the age of the plants and the time of treatment used by the authors.

To determine if NO was the compound that reduced wheat root growth in SNP (**8**) treatments, control experiments were carried out with potassium ferrocyanide (**10**) (Fe(II)CN) and potassium ferricyanide (**11**) (Fe(III)CN), two compounds that have structures similar to SNP (**8**) but lack the ability to produce NO. Both compounds inhibited root elongation to a similar extent to that of SNP (**8**), however, when the NO scavenger, cPTIO (**9**), was included in the medium, the inhibitory effect of ferricyanide (**11**) or ferrocyanide (**10**) was partially reverted. Bethke et al (2006), in accordance with our results, demonstrated that SNP (**8**), Fe(III)CN (**11**), Fe(II)CN (**10**) and KCN produced the break of dormancy of *Arabidopsis* seeds and this effect was prevented by cPTIO (**9**), suggesting the possibility that CN^- brings about an increase in NO concentrations in seeds thus reducing break dormancy. We demonstrated that both, ferricyanide (**11**) and ferrocyanide (**10**), were inhibiting wheat root growth by enhancing NO formation (Fig 2B). The increase of NO could be a consequence of an inhibition of NO detoxifying enzymes or by activation of nitric oxide synthase (NOS), an enzyme found in roots of *Lupinus* (Cueto et al., 1996) and pea (Corpas et al., 2006).

The increased GSH pool found in wheat roots (Fig. 3) in response to Cd, Spm (1) or 0.1 mM SNP (**8**) could be attributed to the increased NO levels found under all these treatments. Several studies have evidenced that GSH biosynthetic pathway is stimulated in response to NO in animal cells and yeast (Moellering et al. 1998; Kim et al. 2004) and very recently, Innocenti et al. (2007) reported that a significant threefold increase of GSH content over control was observed 24 h after SNP (**8**) application in *M. truncatula* roots. In wheat roots, this rise was neither enough to prevent the oxidative damage observed in roots of either Cd or Spm (**1**)-treated plants (evidenced with an elevated TBARS content) nor the significant inhibition of root growth in SNP (**8**)-treated roots (Fig. 1). Meanwhile, Cd enhances lipoxygenase activity (Aravind and Prasad, 2003) and the products of the lipoxygenase reaction, mainly peroxy, alkoxy and hydroxyl radicals, are themselves reactive and result in further membrane lipid deterioration leading to membrane permeability and subsequent growth inhibition. However, SNP (**8**)-treated roots did not show signs of lipid oxidation, despite the evident root growth reduction observed, suggesting that NO increased levels and not lipid peroxidation were the main cause of root growth inhibition, as occurred in rice plants colonized by *R. leguminosarum*, which produced toxic levels of NO (Perrine-Walker et al., 2007).

It has been reported that nitric oxide can react with superoxide anion to generate peroxynitrite ($ONOO_2^-$), a chemical species with potent oxidizing power (Blough and Zafiriou, 1985). Moreover, other ROS such as H_2O_2 (a product of PAs catabolism) or O_2^- acting by themselves might also be involved in Cd and Spm (**1**) phytotoxicity. Allan and Fluhr (1997), using a commercially available type I horseradish peroxidase, examined ROS production "in vitro" and readily detected a catalase-sensitive ROS increase with L-arginine (**4**) (a Put (**3**) precursor) and Put (**3**), but they did not test either Spm (**1**) or Spd (**2**). The specific contribution of each source to the oxidative burst in different tissues or plants and/or in response to different stimuli is still a matter of debate.

4. Conclusions

Cadmium stress triggers several physiological responses as indicated by different cellular markers. The different possible responses can be grouped in a multi-component "fan shaped" model (Sanitá di Toppi and Gabbrielli, 1999), where the importance of each ray of the fan varies according to the plant system or environmental conditions. In our system, wheat roots treated with either cadmium or Spm (**1**) shares a common response, an increased NO formation, that mediates the toxicity exerted by these compounds and results in root growth inhibition. Future research needs to be conducted to clarify the signaling pathways that connect such responses to formation of toxic and protective compounds in plants.

5. Experimental

5.1. General

Put (3), Spd (2), Spm (1), 1,6-hexanediamine, cPTIO (9), GSH (12), GSSG (13), 5,5′-dithiobis(2-nitrobenzoate) (DTNB), butyl hydroxitoluene (BHT), thiobarbituric acid (TBA), and GR were from Sigma Chemical Company (St. Louis, MO). 4-Amino-5-methylamino-2,7-difluorescein diacetate (DAF-FM diacetate) was from Invitrogen, Molecular Probes. All other chemicals were of analytical grade.

5.2. Plant material and treatments

Wheat (*Triticum aestivum* L.) seeds (provided by Buck S.A.) were germinated on wet Whatman paper over a 48 h period. After this time, seedlings were transferred to a hydroponic system with Hoagland (Hoagland and Arnon, 1950) solution containing: 0.1 mM $CdCl_2$, 0.01 or 0.1 mM of the NO-donor SNP (8) and 1 mM Spm (**1**), Spd (**2**) or Put (**3**). Inhibitors of Put (**3**) biosynthesis were also used (1 mM DFMA (**6**) or DFMO (**7**)). As control of CN^- release by SNP (**8**), 0.1 mM potassium ferrocyanide (**10**) and 0.05 mM potassium ferricyanide (**11**) were used. The NO-trap cPTIO (**9**) (300 μM) was added to the treatment solutions as a negative control of NO effects. Treatment solutions were replaced daily.

Plants were grown with a 16/8 h photoperiod at 26/20 °C, under fluorescent white light (photon flux density: 175 μmol m^{-2} s^{-1}) in a controlled environmental growth chamber. They were harvested after five days of growth on each treatment solution. Root length, NO formation, polyamine content, TBARS and glutathione content were determined.

5.3. Root length

All samples were taken randomly from plants with comparable growth rate. A sample of 15 to 20 plants per treatment was used to record root length at indicated time.

5.4. NO detection

Endogenous NO formation was detected by incubating 1.5–2 cm of the apical portions of the primary roots with 10 μM of the positive fluorescent probe (DAF-FM diacetate, Molecular Probes) prepared in 10 mM Tris–HCl (pH 7.4) over 30 min, according to Pagnussat et al. (2003) with some modifications. Thereafter, roots were washed twice with the above buffer, placed horizontally on a slide and squashed using a cover slip. Fluorescence due to NO was examined in a fluorescence microscope (Olympus BX50, excitation 490 nm, emission 515–560 nm, Olympus Optical Co., Tokyo, Japan). Experiments were repeated at least five times and similar results were obtained. Photographs are representative of the observations made. All manipulations were performed under dim green light.

5.5. Analysis of polyamines

Roots (300 mg FW) were homogenized with 5% (v/v) $HClO_4$, maintained 30 min on ice and centrifuged at 3000*g* for 10 min.

The supernatants were derivatized using the dansylation method described by Smith and Meeuse (1966) and 1,6-hexanediamine was used as an internal standard. Standards of Put (**3**), Spd (**2**) and Spm (**1**) were dansylated simultaneously. The dansylated derivatives were extracted with EtOAc (1 ml). Polyamines were separated and identified by TLC performed on high resolution silica gel plates (JT Baker, silica gel plates IB 2-F) using a *n*-hexane:EtOAc (1:1) solvent system. Dansylated polyamines were identified by comparing Rf values of dansylated standards. Silica plates were observed under UV light and bands corresponding to the PAs in the samples and standards were scraped off the plates and eluted with EtOAc (1 ml). Fluorescence measurements were made at 365 nm excitation and 510 nm emission, in an spectrofluorometer (Aminco Bowman).

5.6. Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was determined as the amount of TBARS, measured by the TBA reaction as described by Heath and Packer (1968). Fresh roots of control and treated plants (300 mg FW) were homogenized in 3 ml of 20% (w/v) TCA. The homogenate was centrifuged at 10,000*g* for 20 min. To 1 ml aliquot of the supernatant, 1 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA and 100 μl 4% (w/v) butylated hydroxytoluene (BHT) in EtOH were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 1000*g* for 3 min and the absorbance was measured at 532 nm. The value for non specific absorption at 600 nm was substracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

5.7. Determination of GSH

Non-protein thiols were extracted by homogenizing leaf fragments (300 mg FW) in 2 ml of 5% sulfosalicylic acid (pH 2), containing 1 g polyvinylpyrrolidone (PVP). After centrifugation at 10,000*g* for 20 min at 4 °C, the supernatants were used for the analyses. Total glutathione (GSH (**12**) plus GSSG (**13**)) content was determined by the recycling method according to Anderson (1985), using GSH (**12**) as standard by measuring the absorbance increment at 412 nm, with glutathione reductase, DTNB and NADPH. To quantify GSSG (**13**), 2-vinylpyridine was added to the extract.

5.8. Statistics

All data presented are the mean values of two independent set of experiments. Each value was presented as means ± standard errors of the mean (SE), with a minimum of three replicates. Statistical analysis was carried out by one-way ANOVA using the Tukey test to evaluate whether the means were significantly different, taking $p < 0.05$ as significant.

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