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ORIGINAL PAPER

Nitric oxide and hydrogen cyanide as regulating factors of enzymatic antioxidant system in germinating apple embryos

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Abstract Short-term (3 or 6 h) pre-treatment of apple (*Malus domestica* Borkh.) embryos with nitric oxide (NO) or hydrogen cyanide (HCN) induces transient accumulation of reactive oxygen species (ROS) leading to dormancy removal and germination. We demonstrated that enhanced NO emission by apple embryos during early phase of germination "sensu stricto" is required for seed transition from dormant into non-dormant state, and may be described by the model of "nitrosative door", analogous to "oxidative window". Cellular ROS concentration, resulting from NO or HCN embryo pre-treatment, seems to be under severe control of antioxidant system. Activity of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX) and total peroxidases (Prxs) was determined during NO and HCN-mediated germination "sensu stricto" of embryos. CAT and SOD activity increased transiently 24 h after embryos pre-treatment, while GR and Prx activity was stimulated mainly after 96 h. The most evident alterations were detected in GPX activity, being more than threefold stimulated by NO or HCN. Based on this results, we conclude that these reactive molecules act simultaneously crossing their signaling pathways and we propose that ROS, reactive nitrogen species, HCN at accurate level are essential during seed germination as signaling factors.

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Introduction

Reactive oxygen species (ROS) are considered as main regulating factors in seed dormancy, germination and aging (Bailly et al. 2008). Recently proposed model of the "oxidative window" describes dual (cytotoxic and signaling) role of ROS in seeds (Bailly et al. 2008). These molecules act as positive signal in seed dormancy release and are necessary in seed transition from dormant into non-dormant state. Completion of germination (even under appropriate conditions such as moisture, temperature, light) requires adequate level of ROS. Controlled accumulation of ROS during germination seems to be crucial for the next developmental stage e.g. radicle protrusion (Müller et al. 2009). In the opposite, too high concentration of these molecules provides attack on structural components: proteins, sugars, lipids and nucleic acids and can lead to cell damage or death. Therefore, both ROS production and scavenging should be distinctly controlled. ROS metabolic stability is provided by the cellular enzymatic and non-enzymatic antioxidant system. Superoxide dismutases (SODs; EC 1.15.1.1), catalases (CAT; EC 1.11.1.6) and peroxidases (Prxs) are included into the group of enzymes responsible for ROS scavenging. SODs dismutate two superoxide radicals ($O_2^{-\cdot}$) into hydrogen peroxide (H_2O_2) (Alscher et al. 2002; Misra and Fridovich 1972). CAT catalyzes the next reaction—conversion of two molecules of H_2O_2 into water and O_2 (Mhamdi et al. 2010).

Seeds of apple (*Malus domestica* Borkh.) belong to orthodox type and undergo and tolerate dehydration during maturation. Dry seeds of this category are characterized by

low amount or even absence of ascorbate (ASC) in reduced form and the absence of ascorbate peroxidase (APX; EC 1.11.1.11) activity (Tommasi et al. 2001). Thus, in orthodox seeds antioxidant system is supported mainly by glutathione oxidation/reduction cycles. The glutathione peroxidase (GPX; EC 1.11.1.9) utilizes reduced form of glutathione (GSH) to remove ROS (including also H_2O_2). Until now genes encoding only GPX-like proteins were found in plants (Fu et al. 2002). GSH is oxidized to glutathione in reduced form (GSSG), which is then converted to GSH by glutathione reductase (GR; EC 1.6.4.2) in reaction requiring NADPH (Kranner et al. 2006). Although steady state level of ROS in cells is regulated by antioxidant system, transcription of genes encoding components of this system remains under ROS control (Desikan et al. 2001).

Recently, several molecules (including ROS) were identified as seed dormancy breaking agents. Gaseous molecules acting as inhibitors of mitochondrial respiration chain, mainly inhibitors of cytochrome c oxidase, such as hydrogen cyanide (HCN) or nitric oxide (NO) may alleviate seed dormancy and/or stimulate germination (Bailly et al. 2008; Bethke et al. 2006; Gniazdowska et al. 2010a, b; Kranner et al. 2010; Oracz et al. 2009). Moreover, both HCN and NO trigger ROS production in germinating embryos of sunflower (*Helianthus annuus*) (Oracz et al. 2009) and apple (Gniazdowska et al. 2010b). In our previously published papers we demonstrated that HCN and NO short-term pre-treatment of dormant apple embryos results in transient increase in ROS production mainly in embryo axis (Gniazdowska et al. 2010a, b). Additionally, during "sensu stricto" germination of HCN and NO pre-treated apple embryos enhanced production of ROS-stimulated ethylene biosynthesis. Germination "sensu stricto" is defined as processes associated with the initiation and completion of embryo emergence and refers to the progress of a seed from imbibition through radicle emergence. According to Oracz et al. (2009) HCN-mediated dormancy removal and germination of sunflower embryos depends on modulation of ROS metabolism: activation of NADPH oxidase, and inhibition of CAT and SOD activity. Taking to account, that the effect of pre-treatment of dormant apple embryos with HCN or NO results in dormancy breakage, we may suspect an analogous mode of action of both molecules. Although participation of NO in regulation of seed dormancy and germination was confirmed by numerous authors (Bethke et al. 2006; Sarath et al. 2005; Simontacchi et al. 2004), its mode of action in these processes is identified only fragmentary. There is still a lack of data on the role of reactive nitrogen species (RNS, including also NO) in regulation of enzymatic activity modulating ROS level during early stages of germination of orthodox seed. In our experiment we compared the physiological effect of most popular NO donor—nitroprusside (SNP), which decomposition in light leads to production of NO^+ and CN^- (Bethke et al. 2006) with the influence of embryo fumigation with HCN or NO released as vapors of acidified nitrite. Based on the earlier observation on elevated concentration of ROS in apple embryos pre-treated with NO, SNP or HCN (Gniazdowska et al. 2010b), we pointed to modification of antioxidant enzymatic system during germination "sensu stricto" of embryos of orthodox seeds. The effect of NO, SNP and HCN on activity of ROS scavenging enzymes: CAT, SOD, GR in germinating apple embryos were investigated. Moreover, we demonstrated enhanced NO emission by the embryos during early phases of germination "sensu stricto" and its involvement in modulation of ROS concentration. The presented data allowed us, for the first time, to propose the concept of the "nitrosative door" for seed germination, equivalent to model of the "oxidative window" (Bailly et al. 2008).

Materials and methods

Plant material

The experiments were carried out on apple (*M. domestica* Borkh., cv. Antonówka) embryos isolated from seeds harvested in 2008 and 2009. Apples were provided by Kordel fruit producer at Tarczyn (Poland). Seeds were isolated from fruits at harvest time and stored in dark glass containers at 5°C. The seed coat and endosperm were removed from seeds after 24-h imbibition in distilled water at room temperature (20°C). The embryos were shortly pre-treated with water solution of SNP (5 mM, 3 h) in light, vapors of acidified nitrite (NO, 3 h) and HCN (1 mM, 6 h) according to Gniazdowska et al. (2007, 2010b). Acidified nitrite was prepared using 20 mM sodium nitrite ($NaNO_2$) and 0.1 M HCl as described by Gniazdowska et al. (2010b). Control (non-treated) and NO, SNP, HCN pre-treated embryos were germinated for 96 h (4 days) in Petri dishes (20 embryos per dish) at 25°C with 12/12 h (light/dark) photoperiod, under 150 μmol PAR m^{-2} s^{-1}. Dormant (control, non-treated) embryos were taken for enzymatic determination immediately after isolation (0) and after 3, 6, 24 and 96 h of germination. Embryos pre-treated with HCN and NO or SNP were taken immediately after treatment (6 or 3 h, respectively) and then after 24 and 96 h of culture.

Germinating embryos were counted 4 and 9 days after sowing. Embryos were considered to have germinated when radicles were 2–3 mm long with characteristic gravitropic bending.

Measurement of electrolyte leakage

The conductometric method was used to measure relative electrolyte leakage. Apple embryos (0.2 g) after

pre-treatment (3 or 6 h with NO, SNP and HCN, respectively) and after 24 h of culture were placed in separate test tubes with 10 ml de-ionised water (Milipore Simplicity 185) for 1 h at 20°C in darkness. After every 15-min incubation, the water solution in the test tubes was shaken gently. Initial electrolyte leakage was measured after 1 h of incubation using a conductivity meter (Elmetron CPC-505, Poland). Next, the test tubes with embryos were heated for 5 min at 100°C (water bath) and the final electrolyte leakage was determined. Relative electrical conductivity (EC) was calculated as the ratio of the initial EC to the final EC.

Determination of enzymatic activity

Preparation of enzymatic extract

Apple embryos (0.3 or 0.5 g for SOD) were homogenized in 5 ml 0.1 M potassium phosphate buffer (pH 7.0) with 1 mM EDTA, 5 mM DTT, 0.01 mM PMSF and 2% PVPP, on ice. After centrifugation 10,000*g*, 15 min at 4°C (High Speed Centrifuge MPW-350R, Poland) supernatant was desalted on Sephadex G-250 column equilibrated with 0.1 M potassium phosphate buffer (pH 7.0), only for SOD extraction Sephadex G-250 column (BioRad) was equilibrated with 50 mM glycine buffer (pH 9.5).

Superoxide dysmutase (SOD)

Measurement of SOD activity was done according to Misra and Fridovich (1972). Enzymatic extract (50, 100 or 200 µl) was added to 1.8 ml of the reaction medium [0.05 M glycine buffer pH 9.5, 100 µl 120 mM epinephrine solution (Sigma E1635) in 40 mM HCl]. Reaction was started by adding an aliquot of acidified epinephrine. The oxidation of epinephrine to adrenochrome was monitored at 480 nm (spectrophotometer Shimadzu UV 1700, Japan). One unit of SOD activity was defined as the amount of extract which inhibited the rate of adrenochrome formation by 50%.

Catalase (CAT)

Measurement of CAT activity was performed according to Aebi (1984). Reaction mixture (1.85 ml) contained 0.05 M potassium phosphate buffer (pH 7.0) and 50 µl of enzymatic extract. Reaction was started by adding 100 µl of 6% H_2O_2. CAT activity was recorded as decrease of absorbance at 240 nm using spectrophotometer Shimadzu UV 1700, Japan.

Peroxidase (Prx)

Measurement of Prx activity was done according to Saunders et al. (1964). Enzymatic extract (50 µl) was incubated with 5 mM pyrogallol in 1.85 ml 50 mM potassium phosphate buffer (pH 7.0) at 20°C, in darkness. After incubation, Prx activity was measured by adding 100 µl of 10% H_2O_2. Prx activity was determined as absorbance increase at 430 nm using spectrophotometer Shimadzu UV 1700, Japan.

Glutathione peroxidase-like (GPX-like)

Measurement of GPX-like activity was done according to Flohe and Günzler (1984) with some modifications (Fontaine et al. 1994). Enzymatic extract (0.1 ml) was incubated in 0.7 ml reaction mixture (0.05 M potassium phosphate buffer (pH 7.0) with 0.1 M aminotriazole, 2.5 mM EDTA, 0.1 ml 2.5 mM GSH, 2.5 U of GR (Sigma G3664) at 25°C for 10 min. After incubation, 0.1 ml 2 mM H_2O_2 was added. Reaction was started by adding 0.1 ml of 2.5 mM β-NADPH (Sigma N1630). GPX-like activity was determined as absorbance decrease, monitored at 340 nm using spectrophotometer Shimadzu UV 1700, Japan.

Glutathione reductase (GR)

Measurement of GR activity was performed according to Esterbauer and Grill (1978). Enzymatic extract (0.1 ml) was incubated in 0.8 ml reaction mixture [0.05 M potassium phosphate buffer (pH 7.0) with 0.1 ml 5 mM GSSG (Sigma G4501)] for 10 min at 20°C. Measurement of GR activity was started by 0.1 ml 2 mM β-NADPH. GR activity was measured as decrease of absorbance at 340 nm, using spectrophotometer Shimadzu UV 1700, Japan.

Protein determination

Protein concentration was estimated spectrophotometrically according to Bradford (1976) with BSA (Sigma-Aldrich) used as a standard.

Detection of NO production by apple embryo axes

NO production was detected in isolated axes of apple embryos pre-treated with SNP, NO and HCN and in axes of control (non-treated) embryos immediately after treatment and after 24 h of culture according to Gniazdowska et al. (2010c). After isolation, axes were rinsed in 10 mM Hepes–KOH (pH 7.4) for 15 min. Three axes (total fresh weight of the tissue used for one assay was approximately 0.003 g), were transferred to 100 µl of 10 mM Hepes–KOH (pH 7.4) buffer containing 20 µM specific NO fluorescent probe 4,5-diaminofluorescein diacetate (DAF-FM DA; Molecular Probes, Eugene, OR, USA). After 45-min incubation in darkness, the axes were washed three times in

1 ml 10 mM Hepes–KOH (pH 7.4) and transferred to the cuvette containing 0.8 ml fresh Hepes–KOH (pH 7.4) buffer. Fluorescence was measured and recorded for 1,000 s using Hitachi F-2500 fluorescence spectrophotometer (excitation 495 nm and emission 515 nm). All measurements were carried out at least in 5 repetitions, and their exact reproducibility was confirmed. Fluorescence was normalized per milligram FW and expressed in arbitrary units. Maximal normalized fluorescence from axes of dormant (non-treated) embryos after their isolation from seed coats was estimated as one arbitrary unit.

Statistical analysis

The data are based on three to five sets of experiments with the assay conducted in triplicate. Standard deviation of mean was calculated for all data set.

Results

NO, SNP and HCN-short-term pre-treatment alleviates dormancy of apple embryos

Germination of dormant apple embryos was very slow, as it was described in our previous papers (Gniazdowska et al. 2007, 2010a, b). Till the fourth day of culture no one of dormant (control) embryos germinated (Table 1). The effect of acidified nitrite (NO) and SNP was similar to that observed earlier using S-nitroso-N-acetylpenicillamine (SNAP) and reversed by NO scavenger (0.3 mM cPTIO), which totally abolished germination of apple embryos (Gniazdowska et al. 2010b). After 4 days of imbibitions in water dormant embryos (control) differed visually from non-dormant (pre-treated) ones, although both of them did not germinate or germinated in less than 20% as it was observed for NO, SNP and HCN pre-treated ones (Table 1). Pre-treated embryos started to be light green, their cotyledons did not lie tightly on each other and begun to half-open as it was described in one of our last papers (Gniazdowska et al. 2010b). Prolonged culture of pre-treated embryos resulted in six- to sevenfold stimulation of germination. Nine days after sowing 60–70% of pre-treated embryos were germinated (Table 1).

Table 1 Germination of control (non-treated) apple embryos or embryos pre-treated with NO, SNP or HCN determined 4 and 9 days after sowing

	Days	Control	NO	SNP	HCN
Embryo germination (%)	4	0	15 ± 5	21 ± 3	15 ± 2
	9	10 ± 2	61 ± 6	71 ± 4	58 ± 5

Values are mean ± SD of 3 replicates from 5 independent experiments of approx. 60 embryos each

NO, SNP and HCN-short-term pre-treatment modifies cellular enzymatic antioxidant system in apple embryos

SOD activity

The highest SOD activity (9.5 U min^{-1} mg^{-1} protein) was determined in control apple embryos just after isolation (Fig. 1a). It decreased during the culture period, reaching finally low value—around 2 U min^{-1} mg^{-1} protein at the fourth day of culture. The similar pattern of enzyme activity was observed in pre-treated embryos (Fig. 1a). The only difference was mentioned for NO, SNP and HCN pre-treated embryos after 24 h of germination. In this embryos, SOD activity was about 25–30% higher than in control ones.

CAT activity

Catalase activity was on steady-stayed level (around 10 nmol H_2O_2 min^{-1} mg^{-1} protein) in control non-treated embryos for the entire period of culture. It decreased insignificantly only after 24 h of imbibitions to the value around 7.6 nmol H_2O_2 min^{-1} mg^{-1} protein (Fig. 1). NO, SNP and HCN pre-treatment resulted in slight increase in CAT activity in embryos, observed after 24 and 96 h of germination to about 14 nmol H_2O_2 min^{-1} mg^{-1} protein. CAT activity measured just after embryo fumigation with NO or HCN was slightly lower than in control embryos (Fig. 1b).

Prx activity

Prx activity in dormant embryos was about 0.5 μmol H_2O_2 min^{-1} mg^{-1} protein (Fig. 1c). It increased to 750 nmol H_2O_2 min^{-1} mg^{-1} protein during 3 h of imbibition, and then declined to the value 250 nmol H_2O_2 min^{-1} mg^{-1} protein, being stable till the end of experiment. Pre-treatment of embryos with NO, SNP and HCN also resulted in transient increase in Prx activity, which was detected just after fumigation (3 h), but it was twice lower than that one observed in control embryos (Fig. 1c). Prolonged germination of pre-treated embryos did not alter Prx activity in SNP and HCN pre-treated embryos, and only slightly lowered its activity in NO-treated ones (Fig. 1c).

GPX-like activity

GPX-like activity in control (dormant) embryos remained almost constant (around 10 nmol H_2O_2 min^{-1} mg^{-1} protein) till the end of the experiment (Fig. 1d). It increased

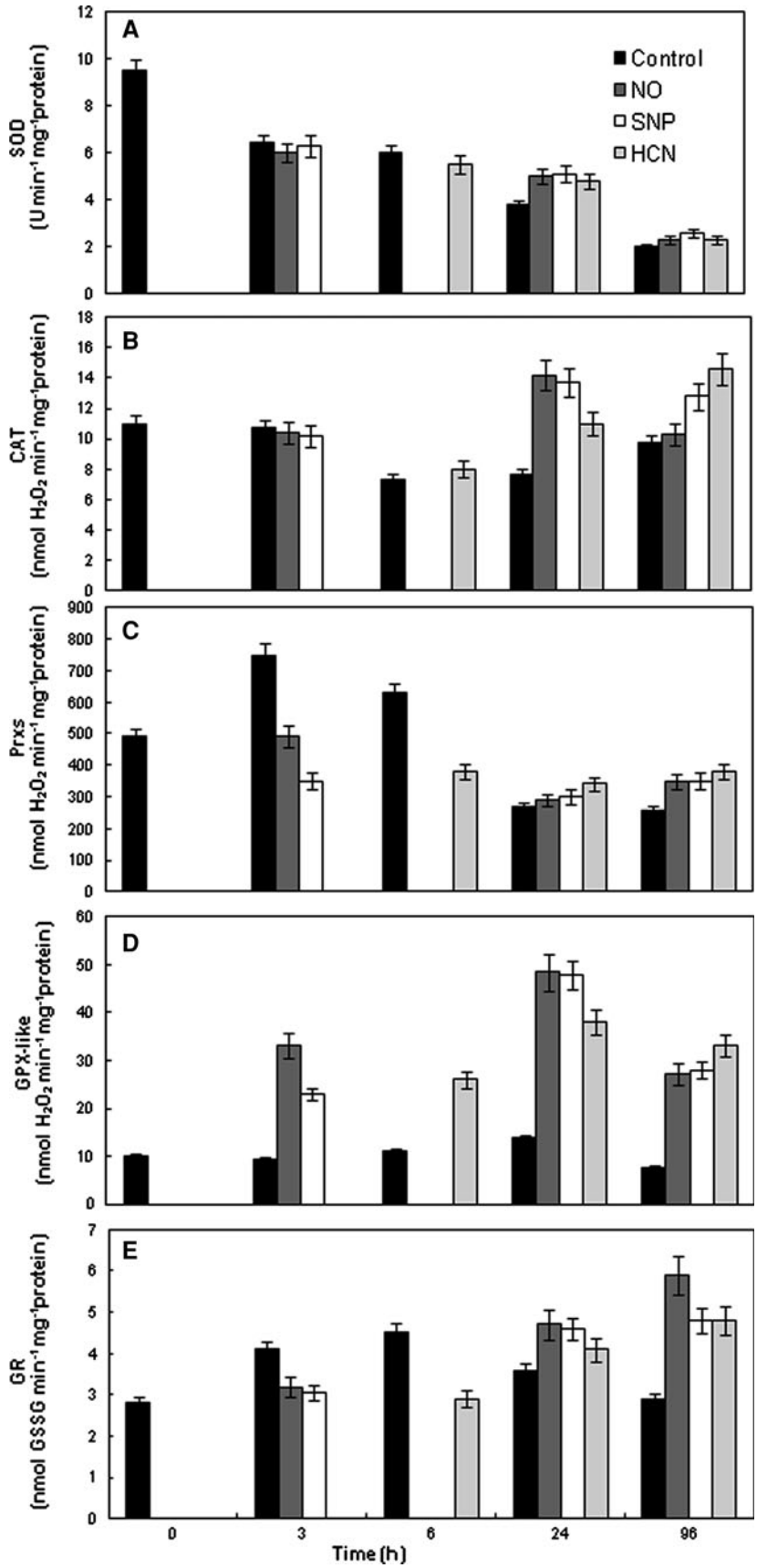

Fig. 1 Changes in the activities of SOD (**a**), CAT (**b**), Prx (**c**), GPX-like (**d**) and GR (**e**) in dormant apple embryos (control) and dormant apple embryos pre-treated with NO, SNP or HCN. Enzymatic activity was measured after seed imbibition and seed coat removal (0), just after embryo treatment with NO or SNP (3 h) or HCN (6 h), and 24 h and 96 h after sowing. Means and SD were calculated from at least three to five independent experiments. *Vertical bars* indicate SD

insignificantly at the first day of germination, reaching value 13.7 nmol H_2O_2 min^{-1} mg^{-1} protein and after that decreased slightly to about 8 nmol H_2O_2 min^{-1} mg^{-1} protein. NO, SNP and HCN-pretreatment resulted in marked enhance of GPX-like activity in embryos. It enlarged mainly in the initial period of culture (24 h), and in NO and SNP pre-treated embryos were more than threefold higher in comparison to the control (Fig. 1d). The prolonged germination of NO and SNP pre-treated embryos resulted in 40% decrease in GPX-like activity, but it was still markedly higher than in control. Similar alterations in GPX-like activity were detected in HCN pre-treated embryos (Fig. 1d). It was only a little bit lower (20%) in 24-h-old HCN pre-treated embryos than in NO or SNP pre-treated ones.

GR activity

The lowest activity of GR (2.8 nmol GSSG min^{-1} mg^{-1} protein) was observed in control embryos just after their isolation. In dormant embryos it increased in 30% during the first day of germination, and then decreased to the initial rate after additional 3 days (Fig. 1e).

GR activity in pre-treated embryos enhanced continuously during germination. The highest GR activity was observed in 4-day-old NO-pre-treated embryos, and it's value was about twice as compared to dormant (control) embryos (Fig. 1e).

Electrical conductivity

Electrical conductivity of control embryos after 3 h of imbibition in water was less than 7% of total leakage (data not shown). Short pre-treatment of the embryos with HCN and SNP caused approximately fourfold increase in electrolyte leakage (Table 2). Pre-treatment of the embryos with gaseous NO resulted in sevenfold increase in electrolyte leakage (Table 2). Electrical conductivity of 24-h-old pre-treated embryos did not differ significantly from that observed in control ones (Table 2).

NO emission by embryo axes

The NO emission, measured as increase in DAF fluorescence was detected for embryos axis isolated from control (dormant) or pre-treated embryos just after pre-treatment (3 or 6 h) and after additional 24 h (Table 3). In axis of control (non-treated) embryos, NO emission was relatively low, around 2–3 units. Fumigation of embryos with HCN or NO resulted in enhanced DAF fluorescence. Its value was the highest for axes isolated from embryos just after pre-treatment (3 or 6 h). Germination on water for next 24 h resulted in slowing down NO emission by the axes.

Table 2 Electrolyte leakage from embryos isolated from dormant apple seeds (control) or embryos shortly pre-treated with NO, SNP or HCN immediately after treatment (3 or 6 h) and after additional 24 h of germination

	Electrolyte leakage (% total leakage)
Control 3/6 h	3.5 ± 1.0
NO 3 h	25.0 ± 4.0
SNP 3 h	12.5 ± 3.5
HCN 6 h	13.5 ± 2.0
Control 24 h	8.0 ± 1.5
NO 24 h	11.0 ± 2.0
SNP 24 h	11.5 ± 2.5
HCN 24 h	15.0 ± 3.0

Values are the average ± SD of five replicates

Table 3 Nitric oxide emission from embryonic axes of non-treated (control) dormant embryos (after seed coat removal—0 and after 3, 6 or 24 h of germination) and from axes of embryos pre-treated with NO, SNP or HCN, immediately after treatment (3 or 6 h—NO 3 h, SNP 3 h, HCN 6 h or 24 h of germination)

	NO emission (arbitrary units)
Control 0	1.0
Control 3 h	2.2 ± 0.3
Control 6 h	2.1 ± 0.2
NO 3 h	5.7 ± 0.6
SNP 3 h	12.3 ± 2.4
HCN 6 h	8.0 ± 1.6
Control 24 h	3.1 ± 0.7
NO 24 h	4.3 ± 1.0
SNP 24 h	4.1 ± 0.7
HCN 24 h	6.2 ± 1.2

Values are mean ± SD of 5 replicates

The greatest drop of DAF fluorescence was observed for SNP pre-treated embryos, while it still remained pretty high for HCN pre-treated ones (Table 3).

Discussion

ROS, RNS and HCN are small molecules and byproducts of the regular metabolism in plant tissues, playing a dual (toxic or signaling) function in seeds physiology. Numerous studies on seeds of various plants confirmed their stimulatory effect on germination (Bailly et al. 2008; Bethke et al. 2006; Bogatek and Gniazdowska 2006; Oracz et al. 2009; Sarath et al. 2005). In this work, we demonstrated that dormancy alleviation in apple embryos correlated with NO

production in embryonic axes (Table 3). Furthermore, increased NO emission was temporary and occurred immediately after treatment, indicating its signaling role (Fig. 2). Moreover, it has been previously reported that NO scavenger (cPTIO) significantly arrested embryo germination and growth of seedlings developed from NO as well as HCN shortly pre-treated apple embryos (Gniazdowska et al. 2010b). Therefore, we may assume that HCN signaling pathway involves not only ROS synthesis but also transient NO emission. Oracz et al. (2009) describing some similarities between HCN and ROS mode of action in sunflower seed dormancy alleviation proposed that HCN signaling pathway starts from enhanced ROS production in seeds. This suggestion was confirmed also for apple embryos in our previously published papers and proved for NO-mediated germination (Gniazdowska et al. 2010a, b). We demonstrated that both H_2O_2 and $O_2^{-\cdot}$ concentrations in 24-h-old apple embryos were two- or threefold higher in NO, HCN pre-treated embryos that in control ones (Gniazdowska et al. 2010a). ROS and RNS content in seeds may modify inhibitory impact of abscisic acid (ABA) on dormancy and germination. SNP partially overcame effect of ABA on switchgrass seeds (*Panicum virgatum*) germination (Sarath et al. 2005). Similarly NO or HCN pre-treatment of dormant apple embryos lowered tissue sensitivity to ABA (Gniazdowska et al. 2007, 2010a) and decreased ABA content particularly during early phase of germination (Bogatek et al. 2003). Reduced ABA concentration was observed also during imbibition of *Arabidopsis* seeds treated with NO or H_2O_2 (Liu et al. 2009, 2010). On the other hand, ROS overproduction can lead to loss of viability due to membrane lipid peroxidation or DNA/RNA and protein irreversible damage (Bailly et al. 2008). Although NO pre-treatment of dormant apple embryos enhanced ROS accumulation only temporary, it did not cause significant loss of membrane integrity in prolonged culture (Table 2). It was postulated for sorghum (*Sorghum bicolor*) embryonic axes that NO may act as antioxidant decreasing electrolyte leakage (Jasid et al. 2008). Protective role of NO was described also in mitochondria of winter wheat (*Triticum aestivum*) seeds germinating under salt stress (Zheng et al. 2009). In apple embryos, enhanced electrolyte leakage was observed only immediately after NO fumigation (Table 2), such temporary arise of membrane permeability may enhance germination, and allow H_2O_2 leakage into the germination medium, which was described earlier (Gniazdowska et al. 2010b). Alterations in extracellular redox potential might protect germinating seeds and young seedlings against pathogen attack (Schopfer et al. 2001). Elevated amount of ROS in the surrounding medium of germinating embryos can also provide cell wall loosening to facilitate radical protrusion (Kranner et al. 2010; Müller et al. 2009).

The concept of H_2O_2 steady state level during early seed germination was created by Puntarulo et al. (1991). Thiol containing tripeptide—glutathione in its reduced form (GSH) is the most important antioxidant in orthodox seeds at the beginning of germination (Tommasi et al. 2001). GSH is used as an electron source for GPX-like activity and is converted to GSSG. Glutathione reductase (GR) prevents GSSG over accumulation by reducing this molecule to GSH (Kranner et al. 2006). Studies on sunflower seeds indicated close relationship between seed vigour and activity of CAT or GR (Bailly et al. 2002).

Although the stimulatory effect of NO on seed dormancy removal and germination was proved by several authors, the involvement of this molecule in regulation of activity of antioxidant system in this process has never been investigated. SODs are the primary enzymatic antioxidants, they are classified into three groups: iron SOD (FeSOD), manganese SOD (MnSOD) and copper–zinc SOD (Cu,ZnSOD) (Alscher et al. 2002). Cytological localization of SODs suggests that during seed imbibition mostly mitochondrial, peroxisomal and cytosolic isoforms play a crucial role. Worthy to mention, MnSODs occurring in mitochondria and peroxisomes are insensitive to HCN and H_2O_2, as compared to Cu,ZnSODs. Results of our work indicate that pre-treatment of apple embryos with HCN, NO or SNP did not significantly change SOD activity immediately after exposure to these gases. It is in contrast to SOD and CAT activity in sunflower embryos which was inhibited by HCN (Oracz et al. 2009). Increased SOD activity in pre-treated apple embryos, was observed only after 24 h of culture (Fig. 1a). It points to MnSOD as the main SODs isoform modulating ROS content at the early phase of germination. It is also possible that this isoform was not affected by NO, since enhancement of SOD activity by NO was detected in mitochondria of germinating wheat seeds (Zheng et al. 2009). Therefore, HCN, ROS and RNS could modify activity of SODs isoforms to adjust ROS accumulation in diverse cell compartments. MnSOD prevents uncontrolled ROS increase in mitochondria and peroxisomes (main sources of ROS and RNS during seed germination). On the other hand Cu,ZnSOD (isoform sensitive to ROS and HCN), localized also in the cytosol alters ROS content and, in consequence probably ethylene production. Baker (1976) demonstrated that ethylene production was inhibited by SOD. We proved, in our previous papers, that ethylene plays a beneficial role in dormancy breakage of apple embryos and postulated that during early phases of germination ethylene is synthesized by direct ROS or RNS attack on ethylene precursor (Gniazdowska et al. 2010a).

CAT belongs to metaloenzymes (haem group containing protein) localized mainly in peroxisomes. CAT and Prx differ in their affinity to H_2O_2. CAT affinity to H_2O_2 is

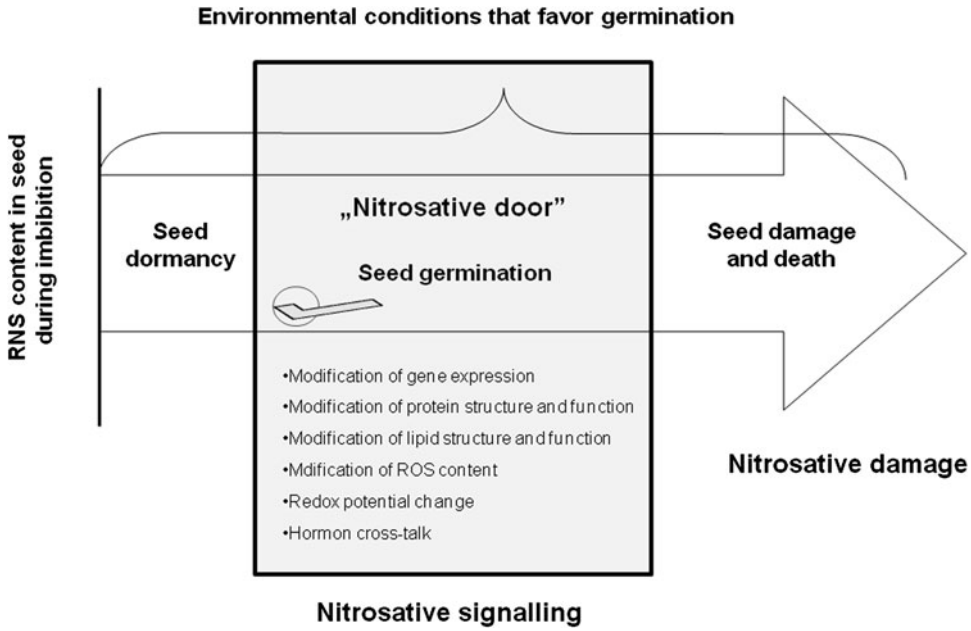

Fig. 2 The model of "nitrosative door" in seed physiology. Seed germination is possible when RNS content in imbibed seeds is enclosed in the "nitrosative door". Below this door, the amount of RNS is too low to promote germination. Dormancy breakage leads to an increased level of RNS in imbibed seed and allows to complete germination. Above this door, too high RNS content induces damages of cellular components that prevent or delay germination. The scheme is based on the model of "oxidative window" proposed by Bailly et al. (2008) with some modifications

lower, therefore its action seems to be linked rather to plant reaction to stresses nor to modulation of ROS signaling (Mhamdi et al. 2010). NO pre-treatment of dormant apple embryos slightly stimulated CAT activity after 24 h of culture (Fig. 1b). Neither HCN nor NO significantly altered CAT activity, which was rather low (Fig. 1b), particularly during early phases of germination (24 h after sowing). It probably reflects to period needed for storage lipids mobilization and oxidation in peroxisomes. Additionally, to our surprise both NO and HCN did not reduce CAT activity in embryos immediately after fumigation (Fig. 1b). A presence of another CAT isoform containing manganese instead of haem group has been postulated. Until now, this pseudocatalase or manganese catalase (resistant to HCN) was found only in some microorganisms (Yasuhisa and Fridovich 1983). It is possible, that higher organisms e.g. plants posses also this type of CAT. Our data suggest that activity of such CAT insensitive to HCN could be linked to ROS metabolism during only short period, at the beginning of seed germination characterized by ROS burst.

Peroxidases (Prxs) of class III are haem-containing glycoproteins acting in ROS scavenging and production. Enhanced Prxs activities were detected during germination of cress seeds (Linkies et al. 2010) and led to cell wall modification resulting in acceleration of radical protrusion (Müller et al. 2009). Rehydratation during apple seed imbibition and later on during seed coat removal results in the oxidative burst. Such temporary ROS unbalance is accompanied by high Prxs activity observed even in dormant apple embryos (Fig. 1c). HCN and NO pre-treatment decreased Prxs activity immediately after embryo fumigation probably because of haem group instability. It is also possible that high ROS/RNS content after application declines Prxs activity to prevent over accumulation of ROS. Moreover, Prxs have been suggested to be involved in RNS metabolism (Almagro et al. 2009).

As it was mentioned earlier, APX activity in dry orthodox seeds is absent but it increases during imbibitions (Tommasi et al. 2001). In apple embryos, activity of APX was undetectable after seed coat removal as well as during early phase of imbibition (results not shown). Thus, GSH is main molecular ROS scavenger. While glutathione peroxidases (GPXs) that structurally differed from Prxs are another important ROS regulators. GPXs react with organic peroxides (also H_2O_2) and protect against lipid peroxidation. Experiments carried out on various plant tissues indicated that GPX molecule contains Cys instead of modified SeCys typical for mammalian GPX. Moreover, GPX is not constitutively presented in plant cells, its existence and catalytical activity is regulated by stress conditions (Fu et al. 2002). NO or HCN fumigation of dormant apple embryos enhanced GPX-like activity (Fig. 1d) and the highest GPX-like activity was observed 24 h after sowing (Fig. 1d). Due to modification in GSH/GSSG ratio in the imbibed seeds and higher availability of GSH, GPX-like enzymes are synthesized to protect membranes and also storage lipids. In apple embryos, GPX-like activity decreased after radical protrusion (data not shown) probably because of higher activity of other antioxidants e.g. APX. Increased content of GSSG in seed cells correlates with viability loss and PCD (Kranner et al. 2006) and also may contribute to inhibition of protein synthesis. GSSG is reversed to GSH by glutathione reductase (GR). It points at key role of GR in the establishment of redox

potential during seed germination. HCN did not inhibit but rather stimulate GR activity in germinating apple embryos (Bogatek et al. 2003) and sunflower embryonic axes (Oracz et al. 2009). Data obtained in our work indicated slight and transient inhibition of GR activity in apple embryos by NO or SNP and HCN only immediately after treatment (Fig. 1e).

Our results confirm the hypothesis that ROS, NO and HCN can act simultaneously. Small signaling molecules (toxic in high concentration) play important role in dormancy alleviation and are under severe control of antioxidant system. We propose that ROS, RNS and HCN at accurate level are essential in the seed germination and all together play the important role as developmental regulators.

Model of the "oxidative window" describes a dual role of ROS in seed physiology (Bailly et al. 2008). The evidence presented in this one and our previously published papers suggests RNS double function in seed dormancy alleviation and seed germination, which may be illustrated by "nitrosative door" (Fig. 2). Role of RNS in seed dormancy and germination is concentration-dependent. The arrow at the scheme presents a raised concentration of RNS in seeds during germination. Below the "nitrosative door", RNS level is too low to allow the seeds to get to transition from dormant state to non-dormant. On the other hand, RNS level above the "nitrosative door" leads to nitrosative stress and even cell death. Seed dormancy alleviation is achieved when RNS concentration reach the "nitrosative door" level. Both models of the "oxidative window" and "nitrosative door" show a beneficial effect of small, very reactive molecules in transition of seeds to seedlings, and particularly at the beginning of germination. This ROS and RNS-dependent conversion from dormant state to non-dormant is strictly regulated by enzymes of antioxidant system. We suggest that based on many data, this enzymatic scavengers should be termed as ROS and RNS modulators, while reactions catalyzed by them—ROS and RNS modulating system. Moreover, HCN (also as co-product of ethylene synthesis) has been shown to cross-talk with ROS and RNS playing an important role in this modulating system.

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