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RAPID COMMUNICATION

The beneficial effect of small toxic molecules on dormancy alleviation and germination of apple embryos is due to NO formation

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Abstract Deep dormancy of apple (*Malus domestica* Borkh.) seeds is terminated by a 3-month-long cold stratification. It is expressed by rapid germination of seeds and undisturbed growth of seedlings. However, stimulation of germination of isolated apple embryos is also observed after applying inhibitors of cytochrome *c* oxidase: nitric oxide (NO) or hydrogen cyanide (HCN) during the first 3–6 h of imbibition of dormant embryos. The aim of this work was to compare the effect of yet another toxic gaseous molecule carbon monoxide (CO) with the effects of HCN and NO on germination of apple embryos and growth and development of young seedlings. We demonstrated that stimulation of germination after short-term pre-treatment with HCN, NO or CO was accompanied by enhanced NO emission from the embryo axes during their elongation. Moreover, similarly high NO production from non-dormant embryos, after cold stratification, was detected. Therefore, we propose that NO may act as signaling molecule in apple embryo dormancy break.

Keywords Carbon monoxide · Hydrogen cyanide · Nitric oxide · Seed dormancy · Seed germination

Abbreviations

ABA	Abscisic acid
CO	Carbon monoxide
cPTIO	2-(4-Carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide
HCN	Hydrogen cyanide
NO	Nitric oxide
SNP	Sodium nitroprusside

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Introduction

Plant growth and development is regulated by various signals that lead to creation of a network of signaling pathways. It is well known that small toxic molecules in low concentration and/or used only transiently may induce positive effects on living organisms. Nitric oxide (NO) is an important regulatory molecule in animals and plants. It is involved in the regulation of many physiological processes in plants: seed germination, root development, flowering, senescence and responses to abiotic stresses and pathogens (reviewed by Shapiro 2005). Hydrogen cyanide (HCN) acts as a protective molecule against herbivores (Zagrobelny et al. 2004). It also plays a regulatory function in seed germination (Bogatek et al. 1991, 1999) or nitrate assimilation (Salomonson and Barber 1990). Another toxic molecule, carbon monoxide (CO), despite its reputation as a lethal gas, manifests a regulatory function in lateral root development, seed germination or stomata movement (Siegel et al. 1962; Guo et al. 2008; Song et al. 2008). All those molecules are endogenously synthesized in plant organisms in micromolar quantities. Hydrogen cyanide, in spite of its evolution from cyanide glucosides (e.g. amygdalin, prunasin) (Siegień and Bogatek 2006), is produced as a co-product during ethylene synthesis in the last reaction of a pathway catalyzed by 1-aminocyclopropane-1-carboxylic acid oxidase

(Yip and Yang 1988). CO is formed from heme molecules in a reaction catalyzed by heme oxygenase (Muramoto et al. 2002), while NO biosynthesis depends on activity of nitric oxide synthase-like enzymes and/or nitrate reductase (reviewed by Besson-Bard et al. 2008). Interestingly, some activities of NO, HCN and CO are similar. All of them slow mitochondrial respiration by inhibiting activity of cytochrome *c* oxidase. On the other hand, NO and CO are suggested to act as cytoprotectants against oxidative stress induced by salinity or heavy metals (Kopyra and Gwóźdź 2003; Liu et al. 2007). Recent studies also suggest interaction of those toxic gases with classical plant hormones, e.g. auxins, ABA and ethylene (Neill et al. 2002; Pagnussat et al. 2003; Lombardo et al. 2006; Oracz et al. 2008; Xuan et al. 2008).

The aim of our work was to compare the involvement of those three gases in regulation of apple embryo dormancy removal and germination. Additionally, we present evidence that NO is endogenously produced in germinating embryos. The results suggest that NO acts not only in dormancy alleviation but is necessary for growth of embryonic axes in the last phase of seed germination.

Materials and methods

Plant material

The experiments were carried out on apple (*Malus domestica* Borkh. cv. Antonówka) seeds harvested in 2007–2009. Dormant seeds were stored in dark glass containers at 5°C. Stratification of seeds was done in sterile sand at 60% of full water capacity at 5°C for 90 days.

Seed coat and endosperm were removed from seeds imbibed for 24 h in distilled water at room temperature (20°C). The isolated embryos were taken for determination.

Germination tests

Embryos isolated from dormant seeds were exposed to 1 mM HCN for 6 h as described by Bogatek et al. (1991); the influence of NO on embryo germination was determined using sodium nitroprusside (SNP, 5 mM for 3 h; Gniazdowska et al. 2007) and vapors of acidified nitrite (NO; Gniazdowska et al. 2010). Acidified nitrite was prepared using 20 mM sodium nitrite ($NaNO_2$) and 0.1 M HCl as described by Gniazdowska et al. (2010). During treatment the embryos were placed on filter paper moistened with 0.05 M Hepes–KOH buffer, pH 7.0. Hepes buffer was used to avoid acidification of the embryo culture solution since a low pH may act as another dormancy-breaking agent. Two artificial CO donors were used: hematin ($C_{34}H_{33}N_4O_5Fe$; Sigma, St. Loius, MO, USA; Liu et al. 2007) and hemin ($C_{34}H_{32}ClFeN_4O_4$, Sigma; Xuan et al. 2008) at different concentrations of 0.05–5 mM. Embryos isolated from dormant seeds were exposed to either hematin for 1–6 h or to hemin for 3 h in the light.

After treatment, embryos were rinsed in distilled water and placed in 9-cm Petri glass dishes containing filter paper wetted with distilled water. Additional experiments were conducted with NO scavenger 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Control (non-treated), stratified and NO, CO or HCN pre-treated embryos were placed in Petri dishes with filter paper wetted with 0.3 mM cPTIO and germinated for 7 days. Control (dormant, non-treated), pre-treated embryos and embryos isolated from stratified (non-dormant) seeds were germinated in 9 cm Petri dishes (20 embryos per dish) in growing chamber at 25°C with 12/12 h (light/dark) photoperiod, under 150 μmol PAR m^{-2} s^{-1}. Germination tests were repeated 3 times in 3–4 independent experiments.

Embryos were considered to have germinated when radicles were 2–3 mm long and exhibited typical gravitropic bending.

Detection of NO production by embryo axes

NO production was detected in isolated axes of germinating pre-treated embryos and in axes of control (non-treated) embryos. Additionally, experiments were performed with axes isolated from control, stratified or pre-treated embryos imbibed in cPTIO (as was described above). Axes were isolated from embryos of the same physiological stage: 2-day-old stratified or 4-day-old pre-treated or control. After isolation, axes were rinsed in 10 mM Hepes–NaOH (pH 7.4) for 15 min. One or three axes, depending on their weight (total fresh weight of the tissue used for one assay was approximately 0.003 g), were transferred to 100 μl of 10 mM Hepes–NaOH (pH 7.4) buffer solution 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 Hepes–NaOH buffer (pH 7.4) and transferred to the cuvette containing 0.8 ml fresh Hepes–NaOH 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 5–8 repetitions, and their exact reproducibility was confirmed. Fluorescence was normalized per milligram FW and maximal fluorescence was expressed in arbitrary units. The data shown in the figure are representative of these experiments.

Results

Short pre-treatment of apple embryos with CO donor terminates their dormancy similar to NO, HCN and cold stratification

Dormant apple embryos germinated very slowly (Table 1). After 7 days, only 11% of the embryos germinated. Cold stratification led to dormancy removal, hence non-dormant embryos germinated quickly; in 95% just after 4 days and after 7 days all were germinated (Table 1). HCN, and NO (derived from acidified nitrite or SNP) alleviated apple embryos dormancy and after 7 days almost 60–65% of the embryos were germinated (Table 1). Since the effect of NO released from acidified nitrite was comparable to that observed after *S*-nitrosopenicillamine (SNAP) pre-treatment described previously (Gniazdowska et al. 2007), we resolved to use that reaction as a source of gaseous NO in the further experiments.

Table 1 Germination rate of non-treated dormant apple embryos (control), non-dormant embryos (stratification) or dormant embryos pre-treated with hydrogen cyanide (HCN, 1 mM, 6 h), sodium nitroprusside (SNP, 5 mM, 3 h), acidified nitrite (NO, 3 h), hematin (2 mM, 3 h) or hemin (4 mM, 3 h) after 4 or 7 days culture in water (H_2O) or in the presence of NO scavenger cPTIO

Pre-treatment	Germination (%)	
	After 4 days	After 7 days
Control		
H_2O	2 ± 2	11 ± 3
+cPTIO	0	0
Stratification		
H_2O	95 ± 3	99 ± 1
+cPTIO	15 ± 2	27 ± 2
HCN		
H_2O	20 ± 5	60 ± 11
+cPTIO	0	1 ± 1
SNP		
H_2O	30 ± 5	65 ± 10
+cPTIO	0	1 ± 1
NO		
H_2O	23 ± 6	60 ± 8
+cPTIO	0	0
Hematin		
H_2O	35 ± 5	62 ± 5
+cPTIO	0	0
Hemin		
H_2O	22 ± 4	51 ± 6
+cPTIO	0	0

Means of 3–5 independent experiments ± SD. Data on germination in water after pre-treatment with HCN, SNP and NO were presented previously by Gniazdowska et al. (2010)

Hematin used as CO donor stimulated apple embryo germination in dose-dependent manner (Fig. 1). Hematin at the highest concentration (5 mM) stimulated embryo germination similarly as hematin at a 10-times lower concentration (0.5 mM; Fig. 1). The highest total embryo germination was detected with 2 mM hematin. Treatment with 2 mM hematin solution for 3 h resulted in alleviation of embryo dormancy and after 7 days almost 62% of the embryos were germinated (Table 1). A little lower stimulation of germination of apple embryos was observed after 3 h pre-treatment with hemin, the other CO donor (Table 1). Beneficial effect of all tested gases (HCN, NO and CO) as well as that of cold stratification on germination of apple embryos was reversed by NO scavenger cPTIO (Table 1), whose presence totally blocked the germination. Only embryos isolated from stratified seeds were able to germinate to some extent (less than 20%) in the presence of cPTIO (Table 1). Stimulation of embryo germination by hematin was dependent on timing of pre-treatment. Short (1–2 h) or prolonged (4–6 h) exposure to 2 mM hematin was less effective than 3 h treatment (Fig. 2). Therefore, for the second CO donor, hemin, only 3 h treatment was chosen and the dose-dependent effect on embryo germination was checked using various concentrations (0.5–5 mM; Fig. 3). The best stimulation of germination (up to 51% after 7 days of culture) was noticed for 4 mM hemin, while lower concentrations were less effective (Fig. 3). Higher concentration of hemin inhibited embryo germination. Therefore, embryos pre-treated with 2 mM hematin and 4 mM hemin for 3 h were chosen for the detection of NO emission.

Seedlings developed from stratified seeds or pre-treated embryos did not exhibit any morphological anomalies characteristic for seedlings developed from dormant, control embryos such as: asymmetric growth and greening of

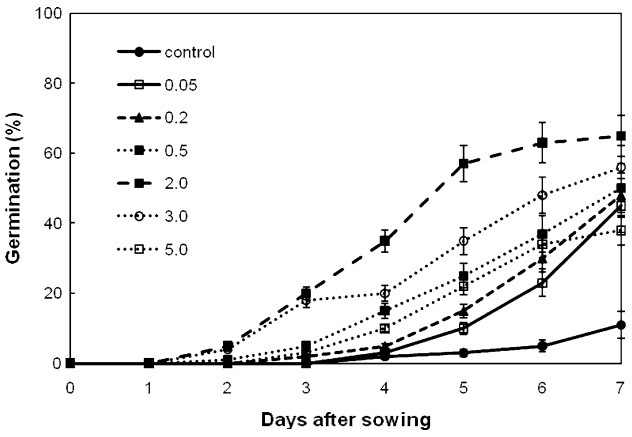

Fig. 1 Germination rate of dormant (control) apple embryos and dormant apple embryos after 3 h pre-treatment with various concentration of hematin (0.05–5 mM). Data are means of nine independent replicates ± SD

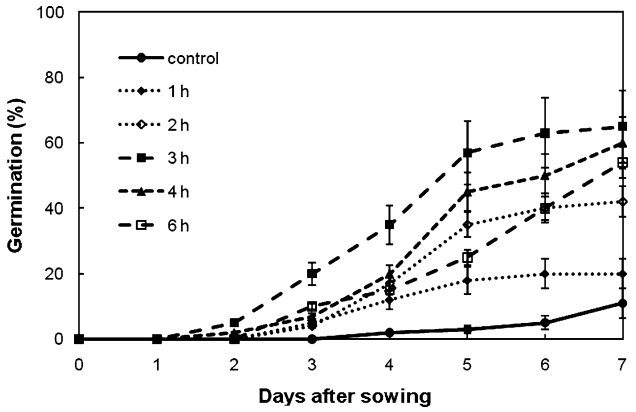

Fig. 2 Germination rate of dormant (control) apple embryos and dormant apple embryos after pre-treatment with 2 mM hematin for 1–6 h. Data are means of six independent replicates ± SD

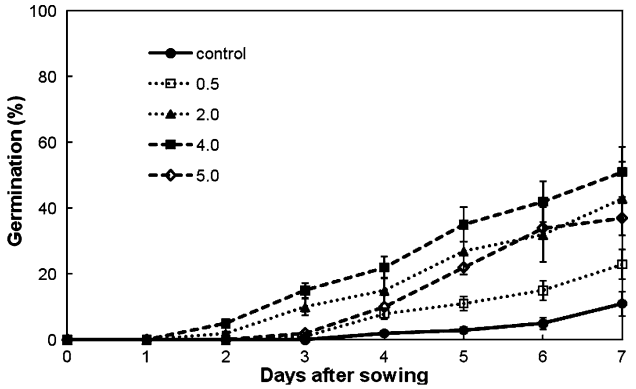

Fig. 3 Germination rate of dormant (control) apple embryos and dormant apple embryos after 3 h pre-treatment with various concentration of hemin (0.5–5 mM). Data are means of three independent replicates ± SD

cotyledons, inhibition in hypocotyls growth, stunting of the roots (Fig. 4). It should be mentioned, however, that the influence of hematin or hemin on removal of morphological anomalies of the seedlings was less pronounced compared to that subjected to HCN and NO or SNP pre-treatment.

Germination after removal of embryonic dormancy of apple embryos by cold stratification or short pre-treatment by toxic gases (HCN, NO, CO) is associated with emission of NO

Pre-treatment of apple embryos by HCN, NO or CO resulted in dormancy removal, so after 4 days approximately 25–30% of the pre-treated embryos were considered as germinated. Their radicles were 3–4 mm long and the characteristic gravitropic bending was visible. Moreover, their cotyledons enlarged and were light green. Embryos isolated from non-dormant, stratified seeds germinated quicker; therefore, embryo radicles 2 days after sowing were collected for detection of NO evolution. Four days after sowing dormant embryos did not germinate, neither was any greening of the upper cotyledons detected (Fig. 4).

The NO emission measured as increase in DAF fluorescence was detected for embryo axes isolated from all pre-treated embryos (Fig. 5). The NO emission was detected for axis isolated from non-dormant stratified seeds, while NO emission observed from axes of control, non-treated embryos and embryos cultured on cPTIO was low (lower than 0.2 arbitrary units mg^{-1} FW; Fig. 5). NO fumigation of embryos resulted in enhanced DAF fluorescence, although it should be mentioned that it was lower in comparison to fluorescence detected for stratified, non-dormant seeds. HCN and hematin or hemin also increased NO emission by the axes (Fig. 4). Hemin pre-treatment resulted in less pronounced effect on NO production, which corresponded to relatively less stimulation of germination (Table 1).

Discussion

Recent studies have demonstrated that small toxic molecules such as HCN, NO and CO are produced by plants as essential signaling molecules (Xuan et al. 2007, 2008; Moreau et al. 2010). All of them probably induce physiological responses by regulation of mitogen-activated protein kinases (MAPK) or by ROS generation (Boczkowski et al. 2006). Seed dormancy and germination are controlled by many endogenous factors, among them also small gaseous molecules used in our experiment. Embryos isolated from dormant apple seeds do not germinate, although the seed coat is removed and they are exposed to environmental conditions favorable for germination (Bogatek et al. 1991). Moreover, deep dormancy of apple embryos could be characterized by other criteria such as abnormalities of developing seedlings, which are expressed as asymmetric growth and greening of cotyledons. Toxic gases NO and HCN stimulated apple embryo germination similarly, as mentioned earlier (Bogatek and Gniazdowska 2006; Gniazdowska et al. 2010), while this effect was hindered by NO scavenger (Table 1). Seedlings developed from pre-treated embryos grew well; both cotyledons were at the same size and green (Fig. 4). The analogous effect of NO and cyanide on seed germination and dormancy removal was detected for other seeds (Giba et al. 2003; Bethke et al. 2006), while reports on the function of CO as dormancy-breaking factor are rare. Our data confirmed some previously reported results demonstrating that CO stimulates germination of dormant seeds (rice, barley, giant foxtail) (Roberts 1964; Dekker and Hargrove 2002) or counteracts inhibition of wheat seed germination in salt stress (Xu et al.

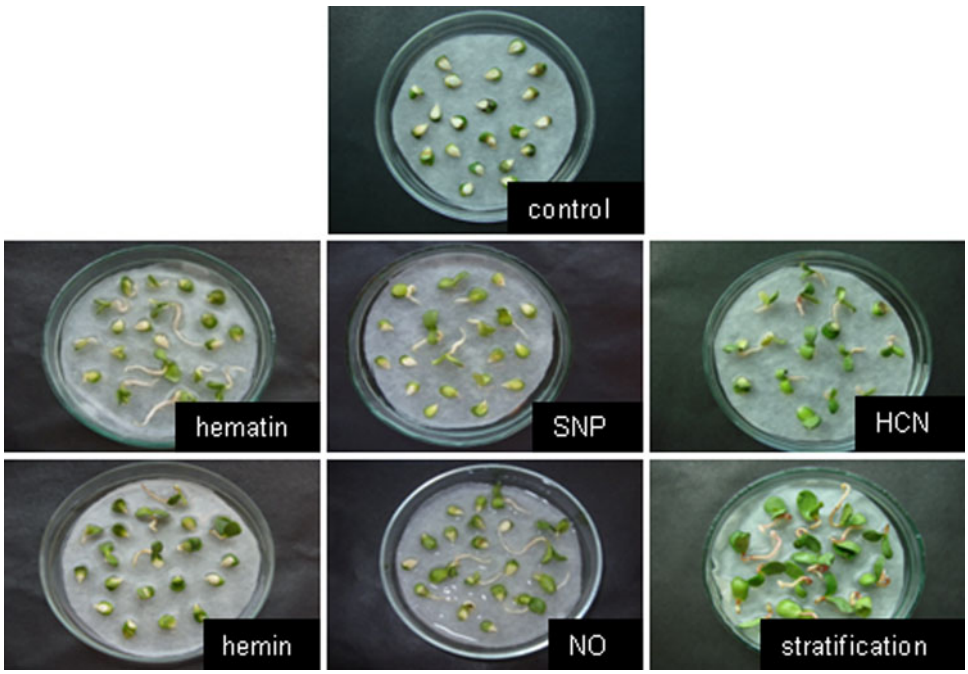

Fig. 4 Growth and development of apple seedlings. Pictures showing the differences in growth of 7-day-old seedlings developed from dormant (*control*) embryos, non-dormant embryos (*stratification*), or embryos pre-treated with hydrogen cyanide (HCN, 1 mM, 6 h), sodium nitroprusside (SNP, 5 mM, 3 h), acidified nitrite (NO, 3 h) hematin (2 mM, 3 h) or hemin (4 mM, 3 h)

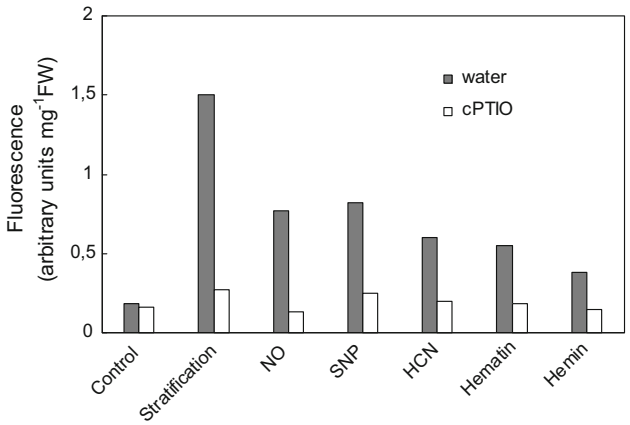

Fig. 5 Nitric oxide emission from embryonic axes of 4-day-old non-treated dormant embryos (control), 2-day-old embryos isolated from non-dormant stratified seeds (stratification), or 4-day-old dormant embryos pre-treated with hydrogen cyanide (HCN, 1 mM, 6 h), sodium nitroprusside (SNP, 5 mM, 3 h), acidified nitrite (NO, 3 h), or artificial CO donors hematin (2 mM, 3 h) and hemin (4 mM, 3 h) after germination on water or cPTIO. Non-dormant embryos and pre-treated embryos were at the same stage of germination

2006). Pre-exposure of cocklebur seeds to other inhibitors of cytochrome *c* oxidase (e.g. KCN or azide) also promoted germination (Eshasi et al. 1982). We used two artificial donors of CO: hemin and hematin. The CO-induced germination of apple embryos was concentration and time dependent (Figs. 1, 2, 3). The best beneficial effect of CO donor was observed after treatment with 2 mM hematin. Increasing hematin concentration (up to 5 mM) did not lead to a stimulation of embryo germination (Fig. 1). The possible reason for such a reaction may be that the toxicity of CO at a certain level is in excess of the physiological requirement. Similarly, prolonged (6 h) exposure to hematin (Fig. 2) may be poisonous to embryos due to disorder of the respiratory metabolism. Both CO donors induced growth and greening of cotyledons, leading to the expansion of well-developed apple seedlings in a fashion similar to that observed for NO and HCN (Fig. 4). Likewise, beneficial effect of short-term (3 h) pre-treatment of dormant embryos with hemin and hematin was impeded by the NO scavenger cPTIO (Table 1), indicating that NO may act downstream in CO signaling pathway. A similar interaction between CO and NO was described in the process of lateral root formation (Guo et al. 2008), or elongation growth of wheat root segments (Xuan et al. 2007). Both molecules seem to be involved in the regulation of many processes via guanylate cyclase, the enzyme responsible for cyclic GMP biosynthesis (Wendehenne et al. 2001; Xuan et al. 2007). Furthermore, it was suggested that in animals those two gases modulate each others activity (Hartsfield 2002).

In our experiment, NO production by the axes of apple embryos was measured during the transition from germination "sensu stricto" to post-germination growth. Germination "sensu stricto" is defined as the process associated with the initiation and completion of embryo emergence, and refers to the progress of a seed from imbibition to radicle emergence (Nonogaki et al. 2007). The highest NO emission, detected as fluorescence of the specific fluorescence probe DAF-FM DA, was measured for axes isolated from non-dormant seeds (after stratification), also suggesting a regulatory role of NO in dormancy

alleviation in natural conditions. In axes isolated from embryos pre-treated with HCN, NO or CO donors, the DAF-FM DA fluorescence was lower than that measured after cold stratification, but still more than double in comparison to axes from dormant non-germinating embryos (Fig. 5). We may conclude that induction of root growth and gravitropic bending of axes isolated from pre-treated embryos was accompanied by increased NO emission. It may be suggested that increased NO emission occurring during the last phase of germination is required for induction of post-germination growth, since cPTIO reduced not only NO emission from axes but also blocked embryo germination (Table 1; Fig. 5). The presented results are also in accord with the data indicating NO biosynthesis in germinating sorghum seeds (Simontacchi et al. 2004).

This is the first report presenting the close interaction between CO- and HCN-mediated dormancy breakage and NO production by the embryos. We suggest that NO operates downstream of CO and HCN and acts as signaling molecule, probably together with ROS in determining seed germination and development of a young seedling. Transient increase in ROS accumulation in embryos pre-treated with HCN or NO was reported by Gniazdowska et al. (2010). Those results also indicate that CO represents another signal for dormancy removal and germination, and we conclude that HCN, NO or CO short pre-treatment in the light may be used for dormancy alleviation of apple embryos instead of long-term cold stratification.

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