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ORIGINAL ARTICLE

Do nitric oxide donors mimic endogenous NO-related response in plants?

J. Floryszak-Wieczorek · G. Milczarek · M. Arasimowicz · A. Ciszewski

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Abstract Huge advances achieved recently in elucidating the role of NO in plants have been made possible by the application of NO donors. However, the application of NO to plants in various forms and doses should be subjected to detailed verification criteria. Not all metabolic responses induced by NO donors are reliable and reproducible in other experimental designs. The aim of the presented studies was to investigate the half-life of the most frequently applied donors (SNP, SNAP and GSNO), the rate of NO release under the influence of light and reducing agents. At a comparable donor concentration (500 μM) and under light conditions the highest rate of NO generation was found for SNAP, followed by GSNO and SNP. The measured half-life of the donor in the solution was 3 h for SNAP, 7 h for GSNO and 12 h for SNP. A temporary lack of light inhibited NO release from SNP, both in the solution and SNP-treated leaf tissue, which was measured by the electrochemical method. Also a NO, selective fluorescence indicator DAF-2DA in leaves supplied with different donors showed green fluorescence spots in the epidermal cells mainly in the light. SNP as a NO donor was the most photosensitive. The activity of PAL, which plays an important role in plant defence, was also activated by SNP in the light, not in the dark. S-nitrosothiols (SNAP and GSNO) also underwent photodegradation, although to a lesser degree than SNP. Additionally, NO generation capacity from S-nitrosothiols was shown in the presence of reducing agents, i.e. ascorbic acid and GSH, and the absence of light. The authors of this paper would like to polemicize with the commonly cited statement that "donors are compounds that spontaneously break down to release NO" and wish to point out the fact that the process of donor decomposition depends on the numerous external factors. It may be additionally stimulated or inhibited by live plant tissue, thus it is necessary to take into consideration these aspects and monitor the amount of NO released by the donor.

Keywords Nitric oxide donors · Light · Ascorbic acid · Glutathione · Phenylalanine-ammonia lyase · Ivy pelargonium leaves

Abbreviations

NO	Nitric oxide
SNP	Sodium nitroprusside
RSNOs	S-nitrosothiols
GSNO	S-nitrosoglutathione
SNAP	S-nitroso-*N*-acetyl-D-penicillamine
PAL	Phenylalanine ammonia lyase
GSH	Reduced glutathione
CPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAF- 2DA	4,5-diaminofluorescein diacetate

Introduction

Studies conducted during the last ten years have shown that nitric oxide (NO) is a simple molecule "for special

J. Floryszak-Wieczorek (✉) · M. Arasimowicz
Department of Plant Physiology, Agricultural University, Wołyńska 35, 60-637 Poznań, Poland
e-mail: florysza@jay.au.poznan.pl

G. Milczarek · A. Ciszewski
Institute of Chemistry and Technical Electrochemistry, Poznań University of Technology, Piotrowo 3, 60-965 Poznań, Poland

assignments", acting in numerous metabolic fields. As it has been documented, NO controls the growth and development of the plant throughout its vegetation, starting from germination (Beligni and Lamattina 2000) and ending with flowering, ripening of its fruits and senescence of its organs (Leshem et al. 1998). Also under environmental stress conditions, both of abiotic and biotic origin, elevated generation of NO occurs in various organs of the plant (del Rio et al. 2004; Wandehenne et al. 2004; Delledonne 2005). The participation of NO in so many biological functions changed our understanding of the course and coordination of important physiological and pathological processes.

The effect of cytoprotective or cytotoxic action of NO on plant metabolism (Beligni and Lamattina 2001) depends to a large extent on the local concentration of the molecule and is affected by the rate of synthesis, displacement and efficiency of removal of this reactive nitrogen species (Wojtaszek 2000; Romero-Puertas et al. 2004).

In order to fully understand the diverse bioregulatory functions of nitric oxide, metabolic responses in the plant are induced experimentally by the administration of exogenous NO. Such a relatively simple methodological approach has yielded numerous valuable pieces of evidence for specific physiological functions of NO, although some of them seemed rather ambiguous and controversial. Several contradictory findings, which have appeared in the studies published so far, are partly the effect of non-reproducible experimental designs, to a large extent consisting in differing methods of treating plant tissue with NO donors of varying concentrations. As it has rightly been proposed by Neill et al. (2003), in order to make the obtained data reliable it is necessary, simultaneously to the NO treatment of the plant, to apply different inhibitors of NO synthesis, NO-scavengers and to monitor precisely the current concentration of this compound in the tissue.

Nitrogen oxide donors are compounds, which produce NO when applied to the biological systems and are able to either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency. The pathways leading to the formation of NO differ greatly among the various groups of compounds, some of which require enzymatic catalysis, while others produce it non-enzymatically.

Transition metal NO complexes represent an important class of NO donors. The most commonly used is sodium nitroprusside (SNP), an NO^+ donor. Here NO acts as a powerful ligand, in which the nitrogen rather than oxygen binds the metal. The mechanism of NO release from SNP is not clear, although it has been known and used in clinical therapy for over 70 years. The SNP solution is extremely photosensitive and its degradation is promoted, e.g. by oxygen and temperature (Wang et al. 2002). According to the cited authors NO release from the donor requires illumination or a single-electron reduction, which under physiological conditions may depend additionally on many reducing agents present in biological systems, such as, e.g. ascorbates, thiols, hemoproteins, as well as NADH and NADPH.

S-nitrosothiols (RSNOs) belong to an important group of NO donors. Nonreductive decomposition of RSNOs leads to the formation of disulphides and release of NO and it is dependent on such parameters as, e.g. light, temperature and pH (Hou et al. 1999). In the solution RSNOs decomposition is promoted by trace amounts of transition metal ions, such as Cu^+ and Fe^{*+} (Stamler and Toone 2002). In biological systems it is promoted additionally by the reductive decomposition by L-ascorbic acid (Smith and Dasgupta 2000) and by thiols present, e.g. in glutathione, seleno compounds contained in selenocystamine and seleno-D,L-cystine, as well as glutathione peroxidase (a selenium-containing antioxidant enzyme) (Hou et al. 1996). The most frequently applied donors from the group of S-nitrosothiols are nitrosoglutathione (GSNO) and S-nitroso-*N*-acetyl-D-penicillamine (SNAP).

While treating tissue with a compound mimicking the action of endogenous NO we should take into consideration the mechanism and kinetics of NO release from the donor, dependent on the external factors, toxicity of accumulated products, etc. Similarly to phytohormones, the effect of NO action to a large extent will depend on its concentration. Hence, at a too high NO donor concentration—instead of stimulation—an inhibition of the process may be observed and thus the obtained results will not reflect the action of endogenous NO in the cell. Commonly applied donor concentrations range from 10 to 500 μM, although there are reports on much higher amounts of the NO donor, maximum 10 mM (Modolo et al. 2002).

In relation to the above-mentioned issue numerous questions may be raised, namely whether the NO donor applied to a plant in different forms and at different doses, in the light and in the dark, can properly reflect functions fulfilled by endogenous NO in the tissue. It is a common opinion that current NO donors are normally thermodynamically unstable, especially in solutions and photosensitive.

The aim of the presented studies was to examine the half-life of SNP, SNAP and GSNO in solution, the effect of light and reducing agents on the release of

nitric oxide by these donors. PAL (phenylalanine ammonia lyase, EC 4.3.1.5)—the enzyme of defense reaction to stress, especially biotic stress, was selected as a marker of physiological changes stimulated by NO and dependent on light.

Materials and methods

Plant material

Experiments were conducted on an ivy-leaved pelargonium (*Pelargonium peltatum* L.) 'Shiva', susceptible to *Botrytis cinerea*. Rooted seedlings of pelargonium came from Fischer GmbH and Co. KG Company, Hillscheid, Germany. Plants were placed in a growth chamber with constant air temperature of 21 ± 2°C at light intensity of 120 μmol photons m^{-2} s^{-1} (fluorescent lamps, cool-white type, TLD 36/64 Philips) with a 12-h photoperiod. Analyses were performed on plants which developed ten leaves.

Nitric oxide (NO)—donors and NO-scavenger treatment

Discs with the diameter of 1 cm cut out from leaf blades were placed on the surface of the following solutions: 100 μM sodium nitroprusside (SNP), 200 μM S-nitroso-*N*-acetyl-D-penicillamine (SNAP), 200 μM S-nitrosoglutathione (GSNO) and H_2O. After 6 h incubation in the light (120 μmol photons m^{-2} s^{-1}) or in the dark discs were transferred either directly to the DAF-2DA solution for 1 h or first to 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) also for the period of 1 h and next to the DAF-2DA solution (1 h).

Crude leaf extract

Leaves cut off from pelargonium plants were washed with distilled water and dried. Next leaf blades were placed in cuvettes on solidified agar (pH of 6.3). Leaves were point inoculated, by placing on the upper side of leaf blades three drops (40 μl) of a *B. cinerea* spore suspension with the concentration of $7.5 \cdot 10^5$ per 1 ml 0.1 M glucose and 0.05 M KH_2PO_4. Control leaves were treated with 0.1 M glucose and 0.05 M KH_2PO_4. Cuvettes with leaves were placed in a growth chamber at 20 ± 2°C and relative humidity of approximately 95%. Plant material was analyzed on the 6 day after pathogen treatment.

In order to prepare a crude leaf extract the tissue (2 g) was homogenized in 2 ml 0.05 M phosphate buffer of pH 7.4 with the addition of 0.05 g Polyclar AT at the temperature of 4°C, and the extract was centrifuged at 15,000*g* for 30 min at 4°C.

Phenylalanine-ammonia lyase (PAL) assay

Phenylalanine-ammonia lyase activity was determined spectrophotometrically according to the modified method by Ward et al. (1989). In order to determine PAL activity fresh material (400 mg) was homogenized in 5 ml of 100 mM borate buffer (pH 8.8) containing 0.5 mM EDTA and 2 mM β-mercaptoethanol. The homogenate was centrifuged at 18,000*g* for 25 min and the supernatant was immediately assayed for PAL activity. The reaction mixture (2 ml), containing 1 ml of 100 mM borate buffer (pH 8.8), 0.5 ml of 0.4 mM L-phenylalanine and 500 μl of the extract, was incubated at 35°C for 24 h. For each analyzed sample a reference sample was prepared, containing a borate buffer and an enzymatic extract. The reaction was stopped by 0.1 ml 5 M HCl and next the mixture was centrifuged at 15,000*g* for 15 min.

The size of absorbance was measured at 290 nm and the result was expressed in micromolar of cinnamic acid per gram fresh weight. Each measurement was performed in five replications.

The H_2O_2 generating system was prepared by adding 0.5 mM glucose plus 2 u ml^{-1} glucose oxidase (GOG) according to Pinto et al. (2002)

NO detection by confocal-laser scanning microscopy

Nitric oxide formation was detected using fluorescent 4,5-diaminofluorescein diacetate (DAF-2DA) (Calbiochem). The leaf sections previously treated with NO-donors and/or CPTIO were placed in 1 ml of buffer solution (10 mM Tris-HCl, pH 7.2). They were then incubated for 1 h at room temperature with 1 ml of DAF-2DA at a final concentration of 10 μM in loading buffer (10 mM Tris-HCl, pH 7.2) added from a 5 mM stock in DMSO. The incubation solutions were then eliminated and the leaf sections were washed three times with fresh loading buffer to remove excess fluorophore. After several minutes sections were affixed to the cover slip bottom of a chamber slide with silicon grease, where they remained immersed in 250 μl of fresh loading buffer.

A Zeiss Axiovert 200 M inverted microscope equipped with a confocal laser scanner (Zeiss LSM 510) was used in this study and sections were excited with the 488 line of an argon laser. Dye emissions were recorded using a 505–530 nm band-pass filter and the autofluorescence of chloroplasts was captured with a

585 nm long-pass filter. Microscope, laser and photomultiplier settings were held constant during the course of the experiment in order to obtain comparable data. Images were processed and analyzed using the Zeiss LSM 510 software.

NO detection and quantification by electrochemical method

All electrochemical measurements were performed using a universal electrochemical analyzer PGSTAT 30 (EcoChemie, Utrecht, the Netherlands) and all potential values reported below were referred versus an Ag/AgCl. NO generation in solutions was monitored by constant current amperommetry at a NO-selective electrode (Ciszewski and Milczarek 2003). The electrode was prepared by electropolymerizing a thin film of poly-eugenol on a cleaned Pt electrode. This was done by repetitive scanning of the electrode potential between −0.2 and 0.6 V in 10 mM solution of eugenol (Fluka) in 0.1 M NaOH. The as modified electrode was then conditioned by applying a constant potential of 0.9 V in phosphate buffer (pH 7.4) until a stable constant background current was reached. Both photostimulated NO release from donors and NO generation from donors in the presence of reducing species donors (GSH, leaf extracts) were monitored by recording the anodic current at the modified electrode at 0.9 V. The current was recalculated into concentration units on the basis of a calibration curve.

The calibration curve was constructed by measuring current responses due to the addition of freshly prepared NO aqueous solutions within the range of 0.05–50 μM. Reactions were carried out in a borosilicate glass reaction vessel. To provide appropriate mixing and enhance sensitivity the electrode was rotated at 2,000 rev/min. It was found that using a rotating electrode instead of a routinely used magnetic stirrer produces much lower background current noise and thus enhances the detection limit of the method. In case of the photochemical NO generation from NO donors, a 17 W fluorescent lamp was used as a polychromatic light source. Solutions were illuminated through the glass face of the reactor. The light source caused some warming up of the reaction mixture, thus the starting and final temperatures are indicated in legends to figures. Illumination intensity was measured with a light power meter to be 35 μmol photons $m^2 s^{-1}$.

Generation of NO in leaves was monitored electrochemically at a needle-type, Nafion coated Pt. The electrode was prepared as follows. The Pt needle was immersed in a commercially available 5% Nafion solution (Aldrich). After withdrawal the electrode was air-dried and then heat-treated in an oven at 60°C for 1 h. Such a modified electrode was then conditioned electrochemically by applying a constant potential of 0.9 V in phosphate buffer (pH 7.4) until a stable constant background current was reached. Since no calibration is possible in the leaf tissue, the response is given in current units.

All measurements of NO generation were performed three times and the numerical values given represent the mean values.

Results

The effect of light on half-life of NO-donors in solution

Kinetics of NO release from the most frequently applied donor solutions, i.e. SNP, SNAP and GSNO, was analyzed under continuous illumination conditions and the results are presented in Fig. 1. The above-mentioned donors showed very diverse dynamics of NO release. In the SNP solution, 2 h after turning on the light, maximum NO concentration of 6.0 μM was recorded, after which time the amount of released nitric oxide gradually decreased. Half-life for SNP was $t_{½}$ = ca.12 h. In turn, the SNAP solution almost immediately after turning on the light released five times more NO (30 μM) and half-life for this donor was assessed to be $t_{½}$ = over 3 h. In contrast to SNAP, in the GSNO solution maximum NO emission (at 12 μM) was recorded as late as 5 h after turning on the light and half-life was $t_{½}$ = ca.7 h.

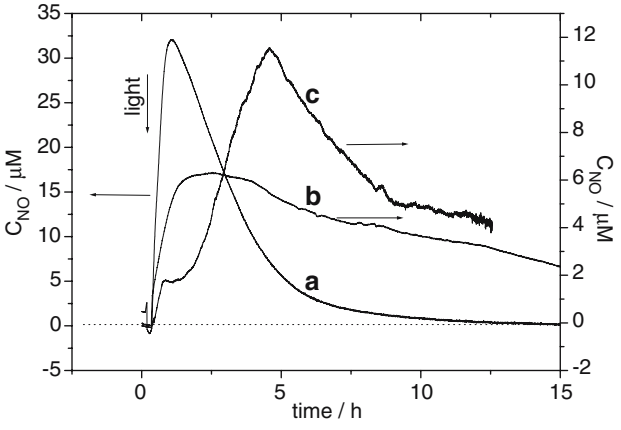

Fig. 1 *a–c* Concentration-time traces of photolytic NO generation by various NO donors under continuous illumination. *a* SNAP; *b* SNP; *c* GSNO. Conditions: donor concentration, 0.5 mM; electrolyte, phosphate buffer pH 7.4; light, polychromatic (*white*), illumination intensity 35 μmol photons $m^2 s^{-1}$; starting temperature , 20°C; final temperature, 30°C

Effect of chopped light on NO release from donors

The results obtained in the experiment with the light being alternately turned on and off (30 min light/30 min dark) confirmed that all the applied donors are photosensitive (Fig. 2a–c). Comparing the rate of decomposition of individual donors it was shown that only the SNP solution fully inhibited NO emission in the dark, while the other two donors, i.e. SNAP and GSNO, visibly reduced NO release after turning off the light.

A similar trend in changes in NO emission rate in the light and dark was found in situ in pelargonium leaf tissue previously treated with 100 μM SNP (Fig. 3).

Additionally, using a fluorescent dye DAF-2DA the NO visualization in tissue was performed, after the application of an appropriate donor to the leaf. The obtained image of NO emission from the donor, in the light and in the dark, is presented in Fig. 4a–i. Intensive green fluorescence spots detected in the epidermal cells, indicating the presence of exogenous NO, were visible for all the applied donors, i.e. SNP, SNAP and GSNO exposed to light (Fig. 4a, d and g). Leaves treated with the donor, in the dark, exhibited either a lack of NO for SNP (Fig. 4b), or a distinctly lighter coloration indicating lower NO contents for SNAP and GSNO (Fig. 4e–h). Additionally applied CPTIO scavenged NO effectively for all the donors, inhibiting or drastically reducing the intensity of green coloration of the applied indicator (Fig. 4c, f and i).

Effect of the reducing agents and leaf extracts on donor decomposition

In order to illustrate the potential effect of reducing agents on donor decomposition, NO emission was analyzed successively from SNP, SNAP and GSNO solutions in the presence of ascorbic acid and reduced glutathione (GSH).

In the dark 500 μM SNP solution did not generate measurable amounts of NO, either in the presence of ascorbic acid or GSH (data not shown). Under identical conditions donors belonging to the group of S-nitrosothiols, i.e. SNAP and GSNO, underwent a certain degree of decomposition and emitted slight amounts of NO, as it is presented in Figs. 5 and 6. After the addition to the SNAP solution (500 μM), irrespective of whether it was GSH (1 mM) or ascorbic acid (1 mM), an immediate donor decomposition was recorded, in the amount of 1.5–2.5 μM initially released NO (Fig. 5). Similarly, GSNO (500 μM) after the addition of reducing agents within a short time generated NO in the amount of 0.25–0.5 μM (Fig. 6).

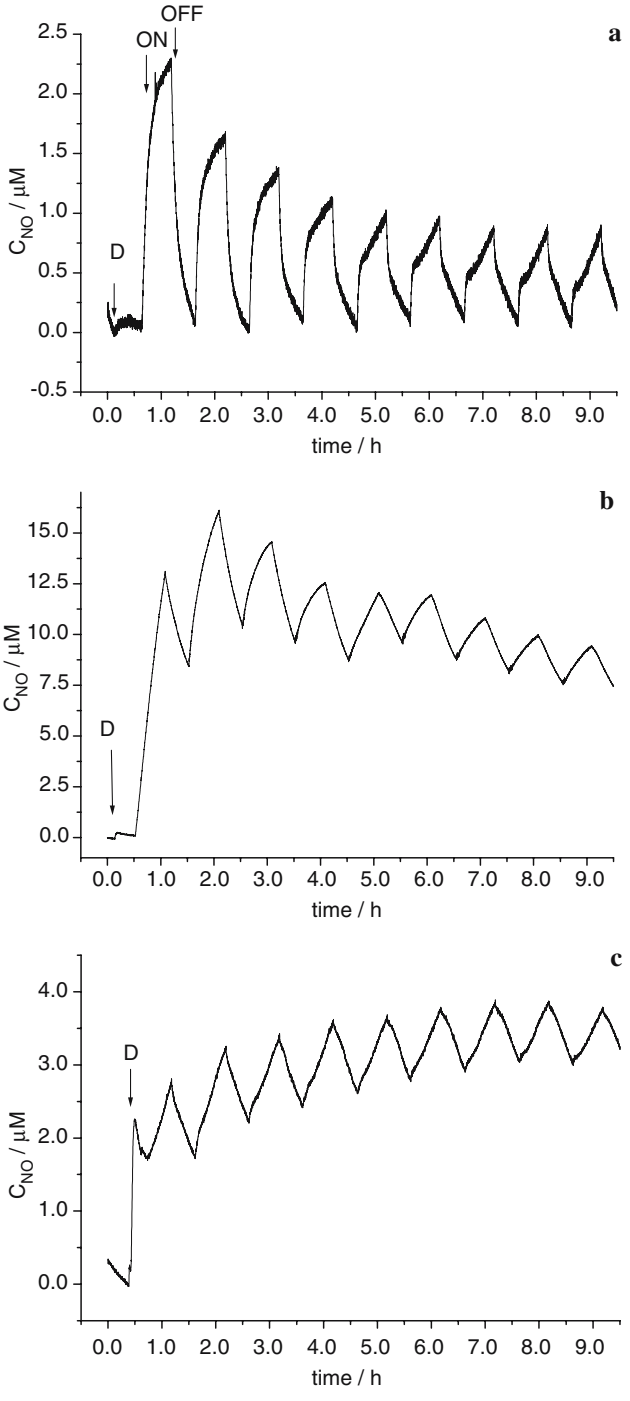

Fig. 2 **a–c** Concentration-time traces of photolytic NO generation from various NO donors under chopped light conditions. **a** SNP; **b** SNAP; **c** GSNO. Illumination and darkness intervals are 30 min in all cases. "D" indicates addition of donor and "ON" and "OFF" switching the light on and off, respectively. Other conditions as in Fig. 1

The next experiment investigated the effect of extracts obtained from pelargonium leaves on the rate of GSNO decomposition in the dark. As it was presented in Fig. 7 extracts exhibited varying NO genera-

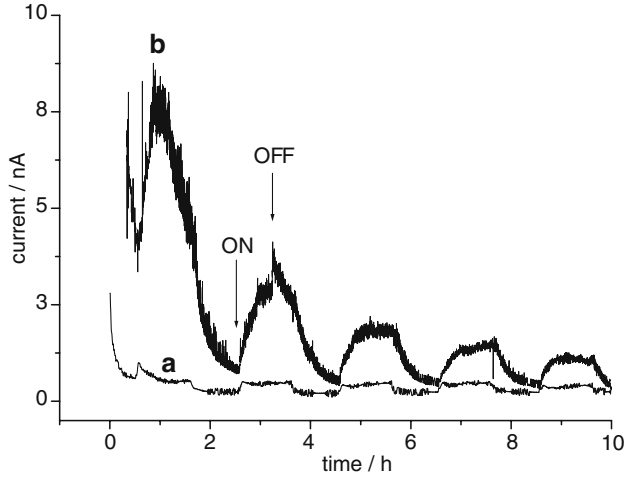

Fig. 3 *a*, *b* NO release from H_2O and SNP (0.1 mM) treated leaf tissue under changing light and dark conditions. *a* H_2O; *b* SNP. NO generation was monitored with the use of polymer modified microelectrode (for details see Material and methods)

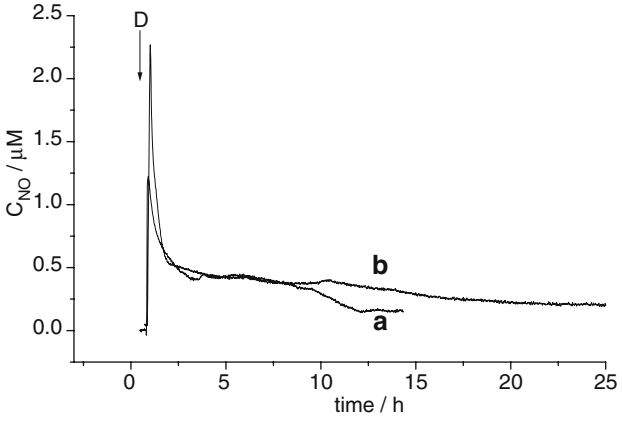

Fig. 5 *a*, *b* Concentration-time traces of chemical NO generation from SNAP in the presence of ascorbic acid and GSH as reducing species. *a* Ascorbic acid; *b* GSH. Conditions: electrolyte, phosphate buffer pH 7.4; temperature 20°C; SNAP 0.5 mM; ascorbic acid and GSH conc. 1 mM. "*D*" marks addition of donor (SNAP)

tion capacity by GSNO. The highest level of nitric oxide was recorded after the addition of the extract obtained from healthy leaves, next for leaves infected with *Botrytis cinerea* (almost three times lower), while the lowest (seven times) for leaves previously treated with SNAP. However, it needs to be stated that the level of NO generated from the GSNO solution in the

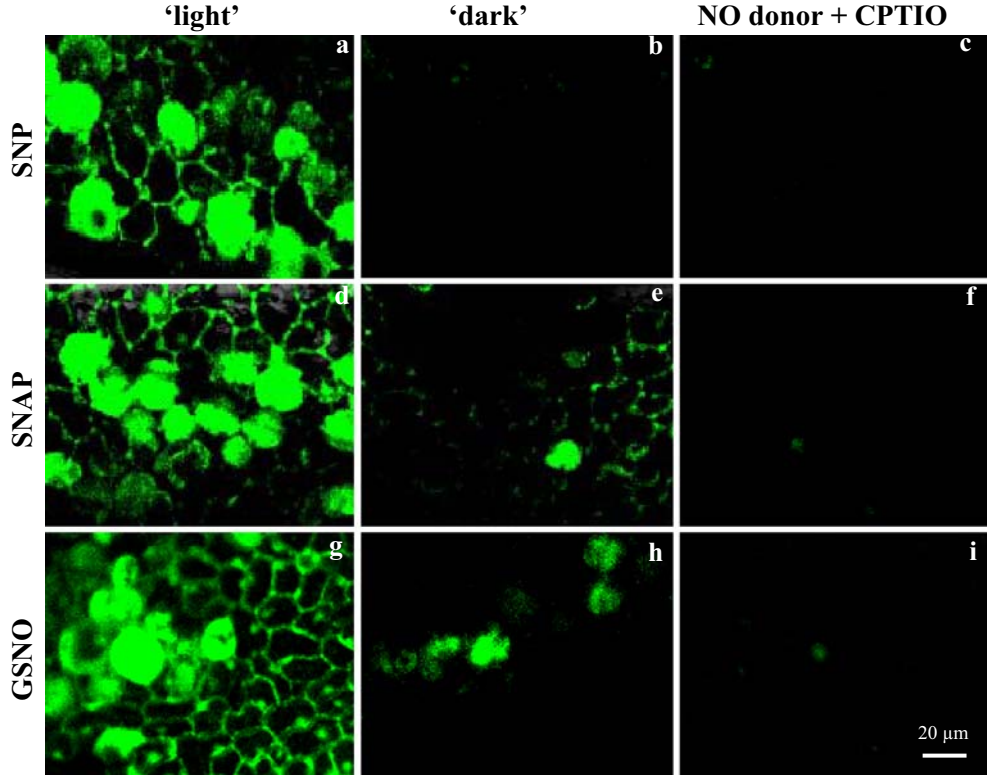

Fig. 4 **a–i** Nitric oxide (NO) visualization in pelargonium leaves by DAF-2DA, 6 h after treatment of leaf tissue with used NO donors in the light and in the dark. **a**, **d** and **g** DAF-2 DA positive (*green fluorescence*) stained for NO in the light condition was observed for each donor treatment, SNP (0.1 mM), SNAP (0.2 mM) and GSNO (0.2 mM); **b** DAF-2 DA negative for SNP in the dark; **e** and **h** DAF-2 DA slight positive for SNAP and GSNO in the dark; **c**, **f**, and **i** NO negative effects after treatment of leaf tissue with SNP, SNAP and GSNO in the light (6 h), then transferred to 200 µM CPTIO (1 h). All images are confocal-laser scanning micrographs. Magnification is identical for **a–i**, *bar* = 20 µm

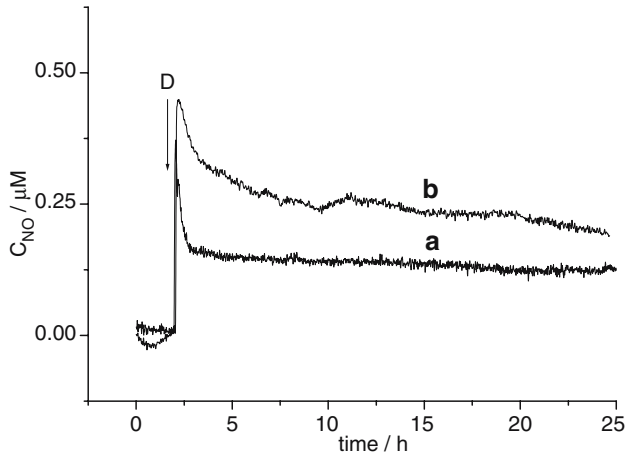

Fig. 6 *a*, *b* Concentration-time traces of chemical NO generation from GSNO in the presence of ascorbic acid and GSH as reducing species. *a* Ascorbic acid; *b* GSH. Conditions as in Fig. 5. "*D*" marks addition of donor (GSNO)

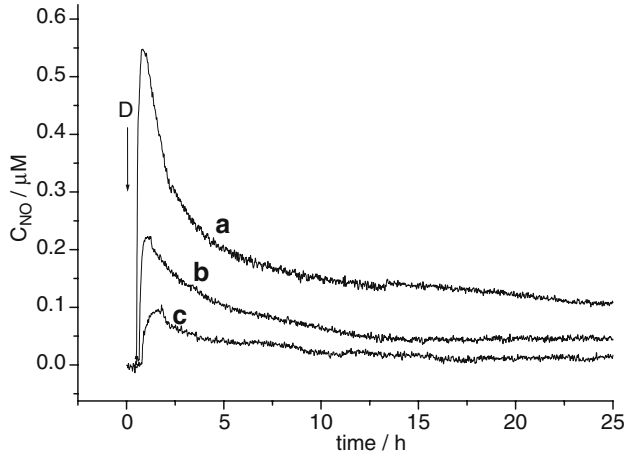

Fig. 7 *a–c* Concentration-time traces of leaf extract, stimulated NO generation from GSNO in darkness. Conditions: donor concentration, 0.5 mM; electrolyte, phosphate buffer pH 7.4; temperature 20°C; extract concentration, 50 µl in 5 ml P/K buffer. *a* Healthy leaf extract; *b* leaf extract infected with *Botrytis cinerea*; **c** extract of SNAP (0.2 mM) treated leaves (for details see Material and methods). "*D*" marks addition of donor (GSNO)

dark after the addition of the extract from healthy leaves was many times lower (over twenty times) than that of the previously reported level of NO released from GSNO in the light (Fig. 1).

PAL activity under NO and light conditions

Pelargonium leaves treated with SNP in the light showed a rapid stimulation of PAL activity starting from 1 h of tissue incubation with the donor (Fig. 8, light). During successive hours the activity continued to increase and the highest, i.e. approximately 50-fold PAL induction was observed 24 h after leaf tissue

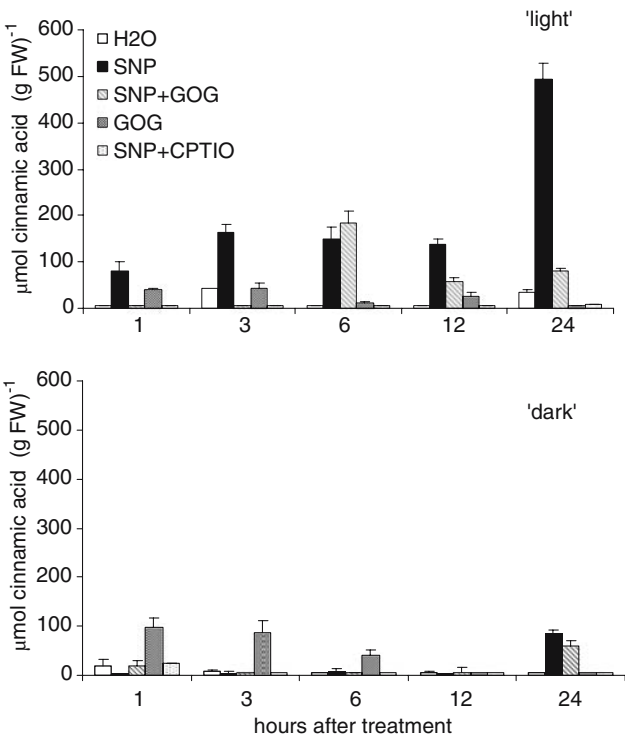

Fig. 8 The effect of exogenous NO and/or H_2O_2 on PAL activity in pelargonium leaves maintained in the light and in the dark. The levels of NO and H_2O_2 were altered in leaf discs by adding SNP (0.1 mM), and/or CPTIO (0.1 mM) and GOG (0.5 mM glucose plus glucose oxidase 2 units ml^{-1}). Values represent the mean ± SE of two independent experiments, each done in triplicate

donor treatment. In successive hours PAL activity was decreasing systematically (data not shown). Inactivation of NO by the application of CPTIO effectively inhibited the activity of the enzyme in all the analyzed times. A much lower PAL stimulation, maintained for 12 h, was found for the H_2O_2-generating system of glucose–glucose oxidase (GOG) in the light. Combined tissue treatment with SNP/GOG caused an almost 20-fold PAL stimulation at 6 h. At that time the recorded increase in enzymatic activity was slightly higher than that for SNP only and significantly lower than for GOG alone at the identical times.

In contrast to the light conditions, in the dark no PAL activation was recorded within 24 h SNP tissue treatment (Fig. 8, dark). Only exogenous H_2O_2, also at the absence of light, for the first 6 h exhibited effectiveness in the slight PAL activation.

Discussion and conclusion

All the NO donors studied were found to be photosensitive, but the effect of light was quite different for SNP

comparing to S-nitrosothiols (SNAP, GSNO). In the former case no NO release was observed in the dark. In such a case the presence of light is essential for SNP to be able to mimic effects of endogenous NO in leaf tissue. This is confirmed by the decrease of NO level to zero both in solution and in leaf blades after the light is switched off. Moreover, no PAL activation was observed for leaves SNP- treated in the dark (see below). In contrast, both SNAP and GSNO showed some NO release even in the dark which might be due to their autodecomposition promoted by room temperature (Seabra et al. 2004). Light might only be necessary to initiate the decomposition process. In either case the presence of light would only facilitate the kinetics of NO release and after switching off the light the NO level would remain relatively high for a long time.

The effect of reducing species (ascorbic acid, GSH) is also different for SNP comparing to S-nitrosothiols studied. Although, there is literature evidence that both ascorbic acid (Smith and Gupta 2001) and thiols (Aleryani et al. 1999; Grossi and D'Angelo 2005) can stimulate NO evolution from SNP, no NO release was detected in the presence of ascorbic acid or GSH under experimental conditions applied in the presented study. This inconsistency may be due to the fact that the aforementioned reports were based on experiments performed under anaerobic conditions, which in fact do not mimic the reality. In air-equilibrated samples the rate of NO autooxidation may be high enough to keep the level of released NO below the detection limit of the electrochemical method. Moreover, SNP reduction by ascorbic acid may also lead under certain conditions to nitrous oxide (N_2O) instead NO (Smith and Gupta 2001). In the case of thiol-stimmulated SNP decomposition under aerobic conditions, peroxynitrite can be formed, thus inhibiting the release of free NO (Aleryani et al. 1999). These effects may explain the observations made by Ioannidis et al. (1996), who found that SNP solutions in the absence and presence of endothelial cell suspensions emitted comparable amounts of NO and concluded that reducing species contained in cells do not promote SNP decomposition. In contrast, the two S-nitrosothiols studied emitted detectable amounts of NO in the presence of ascorbic acid, GSH and leaf extracts. However, the effectiveness of NO emission from those donors in the presence of ascorbic acid and GSH was 15–30 times lower than in the light. In turn, a higher rate of NO generation from GSNO (at the absence of light), after the addition of the extract coming from healthy tissue, in comparison to the extract from infected leaves undoubtedly is connected with varying contents of reducing metabolites present in these tissues. The extract obtained from diseased leaves contained lower amounts of antioxidants in comparison to that of healthy ones (data not shown).

So far, the mechanism of decomposition of S-nitrosothiols donors, both in plant tissue and in solution, still remains unknown, although an essential role is ascribed in this respect to reductive mechanisms. Stamler and Toone (2002) were of the opinion that under physiological conditions, apart from ascorbate, thiol and superoxide, also oxydoreductases, including glutathione peroxidase, might participate in the reduction of such donors (Hou at al. 1996). The same might be true of xanthine oxidase (Trujillo et al. 1998), thioredoxin and the glutathione–dependent formaldehyde dehydrogenase or GSNO reductase (Liu et al. 2001). Most of S-nitrosothiols, e.g. GSNO, can also undergo catalytic breakdown in the presence of transition metal ions, especially copper (Smith and Dasgupta 2000).

Recently Murgia et al. (2004) indicated the possibility of varying effects of different NO-donors releasing NO with either NO^+ (SNP) or NO (SNAP, GSNO, NOC-18) on plant metabolism. The authors cited above showed that SNP exhibited different or even opposite effects than the other tested donors.

When long-term admission of NO is necessary in studying certain response processes the stability of NO donors becomes a crucial issue. Our findings show that the stability of the studied compounds (expressed as their half-life) decreases in the order SNP > GSNO > SNAP and as a consequence peak-levels of NO in solution follow the opposite sequence. This observation is generally consistent with experimental data published so far. For instance Ioannidis et al. (1996) made an observation that under hypoxic conditions in N_2-saturated Krebs-Henseleit buffer (pH 7.4) after 15-min incubation, a two times higher rate of NO generation from 5 mM SNAP is observed, in comparison to 20 mM SNP. NO concentrations were determined spectophotometrically by the oxyhaemoglobin method. On the other hand, Conrath et al. (2004) found that the rate of NO release was five times higher with SNAP (180 μM) than it was with GSNO (180 μM). The above-mentioned authors used the MIMS/RIMS technique to quantify NO. Murgia et al. (2004) also reported a higher generation of NO_X by SNAP and GSNO than by SNP. They assessed indirectly the amount of NO released by various NO donors using the Greiss method (Privat et al. 1997).

Thus it is necessary, when applying SNAP as a NO donor in biological systems, to take into consideration the possibility of faster stimulation of metabolic response via SNAP in comparison to other donors.

Generally, it is known that a majority of evidence on the functions which may be played by NO in the plant was obtained via synthetic NO donors (Wandehenne et al. 2004). Induction of metabolic response through the application of NO to the plant and drawing conclusions on the mediatory role of endogenous NO on the basis of the observed physiological and biochemical changes may be risky. For example Murgia et al. (2004) indicated the possibility of varying effects of different NO-donors releasing NO with either NO^+ (SNP) or NO (SNAP, GSNO, NOC-18) on plant metabolism. The authors cited above showed that SNP exhibited different or even opposite effects than the other tested donors.

PAL is an important enzyme of the phenylpropanoid pathway, the activity of which, controlled by light (Duke and Naylor 1974), increases significantly under stress conditions (Hahlbrock and Scheel 1989). As it was found in this study, the rate of NO generation from SNP is especially dependent on the light. Thus as it was expected, the induction of PAL activity via NO was detected only in the light, beginning from the first hour after SNP treatment. The NO inhibitor CPTIO completely inactivated PAL. On the other hand, Durner et al. (1998) reported induction of PAL gene expression in tobacco cells via NO by SNAP and GSNO in the dark. This is consistent with our finding that for the decomposition of SNAP and GSNO light is not essential. However, one may expect that the admission of these donors in the presence of light would make the observed effects more pronounced. Guo et al. (2004) noted induction of PAL activity in wheat leaves under 0.5 and 2.0 mM SNP. It needs to be stressed that the cited authors controlled the rate of NO generation at the level of 2–4.5 μM. The application of CPTIO inhibited PAL activity.

Differently from our results, a lack of effect of SNP on PAL activity in a tobacco cell culture was observed by Pinto et al. (2002). However, those authors did not report under what light conditions their experiments were carried out and amounts of NO released from SNP were not monitored, either.

In conclusion, we would like to polemicize with the frequently cited statement that "donors are compounds that spontaneously break down to release NO" and indicate that the process of donor decomposition depends on numerous external factors. It may additionally be stimulated by a live plant cell, thus it is necessary to take these aspects into consideration and different techniques should also be used to quantify NO within tissues.

In order to overcome the deficiency of usually used donors, the current focus should be put on creating new class donors, with enhanced stability, controllable NO release and high tissue or cell specificity. The development of specific NO targeting donors would be especially valuable in the elimination of the diversity in NO effects on the plant cell and in emphasizing individual physiological functions. Relatively stable donors accumulated in the cell would exhibit enhanced NO generation only after intracellular decomposition via specific metabolites or enzymes, e.g. esterases, glycosidases or specific proteinases (Wang et al. 2002 and Wang et al. 2005). Such an approach seems to be especially promising in studies, e.g. on induced defense response of plants to pathogens.

At the same time, along with the improvement of NO donors, in situ measurement technologies of endogenous NO should be further developed, which would make it possible to follow real-time NO changes in the target plant tissue or cell. In this respect the application of the electrochemical methods seems very promising.

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