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Nitric oxide preserves the level of chlorophyll in potato leaves infected by *Phytophthora infestans*

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Abstract

Nitric oxide (NO) is a bioactive molecule involved in many physiological processes. Among its biological function, NO has been proved to be cytotoxic against microorganisms in cells of the immune response, thus preventing infection. We have specifically studied the effect of a NO donor, sodium nitroprusside (SNP), on the chlorophyll content in potato leaves infected with the pathogenic fungus *Phytophthora infestans* (*Pi*). Fifteen days after infection, chlorophyll content strongly decayed in water-treated potato leaf sections. SNP was able to partially revert that loss in a dose-dependent manner, being the effective SNP concentrations between 10 μM and 100 μM. $NaNO_2$ and $NaNO_3$, the SNP-derived residual products, were unable to prevent the chlorophyll loss. Treatments with SNP did not affect the survival of *Pi* and the fungus was able to grow in a V_8-agar medium containing 100 μM SNP. Both the amount and the extent of germination of *Pi* sporangia resulted similar in the absence and in the presence of SNP. Respiratory inhibitors of the cyanide-sensitive and cyanide-resistant pathways, 2,4-dinitrophenol and salicylhydroxamic acid respectively, did not change the chlorophyll levels in infected potato leaves, suggesting that NO effect should not be on mitochondrial respiration. These results indicate that NO could be a protective molecule, either preserving the chloroplast membrane of infected leaf sections against the toxicity of reactive oxygen species or being directly involved in any step of the chlorophyll metabolic pathway.

Abbreviations: 2,4-DNP – 2,4-dinitrophenol; NNLA – N-nitro-L-arginine; NO – Nitric oxide; NOS – Nitric Oxide Synthase; O_2^- – superoxide; $OONO^-$ – peroxynitrite; *Pi* – *Phytophthora infestans*; ROS – Reactive oxygen species; SHAM – salicylhydroxamic acid: SNP – Sodium Nitroprusside

Introduction

Nitric oxide (NO) has been implicated in several biological and physiological processes (Stamler et al., 1992). It serves as a short-lived messenger molecule involved in vasorelaxation, smooth muscle relaxation, platelet inhibition, neurotransmission, cytotoxicity and immunoregulation of pathophysiological processes (Hibbs, 1988; Ignarro, 1990; Moncada et al., 1991; Snyder, 1992; Marletta et al., 1993; Anbar, 1995).

From a biological standpoint, the best known reactions of NO are those with (di) oxygen in its various redox forms and with transition metal ions (Lepoivre et al., 1991; Stamler et al., 1992). Moreover, many of the biological effects of NO are chemically based on direct interactions with iron-containing proteins, such as guanylyl cyclase (heme iron), ribonucleotide reductase (non-heme iron) or aconitase (iron sulfur) (Drapier and Hibbs, 1988; Lepoivre et al., 1991; Schmidt et al., 1991; Weinberg, 1992). The interaction with iron sulfur clusters and heme proteins can lead to either a decrease or an increase in protein biological activities. NO can also serve a protective function by diverting superoxide (O_2^-) away from metal clusters toward

disarming sulfhydryl centers, which are targets of peroxynitrite ($OONO^-$) formed from NO and O_2^- (Stamler, 1994).

Evidence has been reported supporting the idea that NO may also be part of the functional complexity in the plant kingdom: (a) Noritake et al. (1996), have described the accumulation of potato phytoalexins by a NO-releasing compound, (b) data reported by Pfeiffer et al. (1995) suggest the presence of NO/cyclic guanylyl monophosphate (cGMP)-sensitive transduction pathway in potato, (c) western blot analysis revealed positive immunoreactivity with rabbit anti-brain Nitric Oxide Synthase (NOS) antibodies in pea embryonic axes and wheat germ (Sen and Cheema, 1995), (d) in senescing pea foliage NO emission was promoted by the addition of an ethylene precursor whereas in rapidly growing pea foliage three NO-releasing compounds inhibited expansion, suggesting a novel pathway in regulation of plant growth (Leshem and Haramaty, 1996). In animals, where the effects produced by NO have been better characterized, it has been proved to be cytotoxic against microorganisms in cells of the immune system, thus preventing infection (Moilanen and Vapaatalo, 1995). Knowing the role of NO against pathogenic agents in animal systems and its putative presence and biological activity in the plant kingdom, this work was directed to study the effect of NO during plant disease development and its putative role in preventing infection. We wished to determine if NO had any visible effect on potato leaves infected by the fungus *Phytophthora infestans* (Mont.) de Bary, the late blight disease agent. The results reported in this work show that chlorophyll levels are strongly maintained by NO in infected potato leaves.

Materials and methods

Plant and fungal material

Potato tubers (*Solanum tuberosum* L cv. Pampeana) were grown in 20 cm diameter pots in a sterile mixture of soil:vermiculite (3:1) and maintained for 15 days at 25 °C and with a 14 h photoperiod. Then, the plants were grown for other 15 days at 18 °C with the same photoperiod. Potato leaves from plants of approximately 30 days were used for experiments. The fungal material consisted in an isolate of the pathogenic fungus *Phytophthora infestans* (*Pi*) (race 1, 4, 7, 8, 10, 11, mating type A2) provided by EEA, INTA-Balcarce, Argentine. Mycelia of *Pi* were grown on V_8-agar medium and on potato tuber slices. Mycelia were harvested into sterile water and immediately placed at 4 °C for 30 min. Then, the suspension was transferred to 18 °C for additional 2–3 h. After filtration with muslin, the suspension was observed under optic microscope for the detection and quantification of sporangia. The concentration was adjusted to 10^5 sporangia/ml.

NO treatments and chlorophyll quantification

Fully expanded potato leaves were excised, midribs removed and cut into approximately 1 cm^2 pieces. Then, leaf pieces were floated in petri dishes containing water or, alternatively, solutions of SNP, N-nitro-L-arginine (NNLA) as a competitive inhibitor of NOS or SNP + NNLA, all of them in the presence or absence of 10^4 sporangia/ml of *Pi*. SNP was used at various concentrations in a range between 10^{-6} and 1 mM. The SNP used in this study has been reported to release nanomolar to micromolar amounts of NO (Noack and Feelisch, 1991). We also used a solution containing 100 μM ascorbic acid and 200 μM $NaNO_2$ as another NO donor as reported previously (Kröncke and Kolb-Bachofen, 1996). Leaf pieces were incubated for different lengths of time at 18 °C with a 14 h photoperiod; then were harvested, frozen and powdered with liquid N_2 and homogenized in 80% acetone. Chlorophyll quantification was performed by measuring $Abs_{652\,nm}$, considering that Chl (μg/ml) = 27.7 $A_{652\,nm}$ (Arnon, 1949). Similar treatments were done with a 10-days old SNP solution and with 100 μM $NaNO_2$ or 100 μM $NaNO_3$ solutions as control treatments for SNP-derived products.

Growth of Pi *and determination of sporangia germination*

Pi mycelia were used as source of inoculum on Petri dishes containing V_8-agar medium in the presence or absence of 100 μM SNP. Petri dishes were incubated for six days at 18 °C in the dark. Germination capability of *Pi* sporangia was analyzed by incubating them with water or 100 μM SNP in microslide plates. After 14 h at 18 °C, the percentage of germinated sporangia was quantified under optic microscope and a picture was taken.

Treatments with respiratory inhibitors

We used 2,4-dinitrophenol (2,4-DNP) as an inhibitor of the cytochrome-dependent respiratory pathway and salicylhydroxamic acid (SHAM) as an inhibitor of the alternative oxidase pathway. Experiments were performed as described for SNP treatments. 2,4-DNP was used at concentrations of 10^{-6}, 10^{-3}, 10^{-1} and 1 mM. SHAM was used at concentrations of 10^{-6}, 10^{-3}, 10^{-2}, 10^{-1} and 1 mM.

Results

SNP preserves the chlorophyll content in infected potato leaf sections

Taking into account the putative presence and biological roles of NO in the plant kingdom, we wished to determine if NO had any visible effect on potato leaves infected by the fungus *Phytophthora infestans* (*Pi*). With that purpose, potato leaf pieces were floated on a solution containing 100 μM SNP in the presence or absence of *Pi* as described in Methods. Fifteen days after beginning the experiment, the chlorophyll content markedly decayed in infected potato leaf pieces in absence of SNP. In contrast, it was strongly maintained by SNP in infected potato leaves. No differences were found in the absence of *Pi* between H_2O and SNP treatments (not shown). Since the effect produced by SNP on the chlorophyll amount was expected to occur in a dose-dependent manner, we tested various SNP concentrations. Figure 1 shows the chlorophyll relative content of potato leaf pieces treated with different SNP concentrations. Values were expressed as the chlorophyll relative content between SNP and control (H_2O) treatments, for both infected and non-infected leaf pieces. Figure 1 shows that, after 15 days, the chlorophyll amount in infected potato leaves maintained in water (0 μM SNP) was of 0.15 relative to non-infected ones. When they were treated with SNP, none of the lowest SNP concentration (between 1 nM and 1 μM) were able to revert the chlorophyll decay produced by *Pi*. In contrast, SNP concentrations ranging from 10 μM to 100 μM had a significative effect in maintaining the chlorophyll content, with a maximum effect at 100 μM. At this SNP concentration the chlorophyll level was nearest to that of the non-infected leaves (0.80). Greater SNP concentrations (500 μM and 1 mM) had no effect on the preservation of chlorophyll levels. Moreover, as was previously reported (Leshem, 1996), a toxic rather than a protective effect is taking place at high concentrations, as can be seen in non-infected SNP treatment. We also tested a mixed solution of $NaNO_2$ and ascorbic acid at a 2:1 ratio as another NO donor (Kröncke and Kolb-Bachofen, 1996). Table 1 indicates the chlorophyll content in treated potato leaf pieces relative to untreated (H_2O) ones, both in the absence and in the presence of *Pi*. When treating them with ascorbic acid + $NaNO_2$, chlorophyll amount reached a percentage of 79.2±9.2% in infected potato leaf pieces, very similar to the results obtained with 50 and 100 μM SNP (Figure 1). In contrast, the percentages were of 22.7±8.4% and 16.15±1.85% for ascorbic acid – and $NaNO_2$-treated leaf pieces, respectively. Thus, another NO donor was able to prevent *Pi*-mediated chlorophyll loss and this was apparently due to NO generation, since none of the reagents alone were able to produce such an effect.

Table 1. Effect of NO donors and SNP-derived residual products on chlorophyll content in *Phytophthora infestans*-infected potato leaf pieces[a]

Reagent	Chlorophyll content (% of water treatment)[b]	
	− *Pi*	+ *Pi*
100 μM SNP	105.4 ± 12.4	80.3 ± 14.1
100 μM $NaNO_2$	89.0 ± 14.1	18.1 ± 3.8
100 μM $NaNO_3$	94.5 ± 7.8	16.58 ± 5.32
100 μM ascorbic acid + 200 μM $NaNO_2$	101.3 ± 9.7	79.2 ± 9.2
100 μM ascorbic acid	92.8 ± 5.2	22.7 ± 8.4
200 μM $NaNO_2$	97.6 ± 16.3	16.15 ± 1.85

[a] Potato leaf pieces were prepared as described in Materials and methods and treated with different reagents. After 15 days of treatment, the chlorophyll was extracted and quantified as described.

[b] Values represent the means ± SE of four different experiments. 100% (water treatment) corresponds to 300 μg of chlorophyll/g FW.

To rule out a possible effect produced by SNP-derived residual products over chlorophyll content, controls with an olded SNP solution and with $NaNO_2$ or $NaNO_3$ solutions were done, maintaining the same experimental conditions. No preservation of the chlorophyll content occurred in infected potato leaves, both for the old SNP solution (not shown) and for NO_2^- and NO_3^- solutions (Table 1: 18.1±3.8% and 16.58±5.32% for $NaNO_2$ and $NaNO_3$ treatments, respectively, referred to H_2O). These results clearly indicate that the chlorophyll maintenance due to SNP treatment is dependent on NO release from fresh SNP solution.

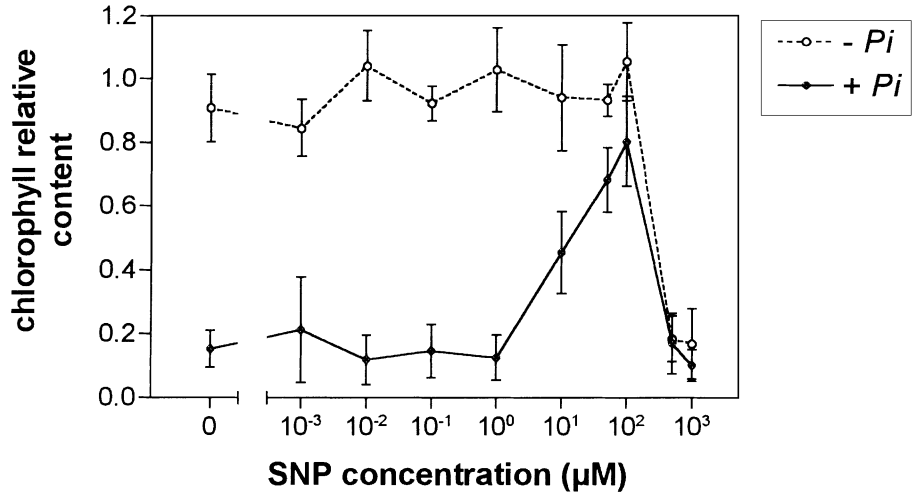


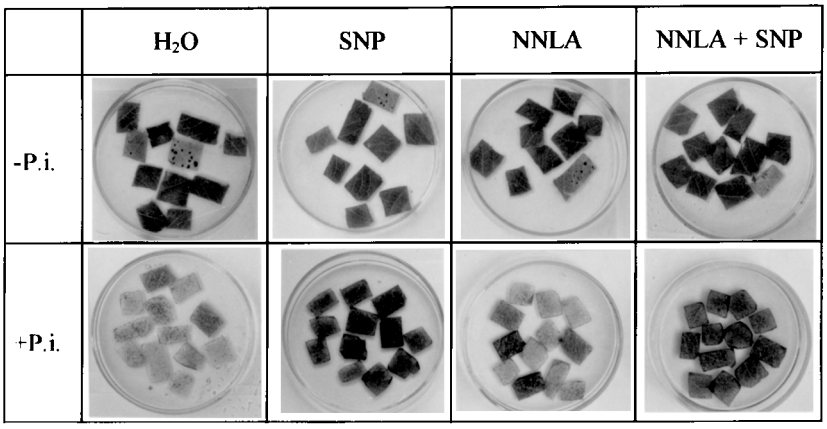
Figure 1. Dose-response curve showing the effect of sodium nitropursside (SNP) on the chlorophyll content of potato leaf pieces infected by *Phytophthora infestans* (*Pi*). Chlorophyll content values are expressed relative to H_2O treatment. ○- - -○ (– *Pi*), ●—● (+ *Pi*). Each point corresponds to the mean value of three independent experiments; bars indicate standard deviation. The *x* axis is broken in order to compare the chlorophyll relative content of *Pi* + SNP treatments with that of *Pi* treatments alone.

NO has been shown to be endogenously generated from L-arginine by Nitric Oxide Synthase (NOS) in animal systems. Positive immunoreactivity with rabbit anti-brain NOS antibodies was detected in pea embryonic axes and wheat germ (Sen and Cheema, 1995). Then, it was interesting to test if in plant systems N-nitro-L-arginine (NNLA), a NOS inhibitor, might influence the chlorophyll content in infected potato leaf pieces. Figure 2a shows the potato leaf pieces after initiating the experiment. The three different treatments (SNP, NNLA and SNP + NNLA) performed in absence of *Pi* presented similar chlorophyll content than control leaf pieces (H_2O). In contrast, the chlorophyll amount of *Pi*-inoculated leaf pieces maintained in water and in NNLA (Figure 2b) decayed to 8% and 5% of the initial values, respectively. Again, treatments with 100 μM SNP were able to largely revert this effect. The chlorophyll content in SNP-treated and infected leaf pieces was 73% of the chlorophyll present in non-infected potato leaves (Figure 2b). This effect was also observed in SNP + NNLA treatment of infected leaf pieces (64% of chlorophyll relative to non-infected leaves). Thus, NNLA, a NOS inhibitor, was not able to revert the preservation of the chlorophyll produced by exogenously-added NO. Moreover, these results could suggest that putative endogenous NOS activity, if present, was not relevant in these experimental conditions.

Has the NO donor antifungal effects?

To eliminate the possibility that the observed differences were due to an effect of SNP treatment on *Pi* infectivity, we tested the ability of *Pi*-treated leaf pieces to infect tuber slices. Potato leaf pieces from water, *Pi* and *Pi* + SNP treatments were used as source of inoculum. Figure 2c shows that SNP had no effect on fungus survival, which kept the ability to develop mycelia. Moreover, mycelia were visible on SNP-treated leaf pieces during the experiments in the same extent as in control water-treated leaf pieces (not shown). To evaluate whether *Pi* could grow in a medium supplied with SNP, *Pi* inoculum was grown on Petri dishes containing V_8-agar medium in the presence or absence of 100 μM SNP. Figure 3a shows that, 6 days after inoculation, *Pi* had already grown appreciably, both in the presence or absence of SNP. The radius of the fungus-growing halo was approximately 4.5 cm without SNP and 6 cm with 100 μM SNP. Moreover, observations under optic microscope detected similar amounts of sporangia in both conditions (not shown), suggesting that *Pi* would have a similar ability not only to grow in the presence of SNP, but also to develop reproductive structures. To obtain more solid hints for the absence of NO antifungal effects, the germination of *Pi* sporangia was quantified, showing no differences between 100 μM SNP and water treat-

a



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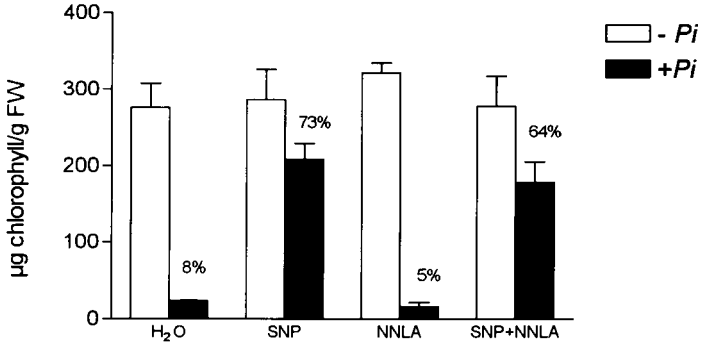

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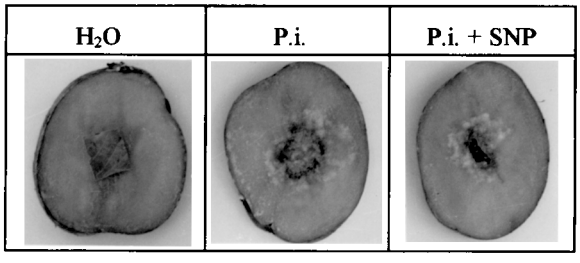

Figure 2. Effect of sodium nitroprusside (SNP) and N-nitro-L-arginine (NNLA) on potato leaf pieces in the presence or absence of *Phytophthora infestans* (*Pi*). (a) Potato leaf pieces were floated in petri dishes containing either water or a solution of 100 μM SNP, 100 μM NNLA or SNP + NNLA, in the presence (+ *Pi*) or absence (− *Pi*) of 10^4 sporangia/ml of *Pi*. Pieces were incubated for 15 days in a 14 h photoperiod at 18 °C. The picture was taken 15 days after initiating the experiment. (b) Chlorophyll quantification of potato leaf pieces. Chlorophyll content was expressed as μg of chlorophyll per gram of fresh weight. Percentages were calculated considering treatments without *Pi* (− *Pi*) as 100%. Bars indicate standard deviation of three independent experiments. (c) Fifteen days after initiating the experiment leaf pieces from H_2O, H_2O + *Pi* and SNP + *Pi* treatments (Figure 2a) were placed on potato slices in order to analyze the infective ability of *Pi*. The picture was taken 5 days after inoculation and shows the growth of *Pi* mycelia.

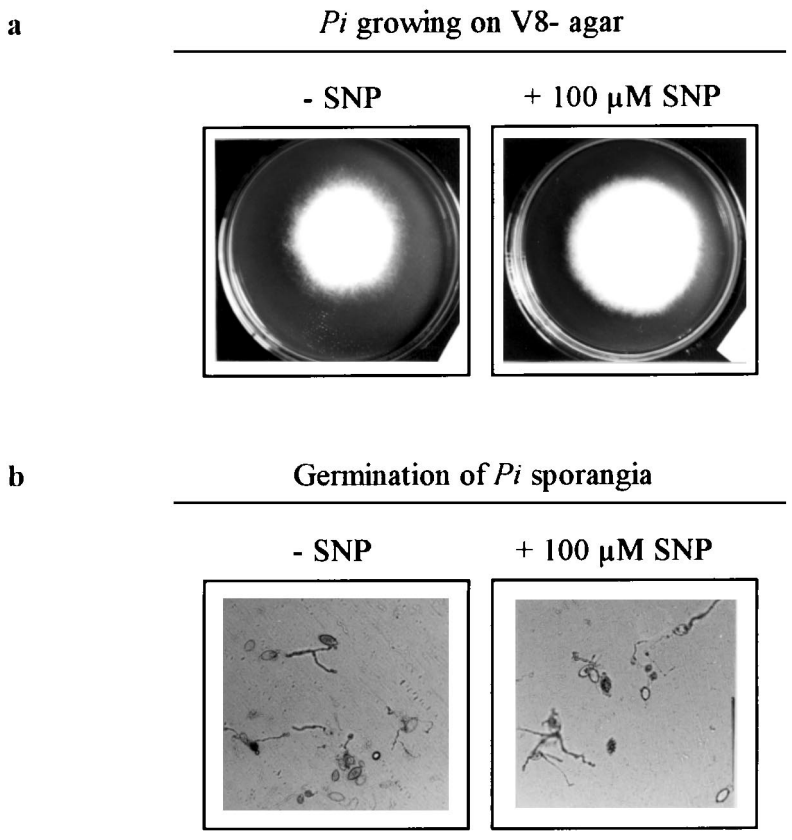

Figure 3. (a) Growth of *Phytophthora infestans* (*Pi*) in V_8-agar medium containing SNP. An inoculum of *Pi* was grown on petri dishes with V_8 medium containing 1.4% agar with or without 100 μM SNP. Petri dishes were incubated at 18 °C in the dark. The picture was taken on a 6-days old culture. (b) Germination of *Phytophthora infestans* sporangia in microslide plates. Sporangia of *Pi* were prepared at a concentration of $10^5.ml^{-1}$ and incubated in H_2O or 100 μM of SNP. After 14 h at 18 °C, the number of germinated sporangia was quantified and a representative picture of the results are shown.

ments (Figure 3b, about 60% of germination for both treatments).

Is mitochondrial respiration involved in NO effect?

Poderoso et al. (1996) and Borutaité and Brown (1996) demonstrated that NO could cause a multiple inhibition of mitochondrial electron transfer at cytochrome oxidase and at the ubiquinone-cytochrome b region of the respiratory chain by damaging iron-sulfur centers, thus inhibiting respiration in isolated rat mitochondria. On the other hand, early reports in plants (Satler and Thimann, 1983) have described that during senescence chlorophyll degradation depends on respiration. Then, it could be possible that the maintenance of chlorophyll content caused by SNP in *Pi*-infected potato leaves were due to a NO-mediated respiratory inhibition. To test this possibility, potato leaf pieces were floated on Petri dishes containing two different respiratory inhibitors. We used, 2,4-dinitrophenol (2,4-DNP) as an inhibitor of the cytochrome-dependent respiratory pathway and salicylhydroxamic acid (SHAM) as an inhibitor of the alternative oxidase. Figure 4 shows that none of the 2,4-DNP and SHAM concentrations that were tested had a significant effect on the chlorophyll content in infected potato leaf pieces. All the relative values corresponding to the chlorophyll amount in the presence of *Pi* referred to that in the absence of *Pi* were similar between inhibitor-treated and untreated potato leaf pieces.

Discussion

In the present study, we demonstrate that chlorophyll levels of infected potato leaf pieces are strongly

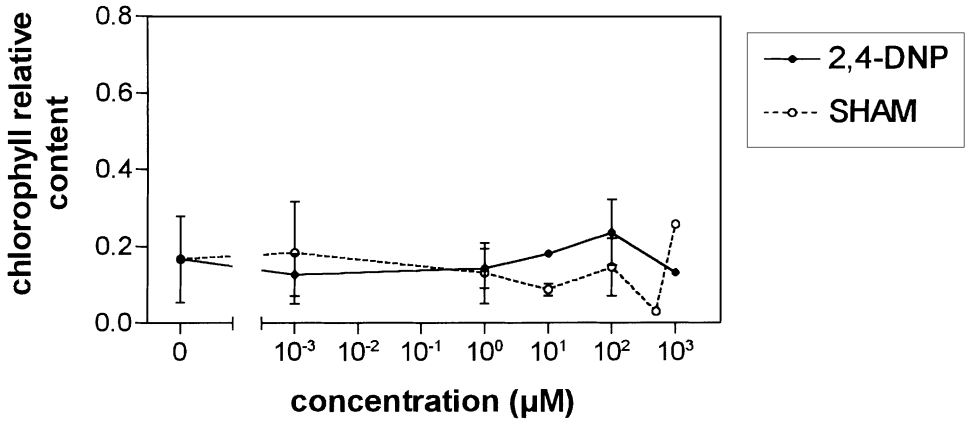

Figure 4. Dose-response curve showing the effect of respiratory inhibitors (SHAM and 2,4-DNP) over the chlorophyll content of potato leaf pieces infected with *Pi*. Chlorophyll content in inhibitor-treated leaf pieces is expressed as relative values to non-infected leaf pieces. Each point and bar correspond to the mean value and SD of three independent experiments. The gap on x axis is used in order to compare the chlorophyll relative content of *Pi* + inhibitor treatment with that of *Pi* treatment alone.

preserved by NO when supplied exogenously. The chlorophyll content was not modified by NNLA, suggesting that the participation of endogenous NOS activity, if it is present, had not enough relevance to be detected in our experimental conditions. On the other hand, it was reported that derivatives of L-arginine with NOS inhibitor activities, are not always effective in inhibiting NOS expression (Miller et al., 1996; Larkman and Jack, 1995). In addition, alternative reaction mechanisms to NOS-catalyzed NO production have been postulated (Schmidt et al., 1996; Modolell et al., 1997).

Kunert (1995) has recently described the effect of NO donors on the survival of conidia, germination and growth of *Aspergillus fumigatus*, an opportunistic fungus pathogen for animals. The report concludes that NO is probably not a major effector molecule in killing phagocytized elements of that fungus by the host's immunocytes. Accordingly to this, our experimental data showed that the survival, sporangia germination, growth and infective capability of *Pi* was not altered by the addition of the NO donor.

NO is a small, highly diffusible and short-lived molecule. It rapidly crosses biological membranes and triggers various different processes in a short period of time. In this context, NO can affect functions of microsomes and organelles such as mitochondria. NO has shown to inhibit mitochondrial function in various types of cells (Poderoso et al., 1996; Takehara et al., 1996). In *P. infestans*-infected potato leaves we found that the NO-mediated prevention of the chlorophyll loss seems not to be arrested through inhibition of mitochondrial respiration. Previous observations done by Doke and Tomiyama (1977) and Nozue et al. (1978) demonstrated that 2,4-DNP uncoupled phosphorylation in potato tubers and delayed the hypersensitive response (HR) caused by *Pi*. As this effect was reverted by ATP, the authors considered that *de novo* protein synthesis was required for HR development. Additionally, a membrane derangement of tobacco leaf chloroplasts in tissues undergoing HR has been seen (Goodman, 1972). Nevertheless, our experimental results show no relationship between uncoupled phosphorylation and chlorophyll breakdown in infected potato leaves.

It is known that during plant-pathogen interactions, different kinds of reactive oxygen species (ROS), e.g., H_2O_2, O_2^-, are produced (Khan and Wilson, 1995; Baker and Orlandi, 1995; Sutherland, 1991). In general, ROS are overproduced early in incompatible plant-pathogen interactions. However, there is evidence supporting the idea that both in incompatible, and to a lesser extent in compatible potato-*Pi* interactions, the growth and development of the fungus is restricted by rapid host cell death during the hypersensitive reaction (HR) (Schröder et al., 1992; Freytag et al., 1994). This HR is frequently triggered by a rapid and transient production of ROS (Hammond-Kosack and Jones, 1996). Moreover, early reports have supplied good evidence showing that the membrane damage that occurs during HR is due to peroxidation. However, it is not yet clear whether lipoxygenase activity, phospholipase action or direct oxidation by ROS are involved (Croft et al., 1990; Goodman and

Novacky, 1994). NO, which is itself a reactive species due to its unpaired electrons, reacts with ROS, diverting them from the medium (Stamler, 1994). Reactions of NO with target ROS may be toxic or protective, depending on the nature of the insult. In systems where toxicity is incurred predominantly from ROS, NO may act as a chain breaker and thus limit damage. This could be the case for systems involving plant-pathogen interactions. However, more evidences are required to clearly establish the interferences of NO with ROS produced during this kind of processes.

On the other hand, it is also known that NO interacts with molecules with metallo-groups, changing their stability. The chlorophyll has a Mg^{2+} in a heme group, thus being a putative NO target. Then, the observed maintenance of chlorophyll content could also be due to changes in its stability, turnover, synthesis and/or degradation rates. Furthermore, chloroplast development and anthocyanin pigment biosynthesis seem to be induced by cGMP and Ca^{2+}. Interestingly, cGMP/Ca^{2+} takes part in a NO-responsive transduction cascade in animals (Neuhaus et al., 1993; Bowler et al., 1994). In conclusion, a NO-mediated effect on chlorophyll metabolic pathway cannot be ruled out either.

Another biological effect of NO is the interaction with transcription factors to increase or reduce the transcription of specific genes (Stamler, 1994). Preliminary results in our laboratory show an increase in the levels of transcripts of 1,3-β-glucanase, phenylalanine-ammonia lyase and glyceraldehyde-3-phosphate dehydrogenase in potato leaf pieces 6 h after treatment with SNP at a concentration as low as 10^{-9} M. These enzymes are induced during infection and have been characterized as proteins related to pathogenic processes (Fritzemeier et al., 1987; Schröder et al., 1992; Stintzi et al., 1993; Laxalt et al., 1996). Therefore, NO might prevent chlorophyll breakdown by inducing plant defense-related mechanisms, thus limiting the damage caused by the infection.

It is difficult to assess whether NO-mediated chlorophyll protection is a cellular response or just a chemical reaction. Although some of the NO-dependent cellular responses reported in other cell systems may serve us for explaining our results, we cannot directly connect them with chlorophyll protection. Further investigation is being carried out in our laboratory in order to test the hypothesis formulated in the present report.

Taken together, these results strengthen the idea that suggests the existence of a NO-sensitive transduction pathway in plants, in addition to a putative protective role of NO in plant defense responses against pathogenic microorganisms. It will be useful, in the future, to transform plants with a NOS gene under a regulated promoter and study the expression of defense responses under different biotic stress conditions.

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