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Impact of Physiological Stresses on Nitric Oxide Formation by Green Alga, *Scenedesmus obliquus*

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Abstract The rate of apparent nitric oxide (NO) release, as measured in the exhaust gas of green alga, *Scenedesmus obliquus*, depended on the light intensity and pH. It doubled after lowering the temperature from 25°C to 15°C and strongly decreased from 35°C to 42°C. The *Scenedesmus* cells, deficient in nitrogen or phosphorus, demonstrated a significant increase in NO production following their transfer to nitrate- and phosphate-rich media. The addition of herbicides (DCMU and glyphosate) or toxic concentrations of Cu^{2+} or Fe^{3+} produced strong NO peaks, resembling those that occurred after sudden darkening. An increase in the Ni^{2+} concentration to 20 ppm resulted in a gradual increase of NO release from the initial ~1.5 ppbv to >20 ppbv, whereas Cd^{2+} instantaneously suppressed the NO production. Presumably, the nitrite-dependent release of NO by the cultures of *Scenedesmus* was not altered by L-NNA, an inhibitor of nitric oxide synthase (NOS), or by its substrate, L-arginine. This seems to exclude the role of NOS in the NO formation under study. Accordingly, it can be assumed that the rate of NO formation is mainly a function of dynamic nitrite pool sizes and environmental factors significantly affect the NO production in algae.

Key words: *Scenedesmus*, NO production, nitrite, temperature, heavy metals, herbicides

The current strong interest in the biochemical signal transductions that are triggered by NO has now also reached to the plant sciences [4]. Like animals, where NO is generated by the conversion of L-arginine into L-citruline +NO, catalyzed by NO synthase (NOS), plants are also found to harbor NOS activity [2, 4, 22]. NO is a free-radical molecule formed endogenously in many biological systems, where it plays a variety of physiological roles [27]. NO can also be generated in tissues by either direct disproportion or a reduction of nitrite to NO under acidic or highly reduced conditions [34]. More interestingly, healthy nitrate-nourished higher plants always emit some NO, at least in the light, the measured rates being correlated with irradiance and photosynthesis [31].

Turning to microalgal suspensions as model systems where NO is stripped from the aqueous phase by a continuous gas stream, Rai *et al.* [23] identified a close relationship between nitrite content and NO release. Furthermore, Mallick *et al.* [18], employing 2,4-DNP, antimycin A, and rotenone, demonstrated a close linkage between nitrite accumulation and NO production. Their additional experiments with the conversion of active molybdenum-nitrate reductase (Mo-NR) into inactive tungsten-nitrate reductase (W-NR) failed to produce NO either in the light or the common 'light off' peak. Since the addition of nitrite to a W-NR-containing system resumed NO synthesis, the enzymatic role of active Mo-NR in the formation of NO in *Scenedesmus* was ruled out [18]. In total contrast to this, Dean and Harper [3], on the basis of their study on *Phaseolus*, a tribe belonging to the family leguminosae, showed the involvement of constitutive nitrate reductase in NO production. After a gap of almost one decade, Yamasaki *et al.* [33] through a recent communication offered support for the earlier work of Dean and Harper [3], and clearly demonstrated NR-dependent NO production using a maize plant. Although these investigations have undoubtedly had tremendous bearing for plant and environmental scientists, currently there is a more pressing need to explore whether other plants use NR for NO production.

Algae constitute a major group of primary producers and account for over half of the fixed total annual carbon [9]. In view of their NO formation potential [18, 23] and enormous biomass in aquatic ecosystems, the possible contribution of algae to the global NO budget cannot be overlooked. It is worth emphasizing that algal proliferation in aquatic habitats is regulated by a multitude of environmental variables, also known as environmental resources, which

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can be either both physical or chemical. The most widely acclaimed factors include light intensity, temperature, pH, availability of nutrients (especially nitrogen and phosphorus), and toxicants like heavy metals and pesticides. Any attempt to project the contribution of algae to the NO budget of the biosphere would be futile if environmental variables are not given due consideration. Accordingly, this study was undertaken to provide a first hand information on NO production by a chlorophycean alga, *Scenedesmus obliquus*, under varied environmental and physico-chemical stresses. An attempt was also made to determine if NOS is involved in algal NO formation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The experiments were conducted with axenic cultures of *Scenedesmus obliquus* (Turpin) Brebisson, SAG strain 276-3a [6]. If not stated otherwise, the cultivation in a nutrient solution N11 (please refer to Mallick *et al.* (1999) for detailed composition) at 25°C and experimental conditions were the same as described by Mallick *et al.* [18]. The thermostatized culture flasks (5 l stirred liquid volume) were continuously illuminated each with one or two high-pressure mercury vapor lamps (Osram HQI, 400 W), yielding 1,200 or 2,200 μE m^{-2} sec^{-1}, respectively, in the center of the empty culture vessel. The photolysis of nitrite by unfiltered light was reduced by 90% by placing two 3-mm polymethacrylate sheets (Type GS 303 DIN 4102, Röhm GmbH, Darmstadt, Germany) as cut-off filters (transmission >410 nm) between the lamps and the illuminated algal cultures.

To avoid any carryover of nitrate or nitrite from previous incubation media to newly started experimental batches, the algal suspensions were always precipitated in a vacuum on 0.2 μm Millipore filters, washed with sterile Ringer's solution under a cleanbench, and then reincubated in fresh culture solution at an initial optical density (O.D.) of 0.14± 0.01 at 540 nm. Under standard conditions, the O.D. increased to about 0.56 within 24 h. In order to determine the N and P deficiency in the test alga, *Scenedesmus* cells were grown separately in a N- or P-deficient medium for 10 days. These cells were then harvested, washed, and used for further experiments.

Chemical Analysis

The nitrite concentration in the culture medium was determined at 1 min intervals using a particle-free culture filtrate obtained from a continuous filtration unit by means of flow-injection analysis [7]. For measuring the gaseous NO, ambient air was purified by charcoal and $KMnO_4$ (Purafil, Dosaville, U.S.A.) and then analyzed for NO and NO_2 at the gas inlet and outlet of the algal cultures using a chemoluminiscence detection instrument (Tecan CLD 770 AL PT, Ecophysics, Münich, Germany) as described by Neubert *et al.* [21].

The amounts of NO in the gas phase are given as mixing ratios and expressed as parts per billion per volume (ppbv). The background mixing ratios of both NO and NO_2 were always below 0.1 ppbv. A NO mixing ratio of 1.0 ppb is equivalent to 1.25 μg m^{-3}, since at 25°C with a normal air pressure, 100 ppb of NO corresponds to about 125 μg NO m^{-3} or 4×10^{-6} mol m^{-3}. Considering a gas flow of 180 l h^{-1}, an NO mixing ratio of 1.0 ppbv represented a momentary NO emission of 3.75 ng min^{-1} from the total suspension volume under study.

All the experiments were repeated in triplicate to confirm reproduciblity.

RESULTS

The filtrates of the nitrate-grown cultures of *Scenedesmus* always contained nitrite in the order of 10 μM (data not shown). Due to the cut-off of most of the light <410 nm, the rates of NO release became at least one order of magnitude lower than previously reported [23] and were also dependent on the light intensity (see Table 1 and Fig. 1 inset). The same held true for the height of the characteristic 'light-off' peak that always occurred upon darkening (Fig. 1). A light-intensity-dependent rise in NR activity was also observed (data not shown).

Table 1. NO production potential of *Scenedesmus obliquus* under varied conditions of light intensity, pH, and temperature.

Treatment	NO release rate (mol 10^{-8} NO g^{-1} dry biomass h^{-1})	Time elapse after change (h)	"light off" peak height (ppb)
Light intensity (μE m^{-2} sec^{-1})			
1,200	1.8±0.05	4.0	38.8±1.3
2,200	3.0±0.08	4.0	54.2±1.2
pH			
5.0	0.6±0.04	0.5	6.3±0.9
6.0	1.6±0.04	1.0	16.5±1.1
6.8	3.0±0.06	control	55.3±1.3
8.0	2.0±0.03	1.0	31.8±1.1
9.0	1.4±0.04	1.0	18.2±1.2
Temperature (°C)			
15	5.9±0.05	1.0	113.7±1.5
25	3.1±0.03	control	52.5±1.3
35	1.1±0.04	1.5	7.8±1.1
42	0.3±0.03	1.5	-

't' significant at P<0.05.

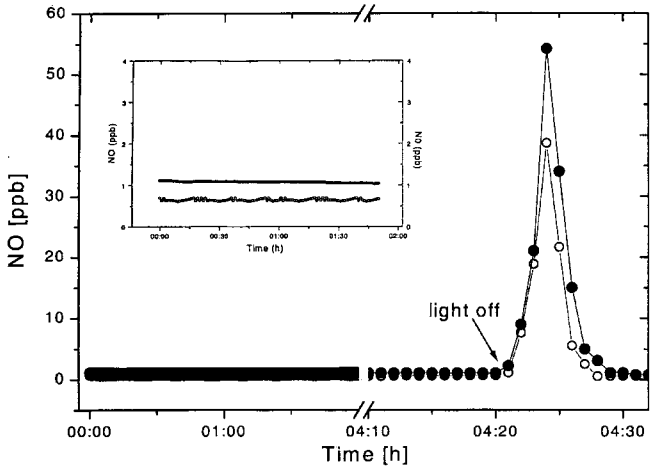

Fig. 1. NO mixing ratio in effluent gas from *Scenedesmus* suspensions receiving light intensity of 1,200 μE m^{-2} sec^{-1} (open circles) or 2,200 μE m^{-2} sec^{-1} (black dots).
Darkening after 4.20 h produced light-off peaks (inset shows the kinetics of NO release).

Figure 2 presents data on the external nitrite concentration during the light-dark transition. In contrast to the findings of Strotmann [28], no sudden rise in the external nitrite pool was observed upon the darkening of the culture suspension. Instead, the rise followed the normal trend and leveled off approximately after 15 min of darkening.

For studying the pH effects on NO production by algal suspensions, appropriate amounts of hydrochloric acid or sodium hydroxide were added to the medium fitted with a pH electrode. After changing the pH value, new approximate steady states were reached after 1 h (0.5 h only for pH 5.0). Thereafter, the NO release rates and heights of the 'light-off' peaks were measured (Table 1). It was interesting to note that any substantial deviation from the standard pH (pH 6.8) decreased the NO production by the algae; finally, the NO release was almost completely suppressed at pH 5.0.

Temperature stress affected the mixing ratio of NO in the exhaust gas more strongly than pH and light intensity

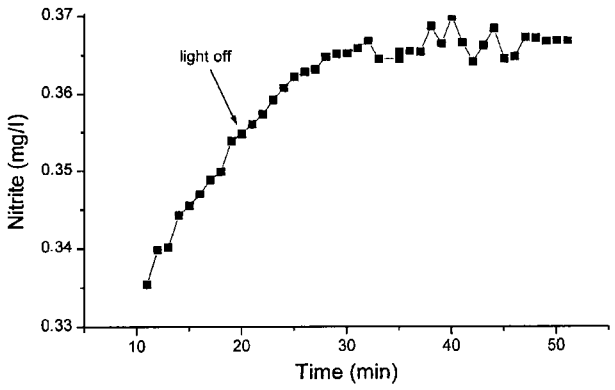

Fig. 2. Changes of nitrite concentration in culture solution in light (2,200 μE m^{-2} sec^{-1}) and after sudden darkening, i.e. during the "light-off" peak.

Table 2. NO release by N- and P-deficient cells of *Scenedesmus* under light (2,200 μE m^{-2} s^{-1}), 10 h after transfer to medium containing nitrogen and phosphorus.

Treatment	Rate of NO release (mol 10^{-8} g^{-1} dry biomass h^{-1})
Control	3.1±0.08
N-starved	7.9±0.09
P-starved	10.8±0.10

(Table 1). In the control (25°C), the mixing ratio amounted to 3.1×10^{-8} mol NO per gram dry biomass per hour and was twice as high ($P<0.05$) 1 h after lowering the temperature to 15°C. Almost the same applied to the size of the "light-off" peak. A shift from 25°C to the supraoptimal temperature of 35°C resulted in a significant decrease of NO production which became almost negligible at 42°C.

Phosphorus-deficient cells, after 10 h of their transfer into a P-containing medium (where no additional nitrite accumulation occurred), released NO at a 3.6 times higher rate than in the nondeficient control (Table 2), namely 10.8×10^{-8} mol NO g^{-1} dry biomass h^{-1}. After 16 h the NO emission started to slow down and finally reached the control value (3×10^{-8} mol NO g^{-1} h^{-1}) after 48 h (data not shown). Nitrogen-deficient cells, on transferring to a N-containing medium, also depicted a significant increase in NO production (Table 2).

The addition of DCMU, an inhibitor of photosynthetic electron transport, instantaneously produced concentration-dependent NO peaks (Fig. 3). Surprisingly, a similar fast effect was obtained with glyphosate, an inhibitor of the enzyme EPSP synthetase in the shikimate pathway (Fig. 4). On a molar basis, however, DCMU was far more effective than glyphosate. In the tested range of herbicide concentrations, the heights of the NO peaks increased more or less exponentially with the herbicide dosage. The

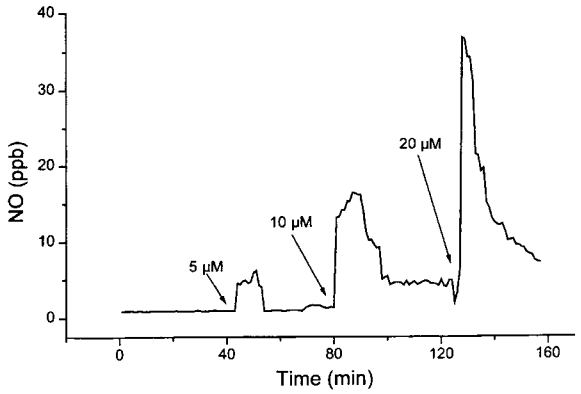

Fig. 3. Stimulation of apparent NO release by various concentrations of DCMU, an inhibitor of photosynthetic electron transport, added at times indicated by the virtual intercept of arrows with the curve. Standard conditions: 2,200 μE m^{-2} sec^{-1}.

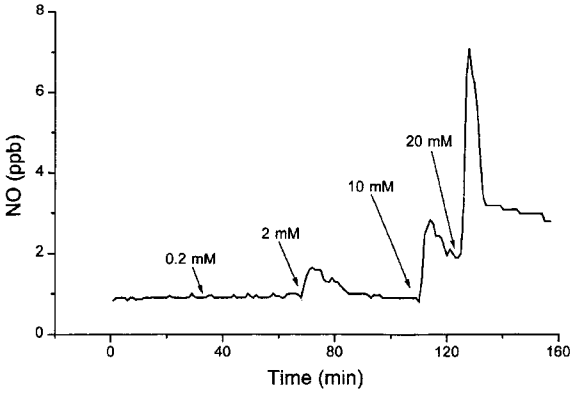

Fig. 4. Effect of inhibitor glyphosate on apparent NO release. Otherwise as in Fig. 3.

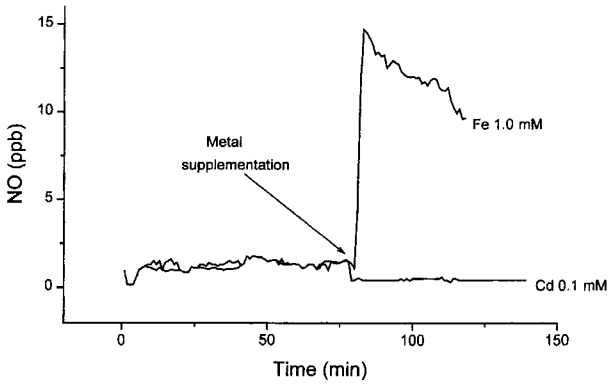

Fig. 6. Apparent NO release before and after spiking medium with Cd^{2+} (10 μM) or Fe^{3+} (1 mM). Otherwise as in Fig. 3.

half-life-times of NO peaks were 5.2 min, 8.4 min, and >15 min after spiking with 5 μM, 10 μM, and 20 μM of DCMU, respectively.

Considering the high sensitivity of planktonic algae to some toxic metals [17], it was assumed that these chemicals might also affect NO production. Treatment of the *Scenedesmus* suspensions with 10 μM of Cu^{2+} produced a sudden NO peak, which then decayed slowly after about 10 min (Fig. 5). At 10 μM, the Ni^{2+} did not cause a significant change in NO release (data not shown), whereas it induced a gradual and persistent rise at 20 μM (Fig. 5). In contrast, Cd^{2+} instantaneously stopped any NO release (Fig. 6), while 1 mM Fe^{3+} caused a strong and rather slowly decaying NO peak. Addition of Cd^{2+} 10 min after spiking the algal suspension with 1 mM Fe^{3+} was found to have a drastic effect on the decaying NO peak, which reached the basal level 3 min after Cd supplementation, and a complete suppression of NO release was noticed after 5 min (data not shown). In the absence of the test alga, Cd^{2+} did not affect photolytic NO release from dilute nitrite solutions.

To verify the possible involvement of NOS in NO production by the test alga, several experiments were conducted. The addition of L-NNA, a potent inhibitor of NOS, at concentrations of 10 μM, 100 μM, and 1 mM had no effect on the plateau level of NO production in the light or the kinetics of the "light-off" peak (Fig. 7a, effect of 1 mM L-NNA only shown). Furthermore, L-NNA did not interfere with the effects of toxic substances like Cu (Fig. 7b), DCMU, and glyphosate (data not shown). Although a better growth of the alga was observed in an L-arginine-supplemented medium than in an N11 medium, it still failed to produce NO or the most common "light-off" peak (data not shown).

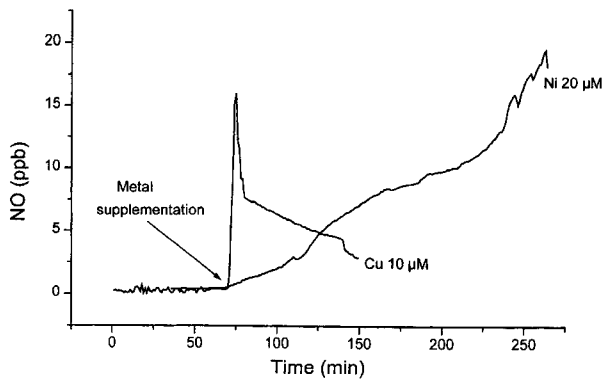

Fig. 5. Impact of spiking medium with copper ions (10 μM) or nickel ions (20 μM) on apparent NO release. Otherwise as in Fig. 3.

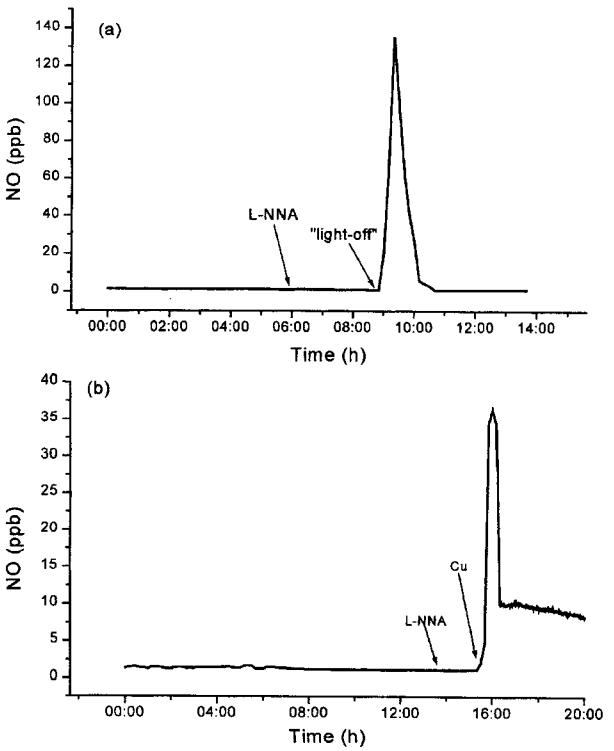

Fig. 7. (a) Effect of L-NNA (1 mM) on nitric oxide production and "light-off" peak of green alga, *Scenedesmus*. (b) Impact of L-NNA (1 mM) on Cu-induced NO production by *Scenedesmus*.

DISCUSSION

Nitric oxide has been a subject of immense interest to environmental scientists due to its dual role in catalytic production and destruction of ozone. As a short-lived trace gas, NO below the concentration of 8 ppb catalyzes the destruction of ozone, whereas above 8 ppb it catalyzes its formation and accumulation [32]. Besides this, the physiological role of NO remains a topic of extreme importance as numerous studies have implicated the role of NO in a wide array of important physiological events. For example, NO has been found to play a role in the vascular system, in the central and peripheral nervous systems, and in immune system response. Moncada *et al.* [19] and Nathan [20] provided comprehensive reviews on the multifarious role of NO in biological systems.

Although the formation of NO by nitrate-grown cultures of *Scenedesmus* was found to be a common attribute, its rate was highly dependent on the intensity of light (Table 1, Fig.1). Keeping in mind that NO formation is linked with nitrite accumulation [18, 24], the development of a light-intensity-dependent NO peak indicates a close integration of photosynthesis and nitrogen metabolism [12]. Light has been demonstrated to stimulate NR activity in higher plants [15] as well as in the cyanobacterium, *Anabaena doliolum* [25]. It enhances the synthesis of NR *via* the development of an active protein synthesizing apparatus. At a molecular level, light has also been shown to induce both NRP and NR mRNA [16]. Thus, the enhanced activity of NR under an optimal light intensity (data not shown) may be the cause of increased nitrite production *vis-a-vis* the high rate of NO formation. The lack of an instantaneous increase in the external nitrite concentration, as well as the non-occurrence of a common 'light-off' peak for nitrite following the darkening of the algal suspension (Fig. 2), indicates a need for further studies on the measurement of intracellular nitrite concentrations.

The lower NO production by *Scenedesmus* cells under pHs of 5.0, 6.0, 8.0, and 9.0, as compared to a pH of 6.8, may be due to the reduced activity of NR (thereby reducing the accumulation of nitrite), which has a pH optima of 7.5 [8]. The severe decline in NO formation at pH 5.0 (Table 1) could be ascribed to the known sensitivity of NR to an acidic pH [24].

Although the exact mechanism is still undetermined, interesting results were obtained when the test alga was subjected to varied temperatures (Table 1). The significant reduction in NO production as well as the complete elimination of the NO peak at 42°C could be due to the higher temperature sensitivity of NR than NiR [13]. In such a situation, NR may fail to reduce the nitrate into nitrite, thereby resulting in a decreased NO production. However, the two-fold rise in NO production at 15°C, as compared to 25°C, could be due to a significant decrease of growth [6], and hence a reduction of protein synthesis and amino acid production plus its utilization. This may result in still higher pool sizes of nitrite and reductant, which would then account for the remarkable increase of NO release.

Recovery from mineral deficiency following the transfer of N- or P-deficient cells to an N- and P-rich medium was accompanied by stimulated NO production (Table 2). Considering the high energy requirement for nitrite reduction [30] and the low energy status in phosphate-starved *Scenedesmus* [29], a more severe decrease in the nitrite reduction rate than in the nitrate reduction would seem quite likely (data not shown). Because of the frequent occurrence of nutrient deficiencies in nature, these aspects with regard to the ecological fluxes of NO deserve further studies.

In algal systems, the evolution of NO has been reported to be linked with the accumulation of nitrite [18]. It is well known that photosynthetic inhibitor herbicides interfere with the electron flow within chloroplast and block nitrite reduction without affecting the reduction of nitrate [1]. This differential inhibition leads to nitrite accumulation in the cell [14]. The appearance of an instantaneous NO peak following the supplementation of DCMU (Fig. 3) provides evidence to support our earlier report [18] on nitrite-dependent NO production in *Scenedesmus* and *Anabaena*. The appearance of a NO peak following the supplementation of the herbicide glyphosate (Fig. 4) was also noticed, although at a concentration far greater than that of DCMU. Glyphosate is a potent inhibitor of the enzyme enolpyruvyl shikimate phosphate synthase. It apparently binds at the active site in place of PEP, thereby blocking the synthesis of the amino acids of the shikimate pathway. This result suggests that any inhibition to reduce the utilization of nitrite products will lead to a build up of nitrite and hence an increased NO formation.

Exposure to toxic concentrations of Cu^{2+}, Ni^{2+}, and Fe^{3+} produced strong NO bursts (Figs. 5 and 6). Although the inhibition caused by these minerals may have occurred in various ways, interference with the photosynthetic electron transport would seem the most likely [17]. In contrast, an immediate and complete suppression of NO release by Cd^{2+} may conceivably be due to the inhibition of NR, which contains functional sulfhydryl groups [26] with a high affinity for Cd^{2+} [11].

Some interesting occurrence such as NO formation in tobacco and soybean plants after viral infection, the induction of defence genes in cell cultures involving the same signal transduction cascade (*via* cGMP) as in mammals, and stimulation and suppression of these responses by arginine L-NNA and also by other NOS inhibitors led Delledonne *et al.* [4] and Durner *et al.* [5] to link NO production to the activation of NOS. It is, nevertheless, worth mentioning that NOS has not yet been purified from plants [10].

However, in the present investigation (see Fig. 7a) the presence of L-NNA did not interfere in the NO production or the development of the "light-off" peak by the test alga. The effects of toxic substances like Cu (Fig. 7b), DCMU, and glyphosate were also unaltered in the presence of L-NNA. These results eliminate a possible involvement of NOS in nitric oxide production in the current test organism. Nevertheless, the complete suppression of NO production plus the absence of the most common "light-off" peak in the L-arginine-grown alga gave further support to the non-participation of NOS in NO production in the chlorophycean alga, *Scenedesmus*. In conclusion, nitrite-dependent NO release by *Scenedesmus* is strongly influenced by both environmental factors and a number of toxic chemicals.

Acknowledgments

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