Reversible inhibition of photophosphorylation in chloroplasts by nitric oxide

Shunichi Takahashi[a], Hideo Yamasaki[a,b,*]

[a] *Laboratory of Cell and Functional Biology, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*
[b] *Division of Functional Genomics, Center of Molecular Biosciences (COMB), University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan*

Received 13 December 2001; accepted 13 December 2001

First published online 18 January 2002

Edited by Barry Halliwell

Abstract Nitric oxide (NO) is a bioactive molecule involved in diverse physiological functions in plants. Here we demonstrate that NO is capable of regulating the activity of photophosphorylation in chloroplasts. The electron transport activity in photosystem II determined from chlorophyll *a* fluorescence was inhibited by NO. NO also inhibited light-induced ΔpH formation across the thylakoid membrane. High concentrations of nitrite and nitrate did not show such inhibitory effects, suggesting that the inhibition is not due to uncoupling effects of the oxidized products of NO. ATP synthesis activity upon illumination was severely inhibited by NO (IC_{50} = 0.7 μM). The inhibition was found to be temporary and the activity was completely recovered by removing NO. Bovine hemoglobin and bicarbonate were effective in preventing NO-dependent inhibition of photophosphorylation. These results indicate that NO is a reversible inhibitor of photosynthetic ATP synthesis.

Key words: ATP synthesis; Chloroplast; Electron transport; Nitric oxide; Photosynthesis

1. Introduction

Nitric oxide (NO), which can be produced from L-arginine by the enzyme nitric oxide synthase (NOS), is involved in the reactions of a wide variety of physiological functions in mammalian systems [1,2]. One of the important reactions of NO in biology is interaction with metal complexes [1]. Because NO possesses a high affinity to transition metals to form metal–nitrosyl complexes [1], proteins containing transition metal ions in either heme or non-heme complexes can be the targets for NO [1]. Guanylate cyclase [3], cytochrome P450 [4], lipoxygenase [5] and cyclooxygenase [5] are reported as the relevant targets of NO in biological systems (e.g. NO activates guanylate cyclase to produce the second messenger cGMP via formation of Fe–nitrosyl complex [3]). It is now evident in animal systems that NO is involved in the 'switching mechanism' of metabolic cascades through activation and inactivation of a broad spectrum of enzymes [1,2].

The mitochondrial respiratory electron transport chain includes abundant proteins containing transition metals such as heme, thereby being a major target of NO in animal cells [1]. In fact, NO inhibits the ATP synthesis (oxidative phosphorylation) in both animal [6] and plant [7] mitochondria. Before the discovery of NOS in animal systems, NO had been considered a respiratory inhibitor produced through air pollution [8]. However, our recognition of NO has changed from simply an inhibitor to a regulator of oxidative phosphorylation because inhibition by NO is temporary and the activity can be completely recovered by removal of NO [1].

In addition to mitochondria, chloroplasts are important organelles for energy transduction in plants (photophosphorylation). Although air pollution studies have suggested that NO may reduce the activity of net photosynthesis [9,10], the effects of NO on photophosphorylation in chloroplasts have not been addressed to date. The aim of this study was to directly examine the effects of NO on the energy transduction system in chloroplast thylakoids. Here we demonstrate that NO is capable of inhibiting electron transport, ΔpH formation and photophosphorylation in a manner of reversible inhibition. In terms of co-regulation of nitrogen and carbon assimilation, we discuss a possible physiological significance of NO-dependent inhibition.

2. Materials and methods

Thylakoid membranes were prepared from spinach leaves according to a previous method [11] with slight modifications. Leaves (20 g) were homogenized with a Waring blender for 20 s in a grinding buffer (200 ml) that contained 0.3 M sorbitol, 50 mM Tricine–KOH (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$ and 5 mM sodium L-ascorbate. The homogenate was filtered through eight layers of cheesecloth. The filtrate was centrifuged at 500×*g* for 3 min and then the supernatant was centrifuged at 2000×*g* for 10 min. The precipitate obtained was suspended in a suspension buffer that contained 0.3 M sorbitol, 20 mM Tricine–KOH (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA and 1.5 mM K_2HPO_4. The suspension was centrifuged again at 2000×*g* for 10 min to wash the thylakoids. The pellet was resuspended in the suspension buffer and used as the thylakoid preparation.

Maximum efficiency of photosystem II (PS II) (F_v/F_m) and electron transport rate (ETR) were measured with a PAM chlorophyll (Chl) *a* fluorometer (Diving-PAM/B, Heinz-Walz, Effeltrich, Germany) [12]. Dark-adapted thylakoids were suspended at 20 μmol ml^{-1} Chl in a reaction mixture (3 ml) that contained 0.1 M sucrose, 10 mM $NaCl_2$, 0.1 M HEPES–KOH (pH 8.0), 50 μM methylviologen, 50 μM ATP and 30 mM L-ascorbate. The optical fiber of the PAM fluorometer was placed on a side of a cuvette (1×1 cm) equipped with a magnetic stirrer device.

*Corresponding author. Fax: (81)-98-895 8576.
E-mail address: yamasaki@comb.u-ryukyu.ac.jp (H. Yamasaki).

Abbreviations: IC_{50}, 50% inhibitory concentration; ETR, electron transport rate; F_v/F_m, maximum efficiency of photosystem II; NOR2, (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide; NOS, nitric oxide synthase; NR, nitrite reductase; PS II, photosystem II; 9-AA, 9-aminoacridine; *pmf*, proton motive force; SNAP, *S*-nitroso-*N*-acetylpenicillamine

0014-5793/02/$22.00
PII: S0014-5793(02)02244-5

Fluorescence quenching of 9-aminoacridine (9-AA) was measured to estimate the extent of ΔpH across thylakoid membranes [13]. For the measurement, we set a fluorescence spectrophotometer (RF-5300, Shimadzu, Kyoto, Japan) as follows: excitation wavelength (and slit width) 400 nm (5 nm) and emission wavelength (and slit width) 450 nm (5 nm). Measurements were carried out with a reaction mixture (3 ml) containing 20 μmol Chl ml^{-1} thylakoids, 0.1 M sucrose, 10 mM $NaCl_2$, 0.1 M HEPES–KOH (pH 8.0), 50 μM methylviologen, 50 μM ATP, 30 mM L-ascorbate and 2 μM 9-AA.

Photophosphorylation (ATP synthesis) activity was determined by a bioluminescence assay. The reaction mixture (3 ml) contained 20 μmol ml^{-1} Chl thylakoids, 0.3 M sorbitol, 20 mM Tricine–KOH (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA, 1.5 mM K_2HPO_4, 50 μM methylviologen, 100 μM ADP and 30 mM L-ascorbate. After illumination the reaction mixture was immediately passed through a 0.2 μm syringe filter (Dismic-25 mixed-cellulose ester, Advantec, Tokyo, Japan) to separate the soluble fraction from the suspension. The obtained soluble fraction was subjected to an ATP assay to determine the activity of photophosphorylation. The assay of ATP was carried out with a luminometer (Lumicounter 700, Microtech Nitti-on, Chiba, Japan) using a luciferin/luciferase assay kit (SZ101, Yamato, Tokyo, Japan).

Actinic light of 150 μmol quanta m^{-2} s^{-1} was used to excite chlorophylls for all experiments described above. The reaction temperature was maintained at 26°C using a temperature-controlled water bath. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) and (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR2) were used to produce NO in the reaction mixtures. NO concentration was electrochemically determined with a nitric oxide electrode (ISO-NO Mark II, World Precise Instruments, Sarasota, FL, USA) in conjunction with a Duo-18 data acquisition system [14]. SNAP and NOR2 were purchased from Dojindo (Kumamoto, Japan). For other experimental conditions see figure legends.

3. Results

Fig. 1 shows effects of NO on electron transport activities of thylakoid membranes measured by PAM Chl *a* fluorescence. In the presence of NO, the emission of Chl *a* fluorescence from PS II was increased in saturation pulse analysis

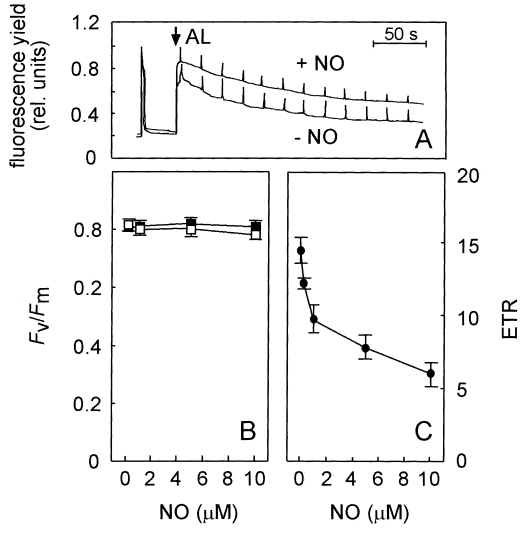

Fig. 1. Effects of NO on electron transport in PS II. A: Kinetics of PAM fluorescence quenching in the saturation pulse analysis. Thylakoids were incubated in the presence (+NO) or absence (−NO) of 10 μM NO for 1 min before the measurements. Actinic light (AL, 150 μmol m^{-2} s^{-1}) was turned on at the arrow. B: F_v/F_m was measured in the NO-treated thylakoids before (■) or after (□) illumination with actinic light for 3 min. C: The overall photosynthetic transport rate (ETR) was estimated from effective PS II quantum yield after illumination with AL for 3 min. Mean values with S.E.M. of three replicates are given in B and C.

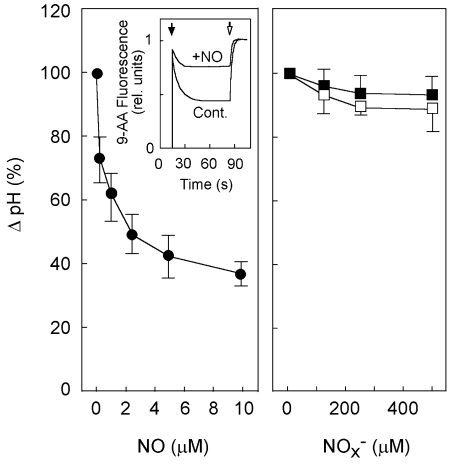

Fig. 2. Inhibitory effect of NO on the light-induced ΔpH formation. ΔpH-dependent uptake of 9-AA was measured in the presence of NO (● in panel A), NO_2^- (□ in panel B) or NO_3^- (■ in panel B). Inset: Kinetics of 9-AA fluorescence in the presence of 10 μM NO (+NO) or the absence of NO (Cont.). The actinic light and fluorometric measuring beam were simultaneously switched on at the black arrow. The white arrow indicates the addition of 2 μM nigericin. Mean values with S.E.M. of three replicates are given in A and B.

(Fig. 1A). Because NO did not show any inhibitory effects on maximum photochemical efficiency of PS II that can be measured by F_v/F_m either before or after the illumination (Fig. 1B), photooxidative damage in PS II cannot account for the fluorescence increase. Nevertheless, the ETR was found to be severely inhibited (Fig. 1C). The inhibition showed a clear dependence on NO concentration (IC_{50} = 3 μM NO).

The linear electron transport from water to $NADP^+$ (substituted by the artificial acceptor methylviologen in this study) is essential to generate the proton motive force (*pmf*, almost synonymous with ΔpH across thylakoid membranes in chloroplasts). If electron transport is inhibited by NO, the concomitant ΔpH formation should also be inhibited. Fig. 2 demonstrates effects of NO on the activity of ΔpH formation that can be detected as fluorescence quenching of the ΔpH indicator 9-AA [15]. The inset shows kinetic traces of the ΔpH-dependent fluorescence quenching of 9-AA in the presence or absence of NO. Like the results of Fig. 1C, ΔpH formation was severely inhibited by NO (Fig. 2A, inset) and the inhibition depended on NO concentration (Fig. 2A). Because the uncoupler nigericin completely recovered fluorescence intensity to the original level, the effect of NO cannot be attributed to direct reactions between NO and 9-AA that might suppress the quenching (Fig. 2A, inset). The value of IC_{50} (2 μM NO) was very similar to that in Fig. 2C.

NO is a short-lived radical species and can be degraded to NO_2^- and NO_3^- in oxygen-containing aqueous solution as follows [16]:

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (1)$$

$$2NO + O_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (2)$$

To exclude possible uncoupling effects caused by degraded products of NO, the effects of NO_2^- and NO_3^- on ΔpH formation were examined (Fig. 2B). Because the chemical NO donor SNAP has the potential to produce twice the amount

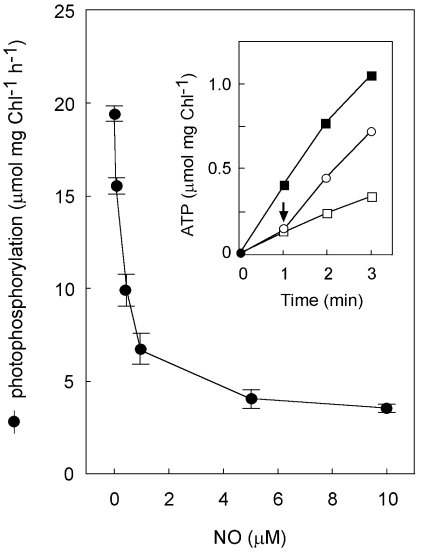

Fig. 3. Effects of NO on photophosphorylation activity in thylakoid membranes. Photophosphorylation activity (●) was measured as ATP synthesis rate under illumination (150 μmol m^{-2} s^{-1}). Inset: Kinetics of ATP synthesis in the presence of 20 μM chlorophyll with (□) or without 1 μM NO (■) and with 1 μM NO and 1 mg ml^{-1} hemoglobin (Hb, ○). Arrow indicates addition of hemoglobin. Mean values with S.E.M. of three replicates are given.

of NO on a stoichiometric basis, 250 μM SNAP used to produce 10 μM NO in the steady state should theoretically produce a maximum of 500 μM NO. If the total amount of NO were rapidly degraded by Eq. 1 or 2, NO_2^- or NO_3^- would be produced in the reaction mixture. However, even in the supposed presence of such high concentrations of supplemental NO_3^- or NO_2^-, substantial inhibition of ΔpH formation was not observed (Fig. 2B). Thus, the inhibitory effect of NO on ΔpH formation is not due to the uncoupling effect of the degradation products of NO as suggested before [8].

Photosynthetic ATP synthesis in chloroplasts (photophosphorylation), catalyzed by H^+-ATPase (CF_0–CF_1 complex), is coupled with vectorial proton translocation across thylakoid membranes [11]. Because light-induced ΔpH formation is the driving force for ATP synthesis in thylakoids, NO, which can

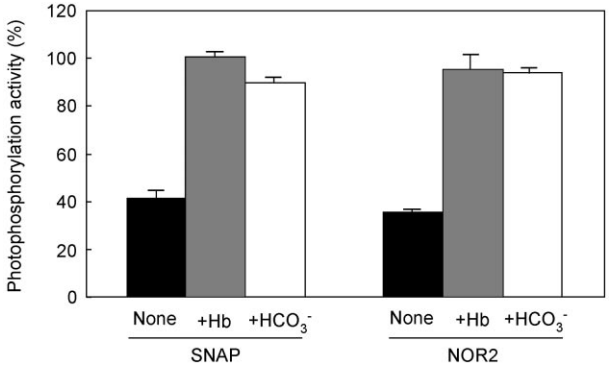

Fig. 4. Effects of hemoglobin and bicarbonate on NO-dependent inhibition of photophosphorylation. Photophosphorylation activity is represented as relative value (%) to the control (19.4 μmol mg Chl^{-1} h^{-1} in the absence of NO). To produce 1 μM NO, 25 μM SNAP or 400 μM NOR2 was added to the reaction mixture. +Hb, in the presence of 1 mg ml^{-1} hemoglobin. $+HCO_3$, in the presence of 10 mM $NaHCO_3$. Mean values with S.E.M. of four replicates are given.

suppress ΔpH formation, could also inhibit photophosphorylation activity. Indeed, NO exerted a strong inhibitory effect on photophosphorylation (Fig. 3). The inset of Fig. 3 shows time courses of the light-induced ATP synthesis in the presence or absence of 1 μM NO. NO significantly suppressed the rate of ATP synthesis upon illumination (□) but the effect was canceled by the addition of hemoglobin (○), a powerful NO quencher [1], to the reaction mixture. The results demonstrated here have clearly shown that the inhibition is temporary and reversible without showing any hysteresis. The IC_{50} for photophosphorylation activity (0.7 μM NO) was much lower than those for ETR and ΔpH formation. It should be noted that NO also inhibited ATPase (ATP hydrolysis) activity that can be measured in the absence of *pmf*. However, the inhibition required a much higher concentration of NO (IC_{50} = 50 μM). The significant difference in IC_{50} between ATP synthesis and ATP hydrolysis activities suggests that NO-induced inhibition of photophosphorylation cannot be attributed to inactivation of the H^+-ATPase.

A suppressive effect apparently similar to hemoglobin was observed when $NaHCO_3$ was added to the reaction mixture (Fig. 4), suggesting that bicarbonate (HCO_3^-) is also capable of preventing the inhibitory action of NO. One may consider that unknown chemistry between the NO donor SNAP and thylakoid membranes might be involved in these effects. To exclude the possible concern of SNAP other than NO production, we applied NOR2, a recently developed chemical NO donor. In Fig. 4, the concentrations of SNAP (25 μM) and NOR2 (400 μM) in the reaction mixture were adjusted to produce an equal amount of NO (1 μM NO at steady-state condition in the absence of thylakoids). Similar to SNAP, NOR2 exerted strong inhibition of photophosphorylation under this condition. Moreover, the inhibition induced by NOR2 was also prevented by the presence of hemoglobin or bicarbonate.

4. Discussion

The present study has demonstrated that NO reversibly inhibits photophosphorylation in thylakoid membranes. Air pollution studies in plants have shown that NO inhibits photosynthetic CO_2 assimilation [9,10]. Because NO is eventually degraded to nitrite (or nitrate) in an aqueous solution under ambient air, it has long been presumed that the harmful effects of NO on photosynthesis could be due to nitrite toxicity [8]. The results shown in this study suggest that nitrite is not involved in the mechanism for NO-dependent inhibition of in vitro photophosphorylation and confirm that the NO molecule itself does have a suppressive effect on the electron transport activity.

Because NO potentially alters the electron paramagnetic resonance (EPR) signal from transition metals contained in electron transfer components, NO has sometimes been applied for EPR studies to characterize photosynthetic electron transport systems. Using NO for releasing bound bicarbonate (CO_2/HCO_3^-), Diner and Petrouleas identified the bicarbonate binding site of non-heme iron complex in PS II [17,18]. It is well known that electron transfer at PS II can be stimulated by bicarbonate [19], which binds to several sites in PS II (e.g. non-heme iron complex, Q_B binding site, water-oxidizing complex) [20]. In contrast, carboxylate anions (–COO^-) including formate ($HCOO^-$) can slow the rate of electron trans-

fer because these anions can replace bound bicarbonate at these binding sites [19]. It is plausible that the reduced rate of electron transport, due to the NO-induced dissociation of bicarbonate from the specific sites of thylakoids, is involved in the mechanism of the inhibitory effect of NO on photophosphorylation. In fact, the rate of electron transfer was slowed by the addition of NO (Fig. 1), the results well consistent with those of a previous EPR study [18].

As shown in Fig. 4, the inhibitory effect of NO on the photophosphorylation can be prevented by a supplemental high concentration of bicarbonate. It is reported that high concentrations of bicarbonate enable the bound NO to liberate from the reaction center and recover electron transport activity [18]. Thus, sensitivity of photosynthesis against NO would depend on a local concentration of bicarbonate within thylakoid membranes. Consistent with this idea, air pollution studies have shown that the inhibitory effects of NO on photosynthesis can be prevented by higher concentrations of CO_2 [10,21].

Emission of NO has been reported in green algae [22–24], cyanobacteria [22] and a variety of higher plants [25,26]. However, the source of NO in plant cells is still controversial [27]. Although there have been a number of reports suggesting that plant cells contain a mammalian-type NOS [28,29], neither a gene nor a protein of NOS has been conclusively identified from plants to date. We have recently shown in vitro evidence that nitrate reductase (NR), a key enzyme of assimilatory nitrogen metabolism in plants and algae, produces NO when its normal product nitrite is provided as the substrate [14,30]. In fact, emission of NO from plants has been suggested to be linked with the accumulation of nitrite in many cases [22,23,26]. Under optimal conditions, nitrite is transported into the chloroplasts and is converted to ammonium by nitrite reductase. Nonetheless, nitrite can be accumulated in the cells when photosynthetic electron transport [31] or nitrogen metabolism [23] is not operational. These observations imply that photophosphorylation would be inhibited by endogenously produced NO at least under unfavorable conditions for nitrite conversion. It is well known that activities of nitrogen assimilation and carbon assimilation are clearly interdependent [32,33]. Because NO reversibly (Fig. 3) and harmlessly (Fig. 1B) inhibits photosynthetic activity, NR-produced NO may down-regulate the photosynthetic activity when nitrite reduction is not operational. In this context, we consider it plausible that NO acts as a transmembrane messenger to alert dysfunctional nitrogen assimilation so that photosynthetic activity can be quickly down-regulated.

Acknowledgements: We are grateful to Dr. M.F. Cohen of the University of the Ryukyus for critical reading of the manuscript. This work was supported by a Grant-in-Aid for Scientific Research (B) and (C) from the Japan Society for the Promotion of Science to H.Y.

References

[1] Wink, D.A. and Mitchell, J.B. (1998) Free Radical Biol. Med. 25, 434–456.
[2] Bogdan, C. (2001) Trends Cell Biol. 11, 66–75.
[3] Murado, F. (1994) Recent Progr. Horm. Res. 49, 239–248.
[4] Stadler, J., Trockfeld, J., Shmalix, W.A., Brill, T., Siewert, J.R., Greim, H. and Doehmer, J. (1994) Proc. Natl. Acad. Sci. USA 91, 3559–3563.
[5] Kanner, J., Harel, S. and Granit, R. (1992) Lipids 27, 46–49.
[6] Brookes, P.S., Bolanõs, J.P. and Heales, S.J.R. (1999) FEBS Lett. 446, 261–263.
[7] Yamasaki, H., Shimoji, H., Ohshiro, Y. and Sakihama, Y. (2001) Nitric Oxide Biol. Chem. 5, 261–270.
[8] Wellburn, A.R. (1990) New Phytol. 115, 395–429.
[9] Hill, A.C. and Bennett, J.H. (1970) Atmos. Environ. 4, 341–348.
[10] Bruggink, G.T., Wolting, H.G., Dassen, J.H.A. and Bus, V.G.M. (1988) New Phytol. 110, 185–191.
[11] Yamasaki, H., Furuya, S., Kawamura, A., Ito, A., Okayama, S. and Nishimura, M. (1991) Plant Cell Physiol. 32, 925–934.
[12] Schreiber, U., Gademann, R., Ralph, P.J. and Larkum, A.W.D. (1997) Plant Cell Physiol. 38, 945–951.
[13] Gilmore, A.M. and Yamasaki, H. (1998) Photosynth. Res. 57, 159–174.
[14] Yamasaki, H. and Sakihama, Y. (2000) FEBS Lett. 468, 89–92.
[15] Hope, A.B. and Hatthews, D.B. (1985) Aust. J. Plant Physiol. 12, 9–19.
[16] Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E. and Byrns, R.E. (1993) Proc. Natl. Acad. Sci. USA 90, 8103–8107.
[17] Petrouleas, V. and Diner, B.A. (1990) Biochim. Biophys. Acta 1015, 131–140.
[18] Diner, B.A. and Petrouleas, V. (1990) Biochim. Biophys. Acta 1015, 141–149.
[19] van Rensen, J.J.S. and Snel, J.F.H. (1985) Photosynth. Res. 6, 231–246.
[20] van Rensen, J.J.S. and Curwiel, V.B. (2000) Indian J. Biochem. Biophys. 37, 377–382.
[21] Anderson, L.S. and Mansfield, T.A. (1979) Environ. Pollut. 20, 113–121.
[22] Mallick, N., Rai, L.C., Mohn, F.H. and Soeder, C.J. (1999) Chemosphere 39, 1601–1610.
[23] Mallick, N., Mohn, F.H., Rai, L. and Soeder, C.J. (2000) J. Microbiol. Biotechnol. 10, 300–306.
[24] Mallick, N., Mohn, F.H., Rai, L.C. and Soeder, C.J. (2000) J. Plant Physiol. 156, 423–426.
[25] Wildt, J., Kley, D., Rockel, A., Rockel, P. and Segschneider, H.J. (1997) J. Geophys. Res. 102, 5919–5927.
[26] Klepper, L. (1990) Plant Physiol. 93, 26–32.
[27] Wojtaszek, P. (2000) Phytochemistry 54, 1–4.
[28] Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Proc. Natl. Acad. Sci. USA 95, 10328–10333.
[29] Ribeiro, E.A., Cunha, F.Q., Tamashiro, W.M.S.C. and Martins, I.S. (1999) FEBS Lett. 445, 283–286.
[30] Yamasaki, H., Sakihama, Y. and Takahashi, S. (1999) Trends Plant Sci. 4, 128–129.
[31] Shingles, R., Roh, M.H. and McCarty, R.E. (1996) Plant Physiol. 112, 1375–1381.
[32] Foyer, C.H., Valadier, M.H. and Ferrario, S. (1995) in: Environment and Plant Metabolism (Smirnoff, N., Ed.), pp. 17–33, BIOS Scientific, Oxford.
[33] Huppe, H.C. and Turpin, D.H. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 577–607.