Detection of Nitric Oxide in Plants by Electron Spin Resonance

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Accepted for publication 28 December 2003.

ABSTRACT

Xu, Y. C., Cao, Y. L., Guo, P., Tao, Y., and Zhao, B. L. 2004. Detection of nitric oxide in plants by electron spin resonance. *Phytopathology* 94:402-407.

Three methods to detect nitric oxide (NO•) are reported here. The first method was determining NO• in extracted plant tissue. NO• was trapped by spin trapping reagent containing diethyldithiocarbamate (DETC) and FeSO₄, extracted by ethyl acetate, and determined with an electron spin resonance (ESR) spectrometer. The second method was indirectly determining NO• in live wheat leaves. Seedlings were cultured in a medium containing FeSO₄, and the leaves were brushed by DETC. Then, the leaves were ground and the complex of (DETC)₂-Fe²⁺-NO was extracted and determined with an ESR spectrometer. The third method was directly

determining NO• in live wheat leaves. After treating plant materials as in the second method, part of the water in leaves was transpired, and the leaf disks were inserted directly into quartz tubes to determine NO• with an ESR spectrometer. The NO• scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) decreased NO• signal detected either by an indirect or a direct method. This result indicates that both methods could detect NO• in the live plant. Using the first methods, we detected NO• change in wheat infected by *Puccinia striiformis* race CY22-2 pathogen (incompatible interaction) at different inoculation times, and it was found that the NO• content dramatically increased at 24 h postinoculation, quickly decreased at 48 h, and increased again at 96 h.

Additional keyword: free radicals.

In 1987, it was discovered that vascular endothelial cells could synthesize the free radical gas nitric oxide (NO•) (26), and NO• could act as a vasodilatory messenger (12). From then on, much attention was given to the study of NO• in biological systems (32), and many other important physiological functions of NO• were discovered (21). In plants, although NO• research is more recent than in animals, mounting evidence suggests that NO• plays important roles in diverse physiological processes such as growth and development (1,9,40), plant disease resistance (6,8,25), abiotic stress (17,19,38), and signal transduction (5–7).

Therefore, the direct detection of NO• has become attractive, especially in biological models. NO• detection assays include the following: (i) chemiluminescence detection using the reaction of NO• with ozone or luminol; (ii) fluorometry using a fluorescent NO• indicator; (iii) spectrophotometry using the formation of NO-hemoglobin (NOHb); (iv) electron spin resonance (ESR) spectrometry using spin (NO•) traps such as Hb, organic compounds, and iron-dithiocarbamate complexes; and (v) an electrochemical detection using microelectrodes specific to NO•. Other indirect assays include spectrophotometry, fluorometry, and radiometry of stable biological metabolites of NO• or L-arginine such as nitrite, nitrate, and L-citrulline. We can apply any method of choice to detect and quantify NO•, but we need to choose the one that is most suitable for a specific purpose, because each method described previously has both merits and disadvantages. The chemiluminescence method, for instance, requires a gas-phase reaction of NO• with ozone. Although very sensitive and selective for NO•, the method required purging of aqueous samples with an

inert gas to strip NO• into an analyzer. Therefore, it is incapable of monitoring intracellular NO•. The other chemiluminescence method with luminol has limitations for the functional analysis of NO•, such as the use of cytotoxic H₂O₂. Electrochemical sensing using microensors provides in situ real-time detection of NO•. However, the only spatial information is directly at the electrode tip and is therefore influenced by the placement of the probe (13,23,24).

ESR is a direct method to detect free radicals, and some efforts have been made to detect NO• (15,27). Mordvintcev et al. (22) employed the high affinity of NO• for transition metal ions to develop a method in which they used the complex of Fe²⁺ with diethyldithiocarbamate (DETC) to trap NO• and form a stable ternary complex (DETC)₂-Fe²⁺-NO. This ternary complex was then detected by ESR spectroscopy at 77 K (29). The spectral feature of the ternary complex (DETC)₂-Fe²⁺-NO is an axial signal with an easily recognized hyperfine structure triplet at $g_{\perp} = 2.035$. However, the detection threshold is higher and the ternary complex (DETC)₂-Fe²⁺-NO is not soluble in water. These drawbacks restrict this methods application in certain cases. Our laboratory improved this method by extracting the ternary complex (DETC)₂-Fe²⁺-NO from the water phase into an organic phase. By this means, the detection threshold was improved to lower than 50 nM, which was 10-fold more sensitive than the usual method. Using this method, the NO• in animal tissue extraction was successively detected at room temperature (36).

Reports on measuring NO• by ESR in plants are very few. Mathieu et al. (20) detected the spectra of leghemoglobin-NO using ESR at 77 K in soybean nodules. To our knowledge, there is not an appropriate method to measure in vitro and in vivo NO• generated in plants by ESR at room temperature. The study reported here had two objectives: (i) modifying the method set up by our laboratory to detect NO• in plant tissue extraction at room temperature; and (ii) developing a method to detect NO• directly or indirectly in plants.

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Publication no. P-2004-0224-01R

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MATERIALS AND METHODS

Plant materials. Wheat seeds (*Triticum aestivum* L. cv. Hanxuan 10) were sown in plastic pots and irrigated with Hoagland's solution. Seeds were allowed to germinate and develop in the greenhouse. The growth chamber was set at a relative humidity of 60 to 80%, day/night temperatures of 25/20°C, 14 h photoperiod (600 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ photosynthetically active radiation) supplied by fluorescent lamps, and 10 h dark. Six-day-old seedlings were used as experimental materials. In the stripe rust disease infection experiment, wheat seedlings were inoculated with *Puccinia striiformis* Westend. race CY22-2 (provided by North-West Science-Technology University of Agriculture and Forestry) and incubated at 18/12°C (day/night).

Reagents and apparatus. DETC and PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) were purchased from Sigma. All other reagents were purchased in China and were analytical grade.

ESR experiments were performed on a spectrometer (200D-SRC; Bruker Instruments, Germany). The $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$ complex both in solution and in intact leaves was measured in a 2.5-mm (for solution) and 3.5-mm (for leaf disks) internal diameter quartz tube at 25°C, respectively. For a control of $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$ complex in frozen solution, the ESR spectrum was measured at 100 K. The volume of organic solvent was 120 μl , and the height of leaf disks was 2 cm. The conditions for measurement were as follows: X-band; 100 kHz modulation with 3.2 G amplitude; microwave power, 20 mW; and central magnetic field 3,385 G, scan width 400 G, time constant 0.3 s, and scan time 4 min. The whole height of triplet hyperfine structure, namely the peak at $g = 2.035$ and its two neighbor peaks, was taken as relative intensity of the ESR signal.

Trapping $\text{NO}^{\bullet}$ in wheat seedling extract. Fresh leaf tissue samples (0.5 g) were ground in a chilled mortar with 1.0 ml of 100 mM phosphate buffer solution or 1.0 ml of 100 mM HEPES buffer solution. Both of them contained 0.32 M sucrose, 0.1 mM EDTA, and 5 mM thioethylenglycol. The homogenate was centrifuged at $13,201 \times g$ for 20 min (4°C). The supernatant contained 5.7 mg of protein and was incubated with spin trapping reagent (7.5 mM FeSO_4 and 25 mM DETC). Then, 300 μl of ethyl acetate was added to the mixture, shaken for 3 min, and centrifuged at $13,201 \times g$ for 6 min. The organic solvent layer was used to determine $\text{NO}^{\bullet}$ on an ESR spectrometer.

Trapping $\text{NO}^{\bullet}$ in wheat seedlings. Six-day-old wheat seedlings were transferred to a culture medium containing 15 mM FeSO_4 , and the leaves were brushed with 40 mM DETC. After 24 h, the leaf disks were divided into two parts. Part one, 0.5 g of leaf tissue was ground in a chilled mortar with 1.0 ml of 100 mM phosphate buffer solution (pH 7.0). After adding 300 μl of ethyl acetate to the homogenate, the mixture was shaken, centrifuged, and separated as described previously. The organic solvent layer was used to determine $\text{NO}^{\bullet}$ on the ESR spectrometer (indirect method). Part two, 0.20 g leaves were treated in a 700 W microwave for 30 s to inactivate the enzymes in the leaves and kept at 40°C to partially dehydrate. Thereafter, the leaf disks were inserted directly into a quartz tube to determine $\text{NO}^{\bullet}$ on an ESR spectrometer. The lost water ratio was calculated by measuring the weight of the leaves before and after the drying treatment.

Nitric oxide standard curve preparation. $\text{NO}^{\bullet}$ gas was prepared as described by Zhao et al. (37) and its saturated concentration in water was calculated as described by Borutaite and Brow (2). The reaction mixture (638 μl) contained 100 mM phosphate buffer solution (pH 7.0), 5.7 mg of bovine serum albumin, 7.5 mM FeSO_4 , 25 mM DETC, and H_2O or various concentration of $\text{NO}^{\bullet}$ solution. Then, the procedures of incubation, extraction, shaking, separation, and measurement were done as described previously and an $\text{NO}^{\bullet}$ standard curve was prepared.

Statistical analysis. Data were analyzed by analysis of variance and Student's *t* test. Values are means of three independent experiments; standard deviations are indicated by error bars. At least 12 wheat seedlings were used per experiment.

RESULTS

Nitric oxide standard curve. Figure 1 shows an ESR spectrum where authentic nitric oxide gas was prepared by chemical process. The peak at $g = 2.035$ and its two neighbor peaks is attributed to the $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$ complex. This triplet hyperfine structure is due to the interaction between the unpaired electron and the nitrogen nucleus of $\text{NO}^{\bullet}$. The spectra recorded in solution at room temperature have an isotropic spectrum as a result of rapid molecular motion. In addition to the ESR signal due to $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$, the signal of DETC with copper is also presented (16,30). It has been shown that the fourth peak at $g = 2.02$ is attributed to the complex of DETC and copper (39). In the process of preparing a nitric oxide standard curve, we also found that the height of the peak at $g = 2.02$ did not change with the increase of $\text{NO}^{\bullet}$ concentration (Fig. 1a and b). It is possible that this copper comes from reagents such as FeSO_4 , bovine serum albumin, or the phosphate buffer solution. However, the height of the triplet hyperfine structure increased linearly with the $\text{NO}^{\bullet}$ concentration. After linear regression analysis, the correlation coefficient rate was 0.9952 (Fig. 1).

Detection of $\text{NO}^{\bullet}$ in wheat seedlings extract. Typical ESR spectrometers have been developed to operate at microwave frequencies of approximately 9 GHz (X-band). However, the presence of more water with a high dielectric constant in biological samples has hampered the development in this field. We used ethyl acetate to extract $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$ complex from plant samples and then easily detected the complex spectra by ESR spectrometer (Fig. 2a), which was similar to that of control in Figure 1 and that reported in animal samples (36). This indicated that the signal came from $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$. HEPES buffer was often used as a solution (pH 7.4) medium in animal tissue tests. We compared the effect of 100 mM phosphate buffer solution (pH 7.0) and 100 mM HEPES buffer solution (pH 7.0) on the ESR signal in wheat leaf extract and found that ESR signal was higher in

Fig. 1. Standard curve of the $(\text{DETC})_2\text{-Fe}^{2+}\text{-NO}$ complex that was obtained by the reaction of $\text{NO}^{\bullet}$ solution with $(\text{DETC})_2\text{-Fe}^{2+}$. Insert is the electron spin resonance (ESR) spectra that represent $\text{NO}^{\bullet}$ concentrations at **a**, 500 nM and **b**, 100 nM.

phosphate buffer solution than in HEPES buffer solution (Fig. 3a and b). This indicated that phosphate buffer solution was more suitable for plant tissue. The effect of different temperatures also was studied and Figure 3c, d, and e show the NO[•] content when the reaction mixture was incubated at 25, 30, and 37°C, respectively, for 60 min. The highest signal was detected at 30°C and this temperature was therefore best for plant tissue extraction. In addition, the effect of incubation periods was tested and it was found that NO[•] signal gradually increased with extension of the incubation period (Fig. 3f to h). The effect of pH was studied and it was found that the NO[•] content had no significant difference at pH 7.0 and 7.4 (Fig. 3i and j). Therefore, the optimum condition for determining NO[•] in plant tissue extract was when plant tissue was ground in phosphate buffer solution (pH 7.0 to 7.4) and the extract was incubated with trapping reagent containing 7.5 mM FeSO₄ and 25 mM DETC at 30°C for 60 min.

Indirect detection of NO[•] in live plants. After DETC and FeSO₄ were absorbed by the leaves and roots of wheat seedlings, respectively, NO[•] produced in vivo was trapped by (DETC)₂-Fe²⁺, and it then formed the (DETC)₂-Fe²⁺-NO complex. Figure 2b

Fig. 2. The electron spin resonance (ESR) spectra of (DETC)₂-Fe²⁺-NO detected by different methods. **a**, The (DETC)₂-Fe²⁺-NO complex in the leaf homogenate was extracted by ethyl acetate and then measured on an ESR spectrometer. **b**, The (DETC)₂-Fe²⁺-NO complex in live wheat leaves was extracted by ethyl acetate and then measured on an ESR spectrometer. **c**, 2-Phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO; 0.5 mM) was supplied for 4 h prior to providing trapping reagent to wheat seedlings. **d**, PTIO (1.0 mM) was supplied for 24 h prior to providing trapping reagent to wheat seedlings. The (DETC)₂-Fe²⁺-NO signal was measured as in the procedure of **b**. **e**, (DETC)₂-Fe²⁺-NO complex was obtained by the reaction of NO[•] solution with (DETC)₂-Fe²⁺, as described in the text, and 120 µl of mixture solution was introduced into quartz tube and put into liquid nitrogen immediately, and was measured on an ESR spectrometer at 100 K. **f**, The live wheat leaves (0.2 g) that contained (DETC)₂-Fe²⁺-NO complex were dried to lose water equal to approximately 30% of total weight, and the leaf disks were inserted directly into a quartz tube of 3.5-mm internal diameter packed 2 cm in height and (DETC)₂-Fe²⁺-NO signal was measured on an ESR spectrometer. **g**, PTIO (0.5 mM) was supplied for 4 h prior to providing trapping reagent to wheat seedlings. **h**, PTIO (1.0 mM) was supplied for 24 h prior to providing trapping reagent to wheat seedlings. The (DETC)₂-Fe²⁺-NO signal was measured as in the procedure of **f**.

presents the spectrum which shows a ternary complex (DETC)₂-Fe²⁺-NO with a hyperfine structure triplet at $g = 2.035$ with $a_N = 12.5$ G. What is more, this spectrum was similar to that of the NO[•] standard curve (Fig. 1). In order to further ascertain the source of the signal, a PTIO (scavenger of NO[•]) experiment was conducted. When 0.5 mM PTIO was provided for 4 h before uptake of trapping reagent, the signal decreased significantly (Fig. 2c). When 1.0 mM PTIO was provided for 24 h before uptake of trapping reagent, almost no signal could be detected (Fig. 2d). These results offer proof that the signals detected by ESR spectrometry came from NO[•] in the plant. Because NO[•] is a labile free radical and its half-life is very short (approximately 6 s), the detected NO[•] content represents an accumulation of the trapped NO[•] in leaves during 24 h. Naturally, the shorter this period is, the lower the NO[•] content. In addition, the trapping reagent that entered the plant should be sufficient for trapping in vivo NO[•]. Incubating 6-day-old wheat seedlings with 15 mM FeSO₄ and 40 mM DETC for 6 h was enough for trapping in vivo NO[•] produced during 24 h (data not shown). DETC easily combines with FeSO₄ to form a precipitating complex, which is difficult for plant absorption. Therefore, DETC and FeSO₄ must be absorbed by different parts of the plant, such as roots and leaves.

Direct detection of NO[•] in live plants. As mentioned previously, water in biological samples restricted ESR detection in certain cases. After DETC and FeSO₄ were absorbed and the (DETC)₂-Fe²⁺-NO complex accumulated to a specific level in the leaf, the intact leaf was heated by microwave for 30 s to inactivate in vivo enzyme activity and the leaves were kept at 40°C for various times to partially dehydrate them. Figure 2f shows the spectrum in intact leaves when the leaves lost water equal to approximately 30% of their total weight, which has similar spectrum parameters to that of Figure 2e in frozen solution at 100 K ($g_{\perp} = 2.035$ with $a_N = 12.5$ G) and to the report by Goodman et al. (10). This indicates that the three peaks were also ascribed to (DETC)₂-Fe²⁺-NO. The baseline of the spectrum was not parallel with the x axis, similar to what had been found with that of nitric oxide-leghemoglobin complex in intact soybean nodules (20). In addition, when the seedlings were treated by PTIO as described previously, the ESR signal in the intact leaf decreased significantly (Fig. 2f to h). This indicates that the signals detected in the intact leaf also came from NO[•]. Comparing with the spectra of Figure 2a to d in solution, the peaks in the spectra of Figure 2e to h in

Fig. 3. The NO[•] content in wheat seedling extract under various experimental conditions: **A**, different media; **B**, different temperatures; **C**, different incubation periods; **D**, different pH levels; **a**, phosphate buffer solution; **b**, HEPES buffer solution; **c**, 25°C; **d**, 30°C; **e**, 37°C; **f**, 30 min; **g**, 60 min; **h**, 90 min; **i**, pH 7.0; and **j**, pH 7.4. * Denotes significant differences from the first column of each group at the 5% confidence interval.

frozen solution and leaves are broadening. When the excised leaves of wheat seedlings were kept at 0 to 4°C for 24 h after finishing the 40°C treatment, the NO• signal had no significant change (Fig. 4d). This indicated that the complex of (DETC)₂-Fe²⁺-NO in intact leaves was relatively stable. In addition, when the leaves lost water equal to about 30, 40, and 50% of their total weight (0.20 g), the ESR signal slightly increased with the increase of lost water, but no statistically significant differences were observed (Fig. 4a to c). This indicated that when the leaves lost water equal to about 30 to 50% of their total weight, the effect of water in leaf on (DETC)₂-Fe²⁺-NO signal was almost the same. Therefore, the NO• in leaf can be relatively constantly detected by this method under the above conditions. When the leaves lost water equal to less than 20% of their total weight, the ESR spectrometer could scarcely detect the (DETC)₂-Fe²⁺-NO signal at room temperature (data not shown).

Changes of NO• contents in wheat seedlings infected by *P. striiformis* at different times. The stripe rust (or yellow rust) caused by *P. striiformis* is a serious disease of wheat in many areas of the world. It has been shown that NO• plays an important role in plant disease resistance (6,8,25). Wheat seedlings infected by CY22-2 show an incompatible interaction (identified by North-West Science-Technology University of Agriculture and Forestry). We found no stripe rust symptoms on leaves after inoculation (data not shown). When monitoring NO• contents in wheat seedlings infected by CY22-2 at different inoculation times, it was found that the NO• content dramatically increased at 24 h, quickly decreased at 48 h, and increased again at 96 h (Table 1). These results suggest that NO• may play an important role in wheat stripe rust disease resistance. The mechanism is under study in our laboratory.

DISCUSSION

In general, ESR is considered the most direct and efficient technique available for detection and measurement of free radicals. Spin trapping is a common technique to detect nitric oxide free radicals. DETC, MGD (*N*-methyl-D-glucamine dithiocarbamate), and DTCS (dithiocarboxy sarcosine) are often used as trapping reagents (31,35). They have been applied extensively to detect NO• produced in animal systems. However, the concentration of NO• generated in some biological systems is too low to be detected. In addition, the water in biological samples also affects

ESR detection. These features impair the sensitivity of ESR and restrict its application. (DETC)₂-Fe²⁺-NO is a hydrophobic complex that can be extracted by organic solvents. Our laboratory used ethyl acetate as an extract solvent to concentrate NO• from tissue and successively detected NO• content in animal tissue (36). NO• is generated almost exclusively by nitric oxide synthase (NOS) in animal cells. However, NO• is produced in many ways by plant cells (33). What is more, there are several differences in NO• metabolism between animal tissue and plant tissue. Therefore, the experimental conditions tested here in plants such as temperature, extract buffer solution, pH, and so on were different from those employed in animals. In this study, we improved the method to measure NO• content in plant tissue extract. The optimum condition of determining NO• concentration in plant tissue extract was when the plant tissue was ground in phosphate buffer solution (pH 7.0 to 7.4) and the extract was incubated with trapping reagent containing 7.5 mM FeSO₄ and 25 mM DETC at 30°C for 60 min. Previous evidence indicates that NOS exists in plants (28,33) and that the main pathway of producing NO• in the plant was via nitrate reductase (NR) (14,18,34). The optimum pH of both NOS and NR was about 7.4 (3,8). Our results show that there was no significant difference in NO• content in wheat leaves extract at pH 7.0 and at pH 7.4. This possibly relates to the many means of NO• formation in plants (33) and the complex components in the extraction. Because cell structure was destroyed during the extraction and the physiological status was changed, NO• detected by this method represents the NO• level in the extracted plant tissue but not in the live plant. However, it is still a useful method for the study of NO• in plants, such as NO• source in plant.

In order to detect NO• in live plants, the trapping reagent must enter into the foliage. However, DETC easily combines with FeSO₄ to form hydrophobic complexes, which was difficult for plant absorption. Therefore, DETC and FeSO₄ must be absorbed by different parts of plants, such as roots and leaves. The complex of (DETC)₂-Fe²⁺-NO in leaf was relatively stable at 0 to 4°C (Fig. 4d), which was similar to the complex in ethyl acetate solvent (36).

When (DETC)₂-Fe²⁺-NO accumulates to a certain level in the leaf, the complex can be extracted by ethyl acetate and then measured on an ESR spectrometer (indirect method) or it can be measured directly on an ESR spectrometer (direct method). Because the water in the leaf absorbs microwaves, the (DETC)₂-Fe²⁺-NO signal in fresh leaves cannot be detected by the direct method at room temperature. Therefore, part of the water in fresh leaves must be eliminated. Theoretically speaking, more (DETC)₂-Fe²⁺-NO signal should be detected as leaves lose water. However, if all water in leaves is transpired, it is difficult for these completely dehydrated leaves to be correctly inserted into quartz tube. The lost water rate was related to plant species, plant organ, growth status, and other factors. For 6-day-old wheat seedlings, the NO• signal was relatively stable when leaves lost water of about 30 to 50% of their total weight (Fig. 4). The baseline of the spectrum was not parallel with the x axis (Fig. 2f to h). Probably, Fe³⁺ (as ferritin) and Cu²⁺ (as mononuclear complexes) produce absorptions in this region. The broadened peaks in these spectra could be explained by restricted molecular motion of the (DETC)₂-Fe²⁺-NO complex. Although the direct method has some shortfalls, it was superior to the indirect method. First, the procedure was simple, which reduces test error caused by the experimenter. Second, the experi-

Fig. 4. The electron spin resonance (ESR) signal intensity in intact leaves under various experimental conditions. Excised leaves lost water to about **a**, 30%; **b**, 40%; and **c**, 50% of their total weight by keeping the leaves at 40°C for various times. **d**, After the treatment of **a**, excised leaves were kept at 0 to 4°C for another 24 h.

TABLE 1. NO• contents in wheat seedlings infected by CY22-2 at different inoculation times

Pathogen	NO• content (nmol g ⁻¹ free weight)			
	0 h	24 h	48 h	96 h
CY22-2	114.3 ± 5.6	256.3 ± 6.2	119.8 ± 7.5	326.5 ± 6.3

mental materials of 2 cm long was enough for plant disks in quartz tube, so the NO[•] contents in different parts of plant, such as roots, shoots, leaf, flower, pathogen-infected areas, or noninfected areas, could be analyzed directly. The NO[•] scavenger PTIO decreased the ESR signal detected either by the indirect or by the direct method (Fig. 2). This indicated that both the indirect method and the direct method could detect NO[•] in live plants.

Previous evidence showed that NO[•] plays an important role in plant disease resistance (6,8,25). We found the NO[•] content in wheat leaves infected by *P. striiformis* race CY22-2 show an up-down-up pattern of variation (Table 1). As described previously, NO[•] has many means of formation in plants (33). Part of the NO[•] may be produced by NOS (11,28,38); part of the NO[•] was possibly produced by the way of NR (14,18,34); part of the NO[•] may come from carotenoids pathway (4); and part of the NO[•] was possibly produced by a chemical pathway, such as the change of pH (33). It is possible that different routes to NO[•] formation have different sensitivity to biotic and abiotic stresses. When wheat seedlings were infected by *P. striiformis* race CY22-2, the rapid response route was probably activated first and then the slow response was activated, so the NO[•] content shows two phases of increase (Table 1). Besides the signal function, NO[•] also acts as a toxic molecule. In evolution, plants potentially developed some mechanisms to inhibit NO[•] content increases. This is possibly the reason for initial NO[•] content increase followed by a decrease after some time (Table 1).

In summary, these methods may not only be applied to phytopathological studies but also help us to understand pathophysiological processes, especially NO[•] roles in the plant disease response.

ACKNOWLEDGMENTS

We thank R. L. Jing from the Chinese Academy of Agricultural Science for providing wheat seeds and the National Science Foundation of China (30070196) for financial support.

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