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RESEARCH PAPERS

Azide-Dependent Nitric Oxide Emission from the Water Fern *Azolla pinnata*[1]

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Abstract—Nitric oxide (NO) is involved in versatile functions in plant growth and development as a signaling molecule. To date, plants have been reported to produce NO following exposure to nitrite (NO_2^-), the amino acid L-arginine, hydroxylamine, or polyamines. Here we demonstrate azide-dependent NO production in plants. The water fern *Azolla pinnata* emitted NO into air upon exposure to sodium azide (NaN_3). The NO production was dependent on azide concentration and was strongly inhibited by potassium cyanide (KCN). Incubation of *A. pinnata* with the catalase inhibitor 3-aminotriazole (3-AT) abolished the azide-dependent NO production. Although nitrite-dependent NO production was inhibited by sodium azide, azide-dependent NO production was not affected by nitrite. These results indicate that *A. pinnata* enzymatically produces NO using azide as a substrate. We suggest that plants are also capable of producing NO from azide by the action of catalase as previously reported in animals.

Keywords: Azolla pinnata, nitric oxide, nitrite, azide, catalase, ROS

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INTRODUCTION

Nitric oxide (NO) is a gaseous signaling molecule that is involved in a multitude of physiological processes in plants [1] and animals [2]. In the late 1980s, it was discovered that NO is synthesized in animal cells by the enzyme nitric oxide synthase (NOS) using the amino acid L-arginine as the substrate (arginine pathway). Later, NO was found to also be produced from nitrite in animals by distinctive mechanisms (nitrite pathway) [3]. Since the regulation of NO production is important in physiological responses, the sources of NO and its production mechanisms are of particular interest in biology and medicine.

In plants, it has been shown that NO is produced from nitrite through enzymatic as well as non-enzymatic mechanisms, whereas the arginine pathway has yet to be elucidated due to the lack of a NOS homolog in plants [1]. Nitrate reductase (NR) is the first enzyme, whose NO producing activity was confirmed in plants by both in vitro [4] and in vivo [5] studies. In contrast to the arginine pathway that is catalyzed by NOS enzymes, the nitrite pathway involves multiple routes and mechanisms. More recently, many plant enzymes other than NR have been reported to produce NO from nitrite: peroxisomal xanthine oxidase [6], plasma membrane bound nitrite:NO reductase [7], and nonsymbiotic hemoglobin [8]. One electron reduction of nitrite by electron transport systems also produces NO in chloroplasts [9] and mitochondria [10]. In addition to these enzymatic mechanisms, non-enzymatic NO production in acidic and reducing environments, that may occur in the apoplast [11] and plastids [12], has physiological relevance.

Compounds other than L-arginine and nitrite have been shown to induce NO production in plants: the polyamines spermine and spermidine in *Arabidopsis* [13] and hydroxylamine in NR-free plant cells [14]. These studies imply that plants may have the potential to utilize a variety of chemicals to produce NO.

Sodium azide (NaN_3) has been applied for research purposes as a vasodilator [15, 16], but it is cytotoxic because it inhibits a range of metal-containing enzyme activities [17]. It is now evident that the vasodilative activity of azide is due to its function as a precursor of NO in animals [16, 18]. However, there is no literature available to confirm the presence of azide-dependent NO production in plants.

In this study we used the floating fern *Azolla pinnata* as a plant model to investigate azide-dependent NO production. *A. pinnata* is a fresh water fern in symbiotic relationship with the nitrogen-fixing cyanobacterium *Nostoc* (*Anabaena*) *azollae*. Its small size and aquatic habitat made *A. pinnata* ideally suited for this

[1] The article is published in original.

Abbreviations: 3-AT—3-aminotriazole; NOS—nitric oxide synthase; NR—nitrate reductase.

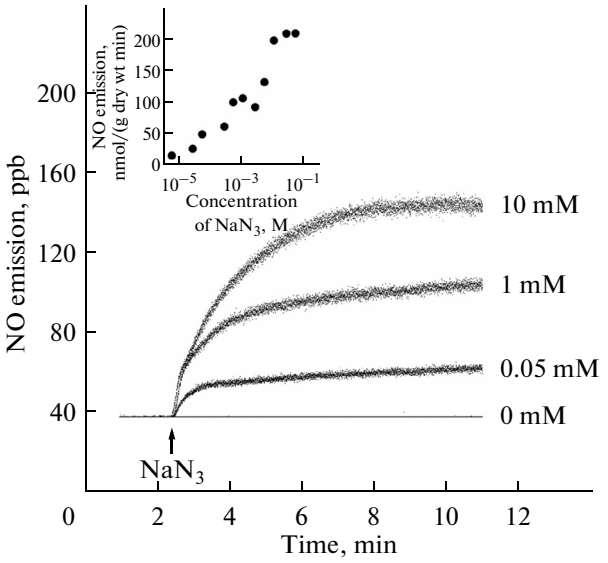

Fig. 1. Time courses of azide-induced NO emission from *A. pinnata*.

Fronds of *A. pinnata* were placed onto a plastic Petri dish that contained 10 mM potassium phosphate buffer (pH 7.0). NO concentration of the headspace was monitored with a chemiluminescence technique. Sodium azide (NaN_3) at various concentrations was added at the arrow indicated. The inset figure shows azide concentration dependence of NO emission from *A. pinnata*. The correlation and regression coefficients were $R = 0.95$ and $R^2 = 0.91$, respectively.

study, facilitating the delivery of exogenous chemicals to the plants in solution. Employing the water fern, we show here the first experimental evidence to verify that plants are capable of producing NO from azide.

MATERIALS AND METHODS

Azolla pinnata was collected from a local taro field in Okinawa, Japan and surface-disinfected in a solution containing 0.12% (v/v) sodium hypochlorite and 0.01% (v/v) Triton X-100. The plants were then cultured in autoclaved cobalt-supplemented 40% nitrogen-free Hoagland E-medium under laboratory conditions (27 ± 1°C, a 16 h photoperiod, and light intensity of 50 μmol/(m^2 s). For experiments, manually derooted fronds were cultured in nutrient medium that was replaced with a fresh-autoclaved one every four days. Details of the method of disinfection, composition of the culture medium, and plant growth conditions have been previously described [19].

A confluent layer of water ferns (10–13-day-old) enough to cover the surface area was placed in a 10-cm in diameter plastic Petri dish that contained 20 mL of 10 mM potassium phosphate buffer (pH 7.0). Two pinholes were made on the Petri dish cover: one at the side was for inserting the outlet pipe to the nitric oxide analyzer and the other on the top cover served as the inlet of air and various chemical solutions. To facilitate mixing of the medium, the Petri dish apparatus was rotated on a shaker.

NO was measured with a chemiluminescence technique that can monitor real-time emission of gaseous NO [20]. The measurement was carried out at room temperature (23 ± 1°C) with a Sievers Nitric Oxide Analayzer (NOA) 280i having a 50 mL/s intake flow rate and the data collected by NO Analysis software (GE Analytical Instruments, United States). The sampling frequency was 8/s.

RESULTS

Figure 1 shows time courses of NO production in *A. pinnata* monitored by the chemiluminescence technique. Real-time measurements, as well as high specificity for NO, are advantages of the chemiluminescence technique [20]. In control experiments, the fronds of *A. pinnata* incubated only with phosphate buffer emitted negligible basal amounts of NO (<0.5 ppb). The signal of NO increased rapidly when azide (NaN_3) was supplied into the medium (Fig. 1). The initial rate of NO production and the extent of apparent steady-state level strongly depended on the concentrations of azide added (Fig. 1, inset).

We next examined whether azide-dependent NO production in *A. pinnata* arises from enzymatic or non-enzymatic (chemical) reactions. The effect of enzyme inhibitors was assessed to test for the involvement of enzymatic activity in the NO production (Fig. 2). Cyanide is a strong inhibitor that binds to many metal-containing enzymes [21]. Figure 2a shows the effects of potassium cyanide (KCN) on azide-dependent NO production in *A. pinnata*. When cyanide was added to plants maintaining apparent steady-state production of NO, the NO production rapidly declined to the basal level. 5 mM KCN completely abolished the 1 mM azide-induced NO production (Fig. 2a, trace *1*). When the fronds were pretreated with cyanide (5 mM) before the addition of azide (1 mM), we observed negligible NO production, which was identical to the basal level (Fig. 2a, trace *2*). The addition of cyanide alone did not induce NO production by *A. pinnata* (data not shown).

Figure 2b demonstrates effects of the catalase inhibitor 3-aminotriazole (3-AT) on azide-dependent NO production. When 3-AT was added to plants that had reached an apparent steady-state azide-dependent NO production, there was only a little inhibitory effect of the catalase inhibitor on NO production (Fig. 2b, trace *1*). Previous studies reported that the inhibitory effect of 3-AT on catalase activity in plants becomes apparent only after a long incubation time of several hours [22]. Therefore, we incubated the plants with 3-AT (10 mM) for 15 min, 1, 3, and 5 h to evaluate its effect. In good agreement with previous reports on the inhibition of catalase, azide-dependent NO production was strongly suppressed depending on the incubation time with 3-AT (Fig. 2b, traces *2* and *3*).

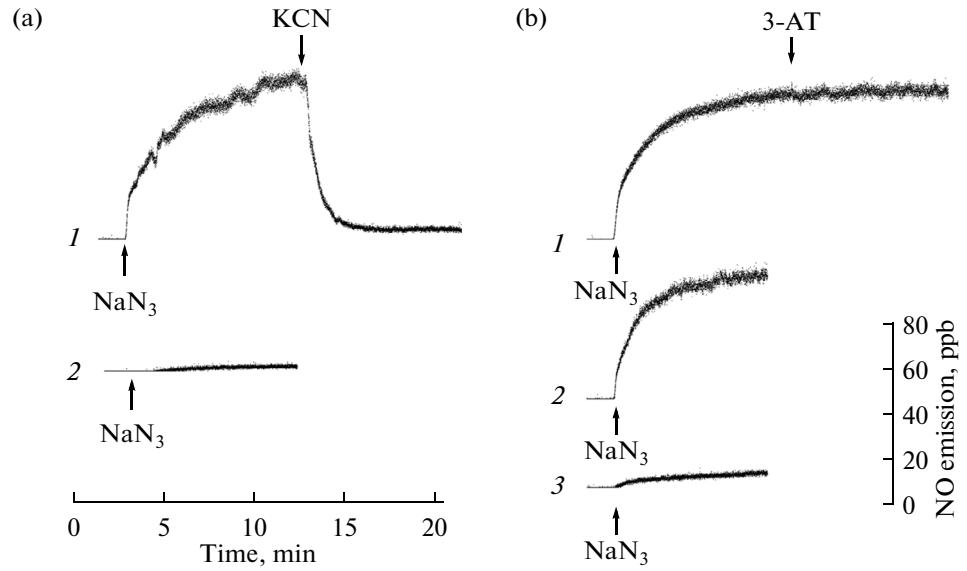

Fig. 2. Effects of enzyme inhibitors on azide-dependent NO production in *A. pinnata*.
(a) The effects of potassium cyanide (KCN) on the NO production. Trace *1*—time course of NO production induced by 1 mM NaN_3. At the arrow indicated, 5 mM KCN was added. Trace 2—NO production of the KCN-pretreated sample. Prior to the NO measurement, *A. pinnata* had been incubated with 5 mM KCN for 15 min. (b) The effects of the catalase inhibitor 3-AT on azide-dependent NO production. Trace *1*—a condition similar to those in Fig. 2 (a) trace *1* except that 3-AT (10 mM) was added instead of KCN. Trace *2*—a condition similar to those in Fig. 2 (a) trace *2* except that the 15 min pre-incubation was made with 3-AT (10 mM) instead of KCN. Trace *3*—time course of NO production in *A. pinnata* pre-incubated with 3-AT (10 mM) for 3 h. Experimental conditions were similar to those in Fig. 1.

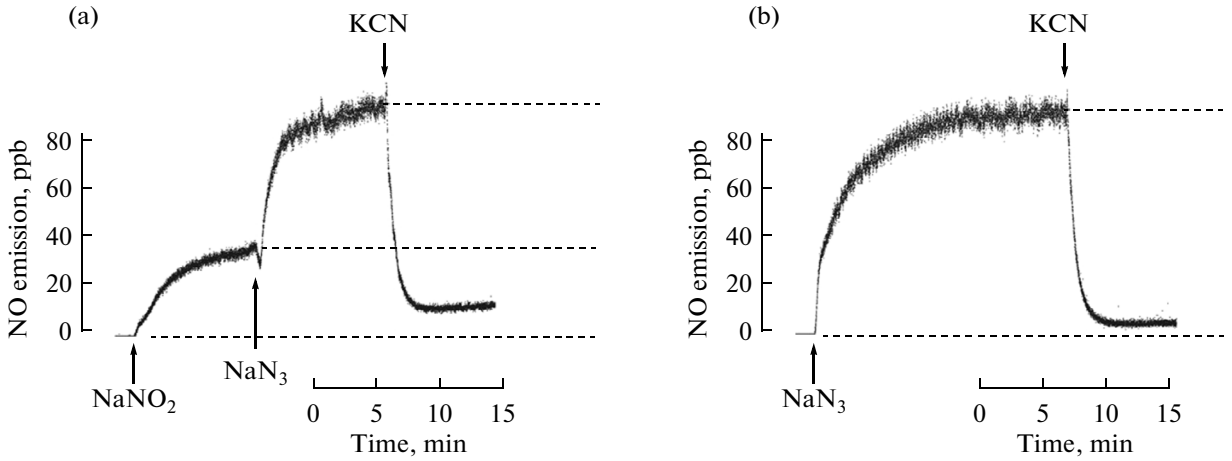

Fig. 3. Nitrite- and azide-dependent NO productions in *A. pinnata*.
(a) The effects of NaN_3 on nitrite-dependent NO production; (b) time course of NO production induced by 1 mM NaN_3 (control). The nitrite-dependent NO production was induced by the addition of 2 mM sodium nitrite ($NaNO_2$) at the arrow indicated. 1 mM NaN_3 was added when the NO production reached the apparent steady-state level. To verify that NO production was enzymatic, 5 mM KCN was added at the arrows indicated. Other experimental conditions were similar to those in Fig. 1.

Incubation of fronds with 3-AT for three hours resulted in 90% inhibition of azide-dependent NO production (Fig. 2b, trace *3*).

We previously reported that NaN_3 inhibits nitrite-dependent NO production catalyzed by the NR enzyme [4]. Thus, it appears that azide plays two opposite roles in plants, i.e., as an inhibitor and inducer of NO production. Therefore, we investigated the relationship between nitrite-dependent and azide-dependent NO production in *A. pinnata*. As has been shown in other vascular plants [5, 23], *A. pinnata* produced NO when 2 mM sodium nitrite was added (Fig. 3a), confirming the presence of the nitrite pathway for NO production in the fern [19]. Upon adding

azide to plants already producing nitrite-induced NO, the level of NO emission rose to a higher steady-state level (Fig. 3a) that was approximately the same as that reached by azide-induced plants without the presence of nitrite (Fig. 3b). Therefore, like its effect on NO production by purified NR enzyme [4], azide inhibited nitrite-induced NO production *in planta*. These results suggest that azide-dependent NO production is independent of the nitrite one; azide inhibits nitrite-dependent NR-catalyzed NO production while simultaneously inducing another route for NO production in *A. pinnata*.

DISCUSSION

In this study we have demonstrated that *A. pinnata* produces NO in response to sodium azide. To the best of our knowledge, this is the first evidence for azide-dependent NO production activity in plants. It should be noted again that the azide-dependent NO production itself is not a novel phenomenon; it has been known for several decades in animal systems. In fact, a vasorelaxant activity similar to that of nitroglycerin has been recognized; sodium azide is a classical physiological NO donor [16]. The present study confirms that plants are also capable of producing NO from azide.

Figure 2 clearly shows that the NO producing activity is completely inhibited by KCN, suggesting that the azide-dependent NO production in *A. pinnata* is due to an enzymatic reaction in plants. In mammalian systems, biotransformation from azide to NO has been ascribed to the enzyme catalase [18]. Catalase, a ubiquitous enzyme with wide phylogenic distribution, including plants, is part of an antioxidant system that detoxifies potentially harmful reactive oxygen species (ROS) produced in cells. The enzyme primarily catalyzes the dismutation reaction of hydrogen peroxide (H_2O_2) [24]. The chemical 3-AT is known to be a specific inhibitor for the catalase reaction [24]. As shown in Fig. 2b, 3-AT effectively suppressed azide-dependent NO production by *A. pinnata*. Its inhibitory effect was maximized with a long incubation time that was presumably needed for uptake of the chemical by the plant. The results agree well with previous studies that observed inhibition of plant catalase activity by 3-AT [22]. Although the identification of the catalase responding to azide awaits future research, it is logical to conclude that catalase in *A. pinnata* produces NO using azide as a substrate.

Although the reaction mechanism, by which catalase forms NO, has yet to be convincingly demonstrated, it is evident that azide acts as a physiological NO donor for plants. Other known mechanisms of NO formation are not likely to be involved in azide-dependent NO production as they have only been found to be inhibited by azide [4, 25]. The results presented in this study (azide-dependent NO production) advance our knowledge of the physiological actions of azide in plants. In plant sciences, sodium azide has been used as an enzyme inhibitor, including of NR [4]. In addition to this inhibitory action, multiple effects of azide on plants have been reported, e.g., mutagen for genetic crop improvement [26], seed germination stimulator [27], or radical scavenger [28]. It was reported that sodium azide displays a protective effect against herbicide toxicity [28], although this was not attributed to NO production. Since the azide-dependent NO producing activity found in animals has not been taken into the consideration in plant sciences, some previous interpretations of the effect of azide may need to be reconsidered in light of our finding.

Reconsideration will be also required to account for the known effects of azide on *A. pinnata*. The fern *Azolla* used in this study has been of particular interest to researchers due to its unique stress evading strategy referred to as "rapid root abscission" that is characterized by the loss of roots in response to multiple stimuli [19, 29]. The phenomenon can be induced experimentally by various types of chemicals, such as nitrite [30], sodium azide, 2,4-dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [29], and the polyamines spermine and spermidine [19]. It is yet unknown how such diverse stimuli cause an identical physiological response, namely, rapid root abscission in *Azolla* plants. Interestingly, azide is an especially strong inducer of rapid root abscission [29]. Together with the facts that nitrite and azide are substrates for plant NO production [4, 5], we consider it plausible that NO is a missing link in the root abscission mechanism to bridge diverse stimuli and chemicals. Further investigation of azide-dependent NO production in plants could shed light on such unsolved mechanisms of environmental responses.

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