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Nitric oxide: A common antipathogenic factor of plants

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In the present study, nitric oxide synthase/nitric oxide (NOS/NO) status was tested in the host plants infected with fungi, bacteria and virus. In each case cytosolic nitric oxide synthase (Cyt-NOS) of diseased plants was inhibited and inhibition was competitive in nature in respect to l-arginine, the substrate for the enzymic activity. Elevation of host nitric oxide (NO) level before infection using nitric oxide (NO) donor protected disease initiation significantly. The nature of enzyme kinetics and the manner of disease protection by nitric oxide donor (NO-donor) was similar in all the three cases of infection. It was concluded that nitric oxide was a common antipathogenic factor of plants.

Keywords: Nitric oxide/nitric oxide synthase, Plant defense

Disease resistance in plants is usually associated with different kinds of passive and active responses. Current concept of active defense mechanism includes programmed cell death (PCD)[1], hypersensitive reaction (HR)[2,3] production of different diffusible signaling molecules like salicylic acid (SA), jasmonic acid (JA), ethylene etc[4] and ultimately the establishment of systemic acquired resistance (SAR)[3,5]. Recently a new concept ie, the concept of nitric oxide (NO) has been introduced in this field[6,7]. It is a potent signaling molecule[8]. NO is well studied in animal system and was first identified as endothelial derived relaxing factor (EDRF)[9]. It is a versatile and powerful effector of animal redox regulated signaling system and immune responses[10] and it also functions as a second messenger of insulin[11]. Nitric oxide synthase (NOS) is the key enzyme for the production of NO from L-arginine in a system[10,11]. In contrast to extensive studies throughout the last two decades or more on role of NO in the mammalian immunity, recently its involvement in the plant defense system has been addressed[6,7]. Some basic analogies at the molecular level regarding the signal transduction of NO in animals to that of plants focus interest to study the involvement of this molecule in plant defense mechanism[12]. It has been reported that NO is produced by plants[6,7]. It mediates leaf expansion[13], root growth[14], phytoalexin production[15] and defense responses against pathogens[12]. In our earlier papers, we have reported the status of NOS in different healthy and diseased plants[16], impact of disease initiation on host NOS[17] and involvement of NOS in host pathogen interaction[18]. In this report we have demonstrated NO as universal effector molecule of plant defense responses. By inhibiting the host NOS activity and thereby restraining NO production, a pathogen makes its way more effective to establish a disease. Furthermore, this finding has been supported by opposite experiments where elevated level of host NO provide significant disease protection after challenging with efficient pathogen (s).

Chemicals – N^G methyl-l-arginine acetate ester (NAME) was purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade.

Collection of healthy and diseased plants – Three different host plants of *Brassica campestris* L. var. sarson Prain, *Citrus aurantifolia* Swingle and *Ammomum subulatum* Roxburg were collected from field in their healthy as well as pathologically disordered condition caused by *Alternaria brassicae* (Bark.) Sacc., *Xanthomonas citri* Hasse. and chirke (mosaic streak) virus, a fungal, bacterial and viral pathogen, respectively.

Assay of NOS activity of intact tissue and homogenates – The reaction mixture containing intact leaf tissue (100 mg) or tissue homogenate (200 – 300 μg of protein) or cytosolic fraction (150 – 200 μg of protein) or soluble particulate fraction (250 – 400 μg of protein) of both healthy and diseased sample was incubated with 10 μM of l-arginine, 64 mM of haemoglobin, in a total volume of 2.5 ml Tris – HCl buffer (pH 7.4) for different periods at 25° ± 1°C. At different time periods, portions of reaction mixture was centrifuged at 10,000 × g for 5 min and NO content of the supernatant was determined and compared with an appropriate control set.

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Determination of NO – Nitric oxide was determined according to Jia *et al.*[19] using scanning Beckman spectrophotometer (Model DU6). Typically, NO content was determined by adding 64 m*M* of oxyhemoglobin to the isolated supernatant and the formation of NO was quantified by determining the conversion of oxyhemoglobin to methemoglobin[19].

Preparation of tissue homogenate – Leaf tissue (100 mg) of diseased and healthy plants was taken separately in a morter with 1 ml of chilled 50 m*M* ofTris-HCl buffer (*p*H 7.4) and homogenized by pestle. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4°C and then the supernatant was further centrifuged at 20,000 × *g* for 30 min at 4°C for the separation of cytosolic and particulate fractions. Both the cytosolic and particulate fractions were collected separately and stored at 0°C. Before further use, particulate fraction was resuspended in equal volume of the same buffer. All the experiments were done at 4^0C. Protein was estimated using Folin-ciocalteu reagent, according to the method of Lowry *et al*[20].

Determination of enzyme kinetics – K_m and V_{max} values of the cytosolic NOS were determined by Lineweaver-Burk Plot.

Statistical analyses – Results presented as mean ± SD (Standard deviation) of at least six individual experiments. Data were analyzed by Student's *t* test and values of $P< 0.01$ were considered significant.

In each cases of pathological conditions irrespective of fungi, bacteria and virus attack, the host NOS activity of the intact tissue declined, as determined by NO production from 0.594 ± 0.027 to 0.333 ± 0.012; 0.462 ± 0.024 to 0.379 ± 0.035 and 0.810 ± 0.051 to 0.525 ± 0.033 nmole/100 g of tissue/hr, respectively when compared with healthy subjects. To determine whether the broken cell preparation could possess NOS activity, tissue homogenate and broken cells was prepared from diseased plants as described earlier and observed enzyme inhibition as found in the intact or whole tissue of disease plants. Addition of 10 μM of NAME to the reaction mixture in all cases completely inhibited NO production. Furthermore, both the particulate and soluble cytosolic fractions of healthy tissue homogenate prepared by ultracentrifugation contained detectable amount of NOS activity. But in case of diseased host only the cytosolic NOS activity was inhibited. The basal NOS activity of the cytosolic fractions of healthy plants were 1.107 ± 0.063; 1.95 ± 0.02; 2.08 ± 0.033 nmole NO produced/mg protein/hr (n=3), respectively, decreased to 0.888 ± 0.022; 1.70 ± 0.04 and 1.69 ± 0.049 nmole NO produced/mg protein/hr, respectively in case of diseased plants. In contrast, NOS activity of the particulate fraction of the host tissue was 0.490 ± 0.016; 0.908 ± 0.029 and 0.751 ± 0.087 nmole NO produced/mg protein/hr respectively at their healthy condition was likely to be changed to 0.475 ± 0.027; 0.900 ± 0.046 and 0.760 ± 0.021 nmole NO produced/mg protein/hr, respectively at their diseased condition.

Lineweaver-Bark plot of the cytosolic NOS activity of the diseased and healthy plants demonstrated that inhibition of NOS activity during diseased phase was related to the increase of K_m of l-arginine from 0.480, 0.344, 0.442 μM (n=6) to 1.0, 0.485, 0.757 μM, respectively when compared to diseased phase with simultaneous decrease of V_{max} from 1.05, 1.98 and 2.17 nmole NO produced/mg protein/hr to 0.869, 1.84 and 1.96 nmole NO produced/mg protein/hr (Table 1). Addition of 10 μM N^G nitro-l-arginine-methyl ester

Table 1 — Effect of diseased condition of the plants on NOS activity of cytosolic fraction. Cytosolic fraction of healthy and diseased plant tissue homogenate (n = 6) was incubated with different concentration of l-arginine for 1 hr. The NOS activity of the cytosolic fractions were analyzed by Lineweaver-Burk plot as described in materials and methods

Host name	Cytosolic fraction of	K_m (μM)	V_{max} (nmole NO produced/mg protein/hr)
Brassica campestris	A	0.480 ± 0.037	1.05 ± 0.05
	B	1.00 ± 0.06*	0.869 ± 0.056*
Citrus aurantifolia	A	0.344 ± 0.042	1.98 ± 0.017
	B	0.485 ± 0.036*	1.84 ± 0.016*
Ammomum subulatum	A	0.442 ± 0.047	2.17 ± 0.039
	B	0.656 ± 0.023*	2.04 ± 0.018*

A – Healthy plant; and B – Diseased plant.
*Significant at $P<0.01$

(NAME) to reaction mixture containing various concentrations of l-arginine (added for the demonstration of K_m and V_{max}) completely inhibited NO production. Durner *et al*[12] have reported that NO induces PR-1 gene expression through salicylic acid. Our study complements their findings by demonstrating the present work that pathogen might somehow be blocking the NOS activity mediating a competitive inhibition and thus, inhibiting the defense gene expression to make the plant more vulnerable for susceptibility. Interestingly, the NOS activity was found to be present in both particulate and cytosolic fraction, but results suggested that only cytosolic NOS participates in this mechanism. Further work on pathogen blocking the NOS activity is going on.

Since NOS activity was inhibited in a similar manner in each cases of infection, we tested the possibility of elevated level of NO to protect plants from disease initiation. Sodium nitroprusside (SNP) is well known for its NO generating character. Other workers[2] have also demonstrated the production of NO (~2 μM) from SNP (0.5 mM). To determine whether SNP could be substantiated for NO production of plants against infection, we experimented the effect of SNP on the progression of pathogenic activity. Administration of SNP (0.1 mg/ml) by foliar spray before 24 hr of infection protected fungal diseases up to 72% and bacterial diseases up to 65% as resulted from cell count of micro-HR detected by Evans Blue staining[21]. Since SNP did not generate NO spontaneously in aqueous medium[22], its effect was quite less. However, there are many other NO donors available which are known to generate NO spontaneously in aqueous medium[21] and it is possible that some of these NO donors may be useful for protection of the disease. Mechanism of this NO protection might be related to self amplifying character of NO as described by Klessig[4]. Therefore, NO donor can possibly be used as an antipathogenic agent and feasibility of using NO donors as a routine adjunct in the crop field can be considered. Thus, a new concept of internal scenario of diseased plants has been exposed in this paper denoting: (i) insufficient production of NO through suppression of cytosolic NOS activity: (ii) exhibition of a competitive inhibition of a cytosolic NOS; and (iii) partial protection from disease by elevating host NO level using NO donor which furnish strength to conclude that NO may play a direct protective role as a general antipathogenic molecule to resist disease occurrence in plants. Further work is in progress to evaluate the potentiality of other NO donors on disease protection in the field.

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