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Involvement of nitric oxide synthase in *Solanum tuberosum-Phytophthora infestans* interaction

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Establishment of *Phytopthora infestans* (Mount.) de B., a potent pathogen on *Solanum tuberosum* L., causes significant inhibition of nitric oxide synthase (NOS) activity, nitric oxide (NO) production. This infection inhibitable NOS activity is very specific and found only in the cytosolic fraction of plant tissue. A Lineweaver-Burk plot of the enzymatic activity demonstrated that the inhibition of the NOS activity by the pathogen was related to the increase of K_m of l-arginine, substrate of NOS, with simultaneous decrease of V_{max}. Furthermore, addition of N^G methyl-l-arginine acetate ester (NAME), a competitive inhibitor of NOS, in the reaction mixture completely inhibited NO synthesis in all cases, indicating decreased production of NO is due to the inhibition of NOS.

Key words : Nitric oxide (NO), nitric oxide synthase (NOS), plant defense.

INTRODUCTION

Nitric oxide has been attracted a great deal of attention to the scientists due to its physiological functions and general ubiquity. Nitric oxide (NO) was first identified as endothelial derived relaxing factor (EDRF) (Palmer *et al.* 1987) and it acts as a signal molecule in immune, nervous and vascular system (Schmidt *et al.* 1994) in mammals. It has also been reported as the second messenger of insulin (Kahn *et al.* 2000). Most of the works related to NO were performed in animal system. Only recently, NO was shown to be synthesized by plants (Wildt *et al.* 1997) and mediate defense responses against pathogen (Durner and Klessing, 1999). Reports reveal that NO induce leaf expansion, root growth and phytoallexin production (Leshem, 1996; Noritake et al. 1996). Nitric oxide synthase (NOS) which is the key enzyme for NO production from l-arginine, activity was found in the roots and nodules of leguminous plants, soybean cells and also from tobacco (Cueto *et al.* 1996; Durner *et al.* 1998; Mathieu *et al.* 1998). Several hypotheses have been put forwarded by scientists to explain the involvement of NO in the development of disease resistance power in plants (Dangel, 1998; Durner and Klessing, 1999; Hausladen and Stamler, 1998). Still we are at a very early stage to draw a conclusion about NO's action in plant (Durner and Klessing, 1999). In this paper we have reported the impact of disease initiation by *Phytophthora infestans* (Mount.) de B. on the activity of NOS in *Solanum tuberosum* L. plants.

MATERIALS AND METHODS

Collection of plants and identification of pathogen

Healthy and diseased plants of *Solanum tuberosum* L. were collected from the field. The causal organism of the disease i.e. *Phytophthora infestans* (Mount.) de B. was confirmed by microscopic study.

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 l-arginine, 64 mM 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 five minutes and the NO content of the supernatant was determined and compared with an appropriate

Control set.

Determination of NO

Nitric oxide was determined according to Jia *et al* (1996) by using scanning Beckman spectrophotometer (Model DU6). Typically, NO content was determined by adding 64 mM oxyhemoglobin to the isolated supernatant and the formation of NO was quantified by determining the conversion of oxyhemoglobin to methemoglobin.

Preparation of tissue homogenate

Leaf tissue (100 mg) of diseased and healthy plants was taken separately in a motor with 1ml of chilled 50 mM Tris-HCL buffer (pH 7.4) and homogenized by pestle. The homogenate was centrifuged at 10,000 × g for 30 minutes at 4°C and the supernatant was further centrifuged at 20,000 × g for 30 minutes 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°C. Protein was estimated using Folin-ciocalteu reagent, according to the method of Lowry *et al*. (1951).

Determination of enzyme kinetics

The K_m and V_{max} values of the cytosolic NOS were determined by Lineweaver-Burk Plot.

Statistical analysis

Results shown are 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.

Chemicals

N^{ω} methyl-l-arginine acetate ester (NAME) was purchased from Sigma Chemical Company, St. Lous, MO, USA. All other chemicals used were of analytical grade.

RESULTS

Results exhibited that there was a significant decrease in the production of NO by the leaf tissue of diseased set in comparison with control (healthy) set. It was found that the healthy plants showed 345 ± 19 pmol NO production/100 mg of leaf tissue/h (n=6, $p<0.001$). In the broken tissue preparation the effect was similar to that of intact tissue. The production of NO was 22% (n=10) lower than that of control set in the diseased tissue homogenate. Addition of 10 µM NAME, a competitive inhibitor of NOS, in the reaction mixture showed complete inhibition of NO production in all cases. Results revealed that both the cytosolic and particulate fractions contained NOS activity. But the infection inhibitible NOS was found only in cytosolic fraction. The NOS in particulate fraction was not affected by disease initiation. The NOS activity of healthy particulate fraction was 760 ± 35 pmol NO production / mg of protein / h and remained found to be essentially unchanged as in diseased set showing 755 ± 25 pmol NO production / mg of protein / h (n=6). In contrast, in healthy tissue the NOS activity of the cytosolic fraction was 1.29 ± 0.056 nmol NO production / mg of protein / h and decreased to 0.960 ± 0.045 nmol NO production / mg of protein / h in the diseased case (n=6).

Lineweaver-Burk Plot of NOS activity of the cytosolic fraction showed that the inhibition of NOS activity in the diseased plant was related to the increase of K_m of l-arginine from 0.370 ± 0.037 µM to 0.819 ± 0.067 µM (n=6) when compared to control, with simultaneous decrease of V_{max} from 1.052 ± 0.031 nmol NO synthesized / mg of protein / h to 0.869 ± 0.026 nmol NO synthesized / mg of protein / h in the cytosolic preparation of diseased plant (Figure 1). Addition of 10 µM NAME to the reaction mixture containing various concentrations of l-arginine added for the determination of K_m and V_{max} resulted in complete inhibition of NO production.

CONCLUSION

Our results suggest that establishment of *Phytophthora infestans* (Mount.) de B. on *Solanum tuberosum* L. caused a significant inhibition of NOS activity or NO production. This 'specific' infection inhibitible NOS is cytosolic in nature. A Lineweaver-Burk plot of the enzymatic activity demonstrated that the inhibition of the NOS activity by the pathogen was related to the increase of K_m of l-arginine, substrate of NOS, with simultaneous decrease of V_{max}. Furthermore, the inhibitory effect of NAME in the NO production in all the cases indicated that the

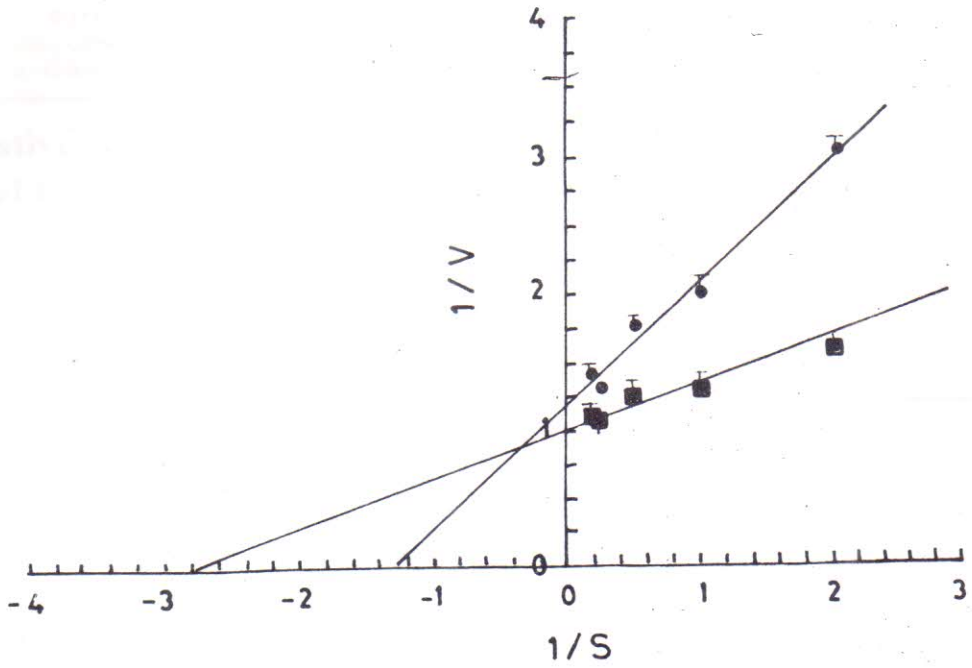

Fig. 1. Line weaver-Burk plot of Nitric oxide synthase activity of cytosolic fraction of diseased and healthy plants.

decreased production of NO by the diseased plants is due to the inhibition of NOS activity by the pathogen. All the above results led us to speculate that pathogen probably makes a plant susceptible by lowering or particularly blocking NOS activity, causing decreased production of NO which is one of the signaling molecule of plant defense. Further work is in progress to determine the status of NOS activity during bacterial and viral infection.

REFERENCES

Cueto, M., Hernandez-Perera, O., Martin, R., Benchura, M.L., Rodrigo, J., Lamas, S. and Geolvano, M.P. (1996). Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. *FEBS Letts.* **398** : 159 – 164.

Dangel, J. (1998). Plant just say NO to pathogen. *Nature* **394,** 525 – 527.

Durner, J. and Klessing, D.F. (1999). Nitric oxide as a signal in plants. *Curr. Opinion Plant Biol.* **2,** 369 – 374.

Durner, J., Wendehenne, D. and Klessing D.F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP ribose. *Proc. Natl. Acad. Sci. USA* **95** : 10328 – 10333.

Jia, L., Bonaventura, C., Bonaventura, J. and Stamler, J.S. (1996). S – nitrosohemoglobin: a dynamic activity of blood involved in vascular control. *Nature* **380** : 221 – 226.

Hausladen, A. and Stamler, S.J. (1998). Nitric oxide in plant immunity. *Proc. Natl. Acad. Sci. USA* **95:** 10345 – 10347.

Kahn, N.N., Acharya, K., Bhattacharya, S., Acharya, R., Mazumder, S., Bauman, W. A. and Sinha, A.K. (2000). Nitric oxide: the "second messenger" of insulin. *IUBMB Life* **49** : 441 – 450.

Leshem, Y.Y. (1996). Nitric oxide in biological system. *Plant growth regul.* **18** : 155 – 159.

Lowry, O. H., Rosebrough, N.J., Farr, A.L. and Randel, R.J. (1951). Protein measurement with folin – phenol reagent. *J. Biol. Chem.* **193** : 265 – 275.

Mathieu, C., Moreau, S., Frendo, P., Puppo, A. and Davies, M.J. (1998). Direct detection of radicals in intact nodules: presence of nitric oxide – leghemoglobin complexes. *Free Rad. Biol. Med.* **24** : 1242 – 1249.

Noritake, T., Kawakita, K. and Doke, N. (1996). Nitric oxide induces phytoalexin accumulation in potato tuber tissue. *Plant Cell Physiol.* **37** : 113 – 116.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium factor. *Nature* **327** : 524 – 526.

Schmidt, H.H.H.W., Lohmann, S.M. and Walter, U. (1993). The nitric oxide and cGMP signal transduction system-regulation and mechanism of action. *Biochim Biophys. Acta.* **1178** : 153 – 175.

Wildt, J., Kley, D., Rockel, A., Rockel, P. and Segschneider, H.J. (1997). Emission of NO from higher plant species. *J. Geophys. Res.* **102** : 5919 – 5927.

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