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Effect of L-Arginine and Its Analogues on the Hypersensitive Response of Tobacco (*Nicotiana tabacum* L. cv. TT-5) Induced by Avirulent Ps61$^-$ Strain of *Ralstonia solanacearum*

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ABSTRACT

Chou, C. C.[1], Lee, M.H., Tzeng, K. C., Tzeng, D. D. S.[1,2] Effect of L-Arginine and Its Analogues on the Hypersensitive Response of Tobacco (*Nicotiana tabacum* L. cv. TT-5) Induced by Avirulent Ps61$^-$ Strain of *Ralstonia solanacearum*. ([1]Department of Plant Pathology, National Chung-Hsing, University, Taichung, Taiwan, R.O.C.; [2]Corresponding author, Email: dstzeng@nchu.edu.tw; Fax: +886-4-22851038)

An L-arginine enhancement effect on hypersensitive response (HR) was observed in tobacco (*Nicotinana tabacum* cv. TT-5) challenge-inoculated with an avirulent Ps61$^-$ strain of *Ralstonia solanacearum*. The enhancement effect was dosage dependent, and symptom expression appeared to be a function of the challenging bacteria. The effect of arginine became prominent at concentration greater than 1 mM, which was manifested by the development of earlier and severer necrotic HR symptoms compared to that of the controls. The accelerated necrogenic response reflected the functioning of a promoted active defense triggered by the challenging bacterium. However, there was no indication of increased toxigenic effect since the rate of population decline of Ps61$^-$ during the course remained nearly unchanged when treated with L-arginine. In contrast to the arginine promoting effect, the application of arginine analogues (NMA, NAME or canavanine), and guanidino compounds (methyl-guanidine or aminoguanidine), greatly retarded and reduced the necrotic response. Canavanine and aminoguanidine were among them the most effective, whereas the supplementation of L-arginine reversed the inhibitory effect of canavanine. The observed enhancement effect of arginine and the differential inhibitory activity of arginine analogues and guanidine compounds suggested that the role of an inducible nitric oxide synthase may play a role in the studied HR in tobacco.

Key words: L-arginine, hypersensitive reaction, nitric oxide.

Abbreviations: HR (hypersensitive reaction), NAME (N-nitro-arginine methyl ester), NMA (N-methyl-arginine), NO (nitric oxide radical), ROS (reactive oxygen species), TTC (2,3,5-triphenyltetrazolium chloride), SDW (sterile distilled water)

INTRODUCTION

The importance of hypersensitive response (HR) in plant resistance against invasion by an incompatible pathogen was well documented. The main characteristic of HR is the quick development of localized necrosis resulted from rapid cell death. The attempted pathogen is thus constrained in the infection sites where series of accompanied defense mechanisms are activated [15]. The phenomenon of HR has been known for decades. Although large efforts have been devoted to explore its mode of action, the involved biochemical basis remained largely unknown. As regards to the rapid cell death in HR, production of reactive oxygen species (ROS) was shown to be one of the critical factors involved [3, 11, 12, 13, 25, 26]. The implicated role of ROS includes the peroxidation which contributes to killing of pathogen with the expense of certain damages on plant host and the cellular signaling which triggers a battery of defensive gene expression (cell wall lignification, PR protein, phytoalexin, etc.). The exclusive role of ROS in HR related cell death, however, was discouraged by quite a few contradictory evidences. The prevention of membrane peroxidation of soybean cells by butylated hydroxytoluene, for example, did not contribute to protection of HR related cell death [42]. Likewise, the requirement of salicylic acid, the well recognized secondary signal leading to systemic acquired resistance, in HR cell death was found not necessarily due to the inhibition of catalase activity [42]. Furthermore, Glezener et al. [14] demonstrated that an incompatible pathogen induced ROS surge may not lead to HR related cell death. The contributing factor(s) of HR cell death remains to be explored.

In active defense of mammalians, arginine-derived nitric oxide radical (NO) production has been proven to be an essential factor working synergistically with ROS in killing an invading pathogen [38]. The discovery of this biosynthetic pathway of NO led to active research and many important discoveries during the last decade regarding to its great significance in biological system. The determinative physiological role of NO in endothelial membrane relaxation, neural transmission, and immuno-defenses are now well recognized in medical science. Up to now, in addition to that in mammalian system, the arginine-derived NO production has been demonstrated in a fairly wide spectrum of biological systems including insects, fungi, and bacteria [25]. The wide distribution indicates that the biosynthetic activity one of the well conserved physiological functions among most living entities. Unlike that in animal system, knowledge regarding to the production of NO and its possible physiological function in plant system is very limited. The production of NO appears to be a function associated with nitrogen fixation; the production of NO was demonstrated from leaf [9, 24, 30] as well as root nodules [21, 34], respectively. Convincing evidence had indicated the presence of NO synthase (NOS) activity in plant tissue [8; 10; 13, 29, 36, 41]. The explicit role of NO in plant defense was indicated by the facts that exogenous application of NO induced serial defense gene expression in tobacco [10]. An increased NOS like activity was detected from tobacco during the hypersensitive response (HR) development in *Ralstonia solanacearum*-tobacco interactions [18, 19]. A pathogen inducible NOS-like enzyme (iNOS) was detected from *Arabidopsis thaliana* and tobacco [4], and the elicitor treatment induced NO burst and subsequent defense gene expression in tobacco [20]. The growing evidence seems to suggest NO as a signal molecule which interacts with the salicylic acid and jasmonic acid signalling pathways [45]. Unlike that of animal system wherein NOS-mediated function appears to be the primary source of NO production, it was noted that plant might also produce NO through the function of a constitutive NAD(P)H nitrate reductase (NR) [9]. Yamamoto *et al.* [46] recently demonstrated that treatment of potato tubers with the *Phytophthora infestans* elicitor hyphal wall components induced the NR gene at the transcriptional and protein level. The determinative role of NO as a signal molecule contributing to defense reaction has been primed focus of research in plant pathology during the past decade.

The main objective of this investigation was to learn whether the arginine-derived NO production is a factor involved in HR derived cell death of plant. As above mentioned, an increased NOS activity was demonstrated in tobacco undergoing HR [18]. In HR of tobacco (*Nicotiana tabacum* L. cv.TT-5) triggered by challenge-inoculation with an incompatible *Ralstonia solanacearum* Ps61^{-} strain, we found that the supplementation of arginine greatly stimulated the development of necrotic symptoms. The arginine promoting effect was inhibited by the addition of arginine analogues and guanidino compounds. We discuss herein the significance of the arginine enhancement on plant HR and its implication regarding to arginine-derived NO production in plant active defense.

MATERIALS AND METHODS

Chemicals

L-arginine, NMA, NAME, canavanine, methylguanidine, and aminoguanidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals including TTC, hydrogen chloride, sodium hydrochloride were obtained from Merck (Damstadt, Germany). Freshly made doubled distilled water was used for the preparation

of medium and reagents.

Preparation of bacterial inocula

The Ps61 strain of *Ralstonia solanacearum* (Smith 1896), originally isolated from tomato plant, was obtained from the Bacteriology Laboratory of the Plant Pathology Department at National Chung Hsing University. The bacterium was stored in sterile distilled water in screw cap tubes at room temperature. For each test, the bacterial suspension was streaked on TTC medium of Kelman [22] and allowed to grow at 30℃ for 48 hours. The avirulent mutant (Ps61$^-$) colony, typically smooth circular, mucoid and dark red in morphology, was transferred to 523 agar slants. After 24 hours, sterile distilled water was added to each slant cultures to suspend the bacteria. The suspension was adjusted to A_{620} = 0.3 (about $5X10^8$ cfu/ml) and used as inoculum for the proceeded experiment. Bacterial suspensions with other concentrations were made by proper dilution from the above suspension.

Infiltration-inoculation of test plants

Nicotiana tabacum cv. TT-5, a tobacco cultivar bred in Taiwan with moderate resistance to *R. solanacearum* [5] was used for the experiment. The test plants were grown in plastic pots (5 inches in diameter) in a greenhouse with natural sunlight. For the inoculation trials, the test plants were transferred to a greenhouse with temperature setting at 24-28℃. The second and third fully developed leaves of the 8 to 10 week-old plants were punctured with a needle and infiltrated with the bacterial suspension according to that described by Lin et al. [32]. For the temperature effect trial where that different setting of temperature was required, the detached leaf system was used instead. The second and third leaves were cut off from the greenhouse grown plants with a sharp razor blade and were kept in 500 ml beakers each separately for maintaining water supply. The leaves were kept in incubator(s) with the same temperature setting for 2-4 hours before the infiltration inoculation test was performed.

Effect of arginine and its analogues on HR

L-Arginine, arginine analogues [NAME, NMA, and canavanine (can)], and guanidino compounds (aminoguanidine and methylguanidine) were each applied together with the bacteria for inoculation. All the test chemicals were adjusted to pH 7.0 with 0.1N NaOH or HCl right before mixing with the bacterial suspension. To avoid the artifact due to diffusion of applied chemicals, each test leaf was used only once.

Necrotic index determination

The necrogenic responses of Ps61$^-$-inoculated leaves undergoing HR process were assessed as that described by Wang et al. [44]. Necrosis severity was rated at six levels based on the proportion (%) of infiltration inoculated area which became necrotic: 0, no necrosis whereas the treated area generally became chlorotic; 1, <20%; 2, 20-40%; 3, 41-60%; 4, 61-80%; and 5, >80% of the inoculated area became necrotic. The necrotic index was calculated by the following equation: necrotic index = Σ[(no. of necrotic rating) $\times$(nos. of plants at the same rating)]/total number of test plants.

Determination of bacterial survival in test leaves undergoing HR

Following a time course after inoculation, leaf discs (approximately 6 mm in diameter) were sampled from the inoculated region with a stainless cork borer. A total of 10 discs from each treatment were homogenized with 1ml sterile distilled water by a pestle and mortar. The homogenates were then diluted in 10 X series and thereafter plated on TTC medium [22]. Numbers of bacterial colonies developed were counted 48 hrs after incubation at 30 ℃.

RESULTS

Effect of arginine supplementation on HR symptom development

Upon infiltration inoculation, all the tested pot-grown plants started to show typical HR symptoms on leaves 12 hrs after treatment (Fig. 1). The addition of arginine appeared to enhance the necrotic symptom development. The effect of arginine was dose-dependent at the concentration ranged from 0-10 mM. The enhancement effect was manifested by the fact that the observable symptoms developed earlier and appeared to be severer (data not shown). At 35 hours after inoculation, a 100% necrotic rate of necrogenic response was observed among treatments with more than 5 mM arginine supplementation, whereas that on the compared control treatment was only about 60%. In parallel to the experiment, arginine at the tested range of concentrations were also applied to an extra set of tobacco plants without the challenging bacterium as an additional control, none of these plants showed any symptoms (data not shown).

Effect of inoculum concentration on arginine effectiveness

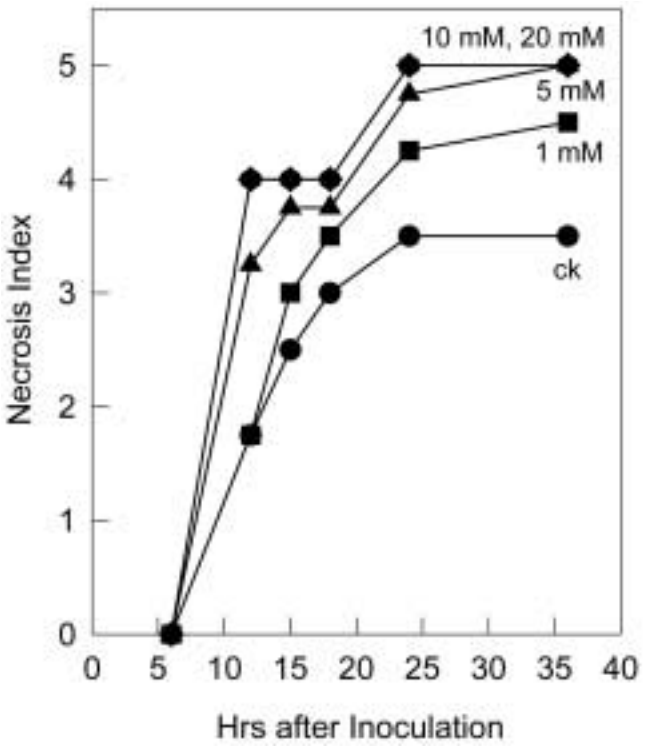

Fig. 1. Effect of L-arginine supplementation on the necrotic response of tobacco leaf (*Nicotiana tabacum* cv. TT-5) upon infiltration inoculation with the avirulent Ps61- isolate of *Ralstonia solanacearum*. L-arginine at 1 (■), 5 (▲), 10 (▼) and 20 mM (◆), respectively, were added to the bacterial suspension (final concentration $5X10^8$ cfu/ml) right before inoculation; the control plants (ck, ●) were inoculated without L-arginine addition. The test plants were then incubated at 24-28 ℃. Data shown were mean of 6 replicates.

Leaves of pot-grown tobacco were infiltrated with the test bacteria at concentration ranging from 3.5×10^7 to 2.9×10^8 cfu/ml. Arginine was added to each test inoculum (final concentration 1 mM) as above described. The symptom development 14 hrs after inoculation indicated that the arginine enhancement on the necrogenic response was evident among all treatments with inoculum concentration greater than 7.0×10^7 cfu/ml (Fig. 2). The necrotic rating among these leaves all exceeded 60% of the inoculated region, while those among the compared control leaves were all less than 40%. In leaves treated with 3.5×10^7 cfu/ml bacterial suspension, although a slight enhancement of necrotic response was observed, the effectiveness was not significant.

Effect of arginine treatment on survival of bacteria in leaf tissue undergoing HR

Following a time course, the bacterial population in the inoculated region was monitored when 5 mM arginine were added while performing inoculation. The necrogenic response started about 6-8 hrs after inoculation. Significant decline of bacteria numbers in these tissues, however, was not evident up to 8-12 hrs after inoculation (Fig. 3). Moreover, the rate of population decline with arginine

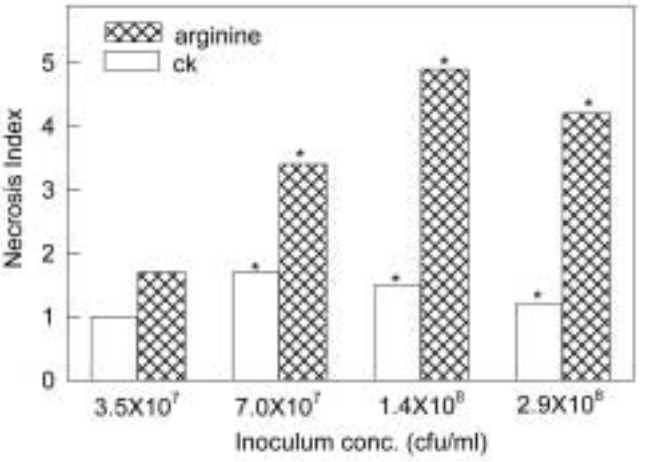

Fig. 2. Effect of inoculum concentration on the arginine-promoted hypersensitive necrogenic response of tobacco (*N. tabacum* cv. TT-5) leaves challenge-inoculated with Ps61- isolate of *Ralstonia solanacearum*. L-arginine (1 mM) was applied together with the bacteria adjusted to the indicated concentration. The control treatments (ck) were inoculated without arginine amendment. Data shown were mean necrosis index of 10 replicates determined 14 h after inoculation. "*" indicates significant difference by Student t-test. (P<0.05, n=10).

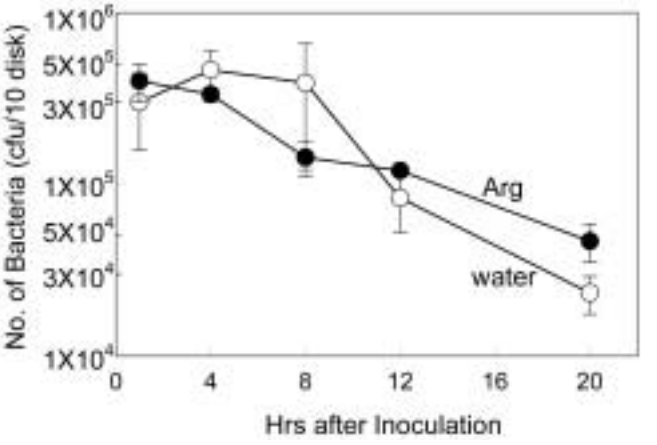

Fig 3. Effect of a 5 mM L-arginine supplementation (Arg) on the population dynamic of *Ralstonia solanacearum* ($Ps61^-$ isolate, adjusted to 5×10^8 cfu/ml) in tobacco (*Nicotiana tobacum* cv. TT-5) leaf tissue in which an enhanced HR process was observed. The control (ck) leaves were treated with only test bacteria without L-arginine supplementation. Data presented were average counts of colony forming units detected from 10 leaf disks (6 mm in diameter) at the indicated sampling time.

treatment appeared to be similar to that of water treated control. Throughout a 20-hr experimental period, the bacterial concentration within the inoculated tissue of both arginine supplemented and control treatment were both reduced by approximately 10-fold.

Effect of nitric oxide synthase inhibitors on HR

The addition of the arginine analogues NAME, NMA and canavanine each at 20 mM resulted in reduction of the HR-derived necrosis. On control leaves, HR symptoms became visible about 6-8 hrs after inoculation, which then very rapidly reached the top scale severity at 10 hrs after inoculation (Fig. 4). The 3 arginine analogues were all inhibitory to the bacteria induced HR; and among them canavanine appeared to be the most effective. The observable initiative HR symptom on canavanine treated leaves was detected not until 14 hrs after inoculation. Symptoms developed on these leaves then remained very mild throughout the experimental period; no visible necrotic response was detected until 24 hrs after inoculation. The addition of NMA and NAME was in the order less effective in alleviating the HR symptom development. The effectiveness was clearly shown by the time delay for and the final outcome of the symptom development. In order to know whether or not the observed canavanine effect was due to its competition with arginine for NOS, a 10 mM arginine was applied together with 20 mM canavanine to the inoculum while doing inoculation. The leaves treated solely by arginine (10 mM) or canavanine (20 mM) served as the compared control to show the competitive effect. As shown in Fig. 5, the enhancement effect of arginine and the inhibitory effect of canavanine were similar to that shown above; whereas the addition of arginine greatly reduced the inhibitory effect of canavanine on HR symptom development. The symptom development on leaves treated with canavanine-arginine mixture (can+Arg) was comparable to that of control (ck) treatment in which no amendment was added to the inoculum.

Aminoguanidine and methylguanidine each at 0.1, 1, and 10 mM, respectively, were added to the test inoculum for the evaluation of their effectiveness on the HR symptom development. Both aminoguanidine and methylguanidine appeared to delay the time of and reduce the severity of the HR symptom development (Fig. 6). The inhibitory effect was dose dependent. For aminoguanidine, more than 1 mM seemed to be required, whereas for methylguanidine, the effectiveness by 0.1 mM treatment appeared to be similar to that by 1 mM treatment.

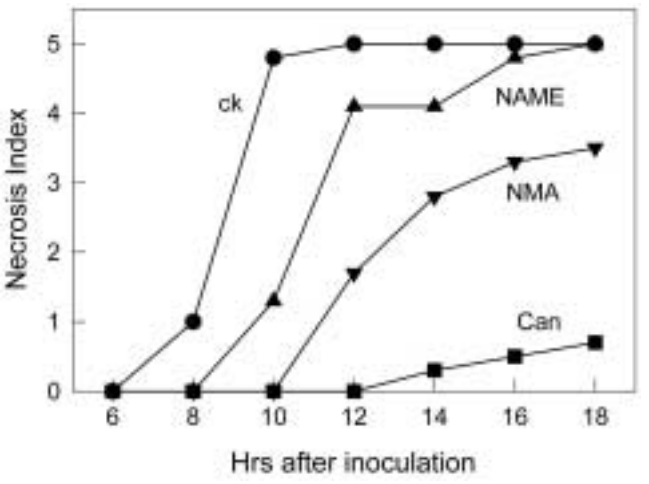

Fig. 4. Effect of N-nitro-L-arginine methyl ester (NAME, ▲), N-methyl-L-arginine (NMA, ▼) and canavanine (can, ■), on the hypersensitive necrogenic response of tobacco (*Nicotiana tobacum* cv. TT-5) leaves infiltration inoculated with Ps61$^-$ isolate of *Ralstonia solanacearum* (5×10^8 cfu/ml). The L-arginine analogues each at 20 mM were applied together with the bacterial suspension while doing inoculation. The control (ck, ●) leaves were treated with only bacteria suspension. Data presented were mean of 8 replicates.

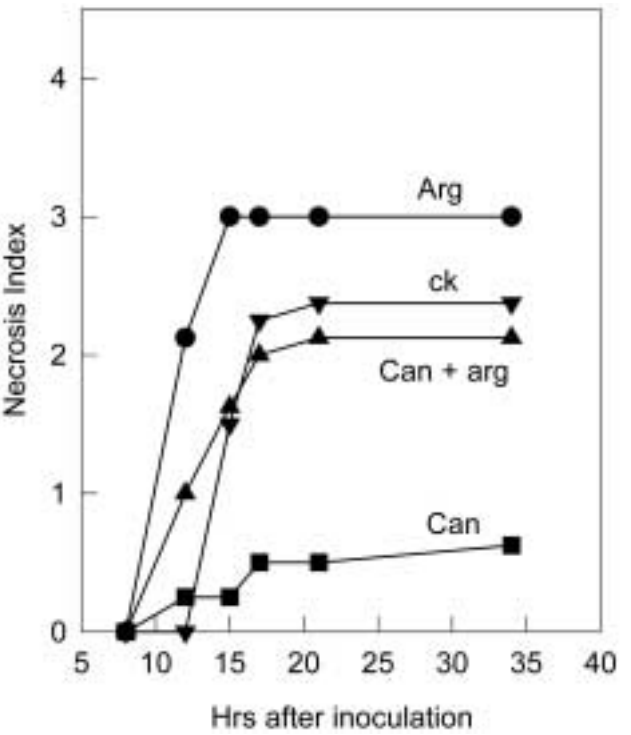

Fig. 5. Effect of L-arginine (Arg, 5 mM) supplementation in reversing the effect of canavanine (Can, 20 mM) inhibition on the hypersensitive necrogenic response of tobacco (*Nicotiana tobaccum* cv. TT-5) caused by artificial inoculation with Ps61$^-$ isolate of *Ralstonia solanacearum* (5×10^8 cfu/ml). The control leaves (ck) were treated with only test bacteria. Data shown were mean of 8 replicates.

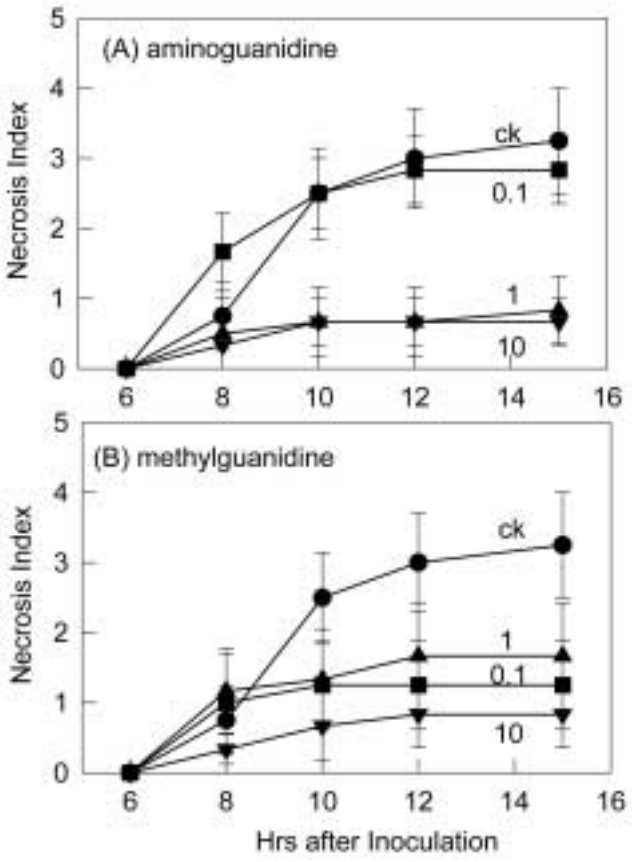

Fig. 6. Effect of aminoguanidine (A) and methylguanidine (B) supplementation (each at 0.1, 1 and 10 mM, respectively) on the hypersensitive necrogenic response of tobacco (*Nicotiana tobaccum* cv. TT-5) leaves infiltration inoculated with Ps61$^-$ isolate of *Ralstonia solanacearum* (5 $\times 10^8$ cfu/ml). The chemicals were applied together with the bacterial suspension while doing inoculation. The control (ck) leaves were treated with only bacterial suspension. Data presented were mean of 8 replicates.

DISCUSSION

Arginine is an essential amino acid in most living organisms. Important physiological functions known for arginine participation include protein synthesis, polyamine synthesis, nitric oxide synthesis, and the urea cycle metabolism. The dose-dependent stimulatory effect of arginine supplementation on necrogenic response of tested tobacco challenged with Ps61- bacteria (Fig. 1) clearly indicated the production of certain cell lethal metabolites. Using epidermal peel of tobacco (*Nicotiana tabacum* cv. *Xanthi*) leaf, Allan and Fluhr [1] demonstrated that application of 1 mM arginine by itself may lead to increased ROS production via the function of the ubiquitous cell wall amine oxidases, and the cellular killing activity was thus observed. For the tobacco cultivar (cv. TT-5) used in this investigation, either the whole plant or the detached leaf system, the infiltration application of arginine alone at the tested concentration (1 to 20 mM) did not show any damaging effects on the tobacco tissues throughout the test (data not shown). The enhanced killing on tested plant cell was apparently dependent on the presence of the challenging bacteria Ps61$^-$. A plausible explanation for the discrepancy between the two works appeared to be the different cultivars and different experimental systems applied. As arginine is a ready precursor for NO biosynthesis, the possible involvement of both amine oxidase-mediated ROS production and NOS-mediated NO production were both likely reasons responsible for the observed necrogenic response enhancement. The dose-response effect of arginine shown in this experiment implicated the production of NO a likely cause of the enhanced necrogenic response.

In the studied HR model system, the main contribution of challenging bacteria was obviously the provision of elicitor that triggers the hypersensitivity. For *R. solanacearum*, involvement of harpin in eliciting HR has been a popular researched subject [2]. The effect of inoculum concentration on arginine efficacy shown in Fig. 2 suggested the need of an optimized elicitor concentration for better signal transduction and the resulted gene expression. The development of rapid necrogenic response among the host tissue undergoing HR reflected the suicidal reactivity of host cells and by that invasion of the attempted pathogen was stopped. An obvious reason for the stopped invasion was the production of certain microbicidal metabolite and a reduction of the bacterial population during the course of reaction was anticipated. The reduction of viable bacterial propagules after HR symptom became conspicuous shown in Fig. 3 simply indicated this fact. In active defense of mammalian, the microbicidal reactivity was known to be a function of the synergistic effect of ROS and NO generated during the incompatible host parasite interaction [31]. For active defense of plant system, convincing evidence has demonstrated the involvement of ROS [11, 12, 23]. In the tested system, the supplementation of arginine was expected to increase NO-mediated reactivity and thus a faster decline of the applied bacteria. The results shown in Fig. 3, however, indicated that might not be true. In regarding to cellular toxicity of ROS, the participation of transition metal is critical for the production of primary toxicant hydroxyl radical via the Haber Weiss reaction. The damage incurred thus resides mainly upon the availability and the specific-location of transition metals rather than the concentration of ROS [43]. For tobacco tissues undergoing HR, malfunctioning of the plasmalemma proton pump and thus the cytoplasmic acidification appears to be one of the earliest responses commonly observed. Whether or not this

led to the increased release of transition metals from their binding posts (eg. transferitin, etc) and thus making the affected plant cells more vulnerable to oxidative damage remained to be illustrated.

The arginine-derived NO production is a function of NOS activity as demonstrated in animal system. As NO was supposedly derived from the guanidino group of arginine [33, 39], various arginine analogues and guanidino group containing chemicals were often applied as competitive inhibitor for the enzymatic function to illustrate the involvement of NOS in a physiological or pathological event [7, 25]. The same methodology has recently also been successfully applied in potato, tobacco, soybean, arabidopsis, lupin, pea, *Mucuna hassjoo*, as well as *Neurospora crassa*, to demonstrate the participation of NOS in certain physiological and pathological function [8; 10; 13, 28, 30, 36, 37]. The inhibitory effect of NMA, NAME, canavanine, aminoguanidine, and methylguanidine on HR symptom development (Figs. 4, 5 and 6) provided evidence that the NOS-mediated NO production like that in mammalian was also functioning in the tested HR reaction system. The reversing effect of arginine on canavanine inhibition (Fig. 5) clearly indicated the functioning of substrate competition as a likely reason for the observed inhibitory effect. As regard to the efficacy of these chemical inhibitors, canavanine and aminoguanidine were both known to be potent inhibitors specifically for iNOS (inducible iso-form) [7, 25]; although canavanine was also known to be an inhibitor to all enzymes relating to arginine metabolism [33, 40]. Moreover, NMA was known to be an inhibitor for all iso-forms of NOS including eNOS (endothelium), bNOS (brain) and iNOS [16, 17, 27, 35]. And NAME was known to have stronger inhibition on eNOS or neuron NOS rather than iNOS [16]. The superior inhibitory efficacy of canavanine and aminoguanidine on HR symptom development (Figs. 4 and 6) implied the importance of iNOS-like enzyme in the observed tissue damage. The better efficacy of NMA as compared to that of NAME also supported this view. The critical importance of iNOS in active defense of mammalian is well documented [7, 17]. The evidence herein provided suggest the alike mode of action may be also functioning in higher plants. During the course of this study, efforts have also been devoted to explore the molecular basis of NO production during HR symptom development. The production of NO has been demonstrated from the studied incompatible host-parasite interaction by chemical reaction, nitric oxide analyzer, as well as electron spin resonance spectroscopy [6]. The possible involvement of NO in the incompatible plant-pathogen interaction will be further addressed.

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摘 要

周俊吉、李敏惠、曾國欽、曾德賜 2006. 精氨酸與其結構類似物對無毒力青枯病菌 (*Ralstonia solanacearum*) Ps61$^-$ 菌株在菸草所誘發過敏性反應之影響。植病會刊 15:97-105 (國立中興大學植物病理學系；[1]聯絡作者，電話：886-4-2851038；傳眞：886-4-2851038；電子郵件：dstzeng@dragon.nchu.edu.tw)

在以無毒力青枯病菌 (*Ralstonia solanacearum*) Ps61$^-$ 菌株行人工接種菸草 (*Nicotinana tabacum* cv. TT-5) 植株的同時，精氨酸 (L-arginine) 的添加對所誘發的過敏性反應具有明顯的促進作用。此一促進現象明顯與添加濃度有關，其病徵表現明顯爲接種細菌的作用所造成；精氨酸的影響在添加濃度達 1 mM 以上即可明顯看出，與未添加處理之對照組比較，其主要可明顯提高過敏性反應壞疽病徵的嚴重程度。壞疽病徵的加速，顯示由接種細菌 Ps61$^-$ 菌株所誘發的主動防衛反應有受到促進的現象，然此並非其對病原毒害作用的強化，在反應過程中，精氨酸的添加對 Ps61$^-$ 菌株在寄主體內族群的衰減與對照處理比較並無明顯差異。相對於精氨酸添加的促進作用，添加包括甲基精氨酸 (NMA)、硝基精氨酸甲基酯 (NAME) 與刀豆氨基酸 (canavanine) 等精氨酸結構類似物，以及 methyl-guanidine 或 aminoguanidine 等亞氨基單二胍類 (guanidine) 複合物，均可使此一過敏性反應壞疽病徵的顯現大爲延遲與減輕，其中並以刀豆氨基酸與 aminoguanidine 的添加抑制效果最爲明顯，而精氨酸的添加則可回復刀豆氨基酸添加所顯現的抑制效果。由試驗中所見精氨酸的添加對過敏性反應的明顯促進作用，以及不同精氨酸結構類似物與亞氨基單二胍類複合物的添加對病徵表現所顯現的差異性抑制效果顯示，在供試菸草之過敏性反應中，誘導型一氧化氮自由基生合成酶的參與作用值得進一步瞭解。