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INFLUENCE OF SUPEROXIDE ANION AND HYDROGEN PEROXIDE IN THE HYPERSENSITIVE REACTION AND THE BACTERIAL MULTIPLICATION OF BEAN/ *Pseudomonas syringae pv. phaseolicola* INTERACTION

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ABSTRACT

The role of the oxidative burst, transient production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), in elicitation of hypersensitive reaction (HR) was investigated in bean (*Phaseolus vulgaris* L. cv. Bronco) inoculated with compatible, wild type strain (WT), or incompatible strain (Tn5 mutant) of *Pseudomanas syringae* pv. *phaseolicola* (Burkholder). Inoculation with the WT strain caused water-soaked lesions, while Tn5 strain elicited HR necrosis started to appear 12 h post-inoculation.

Biochemical parameters, such as highly increase of the conductivity (electrolyte leakage), which is a well-known indicator of permeability changes associated with HR, was found in the incompatible bean/bacteria (Tn5 strain) interaction. The conductivity of this incompatible combination was early induced and markedly increased, which recorded 1.5 & 6.25-times higher than the control at 4 & 32 h post-inoculation, respectively. Also, lipid peroxidation, which is a well-known parameter of HR development, was markedly increased in the incompatible bean/bacteria interaction. It recorded, approximately, 3-times higher than the control treatment at 16, 20, & 24 h and almost 4-times at 32, 36 h post-inoculation.

It was a highly reduction in the growth rate of the Tn5 bacterial strain as compared to that in the compatible combination, WT strain, in bean leaf tissues as a result of superoxide anion and hydrogen peroxide generation. Multiplication of Tn5 strain slightly increased by 1.3 and 0.5 log, however WT strain increased by 4.6 and 5.6 log at 24 and 48 h post-inoculation, respectively.

Either $O_2^{\cdot-}$ or H_2O_2 were highly significant generated in bean leaf tissues infiltrated with the incompatible, Tn5 bacterial strain, as compared with both, tissues infiltrated with the compatible WT strain or control, at all time detection. $O_2^{\cdot-}$ level in tissue, infiltrated with Tn5 strain, reached 320 & 464% of the control treatment at 6 & 24 h, respectively, post-infiltration. However, H_2O_2 level recorded 311 & 540% of the control values at 6 & 36 h, respectively post-infiltration.

These results suggest that an oxidative burst consisting of, at least, $O_2^{\cdot-}$ and H_2O_2 occur during the hypersensitive defense response, and may be it is influenced in HR necrotization, as well as counteracted the bacterial multiplication in bean leaf tissues.

Keywords: Hydrogen peroxide (H_2O_2), hypersensitive reaction (HR), Lipid peroxidation, *Phaseolus vulgaris*, *Pseudomanas syringae* pv. *Phaseolicola*, and superoxide anion ($O_2^{\cdot-}$).

INTRODUCTION

Pseudomanas syringae pv. *phaseolicola* (Burkholder) Young *et al.* causes water-soaked disease spots in bean *Phaseolus vulgaris* L., but induces a hypersensitive reaction (HR) in non-host plant species (Klement,

1971). However, Somlyai, *et al.*, (1986), isolated some transposed mutants which are not able to incite diseases symptoms in bean plants, but induced HR (Hri$^+$ Path$^-$ mutants).

Hypersensitivity is a complex response occurring in plants that are challenged by incompatible micro-organisms (Anderson, 1988). Changes in membrane potential and ion permeability occur during the hypersensitive response caused by incompatible bacteria (Atkenson *et al.*, 1985 and Pavlovkin *et al.*, 1986). Changes in this flux may result from damage to the plasmalemma caused by lipid peroxidation, an event documented to occur in elicitor-treated tissue (Rogers, 1988) or in hypersensitivity when triggered by bacteria (Adam *et al.*, 1989 and Keppler & Novacky, 1987). Király *et al.* (1991), summarized that, elicitation of rapid plant cell death (HR) is probably caused by the following sequence of events:

Reactive free radicals (e.g. $O_2^{\cdot-}$) › Lipid peroxidation › Membrane damage › Stress compounds (e.g. phytoalexins) › Cell death.

The bactericidal mechanism by the hypersensitive plant tissue is still unknown. The bacterial death concurrent with the increase in conductivity may be due to bactericidal substances released by hypersensitive plant cells (Klement, 1982). However, Doke (1983), regarded that $O_2^{\cdot-}$ generation in plant tissues is a primary defense reaction upon recognition of alien organisms comparable to that in leukocytes or macrophages of animals. Since a long time, it has been established that bacterial death and a strong increase in conductivity due to electrolyte leakage are known markers of plant tissue HR against incompatible phytopathogenic bacteria (Cook and Stall, 1968; Goodman, 1968; Stall and Cook, 1979).

The present study aimed to test the hypothesis of a direct bacterial effect of some reactive oxygen species (ROS), specially superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), generation during bean hypersensitive reaction (HR) against *P. syringae* pv. *phaseolicola*, as well as the involvement of these ROS in HR necrotic development of bean/bacteria incompatible combination.

MATERIALS AND METHODS

Bacterial strains:

Two strains of *Pseudomanas syringae* pv. *phaseolicola* (Burkholder) Young *et al.* were used. Compatible strain, is wild type (WT), isolated in March & April 2000 from naturally infected beans grown at Kafr El-Zayat district, El-Gharbia Governorate, Egypt. The second strain (incompatible one) was Tn5 transposon mutant (Tn5), which induces hypersensitive reaction and unable to induce water-soaked lesions (Hri$^+$ Path$^-$), provided by Prof. Z. Klement, Plant Protection Institute, Budapest, Hungary. Strains were routinely subcultured on NA medium. Some slants were maintained at 4°C, and the cultures were grown at 27°C.

Inoculum preparation:

Compatible and incompatible, two strains of *P. syringae* pv. *phaseolicola* were grown on NA for 24 hr. at 27°C and harvested by washing the culture with sterile saline. Cell concentration was adjusted to an optical

density of 0.06 at 660 nm which was equal to 10^8 cfu. ml^{-1}. Actual inoculum (10^7 cfu/ml) was prepared by ten-fold dilution of the bacterial suspension.

Plants and Inoculation:

Ten bean seeds (*Phaseolus vulgaris* L. cultivar Bronco) were sown in 15cm plastic pots containing sandy soil and grown under normal greenhouse condition. When plants were 10-days old, they were thinned to 2 plants/pot. Two days later, One-third-expanded trifoliate leaves were used for inoculation. The inoculation process was carried out according to Ercolani & Crosse, (1966) and Király *et al.*, (1997). Inoculum was sprayed onto the abaxial leaf surface with high-pressure atomizer until the exposed area became water-soaked. Any excess inoculum was carefully blotted from the leaf surface. Sterile saline was treated to another plants as control treatments. When all water-soaking had disappeared (20-30 min after inoculation), plants were maintained at 20°C ± 2 and 80 % ± 5 relative humidity.

Hypersensitive reaction (HR) test:

The appearance of HR necrosis was monitored by visual observation according to Somlyai *et al.*, (1988). Devlin and Gustine, (1992) were rated the HR to three categories (none, partial, or full) according to rate and extent of response, no visible response, a mottled and incomplete necrosis, or a solid patch of necrosis, respectively.

Determination of conductivity:

From treated and control tissues, samples were taken as 20 discs (7- mm diameter) at regular intervals (4 h), and floated on 20 ml deionized water in erlenmeyer flasks. Flasks were shaken at (90 rpm), conductivity was measured directly by Radiometer conductimeter as described by Peever and Higgins (1989).

Lipid peroxidation assay:

Lipid peroxidation was assayed by measuring malondialdehyde (MDA) accumulation, a product of unsaturated fatty acid peroxidation. The MDA content was determined by the thiobarbituric acid (TBA) reaction according to Dhindsa *et al.*, (1981). Each sample (0.1gm fresh weight) was frozen in liquid nitrogen and homogenized in 2 ml citrate-phosphate buffer (0.2 M pH 6.5) containing 0.5% Triton X-100. The homogenate was strained through three layers of cheesecloth and then centrifuged for 15 min at 15000 *g*. One ml, from the supernatant fraction, was added to an equal volume of 20 % (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. Tubes were vortexed, placed in water bath (at 95 °C) for 40 min, and then immediately cooled on ice for 15 min. Samples were centrifuged at 10000 *g* for 15 min. Spectrophotometrically assay for the supernatant was determined by subtracted the absorbency values at 532 nm from the nonspecific absorbency at 600 nm. The MDA concentrations were calculated with the extinction coefficint value of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} fresh weight of leaf tissue.

Bacterial multiplication assay *in vivo*:

The multiplication of the bacteria in the inoculated leaf tissues was determined with the plate-count technique at different time (0, 4, 8, 12, 16, 20, 24, 28, 32,36, and 48 hours) post-inoculation. Three discs (1cm in diameter) from the inoculated leaf tissues, were triturated in 0.5 ml sterile saline. From the suspension, 100µl from the tenfold dilution series were plated on nutrient agar plates. The bacterial cells number for 1 cm^2 leaf area was calculated as colony forming unit (CFU) after incubation for 48 hours at $27^{o}C$.

Determination of reactive oxygen species (ROS):

For each sample, 20 discs (7-mm diameter) were cut out with a cork borer and incubated in 5ml of potassium phosphate buffer (10mM and pH 7.8). The buffer was vacuum infiltrated into the intercellular spaces for 5 min. After 20 min incubation on a rotary shaker (90 rpm) at room temperature, samples of liquid were taken to determine ROS ($O_2^{\cdot-}$ & H_2O_2) (Minardi and Mazzucchi, 1988).

Superoxide anion ($O_2^{\cdot-}$) assay:

The presence of superoxide anion ($O_2^{\cdot-}$) was detected spectrophotometrically by cytochrome *c* reduction at 550 nm as described by McCord and Fridovich (1969). $O_2^{\cdot-}$ was detected in 0.5 ml from the liquid incubated buffer with 20 mM cytochrome *c* in 0.1 M potassium phosphate buffer (pH 7.8). Xanthine-xanthine oxidase system (which produce $O_2^{\cdot-}$) was used as a positive control, and superoxide dismutase (SOD) was used (at 1.0 unit) to calculate the specific reduction of cytochrome *c* (El-Zahaby, 1995).

Hydrogen peroxide (H_2O_2) assay:

Hydrogen peroxide (H_2O_2) presence was detected spectrophotometrically at 590 nm by using 3-methyl-2-benzothiazolinone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB) according to Ngo and Lenhoff (1980). Reaction mixture contained 0.5 ml liquid incubated buffer, 3.33 mM DMAB, 0.7 mM MBTH and 0.25 unit peroxidase in 0.1 M potassium phosphate buffer (pH 7.8). Xanthine-xanthine oxidase system supplemented with SOD (which react H_2O_2) was used as a positive control, and catalase (CAT) was used (at 1.0 unit) to calculate the presence of H_2O_2 (El-Zahaby, 1995). All spectrophotometrically assays were carried out with CE CIE C=1020 apparatus.

Experimental design and statistical analysis:

All experiments were performed with a minimum of three replicates per treatment per time point. Each experiment was done three times. Data were expressed as the means ± SE (unless otherwise stated).

RESULTS

Hypersensitive reaction (HR):

As a symptom expression, inoculation of bean leaves with the wild type race (WT) of *P. syringae* pv. *phaseolicola* caused water-soaked lesions

appeared in the inoculated leaf areas. However, inoculation with the Tn5 race induced HR necrosis. These phenotypic symptoms were recorded by visual observation as seen in (Table 1). Data indicated that water-soaked lesions appeared on inoculated leaf areas, 18 h after inoculation with the WT race, and three days post-inoculation, incomplete HR started to appear on the water-soaked lesions. On the other hand, infiltration with incompatible race (Tn5) elicited rapid HR started as mottled and incomplete necrosis 12 h after infiltration, however it turned to be partial necrotic and solid batches of necrosis at 18 and 36 h post-inoculation. In the control leaf tissues, infiltrated with sterilized saline, no visible symptoms, neither water-soaked lesions nor HR necroses, appeared.

Table 1: Visual observations of hypersensitive reaction (HR) in bean leaves (*P. vulgaris* cultivar Bronco) at different time after inoculation with compatible strain (WT) or incompatible (Tn5) strain of *P. syringae* pv. *phaseolicola*.

Bacterial strain	Symptom expression at different time after inoculation						
	6 hours	12 hours	18 hours	24 hours	36 hours	48 hours	72 hours
WT	-	-	WS	WS	WS	Ws	WS&+
Tn5	-	+	++	++	+++	+++	+++
Control	-	-	-	-	-	-	-

+++ Solid batch of necrosis, ++ partial necrosis, + mottled and incomplete necrosis, - no visible response, and WS water-soaked lesions. Five plants per treatment were evaluated.

Cunductivity:

Conductivity measurements, which is well-known indicator of permeability changes associated with HR, indicates that in the compatible bean/bacteria (WT) interaction, revealed later and increase in the conductivity after appearance of visible water-soaked symptoms. However, in the incompatible bean/bacteria (Tn5) interaction, early induction of the conductivity (4-8 hours post-inoculation) was obtained parallel to expression of HR necrosis (Fig. 1). During the HR development in the hypersensitive tissue, conductivity marked increase, it was one and half-times higher than both compatible and control tissues at 4 h post-inoculation. However, it was approximately 3–times and 6.25-times higher than the compatible and control tissues, respectively at 32 hours after inoculation.

Lipid peroxidation

The level of lipid peroxidation was followed by measuring malondialdehyde (MAD) accumulation in bean leaves after inoculation with the compatible or incompatible bacterial race (Fig. 2). The level of lipid peroxidation in leaves inoculated with the incompatible race (Tn5) was usually higher than in leaves inoculated with the compatible one (WT) at all times sampled. It was approximately 2–times higher than the compatible interaction at 20, 24, 28, 36 h post inoculation, while, it was higher than the control treatment (infiltrated with sterilized saline) approximately 3-times at 16, 20, 24h and almost 4-times at 32 and 36h post inoculation.

Bacterial multiplication *in vivo*:

The rate of multiplication of *P. syringae* pv. *phaseolicola* (compatible "WT" and incompatible "Tn5" races) in bean leaf tissues, was followed by calculation the colony forming units (CFU) using the plate-count technique (as described in the materials and methods). Results of the comparative growth studies are given in Figure 3. There was a large reduction in growth rate of the bacterial multiplication in the hypersensitive combination (Tn5 strain) as compared to that in the compatible combination (WT strain). The multiplication of the Tn5 slightly increased by 1.3 log at 24h after inoculation then started to be declined, which recorded only 0.5 log increase after 48h from inoculation. However WT bacterial strain highly multiplied after infiltration, which increased by 4.6 and 5.6 log at 24 and 48h, respectively.

Superoxide anion and hydrogen peroxide generation:

Results of, superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) production, in bean leaves infiltrated with *P. syringae* pv. *phaseolicola* indicated that significantly more $O_2^{\cdot-}$ was generated in leaves infiltrated with the Tn5 race than in the case of infiltration with the WT race or in the control leaves infiltrated with the saline, at all times sampled (Fig. 4). $O_2^{\cdot-}$ was substantially generated in leaves infiltrated with Tn5 bacterial race, which reached 320, 450, 413, 464, 432, and 417 % of the control treatment at 6, 12, 18, 24, 30, and 36 h post-infiltration, respectively. Less noticeable, but still significant, increases in $O_2^{\cdot-}$ generation were found in leaves infiltrated with the WT bacterial race. It recorded 140, 190, 182, 204, 208, and 242 % of the control values at 6, 12, 18, 24, 30, and 36 h post-infiltration.

The production of hydrogen peroxide (H_2O_2) was illustrated in (Fig. 5). Infiltration with the incompatible bacterial race (Tn5) dramatically generated H_2O_2 in bean leaf tissues than in the case of WT race or saline infiltration. Increases of H_2O_2 were started, in the case of hypersensitive reaction, directly after infiltration. Which recorded 168 n mol/g fresh weight at 6 h post-infiltration, approximately 311% and 247% of the control and compatible interaction, respectively. Later, 12, 18, 24, 30, and 36 h post-infiltration it reached 285, 280, 388, 451 and 540% respectively of control and 195, 253, 265, 233, 265% respectively of the WT compatible race values. However, infiltration with the WT race slightly, but not significantly, increased the production of H_2O_2 at 6, 12, 18 h post-infiltration. Thereafter, it significantly produced at 24, 30 and 36 h post-infiltration, which recorded 147, 193 and 203%, respectively of the control saline infiltration.

DISCUSSION

In recent years attention has focused increasingly on the general process of plant-pathogen recognition. As a symptom expression, the incompatibility of *P. syringae* pv. *phaseolicola* (Tn5 transposon mutant) towards bean leaves (*P. vulgaris* L.) was revealed by its ability to induce confluent necrosis after 24 h, this result is in agreement with the finding of Klement (1971).

In the bacteria induced hypersensitive reaction (HR) of plant tissues, K^+ efflux with concurrent membrane depolarization was detected 4 h prior to increased conductivity, an accepted measure of electrolyte leakage (Pavlovkin *et al.*, 1986). Also, Keppler & Novacky (1986) reported that, lipid peroxidation accompanied a dramatic membrane depolarization and both were occurred about 1 h before conductivity increased.

The timing of lipid peroxidation and electrolyte leakage observed in the present study suggest that structural membrane damage occurred in bean tissue infiltrated with bacteria-induced HR, induced by some mechanism! This mechanism may be is ROS, this results confirms the order of events reported by Anderson *et al.* (1991), who published that lipid peroxidation, in bean/*Colletotrichum lindemuthianum* combination, may be a consequence of the rapid production of activated oxygen species.

According to Klement (1982), the bacterial death concurrent with the increase in conductivity may be due to bactericidal substances released by hypersensitive plant cells. In the present work result's, it was a highly reduction in the growth rate of the bacterial multiplication in the hypersensitive combination (Tn5) strain.

Also results show that there is a highly induction of $O_2^{\cdot-}$ generation and H_2O_2 accumulation in the bean tissues infiltrated with the Tn5 incompatible bacterial strain. This observation is consistent with other reports (cf. Kiraly *et al.*, 1991) who reviewed that, ROS are associated with bacterially hypersensitive rapid cell collapse (Keppler and Novacky, 1987; Ádám *et al.*, 1989, 1990; Keppler and Baker, 1989; Croft *et al.*, 1990). These results contradict the publication of Minardi & Mazzucchi (1988), in which they indicated that, the death of incompatible bacteria, *P. syringae* in tobacco leaf tissue, during HR is not directly linked to $O_2^{\cdot-}$ generation.

Anderson *et al.*, (1991) suggested that the cellular necrosis in bean plants treated with *C. lindemuthianum*-elicitor, may result from altered membrane functions that are the consequence of increasing levels of free radical accumulation, they observed a maximum increase in activated oxygen within 30 min, therefore they reported that this event could initiate the other changes in membrane properties that are apparent later such as lipid peroxidation.

Rapid generation of superoxide and accumulation of H_2O_2 is a characteristic early feature of the hypersensitive response following perception of pathogen avirulence signals (Lamb and Dixon, 1997).

One can concluded that, rapid and highly generation of superoxide $O_2^{\cdot-}$ and markedly accumulation of H_2O_2, found in this investigation in the incompatible combination, can be considered as elicitors of plant cell death (HR necrotic) as well as inhibited the bacterial multiplication *in vivo*.
Further studies will be needed to determine more about the nature of the ROS effect on both host and pathogen.

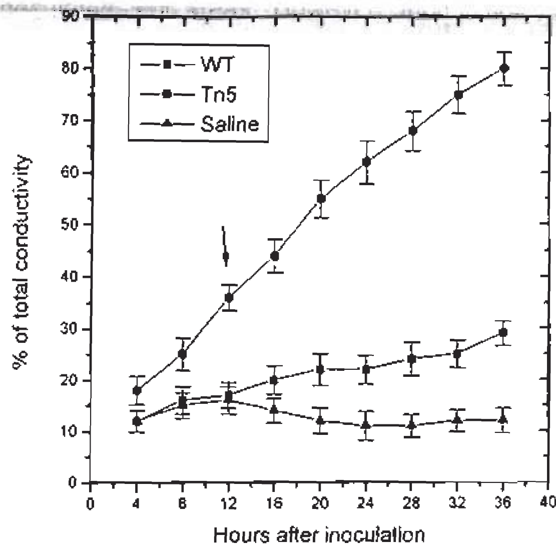

Figure 1: Electrolyte leakage measurements of bean leaf tissues infiltrated with compatible (Wt) or incompatible (Tn5) strains of *P. syringae* pv. *phaseolicola* at different time post inoculation. For control treatment leaves were infiltrated with sterilized saline. Arrow is denotes time of HR lesion formation, and error bars are (SE). Values are per cent of total conductivity, and each value is mean of three independent experiments.

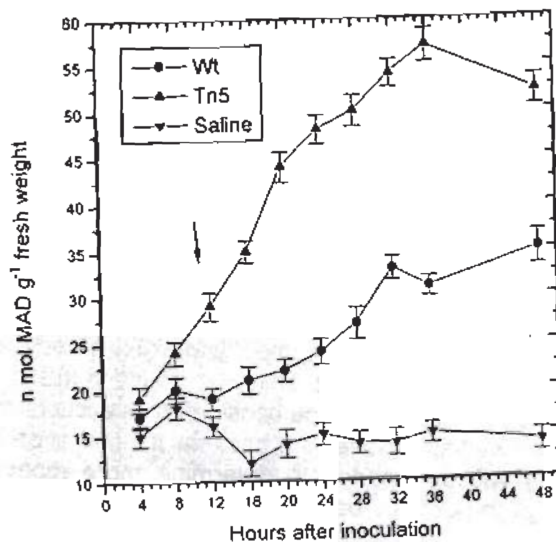

Figure 2: Lipid peroxidation assayed as malondialdehyde (MDA) content of bean leaf tissues infiltrated with compatible (Wt) or incompatible (Tn5) strains of *P. syringae* pv. *phaseolicola*, measured various time post treatment. Values are expressed in nmol g^{-1} fresh weight of leaf tissue and data are mans of four experiments. Arrow is denotes time of HR lesion formation, and error bars are (SE).

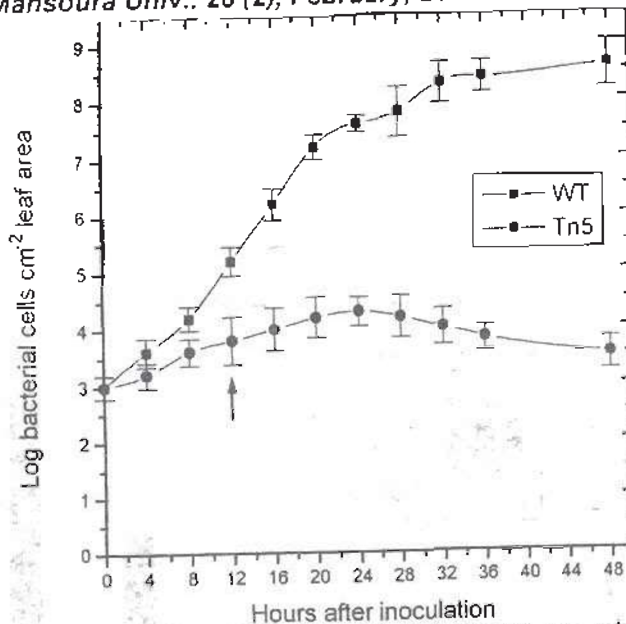

Figure 3: *In vivo*, bacterial multiplication of *P. syringae*. pv. *phaseolicola* compatible and incompatible with *Phaseolus vulgaris* (cv. Bronco), at various time post inoculation. Data presented are log CFU sm^{-2} inoculated leaf area, and values are means of three replicate experiments. Arrow is denotes time of HR lesion formation and error bars are (SE).

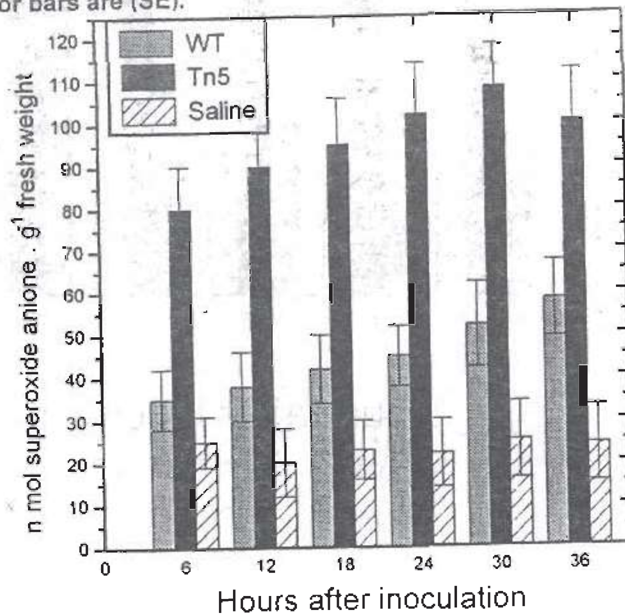

Figure 4. Level of superoxide (O_2^{-}) generation from bean leaf tissues (*Phaseolus vulgaris* cv. Bronco) infiltrated with compatible (WT) or incompatible (Tn5) strains of *P. syringae* pv. *phaseolicola*, measured various time post treatment. Values are expressed in n mol g^{-1} fresh weight of leaf tissue and data are mans of three replicate experiments.

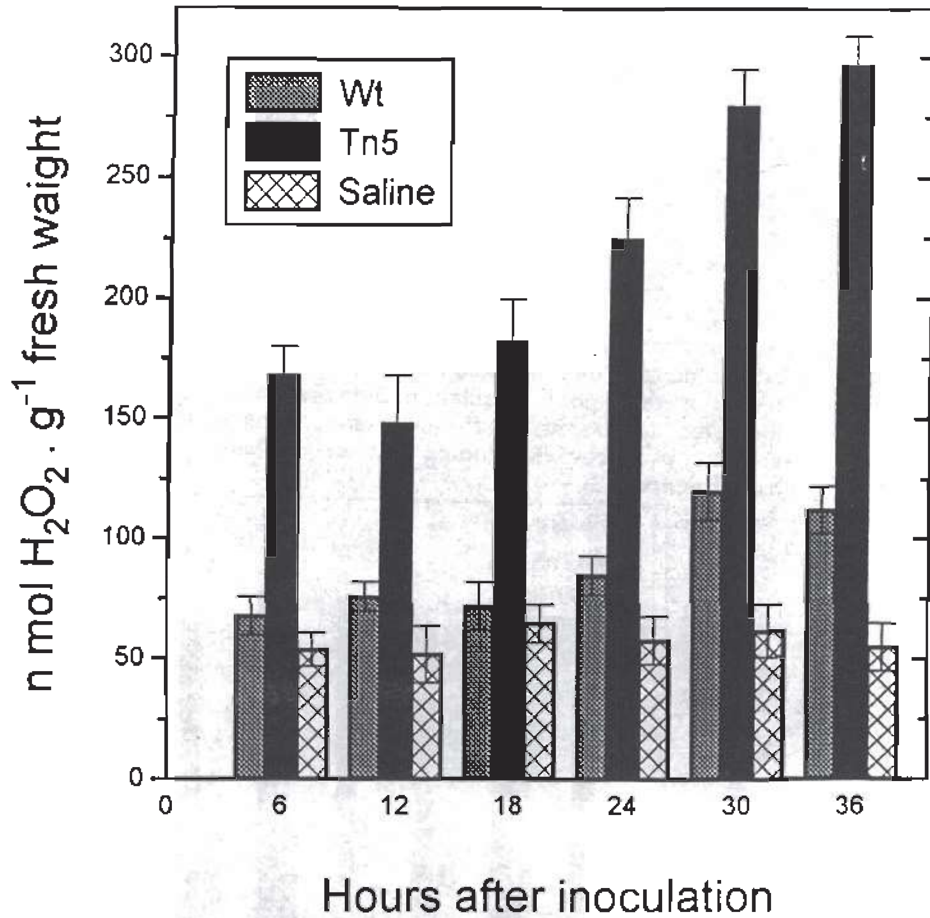

Figure 5: Level of hydrogen peroxide (H_2O_2) generation from bean leaf tissues (*Phaseolus vulgaris* cv.Bronco) infiltrated with compatible (WT) or incompatible (Tn5) strains of *P. syringae* pv. *phaseolicola,* measured various time post-treatment. Values are expressed in n mol g^{-1} fresh weight of leaf tissue and data are mans of three replicate experiments.

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تأثير أنيون السوبر أكسيد و فوق أكسيد الهيدروجين على تفاعل الحساسية المفرطة و تكاثر البكتريا سيدوموناس سيرنجي فاصيوليكولا في أوراق نبات الفاصوليا

حسان محمد الذهبى

قسم النبات الزراعى ، كلية الزراعة بطنطا ، جامعة طنطا ، جمهورية مصر العربية

تم دراسة دور بعض أنواع الأوكسجين الحر النشط (ROS) مثل أنيون السوبر أكسيد ($O_2^{\cdot-}$) و فوق أكسيد الهيدروجين (H_2O_2) فى إظهار تفاعل الحساسية المفرطة (HR) في أوراق نبات الفاصوليا (صنف برونكو) تم تلقيحها بسلالتين من بكتريا سيدوموناس سيرنجى فاصيوليكولا ، هاتان السلالتان كانتا مختلفتين في تفاعلهما مع صنف الفاصوليا تحت الاختبار ، حيث كانت إحداهما متوافقة وهى السلالة البرية (WT) حيث ينتج عن تلقيحها ظهور الأعراض المرضية ، أما الثانية فقد كانت غير متوافقة وهى السلالة المطفرة (Tn5) حيث ينتج عن التلقيح بها موت موضعى للأنسجة على شكل بقع ناتجة عن تفاعل الحساسية المفرطة HR التى تبدأ فى الظهور بعد ١٢ ساعة من التلقيح.

تم دراسة بعض المؤشرات البيوكيميائية المرتبطة بتفاعل الحساسية المفرطة ، وقد أوضحت النتائج المتحصل عليها من دراسة معامل التوصيل الكهربائى (ارتشاح الإلكتروليتات) - والذى يعتبر مؤشرا جيدا لمدى التغير فى النفاذية والمرتبط بتفاعل الحساسية المفرطة - أن التلقيح بالسلالة المطفرة (الغير متوافقة Tn5) قد ادي الي زيادة مبكرة وملموسة فى التوصيل الكهربائى ، حيث سجلت ١,٥ & ٦,٢٥ ضعفا مقارنة بمعاملة الكنترول بعد ٤ & ٣٢ ساعة من التلقيح علي التوالي. أيضا فإن أكسدة الدهون Lipid peroxidation ، والتى تعتبر مقياس جيد لتطور HR ، قد ازدادت نتائجها بدرجة عالية فى حالة تلقيح أوراق نبات الفاصوليا بسلالة البكتريا الغير متوافقة ، حيث ارتفعت درجة الأكسدة للدهون بمعدل ٣ أضعاف تقريبا بعد ٦ & ٢٠ & ٢٤ ساعة وكان الارتفاع يعادل ٤ أضعاف الكنترول تقريبا بعد ٣٢ & ٣٦ ساعة من التلقيح.

أوضحت نتائج دراسة نمو البكتريا فى داخل النسيج النباتى أن هناك انخفاضا شديدا فى معدل تكاثر سلالة البكتريا المطفرة (Tn5) مقارنة بالسلالة البرية (WT) نتيجة لتولد أنيون السوبر أكسيد و فوق أكسيد الهيدروجين ، حيث لوحظ زيادة لوغاريتمية طفيفة فى معدل النمو اللوغاريتمى للسلالة (Tn5) حيث سجلت زيادة قدرها ١,٣ & ٠,٥ لو مقارنة بالسلالة البرية (WT) التي سجلت زيادة قدرها ٤,٦ & ٥,٦ لو بعد ٢٤ & ٤٨ ساعة من التلقيح علي التوالي.

أوضحت نتائج قياس أنيون السوبر اكسيد وفوق أكسيد الهيدروجين أن هناك كمية عالية المعنوية قد تم إنتاجها داخل أنسجة الأوراق الملقحة بالسلالة الغير متوافقة (Tn5) مقارنة بالسلالة البرية أو معاملة الكنترول في كل مراحل الكشف. حيث سجل إنتاج $O_2^{\cdot-}$ في الأنسجة الملقحة بالسلالة Tn5 ٣٢٠ & ٤٦٤ % من قيمة معاملة الكنترول بعد ٦ & ٢٤ ساعة من التلقيح علي التوالى. بينما كان معدل إنتاج H_2O_2 فى الأنسجة الملقحة بالسلالة البرية ٣١١ & ٥٤٠ % من المعاملة الكنترول بعد ٦ & ٣٦ ساعة من التلقيح علي التوالى.

هذه النتائج تشير إلى أن كل من أنيون السوبر أكسيد ($O_2^{\cdot-}$) و فوق أكسيد الهيدروجين (H_2O_2) يظهران فى أثناء الاستجابة الدفاعية للحساسية المفرطة. ومن الممكن أن يكون لهما تأثير فى ظهور الموت الموضعى لتفاعل الحساسية المفرطة HR ، وفى نفس الوقت يكون لهما تأثير مضاد لتكاثر البكتريا فى أنسجة أوراق نبات الفاصوليا.