Acta Phytopathologica et Entomologica Hungarica 32 (3–4), pp. 265–280 (1997)

Submicroscopic Evidence of Bacterially Induced Resistance in Tobacco Leaves

P. G. OTT[1], L. SZABÓ[2], E. BALÁZS[2] and Z. KLEMENT[1]

[1] Department of Pathophysiology, Plant Protection Institute, Hungarian Academy of Sciences, H-1525 Budapest, P.O. Box 102, Hungary
[2] Institute for Plant Sciences, Agricultural Biotechnology Center, H-2101 Gödöllő, P.O. Box 411, Hungary

A prototrophic HR (hypersensitive reaction)-negative mutant of *Pseudomonas syringae* pv. *phaseolicola* was used to induce the early induced resistance (EIR) and the late induced resistance (LIR) in tobacco. This mutant also served as an indicator of submicroscopic host responses in non-treated, heat shocked, and dark-treated leaves. Heat shock (50 °C, 20 s) and dark are known to suppress EIR and LIR, respectively. Intercellular spaces of the non-treated and heat-shocked leaves contained dividing bacteria at 6–12 hpi. At 24–48 hpi the ratio of dividing bacteria decreased with a concomitant increase of an intensely electron-dense, distorted bacterial form frequently embedded in an amorphous, electron-dense matrix. Around 12 hpi paramural papillae appeared as site-specific plant responses to the neighbouring bacterial cells. Occurrence of papillae correlated closely with the distorted, possibly damaged bacterial form. The highest number of dividing bacteria and the largest microcolonies indicated the partial lack of EIR in heat-shocked leaves, but neither the distorted bacteria nor papillae could be connected with EIR. In the dark, compared to the other two treatments, less distorted bacteria constituted looser microcolonies after 24 hpi, while papilla formation was suppressed. Thus a relationship was found between papilla formation, the distorted bacterial cells and LIR.

Plant cells recognize the presence of bacteria and rapidly develop localized types of responses. Ultrastructural studies have shown that different types of bacteria, such as saprophytes, incompatible pathogens, or pathogenic mutants, induce similar responses (Al-Mousawi et al., 1983; O'Connell et al., 1990; Brown and Mansfield, 1988; Brown et al., 1995). These bacteria multiply in the extracted plant intercellular fluid, but can not within a responding plant tissue (Somlyai et al., 1986; Willis et al., 1990). Not only pathogens but also saprophytes trigger expression of plant defense genes (Jakobek and Lindgren, 1993; Meier et al., 1993). These facts imply an operative, non-specific, locally induced resistance mechanism apart from the incompatible-specific hypersensitive reaction (HR), that results in the decline of bacterial population (Hevesi et al., 1981).

Two locally induced forms of resistance have been described in tobacco: the early induced resistance (EIR) and the late induced resistance (LIR). The EIR exists from 3–6 hpi till about 20 hpi (Burgyán and Klement, 1979), and is inhibited by a short heat-shock (Visnyovszky et al., 1983) or a protein synthesis inhibitor, cycloheximide (Bozsó et al., 1997). The EIR is able to prevent induction of HR (Burgyán and Klement, 1979; Klement et al., 1997), while the LIR, that corresponds to the "induced resistance" described by Sequeira (1975) requires more time (about 24 h) and intense illumination to develop, but it is more effective for a longer time (about six days) than EIR since it suppresses

0238–1249/97/$ 5.00

both the incompatible (HR) and compatible (disease) symptoms (Lovrekovich and Farkas, 1965).

A number of electron microscopic studies on plant-bacterium interactions involve saprophytes (Smith and Mansfield, 1982; Brown and Mansfield, 1988; O'Connell et al., 1990; Brown et al., 1995) or *hrp* (hypersensitive reaction and pathogenicity) mutants (Bestwick et al., 1995; Brown et al., 1995; Fett and Jones, 1995) as a bacterial partner. While similar ultrastructural phenomena (e.g. appearance of a pellicle and granular material around bacterial cells) are described, the interpretation of the results varies considerably due to the individuality and complexity of the model systems. For example, very different roles and importance are attributed to the envelopment of bacteria at the plant cell wall (cf. e.g. Bestwick et al., 1995; Hildebrand et al., 1980; Bonatti et al., 1979; Mazucchi and Bazzi, 1982) in the plant defense reaction. Results are more congruent if reference is made to localized alterations within plant cells affected by bacteria: the changes usually begin with a localized convolution of the plasma membrane, increased synthetic and transport activities and sometimes end up with a deposition of differently shaped and structured cell wall appositions, papillae. Papillae are regarded as protective structures involved in creating an antibacterial environment (Peng and Kuć, 1992; Bestwick et al., 1995; Brown et al., 1995).

Our aim was to examine the ultrastructural nature of EIR and LIR. To separate EIR and LIR, tobacco leaf samples from the following three treatments were compared: heat-shock (to inhibit EIR); dark (to inhibit LIR) and control (normal growth condition where both EIR and LIR are active). We used a single inoculum prepared from a HR-negative mutant of *Pseudomonas syringae* pv. *phaseolicola* to induce both of these resistance forms and to indicate their effect.

Materials and Methods

Bacteria

We used a HR-negative mutant (No. 1250), isolated by Somlyai et al. (1986), of *Pseudomonas syringae* pv. *phaseolicola* for the inoculations in order to avoid the hypersensitive collapse of the infected leaves. Mutant No. 1250 causes neither the HR in the non-host tobacco, nor any disease symptom in a susceptible bean host. However, it is capable of inducing the EIR and LIR (unpublished results). Although this mutant was not characterized genetically, it is probably a *hrp* mutant. To prepare the inoculum, late exponential-phase bacterial cells grown at 25 °C in King's B broth (King et al., 1954) were collected in a microfuge and resuspended in sterile distilled water at a density of 1–2 × 10^9 CFU/ml.

Plants

Tobacco *Nicotiana tabacum* cv. "Samsun" plants were kept in a greenhouse until 2–3 months of age. They were transferred to growth chambers (set to 20 °C, 16 h photoperiod; no light for the dark treatment) one day before inoculation. We inoculated intervenial areas from the middle part of fully expanded, young leaves with a hypodermic syringe fitted with a 26 gauge needle (Klement, 1963). The experiments were carried out at 20 °C. The treatments of plants were as follows:

1. control (only inoculated with bacteria, both types of induced resistance are expressed): continuous light after inoculation;

2. heat-shock treatment (to inhibit EIR for at least 10 h): attached leaves were submerged into a 51 °C water bath for 15 s, inoculated and then the plants were put under continuous light;

3. dark treatment (to inhibit LIR): plants were kept in complete darkness throughout the 24 h preinoculation and the experiment, and in minimal light for inoculations and sampling.

Samples were taken 2, 4, 6, 12, 24 and 48 h after inoculation, using a 9 mm (diameter) cork borer.

Sample preparation and electron microscopy

Leaf discs were fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 12 h in sealed vials at 4 °C, with a change of buffer after 2 h. Small (ca. 1 mm^2) pieces were cut and post-fixed in 1% osmium tetroxide. Tissue was stained in 5% uranyl acetate for 1 h and dehydrated in a series of ethanol concentrations, and finally in propylene oxide. It was impregnated in propylene oxide: SPI Pon Araldite resin (1:1) for 1 h and then in the undiluted resin overnight. Blocks directed in BEEM® capsules were polymerized at 60 °C for 48 h. With an MT 7000 ultramicrotome, ultra thin (50 nm) sections were made for examination under a Zeiss EM 910 transmission electron microscope. For quantitative assessments we examined at least 30 bacterial attachment sites per sample. Every sample derived from a combination of a treatment and a sampling time. Fifteen attachment sites were selected from two separate blocks of leaf tissue prepared from the same inoculation site.

Results

The *P. syringae* pv. *phaseolicola* HR-negative mutant did not cause macroscopic symptoms in tobacco even at a density of 10^9 CFU/ml, however, there were distinctive changes seen in the bacterial cells and their environment, the plant's inter- and intracellular spaces. The changes were followed in heat-shocked leaves (inhibition of EIR), in leaves kept in the dark (LIR is inhibited) and in control leaves (neither EIR nor LIR are inhibited) from 2 to 48 hpi.

Changes in bacterial number and morphology in intercellular spaces

Two main bacterial types could be distinguished: 1. one type was only slightly electron-dense, had a normal, elongated and rounded shape with discernible inner structure (thereafter: normal cells, e.g. Fig. 1A). The bacteria showing signs of division (thereafter: dividing cells, e.g. Figs 2A, 2B and 3A) or signs of blebbing (i.e. 20–30 nm dia. vesicles were extruded from their surface, e.g. Figs 2A and 2C) also belonged to this type. 2. The other type was usually strongly electron-opaque and had an irregular, concave shape with sometimes lobed contour (hereafter: distorted cells, e.g. Figs 1D and 2D). Between these two types there were transitional stages (thereafter: transient forms, e.g. Figs 1B, 1C and 3C) as well.

In the early period (2–6 hpi) in all treatments the bacterial cells were normal, most of them stayed alone but later (12–48 hpi) the majority established microcolonies (e.g. Figs 1B and 3B) that were largest in the heat-shocked (9–10 cells/colony/section) and smallest in the dark (5–6 cells/colony/section) treatment. Most dividing cells (at 6 and 12 hpi) were seen in the heat-shocked cells (Fig. 4). In the control and heat-shocked tissue, from about 12 hpi the percentage of dividing cells began to decrease steadily, while the percentage of distorted bacterial cells increased drastically to above 40% (Fig. 5) till 48 hpi. This trend did not apply to the dark treatment, where from 24 hpi the ratio of distorted cells remained around 12% with a concomitant increase in the percentage of dividing cells (Fig. 4). However, the decrease of the ratio of dividing cells was also seen in the dark between 12 and 24 hpi, when many transient forms appeared. In all treatments, some bacteria were blebbing, at a maximal ratio (20–25%) between 12 and 24 hpi.

We assessed bacterial density of the microcolonies, i.e. the average distance between bacterial cells in a colony. These distances decreased during the experiment in all treatments: from 1.7 to 0.3 μm in the control; from 2.7 to 0.4 μm in the heat shocked and from 3 to 1.1 μm in the dark. It was obvious that the distorted bacterial cells were three times more densely packed within colonies (e.g. Figs 1D and 2D) than the normal cells (e.g. Fig. 3D).

Envelopment of bacteria

We recorded two basic kinds of envelopment located around and among bacterial cells: 1. a membrane-forming material (hereafter pellicle) around bacterial cells or microcolonies and connected to plant cell walls (e.g. Figs 1A and 2D); 2. differentially electron-dense granular or fibrillar matrix appearing among bacteria or on the outer side of the plant cell wall (e.g. Figs 1A, 1B and 2C). The amount and frequency of both forms increased with time in all treatments (Table 1). Most pellicles and matrix were seen in the control and least in the dark treatment. The extremely electron-opaque granular matrix was especially common at 24 and 48 hpi in the control and heat-shock treatment (Figs 1C and 2D, Table 1), in frequent association with the distorted bacterial types. On the contrary, this form of envelopment was rare and the ratio of uncovered cells was twice as high in the dark, as compared to the control or heat-shocked treatments.

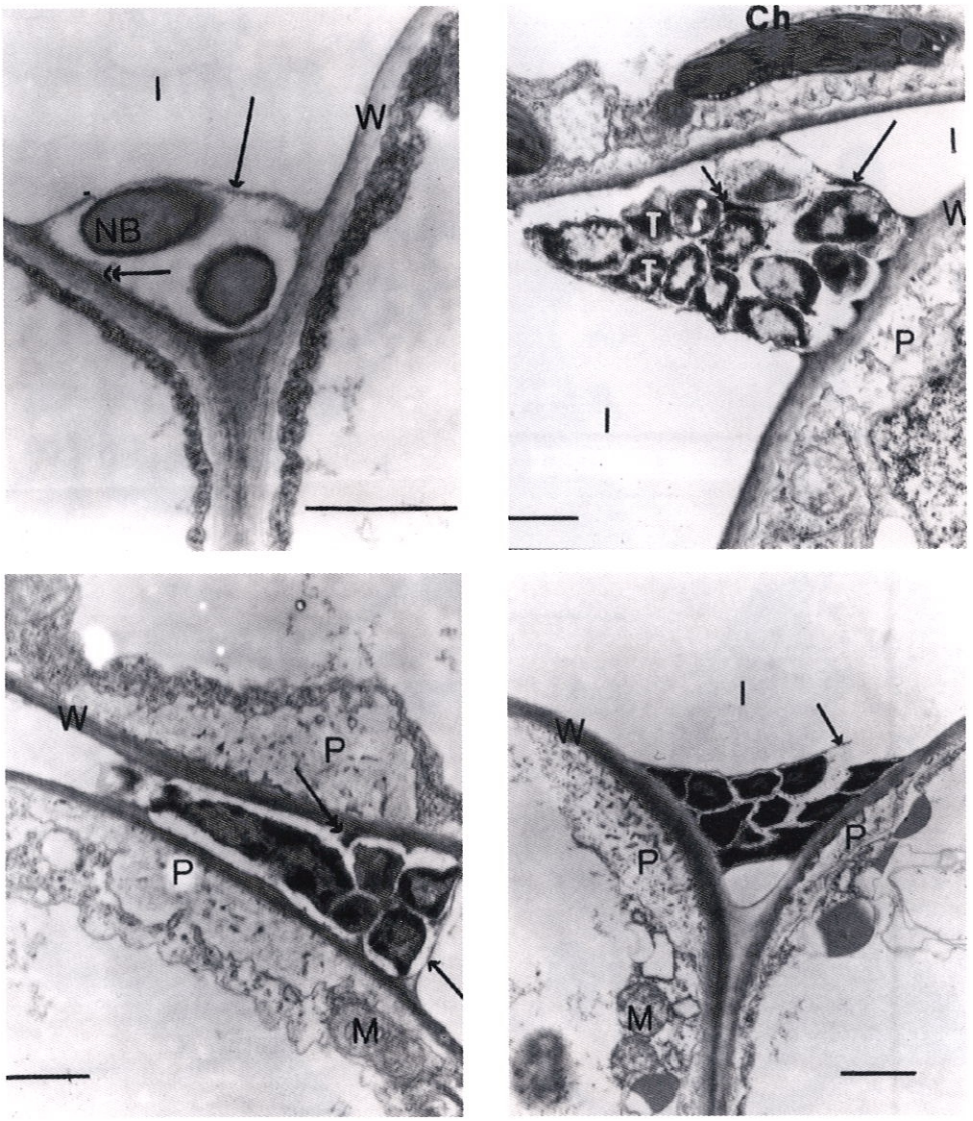

Fig. 1. Interaction between tobacco leaf cells and an HR-negative mutant of *P. syringae* pv. *phaseolicola*. Control (light) treatment. A, 6 hpi. B, 12 hpi. C, 24 hpi. Most bacteria are of the transient or distorted type. D, 48 hpi. Colony of distorted, intensely electron-dense bacterial cells in association with a papilla. Ch, chloroplast, I, intercellular space, M, mitochondrion, NB, normal bacterial cell, P, papilla, T, transient bacterial cell from between normal and distorted, W, plant cell wall. Single-headed arrows, pellicle, double-headed arrows, granular-fibrillar matrix. Horizontal bars, 1 μm

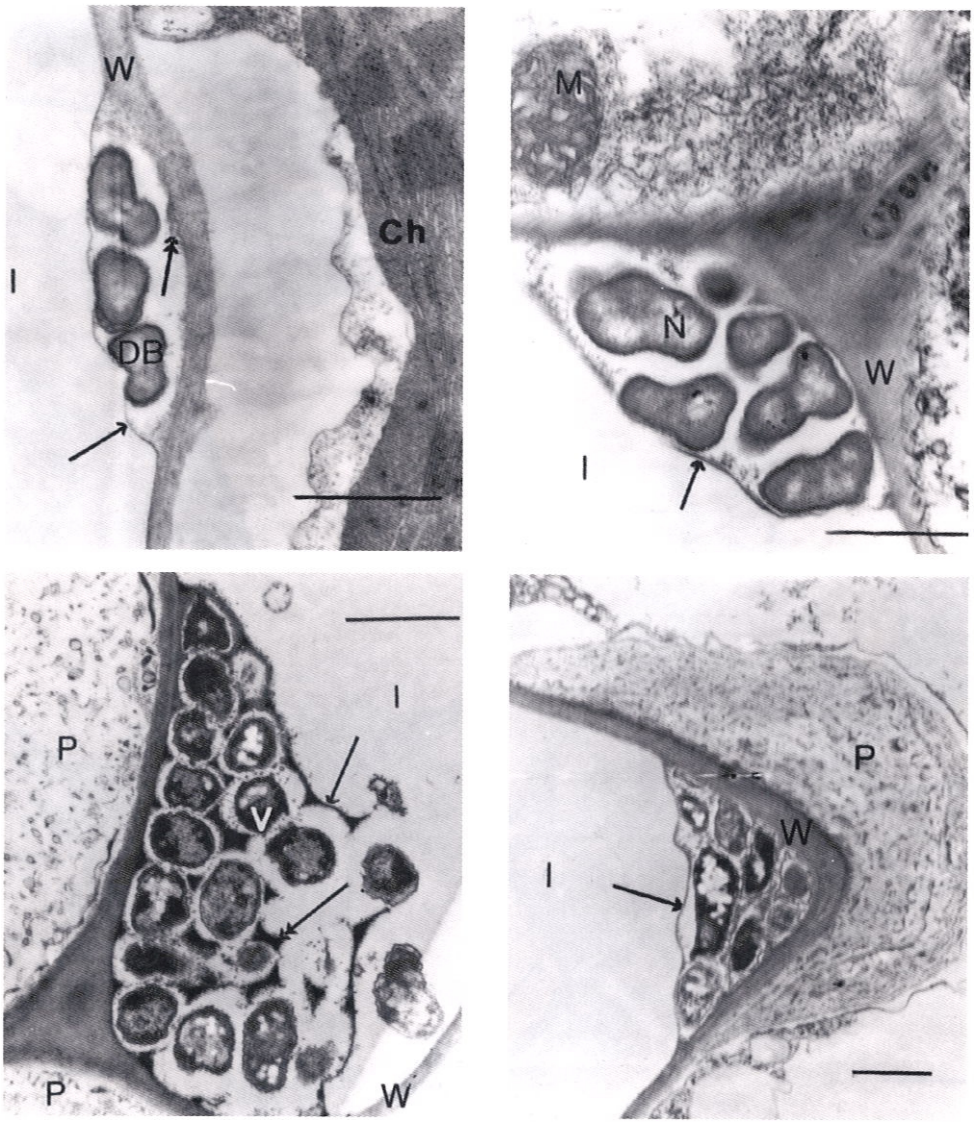

Fig. 2. Interaction between tobacco leaf cells and an HR-negative mutant of *P. syringae* pv. *phaseolicola*. Heat shock treatment. A, 6 hpi. B, 12 hpi. C, 24 hpi. D, 58 hpi. Ch, chloroplast, DB, dividing bacterium, I, intercellular space, P, papilla, V, blebbing bacterial cell, W, plant cell wall. Single-headed arrows, pellicle double-headed arrows, granular-fibrillar matrix. Horizontal bars, 1 μm

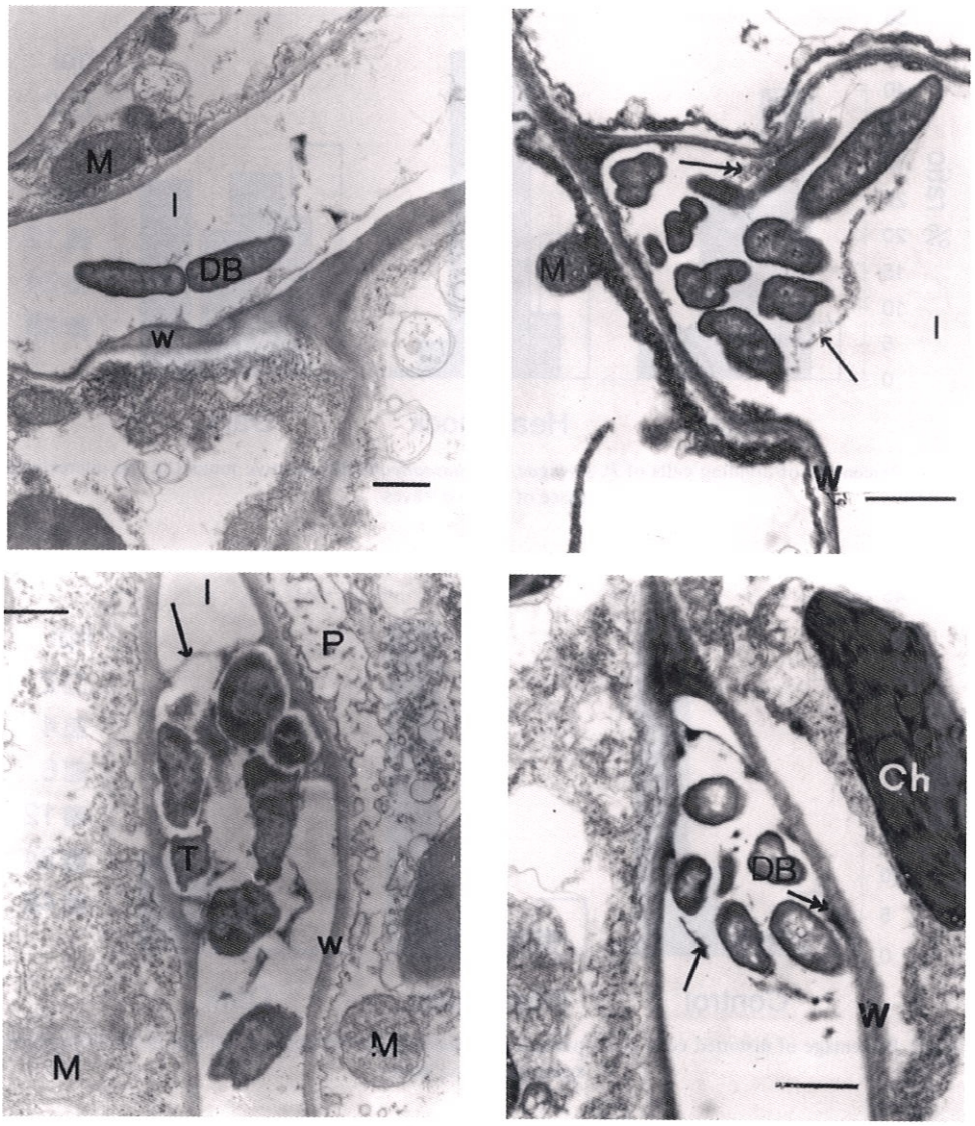

Fig. 3. Interaction between tobacco leaf cells and an HR-negative mutant of *P. syringae* pv. *phaseolicola*. Dark treatment. A, 2 hpi. The water from the inoculum has not yet evaporated, there is much of a loose granular-fibrillar material in the intercellular space. B, 12 hpi. The pellicle looks similar to the matrix. C, 24 hpi. Many transient bacteria and a poorly developed papilla. D, 48 hpi. Bacteria look normal, there is no papilla. DB, dividing bacterium, I, intercellular space, M, mitochondrion, N, normal bacterial cell form, P, papilla, T, transient bacterial cell from between normal and distorted, W, plant cell wall. Single-headed arrows, pellicle, double-headed arrows, granular-fibrillar matrix. Horizontal bars, 1 μm

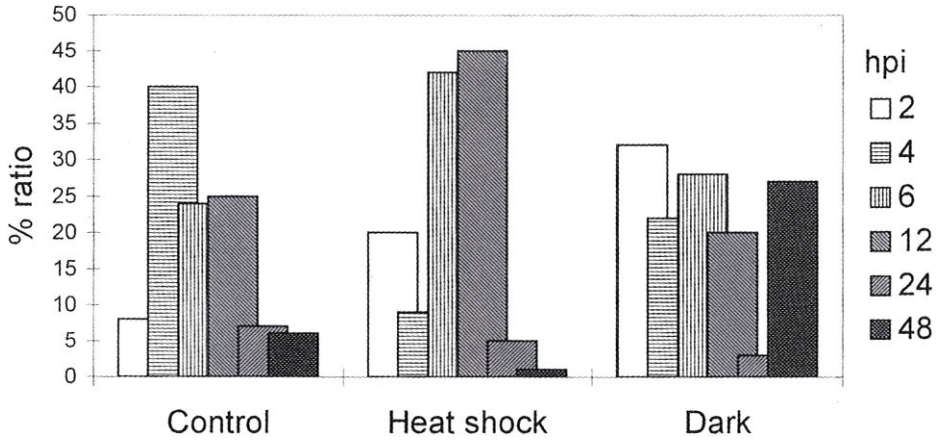

Fig. 4. Percentage of dividing cells of *P. syringae* pv. *phaseolicola* HR-negative mutant in the intercellular space of tobacco leaves

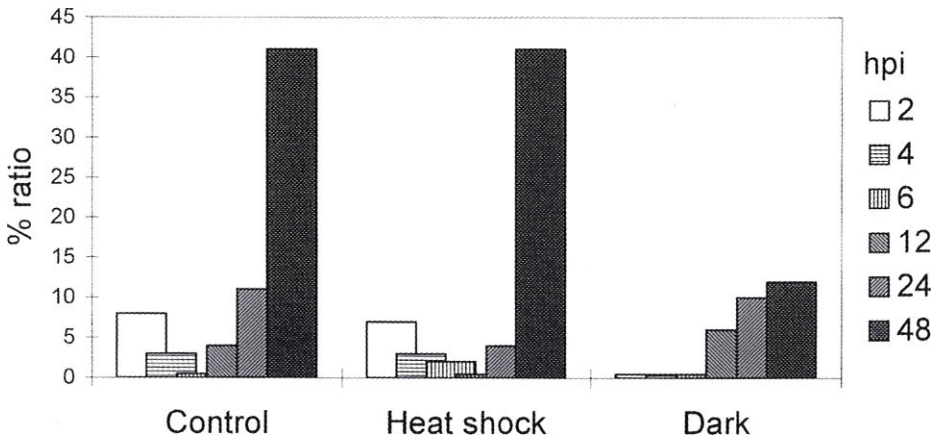

Fig. 5. Percentage of distorted cells of *P. syringae* pv. *phaseolicola* HR-negative mutant in the intercellular space of tobacco leaves

Changes in the infected plant cells

The most evident feature of the responding plant cells to this HR-negative mutant was the highly localized formation of papillae, i.e. layered deposits inside the cell wall opposite the bacterial attachment site. In this study we concentrated primarily on the papillae, although some other alterations, such as damage of plant cells, were also observed. The papillae were seen at or after 12 hpi in the control leaves (Figs 1B, 1C, 1D

Table 1

Overview of ultrastructural changes in tobacco leaves inoculated with a HR-negative mutant of *P. syringae* pv. *phaseolicola*

hpi	Dividing bact.[a]	Distorted bact.[b]	Very distorted bact.[c]	Papilla development[d]	Opaque covering matrix[e]	Pellicle[f]
			Control			
2	–	–	–	–	–	+
4	+++	–	–	–	–	++
6	++	–	–	–	–	++
12	++	+	–	+	–	++
24	–	+++	+	+++	++	++
48	–	+++	+++	++	+++	+++
			Heat-shock			
2	+	–	–	–	–	–
4	–	–	–	–	+	++
6	+++	–	–	–	–	++
12	+++	–	–	–	–	++
24	–	+++	–	+++	+++	++
48	–	+++	+++	+++	++	++
			Dark			
2	+++	–	–	–	–	++
4	++	–	–	–	–	+
6	++	–	–	–	–	+
12	+	++	–	–	+	++
24	–	+++	+	+	+	++
48	++	+	+	–	–	++

[a] Data according to Fig. 2: –, 0–10%; +, 10–21%; ++, 22–30%; +++, above 30%
[b] Data according to Fig. 3a: –, 0–15%; +, 16–30%; ++, 31–45%; +++, above 45%
[c] Data according to Fig. 3b: –, 0–8%; +, 9–16%; +++, above 33%
[d] Data according to Fig. 4: –, 0–1; +, 1–2; ++, 2–3; +++, above 3
[e] Ratio of attachment sites: –, 0–10%; +, 11–20%; ++, 21–30%; +++, above 30%
[f] Quantity in abitrary units: –, 0 or very low; +, low; ++, medium; +++, high

and Fig. 6). The heat shock seemed to delay this process, however after 24 hpi we saw more complex papillae in the heat shocked (Figs 2C and 2D) than in the control tissue (cf. Fig. 6). In the dark the development of papillae was remarkably suppressed (cf. Figs 1D, 2D and 3D, see also Fig. 6). The thickness of papillae for one attachment site (sum of thickness/sum of all attachment sites per treatment) was 270, 420 and 50 nm in the control, heat-shocked and dark-treated leaves, respectively. This was in part attributable to the lower frequency of papillae in the dark (it was 8% compared to 26% in the control

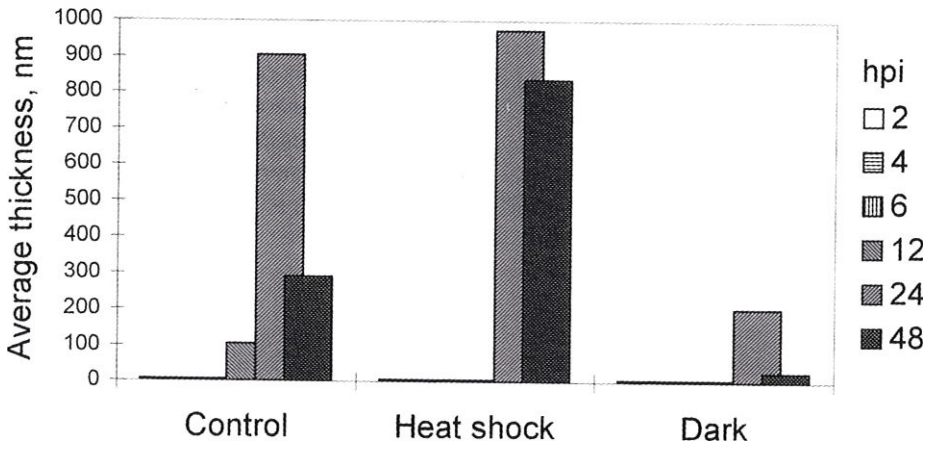

Fig. 6. Development of papillae as a localized response of tobacco cells to a *P. syringae* pv. *phaseolicola* HR-negative mutant

and 42% in the heat-shock treatment), but existing papillae were also 50% thinner (500 nm in average) than in the other two treatments (both around 1 μm).

During the development of a plant response the appearance of papillae changed. Early (about 12 hpi), complicated vesicular structures between the cell wall and cytoplasmic membrane appeared (Fig. 1B); later ornate, usually thick appositions emerged having alternate layers of electron-opaque and lucent materials (e.g. Figs 1D and 2D). Only the amount (average thickness) of the early papilla forms found in the dark was comparable to the values of the other two treatments: all late forms in the dark were either severely depressed or even lacking.

Discussion

When a tobacco leaf is infiltrated with bacteria other than compatible pathogens, EIR and later LIR are induced. Both host responses are macroscopically symptomless. EIR is known to suppress growth and HR-inducing activity of a challenge incompatible bacterium (Burgyán and Klement, 1979; Hevesi et al., 1981), LIR was shown to be effective against both incompatible and compatible challenge bacteria (Lovrekovich and Farkas, 1965; Sequeira, 1975). It is probable that induced plant responses like EIR or LIR are responsible for the failure of pathogenic mutants or saprophytes to establish themselves within leaves. One can conceive that the inhibitory effects of EIR and/or LIR may be evidenced by changes in the ultrastructure of bacterial cells. In the present study we used an HR-negative prototrophic strain of *P. syringae* pv. *phaseolicola* not only to induce EIR and LIR but also as an indicator of their effect. Besides bacterial

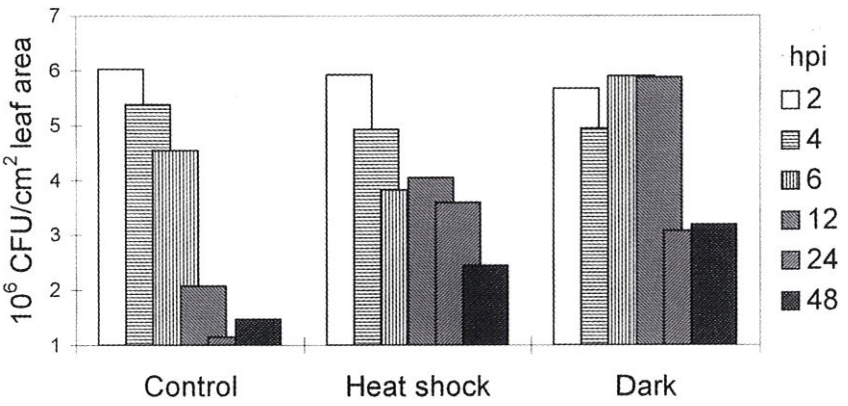

Fig. 7. Culturable cell numbers of *P. syringae* pv. *phaseolicola* HR-negative mutant obtained from tobacco leaf tissue

morphology we examined other submicroscopical events like envelopment of bacteria in the plant intercellular spaces and localized formation of wall appositions within plant cells.

Changes in bacterial morphology reflect a resistance mechanism

In this study, many signs implied a resistance in effect (Table 1). The bacteria had not lost their capacity to multiply in the plant, which means that the intercellular space was suitable for their multiplication up to 12 hpi. The ratio of dividing bacterial cells to the total cell number was found to be highest when EIR was inhibited by heat-shock. Between 12 and 48 h, presumably due to activated plant defense mechanisms, the distorted bacterial form appeared and there seemed to be a transition toward this form in the control and heat-shocked leaves (heat shock is able to delay the EIR only for about 6–8 hpi). The intercellular environment contrarily affected the ratio of the dividing and the distorted bacterial cells. The distorted, electron-opaque cells became predominant between 24 and 48 hpi in the control and heat-shocked leaves. Moreover, at 24 and 48 hpi about half of those cells that were not distorted also lost their dividing activity (data not shown). Especially in the control leaves, by this time the majority of the bacterial population comprised non-culturable or non-viable cells (Fig. 7). Therefore we regard the distorted bacteria as damaged or dead.

The ratio of the homogeneously electron-dense (no inner structure discernible) distorted cells to the total cell number did not increase when the LIR was suppressed (dark treatment), in contrast to the control or heat-shock treatment, after 24 hpi (Table 1). However, there was a high ratio of transient forms in the dark at 24 hpi that suddenly dropped until 48 hpi. Parallelly the normal, dividing cells reappeared. This suggests that at least the transient bacteria are not necessarily dead, they may be static, with a chance

of recovery. And this chance seems to be given only in the dark, although we saw only quantitative (not morphological) differences in distorted cells between the control and the dark treatment.

Possible role of papillae and LIR in generating the distorted bacterial form

There were several other changes in correlation with the ratio of the distorted cells and the presence (or absence) of EIR or LIR. The frequency of the distorted bacterial cells and the amount of papillae was high in the heat-shocked and control leaves and low in the dark treatment. In the heat-shocked and control leaves, where both EIR and LIR are present at 24 and 48 hpi, most transient and distorted bacterial cells (78% and 71%, respectively) were associated with papillae. From our results it is not possible to think far beyond correlation, but given the fact that many types of bacteria (heat-killed, saprophytic, *hrp* mutant) can induce papilla formation it is tempting to imply a role of papillae in the generation of the distorted bacterial form, i.e. in the change toward irreversible damage. This would also be consistent with suggestions made by other investigators, i.e. paramural deposits may restrict diffusion of either microbial or plant-derived (e.g. nutrients) metabolites through the plant cell wall (O'Connell et al., 1990), thus influencing bacterial metabolism. Moreover, constituents (hydroxyproline-rich glycoproteins) of papillae have also been found on the outer side of the plant cell wall, within the matrix covering the bacteria (Bestwick et al., 1994; Brown et al., 1995). The fact that in the absence of LIR (dark treatment) 67% of transient cells were observed without any papilla response at 24 hpi and that the ratio of transient cells dropped in favour of normal cells (Figs 4, 6 and Table 1) at 48 hpi suggests that papillae are not responsible for the initial damage of bacteria (reflected by the transient form), but their presence is needed for a more effective antibacterial activity seen in the control and the heat-shock treatment at 48 hpi.

In the heat-shocked leaves there was a delayed but stronger papilla response compared to the control. This may have been triggered by the bigger microcolonies resulting from the inhibition of EIR, since we found that more attaching bacteria were more "successful" in inducing a papilla than less bacteria. Papillae developed later than the EIR. Therefore we conclude that papillae are not directly connected to the EIR. Our results suggests that the papilla formation may be connected with the development of the LIR and the bacterial cell damage is a consequence of this process. This conclusion is further confirmed by the following observations:

1. Different (saprophytic, heat-killed pathogenic or pathogenic mutant) bacteria that trigger papilla formation can also induce LIR in tobacco leaves, indicating an aspecific interaction.
2. The time sequence of the development of LIR coincides with the appearance of papillae (after 12 hpi).
3. Compatible bacteria suppress both LIR (otherwise they were unable to cause disease) and papilla development (Brown et al., 1995).

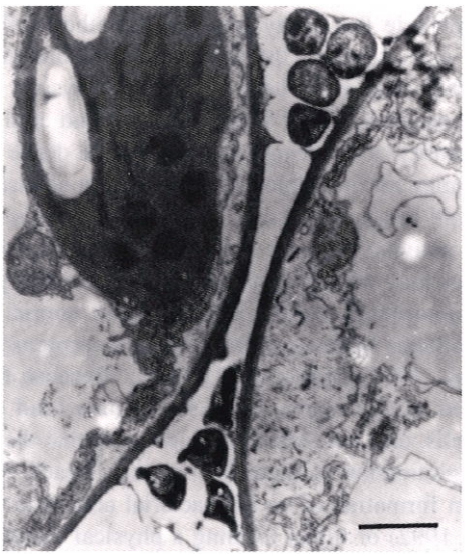

Fig. 8. A papilla response and its putative effect is highly localized. The group of distorted bacteria are associated with a large papilla, the group of normal cells above are not. Control treatment, 24 hpi. Bar, 1 μm

4. Electron micrographs show that the plant cell response is highly localized to the attachment site of distorted bacterial cells. There are irregular distorted bacteria close to a papilla and to normal cell types only two micrometers away (Fig. 8). Therefore, not all bacterial cells are damaged by this local plant cell response in the inoculated tissue. This was established when the live bacterial number in inoculated tobacco leaves was measured by plate count method. These investigations also proved that the live bacterial number was the highest when the LIR was suppressed in the dark (Fig. 7).

Possible role of envelopment

Earlier studies regarded the envelopment of bacteria (the pellicle and the amorphous, electron-dense matrix among bacterial cells) as signs or means of active immobilization (Goodman et al., 1976; Sequeira et al., 1977; Benhamou, 1991; Bestwick et al., 1995), an insignificant phenomenon in terms of defense (Cason et al., 1978; Bonatti et al., 1979; Al-Issa and Sigee, 1982; Ebrahim-Nesbat and Slusarenko, 1983; Jones and Fett, 1985), an advantageous environment for the bacteria enclosed (Mazzuchi et al., 1982) or even an artifact of inoculation (Hildebrand et al., 1980). In our experiments the pellicle appreared early (at 2–4 hpi). It was more frequent and more manifest with time (Table 1). Later, around the bacteria enclosed in the pellicle, an electron-opaque, granular matrix emerged, embedding the bacterial cells. This matrix was seen frequently around the distorted cells in the control and heat-shocked treatment and not so often in

the dark. Though the pellicle frequently appeared together with the dense amorphous matrix among bacterial cells, it seems that they are not results of the same or correlated process. Thus, the pellicle may have been shaped by physical forces working on air-water-interfaces, which supports earlier studies (Hildebrand et al., 1980; Jones and Fett, 1985). The growing quantity of covering material and the dense granular matrix at later stages (24 and 48 hpi) may not be explained with only condensation of a water-soluble material of plant cell wall origin. It possibly involves accumulation of substances originating from biological activity. Because pellicles are common in both control and the dark treatments, pellicles may not correlate with the concurrently developing resistance, like LIR or EIR. The amorphous matrix in which both normal and distorted bacterial cells were embedded contains hydroxyproline-rich glycoproteins that are also present in papillae (Bestwick et al., 1995; Brown et al., 1995). Because the number of distorted bacterial cells embedded in this matrix increases in time (and they are closer to each other than the normal cells, data not shown), it is possible that the amorphous matrix may play a role i) in the agglutination of invading bacteria (Leach et al., 1982; Swords and Staehelin 1993) or ii) in formation of an antibacterial condition within the encapsulated colony (Peng and Kuć, 1992) or iii) in forming a physical barrier that makes plant's inhibitory substances more and bacterial attempts to obtain nutrients less effective (Bestwick et al., 1995).

Since the EIR and LIR overlap in time, we had to inhibit either of them for distinction. Our treatments are not completely inhibitory to EIR or LIR and might affect bacteria not only through the inhibition of EIR and LIR. We could see weak papilla formation as well as distorted bacterial cells in the dark treatment. Rather than inhibiting resistance, the heat-shock may stimulate bacterial growth via e.g. leakage of nutrients into the intercellular space. However, we only could measure low and transient (from 1–4 hpi) leakage of electrolytes (data not shown) from the heat-shocked tissue, which alone probably does not support sustained bacterial growth. But quantitative assessments of the micrographs showed clearly that when the EIR was inhibited the ratio of dividing and the number of culturable bacterial cells was highest. Working with *hrp* mutants of *P. syringae* pv. *syringae* we also observed attenuation of an otherwise normal bacterial growth suppression and superinduction of *hrp* genes in leaves after the leaves were heat-shocked (Bozsó et al., 1997). The micrographs also showed that when the LIR was inhibited the frequency of papillae and the percentage of distorted bacteria was the lowest.

We conclude that the submicroscopic changes (papilla formation – bacterial cell damage) are independent from the HR and can be outward signs of the LIR.

Acknowledgements

This study was supported by the Hungarian Science Foundation (OTKA, ID number T013328) and the Hungarian Science Instrument Center, OTKA A334.

Literature

Al-Issa, A. N. and Sigee, D. C. (1982): The hypersensitive reaction in tobacco leaf tissue infiltrated with *Pseudomonas pisi* I. Active growth and division in bacteria entrapped at the surface of mesophyill cells. Phytopath. Z. 104, 104–114.

Al-Mousawi, A. H., Richardson, P. E., Essenberg, M. and Johnson, W. M. (1983): Specificity of the envelopment of bacteria and other particles in cotton cotyledons. Phytopathology 73, 484–489.

Benhamou, N. (1991): Cell surface interactions between tomato and *Clavibacter michiganense* subsp. *michiganense*: localization of some polysaccharides and hydroxyproline-rich glycoproteins in infected host leaf tissues. Physiol. Mol. Plant Pathol. 38, 15–38.

Bestwick, C. S., Bennett, M. H. and Mansfield, J. W. (1995): *Hrp* mutant of *Pseudomonas syringae* pv *phaseolicola* induces cell wall alterations but not membrane damage leading to the hypersensitive reaction in lettuce. Plant Physiol. 108, 503–516.

Bonatti, P. M., Dargeni, R. and Mazucchi, U. (1979): Ultrastructure of tobacco leaves protected against *Pseudomonas aptata* (Brown et Jamieson) Stevens. Phytopath. Z. 96, 302–312.

Bozsó, Z., Ott, P. G. and Klement, Z. (1977): HR-positive phenotype of the *Pseudomonas syringae* pv. *syringae hrpK* mutant and *hrp* gene superinduction in tobacco leaves treated with protein synthesis inhibitors. In: K. Rudolph, T. J. Burr, J. W. Mansfield, D. Stead, A. Vivian and J. von Kietzell (eds): Proc. 5th Int. Conf. on *Pseudomonas syringae* Pathovars and Related Pathogens. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 122–126.

Brown, I. R. and Mansfield, J. W. (1988): An ultrastructural study, including cytochemistry and quantitative analysis of the interactions between pseudomonads and leaves of *Phaseolus vulgaris*. Physiol. Plant Pathol. 33, 351–376.

Brown, I. R., Mansfield, J. and Bonas, U. (1995): *Hrp* genes in *Xanthomonas campestris* pv *vesicatoria* determine ability to suppress papilla deposition in pepper mesophyll cells. Mol. Plant Microbe Interact. 8, 825–836.

Burgyán, J. and Klement, Z. (1979): Early induced selective inhibition of incompatible bacteria in tobacco plants. Phytopathologia Mediterranea 18, 153–161.

Cason, E. T., Richardson, P. E., Essenberg, M. K., Birkerhoff, L. A., Johnson, W. M. and Venere, R. J. (1978): Ultrastructural cell wall alterations in immune cotton leaves inoculated with *Xanthomonas campestris*. Phytopathology 68, 1015–1021.

Ebrahim-Nesbat, F. and Slusarenko, A. J. (1983): Ultrastructure of the interaction of cells of *Pseudomonas phaseolicola* with cell walls of a resistant and susceptible bean cultivar. Phytopath. Z. 108, 148–159.

Fett, W. F. and Jones, S. B. (1995): Microscopy of the interaction of *hrp* mutants of *Pseudomonas syringae* pv. *phaseolicola* with a nonhost plant. Plant Sci. 107, 27–39.

Goodman, R. N., Huang, P. Y. and White, J. A. (1976): Ultrastructural evidence for immobilisation of an incompatible bacterium. Phytopathology 66, 754–764.

Hevesi, M., Mehiar, F. F. and Klement, Z. (1981): Suppression of challenge bacteria in tobacco leaves in the early and late period of induced (acquired) resistance caused by *Pseudomonas fluorescens*. Acta Phytopathol. Acad. Sci. Hung. 16, 355–364.

Hildebrand, D. C., Alosi, M. C. and Schroth, M. N. (1980): Physical entrapment of pseudomonads in bean leaves by films formed at air water interfaces. Phytopathology 70, 98–109.

Jakobek, J. L. and Lindgren, P. B. (1993): Generalized induction of defense responses in bean is not correlated with the induction of the hypersensitive reaction. Plant Cell 5, 49–56.

Jones, S. B. and Fett, W. F. (1985): Fate of *Xanthomonas campestris* infiltrated into soybean leaves: an ultrastructural study. Phytopathology 75, 733–741.

King, E. D., Ward, M. K. and Raney, D. E. (1954): Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44, 301–307.

Klement, Z. (1963): Method for the rapid detection of the pathogenicity of phytopathogenic *Pseudomonas*. Nature 199, 299–300.

Klement, Z., Bozsó, Z., Ott, P. G. and Rudolph, K. (1997): The mechanism of symptomless reaction of plants induced by phytopathogenic pseudomonads. In: K. Rudolph, T. J. Burr, J. W. Mansfield, D. Stead, A. Vivian and J. von Kietzell (eds): Proc. 5th Int. Conf. on *Pseudomonas syringae* Pathovars and Related Pathogens. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 127–132.

Leach, J. E., Cantrell, M. A. and Sequeira, A. L. (1982): Hydroxyproline-rich bacterial agglutinin from potato. Plant Physiol. 70, 1353–1358.

Lovrekovich, L. and Farkas, G. L. (1965): Induced protection against wildfire disease in tobacco leaves treated with heat-killed bacteria. Nature 205, 823–824.

Mazzuchi, U., Bazzi, C. and Bonatti, M. B. (1982): Encapsulation of *Pseudomonas syringae* pv. *tabaci* in relation to its growth in tobacco leaves both pretreated and not pretreated with protein-lipopolysaccharide complexes. Physiol. Plant Pathol. 21, 105–112.

Meier, B. M., Shaw, N. and Slusarenko, A. J. (1993): Spatial and temporal accumulation of defense gene transcripts in bean (*Phaseolus vulgaris*) leaves in relation to bacteria-induced hypersensitive cell death. Mol. Plant Microbe Interact. 6, 453–466.

O'Connell, R. J., Brown, I. R., Mansfield, J. W., Bailey, J. A., Mazau, D., Rumeau, D. and Esquerre-Tugaye, M. T. (1990): Immunocytochemical localisation of hydroxyproline-rich glycoproteins accumulating in melon and bean at sites of resistance to bacteria and fungi. Mol. Plant Microbe Interact. 3, 33–40.

Peng, M. and Kuč, J. (1992): Peroxidase-generated hydrogen peroxide as a source of antifungal activity *in vitro* and on tobacco leaf disk. Phytopathology 82, 696–699.

Sequeira, L. (1975): Induction and suppression of the hypersensitive reaction caused by phytopathogenic bacteria: specific and non-specific components. In: R. K. S. Wood and A. Graniti (eds): Specificity in Plant Diseases. Plenum Press, New York, pp. 289–306.

Sequeira, L., Gaard, G. and De Zoeten, G. A. (1977): Interaction of bacteria and host cell walls: its relation to mechanisms of induced resistance. Physiol. Plant Pathol. 10, 43–50.

Smith, J. J. and Mansfield, J. W. (1982): Ultrastructure of interactions between pseudomonads and oat leaves. Physiol. Plant Pathol. 21, 259–266.

Somlyai, G., Hevesi, M., Bánfalvi, Zs., Klement, Z. and Kondorosi, Á. (1986): Isolation and characterization of non-pathogenic and reduced virulence mutants of *Pseudomonas syringae* pv. *phaseolicola* induced by Tn5 transposon insertions. Physiol. Plant Pathol. 29, 369–380.

Swords, K. M. M. and Staehelin, L. A. (1993): Complementary immunolocalization patterns of cell wall hydroxyproline-rich glycoproteins studied with the use of antibodies directed against different carbohydrate epitopes. Plant Physiol. 102, 891–901.

Visnyovszky, É., Hevesi, M. and Klement, Z. (1983): Early and late induced resistance of plants against bacteria. Tag. Ber. Akad. Landwirtsch. Wiss. DDR Berlin 216, 357–364.

Willis, K., Rich, J. J. and Hrabak, E. M. (1990): The *hrp* genes of phytopathogenic bacteria Mol. Plant Microbe Interact. 4, 132–138.