Effect of Plasmolytica on the Hypersensitive Reaction Induced by Bacteria in Tobacco: a Comparison With the Virus-Induced Hypersensitive Reaction

A. Gulyás, B. Barna, Z. Klement, and G. L. Farkas

First and fourth authors are research scientist and director, respectively, Institute of Plant Physiology, Hungarian Academy of Sciences, H-6701 Szeged, Hungary; second and third authors are research scientist and principal plant bacteriologist, respectively, Research Institute for Plant Protection, Budapest, Hungary.

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ABSTRACT

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Since plasmolysis inhibits the hypersensitive reaction (HR) induced by viruses, a study was undertaken to investigate the effect of solutions of selected plasmolyzing agents (plasmolytica) on the HR induced by incompatible bacteria (*Pseudomonas pisi, Pseudomonas phaseolicola,* and *Xanthomonas vesicatoria*) in Xanthi tobacco leaves. Leakage of electrolytes from disks of tissue from leaves injected with bacterial suspensions was measured in the presence of different concentrations of nonpermeating (mannitol), slow-permeating (sucrose), and rapid-permeating (ethylene glycol) plasmolytica, respectively. Only the nonpermeating and slow-permeating plasmolytica inhibited the bacterial HR. Although the development of viral HR is sharply inhibited by osmotica above the iso-osmotic value, the inhibition of bacterial HR (as a function of the concentration of the plasmolytica) was gradual. The bacterial HR, therefore, does not seem to be affected by the turgor of the host cells. The onset of bacterial HR was inhibited by the plasmolytica irrespective of the time of treatment after inoculation.

Additional key words: incompatibility, stress.

The hypersensitive reaction (HR) is a widespread response of plants to invasion by fungal (23), viral (8), and bacterial (12) pathogens. Rapid cell collapse is induced in incompatible, but not compatible, host-parasite combinations and often is associated with the localization of the pathogen near its entry point. The HR symptoms induced by fungi, viruses, and bacteria appear to have much in common; however, it remains to be seen whether the processes leading to these HRs are cytologically and biochemically similar. Therefore, comparative studies on various aspects of the HRs induced by different pathogens are warranted.

The observation by Otsuki et al (16) that the virus-induced HR is not expressed in TMV-infected tobacco leaf protoplasts has led to the hypothesis that (at least with virus diseases) cell-to-cell contact is necessary for the development of the HR. Indeed, plasmolysis, leading to the disruption of cell-to-cell contact, inhibits the formation of local lesions in leaves of virus-infected hypersensitive host plants (3,7,9). More significantly, the increase in permeability, an early characteristic of the HR (1,5,15), also is inhibited if cells of the TMV-infected tissues become plasmolyzed (7). These observations are in line with the "cell-to-cell contact hypothesis," although other alternatives, like inhibition of the HR by osmotic stress and/or protection of the cell membranes by the osmotic agents cannot be excluded. Investigations of the mode of action of solutions of plasmolyzing agents (plasmolytica) affecting the viral HR are in progress (19).

No data are available on the effect of plasmolytica on the bacterial HR. The present studies were undertaken to test whether or not the HR induced by bacteria is affected by hypertonic media. To provide quantitative data, the effect of plasmolytica on the HR-associated leakage of electrolytes from the tissues was measured.

MATERIALS AND METHODS

Plant material and inoculation techniques.—*Nicotiana tabacum* 'Xanthi' plants were grown under ordinary greenhouse conditions. Fully expanded leaves of 3- to 4-mo-old plants were used for the experiments.

The intercostal areas of the leaves were infiltrated with a suspension of bacteria previously grown in nutrient broth for 24 hr and diluted with sterile tap water to a concentration of 5×10^7 cells/ml, by the method of Klement (10). Leaf areas injected with sterile tap water served as untreated controls. Except where otherwise stated, the plants were kept at 30 C after injection and exposed to an air stream to facilitate the evaporation of water from the intercellular spaces. After complete evaporation of the excess water (usually after 1 hr) disks 1.5 cm in diameter were punched from the injected leaf areas and floated on water or various plasmolytica in petri dishes at 25 C. To reduce variability, care was taken to use an identical number of disks from the same leaf for each treatment. Six disks were used per treatment.

Measurement of host-cell permeability.—The conductivity of the solution on which the leaf disks were floated was measured at intervals with a Type OK-102/1 μ Siemens-range RADELKISZ conductometer (Budapest, Hungary) at 25 C. In the figures the increase in conductivity is shown as a function of time after the start of floating. Since the various plasmolytica might have an effect not only on the HR but also on the permeability of healthy cells, all values pertaining to the effect on the HR of a plasmolyticum were corrected for the leakage from comparable noninoculated leaf disks in the presence of the same plasmolyticum. The experiments were repeated four to six times.

Plasmolytica.—Different plasmolytica were tested for effect on the bacterial HR: (i) mannitol, a nonpermeating plasmolyticum, (ii) sucrose, a slow-permeating plasmolyticum, and (iii) ethylene glycol, a rapid-permeating plasmolyticum.

Bacteria.—*Pseudomonas pisi* Sackett (ATCC 11055), *P. phaseolicola* (Burkholder) Dowson, race 1 (Hungarian isolate), and *Xanthomonas vesicatoria* (Doidge) Dowson 1956 (Hungarian isolate from tomato) are known to induce a typical HR, resulting in the collapse of the leaf tissue, if injected into tobacco leaves in concentrations at or above 5×10^7 cells/ml. *Pseudomonas fluorescens* Migula (ATCC 13525), a typical saprophyte, which does not induce the HR in tobacco, was used as control. The HR induced by the three incompatible bacteria in leaves in air appeared at different times after inoculation. Cell collapse was observed in tobacco leaves infected with *P. pisi* within 4–5 hr, with *P. phaseolicola* within 6–7 hr, and with *X. vesicatoria* within 10–12 hr. The approximate induction times (time necessary for the

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irreversible triggering of the HR) for leaves in air were: *P. pisi* 1.5 hr, *P. phaseolicola* 3–4 hr, and *X. vesicatoria* 5–6 hr. The floating of leaf disks on water or aqueous media delayed the onset of HR by about 1–2 hr as indicated by the delayed start of the increase in conductivity as compared to the reaction in air.

RESULTS

Dependence of HR inhibition on the nature of osmotica.—*Pseudomonas fluorescens* did not induce the HR in tobacco leaves (Fig. 1-A). A very sharp increase in permeability was observed in tissues injected with *P. phaseolicola* and *P. pisi* (Fig. 1-C, D). *Xanthomonas vesicatoria*, probably because of its higher temperature optimum (30 C), gave only a slight response (Fig. 1-B).

Only the slow-permeating (sucrose) and nonpermeating (mannitol) plasmolytica inhibited the HR (leakage of electrolytes) (Fig. 1-C,D). Sucrose at the same concentration inhibited the HR to the same extent as, or even more effectively than, mannitol in some experiments (Fig. 1–3).

Concentration-dependence of the effect of plasmolytica.—Sucrose and mannitol tended to decrease the HR-associated leakage more at high (0.4–0.6 M) than at lower (0.1–0.3 M) molarities (Fig. 2,3). The dose-response effect (concentration dependence) was gradual. Complete inhibition of the bacterial HR was not observed at any concentration of the plasmolytica tested (up to 0.6 M). There was no indication of a stimulation of the HR around the iso-osmotic range (Fig. 2,3).

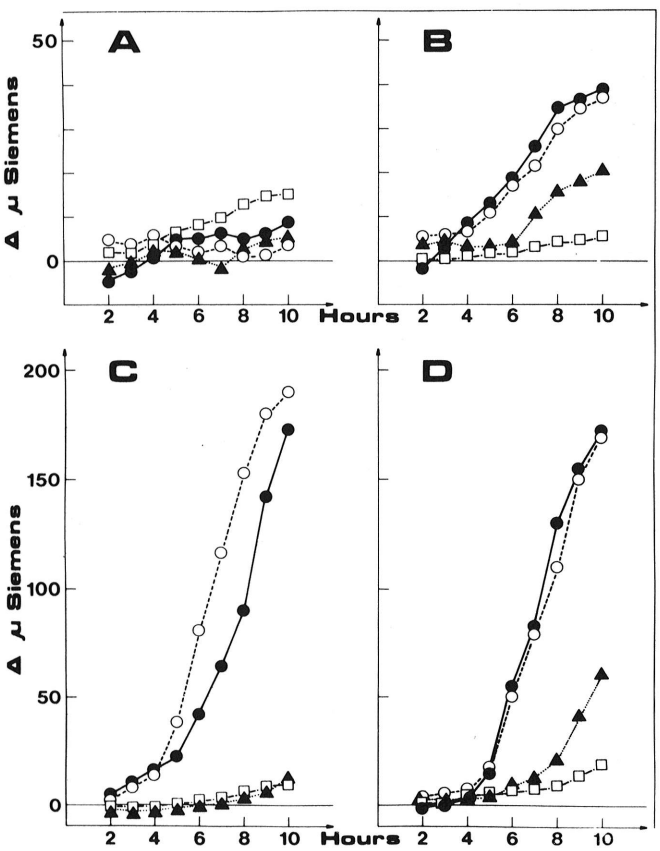

Fig. 1-(A to D). The effect of hypertonic solutions (0.6 M) of plasmolytica on the hypersensitive reaction induced by phytopathogenic bacteria in tobacco leaves. The curves represent the time-course of change in host cell permeability after injection of bacterial suspensions into the intercellular spaces: **A)** *Pseudomonas fluorescens* (saprophytic control), **B)** *Xanthomonas vesicatoria,* **C)** *Pseudomonas phaseolicola,* and **D)** *Pseudomonas pisi.* The leakage of electrolytes from disks floated on water (●——●), ethylene glycol (○ - - - ○), mannitol (▲ ···· ▲), and sucrose (□ - · - □) was monitored from the 2nd hr after injection. Note the different ordinate scales of the upper and lower pairs of figures.

Dependence of the effect of plasmolytica on the time of application.—After the injection of bacterial suspensions into the leaves the excess water had to be evaporated from the intercellular spaces before the leaf disks were floated on the surface of the plasmolytica. Unless this precaution was taken, the high water content of the intercellular spaces interfered with the appearance of the HR (2) probably because the bacteria have to be firmly attached to the cell wall in order to be able to elicit the HR (6, 21,22). It took about 1 hr to evaporate the excess intercellular water. This may be important because the bacterial HR becomes irreversibly triggered during the first few hours ("induction period") after inoculation although a longer latent period precedes the appearance of visible cell collapse (11). Since the induction period is 1.5 hr with the tobacco/*P. pisi* system and full plasmolysis of the tobacco leaf tissues in 0.6 M mannitol was reached only within 2.5–3.0 hr, in the experiments summarized in Fig. 1 and 2 the plasmolytica may have affected the latent rather than the induction period. To circumvent this problem, *X. vesicatoria*, which requires a longer induction and latent period to induce the HR, was also included in the experiments. Moreover, *X. vesicatoria* does not induce HR at 38 C (4). Thus, after inoculation with *X. vesicatoria,* the excess water could be evaporated from leaves at 38 C and the HR was evoked later by lowering the temperature. Consequently, the effect of plasmolytica could be tested in two treatments: (i) plasmolytica were applied *simultaneously* with the 38 C→ 25 C temperature shift which induced the development of HR and (ii) leaf disks were treated with plasmolytica at 38 C, *before* the temperature shift.

The results obtained with both treatments were essentially the same as those obtained with the tobacco/*P. pisi* system (Fig. 3, Table 1). We concluded that the nonpermeating osmotica inhibit the bacterial HR irrespective of the time of treatment.

Effect of plasmolytica on the growth of *P. pisi* in culture medium.—Since the HR is evoked only by living (perhaps only by multiplying) bacteria (11,13), and the plasmolytica may affect bacterial growth, the effect of the compounds used was tested on the multiplication of *P. pisi* in culture medium. Although some of the plasmolytica, at high concentrations, decreased bacterial

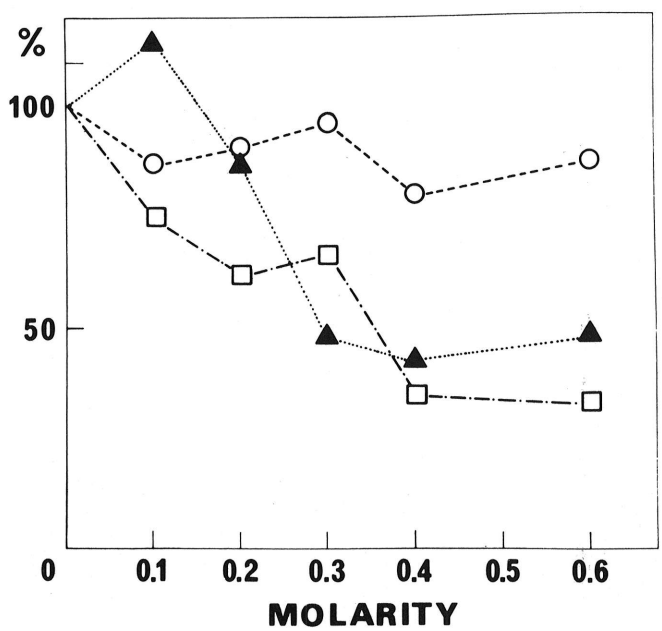

Fig. 2. Concentration dependence of the effect of solutions of plasmolytica on the hypersensitive reaction induced by phytopathogenic bacteria in tobacco leaves. Disks were punched from leaf tissues injected with *Pseudomonas pisi* and floated on plasmolytica of various concentrations. The change in permeability of the host cells (leakage of elektrolytes) was monitored as a function of time after inoculation. The conductometric values (μ Siemens), measured in the incubation medium at the 8th hr after inoculation, are expressed as percentages of the water control (100%). Ethylene glycol (○ - - - ○), mannitol (▲ · · · ▲), and sucrose (□ - · - □).

growth (Fig. 4), this cannot explain their effect on the HR because the hypersensitive reaction also was affected at low concentration ranges (0.1–0.3 M), which had little or no effect on bacterial growth. Furthermore, due to dilution, the concentration of the plasmolytica was less in the intercellular spaces than in the solutions used for treatments.

TABLE 1. Effect of osmotica on the "delayed" hypersensitive reaction induced by *Xanthomonas vesicatoria* in tobacco leaves at high temperature[a]

Osmoticum	Permeability (conductivity)[b] of leaf tissue exposed to osmotica at:		
	0.3 M (%)	0.4 M (%)	0.6 M (%)
Ethylene glycol	111.3	90.8	80.4
Mannitol	22.2	15.0	5.5
Sucrose	72.2	38.3	23.8

[a]Tobacco plants, after injection with the bacteria, were kept at 38 C for 15 hr. Disks were punched from the still symptomless leaves and floated on solutions of the plasmolytica or on water controls at 38 C for 3 hr. The experimental material then was transferred to 25 C and the change in permeability of the cells was monitored. The values presented in the table were calculated from the absolute figures of conductivity measured 10 hr after the temperature shift. Figures were corrected for effects of the osmotica alone.

[b]Permeability (conductivity) is expressed as percent of similar measurements of water-treated controls.

DISCUSSION

The results presented show that hypertonic (plasmolyzing) concentrations of the nonpermeating plasmolytica inhibit the bacterial HR. The inhibition of the viral HR by nonpermeating plasmolytica also has been reported (3,7,9). However, the inhibition of viral HR under plasmolyzing conditions seems to be complete, but the inhibition of bacterial HR, on the basis of permeability data, appears to be partial.

Another major difference between the viral and bacterial HR emerged from our studies. The osmotica "protect" the host tissues against the manifestation of both viral and bacterial HR. However, with the bacterial HR the concentration-dependency of the effect of plasmolytica is gradual: the more concentrated the osmoticum the more pronounced is the inhibitory effect. It does not appear to be important whether or not the host cells are plasmolyzed. In contrast, the development of viral HR (judged by the number of lesions and their diameters) exhibits a sharp dependency on the concentration of the osmoticum near and slightly above the isoosmotic range. Iso-osmotic conditions favor the development of the HR, but even slight plasmolysis results in a discontinuous response; ie, a drastic decrease in number and size of lesions.

Although the discontinuous response of the viral HR to increasing osmotic pressure strongly supports the importance of plasmolysis in the inhibition of the viral HR, and this is in line with the cell-to-cell contact hypothesis of Otsuki et al (16), there are other attractive alternatives which deserve attention. The osmotic shock induced by hypertonic media induces a number of metabolic changes in the cell (14,17–20) and these might interfere with the HR. It should be stressed that some of these changes have also been

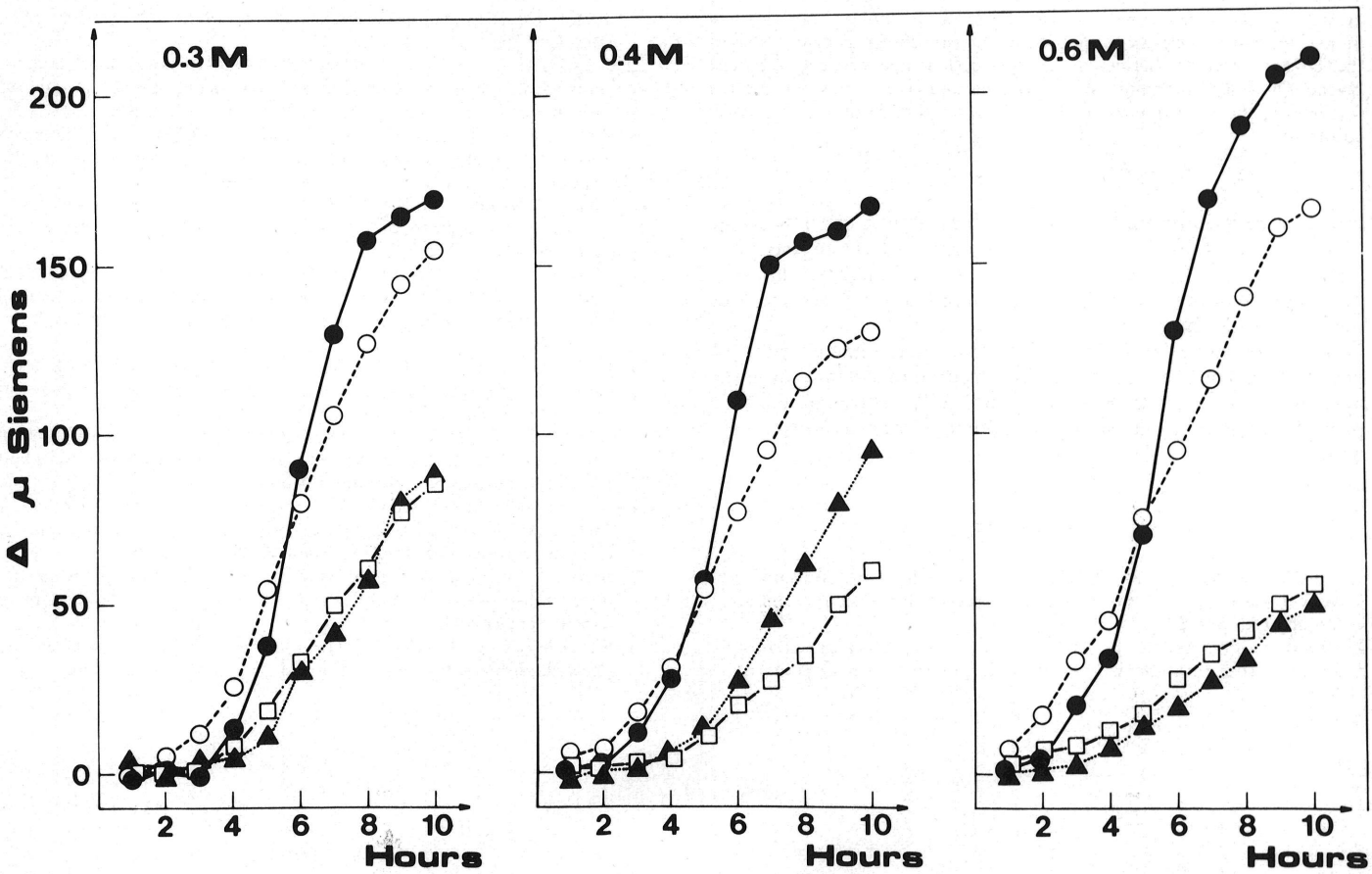

Fig. 3. The effect of osmotica on the "delayed" hypersensitive reaction evoked by *Xanthomonas vesicatoria* in tobacco leaves transferred to 25 C, following a prolonged latent period induced by high-temperature (38 C) treatment. Effect of the application of plasmolytica simultaneously with the 38 C → 25 C temperature shift. Tobacco plants after injection with the bacteria were kept at 38 C for 15 hr. Disks were then punched from the still symptomless leaves and floated at room temperature on plasmolytica of various concentrations. The change in permeability of the host cells was monitored, as a function of time. Water (●——●), ethylene glycol (○- - -○), mannitol (▲ · · · ▲), sucrose (□ - · - □).

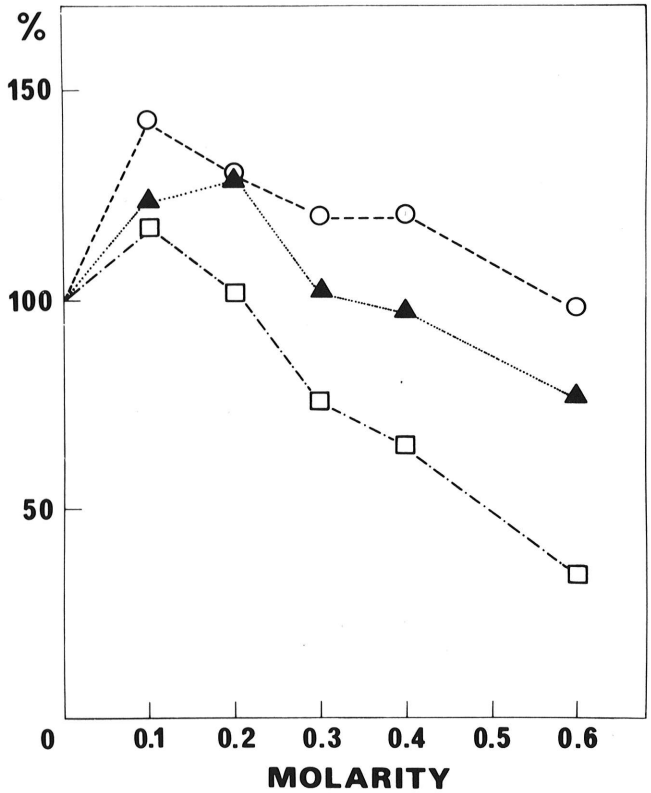

Fig. 4. The effect of osmotica on the multiplication of *Pseudomonas pisi* in vitro. Nutrient broth, nonsupplemented and supplemented with various amounts of plasmolytica, was seeded with *P. pisi* and the extent of bacterial growth was measured turbidimetrically. The values obtained after growth at 30 C for 5 hr in the presence of plasmolytica are shown as percentages of the "control" (figures obtained in the nonsupplemented medium). Ethylene glycol (○- - -○), mannitol (▲ · · · ▲), sucrose (□ - · - □). During the incubation, the cell number quadrupled in the nonsupplemented control cultures.

shown to be discontinuous, ie, they occur abruptly upon the onset of plasmolysis when the cells lose their turgor (20). In any case, our investigations (7) and those of Coutts (3) suggest that the development of the viral HR depends on cell turgor. The same does not seem to apply to the bacterial HR.

With the bacterial HR, the membrane-stabilizing effect of osmotica can be a major mechanism leading to the inhibition (or masking) of the HR. With the viral HR, although osmotic protection might be one factor, some other mechanism(s) also must be involved.

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