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EFFECTS OF MONOPARACOUMARYLPUTRESCINIUM CHLORIDE ON THE HYPERSENSITIVE REACTION OF *GOMPHRENA GLOBOSA* LEAVES TO TOMATO BUSHY STUNT VIRUS

S. PENNAZIO, *G. DELLAMONICA, P. REDOLFI

Institute of Applied Plant Virology, National Research Council, 10135 Torino, Italy;
and *Laboratory of Biological Chemistry, C. Bernard University, Lyon 1, Villeurbanne, France

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Summary. — Monoparacoumarylputrescinium chloride (pCPH), supplied to detached leaves of *Gomphrena globosa* via the petiole, induced interference with tomato bushy stunt virus infection by reducing the number and size of the necrotic local lesions. The phenolic compound neither inactivated directiy the virus in vitro, nor induced interference when supplied just after virus inoculation, all this indicating an effect on cell metabolism. The interference was positively correlated to both pCPH concentration and time of induction (viz., the intervals between pCPH supply and inoculation). Coumaric acid did not, whereas putrescine, but not spermidine or spermine, did induce interference. Disc electrophoresis in polyacrylamide gels revealed no changes in the soluble protein constitution between pCPH-treated and control leaves.

Key words: cinnamic acid-diamine conjugates; antiphytoviral effect; hypersensitivity; Tombusvirus

Introduction

Induced interference with virus infection can occur when particular chemicals are supplied to leaves or plants from outside (Hirai, 1977). A distinctive characteristic of these chemicals is to induce in leaves or plants a resistance to viral infection persisting for some time after administration. In particular, resistance to viral infection in hypersensitive combinations, easily assessable by reduction in number and size of necrotic local lesions, has been induced by several different chemicals (Stein and Loebenstein, 1970, 1972; Gianinazzi and Kassanis, 1974; Kassanis and White, 1975; Cassells *et al.*, 1978; White, 1979; Pennazio and Redolfi, 1980). The mechanism of interference of these chemicals with viral infection is still unknown.

During the hypersensitive reaction to viruses, plant tissues accumulate large amounts of different phenolic compounds (Fritig *et al.*, 1972; Tanguy-Martin and Martin, 1972; Redolfi *et al.*, 1978). The significance of the presence of phenolics in plant tissues hypersensitive to viruses should be therefore evaluated in connection with the host resistance, by analogy with the hypersensitive reaction to fungi and bacteria (Kosuge, 1969). In fact, many phenolics are known to have a virucidal activity in both plants (Krylov and Usoltseva, 1976; Hirai, 1977) and animals (Béládi *et al.*, 1977).

In the present paper we describe the effect of monoparacoumarylputrescinium chloride (pCPH) as a resistance inducer in *Gomphrena globosa* leaves hypersensitive to tomato bushy stunt virus (TBSV; *Tombusvirus* group). The peculiar interest of pCPH rests on the finding that it is a natural phenolic compound isolated from tobacco callus tissue cultures (Mizusaki *et al.*, 1971) and found in a large amount in the ovaries of several plant species (Martin-Tanguy *et al.*, 1978).

Materials and Methods

Plant and virus. G. globosa growing conditions and TBSV purification have been described (Pennazio *et al.*, 1976). Plants having a plastochron index 5 (Pennazio *et al.*, 1976) were preconditioned for 24 hr at 22 °C, 1,000 lux of continuous fluorescent light (Philips TL 55) and 65 % relative humidity.

Chemical treatment of detached leaves. Leaves of the 3rd pair, counted from the plant base, with a leaf plastochron index 2 (Pennazio *et al.*, 1976) were detached, their petioles immersed for 24 hr in an aqueous solution of the test chemicals and then transferred to distilled water. The opposite leaves of the same pairs were kept in distilled water as controls. All leaves were kept in a growth chamber at environmental conditions reported above. On the average, each leaf took up 1.5 ml solution (or water) per day. The following chemical compounds were tested: pCPH, synthesized as described below; coumaric acid (Merck); putrescine dihydrochloride (Calbiochem); spermidine trihydrochloride (Calbiochem); and spermine tetrahydrochloride (Calbiochem). Immediately after detachment or at daily intervals after the chemical treatment, the leaves were gently rubbed with carborundum and 10 μg/ml purified TBSV and rinsed with distilled water. The resulting necrotic local lesions were counted and measured 6 days later (Pennazio *et al.*, 1976). For each assay, 10 to 20 leaves were inoculated and several hundres of lesions counted and measured. The induced interference was expressed as per cent reduction in lesion number and size produced on treated leaves, in comparison with their controls. The time necessary to induce interference (time of induction) was calculated starting from the beginning of the chemical treatment (0 day time), just after leaf detachment.

Synthesis and properties of pCPH. pCPH was prepared by the procedure described by Mizusaki *et al.* (1971) for hydroxycinnamic amides. Acetylparacoumaryl chloride was added to a solution of putrescine in $CHCl_3$ and the desired product was obtained by extraction of $CHCl_3$ with 1 % HCl, which is hydrolyzing the acetyl derivative, followed by crystallization from ethanol. The element analysis found was (%): C, 55.83; H, 7.21; N, 10.08; 0, 13.95; Cl, 12.88. That calculated (1/2 molecule H_2O) was: C, 55.81; H, 7.51; N, 10.02; O, 14.31; Cl, 12.70. Infrared spectra, determined in KBr pellets by Perkin Elmer Infracord; mass spectrometry carried out by AEI MS902 at 70 eV; and paramagnetic resonance, carried out by Bruker 80 MHz (TMS internal reference) confirmed the structure shown in Fig. 1.

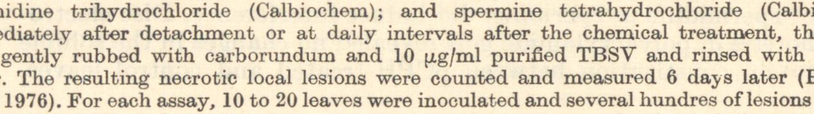

Fig. 1.
Structure of monoparacoumarylputrescinium chloride

Extraction and gel electrophoresis of soluble leaf proteins. Detached leaves were kept for 24 hr in a 1 mM pCPH solution. At the end of the treatment, some of the leaves were collected and processed as reported below. The others were transferred to distilled water and, at daily intervals, extracted at 4 °C by grinding 1 g fresh tissue with 1 ml citrate-phosphate buffer, pH 2.8 (Mac Ilvaine) or 0.1 M acetate buffer, pH 4. The homogenates were centrifuged at 105,000 × g for 60 min and 50 μl supernatant were immediately electrophoresed on 7.5 and 10 % polyacrylamide gels. Electrophoresis was carried out at 4 °C using the Davis' (1964) procedure in glass tubes, at constant current of 3 mA/tube applied for 2 hr. Some μg of solid bromophenol blue were added as marker. After electrophoresis, the gels were stained with 0.05 % Coomassie blue overnight, destained with ethanol: acetic acid: water (2 : 1 : 7), and scanned at 580 nm in a Chromoscan MK II recording densitometer.

Leaves kept in distilled water were collected at corresponding time intervals and processed in the same way as controls.

Results

Induced interference

On feeding 1 mM pCPM solution to *G. globosa* detached leaves statistically significant (t test) reductions in lesion number and size were observed when TBSV inoculation was carried out 1 to 2 days after the end on the treatment (time of induction: 1 to 2 days). The level of the induced interference increased with the time of induction (Fig. 2-I) (correlation coefficient 0.98). No interference was observed when leaves were inoculated with TBSV just before pCPH treatment (time of induction: 0 day).

In a secound experiment, detached leaves were kept for 24 hr in pCPM solutions of different concentrations, just after detachment and then transferred to distilled water for 2 days before TBSV inculation (time of induction: 3 days). Corresponding control leaves were kept for 3 days in distilled water before inoculation. Fig. 2-II shows that the interference increased with pCPH concentration. The highest concentration tested (1 mM) caused a slight wilting of the leaves, later disappearing on keeping the leaves in distilled water. Higher concentrations were toxic to the detached leaves.

One mg/ml pCPH did not inactivate 50 μg/ml TBSV following in vitro incubation at room temperature for 2 hr. The recovered infectivity of the treated TBSV suspension after 24 hr dialysis against 0.01 M phosphate buffer, pH 7, was not significantly different from that of the control suspension diluted with phosphate buffer.

To verify whether the moieties of pCPH could be responsible for the induction of interference, solutions of coumaric acid buffered to pH 6 and putrescine dihydrochloride were separately supplied to *G. globosa* detached leaves via the petiole, following the same procedure as described for pCPH. Coumaric acid had no effect on TBSV infection. Putrescine dihydrochoride induced interference with TBSV infection, but the correlation between the time of induction and the level of interference was different from that found for pCPH (Fig. 3-I). A positive correlation ($r = 0.98$) was found between the level of interference and the concentration of pCPH supplied to the detached leaves (Fig. 3-II), but the reduction of the lesion size was smaller than that obtained with pCPH.

Spermidine trihydrochloride and spermine tetrahydrochloride solutions were toxic when supplied to the detached leaves at 1 mM concentration. Lower concentrations induced no significant levels of interference.

Protein constitution after pCPH treatment

Extracts from pCPH-treated and control leaves were electrophoresed on polyacrylamide gels. No difference in protein constitution was found.

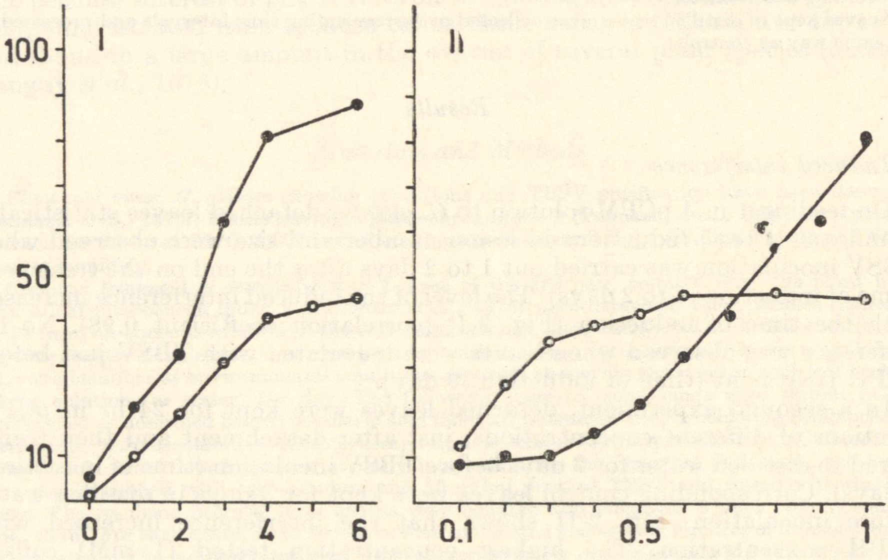

Fig. 2.

Interference with TBSV infection induced in *G. globosa* leaves by pCPH as a function of time of induction (I) and molar concentration (II)

The interference (ordinate) is expressed as per cent reduction in lesion number (●) and size (○) of the treated leaves in comparison with the controls.

Abscissae: I — time of induction (in days); II — mM pCPH

Fig. 4 (plate XIX) shows the protein patterns of MacIlvaine buffer extracts from control and pCPH-treated leaves 4 days after treatment. These extracts were run on 10 % gels and the patterns obtained can be considered representative of the whole series of experiments.

Discussion

pCPH affected TBSV localized infection by inducing interference in detached *G. globosa* leaves, but it did not directly inactivate the virus. A host-mediated mode of action seems therefore to occur by preventing the cell-to-cell spread of TBSV and/or by reducing cell susceptibility to TBSV infection. Martin-Tanguy *et al.* (1976) reported an antiviral activity of para-coumarylputrescine, but they supplied a very high concentration of the chemical to tobacco disks, hypersensitive to tobacco mosaic virus, just after virus inoculation.

The effectiveness of pCPH as a resistance inducer depended on concentration and time of induction. The positive correlation found between the time of induction and the level of interference, within the considered time interval, seems to be peculiar to pCPH since the other chemicals so far tested attained their maximum effect more quickly (Stein and Loebenstein, 1970, 1972; Gianinazzi and Kassanis, 1974; Stein *el al.*, 1979; Pennazio and Redolfi, 1980). A process involving a high number of metabolic steps or a particular step slowing down the whole process, may explain the delay in acquiring the resistance.

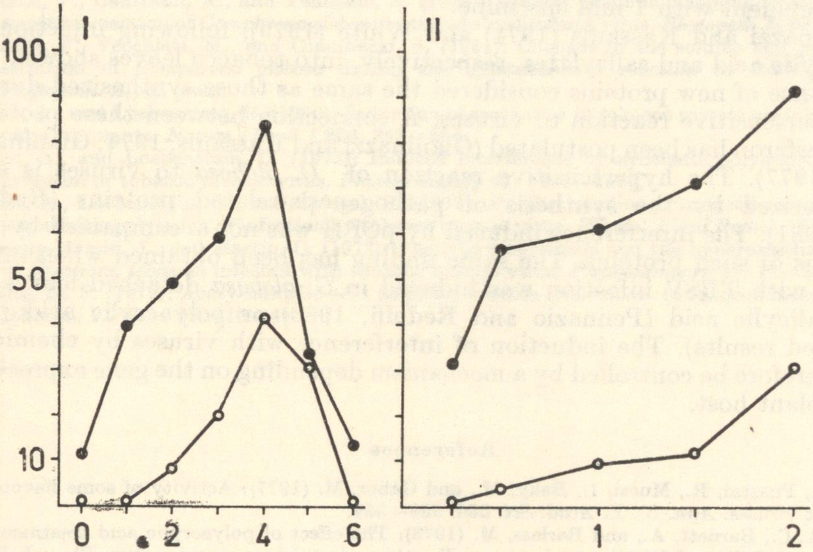

Fig. 3.

Interference with TBSV infection induced in *G. globosa* leaves by putrescine dihydrochloride as a function of time of induction (I) and molar concentration (II)

The interference (ordinate) is expressed as per cent reduction in lesion number (●) and size (○) of the treated leaves in comparison with the controls.

Abscissae: I — time of induction (in days); II — concentration of putrescine dihydrochloride (mM)

Putrescine was found effective in inducing interference with TBSV. This result suggests that the diamine could be the active moiety of pCPH. However, the differences in the correlations between time of induction, concentration and the levels of interference shown by putrescine dihydrochloride and pCPH indicate that the conjugation of the diamine to coumaric acid can result in some changes in its mode of action, in particular by increasing the level of interference with the time of induction (compare Figs 2-I and 3-I) and by limiting the cytopathic effect of TBSV infection (compare Figs 2-II and 3-II).

Spermidine and spermine were toxic when a 1 mM solution was supplied to detached leaves. These leaves became chlorotic within 24 hr and later wilted. Kaur-Sawhney and Galston (1979) found that in the light all polyamines promoted the disappearance of chlorophyll, this effect having been particularly evident with 1 mM spermine.

Gianinazzi and Kassanis (1974) and White (1979), following injection of polyacrylic acid and salicylates, respectively, into tobacco leaves showed the appearance of new proteins considered the same as those synthesized during the hypersensitive reaction to viruses. A connection between these proteins and interferon has been postulated (Gianinazzi and Kassanis, 1974; Gianinazzi *el al.*, 1977). The hypersensitive reaction of *G. globosa* to viruses is also characterized by the synthesis of pathogenesis-related proteins (Redolfi *et al.*, 1981). The interference induced by pCPH was not accompanied by the synthesis of such proteins. The same finding has been obtained when interference with TBSV infection was induced in *G. globosa* detached leaves by acetylsalicylic acid (Pennazio and Redolfi, 1980) or polyacrylic acid (unpublished results). The induction of interference with viruses by chemicals may therefore be controlled by a mechanism depending on the gene expression of the plant host.

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