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ANTIVIRAL ACTIVITY IN PLANTS OF A MYCOVIRAL DOUBLE-STRANDED RNA FROM *TRICHOTHECIUM ROSEUM*

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Summary. — Hexagonal virus-like particles (VLPs) measuring 45 nm across were detected in mycelial extracts from *Trichothecium roseum* Himachal strain, the source fungus for the production of T-poly (Trichothecium polysaccharide), a known inhibitor of plant viruses. VLPs were found to contain double-stranded RNA (ds RNA) and the purified ds RNA was capable of inhibiting tobacco mosaic virus infection in *Nicotiana glutinosa plants*. Active preparations of T-poly were found to contain traces of ds RNA, probably of mycoviral origin.

Key words: Trichothecium roseum; polysaccharide; virus-like particles; double-stranded RNA; virus inhibition

Introduction

The mold fungus *Trichothecium roseum* produces in culture a potent, non-toxic, heat stable, complex polysaccharide (T-poly) which is capable of inducing resistance against a number of unrelated viruses in their hypersensitive host plants (Gupta and Price, 1950, 1952; Bawden and Freeman, 1952; Gendron and Kassanis, 1954; Khurana *et al.*, 1978). T-poly is the first antiviral agent of microbial origin to be shown to act through the host (Bawden, 1954), the action being time, dose and host dependent. T-poly induces not only local but also systemic antiviral resistance and this resistance inducing action of T-poly could be partly reversed by timely treatment of host plants (*Nicotiana glutinosa*) with actinomycin D (Gupta *et al.*, 1974).

Bawden and Freeman (1952) concluded that the active antiviral substance obtained from *T. roseum* is a complex polysaccharide that invariably contained nitrogen (0.9 to 2.4% w/w). They separated the polysaccharide complex into five fractions, all containing nitrogen, and having antiviral activity. In agreement with this finding, T-poly prepared by us has been found to contain traces of nitrogen (0.4 to 1.0% w/w). While exploring the source of this nitrogen, we carried out electron microscopy of potent preparations of T-poly as well as of the mycelial homogenates. This led to the discovery of virus-like particles in the fungal cultures detectable during the log phase

of its growth in liquid medium. Significance of this finding in relation to the chemical nature of T-poly and the antiviral resistance induced by it in host plants is discussed.

Materials and Methods

Fungus. T. roseum Himachal strain was maintained in modified Shope's medium (Gupta *et al.*, 1974) as slant cultures.

T-poly. For production of T-poly, the fungus was grown at 28 ± 1 °C in one-litre stationary conical flasks each containing 150 ml of modified Shope's medium for a period of 28 days. Thereafter, the mycelial mats were pooled and homogenized in the spent medium. The slurry was centrifuged at 2000 rev/min (IEC International) for 20 min and the supernatant was passed through a double-layer muslin cloth. T-poly was prepared from this clarified culture homogenate as described (Gupta *et al.*, 1974).

Virus-like particles (VLPs) were concentrated and purified from the clarified culture homogenate by differential centrifugation: 40,000 rev/min for 90 min to pellet the VLPs, 18,000 rev/min for 20 min to clarify the resuspension and again at 40,000 rev/min for 90 min to repellet the VLPs (Beckman, rotor 40). The final pellet was resuspended in a suitable volume of phosphate buffered saline (PBS) pH 7.2 and centrifuged at 3000 rev/min (Janetzki T 23) for 30 min. The supernatant, containing VLPs, was collected and stored at −20 °C until used. Large scale purification of VLPs was carried out by precipitation with polyethylene glycol (PEG) 6000 by a combination of the methods of Tuveson and Peterson (1972) and Detroy and Still (1975). Briefly, 0.5 M NaCl and 6% (w/v) PEG were added to the clarified culture homogenate and stirred continuously for 2 hr at 2 ± 1 °C. The precipitated VLPs were collected by centrifugation at 6000 rev/min (Janetzki T 23) for 30 min and resuspended in PBS. Further purification was carried out by differential centrifugation as described above. Samples of active T-poly dissolved in PBS (2.5 mg/ml) were similarly subjected to differential centrifugation for recovery and detection of VLPs in it.

Electron microscopy. VLP preparations were examined under a Hitachi HV IIE-1 electron microscope on carbon-coated copper grids (400 mesh) after negative staining with 2% (w/v) potassium phosphotungstate or 1% (w/v) aqueous uranyl acetate. Micrographs were taken at an instrumental magnification of ×59,000 at 70 kV.

Extraction of nucleis acid from VLPs. Sodium dodecyl sulfate (Koch-Light, U.K.) was added to the VLP preparation to a final concentration of 1% (w/v). An equal volume of 90% aqueous phenol containing 0.1% 8-hydroxy quinoline was then added (Cox *et al.*, 1970) and the mixture shaken for 20 min at 30 °C. The aqueous phase was separated by centrifugation and mixed with 2 volumes of chilled methanol containing 0.1 M sodium acetate. After storage at −20 °C for 10−15 hr, the precipitated nucleic acid was collected by centrifugation at 4000 rev/min (Janetzki T 23) for 15 min and dissolved in 0.2 M sodium acetate. The final precipitate, obtained after repeated precipitation to remove residual phenol, was dissolved in saline sodium citrate buffer (SSC = 0.15 M NaCl + 0.015 M trisodium citrate) pH 7.4, placed at 4 °C and used without delay for further experiments. This preparation gave a UV absorption spectrum characteristic of nucleic acids with λ max at 256 nm.

Characterisation of the nucleic acid. Determination of the sugar moiety was carried out by differential staining with orcinol (Ogur and Rosen, 1950) or diphenylamine (Burton, 1956). Sensitivity to ribonuclease was determined by incubation with pancreatic ribonuclease A (Sigma; 4 μg/ml) for 30 min at 37 °C in SSC and 0.1 SSC followed by measurement of hyperchromicity at 260 nm in a Unicam spectrophotometer. Deoxyribonuclease (bovine spleen, Sigma; 20 μg/ml) treatment was carried out in 0.1 M sodium acetate buffer containing 0.001 M $MgSO_4$ (Lemke and Ness, 1970) and incubated at 28 °C for 30 min. The thermal denaturation of the nucleic acid was studied by measuring the increase in absorption at 260 nm in SSC and 0.1 SSC as a function of temperature using a Pye Unicam Sp 8000 spectrophotometer attached with a temperature programme controller. A drop of ethylene glycol was added to the cuvettes to attain temperature up to 104 °C (Marmur and Dotty, 1962).

Assay for antiviral activity. T-poly, VLP and nucleic acid preparations were tested for antiviral activity in *Nicotiana glutinosa* plants. Leaves were sprayed/rubbed on the upper surface with the test material and 48 hr later challenged with tobacco mosaic virus.

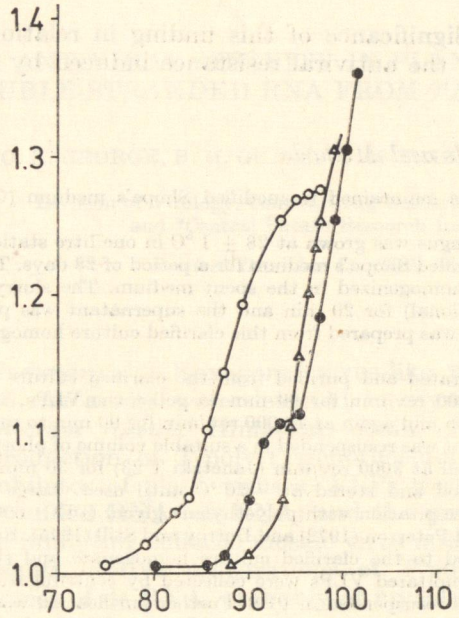

Fig. 2.
Thermal denaturation curves of ds RNA from VLPs at 0.1 SSC (○) or SSC (●) deoxyribonuclease-treated T-poly (△)
Abscissa: temperature (°C); ordinate: relative absorbancy at 260 μm
Heating was done at a rate of 1 °C/min

Results

Virus-like particles

VLPs were not detected in any of the potent antiviral preparations of T-poly. However, they were abundant in the fungal culture homogenate. Concentration of VLPs in the clarified culture homogenate increased with increasing fermentation period of the fungus, with a maximum after 28 days of growth. Ten g wet weight of mycelia harvested after 28 days of growth yielded about 2.5 mg of VLPs.

Under the electron microscope, the VLPs appeared hexagonal in outline (Fig. 1, Plate LVII) and measured on the average 45 nm across both in phosphotungstate- and uranyl acetate-stained preparations. An outer coat and a dense inner core could be clearly seen in most particles while some appeared to be devoid of the outer coat (Fig. 1, arrow).

Nucleic acid from VLPs

VLPs were found to contain RNA and not DNA as confirmed by the positive orcinol and negative diphenylamine reactions and further ascertained by resistance of the nucleic acid to deoxyribonuclease. The viral RNA was shown to be double-stranded by its characteristic melting curve (Fig. 2) and by its response to ribonuclease treatment. The rapid melting over a narrow temperature range is considered characteristic of ds nucleic acids (Richards *et al.*, 1965; Banks *et al.*, 1970). The transition mid point (Tm)

Table 1. Activity of T-poly, VLP and ds RNA in N. glutinosa plants against TMV

Leaves treated with	Lesions per leaf) mean ± S.E.)	% inhibition
T-poly	14 ± 1.96	94
VLP	226 ± 7.17	2
ds RNA	112 ± 15.56	51
PBS	230 ± 15.96	0

From 8—12 weeks old healthy plants (5 plants per group) were decapitated and pruned to have only 4 middle order leaves. T-poly (2.5 mg/ml), VLP (200 μg/ml), ds RNA extracted from VLP (20 μg/ml) or PBS pH 7.2 were sprayed/rubbed onto the upper surface of two basal leaves. After 48 hr, all the four leaves of a plant were washed, blotted dry and dusted with carborundum powder (600 mesh) on the upper surface and mechanically inoculated with a standard preparation of TMV. Local lesions of the virus were counted after 3 days.

of the ds RNA was about 88 °C in 0.1 SSC while in SSC it was beyond 100 °C. But the melting curve was not uniform, probably due to heterogeneity of the ds RNA species. With ribonuclease there was no detectable hyperchromic effect in SSC. But if the RNase treatment was carried out in 0.1 SSC, a 22% increase in absorption was recorded at 260 nm. Stability to ribonuclease at high and susceptibility at low ionic strength are considered characteristic of ds RNA (Richards *et al.*, 1965; Banks *et al.*, 1970.

Nucleic acid in T-poly

Eventhough VLPs were absent from T-poly preparations, presence of VLP-derived nucleic acid in them was a possibility. To check this, potent preparations of T-poly were extracted with trichloroacetic or perchloric acid. For extraction with trichloroacetic acid, 2.5 volumes of 10% trichloroacetic acid were added to an aqueous solution of T-poly and the mixture was kept at 4 °C for 10—15 hr following the method of Schneider (1945). The resultant precipitate was washed first with 5% trichloroacetic acid followed by cold and hot (60 °C) ethanol, boiled for 15 min in 5% trichloroacetic acid and centrifuged. RNA in the supernatant was estimated by the orcinol method (Ogur and Rosen, 1950). Perchloric acid extraction was carried out following the procedure of Schmidt and Thannhauser as modified by Munro and Fleck (1966). The precipitate obtained with cold 0.6 M perchloric acid (1/2 vol) was hydrolysed with 0.3 M KOH. Subsequently, the acid-soluble fraction was separated and its RNA content estimated by UV absorption at 260 nm and by the orcinol method. Both trichloroacetic acid and perchloric acid methods showed that 1 mg of T-poly contained approximately 6—8 μg of RNA.

Once the presence of RNA in T-poly was confirmed, thermal hyperchromicity studies were conducted with T-poly to determine the strandedness of the RNA present in it. For this, first the UV absorption of T-poly (1 mg/ml in 0.1 SSC) at 260 nm was measured at ambient temperature (30 °C); the temperature was gradually raised (1 °C/min) and the absorption at 260 nm was measured as a function of temperature. There was a rapid increase in UV absorption of T-poly at 260 nm between 90 and 100 °C (Fig. 2). To determine if the thermal hyperchromicity exhibited by T-poly was due to ds RNA and not to contaminating fungal DNA or some other component, T-poly was treated with ribonuclease and deoxyribonuclease prior to measurement of hyperchromicity: 1 mg/ml of T-poly was incubated with 100 μg/ml of deoxyribonuclease (bovine spleen, Sigma) for 15 hr at 28 °C or with bovine pancreatic ribonuclease A (Sigma, 50 μg/ml) for 15 hr at 37 °C. Before ribonuclease treatment, T-poly solution was heated for 30 min in a boiling water bath and then rapidly cooled.

Treatment of T-poly with deoxyribonuclease could not abolish its hyperchromicity; however, if treatment was carried out with deoxyribonuclease first followed by ribonuclease, T-poly showed no increase in UV absorption

Table 2. Relationship between fermenattion period of T. roseum and antiviral activity of T-poly

Length of fermentation (days)	Lesions per leaf* mean ± S.E.	% inhibition
7	288 ± 107	50
14	166 ± 69	61
21	42 ± 13	93
28	35 ± 9	94
PBS	579 ± 31	0

* Average number of lesions per leaf of TMV on *N. glutinosa* plants; 5 plants with a total of 20 leaves per group.

T-poly = 2.5 mg/ml.

with temperature. These results provided ample evidence that the RNA present in T-poly is double-stranded.

Antiviral activity

Treatment of *N. glutinosa* plants with purified VLP-derived ds RNA made them resistant to infection with TMV (Table 1). In contrast, treatment of plants with VLP preparations had no effect on the appearance of TMV lesions.

Antiviral resistance-inducing activity of T-poly increased with increasing fermentation period (7, 14, 21, and 28 days) of the fungus (Table 2). Even-though there was no significant difference in the yield of T-poly at these intervals, the one prepared after 28 days of fungal growth was found to be the most potent. There was also an indication of progressive increase in nitrogen content of the samples with time, maximum (1% w/w) having been recorded in the 28-day material as determined by the micro Kjeldahl method.

Discussion

Virus-like particles have been reported in a number of fungi notably several species of *Penicillium* and *Aspergillus* (Ellis and Kleinschmidt, 1967; Banks *et al.*, 1968, 1969*a*, *b*, 1970). These fungi yield antiviral substances capable of inducing interferon in vertebrate hosts (Kleinschmidt *et al.*, 1964; Kleinschmidt and Murphy, 1967; Banks *et al.*, 1969*b*, 1970). The active ingredient in these substances have been found to be either the VLPs or their ds RNA genomes (Kleinschmidt *et al.*, 1968; Banks *et al.*, 1969*b*, 1970). T-poly is a fungal product obtained from *T. roseum* and is capable of inducing antiviral resistance in plants. As with known interferon inducers, the antiviral action of T-poly can be reversed by timely treatment of host plants with actinomycin D (Gupta *et al.*, 1974). The discovery that mycelial extracts of *T. roseum* contain numerous ds-RNA-containing VLPs raised the possibility that these may have either a direct or an indirect role in the expres-

sion of a part or whole of the antiviral resistance inducing activity of T-poly. Eventhough VLPs were absent in T-poly, the experimental evidence obtained by us suggests that T-poly samples prepared according to the procedure described, do contain traces of ds RNA that either may represent the non-encapsulated pool existing in the fungal cells or was released by disruption of VLPs during the process of extraction. According to Sansing *et al.* (1973), at least 10% of the viral ds RNA remains non-encapsulated in *Penicillium stoloniferum*. Lemke and Ness (1970) suggested a sizable extra-particulate pool of ds RNA accumulating in cultures of *P. chrysogenum*. The thermal hyperchromicity profile at 260 nm exhibited by T-poly closely resembled that of the ds RNA derived from VLPs (Fig. 2) and supports the presence of viral ds RNA in it in mixture with the polysaccharide component. This is further confirmed by the abolition of hyperchromicity following ribonuclease but not deoxyribonuclease treatment.

According to Bawden and Freeman (1952), all active preparations of T-poly contained nitrogen. We found that the potency of T-poly samples increased with increasing period of fermentation of the fungus. There was also a parallel increase in the concentration of VLPs in the fungal culture and in the amount of nitrogen in T-poly. The source of nitrogen in T-poly may be the viral ds RNA which may also have a role in the antiviral resistance-inducing activity. This was suggested by the fact that purified viral ds RNA, by itself, was capable of inducing antiviral resistance in *N. glutinosa* test plants (Table 1).

To our knowledge, this is the first report of a mycoviral ds RNA capable of inducing antiviral resistance in plants. Further work is in progress to analyse and confirm the relative roles of polysaccharide and ds RNA in the antiviral resistance-inducing activity of T-poly in plants. Attempts are also being done to develop a virus free strain of *T. roseum*.

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