Properties of a Tobacco Necrosis Virus Isolate from *Pogostemum patchuli* in Brazil

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ABSTRACT

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An isometric virus isolated from leaves of patchouli (*Pogostemum patchuli*) was identified as tobacco necrosis virus (TNV). Infection in patchouli was systemic, but symptomless. Identification of TNV was based on host range, symptomatology, in vitro properties, morphology, and serology. *Celosia thompsonii*, *Leonotis nepetaefolia*, *Hibiscus esculentus*, and *Physalis floridana* were previously unreported hosts of TNV. The virus was purified and a specific antiserum was produced. Concentration of this virus in tobacco leaves was critical in serodiagnosis, since the antiserum did not react in agar gel with sap prepared from leaves with less than five lesions per square centimeter.

Patchouli (*Pogostemum patchuli* Pelles), a bush of the Labiatae family, contains an aromatic oil that is widely used in the soap and perfume industry.

During study of a virus complex infecting patchouli, an isolate of tobacco necrosis virus (TNV) was obtained. In patchouli, TNV caused a systemic but symptomless infection. We report here the host range, symptomatology, physical properties in vitro, electron microscopy, purification, and serology of this TNV isolate.

MATERIALS AND METHODS

Virus isolation and host range study. Six cultivars of patchouli (Campinas, Vietnan, Pará, Ituberaba, Ubatuba, and Amazonas) with mosaic symptoms were obtained from the Department of Medicinal and Aromatic Plants of the Instituto Agronômico de Campinas (IAC), São Paulo, and were used as source plants for virus isolation. Electron microscopic observation of the leaf dips prepared from these plants showed flexous potyviruslike particles and bacilliform rhabdoviruslike particles (7). Saps prepared by macerating 1 g of leaves in 4 ml of 0.001 M phosphate buffer, pH 8.0, with 0.1% Na_2SO_3 were mechanically inoculated to a range of plants commonly used in virus study. Two types of lesions appeared in inoculated leaves of *Chenopodium amaranticolor* Coste & Reyn. The first type, necrotic local lesions 2 days after inoculation, was induced by an isometric virus. The second type of necrotic lesion, which appeared 5 days after inoculation and later was surrounded by a red ring (7), was caused by a potyvirus. The rhabdoviruslike particles were not recovered. A single-lesion isolate of the isometric virus from patchouli cultivar Campinas, which was later identified as an isolate of TNV, was used in this study. The virus was maintained and propagated in *Nicotiana tabacum* L. 'TNN.' In the later trials, patchouli plants collected from Montes Claros (Minas Gerais) and Belém (Pará), were assayed for the presence of TNV by inoculation in *C. amaranticolor*. For the host-range study, 87 species of plants in 18 (17 dicotyledonous and one monocotyledonous) families were mechanically inoculated with this virus. Plants that did not develop symptoms were back-inoculated to *C. amaranticolor*.

Physical properties in vitro. Physical properties were studied by standard procedures. The inoculum used to determine the thermal inactivation point and longevity was obtained from *C. amaranticolor* (1 g/ml of buffer). For dilution end point the inoculum was prepared from *N. tabacum*, *Gomphrena globosa*, or *Chenopodium murale*.

Electron microscopy. Virus morphology was studied in leaf dip preparations of infected leaves of *C. amaranticolor*, *C. murale*, or *N. tabacum*. Cytopathologic studies were carried out in ultrathin sections of foliar tissue doubly fixed in glutaraldehyde and osmium tetroxide, both in phosphate buffer, and embedded in Epon or Spurr's medium. Examinations were made with a Jeol JEM-100C electron microscope.

Purification. Purification process was based on the method of Kassanis (11) with some slight modifications. The virus was increased in *N. tabacum* 'TNN.' The inoculated plants were kept inside a screenhouse (temperature 18–42 C with an average of 30 C). Five days after inoculation, the inoculated leaves were collected and stored in a freezer for 10 days before they were homogenized in a blender with an equal volume of 0.001 M phosphate buffer pH 8.0 containing 0.1% Na_2SO_3. The sap was squeezed through cheesecloth, and then clarified by using one of the following four methods: (i) Addition of *n*-butanol (8 ml/100 ml sap) followed by agitation for 40 min. (ii) Sap was frozen at −10 C for 24 hr and thawed (19). (iii) Same as ii, but followed by heating with agitation at 50 C for 10 min. (iv) Addition of ethanol (20 ml/100 ml of sap) and agitation for 40 min (14). After one low-speed centrifugation, virus was concentrated by adding of ammonium sulphate (0.25 g/ml) (11) followed by a low-speed centrifugation and resuspension of pellets in buffer (one-tenth of the original volume). After two additional cycles of low- and high-speed centrifugations, the final pellet was resuspended in water. Purity and concentration of the preparations were determined by electron microscopy and UV spectrophotometry (Spectronic 200 UV), using an extinction coefficient of 5/mg/ml/cm at 260 nm (11). Estimation of nucleic acid content was based on A_{280}/A_{260}, according to Layne (13).

Serology. To produce specific antiserum, a rabbit received two intramuscular injections of purified virus (2 mg and 0.75 mg, respectively), with a 15-day interval. Virus preparations were emulsified with equal volume of Freund's complete adjuvant (Difco Lab., Detroit, MI 48232). Antiserum was collected four times, at weekly interval, through heart puncture; the first bleeding was done 27 days after the last injection. Agar gel double diffusion was ued to detect virus. The agar gel consisted of 0.75% Noble agar, 0.85% NaCl, and 0.02% sodium azide. Antiserum titers was determined by testing it against crude sap or purified TNV at

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0.125 mg/ml. To confirm the serological identification of the virus, crude sap of infected tobacco leaves or a purified preparation at 0.125 mg/ml was tested against the following antisera: Californian isolate of TNV (from R. N. Campbell, University of California, Davis 95616), Canadian isolate of TNV and cucumber necrosis virus (from H. F. Dias, Agriculture Canada, Vineland Station, Ontario) and carnation mottle virus (from O. Lovisolo, Laboratorio di Fitovirologia Applicata, Torino, Italy).

RESULTS

Virus isolation and host range. TNV was isolated from all six cultivars of patchouli from IAC, but not from seven samples received from Montes Claros (Minas Gerais) and Belém (Pará). The virus was recovered from roots, stems, leaves, and shoots of infected patchouli, but the highest concentration was in shoots and leaves. Inoculation of virus-free patchouli plants (obtained by meristem culture [8]) with TNV produced a systemic and symptomless infection.

Among the 87 species of plants tested, the virus induced necrotic local lesions, but not systemic infections, in 36 species and was recovered from nine symptomless plant species. The host range and symptomatology of this TNV isolate agree with those previously reported (6,15,17,18). Plants previously unreported as hosts for TNV were *Celosia thompsonii* Hort., *Leonotis nepetaefolia* L., *Hibiscus esculentus* L., and *Physalis floridana* Rydberg. The plants previously untested for TNV that did not show symptoms and from which no virus was recovered were: *Dahlia variabilis* Desf., *Bidens pilosa* L., *Eruca sativa* Mill., *Scabiosa maritima* L., *Euphorbia ifolia* runJacq., *Manihot utilissima* Pohl., *Leonorus sibiricus* L., *Mentha viridis*, *Phaseolus lunatus* L., *Malva parviflora* L., *Sida rhombifolia* L., *Solanum gilo* Reddi, *Capsicum annuum* L., *Apium graveolens* L., *Coriandrum sativum* L., *Foeniculum vulgare* Hill, and *Petroselinum sativum* Hoffn.

In vitro physical properties. The patchouli isolate of TNV had a thermal inactivation point of 85–90 C, a dilution end point of 10^{-5}–10^{-6} in *N. tabacum* or *G. globosa* and 10^{-6}–10^{-7} in *C. murale*. In crude sap, the virus was infective for 4 days, but not for 7 days, at room temperature (25–26 C) and for more than 28 days at 3–4 C.

Electron microscopy. Examination of leaf dip preparations of some infected plants revealed the presence of isometric particles 25–30 nm in diameter (Fig. 1). In patchouli, probably due to the relatively low concentration, TNV particles were not observed either in leaf dip preparations or in thin sections. Similar particles were the sole component of highly purified and infectious preparations (Fig. 2). These particles could also be seen in thin sections of local lesions from tobacco, *C. amaranticolor*, and *C. murale*. Although TNV particles were difficult to distinguish from ribosomes, they were observed in the cytoplasm, occasionally in a crystalline array (Fig. 3). Eventually the particles appeared aligned, forming short strings (Fig. 3, insert). In rare instances, viruslike particles were found in the nucleoplasm (Fig. 4). No satellite viruslike particles were ever found.

Purification. Among the four methods of clarification tested, the ethanol method gave the most satisfactory results. Electron microscopical examination of purified TNV, prepared by using ethanol as the clarifying agent, showed numerous intact and empty isometric particles ~28 nm without apparent contamination of host materials (Fig. 2). The yield of the purified virus was 2.8 mg/kg of tobacco leaves. The purified preparation gave a typical nucleoprotein spectrum with the maximum and the minimum absorption at 260 nm and 244 nm, respectively, and a ratio of A_{260}/A_{240} equal to 1.215. The nucleic acid content of the purified virus was estimated to be about 12%.

Serology. The antisera obtained from the four bleedings did not react with healthy tobacco sap, but did react with undiluted sap of tobacco infected with the virus or with purified virus preparations in agar gel double-diffusion tests, forming one curved precipitin line. Antisera obtained from the first three bleedings reacted with undiluted infected sap at a dilution of 1/256 and at a dilution of 1/2,048 when tested with a purified preparation. The concentration of this virus in tobacco leaves appeared to be critical in serodiagnosis of this virus. For example, sap prepared from tobacco leaves that had less than five lesions per square centimeter did not form a visible line with the antisera in agar gel. However, sap prepared from leaves with 5–10 lesions per square centimeter formed a visible line with 1:2 dilution of the antisera. Crude sap of infected patchouli did not react with the antisera in agar gels. After the virus in the sap was concentrated 40-fold by ultracentrifugation, it gave positive reactions, even at a 1:32 dilution. The patchouli isolate of TNV either in crude sap of tobacco leaves or in purified preparation, reacted positively with the antisera to Californian and Canadian isolates of TNV, but not with cucumber necrosis virus or carnation mottle virus antisera.

DISCUSSION

Based upon these results, the isometric virus from patchouli was identified as an isolate of TNV. The infection of patchouli by TNV is systemic and symptomless as it also is in *Primula obconica* (1), *Vitis vinifera* L. (4), and *Malus sylvestris* Mill. (21). Thus, patchouli is one of the few species known to be infected systemically by TNV (1,10,11,17).

Tobacco necrosis virus was first described in Cambridge, England, infecting *N. tabacum* and *N. glutinosa* seedlings (18). It was later demonstrated to be soilborne and transmitted by the fungus, *Olpidium brassicae* (20). It was common in irrigated fields and in unsterilized soil in glasshouses (11). Although TNV is widespread, it causes only a few diseases (10); eg, tulip necrosis (Augusta disease), bean stipple streak, ABC disease of potato tubers, and a form of cucumber necrosis (10,11).

All six cultivars of patchouli from IAC, Campinas, but not from Montes Claros and Belém, were infected with TNV. This indicates that the infection probably occurred in Campinas, where TNV was detected in tobacco and strawberry roots by Costa (5).

In our host reaction study, translocation of the virus in leaf veins of *Lycopersicum esculentum* Mill. and *Vigna unguiculata* (L.) Walp. (reported by Smith [18]), was not observed; its translocation to uninoculated leaves in *Phaseolus vulgaris* L., (reported by Smith [18] and Natti [15]) did not occur and it became localized and formed a dark net in inoculated leaves; and it infected *Calendula officinalis* L. and *Chrysanthemum* sp., which were described as immune to TNV by Price (16) and Natti (15). The opposite occurred in *Mirabilis jalapa* L.. *Brassica oleracea* var. *capitata* L., which was reported by Fulton (6) to be susceptible to TNV, was not infected by the patchouli isolate.

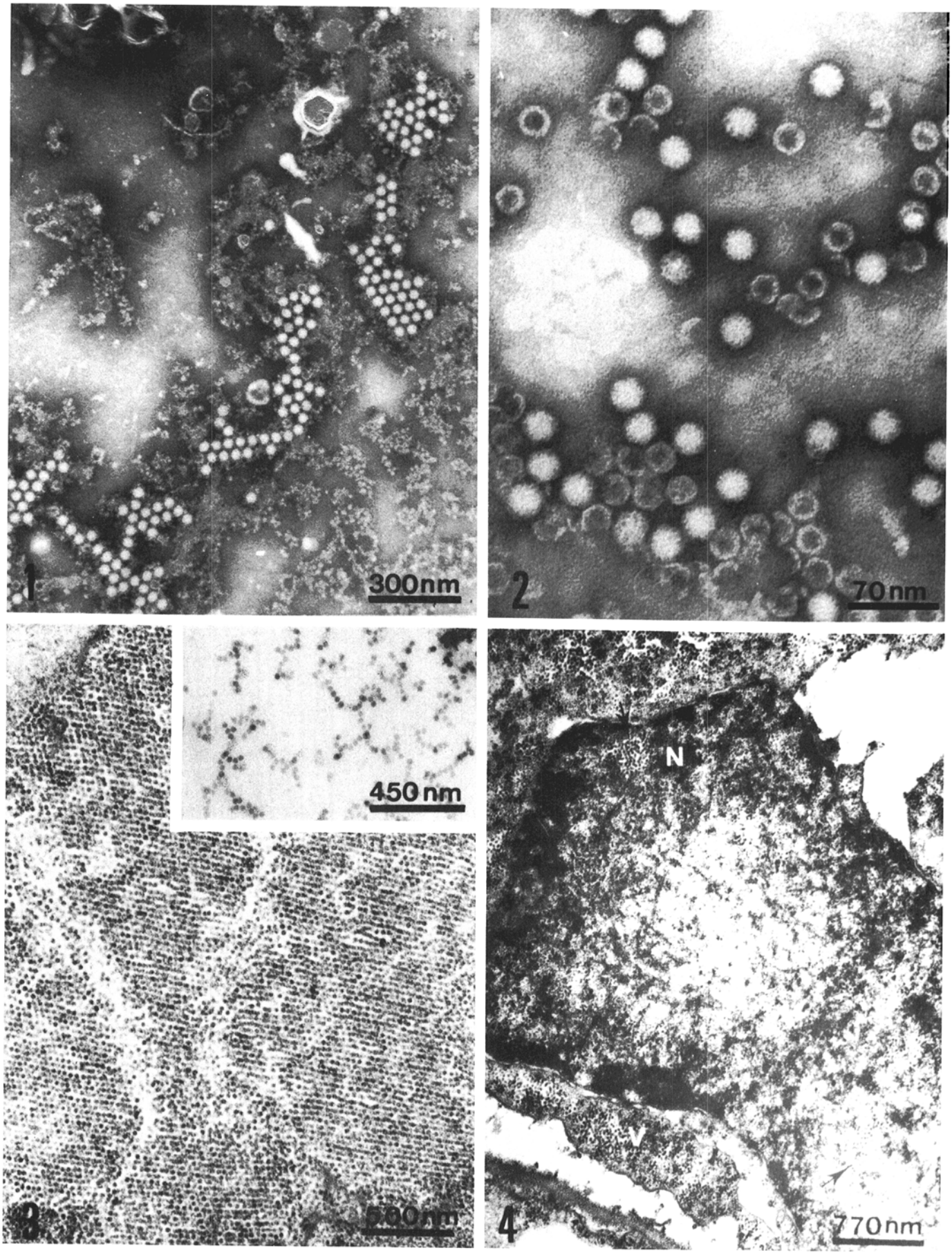
Among the plants susceptible to the patchouli TNV isolate, *Zinnia elegans* Jacq., *Leonotis nepetaefolia* L., *Datura stramonium* L., *Sonchus oleraceus* L., *Lycopersicum esculentum* Mill., *Nicandra physaloides* Gaertn., and *Physalis floridana* Rydberg showed symptoms only when kept at lower temperature (18–23 C). The effect of temperature on the susceptibility of plants to TNV has been observed by Bawden and Pirie (3).

The in vitro physical properties of the patchouli isolate are very similar to those reported for TNV (11).

Isometric virus particles usually are difficult to detect in leaf-dip preparations, but in some of our tobacco leaf-dip preparations there were many 25–30 nm particles. The assumption that they were TNV particles was confirmed by observing purified preparations. The nature of the stringlike array of the virus particles, also observed by Kassanis et al (12), is unknown. On rare occasions, particles were seen within the nucleus, a feature so far unreported for TNV (11).

Bawden and Pirie (2,3) observed that when plants used to increase TNV were kept at high temperature and light intensity, susceptibility dropped sharply and the quality and yield of purified virus preparations were very poor. This could explain the relatively low yield of virus in our purifications, since no special care was taken to keep plants at low temperature and light intensity. Harrison (9) suggested that the decrease of number of TNV particles in leaves kept at temperature >22 C could be explained by the difference between the inactivation and multiplication rates of virus particles.

The difference between the RNA content found in this work



Figs. 1–4. Electron micrographs of an isolate of TNV from patchouli. **1,** Isometric particles in leaf-dip preparations from infected tobacco plants and **2,** in a purified preparation. **3,** Crystalline aggregate of virus in the cytoplasm of *Chenopodium murale* leaf cell and (inset) a stringlike array of particles in the cytoplasm of a *C. amaranticolor* leaf cell; and **4,** viruslike particles in the nucleoplasm of a *C. murale* leaf cell.

(12%) and the published data, 19%, as well the ratio of absorptions at 260 and 280 nm (1.515 against 1.7 reported) must be due to loss of RNA by some particles during purification. The maximum and minimum absorptions are in close agreement with the reported values (4). Electron microscopic examination of purified preparations showed that many particles of virus were penetrated by stain, which suggests that RNA had been lost (Fig. 2).

Positive results obtained from serological tests with antisera against isolates of TNV from California and Canada confirmed the identity of the virus isolate from patchouli.

Because TNV concentration in plants are usually low (1,21), samples collected from the field, which give negative results in serodiagnosis, should be mechanically inoculated to tobacco plants for bioassay or the virus concentrated prior to seroassay. As shown in this work, saps prepared from tobacco leaves with more than five lesions per square centimeter have sufficient virus antigen to be detected by the agar gel method.

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