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Purification and serology of virions of impatiens necrotic spot tospovirus

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Abstract

Impatiens necrotic spot tospovirus (INSV) virions were purified using a procedure devised for tomato spotted wilt tospovirus (TSWV) from systemically infected *Nicotiana benthamiana* plants grown at 33 °C day/26 °C night and a photoperiod of 14 hours. With plants grown at 24/18 °C purification was unsuccessful. In SDS-PAGE the protein pattern of INSV was similar to that reported for TSWV, except the appearance of a single G2 protein band. A polyclonal antiserum, prepared against virions, reacted in Western blots with INSV nucleoprotein and glycoproteins but only with TSWV glycoproteins. In DAS ELISA the antiserum reacted with both INSV and TSWV infected plant sap and, after absorption with TSWV, only with INSV. In TAS ELISA the antiserum trapped both INSV and TSWV nucleoproteins and glycoproteins as detected by specific monoclonal antibodies, and, after absorption with TSWV, only the homologous proteins. This appears to be the first report of the purification of INSV virions and the production of an antiserum reacting with both nucleoprotein and glycoprotein antigens.

The genus *Tospovirus*, family *Bunyaviridae*, includes tomato spotted wilt virus (TSWV), the type species, and impatiens necrotic spot virus (INSV) (Murphy et al., 1995) and others such as groundnut ringspot virus (GRSV), tomato chlorotic spot virus (TCSV) and groundnut bud necrosis virus (GBNV). These viruses have recently been recognised as distinct on the basis of differing genome sequences and serological unrelatedness of the N proteins (Adam et al., 1993, 1995; de Haan, 1994).

INSV was first described in the USA and later reported in several European countries, mostly infecting ornamentals (Law and Moyer, 1990; Lisa et al., 1990; de Avila et al., 1992; Vaira et al., 1993). So far only the nucleocapsid (NC) of INSV has been purified and antisera to it raised (Law and Moyer, 1990; de Avila et al., 1992), whereas with TSWV the virion has been purified, antisera to the whole particle prepared and the viral proteins characterized (Mohamed et al., 1973; Tas et al., 1977a and b). The nucleoprotein (N) of INSV is serologically unrelated to TSWV N but INSV membrane glycoproteins (G1 and G2) react with polyclonal and monoclonal antibodies to TSWV glycoproteins (Law and Moyer, 1990; de Avila et al., 1992; Vaira et al., 1993, Adam et al., 1995). Lawson et al. (1993) found that an isolate of INSV producing few virions at 20 °C, gave a much higher yield at 27 °C. In this paper we report the purification of INSV virions from *N. benthamiana* grown at high temperature, the preparation and characterization of a polyclonal antiserum to the virion, and analysis of the viral proteins by SDS-PAGE.

INSV was isolated from statice (*Limonium sinuatum*) in Liguria (north western Italy). The isolate was similar in host range to other INSV isolates (de Avila et al., 1993; Vaira et al., 1993) and was serologically confirmed as INSV (Adam et al., 1995). Sap of infected glasshouse-grown *N. benthamiana* was seen by EM after negative staining to contain virions, confirming that the isolate was not morphologically defective.

TSWV-L3 (DSM PV-0182) was a typical TSWV, isolated from tobacco (Adam et al., 1995). Both viruses were maintained by sap inoculation of *N. benthamiana* and *N. rustica* grown in the glasshouse.

INSV was purified from systemically infected *N. benthamiana* leaves 12–15 days post-inoculation.

Inoculated plants were kept either in the glasshouse at 24–18 °C (± 2) day/night temperature, with natural light, from July to September, or in a growth chamber at 14 h photoperiod, 150 μmol m $^{-2}$ s^{-1} PAR with Philips TL 95 at 33 °C (± 1) in the light and 26 °C in the dark. Virus was also purified from roots of glasshouse plants.

TSWV was purified from systemically infected *N. rustica* leaves collected about 10 days post-inoculation from plants grown in the glasshouse at 24/18 °C.

Both viruses were purified by the method of Black et al. (1963), modified by J. R. Diaz-Ruiz (pers. com., 1992), as reported by Adam et al. (1995). Infected leaves were homogenized with 3 volumes (w/v) of 0.1M Na phosphate buffer, pH 7, containing 0.01M Na_2SO_3. The slurry was filtered through nylon stocking and centrifuged for 10 min at 16,300 × g. The pellets were resuspended in 2 volumes (v/w leaf weight) of 0.01M Na phosphate buffer, pH 7, containing 0.01M Na_2SO_3 (resuspension buffer, RB) and stirred for 30 min, then centrifuged at 1,000 × g for 15 min. The supernatant was carefully removed and centrifuged at 70,000 × g for 30 min. Pellets were resuspended in RB and centrifuged at 1,100 × g for 10 min. The supernatants were layered on a 5–40% sucrose density gradients prepared in RB, and centrifuged at 72,000 × g for 45 min. The virus-containing band was removed with a syringe, diluted with one volume of RB and concentrated by centrifugation at 70,000 × g for 30 min. The pellets were resuspended in RB. The protocol was completed in about five hours at about 4 °C. Steps in purification were evaluated by negative stain EM and SDS-PAGE.

For electrophoresis, samples were denatured at 100 °C for 5 min with 0.02 M Tris-HCl buffer, pH 8, containing 1% SDS and 1% 2-mercaptoethanol. SDS-PAGE was done using the PhastSystem apparatus, PhastGel Gradient 8–25, and low molecular weight markers (Pharmacia, Uppsala, Sweden) according to manufacturer's instructions.

For electron microscopy, crude sap or purified preparations were absorbed to Formvar-carbon coated grids, rinsed with water and negatively stained in 1% uranyl acetate. Filmed grids were subjected to glow-discharge before use with purified preparations, to obtain better adhesion of the sample and negative stain.

To produce antiserum against INSV virions, one rabbit was injected intramuscularly with purified material emulsified with Freund's adjuvant, complete for the first injection and incomplete for the subsequent three injections. The first injection was made with partially purified material from *N. benthamiana* grown at 24/18 °C, later injections were made with purified virus preparations from the same host, but grown at 33/26 °C. Bleedings were taken at two week intervals and their titres measured by agar gel double diffusion. The bleeding with the highest titre (1/32 against INSV and 1/4 against *N. benthamiana* sap), obtained 2 months after the last injection, was used for subsequent work. An ELISA kit against INSV NC was purchased from Loewe (Otterfing, Germany); other antisera or monoclonal antibodies used against the NC and glycoproteins of TSWV and the NC of INSV are listed in Table 1 and detailed in Adam et al. (1995).

Table 1. Antibodies used

Antibodies	Immunogen
A310HA[a]	INSV virion
A310TA[b]	INSV virion
INSV Loewe	INSV nucleocapsid
DSM As-105	TSWV virion
Mab 5E4	INSV nucleocapsid
Mab 4F2	TSWV nucleocapsid
Mab 2B6	TSWV virion
Mab APO 2B3/2D11	TSWV glycoproteins

[a] absorbed with healthy sap.
[b] absorbed with TSWV-infected sap.

The antiserum against INSV virions (A310) was cross-absorbed with healthy preparations (A310HA) or with partially concentrated preparations of TSWV from *N. benthamiana* (A310TA). Healthy or TSWV-infected *N. benthamiana* leaves were homogenized with 0.1 M phosphate buffer pH 7 containing 20 mM Na_2SO_3, filtered through nylon stocking and centrifuged at 27,000 × g for 30 min. The pellets were resuspended in the antiserum diluted 1/4 with PBS, incubated overnight with stirring and centrifuged at 250,000 × g for 30 min.

For ELISA, antibodies were purified from A310HA and A310TA using protein G (Pharmacia, Uppsala, Sweden) and conjugated with alkaline phosphatase using glutaraldehyde in a two-step procedure. Antiserum reactivity was evaluated by agar gel double diffusion and by antigen-coated plate trapping ELISA, DAS-ELISA and TAS ELISA, using crude sap from healthy, INSV- or TSWV-infected *N. benthamiana*.

For Western blots, proteins were separated by SDS-PAGE as above and transferred to nitrocellulose by diffusion at 50 °C for 30 min on PhastSystem apparatus; the membranes were blocked for 2 min with 2% Tween–20 in PBS. The antisera were diluted with PBS

containing 0.05% Tween and 2% polyvinylpyrrolidone and the bound antibodies detected using anti-rabbit IgG alkaline phosphatase conjugate. Low molecular weight markers from Pharmacia were biotinylated using N-hydroxy-succinimido-biotin; after denaturation with SDS, their MWs were estimated by comparison with the original markers. They were used as markers on Western blots and detected using streptavidin-conjugated with alkaline phosphatase. The development solution contained 0.165 mg/ml of BCIP and 0.33 mg/ml of NBT.

The purification method adopted proved suitable for the purification of TSWV virions from *N. rustica* grown at 24/18 °C. After density gradient centrifugation, a light-scattering band rich in virions formed in the upper third of the tube. This procedure with INSV from *N. benthamiana* grown at 24/18 °C gave no visible bands and the zone equivalent to that containing TSWV virions contained a few virions and large amounts of host membrane; upon SDS-PAGE, such preparations formed several faint bands with only the nucleoprotein band clearly visible. Preparations from roots of INSV-infected *N. benthamiana* grown in the glasshouse produced a faint light-scattering zone in the sucrose density gradient, containing relatively more virions and less membrane material, but the yield of virus per unit fresh weight was poor and the yield of roots was also small. Preparations from infected plants grown at 33/26 °C were less contaminated with green host material, and a distinct light-scattering zone formed in the gradient, at the same position as that of TSWV. Concentrated preparations from this zone contained large amounts of virus particles, slightly contaminated by host membranes (Figure 1). In SDS-PAGE the purified preparations formed three sharp bands (Figure 2), that, by analogy with TSWV, were identified as N, G2 and G1 in order of increasing MW estimated to be 27,000, 56,000 and 83,500 respectively.

In antigen-coated plate-trapping ELISA, A310HA reacted with both INSV and TSWV; A310TA reacted with INSV but the reaction with TSWV was greatly reduced (Figure 3).

In Western blots, A310HA reacted with homologous N, G1 and G2, and with TSWV G1 and G2. INSV IgG from the Loewe kit reacted with INSV N and faintly with INSV G1 and G2 and TSWV G1. DSM-As105 (against TSWV virions) reacted with the homologous TSWV N and with the glycoproteins of both viruses. TSWV usually formed a double G2 band, while INSV formed a single G2 band of slightly lower mobility (Figure 4).

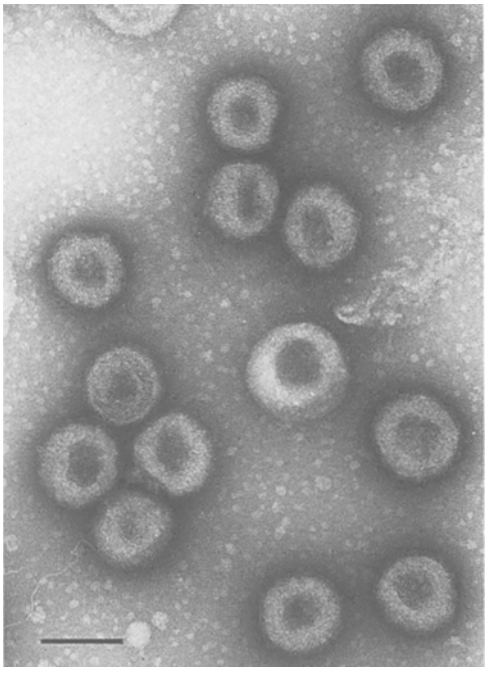

Figure 1. Purified preparation of INSV negatively stained in 1% uranyl acetate. Bar = 100 nm.

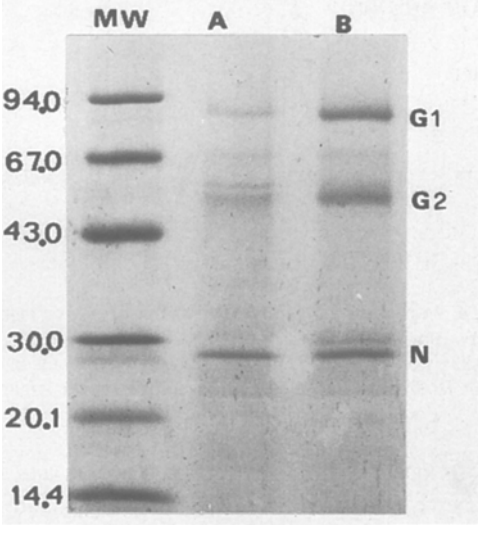

Figure 2. SDS-PAGE of two INSV purified preparations (lanes A and B). A came from roots, B from leaves. MW = molecular weight markers. N, G2 and G1 proteins are indicated on the right and molecular weight of the markers (kDa) on the left. The gel was stained with Coomassie Brilliant Blue.

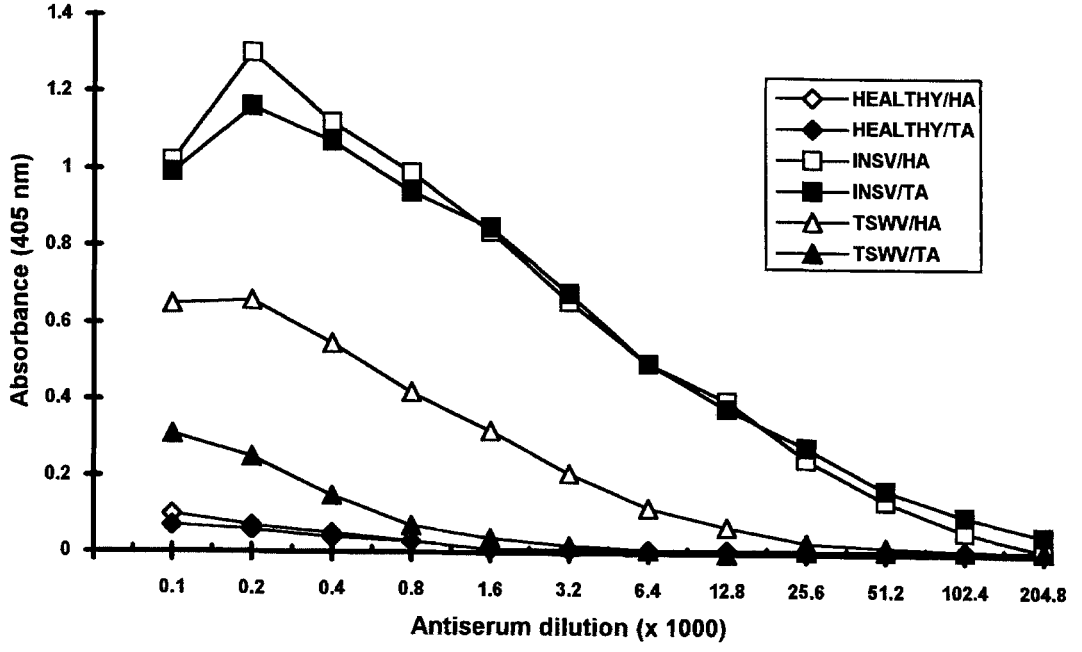

Figure 3. ELISA reaction on plates coated with sap, diluted 1/100, from healthy, INSV- or TSWV-infected *N. benthamiana*. The detecting antibody was from INSV antiserum absorbed with healthy sap (A310HA) or with TSWV-infected sap (A310TA).

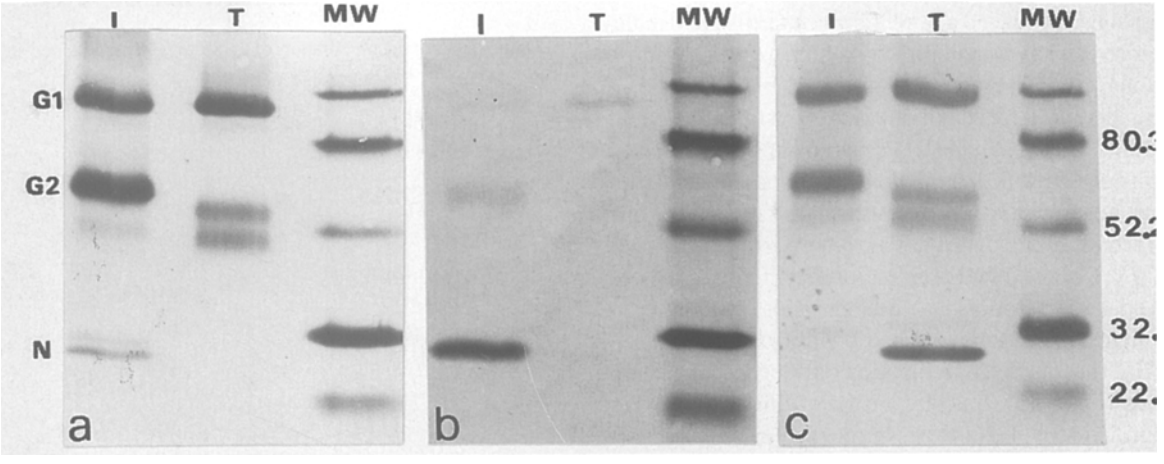

Figure 4. Western blots of purified INSV (I) and TSWV (T) preparations after separation by SDS-PAGE. MW = biotinylated molecular weight markers. Blots were probed with INSV A310 HA antiserum (a), antibodies from the Loewe INSV kit (b) and TSWV antiserum (c). Bound antibodies were detected with goat anti-rabbit AP conjugate and biotinylated markers with streptavidin-AP conjugate. The substrate was NBT/BCIP. Proteins N, G2 and G1 are indicated on the left and molecular weights of the markers (kDa) on the right.

IgGs purified from A310HA, A310TA and from the Loewe INSV kit were tested by DAS and TAS ELISA, for the detection of INSV and TSWV (Figure 5). The results agreed with those of the previous tests: in DAS ELISA A310HA detected both INSV and TSWV, while A310TA and the Loewe kit detected only INSV.

When these antibodies were used as coating in TAS ELISA, INSV NC Mab 5E4 reacted with INSV in all cases; TSWV NC Mab 4F2 reacted with TSWV only when A310HA was used for coating. The two Mabs DSM 2B6 and APO 2B3/2D11, produced with TSWV glycoproteins, detected both viruses when the coating antibody was A310HA, but only INSV when the coat-

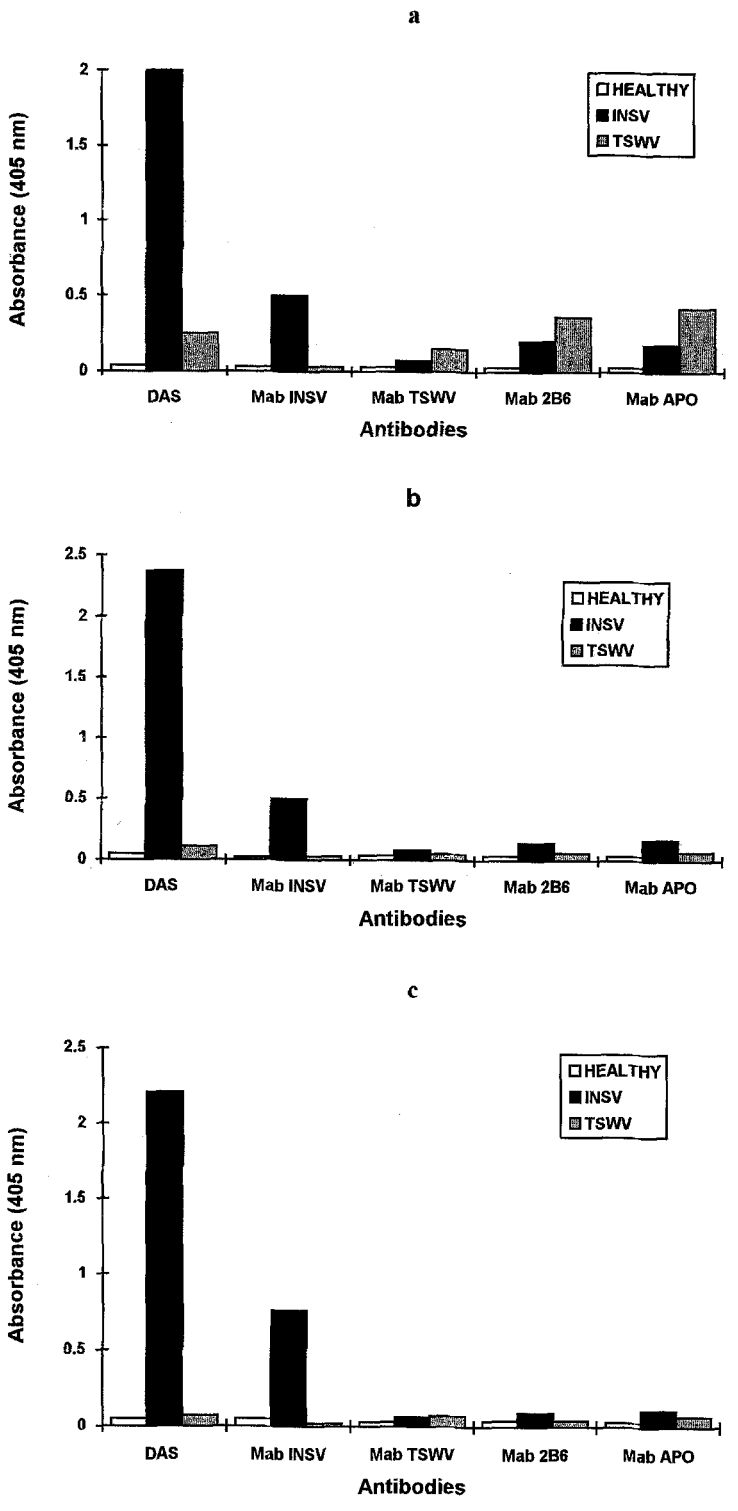

Figure 5. DAS and TAS ELISA reactions of antibodies from A310HA (a), A310TA (b) and from the Loewe INSV kit (c) with INSV, TSWV and healthy plant sap diluted 1/100. Plates were coated with the indicated antibodies. For DAS ELISA the antigens were detected using the same antibodies conjugated with alkaline phosphatase (DAS). For TAS ELISA the antigens were detected with Mab 5E4 against INSV nucleoprotein (MAb INSV), Mab 4F2 against TSWV nucleoprotein (Mab TSWV), Mab 2B6 against TSWV glycoproteins (Mab 2B6) and Mab APO 2B3/2D11 against TSWV glycoproteins (Mab APO). Substrate incubation was 60 min for DAS ELISA and 20 min for TAS ELISA.

ing antibody was A310TA. There was no reaction with IgG from the Loewe kit.

IgG from A310HA gave positive reactions in DAS ELISA with GRSV, TCSV and GBNV from *N. benthamiana* crude sap (data not shown).

This report appears to be the first on purification of INSV virions and the production of an antiserum reacting with both nucleoprotein and glycoprotein antigens. We were able to purify the virus from plants grown at high temperature using a method devised for TSWV, which, however, was not successful for INSV-material grown at normal glasshouse temperatures.

The pattern of structural proteins obtained with INSV in SDS PAGE is similar to that reported for TSWV, but with INSV, G2 was present as a single band, whereas with TSWV two G2 bands appear. INSV G2 migrated a little more slowly than TSWV G2a. The latter may be more susceptible to degradation than INSV G2 and thus give rise to a second band.

The reaction of our antiserum to INSV virions with TSWV glycoproteins confirms that the G proteins are serologically related, a result previously obtained using TSWV antisera only. The presence of common glycoprotein epitopes under native conditions was confirmed using the two TSWV glycoprotein-specific Mabs.

Under native conditions, in DAS ELISA, our INSV antiserum clearly detected GRSV, TCSV and GBNV. As previously reported (Adam et al., 1995), the three viruses were also detectable in DAS ELISA using polyclonal antibodies against TSWV G proteins. These two results confirm that all tospovirus glycoproteins so far tested have common antigenic determinants.

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