Effects of Passaging a Defective Isolate of Impatiens Necrotic Spot Virus at Different Temperatures

R. H. Lawson, M. M. Dienelt, and H. T. Hsu

U. S. Department of Agriculture, Agricultural Research Service, Florist and Nursery Crops Laboratory, Beltsville, MD 20705-2350.
We thank J. W. Moyer, North Carolina State University, Raleigh, for the INSV-Igg and TSWV-D cultures and D. Thomas of Goldsmith Seeds, Inc., Gilroy, CA, for the TSWV-NC4 culture.
Accepted for publication 20 February 1993.

ABSTRACT

Lawson, R. H., Dienelt, M. M., and Hsu, H. T. 1993. Effects of passaging a defective isolate of impatiens necrotic spot virus at different temperatures. Phytopathology 83:662-670.

The effects of two temperatures on the cytopathology of a defective isolate of impatiens necrotic spot virus (INSV-Igg) were compared with the effects on two normal isolates of tomato spotted wilt virus (TSWV-NC4 and TSWV-D). INSV-Igg produced very few enveloped virions in plants grown at approximately 20 C. Instead, masses of nucleocapsid (N) protein appeared in characteristic chainlike formations. The N protein was serologically distinct from that of TSWV. In five experiments with eight to 10 mechanical transfers, each at elevated temperatures, one experiment in a greenhouse during summer and the other four in controlled environment chambers (27/24 C, light/dark), virions appeared in *Nicotiana benthamiana* plants infected with INSV-Igg between the first and fourth passages and thereafter, were consistently produced. In two out of four growth-chamber experiments, extracts from infected plants grown at 27/24 C (light/dark) did not react in enzyme-linked immunosorbent assay (ELISA) with antisera to INSV N protein after the third passage. This serological change was correlated in one experiment with a change in the appearance of the N protein: Characteristic chains were no longer observed in infected cells, but large, amorphous, electron-dense masses appeared in which virions were visible. These masses failed to react with antisera to INSV N protein in immunogold-labeling experiments. In parallel serial passages at 21/18 C (light/dark), virions were not observed in infected plants. Cytopathology and serological reactivity of the N protein remained unaltered. Passaging at different temperatures did not alter the cytopathology of plants infected with TSWV-NC4 or TSWV-D. The high temperature triggered an increase in production of virions in the INSV-Igg culture and was sometimes accompanied by an antigenic change in the N protein.

The tospovirus genus of Bunyaviridae currently contains two viruses, tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV). Polyclonal antisera to the nucleocapsid (N) protein have differentiated the genus into three serogroups, two in TSWV and one in INSV (2,3,12,14,15). INSV, once thought to be a strain of TSWV, has been further separated from TSWV on the basis of its RNA structure and cytopathology (13,22,23). Although both viruses have wide host ranges, INSV is found mainly in ornamental species (12,23).

The tospovirus genome consists of three single-stranded RNA molecules: small (S), medium (M) and large (L) (19,21). L RNA codes for the polymerase (4,5), and S RNA codes for the N protein (4) and nonstructural (NSs) proteins found in filamentous inclusions or dispersed within the cytoplasm (11). Glycoproteins in the envelope of the intact virion are encoded by the M RNA (16). TSWV isolates lacking particles are thought to be defective forms created by mutations or deletions of the M RNA (21). This may occur after repeated mechanical transfer through the same (9) or different (21) hosts.

When INSV was first reported, masses of N protein were observed without enveloped virions (12,22). Although these isolates were defective, additional isolates have been reported that form mature virions (2,23,24).

We observed numerous virions associated with a defective isolate of INSV from gloxinia (INSV-Igg) propagated in infected *Nicotiana benthamiana* Domin. plants growing at high temperatures in a greenhouse during summer. The objective of this study was to assess the effects of temperature on the cytopathology and serological behavior of INSV-Igg. Two normal isolates of TSWV also were evaluated.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1993.

MATERIALS AND METHODS

Virus cultures and antisera. INSV-Igg was isolated from gloxinia and typically produced N protein in chainlike formations, paracrystalline filamentous inclusions, few, if any, virions, and amorphous inclusions (22). The composition of amorphous inclusions is unknown; however, antibody labeling indicated that it is not N protein (22).

TSWV-NC4 was isolated from *Lobelia*. TSWV-D was received in *Datura stramonium* L. All cultures were propagated in *N. benthamiana* and stored frozen at −70 C, as were the original cultures.

Polyclonal antisera were obtained (Agdia, Inc., Elkhart, IN). Antiserum to the N protein of INSV was prepared prior to the taxonomic separation of INSV from TSWV and was termed TSWV-I. We refer to this antiserum as INSV-N antiserum. TSWV-L antiserum was prepared to purified TSWV. Both antisera were cross-absorbed with healthy *N. benthamiana* sap in 0.02 M $Na_2HPO_4{\cdot}KH_2PO_4$, pH 7.0.

Growing environments. INSV-Igg and TSWV-D were transferred to *N. benthamiana* plants and were initially grown in a greenhouse during summer, with temperatures fluctuating between 24 and 37 C. Cultures were transferred every 6–8 days for eight to 10 passages. *N. benthamiana* seedlings grown in a greenhouse were inoculated at the four- to six-leaf stage. Virus inoculum was prepared in 0.1 M $K_2HPO_4{\cdot}KH_2PO_4$ and 0.01 M $NaSO_3$, pH 7.2 (inoculation buffer) (1).

After virions were observed in *N. benthamiana* plants infected with INSV-Igg in the greenhouse during summer, the effects of high temperatures were evaluated under controlled environment conditions. INSV-Igg, TSWV-NC4, and TSWV-D were each transferred to 10 *N. benthamiana* plants from the original cultures stored frozen at −70 C. Half the plants were placed in a growth chamber at 21/18 C (light/dark; low temperature [LT]), and the other half were placed in a chamber at 27/24 C (light/dark; high temperature [HT]). Plants were grown with a 16-h photoperiod with fluorescent and incandescent illumination at 19 W/m^2. The virus was passaged every eight to 10 days at LT and every 6

to 7 days, after the third passage, at HT, for a total of eight to 10 passages.

Each series of mechanical transfers in the two chambers was performed four times. These series were designated GC-1, -2, -3, and -4. In a separate experiment, INSV-Igg from GC-1 was transferred for five passages at LT, after seven initial passages at HT.

Electron microscopy. Tissue samples were taken from the first passage of each virus and periodically thereafter through the tenth passage. Samples were taken from plants grown in the greenhouse, GC-1, -2, and -4. Seven or eight samples were cut from two or three plants per treatment. Leaf pieces about 2 mm square were cut from inoculated leaves 4–6 days after rubbing, when symptoms first appeared. Systemically infected leaves showing veinal chlorosis were sampled 1 or 2 days later. Tissue was fixed in 2% glutaraldehyde and 1.5% acrolein in 0.05 M PO_4, pH 7.0. After vacuum infiltration on ice for 2 h, samples were rinsed in 0.05 M PO_4 and dehydrated in a graded series of ethanol (30, 50, 70, 90, and 100%), followed by propylene oxide. Half the samples taken from the first passage in the greenhouse experiment also were postfixed in 1% OsO_4 in 0.05 M PO_4, pH 7.0, prior to dehydration. All samples were embedded in LX112 epoxy resin. Ultrathin sections were stained 45 min in uranyl acetate and 12 min in lead citrate.

Tospovirus inclusion terminology. Tospoviruses produce several inclusions, and the terminology used to describe these inclusions is not always consistent. We reserve the term nucleocapsid (N) protein for inclusions (other than virions) that react with antibody to INSV-N protein. Aggregated N protein refers to electron-dense masses similar to those referred to as amorphous masses (9,25), dark diffuse masses (8), nucleocapsid aggregates (2,10,25), dense aggregates (21), viroplasm (6,17), and dense masses (20). Unaggregated N protein refers to a diffuse structure similar to one previously described as viroplasm (10). The term amorphous inclusion is retained for the unique electron-dense inclusions previously reported only in INSV-Igg infections (22). Filamentous inclusions are classified as types I and II; type I filamentous inclusions are nonparacrystalline arrays produced in TSWV infections, and type II filamentous inclusions are paracrystalline inclusions representative of INSV infections (23).

Virions known to occur in single-enveloped forms clustered within the ER (endoplasmic reticulum) when mature (6,8,17,18) or as nonclustered, double-enveloped forms when immature (10,17,22) are described only as clustered or individual. This reflects the difficulty in distinguishing viral envelopes from ER membranes in nonosmicated tissue.

Immunogold labeling. Sections from the original culture, the greenhouse experiment, and the low- and high-temperature chambers of GC-1 and -4 were used for immunogold labeling. Sections on Formvar-coated nickel grids or uncoated gold grids were blocked in 0.01 M Tris-HCl, 0.5% NaCl, 0.3% Tween 20, and 1% bovine serum albumin (BSA), pH 7.4 (Tris-buffered saline [TBS]-BSA), with 5% goat normal serum for 1 h. After rinsing in TBS-BSA, grids were coated with polyclonal antiserum (undiluted), 1 mg/ml, for 1.5–2 h. Coated grids were rinsed in TBS-BSA, blocked for 5 min, and incubated for 1 h with goat antirabbit 5- or 10-nm gold label (Amersham Life Sciences, Arlington Heights, IL). Gold conjugate was diluted 1:10 in TBS-BSA containing 1% goat normal serum. After rinsing four times in TBS-BSA and three times in distilled H_2O, sections were stained in uranyl acetate and lead citrate.

Tissue blotting. Infected *N. benthamiana* plants grown in the greenhouse during summer were monitored serologically at each passage to verify purity of the isolates. Tissue blots were prepared from systemically infected plants, using the method previously reported (7). Plants infected with INSV-Igg, TSWV-NC4, and TSWV-D were tested with antisera to N protein of INSV-Igg and to TSWV-L.

Enzyme-linked immunosorbent assay (ELISA). Relative concentrations of viral antigens in infected tissue were determined by double-antibody sandwich ELISA with Nunc-Immuno MaxiSorp plates (A/S Nunc, Roskilde, Denmark). Alkaline phosphatase-labeled immunoglobulins were used at dilutions recommended by the manufacturer (Agdia, Inc.). One part of systemically infected leaf tissue was ground in 9 parts of inoculation buffer. From the homogenate, a 1–3 serial dilution was made in phosphate-buffered saline solution (0.02 M $Na_2HPO_4 \cdot KH_2PO_4$, 0.15 M NaCl, pH 7.4). Tests were made for each passage of INSV-Igg, TSWV-NC4, and TSWV-D, and the ELISA dilution endpoints were determined. The ratios in dilution endpoints between the samples from HT and LT regimes were noted for each passage and were normalized for comparison of the virus titers in serial passages at high temperatures.

RESULTS

Symptomatology. Symptoms produced by INSV-Igg, TSWV-NC4, and TSWV-D on *N. benthamiana* were identical. Chlorotic lesions appeared on inoculated leaves approximately 4–5 days after inoculation, when plants were incubated at LT. Systemic vein yellowing developed after 6–7 days and was followed by wilting and collapse of tissue. Initial symptoms were similar on plants at both temperatures, but at HT, they appeared 1–2 days earlier, and tissue collapse occurred more rapidly.

TABLE 1. Temperature-induced cytopathological changes in *Nicotiana benthamiana* infected with a gloxinia isolate of impatiens necrotic spot virus (INSV-Igg)

Cytopathological structure	Original culture[a]	Greenhouse during summer			GC-1[b]						GC-2	GC-4
					21/18 C[d]			27/24 C			27/24 C	27/24 C
		1[c]	3	10	1	4	10	1	4	10	7	7
Virions												
Clustered	−	−	+	+	−	−	−	−	+	+	+	+
Individual	+/−	−	+	+	−	−	−	−	+	+	+	+
Nucleocapsid (N) protein[e]												
Aggregated	+	+	+	+/−	+	+	+	+	−	−	+	+
Unaggregated	+	+	+	+	+	+	+	+	+	+	?[f]	+
N-proteinlike inclusions[g]												
Aggregated	−	−	+	+	−	−	−	−	+	+	+	+
Unaggregated								−	+	+	?[f]	+
Paracrystalline filamentous inclusion	+	+	−	+	+	+	+	+	−	−	+	+
Amorphous inclusion	+	+	−	−	+	+	+	+	−	−	−	−

[a] + = present; − = absent; and +/− = uncommon.
[b] Growth-chamber experiment number.
[c] Passage number.
[d] Light/dark temperatures.
[e] Labeled with INSV-N antiserum.
[f] Electron-dense material present but identity not confirmed by immunolabel.
[g] Not labeled by INSV-N antiserum.

Cytopathology of INSV-Igg. Cytopathology of the original INSV-Igg culture appeared as previously described (22) (Table 1). The dominant viral inclusions were distinctive, chainlike aggregates of N protein (Fig. 1A) showing regular periodicity. Nucleocapsid material was often surrounded by ER. Unaggregated N protein was seen less often. Electron-dense amorphous inclusions, staining more intensely than N protein, also were present (Fig. 1A). Amorphous inclusions showed no regular periodicity and were not typically surrounded by a membrane. Filamentous inclusions formed type II arrays (Fig. 1B). A few enveloped virions were observed occasionally.

By the third greenhouse passage, when temperatures averaged 32 C or higher during the day, there were significant cytopathological changes (Table 1). Virions appeared in clusters (Fig. 2A) or individually, often close to aggregated N protein (Fig. 2A and B). In later passages, less nucleocapsid material was seen, and amorphous inclusions were absent. Type II filaments appeared only sporadically. These changes remained constant through the tenth passage.

In the first series of transfers under controlled conditions (GC-1), plants at LT showed a cytopathology similar to that of the original INSV-Igg, even after seven or more passages (Table 1). At HT, the cytopathology in the first through third passages appeared similar to that at LT (Table 1), but after the third passage, virions were more commonly observed.

By the fourth HT passage, chainlike N protein was no longer observed. Instead, an electron-dense material appeared that contained individual virions randomly dispersed within the matrix and on the periphery (Fig. 2C). This inclusion differed from aggregated N protein and amorphous inclusions. It was frequently associated with virions, lacked the striations of aggregated N protein, and appeared less homogenous than amorphous inclusions. Individual particles also were observed close to inclusions (Fig. 2D) resembling unaggregated N protein seen in the original INSV-Igg culture. Virions appeared less frequently in large clusters (Fig. 2E). Amorphous and type II inclusions were not observed. These effects were not reversed in five subsequent passages at LT.

Results of the second series of passages at HT (GC-2) differed from those of GC-1 (Table 1). Instead of disappearing, N protein with a chainlike appearance remained. Large numbers of individual virions were present, with clusters observed less frequently. Some cells contained the electron-dense material seen in GC-1 (HT). Amorphous inclusions could not be definitely identified, but type II inclusions were present.

Samples from GC-3 were not embedded; however, HT samples from GC-4 revealed a cytopathology more like those of greenhouse and GC-2 HT plants than like GC-1 HT plants. Virions appeared in large clusters in a few sections from the first passage and in all sections from subsequent passages. By the 3rd passage, electron-dense material resembling that prominent in GC-1 HT plants developed. Chains of aggregated N protein, unaggregated N protein, and filamentous inclusions remained present through the ninth passage. Amorphous inclusions were not observed after the first passage.

Cytopathology of TSWV-NC4. Plants grown in a greenhouse during summer primarily contained clustered virions in the first through seventh passages (Fig. 3A). Short type I filaments (Fig. 3B), aggregated N protein (Fig. 3C), and unaggregated N protein (not shown) also were present. Amorphous inclusions were absent. By the eighth passage, the amount of N protein apparently increased, and there were fewer clusters of virions.

Plants infected with TSWV-NC4 grown at both LT and HT showed similar cytopathology through the eighth passage. Infected cells contained membrane-bounded, clustered virions, aggregated and unaggregated N protein, and scattered type I filaments.

Cytopathology of TSWV-D. Plants infected with TSWV-D appeared almost identical to plants infected with TSWV-NC4. Filamentous inclusions could not be definitely identified, however. Membranes, frequently Golgi apparatus, were associated with virions and with electron-dense amorphous material. These formations, which often appeared to be associated with developing virions, were similar to those reported previously (6,8,10,17,18). Virions appeared dispersed, individually or clustered within cisternae. Aggregated and unaggregated N proteins were present. Aggregated N protein displayed the chainlike formation present in INSV-Igg and TSWV-NC4 infections.

Repeated mechanical transfer through 10 passages in *N. benthamiana* revealed no changes in viral structure in plants grown in a greenhouse during summer or in plants grown at LT and HT in the controlled environments.

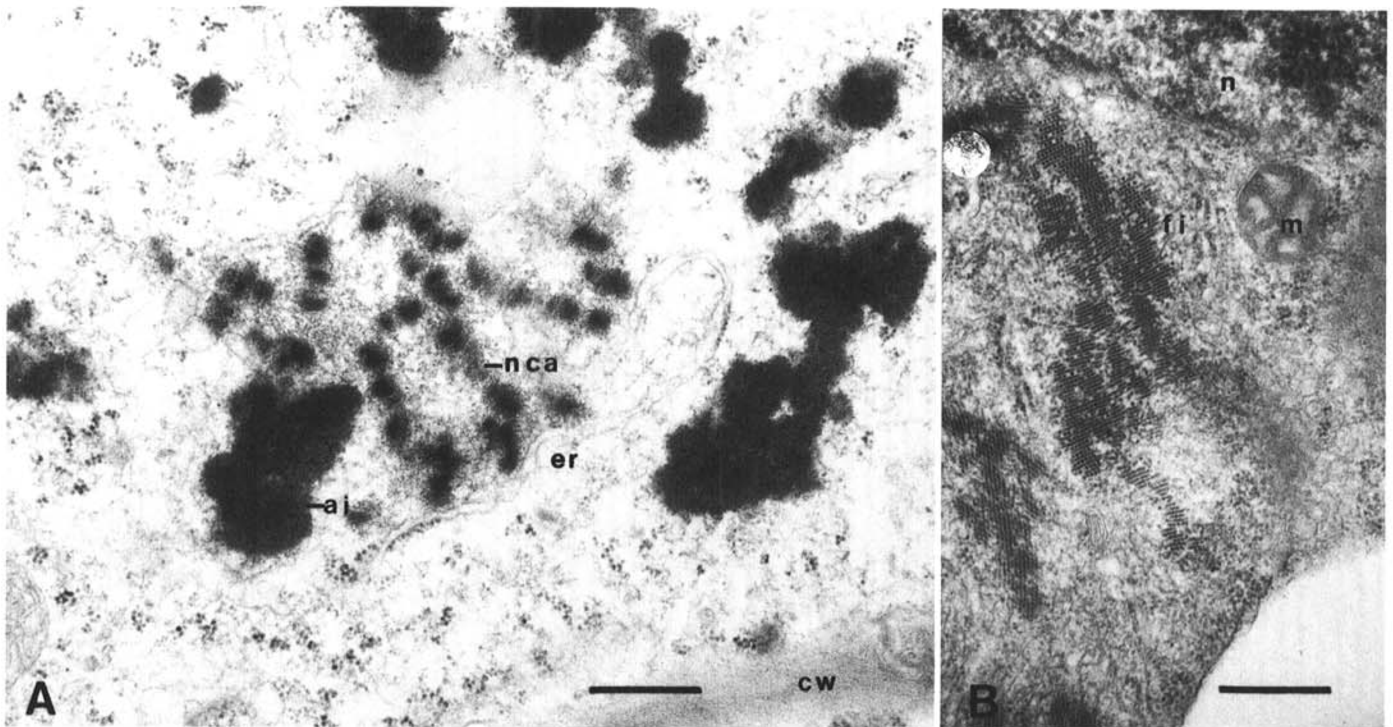

Fig. 1. Impatiens necrotic spot virus (INSV)-Igg in *Nicotiana benthamiana*, first passage from original culture (OsO_4 fixation). **A,** aggregated nucleocapsid protein (nca) forms a characteristic chainlike formation. Amorphous inclusions (ai) are also present. Bar = 420 nm; er = endoplasmic reticulum. **B,** filamentous inclusions (fi) form paracrystalline arrays. Bar = 455 nm; m = mitochondrion, cw = cell wall, and n = nucleus.

Cytology of healthy controls. Noninoculated *N. benthamiana* plants remained free of the viral inclusions present in plants inoculated with INSV and TSWV cultures.

Immunolabeling INSV-Igg. INSV-N antiserum reacted with aggregated (Fig. 4A) and unaggregated (Fig. 4B and C) N protein produced in early passages but not with amorphous (Fig. 4D) and filamentous inclusions (Fig. 4A). No cross-reaction occurred between INSV-Igg N protein and antiserum to TSWV-L.

During summer passages, virions in cells infected with INSV-Igg were labeled with antiserum to INSV N protein, although the reaction was weaker than with aggregated N protein (Fig. 5A). In contrast, virions in INSV-Igg-infected tissues showed a stronger cross-reaction with antiserum to TSWV-L (Fig. 5B).

In the GC-1 experiment, N-protein aggregates formed by INSV-Igg at LT also reacted with INSV-N antiserum (Fig. 5C). At HT, there was no reaction with virions or electron-dense aggregated material (Fig. 5D). Antiserum to TSWV-L did not label INSV-Igg virions. Diffuse material indistinguishable visually from unaggregated N protein also remained unlabeled. In the GC-4 experiment, aggregated and unaggregated N protein present in

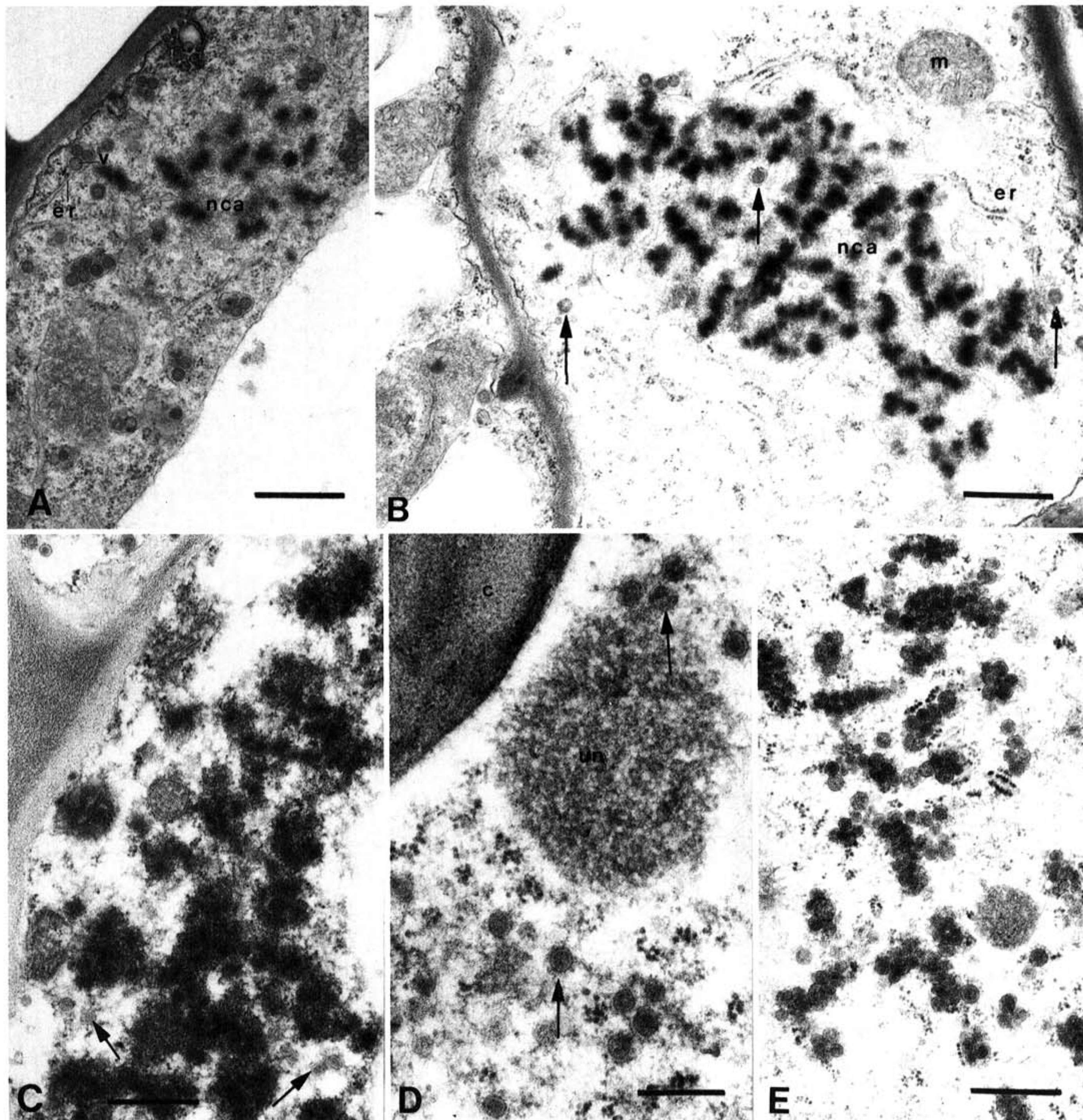

Fig. 2. Impatiens necrotic spot virus (INSV)-Igg in *Nicotiana benthamiana*, late passages. **A and B,** infected plants grown in a greenhouse during summer (OsO_4 fixation). Aggregated nucleocapsid (N) protein (nca) retains the chainlike appearance of the original culture, but virions (v) clustered within the **A,** endoplasmic reticulum (er) and **B,** individual virions (arrow) are present. **A,** bar = 580 nm. **B,** bar = 640 nm; m = mitochondrion. **C–E,** infected plants grown at 27/24 C, light/dark (fixation without OsO_4). **C,** electron-dense material is more diffuse and less structured than N protein and amorphous inclusions in original INSV-Igg culture. Arrows indicate virions. Bar = 420 nm. **D,** individual virions (arrows) and material of unknown composition (un) resembling unaggregated N protein are present. Bar = 250 nm; c = chloroplast. **E,** clustered virions are irregular in size and shape. Bar = 420 nm.

late HT passages were labeled strongly by INSV-N but not by TSWV-L. Virions were weakly labeled by both antisera.

Immunolabeling TSWV-NC4. In all growing environments, antiserum to TSWV-L strongly labeled TSWV-NC4 aggregated N protein and virions (Fig. 6A) and unaggregated N protein. Filamentous inclusions were not labeled. No reaction occurred between TSWV-NC4 and INSV-N antiserum (Fig. 6B).

Tissue blotting. Tissue blots confirmed that plants containing virions and infected with INSV-Igg were not contaminated with TSWV. INSV-N antiserum reacted with INSV-Igg-infected plants but not with those infected with TSWV-NC4 or TSWV-D. In contrast, TSWV-L antiserum produced a strong homologous reaction and reacted weakly with blots from INSV-Igg-infected plants.

ELISA. In GC-1, extracts from plants infected with INSV-Igg and grown at LT and HT showed similar homologous dilution endpoints for the first three passages. After the fourth passage, a higher ELISA reading was obtained from LT plants compared to HT plants at the same dilution (Table 2; Fig. 7). By the sixth passage, A_{405nm} values remained high for LT plants but decreased to a very low level for HT plants (Table 2). In contrast, homologous ELISA values from extracts of plants infected with TSWV-NC4 and TSWV-D grown at both LT and HT showed no significant difference between the two temperature treatments.

INSV-Igg, which lost the ability to react with INSV-N antiserum after seven passages at HT, was passaged five times at LT. No reaction occurred in extracts from these plants when they were tested with this antiserum.

Determination of ELISA dilution endpoints was repeated three times in controlled environment chambers (Fig. 7). In GC-2, a decrease of INSV-Igg titer in HT plants was observed in the fourth passage when compared to that of LT. However, after the fourth passage, the titers of INSV-Igg were similar in plants grown under both temperature regimes, although there was a slight decrease at the sixth HT passage. In the GC-3 experiment, the INSV-Igg titer began to decrease at the fourth passage in HT plants and decreased to a very low level by the sixth passage. In the GC-4 experiment, no difference occurred between titers of LT and HT plants throughout the different passages.

DISCUSSION

High temperatures in greenhouse or controlled environments were associated with at least two changes in INSV-Igg when the culture was maintained in *N. benthamiana*. Infected plants grown at HT consistently developed large populations of virions that did not occur in infected plants grown at LT. In two out of five HT trials, cultures serologically distinct from INSV-Igg were obtained.

Although it has been recognized for many years that the presence or absence of tospovirus virions can be experimentally manipulated (9,21), temperature has not been implicated previously as a factor. Furthermore, the change usually involves the formation of defective isolates from nondefective isolates, rather than the other way around. Thus, defective isolates have been produced after repeated mechanical transmission through the same (9) or different hosts (21).

Regardless of whether an absence of virions occurs experimentally, as with some TSWV isolates (21), or naturally, as with INSV-Igg (12), an absence of virions has been associated with a reduction in the amount of G proteins (glycoproteins) (12,21). Because these are components of the viral envelope, a reduction in them may interfere with formation of virions (21). It seems probable that the reverse also is true and that the increased numbers of virions seen at HT reflect an increased production of G proteins.

The mechanisms by which heat promotes virion formation are unknown. It does not appear that HT switches genes on and off for an immediate and widespread alteration in gene function, however. Particles usually do not appear until the third passage. The exception, in the GC-4 experiment, was the formation of virions in the first passage of plants grown at HT. In this experiment, the concentration of virus in the original inoculum was low, there was a delay in symptom development and the plants were larger and more mature than the plants used in other tests. The combination of these factors may account for the presence of the virions in the earlier passage.

In contrast to greenhouse results, GC-2 and -4 experiments, virions in GC-1 did not label with INSV-N antiserum. This result may reflect a true difference in the antigenicity of the virions

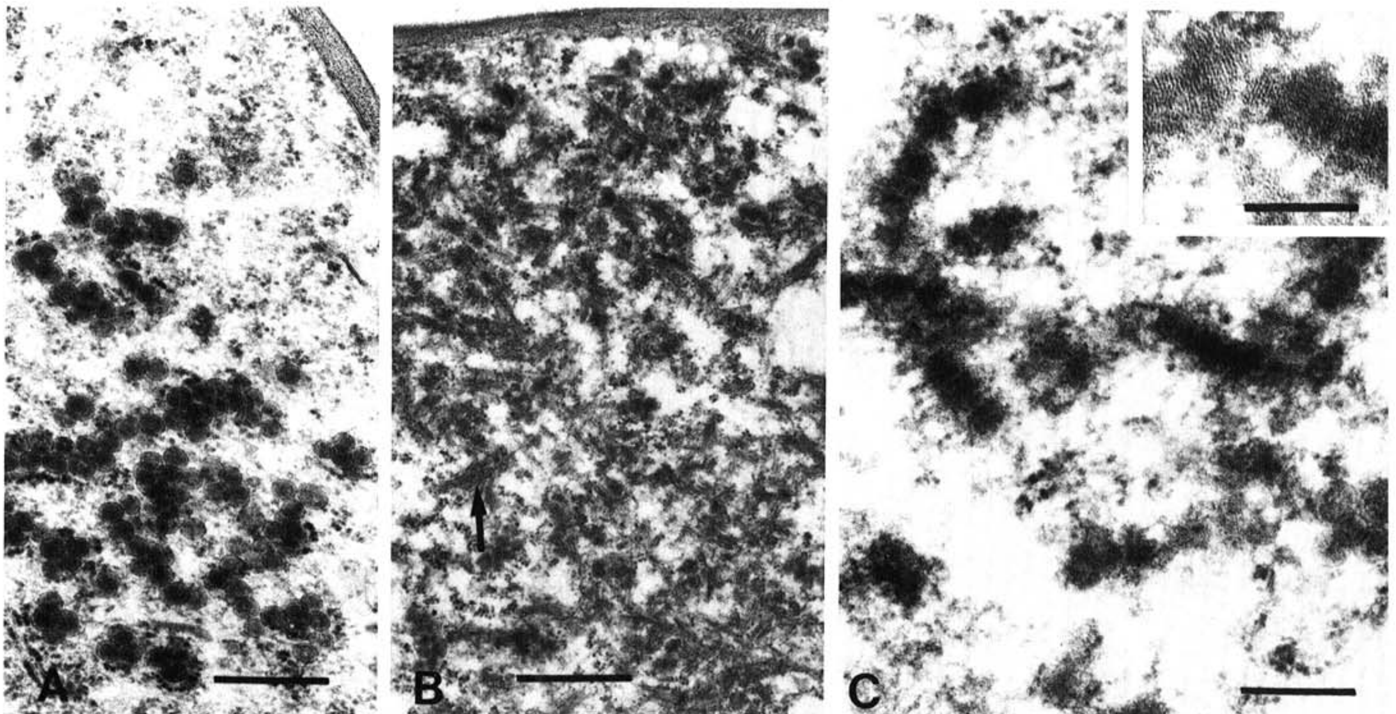

Fig. 3. Tomato spotted wilt virus (TSWV)-NC4 in *Nicotiana benthamiana* (fixation without OsO_4). **A,** clustered virions are the most frequently observed inclusion. Bar = 420 nm. **B,** short dispersed filaments (arrow) also are present. Bar = 320 nm. **C,** nucleocapsid protein displays chainlike appearance and faint striations (inset). Bar = 250 nm; inset bar = 160 nm.

based on a preferential selection of a different form of N protein at HT. The GC-1 HT isolate did not react in ELISA against INSV-N antiserum, possibly because it lacked the chainlike N protein that labels strongly with this antiserum.

The source of N protein for virion formation in GC-1 may be the electron-dense amorphous material present in infected cells. This material lacked the chainlike appearance and regular striations present within N protein of INSV-Igg. It more closely resembled the amorphous inclusions of INSV-Igg (22) but appeared more diffuse and was often associated with virions. Amorphous inclusions and the HT electron-dense material were not observed in the same samples, however, which may indicate the HT material is another form of amorphous inclusions. The literature contains numerous descriptions of the cytopathology of different tospovirus infections, mostly featuring electron-dense masses of N protein (2,3,6,8–10,12,17,20,21). The absence of any alternative suggests that the electron-dense masses found in plants at HT are also N protein. Their failure to react with INSV-N

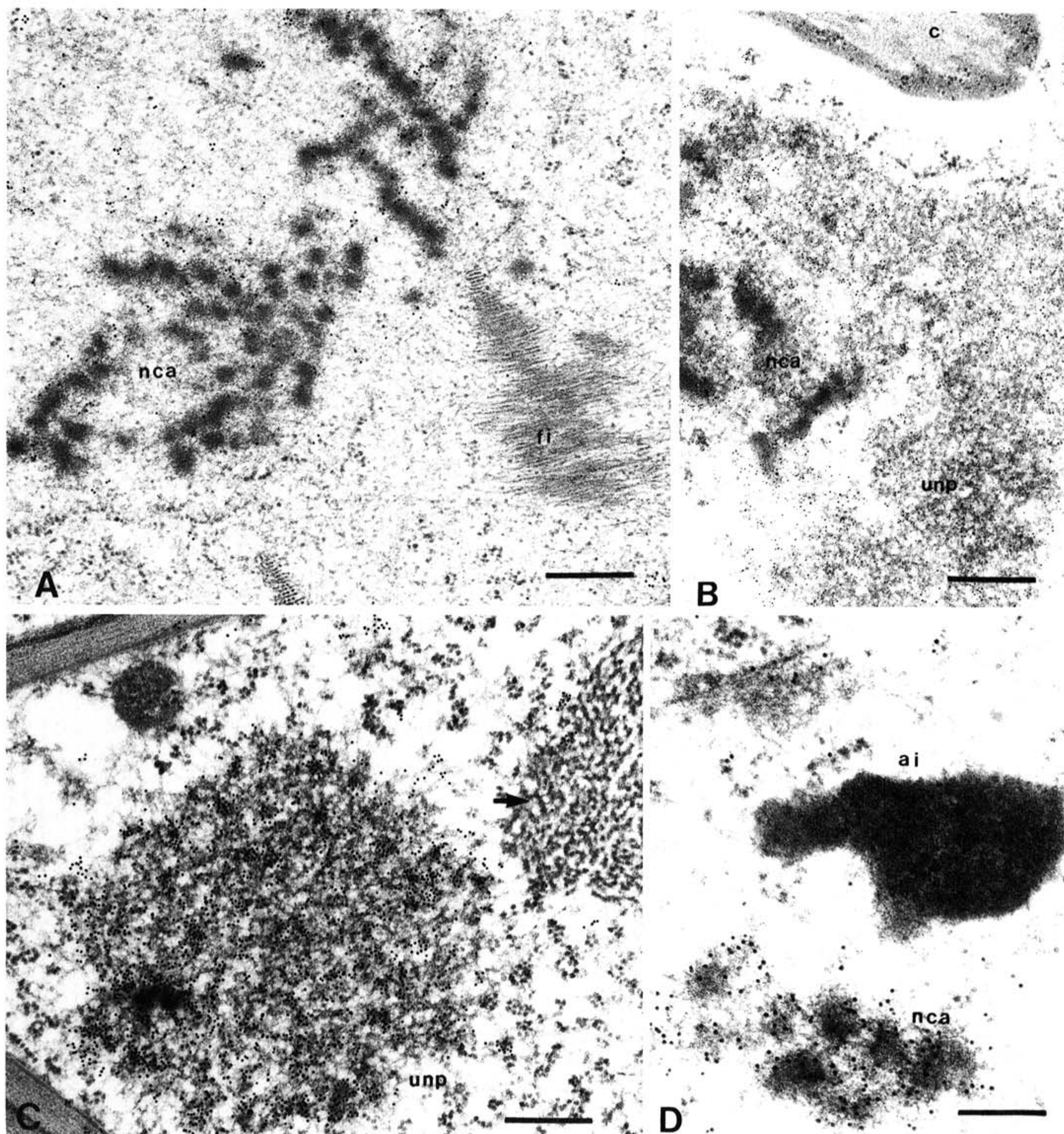

Fig. 4. Immunogold labeling of impatiens necrotic spot virus (INSV)-Igg structures in the first high-temperature passage in *Nicotiana benthamiana*. Antibody is to the nucleocapsid (N) protein of an INSV isolate (fixation without OsO_4). **A,** antibody labels aggregated N protein (nca) but not filamentous inclusions (fi). Bar = 320 nm. **B,** antibody labels the unaggregated N protein (unp) often associated with electron-dense N-protein aggregates. Bar = 230 nm; c = chloroplast. **C,** unaggregated N protein also is labeled by antibody when not associated with aggregates. N protein is in a developing sieve element; arrow indicates P protein. Bar = 310 nm. **D,** antibody labels N-protein aggregates but not amorphous inclusions (ai). Bar = 180 nm.

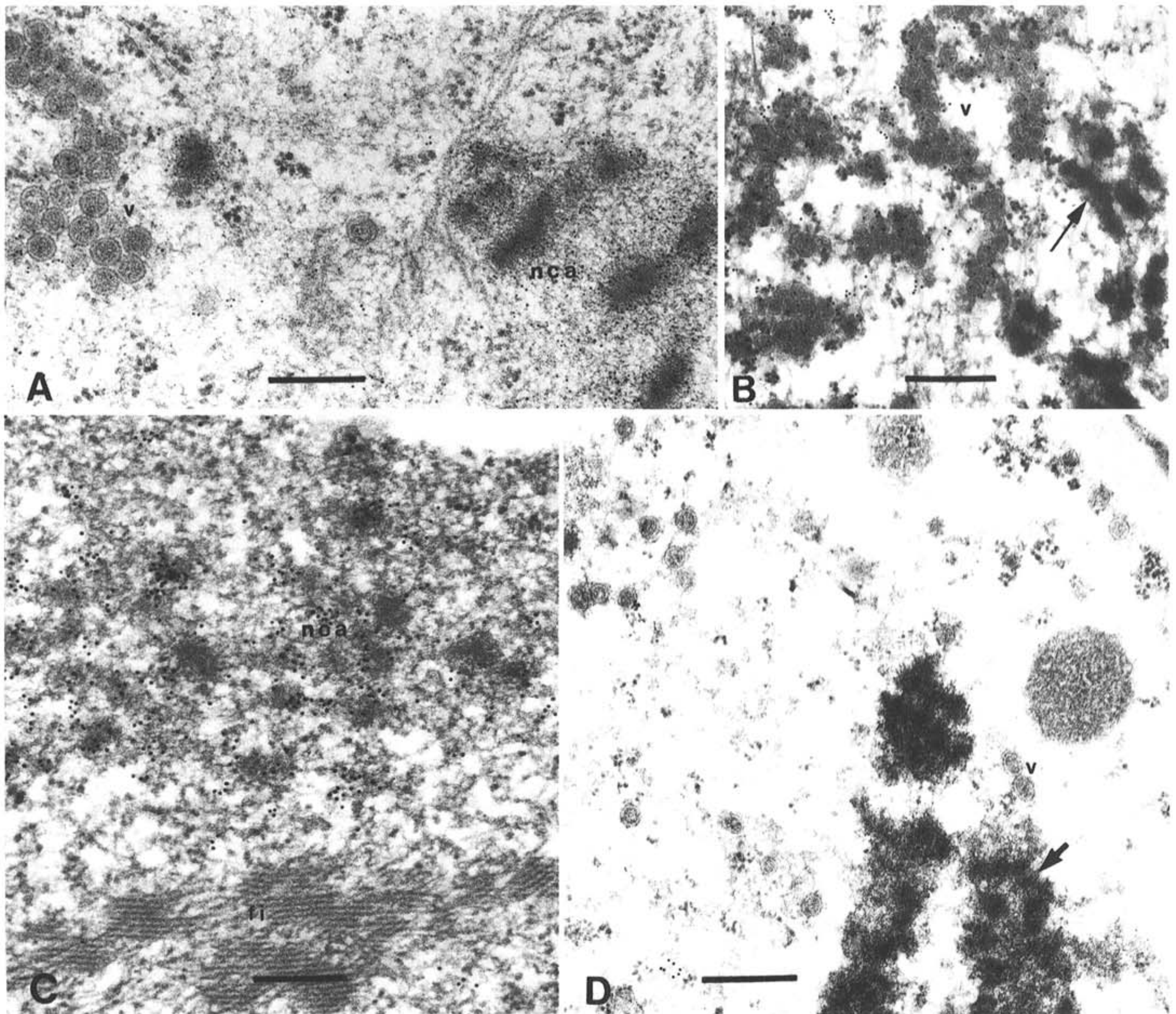

Fig. 5. Immunogold labeling of impatiens necrotic spot virus (INSV)-Igg structures in late passages in *Nicotiana benthamiana* (fixation without OsO_4). **A and B,** summer greenhouse; **C and D,** growth chambers. **A,** INSV-N antibody labels nucleocapsid (N)-protein aggregates (nca) more strongly than it labels particles (v). Bar = 230 nm. **B,** tomato spotted wilt virus (TSWV)-L antibody labels virions but not electron-dense aggregates (arrow). Bar = 375 nm. **C,** INSV-N antibody labels N protein in infected plants grown at 21/18 C, light/dark, for 10 passages but not **D,** electron-dense material (arrow) in infected plants grown at 27/24 C for 10 passages. **C and D,** Bar = 230 nm; fi = filamentous inclusions.

TABLE 2. Enzyme-linked immunosorbent assay (ELISA) titration (A_{405nm}) of tomato spotted wilt virus (TSWV-NC4) and impatiens necrotic spot virus (INSV-Igg) antigens during passages in *Nicotiana benthamiana* at two temperature regimes[a]

Passage	INSV/ TSWV isolates	Temperature (C)	Dilution series										
			1	2	3	4	5	6	7	8	9	10	11
4	Igg	21/18	+	+	+	+	+	+	1.94	0.84	0.29	0.07	0
		27/24	+	+	+	+	+	1.58	0.52	0.15	0.05	0.02	0
	NC4	21/18	+	1.80	1.60	1.55	1.52	1.21	0.88	0.47	0.20	0.03	0
		27/24	+	+	+	1.99	1.89	1.57	1.07	0.56	0.22	0.05	0
6	Igg	21/18	+	1.82	1.72	1.76	1.78	1.43	0.90	0.49	0.22	0.09	0.04
		27/24	0.18	0	0	0	0	0	0	0	0	0	0
	NC4	21/18	+	+	+	186	1.22	0.84	0.40	0.20	0.09	0.05	0.02
		27/24	+	1.76	1.59	1.40	1.09	0.87	0.59	0.37	0.17	0.09	0.04
7	Igg	21/18	1.32	1.20	1.05	0.81	0.68	0.36	0.22	0.09	0.03	0	0
		27/24	0	0	0	0	0	0	0	0	0	0	0
	NC4	21/18	+	+	1.47	1.41	1.43	1.08	0.74	0.29	0.15	0.05	0.02
		27/24	+	+	1.86	1.85	1.55	1.35	1.00	0.59	0.31	0.11	0.06

[a]Inoculated plants were kept at 21 C day/18 C night or 27 C day/24 C night with 16 h photoperiods. Leaves with systemic symptoms, which usually developed 5–7 days after inoculation, were sampled and TSWV and INSV antigens were titered by double-antibody sandwich ELISA with I-specific and L-specific antisera. The NC4 isolate did not react with INSV-N-specific antiserum, and the Igg isolate did not react with L-specific antiserum. The A_{405nm} values of healthy controls for each dilution ranged from 0.001, highest dilution, to 0.05, lowest dilutions, for each test and were used to correct the A_{405nm} values. Positive signs indicate the A_{405nm} values were greater than 2.0.

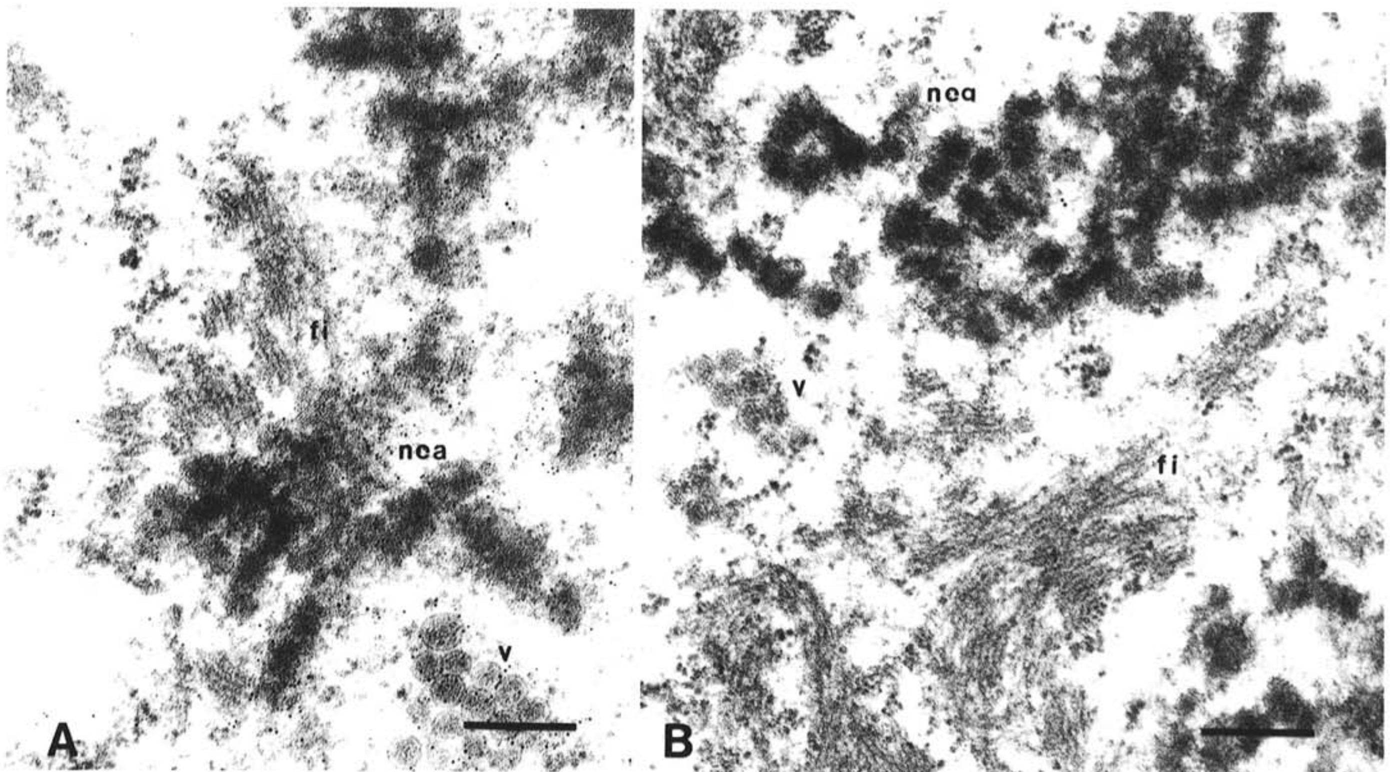

Fig. 6. Immunogold labeling of tomato spotted wilt virus (TSWV)-NC4 structures in infected *Nicotiana benthamiana* plants (fixation without OsO_4). **A,** antibody to TSWV-L labels aggregated nucleocapsid (N) protein (nca) and virions (v), but not filamentous inclusions (fi). Bar = 230 nm. **B,** antibody to N protein of impatiens necrotic spot virus (INSV)-Igg fails to label any structure. Bar = 230 nm.

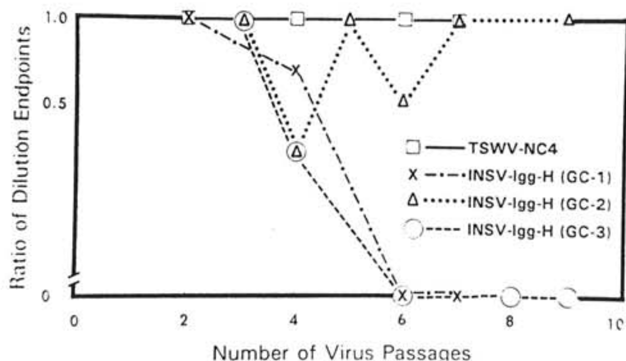

Fig. 7. Effects of high temperature on enzyme-linked immunosorbent assay (ELISA) titers of impatiens necrotic spot virus (INSV)-Igg in *Nicotiana benthamiana* compared with tomato spotted wilt virus (TSWV)-NC4. Virus cultures were maintained by serial passages at low temperatures, 21/18 C, light/dark, or high temperatures, 27/24 C, light/dark. The ratios of double-antibody sandwich ELISA dilution endpoints at high temperatures to those at low temperatures were determined for each passage, providing an indication of the relative concentrations of the virus at the two temperatures, as were changes in virus titers propagated at high temperatures. The titer of INSV-Igg decreased after the third passage in two of the three experiments in plants grown at high temperatures; TSWV-NC4 was unaffected.

antiserum may reflect the fact that this antiserum was made to an INSV isolate that did not contain amorphous inclusions (12). Further characterization of GC-1 and amorphous inclusions is necessary to define their chemical makeup.

Several explanations are proposed for the changes in INSV-Igg cytopathology at HT. Contamination of the original INSV-Igg culture with a nondefective INSV isolate is possible but seems unlikely. Clusters of mature virions were never observed at LT. Possibly, HT supports multiplication of preexisting variants that thrive in heat and are suppressed at lower temperatures. This explanation is supported by the fact that HT did not consistently produce a serologically modified culture. Another explanation is that heat induces genomic changes perpetuated by selective multiplication. This is unlikely, however, because 27 C is probably too low to induce mutations. Perhaps selection occurs resulting in preferential synthesis of preexisting RNAs, without specific alterations like mutations or deletions. Another simpler explanation is that the G protein in the envelope is produced more efficiently at higher temperatures. If this were true, however, the HT culture produced in the GC-1 series should have produced fewer virions when grown at lower temperatures.

In conclusion, serial passages at HT may affect the cytopathology and serological behavior of INSV-Igg in *N. benthamiana* to such an extent that characteristic cytopathology and serological diagnostic features are no longer recognizable. In some cases, a culture may even result that is serologically distinct and lacks the chainlike N protein, type II filamentous inclusions, and amorphous inclusions usually present in plants infected with INSV-Igg.

This may have important implications for certification programs relying on polyclonal antisera to detect INSV and TSWV. Infections may exist under certain conditions that do not react with antisera to N protein from either virus, resulting in false negatives.

More research on the impact of temperature and host on other strains of INSV is necessary to determine whether other defective forms in the INSV serogroup are temperature sensitive. The two TSWV isolates examined in this report showed little or no temperature-related changes. If other defective isolates of INSV are temperature sensitive, this may represent another feature by which INSV can be differentiated from TSWV.

LITERATURE CITED

1. Black, L. M., Brakke, M. K., and Vatter, A. E. 1963. Purification and electron microscopy of tomato spotted wilt virus. Virology 20:120-130.
2. de Avila, A. C., de Haan, P., Kitajima, E. W., Kormelink, R., Resende, R. de O., Goldbach, R. W., and Peters, D. 1992. Characterization

of a distinct isolate of tomato spotted wilt virus (TSWV) from *Impatiens* sp. in the Netherlands. J. Phytopathol. 134:133-151.
3. de Avila, A. C., Haquenot, C., Resende, R. de O., Kitajima, E. W., Goldbach, R. W., and Peters, D. 1990. Serological differentiation of 20 isolates of tomato spotted wilt virus. J. Gen. Virol. 71:2801-2807.
4. de Haan, P., Kormelink, R., Peters, D., and Goldbach, R. 1990. Genetic organization and expression of the tomato spotted wilt virus genome. Pages 60-66 in: Virus-Thrips-Plant Interactions of Tomato Spotted Wilt Virus, Proceedings of a USDA Workshop. U. S. Dep. Agric., Agric. Res. Ser., ARS-87. 170 pp.
5. de Haan, P., Kormelink, R., Resende, R. de O., van Poelwijk, F., Peters, D., and Goldbach, R. 1991. Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J. Gen. Virol. 71:2207-2216.
6. Francki, R. I. B., and Grivell, C. J. 1970. An electron microscope study of the distribution of tomato spotted wilt virus in systemically infected *Datura stramonium* leaves. Virology 42:969-978.
7. Hsu, H. T., and Lawson, R. H. 1991. Direct tissue blotting for detection of tomato spotted wilt virus in *Impatiens*. Plant Dis. 75:292-295.
8. Ie, T. S. 1971. Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. Virology 43:468-479.
9. Ie, T. S. 1982. A sap-transmissible, defective form of tomato spotted wilt virus. J. Gen. Virol. 59:387-391.
10. Kitajima, E. W., de Avila, A. C., Resende, R. de O., Goldbach, R. W., and Peters, D. 1992. Comparative cytological and immunological labelling studies on different isolates of tomato spotted wilt virus. J. Submicrosc. Cytol. Pathol. 24:1-14.
11. Kormelink, R., Kitajima, E. W., de Haan, P., Zuidema, D., Peters, D., and Goldbach, R. 1991. The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus (TSWV) is associated with fibrous structures in TSWV infected plant cells. Virology 181:459-468.
12. Law, M. D., and Moyer, J. W. 1990. A tomato spotted wilt-like virus with a serologically distinct N protein. J. Gen. Virol. 71:933-938.
13. Law, M. D., and Moyer, J. W. 1991. Nucleotide sequence of the 3′ non-coding region and N gene of the S RNA of a serologically distinct tospovirus. J. Gen. Virol. 72:2597-2601.
14. Law, M. D., Speck, J., and Moyer, J. W. 1991. Genomic organization of the impatiens necrotic spot virus M RNA. (Abstr.) Phytopathology 81:1192.
15. Law, M. D., Speck, J., and Moyer, J. W. 1991. Differences in the N protein nucleotide sequence between tospovirus serogroups. (Abstr.) Phytopathology 81:1192.
16. Law, M. D., Speck, J., and Moyer, J. W. 1992. The M RNA of impatiens necrotic spot tospovirus (Bunyaviridae) has an ambisense genomic organization. Virology 188:732-741.
17. Milne, R. G. 1970. An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. J. Gen. Virol. 6:267-276.
18. Milne, R. G., and de Zoeten, G. A. 1967. A comparison of some methods of preparation of thin sections of virus-infected leaves for electron microscopy. J. Ultrastruct. Res. 19:398-407.
19. Mohamed, N. A., Randles, J. W., and Francki, R. I. B. 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21.
20. Peters, D., de Avila, A. C., Kitajima, E. W., Resende, R. de O., de Haan, P., and Goldbach, R. W. 1990. Pages 1-14 in: Virus-Thrips-Plant Interactions of Tomato Spotted Wilt Virus, Proceedings of a USDA Workshop. U. S. Dep. Agric., Agric. Res. Ser., ARS-87. 170 pp.
21. Resende, R. de O., de Haan, P., de Avila, A. C., Kitajima, E. W., Kormelink, R., Goldbach, R., and Peters, D. 1991. Generation of enveloped and defective interfering RNA mutants to tomato spotted wilt virus by mechanical passage. J. Gen. Virol. 72:2375-2383.
22. Urban, L. A., Huang, P.-Y., and Moyer, J. W. 1991. Cytoplasmic inclusions in cells infected with isolates of L and I serogroups of tomato spotted wilt virus. Phytopathology 81:525-529.
23. Vaira, A. M., Rogzero, P., Luisoni, E., Mesenga, V., Milne, R. G., and Lisa, V. Two tospoviruses in Italy. Characterization of two tospoviruses in Italy, tomato spotted wilt and *Impatiens* necrotic spot. J. Phytopathol. In press.
24. Vali, R. J., and Gildow, F. E. 1992. Symptomatology, cytopathology and distribution of tomato spotted wilt virus in infected greenhouse ornamentals. (Abstr.). Phytopathology 82:248.
25. Verkleij, F. N., and Peters, D. 1983. Characterization of a defective form of tomato spotted wilt virus. J. Gen Virol. 64:677-686.