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SPINACH LATENT VIRUS: SOME PROPERTIES AND COMPARISON OF TWO ISOLATES

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The virus earlier reported from spinach *(S. oleracea)* from Yugoslavia under the tentative name of spinach latent virus has been shown — by symptomatology in test plants, properties in sap, transmission through seed and serological reactions — to be identical with the ilarvirus characterised under the same name in the Netherlands.

After mechanical inoculation the Yugoslav isolate (SpLV/ /H) of spinach latent virus infected 22 out of 32 species from nine families, causing reactions somewhat stronger than those described for two Netherlands isolates (GE36 and Sp20—9). The sap from infected *C. quinoa* was infective after 10 min at 55 but not at 60° C, after dilution to 10^{-3} but not to 10^{-4}, and after 6 but not 8 days at 22° C. The sap extracted with phosphate buffer containing TGA retained infectivity considerably longer. SpLV/H was transmitted through a high rate of seed from infected *C. quinoa* (60%), *N. clevelandii* (90%), *N. megalosiphon* (95%) and *S. oleracea* (60%). It was also transmitted through pollen, causing infection of seed [*C. quinoa* (21%), *S. oleracea* (56%)] and of pollinated spinach plants. Electron microscopy of infected *C. quinoa* and *B. vulgaris saccharifera* leaf cells revealed, in the cytoplasm and in 'small vacuoles', groups of small vesicles surrounded by a unit membrane. In the cells of control plants the vesicles were found sporadically, but only inside the 'small vacuoles'. The ultrastructural modifications mentioned have not been described in other ilarvirus infections.

Introduction

A mechanically transmissible seed- and pollen-borne virus differing from other viruses detected in spinach was isolated from spinach plants and seed in Yugoslavia in 1977 and briefly described under the provisional name of spinach latent virus (SpLV) (Štefanac 1978). During further analysis some properties of the virus were found similar to those of the ilarvirus group and it was suspected to be a new member of this group (Štefanac 1979). A similar virus, also named SpLV, was isolated from spinach seed originating from a number of countries and studied independently in the Netherlands (Bos, Huttinga and Maat 1980). Bos et al. (1980) analysed in detail and fully described their SpLV providing the evidence that the virus is a distinct and definitive member of the group of ilarviruses (cf. also Matthews 1982). The same authors found their SpLV identical with the virus encoded GE36, earlier detected in *C. quinoa* and supposed to have been isolated from apple and pear (Van der Meer 1968, Maat and Vink 1971).

The results of the studies on the host range and some other properties of SpLV from Yugoslavia, which were not previously described, and of its serological comparison with SpLV from the Netherlands are presented in this paper.

Material and Methods

Virus isolates. Unless otherwise stated studies were done with an isolate earlier obtained from leaves of spinach (*Spinacia oleracea* 'Matador') plant collected near Zagreb (Štefanac 1978), marked in this paper as SpLV/H. The Sp20-9 (type) isolate of SpLV (Bos et al. 1980) used for comparative serological tests was obtained from Dr L. Bos (Wageningen).

Biological studies. Extracts for inoculation were prepared by grinding leaves in 0.06 M phosphate buffer at pH 7.6 with 0. 1% (v/v) TGA. Test plants were grown in a glasshouse at c. 18—23°C (sometimes higher in spring). The isolate SpLV/H was propagated in *C. quinoa* plants to provide inoculum for host range tests and the source of virus for purification and serological experiments; the isolate Sp20—9 was also cultured in *C. quinoa*. Quantitative assays were made in *C. amaranticolor*. Back inoculations were made to *C. amaranticolor* and/or *C. quinoa* (3—4 plants altogether in each experiment) from inoculated and non-inoculated leaves, or virus was checked serologically.

Tests for seed and pollen transmission were done as described previously (Štefanac 1978): seeds were sown in sterilized soil and seedlings tested by sap inoculation to *Chenopodium* species.

The test for transmission through infected pollen to the pollinated plants was conducted with 'Matador' spinach. Twelve days before collecting samples of leaves and stems from pollinated spinach for infectivity assays, pollinators were removed to avoid the presence of infected pollen on the surface of tested spinach. Nevertheless, healthy *C. quinoa* plants kept together with the spinach close to infected spinach pollinators were used as control for possible contamination with infected pollen.

L i g h t m i c r o s c o p y. Epidermal strips taken from parts of the leaf lamina with chlorotic symptoms were mounted in water and examined at magnification of x600 using bright field illumination.

E l e c t r o n m i c r o s c o p y. Small pieces of chlorotic foliar tissue, taken from local lesions of infected *C. quinoa* and from systemically infected leaves (chronic phase) of *C. quinoa* and *B. vulgaris saccharifera,* were fixed for 60 min in 1% (v/v) glutaraldehyde in cacodylate buffer pH 7.2 and postfixed for 2 hr in 1% (w/v) osmium tetroxide. After dehydration through a series of ethanol, the pieces were embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate before examination in a Siemens Elmiskop I. The adequate foliar tissue from healthy plants was used as control.

V i r u s p u r i f i c a t i o n. SpLV/H and Sp20—9 were partially purified using the first part of the purification procedure developed for SpLV (B o s et al. 1980), i.e. by two cycles of differential centrifugation followed by one cycle of sucrose-gradient centrifugation. The final pellets were resuspended in 0.018 M phosphate-citric acid buffer at pH 7 (PCA buffer), containing 2% formaldehyde when the virus was used for immunisation of a rabbit. Formaldehyde was removed by dialysis against PCA buffer.

S e r o l o g y. A rabbit was injected intramuscularly on each of two occasions, 10 days apart, with c. 0.3 ml of partially purified SpLV/H using Freund's complete adjuvant (an equal volume). The serum collected 2 wk after the second injection had a poor virus titre (1/2), indicating that SpLV/H is, like GE36 and Sp20—9 (M a a t and V i n k 1971, B o s et al. 1980), weakly immunogenic. The serum did not contain antibodies against normal plant proteins, and was used for detection of the virus in host range studies.

Antiserum against Sp20—9 (homologous titre 1/32) for comparative serological tests was provided by Dr L. B o s.

Serological reactions were made by double diffusion in 0.9% (w/v) agar in 0.85% (w/v) NaCl or in PCA buffer (B o s et al. 1980), each containing 0.3% sodium azide.

R e s u l t s

Host range and symptomatology

SpLV/H was transmitted by manual inoculation of sap to 22 of 32 species of nine botanical families (Table 1). Most plants were infected systemically showing mild symptoms or no symptoms. Reactions were essentially similar to those described for GE36 and Sp20—9 isolates of SpLV (V a n d e r M e e r 1968, B o s et al. 1980), although somewhat stronger. Temperatures around 20°C generally supported infections; the plants developed obvious symptoms or were infected more consistently than above 23°C. By slightly raised temperature infected plants often recovered, either completely or only from the symptoms, and some species became immune to infection.

Test species which reacted with rather characteristic symptoms were:

Chenopodium amaranticolor. Pin-point cholorotic local lesions sometimes with a diffuse green halo, appearing 3—5 days after inoculation (Fig. 1B).

Table 1. Reaction of test plants to SpLV/H

Family and species	Inoculated leaves	Uninoculated leaves
AIZOACEAE		
Tetragonia expansa	+	(+—) s
AMARANTHACEAE		
Amaranthus retroflexus	—	+— s^0
Celosia argentea	—	+— s^0
C. cristata	+ s	+— s^0
Gomphrena globosa	+	+^{0}
CHENOPODIACEAE		
Beta vulgaris		
saccharifera 'Mano'	+	+— (s)
cicla 'Egypat'	+	+— (s)
'Detroit'	+	+ (s)
Chenopodium album	+	—
**C. amaranticolor*	+	—
C. murale	+	+—
**C. quinoa*	+	+
**Spinacia oleracea* 'Matador'	(+) s	(+) s
COMPOSITAE		
Lactuca sativa 'Vanguard'	+ s^0	—
Zinnia elegans	—	—
CRUCIFERAE		
Brassica chinensis	—	—
B. rapa rapa	—	—
Sinapis alba	—	—
CUCURBITACEAE		
Cucumis sativus 'Delicatess'	—	—
FABACEAE		
Phaseolus vulgaris	+—	—
Pisum sativum 'Provansalec'	—	—
Vicia faba	+—	
Vigna cylindrica (Fig. 1C)	+—	—
V. sinensis	+—	—
LAMIACEAE		
Ocimum basilicum	—	—
SOLANACEAE		
Datura stramonium	—	—
Hyosciamus niger	—	—
Lycopersicon esculentum	—	—
Nicotiana bigelovii	—	+ s
N. clevelandii	(+) s	(+) s
**N. glutinosa*	+— s	(+—) s
**N. megalosiphon*	+—	+— (s)
**N. tabacum* 'Samsun'	+— s	(+—) s
'White Burley'	+— s	(+—) s
Petunia hybrida	+—^{0}	(+—) s

C. quinoa. Diffuse chlorotic local lesions 2 mm in diameter, c. 3—5 days after inoculation. One to 2 days later systemic symptoms consisting of chlorosis, chlorotic lesions and necrosis spreading from the base of young leaves to their tips (Fig. 1A), often followed by top necrosis.

Nicotiana glutinosa and *N. tabacum.* At low temperatures in some plants systemic mild chlorosis (later fine necrosis) along small veins in the form of a fine net (Fig. 2A), or mild mosaic. Symptoms usually appeared in only one or two leaves. After their disappearance plants remained infected if temperatures were not too high but the virus was often in low concentration.

N. megalosiphon. Chlorotic local lesions soon turning necrotic (Fig. 2B), appearing 3—4 days after inoculation. Systemic chlorotic and necrotic 'oak-leaf' patterns, accompanied by few rings, spreading from the base of leaves (Fig. 2C), and sometimes top necrosis. Plants susceptible to infections exclusively during winter.

Virus failed to infect some species (*Cucumis sativus, Datura stramonium, Hyosciamus niger, Momordica balsamina*) used in identification of other ilarviruses.

Five other SpLV isolates obtained from spinach plants and seed in our laboratory were not analysed concerning the reaction of test plants. Somewhat better expressed transient mosaic and vein clearing symptoms developed in spinach mechanically inoculated by one of these isolates could also be attributed to the differences in conditions of the glasshouse.

Properties in vitro

Crude sap from systemically infected *C. quinoa* retained some infectivity after heating for 10 min at 55 but not at 60°C, and after dilution with distilled water to 10^{-3} but no to 10^{-4}. Aging in vitro at c.22°C was between 6 and 8 days in cude sap, and between 16 and 18 days when sap was extracted in buffer for inoculation. In the same buffer sap exhibited good infectivity after 30 days kept at 1°C, but only 1/5 of it when TGA was replaced by DIECA + 2-mercaptoethanol (0.02 M).

In desiccated leaves of infected *N. megalosiphon* and *T. expansa* stored under $CaCl_2$ (at 4°C) some infectivity still remained after five and six years of storage, respectively.

Transmission through seed and pollen

In earlier experiments seed from infected spinach and from healthy plants pollinated with infective pollen gave rise to a high proportion of infected seedlings (Štefanac 1978). Subsequently, seed and pollen transmissions in experimental host species were analysed. In seed samples from infected *C. quinoa, N. bigelovii, N. clevelandii* and *N. megalosiphon,*

+ = consistently infected
+— = sometimes infected
— = no symptoms, infection not demonstrated
—— = no symptoms, infection not tested
s = symptomless infection
( ) = sporadic and/or transient symptoms, sporadic symptomless infection
⁰ = low concentration of virus
* = symptoms described previously (Štefanac 1980)

SpLV/H was detected in 12/20 (60%), 0/20 (0%), 18/20 (90%) and 19/20 (95%) seeds, respectively. The virus was also detected in 4/19 (21%) seeds from healthy *C. quinoa* plants pollinated with infected pollen. In all cases, except for *C. quinoa*, progeny seedlins did not show symptoms. The reaction of *C. quinoa* was characterised by symptoms differing from those obtained after mechanical transmission which coincided with those described earlier for Sp20—9 in similar experiments (Fig. 5 of B o s et al.).

Tests were also made to detect whether the virus was transmitted by pollen to the pollinated plants, a property characteristic of some ilar- but not of nepoviruses. In one experiment, the virus was detected in the stage of fructification in 5 of a batch of 6 previously healthy plants of spinach which, at the time of flowering, were placed for pollination next to infected male spinach plants. Leaves from control *C. quinoa* plants, exposed at the same time to the pollen of infected spinach, did not show infectivity.

Finally the persistence of the virus in seed was tested. In 5 years old samples of spinach seed, kept at room temperature in a polyethylene bag, the virus was detected in 55% of viable seed, the percentage being somewhat lower than the one detected 5 years earlier in the same seed lot (cf. Š t e f a n a c 1978).

Cytopathological analysis

Neither inclusion bodies nor obvious alterations of cell organelles, except when the cells were killed, were observed by light microscopy in the epidermal cells from infected plants belongig to *Solanaceae* and *Chenopodiaceae*. Strips from plants grown in optimal conditions for multiplication of the virus were checked several times. Absence of granular cytoplasmic inclusion bodies visible by light microscopy, which often accompany infections with nepoviruses, and especially the possibility of transmission with pollen to the pollinated plants indicated that SpL/H could belong to the ilarviruses.

In ultrathin sections through infected leaf cells of *C. quinoa* and *B. vulgaris saccharifera* no virus inclusions were found, either. In sections, virus particles could not be distinguished from ribosomes .Groups of small vesicles (mostly 30—80 nm diameter) surrounded by unit membrane were often present in the cytoplasm of infected cells (Fig. 3A—C). Similar vesicles were commonly observed inside 'small vacoules' in the cytoplasm (Fig. 3A,B,D) and in the central vacuole (Fig. 3E). Vesicles within the 'small vacuoles' were also seen in the control material, but only once in *C. quinoa* and on one occasion in sugar beet (Fig. 3F) placed inside vacuoles.

Partial purification

Partially purified preparations showed good infectivity. As established by quantitative serological tests, 1/2 to 2/3 of the initial virus were lost during purification. The shape and size of the virions of SpLV/H (Fig. 4A) correlated with those of Sp20—9 (cf. B o s at al. 1980).

Serological comparison

Gel-diffusion precipitin tests were done to compare the Yugoslav isolate SpLV/H with the Dutch type isolate Sp20—9 of SpLV. In infective crude sap or in partially purified preparations, the two isolates reacted

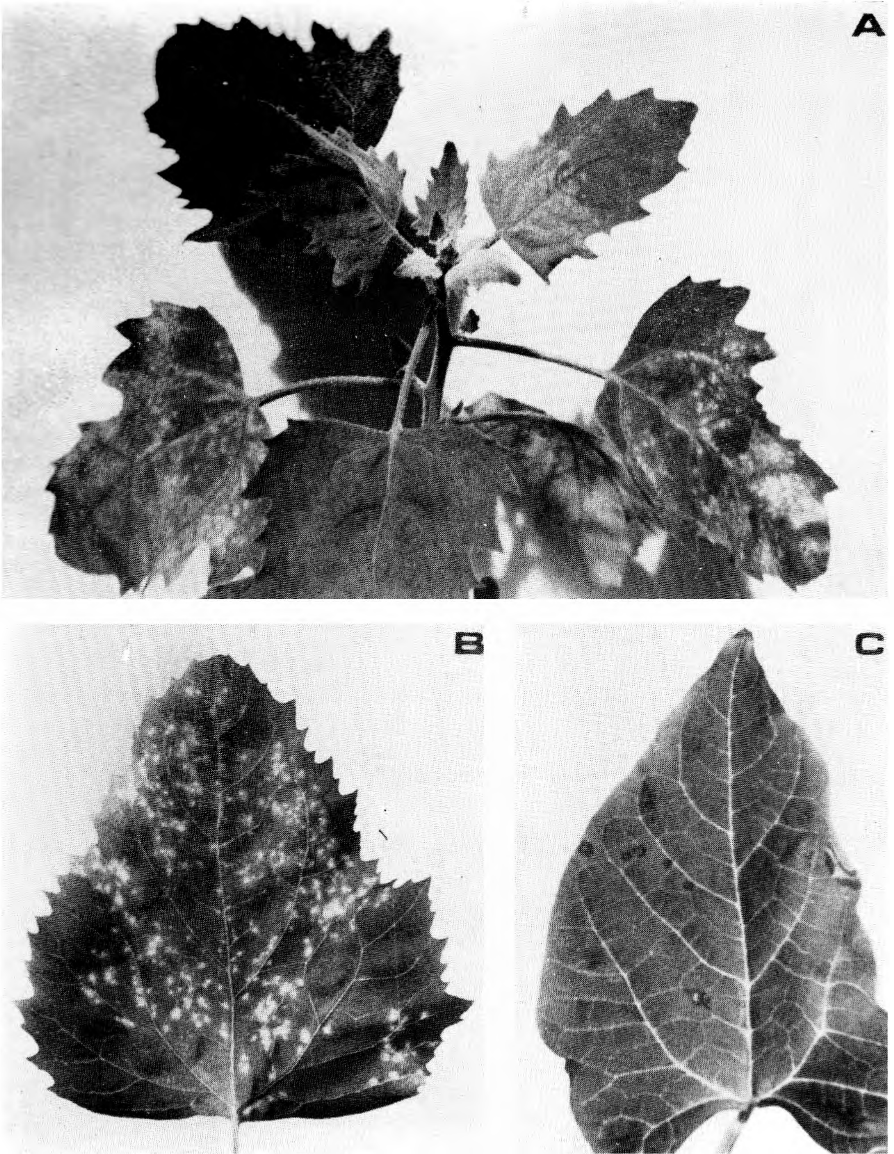

Fig. 1. A. Local lesions and systemic chlorosis induced in *Chenopodium quinoa*. B. Local chlorotic lesions in *C. amaranticolor*. C. Local necrotic lesions in *Vigna cylindrica*.

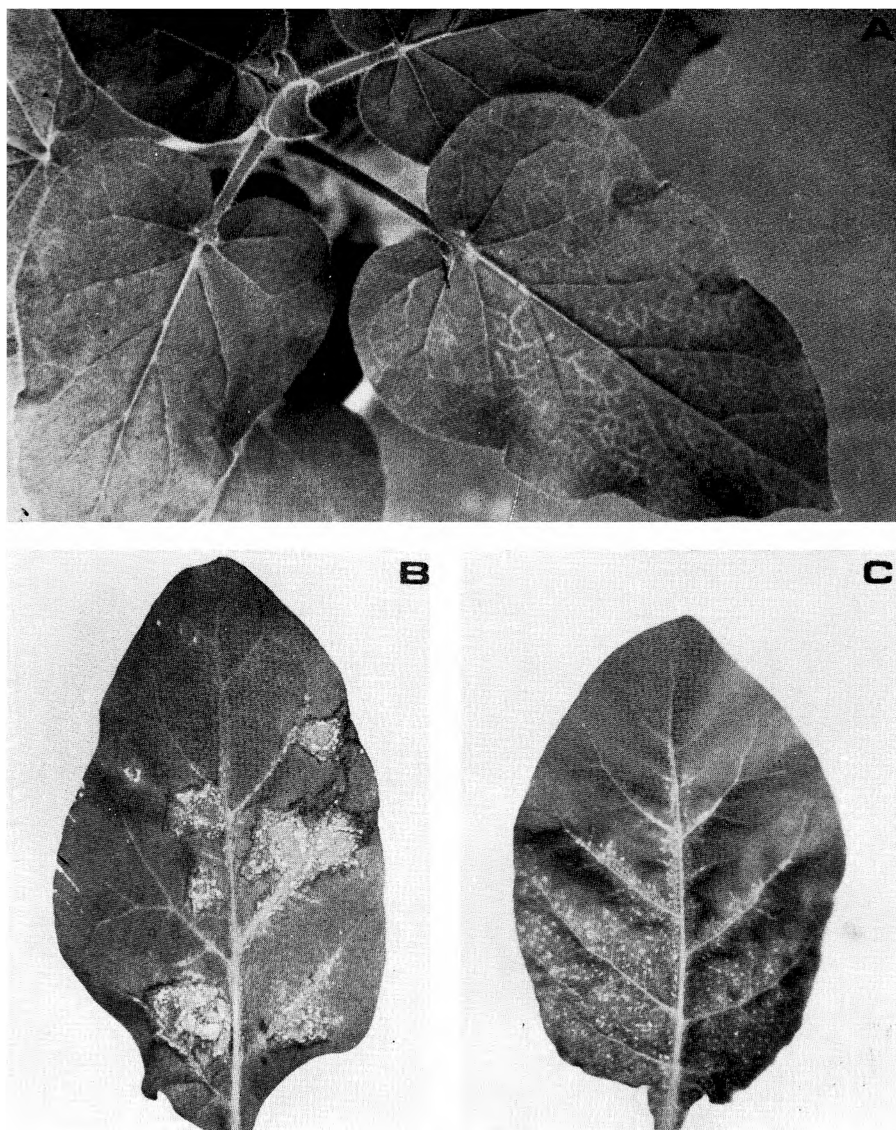

Fig. 2. A. Systemic necrotic net along small veins and 'recovery' of *Nicotiana glutinosa*. B, C. Local necrotic lesions (B) and systemic 'oak leaf' pattern symptoms (C) in leaves of *N. megalosiphon*.

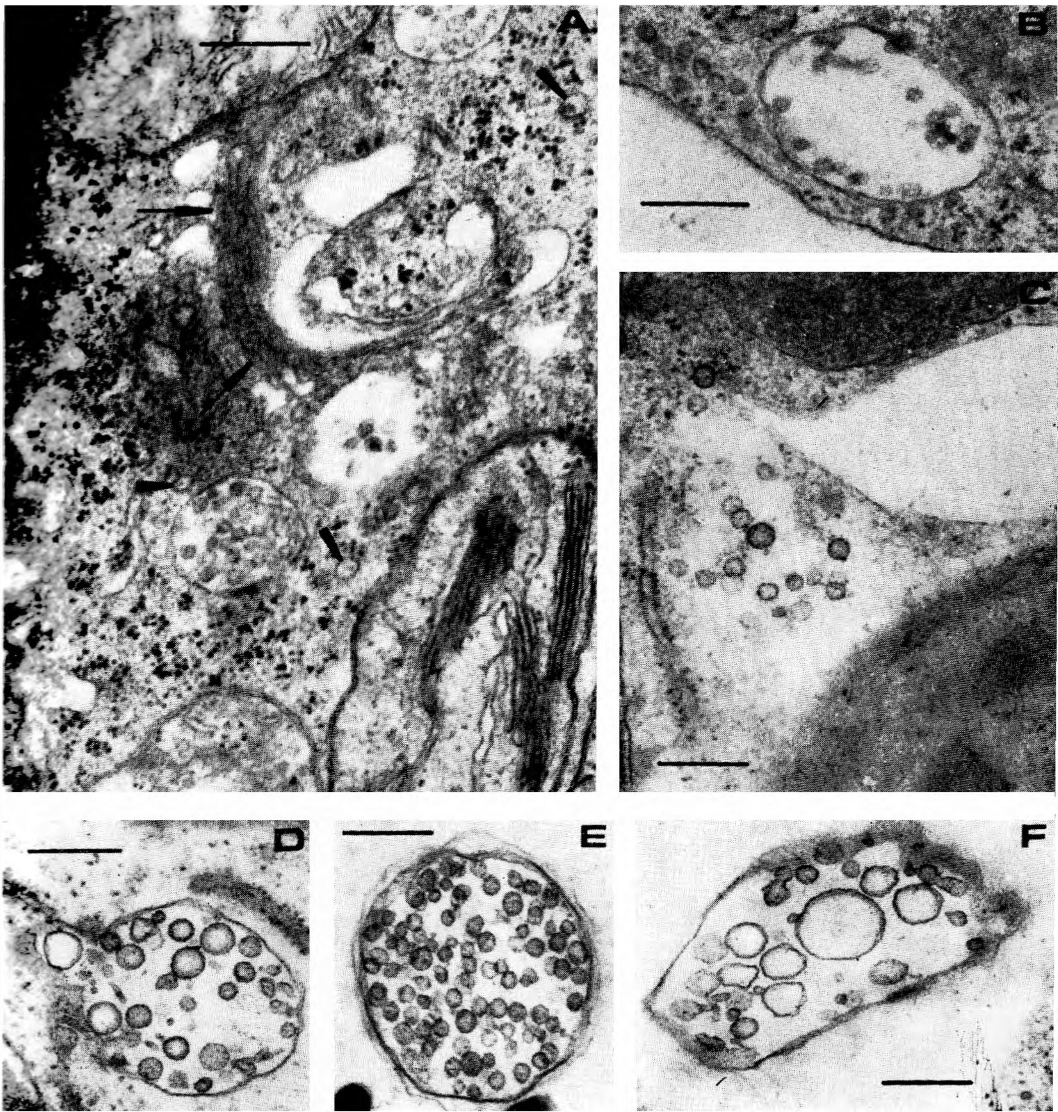

Fig. 3. Vesicular structures in leaf parenchyma cells. A, B. *Chenopodium quinoa*. C—F. *Beta vulgaris saccharifera*. A—E. Virus infected material. F. Healthy material. A. Part of infected cell with highly active dictyosome (arrows), 'small vacuoles' with vesicles, and vesicles lying freely in the cytoplasm (arrow heads). B. 'Small vacuole' with vesicles, and vesicles in the cytoplasm. C. Vesicles in the cytoplasm. D. Vesicles in a 'small vacuole' within the cytoplasm. E, F. Vesicles surrounded by membranes, lying in central vacuole. Bars represent 300 nm.

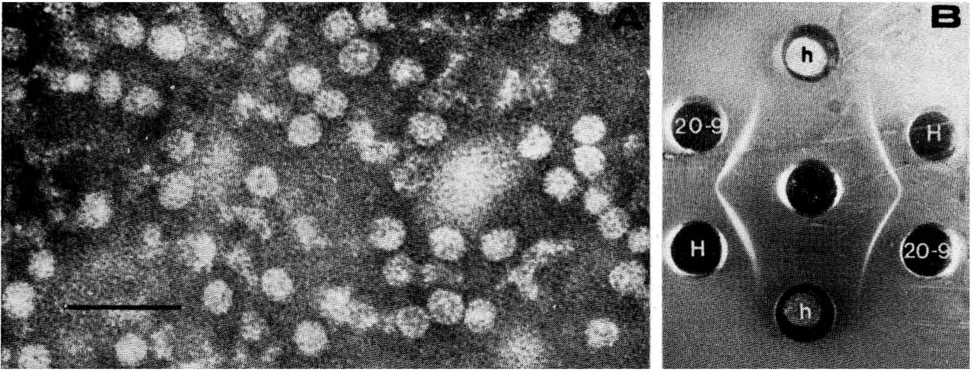

Fig. 4. A. Partially purified preparation of SpLV/H, fixed in formaldehyde and stained with 2% potassium phosphotungstate (pH 6.5). The bar represents 100 nm. B. Gel-diffusion serological test comparing SpLV/H (H) and Sp20-9 (20-9) isolates of spinach latent virus. Central wall contains antiserum to the isolate Sp20-9. Wells (h) contain virus-free sap from *C. quinoa*.

to antiserum against Sp20—9 type isolate forming continuous lines without producing spurs (Fig. 4). A heterologous titre of the Sp20—9 antiserum was 1/32 corresponding to the homologous titre of this serum in parallel experiments. These tests show that the two isolates are serologically very closely related and probably identical.

In NaCl-agar both virus isolates, SpLV/H and Sp20—9, usually reacted with two distinct lines (cf. Fig. 11 of Bose et al.) presumably due to certain damage of virus particles. When the agar in PCA buffer was used, only a single specific virus line was formed (Fig. 4).

Discussion

The host range, symptomatology, high rates of transmission by seed and properties in vitro of SpLV obtained from Yugoslavia are very similar to those of SpLV described by Bos et al. (1980). Moreover, comparative serological tests indicate that the two virus isolates are serologically closely related if not completely identical. This is in accordance with previous findings of Bos et al. (1980) who had not detected any appreciable differences between their isolates of SpLV originating from different countries, and among their type isolate Sp20—9 and GE36 virus earlier obtained by Van der Meer (1968). Since environmental conditions greatly influence the symptom severity (cf. also Van der Meer 1968, Bos et al. 1980), it cannot be said whether the somewhat stronger reactions obtained in our experiments with isolate SpLV/H express the real difference between this isolate and the isolate Sp20—9 or they are a result of differences in environmental conditions.

SpLV had earlier been found to be more stable in crude sap than most other ilarviruses (Van der Meer 1968, Štefanac 1979, Bos et al. 1980), and in this respect it shows similarity to elm mottle virus (Jones and Mayo 1973). With SpLV/H we have now shown that infectivity in sap of SpLV can be considerably stabilised by extraction in 0.06 M phosphate buffer at pH 7.6 with 0.1% TGA.

Although it was found earlier that the pollen infected with SpLV gave rise to infected seed (Štefanac 1978), we now give evidence that SpLV is transmitted through pollen to pollinated plants, a feature known to occur by ilarviruses prune dwarf and Prunus necrotic ringspot (cf. Fulton 1981). Whereas this manner of spreading is very important in the epidemiology of the two viruses, in the case of SpLV it is only of academic interest.

Groups of small vesicles frequently found in the cytoplasm of cells infected with SpLV could be connected with intensified activity of dictyosomes. These vesicles are comparable to small single--membraned vesicles observed in cells infected by Pelargonium zonate spot virus (PZSV) (Castellano and Martelli 1981), a virus with some properties resembling those of ilarviruses and others suggesting that it may not be a member of this group (Castellano and Martelli 1981, Gallitelli 1982). Similar vesicles were earlier also described in cells affected by barley yellow dwarf virus (Gill and Chong 1979). The vesicles we observed within 'small vacuoles' could represent paramural bodies which often occur at the cell wall-plasmalemma interface connected with different virus infections (cf. Martelli and Russo 1977) and also found in PZSV-infected cells (Castellano and Martelli

1981). However, the lack of their join with the cell wall in our sections does not permit a reliable conclusion about their paramural origin. The rows of virus particles within the membrane-walled tubules which were reported in the cytoplasm of cells infected by two ilarviruses, Citrus variegation (Gerola, Lombardo and Catara 1969) and tobacco streak (Edwardson and Purcifull 1974), were not found in plants infected by SpLV. The reason for not finding them was probably not young enough leaf tissue which was used for analysis.

*

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SAŽETAK

LATENTNI VIRUS ŠPINATA: NEKA SVOJSTVA I USPOREDBA DVAJU IZOLATA

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S pomoću reakcije pokusnih biljaka, svojstava u sirovom soku, prenošenja sjemenom i seroloških reakcija utvrđeno je da je virus izdvojen iz špinata (S. *oleracea*) u Jugoslaviji i privremeno opisan pod nazivom latentnog virusa špinata (spinach latent virus) identičan ilarvirusu karakteriziranom u Nizozemskoj pod istim nazivom.

Jugoslavenski izolat (SpLV/H) latentnog virusa špinata nakon mehaničke inokulacije zaražavao je 22 vrste biljaka iz devet porodica, izazivajući u njima nešto jače reakcije od onih koje su opisane za nizozemske izolate (GE36 i Sp20—9). Sirov sok zaraženog *C. quinoa* bio je infektivan nakon zagrijavanja 10 min na 55 ali ne 60°C, nakon razrjeđivanja od 10^{-3}, ali ne 10^{-4}, i nakon stajanja od 6, ali ne 8 dana na 22°C. Sok ekstrahiran s pomoću fosfatnog pufera zadržavao je infektivnost znatno duže. SpLV/H se prenosio u visokom postotku sjemenom vrsta *C. quinoa* (60%), *N. clevelandii* (9%), *N. megalosiphon* (95%) i *S. oleracea* (60%). Virus se prenosio također polenom, uzrokujući zarazu sjemenki [*C. quinoa* (21%), *S. oleracea* (56%)] i oprašenih biljaka špinata. S pomoću elektronskog mikroskopa ustanovljene su u stanicama listova zaraženih biljaka *C. quinoa* i *B. vulgaris saccharifera* u citoplazmi i u 'malim vakuolama' skupine mjehurića promjera c. 40—80 nm. U stanicama kontrolnih biljaka navedeni mjehurići bili su izuzetno rijetki i prisutni samo u 'malim vakuolama'. Navedene ultrastrukturne promjene dosad nisu zabilježene pri infekcijama drugim ilarvirusima.

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