BIOLOGIA PLANTARUM (PRAHA)

19 (3) : 196–204, 1977

Experience with the Diagnostics and Purification of Plum Pox Virus

LIANA ALBRECHTOVÁ, J. CHOD and J. POLÁK

Institute of Plant Protection, Praha*

Abstract. Some diagnostic methods devised for the demonstration of the presence of plum pox virus in plum (*Prunus domestica* L.) and apricot (*Armeniaca vulgaris* LAM.) leaves were examined. The method of radial diffusion in agar can be recommended as the simplest and the least time consuming method which can be used during the entire vegetation period. In order to obtain antisera, some preparation methods of PPV antigen were verified. The best preparation method was a modification of Van Oosten's method in which HEPES buffer pH 6.7 was used for the homogenization of leaves from infected *Nicotiana clevelandii* GRAY plants and for the solution of sediments after ultracentrifugation. In this way, antigens with titre up to 1 : 2048 and during the immunization of rabbit antisera with titre 1 : 512 to 1 : 1024 were obtained. After the saturation of antisera according to Uyemoto, the titre of the antisera was 1 : 64 to 1 : 256. Antisera were used for agar preparation in transparent plastic boxes. 0.2 to 1 g portions of leaf material were homogenized with 0.05 M Tris-HCl buffer pH 7.2, 3 % pyrrolidine and 1 % polyvinylpyrrolidone in the ratio of 1 : 3 for the determination of PPV in plum and apricot leaves.

The determination of plum pox virus (PPV) in the leaves of fruit trees is relatively difficult. Mechanical transmission of PPV from the leaves of diseased plums is possible only from young leaves and buds because of the high polyphenol content and it requires exactly defined conditions (SCHADE 1968). An easy mechanical transmission of PPV from ripe plum fruits was described by VAN OOSTEN (1970), while KRÖLL (1974) did not obtain positive results which he attributed to low pH of expressed sap from the fruits in the unfavourable climatic conditions of Central Europe. Fruit growers can obtain data on the health conditions of their trees most rapidly and simply only by means of serological methods. The preparation of antigens in order to obtain antiserum against PPV has been attempted by numerous workers as *e.g.* DOUNINE and MINOIU (1968), GYÖRGY and NÉMETH (1968), SCHADE (1969), RANKOVIĆ and JORDOVIĆ (1970), BABOVIĆ *et al.* (1971), VAN OOSTEN (1972). SCHADE (1969) was successful in detecting PPV in plum leaves by means of the latex test. CASPER (1972, 1973, 1975) and KRÖLL (1973) proved the presence of PPV in plum leaves by means of radial diffusion in agar after SHEPARD (1972).

Received August 5, 1976

**Address:* VÚRV, 161 06 Praha 6-Ruzyně, 507, Czechoslovakia.

In the years 1970—1975, we examined some diagnostic methods with respect to their applicability for PPV detection in plum and apricot leaves and compared some purification methods for the preparation of a specific antiserum.

Material and Methods

Plum buds germinated either in the greenhouse or in the open were used in the experiments with mechanical transmission of PPV. The buds were homogenized first with one volume and later with a two-fold volume of 0.015 M phosphate buffer pH 7.8 containing 0.01 M DIECA (diethyldithiocarbamate, sodium salt) and 0.01 M caffeine (KEGLER and OPEL 1963). Diseased plum trees were from three localities near Prague and from Bulgaria. The scions from Bulgaria, sent to us by Dr. Trifonov, were grafted prior to the experiments onto healthy plum trees. *Myzus persicae* (SULZ.) was used for the transmission of PPV by aphids, acquisition feeding lasted 2—5 min and inoculation feeding 4 h. The symptoms on *Chenopodium foetidum* were compared visually with the symptoms caused by three isolates of PPV supplied by Prof. Dr. Šutić.

Electronmicroscopic preparations from the host plants were prepared by the dip method. To check purification methods, PPV antigens were diluted with distilled water in the ratio of 1 : 5 to 1 : 20 and the samples were transferred onto electronmicroscopic nets coated with formvar membrane. The preparations were shadowed with Au under 20° angle and observed and photographed by means of an electron microscope Tesla BS 242.

The PPV isolate received from Dr. Casper with which *Nicotiana clevelandii* plants were infected, was used for the preparation of the antigen. The leaves were harvested always 21 days after the infection and immediately used for PPV purification. The properties of the isolate were examined by infecting the plants of *C. foetidum* SCHRAD., *N. clevelandii* GRAY, *N. acuminata* GRAH., *N. excelsior* BLACK, *N. bigelovii* var. *quadrivalvis* PURSH and by determining PPV particles by the dip method from *N. clevelandii* leaves by means of electron microscopy.

Purification Procedures

1. The purification procedure of GYÖRGY and NEMÉTH (1968) was modified in such a way that 250 g of fruit flesh with the symptoms were used and 0.01 M DIECA was added to the buffer solution to prevent darkening of the homogenate. The sap was centrifuged at $9000 \times g$, 15 min, instead of preliminary gel filtration. 200 ml of sap were layered on a Sephadex G-200 column (7 × 15 cm). 2.5 ml fractions were collected, 5 fractions were pooled and the virus was concentrated by means of ultracentrifugation.

2. The purification procedure of GILLS (1971) was used only with minor modifications for PPV purification from infected *N. clevelandii* plants. The leaves were frozen in the mortar before homogenization for only 1 h, because greater losses of the virus occurred with prolonged freezing in the leaves of *N. clevelandii* plants. The purified preparation was dissolved in 1/50 volume (of the initial leaf sap volume) of citrate buffer pH 6.2 which contained 0.5 M urea (Lachema).

3. The purification procedure described by RANKOVIĆ and JORDOVIĆ (1970) was not modified and was used with only one difference, *i.e.* the purified preparation was dissolved only in 1 ml of 0.02 M phosphate buffer pH 7.0.

4. Urea concentration in buffer was lowered to one half in the purification procedure according to VAN OOSTEN (1972) and gradient centrifugation in 10—50% sucrose gradient was omitted.

5. The purification procedure modified by Casper. This procedure differs from that described by VAN OOSTEN only in that 0.02 M HEPES buffer with 0.01 M DIECA was used (also in the ratio of 1 : 4) instead of citrate buffer. The sediments obtained after centrifugation were dissolved in 0.02 M HEPES buffer pH 6.7 and 20% sucrose solution was also added in 0.02 M HEPES buffer pH 6.7.

Janetzki K-24 centrifuge was used for low speed centrifugation (up to 15 000 $\times g$) and ultracentrifuge UC Spinco L for high speed centrifugation. Centrifugation was performed at 4 °C.

Serology

Rabbits were immunized intramuscularly with the obtained antigen in one injection with the complete Freund adjuvans, and in 6 injections with the incomplete Freund adjuvans at one-week intervals. Blood was taken from the ear vein 10 days after the last injection. Antiserum titre was determined by means of drop precipitation reaction. Because the antisera reacted with protein from healthy *N. clevelandii* plants, they were saturated according to the method of UYEMOTO *et al.* (1972) and then used for serological diagnosis.

PPV from plum and apricot leaves was tested in agar plates in square transparent plastic boxes, 4.5 $\times$ 4.7 cm, provided with a lid. Agar containing boxes were kept in a humid chamber at room temperature. 4 ml of agar formed a 2 mm thick layer. The agar mixture contained 2 ml of 2% ionagar No. 2 in 0.05 M Tris-HCl buffer pH 7.2, 1 ml of 0.05 M Tris-HCl buffer pH 7.2 and 1 ml of saturated antiserum with titre 1 : 64 with 0.08 ml of 5% sodium azide. Antiserum and sodium azide were warmed on a water bath to 45 °C. Agar was boiled prior to this in an Erlenmeyer flask stoppered with a cellulose plug, and left to cool to 45 °C on a water bath. After mixing, the agar mixture was pipetted into the boxes and allowed to cool. 20 to 25 holes, 3.5 mm in diameter, were cut into the agar. The holes were situated 5 mm from each other. When the distance was shorter than 5 mm, no precipitation rings were formed in some cases. Plum and apricot leaves were tested during the entire vegetation period. Leaf parts with virus symptoms were cut from the leaves with scissors. 0.2—1 g samples were ground in a mortar with 0.05 M Tris-HCl buffer pH 7.2 which contained 3% pyrrolidine and 1% polyvinylpyrrolidone (SHEPARD 1972). The thick and slimy homogenate thus obtained was squeezed through small pieces of rhovyl paper (Schleicher and Schüll 1002); filtrate drops were immediately sucked into Pasteur pipettes and applied to the holes. At low virus concentrations, the holes were filled again with the same sap after 2 h. Assessment is possible only after 12 h, final evaluation after 48 to 72 h. Assessment is sometimes difficult when sap is spilt over the hole edges. For this reason agar was rinsed with distilled water and excessive water was blotted up. The reaction was observed in obliquely directed light.

Results

Transmission of PPV on Host Plants

The transmission of PPV from germinated plum buds on host plants of *C. foetidum*, *N. clevelandii* and *N. bigelovii* var. *quadrivalvis* was attempted in

the years 1971—1973 from three localities near Prague and from Bulgaria. We attempted to transmit PPV with aphids and mechanically. Aphid transmission was successful at most in 40% of *N. clevelandii* plants, while mechanical transmission only in 10 to 20% of *C. foetidum* and *N. clevelandii* plants. At first, no symptoms appeared in *N. clevelandii* plants after both mechanical- and aphid- transmission, and only a weak, less conspicuous mosaic appeared as late as 4 weeks after the infection. No typical brown-yellow local lesions but only yellow spots appeared in *C. foetidum* plants after the mechanical transfer of PPV from plum leaves. These symptoms resembled those described by ŠUTIĆ *et al.* (1971) for yellow PPV strain. Infected *C. foetidum* plants developed brown-yellow lesions, typical of PPV, only after at least a double passage of the virus through *N. clevelandii* plants. Mechanical transmission of the virus from germinated plum buds on *N. bigelovii* var. *quadrivalvis* plants, on which a very weak mosaic appeared on more mature leaves, was also successful. The attempts to obtain a typical necrotic isolate were not successful.

Preparation of PPV Antigen

Serological methods are suitable for the detection of PPV in the leaves of fruit trees, but they require the preparation of a specific antiserum. The preparation of the PPV antigen requires as virus source an isolate with higher virus concentration and because our isolates did not fulfil these conditions, we used the PPV isolate supplied by Dr. Casper.

In 1970, we first attempted the preparation of PPV antigen from ripe plum fruits by means of gel filtration (GYÖRGY and NEMÉTH 1968). Only very few particles (2—10 per net mesh) could be found in the resulting purificates by means of electron microscopy and no symptoms appeared after mechanical inoculation of *C. foetidum* plants. For this reason, we used for the preparation of PPV antigen from the leaves of infected *N. clevelandii* plants the method GILLS (1971) used for the preparation of oat necrotic mottle virus which has approximately the same length as PPV. When 50 g leaf samples were used, the PPV purificate obtained (1 ml) had its titre 1 : 32 and reacted with the antiserum against protein from healthy plants when diluted 1 : 2 to 4. After saturation with the antiserum against protein from healthy plants, the titre was 1 : 16. A small amount of particles, slightly degraded, probably due to the action of 0.5 M urea contained in the buffer, could be observed by means of electron microscopy. The purificate (1 ml) prepared by the method of RANKOVIĆ and JORDOVIĆ (1970, obtained from a 25 g leaf sample, had its antigen titre 1 : 16 to 1 : 32. The purificate reacted with the antiserum against protein of healthy *N. clevelandii* plants at the dilution 1 : 2. VAN OOSTEN (1972) reported a method of PPV preparation in which Triton X-100 was used to clear the sap from *N. clevelandii* leaves and 0.01 M citrate buffer with 1 M urea and with 0.1% mercaptoethanol for the solubilization of sediments. Because we had established degradation of potato Y virus under the influence of urea when preparing antiserum against the Y virus (POLÁK *et al.* 1975), we used only 0.5 M urea during PPV purification. In spite of this we obtained PPV antigens which reacted only weakly or did not react at all with a specific antiserum. No virus particles or only very few particles could be found by means of electron microscopy. We then followed and examined individual steps of the purification method of VAN OOSTEN (Table 1) and established

TABLE 1

Investigation on the purification method of VAN OOSTEN by means of electron microscopy and by means of serology
Buffer 1 — citrate buffer pH 6.7
Buffer 2 — citrate buffer pH 6.7 + 0.1%ME + 0.5 M urea

	Homogenization of *N. clevelandii* leaves (1 : 4) + 0.01 M citrate buffer pH 6.7 + 0.05 M EDTA + 0.015 M DIECA		5% Triton X-100 + UC (R 30) 78 000×*g*, 90 min, sediment +		UC (SW 25), 90 000×*g*, 120 min, 20% sucrose sediment +	
EM particle number per mesh	before centrifugation: more than 100	after centrifugation: more than 50	buffer 1: more than 100	buffer 2: 5 degraded	buffer 1: more than 100	buffer 2: 0
serological precipitation reaction	++++	+++	++	+	++	?

that particle degradation and disintegration was caused by urea, above all when antigen suspension was exposed to it for 2 h before gradient centrifugation, in the same way as was described in the original procedure. On the recommendations of Dr. Casper we examined his modification of Van Oosten's method (CASPER 1972, 1973, 1975), in which HEPES buffer is used instead of

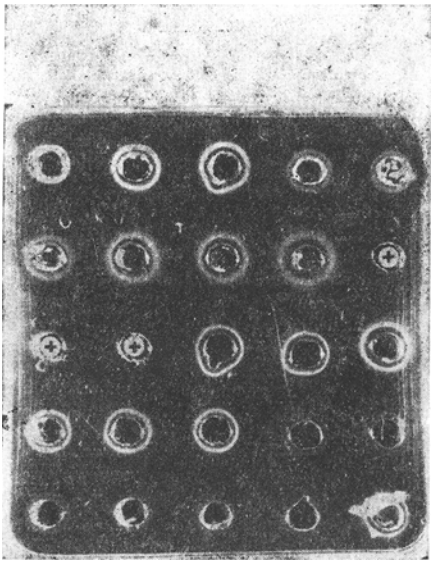

Fig. 3. Serial demonstration of PPV by means of radial diffusion in agar in the leaves of diseased and healthy (+) plum and apricot leaves.

citrate buffer with urea. PPV antigen was prepared according to this modification with the only difference that 0.01 M DIECA was added to the homogenization medium which prevented polyphenol oxidation and sap browning during the first steps of purification. DIECA did not influence the yield of the virus. We also tested the influence of some homogenization buffers on PPV yield. HEPES buffer with or without DIECA and homogenization buffer after Van Oosten were equal. Antigens with high titre (Fig. 2) were obtained with the method modified by Casper. Titre values obtained with drop precipitation reaction were 1 : 256 to 1 : 2048 according to the amount of plant material used. Virus yields were 8—16 times higher than the yields obtained by the method described by RANKOVIĆ and JORDOVIĆ (1970), when the same amount of plant material was used (ALBRECHTOVÁ 1976). These virus isolates reacted with the antiserum against proteins from healthy *N. clevelandii* plants in the dilution 1 : 4 to 1 : 8. The antigens were used directly for the immunization of rabbits. The antisera obtained had their titres from 1 : 256 to 1 : 1024. They reacted with the sap of healthy *N. clevelandii* plants maximally at the dilution 1 : 4. The titre dropped to 1 : 64 to 1 : 256 after saturation according to UYEMOTO *et al.* (1972) and then the antisera did not react with proteins from healthy plants.

Serology

We examined the significance for practical use of radial diffusion in agar according to CASPER (1972, 1973, 1975), which has many advantages. One of the advantages is that agar plates can be prepared in advance in small transparent polystyrene boxes with a lid 4.5 × 4.7 cm (FRG), or 6.5 × 4 cm (GDR). The lid prevents desiccation of the agar gel and the boxes with agar can be kept in a wet chamber at room temperature for 3 and more months. Saturated antiserum for the preparation of agar gel has to have its titre 1 : 64 in drop precipitation reaction. Dilution ratio with respect to the total agar and buffer volume in the box is 1 : 4 (1 ml of the antiserum (titre 1 : 64) + + 1 ml of buffer + 2 ml of 2% agar). The optimum ratio of buffer to plum leaves is 1 : 3. Pipetting of the concentrated homogenate is difficult, as the homogenate is very slimy. On the other hand, when during the homogenisation the ratio of leaf material and buffer was 1 : 4, it was not possible to demonstrate lower virus concentrations. The optimum ratio of buffer and epidermis or flesh is 1 : 2. Samples with lower virus concentrations are pipetted again 1 h after the diffusion of the first portion. The solutions used for homogenisation must not be stored longer than 4 to 6 weeks. During radial diffusion in agar, antigen diffuses radially into the gel and dilutes simultaneously continually, and at an optimum ratio of antigen and antibody a white precipitation ring precipitates, the intensity of the ring being dependent on the concentration of PPV antigen. A magnifying glass and observation after 72 h are recommendable for the observation of very weak rings near the hole. The reaction can be observed already after 12 to 24 h in some cases, but it is better to postpone final evaluation till after 72 h because of weaker reactions. In 1975, we examined with our antisera and with Dr. Casper's antiserum 75 samples of plum and apricot leaves, of plum fruits, and of host plants. The reaction with the sap from healthy leaves of *N. clevelandii* and *C. foetidum* plants as well as with the sap from healthy plum and apricot leaves did not occur in any case. A positive reaction was obtained if the leaves showed symptoms, and also in those cases when the symptoms were weak and atypical or with the sap from the epidermis of diseased fruits. We could not obtain a positive reaction with fruit flesh, even when clearly red and necrotic flesh parts were homogenized. No reaction was obtained with the sap from the leaves lacking symptoms, *i.e.* or from diseased trees on which symptoms appeared on other shoots. Neither was positive reaction obtained from the leaves of plum and apricot trees which had distinct symptoms, caused probably by other viruses. The leaves could be tested successfully from the middle of May till leaf drop, *i.e.* during the entire vegetation period. Precipitation rings with the sap from plum leaves were more intense and often diffused, while precipitation rings with the sap from apricot leaves were weaker and well differentiated.

Discussion

According to our experience, mechanical transmission and transmission with aphids are not suitable for series demonstration of PPV presence in plum leaves. Mechanical transmission is difficult, the results are uncertain, and it requires exactly defined temperatures which can be best ensured in controlled-environment chambers. The results can be influenced by exces-

sively high temperatures which can occur in glasshouses, because the heat inactivating point of PPV is low; it vacillates between 40 and 55 °C according to the isolate. Our isolates were in no case necrotic. Similar results were also obtained by PAULECHOVÁ (1974) in Slovakia.

The most rapid and simplest methods for direct diagnosis of PPV in plum and apricot leaves are serological methods, provided a good and specific antiserum is available. To obtain the antiserum, we examined some purification methods for the preparation of PPV antigen. The antigens with the highest titre (1 : 2048) were obtained by the method of VAN OOSTEN (1972) as modified by CASPER (1975). HEPES buffer which is used here, is relevant for the high yield of PPV; it probably prevents PPV aggregation with proteins of host plants (ALBRECHTOVÁ 1976) and in this way virus losses during low speed centrifugation. Extract clearing did not influence the yield of PPV.

We observed, similarly as SCHADE (1969) did that PPV has relatively poor antigen properties. DOUNINE and MINOIU (1968) and KRÖLL (1973) were successful in stimulating antibody formation with vitamin B 12 and when mixing antigen with bactobentonite. Vitamin B 12 was not at our disposal and for this reason we could not verify their finding.

Serological diffusion method in agar has great advantages for the detection of PPV in plum and apricot leaves over biological methods. Plum and apricot leaves can be tested with this method during the entire vegetation period. We also successfully tested those leaves which had been kept one week in a refrigerator prior to homogenization as well as frozen leaves, which is in agreement with the results obtained by KRÖLL (1973) and CASPER (1973, 1975). We further established, similarly as CASPER (personal communication), that when the distance between the holes was shorter than 5 mm, no precipitation ring was formed. The disadvantage of this method is that the virus can not be demonstrated in the leaves of diseased plum trees which are situated on symptomless shoots. Virus concentration may be too low in them or the shoots may be free of the virus; virus-free shoots can occur on diseased trees (TRIFONOV 1969). For this reason it is necessary to test the trees in subsequent years.

The presence of PPV can also be determined in leaves with atypical symptoms, where plum pox disease can be camouflaged by other virus diseases or by damage caused to the leaves by the treatment with some pesticide. Thus serological diagnosis is of major importance in those trees and rootstocks, in which visual diagnosis of plum pox disease is difficult or impossible.

Acknowledgement

The authors wish to thank Dr. R. Casper for valuable advice on PPV problematics, for supplying them with PPV isolates and antisera and for other material help; Dr. D. Trifonov for sending them scions and PPV isolates from Bulgaria and Prof. Dr. D. Šutić for sending them PPV isolates from Jugoslavia. The skilled technical assistance of Mrs. Jitka Pívalová is also gratefully acknowledged.

References

ALBRECHTOVÁ, L.: Untersuchungen über die Anwendung von Good-Puffern in der Pflanzenvirologie. — Arch. Phytopathol. Pflanzenschutz (in press, 1976).

BABOVIĆ, M., ŠUTIĆ, D., BODE, O.: Purification and some properties of sharka virus. — Ann. Phytopathol. **192** : 203—209, 1971.

CASPER, R.: Serologische Untersuchungen über das plum pox virus (Scharka-Virus). — Jahresber. Biol. Bundesanst. Land. Forstwirtschaft. P. 59, 1972.

CASPER, R.: Serologischer Routinennachweis von plum pox virus (Scharka-Virus). — Jahresber. Biol. Bundesanst. Land. Forstwirtsch. P. 81, 1973.

CASPER, R.: Serodiagnosis of plum pox virus. — Acta Hort. **44** : 171—172, 1975.

DOUNINE, M. S., MINOIU, N.: On serodiagnosis of plum pox virus. — Tagungsber. DAL (Berlin) **97** : 259—267, 1968.

GILLS, C. C.: Purification of oat necrotic mottle virus with silver nitrate as clarifying agent. — J. gen. Virol. **12** : 259—270, 1971.

GYÖRGY, B., NÉMETH, M.: Serologische Diagnose des Scharka-Virus an Aprikosen. — Tagungsber. DAL (Berlin) **97** : 269—271, 1968.

KEGLER, H., OPEL, H.: Ein verbessertes Verfahren zum Nachweis von Ringfleckenviren der Kirsche mit krautigen Testpflanzen. — Thaer Arch. **7** : 237—244, 1963.

KRÖLL, J.: Versuche zum serologischen Nachweis des Scharka-Virus der Pflaume. — Arch. Phytopathol. Pflanzenschutz **9** : 415—416, 1973.

KRÖLL, J.: Versuche zum Nachweis des Scharka-Virus in den Früchten, der Rinde und den Wurzeln von *Prunus domestica* L. — Arch. Phytopathol. Pflanzenschutz **10** : 349—350, 1974.

OOSTEN, H. J. VAN: The isolation of sharka (plum pox) virus from leaves and fruits of plum with herbaceous plants. — Neth. J. Plant Pathol. **76** : 99—103, 1970.

OOSTEN, H. J. VAN: Purification of plum pox virus with use of Triton X-100. — Neth. J. Plant Pathol. **78** : 33—44, 1972.

PAULECHOVÁ, K.: [Mechanical transmission of plum pox virus.] In Czech. — In: Sbor. věd. prací z V. celost. konf. o ochr. rostl. Pp. 215—218. Brno 1974.

POLÁK, J., ALBRECHTOVÁ, L., CHOD, J., JOKEŠ, M.: Partielle Reinigung des Kartoffel Y-Virus zur Antiserumherstellung. — Arch. Phytopathol. Pflanzenschutz **11** : 179—188, 1975.

RANKOVIĆ, M., JORDOVIĆ, M.: Jedan pogodan metod za prečišćavanje virusa šarke. [A suitable method for purification of Sharka virus.] In Croat. — Zaštita Bilja **109** : 195—199, 1970.

SCHADE, C.: Nachweis von Viren in Pflaumenbäumen zu verschiedenen Jahreszeiten. — Tagungsber. DAL (Berlin) **97** : 283—288, 1968.

SCHADE, C.: Eigenschaften und Serologie des Scharkavirus der Pflaume. — Zentralbl. Bakt Abt. II, **123** : 299—304, 1969.

SHEPARD, J. F.: Gel-diffusion methods for the serological detection of potato viruses X, S and M. — Bull. 662, Montana Agr. Exp. Stat. Montana State Univ. Bozeman, USA 1972.

ŠUTIĆ, D., JORDOVIĆ, M., RANKOVIĆ, M., FESTIĆ, H.: Comparative studies of some sharka (plum pox) virus isolates. — Ann. Phytopathol. **3** : 185—192, 1971.

TRIFONOV, D.: Lokalisierung und Wanderung des Scharka Virus (plum pox virus) in Pflaumenbäumen. — Zentralbl. Bakt. Abt. II, **123** : 340—347, 1969.

UYEMOTO, J. K., PROVIDENTI, R., SCHROEDER, W. T.: Serological relationship and detection of bean common and b ean yellow mosaic viruses in agar gel. — Ann. appl. Biol. **71** : 235—242, 1972.

Figures at the end of the issue

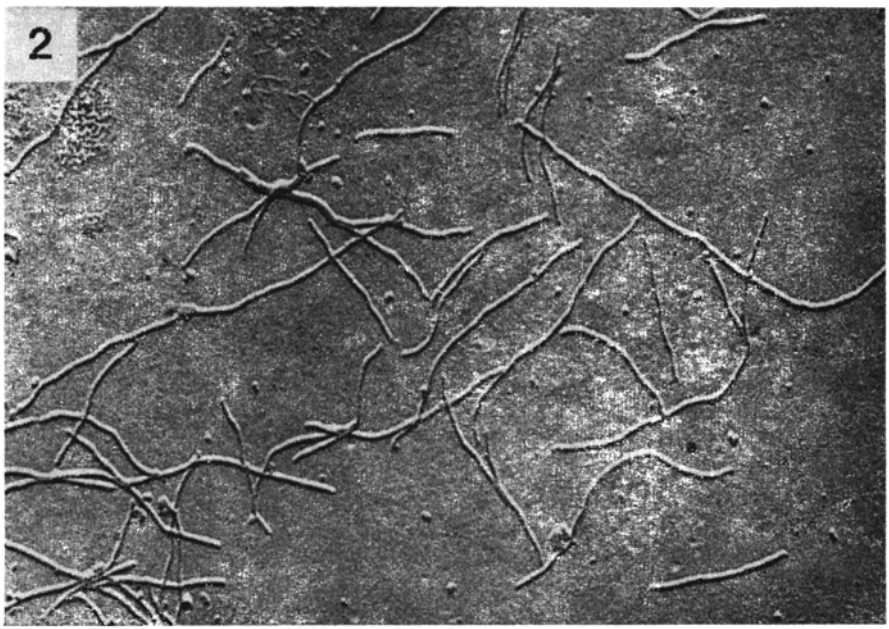

Fig. 1. Plum pox virus particles prepared according to GILLS with 0.5 M urea; shorter particles slightly degraded; total magn. 25 400×.
Fig. 2. Plum pox virus particles prepared by the method of VAN OOSTEN, modified by CASPER, with HEPES buffer. Undamaged particles. Total magn. 25 400×.