Purification and Properties of Arabis Mosaic and Tomato Bushy Stunt Viruses Isolated from Lilac (Syringa vulgaris L.)

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Abstract. The paper gives more detailed characteristics of Arabis mosaic virus (AMV) and tomato bushy stunt virus (TBSV) isolated from lilac, the latter being identified in lilac (from plants suffering from yellow ring disease) for the first time. The isolate of TBSV from lilac, from which an antiserum with a titre of 1024 was prepared, is closely related to the artichoke strain. Information is given about two types of ringspot disease and about chlorotic ringspot of lilac. Whereas in the leaves of lilac suffering from ringspot disease (of ring mosaic type) the presence of AMV was demonstrated, the sap transmission from the leaves diseased with ring spot of line-pattern (and wave-like mosaic) type failed; from the leaves affected by chlorotic ringspot a mixture of AMV and cherry leaf roll virus was identified. In addition, the polyetiological nature of "spring" mosaic and necrotic mosaic of lilac, in which bacterium Pseudomonas syringae van Hall was found is dealt with. The TBSV was also identified in the isolate of necrotic mosaic.

Additional index words: Lilac ringspot, chlorotic ringspot, yellow ring, "spring" mosaic, necrotic mosaic, cherry leaf roll virus, Pseudomonas syringae van Hall.

The present paper is a continuation of a previous contribution dealing with the symptoms of some virus diseases of lilac occurring in Czechoslovakia, as well as with some sap inoculation experiments and transmission tests by means of grafting and by aphids (NOVÁK 1958, 1966, 1969) and giving a preliminary report on the isolation and serological detection of Arabis mosaic virus (AMV) and cherry leaf roll virus (CLRV) — NOVÁK and LANZOVÁ 1974, 1975a. In 1972—1976 further studies were aimed at explaining the causal agent of the yellow ring disease, which was found for the first time in 1952 and in the next two years was transmitted by grafting to a few lilac seedlings. Moreover, two other lilac diseases hitherto undescribed in Czechoslovakia, which seemed to be partially of bacterial origin, were studied. Whereas one of these, which, due to its occurrence, was given the name of "spring" mosaic, was observed for the first time in 1951, the second disease, tentatively named necrotic mosaic, was ascertained in 1973. The attempts to transmit "spring" mosaic by grafting were carried out in the years 1952—1954. Since the time when further research of lilac viruses had to be interrupted, only limited experiments with their transmission to lilac and to other members of the Oleaceae family have been performed (NOVÁK

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1958). In spite of that a systematic observation of the way of spreading individual diseases in their original localities has continued.

Material and Methods

Ringspot and Chlorotic Ringspot

The virus was isolated from a greater number of various plants several times in the course of the experimental years in various growing seasons, from the young leaves as well as from the buds and flowers. The plants with most typical symptoms were used as source plants for virus assaying.

In our experiments with ringspot disease, two lilac shrubs, each of which showed slightly different symptoms, were used as source plants. One shrub ("Syr 2") was affected by ringspot of line-pattern type or wave-like mosaic, the second ("Syr 8") showed yellow rings of different size, of which some were linked to the main or lateral vein (Fig. 3). Neither of the source plants used showed symptoms of both types on any leaf. For examination of chlorotic ringspot a source plant ("Syr 1") with symptoms of yellow oak-like pattern, which are connected with chlorosis of a part of the blade inside the pattern, was used (Fig. 4).

For isolation of the virus Chenopodium quinoa, C. amaranticolor, Nicotiana tabacum ev. White Burley, Petunia hybrida and Cucumis sativus cv. Gele Tros were used. Inoculum was prepared by homogenizing plant material in a mortar by application of a stabilization solution according to Kegler and Opel (1963) or of 1% solution of nicotine. Mechanical inoculation of plants was performed using the carborundum method. For maintaining isolates, Chenopodium amaranticolor, Nicotiana clevelandii and Cucumis sativus were used. The host range was studied on 40 herbaceous test plants. To confirm the systemic reaction on single hosts the back inoculation tests on C. quinoa or N. tabacum cv. White Burley were made.

Stability of the virus in crude sap was assayed after heating for 10 min, storage in vitro and dilution of the sap by means of the usual methods on C. quinoa. Electron-optical investigation was made on samples negatively stained with 1% potassium phosphotungstate pH 6.5 in water. The electron microscope TESLA BS 513 was used for the examination of specimens.

The biological properties found, the failure of experimental transmission of the disease by aphids (Novák 1966) and the absence of elongated particles under the electron microscope resembled the features ascertained in some of the viruses of the nepovirus group. That's why for the demonstration of the presumed identity, standard antisera against the following viruses were used for serological detection: AMV, CLRV, tomato black ring, strawberry latent ringspot and raspberry ringspot viruses. The antisera to AMV were supplied by B. D. Harrison (Dundee) and D. Z. Maat (Wageningen), to CLRV from Sambucus nigra by Z. Stefanac (Zagreb) and from Cerasus avium ("Eckelrade 216" isolate) and to the other above-mentioned viruses by D. Z. Maat. In addition, also cucumber mosaic virus antisera (supplied by D. H. M. van Slogteren, Lisse), antisera against tobacco necrosis virus — serotype A (supplied by G. H. Gibbs, Rothamsted) and antisera to CLRV prepared from Sambucus nigra (isolate San 1) by the authors. The Ouchterlony double-diffusion test using 1% agar Difco Noble in 0.85% saline with addition of 0.02 sodium azide was applied for all serological detection tests.
With respect to the results of previous examinations, the virus purification was made from *Nicotiana clevelandii* plants by using Steere's butanol/chloroform method in modification described by Harrison and Nixon (1960), which was only slightly modified by us according to our possibilities. Differential and density gradient centrifugation was made on a SPINCO ultracentrifuge, using L 50 and SW 25.1 rotors, respectively.

For antiserum preparation two rabbits were immunized with purified virus preparations. At an interval of one week each of the rabbits were given three intramuscular injections with an emulsion containing 1 ml of virus suspension and 1 ml of Freund's incomplete adjuvant. These antigen rations were supplemented by intravenous injections applied in an amount of 1 ml per 24 h after application of the second and third intramuscular injections. The antisera obtained were preserved with sodium azide. Their reaction with purified antigen as well as with crude sap from different plant hosts was proved and compared with the reaction of standard antisera (with the same antigens).

**Lilac Yellow Ring**

In isolating the virus and in the study of its biological properties the same methods and material as described above were used. The back inoculation tests were made on *Chenopodium quinoa*. Due to the results of preliminary diagnostic tests and to the ascertained biological properties of the isolate, distinct from those of the viruses of the nepovirus group, antisera against the following viruses (besides formerly used antisera) were used for serological detection: tobacco necrosis virus (bean isolate and *Lonicera* isolate supplied by D. Z. Maat), and tomato bushy stunt virus (TBSV), petunia and artichoke strains, supplied by G. P. Martelli (Bari).

The *Chenopodium quinoa* plants grown under artificial light at 16—24 °C were used for virus purification. The frozen leaves from infected plants with severe disease symptoms after refrigeration were homogenized in a laboratory blender with an equal volume of distilled water. The homogenate was pressed through cheesecloth and after another addition of the same amount of distilled water the pH value of the sap was adjusted to pH 5.0 and the extract was centrifuged for 10 min at 10 000 rpm. The resulting supernatant was decanted and centrifuged in a TI 50 rotor of SPINCO ultracentrifuge for 120 min at 40 000 rpm. The pellets containing virus were resuspended in 0.05 M phosphate buffer pH 7.2 in about 1/10 of the original volume — and equal volumes of suspension were layered on 10, 20, 30, 40 and 50 % sucrose gradients in 0.05 M phosphate buffer pH 7.2 and centrifuged in a SW 25.1 rotor of SPINCO ultracentrifuge at 25 000 rpm for 150 min. The gradient tubes were then punctured and the content was collected dropwise into fractions of 1 ml. The absorbancy of each fraction was then measured on a VSU2 spectrophotometer at 260 nm wave-length. Virus samples in resulting gradient fractions were bioassayed after the remaining sucrose was removed by dialysis and checked for purity (and particle type) with an electron microscope. Then decanted T and B fractions were used for the immunisation of rabbits by the above-described method.

**"Spring" Mosaic and Necrotic Mosaic of Lilac**

The first step in the diagnosis of these two diseases was to confirm the opinion that bacteria participate in the origin and development of the disease
and, at the same time, to isolate from diseased plants the viruses which are also potential causal agents of these diseases. The identification of isolated pathogens and, if need be, the nature of their mutual relationships was the next program of this investigation.

The isolated bacteria were identified serologically after demonstration of their infectivity by means of the hypersensitivity response on tobacco plants after KLEMENT (1963). The bacteria *Pseudomonas syringae*, which can be considered as practically the only possible bacterial pathogen of lilac and which in some years also occur frequently in our country were serologically detected by means of a specific antiserum, which was prepared by the authors (Novák et al. 1976).

### Results

#### Ringspot Disease

Whereas from the “Syr 2” source plant (suffering from ringspot of line-pattern and wave-like type) the transmission by means of mechanical inoculation failed even in spite of repeated isolation, the transmission from the “Syr 8” source plant (with symptoms of ring mosaic type) was successful even in the first inoculation experiment. Symptoms produced on herbaceous host plants as well as the ascertained stability of the virus in expressed sap (TIP — 56-58 °C, DEP — 10⁻⁴, LIV — 12 days) resembled those of the nepoviruses. By means of serological tests, the AMV was demonstrated in various test plants with both available antisera against AMV.

As the highest concentration of the virus was found in systemically diseased plants of *Nicotiana clevelandii*, these host plants were used for
puriﬁcation of the virus. AMV preparations puriﬁed by density gradient centrifuging showed typical UV absorption spectrum. The distribution of proteins in sucrose gradients is shown in the record curve (Fig. 1). Decanted bottom fractions (11—15) and top fractions (16—17) were separately injected, each into one rabbit. The antiserum prepared had a titre of 64 and 128, respectively. In the Ouchterlony double-diﬀusion tests they reacted with a single precipitin band with puriﬁed preparations as well as with crude extracts from infected *Nicotiana clevelandii* and also from other tested plants.

**Chlorotic Ringspot**

Although in lilac this disease originating from “Syr 1” source plant showed symptoms diﬀering signiﬁcantly enough from ringspot disease (“Syr 8”), the symptoms on herbaceous indicators resulting from sap inoculation experiments were almost the same in both isolates, and diﬀered mainly only in manifested symptom intensity, which was more severe in the chlorotic ringspot. Also, the stability of the virus found in crude sap (TIP — 54–56°C, DEP — 10^-4, LIV — 10 days) did not suggest the presence of a virus too dissimilar in its biological properties.

In serological detection tests of this isolate CLRV was found besides AMV. Owing to this fact the collection of investigated indicators was supplemented with *Chenopodium capitatum* and *Vicia faba*, which are diﬀerential hosts of these viruses (KEGLER et al. 1966). On elimination of individual viruses from the mixture, another serological detection was performed with all tested antisera. In the sap of broad bean plants, in which symptomless CLRV was present, positive reactions with CLRV antisera prepared from *Sambucus nigra* (of Yugoslav and our own origin) were demonstrated, but not with the antisera from cherry (of Dutch origin). Other viruses than those which corresponded to the respective diﬀerential hosts were not found.

**Lilae Yellow Ring**

The symptoms of this disease, which was tentatively named as yellow ring, were very diﬀerent from those evoked by ringspot and chlorotic ringspot. They were characterized by roundish spots of various sizes ranging from light green to yellow, at ﬁrst diﬀused (Fig. 5a), later with bright yellow or necrotic borders, sometimes with pale green roundish areas with yellow borders (Fig. 5b).

The symptoms on herbaceous indicators produced by this virus also diﬀered signiﬁcantly from those evoked by ringspot and chlorotic ringspot. On assayed hosts they were mostly only local and as far as they manifested themselves by systemic reactions they were found, as a rule, only in a small or very small proportion of plants. Thus *e.g.* in *C. quinoa* a systemic reaction was proved by back inoculation tests only in 5 tests (on 11 plants of 24 plants tested) of 107 tests (364 plants). A small proportion of systemic infection (about 16 %) was also found in cucumber, on which mostly only local spots in cotyledons were observed. The hosts on which systemic infection was more frequent, often showed only very slight systemic symptoms that easily escaped attention (*e.g.* *Lycopersicum esculentum, Spinacia oleracea, Atriplex hortensis*), or they were completely symptomless.
A further characteristic of the isolate from yellow ring lilac plants ("Syr 5") were the reactions on some specimens of Chenopodium, which were very different from the symptoms caused by AMV and CLRV. Whereas the isolates of these two viruses on C. quinoa were characterized by a systemic mottle and top wilting, or, in some cases, by dying of whole plants from the top, the isolate "Syr 5" caused in the course of 4--6 days necrotic lesions on inoculated leaves, which gradually died in the central or bottom leaf floor. In places where hanging dead or wilted leaves were connected with the stem, more or less extended necroses of the stem were formed (after 20 to 26 days), which in the course of time broke down. Young plants, as a rule, died completely from the base in a short time. Similar symptoms were observed also on C. foetidum and C. murale; in the latter, however, necroses developed more slowly. In C. amaranticolor, in which top necrosis also sometimes occurred, the reaction was not so typical.

Distinctly developed systemic reactions, which arose almost regularly were observed only on Nicotiana clevelandii, N. megalosiphon and Celosia plumosa plants.

By assaying the stability of the virus in expressed sap, which was repeated 5 times at various periods, the following values were obtained: TIP — 84-85 °C, DEP 10^-4, LIV 32 days (a longer time of storage was not tested). In these properties, as well as in the nature of reaction on herbaceous indicators, isolate "Syr 5" resembled the TBSV.

In serological tests, in which N. megalosiphon and Celosia plumosa were used, a strong positive reaction (in titre 1 : 512) with the antiserum of the artichoke crinkled mottle strain of TBSV was obtained, whereas the reaction with the antiserum against Petunia asteroid mosaic strain failed. No other antiserum used for detection reacted with the tested antigens.

Purification. Owing to a high amount of virus suspension all three tubes of the rotor with sucrose density gradients could be filled right after the first ultracentrifuging. After one cycle of sucrose-gradient centrifuging all three preparations showed typical UV absorption spectrum and practically did not differ from one another. The distribution of proteins and the separation of T and B components in sucrose gradients is shown on the record curve (Fig. 2). After control of their biological activity, these fractions were decanted and used for the immunization of two rabbits. The titre of both prepared antisera was 1024. In the Ouchterlony double-diffusion test they reacted with a single precipitin band with the purified preparation of the virus as well as with crude extracts from infected N. megalosiphon and Celosia plumosa plants.

"Spring" Mosaic and Necrotic Mosaic of Lilac

From the leaves of lilac shrubs affected by "spring" mosaic (Fig. 6) as well as from those attacked by necrotic mosaic, in both of which the viruses were confirmed by means of sap inoculation tests, the bacteria Pseudomonas syringae van Hall were also isolated.

The results of transmission tests by grafting were positive with both these diseases. After grafting diseased scions (originating from shrubs affected by the first or by the second disease) on lilac root-stocks, the symptoms corresponding to the respective disease were obtained. Using the method of chip-budding, however, the results of transmission tests were in some cases
significantly different from those in which the method of grafting was used, while the transmission of bacteria failed.

In the case of "spring" mosaic, the symptoms resulting from chip-budding experiments somewhat resembled the disease symptoms which were described earlier under the name of streak mosaic (ŠMOLÁK and NOVÁK 1950). The virus isolated from these streak-mosaic plants by means of sap inoculation, has not been identified so far. The symptoms of necrotic mosaic observed on root-stocks after chip-budding tests were similar to the symptoms of yellow ring disease. The TBSV was isolated from their leaves and serologically identified.

Discussion

The two types of lilac ringspot mentioned in this paper were originally considered as identical, on the basis of experiments in which after grafting of scions of individual ringspot types on parallel root-stocks (lilac seedlings and various cultivars), the same or very similar results were obtained as to symptoms (NOVÁK 1966). The sap inoculation tests with these two types of ringspot however showed their different etiology. The results of our experiments showing that the line-pattern and wave-like type of ringspot (which is probably the most common virus disease of lilac in the world) is not transmissible by sap, correspond fully with the results of SCHMELZER (1970) and VAN DER MEER (1976). In a few of these experiments symptoms were observed on the root-stock, similar to those of lilac ring mottle virus, which has recently been identified as new virus of the ILAR virus group (VAN DER MEER et al. 1976, HUTTINGA and MOSCH 1976). Our demonstration of AMV in lilac plants diseased with the second type of ringspot, which was preliminarily reported by NOVÁK and LANZOVÁ (1975a), was confirmed in a recent paper by VAN DER MEER (1976); hitherto the AMV has been known in lilac only together with the tomato black ring virus (SCHMELZER 1970).

The identification of TBSV in lilac is probably the first evidence that the lilac is a new natural host of this virus. The TBSV was isolated from plants suffering from a disease which was tentatively named as yellow ring and which was similar to the disease described in Poland by KOCHMAN et al. (1964). According to our experiments the isolate of TBSV from lilac is closely related to the artichoke strain of this virus (MARTELLI et al. 1971). On the other hand, our isolates from cherry and plum were ascertained as a complex of artichoke and Petunia strains (NOVÁK and LANZOVÁ 1977), whereas our isolates of this virus from grapevine and hop were related to the Petunia strain (NOVÁK and LANZOVÁ 1976). These findings, as well as the foregoing demonstration of TBSV in cherries (ALBRECHTOVÁ et al. 1975, NOVÁK and LANZOVÁ 1975b) and the evidenced Pelargonium strain of TBSV isolated in our country from Pelargonium zonale (CHÔD et al. 1974) testify that TBSV is of great economic importance in Czechoslovakia.

According to the results of our experiments with lilac "spring" mosaic and lilac necrotic mosaic it is not possible, for the time being, to decide whether the primary pathogen is a virus or a bacterium. In respect of the observed syndrome, which significantly differs from diseases due to only one of these pathogens, it can be believed that the disease is evoked by both pathogens. Similar results were obtained in the study of etiology of cherry detrimental canker, whose development is influenced by both TBSV and the bacterium Pseudomonas syringae VAN HALL. (NOVÁK and LANZOVÁ 1975b).
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References


Figures at the end of the issue.
Fig. 5. Elongated mycoplasma-like bodies and some spherical forms bounded by a smooth unit-membrane and containing ribosome-like granules and deoxyribonucleic acid-like strands. (70 400 ×; 110 000 ×).

Fig. 6. A crystal (C) lying in a microbody surrounded by a membrane (M) in the protoplasm of phloem parenchyma cell of Solanum laciniatum Arr. plant infected with potato witches’ broom; the crystal is about 750—800 nm long and has a lattice spacing of about 12 nm (CW cell wall). (48 000 ×).
Fig. 3. Ringspot of ring mosaic type of lilac from which AMV was isolated.
Fig. 4. Symptoms of chlorotic ringspot of lilac from which AMV and CLRV were isolated.
Fig. 5. Lilac yellow ring from which TBSV was isolated. Symptoms on young leaves (5a) and on older leaves (5b).
Fig. 6. Symptoms of lilac "spring" mosaic, in which Pseudomonas syringae participates.
Figs. 1 to 6: The analysis of the antigens of the individual species by means of immunoelectrophoresis in agarose-starch gel. In Figs. 1, 2, 3 — detection with the antiserum against albumin fraction from cotyledons. In Figs. 4, 5, 6 — detection with the antiserum against globulin fraction. Sample specification in Figs. 1 to 6:

In Figs. 1 and 4: Ph. caracalla and the group of American endemics: A — Ph. caracalla; B — Ph. acutifolius; C — Ph. coccineus; D — Ph. lunatus; E — Ph. vulgaris ssp. aborigineus; F — Ph. vulgaris ssp. vulgaris.