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OCCURRENCE OF PEA ENATION MOSAIC VIRUS IN YUGOSLAVIA

DAVOR MILIČIĆ, RUŽICA LEŠIĆ, ĐORĐE MAMULA, NADA PLEŠE, and VLADIMIR HRŠAK

(Institute of Botany, University of Zagreb, and Faculty of Agriculture, University of Zagreb)

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Introduction

Pea enation mosaic virus (PEMV) spreads over all parts of the northern temperate region. According to the data of Quantz (1968), this virus is present in countries adjacent to Yugoslavia, e. g. in Hungary, Italy and Austria, but it has not been previously found in Yugoslavia.

The PEMV is easily transmissible mechanically and by means of some insects belonging to the group of *Aphidae*, especially by *Acyrtosiphon pisum* and *Myzus persicae*. The insects transmit the PEMV in a persistent manner, but most of them cease to transmit it after several days. It is not quite certain that the virus can multiply in insects (Shepherd 1971).

The PEMV particles are polyhedral, and there are two different kinds of particles. Both sorts of particles have a diameter of about 29 nm. The larger particles are settled at 112 S and the smaller ones at 99 S. They have a different quantity of RNA; the molecular weight of the larger component is 1.6×10^6 and of the smaller one 1.3×10^6 (Hull and Lane 1973). The capsid of the larger component is built of 180 protein subunits and of the smaller one of 150 subunits. The presence of both components is necessary for the beginning of the infection process.

The PEMV provokes a very common disease of pea and broad bean. A very strong mosaic and transparent spots appear on infected plants, especially on peas. The virus name derives from enations which arise on infected leguminous plants in the later stages of infection. The enations are ordinarily not visible on plants growing in fields, but they are regularly present on plants cultivated in a glasshouse. Higher temperatures in the glasshouse have perhaps a positive influence on the appearance of enations. They have the character of outgrowths formed in the region of veins and placed on the lower leaf side.

Material and Methods

The investigations were performed with two Yugoslav PEMV isolates derived from pea. The first isolate G1 was obtained from pea plants growing on the experimental field of the Faculty of Agriculture in Zagreb (Maksimir). The second isolate G2 was acquired from a private garden in Delnice (Yugoslavia). In order to compare them with Yugoslav isolates we obtained from abroad the wild type of this virus and the isolate P3. Dr. G. Adam (Tübingen) kindly sent us the foreign strains and the serum against PEMV through Dr. Z. Štefanac (Zagreb). An other serum against PEMV was received from Prof. G. P. Martelli (Bari). We are indebted very much to all the mentioned researchers.

Purification of the isolate G2 was performed according to a modified method of Hull and Lane (1973).

The spectrophotometric analysis of the partially purified virus suspension was performed with the Model Varian-Techtron 635 D spectrophotometer.

The antiserum against the G2 isolate of PEMV was prepared so that a rabbit was injected four times in intervals of two to three days. All together four intravenous injections were given (I 0,5 mg, II 0.9 mg, III 0.5 mg, IV 0.25 mg) with the total quantity of 2.15 mg of virus. The rabbit was bled four weeks after the first injection.

The serological investigations were performed by means of double diffusion tests in agar-gel.

Results

Finding places

Only two finding places of PEMV in Yugoslavia have been found till now, i. e. Zagreb and Delnice. The isolate G1 from Zagreb was cultivated in *Vicia faba* during the winter. This isolate was obtained from pea cultivar Stern. Bright spots, which were transparent and very prominent on this cultivar, were situated especially in the leaf vein region (fig. 1A). These symptoms caused the effect of a very distinct mosaic on account of which this disease got its German name "scharfes Mosaik". Moreover, the leaves were deformed and the internodes shorter than in healthy pea plants. The pods were small and usually curved. This isolate was conserved according to a modified method of McKinney (1947) and was preserved in a refrigerator. A whole year this material has remained infective and served as source of the virus.

Fig. 1. *Pisum sativum* with symptoms of pea enation mosaic virus. A. Strong mosaic on stipules. B — D. Enations on the underside of stipules.

Fig. 2. A. Lesions on the inoculated broad bean leaf after infection with pea enation mosaic virus. C. Curve showing the UV absorption of G2 virus suspension. B. Serological reaction performed with the method of double diffusion in agar gel. The antiserum against PEMV was put in the central basin. Infected plant sap was placed in three upper basins (V), and the healthy control sap in lower basins (H). The reaction appeared only between the upper basins and the basin with PEMV antiserum showing that infected plant contained virus particles. D. Electron micrograph of PEMV particles. Bar represents 150 nm.

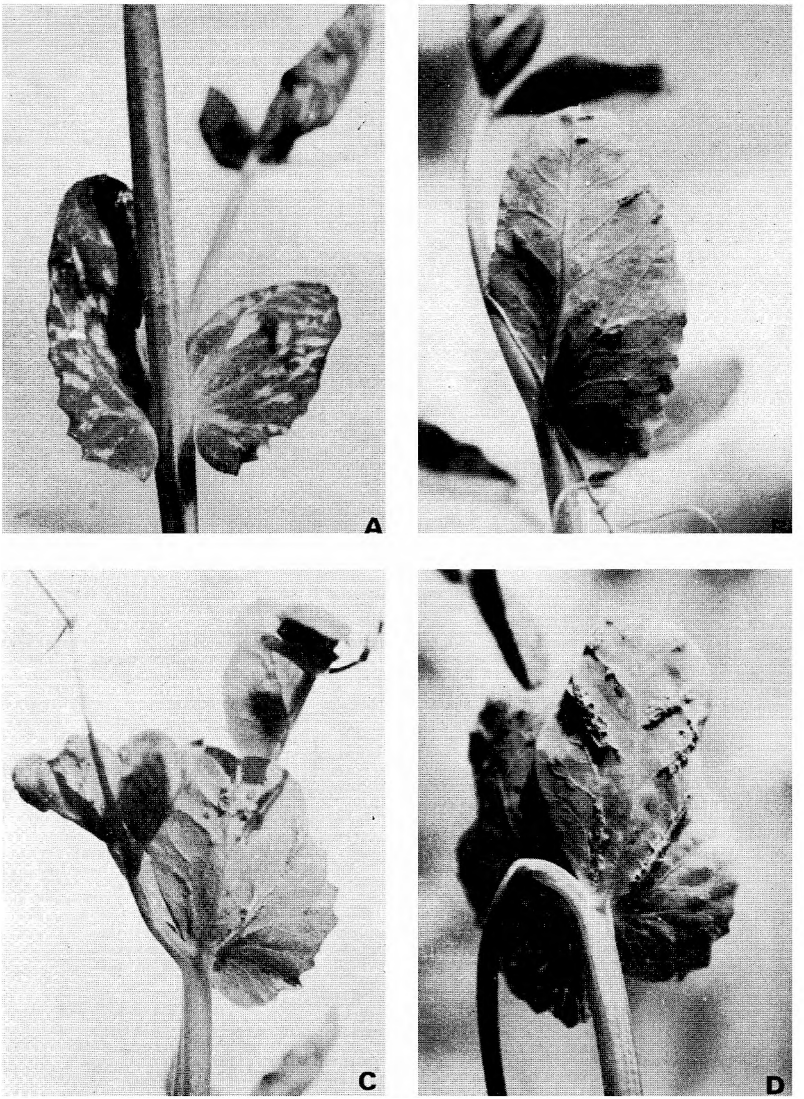
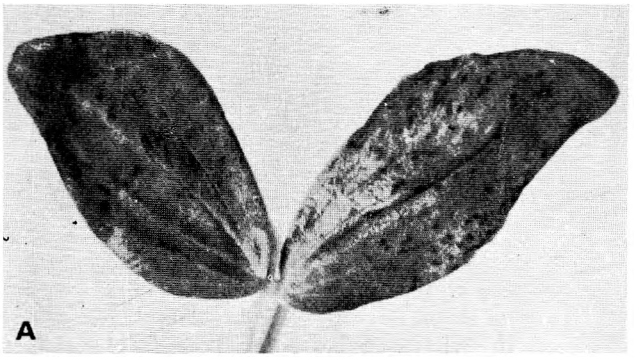
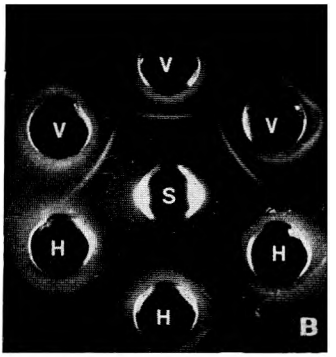
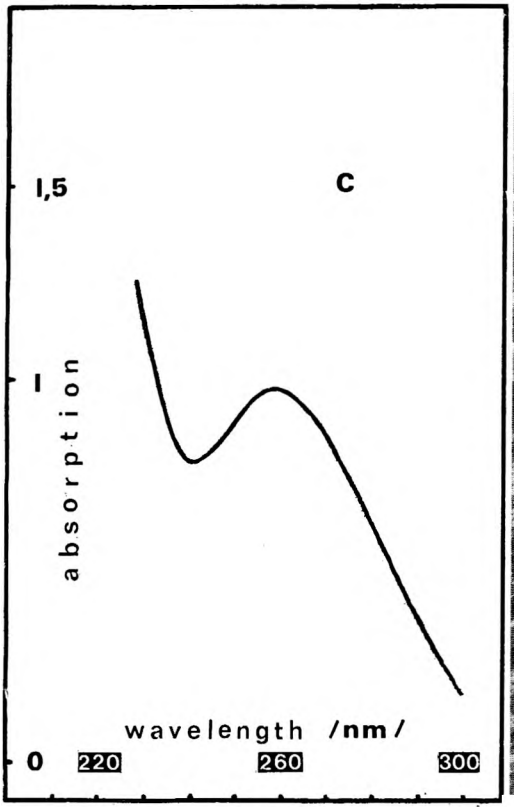


Fig. 1.







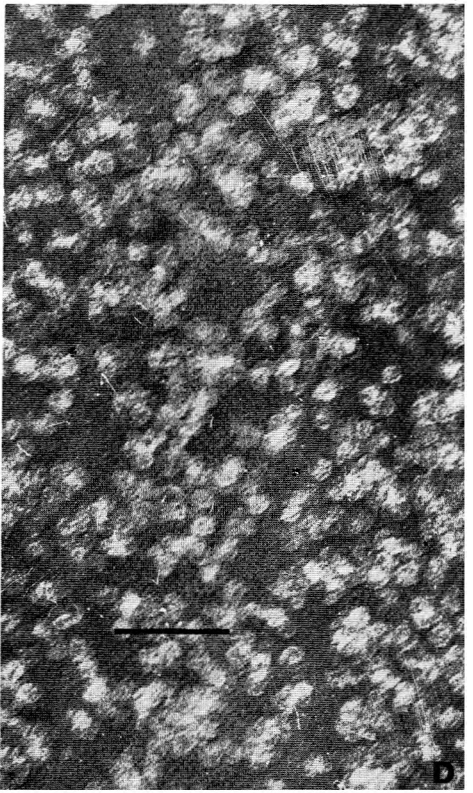


Fig. 2.

Symptoms on herbaceous plants

Symptoms of G1 and G2 on broad bean (*Vicia faba*) were weak in the glasshouse during the winter. However, in spring the symptoms were stronger, probably under the influence of higher temperature. Sometimes local lesions appeared in form of brown spots on inoculated leaves (fig. 2A). Systemic symptoms in form of elongated and transparent spots arose on some leaves approximately 20 days after inoculation. On the same leaves it was possible to find characteristic enations but their number was not great.

The sequence of symptoms was specially interesting on pea plants cultivated in a glasshouse. The first symptoms were observed in form of vein clearing and mild mottling on leaves placed above the inoculated leaves approximately 6 days after inoculation. The next symptom form was revealed about a fortnight after inoculation. The leaves were slightly deformed and had a large number of round and elongated spots, which were sometimes transparent (fig. 1A). At this time the enations were not yet present although sometimes small tissue proliferations were visible on places where usually enations arise. New basal shoots appeared together with very strong symptoms about 20 days after inoculation. The new leaves were deformed and epinastic curved, and the enations grew out from the lower leaf surfaces. They were often present in a large number nearly on every leaflet (fig. 1B-D).

As the enations were very characteristic for this virus, it is useful to stress that the enations were very rare on infected peas cultivated in open fields. We have got the impression that the symptoms in the field appear slower on account of lower temperatures. It is obvious that the high temperature intensifies the symptoms in the glasshouse and makes also the good development of enations possible.

Chenopodium amaranticolor is also an important test plant for PEMV. On that plant appear chlorotic local lesions in form of very small spots. When the weather is favourable the lesions become red and more obvious.

Some authors stress that *Chenopodium quinoa* is a suitable plant for quantitative experiments with PEMV (H a g e d o r n et al. 1964). On the contrary, our specimens of this species were not favourable for such investigations.

Purification of PEMV

Pea leaves were homogenized in a mortar with the same quantity of 0.2 M sodium acetate buffer, pH 6, which contained 0.1 M of ascorbic acid. The total amount of the homogenate obtained was 50 ml. To this solution 50 ml of chloroform and butanol in proportion 1 : 1 was added and then the fluid was shaken during 3 min at 4° C. Afterwards this suspension was centrifuged 6 min at low speed by means of a swing out bucket rotor. Then the aqueous phase was centrifuged during 5 min at 5,000 rev./min using an angle rotor. The supernatant was further centrifuged 90 min at 30,000 rev./min again employing an angle rotor. The sediment was dissolved in 17 ml of 0.1 M acetate buffer pH 6 which contained 5 % sucrose and 0.1 M ascorbic acid.

This suspension was centrifuged during 5 min at 5,000 rev./min but the sediment did not appear. Therefore, the supernatant was twice centrifuged during 6 min at 12,000 rev./min. At the end, the last supernatant

was centrifuged during 90 min at 36,000 rev./min using also an angle rotor. In such a way rich virus sediments were got. One part of this sediment was dissolved in 0.33 ml of distilled water, and the other in 0.33 ml of 0.05 M acetate buffer pH 6.0. These virus suspensions represented partially purified virus and were used for further experiments (fig. 2 D).

Spectrophotometric investigations

It was found that the UV light absorption of the virus suspension was characteristic of nucleoprotein (fig. 2 C), and the relation E 260/280 was 1.61. As it is known, the absorbance of virus suspension which has the concentration 1 mg/ml and the thickness 1 cm is 7.5 (Shepherd 1970). It was also found on the basis of the mentioned data that the concentration of the virus particles in plant sap was about 0.3 mg/ml. This concentration corresponds to the data in the literature (Shepherd 1970).

Serological experiments

The first serological tests were made at the end of spring with the aim to identify our isolates G1 and G2. On this occasion these isolates positively reacted with the German serum against PEMV. Between the virus and antiserum basins a strong precipitation line was formed (fig. 2 B). On the other hand the line did not appear between the control basin filled with healthy sap and the antiserum basin. In this experiment it was ascertained that our isolates belonged to the PEMV. The serum titre with G1 isolate was 1/4.

During the following winter it was found that the newly prepared serum contained antibodies against the homologous virus. The homologous virus (G2) and the newly prepared antiserum were put in two basins, and after a while it was established that precipitation lines appeared between the basins. One line was very distinct and it belonged to the virus, and the other one was weak and it belonged to a virus protein. This conclusion was reached because the first line was placed close to the virus basin which made it clear that it was provoked by a protein of great molecular weight, i. e. by virus particles. The second line was placed far from the virus basin which showed that it was caused by a small protein able to diffuse faster. However, both lines were provoked by virus because both lines were absent in control tests performed with healthy sap.

Besides that it was established that both the German serum against PEMV and the G2 antiserum reacted in agar-gel with G2 virus. On this occasion, also two precipitation lines appeared, i. e. the virus line and the small protein one. Both the corresponding virus and small protein precipitation lines coalesced with one another. It seems therefore that not only the viruses are related but their small proteins too.

It is interesting to note that during the first experiment performed in spring only one precipitation line was visible.

A latent pea virus

The pea cultivar Stern from which the isolate G1 was obtained contained yet another virus tentatively named latent pea virus. Its name resulted from the fact that it did not produce obvious and durable symp-

toms on pea plants. When the pea was for a long time infected with this virus, it showed no or very weak symptoms. However, the freshly infected pea displayed a vein clearing about 6 days after inoculation. This vein clearing gradually extended toward the intercostal leaf regions so that soon a fairly thick light green net was visible on the upper pea leaves. These symptoms quickly disappeared so that after one to two days the leaves had their normal appearance again.

Contrarily the broad been is a favourable plant for investigating this virus. The pea latent virus often produces small brown local lesions followed by a strong systemic infection. However, this strong reaction on broad bean appeared only in late spring and summer, and the symptoms were very mild or entirely disappeared during the winter.

The PEMV and latent pea virus provoke different local lesions on *Chenopodium amaranticolor*. While the first virus produces minute local lesions, the lesions of latent pea virus are conspicuous with a diameter of 1.5 mm. The sap from the latter lesions was investigated with the dipping method in an electron microscope, and elongated, about 750 nm long virus particles were found.

Discussion

Yugoslav virologists began to study viruses of leguminous plants ten years ago on account of the importance of these plants as sources of proteins. During this period viruses of various clover plants (Babović 1969b, 1974; Grbelja 1974; Malak 1974; Šutić i Babović 1965), lucerne (Babović 1968), bean (Aleksić 1967) and birdsfoot-trefoil (Buturović 1974) were investigated in particular. Besides that a mycoplasma-like microorganism which is the cause of the disease clover phyllody was also studied (Grbelja and Ljubešić 1974). The number of all papers about this subject is not exhausted with the above citations.

On the contrary, the viruses of pea have not been investigated in Yugoslavia up to now. In this paper we therefore reported on the occurrence of the pea enation mosaic virus (PEMV) on pea in this country. This virus has been recently found on pea (Martelli 1969), lentil (Vovlas e Rana 1972) and broad bean (Vovlas et al. 1973) in Italy.

In this paper the hosts of PEMV have not been studied in detail because the virus symptoms on leguminous hosts in form of enations were very characteristic for this virus. On account of this fact the presence of PEMV on pea was relatively easily established. The host range of this virus was investigated by Hagedorn et al. (1964).

As mentioned in the *Introduction*, PEMV is a persistent virus which can for a relatively long time remain infective in its aphid vectors (Osborn 1935). It can multiply in the cells of its plant hosts, specially in nuclei which sometimes contain large quantities of virus particles. It is characteristic for PEMV that with some host plants it causes a vesiculation in perinuclear space (De Zoeten et al. 1972).

PEMV has several times been well purified (Gibbs et al. 1966, Izadpanah and Shepherd 1966, Shepherd 1970). This gave us the opportunity to prepare a partially purified virus suspension from a comparatively small amount of infected plant material.

Summary

Pea enation mosaic virus (PEMV) was found in two localities in Yugoslavia, i. e. in Zagreb and Delnice. The isolates G1 (Zagreb) and G2 (Delnice) were transmitted to pea, broad bean, and *Chenopodium* species. Characteristic enations often arose on pea and broad bean, specially under glasshouse conditions. Other symptoms like deformations of leaves, stems and pods, and strong mosaic were also present, specially on peas. The presence of PEMV in these species was proved by means of antisera obtained from Germany and Italy. The isolate G2 was partially purified and photographed with the electron microscope. The virus particles had a diameter of about 30 nm. A rabbit was injected the partially purified virus and a serum against the Yugoslav G2 isolate was prepared after a month.

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SADRŽAJ

NALAZ VIRUSA ENACIJA I MOZAIKA GRAŠKA U JUGOSLAVIJI

Davor Miličić, Ružica Lešić, Đorđe Mamula, Nada Pleše i Vladimir Hršak
(Institut za botaniku Sveučilišta i Poljoprivredni fakultet Sveučilišta u Zagrebu)

Virus enacija i mozaika graška (pea enation mosaic virus) raširen je u svim sjevernim umjerenim regijama. On je rasprostranjen u Italiji, Austriji i Madžarskoj, ali u Jugoslaviji nije bio dosad pronađen.

U ovom radu prikazan je prvi nalaz tog virusa u Jugoslaviji. Nađen je na dva lokaliteta u Hrvatskoj, i to u Zagrebu i Delnicama. Izolati G1 (Zagreb) i G2 (Delnice) tog virusa preneseni su na grašak, bob i vrste *Chenopodium.* Na grašku i bobu nastale su karakteristične enacije, naročito često kad su se inficirane biljke uzgajale u stakleniku. I drugi simptomi, kao što su deformacije lista, stabljike i ploda te jaki mozaik, očitovali su se osobito na grašku.

Nazočnost tog virusa u navedenim biljkama dokazana je s pomoću antiseruma koji je dobiven iz Njemačke i Italije. Elektronskomikroskopski pregled djelomično purificirane virusne suspenzije pokazao je da čestice virusa imaju dijametar oko 30 nm.

Purifikacija virusa izvršena je prema ponešto modificiranoj metodi koju su opisali Hull i Lane. Da bi se uklonili mnogi sastavni dijelovi stanice, sirovi infekcioni sok obrađen je smjesom kloroforma i butanola. Vodena faza izvrgnuta je dvama turnusima diferencijalnog centrifugiranja uz upotrebu acetatnog pufera i askorbinske kiseline. Na kraju postupka dobiven je veliki talog koji je predstavljao djelomično purificirani virus.

Mjerenjima apsorpcije ultravioletnog svjetla, izvršenim s pomoću spektrofotometra, dobila se apsorpcijska krivulja koja je karakteristična za viruse. To je pokazalo da se priređena virusna suspenzija sastojala većinom od virusnih čestica. Na osnovi mjerenja ustanovljeno je također da je koncentracija virusa u biljnom soku iznosila 0,3 mg/ml.

Djelomično purificirani virus injiciran je u tijelo kunića, pa je poslije mjesec dana dobiven serum protiv jugoslavenskog izolata G2 virusa enacija i mozaika graška.

Prof. dr Davor Miličić, Đorđe Mamula, mr biol., dr Nada Pleše i *Vladimir Hršak,* stud. biol.,
Institut za botaniku Sveučilišta
Marulićev trg 20/II
41000 Zagreb (Jugoslavija)

Prof. dr ing. Ružica Lešić,
Poljoprivredni fakultet
Šimunska cesta 25
41000 Zagreb (Jugoslavija)