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ALFALFA MOSAIC VIRUS ON CLOVERS IN YUGOSLAVIA

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Introduction

Populations of alfalfa mosaic virus (AMV) consist of particles of different sizes (Bancroft and Kaesberg, 1958). The particles are either spherical, of about 18 nm in diameter, or bacilliform; the latter have different lengths so that the longest ones reach about 60 nm (Bos and Jaspars, 1971). AMV shows a peculiarity both with regard to the numerous strains in which it appears and to other properties for which it has been classified in a special group of monotype viruses in the system of Harrison et al. (1971).

AMV is widely spread and attacks a large number of plant species; according to Hull's data (1969) it infects 305 species from 47 plant families. A detailed list of clover species which can be virus hosts and a list of clover viruses were published by Schmelzer and Wolf (1971).

While in England infections with AMV on red and white clovers are very rare, AMV is widely spread in the USA and other countries. AMV causes a considerable loss of yield in alfalfa and clover cultures (Kreitlow et al., 1957). According to Hanson and Hagedorn (1952), red clover is a reservoir of many legume viruses, especially of AMV.

In Yugoslavia there are few data about virus diseases of clovers. Šutić and Babović (1965) isolated the following viruses from red clover: white clover mosaic virus, bean yellow mosaic virus and pea common mosaic virus. Pea common mosaic virus is related to bean yellow mosaic virus so that it is considered a strain of bean yellow mosaic (Bos, 1970). Thus, only two viruses on clovers have been discovered in Yugoslavia so far.

As the viruses on clovers have been little studied in our country, we have started to investigate these viruses. In this paper our first results are reported.

Material and Methods

During 1972 and 1973 we noticed the symptoms of a virus disease on *Trifolium pratense* and *T. fragiferum*. The diseased clovers were found in the Sarajevo Valley and in the environments of Zagreb where clovers were grown as forage, and in the fields of Solin near Split where they grew spontaneously (Fig. 1).

We succeeded in transmitting the viruses from clover to herbaceous plants by mechanical inoculation adding 0,1 M phosphate buffer pH 7,2. From the infected clovers several AMV isolates were obtained. Virus isolates from *T. pratense* are marked as abbreviations Sa and So, and isolates from *T. fragiferum* as FrS and FrZ.

For purification of Sa the method of Gibbs et al. (1963) was used, which was employed also by Babović (1968) in a modified way. Ultracentrifugation was done in a highspeed Spinco Model L 50 centrifuge, by using a rotor No 40 at 35,000 r. p. m., i. e. 81,000 g. Owing to a shortage of material, only two cycles of differential centrifugation were performed. At the end of procedure the virus was suspended in phosphate buffer.

For identification of the virus an AMV antiserum was used, which was kindly provided by Dr. M. Babović (Belgrade). Serological experiments were performed by double diffusion method in agar-gel.

Results

Localities and symptoms

The first attempts to isolate a virus from *T. pratense* were performed on the material found at Solin. A great number of specimens of that species of which 20% had yellow leaf parts (Fig. 1 A, B, 2 A) were found in two fields. The yellow leaf parts in the form of lines frequently stretched from the central vein towards the margins. Sometimes most of the leaf surface was yellow; the yellow parts were less developed, more or less deformed and uneven (Fig. 2 A). Sa and So isolates were taken from such specimens of red clover.

At the same locality grew several specimens of *T. fragiferum*. A certain number of plants had yellow spots and lines on the leaves (Fig. 1 C). No deformation was noticed on the diseased leaves. A few infected specimens were collected and FrS isolate was taken from them.

Last autumn *T. fragiferum*, collected in the valley of the Ribnjak Brook at Gračani near Zagreb, was investigated. FrZ virus was isolated from the specimens which had the same symptoms as those from Solin (Fig. 1 C).

The fact, that the first collected virus isolates from clovers belonged to AMV, shows that the virus is quite frequently present on clovers and that the infected clovers in our country too are an important reservoir of that virus.

Analysis of isolates on herbaceous plants

Investigations of the host range and the reactions of herbaceous plants were mainly performed with Sa (Table 1). The other isolates were examined only on test plants for AMV, i. e. on *Chenopodium quinoa, Ocimum basilicum* and *Phaseolus vulgaris.*

Table 1. Reactions of herbaceous plants after infection with isolates of alfalfa mosaic virus from clover

Local simptoms on incolulated leaves are marked with sing I placed before the description of symptoms; systemic symptoms on younger leaves are marked II. Plants marked only I are locally infected, and only II are systemically infected.

CHENOPODIACEAE

Chenopodium amaranticolor Coste et Reyn. I local lesions, chlorotic and necrotic spots, II vein yellowing, epinasty of leaves.

Ch. quinoa Willd. I chlorotic and necrotic lesions; II chlorotic spots and mosaic, epinastic curving of leaves.

Ch. album L. I local lesions; II chlorotic spots.

CUCURBITACEAE

Cucumis sativus L. I chlorotic spots and rings.

LABIATAE

Ocimum basilicum L. II yellow spots spreading later and joining so that considerable light-yellow areas appear on leaves.

LEGUMINOSAE

Phaseolus vulgaris L. I two days after inoculation small brown necrotic spots 1—2 mm in diameter; later on, the spots either enlarge or brown necrotic rings develop round them; sometimes the lesions join causing leaf wrinkling; red lines along the length of the veins.

Vicia faba L. I small brown local lesions, necrosis; II brown lines and necrosis on stem, withering of the plant.

SOLANACEAE

Datura stramonium L. I chlorotic and necrotic rings and half-rings grouped very densely; epinastic leaf curving; II vein yellowing.

Nicandra physaloides Gärtn. I necrotic lesions.

Nicotiana glutinosa L. I necrotic rings and half-rings about 2 mm in diameter; II chlorotic variegation.

N. megalosiphon Heurck et Muell. Arg. I necrotic rings; II mosaic.

N. rustica L. I chlorotic variegation; II chlorotic variegation, necrotic spots, recovery.

N. tabacum L. var. Samsun, White Burley and Hicks resistant. I necrotic spots, rings and half rings; II necrotic lines trimming the veins, intercostal rings and half rings (Fig. 2 B, C), recovery.

Solanum nigrum L. II small necrotic rings and half rings.

Of all isolates Sa provoked the strongest symptoms. Under the influence of Sa systemic symptoms appeared on *Chenopodium* species. *Ocimum basilicum* developed intensive yellow spots and variegation

which are very characteristic of infection with AMV. The analysis of viruses on herbaceous plants suggests that AMV is the cause of the disease.

Purification of virus and proofs of its presence in suspension

Leaves of infected *N. glutinosa* plants were used for the purification. Plant sap from those leaves was ground in a mortar. After the treatment with cloroform, the solution had a yellowish-brown colour which it did not lose even after two differential centrifugation cycles. The yellowish--brown colour of the final suspension proves that N. glutinosa, at least in advanced stages of its development, is not particularly suitable for purification.

However, the suspension obtained could be used for electron microscopical investigations. After the treatment of the suspension with phosphotungstate acid, a great number of bacilliform particles with round ends which are characteristic of AMV population were noticed in the electron microscope. Since these particles had dimensions of AMV, we were sure that they belonged to AMV.

An analysis of the suspension in spectrophotometer showed that the main part of purified suspension was composed of virus particles. It also established that the ratio between absorption of UV light at 260 and 280 nm, i. e. A 260/280 was 1.63 (Fig. 3). This datum points out that the suspension was purified well enough and that UV absorption corresponds to that of AMV (Bos and Jaspars, 1971). Apart from this, the absorption curve was characteristic of a virus, which also points out that on the whole virus suspension did not have other impurities (Fig. 3). On the basis of UV light absorption at 260 nm wave lenght, we could calculate the absolute virus concentration in plant sap which was about 1 mg/ml.

Fig. 1. Symptoms of alfalfa mosaic virus. A and B *Trifolium pratense,* yellow lines and bands along the veins; C *Trifolium fragiferum,* yellow spots and mosaic.

Sl. 1. Simptomi virusa mozaika lucerne. A i B *Trifolium pratense,* žute linije i vrpce duž nerava. C *Trifolium fragiferum,* žute pjege i mozaik.

Fig. 2. A — C Symptoms of alfalfa mosaic virus. A *Trifolium pratense;* yellow patches and bands, leaf deformation. B *Nicotiana tabacum,* White Burley; lines along the veins. C *N. tabacum,* Samsun; line pattern. D Serological diffusion test. In centre well, serum against AMV is placed (As); in right peripheral wells infective plant sap diluted 1 : 1, 1 : 2, 1 : 4 (Inf); in left peripheral well healthy plant sap in the same dilution grades (H).

Sl. 2. A — C Simptomi virusa mozaika lucerne. A *Trifolium pratense;* žute pjege i vrpce, deformacija lista. B *Nicotiana tabacum,* White Burley; linije duž nerava. C *N. tabacum,* Samsun; isprekidane linije. D Serološki difuzijski pokus. U centralni bazen stavljen je serum protiv virusa mozaika lucerne (As). U desne periferne bazene stavljen je infekciozni sok u razrjeđenju 1 : 1, 1 : 2 i 1 : 4 (Inf); u lijeve periferne bazene zdravi sok u istim stupnjevima razrjeđenja (H).

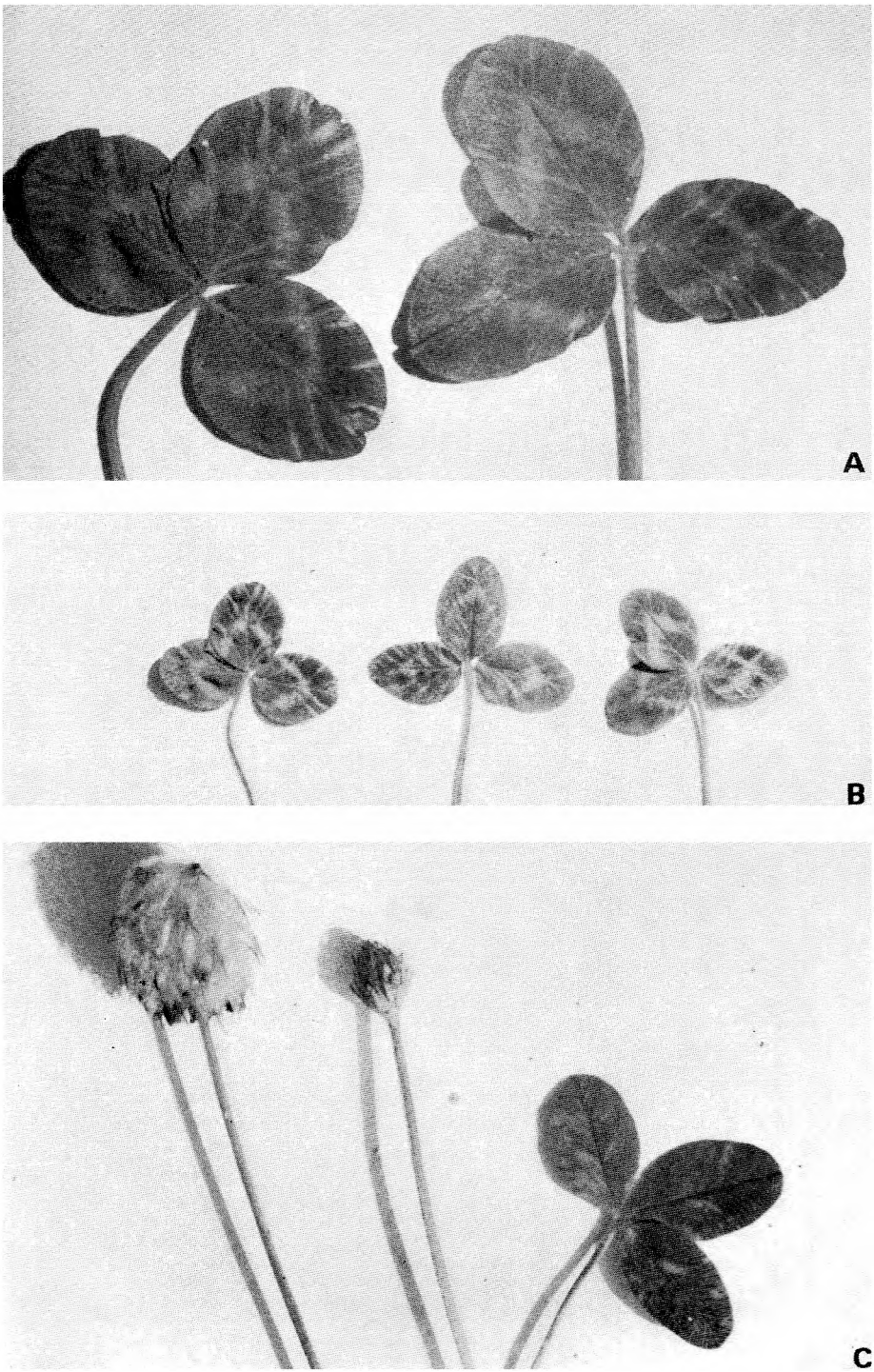

Fig. 1. — Sl. 1.

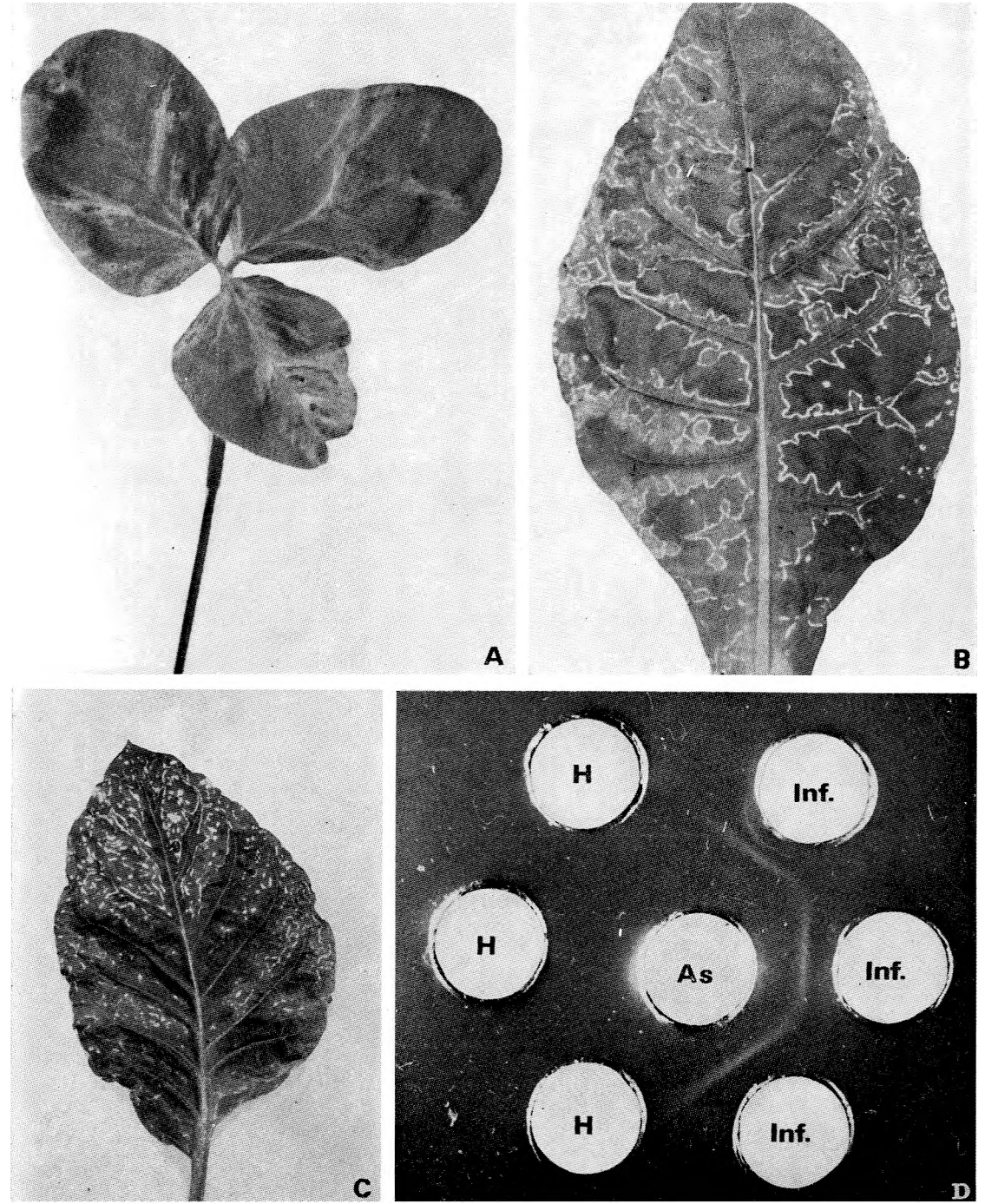

Fig. 2. — Sl. 2.

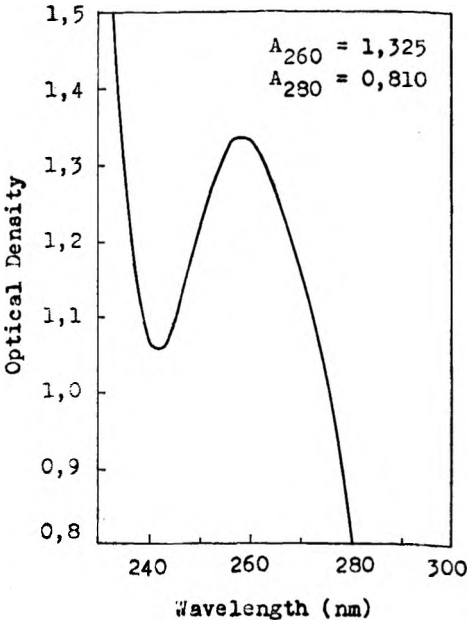

Fig. 3. Ultraviolet absorption of purified Sa isolate of alfalfa mosaic virus.
Sl. 3. Ultravioletna apsorpcija purificiranog izolata Sa virusa mozaika lucerne.

Serological investigations

Serological experiments were also performed and they proved too that the isolated virus belonged to AMV. The antiserum of AMV had a homologous titre 1 : 256, and for the identification of Sa it was diluted to 1 : 8. That serum was placed into a centre well, while the infectious sap and the control healthy sap were in the peripheral wells. A day after, the precipitation line appeared only between the wells containing the antiserum and infectious sap (Fig. 2 D). The appearance of that line is an additional proof that Sa really belongs to the AMV.

Discussion

As it has already been said, a small number of viruses on clovers has been found in Yugoslavia till now. For that reason it was possible, even after the first attempt, to isolate a virus from clovers which had not been identified on them before in our country. Since a great number of various viruses have been discovered on clovers in Europe so far (Schmelzer and Wolf, 1971), it is certain that many different interesting viruses will be isolated from our clovers too.

AMV is probably quite widely spread over the areas we were investigating. It seems that our country, in this respect, is similar to the areas in America and other regions where clovers cultivated as forage

represent reservoirs of AMV. *Ballota nigra,* for instance, which is widely spread in Yugoslavia is probably often infected with AMV too (Milič i ć et al., 1974).

Although Sa and So originated from the same locality, they differed in virulence. Sa was a considerably stronger strain showing very obvious symptoms (Fig. 2 A, B) after several passages through tobacco (Hull, 1969). For this reason Sa was more fully investigated than the other strains. *Phaseolus vulgaris* which reacted locally to our isolates, showed different reactions to some isolates on primary leaves. Particularly suitable for identification purposes was *Ocimum basilicum* which reacted to infection with intensive yellow spots and variegation (Lovisolo, 1966).

Summary

On the leaves of *Trifolium pratense* and *T. fragiferum,* symptoms of virus disease were noticed in the form of yellow spots, lines and patches. Diseased specimens were found in the environments of Zagreb, in the Sarajevo Valley and among wild grown clover at Solin. From diseased clovers a virus was isolated mechanically and identified as alfalfa mosaic virus (AMV) on the basis of reaction on 14 differential herbaceous plants. This result was confirmed by electron microscopical investigations. At the same time characteristic spherical and bacilliform particles were established in purified sap. The identification of virus was also confirmed by positive results of serological tests performed by double diffusion method in agar-gel.

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SADRŽAJ

Na listovima vrsta *Trifolium pratense* i *T. fragiferum* zapazili smo simptome virusne bolesti u obliku žutih pjega, crta i ovećih šara. Oboljeli primjerci nalazili su se među djetelinom uzgajanom na nekoliko prostranih površina u okolici Zagreba i Sarajevskom polju ili među djetelinom izraslom samoniklo na manjim livadama u Solinu. Iz bolesnih djetelina mehanički smo izolirali virus mozaika lucerne (VML), koji smo identificirali na temelju reakcije 14 diferencijalnih zeljastih biljaka. Da je bio izoliran stvarno VML, ustanovili smo i na temelju elektronsko-mikroskopskih istraživanja prilikom kojih su u pročišćenom soku zapažene karakteristične sferoidne i baciliformne virusne čestice. Identifikaciju virusa potvrdio je i pozitivni ishod serološkog pokusa koji smo izveli metodom dvostruke difuzije u agarskom gelu.

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