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FURTHER INVESTIGATION OF THE DEFECTIVE KAZAKHSTAN STRAIN OF TOBACCO MOSAIC VIRUS

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Introduction

In Goldin's book (1963) dealing with intracellular inclusions induce in plants by various viruses a special chapter is devoted to an interesting tobacco mosaic virus (TMV) strain from Kazakhstan. The Kazakhstan strain (Ka strain) caused different inclusions not only in the cytoplasm but also in the nucleus. Goldin's investigations were performed only with light microscope so that the ultrastructure of inclusions remained unknown.

A important contribution to the knowledge of Ka strain derived from Brčak (1978) who ascertained that the Ka strain was a defective strain of TMV. Brčak (1978) based his opinion on the following facts: 1. the Ka strain rarely formed virus particles of the normal length of 300 nm, but more frequently shorter or longer particles; 2. the Ka strain, in comparison with normal TMV, had a lower thermal inactivation point, and spread very slowly to the upper leaves of tobacco plants leaving them without symptoms; 3. the Ka strain induced inclusions in the form of long coiled fibres, which frequently appear in plants infected with defective strains.

Already Goldin (1963) described the symptoms provoked by the Ka strain on tobacco plants. He knew that some other herbaceous plants could react in a similar way, i. e. *Solanum lycopersicum, S. luteum, S. aviculare, Lycopersicum hirsutum,* and *Gomphrena globosa.* In all these plants the Ka strain caused a systemic disease. On the other hand, some plants reacted to infection with local lesions (cf. Miličić et al. 1979).

This paper presents some more information about the symptomatology and also about light and electron microscopical structure of various inclusion bodies appearing during the Ka strain infection.

Material and Methods

During this investigation the same Ka strain of TMV was used which Brčak (1978) had already studied. This author kindly sent us this strain many years ago.

The tobacco plants of cv. Samsun were employed for light and electron microscopy. The light microscopic investigations were performed only on living plant material. For electron microscopy leaves with obvious symptoms were employed. Small leaf parts were fixed for 30 min in 1% (v/v) glutaraldehyde in cacodylate buffer and after appropriate washing in buffer were postfixed for 2 h in 1% (w/v) osmium tetroxide. After fixation samples of tissue were dehydrated in ethanol series and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop I.

Results

Investigations on herbaceous plants

On Samsun tobacco the first symptoms appeared about 8 days p. i. in the form of chlorotic spots or vein clearing. The latter symptom was accompanied with a chlorosis which spread from the veins towards the intercostal leaf parts. Later the spots became larger with yellow margins and often with necrotic centres. It is characteristic of this tobacco infection that the virus spreads slowly in the upper parts where the leaves remain without symptoms.

In order to establish whether the tobacco plants infected with the Ka strain can be superinfected with common TMV, we inoculated the upper leaves which were without symptoms with the common TMV. After a few days the upper leaves showed mosaic symptoms characteristic of the common TMV and numerous hexagonal prisms appeared in their epidermiss cells. These prisms are very rare or are not present at all in cells infected with the Ka strain. Consequently, the Ka strain does not protect the tobacco plants from infection with common TMV. The same experiment with the same result was made by Goldin (1963).

Nicotiana megalosiphon reacted with a large number of necrotic local lesions 5 days p. i. The lesions consisted of a grey-black margin and a light centre. Later the necrotic lesions became larger, reached a diameter of 4 to 5 mm and successively joined together. This was a progressive process, and 8 days p. i. the greatest part of the leaf blade became necrotic. After a few days the inoculated leaves dried up. On the leaves situated above the inoculated ones, a large number of very small necrotic lesions appeared, but the top leaves were mostly symptomless.

On the inoculated leaves of *Chenopodium amaranticolor* 30 to 40 local lesions arose, which were about 2 mm large. The lesions consisted of a light centre and a brown-grey ring around the centre. The number of lesions was remarkably smaller than after the inoculation of this plant with the common TMV which caused about a thousand lesions on one leaf.

Chenopodium quinoa reacted locally forming yellow lesions which became gradually green on the margins. The number of lesions was 10 to 20 per one leaf.

Light microscopic investigations

The first intracellular changes appeared in the young tobacco plants 8 days p. i. Crystalline aggregates 10 to 20 μm long were observed in the epidermis cells. They consisted of very fine threads parallelly arranged and disposed in layers. These aggregates were similar to the structures described by Amelunxen (1955, Fig. 15; 1958, Fig. 5 i-k) and Wehrmeyer (1959, Fig. 14 c) during their studies of the first development stages of spindle-like inclusions caused by two elongated viruses. Twenty days p i. the aggregates were similar to grain masses or to plate-like bodies. At that stage of infection it was possible to find similar crystalline inclusions also in the nucleus. They were investigated with polarisation microscope, but only a part of them were birefringent. A month p. i. the inclusions became compact bodies sometimes striped and always strongly birefringent. The bodies showed a positive character of birefringence with regard to the direction of stripes.

About 40 days p. i. crystalline rods were present in the cytoplasm disposed either singly or forming lateral aggregates and expressing a strong optical birefringence. Similar bodies were situated also in the nucleus. Sometimes many elongated or round crystalline bodies were present (cf. Fig. 3). The elongated crystals stretched in various directions and were approximately as long as the diameter of the nucleus.

The same plant material was examined in July, i. e. four months p. i. In the hair cells and in the leaf epidermis there were numerous long coiled fibres or loops in the form of number eight. In addition to that intranuclear crystals were very frequent. The presence of long coiled fibres and frequent intranuclear crystals are specific properties of the Ka strain (cf. Miličić et al. 1979).

During these investigations carried out at normal temperature from 20 to 25° C we did not observe any 'large amorphous X-body' in the cytoplasm. This is necessary to mention because amorphous and electron-dense X-bodies are produced by some defective strains, such as strain flavum, Ni 118, PM1, and English PM2 (Bald 1964, Kolehmainen et al. 1965, Kassanis and Milne 1971, Kassanis and Turner 1972). The bodies of these strains probably represent the insoluble coat protein.

Electron microscopic investigations

Virus particles

Brčák (1978) has established that the Ka strain in distinction from the common TMV forms particles which are often longer or shorter than 300 nm when dipping preparations were shadowed with metals. In order to complete these data we investigated twice the particles in the plant sap treated with potassium phosphotungstate (Figs. 1 and 2). The first examination was made with the sap of tobacco Samsun in April, about one month p. i. In the electron microscope some particles about 300 nm long were visible, but also shorter or longer particles (Fig. 1). The par-

ticles were altered and swollen, and about 21 nm wide. They were, therefore, wider than the normal 18 nm wide TMV particles.

However, a considerable quantity of virus protein was in various stages of disintegration. Very often short fragments were present which had a small number of turns in the helix (cf. Gibbs and Harrison 1976, p. 66) and therefore they were placed flatly with a clearly visible central cavity (Fig 1 d). Other fragments, some tens of nanometers long, were also frequent and showed various stages of disintegration (Fig. 1 f).

Later on — when this article was in print — we established that the degree of virus particle desintegration depends very much on the temperature at which the living leaves are kept before the negative staining. Fig. 1 shows strongly desintegrated virus particles prepared from leaves which were kept in a refrigerator at 10° C during the night before treatment. On the contrary, if the leaves are kept at room temperature before the staining, the desintegration is lower and a large number of virus particles are present from which several are very long (cf. Brčák 1978).

As the first negatively stained preparations gave an interesting result, we repeated the procedure using the same plant in July, approximately four month p. i. In this material many particles, 17 nm wide, were present (Fig. 2). It was remarkable that they were often broken and then only 100 nm long or present in the form of disc-like fragments (Fig. 2 d). Besides, it was apparent that the virus protein in these preparations was in a rather stable condition; no special sign of protein decomposition was visible (Fig. 2). The leaves for this preparation were kept at normal room temperature before the negative staining.

Virus inclusions

The second part of electron microscopic investigation consisted of an analysis of virus inclusions. For this purpose we used tobacco plants cv. Samsun about 40 days p. i. where already during observations of living cells in the light microscope the presence of intracytoplasmic and intranuclear inclusions was proved. In concordance with light microscopic observations many intranuclear inclusions were found in ultrathin sections. Usually one large inclusion was situated in the centre of the nucleus and many smaller ones on the periphery (Figs. 3 and 4). The

Fig. 1. Particles of the defective Ka strain negatively stained with potassium phosphotungstate. Before this treatment the infected tobacco leaves were kept some hours at 10 °C in refrigerator. Many small fragments similar to discs in face view (d), same large fragments in side view (f), and swollen virus particles (s). Bar marker represents 100 nm.

Fig. 2. Negatively stained infective sap with particles of the Ka strain. Many particles are broken and shorter than TMV. Disc-like groups are visible in some places (d) and in the inset. Bar markers represent 100 nm.

Fig. 3. Intranuclear inclusions of the Ka strain. Large central inclusion (l), marginal inclusions (m). Bar marker represents 400 nm.

Fig. 4. Intranuclear inclusions similar to those on Fig. 3. Large, almost empty spaces in the cytoplasm where dissolved inclusions were situated. Bar marker represents 500 nm.

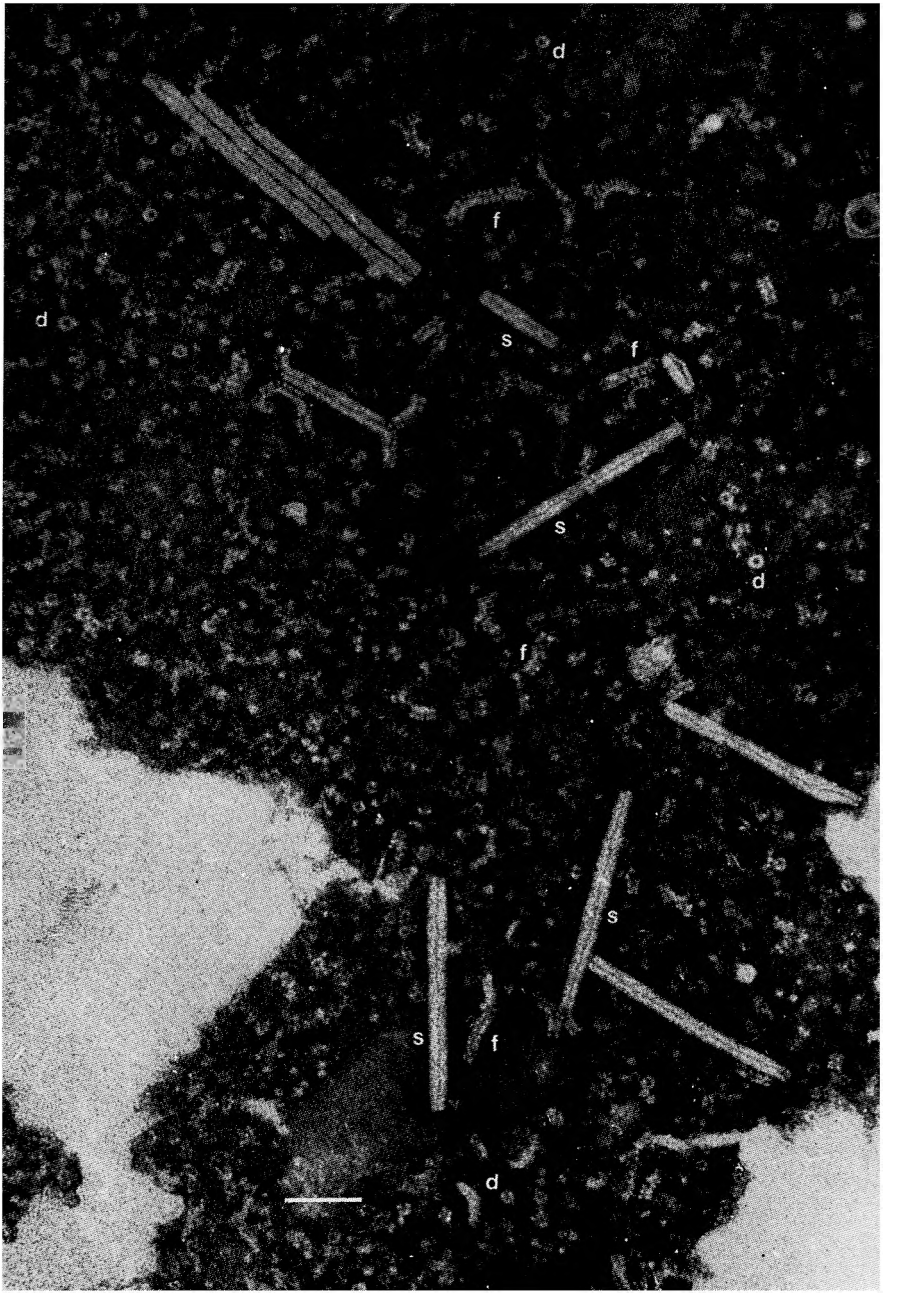

Fig. 1.

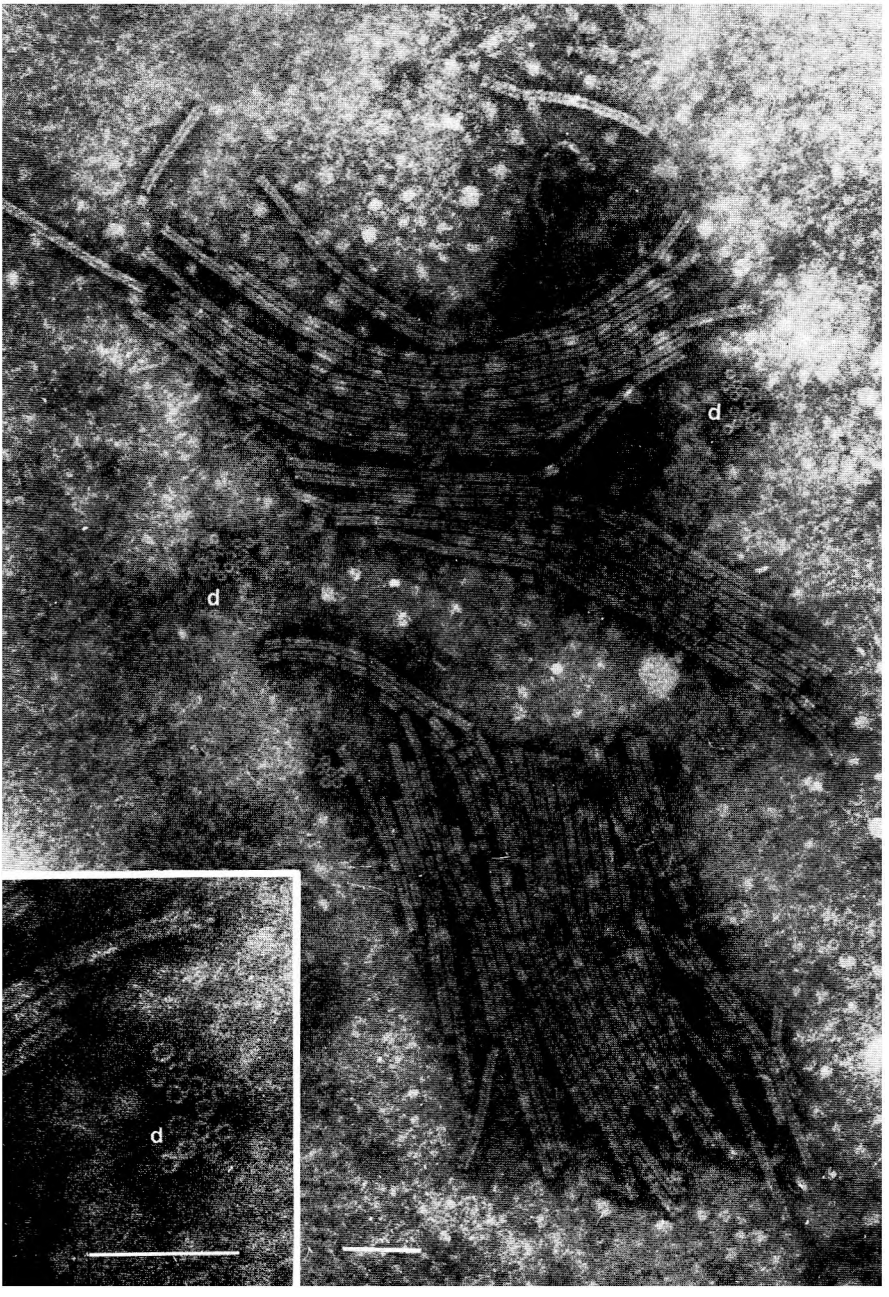

Fig. 2.

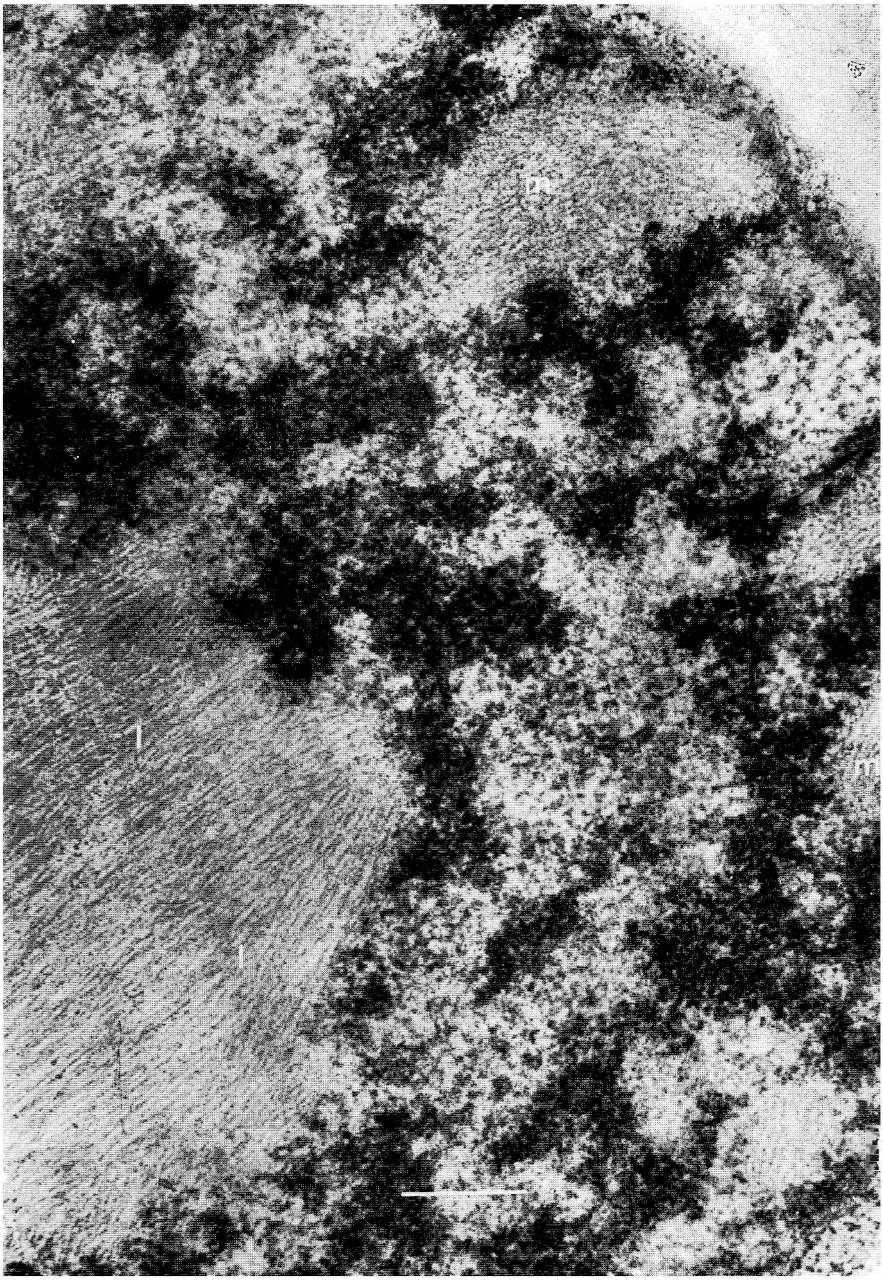

Fig. 3.

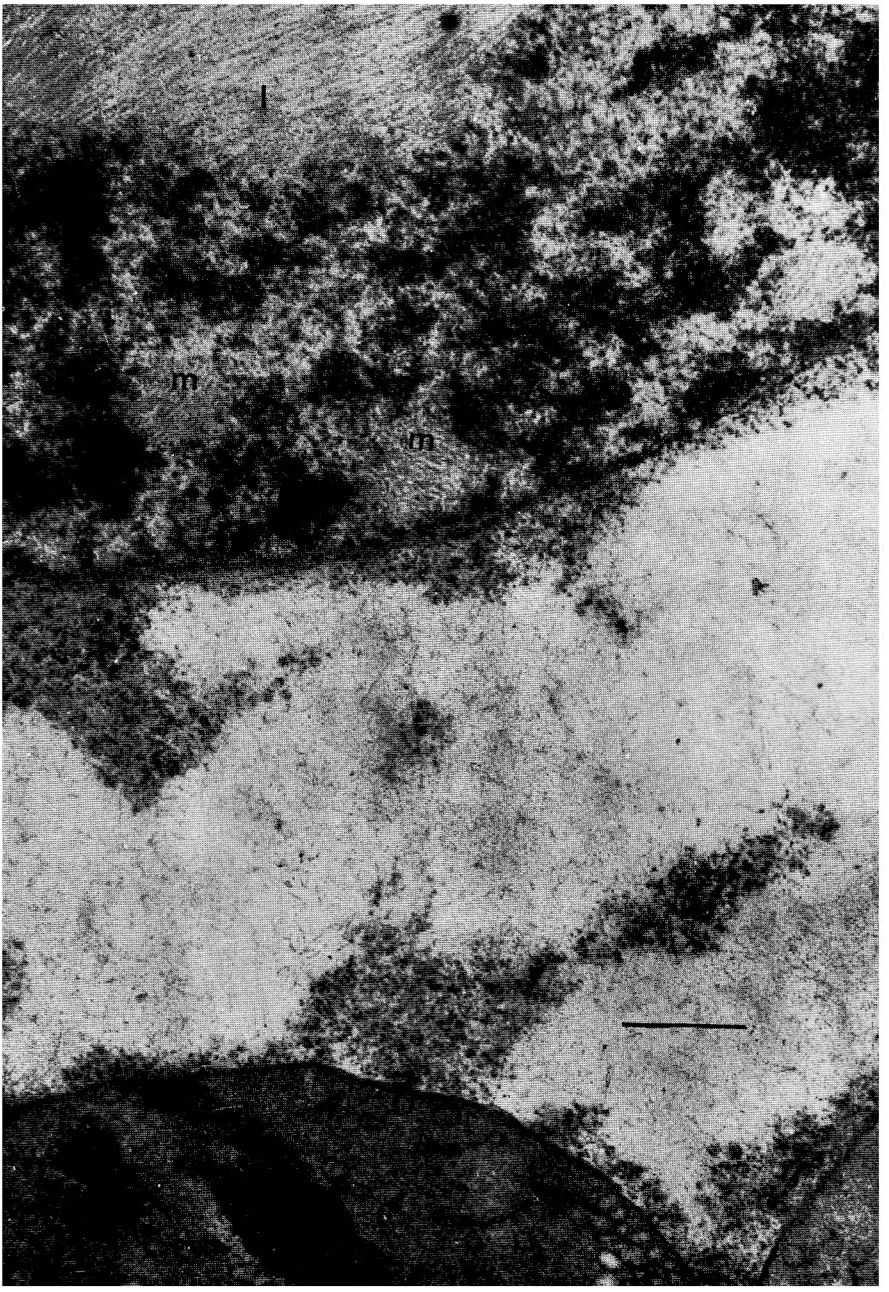

Fig. 4.

inclusions obviously presented aggregates of parallelly arranged virus particles. The central parts of the particles, probably viral RNA, were intensively stained, and it seems that these parts represent the stretched threads observed in the past year (Miličić et al. 1979).

While in the nucleus the virus inclusions were well fixed, in the cytoplasm — in the places where on the basis of light microscopic investigation we could expect virus inclusions — we found almost empty places (Fig. 4). In the past year we also had difficulties in the fixation of inclusions (Miličić et al. 1979). It seems that glutaraldehyde is not a favourable medium for the fixation of inclusions.

The second time we fixed this material 4 months p. i., when beside the usual cytoplasmic and nuclear inclusions, still a large number of long coiled fibres was present in the living cells. In this case, we could find neither intranuclear nor intracytoplasmic inclusions. In the places, where according to light microscopy of living cells we had expected to see the inclusions, almost empty places were present. Accordingly, the inclusions were in a condition that did not allow to fix them well.

Discussion

The results of investigations by Brčák (1978), Miličić et al. (1979) and the results of this paper show that the Ka strain of TMV is indeed a defective strain. It was here established that the virus particles were often broken, which was a consequence of the loose structure of coat protein. The coat protein is probably often built of stacked discs and has not always a normal helical arrangement of subunits.

By means of a light microscope we studied the sequence of appearance of various inclusion types during the process of infection. We established that the first inclusions were small and appeared in the cytoplasm. This is in accord with the investigation of Goldin (1963) who directly observed the appearance of various types of inclusions in one and the same cell by means of long and continual investigations. Unlike Goldin's results, who observed an early appearance of loops (long coiled fibres), in our investigation the loops appeared later. Thus, it is probable that the sequence of inclusion appearance can vary. A special attention was paid to the intranuclear inclusions which regularly appear after the cytoplasmic ones.

During the investigation of loops we established that the optical birefringence was very strong and the character of birefringence was positive with regard to the direction of fibres. This result suggests that the fine structure of coiled fibres of the yellow G2 strain investigated by Wehrmeyer (1959) is not equal to the ultrastructure of fibres of the Ka strain. Wehrmeyer (1959) has established that the coiled fibres of the G2 strain often have a negative character of birefringence with regard to the direction of fibres.

Many defective strains of TMV are artificial and they were obtained after the treatment of common TMV with nitrous acid. However, according to Goldin's data (1963) the Ka strain was found in Kazakhstan in nature, i. e. it is a natural defective strain. The infectivity of the Ka strain is probably not very strong, which is obvious from our data on the number of lesions on the leaves of *Ch. amaranticolor*. On account of its

weak infectivity the Ka strain can survive in nature with difficulty and therefore this strain must be rare in nature. Goldin (1963: p. 106) states that the Ka strain in dried state can retain its infectivity for many years, similarly to TMV. In our experiments the Ka strain also remained infective in dried leaves for many years and this is probably the reason why the Ka strain can maintain itself in nature.

Summary

The paper presents some new data on host plants of the defective Kazakhstan strain (Ka) of tobacco mosaic virus. *Chenopodium amaranticolor* and some *Nicotiana* species can be used as local lesion hosts for detailed investigations. The appearance of various forms of intracellular inclusions during the infection process was analysed by means of light microscope.

The electron microscopic preparations of fresh infective sap treated with potassium phosphotungstate showed that the virus particles rarely had the length of 300 nm, more frequently they were shorter or longer. When before this treatment the infected leaves are kept some hours in a refrigerator, the particles are mostly desintegrated. In the infection phase, when long coiled fibres are present in the cells the virus particles are often broken and consist of fragments about 100 nm long.

Moreover the ultrastructure of intracellular inclusions is described and illustrated. In the late phases of infection it was not possible to fix well various forms of cytoplasmic and intranuclear inclusions.

Although the Ka strain is defective, it can retain its infectivity in dried leaves for many years and maybe in this manner maintain itself in nature.

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SAŽETAK

DALJA ISTRAŽIVANJA DEFEKTNOG KAZAHSKOG SOJA VIRUSA MOZAIKA DUHANA

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U ovom radu donosimo nove podatke o domadarima kazahskog soja (Ka soja) virusa mozaika duhana. *Chenopodium amaranticolor* i neke vrste *Nicotiana* jesu domadari Ka soja koji stvaraju lokalne lezije pa se mogu upotrijebiti za razna podrobnija istraživanja. U radu je proučavana s pomoću svjetlosnog mikroskopa pojava različitih tipova intracelularnih uklopina tijekom infekcijskog procesa. Naročito je važna za poznavanje ovoga soja bila analiza svježeg infekcioznog soka obrađenog kalijevim fosforno-volframatom. U tim preparatima čestice su bile često kraće od 300 nm, tj,. od normalne dužine virusa. Osim toga čestice su bile razlomljene, proteinski omotač bio je često izgrađen od naslaganih diskova a ne od spiralno raspoređenih podjedinica i vidjele su se različite faze dezintegracijskog procesa virusnog proteina. Na kraju je opisana i ilustrirana ultrastruktura intranuklearnih uklopina.

Premda je Ka soj defektan, on može više godina zadržati infekcioznost u suhim listovima i možda se na taj način održava u prirodi.

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