ELECTRON MICROSCOPY OF TWO VIRUSES OF DEADLY NIGHTSHADE (*ATROPA BELLADONNA* L.)

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Summary. – Deadly nightshade plants showing severe necrotic lesions on leaves were observed in southern Bohemia. In negatively stained preparations of spontaneously infected deadly nightshade, artificially inoculated host plants and purified preparations two types of virus particles, isometric ones of about 26 nm in diameter and flexuous ones with length of 765 nm were seen by electron microscopy. The virus with isometric particles was identified as belladonna mottle virus (BMV), indistinguishable serologically from the Hungarian isolate of this virus. Identification of the virus with flexuous particles is discussed. Observations of the ultrastructure revealed the presence of filamentous virus particle aggregates and chloroplasts with peripheral vesicles bounded by double membranes, a feature typical for tymoviruses.

Key words: Atropa belladonna; belladonna mottle virus; electron microscopy; flexuous virus particles; ultrastructure; virus aggregates

Introduction

Deadly nightshade (*Atropa belladonna* L.), a member of the family *Solanaceae*, is an important medicinal plant grown world-wide for its content of valuable alkaloids, particularly of atropine and scopolamine. Several virus and fungal diseases occur in deadly nightshade, but only belladonna mottle virus infection was reported in this crop in the Czech Republic (Pelikánová *et al.*, 1992).

Deadly nightshade plants with necrotic lesion symptoms were discovered in southern Bohemia. Electron microscopy, mechanical transmission and serology were used to elucidate the etiology of this disease.

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Abbreviations: AMMV = atropa mild mosaic virus; BMV = belladonna mottle virus; BMV-H = Hungarian isolate of BMV; HMV = henbane mosaic virus; TEM = transmission electron microscopy

Materials and Methods

Plants. Naturally infected plants of deadly nightshade (*Atropa belladonna* L.) were found in a plant community of *Atropetum belladonnae* (Br.-Bl. 1930) by Dr. J. Pelikánová, Institute of Plant Molecular Biology, České Budějovice in 1989 in Poněšice, southern Bohemia. The infected plants showed necrotic lesions on the leaves.

Inoculation of test plants. Symptomatic leaves of *A. belladonna* were ground in 0.066 mol/l phosphate buffer (Sörensen) pH 7.0 (1:1, w/v), and the resulting sap was rubbed onto carborundum-dusted leaves of herbaceous test plants. The plants were observed for 5 weeks after inoculation.

Virus purification. Virus culture was propagated in *Nicotiana tabacum* cv. Xanthi. Infected leaves were harvested 20 days after inoculation and homogenized in 0.5 mol/l phosphate buffer pH 7.2 with 1% mercaptoethanol. The homogenate was filtered through cheesecloth and emulsified with quarter volume of a 1:1 mixture of n-butanol and chloroform. The juice was stirred for 45 mins at room temperature and then centrifuged for 10 mins at 7,000 rpm in a Janetzki K 24 centrifuge. The clarification and low speed centrifugation were repeated twice. The aqueous phases were collected and pooled and the virus was pelleted by ultracentrifugation for 3.5 hrs at 45,000 rpm in a Beckman Ti 50.2 rotor at 4°C. The pellets were resuspended in 0.01 mol/l phosphate

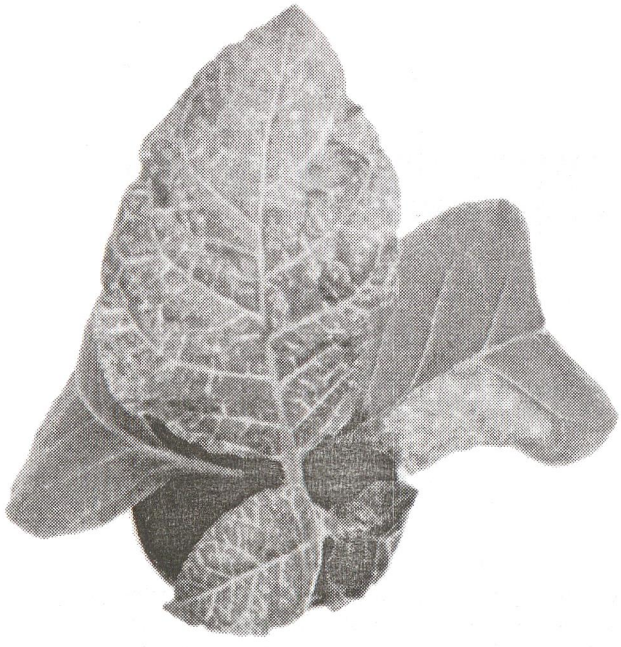

Fig. 1
Reaction of *N. tabacum* L. cv. Samsun 12 days after mechanical inoculation with crude sap from leaves of spontaneously infected *A. belladonna* L.
Necrotic local lesions and vein clearing on young leaves.

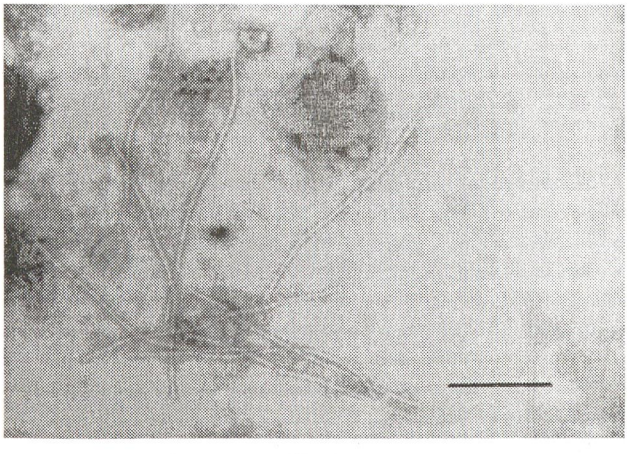

Fig. 2
Negatively stained preparation of flexuous virus-like particles
Bar = 300 nm.

buffer pH 7.2. Further virus purification was conducted by density gradient centrifugation through 10–40% sucrose in 0.01 mol/l phosphate buffer pH 7.2 at 28,000 rpm for 2 hrs in a Beckman SW 28 rotor. The contents of the tubes were analyzed by a flow registration analyzer and fractions of 1 ml were collected, pooled and diluted in 0.01 mol/l phosphate buffer pH 7.2. The virus sample was then concentrated by centrifugation at 45,000 rpm for 3 hrs (Pelikánová *et al.*, 1992).

Transmission electron microscopy (TEM). Ultrathin sections were prepared from small leaf pieces of symptom-bearing *N. tabacum* cv. Xanthi plants as described earlier by Fránová *et al.* (1996). Ultrathin sections, crude sap preparations from diseased and healthy plants as well from purified preparations negatively stained with 2% uranyl acetate, were examined in Philips 420 electron microscope.

Serology. Serological tests were made by the double diffusion technique with 0.7% Difco Noble agar gel which contained 0.018 mol/l citric acid and 0.02% sodium azide. Saps from healthy *N. tabacum* cv. Xanthi and *Atropa* served as controls. A Hungarian isolate of belladonna mottle virus (BMV-H) was kindly provided by Dr. J. Horváth, Research Institute for Plant Protection, Budapest, Hungary. An antiserum against BMV-H was prepared in our laboratory (Pelikánová *et al.*, 1992).

Results

Mechanical virus transmission

Virus-infected *Nicotiana tabacum* L. cv. Samsun (Fig. 1), *N. glutinosa* L., *N. rustica* L., *N. tabacum* L. cv. Xanthi, and *Datura stramonium* L. showed local necrotic lesions, vein clearing of young leaves and green systemic mosaic with dark green blistering. *N. clevelandie* Gray produced necrotic lesions on inoculated leaves and systemic necrotic leaf spots. *Chenopodium murale* L. and *C. amaranticolor* Coste et Reyn. developed local necrotic lesions. No symptoms were observed on *Cucumis sativus* L., *C. quinoa* Willd, *Brasica pekinensis* (Lour.) Rupr., *B. rapa* L. var. *rapa* Thell. and *Raphanus sativus* L. subs. *sativus* var. *radicula* Pers.

Virus purification and TEM

The purified mixture of viruses showed UV absorption characteristic of a nucleoprotein. After the density gradient centrifugation, the virus mixture exhibited two peaks of UV absorbance corresponding to the top and bottom components and the $A_{260/280}$ was 1.1.

In leaf-dip preparations for TEM from naturally diseased *Atropa* and artificially infected host plants, a mixed population of flexuous filaments of about 13 x 765 nm (Fig. 2) and isometric virus-like particles averaging to 26 nm in diameter were observed. Micrographs of purified preparations showed similar isometric and flexuous particles. After purification, the filaments were straight or only slightly flexuous and a lot of particles were halved or more broken, probably as a result of purification procedure.

Ultrastructural studies on *N. tabacum* cv. Xanthi plants confirmed the occurrence of a mixed virus infection. Isometric virions were located in vacuoles and in cytoplasm (Fig. 3). Moreover, alterations typically associated with a tymovirus

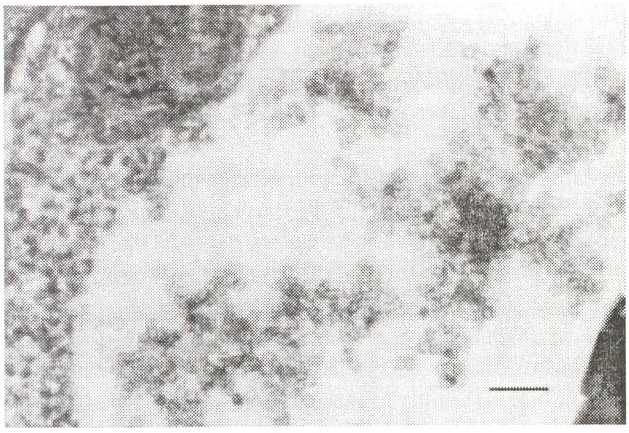

Fig. 3
Isometric BMV particles in central vacuole on ultrathin section from *N. tabacum* L. cv. Xanthi leaf tissue
Bar = 100 nm.

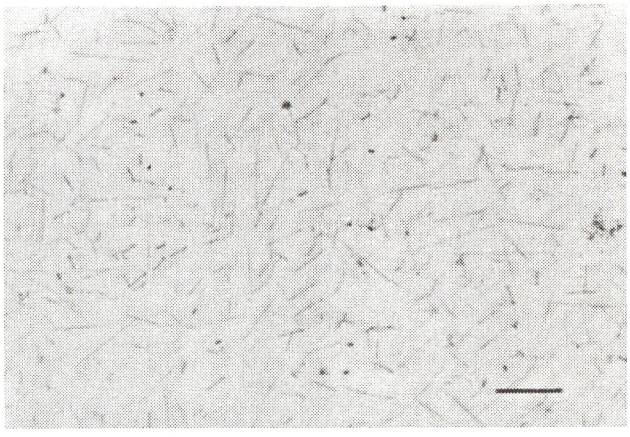

Fig. 5
Filamentous virus-like particles scattered in vacuole of *N. tabacum* L. cv. Xanthi leaf cell
Bar = 200 nm.

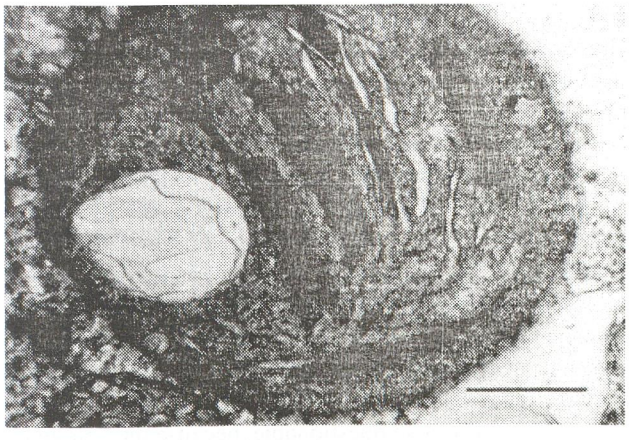

Fig. 4
Ultrathin section of chloroplast in BMV infected *N. tabacum* L. cv. Xanthi cell showing marginal vesicles of various size
Bar = 500 nm.

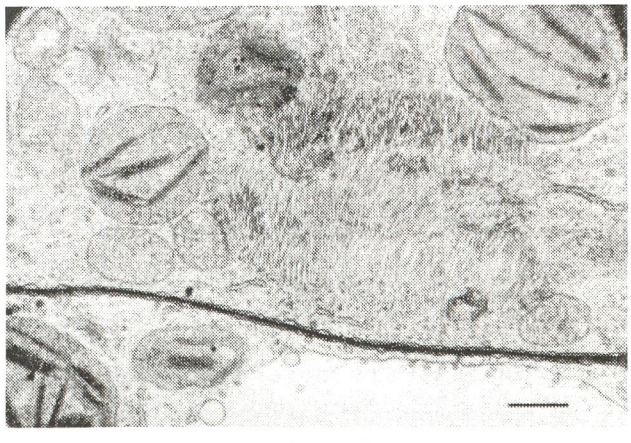

Fig. 6
Large aggregate of filamentous virus particles in cytoplasm of parenchyma cell of *N. tabacum* L. cv. Xanthi
Bar =500 nm.

infection were seen. Chloroplasts developed small peripheral vesicles bounded by double membranes (Fig. 4) and later they rounded and clumped. Filamentous virus-like particles were seen either singularly (Fig. 5) or organized in ovoid aggregates (Fig. 6) in the cytoplasm of phloem, xylem and parenchyma cells. In these aggregates, the particles were arranged parallely when cut in longitudinal sections. These aggregates were also seen in oblique or cross section (Fig. 7) and were often present near mitochondria and chloroplasts. No other pathogenic organisms were observed. Specimens prepared for TEM from healthy *N. tabacum* Xanthi leaves did not reveal any morphological and structural alteration.

Serology

The antiserum against BMV-H did not react with the saps from healthy plants of *Atropa* and *N. tabacum* cv. Xanthi in

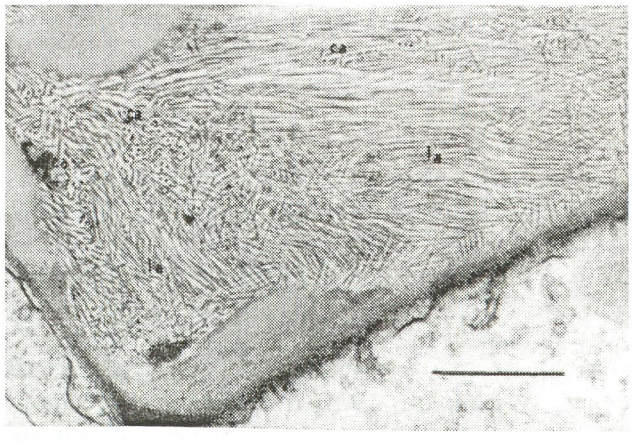

Fig. 7
Filamentous particle aggregates in cross (ca) and longitudinal (la) sections
Bar = 500 nm.

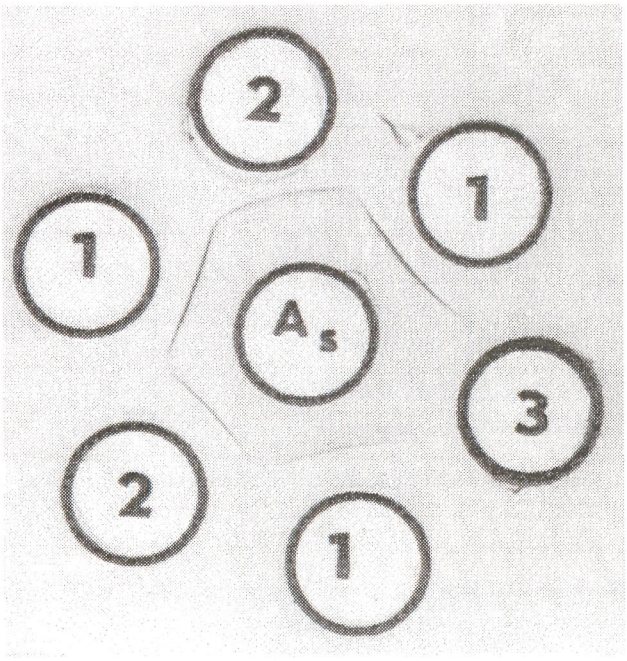

Fig. 8
Double diffusion test
Sap from BMV-H infected *N. tabacum* cv. Xanthi (1), sap from BMV-C infected *N. tabacum* cv. Xanthi (2), sap from healthy *N. tabacum* cv. Xanthi (3), and antiserum to BMV-H (middle well).

the double diffusion test. The saps from diseased deadly nightshade and artificially infected tobacco plants reacted with this antiserum and did not form spur lines (Fig. 8).

Discussion

The ultrastructural studies revealed the presence of a multiple infection (two viruses) in *A. belladonna*. The virus with isometric particles was serologically identified as BMV. BMV was found for the first time in Europe in Germany by Bode and Marcus (1959). It was established as a member of the tymoviruses and belongs to the Andean potato latent virus subgroup (Paul *et al.*,1968; Paul, 1969, 1971; Jankulowa *et al.*, 1968). The virus has also been known in other European countries, namely Bulgaria (Paul *et al.*, 1968), former Yugoslavia (Štefanac, 1974), Hungary (Horváth *et al.*, 1976) and recently in the former Czechoslovakia (Pelikánová *et al.*, 1992). Jankulowa *et al.* (1968), Štefanac (1974) and Horváth *et al.* (1976) compared European isolates of BMV from *A. belladonna* and found them serologically indistinguishable. However, Pelikánová *et al.* (1992) found the Czechoslovakian isolate of BMV coming also from deadly nightshade plants serologically distinguishable from BMV-H. It is reported here that an isolate of BMV coming from the same natural host plant and from the same region was indistinguishable, serologically, from a BMV-H isolate. It is possible that this new isolate is also serologically indistinguishable from other European isolates of BMV from *A. belladonna*.

Although several viruses with filamentous particles were found in *Atropa*, according to the length and a shape of particles only atropa mild mosaic virus (AMMV), a strain of henbane mosaic virus (HMV) apparently reminded of our virus isolate. AMMV is a RNA-containing virus with 800 or 900 nm long filaments and infects mainly solanaceous hosts. It is readily transmitted by inoculation of sap, and by aphids in the non-persistent manner. It was reported only from England, Germany and Italy, but it may be widespread in solanaceous weed hosts (Govier and Plumb, 1972). Nevertheless, we noted the differences in ultrastructure features of AMMV and those observed in our experiments. Harrison and Roberts (1971) described crystalline structures and pinwheels, a feature typical for viruses of the potato Y group (potyviruses), on ultrathin sections of tobacco leaves infected with AMMV. We did not find either the crystalline material or pinwheels in our preparations. The viral aggregates found in our study correspond to carlaviruses (Brunt *et al.*, 1976). Moreover, AMMV is reported to be readily transmissible by aphids *Myzus perzicae* Sulz. in brief feeding periods (Govier and Plumb, 1972; Harrison and Roberts, 1971). We were not successful in freeing of AMMV from BMV, although we used the same vector and the transmission experiments were done repeatedly. Nevertheless, affiliation of our virus isolate to carlaviruses according to the normal length of its particles (about 765 nm) is also questionable, because they range in normal length from 620 to 690 nm (Wetter and Milne, 1981). Unfortunately, appropriate serological tests were not done, because an antiserum against AMMV or HMV was not available. It is possible, that our virus culture contained apparently a new virus infecting deadly nightshade.

As regards the symptomatology observed in our experiments on naturally diseased *A. belladonna* and those previously described for single BMV or AMMV infection, there are also significant differences. The plants infected just with BMV or AMMV showed mosaic, mottling and vein banding symptoms on leaves (Govier and Plumb, 1972; Pelikánová *et al.*, 1992), while deadly nightshade infected synergically with a virus with flexuous particles and BMV in our experiments revealed only severe necrotic lesions. However, differential host plants artificially inoculated with this virus mixture culture revealed symptoms very similar to those described for BMV earlier, although electron microscopy of negatively stained preparations revealed the presence of both isometric and flexuous particles. In view of the fact that we were not able to separate these two viruses either on differential hosts or by aphids transmission, we

cannot clarify, if the symptomatology described is to be attributed mainly to BMV or a virus with flexuous particles. In order to solve this dilemma it will be necessary, in further research, to identify the roles of these viruses, both singularly and in association.

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