DAPHNE DECLINE – WHAT IS THE CAUSAL AGENT(S)?

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Summary. – A double infection of Daphne mosaic virus (DapMV) and an associated bacilliform virus was observed in the samples of diseased *Daphne mezereum* shrubs that showed mosaic patterns, precocious leaves reddening, defoliation, repeated flowering with subsequent declining. Extensive aggregations of bacilliform particles (166–370 x 65 nm and 169–233 x 68–78 nm) occurred in the nucleus or perinuclear space of root and leaf tissues suggesting that the virus might belong to the genus *Nucleorhabdovirus,* family *Rhabdoviridae.* However, the single bacilliform virions appeared occasionally in the cytoplasm of the leaf cells. We supposed that occurrence of the mixed virus infection could be the cause of *D. mezereum* decline in the Czech Republic.

Key words: Daphne mezereum declining, Daphne mosaic virus; electron microscopy, rhabdovirus-like particles

The plants of the genus *Daphne* are disposed to the frequent virus infection. Also mixed virus infections appear to be common, especially in vegetative propagated cultivars. In east and south of Bohemia, epidemic occurrence of DapMV, a putative member of the genus *Potyvirus,* family *Potyviridae* appeared in diseased *D. mezereum* plants that showed light green rings and mosaic pattern on the leaves (Fránová *et al*., 2006). Afterwards, some plants showed also a necrosis predominantly near the leaf edge, precocious leaves yellowing and reddening with branches defoliation (Fig. 1). The shrubs came into blossom two or three times during one year. In the decade from 1996 to 2006, most of the affected shrubs with severe symptoms declined and died out.

The DapMV infecting *D. mezereum* was well characterized and according to the nucleotide sequence of the coat protein gene (Acc. No. AY507723), it was proposed as a putative member of the genus *Potyvirus* (Fránová *et al*., 2006). Complete sequence of DapMV (Acc. No. DQ299908) allowed the identification of Papaya leaf distortion mosaic virus (Acc. No. NC005028) as the most related member of the genus *Potyvirus* with 46.1% amino acid identity (Petrzik and Fránová, 2006). It should be mentioned that DapMV has not been assigned to the genus *Potyvirus* yet (Faquet *et al*., 2005).

The virus was not sap-transmitted to any of the following common herbaceous virus indicator plant species as *Cucumis sativus* L., *Chenopodium album* L., *C. amaranticolor* Coste et Reyn., *C. quinoa* Willd., *Datura stramonium* L., *Nicotiana tabacum* L. cv. Samsun, *N. benthamiana* Domin, *N. clevelandii* Gray, *N. occidentalis* ssp. obliqua P1, *N. occidentalis* Wheeler, accession 37B, *N. rustica* L., *Petunia hybrida* Hort ex Vilm., and *Physalis floridana* Rybd. Furthermore, none of the following nine viruses was detected by direct double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) in crude sap from symptomatic leaves (Loewe Biochemica, Germany). We tested Alfalfa mosaic virus, Apple mosaic virus, Cucumber mosaic virus (family *Bromoviridae*), Arabis mosaic virus, Cherry leaf roll virus, Tomato ringspot virus (family *Comoviridae*), Strawberry latent ringspot (genus *Sadwavirus*), Tobacco mosaic virus (family *Togaviridae*), and Tobacco necrosis virus (family *Tombusviridae*).

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Abbreviations: DapMV = Daphne mosaic virus; DAS-ELISA = double-antibody sandwich enzyme-linked immunosorbent assay

Fig. 1

***D. mezereum* plant affected by mixed infection of DapMV and rhabdovirus(es)**

In the next step, we tested crude sap preparations from symptomatic leaves in potyvirus-specific ELISA. ELISA kit with antigen-coated plates used antibody AS-0573 for the general detection of potyviruses (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The results showed that the crude sap preparations from diseased *D. mezereum* reacted weakly in potyvirus-specific ELISA (data not shown). However, the flexuous filamentous particles sized 13 x 696 nm indicating the presence of potyviruses were detected in negatively stained preparations of all diseased plants examined by transmission electron microscopy (JEOL 1010). The immunoelectronmicroscopical decoration test (Milne, 1984) with two virus samples, one of them prepared from wild infected plant (south Bohemia) and one from infected cultivated plant (east Bohemia) were tested with 65 antisera to various potyviruses diluted 1:50 (Table 1). The antisera to Sweet potato chlorotic stunt virus Brunt (family *Closteroviridae*), Lettuce distortion mosaic, Lettuce poty 96-685, and Luzula leaf streak viruses (unassigned viruses) were used as well. For the heterologous combinations, the antiserum dilution endpoints were determined with two-fold dilution series. The results showed that both virus samples reacted identically and not very strongly with 5 antisera against Chilli veinal mottle, Colombian datura, Papaya ringspot, Tobacco vein mottling and Yam mosaic viruses. The titres of the positive reactions were determined at most to 1:400, although the respective homologous titres were at least 1:6,400. Since none of the antisera reacted as strongly as with their homologous antigens, the results suggested that the tested virus samples appeared to involve a serologically distinct potyvirus. The results of decoration test were in agreement with results of potyvirus-specific ELISA mentioned above, e.g. there were only weak reactions with DapMV infected materials.

However, serology has its essential value in diagnosis, but it has a limited value in taxonomy. There are a growing number of examples, when serological relationships turned out to be inadequate for a clear-cut taxonomical assignment of a new virus. Currently, the most reliable method for assessment of a new virus is the comparison of the nucleotide or amino acid sequences. Correspondingly, the potyvirus Shallot yellow stripe virus was recognized as serologically related to Alstroemeria flower banding virus, but they were very different in their coat protein sequences (Pfeilstetter and Lesemann, unpublished). The analogous cases came out also among tymoviruses and tombusviruses (Koenig *et al*., 2004, 2005; Shukla and Ward, 1988). Conversely, serological data may fail to recognize the distinctiveness of two serologically related viruses, which are clearly distinct according to the sequence data.

The electronmicroscopical examination of ultrathin sections prepared from leaf, branch, and root of the diseased plants showed the presence of bacilliform rhabdovirus-like particles, in addition to the presence of DapMV. The shrubs affected by double virus infection showed not only mosaic patterns as described for DapMV-infected plants, but also the precocious leaves yellowing

and reddening, necrosis, defoliation, and finally dieback of the plants. To our knowledge, the only case of detection of bacilliform rhabdovirus-like particles was detected in the symptomless plants of *D. mezereum* in Czech Republic (Fránová *et al*., 2001). Unfortunately, neither antibodies specific for the members of the family *Rhabdoviridae* nor the group-specific primers for rhabdovirus detection by PCR were available for definite proof of the rhabdovirus presence. Also, serological and PCR analyses of the anticipated rhabdovirus(es) were not accomplished. In addition, repeated mechanical inoculation of crude sap from symptomatic leaves to various host plants was unsuccessful. On the other hand, considering the shape and size of bacilliform virions (166–370 x 65 nm in root tissues, 169–233 x 67–78 nm in leaf tissues), we supposed that the observed bacilliform virus might be a member of the family *Rhabdoviridae.* Bacilliform particles differed in morphological characteristics from those described in healthy-looking *D. mezereum,* which were shorter and relatively wider (162–215 x 62–75 nm) (Fránová *et al*., 2001). They preferentially accumulated in cytoplasmic vesicles, as well as they were within the nucleus and perinuclear space. Moreover, all non-nuclear particles were membrane-bound, either singly or in groups. The unenveloped particles were not observed. In diseased tissues, the viral particles accumulated in large aggregates enclosed by a thin membrane and sporadically scattered directly in the nucleoplasm in the cells of roots, branches and leaves (Fig. 2). Extensive virus aggregations led to the production of nuclear invaginations filled with the virus particles seen in the perinuclear space (Fig. 3). In some cases, nuclei contained the aggregates of virions were abnormally enlarged. Occasionally, mass of the particles invaded through membrane into cytoplasm (Fig. 4). In the parenchymatic cells of the diseased leaves and branches, tubular particles were seen singly and free of the membrane in the cytoplasm (Fig. 5). The co-infection of the same cell by bacilliform and filamentous virions or cylindrical inclusions typical for DapMV was observed rarely (Fránová *et al*., 2006).

In the root tissue, the individual virions appeared to be bacilliform with the length approaching 166–370 nm and with diameter approx. 65 nm. They were randomly orientated or packed side by side (Fig. 6). These particles appeared to be composed of an outer double-layered envelope about 15 nm thick surrounding an electron-translucent zone about 35 nm in diameter that contains a central core of about 12 nm wide. This core seemed to correspond with the axial channel of the presumptive nucleocapsid. The cross-section of the particles exhibited an annular profile. The densely stained, spiked outer zone was separated from the inner stained circle with the central core largely unstained with or without central dot. The longitudinal-section showed bullet-shaped or rod-like patterns.

Table 1. Reactivity of antisera to potyviruses used for the electronmicroscopical decoration test of two isolates DapMV

Antiserum against the virus	Decoration titer*
Species of the genus *Potyvirus*	
Alstroemeria mosaic virus	Neg.
Amaranthus leaf mottle virus	Neg.
Apium virus Y	Neg.
Artichoke latent virus	Neg.
Asparagus virus 1	Neg.
Bean common mosaic necrosis virus	Neg.
Bean common mosaic virus NY 15	Neg.
Blackeye cowpea mosaic virus	Neg.
Peanut stripe virus	Neg.
Bean yellow mosaic virus	Neg.
Beet mosaic virus	Neg.
Bidens mottle virus	Neg.
Carnation vein mottle virus	Neg.
Carrot thin leaf virus	Neg.
Celery mosaic virus	Neg.
Chilli veinal mottle virus	400
Clover yellow vein virus	Neg.
Cocksfoot streak virus	Neg.
Colombian datura virus	400
Cowpea aphid-borne mosaic virus	Neg.
Endive necrotic mosaic virus	Neg.
Groundnut eyespot virus	Neg.
Henbane mosaic virus	Neg.
Hippeastrum mosaic virus	Neg.
Kalanchoë mosaic virus	Neg.
Konjac mosaic virus	Neg.
Leek yellow stripe virus	Neg.
Lettuce mosaic virus	Neg.
Lily mottle virus	Neg.
Moroccan watermelon mosaic virus	Neg.
Narcissus degeneration virus	Neg.
Narcissus late season yellows virus	Neg.
Narcissus yellow stripe virus	Neg.
Papaya ringspot virus	200
Passion fruit woodiness virus	Neg.
Pea seed-borne mosaic virus	Neg.
Peanut mottle virus	Neg.
Pepper mottle virus	Neg.
Pepper veinal mottle virus	Neg.
Peru tomato mosaic virus	Neg.
Plum pox virus	Neg.
Potato virus A	Neg.
Potato virus A, strain 1,2,3	Neg.
Potato virus V	Neg.
Potato virus Y ^{N}	Neg.
Potato virus Y ^{O}	Neg.
Potato virus Y $^{N+O}$	Neg.
Sorghum mosaic virus	Neg.
Soybean mosaic virus	Neg.
Sweet potato feathery mottle virus	Neg.
Tobacco etch virus	Neg.
Tobacco vein banding mosaic virus	Neg.
Tobacco vein mottling virus	200
Turnip mosaic virus	Neg.
Watermelon mosaic virus	Neg.
Wild potato mosaic virus	Neg.
Wisteria vein mosaic virus	Neg.
Yam mosaic virus	400
Zucchini yellow mosaic virus	Neg.
Tentative species of the genus *Potyvirus* and other *Potyviridae*	
Alstroemeria flower banding virus	Neg.
Maclura mosaic virus	Neg.
Narcissus latent virus	Neg.
Pepper mild mosaic virus	Neg.
Sunflower chlorotic mottle virus, Lenardon	Neg.
White bryony virus, Germany	Neg.

*reciprocal value.

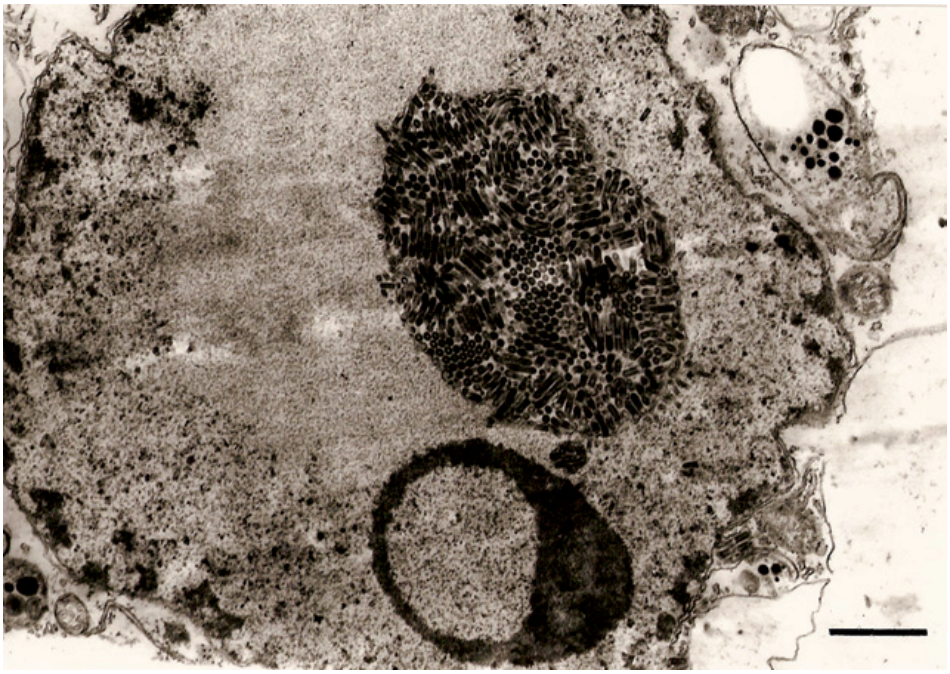

Fig. 2

Ultrathin section of root tissue from diseased *D. mezereum*

Aggregates of rhabdovirus-like particles in the nucleus enclosed by a membrane. Single particles are visible in nucleoplasma and perinuclear space. Bar = 1 μm.

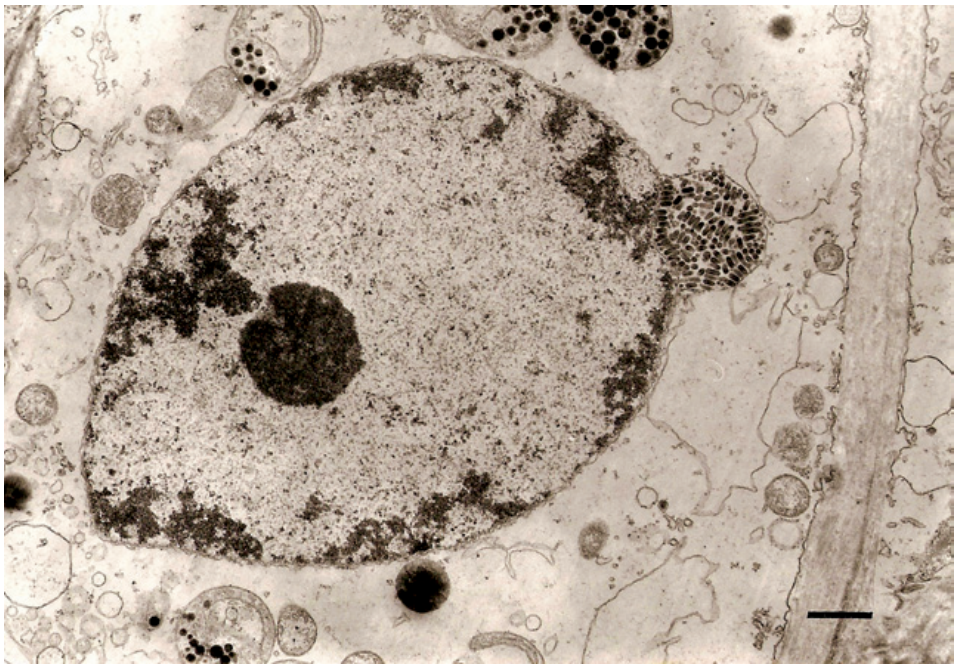

Fig. 3

Ultrathin section of root tissue from diseased *D. mezereum*

Rhabdovirus-like particles accumulated in enlarged perinuclear space between two nuclear membranes. Bar = 1 μm.

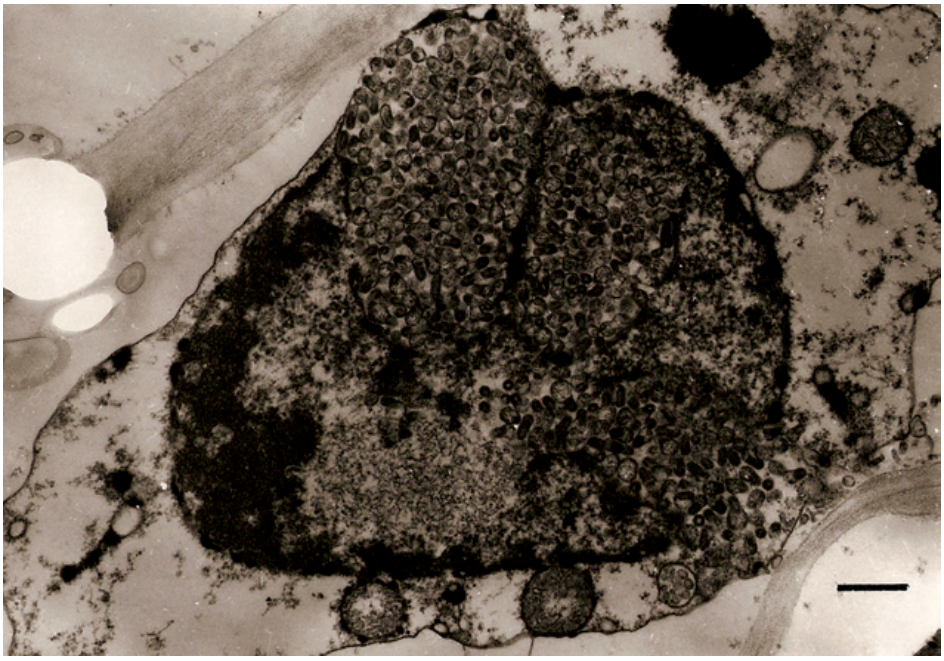

Fig. 4

Ultrathin section of branch tissue sieve tube element from diseased *D. mezereum*

Enlarged and deformed nucleus partially filled with rhabdovirus-like particles mostly bound by a membrane. Bar = 500 nm.

The morphological similarity and size of bacilliform particles in the cytoplasm of leaf and branch tissues corresponded with the particles observed in the nuclei of root tissues (166–370 x 65 nm). However, some differences were observed in their morphology when compared with the particles detected in nuclei of leaves and branches: their length varied from 169–233 nm and the width of 68–78 nm. Particles in nuclei of leaf and branch tissues were mostly bound to a membrane or one end of the particle was abnormally enlarged and formed globular envelope approx. 115 nm in diameter (Fig. 7). We detected no virus-like particles, inclusions or aggregates in preparations of healthy plants of *D. mezereum*.

The members of *Rhabdoviridae* family infecting plants are assigned into two genera *Nucleorhabdovirus* and *Cytorhabdovirus* according to the site of virus maturation (Faquet *et al*., 2005). The cytorhabdoviruses replicate in the

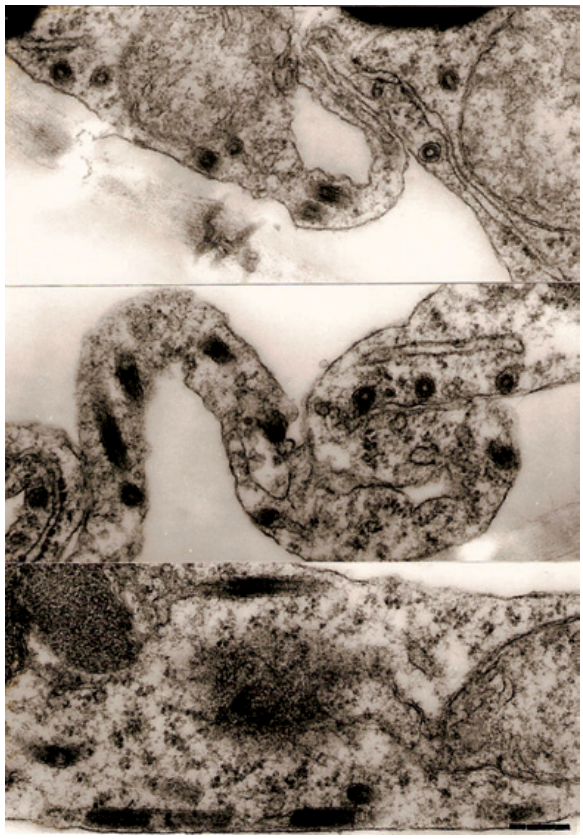

Fig. 5

Ultrathin section of cytoplasm of spongy parenchyma cells of *D. mezereum* leaves

Longitudinally sectioned bacilliform particles with their axial channel and transversally sectioned particles exhibiting their annular profile. Bar = 200 nm.

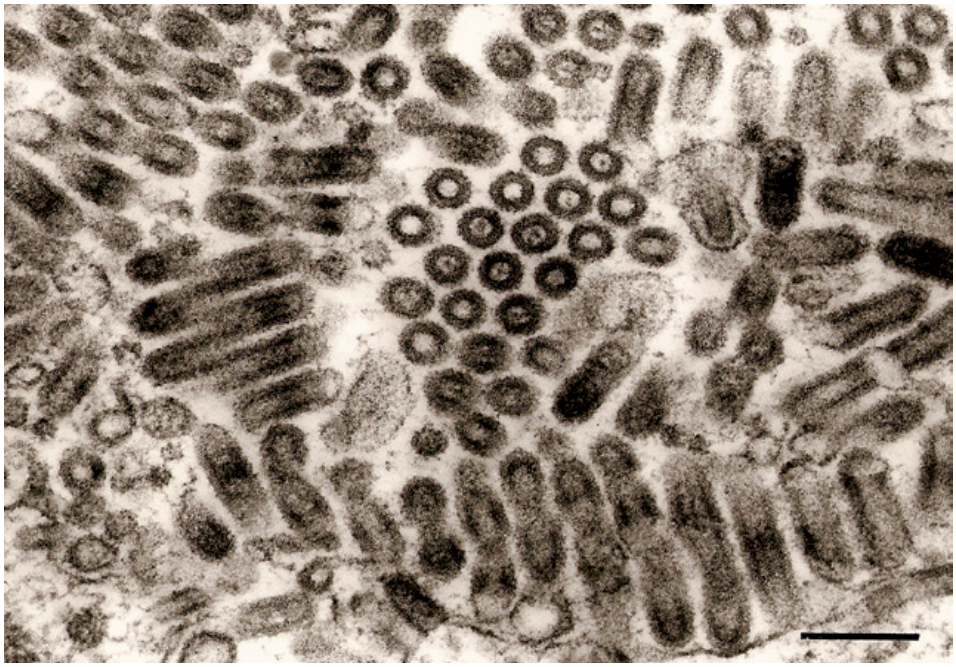

Fig. 6

Cross and longitudinally sectioned rhabdovirus-like particles in nucleus of *D. mezereum* root cell

Bar = 200 nm.

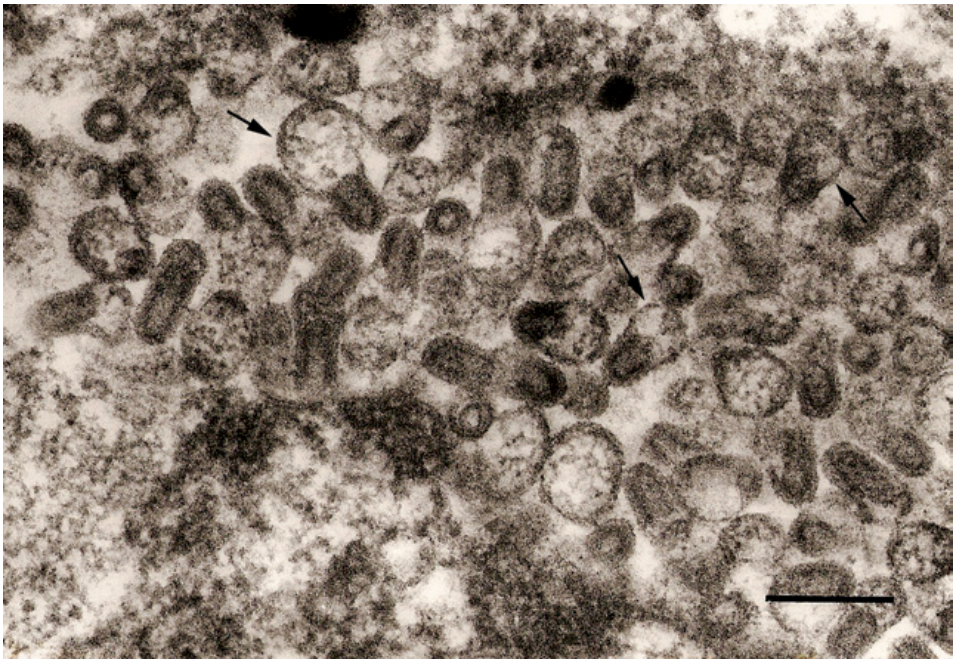

Fig. 7

Rhabdovirus-like particles bounded by a membrane (arrows) in the nucleus of parenchymatous cells in branch tissue of *D. mezereum*

Bar = 200 nm.

cytoplasm and the nucleorhabdoviruses replicate in the nucleus of infected cells. In relation to the presented results, it was not possible to determine, whether the observed virus was a member of the genus *Cytorhabdovirus* or *Nucleorhabdovirus.* We observed the rhabdovirus-like particles associated predominantly with the nuclei suggesting that they might belong to the genus *Nucleorhabdovirus*. However, the rhabdovirus-like particles appearing at the nuclear periphery might be different from those occurring singly in the cytoplasm. It was possible that we detected two different rhabdoviruses. We are aware of the fact that our results did not provide direct proof of the identity of the rhabdovirus-like particles. Since the filamentous virions of DapMV were observed singly in plants with light green rings and mosaic pattern (Fránová *et al*., 2006) and double infection with DapMV and rhabdovirus(es) was detected in declined shrubs, the dieback of *D. mezereum* in the Czech Republic could be attributed to the presence of the mixed virus infection. In order to elucidate this hypothesis, it will be necessary to identify the role of these pathogens in the single as well as in the mixed infection. Nevertheless, this is the first report describing the detection of mixed virus infection of *D. mezereum* in Czech Republic.

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