MIXED INFECTION OF BLACK CURRANT (*RIBES NIGRUM* L.) PLANTS WITH BLACKCURRANT REVERSION ASSOCIATED VIRUS AND RHABDOVIRUS-LIKE PARTICLES WITH SYMPTOMS OF BLACK CURRANT REVERSION DISEASE

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Summary. – Black currant plants of cvs. Black Smith and Karlštejnský dlouhohrozen showing symptoms of severe Russian (R) form of black currant reversion disease (BCRD) were found in 1999–2000 in the Czech Republic. Five selected plants of both cultivars originating from two distant loci were tested by polymerase chain reaction (PCR) for presence of the Blackcurrant reversion associated virus (BRAV), the causal agent of BCRD. In all plants, virus-specific 215 nt cDNA fragments proving the presence of BRAV were obtained. Moreover, in two of those five black currant plants, rhabdovirus-like particles were found in ultrathin sections by electron microscopical examinations. The particles measured 200–347 nm by 64–90 nm. They occurred mostly within nuclei of parenchyma cells of vascular bundles as single particles, rafts of particles, but also in aggregates. They were found also in the perinuclear space and occasionally directly in the cytoplasm. Clusters of particles either within the nucleus or in the perinuclear space were membrane-bound. We bring evidence on the occurrence of the severe (R) form of BCRD and the first evidence of BRAV in the Czech Republic.

Key words: black currant reversion disease; electron microscopy; PCR; rhabdovirus-like particles; Ribes spp.; Blackcurrant reversion associated virus

Introduction

Reversion of black currant, the most important virus disease in commercial *Ribes* spp. has been first described in the Netherlands (Ritzema Bos, 1904) and later in England (Amos and Hatton, 1927). The reversion occurs at least in two forms: the common (European, E form) and the severe (Russian, R form), the latter limited to the eastern and central Europe and Scandinavian Peninsula (Jones, 1993). So far, the forms could be differentiated only by symptoms. Recently, BRAV belonging to the *Blackcurrant reversion associated virus* species, the *Nepovirus* genus, the *Comoviridae* family has been discovered as the causal agent of BCRD (Lemmetty and Lehto, 1999).

In Czechoslovakia the disease has been first reported by Blattný (1930) as causing in 1940es and 1950ies heavy damages in particular in the border regions of Bohemia (Blattný *et al.*, 1956). The early records (Blattný *et al.*, 1956) have indicated only the incidence and symptoms of the disease, which name was commonly used for virus-like disorders in currants by growers.

Since 1998 we have observed in the Czech Republic severe infestations of red and white currants with the full blossom disease, reported by Rakús *et al.* (1974) as a phytoplasma disease. Studying the etiology of the full blossom disease, we have conducted simultaneous comparative PCR experiments and electron microscopical

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Abbreviations: BCRD = black currant reversion disease; BRAV = Blackcurrant reversion associated virus; E = European; R = Russian; PCR = polymerase chain reaction; RT-PCR = reverse transcription–PCR

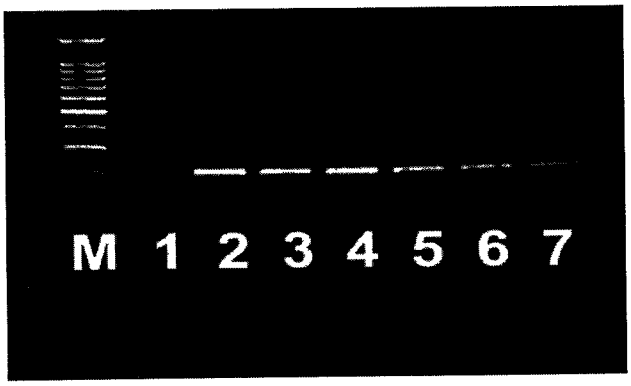

Fig. 1
Detection of BRAV-specific 215 bp fragment amplified from black currant plants by RT-PCR

Agarose gel electrophoresis. A 100 bp DNAsize marker (lane M); negative control, healthy cv. Öjebyn, Finland (lane 1); symptomatic plants of cv Karlštejnský dlouhohrozen, location Chrastiny near Písek (lanes 2, 3 and 4); symptomatic plants of cv. Black Smith, Holovousy (lanes 5 and 6) – BRAV positive control cv. Mortii, Finland (lane 7).

observations on BCRD-affected black currants, which we report in this paper.

Materials and Methods

Plant material. Three plants of cv. Karlštejnský dlouhohrozen were taken from a plantation at Chrastiny near Písek, southern Bohemia in May 1999 and two plants of cv. Black Smith from the Research and Breeding Institute of Pomology, Holovousy in East Bohemia in May 2001. All the five plants showed symptoms typical of the R form of BCRD with severe flower malformations: elongated style, absence of stamens and an increase in the number of petals. The affected flowers were sterile. In comparison with healthy plants, leaves from reverted plants were narrower, contained a lower number of main veins and had larger but fewer marginal serrations. These plants were examined by reverse transcription – PCR (RT-PCR) for BRAV detection and by electron microscopy. As negative control samples from virus-indexed healthy nuclear stock plant of black currant cv. Öjebyn and as a positive control for BRAV back-inoculated black currant cv. Mortii were used. Both controls were obtained from Dr.A. Lemmetty, Agrifood Research Finland, Jokioinen, Finland.

Virus detection. Young leaves of healthy and diseased black currant plants were used for total RNA extraction from about 100 mg of fresh tissue according to the RNeasy Plant Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues (Qiagen). RNA extracts were used in RT-PCR according to the One-Step Protocol for RT-PCR reactions (Ready-To-Go™ RT-PCR Beads, Pharmacia Biotech Inc.) according to the manufacturer's instructions (Lehto and Hirvonen, 1999). The primers 1 (5'-GTAA TACGCTGGTGTCTC-3') and 2 (5'-GAAAGGACATTTCAGA CTC-3') were used to amplify a 215 nt 3'-terminal fragment of BRAV RNA 2 according to Lemmetty *et al.* (2001). RT-PCR beads were designed for a 50 µl reaction volume per tube. Each reaction contained 2 U of Taq DNA polymerase, 10 mmol/l Tris-HCl pH 9.0, 60 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 200 µmol/l dNTPs, Moloney murine leukemia virus (MMuLV) reverse transcriptase (FPL Cpure®), RNAguard® ribonuclease ihibitor (porcine) and stabilizers, including RNase/DNase-free BSA. Each bead was suspended in 43 µl of sterile water and 1 µl (10 pmoles) of each primer and 5 µl of RNA as template. Amplification consisted of 29 cycles and was conducted in a MJ Research Thermocycler (Watertown, USA). The following parameters were used: initial incubation at 42°C for 30 min, initial denaturation at 95°C for 5 mins, 29 cycles 95°C for 30 secs, 54°C for 30 secs, and 72°C for 30 secs and final extension at 72°C for 5 mins (A. Lemmetty, personal communication).

Agarose gel electrophoresis. Thirty µl of each virus-specific 215-nt cDNA fragment was analyzed by electrophoresis in a 2% agarose gel in 40 mmol/l Tris-acetate and 1 mmol/l EDTA pH 8.0, followed by staining with ethidium bromide and visualization of DNA bands on a UV transiluminator (Lemmetty *et al.*, 2001). A 100 bp DNA size marker (Gibco BRL) was employed.

Electron microscopy. Samples of midribs, leaf petioles and flower stalks from the five abovementioned symptomatic shrubs were examined. Pieces (approximately 2 x 2 mm) of tissues were fixed in 0.1 mol/l potassium phosphate buffer pH 7.3, 5% glutaraldehyde and 4% sucrose for 24 hrs at 4°C under mild vacuum. The samples were postfixed in 1% osmium tetraoxide, dehydrated in ethanol in a standard way and embedded in Durcupan resin (Fluka). Thin sections were double-stained with uranyl acetate and examined in a Jeol 100 MB electron microscope.

Results and Discussion

Using the BRAV-specific primers a 215-nt product (Fig. 1) was obtained from all symptomatic plants of both cultivars (Karlštejnský dlouhohrozen and Black Smith) and from the

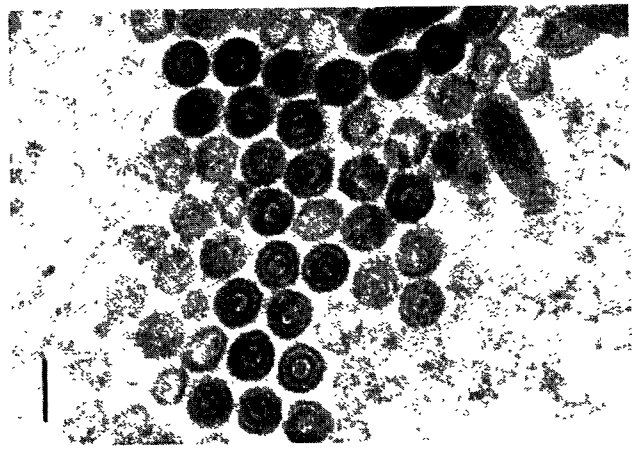

Fig. 2
Rhabdovirus-like particles

Electron microscopy, ultrathin section. The densely stained, spiked outer zone is clearly separated from an inner stained circle, the central core is largely unstained, with central dot. The bar = 100 nm.

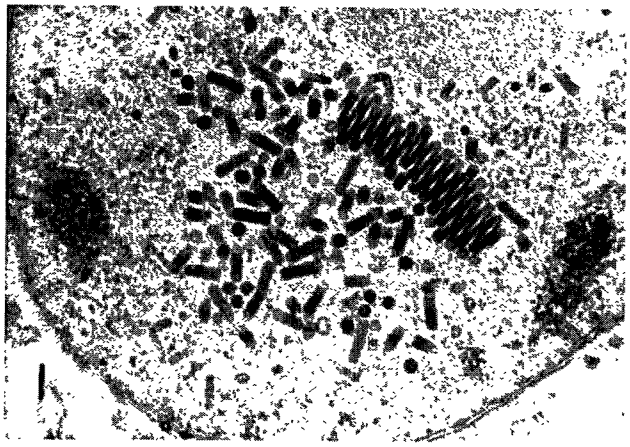

Fig. 3
Bacilliform virions with rounded ends dispersed singly or forming rafts in the nucleoplasm
Electron microscopy, ultrathin section. The bar = 200 nm.

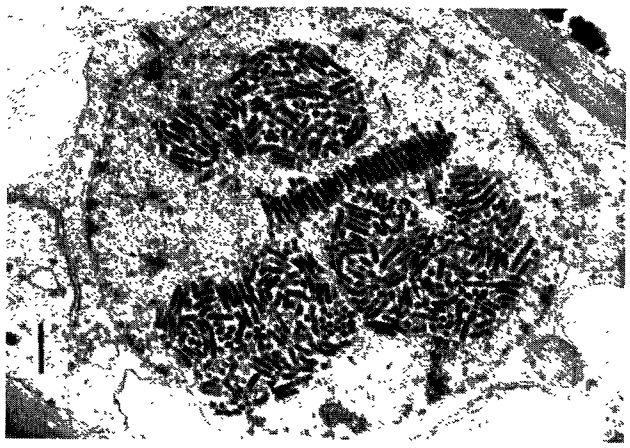

Fig. 4
Membrane-associated rhabdovirus-like particles forming rafts or dispersed singly in the nucleus and in perinuclear space of parenchyma cell of vascular bundle
Electron microscopy, ultrathin section. The bar = 500 nm.

positive but not negative control. In addition, in one of the three plants of cv. Karlštejnský dlouhohrozen and in one of the two plants of cv. Black Smith rhabdovirus-like particles in ultrathin sections of flower stalks were observed. The particles were 200–347 nm x 64–90 nm in size (Fig. 2). They occurred mostly within the nucleus of parenchyma cells of vascular bundles as single particles, rafts of particles (Fig. 3) and aggregates (Fig. 4). They were found also in the perinuclear space (Fig. 5) and occasionally directly in the cytoplasm (Fig. 6). Single particles were often dispersed within the nucleoplasm generally around the nucleolus (Fig. 7). Clusters of particles either within the nucleus or in the perinuclear space were membrane-bound. Neither phytoplasma bodies nor another virus-like particles were observed on electron micrographs. No particles were also found in the negative (healthy) control. Rhabdovirus-like particles have been firstly found in leaf and petal tissues of three plants of black currant cv. Ben Nevis affected with the R form of BCRD in United Kingdom but not in those affected with the E form of BCRD (Roberts and Jones, 1997). Our results are the first confirmation of the presence of rhabdovirus-like particles in BCRD R-form-affected black currants. In addition to the study of Roberts and Jones (1997) we proved BRAV in plants by RT-PCR in another two cultivars and at

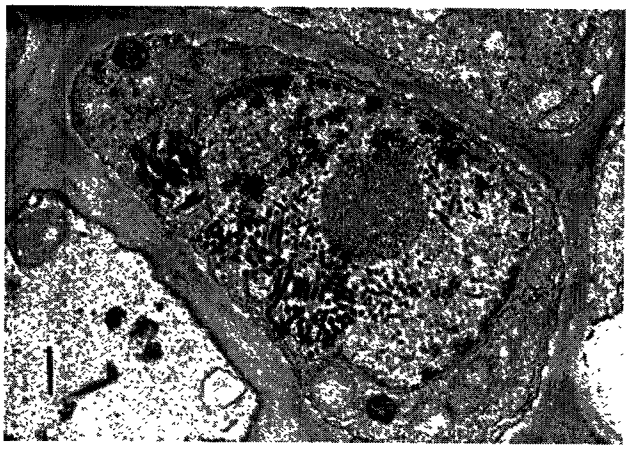

Fig. 5
Nucleus with virions accumulated in enlarged perinuclear space and dispersed in the nucleoplasm
Electron microscopy, ultrathin section. The bar = 500 nm.

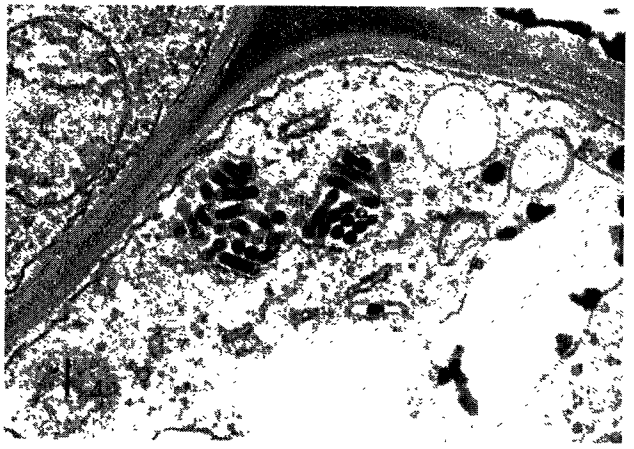

Fig. 6
Rhabdovirus-like particles forming small aggregates in cytoplasm
Electron microscopy, ultrathin section. The bar = 200 nm.

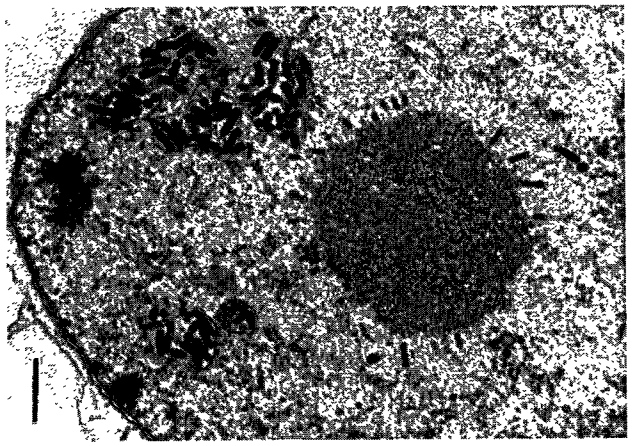

Fig. 7
Rhabdovirus-like particles forming a small groups or dispersed singly within the nucleoplasm
Electron microscopy, ultrathin section. The bar = 500 nm.

two distant loci of continental Europe. Plants of cv. Black Smith were brought to the germplasm collection of the Research and Breeding Institute of Pomology, Holovousy from Slovakia in 2000.

In both cultivars, we did not observe any difference in symptoms between plants infected with BRAV and rhabdovirus, and those infected only with BRAV. As all plants showed typical reversion disease, it seems that the mixed infection with the rhabdovirus does not influence the symptoms.

There is no more recent evidence on the occurrence of BCRD and BRAV in the Czech Republic. Both black currant cultivars, which we found to be infected with BRAV showed malformed flowers and leaves characteristic for the R form of BCRD. We hereby bring additional evidence on the occurrence of the R form of BCRD and the first evidence on the occurrence of BRAV in the Czech Republic.

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