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Occurrence of RBDV in Latvia and virus elimination *in vitro* by chemotherapy

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To determine the incidence and distribution of Raspberry bushy dwarf idaeovirus (RBDV) in Latvia 27 commercial and varietal collection plantations of *Rubus* spp. were surveyed in the spring of 2007. In total 224 leaf samples from 59 genotypes were collected for analyses. A combination of meristem tip culture with different antiviral compounds was used to test virus elimination possibilities *in vitro* from naturally infected plants of cultivar 'Babje Leto 2'. Plant samples for RBDV infection and the efficiency of virus elimination were verified by double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) using polyclonal antibodies. The obtained results showed that RBDV was spread in 70 % of surveyed raspberry plantations. The incidence of RBDV in the tested plants was 35 % and varied greatly among the cultivars. Most of the commonly grown cultivars from Eastern Europe, such as 'Kirzach', 'Balzam' and 'Sputnica', were infected with RBDV. Virus was not detected in plant samples of cultivar 'Tulameen'. RBDV elimination combining meristem culture with ribavirin for all treated plants was unsuccessful. Treatment with azacytidine and dicyanamide was effective only for meristem clones originated from one mother plant. It suggests that the particular plants were infected with a stable virus isolate, which cannot be eliminated with chemotherapy and *in vitro* propagation techniques. To develop effective RBDV elimination procedures more work is necessary to characterize the virus isolates infecting raspberry and to optimise *in vitro* techniques. The experiments are being continued.

Key words: antiviral compounds, chemotherapy, DAS ELISA, meristem culture, *Rubus* spp.

Introduction. Red raspberry (*Rubus idaeus* L.) is one of the most important small fruit crop in Latvia, which occupies about 10 % of commercial fruit plantation area. Raspberry viruses are wide spread and they cause severe losses of yield and quality everywhere raspberry is cultivated. Raspberry bushy dwarf idaeovirus (RBDV) infects wild and cultivated *Rubus* spp. plants throughout the world and is one of the most important pathogens of red raspberry (Natsuaki et al., 1991). Every year viral diseases cause significant raspberry yield loses due to premature defoliation, decreased vigour, leaf curling, necrosis, abortion of drupelets, death of lateral

shoots and increased winter kill (Mavrič Pleško et al., 2009). In some red raspberry, such as *R. idaeus* var. *idaeus* L. and *R. idaeus* var. *strigosus* Maxim, RBDV induces yellows disease, crumbly fruit and decrease of vigour. RBDV isolates are categorised into two groups: Scottish strain (RBDV-S) and resistance breaking strain (RBDV-RB). Most isolates studied worldwide fall into S group strain and some raspberry cultivars are resistant to this strain. Whereas RBDV-RB strain can infect raspberry cultivars that are immune or highly resistant to S type strain (Jones et al., 2000). RBDV is efficiently transmitted via seed and pollen. Other ways of natural transmission are not known. Infected wild and cultivated raspberries act as virus natural reservoirs (Wang et al., 2008). The only way to decrease RBDV spread in raspberry plantations is to use healthy planting material and resistant cultivars. Thermotherapy, chemotherapy, and tissue culture techniques have been used either alone or in combination to eliminate viruses from plants (Spiegel et al., 1993). The meristem-tip culture has been used widely for the production of virus-free plant material in many species propagated mainly by vegetative means (Manganaris et al., 2003). The chemotherapy is one of the newest methods for elimination of plant viruses and is widely used in combination with micro-propagation (Cieslinska, 2003). Compounds such as ribavirin, 5-azacytidine and other antiviral compounds have been successfully utilized for virus elimination in other economically important crops (Nascimento et al., 2003).

The certification program for raspberry planting material has not been established in Latvia. Therefore, the risk that RBDV has spread uncontrolled in raspberry plantations with infected planting material and by natural transmission during the long period of time is very high. Previous research on raspberry viruses was carried out in 1970s and was based only on visual observations and biological indexing. Nowadays data are not available about the spread of viral diseases in raspberry plantations including such important pathogen as RBDV. The aim of this research was to determine the incidence of RBDV in raspberry plantations and to investigate RBDV elimination possibilities by combining micro-propagation and chemotherapy techniques.

Object, methods and conditions. Surveys and plant sampling. During the spring of 2007 twenty seven commercial and varietal collection plantations of red raspberry and blackberry were surveyed in all regions of Latvia. In total 224 leaf samples were collected from 59 genotypes. From them 60 samples were collected in varietal collections. Leaflets were collected randomly from each cultivar in rows approximately ¼, ½ and ¾ of the way across the fields (Strik, Martin, 2003). From plants with visible symptoms additional samples were collected. The samples were transported to the laboratory in ice bag, proceeded immediately for analyses or stored at −80 °C.

Micropropagation and chemotherapy. To test the virus elimination possibilities *in vitro* 23 plants of raspberry cultivar 'Babje Leto 2' were selected from field trial at Pūre Horticulture Research Centre. Plant initiation *in vitro* was carried out by using meristem tip explants from the apical and lateral buds of naturally infected plants with RBDV. Modified M&S basal salt medium (Murashige, Skoog, 1962) containing ¼ of nitrates and double strength Fe salts, supplemented with 170 mg L^{-1} KH_2PO_4 and 0.4 mg L^{-1} thiamine, 2 mg L^{-1} BAP, 0.05 mg L^{-1} ISS and 0.1 mg L^{-1} GA_3 were used for initiation of microplants. The microplants were cultivated *in vitro* for five passages in above described medium containing BAP 1 mg L^{-1}. For

chemotherapy three variants of media supplemented with antiviral chemicals were compared. Antiviral compounds used were ribavirin 30 mg L^{-1} (Sharma et al., 2007), azacytidine 25 mg L^{-1} (Nascimento et al., 2003) and dicyanamide 25 mg L^{-1} (Bittner et al., 1989). The previously used propagation medium without antiviral compounds was used as a control. Twenty microplants were used in each treatment. The effect of antiviral compounds on microplants was recorded as a number of survived plants after 25 days. The data from the chemotherapy treatments were subjected to analysis of variance (ANOVA) and mean values were compared using less significant difference (LSD) at 95 % significance level.

R B D V d e t e c t i o n. For the detection of RBDV in plant material commercially available double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) kit (Bioreba AG, Switzerland) was used in all investigation steps according to the manufacturer instructions with some modifications. The coating and conjugate conditions were changed from manufacturer standard procedure of 4 h incubation at 30 °C to an overnight incubation in the refrigerator at 4–6 °C. The absorbance was read at 405/492 nm with dual filter microplate reader (Asys Expert 96, Hitech, Austria) after 30 min, 1 h and 2 h incubation. A "cut-off" value was calculated according to manufacturer technical information (Bioreba AG, Switzerland).

Results. During the surveys in *Rubus* spp. plantations chlorotic spots on leaves, yellowing, mottling, undersized height and crumbly fruits were observed. The presence of RBDV by DAS ELISA was detected in 70 % of surveyed plantations. From three surveyed raspberry farms all the tested samples were infected with RBDV. The incidence of RBDV in plant samples was 35 %.

Raspberry cultivars 'Balzam' and 'Sputnitsa' were the most infected with RBDV among the other tested cultivars, but all the samples from cv. 'Tulameen' were negative with DAS ELISA (Fig.).

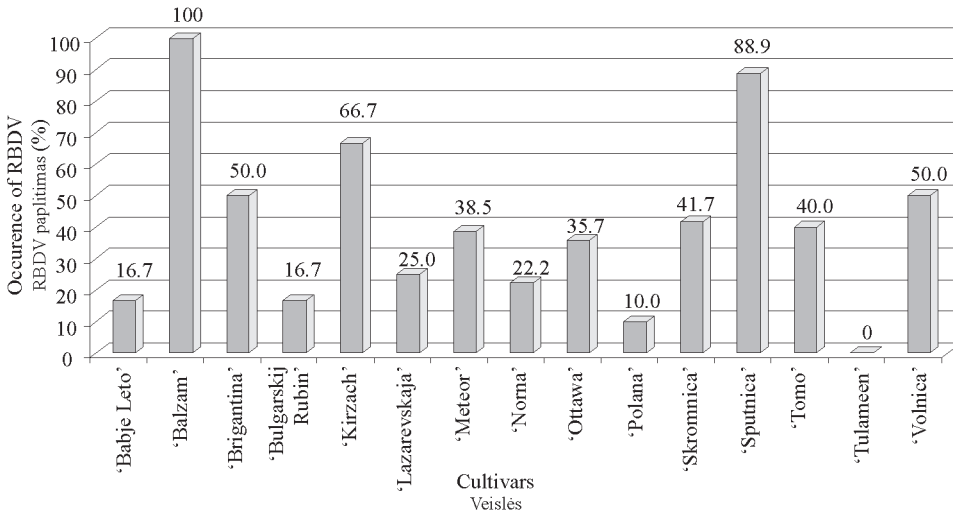

Fig. Occurrence (%) of RBDV in tested raspberry samples
Pav. RBDV paplitimas tirtuose aviečių pavyzdžiuose, %

For the tests for virus elimination possibilities in the field selected raspberry plants of cultivar 'Babje Leto 2' showed typical symptoms of RBDV infection, such as, undersized height, small, yellow foliages and crumbly fruits. According to DAS ELISA test results, it was proved that nine raspberry plants are infected with RBDV. Infected raspberry plants with typical RBDV symptoms and positive reaction in DAS ELISA test were propagated in vitro for five passages and obtained microplants were tested for RBDV infection after third and fifth passage. After third passage RBDV was detected in meristem clones originated from two mother plants, but after fifth passage RBDV was detected in meristem clones from four mother plants (Table 1).

Table 1. RBDV in raspberry meristem clones after third and fifth passage *in vitro*
1 lentelė. RBDV aviečių meristeminiuose klonuose po trečiojo ir penktojo persodinimo *in vitro*

Mother plant No. Motininio augalo Nr.	3rd passage* Trečiasis persodinimas	5th passage* Penktasis persodinimas
1.13	-	-
2.18	+	+
2.19	+	+
2.21	-	-
3.13	-	+
3.14	-	-
3.15	-	+

* + RBDV detected by DAS ELISA; - RBDV not detected by DAS ELISA test
* + RBDV aptikta DAS ELISA testu; - RBDV neaptikta DAS ELISA testu

To improve RBDV elimination from infected microplants, infected meristem clones from three mother plants were treated by chemotherapy in mediums amended with different antiviral compounds. After 25 days different reaction of plants to the amendment of antiviral compounds in the medium was observed. The significantly highest amount of necrotic plants was observed in the medium containing ribavirin. The percentage of survived plants in other two media containing antiviral chemicals was high and did not differ significantly from control medium (Table 2).

Table 2. Amount of survived raspberry microplants (%) after 25 days of chemotherapy
2 lentelė. Išlikusių aviečių mikro augalų kiekis (%) praėjus 25 dienoms po chemoterapijos

Treatment Variantas	Mother plant No. Motininio augalo Nr.		
	2.18	2.19	3.15
Control / Kontrolė	99	96	99
Ribavirin 30 mg L^{-1}	70	60	83
Azacytidine 25 mg L^{-1}	100	100	100
Dicyanamide 25 mg L^{-1}	98	100	100
$\gamma_{0.05}$	0.04	0.04	0.03

After chemotherapy microplants were tested with DAS ELISA for RBDV infection. Meristem clones originated from mother plants 2.18 and 2.19 after chemotherapy showed positive results on RBDV (Table 3).

Table 3. The efficiency of chemotherapy
3 lentelė. Chemoterapijos efektyvumas

Mother plants No. Motininio augalo Nr.	Control Kontroė	Ribavirin	Azacytidine	Dicyanamide
2.18.	+	+	+	n/a
2.19.	+	+	+	+
3.15.	+	+	-	-

The RBDV elimination with ribavirin, azacytidine or dicyanamide for those clones was unsuccessful. Meristem clones originated from mother plant 3.15 remained infected with RBDV after treatment with ribavirin, but treatment with azacytidine or dicyanamide was successful according to DAS ELISA test.

Discussion. The research presented here demonstrated that RBDV is widespread in raspberry plantations in Latvia and that all commonly grown cultivars are infected. In the countries where certification programmes are not established, like in Chile, the incidence of RBDV is 35–68 % (Medina et al., 2006), what corresponds to the data obtained in this study. The percentage of infected plants in commercial plantations was lower than in varietal collections. Possibly it could be explained that nowadays farmers prefer to grow new varieties with *Bu* gene that are resistant to RBDV Scottish strain (RBDV-S). However, another RBDV-RB strain that widely occurs in central Europe, Russia and Siberia is able to infect cultivars that are resistant to S strain (Diekmann et al., 1994; Knight, Barbara, 1999). Although no information is available if Russian raspberry cultivars have gene *Bu*, our research showed that in Latvia common raspberry cultivars from Eastern Europe, such as 'Kirzach', 'Balzam' and 'Sputnica', are highly infected with RBDV, probably with RBDV-RB strain. RBDV infection was not found in collected samples from raspberry cultivar 'Tulameen', which lack resistance gene *Bu* and in other European countries was found that 'Tulameen' is susceptible to both strains (Chard et al., 2001; Wood, Hall, 2001; Martin, 1999). It indicates that this cultivar was probably introduced as virus-free stock in Latvia and had not yet been infected with RBDV in the fields.

The RBDV elimination by combination of micro-propagation and chemotherapy did not give the expected results. As shown in other studies RBDV is difficult or impossible to eliminate from certain genotypes of raspberry by meristem tip culture (Wang, Valkonen, 2009). No one of the used antiviral compounds eliminated the virus in raspberry meristem clones from all the mother plants. Ribavirin has been demonstrated to give highest results in virus elimination in comparison with other antiviral chemicals (Nascimento et al., 2003). However, ribavirin was shown be effective in elimination fruit viruses from apple, raspberries and *Prunus* spp. (Cieslinska, 2007; Sharma et al., 2007). In our work better results were obtained in treatment by azacytidine and dicyanamide for meristem clones originated from mother plant 3.15. This could be explained by the combination of host and virus genotype as shown in other

studies that the efficiency of virus elimination in host species differs depending on the virus and the host genotype (Wang et al., 2008). Possibly the meristem clones 3.15 were infected with other virus strain than clones from mother plants 2.18 and 2.19. Obtained results suggest that plants were infected with stable virus strains, which cannot be readily eliminated with chemotherapy and micro-propagation techniques. To improve RBDV elimination effect it is necessary to combine tissue culture techniques with chemotherapy and thermotherapy. The results obtained in this study will be useful in the establishment of virus-free planting material propagation and certification program in the country. The work is being continued.

Conclusions. 1. In Latvia commonly grown raspberry cultivars from Eastern Europe, such as 'Kirzach', 'Balzam' and 'Sputnica', are highly infected with RBDV.

2. Raspberry cultivar 'Tulameen' possibly was introduced as virus-free stock in Latvia and had not yet been infected with RBDV in the fields.

3. Antiviral compounds ribavirin, azacytidine and dicyanamide alone are not enough effective for RBDV elimination from raspberry *in vitro*.

4. In this experiment treated meristem plants were infected with stable virus strain, which cannot be readily eliminated with micro-propagation techniques and chemotherapy.

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Aviečių žemaūgiškumo viruso (RBDV) paplitimas Latvijoje ir viruso naikinimas *in vitro* chemoterapija

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Santrauka

Siekiant nustatyti aviečių žemaūgiškumo viruso (RBDV) paplitimą ir pasiskirstymą Latvijoje, 2007 metų pavasarį buvo ištirtos 27 *Rubus* spp. komercinės ir veislių kolekcijų plantacijos. Analizei buvo surinkti 224 lapų pavyzdžiai priklausantys 59 genotipams. Siekiant ištirti viruso pašalinimo *in vitro* iš natūraliai užkrėstų veislės 'Babje leto 2' augalų galimybes, buvo panaudotas meristeminės kultūros ir skirtingų antivirusinių cheminių medžiagų derinys. Aiškinantis užkrėstumą šiuo virusu ir pastarojo pašalinimo veiksmingumą, augalų pavyzdžiai buvo patikrinti imunofermentiniu metodu (DAS-ELISA), panaudojant polikloninius antikūnius. Gauti rezultatai parodė, kad RBDV paplitęs 70 % tirtų aviečių plantacijų. RBDV paplitimas tirtuose augaluose siekė 35 % ir atskirose veislėse labai skyrėsi. Daugelis populiariausių Rytų Europos veislių, kaip 'Kirzach', 'Balzam' ir 'Sputnica', buvo užkrėstos AŽV. Viruso nerasta veislės 'Tulameen' pavyzdžiuose. Mėginimas išnaikinti RBDV, derinant meristeminę kultūrą su ribavirinu terpėje, visuose apdorotuose augaluose buvo nesėkmingas. Apdorojimas azacitidinu ir dicianamidu paveikė tik tuos meristemų klonus, kurie buvo kilę iš motininio augalo. Tai rodo, kad kai kurie augalai buvo užkrėsti stabiliu viruso izoliatu, kurio chemoterapija ir *in vitro* dauginimo metodais išnaikinti neįmanoma. Norint sukurti veiksmingas RBDV naikinimo priemones, reikia tiksliai apibūdinti avietes užkrečiančias viruso atmainas ir optimizuoti *in vitro* metodus. Tyrimai bus tęsiami.

Reikšminiai žodžiai: antivirusinės medžiagos, chemoterapija, DAS-ELISA, meristeminė kultūra, *Rubus* spp.