Blueberry red ringspot virus Eliminated from Highbush Blueberry by Shoot Tip Culture

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Abstract

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In order to find an effective elimination method of *Blueberry red ringspot virus* (BRRV), shoot tips of 2–3 mm size originating from buds of highbush blueberry cv. Darrow naturally infected by BRRV and collected in winter months were regenerated and multiplied on woody-plant medium (WPM) with 1.5 mg/l zeatin and 20 g/l sucrose. Developed shoots tested negative by polymerase chain reaction (PCR) for BRRV were transferred onto a rooting medium consisting of WPM supplemented with 1 mg/l indole-3-butyric acid. Rooting plants were transferred into soil and tested twice by PCR 6 months apart. Ninety per cent eradication of BRRV was achieved while isolating shoot tips, multiplying them in one vegetative generation, and regenerating whole 50–60 cm high plants within 30 months. All plants developed rich fruits after their overwintering in a cold greenhouse and were tested BRRV negative in summer 2013.

Keywords: BRRV; *in vitro*; PCR; *Vaccinium corymbosum* L.; virus-free; virus elimination

Red ringspot disease (RRS) of highbush blueberry was identified in the United States in 1954 (Hutchinson & Varney 1954; Ramsdell *et al.* 1987), and its viral origin was confirmed about 30 years later (Kim *et al.* 1981). The causative agent – *Blueberry red ringspot virus* (BRRV) – has a circular dsDNA genome encapsidated in particles 42–46 nm in diameter (Kim *et al.* 1981). The complete genome sequence of the New Jersey isolate has been described (Glasheen *et al.* 2002), and the virus has recently been classified into the genus *Soymovirus*, family *Caulimoviridae.* Although there is no commercially available kit for routine ELISA diagnostics, the virus can be reliably detected by polymerase chain reaction (PCR) using BRRV-specific primers (Glasheen *et al.* 2002; Polashock & Ehlenfeldt 2009).

To date, the virus has been detected outside of North America on imported plants in Japan (Isogai *et al.* 2009), and in the three European countries: Slovenia, Czech Republic, and Poland (Mavrič Pleško *et al.* 2009; Přibylová *et al.* 2010; Kalinowska *et al.* 2012). The infection in Slovenia was found in one plant of cv. Coville introduced with planting material in the 1980s.

In the Czech Republic, surveys of plantations, germplasm collections, and propagating material for BRRV were conducted during 2009–2010. These resulted in finding two infected plants of cv. Darrow about 10 years old among 67 tested plants of 10 cultivars at a single location (Přibylová *et al.* 2010). Infected plants developed typical symptoms of red ringspot on the upper surfaces of leaves and faint ringspot on fruits. The leaves turned completely red in late summer and bushes were reduced in growth. In both Slovenia and the Czech Republic, BRRV occurrence was limited to a few plants putatively generated from

infected propagative material. The infection in the Czech Republic was not actively spreading to the neighbouring plants during 3 years of our monitoring of symptoms and PCR testing (Přibylová *et al.* 2010). Similar observations have been made in Japan, where BRRV infections were specifically restricted to distinct cultivars. Since the possible vectors remain unknown (Cline *et al.* 2008; Polashock & Ehlenfeldt 2009), all these data indicate that the major mode of BRRV spread, at least in the past, has been infected planting material. This hypothesis is also supported by phylogenetic analysis of 3 complete and 22 partial sequences of American, Japanese, and European BRRV isolates (Petrzik *et al.* 2011). This study revealed that European isolates are closely related to the American ones.

As highbush blueberry plants are mainly propagated vegetatively, virus infection can easily spread into new areas. Thus, disease control depends on the production of virus-free propagating rooting stem cuttings. With increasing spread of the virus in Europe there is need for an effective virus-elimination method which has been missing in the literature so far. The aim of our work was to test an *in vitro* method for producing BRRV-free blueberry plants using infected cv. Darrow as a model.

MATERIAL AND METHODS

Plant material. The plant material originated from a field collection for propagation of highbush blueberry (*Vaccinium corymbosum* L.) in South Bohemia, Czech Republic. Branchlets of young shoots from BRRV-infected blueberry plants cv. Darrow (Přibylová *et al.* 2010) were collected in January 2010.

Shoot tip cultures. After sprouting in water at room temperature, the apical and lateral vegetative buds were surface-sterilised with 0.15% mercury chloride along with several drops of Tween 20 and rinsed three times (5 min per rinse) with sterilised water. Buds were thereafter dissected aseptically, outer scales were removed, and the shoot tips of 2–3 mm size bearing inner scales, immature leaves. and stem tissue were used for propagation. Shoot tips were transferred aseptically to 100 ml Erlenmeyer flasks with woody-plant agar medium (WPM) (Lloyd & McCown 1980) including vitamins (Duchefa Biochemie, Haarlem, the Netherlands) and supplemented with 1.5 mg/l zeatin and 20 g/l sucrose. Cultures were maintained under photoperiod of 16 h light, light intensity 55 μmol/m²/s, and temperature of 23°C. After 3–4 months the regeneration of shoots was observed. Shoots were

separated and cultivated individually on the WPM. Portions of shoots were used for detecting BRRV by PCR. Developed plantlets testing negative were transferred onto a rooting medium consisting of WPM supplemented with 1 mg/l indole-3-butyric acid (IBA). Four months later, rooting plants were transferred from the Erlenmeyer flasks to soil and were maintained in a hotbed under greenhouse conditions. After 6 months, when these plants had reached 8–10 cm, they were transferred to pots, maintained under cold greenhouse conditions and tested by PCR. Repeated testing by PCR was conducted 6 months later, when the plants had reached 50–60 cm. Final testing was conducted after their overwintering and development of fruits in summer 2013.

BRRV detection. DNA was extracted from 0.1 g of regenerated shoots from *in vitro* cultures using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) for purification of total DNA from plant tissue according to the manufacturer's instructions. In regenerated plants, leaf samples of individual branches from each plant were used for BRRV detection.

Primer sets RRSV3/RRSV4 (Polashock & Ehlenfeldt 2009) and RR13/RR14 (Glasheen *et al.* 2002) were used in PCR for BRRV detection. Symptomatic leaves of cv. Darrow infected with BRRV from a field germplasm collection (Přibylová *et al.* 2010) were collected, maintained at –20°C, and served as a positive control in PCR assays.

PCR with *Taq* Purple DNA polymerase utilised the following program: 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 49°C for 30 s, and 70°C for 45 s, followed by a final extension at 70°C for 5 minutes. A total volume of 25 μl contained 1 μl of the DNA; 200 μmol/l dNTPs; 0.5 μl of each primer (20 pmol/μl); 75mM Tris-HCl, pH 8.8; 20mM $(NH_4)_2SO_4$; 0.01% Tween 20; 2.5mM $MgCl_2$; 2.5 U of *Taq* Purple DNA polymerase; and stabilisers (Top-Bio, Prague, Czech Republic). Amplification was conducted using an MJ Research (Waltham, USA) thermocycler. Aliquots (4 μl) of each PCR product were analysed by electrophoresis in *Tris*-acetate-EDTA buffer.

RESULTS

In view of the declining state of the BRRV-infected shrub of cv. Darrow, only 18 shoot tips were successfully isolated from sprouting shoots. After 4 months, 13 shoot tips (72%) regenerated and formed multiple shoots. These shoots were separated and cultivated individually on the WPM. Four months later, regenerated shoots were used in the PCR test for BRRV.

Table 1. Numbers of isolated shoot tips, regenerated shoots, and plants tested by PCR and found infected in process of eliminating *Blueberry red ringspot virus* from highbush blueberry cv. Darrow by *in vitro* culture of shoot tips

Shoot tips isolated from BRRV infected plant	Regenerated shoot tips	*In vitro* multiplication		Rooted plants		Plants in soil		Overwintered plants	
		shoots tested	BRRV infected	shoots transferred to soil	plants surviving	plants tested	BRRV infected	plants tested	BRRV infected
18	13	103	2	60	30	30	3	30	0

In the first vegetative generation, 103 shoots were obtained in total. From 11 original regenerated shoot tips, 83 BRRV negative shoots were obtained (Table 1). These shoots were transferred to the rooting medium consisting of WPM supplemented with 1 mg/l IBA. In this medium, 60 shoots formed roots. Six months later, the rooting shoots were transferred from Erlenmeyer flasks to soil and maintained in a hotbed under greenhouse conditions. Thirty plants survived transfer into soil. When these plants reached 8–10 cm (after 4 months), they were transferred to pots, maintained under glasshouse conditions, and tested by PCR. Only one out of 30 plants tested BRRV positive. Two additional BRRV-infected plants were revealed in a repeat test 6 months later, when the plants had reached 50–60 cm in height (Table 1). Thus, 90% eradication of the virus was achieved within 30 months just by isolation of shoot tips, their multiplication in one vegetative generation, and regeneration into whole plants. Final testing of plants was conducted after their overwintering in summer 2013, when they developed rich normal fruits. No symptoms on leaves and fruits were observed. All 30 plants had reached over 100 cm in height and tested BRRV negative (Table 1).

While virus-infected shoots are usually eliminated from further cultivation, we had intended to multiply some *in vitro* BRRV-infected plants for additional experiments and as BRRV positive controls. Therefore, the base of BRRV-infected shoots remaining after PCR testing was used for propagation in the next vegetative generation. To our surprise, only in 3 out of 20 plants originating from two original regenerated shoot tips (Table 2) indexed positive

in PCR assay using the RRSV3/RRSV4 and RR13/RR14 primer sets yielding PCR products of 548 bp and 487 bp, respectively. Twenty-nine shoots were regenerated from 3 BRRV-infected ones of the first vegetative generation. Only 2 of these were shown by PCR to be infected by BRRV. In the third subsequent vegetative generation, 29 shoots derived from those 2 positive were tested and only 3 of those were BRRV infected. In the fourth vegetative generation 34 shoots were tested by PCR and none of them was infected (Table 2). We showed that maintaining and propagating BRRV-infected blueberry shoots *in vitro* as positive controls is impractical due to spontaneous elimination of the virus from explants in tissue culture propagation.

DISCUSSION

Various methods can be exploited for virus elimination from plants, such as apical meristem cultures (CHEONG *et al.* 2012) often combined with thermotherapy (TAN *et al.* 2010), cryotherapy (WANG & VALKONEN 2008), or chemotherapy (ŠPAK *et al.* 2012). In view of the limited source of infected shoots, and to avoid loss of plant material, we decided to start with shoot tip culture and did not pursue thermotherapy or chemotherapy.

To our knowledge, there has been only one published report to date regarding eradication of red ringspot disease (RSS) in blueberry (STRETCH & SCOTT 1977). Plants free of RSS were produced from softwood cuttings of shoots emerging from chip buds from RRS-infected buds inserted into healthy blueberry stock cultivars. Several RRS-free plants of the

Table 2. Numbers of isolated buds and regenerated shoots tested by PCR and found infected in attempts to multiply *in vitro Blueberry red ringspot virus*-infected highbush blueberry cv. Darrow plants as positive controls

Buds isolated from BRRV infected plant	Regenerated shoot tips	*In vitro* generations							
		1.		2.		3.		4.	
		shoots tested	BRRV infected	shoots tested	BRRV infected	shoots tested	BRRV infected	shoots tested	BRRV infected
2	2	20	3	29	2	29	3	34	0

blueberry cultivars Blueray, Burlington, and Darrow were propagated by this method, while heat treatment of infected plants did not inactivate the virus. However, this laborious procedure is not practical in commercial plantings. Moreover, virus-free status of plants was evaluated after 36 months only according to the absence of symptoms, as neither serological nor molecular methods for BRRV detection were available at the time of that work in 1977.

While large-scale production of high-quality plants is needed for commercial plantation establishment, traditional vegetative propagation of highbush blueberry by cuttings has not been successful due to poor rooting ability, considerable demand for large amounts of mother plants, their limited seasonal growth, and relatively high price. In *Vaccinium* species, *in vitro* techniques are quite effective for rapid mass production of high-quality planting material for large-scale cultivation, germplasm improvement, gene conservation, and research purposes. Micropropagation via direct shoot regeneration from meristems is well suited for obtaining genetically homogeneous planting material that is identical with mother genotype (Ostrolucká *et al.* 2007). For our purpose, therefore, we used the method of shoot tip culture utilised in micropropagation of *Kalmia latifolia* (Lloyd & McCown 1980). This species is a member of the family *Ericaceae*, as is blueberry (*Vaccinium* genus). The method was efficient for regenerating and multiplying shoot tips from branchlets collected in winter and for sprouting in the lab.

With respect to possible latency of the virus in plants, repeated virus testing of plants derived from shoot tip cultures is critical to the assumption of BRRV-free status in plants. To verify virus-free status of regenerated plants, a 6-month testing interval is recommended. The PCR method using primer sets RRSV3/RRSV4 and RR13/RR14 has proven to be sensitive enough for detecting BRRV in regenerated shoots and whole plants. Because of the formation of multiple shoots *in vitro* and branching of whole plants, sampling represents a challenge for reliable virus detection. Therefore, we tested all stems regenerated *in vitro* and leaf samples of individual branches of each plant transferred into soil were used for BRRV detection.

In conclusion, the use of apical shoot tip cultures without thermotherapy or chemotherapy was found to be efficient for eliminating BRRV from an infected shrub and producing virus-free propagation material. WPM was found to be effective for regenerating shoot tips, multiplying shoots, and, after adding IBA, also

for rooting of plants. This method is more efficient than is the originally described chip bud grafting of meristem plantlets onto healthy seedlings (Stretch & Scott 1977) and can be useful to save critical one-of-a-kind germplasm of blueberry.

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