

## **YEAR-ROUND BLUEBERRY SCORCH VIRUS DETECTION IN HIGHBUSH BLUEBERRY**

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**Abstract.** Viral diseases are a worldwide problem of blueberry which a major limiting factor for production. A survey for Blueberry scorch virus (BIScV) by DAS-ELISA in various organs of highbush blueberry conducted from May 2010 to April 2011, showed the occurrence of these virus in cvs Bluecrop and Herbert, which showing virus-like symptoms. Samples of plant materials (bud flower, flower, leaf, bark) were collected individually from each highbush blueberry plant of every cultivar. It was established that the detection of virus of each the investigated bushes cvs Bluecrop and Herbert depended on the tested plant materials as well as the period in which the tests were performed. The effectiveness of the virus detection varied for the investigated cultivars. The presence of the BIScV was confirmed in leaves samples with specific primer pair which amplifies a 430 bp fragment of the 5'-proximal ORF I [RNA-dependent RNA polymerase (RdRp)].

**Key words:** blueberry cultivars, BIScV, DAS-ELISA, RT-PCR

### **INTRODUCTION**

Blueberries belong to the genus *Vaccinium* in the heath family (*Ericaceae*). Their popularity has been increasing recently as new research discloses their nutritional benefits. Highbush blueberry have been affected by virus-related diseases that have cause multimillion dollar losses [Jaswal 1990, Caruso and Ramsdell 1995]. Scorch disease, caused by *Blueberry scorch virus* (BIScV; genus *Carlavirus*, family *Betaflexiviridae*) was first identified as a disease of blueberries on 'Berkeley' bushes in a commercial field near Puyallup, WA, in 1980 [Bristow and Martin 1987, Martin and Bristow 1988]. Since then, BIScV has been detected in several other commercial fields in USA [Converse and Ramsdell 1982, Wegener et al. 2006] and Europe: Italy [Ciuffo et al. 2005]

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and Poland [Paduch-Cichal et al. 2011]. Symptoms caused by BScV are visible in the first or second year after infection [Bristow et al. 2000] range from complete necrosis (blighting) of flowers and young leaves and twig dieback in some cultivars (sensitive) to no visible damage in other cultivars (tolerant).

The most economical and common method for virus detection and identification is the double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). It was used in mass-testing of blueberry plants for BScV [Wegener et al. 2006]. The viruses are unevenly distributed in plants, and their titer can fluctuate throughout growing season. The highest DAS-ELISA readings were noted in leaf samples collected from bushes infected with BScV in June-September [Martin and Bristow 1988] or in June-August [MacDonald et al. 1991, Halpern and Hillman 1996, Wegener et al. 2006].

Since DAS-ELISA results depended on the tissue source and the time of year in which samples were collected, RT-PCR appeared a more sensitive method for detecting BScV [Wegener et al. 2006]. Highbush blueberry plants are known of high anti-oxidant activity associated with high capacity of phenolic, anthocyanin, and antioxidant capacity [Prior et al. 1998, Wu et al. 2004]. Due to the presence of high amounts of polyphenols and other virus inhibitors in blueberry plants, nucleic acid extraction is uneasy. The amounts of these components vary in particular tissues in different periods of year [Loomis 1974]. The leaf samples collected from blueberry plants that showed symptoms in general proved to be the most suitable organ for RNA isolation and gave a sufficient level of viral BScV-RNA for detection by RT-PCR [Ciuffo et al. 2005, Wegener et al. 2006, Moretti et al. 2011].

The aim of this work was to check the possibility of BScV detection in various blueberry tissues throughout the vegetation and leafless period by DAS-ELISA.

## MATERIAL AND METHODS

The research was performed at the Department of Plant Pathology (Faculty of Horticulture, Biotechnology and Landscape Architecture, Warsaw University of Life Sciences – SGGW) from May 2010 to April 2011.

**Samples collection.** The investigation material comprised 53 highbush blueberry plants cultivars Bluecrop (15 plants) and Herbert (38 plants) grown on plantation located in central Poland. Bushes were chosen on the basis of DAS-ELISA results obtained in May 2009.

Samples of plant material were collected every four weeks according to the following scheme (tab. 1). The samples of plant material were collected individually from each highbush blueberry plant of every cultivar: 8–10 leaves (leaf sample) from randomly chosen shoots, 8–10 flower buds (bud sample) or flowers (flower sample) from randomly chosen shoots and samples of bark from 2–3 one-year-old and many-year-old shoots.

**Serological detection of virus.** BScV was detected by DAS-ELISA with specific antibodies from Agdia Incorporated (USA). The separate samples were prepared by grinding 0.250 g of fresh plant tissue in general extract buffer 3 (GEB3) in the ratio 1:10 (w:v) and tested according to the manufacturer's protocol. After 1 h of incubation at

room temperature, substrate hydrolysis was measured as a change in absorbance at OD 405 nm using Infinite® 200Pro microplate reader (Tecan, Austria GmbH). Samples were considered positive if their optical density (OD 405 nm) readings were at least twice those of healthy controls.

Table 1. Scheme of collected samples of plant materials for every cultivar

| Organ            | Terms of taking samples  |
|------------------|--------------------------|
| Leaves<br>Flower | May 2010                 |
| Leaves           | June–October 2010        |
| Bark             | November 2010–April 2011 |
| Flower buds      | March–April 2011         |

**Statistical analysis of DAS-ELISA results.** Multifactor analysis of variance was performed with the help of the IBM SPSS Statistics 19 programme for the absorbance values obtained in DAS-ELISA to which were subjected the samples collected in various months from different organs of each of the 15 and 38 highbush blueberry plants of cultivars Bluecrop and Herbert, respectively. Differences between mean values of absorbance were determined by the Tukey's range test at the significance level of  $\alpha = 0.05$ .

**Detection of BISCv using RT-PCR.** RT-PCR with total RNA and appropriate primers was used in order to confirm DAS-ELISA results. Total nucleic acids were isolated from the leaf tissue of DAS-ELISA-positive (30 samples) and negative samples (5 samples) using the silica capture (SC) method described originally by Boom et al. [1990] and adapted to the diagnosis of plant viruses by Malinowski [1997]. For RT-PCR, Titan One Tube RT-PCR System (Roche) was used with the primer pair specific to the 5'-proximal ORF I [RNA-dependent RNA polymerase (RdRp)]: 5'-ATGGCACTCACATACAGAAGTCC-3' and 5'-TGCCTCTTCAATGCACGATGTTC-3'. A primer pair specific to the ribulose 1,5-bisphosphate carboxylase chloroplast (*Rbc1*) gene (*Rbc1-F/Rbc1-R* 5'-TACTTGAACGCTACTGCAG-3' and 5'-CTGCATGCATGTCACGGTG-3') [Sanchez-Navarro et al. 2005] was used as the internal control to amplify the corresponding mRNA in blueberry leaf tissue by RT-PCR. Samples were subjected to a reverse transcription 30 min at 50°C, 2 min of denaturation at 94°C, followed by 35 cycles of 30 s of denaturation, 45 s annealing at 52°C, and 45 s elongation at 68°C with a final extension of 7 min at 68°C. The reaction products were resolved by electrophoresis in the buffer TBE in 1.2% agarose gel. Sequencing were performed in the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. All the sequences will be read at least twice.

**Observation of diseases symptoms.** During the entire experimental period, observation of bushes of the investigated cultivars was carried out and all the observed disease symptoms occurring in the plant appearance were noted.

## RESULTS

**Diseases symptoms.** In each year of the study, initial virus-like symptoms on Herbert and Bluecrop blueberry plants were observed during the second week of May. Symptoms on cv. Herbert consisted of severely reduced vigor, shoot defoliation, distorted and crinkled apical leaves with dark reddish lesion. Some of bushes also showed mosaic and leaf chlorosis. Bluecrop plants showed mosaic, dark reddish lesions on apical leaves and general decline. In late fall (October), blueberry bushes cvs Herbert and Bluecrop developed foliar red line pattern and an oak-line pattern. Symptoms were more severe on cv. Herbert than on cv. Bluecrop.

Table 2. The detection of BScV in different parts of two highbush blueberry cultivars during various periods of the year

| Cultivar | Month     | Average value of absorbance $A_{405\text{ nm}}$ |           |           |          |
|----------|-----------|---|-----------|-----------|----------|
|          |           | leaves  | flowers   | bark      | buds     |
| Bluecrop | May       | 0.334 a <sup>a</sup>                            |           |           |          |
|          | September | 0.364 a   |           |           |          |
|          | October   | 0.296 a   |           |           |          |
|          | November  |   |           | 0.245 a   |          |
|          | January   |   |           | 0.265 a   |          |
|          | February  |   |           | 1.257 d   |          |
|          | March     |   |           | 0.373 ab  | 0.441 ab |
|          | April     |   |           | 0.234 a   | 0.289 a  |
| Herbert  | May       | 0.346 a   | 0.504 abc |           |          |
|          | June      | 0.253 a   |           |           |          |
|          | August    | 0.414 ab  |           |           |          |
|          | September | 0.507 abc                                       |           |           |          |
|          | October   | 0.514 abc                                       |           |           |          |
|          | November  |   |           | 0.673 abc |          |
|          | January   |   |           | 0.286 a   |          |
|          | February  |   |           | 0.791 c   |          |
|          | March     |   |           | 0.518 abc | 0.411 ab |
|          | April     |   |           | 0.243 a   | 0.256 a  |

<sup>a</sup> Means followed by the same letters are not significantly different for  $\alpha = 0.05$

**Virus identification by DAS-ELISA.** During the test period BScV was detected in the leaves (May, September, October), bark (November, January–April) and bud (March, April) collected from cv. Bluecrop and leaves (May, June, August–October), flowers (May), bark (November, January–April) and bud (March, April) on Herbert blueberry plants. Bark samples from February were the most reliable organ from BScV

detection in blueberry bushes cv. Bluecrop and from November, February and March of Herbert blueberry plants. The highest  $A_{405\text{nm}}$  was obtained in bark extracts from Herbert blueberry bushes in February. The mean  $A_{405\text{nm}}$  values obtained while testing bark samples in February were also higher than the mean  $A_{405\text{nm}}$  values obtained for leaf and bud samples collected at various other times. During the testing of bark samples from cv. Herbert, higher mean  $A_{405\text{nm}}$  values were obtained in November, February and March as compared with the mean  $A_{405\text{nm}}$  values noted in January and April. No statistically significant differences were noted between the mean absorbance values obtained in the remaining months for samples collected from cv. Herbert. Differences of statistical significance were observed while testing bark samples of two of the investigated cultivars. In November, higher mean absorbance values were obtained for such samples from cv. Herbert as compared to cv. Bluecrop. On the other hand, the mean absorbance values obtained for bark samples from cv. Bluecrop in February were higher compared to the means obtained while testing samples from cv. Herbert in November, January, February, March and April (tab. 2).

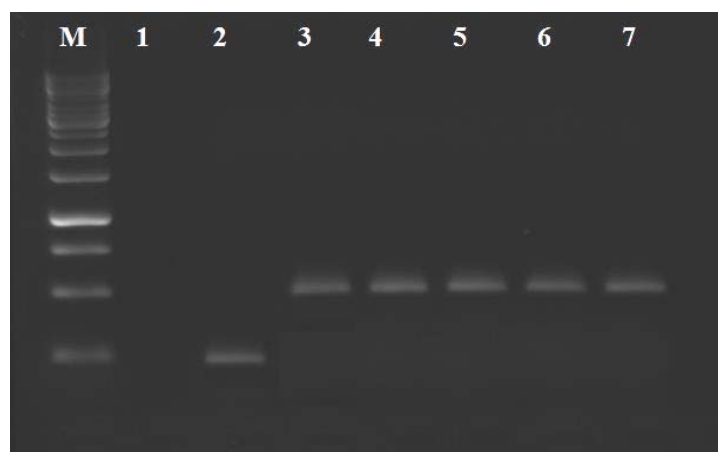


Fig. 1. Agarose gel analysis of B1ScV amplicons obtained by RT-PCR assays. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas); lanes 1–2: healthy highbush blueberry (DAS-ELISA-negative samples); lanes 3–7: samples infected with B1ScV (DAS-ELISA-positive samples). The B1ScV RdRp gene amplicons are shown at 430 bp. The *RbcI* amplicon is shown at 183 bp

**Detection of B1ScV by RT-PCR.** The presence of B1ScV was confirmed by RT-PCR in leaf samples of blueberry bushes (15 cv Bluecrop and 15 cv Herbert) with amplification of a 430 bp fragment. As expected, no product was amplified from total RNA of healthy blueberry leaves while a 183 bp long fragment corresponding to the plant internal control *RbcI* was obtained (fig. 1). The 9 amplicons of the 430 bp size were sequenced, deposited in GenBank with Accession No. KF876235-KF876243 and the nucleotide sequence of the products showed 98–99% identity with the published BC-1 strain (GenBank Accession No. AY941198).

## DISCUSSION

Detection of BISScV by DAS-ELISA in highbush blueberry was possible throughout the year in extracts of one or more of the following bush organs: leaves, flowers, bark or buds. However, the BISScV titer varied between organs and between recording date, so that is important to choose the particular organ that will give the optimal ELISA reading at a given time of year.

The results obtained in our study concerning the detection of BISScV in the blueberry cultivars by ELISA were similar to those obtained by previous workers. Martin and Bristow [1988], Jaswal [1990], MacDonald et al. [1991], Cavileer et al. [1994], Halpern and Hillman [1996], Martin [2001], Bristow et al. [2000], Medina et al. [2006], Wegener et al. [2006] and Paduch-Cichal et al. [2011], detected BISScV in flower buds, leaves, flowers and bark.

The authors of this article found that bark samples from cv. Herbert are the most appropriate for detecting BISScV in February, March and November. Bark from Bluecrop blueberry plants from February was also most reliable for BISScV detection. The presence of the virus in bark or flower bud samples had not been reported earlier. Reduced BISScV detectability after May may be attributed to the rise in temperature during summer which reduced replication of the virus rendering its detection by ELISA more difficult.

Additionally, BISScV infection was confirmed by RT-PCR. According to previous data [Ciuffo et al. 2005, Wegener et al. 2006, Moretti et al. 2011], the virus was easily detected in leaves by RT-PCR.

In our experiment reported here, bushes of cvs Bluecrop and Herbert in which the presence of BISScV was detected showed symptoms similar to those which had been earlier described in the USA and Canada [Martin and Bristow 1988, Caruso and Ramsdell 1995, Bristow et al. 2000] and in Italy [Ciuffo et al. 2005].

From the practical point of view, the assessment of the plants' health based only on their observation is not a sufficient diagnostic method. Symptomless infected plants may comprise a source of infection for the neighbouring healthy plants. It is known from the data provided in literature that aphids are BISScV vectors. They are considered to be the most important pests of highbush blueberry in the USA and Canada [Bristow et al. 2000, Garcia-Salazar 2002, Prodorutti et al. 2007, Pansa and Tavella 2008]. As far as Poland is concerned, this insight results from the investigations carried out by Łabanowska [2010], the beet leaf aphid (*Aphis fabae* Scopoli), peach aphid (*Nectarosiphon persicae* Sulzer) and berry aphid (*Amphorophora borsalis*) were observed on highbush blueberries. Up till now, the relation between aphid and highbush blueberry viruses has not been explained, but the possibility that in our country these species are responsible for spreading BISScV during the vegetation period should be considered.

The results of the research reported in the present paper show that different parts of highbush blueberry may be used throughout the vegetation and the leafless period for the detection of BISScV by DAS-ELISA. Additionally, BISScV can be detected by RT-PCR during the vegetation. These data are very valuable because they allow to prolong the testing period of highbush blueberry plants to cover the entire year. These data could be helpful in establishing the testing schedule according to which candidate

materials for virus-free blueberry plants should be screened for these viruses as part of in sanitary certification programs.

## CONCLUSIONS

1. It was established that the detection of BScV in bushes of the investigated cultivars of highbush blueberry: Bluecrop and Herbert by DAS-ELISA test depended on the tested plant organ as well as the month in which the test was performed.

2. Effectiveness of the detection of BScV varied of the investigated cultivars of highbush blueberry.

3. While testing the highbush blueberry Bluecrop cultivar for the presence of BScV, ‘bark’ samples should be collected in February.

4. Detection of BScV in plants of the highbush blueberry Herbert cultivar should be performed in bark samples in February, March and November.

5. Detection of BScV in leaf samples of blueberry Bluecrop and Herbert cultivars by RT-PCR technique is also feasible.

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## **WYKRYWANIE WIRUSA OPARZELINY BORÓWKI WYSOKIEJ W RÓŻNYCH OKRESACH ROKU**

**Streszczenie.** Celem przeprowadzonych badań było wykrywanie i identyfikacja wirusa oparzeliny borówki wysokiej (Blueberry scorch virus, BScV) w różnych organach pobieranych z krzewów borówki wysokiej odmian Bluecrop i Herbert rosnących na plantacji produkcyjnej zlokalizowanej w centralnej Polsce. Badania były prowadzone w okresie od maja 2010 do kwietnia 2011 r. przy użyciu testu serologicznego DAS-ELISA. Próby materiału roślinnego (pąki kwiatowe, kwiaty, liście, kora) pobierano indywidualnie z krzewów każdej z badanych odmian. Ustalono, że wykrywanie wirusów w krzewach odmian Bluecrop i Herbert zależało od testowanego organu oraz terminu, w którym przeprowadzono test. Obecność BScV w krzewach badanych odmian potwierdzono przy pomocy techniki RT-PCR z wykorzystaniem starterów amplifikujących fragment 5' genu kodującego polimerazę RNA zależną od RNA.

**Słowa kluczowe:** odmiany borówki wysokiej, wirus oparzeliny borówki wysokiej, test DAS-ELISA, technika RT-PCR

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