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# Incidence of viruses and viroids in Polish hop gardens

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**Abstract.** Viruses and viroids cause losses in hop production and are very difficult to eliminate, they spread easily and infected plants become a source of infection for other plants in the hop garden. Hop infected with viruses or viroids usually do not show any symptoms of disease, which makes it difficult to identify infected plants and prevents their elimination from the plantation. Monitoring is of utmost importance to detect and eradicate at an early stage first sources of infection with pathogens that have potentially catastrophic impacts on hop production. The aim of the study was determine the incidence of *Hop latent virus* (HpLV), *Arabis mosaic virus* (ArMV), *Hop stunt viroid* (HSVd), *Apple fruit crinkle viroid* (AFCVd) and *Citrus bark cracking viroid* (CBCVd) in Polish hop gardens. Hop leaves from commercial hop gardens and a propagation facility were tested by RT-PCR. On the basis of the obtained results, HpLV was found to occur in a few hop gardens in all regions of hop cultivation in Poland. HSVd was found in one hop garden and ArMV, AFCVd and CBCVd were not found in any of the tested samples. The results provided knowledge about these pathogens and will be useful in effective management of the risk of these dangerous diseases.

**Keywords:** virus, viroid, hop, HpLV, HSVd

## INTRODUCTION

The hop (*Humulus lupulus*) is one of the two species belonging to the genus *Humulus*. In Poland, hop is grown on an area of about 1420 ha, which ranks our country in the third position in Europe and the fifth in the world, in terms of cultivation area and production volume (IHGC, 2015). Hop is grown mainly for the needs of the brewing industry, as a raw material being the source of the characteristic flavour and aroma. Due to their anti-inflammatory and antioxidant properties, hop is also used in the cosmetics industry as an ingredient in shampoos and creams which delay the process of skin aging. In addition, extracts of hop cones are used in the pharmaceutical industry, for the production of sleep-inducing and sedative drugs, aiding in digestion and soothing ailments linked to menopause (Bohr et al., 2005; Corrêa et al., 2018; Forino et al., 2016).

Hop is a perennial species, cultivated for many years in the same position, without crop rotation. After a garden is established, over time, a gradual accumulation of pathogens in the plants and soil follows. Viruses and viroids are pathogens which accumulate particularly easily in plants because there are no effective chemical methods to combat them. Chemical agents can only reduce the population of vectors transmitting these pathogens. An additional problem promoting infections is the ease with which they are transmitted during agrotechnical procedures when mechanical damage to plants occurs (Skomra, 2015). Viral diseases rarely cause visible symptoms on hop, only in extreme cases can a viral or viroid infection lead to morphological changes of leaves, stunting or death of susceptible hop cultivars (Pethybridge et al., 2008). Despite the fact that infected plants do not show any symptoms, they often provide lower yield of cones with adversely modified chemical composition. The yield and content of alpha acids depend to a large extent on the variety and can be reduced by as much as 35% and 50%, respectively, in infected plants (Barbara et al., 1990; Patzak et al., 2001; Sano, 2013).

In Poland, so far, research has been conducted on the most common viruses: *Hop mosaic virus* (HpMV), *Prunus necrotic ring spot virus* (PNRSV), *Arabis mosaic virus* (ArMV), *American hop latent virus* (AHLV) and *hop latent viroid* (HLVd) (Skomra, 2001; Grudzińska, Solarska, 2005). In 2018, was started monitoring of hop gardens comprising all Polish hop growing regions to detect economically important hop viruses: *Hop latent virus* (HpLV), *Arabis mosaic virus* (ArMV), *Hop stunt viroid* (HSVd), *Apple fruit crinkle viroid* (AFCVd), *Citrus bark cracking viroid* (CBCVd). In the first year of monitoring, the pre-

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sence of HpLV was found in 6, and HSVd in 2 out of 900 tested hop plants (Przybyś et al., 2019). HpLV belonging to the *Carlavirus* genus does not usually cause symptoms in hops, although sometimes it causes chlorotic spots on the leaves (Ziegler, et al. 2014). Its vector is hop aphid (*Phorodon humuli*) (Adams, Barbara, 1982). HpLV is not spread through seeds (Brunt, et al., 1996). Due to the latent nature of the infection, the exact extent of HpLV is unknown, but it has been found in hop gardens in Europe, the United States, New Zealand, Australia, China, South Africa and Japan (Pethybridge, et al., 2008, Seigner et al., 2014). ArMV belonging to the genus *Nepovirus* has a very large group of natural hosts including many weed species and crop species (Brunt, et al., 1996). Its spread depends on the occurrence of nematodes *Xiphinema diversicaudatum, X. coxi,* and *X. bakeri,* which are vectors of this virus, in hop gardens. Unlike HpLV, it can spread through seeds (Brunt, et al., 1996). Its occurrence in Europe has been established in England, France and Germany.

A separate group of the pathogens of hops is constituted by viroids, which are a bare, self-replicating form of single-stranded RNA, without a protein coat. HSVd has already been found in hop gardens in Japan, Korea, China, USA and Europe, which, in addition to hop, can infect grapevine, almond, plum, peach and apricot (Luigi, Faggioli, 2013; Sano, 2013). The infection with this pathogen causes stunting, yellowing and leaf curling in plants, but symptoms often appear several years later, in hop often causing a significant reduction in the content of alpha acids (Sano, 2013). HSVd is spread only mechanically through agrotechnical treatments damaging plants, and the manner of the long-distance spread of the disease is transmission along with infected seedlings (Pethybridge et al., 2008). Another viroid causing similar symptoms to HSVd, which has been found in hop gardens in Japan, is AFCVd (Sano, et al., 2008). Sometimes there occur asymptomatic infections with this pathogen, but invariably the effects of infection include a significant reduction in the content of alpha acids in hop cones. A very dangerous viroid that first appeared in Slovenian hop gardens in 2007 is CBCVd (Jakse et al., 2015). It has been shown to cause HSVd-like symptoms: stunting, yellowing and leaf curling, and plant death, but the incubation period is much shorter with CBCVd infection, and the disease progresses more aggressively. The disease spreads very quickly in hop gardens, usually along rows. Due to the recent epidemic in Slovenia and the level of threat to hop in Europe, a warning about CBCVd was published in June 2015 on the EPPO (European and Mediterranean Plant Protection Organization) alert list.

The aim of the research was to assess the harmfulness of viral diseases for Polish hop gardens by determining the occurrence of viruses (HpLV and ArMV) and viroids (CBCVd, HSVd, AFCVd) in Poland.

## MATERIALS AND METHODS

### Plant material and sampling

The research was conducted during the period of 2018–2019. Samples of hop leaves were collected from gardens located in all of the hop-growing regions in Poland: Lublin, Greater Poland and Lower Silesia (Fig. 1). Samples were collected three times during the growing season from both bitter and aromatic varieties. For this purpose, each plant from which a sample was taken for testing was labelled, so that it was possible to collect two additional samples at a later date. The first term covered the period of training hop plants onto the guiding strings, the second one covered the flowering phase and the third one covered the stage of maturity. The leaves from various storeys of the plant were collected into sterile 50 ml tubes and cooled to 4°C while still being in the garden.

### RNA isolation

In order to perform isolation, 50 mg of material from several leaves was used and placed in 2 ml tubes, filled with 6 ceramic balls with a diameter of 2.8 mm. The samples thus prepared were cooled in liquid nitrogen and powdered in a Tissue Lyser homogeniser (Qiagen, Germany) at a shaking frequency of 30 Hz for 3 minutes. The homogenised material was subjected to total RNA isolation using RNA isolation kits – RNeasy PowerPlant Kit (Qiagen), in accordance with the procedure recommended by the manufacturer with a modification resulting from the high content of phenolic compounds in hop leaves. The modification consisted in reducing the volume of MBL/β-ME lysis buffer from 600 µl to 550 µl and adding 50 µl of Phenolic Separation Solution in order to reduce formation of nucleic acid complexes with oxidized phenolic compounds, which would decrease the amount of isolated RNA. The isolated RNA was additionally purified to remove DNA residue using the DNase Max kit (Qiagen) in accordance to the procedure recommended by the manufacturer.

### Reverse transcription

To obtain cDNA, RNA viruses and viroids were subjected to reverse transcription. 1 µl of oligo $d(T)_{15}$ (50 µM) - ArMV and HpLV or 1 µl of inverted specific primer (2 µM) – HSVd, CBCVd, AFCVd were added to 9 µl of sterile RN-ases free water (Table1), 2 µl RNA (1 µg/µl) and 1 µl of dNTP 10 mM mixture (Invitrogen, USA). The whole mixture was incubated at 65°C for 5 minutes and then cooled on ice for 1 minute. 4 µl of 5x First-Strand buffer (Invitrogen), 1 µl of 0.1M DTT (Invitrogen), 1 µl of RNasin (40 u/µl) – RN-ases inhibitor (ThermoFisher Scientific) and 1 µl of reverse transcriptase M-MLV Superscript III (200 u/µl) (Invitrogen) were added to the cooled mixture. The final volume of the reaction mixture was 20 µl. The reaction of reverse transcription was carried out

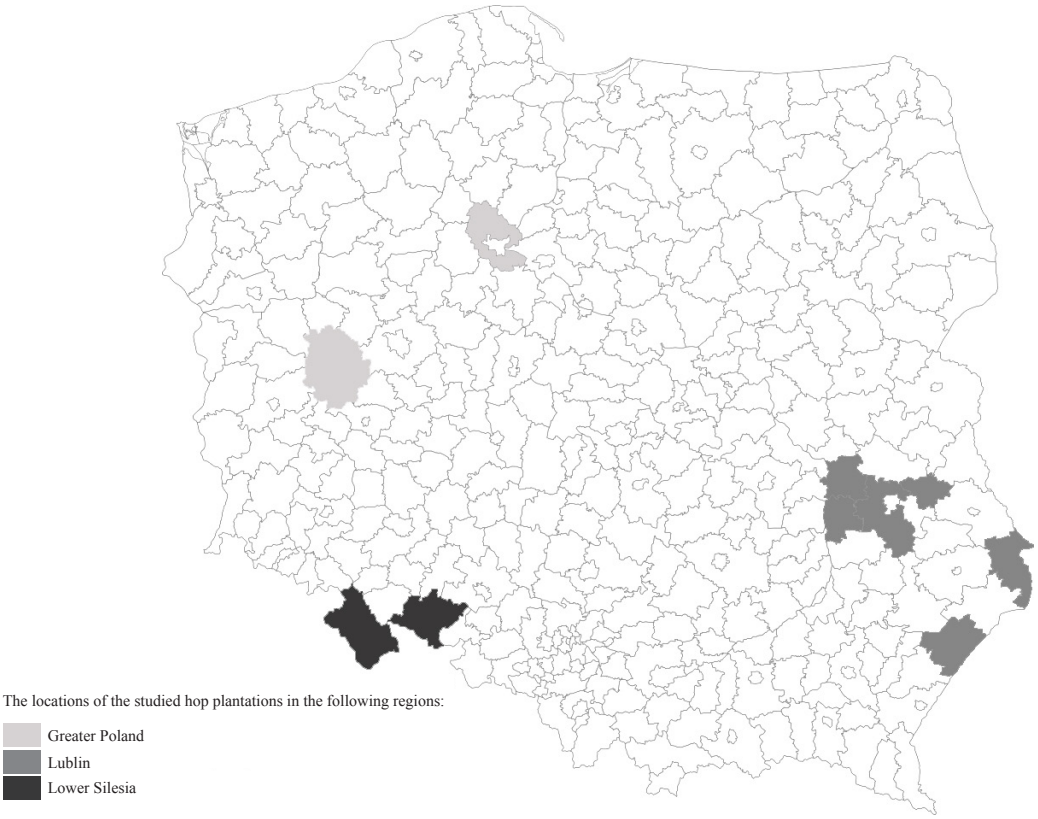



Figure 1. Locations of hop garden from which samples for research were collected, according to hop-growing regions in Poland.

at 50°C for 60 minutes, after which it was heated to 70°C for 15 minutes in order to stop the reaction and denaturation of the reverse transcriptase.

**DNA amplification**

DNA amplification was carried out using 2 µl of cDNA obtained through reverse transcription, 25 µl of Platinum Green Hot Start PCR 2x Master Mix (Invitrogen), 0.2 µM of each of the specific PCR reaction starters (Table 1). The reaction mixture was replenished with nuclease-free water to a final volume of 50 µl. PCR parameters consisted of a denaturation step at 94°C for 2 minutes, followed by 35 cycles: 94°C for 30 seconds, 60°C (ArMV, CBCVd), 56°C (HpLV), 54°C (HSVd), 55°C (AFCVd) for 30 sec, 72°C for 30 or 90 seconds (HpLV), to finish with an extension step at 72°C for 5 minutes. The obtained amplicons were separated on a 2% agarose gel stained with ethidium bromide and visualized in UV light.

## RESULTS AND DISCUSSION

In total, 1800 hop samples were collected over the period of 2018–2019 (Table 2). Samples were obtained from both symptomatic and asymptomatic plants. The research material was collected from 33 gardens with a total area of 55 ha. A larger number of samples collected in the Lublin region resulted from a greater number of gardens located in this region of hop cultivation.

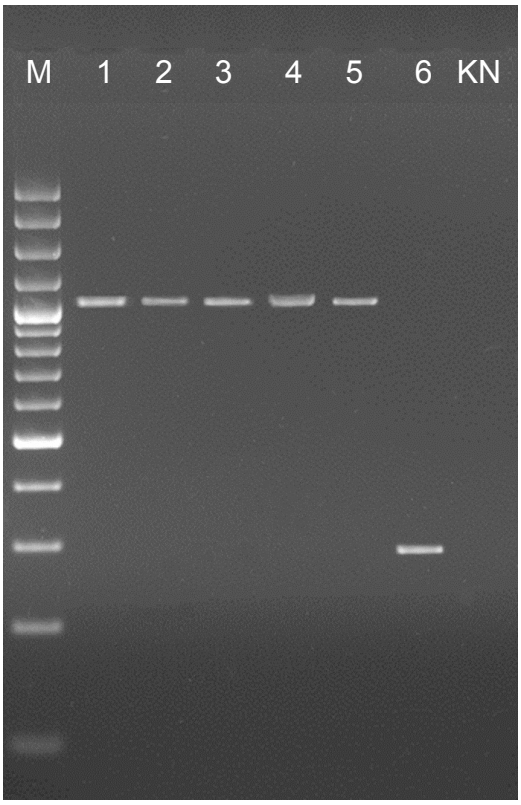
In the research conducted, the occurrence of particular studied pathogens was determined using RT-PCR screening method (Fig. 2). In order to detect HpLV, PCR primers, which amplify the 5'-end of the viral genome, were used. The design of these primers was based on the gene encoding methyltransferase, which is highly evolutionarily conserved. In order to detect ArMV, a highly conservative region encoding coat protein (CP) was amplified, and all of the viroids: HSVd, CBCVd, AFCVd were detected with the use of the primers which amplified their full genomes (Table 1).

Different pathogens can be detectable in infected hops at different stages of plant development. In the case of ArMV detection, it is best to obtain leaf samples during the spring season because only then is it possible to detect infection by this virus, while HpLV is best detected at a later period (Wetzel et al., 2002). In the conducted research, the occurrence of hop latent virus (HpLV) was found in all regions of hop cultivation in Poland (Table 3). In the Lublin and Greater Poland regions, the presence of the virus

was found in two gardens of the cv. Marynka, one per each region. In the Lower Silesian region, infections caused by HpLV were found in two gardens where the cv. Hallertau Tradition and cv. Magnum were cultivated. HpLV was detected solely in the samples obtained during the second and third term. This was in agreement with the observations of Ziegler et al. (2014) and Tsai et al. (2012), where HpLV was also detected when the samples were composed of mature leaves from the lower part of the plant obtained in the second half of the growing season.

As a result of this research, infections with the hop stunt viroid (HSVd) were also established, after having been detected only in the Greater Poland hop cultivation region on the cv. Magnum in one hop garden. As in the case of HpLV, HSVd infestation was found in samples collected during the second and third term. HSVd was first discovered in Japan in the 1970s, but its occurrence was also later reported in South Korea and the USA (Yamamoto et al., 1973; Sano et al., 1989; Lee et al., 1990; Pethybridge et al., 2008). It was found for the first time in Europe in 2012 (Radisek et al., 2012). In the current research, the hop plant from which samples were obtained for testing showed signs of leaf yellowing (Fig. 3). Similar signs were observed by Eastwell and Nelson (2007). Furthermore, 3–5 years after infection they recorded inhibition of the plant growth. The inhibition of the growth of infected plants leads to a decrease in harvest yield and a decrease in the level of alpha acids (Sano, 2003). In the reserach conducted, no stunting of the infected plant was observed, which may suggest that its infection occurred no earlier than 3 years ago. Like other viroids, HSVd is transmitted mechanically. It has many hosts: plum, peach, citrus and vine (Sano et al., 1989, Diener et al., 1988, Matoušek et al., 2003), which may be a reservoir of the pathogen.

In total, as part of the research, HpLV was detected in 11 samples taken from 4 hops, which constitutes 0.6% of the tested samples. This is a low infection rate especially compared to a study by Pethybridge (2005), who found HpLV infections in 6-87% of hop plants located in Australia, depending on the variety. In Europe, in Germany, based on the monitoring studies carried out by Seigner et al. (2014) it was found that HpLV is widely distributed




M – Ladder, lanes 1-5 – hop latent virus (HpLV), 1116 bp, lane 6 – hop stunt viroid (HSVd), 297 bp, KN – negative control.

Figure 2. Electrophoretic analysis of RT-PCR products in 2% agarose gel. The gel was stained with ethidium bromide.

In this research HSVd was found in 2 out of 1800 samples obtained from one hop garden. The first hop infections in Europe were found in 2007 in Slovenia, where 1 to 30% of plants were infected in various gardens (Radisek et al., 2012). The presence of HSVd was also confirmed in monitoring studies carried out in Germany, where infections

Table 1. PCR primers used for the detection of viruses and hop viroids.

| Pathogen | Primer | Sequence (5'-3') | Product (bp) | References |
|---|---|---|---|---|
| ArMV | ArMV-F | ACCAGTGCCTACAAGAGTGTGTCC | 213 | Komínek et al. 2003 |
| | ArMV-R | TTGATTCCAGTTGTTAGTGACCCC | | |
| HpLV | HpLV 5'Mlu | CGCACGCGTGGATAAACAAACATACAA | 1116 | Ziegler et al. 2014 |
| | HpLV 3'-1100 | GCTTAGCAATTGCGGATTGCAC | | |
| HSVd | HpSVd3-160 | GACGATCGATGGTGTTTCGAAG | 297 | Ziegler et al. 2014 |
| | HpSVd5-160 | ATCGATCGTCCCTTCTTCTTTAC | | |
| AFCVd | AF-F | TTGTCGACGAAGGGTCCTCA | 382 | Sano et al. 2004 |
| | AF-R | TTGTCGACGACGAGTCACCA | | |
| CBCVd | CVd-IV-F1 | GGGGAAATCTCTTCAGAC | 284 | Bernad, Duran-Vila, 2006 |
| | CVd-IV-R1 | GGGGATCCCTCTTCAGGT | | |

Table 2. Hop samples used in the tests.

| Region | Cultivar | | | | Total |
|---|---|---|---|---|---|
| | Aroma hop | | Bittering hop | | |
| Lublin | Lomik | 72 | Marynka | 300 | 1080 |
| | Lubelski | 192 | Magnum | 402 | |
| | Perle | 66 | | | |
| | Sybilla | 48 | | | |
| Greater Poland | Lubelski | 30 | Marynka | 60 | 402 |
| | Perle | 30 | Magnum | 252 | |
| | Hallertau Tradition | 30 | | | |
| Lower Silesia | Lubelski | 60 | Marynka | 72 | 318 |
| | Hallertau Tradition | 72 | Magnum | 114 | |
| Total | | 600 | | 1200 | 1800 |

Table 3. Incidence of viruses and viroids in hop garden.

| Region | Pathogen | | | | | |
|---|---|---|---|---|---|---|
| | HpLV | | | HSVd | | |
| | Cultivar | NHG | Samples | Cultivar | NHG | Samples |
| Lublin | Marynka | 1 | 3 | | 0 | 0 |
| Greater Poland | Marynka | 1 | 2 | Magnum | 1 | 2 |
| Lower Silesia | Hallertau Tradition | 1 | 2 | | 0 | 0 |
| | Magnum | 1 | 2 | | | |
| | Total | 4 | 9 | | 1 | 2 |

NHG – number of hop gardens with pathogen detected

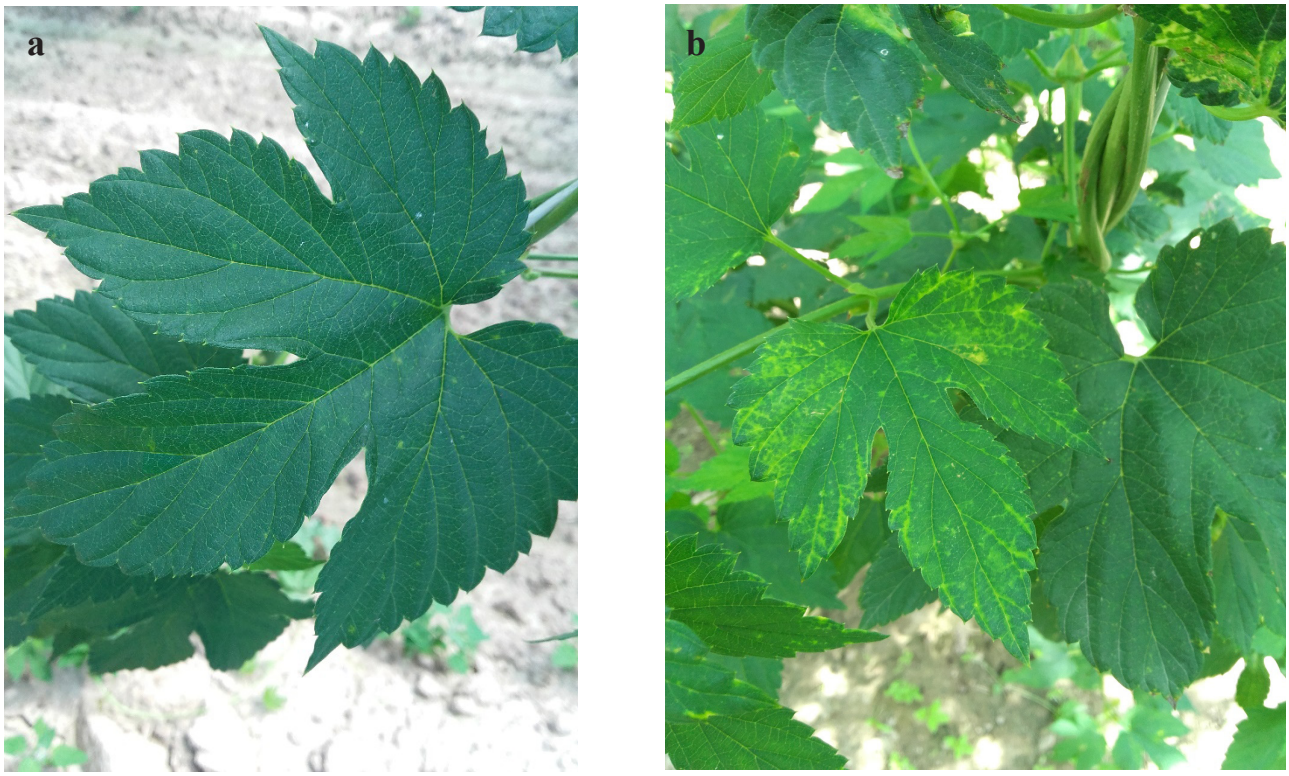



Figure 3. Hop leaf cv. Magnum a) no symptoms, b) with HSVd symptoms.

with this pathogen were detected in 9 out of 1444 tested plants (Seigner et al., 2014).

None of the tested samples showed the presence of *Arabis mosaic virus* (ArMV), *Apple fruit crinkle viroid* (AFCVd) and *Citrus bark cracking viroid* (CBCVd).

Monitoring the occurrence of viral diseases amongst plants is a very important activity, the purpose of which is to support early detection of threats and prevent the subsequent spread of diseases. The results indicate that HpLV and HSVd are the biggest threat to hop cultivations in Poland. However, it should be noted that in the case of HSVd only 2 samples from one infected hop plant was identified. Despite failure to find CBCVd, AFCVd and ArMV, the existence of this problem cannot be ruled out, especially that infections caused by these pathogens are known in Europe (Pethybridge et al., 2008; Radisek et al., 2012). Because genetic sources of resistance to HpLV and HSVd are unknown, it is crucial that the health of a hop garden be maintained at a high level by using high-quality seedlings as well as following phytosanitary recommendations, which will protect hop gardens against an infection and its subsequent spreading.

## CONCLUSIONS

1. HpLV was found in only a few gardens in all of the hop cultivation regions in Poland.
2. HSVd was found in one hop garden in the Greater Poland region in Poland.
3. There were no ArMV, AFCVd and CBCVd in Polish hop gardens.
4. Due to the recent reports on the emergence of new pathogens which have been absent in Europe so far, such as HSVd or CBCVd, constant monitoring of the health of hop plants should be carried out in hop gardens.

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