Hop Stunt Viroid: Effects on Vegetative Growth and Yield of Hop Cultivars, and Its Distribution in Central Washington State

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Abstract

Hop stunt viroid (HSVd) is an economically important pathogen that reduces growth and yield of hops. Visual symptoms of infected hop are highly dependent on cultivar. A study was conducted using six cultivars of hop to determine the impact on yield. Average dry cone yields of infected ‘Glacier’, ‘Cascade’, and ‘Willamette’ were reduced by 62, 14, and 34%, respectively, relative to noninoculated healthy plants. No significant yield reduction was observed for ‘Nugget’, ‘Columbus’, and ‘Galena’. The α -acid and β -acid contents showed a parallel pattern. Horticultural parameters of Willamette

and Nugget were measured in the final year of the study. Internode length, shoot length, and side-arm length were reduced by 29, 26, and 73%, respectively, for infected Willamette bines relative to noninfected bines; no effects were observed resulting from infection of Nugget. To understand the current potential impact of HSVd, a survey was conducted to determine its distribution in central Washington. The survey revealed that 17% of hop plants tested are infected. Hop yield and hop plant longevity will be significantly affected by this level of infection.

Hop stunt viroid (HSVd) is a subviral pathogen belonging to the genus *Hostuviroid*, family *Pospiviroidae* (Flores et al. 1998). Several genotypes of HSVd have been identified (Kawaguchi-Ito et al. 2009; Sano 2003). The number of reported genotypes is variable but there are five clades based on host specificity and sequence: hop–grapevine clade (Sano et al. 1985; Sasaki and Shikata 1977), German grapevine isolates (Puchta et al. 1988), citrus-cucumber clade, citrus cachexia clade (Reanwarakorn and Semancik 1999), and the stone fruit clade (Sano et al. 1989). In hop, HSVd causes hop stunt disease, which results in serious economic damage to some cultivars. Symptoms of hop stunt disease include stunting, moderate epinasty, and delay in climbing toward the top wire (Yamamoto et al. 1970). Hop is cultivated mainly for cones that contain organic acids referred to as α -acids and β -acids used in the brewing industry to add bitterness to beer. Reduced cone yield and an altered α -acid/ β -acid ratio have also been observed as a result of HSVd infection of hop plants (Yamamoto et al. 1973).

The United States is the second largest producer of hops in the world, and the largest producer of α -acid. In 2015, about 17,658 ha of U.S. land was used for hop cultivation. Washington State alone accounts for 75% of U.S. hop production (NASS 2015). HSVd was first identified in Washington State in 2004 (Eastwell and Nelson 2007). At that time, 15% of the plant samples collected in three major hop-growing regions of Washington were found to be infected with HSVd. However, sample size for this survey was limited. A more extensive study was needed to estimate the current distribution of HSVd in these hop-producing areas to gauge the economic impact of HSVd.

A consequence of the long history of hop stunt disease in Japan is that previous investigations of the impact of HSVd focused on hop cultivars that are commonly grown in that area. Variability in symptom expression among cultivars more commonly grown in North America was observed during the initial survey (Eastwell and Nelson 2007). Some cultivars such as ‘Nugget’ did not exhibit visual symptoms or acute reduction in plant growth. To determine the extent to which cultivars respond differently to HSVd infection, a study was

conducted that included six cultivars. Cone yields and α -acid and β -acid contents of HSVd-infected plants and noninoculated control plants were measured for 5 years. In the final year of the study, horticultural parameters of plant growth were measured for tolerant and moderately affected cultivars.

These studies will further advance our understanding of the long-term effects of HSVd on different hop cultivars which, in turn, will prove useful for better cultivar selection and disease management in the future.

Materials and Methods

Block design. The plot used for this study is located at the Irrigated Agriculture Research and Extension Center, Prosser, WA (46.26°N, 119.74°W, and 265 m elevation). A randomized block design plot was used to account for soil differences across the block. Each hill in a block was established from a single rhizome planted on spacing of 2.13 by 2.13 m, with buffer rows of ‘Glacier’ planted on all outside borders and on pole rows separating blocks. The bines were strung to a cross wire at a height of 5.5 m. All bines were tested and found free of all known hop virus-like agents, with the exception of *Hop latent viroid* that uniformly infected all plants in this study. Each block contained nine plants of each cultivar. In total, eight blocks each of ‘Willamette’ and Nugget and six blocks each of Glacier, ‘Cascade’, ‘Columbus’, and ‘Galena’ were planted. A drip irrigation system was used throughout. Standard horticultural practices for commercial production were followed.

All bines in one-half of the blocks of each cultivar were mechanically inoculated with HSVd in 2007. Each year, prior to harvest, RNA was isolated from leaves of each plant using the RNeasy plant mini kit (Qiagen, Valencia, CA) and tested for HSVd using reverse-transcription polymerase chain reaction (RT-PCR) (Eastwell and Nelson 2007) to confirm the HSVd status. Because of spread of HSVd in a portion of the research plot, the trial with Glacier was terminated in 2010 and the trials with Cascade, Columbus, and Galena was terminated in 2012, and those plots removed. In addition to research plots, yield data were collected from two commercial hop yards for Willamette and Glacier cultivars between the 2005 and 2009 growing seasons. Each of the commercial on-farm trials consisted of five paired blocks of four bines.

Measuring growth and yield. In each year of the study, three plants from each of the research blocks and four plants from the on-farm trial blocks were cut 0.6 m above the ground at commercial harvest time for each cultivar. Cones were harvested separately from each block for six consecutive years, except as noted. Cones were kiln dried following commercial practices. In the final year of the

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study, two plants per block were selected from the remaining research blocks and, in addition to cones, leaves and stems were also collected separately from each bine. Shoot length, internode length, and side-arm length of each bine were measured. The leaves and stems were dried for 48 h at 57°C, and dry weight of each plant component was measured. Relative matter partitioning was calculated by taking the ratio of dry weight of cones to dry weight of shoots (leaves and stems).

Measuring organic acids. Hop α -acids and β -acids were determined by following the American Society of Brewing Chemists spectrophotometric method Hops-6A and Hops-12 (American Society of Brewing Chemists 1992). Organic acid content was corrected to 8% moisture content.

Survey sampling. ‘Zeus’, Columbus, Cascade, ‘Super Galena’, Willamette, ‘Apollo’, Nugget, ‘Bravo’, ‘Chinook’, and ‘Centennial’ hops were the 10 most extensively planted varieties in Washington State (NASS 2015). To achieve 95% confidence in the outcome of a survey for a randomly distributed trait of each cultivar, a minimum of 180 samples of each cultivar is needed to be sampled (Hancock and Holler 1993). However, availability and access to samples highly influenced the number of samples collected. Additional proprietary varieties such as Bravo, ‘Palisade’, ‘Simcoe’, and cultivars planted on smaller acreages, including Glacier, ‘Sterling’, ‘Vanguard’, and Galena, were included in this survey.

During a 2-week period of early July 2012, leaf samples from a total of 1,635 hop plants of 20 different cultivars were collected from three distinct hop-growing regions of eastern Washington: lower

Yakima Valley, Moxee Valley, and Toppenish Creek. Four leaves per plant were pressed onto Whatman FTA Plant Saver Cards (Fisher Scientific, Inc., Pittsburgh) following the manufacturer’s recommendations. Nucleic acid collection cards were sealed in plastic bags and stored at –20°C until used for nucleic acid extraction.

At the time of analysis, four to five discs (2 mm) were excised from FTA cards, to which 250 μ l of GEB buffer (0.01 M Na_2CO_3 , 0.03 M NaHCO_3 , 2% polyvinylpyrrolidone, 0.5% Tween-20, and 0.2% bovine serum albumin) was added. After incubating at room temperature for 60 min, 8 μ l of this solution was transferred to 92 μ l of GES buffer (0.1 M glycine, 50 mM NaCl, 1 mM EDTA, and 0.5% Triton X 100, pH 8.0) containing 1% of 2-mercaptoethanol. The mixture was incubated at 95°C for 10 min and rapidly cooled on ice.

RT-PCR. Samples were tested for HSVd using RT-PCR using the SuperScript III one-step system Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Primers HSVd1 (GCCCCGGGGCTCCTTT CTCAGGTAAG) and HSVd2 (GGCAACTCTTCTCAGAATCC) (Eastwell and Nelson 2007) were used for RT-PCR. First-strand synthesis was performed at 60°C for 30 min. Polymerase activation was achieved at 94°C for 2 min; followed by 35 cycles of 94°C for 15 s, 59°C for 30 s, and 68°C for 1 min; followed by a final extension step at 68°C for 10 min. Products were resolved by high-voltage capillary electrophoresis (QIAxcel; Qiagen), and size determined by comparison with a size marker of 50 to 800 bp (Qiagen). Throughout the study, an HSVd-infected positive control, a negative control, and a nontemplate control were included during HSVd testing to monitor for the possible occurrence of false-positive or false-negative assay results.

Fig. 1. Impact of *Hop stunt viroid* infection on cone yield was measured in six hop cultivars. GL = Glacier, WI = Willamette, CA = Cascade, CS = Columbus, NU = Nugget, and GA = Galena. “F” indicates values from on-farm commercial grower plots. Values are the calculated yield per hectare of infected plants relative to noninoculated plants. Error bars indicate 95% confidence intervals; * indicates the statistically significant difference between infected plants and noninoculated plants of each cultivar at $\alpha < 0.05$. Significant difference at $\alpha < 0.05$ between cultivars in response to *Hop stunt viroid* is represented by different letters (A, B, C, and D).

Fig. 2. Relative cone yield of *Hop stunt viroid*-infected plants compared with an uninfected control in Willamette and Nugget from 2008 to 2013. Relative cone yield is assessed by taking the ratio of dry weight of cones from infected hops to dry weight of healthy hops. Dotted line is a reference line to indicate yield in uninfected control hops. Error bars indicate 95% confidence intervals; * indicates the statistically significant difference between infected plants and noninoculated plants of each cultivar at $\alpha < 0.05$.

A second primer pair (HSVd56 [GGTACTTACCTGAGAAAG GAGCCCCGGGGC] and HSVd160 [GAAGGTAAAGAAGAAG GACGATCGATGGTG]) was used to confirm the reliability of RT-PCR with primer pair HSVd1 and HSVd2. RT-PCR conditions for HSVd56 and HSVd160 were the same as described above, except the annealing temperature was reduced to 53°C. In total, 12 samples from FTA card extracts and 20 RNA extracts from hop leaves were tested with both primer pairs. Results were 100% consistent when results of RT-PCR with the two primer pairs were compared. Therefore, further RT-PCR assays of the 1,635 survey samples were performed only with primers HSVd1 and HSVd2.

Results

Effect of HSVd on cone yield of hop cultivars. In the first 3 years after infection, HSVd-infected Glacier consistently exhibited a reduction in production, with an average of 66% reduced dry cone weight by yield per hectare compared with the noninoculated control. This reduction in dry cone weight yield is comparable with the 64% reduction in yield observed from the 3-year on-farm trial. Spread of HSVd within the research block led to removal of Glacier after 3 years. Harvest data from the remaining five cultivars continued for the next two growing seasons. Over 5 years, the average dry cone weight yield of Willamette, Cascade, Columbus, Galena, and Nugget was reduced by 28, 14, 6, 6, and 2%, respectively (Fig. 1). The cone yield of infected Willamette from the commercial on-farm trial was reduced by 35% relative to noninoculated control bines.

In the final year (2013), only Nugget and Willamette were used for harvest data. Infection by HSVd resulted in a significant reduction in cone yield of Willamette in all years of the study (Fig. 2). The average reduction of cone yield of Willamette hops was 34% over the 6 years

of observation. Nugget cone yield was not significantly affected throughout the years of this study.

Effect on α -acid and β -acid content. When the α -acid yield per hectare and β -acid yield per hectare are considered, yield from the cultivars Glacier, Willamette, and Cascade are significantly reduced as a result of infection with HSVd (Figs. 3 and 4). Over 3 years of study, the α -acid and β -acid contents of Glacier cones were reduced 31 and 40%, respectively. Superimposed on the overall reduction in cone yield, this constitutes a reduction in α -acid and β -acid production by 73 and 76%, respectively, for Glacier. Over 5 years, the average α -acid and β -acid contents of Willamette cones were reduced by 37 and 36%, respectively. The major impact of HSVd on the production from Cascade was on cone yield. The α -acid and β -acid contents were not significantly affected but, when the cone yield and organic acid were considered together, the yield of α -acids and β -acids on a per-hectare basis was reduced by 17 and 18%, respectively. Yields from cultivars Columbus, Nugget, and Galena are not significantly affected (Figs. 3 and 4).

In all cultivars included in this study, the ratios of α -acids to β -acids were not significantly affected in response to infection.

Effect on growth. Stunting and growth retardation were observed in HSVd-infected Willamette relative to noninoculated plants. Side-arm length was reduced by 73% relative to uninfected control plants. Internode length and shoot length were also reduced by 29 and 26%, respectively, compared with noninoculated controls. No significant reduction in the number of nodes was observed.

In Nugget, a slight reduction in internode and side-arm length by 4% each was observed compared with the uninfected control; however, differences were not statistically significant. In contrast, the number of nodes per bine was higher in infected Nugget plants (Fig. 5).

Fig. 3. Impact of *Hop stunt viroid* (HSVd) on the yield of α -acids was measured on six different cultivars. GL = Glacier, WI = Willamette, CA = Cascade, CS = Columbus, NU = Nugget, and GA = Galena. Values are the calculated yield per hectare of infected plants relative to noninoculated plants. Each bar is the average of 5 years of harvest data collected from a research plot at Washington State University, with the exception of the two values labeled "F", which are from 5 years of data from on-farm commercial grower plots. Bars represent 95% confidence intervals; * indicates the statistically significant difference between infected plants and noninoculated plants of each cultivar at $\alpha < 0.05$. Significant difference at $\alpha < 0.05$ between cultivars in response to HSVd is represented by different letters (A, B, C, and D).

Fig. 4. Impact of *Hop stunt viroid* (HSVd) on the yield of β -acids was measured on six different cultivars. GL = Glacier, WI = Willamette, CA = Cascade, CS = Columbus, NU = Nugget, and GA = Galena. Values are the calculated yield per hectare of infected plants relative to noninoculated plants. Each bar is the average of 5 years of harvest data collected from a research plot at Washington State University, with the exception of the two values labeled "F", which are from 5 years of data from on-farm commercial grower plots. Bars represent 95% confidence intervals; * indicates the statistically significant difference between infected plants and noninoculated plants of each cultivar at $\alpha < 0.05$. Significant difference at $\alpha < 0.05$ between cultivars in response to HSVd is represented by different letters (A, B, C, and D).

Results showed that relative matter partitioning in response to HSVd infection is similar in Willamette and Nugget. Cone to vegetative tissue dry weight ratio was higher in infected plants compared with noninoculated controls of both cultivars (Fig. 6).

Incidence of HSVd in central Washington. Lower Yakima Valley, Moxee Valley, and Toppenish Creek are three major hop-growing areas in Washington State. HSVd was detected in all three of these regions (Table 1). HSVd infection rates varied from 6 to 72% depending on the cultivar, with an average of 17% of the total 1,635 samples tested (Table 2). Galena, Glacier, and Cascade showed a higher incidence of HSVd relative to other cultivars. Out of 20 cultivars represented in this survey, only 1 cultivar, designated “D”, did not yield any plants that were positive for HSVd. Cultivar D is a propriety variety that was recently released; consequently, the sample size for this cultivar in this survey was limited to 15 bines. Samples of other cultivars that were recently released for commercial production were found to be infected with HSVd to levels comparable with the older public cultivars (14 versus 17% overall).

Discussion

The effect of viroid infection on growth and yield of several crop species was previously studied. Tuber yield in potato infected with severe strains of *Potato spindle tuber viroid* was reduced up to 64% (Singh et al. 1971). Studies also indicated that the quantitative

impact of viroid infection is highly cultivar dependent. For instance, *Avocado sunblotch viroid* infection reduced yield in ‘Fuerte’ by 14 versus 82% in ‘Edranol’ (da Garca 1985). Additionally, the particular strain of the viroid (Itaya et al. 2002) and temperature (Gomez et al. 2008; Harris and Browning 1980; Singh 1983) also influence impact on the host.

Hop stunt disease was first observed in Japan, where ‘Kirin II’ showed severe stunting, and the yield was reduced by 50% (Sano 2013; Yamamoto et al. 1973). During that time, most of the hop industry in Japan was dominated by a single cultivar; this is in contrast to the current situation, where several cultivars of hops are grown worldwide for their distinctive flavors and brewing characteristics. HSVd is now reported in all the major hop-growing areas of the world (Eastwell and Nelson 2007; Guo et al. 2008; Lee et al. 1988) and has emerged as a major threat to hop cultivation (Kawaguchi-Ito et al. 2009; O’Neal 2015; Parkinson and Reed 2013).

Comparison of the response of several cultivars to HSVd reveals that there is a strong cultivar dependence on the impact of HSVd on yield and growth. When individual yield components are compared, it is evident that the cone yields of three sensitive cultivars are reduced by infection with HSVd (Fig. 1). The α -acid and β -acid content of Glacier and Willamette are also significantly reduced whereas the α -acid and β -acid content of Cascade is unaffected.

Fig. 5. Effect of *Hop stunt viroid* (HSVd) on vegetative growth parameters of Willamette and Nugget. Dotted line is a reference line to indicate yield in uninfected control hops. Error bars represent 95% confidence intervals; * indicates the statistically significant difference between infected plants and noninoculated control plants of each cultivar at $\alpha < 0.05$. Significant difference at $\alpha < 0.05$ between cultivars in response to HSVd is represented by different letters (A and B).

Fig. 6. Effect of *Hop stunt viroid* on relative matter partitioning of Willamette and Nugget. Error bars represent 95% confidence intervals. Levels not connected by same letter are significantly different at $\alpha < 0.05$.

Thus, the cultivars investigated in this trial exhibit three distinct patterns in the response to infection by HSVd: (i) reduced cone yield and reduced α -acid and β -acid content, (ii) reduced cone yield only, and (iii) no effect.

The average yield loss in infected Willamette was 34% over the span of 6 years; reduction in growth and canopy development was also observed. Moreover, yield loss was higher as years progressed (Fig. 2); this roughly parallels the pattern reported for other hop plants (Sano 2003). This increased symptom expression over time may be due to increased viroid concentration or more extensive colonization of the root area. Decreased internode, side-arm, and shoot length led to an overall reduction of canopy of infected Willamette bines; this phenomenon was also observed for the sensitive Glacier (Eastwell and Nelson 2007). Side-arm length is the trait that was effected most in infected bines compared with all other physical parameters that were measured in this study. Recent studies showed that HSVd infection caused disturbances in hop regulatory genes in 'Admiral' (Füssy et al. 2013); this might be one of the underlying causes for stunting in some cultivars. The physiological and biochemical basis for the differences in response across cultivars needs further investigation. Interestingly, relative matter partitioning showed similar patterns in both sensitive and tolerant cultivars in response to HSVd infection; that is, infected bines have a higher cone/vegetative growth ratio than healthy bines. This shows that photoassimilate resources of the plants were channeled more toward reproductive tissues rather than vegetative tissues in the presence of HSVd infection.

HSVd spreads mainly through mechanical means and infected propagating material (Pethybridge et al. 2008; Sano 2013). No natural vectors are reported for HSVd transmission. The present survey

revealed that 17% of hops growing in this region are infected with HSVd, which is slightly higher compared with the tests reported in 2007. Our survey shows that HSVd can infect all 20 cultivars used in this survey, which included 10 major cultivars grown in the central Washington growing region. Although symptom expression and significant yield loss occur only in some cultivars, the asymptomatic cultivars can act as reservoirs for the viroid. Moreover, detailed analysis of the impact of HSVd infection on brewing quality of hops remains to be determined. Retention of tolerant but infected hop cultivars may reduce the ability of the industry to respond to future trends in demand for hop varieties. Immediate removal of infected bines will decrease the sources of viroid inoculum. Coupled with modifications in horticultural practices to reduce the mechanical transmission of HSVd, these actions will reduce the spread of hop stunt disease in the future.

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Table 1. Distribution of Hop stunt viroid in hop-growing regions of Washington State

Location	Total samples (n)	Samples positive (n)	Positive (%)
Lower Yakima Valley	625	138	22
Moxee Valley	564	112	20
Toppenish Creek	446	45	10

Table 2. Number of samples collected and tested positive for Hop stunt viroid within each hop cultivar

Cultivar ID	Number of		Positive (%)
	Total samples tested	Samples tested positive	
Cascade	195	61	31
Centennial	188	18	10
Chinook	231	30	13
Columbus	31	4	13
Galena	47	34	72
Glacier	16	9	56
Nugget	57	12	21
Sterling	16	1	6
Super Alpha	65	5	8
Super Galena	126	14	11
Tomahawk	98	24	24
Unidentified	73	13	18
Vanguard	32	3	9
Zeus	179	20	11
Proprietary selections ^a			
Cultivar A	176	28	16
Cultivar B	10	2	20
Cultivar C	15	1	7
Cultivar D	15	0	0
Cultivar E	65	6	9
Total	1,635	285	17

^a Some recent proprietary cultivars are in limited distribution; identities are withheld to preserve data from individual farms.

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