

博士学位論文

Study on the influence of the hop cultivation factors
on its growth, development and qualities

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CHAPTER 1

INTRODUCTION

Hop (*Humulus lupulus* L.) is a perennial, dioecious, and climbing plant. In plantations, and while still underground, hop sprouts from the root during spring and goes up the wires by twining. It reaches a height of 6–8 m only three months after sprouting and in early summer it blooms; hop then develops cones, which are generally harvested within one to two months after blooming.

Hop provides bitterness, fullness, and flavor to beer, all derived from the α -acids, polyphenols, terpenes, etc. present within it (Inui et al., 2013). In order to control beer taste and flavor, different cultivars (Kovacevic et al., 2015) and different processed hop products, such as pellets or hop extracts (each containing different components) are generally used. And controlling the brewing process, especially the boiling process, is another effective means of determining beer quality. In general, hops are added at the beginning of the wort boiling stage to isomerize alpha acid to iso-humulone in order to adjust the bitterness of the beer. However, the addition of hops at the beginning of the wort boiling stage also causes a loss of hoppy aroma due to the increase in evaporation of aroma compounds such as terpenoids. Thus, the timing and the amount of hops added needs to be adjusted in order to achieve the target bitterness and the desired aroma qualities. In addition, the fermentation conditions, the yeast strains used and the temperature, pressure and tank size are also important and must be controlled because they can affect the transition ratio of iso-alpha acid, terpenes and polyphenols from wort to beer and the metabolites present (Van

Opstaele et al., 2006; Schönberger, et al., 2011).

To some extent, it is possible to achieve the desired beer quality simply by selecting specific cultivars, adding hop products, and brewing it properly. However, hop is an agricultural crop and its quality is itself unstable, varying according to climate (Krofta and Kucera, 2010), root properties (e.g. age, viral infections), and cultivation conditions such as fertilizing, pruning, and harvest (Sharp et al., 2014). Therefore, it is important to control cultivation conditions in order to obtain high quality hop.

Many researchers have studied the relationship between hop quality and its cultivation conditions. Krofta and Kucera (2010) reported that hop quality could be affected by the climatic conditions in the cultivation region and year. Mozny et al. (2009) also reported that recent climate changes led to earlier flowering and reduced α -acid content. Kishimoto et al. (2008) reported that the changes observed in the amounts of hop aroma compounds were due to the use of agrochemicals to prevent downy mildew. In addition, the prohexadione calcium treatment applied during blooming period is known to affect some of the secondary metabolites (Kavalier et al., 2014). It has also been reported that viral and viroidal infections reduce the vegetative growth of hops (Pethybridge et al., 2008; Fussy et al., 2012; Sano, 2012). Various cultivation factors can affect hop photosynthesis (Pokorny et al., 2011). Hop root age also affects the aroma quality in hop and beer (Pluhackova et al., 2011). Hence, several cultivation factors can affect hop quality.

Cultivation factors can be classified into three types: 1) 'natural origin', such as the kind of soil, and hop plant age; 2) 'culture-controlled' such as fertilizer application, pruning, and harvest

timings; 3) ' climatic origin', such as temperature, rainfall, and the amount of sunlight.

Table I-1 Classification of cultivation factors

factor type	variety
1) Natural origin	Soil
	Plant age
	Disease (Fungi, B Fungi etc.
2) Culture-controlled	Pruning time
	Harvest time
	Fertilizer application etc.
3) Climatic origin	Temperature
	Rainfall
	Amount of sunlight etc.

In this thesis, we investigated the influence of pruning, blooming and harvest timings (culture-controlled), plant age(natural origin) and virus(culture-controlled and natural origin) on the hop growth, development, qualities and beer qualities for Saaz variety cultivated in Saaz region in Czech Republic. Saaz variety is mainly cultivated in three areas - Saaz, Aucha, and Tirschitz in Czech Republic. Saaz region was selected for this study because it is the largest of the three (**Fig. I-1**).

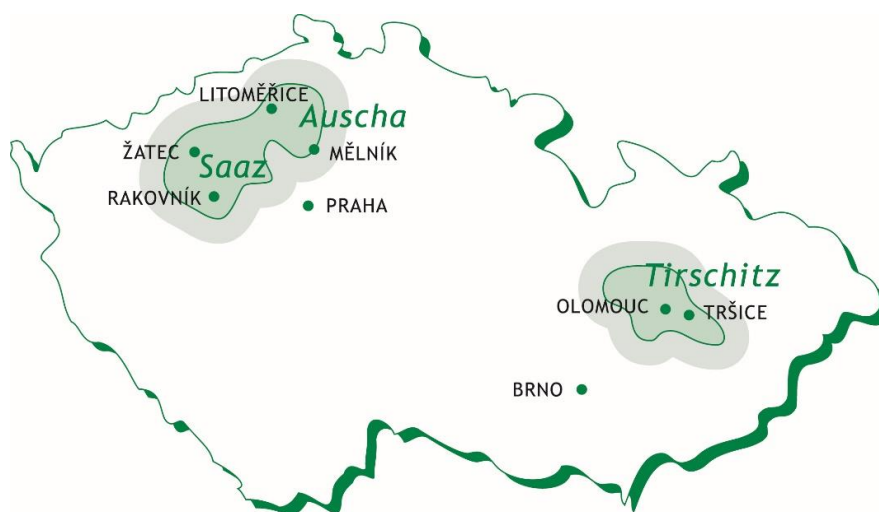


Fig. I-1. The three hop cultivation areas in the Czech Republic

CHAPTER II

The influence of pruning and harvest timing on the growth, development, yield, and qualities of hop

Introduction

In this chapter, we investigated the culture-controlled factors, pruning, blooming, and harvest timings, and analyzed the relationship between these three variables and hop aroma quality. Pruning and harvest were considered as the start and end processes of hop cultivation, respectively. Regarding cultivation timing, the behavior of chemical compounds after blooming has been analyzed previously (Skinner et al., 1974; Menary and Doe, 1983; Murphey and Probasco, 1996). The optimal harvest timing for each hop variety can be estimated from indicators such as yield and appearance (Lutz et al., 2009). It was also confirmed that harvest timing influences hop chemical components, such as terpenes, and these affect the beer's sensory quality (Sharp et al., 2014). In addition, hop storage index (HSI), an indicator of hop's deterioration, was found to increase with a delay in harvest timing (Cocuzza et al., 2013). On the other hand, functional flavonoids, such as desmethyl xanthohumol, were reported to increase during cone formation (Keukeleire et al., 2003).

Although the above-mentioned studies considered hop maturity and harvest timing, none of them included pruning and blooming timing as variables. In the present study, hop quality (α -acid, essential oils, and terpene compounds) and beer sensory quality were studied with the Saaz variety from Saaz area, Czech Republic. In the Saaz variety, pruning is performed during April in order to guide the twining of the new sprout above ground, and it lasts about 20 days; harvest of hop cones occurs from the middle of August to the beginning of September, within about 25 days (**Fig. II-1**).

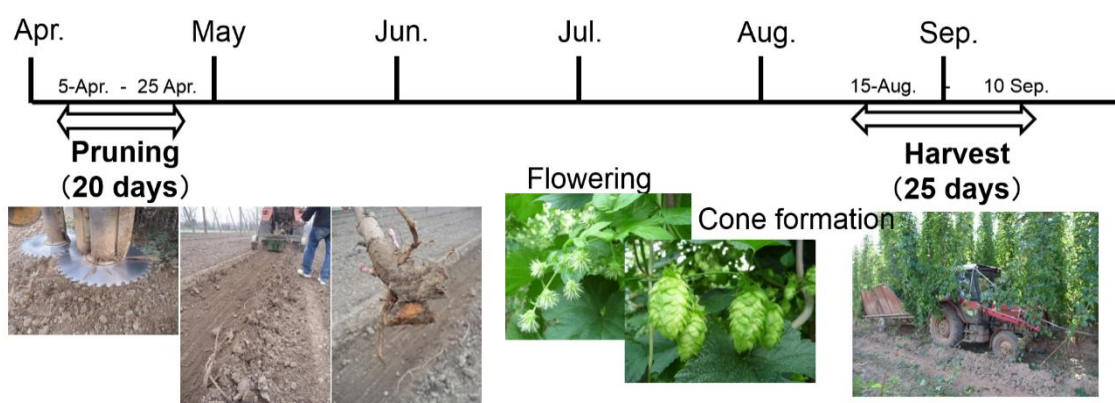


Fig. II-1. Flow of Saaz hop cultivation in Czech Republic

Pruning and harvest seasons are thus longer than those of other varieties in the world, which might be related to the fact that over 90% of the cultivation area in the Czech Republic is occupied by one variety (Saaz). This is a very wide range and might result in the fluctuation of hop and beer quality. Because pruning timing may affect blooming timing, it may also affect the vegetative and reproductive periods as a result (**Fig. II-2.**), which is the main reason for including the effect of pruning timing on blooming timing and hop quality, and the effect of the time span between blooming and harvest on hop quality, yield, and aroma in beer.

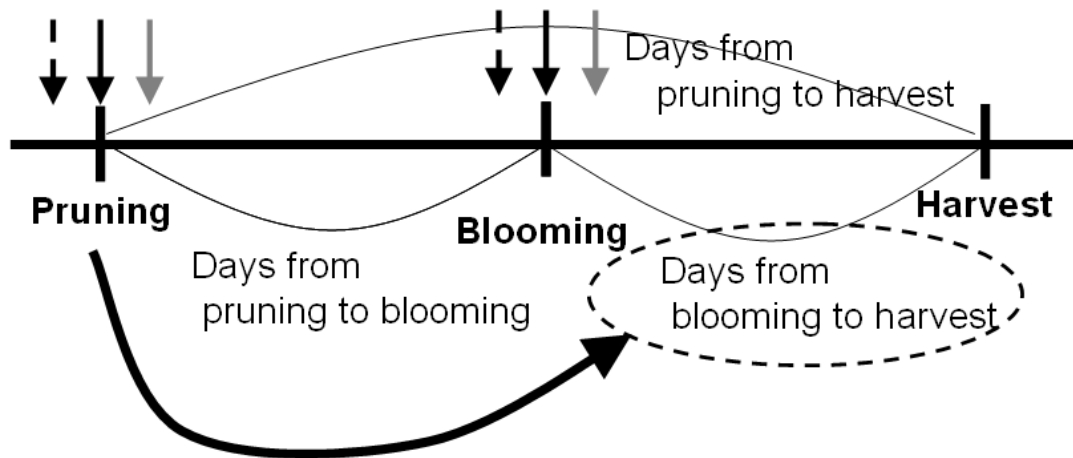


Fig. II-2. Point of view and hypothesis in this study about hop cultivation period

Materials and Methods

Test conditions

Four villages—Rybnany, Lipenec, Blsany, and Kroucova—in the Saaz region were selected and used for experimental cultivation (test gardens). These four are located in the north-west, north, south-west, and south-east side of the Saaz region, respectively (**Fig.II-3**), and are at distance of about 10 km from each other. The kind of soil and plant age differed between the test gardens (**Fig.II-3**). As shown in Table I-1, in 2012, 15 combinations of pruning and harvest timings were tested within each garden: three pruning (Apr. 5, 15, and 25) conditions were combined with five harvest timings (Aug. 15 and 25, Sep. 3, 10, and 20); eight vines were harvested for each combination of pruning and harvest conditions. The influence of harvest timing was also examined in 2010 and 2011, in Rybnany (**Table I-1**).

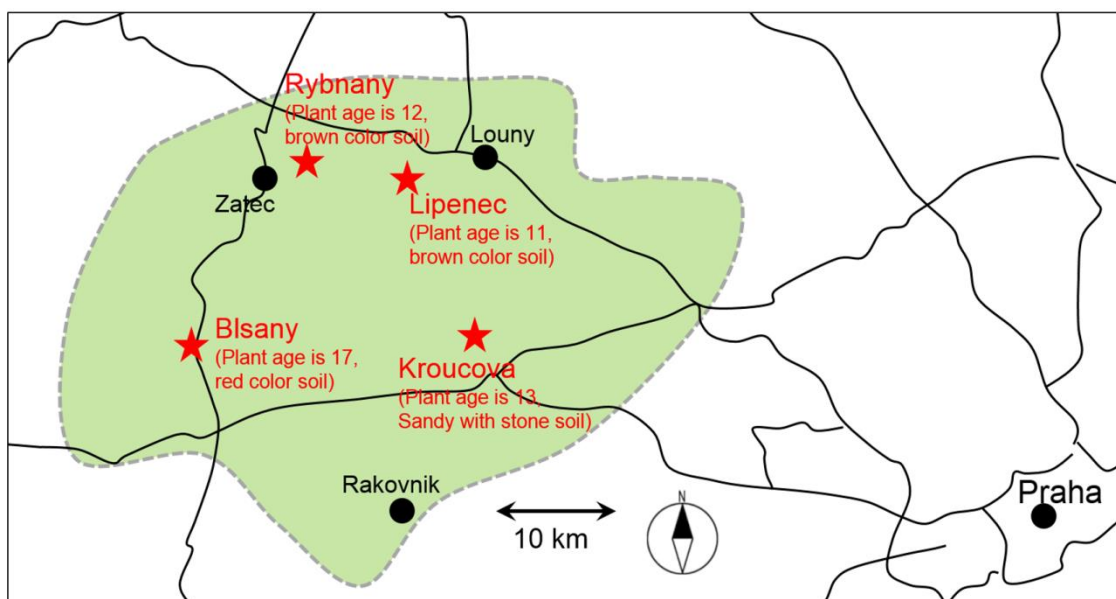


Fig.II-3. Geographic distribution of the four test locations. Saaz region is shown by green color.

Table II-1. Experimental design

Location	Year	Pruning	Harvest
		5-Apr(±1day)	15-Aug
			24-Aug
			3-Sep
			12-Sep
			20-Sep
-Rybnany	2012	15-Apr(±1day)	15-Aug
-Lipenec			24-Aug
-Blsany			3-Sep
-Kroucova			12-Sep
			20-Sep
		25-Apr(±1day)	15-Aug
			24-Aug
			3-Sep
			12-Sep
			20-Sep
-Rybnany	2011	14-Apr	15-Aug
			22-Aug
			29-Aug
			5-Sep
			12-Sep
			19-Sep
-Rybnany	2010	16-Apr	18-Aug
			24-Aug
			30-Aug
			7-Sep
			14-Sep
			21-Sep

Climatic data

Temperature and precipitation data were obtained from the climate observation systems administrated by the Hop Research Institute Co., Ltd., for four locations near the four experimental gardens (Rybnany, Lipenec, Blsany, and Kroucova).

Hop blooming and cone formation

Reproductive development characteristics i.e., flowering and cone formation were recorded every week from the end of June to the middle of August. Flowering and cone formation seasons were defined by the 50 % flowering/ cone formation ratio, calculated from the average of that observed in the eight vines collected in each experiment.

Calculating the yield

A picking machine was used to collect all the raw hop cones and their weight and moisture content were determined. Shortly after harvest, they were kilned at 55 °C for about eight hours until their moisture content reached $10 \pm 1\%$. After kilning, the dry weight of each hop cone was determined in order to calculate the accurate yield.

Storage conditions for hop samples

Hop samples were collected soon after kilning was finished. Kilned hop samples were packed by nitrogen purge and stored below their freezing point until used in chemical analyses and brewing trials.

Chemical analyses of hop samples

The chemical analyses of hop samples were conducted at the Hop Research Institute Co., Ltd. in Saaz, Czech Republic. Hop α -acid was measured using the EBC 7.7 liquid chromatography (High Performance Liquid Chromatography) method with a Nucleosil RP C18 column (Macherey-Nagel, Germany, 5 μ m, 250 \times 4 mm) and a SHIMADZU LC 20A chromatograph (Shimadzu, Japan) with diode array detectors (DAD).

Hop essential oils were obtained by steam distillation and the terpene content of the distilled oil was determined by gas chromatography (GC) on a DB 5 capillary column (Chromservis, Czech Republic, 30 m \times 0.25 mm \times 0.25 μ m film thickness) using a Varian 3400 gas chromatograph together with a Finnigan ITD 800 mass detector. Qualitative criteria were based on a comparison of GC retention indices and mass spectra with those of pure compounds. A semi-quantitative evaluation of the composition of hop oils was performed on the basis of the peak area of individual components and expressed in relation to the total integrated area of all substances involved.

Hop color measurement

A dried hop cone was pulverized using a coffee mill (Melita Japan Ltd.) Milling was adjusted to get identical particle sizes. The color of pulverized hop was evaluated using colorimetric values in a CIE L*a*b system measured with a CM-2022 spectrophotometer (Minolta Co., Ltd, Tokyo, Japan), and the data were calculated under a 10° observer and D65 illuminants. Color data were analyzed using SpectraMagic™ color-control software (Minolta). The closest RHSCC number to hop's cone color, based on its CIE L*a*b value, was obtained by visual discrimination and measurement with the device mentioned above. RHSCC number is a standard reference for plant color identification in the horticultural industry selected by the Color Classification System version 2.1.1 (The Japan Research Institute Co., Ltd.) and can be used for objective selection of the closest RHSCC number by color data.

Hop preparation for sensory evaluation

A dried hop cone was pulverized using a coffee mill (Melita Japan Ltd.), adjusting the milling power in order to get identical particle sizes. One gram of pulverized hop was added to 100 ml of 10 mM citrate buffer solution (pH 5.3) at 100 °C. This mixture was kept at this temperature, without heating, for 5 min and cooled on ice water afterwards. Hop grounds were then removed using a 0.45 µm filter and 5 ml of the resulting filtrate was diluted in 20 ml of distilled water for sensory evaluation.

Brewing trials

Trials were conducted in a pilot scale brewery (100 L) with a malt ratio of 100 %. Hops were added twice: at the beginning (kettle hopping) and at the end (late hopping) of wort boiling. In order to compare differences in hop aroma characteristics, an identical amount of each hop sample was added during the late hopping stage, and only at this stage. The amount of hop extracts added at kettle hopping was decided to be the same, in order to provide identical bitterness to beer samples. Worts were fermented at 10 °C using lager yeasts.

Linalool analyses in beer

Exactly 2.0 g of beer were poured into a 10 ml crimp-top vial; 20 µl of 50 mg/l beta-Damascone were then added (final concentration = 0.5 mg/l) and a Mono trap™ (GL Sciences Inc.) was introduced in the vial, before attaching the cap (Φ 20 mm). The vial was strongly shaken for 30 min, the Mono trap™ was taken out and put into a MT Extract Cup, and 200 µl of methylene chloride were added to the vial as the extraction solvent; water was added outside the glass containers and the MT Extract cup was again set in the vial. Samples were sonicated for 5 min. and 0.2 µl of the extraction solvent were then used in Gas Chromatography-Mass Spectrometry and quantified by the Single Ion Monitoring mode.

Sensory evaluation

Seven well-trained panelists conducted beer sensory evaluation based on hop aroma intensity and five generic descriptions of hop aroma: floral, fruity, citrusy, herbal, and sylvan

(woody). Hop aroma in beer was compared to that of hop essential oil produced by Botanix Ltd. and panelists scored each characteristic on a scale from 0 to 5. All scores were then normalized to remove any panelist bias. 4 or 5 samples from same location and same year were conducted at a single session and it was done at least 2 times in different date. Their order was randomized for each panelist.

Statistical analysis

Statistical tests were conducted using the JMP 10.01 software (SAS, Cary, NC, United States). Statistical differences between means were determined by two-way ANOVAs (Analysis of Variance), considering three significance levels: $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results and Discussion

Influence of the pruning date on the blooming date

Fig. II-4 shows the relationship between pruning and blooming (cone formation) dates at the four locations, in 2012. It can be seen that pruning date does not influence blooming date as the slope of the line corresponding to their correlation is very small. There was no correlation between the date of pruning and the date of cone formation. However, shorter pruning intervals corresponded to shorter blooming intervals. The cumulative temperature calculated from the average temperature in each day (Fig. II-3) was 290 °C in April (pruning period) and 1,942 °C from April to July

(blooming period). Hence, the ratio of cumulative temperature in April is only 15 %, suggesting that the 20 days difference in pruning dates in April did not affect the blooming dates.

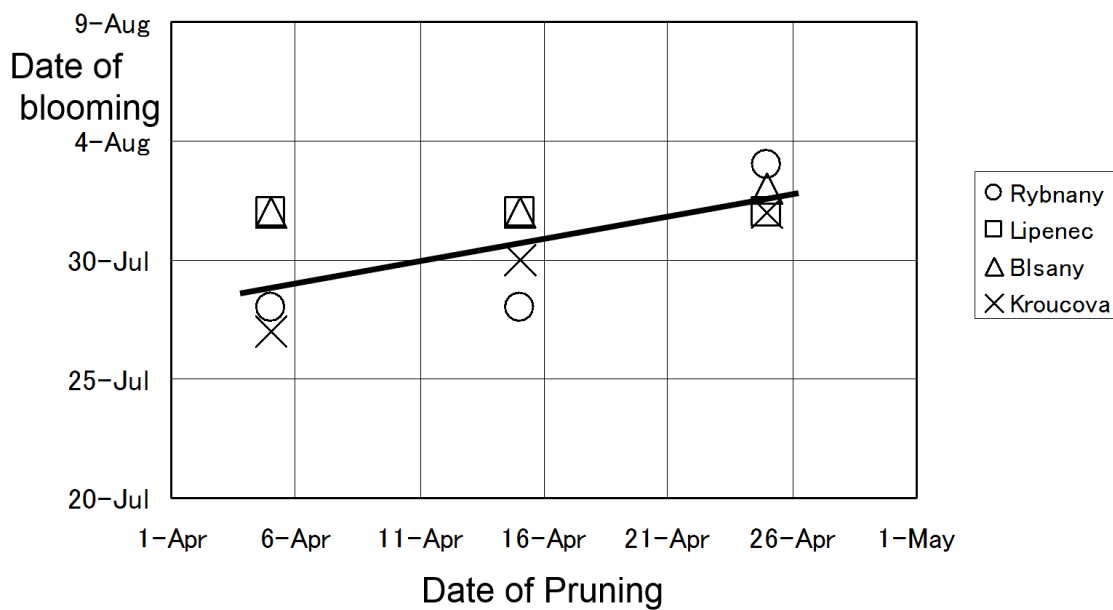
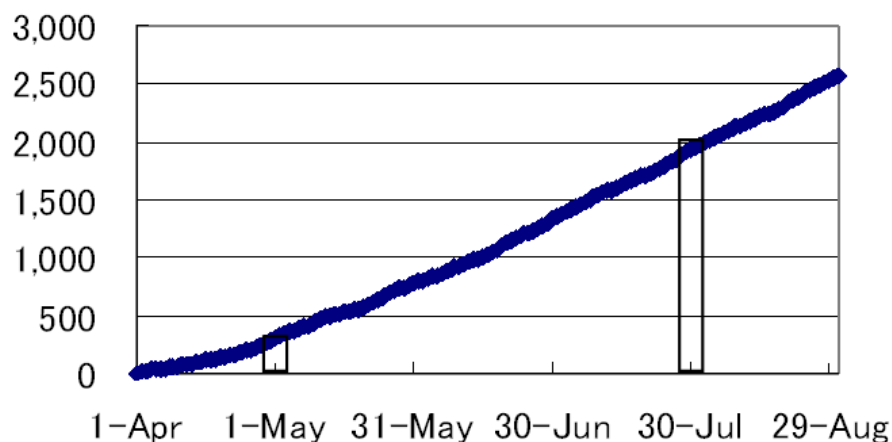


Fig. II-4. Relationships between pruning and blooming times. Both horizontal and vertical axes have 5-day intervals as the unit. Symbols correspond to locations.

(A) Cumulative temperature (°C)
(2012. Apr.–Aug. Rybnany)



(B) Cumulative temperature from pruning to blooming

	Rybnany	Lipenec	Blsany	Kroucova
5-Apr	1,841	1,923	1,923	1,816
15-Apr	1,773	1,855	1,855	1,814
25-Apr	1,812	1,766	1,789	1,766

Fig. II-5. Cumulative temperature calculated from daily average temperature, from Apr. 1 to Aug. 31 (A) and from pruning to blooming (B).

Influence of the pruning date on hop quality and yield

The relationships between pruning date and hop yield, chemical components, and aroma intensity in beer investigated at the four locations in 2012 are shown in **Table II-2**. There were no significant effects of pruning date on hop essential oils, linalool, sensory score, and yield; α -acid was significantly affected ($P < 0.05$). Results confirmed that the amount of essential oils and linalool in hop did not change according to the pruning dates in all four locations (**Fig. II-6**). Hop aroma intensity and yield also showed no difference between pruning dates. In some locations, hop pruned

later tended to have a slightly higher amount of α -acid, and thus significant differences were found (Fig. II-6). The length of the vegetative period, from pruning to blooming, may therefore affect hop's secondary metabolism. However, the data on α -acid behavior presented here is derived from only one year of observations, and the same should be confirmed for several cultivation years. As described before, blooming time may not vary with pruning time, so pruning may not affect the period from blooming to harvest (Fig. II-6). This is reflected in the results of this section, as pruning date rarely affected hop quality.

Table II-2. Results of the Two-way ANOVAs performed, considering locations (A) and years (B). F-values and their significance are shown for each test. L=location, P=pruning date, B=blooming date, B-H=time period between blooming and harvest, Y=year

Attribute	All Locations (2012)						
	L	P	B H	L*P	L*B H	P*B H	L*P*B H
<i>(A)</i>							
Oil	30.24***	3.67	140.36***	0.52	3.12*	0.02	1.87
Linalool	25.40***	1.36	134.28***	1.03	3.12*	0.19	0.25
α acid	48.12***	7.09*	5.61*	2.25	2.33	0.56	1.63
Sensory score	5.83**	1.50	25.96***	2.82*	0.97	0.01	0.38
Yield	44.34***	2.23	1.60	1.22	1.87	0.01	0.27
Attribute	Years in one location (Rybnany)						
	Y	B H		Y*B H			
<i>(B)</i>							
Oil		10.82**		19.44***			0.46
Linalool		46.14***		30.24***			4.69
α acid		0.51		2.87			0.24
Yield		6.20*		0.03			0.23

* Significance level at the 95.0 % level; ** Significance level at the 99.0 % level; *** Significance level at the 99.9 % level.

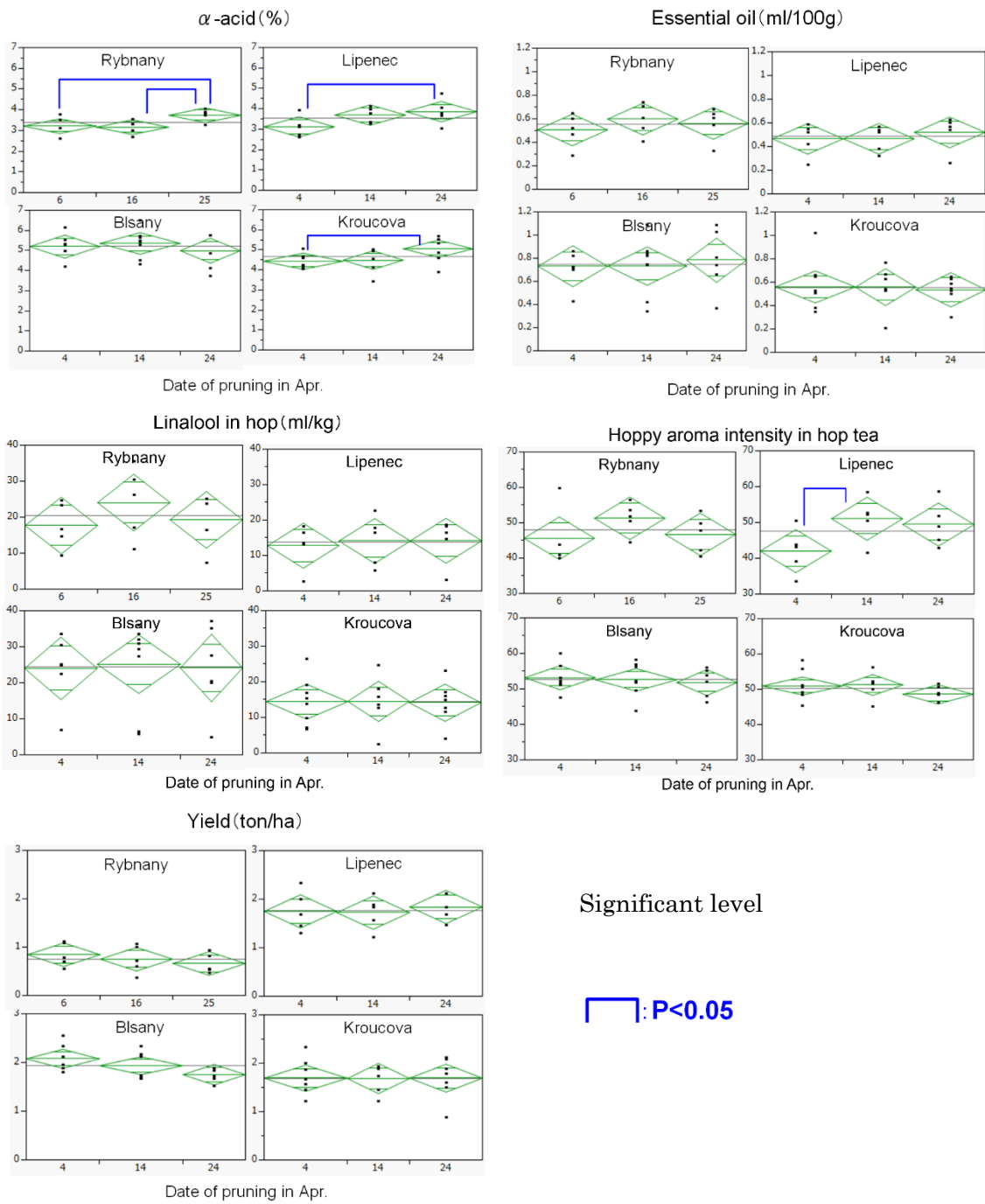


Fig. II-6. Influence of the pruning date on hop quality, yield, and aroma in beer.

Influence of the harvest date on hop quality and yield

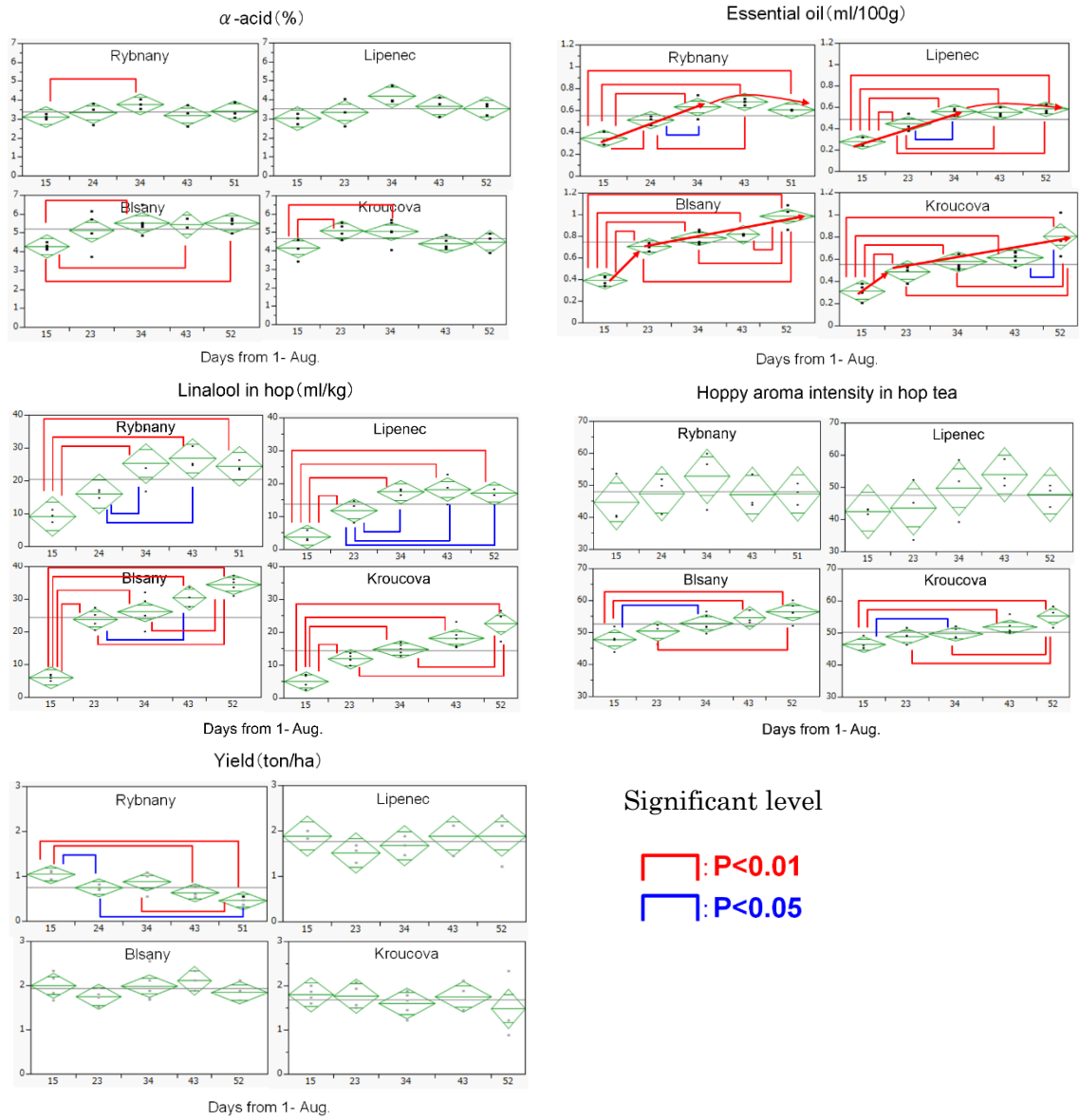
The influence of different harvest times was examined at the four locations in 2012 and at

Rybnany in three consecutive years (**Table II-1**). Significant effects at the 99.9% level were detected in the period from blooming to harvest for essential oils, linalool, and sensory score (**Table II-2**). **Fig.II-7A** shows the behavior of α -acid, oil content, and linalool in hop. As blooming timings were almost identical for all gardens (within 1 week), horizontal axes in **Fig.II-6** correspond to days from the 1st of August. The amount of essential oil and linalool increased at all locations until the middle of September; these increases only continued until the end of September in Blsany and Kroucova. It is difficult to understand why these two patterns exist. In association with the increase in essential oils and linalool, hop aroma intensity also increased; α -acid and yield were almost stable during the harvest period. Six mono-terpenes (linalool, geraniol, myrcene, ocimene, β -pinene, D-limonene) and four sesqui-terpenes (β -farnesene, bergamotene, α -humulene, β -caryophyllene) in hop were analyzed (**Fig.II-8**) In the four locations, sesqui-terpenes were generated early in the harvest period and mono-terpenes were generated later. These results suggest that harvest timing affects both intensity and balance of the hoppy aroma in beer.

We also investigated the influence of the harvest date through three consecutive years (2010, 2011, and 2012) at Rybnany (**Table II-2B**). Significant effects at the 99.9% level were found in the period from blooming to harvest for essential oils and linalool. According to the climatic data, differences in temperature and rainfall between the three years in one location are larger than those observed between the four locations in one year (**Fig. II-9**). Essential oils and linalool content in hop increased during the harvest period in all years; while sesqui-terpenes were mainly generated in the beginning of the harvest period, mono-terpenes were generated towards the end of the harvest period.

This behavior is therefore similar to that found for the four locations in 2012 (Figs. II-7 and II-8).

A



B

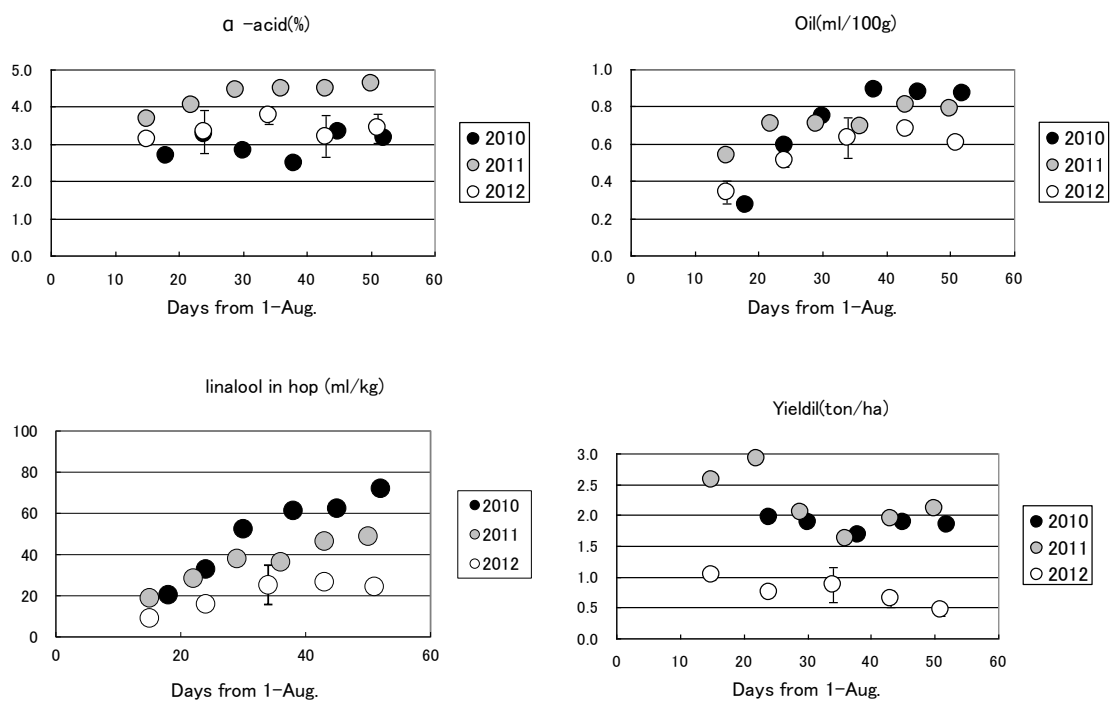


Fig.II-7. Influence of hop harvest time on beer hop quality, yield, and aroma, at the four locations, during 2012 (A) and at one location (Rybnany) in 2010, 2011, and 2012.

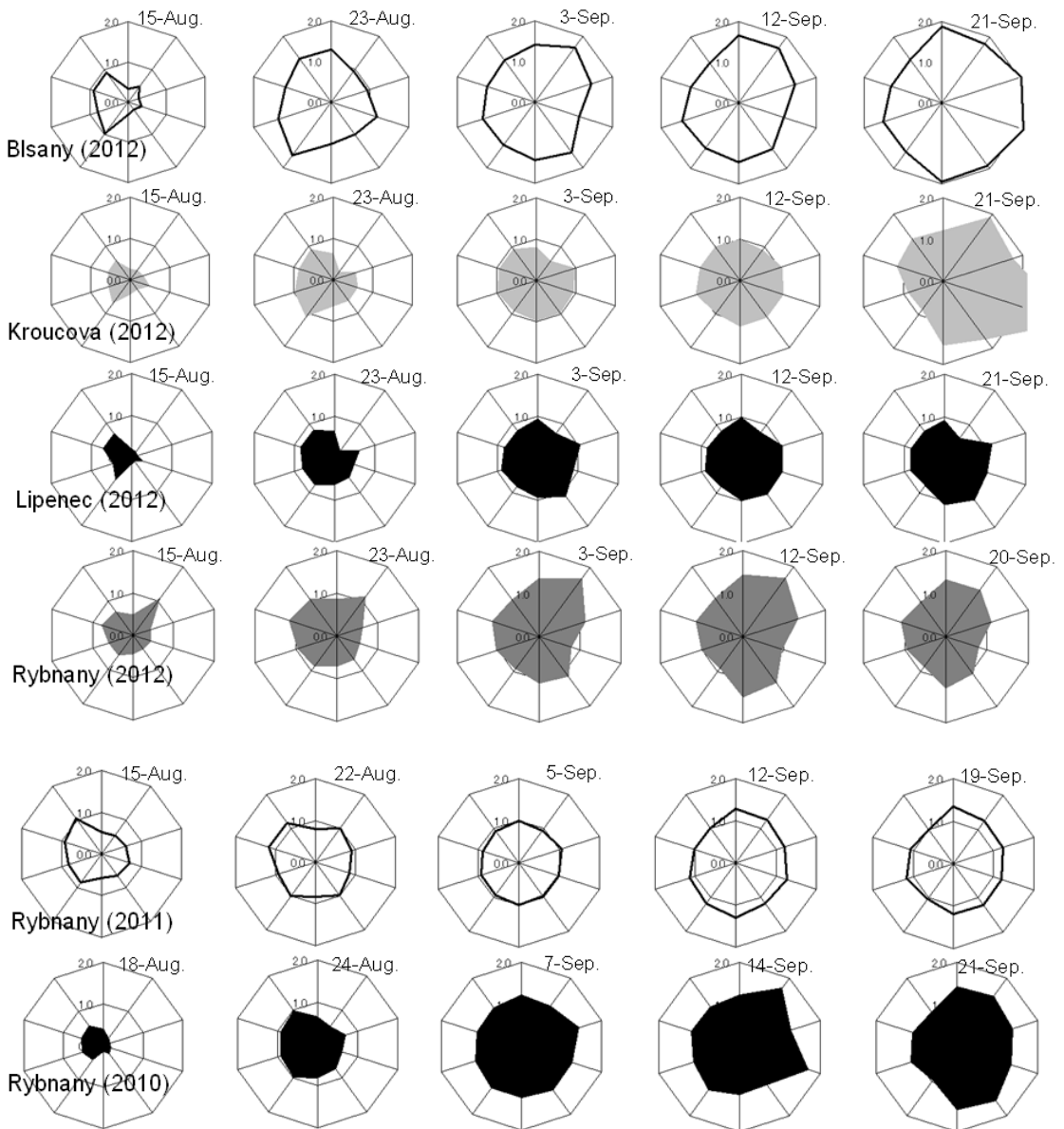
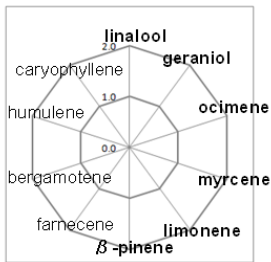


Fig.II-8. Variation of terpenes during hop harvest period. The composition of the radar chart is shown on the top of the figure. The six mono-terpenes are indicated in bold characters; the other four compounds are sesqui-terpenes, Numbers show the ratio of each terpene towards the average amount calculated for each year. The average amount was calculated from 60 samples in 2012 and from five samples in 2010 and 2011.

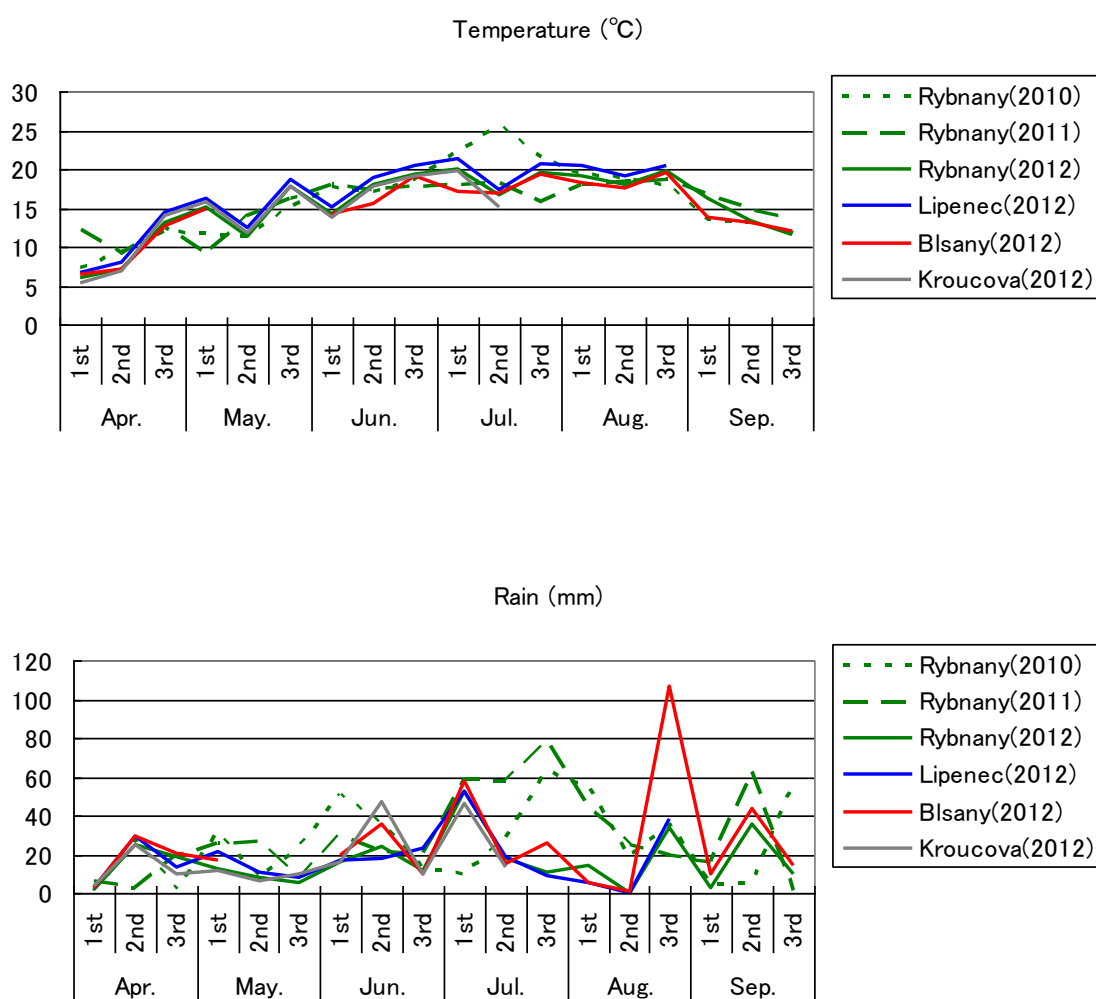
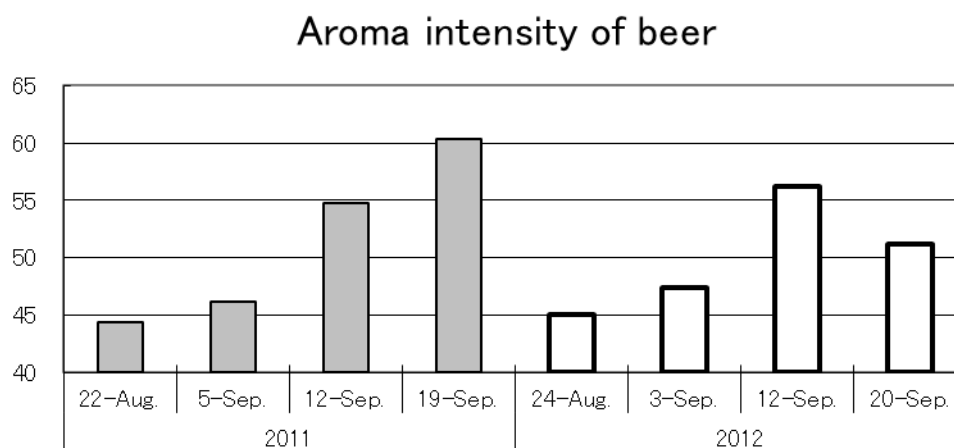


Fig. II-9. Climate data for the four locations studied in 2012 and for the three years (2010, 2011, 2012) in Rybnany.

Influence of the harvest date on beer hoppy aroma

We brewed beer using the hops harvested in Rybnany in 2011 and 2012. Results confirmed that the intensity of hoppy aroma was stronger in beer brewed with later harvested hop compared to that in beer brewed using the hop harvested in an early period. Beer linalool content showed the same tendency as aroma intensity (data not shown). The balance of hoppy aromas is different between harvest times and these aromatic characters also differ between years (**Fig. II-10**). This tendency is generally consistent with that of other varieties (Sharp et al., 2014). Therefore, although hoppy aroma intensity may be an indicator for suitable harvest timing, it may be difficult to predict beer aroma character based on hop aroma intensity alone because of its yearly fluctuation.



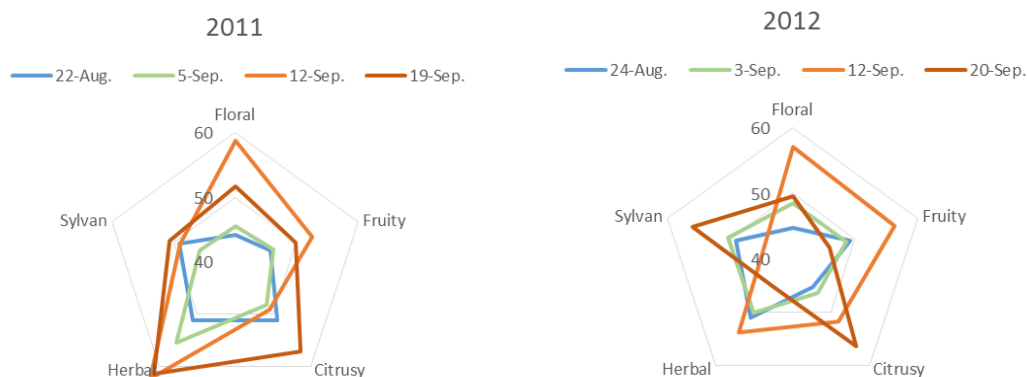
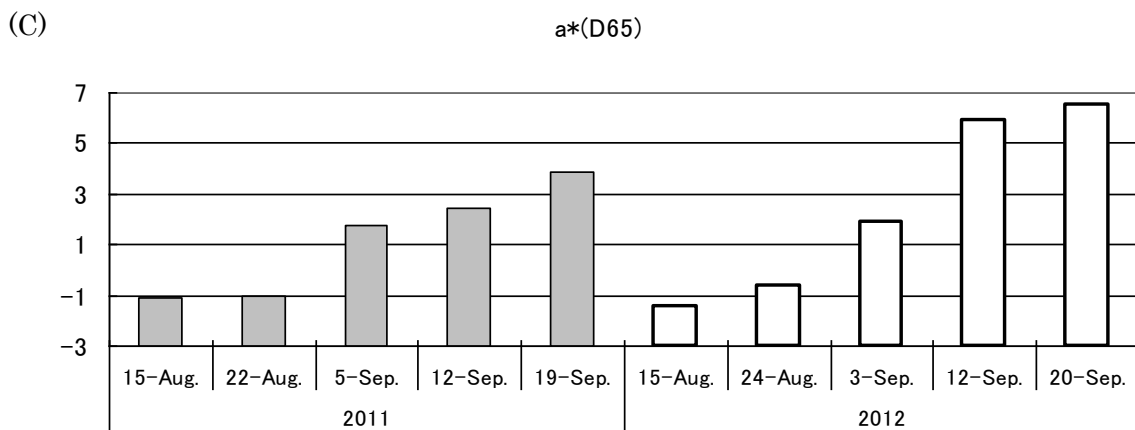
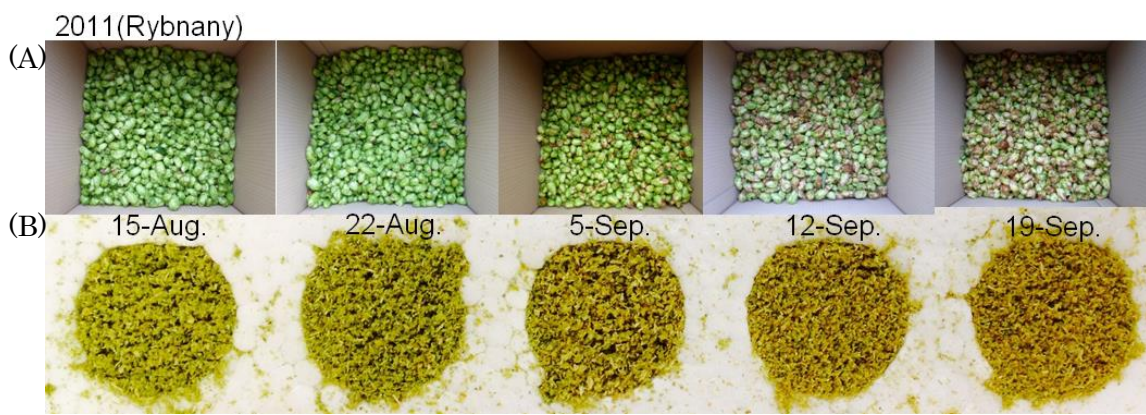


Fig. II-10. Scores of beer sensory evaluation for hoppy aroma.

Change in hop color during the harvest period (Rybnany in 2011)

Historically, many brewers evaluating hop color might prefer bright green color to the brown color from the overall view point. And there are some post-harvest methods to maintain its bright green color such as sulfur treatment at kilning process. In this study, hop color changed during the harvest period and the results of hop color measurements are shown in **Fig. II-11** Early harvested hops showed a bright green color, which gradually changed to yellow-brown towards the end of the period. As shown in **Fig. II-11**, the a-value (indicating color change from green to red) tended to increase during the harvest period whereas the hue angle ‘h’ (indicating the direction of the rectangular coordinates - the horizontal axis is from green to red, the vertical axis is from blue to yellow) decreased during the harvest period. Although the data shown in **Fig. II-11** are only from Rybnany in 2011, the same color changes were recognized in the other samples (three years in

Rybnany and four locations in 2012). These results suggest hop color could be a good indicator to estimate hop aroma quality. The brown colored hop harvested later in September was analyzed for infections by downy mildew using qRT-PCR, but downy mildew infection was not confirmed (data not shown). It can therefore be assumed that the change in hop color is due to the oxidation of polyphenols or to the decrease of chlorophyll in hop bract during the harvest period.



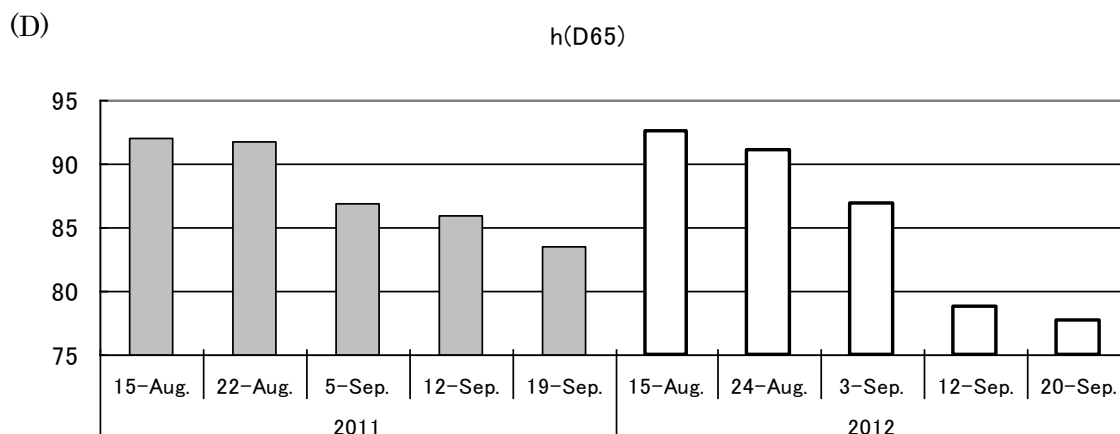


Fig. II-11. Appearance of hop, according to its harvest date. Raw hop cones (A), ground hop cones (B), a-value, showing the direction from green to red (C), and hue angle, showing the direction of the rectangular coordinates (the abscissa is from green to red, the ordinate is from blue to yellow) (D).

Conclusion

In this study, we have evaluated the influence of the pruning and harvest timing on hop aroma characteristics, chemical components, appearance, yield, and beer quality in four locations and in three years. The results indicated that the period from blooming to harvest had the significant impact on the amount of essential oils, mono-terpenes and intensity of beer hoppy aroma in all locations and in all years. On the other hand, pruning timing affect neither hop nor beer quality. These findings should make it possible to select the best time for harvesting in order to give beer

products target aroma in accordance with the type of beer.

In future work, further studies need to be conducted to elucidate how secondary metabolism are influenced by cultivation conditions such as temperature and rainfall. From these studies, we could select the best time of harvest more logically and strategically. Recent studies such as hop's draft genome (Ono et al., 2014) and its secondary metabolism (Nagel et al., 2008; Wang et al., 2008; Li, et al., 2014) would give support to develop genuine cultivation technologies those contribute to hop and beer products.

Acknowledgements

The authors would like to thank all the members of the Hop Research Institute Co., Ltd. in Saaz, Czech Republic for their contribution to hop analyses, and all the members of Zatec Hop Company Ltd. for their kind support to this study. The authors would also like to thank all farmers who allowed authors to observe their hop gardens and provided hop samples.

CHAPTER III

The influence of the age of a hop plant on the growth, development, yield, and qualities of hop

Introdcution

In this chapter, we investigated the natural origin factor, the age of hop plant. Hop is a perennial plant. Same root stock has been used for 20-30 years until it could secure original yield for its variety. Root continues growing after plantation and spread its root area in the soil year by year and its vegetative growth and reproductive development may change in its condition. Although the composition of secondary metabolites such as bitter compounds (humulones) and aroma compounds (terpenes) may change according to hop plant age, such study have not been done by now. In this chapter, we investigated the influence of hop plant age on vegetative growth, blooming time, hop quality and beer quality for Saaz variety cultivated in Saaz region, Czech Republic.

Materials and Method

Test samples

Eight hop gardens (variety Saaz) in the Saaz region of the Czech Republic were selected to provide test samples (**Table III-1**). All of the samples were virus free and collected from the same

clone ('Osvald's clone 72'). Samples from all 8 hop gardens were tested in 2 consecutive years, 2010 and 2011.

Table III-1 Test samples

Area	Variety	Garden	Year of planting	Age	
				2010	2011
Saaz in GZ	Saaz	a	2010	1	2
		b	2009	2	3
		c	2008	3	4
		d	2007	4	5
		e	2006	5	6
		f	2001	10	11
		g	1996	15	16
		h	1991	20	21

All hops are 'virus free' and consist of 'Osvald's clone 72'.

Observations of hop growth and development

Nine different hop plants were selected in each test garden, and observations were carried out on vegetative growth characteristics such as hop height (m), stem diameter (mm) and leaf size (cm) at a height of 1.5 m, every 2 weeks from April to August. Reproductive development characteristics such as flowering and cone formation were recorded every week from the end of June to the middle of August. The dates of flowering and cone formation were defined by the date of 50 % flowering/cone formation.

Cultivation history

Farmers at each of the 8 test gardens recorded the cultivation history - including the date of

pruning, harvest, fertilizer and agrochemical application, and kilning condition after harvest.

Storage conditions for hop samples

Hop samples were collected from each farm as soon as kilning had finished after harvest. They were then vacuum packed and stored at below freezing point until the chemical analysis and brewing trials were ready to be carried out.

Chemical analyses of hop samples

Chemical analyses of hop samples were conducted by the Hop Research Institute Co., Ltd. Saaz in the Czech Republic. Hop resins were measured using the EBC 7.7 liquid chromatography (HPLC) method with a Nucleosil RP C18 column (Macherey-Nagel, Germany, 5 μm , 250 \times 4 mm) and a SHIMADZU LC 20A chromatograph (Shimadzu, Japan) with diode array detectors (DAD). Hop essential oils were obtained by steam distillation and the terpene content of the distilled oil was determined by gas chromatography (GC) on a DB 5 capillary column (Chromservis, CR, 30 m \times 0.25 mm \times 0.25 μm film thickness) using a Varian 3400 gas chromatograph together with a Finnigan ITD 800 mass detector. Qualitative criteria were based on a comparison of GC retention indices and mass spectra with those of authentic compounds. A semi-quantitative evaluation of the composition of hop oils was performed on the basis of the peak area of individual components and expressed relative to the total integrated area of all substances involved.

Brewing conditions

The batch size used for brewing was 100 liters, with a malt ratio of 100 %. Hops were added twice – once at the beginning (kettle hopping) and again at the end (late hopping) after 90 minutes of wort boiling. Only test hops were used for the late hopping and, in order to compare any differences in hop aroma characteristics, the same amount of each test hop sample was added during the late hopping stage. The amount of alpha acid added at the first hopping was decided to be the same bitterness between each beer sample. Each cold wort was fermented at 10 degrees Celsius using lager yeast.

Sensory evaluation

Sensory evaluation was conducted by 7 well-trained panelists, using set criteria. Hop aroma characteristics in the beer were evaluated using 5 generic hop aroma descriptions: floral, fruity, citrusy, herbal and sylvan (woody). These characteristics were compared to the fraction of hop essential oil produced by Botanix Ltd. Beer taste was evaluated in terms of 6 generic beer characteristics: malty, sweet body, mild, sour and astringent. Panelists scored each characteristic on a scale from 0 to 5. All scores were then normalized to remove any panelist bias.

Chemical analyses of terpenes in beer

Each beer sample was prepared by solid phase extraction, using Bond Elut ® JR-C18 500 mg (purchased from Agilent Technologies). A 20 g sample was applied to the column after the

addition of borneol, as an internal standard. CH₂Cl₂ was used for elution and the eluent obtained was concentrated using a vacuum evaporator.

A 0.2 µl sample of extraction liquid was injected into a GC-MS (Agilent 7890 GC & 5975C GC/MSD) and analyzed under the following conditions.

Capillary column: DB-WAX (J&W Co. Ltd, 60 mm × Ø 0.25 mm × 0.5 µm), oven temperature: 40-240 degrees Celsius (6 degrees Celsius/min.), [rising to 240 degrees Celsius for 20 min.,][240 degrees Celsius, 20 min.,] carrier gas: He, 1.5 ml/min., transfer line: 240 degrees Celsius, MS ion source: 230 degrees Celsius, MS 4 polar: 150 degrees Celsius, front inlet: 240 degrees Celsius.

Results and Discussion

Growth and development behavior

There was little difference between 8 test gardens in terms of the date of pruning and harvest or kilning condition after harvest. Vegetative growth data (just before harvest) are shown in **Fig. III-1** In younger hops (except for the 1-year-old sample in 2010), the stem diameter and leaf size at 1.5 m height were larger than in the older hops in both 2010 and 2011. The lower vegetative growth of the 1-year-old hop sample may indicate that the plants were in a transition phase, resulting in smaller size. Reproductive development data are shown in **Fig. III-2**. In younger hops, the number of days from pruning to cone formation was greater than that in older hops, in both years. These results suggest that, in younger hops, vegetative growth is promoted but reproductive

development starts late. Therefore, hop plant age may affect hop secondary metabolism and the generation of aromatic substances such as terpene compounds.

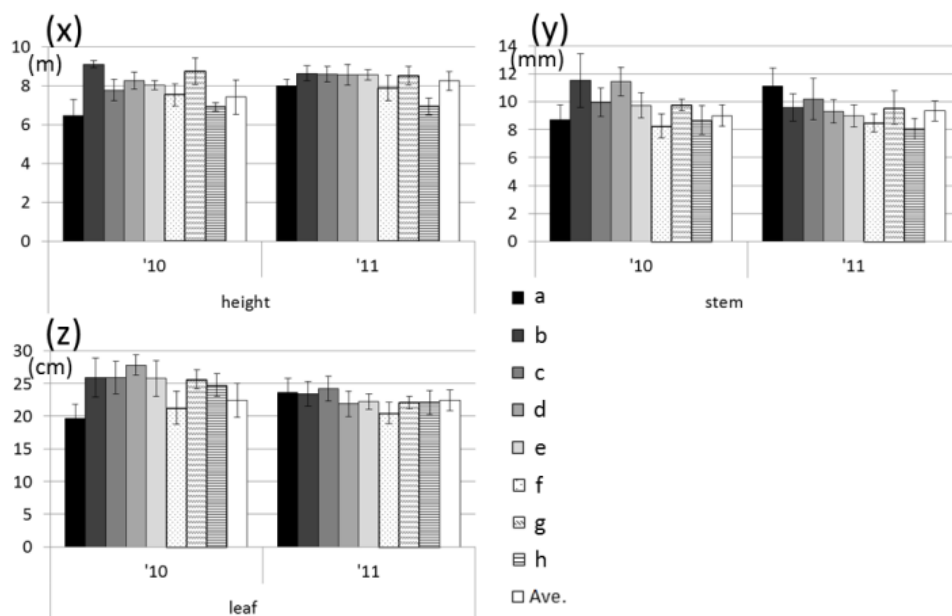


Fig. III-1 Vegetative growth of hops: height (x), stem diameter at 1.5 m (y) and leaf size at 1.5 m above ground (z), just before harvest.

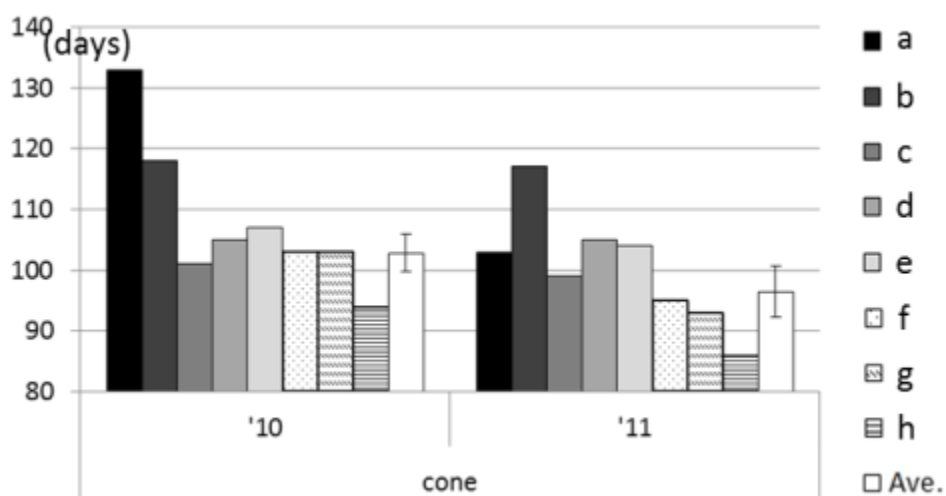


Fig. III-2 Reproductive development - days from pruning to cone formation

Chemical composition of hops

Alpha acid concentrations in the hops sampled are shown in **Fig. III-3**. In younger hops, the alpha acid concentration was higher than that in older hops in both 2010 and 2011.

Fig. III-4 shows the relative concentration of 10 terpenes at different hop plant ages. In these radar charts, monoterpenes such as linalool, geraniol, myrcene, ocimene, β -pinene and d-limonene are shown on the right-hand side. In general, these substances contribute to hoppy aromas that can be described as floral, fruity and citrusy. Sesquiterpenes such as β -farnesene, bergamotene, α -humulene and β -caryophyllene are shown on the left-hand side of the radar chart. In general, these substances contribute to mild aromas that can be described as sylvan. The numerical values shown on the radar charts express the relative ratio compared to the average percentage of each compound in each essential oil. Average values were calculated from about 30 different samples from each crop. The amount of essential oil and the relative percentage of each terpene compound in the hops sampled are shown in **Table III-2**. It is obvious that in young hops, under 5 years old, monoterpenes that may contribute to hoppy aromas are found at lower levels (except for ocimene) whereas sesquiterpenes that may contribute to a mild aroma are present at much higher levels.

Vegetative growth was more luxuriant and flowering was later in the younger hops compared to older ones. The chemical analysis results for alpha acid and terpenes also indicate that

secondary metabolism in younger hops may be different to that in older plants.

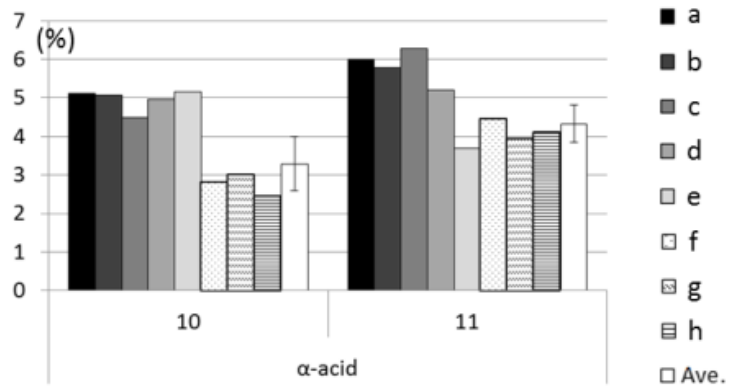


Fig. III-3 Content of α -acid in hops harvested from 8 different gardens in 2 consecutive harvest years, 2010 and 2011.

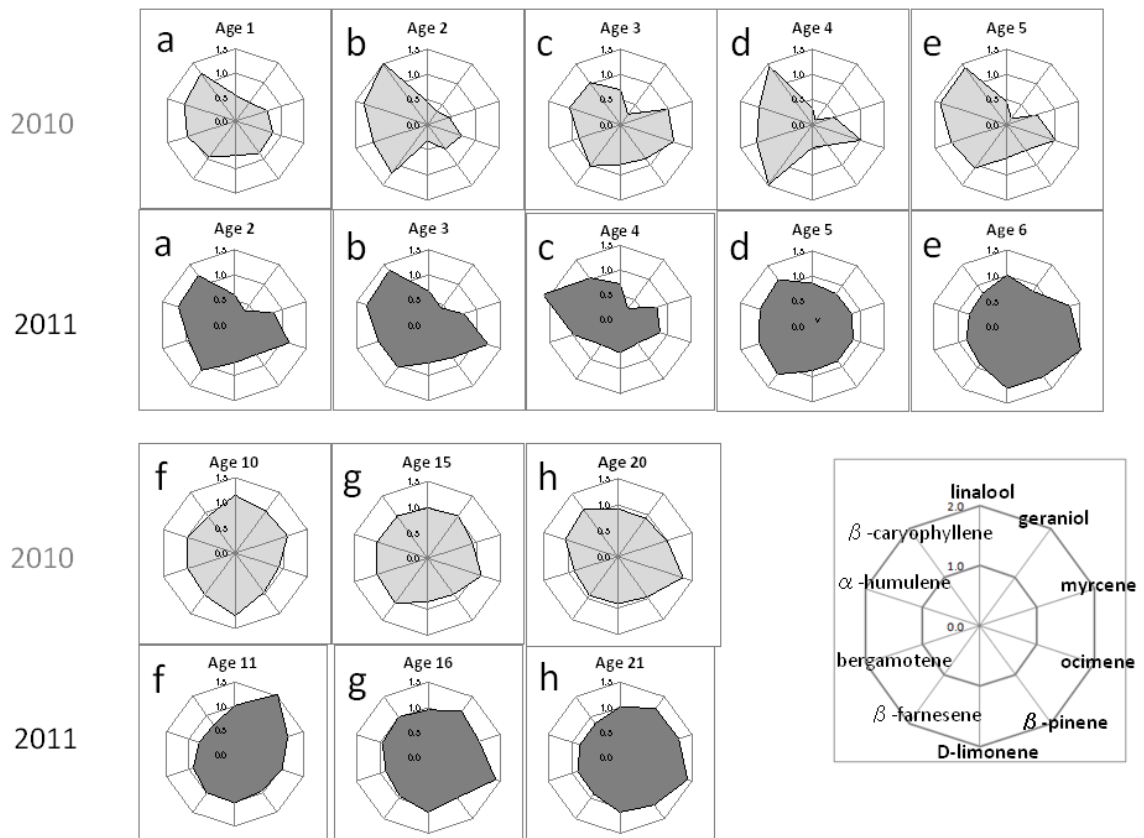


Fig. III-4 Comparison of terpene profiles in hop samples. The 6 compounds indicated on the right-hand side of the chart are monoterpenes (linalool, geraniol, myrcene, ocimene, β -pinene and d-limonene). The 4 compounds indicated on the left-hand side of the chart are sesquiterpenes (β -farnesene, bergamotene, α -humulene and β -caryophyllene). The charts show the relative content of each compound in each age of hop, compared to the average of about 30 different hop samples collected each harvest year.

Table III-2. Amounts of essential oils and the relative % of each terpene in hops from 8 different gardens

		2010								2011							
		a	b	c	d	e	f	g	h	a	b	c	d	e	f	g	h
Oil amount	ml/100g	0.64	0.49	0.62	0.52	0.54	0.75	0.53	0.54	1.04	0.44	0.61	0.64	0.58	0.71	0.57	0.58
Linalool	rel. %	0.33	0.28	0.42	0.20	0.28	0.70	0.61	0.57	0.32	0.36	0.38	0.45	0.54	0.54	0.51	0.54
Geraniol	rel. %	0.13	0.09	0.07	0.03	0.04	0.28	0.28	0.25	0.05	0.06	0.04	0.11	0.12	0.22	0.16	0.17
Myrcene	rel. %	23.90	15.60	33.30	15.40	21.40	37.70	31.70	34.00	28.30	25.50	27.30	28.30	44.70	40.55	37.00	42.50
Ocimene	rel. %	0.08	0.07	0.11	0.10	0.10	0.09	0.11	0.13	0.12	0.13	0.09	0.09	0.16	0.11	0.15	0.15
β -Pinene	rel. %	0.47	0.34	0.48	0.28	0.36	0.55	0.50	0.55	0.39	0.44	0.33	0.46	0.66	0.52	0.54	0.63
D-Limonene	rel. %	0.11	0.05	0.12	0.07	0.10	0.19	0.13	0.14	0.11	0.11	0.10	0.13	0.18	0.15	0.16	0.16
β -Farnesene	rel. %	22.40	28.60	24.10	34.80	25.30	24.50	25.90	22.10	20.00	18.90	11.60	21.50	16.70	18.45	17.60	16.50
Bergamotene	rel. %	0.94	1.00	0.77	1.02	1.02	0.87	0.93	0.78	0.80	0.88	0.83	0.94	0.71	0.77	0.75	0.75
α -Humulene	rel. %	18.20	20.90	16.90	17.60	22.20	15.70	16.50	17.00	27.30	30.10	37.80	25.40	18.20	18.15	22.30	19.40
β -Caryophyllene	rel. %	8.10	9.80	6.80	9.50	9.20	6.00	6.70	7.50	7.86	8.63	6.62	7.38	5.25	4.93	6.42	5.47

Beer analyses

Brewing trials were carried out using 4 randomly selected samples ('b', 'd', 'g' and 'h' in

Table III-1) over 2 consecutive cropping years, 2010 and 2011. The results of beer sensory

evaluation are shown in **Fig. III-5** and **Fig. III-6**. These radar charts show the average of the

normalized values obtained from all 7 panelists. Beers brewed from younger hops such as 'b' and 'd'

tended to have less floral, fruity and citrusy characteristics (except for 'd' in 2011), as shown in **Fig.**

III-5. As shown in **Fig. III-6**, beers brewed from younger hops such as ‘b’ and ‘d’ also tended to have more malty and sweet characteristics (except for ‘d’ in 2011). In the case of ‘d’ in 2011, it may have been difficult for panelists to recognize any malty and sweet taste in the beer because of its floral aroma. Since the hops used in ‘d’ were 5 years old in 2011, it may no longer have been a ‘young’ hop sample. These results indicate that, in general, beers brewed from younger hops tend to have weaker hoppy aromas that can be described as floral, fruity and citrusy, while malty and sweet characteristics can be easily recognized.

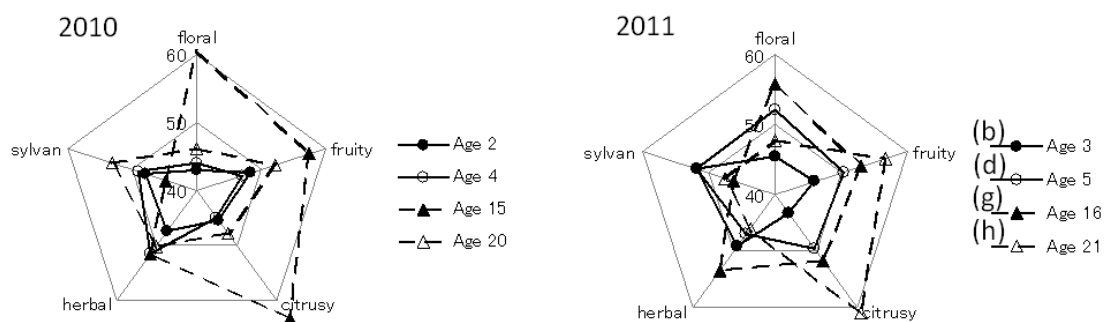


Fig. III-5 Sensory results for hop aroma characteristics in beer. The charts show the average normalized sensory score from 7 panelists.

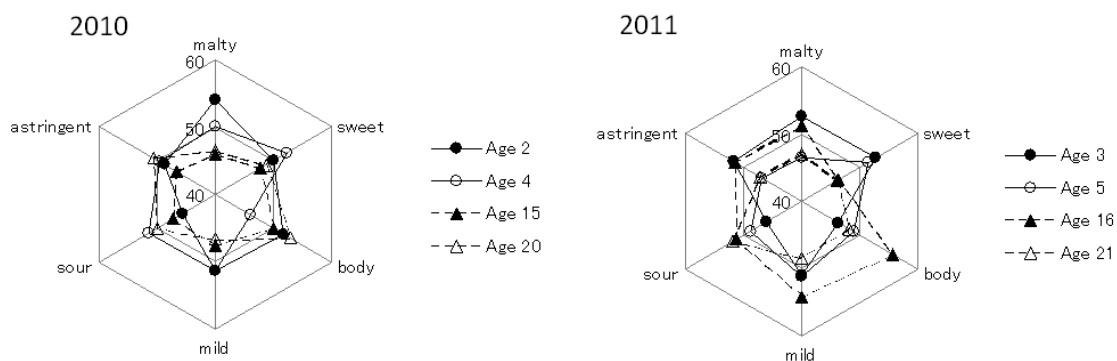


Fig. III-6 Sensory results for beer taste. The charts show the average normalized sensory score from 7 panelists.

A comparison of the terpene compounds found in the hop and beer samples is shown in **Fig. III-7** and **Fig. III-8**. In these graphs, the relative amounts in each of the 4 samples are shown, with the maximum value set at 1.0. The concentrations of aroma compounds in each beer are listed in **Table III-3**, for reference. As mentioned in the ‘chemical composition of hops’ section, monoterpenes such as linalool, geraniol, myrcene and d-limonene were found at relatively low levels in younger hops such as ‘b’ and ‘d’, with the exception of ‘d’ in 2011 (see **Fig. III-7**). In contrast, sesquiterpenes such as β -farnesene, α -humulene and β -caryophyllene were found at higher levels in younger hops. The results show that beers brewed from younger hops such as ‘b’ and ‘d’ had a lower monoterpene content, with the exception of linalool in 2011 ‘d’ (see **Fig. III-8**). In the case of sample ‘d’ in 2011, concentrations of linalool, geraniol, myrcene and d-limonene in hops were higher than those found in 2010 (see **Fig. III-7**), and the linalool content in the beer produced was also relatively high (see **Fig. III-8**). These results indicate that concentrations of monoterpenes in beer could be correlated with those found in hops. However, it was not clear whether sesquiterpene concentrations showed a similar correlation. It was assumed that most sesquiterpenes were difficult to be brought into wort and were easy to be oxidized and hydrolyzed to other compounds. Therefore, considering the behavior and changes of terpene compounds of hop during brewing process, it is very important to precisely define any differences in hop quality caused by various cultivation factors in order to achieve the target beer quality.

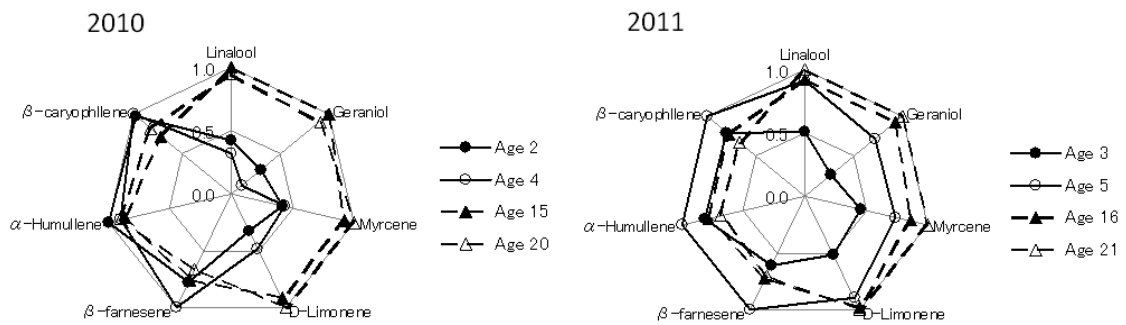


Fig. III-7 Terpene profiles in hop samples. The charts show the relative content of each terpene, compared to the maximum content found in each of the 4 different root ages.

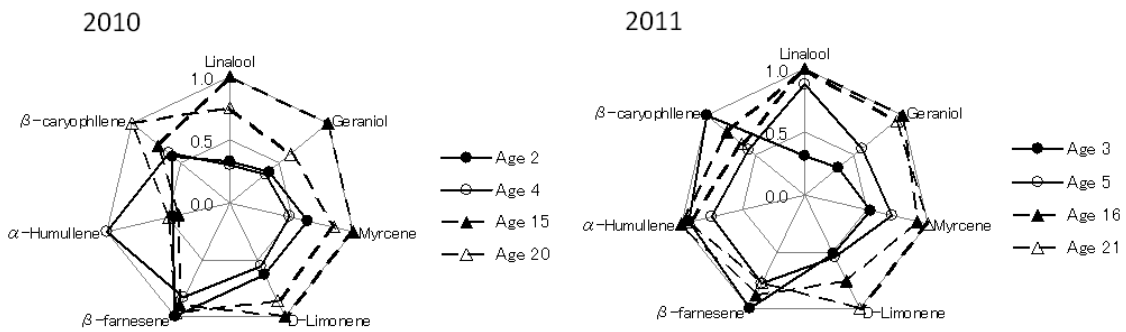


Fig. III-8 Terpene profiles in beer samples. The charts show the relative content of each terpene compared to the maximum content found in each of the 4 different root ages.

Table III-3. Concentration of aroma compounds in beer (ppb)

	2010				2011			
	b	d	g	h	b	d	g	h
Linalool	8.27	7.40	24.80	18.66	9.62	27.20	31.08	30.96
Geraniol	3.96	3.59	9.91	6.04	3.15	5.34	9.18	8.58
Geranyl acetate	0.40	0.36	1.24	0.65	0.18	0.25	0.63	0.58
Myrcene	4.42	3.36	7.03	5.91	5.50	7.35	9.33	10.35
D-Limonene	1.50	1.32	2.37	2.03	1.91	2.00	2.75	3.66
β -Citronellol	6.47	6.54	15.27	10.70	6.51	11.11	21.66	18.21
α -Terpineol	2.63	2.58	4.29	4.23	2.96	4.80	4.92	5.74
Nerol	1.77	1.61	2.49	2.43	1.27	1.99	2.70	2.45
Ethyl 2-methyl butanoate	0.24	0.27	0.28	0.24	0.36	0.32	0.39	0.42
Iso Varellic ethyl ester	0.45	0.44	0.42	0.35	0.58	0.54	1.25	0.56
β -Caryophyllene	0.49	0.52	0.60	0.83	0.29	0.17	0.23	0.19
Caryophyllen oxide	1.68	1.48	1.34	2.39	0.13	0.16	0.09	0.13
β -Farnesene	13.46	11.21	12.10	12.90	8.15	6.27	7.21	6.26
α -Humulene	2.38	5.19	2.14	2.54	1.28	1.03	1.36	1.27
β -Ionone	0.26	0.24	0.13	0.19	0.15	0.11	0.09	0.13
Nerolidol	0.69	0.65	0.43	0.75	0.27	0.36	0.22	0.37

A comparison between the sensory evaluation and chemical analyses carried out on the 2010 crop is shown in **Fig. III-9** and **Fig. III-10**. **Fig. III-9** shows the results of the sensory evaluation for hoppy aromas in beer in 2010 (reproduced from **Fig. III-5**). **Fig. III-10** shows the results for the hoppy aroma compounds that contribute to floral, fruity, citrusy, herbal and sylvan aromas, respectively. **Table III-4** shows the aroma compounds that may affect hoppy aromas (such as floral, fruity, citrusy, herbal and sylvan). The relationships between hop aroma characteristics and compounds in beer derived from hops were compared with the results from our past study using GCXGC-TOF/MS (ASBC congress in 2011). The values shown in Fig. 10 are relative values, with the maximum value from the 8 samples ('b', 'd', 'g' and 'h' from 2 separate years) set at 1.0. As shown in Fig. 9 and Fig. 10, there is a positive correlation between the sensory results and the analytical results. The levels of compounds contributing to floral, fruity and citrusy aromas were lower in younger hops such as 'b' and 'd' and the data showed a good match with the sensory results obtained separately, whereas beer brewed with hop 'g' (15 years old) had more of these aroma compounds. In the case of herbal aromas, there was no significant difference between the various

trials in terms of either sensory or analytical results. However, fewer compounds contributing to sylvan aromas were found in beer 'g' (15 years old). The sensory evaluation produced the same result. Overall, beers brewed from younger hops such as 'b' and 'd' had lower levels of compounds that could contribute to floral, fruity and citrusy aromas. The same trends were found in the results of the 2011 crop trials (data not shown). These findings confirm that sensory results correspond well with the results obtained from chemical analyses.

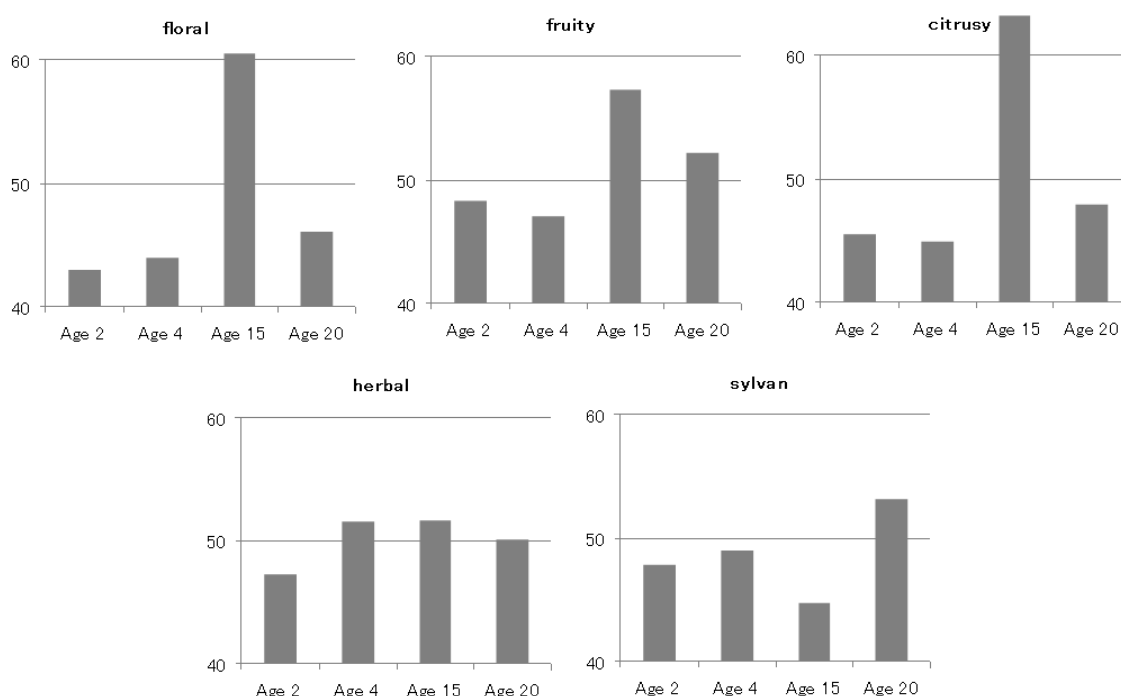


Fig. III-9 Sensory results for hop aroma characteristics in beer samples from 2010 (as shown in Fig. III-5) reworked in the form of a bar graph.

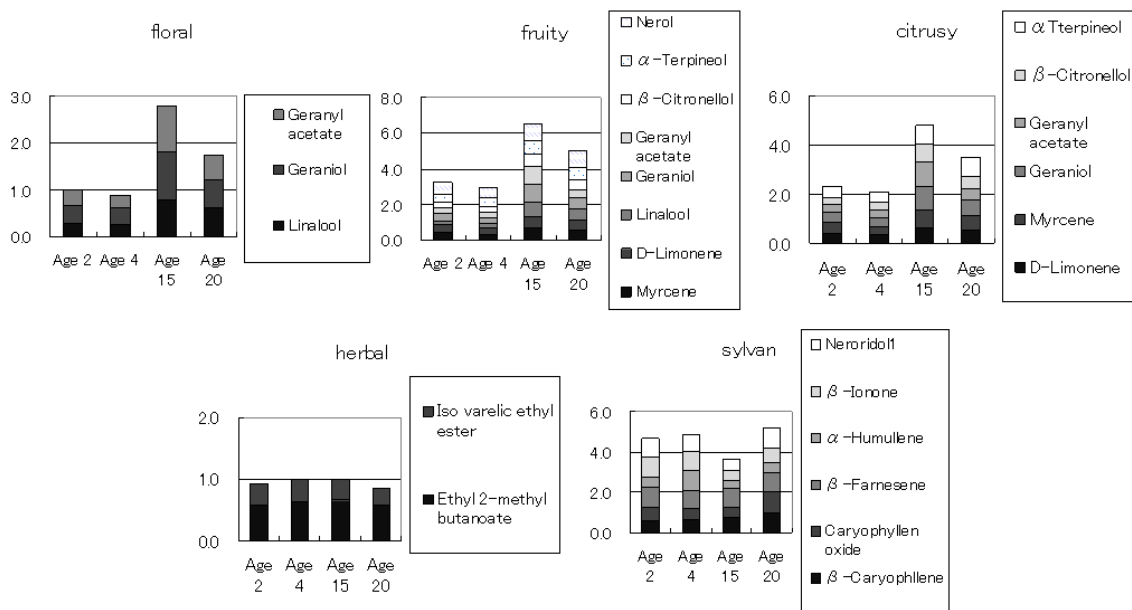


Fig. III-10 Comparison of total relative contents of the hop aroma compounds that are related to floral, fruity, citrusy, herbal and sylvan aromas in beer (using hops from the 2010 crop). The values on the bar graphs show the total relative amounts of hop aroma compounds compared to the maximum amount of each compound found in the 8 hop samples ('b', 'd', 'g' and 'h' from 2 crops).

Table III-4. Hop aroma characteristics in beer and estimated chemical composition and odor descriptions.

Relationships between cultivation, hops and beer quality

As shown in the previous chapter, younger hops (less than 5 years old) have less monoterpenes that contribute to floral, fruity and citrusy aromas. Conversely, they have more sesquiterpenes that contribute to mild aromas (such as sylvan). Beers brewed from younger hops

also contain less monoterpenes - confirming the correlation between aroma compounds in the hops used and the beer produced from them. The chemical analysis results correspond well with the sensory results, showing that beers brewed from young hops have less pronounced hoppy aromas (such as floral, fruity and citrusy).

The levels of monoterpenes and sesquiterpenes present in the hops varied with hop plant age. These results may have been caused by cultivation differences. In younger hops, vegetative growth (both stem and leaf) was luxuriant and was associated with later flowering than hops grown from older roots. This difference in vegetative growth and reproductive development may influence lupulin gland formation and secondary metabolism. And if flowering is late, maturity must be also late. This means that younger hops may sometimes be harvested before they are fully mature.

Overall, these studies confirm that 'hop plant age' is an important cultivation factor that may have a significant effect on hop and beer quality. It is, therefore, important to control the various cultivation factors involved in hop production in order to achieve the target beer quality.

Conclusions

The hop plants produced from young roots possess some unique characteristics. Vegetative growth of both stems and leaves is luxuriant, flowering is later than usual, and less monoterpenes (which contribute to floral, fruity and citrusy aromas) are produced. In contrast, more sesquiterpenes are present - contributing to mild aromas (such as sylvan). Beers produced from hop plants growing from young roots are characterized by a less hoppy aroma, with fewer floral, fruity and citrusy

attributes.

Therefore, in order to achieve the target beer quality, it is important to consider the influence of various factors related to cultivation - including the effects of hop hop plant age on hop aroma quality, as discussed in this study.

CHAPTER IV

Influence of *Apple Mosaic Virus* on the Growth, Development, Yield, and Qualities of Saaz hop

Introduction

In this chapter, we investigated the natural origin factor, *Apple Mosaic Virus* (ApMV) on the growth, development, yield, and qualities of Saaz hop. It has been reported that viral and viroidal infections reduce the vegetative growth of hops (Pethybridge et al., 2008; Fussy et al., 2012; Sano, 2012; Fussy et al., 2013). Pethybridge et al. (2008) summarized the effects of virus and viroids on hops relative to quantity, quality and epidemiology. It has also been reported that the hop stunt viroid detrimentally affects hop growth and quality, and so this viroid has become a significant problem in hop-producing regions of the world (Sano, 2012; Fussy et al., 2013; Matousek et al., 2013). The influences of the hop latent virus (HpLV), hop mosaic virus (HpMV), and apple mosaic virus (ApMV) on hop varieties (Nugget, Opal, Pride of Ringwood, and Victoria) have been studied in Australian hop producing areas. These viruses weaken hop growth and decrease the content of humulone, a key component for beer bitterness (Pethybridge and Wilson et al., 2002). The incidence and spatial distribution of viruses have been studied in hop-producing areas in both the United States and Australia relative to the mode of transmission (e.g., via insect vectors or through sap transferred

by agricultural machinery) (Pethybridge and Hay et al., 2002; Pethybridge and Madden et al., 2002; Pethybridge and Nelson et al., 2002).

The Czech hop variety, Saaz, is known as a fine-aroma hop owing to its high qualities of bitterness and aroma. A comparison of the chemical profiles between virus-free and infected Saaz hop showed that infection may decrease the amounts of humulone and essential oils produced by the plant (Jelinek et al., 2012), but the specific effects of ApMV and HpMV are not yet known. Both viruses are among the most consequential for reducing yield and quality for many varieties of industrial hops (Pethybridge et al., 2008). However, the influence of ApMV and HpMV viruses on plant growth and beer quality have not been investigated for Saaz hops in Europe. Therefore, in this study we investigated infections by ApMV and HpMV in Saaz hop and the influence of ApMV on vegetative growth, yield, chemical profile, and beer aromatic qualities. We also observed the spatial pattern of ApMV and HpMV occurrence in Saaz hop plants in 17 commercial gardens in the Saaz region of the Czech Republic.

Materials and methods

Virus test garden

Two block areas in a test garden in Deštnice village, Saaz, Czech Republic (managed by V. F. Humulus Ltd., Žatec, Czech Republic) were used for this study. Each block contained 40 Saaz hop plants of the Oswald's clone 72 variety planted in the autumn of 1999. In examining the soil, no

nematodes, such as *Xiphimena* and *Longidorus*, were found in the experimental blocks. Virus-free shoots produced by V. F. Humulus Ltd. in accordance with the standards of European and Mediterranean Plant Protection Organization (Certification Schemes, Pathogen Tested material hop, PM 4/16(1)), were planted in one experimental block. ApMV-infected shoots, derived from ApMV infection roots also produced by V. F. Humullus Ltd., were planted in the other experimental block. The two blocks were separated by six meters to prevent the infected plants from infecting the virus-free plants.

All hop plants were subjected to virus and viroid tests in spring 2, 000, described later in the paper. ApMV, HpMV, Arabis mosaic virus (ArMV), cherry leafroll virus (CLRV), hop latent virus (HLV), cucumber mosaic virus (CMV), petunia asteroid mosaic virus (PAMV), tobacco necrosis virus (TNV), and hop latent viroid (HLVd) were not detected in the virus-free plants. ApMV was maintained in all the infected plants, 70 % of which was also infected by HpMV. However, the plants were not infected with ArMV, CLRV, HLV, CMV, PAMV, TNV, or HLVd. The plants in both blocks were prevented from becoming infected by additional viruses by applying mechanical procedures, such as pruning and harvesting. After the year 2000, 10 randomly-sampled plants in each block were subjected to the virus test to confirm that no additional infections had occurred in the blocks. Random sampling, and virus and viroid infection checks described above were undertaken every year to ensure that the initial experimental conditions were preserved throughout the study.

In addition, 17 other hop production gardens in the Saaz region were examined to characterize the

spatial distribution patterns of virus infection. Young shoots were collected in 2010 from the 10 plants (**Fig. IV-1**) from each production garden for virus assay.

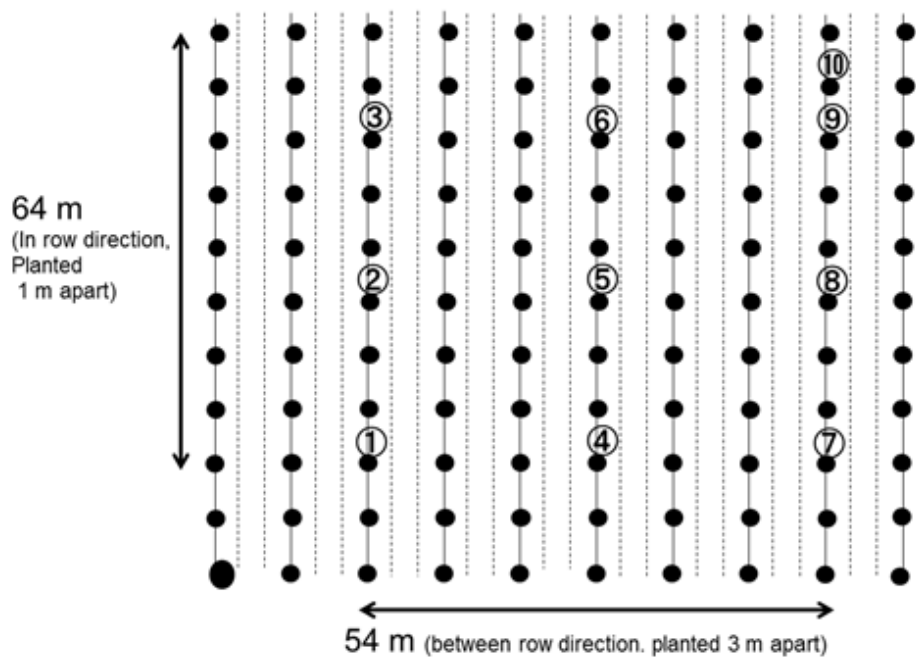


Fig. IV-1. Spatial layout of hop gardens. ApMV and HpMV infection distributions were investigated at 17 commercial hop gardens. Numbers indicate the root position of sampled hop plants. Black dots show poles of hop trellises. Solid and dotted lines represent rows of hop plants.

Commercial gardens

We randomly selected 15 commercial gardens planted with Saaz hop plants (Osvald's clone 72 variety) in the Saaz region to compare the general hop quality with virus infection. We also collected yield data and samples for chemical analyses of humulone, essential oil, and terpene

profiles.

Climatic data

Temperature and precipitation data were obtained from weather observation systems managed by the Hop Research Institute Co., Ltd., located 10 km south of the test garden, at Kneževs village.

Assays for viruses

Young shoots were collected in early April and homogenized in a 1.0-ml solution of 0.01 M phosphate-buffered saline (PBS) containing polyvinylpyrrolidone (20 g/L). The extracts (100 µl) were tested in duplicate by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using polyclonal antisera to ApMV, HpMV, ArMV, CLRV, HLV, CMV, PAMV, TNV, and HLVd (Loewe Ltd., Sauerlch, Germany). Light absorbance at 405 nm was measured for each sample, and compared with the measurements for positive, negative, and buffer-only controls. The positive/negative threshold was set at twice the absorbance of the control wells. This threshold value was consistently higher than the value obtained using the average absorbance of control wells (plus three standard deviations) and so produced a more conservative estimate of virus incidence.

Measurement of hop growth and development

Seven different hop plants were randomly selected in each test block, and vegetative

growth characteristics were measured every 2 weeks from April to August. Measurements included hop height, bine (main stem of hop plant) diameter, and leaf length (at 1.5 m above ground).

Transition from the vegetative to the reproductive phase at a height of 5.0 m from the ground was observed every week from the end of June to the middle of August.

Yield calculation in virus test garden

Raw hop cones from all plants were collected by a pilot-scale picking machine and their weights and moisture contents were determined. Shortly after harvest, the picked hop cones were dried in a kiln at 55°C for approximately 8 h until their moisture content fell to 10% of wet weight. The weight of dried hop cones was measured to calculate an accurate yield.

Storage conditions for hop samples

Dried hop cones were packed in shaded plastic bags with gaseous nitrogen and stored at -20°C in a dark place prior to being used in chemical analyses and brewing trials.

Chemical analyses of hop samples

Chemical analyses of hop samples were conducted at the Hop Research Institute Co., Ltd. (Žatec, Czech Republic). Hop humulone content was measured by the EBC 7.7 liquid chromatography (HPLC) method with a Nucleosil RP C18 column (Macherey-Nagel GmbH & Co. KG, Düren Germany: 5 µm, 250 × 4 mm) and a SHIMADZU LC 20A chromatograph (Shimadzu

corporation, Kyoto, Japan) with a diode array detector (DAD).

The essential oils of hops were extracted by steam distillation, and the composition of terpene content of the distilled oil was determined by gas chromatography (GC) using a Finnigan ITD 800 mass detector (Matsui et al., 2016). The detected compounds were identified from the comparison of GC retention indices and mass spectra with those of pure compounds. A semi-quantitative evaluation of the composition of hop oils was performed on the basis of the peak area of individual components and expressed in relation to the total integrated area of all components.

Brewing

Small-scale brewing mimicking commercial beer production was conducted in a 100-L pilot scale brewery (Suntory Beer Ltd., Tokyo, Japan) with a malt ratio of 100%. Hops were added twice: at the beginning (kettle hopping) and at the end (late hopping) of wort boiling. To compare differences in the characteristics of hop aromas, an identical amount of each hop test sample harvested in 2011 (milled hop) was added only at the late hopping stage. The amount of the hop extract typically used in the commercial brewing process was added at the time of kettle hopping to achieve an identical bitterness among beer samples. Worts were fermented at 10°C, using lager yeasts.

Sensory evaluation

Seven well-trained panelists conducted sensory evaluations, based on hop aroma intensity, of the beers brewed using infected and uninfected hops. The hop aroma in each beer was compared to that of hop oil produced by Botanix Ltd (UK). Five generic descriptions of hop aroma were used as characteristics: floral, fruity, citrusy, herbal, and sylvan (woody). Panelists scored each aroma characteristic on a scale from 0 to 5. All scores were then normalized to remove biases of panelists. An evaluation of each sample was carried out at least twice on different dates. The order in which beers were sampled was randomized for each panelist, and all evaluations were conducted with panelists blind to the status of the hops (infected vs. uninfected) they were testing.

Statistical analysis

Statistical tests were performed using the JMP 10.01 software (SAS, Cary, NC, United States). Statistical differences between means were determined by two-way ANOVAs (analysis of variance), by examining significances at $p < 0.05$, 0.01, and 0.001.

Results

Incidence of infection in the virus test garden

The presence of viruses and viroid were examined in the test garden in Deštnice. Ten hop plants were randomly selected from each test block. ApMV infection rates accounted for 0% and 100% in the uninfected and infected blocks, respectively. Infection rates of HpMV were

comparatively low, at 0% and 10%, in uninfected and infected blocks, respectively. Other viruses, ArMV, CLRV, HLV, CMV, PAMV, TNV, and HLVd were not detected both in uninfected and infected blocks.

Growth and development of hop plants

Heights of ApMV-infected plants were 10 to 15% shorter than those of uninfected plants (**Fig. IV-2a**). Significant differences were first observed between the two groups by the middle of June in 2010 and by the end of May in 2011. Bine diameter at 1.5 m height of ApMV-infected plants was not significantly different in 2010 (**Fig. IV-2b1**). However, in 2011, a significant reduction (20%) in infected plants was observed throughout the cultivation period (**Fig. IV-2b2**). Length of leaves at 1.5 m height from the ground was 10% less in ApMV-infected plants than in uninfected plants in both 2010 and 2011 (**Fig. IV-2c**), with significant differences appearing earlier in 2011 than 2010. Changes in leaf appearance, such as chlorotic rings and necrotic spots, caused by ApMV were not observed in these tests (data not shown). The time between when plants sprouted in spring to the appearance of cones on 50% of plants did not differ between ApMV-infected and uninfected plants in 2010 (**Fig. IV-2d1**), but was significantly longer in infected plants in 2011 (**Fig. IV-2d2**).

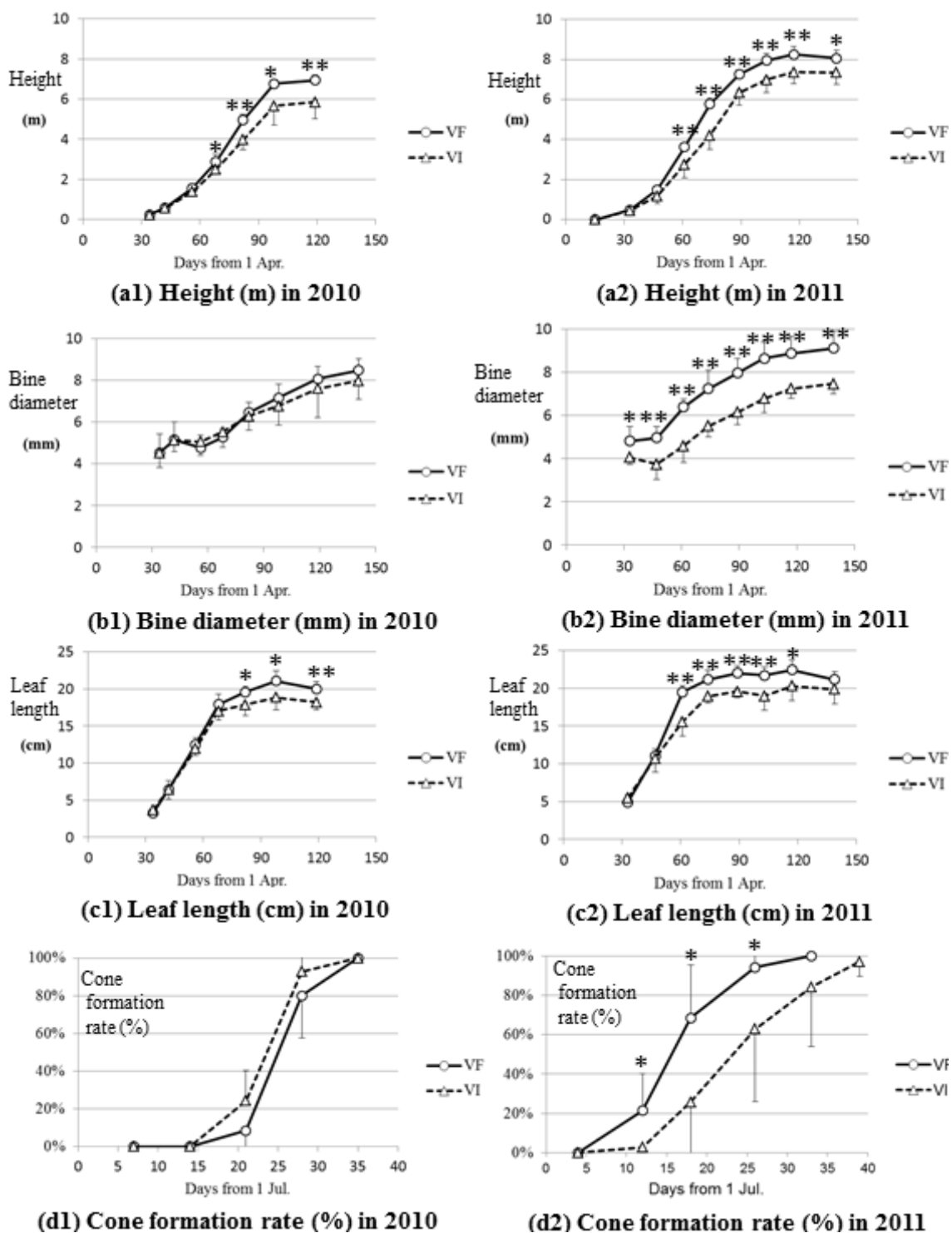


Fig. IV-2 Behaviors of vegetative growth (height, bine diameter and leaf length) and cone formation during cultivation seasons in 2010 and 2011. VF and VI indicate virus-free (uninfected) and

ApMV-infected hops, respectively. Each symbol represents data from 7 plants. Error bars show 1

standard deviation. Asterisk (*) indicates significant difference (* = $p < 0.05$; ** = $p < 0.01$).

Yield, humulone content, and essential oil content

Yield of the ApMV-infected plants (0.6 ton/ha in 2010, 0.9 ton/ha in 2011) was lower than that of uninfected plants (1.9 ton/ha in 2010, 2.0 ton/ha in 2011). ApMV-infected yield was also below average in comparison to 15 commercial hop gardens in both 2010 and 2011 (**Fig. IV-3a**).

Humulone production in ApMV-infected plants (3.25% in 2010, 4.18% in 2011) was also lower than that in uninfected plants (4.95% in 2010, 4.99% in 2011), but was comparable to the average of 15 productive hop gardens (**Fig. IV-3b**). The total amounts of essential oil produced tended to be roughly equal to the amounts of humulone (**Fig. IV-3c**).

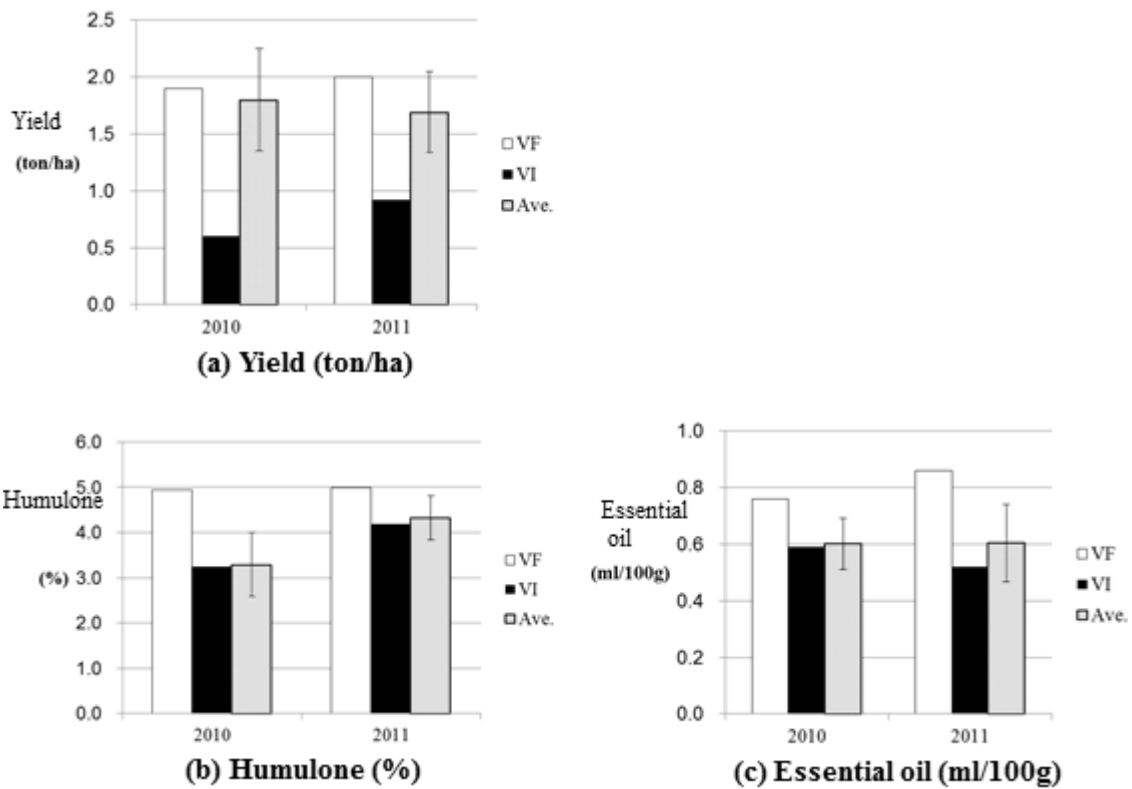


Fig. IV-3 Yield, humulone, and essential oil levels in dried hop cones after harvest. VF and VI indicate virus-free (uninfected) and ApMV-infected hop, respectively. Ave. indicates the mean as obtained from 15 commercial hop gardens in each year. Error bars show 1 standard deviation.

Composition of terpenes

ApMV-infected hop cones had a higher proportion of sesqui-terpenes (farnesene, bergamotene, and caryophyllene) and a lower proportion of mono-terpenes (myrcene and β -pinene) than cones obtained from uninfected plants. This pattern was also true relative to average terpene composition in cones from 15 other productive gardens in the region (**Table IV-1**).

Table IV-1 Composition of aroma compounds in essential oil. Relative percentage was calculated by comparing peak area of each individual compound to the total peak area of all compounds. Mean and standard deviation were calculated using data collected from 15 commercial hop gardens.

			Mono-terpene					Sesqui-terpene				
			linalool	geraniol	myrcene	ocimene	β -pinene	limonene	farnesene	bergamotene	humulene	caryophyllene
2010	VF	(rel %)	0.56	0.21	39.5	0.070	0.73	0.16	21.3	0.90	16.6	5.81
	VI	(rel %)	0.63	0.13	30.1	0.070	0.58	0.22	26.8	1.28	16.9	7.31
	Mean	(rel %)	0.62	0.28	34.3	0.099	0.57	0.15	23.9	0.89	16.0	6.63
	STDEV		0.06	0.07	3.6	0.035	0.07	0.03	1.8	0.13	1.3	0.60
2011	VF	(rel %)	0.45	0.05	31.2	0.100	0.48	0.14	20.7	0.89	23.8	6.87
	VI	(rel %)	0.45	0.09	22.9	0.080	0.39	0.11	21.7	1.03	27.9	8.12
	Mean	(rel %)	0.53	0.14	34.7	0.106	0.55	0.15	18.4	0.85	23.4	6.49
	STDEV		0.11	0.04	5.9	0.027	0.09	0.02	2.3	0.09	3.6	0.93

Hoppy aroma in beer

Beer brewed utilizing the hops derived from the ApMV-infected plants had a significantly weaker fruity aroma than beers brewed from the uninfected plants (**Fig. IV-4**).

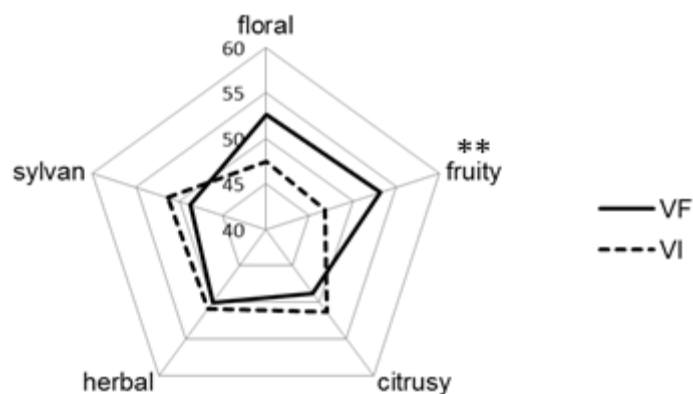


Fig. IV-4 Scores for beer aroma. VF and VI shows virus free and ApMV infected hops, respectively.

(** = $p < 0.01$).

Spatial distribution of viruses in productive hop gardens

Four adjacent plants were found to be infected by ApMV in one commercial hop garden (Table IV-2, garden M), but adjacent infections were not observed in other gardens. Average infection rates varied among hop gardens and growers (Table IV-2 and Fig. IV-5).

Table IV-2 Spatial distribution of ApMV and HpMV infection in 17 commercial hop gardens. Plant number corresponds to the number in Figure 1. Character ‘P’ indicates positive infection of ApMV or HpMV.

(a) ApMV

row	plant No	Hop garden (number indicates plant age)																
		A 1	B 2	C 3	D 4	E 5	F 10	G 10	H 10	I 10	J 10	K 10	L 10	M 10	N 10	O 10	P 15	Q 20
row A	1	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	P	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	P	-	-	-	-	-	-	P	-	-	-	-
row B	4	-	-	-	-	-	P	P	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
row C	7	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	P	-	-	P	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-

(b) HpMV

row	plant No	Hop garden (number indicates plant age)																
		A 1	B 2	C 3	D 4	E 5	F 10	G 10	H 10	I 10	J 10	K 10	L 10	M 10	N 10	O 10	P 15	Q 20
row A	1	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	P	-
	2	-	-	-	-	-	-	-	-	-	-	P	P	-	-	-	-	-
	3	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-
row B	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-
row C	7	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-
	9	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

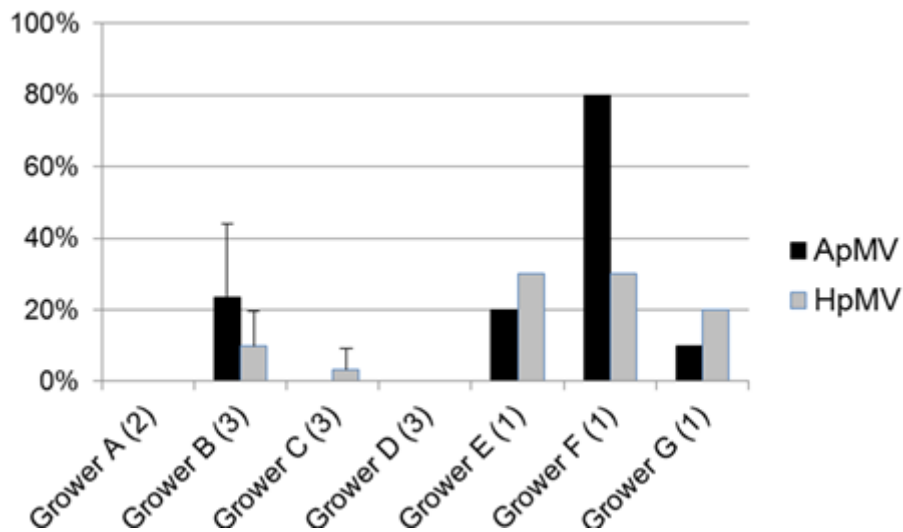


Fig. IV-5 Infection rate of ApMV and HpMV at each grower in 2010. Number in brackets shows how many hop gardens were used to calculate infection rates. Error bars show 1 standard deviation.

Discussion

ApMV affected vegetative growth characteristics, such as height, bine diameter, leaf length, and the time of blooming (**Fig. IV-2**). The difference in the timing of 50% cone formation between ApMV-infected hop plants and uninfected hop plants was within 10 days (**Fig. IV-2d**) of one another. In previous studies, longer maturation periods resulted in an increase in essential oil and monoterpene contents in plants, but did not change the amount of sesquiterpenes and humulone (Matsui et al., 2016). However, in this study, the amounts of humulone in virus-infected hops were less than those in virus-free hops for both years of study (2010 and 2011) (**Fig. IV-3**). And ApMV-infected hop cones had a higher proportion of sesqui-terpenes and a lower proportion of

mono-terpenes than cones obtained from uninfected plants (**Table IV-1**). We surmise that ApMV affects the biosynthetic pathway of secondary metabolites. This subsequently affects beer aroma quality (**Fig. IV-4**), with a decrease in essential oil content and an alteration in the terpene profile (myrcene, β -pinene, farnesene, bergamotene, and caryophyllene). Generally, sesqui-terpenes (such as farnesene, bergamotene, and caryophyllene) confer a 'mild' hoppy aroma to beer, while mono-terpenes (such as linalool geraniol and myrcene) confer a 'floral' hoppy aroma (Inui et al., 2013). The lower content of essential oils and the higher ratio of sesqui-terpenes of ApMV-infected hops resulted in weaker fruity aroma in beer. Therefore, ApMV infections are detrimental to the floral quality of the beer made from Saaz hop.

ApMV infection also lowered hop yield (**Fig. IV-3a**). This result may be partly due to the shorter, and thus smaller leaves (with same number of leaves) in main stems following infection (**Fig. IV-2c**). Although chlorotic rings and necrotic spots in ApMV-infected plants were observed by previous authors (Pethybridge et al., 2008), such symptoms were not observed in our study. The symptoms caused by ApMV may differ according to hop variety.

The difference in growth and development between ApMV-infected and uninfected plants in 2011 was larger than the growth and development difference in 2010, especially bine diameter and cone formation (**Fig. IV- 2**). It should be noted that differences in growth and development characters became evident earlier in 2011 than 2010 may have been due to differences in climatic conditions between years. Temperature in May 2011 was warmer than that in 2010 (**Fig. IV-6**). It may be that ApMV infection affects hop sensitivity to environmental conditions.

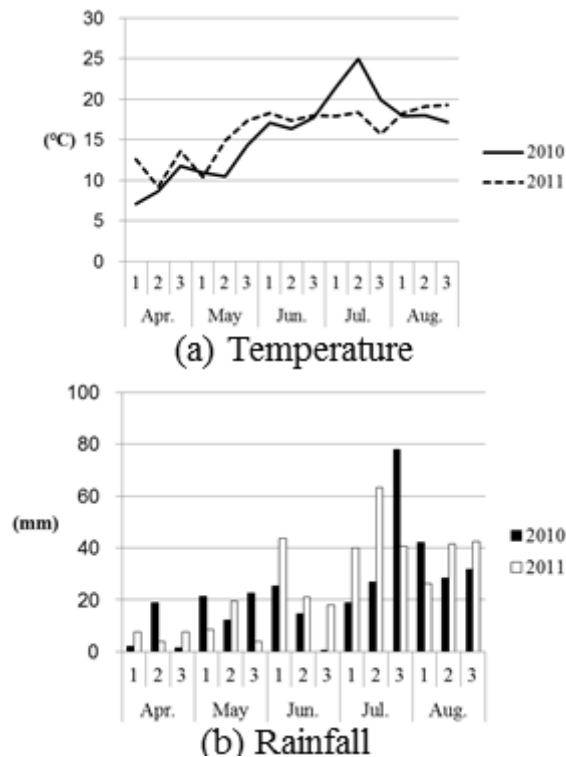


Fig. IV-6. Weather condition in Kneževs village in 2010 and 2011. Kneževs village is located 10 km south from the test garden in Deštnice village.

Pethybridge et al. showed that the transmission of ApMV between plants in commercial gardens occurs predominantly by mechanical means (e.g., during the pruning process in spring (Pethybridge et al., 2008)). This mode of transmission was observed in one garden we sampled (Table IV-2, garden M), but the hop plants in the majority of gardens remained virus-free over the course of our study, and one particular garden has seen no infection of ApMV or HpMV over its 20 years after hop planting (Table IV-2, garden Q). It is likely that Saaz hop plants of the Oswald's clone 72 variety have low susceptibility to ApMV and HpMV. Additionally, we found that the infection rate varied among growers (Fig. IV-5). It suggests that good field practices (GFP) such as

the use of new blade for spring pruning, the protection against hop aphids and the sterilization of harrow and the equipment for harvest could be used to suppress the spread of ApMV and HpMV. A comparison of cultivation methods and field practices between gardens with high or low rates of infection may aid in evaluating the effects of such practices.

HpMV infection rate decreased from 70% in 2000 to 10% in 2010 in the infected block plants. Such decrease of infection rate has been sometimes observed in hop (Sano, personal communication) and may be due to a lack of nutrients or to the original resistance of each hop variety to HpMV. However, the detailed mechanisms have not been elucidated for hops, so this topic should be considered for additional research for maintaining the health of hops.

Our study confirms the detrimental effects caused by ApMV infection on both hop production and subsequent quality of beer derived from Saaz hop plants, which agrees with previous investigations of other hop varieties and production areas (Pethybridge and Madden, 2002; Pethybridge and Nelson, 2002). The negative impacts of ApMV infection include decreased yield, humulone production, and essential oil content, and a change in aromatic compounds. ApMV transmission between plants in commercial hop gardens may occur by transferring viruses on field implements (Pethybridge et al., 2008). Future investigations into analysis of this method of transmission and other epidemiological considerations can contribute to GFP-based management of viral infections.

Acknowledgement

We extend our deep gratitude to Mr. Isao Onoda, who died on April 10, 2016. He introduced the idea of virus-free hops in the Czech Republic in the early 1980s, and contributed to the development of the Czech hop industry for over 50 years, including by his support for hop science studies. We also thank the growers who allowed us to sample and observe their hop gardens.

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