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DOCTORAL COURSE IN CROP SCIENCE

CYCLE: XXVIII

Characterization of different hop (*Humulus lupulus* L.) cultivars: response to drought stress, chemical composition and sensory profile

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

January 31st, 2016 Giulia Bellaio

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Science never solves a problem

without creating ten more

George Bernard Shaw

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Riassunto

I coni di luppolo, le infiorescenze femminili della pianta *Humulus lupulus* L., hanno un ruolo importante nella definizione dell'aroma, della stabilità dell'amaro e della shelf-life della birra. I coni di luppolo sono caratterizzati dalla presenza di particolari sostanze, ad esempio gli α- e β-acidi che contribuiscono al sapore amaro della birra, e gli oli essenziali, responsabili dell'aroma. Attualmente il luppolo viene coltivato principalmente in altri Paesi e importato in Italia come materia prima. Nonostante la coltivazione di luppolo non sia molto diffusa in Italia, il numero di micro-birrifici è in continua crescita e la maggior parte di essi è localizzata nel Nord-Italia. Considerando questi dati, risulta interessante determinare la qualità ottenibile da luppoli coltivati in quest'area. La disponibilità d'acqua è uno dei maggiori fattori ambientali che limita la produzione e nell'area del Mediterraneo il ciclo fisiologico del luppolo coincide con un clima caratterizzato da alte temperature e periodi siccitosi. Perciò, la selezione di cultivar di luppolo più tolleranti allo stress idrico è di cruciale importanza per l'introduzione di questa coltivazione in Italia. Ciò nonostante, le riposte fisiologiche, molecolari e metaboliche attivate in luppolo in risposta allo stress idrico sono poco conosciute. Per questo motivo dieci cultivar di luppolo sono state sottoposte a stress idrico prolungato e durante la cinetica di stress sono stati misurati alcuni parametri fisiologici e la concentrazione di alcuni ioni. Per l'esperimento è stato utilizzato un totale di 8 piante per ogni cultivar (4 piante controllo e 4 piante stressate). I vasi utilizzati (di un volume di 3 L) sono stati sigillati allo scopo di evitare l'evaporazione e, mentre le piante controllo sono state irrigate ogni giorno, le piante stressate sono state lasciate senza acqua fino al raggiungimento del coefficiente di avvizzimento permanente (dopo circa 30 giorni). Sono stati misurati parametri fisiologici e di crescita (peso dei vasi, assi fogliari, lunghezza degli internodi e valori di clorofilla misurati tramite SPAD). I risultati ottenuti hanno evidenziato come, comparate alle piante controllo, le piante stressate non riducono la loro traspirazione fino al raggiungimento di una frazione di acqua traspirabile nel terreno (FTSW) ≈0.7, mostrando in generale un comportamento anisoidrico. L'esperimento ha permesso di individuare le cultivar più tolleranti/sensibili allo stress idrico. Oltre alla selezione di cultivar di luppolo tolleranti allo stress idrico, è importante verificare la qualità ottenibile da luppoli coltivati in Nord-Italia e la sua variabilità tra diverse annate di coltivazione. Nonostante l'effetto delle condizioni

climatiche sulla formazione di α e β-acidi sia ben conosciuto, non ci sono invece informazioni sulla variabilità delle molecole volatili del luppolo fra diverse annate. Per questa ragione, il profilo degli acidi e dei componenti volatili di sedici cultivar di luppolo coltivate nello stesso campo sperimentale a Parma (Emilia-Romagna, Italia) in due diverse annate sono state analizzate via Cromatografia liquida ad alta prestazione (HPLC) e Gas-cromatografia bidimensionale (GCXGC) rispettivamente. È stata evidenziata, in generale, una forte variabilità degli acidi e dei componenti volatili di luppolo in rispetto all'annata. Ciò nonostante, gli acidi del luppolo e i composti volatili chiave mostrano un pattern di base tipico per ogni varietà. In aggiunta, undici varietà di luppolo coltivate nel 2013 sono state sottoposte ad estrazione ad ultrasuoni, e gli estratti ottenuti sono stati utilizzati per aromatizzare una birra stile *Blond Ale*. Ogni birra aromatizzata è stata successivamente analizzata sensorialmente da un panel addestrato. Infine, le proprietà sensoriali delle birre aromatizzate sono state valutate in relazione al profilo volatile delle varietà ed alcune correlazioni sono state evidenziate. Sono state evidenziate correlazioni significative ($p < 0.05$) fra specifici componenti volatili e i descrittori aromatici 'luppolato', 'erbaceo' e 'speziato', mentre poche molecole volatili sono risultate essere correlate con i descrittori 'agrumato', 'fruttato' e 'floreale'. Una caratterizzazione chimica e sensoriale dei componenti volatili in differenti cultivar di luppolo è stata effettuata anche allo scopo di identificare le molecole odorose di luppoli particolarmente agrumati. Infatti, nonostante differenti caratteristiche odorose come note erbacee, floreali/fruttate e speziate siano già state descritte, pochi tentativi sono stati effettuati per correlare chiaramente l'aroma agrumato con il profilo chimico del luppolo. Gli oli essenziali di tre varietà di luppolo con un noto carattere 'agrumato' e tre varietà di luppolo 'speziate'/'luppolate' sono stati analizzati chimicamente e sensorialmente. Ciascun olio essenziale è stato frazionato tramite estrazione in fase solida (SPE) e, grazie all'analisi sensoriale descrittiva delle diverse frazioni, la frazione SPE 70/30 etanolo/acqua (v/v) è risultata essere la più agrumata. Questa frazione è stata successivamente analizzata tramite microestrazione in fase folida in spazio di testa (HS-SPME) e Gas Cromatografia−Spettrometria di Massa/Olfattometria (GC-MS/O). Sono state individuate 59 molecole volatili, 35 delle quali sono risultate essere molecole odorose. In questo studio sono state caratterizzate le molecole odorose della frazione agrumata dell'olio essenziale di luppolo e grazie a clusterizzazione e analisi delle componenti principali (PCA) è stato possibile

evidenziare che luppoli che esprimono un tipico aroma agrumato sono caratterizzati da alti livelli di particolari esteri come ad esempio metil (E)-4-decenoato, nonanoato di metile e decanoato di metile, alti livelli di esteri del geraniolo e nerolo e bassi livelli di sesquiterpeni 'erbacei/speziati' come ad esempio α-umulene e β-cariofillene.

Riassumendo, in questo lavoro sono state evidenziate differenti risposte allo stress idrico in diverse cultivar di luppolo, permettendo di individuare cultivar più tolleranti e sensibili. La qualità di luppoli coltivati in Nord-Italia (espressa come concentrazione di acidi e profilo volatile) e la loro variabilità sono state delineate in due annate diverse ed alcune importanti caratteristiche organolettiche (specialmente la nota 'agrumata') sono state caratterizzate per la prima volta dal punto di vista molecolare. Questo progetto ha dunque aumentato la nostra conoscenza in merito alla risposta allo stress idrico in luppolo e in merito al suo aroma, fornendo informazioni basilari utili per coltivatori e per l'industria del luppolo.

Summary

Hop cones, the immature inflorescences of the female plant of *Humulus lupulus* L., have an important role in defining flavour, bitter stability and shelf life of beer. Hop cones are characterised by a wide range of molecules, such as α- and β-acids that contribute to the bitter taste of beer, and essential oils, which are responsible for the aroma. Nowadays hop is mainly cultivated in other countries and imported in Italy as raw material. Despite hop's cultivation is not widespread in Italy, the number of Italian microbreweries is increasing and most of them are located in Northern-Italy. Taking into accounts this geographical distribution, it is therefore interesting to determine the achievable quality of hops cultivated in the Northern-Italy area. Among factors that limit the crop production, water availability is one of the major constraint since, in Mediterranean areas, the phenological cycle of hops coincides with a climate characterized by high air temperature and drought. For this reason, the selection of hop drought-tolerant varieties is crucial for the introduction of this crop in Italy. Hop physiological, molecular and metabolic responses activated in response to drought are poorly understood. For this reason eleven hop cultivars were subjected to drought and physiological and ionomic parameters were measured throughout water stress development. A total of 8 plants for each cultivar (4 controls and 4 water stressed-plants) were used. 3-L pots were sealed to avoid water evaporation, then control plants were watered every day while stressed plants were left without irrigation till the achievement of permanent wilting (after about 30 days). Physiological and growth parameters (pots weight, leaves axes, shoots length and SPAD chlorophyll meter) were measured. Results highlight that, compared to well-watered plants, most of stressed hop varieties did not reduce their transpiration until the Fraction of Transpirable Soil Water (FTSW) ≈0.7, showing, in general, an anisohydric behaviour. The experiment allowed us to pinpoint the more drought-tolerant/susceptible hop cultivars. Besides the selection of drought-tolerant cultivars, it is important to verify the achievable quality of hops cultivated in Northern-Italy and its variability among different cultivation years. Although the effect of climatological conditions on α and β-acids formation is well-known, no information are available on the variability of hops' volatile profiles among different growing seasons. For this reason, hop acids and volatile compounds profiles of sixteen hop varieties cultivated in the same field in Parma (Emilia-Romagna, Italy) in two

different years were analysed via High Performance Liquid Chromatography (HPLC) and comprehensive two-dimensional gas chromatography (GCXGC) respectively. In general, a strong variability in the dependence of the amount of hop acids and volatile compounds on the climate was found, nevertheless, hop acids and the key compounds of aroma showed a pattern specific for each variety. Moreover, cones of ten hop varieties cultivated in 2013 were subjected to ultrasonic extraction and the extracts were used to flavour a *Blond Ale* beer. Each beer was then sensory analysed by a trained panel. Finally, the sensory properties of flavoured beers were evaluated in relation to hops' volatiles profiles and correlations were pinpointed. High and significant ($p < 0.05$) correlation coefficients were find between specific volatile compounds and aromatic descriptors as 'hoppy', 'grassy' and 'spicy', while few volatiles correlated with the descriptors 'citrusy', 'fruity' and 'floral'. A chemical and sensory characterization of volatile compounds in different hop varieties was also performed to identify the odour-active molecules of particularly citrusy hops. Indeed, although different odour impressions such as grassy, floral/fruity or spicy notes have already been described, few attempts were made to clearly correlate the citrus aroma with the chemical profile of hop oil. The essential oil of three hop varieties with a well-known 'citrus' character and of three 'spicy'/'hoppy' varieties were chemically and sensory analysed. Each essential oil was fractionated by Solid Phase Extraction (SPE) and, according to a descriptive sensory analysis of the different fractions, the 70/30 ethanol/water (v/v) SPE fraction turned out to be the most citrusy one. This fraction was then analysed via Headspace Solid Phase Microextraction (HS-SPME) and Gas Chromatography−Mass Spectrometry/Olfactometry (GC-MS/O). A total of 59 volatiles were identified, 35 of which resulted to be odour-active. In this study the character-impact odorants in the citrusy fraction of hop essential oil were outlined; hierarchical cluster analysis and Principal Component Analysis (PCA) highlighted that hops that expressed a typical citrus aroma are characterized by high levels of specific esters as methyl (E)-4-decenoate, methyl nonanoate and methyl caprate, high levels of esters of geraniol and nerol and low levels of 'grassy/spicy' sesquiterpenes as α-humulene and β-caryophyllene.

Summarizing, in this work different responses to drought stress were highlighted in different hop cultivars, allowing us to determine the most tolerant and drought-sensitive hop cultivars. The quality of hops cultivated in Northern-Italy (expressed as hop acids content and volatile

profile) and its variability were outlined for two cultivation years and some important odour impressions in hops (especially ‘citrusy’ notes) were characterized by a molecular point of view. This project has therefore increased our understanding of hop response to drought stress and of hop aroma, providing initial practical benefits for hop growers and for the hop industry.

1. General background and aims

1.1. *Humulus lupulus* L. plant

Hop (*Humulus lupulus* L.) is a dioecious perennial plant that belongs to the family of Cannabaceae. The female inflorescences of hop are called 'cones' and contain numerous glandular trichomes (lupulin glands) in their bracts (**Figure 1**). Hop cones are widely used as raw material in the brewing industry because they improve flavour-stability of beer and play an important role in bitter stability, beer foam and shelf life of beer (Schönberger and Kostelecky, 2011). Hop aroma is characterized by the presence of a complex mixture of volatile compounds in hop essential oil contained into the lupulin glands, while beer bitterness is mainly due to the reaction products of hop acids. Additionally to their contributions to the aroma and taste of beer, hop-derived compounds have different health-beneficial activities. Hop bitter acids have been reported to exert a wide range of medicinal effects, both in vitro and in vivo; they exhibit potential anticancer and estrogenic activities and are effective against inflammatory and metabolic disorders (Zanoli and Zavatti, 2008; Van Cleemput et al., 2009).

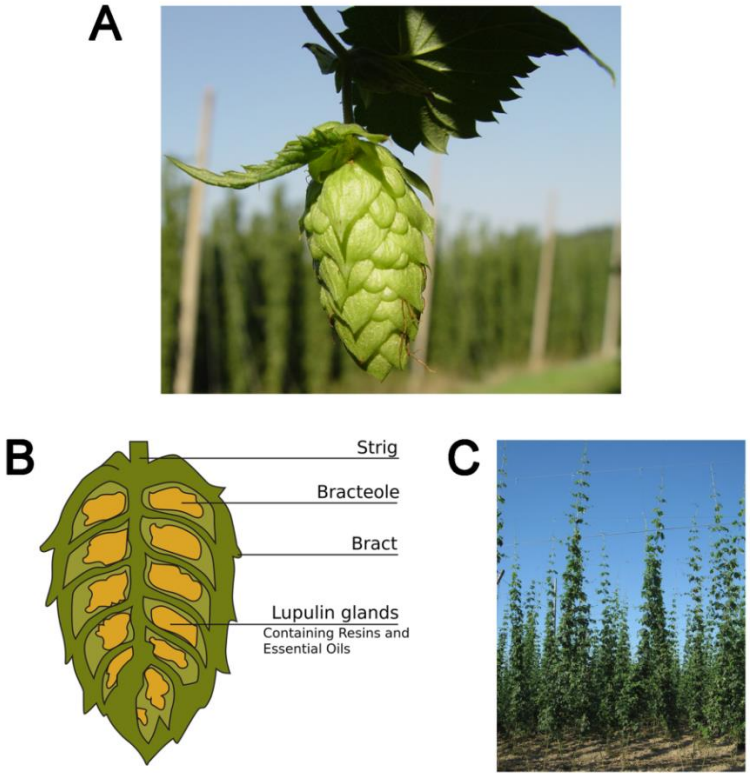


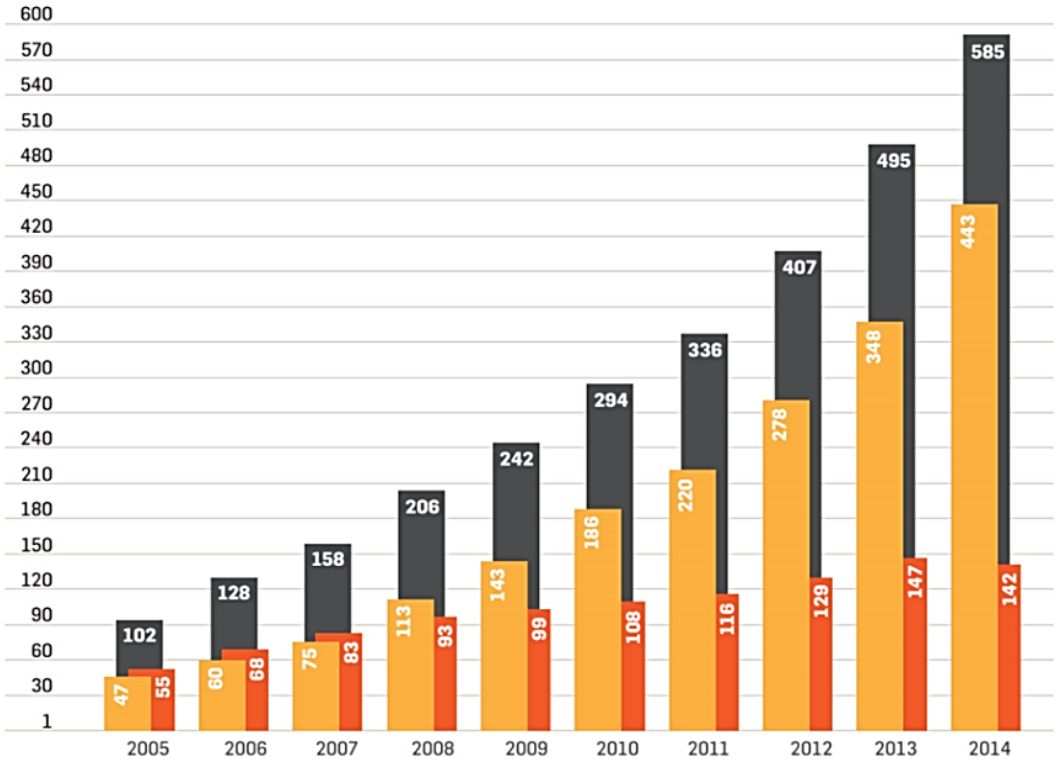
Figure 1 – Hop cone **(A)**, hop cone section **(B)** and hop plants in a field **(C)**

The International Hop Growers' Convention (IHGC) stated that the 2014 world hop production was 94,520 tons of raw hop cones, which was produced on an acreage of approximately 48,100 ha (IHGC Market Report, 2015). It was also highlighted that 2014 hop production and acreage increase of 12% and 4.3% compared to 2013, respectively. The increasing spread of the craft beer markets, is paralleled by a rise of biochemical and molecular research in hop aroma (Schönberger and Kostelecky, 2011). Among European countries, Italy is the tenth for beer production and the total added value related to the production and sale of beer in 2013 was estimated to be approximately €3.2 billion (Assobirra annual report, 2014). Furthermore the number of Italian micro-breweries is increasing (**Figure 2A**), and most of them are located in Northern-Italy (**Figure 2B**) (Assobirra annual report, 2014). Nevertheless Italy is still a hop powder and extracts importer, which are mainly purchased from Germany (**Table 1**) (Assobirra annual report, 2014).

Table 1 – Import of hops in Italy in 2014 (tons). Source: Assobirra annual report (2014), ISTAT data

	Hops powder	Hops extract	Total
Germany	2990.578	124.586	3115.164
Poland	28.966	17.636	46.329
Belgium/Luxemb.	35.685	8.322	44.007
Solvenia	31.826	-	31.826
Spain	23.781	0.346	24.127
United Kingdom	18.230	1.095	19.325
Denmark	-	14.400	14.400
Czech Republic	10.943	-	10.943
France	-	2.071	2.071
Total EU	3165.209	168.183	3333.392
USA	6.000	5.412	11.412
Total	3171.209	173.595	3344.804

A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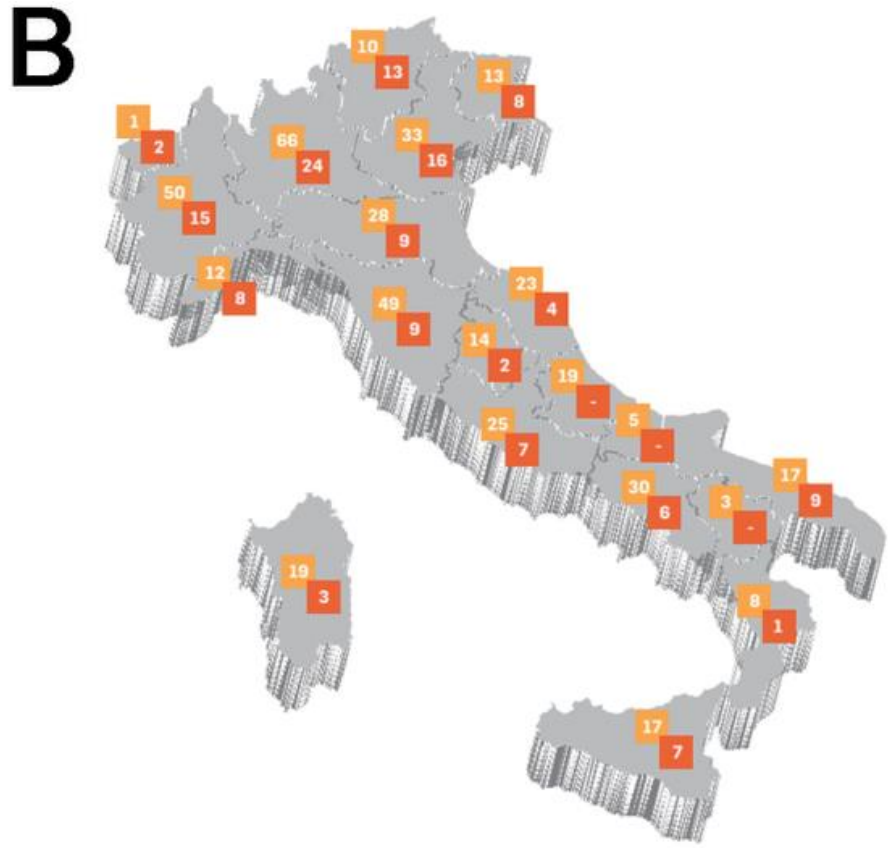
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Figure 2 – **(A)** Number of microbreweries (in yellow) and brew pubs (in orange) in Italy (trend 2005-2014). **(B)** Geographical distribution of microbreweries and brew pubs in Italy in 2014 (Assobirra annual report, 2014)

1.2. Hop cultivation

Hop is a perennial, wind-pollinated climber plant (Neve, 1991). The aerial vegetative part of the plant die off in autumn while the rootstock stays in the soil over winter for many years. In spring the stem tissue of the upper part of the rootstock produces numerous buds from which many shoots develop. The farmer selects the strongest shoots and trains them up the strings. The plant needs a support up which to grow so it is cultivated on strings suspended from a trellis. Therefore, setting up a hop yard requires considerable capital and the structure has to support the weight of the crop also in adverse weather conditions. Hop cultivation system differ between countries and cultivars. For example, the height of hop wireworks is highly variable. Before the advent of mechanical picking, the bine was cut down from the overhead wirework (3.75-4.25 m high) and the hops were picked by hand, while, in mechanical picking, the bine is cut down from a higher wirework and above the rootstock and transported in a trailer to a static picking machine (Briggs et al., 2004). Therefore, mechanical picking allowed to build higher wireworks, such as those in USA (4.0-5.5 m) and Europe (6.0-7.0 m) (Briggs et al., 2004). The rows of plants are usually 2.8-3.2 m apart to allow tractors to pass freely and plants are 0.8-1.1 m apart on rows (Fernández-Pola, 1996). Hop plants have a fair resistance to frost as the root system is very large and deep into the ground (Verzele and De Keukeleire, 2013). The growth of the plant makes heavy demands on soil nutrients which must be restored. Fertilizer treatments should be based on soil analyses (Briggs et al., 2004). In his book, Fernández-Pola (1996), suggest to apply the following fertilizer treatment: 500 kg ha^{-1} of ammonium sulphate plus 300 kg ha^{-1} of sodium nitrate, 400 kg ha^{-1} of calcium superphosphate (in early spring) and 250-300 kg ha^{-1} of potassium sulphate (half at the starting of vegetative growth and half at the starting of flowering). Hops require fertile, well-drained soil and it cannot tolerate excessive moisture (Fernández-Pola, 1996). The plant requires a good depth of soil and a pH above 6.5. In England and Western Europe the water requirements of the hop crop are usually supplied by natural rainfall but elsewhere irrigation is often necessary. For example, in the USA the crop requires 400±500 mm of rain in the

Willamette Valley and 760 mm in the Yakima Valley, which is supplied by irrigation, while in Australia and in Serbia overhead sprinkler systems are widely used (Briggs et al., 2004).

Considering that water stress is one of the major problems affecting crop growth and productivity, irrigation system and selection of drought tolerant varieties is crucial. In Mediterranean climate areas, the phenological cycle of hops coincides with periods of high air temperature combined with drought. The increase in the number of hot days and more frequent occurrence of drought periods will have a direct impact on the yield and quality of crops (Izaurralde et al., 2003). Hops are also susceptible to attack by many pests and diseases (such as aphids, mites, nematodes, mildew and virus) so most hop growers need to use agrochemicals to produce a commercial crop (Briggs et al., 2004). The agrochemicals that can be used are licensed by national or international bodies who also set a maximum allowable residue for the chemical in the final product, but new agents have to be developed and approved periodically (Briggs et al., 2004). Due to the limited hop cultivation, in Italy there are no chemical agents approved for this crop and hop growers are forced to use organic treatments, with the exception of three commercial insecticides characterised by the presence of lambda-cyhalothrin (Sistema Informativo Agricolo Nazionale; www.sian.it).

As for other plants, hop flowering is strongly influenced by the duration and the rate of daylight and is induced after the growing a certain number of nodes (the plant must produce 20-25 nodes before being ready to flower) (Neve, 1991; Briggs et al., 2004). Flowers develop at the terminal buds of lateral branches and female flowers develop into cones. In the Northern hemisphere hop cones ripening occurs in August. In particular, the first traces of resin can be detected in early August and resin synthesis is almost complete by the end of the month (De Keukeleire et al., 2003). Picking time is determined by visual examination of cones, by weather conditions and by commercial considerations (Verzele and De Keukeleire, 2013). The characteristics by which a grower decides that the cones are ready for picking are (Burgess, 1964):

1. The bracts and bracteoles close towards the axis of the cone giving it a compact form;
2. The bracts and bracteoles become firm and slightly resilient. They rustle when squeezed in the hand and are rather easily detached from the axis;
3. The colour of the bracteoles and the bracts changes to a yellow-green;
4. The lupulin glands are completely filled with resins.

1.3. Chemical components in hop cones

Hop lupulin glands contain many secondary metabolites, including resins, essential oils and tannins. The major components present in hop cones are listed in **Table 2** (Kishimoto, 2008).

Table 2 – Major components in hop cones

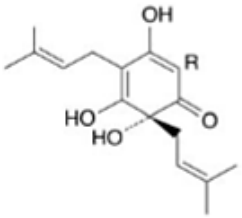
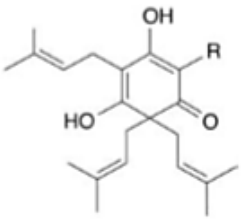
Major components	Concentration (% w/w)
Cellulose-lignins	40.0 – 50.0
Proteins	15
α-acids	2.0 – 17.0
β-acids	2.0 – 10.0
Water	8.0 – 12.0
Minerals	8
Polyphenols and tannins	3.0 – 6.0
Lipids and fatty acids	1.0 – 5.0
Hop oil	0.5 – 3.0
Monosaccharides	2
Pectins	2
Aminoacids	0.1

Hop acids

Hop resins comprise hard resins (including xanthohumol, iso-xanthohumol and flavone, not soluble in hydrocarbon solvents) and soft resins (also called 'hop acids' and soluble in hydrocarbon solvents). Today, the most important analytical determination for hop evaluation is the quantitative analysis of α-acids (Verzele and De Keukeleire, 2013). Indeed, while for a long time hop quality was evaluated according to colour and aroma, gradually α-acids has been used more and more as a quality criterion. Hop acids are composed of α-acids and β-acids, which are both synthetized from prenylated acylphloroglucinol (Briggs et al., 2004). α-acids are divided into five isomers including humulone, cohumulone, adhumulone, prehumulone, and posthumulone, while β-acids are divided into lupulone, colupulone, adlupulone, prelupulone and postlupulone (**Table 3**) (Briggs et al., 2004). From 100 to 800 g of hop cones are added per hectoliter, depending on the wort density, the hop variety and its α-acids content (Verzele and De Keukeleire, 2013). Often, high α-hops are added during the initial stages of wort boiling process, while so-called 'noble' or 'aroma' hops are added only 15-30 minutes before the end of wort boiling (Briggs et al., 2004). In conventional brewing,

hops are boiled with sweet wort, in this way hop acids are isomerized into iso-α-acids, soluble components that represent the main bitter component of beer. The aim is indeed to obtain the necessary bitterness by addition of hops in the first stages of the wort boil and to achieve a desirable aroma contribution of the more expensive aroma hops by reducing the time during which essential oils are removed from the boiling wort (Verzele and De Keukeleire, 2013). Indeed, during boiling the essential oil constituents are vaporized, so brewers may add 'aroma' hops at the end of the boiling to replace this loss. Bittering of beer can instead be achieved also by using pre-isomerized hop extracts; where α-acids have already been isomerized into iso-α-acids (Verzele and De Keukeleire, 2013).

Table 3 – Analogues of the α and β-acids (Briggs et al., 2004)

α-acids					β-acids		
							
Name	Formula	m.p. (°C)	$[\alpha]_0^{24}$	pKa	Name	Formula	m.p. (°C)
Humulone	$C_{21}H_{30}O_5$	64.5°	−211°	5.5	Lupulone	$C_{26}H_{38}O_4$	92°
Cohumulone	$C_{20}H_{28}O_5$	oil	−208.5°	4.7	Colupulone	$C_{25}H_{36}O_4$	93−94°
Adhumulone	$C_{21}H_{28}O_5$	oil	−187°	5.7	Adlupulone	$C_{26}H_{38}O_4$	82−83°
Posthumulone[a]	$C_{19}H_{26}O_5$	oil	–	–	–[d]	$C_{24}H_{34}O_4$	101°
Prehumulone[b]	$C_{22}H_{32}O_5$	oil	−172°	–	–[e]	$C_{27}H_{40}O_4$	91°
Adprehumulone[c]	$C_{22}H_{32}O_5$	–	–	–	–[e]	$C_{27}H_{40}O_4$	90°

Hop essential oil

The composition of the essential oil depends on genetic (cultivar) and cultural factors. By definition, essential oils are volatile in steam, and for this reason, most essential oil will be lost during boiling. To add hop aroma to beer, hops can be added to beer at the end of boiling

(late hopping) or in the conditioning tanks to introduce a particularly strong hop aroma (a process known as ‘dry hopping’).

Dry hops contains about 0.5–3% of essential oil (see **Table 2**). **Figure 3** shows the classification of hop oil components according to Schönberger and Kostelecky (2011). Hop essential oil typically consist of 90% terpenoids, dominated by the monoterpene β-myrcene and the sesquiterpenes α-humulene and β-caryophyllene, but over 300 different volatile compounds has been identified (Eri et al., 2000). As the hop ripens, trace of oxygenated compounds of the essential oil appear first, then caryophyllene and humulene, and finally myrcene is formed (Briggs et al., 2004). Probably the first oxygenated component of hop oil to be characterized was 2-undecanone, which is now known to be accompanied by other methyl ketones. Differences in aroma properties between hop varieties can be attributed to variations in the composition of their essential oil. However, not all character-impact odorants in hop essential oil have been identified, and hop aroma is still not completely characterized. Indeed, recent work using GC×GC with flame ionisation detection has suggested that there may be over 1000 compounds in hop oil including aldehydes, ketones, esters, alcohols, acids, oxygen heterocyclic compounds and sulphur-containing compounds (Eyres et al., 2007).

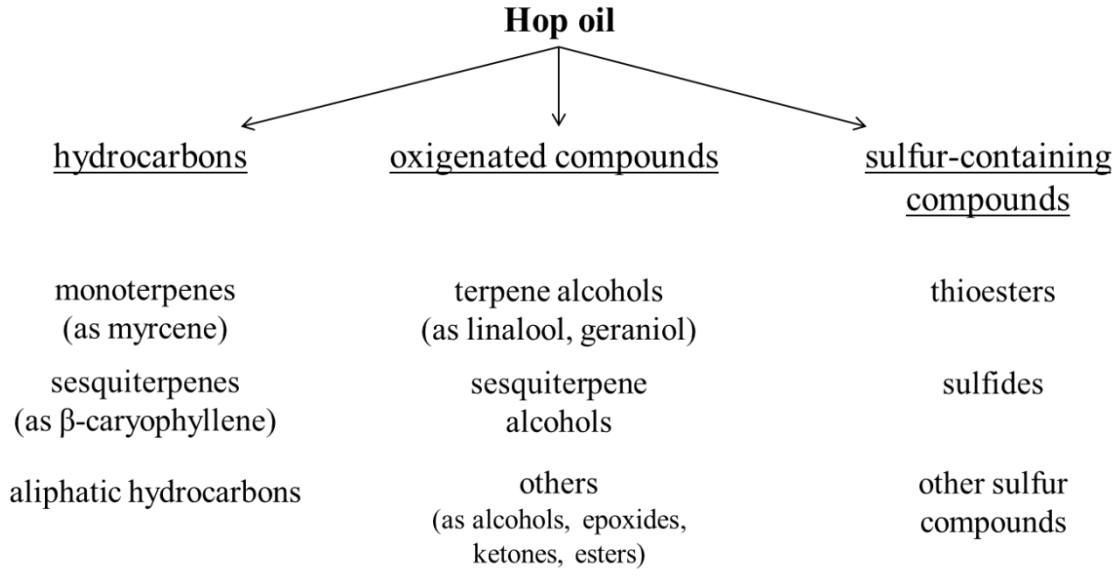


Figure 3 – Hop oil composition (Schönberger and Kostelecky, 2011)

1.4. Analysis of hop aroma

The first aromatic compounds of hop oil were determined at the end of 20th century (Fukuoka and Kowaka, 1983). Since then, several studies were focused on chemical profiles of hops (Eri et al., 2000; Nance and Setzer, 2011; Vázquez-Araújo et al., 2013) and on their odour-active compounds (Steinhaus and Schieberle, 2000; Eyres et al., 2007; Steinhaus et al., 2007; Van Opstaele et al., 2012a; Kankolongo Cibaka et al., 2015) and several biochemical techniques were adopted for their characterization. The most common methods for isolating essential oils from hops are based on vacuum or steam distillation (Kovačevič and Kač, 2001; Hanke et al., 2008), solvent extraction (Perpete et al., 1998; Leonardi et al., 2013), extraction with carbon dioxide (Van Opstaele et al., 2012b; Van Opstaele et al., 2013), direct thermal desorption (DTD) (Eri et al., 2000) and headspace solid phase micro-extraction (HS-SPME) (Gonçalves et al., 2012; Van Opstaele et al., 2012a). SPME apparatus consists of a fused silica fibre coated with an adsorbent organic phase. The SPME fiber is exposed to the headspace of the sample (HS-SPME) or directly into an aqueous sample and concentrates volatile components by adsorption and/or absorption. Then, the fiber release the extracted material into the heated injection port of the gas chromatograph (GC) where the volatile compounds are desorbed from the fiber and transferred to the GC column for separation. Gas Chromatography (GC) and Mass Spectrometry (MS) are generally applied for the identification and quantification of hop essential oil components. Comprehensive two-dimensional gas chromatography (GC×GC) consists, for example, of two columns with different stationary phases that create a two-dimensional separation based on two different column properties. Using GC×GC, two compounds with similar boiling points that co-elute on the first column may be divided by the second column if they have a different polarity.

Despite its extensive applications, a GC detector do not provides information regarding the odour activity of each molecule, and there is not always a strong positive correlation between the peak area and the odour intensity (Eyres et al., 2007). A valuable tool for identifying character-impact odorants is gas chromatography-olfactometry (GC-O), where human assessors are used to detect and characterize volatile compounds as they elute from a column following a GC separation (Delahunty et al., 2006) (**Figure 4**).

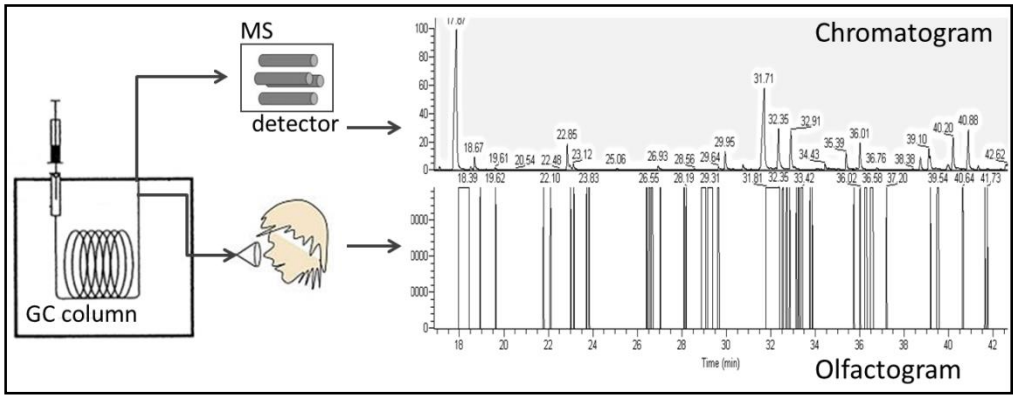


Figure 4 – GC-MS/O workflow diagram

Previous studies used GC-O technique to identify hop's key components to define hop aroma characteristics and a relatively high number of character impact compounds have been proposed in the literature. Organoleptic impressions derived from the essential hop oils are mainly described in terms of floral, citrus, spicy or herbal flavours. As mentioned by Schönberger and Kostelecky (Schönberger and Kostelecky, 2011), green and grassy notes are due to aldehydes (Kishimoto et al., 2006), floral and fruity impressions derived from linalool, geraniol, β-ionon, citronellol, and a variety of ketones, epoxides, and esters (Marriott, 2001; Kishimoto et al., 2006; Van Opstaele et al., 2006) and spicy/herbal flavours can be attributed to oxidized sesquiterpenes (Goiris et al., 2002; Eyres et al., 2007). Some hop-derived sulphur compounds may also play an important role in the flavour of particular hop varieties (Kankolongo Cibaka et al., 2015). Recently, the use of GC-O techniques, such as aroma extract dilution analysis (AEDA) (Steinhaus et al., 2007) and Charm Analysis in combination with quantitative analysis has been reported in the determination of some odour-active components (Kishimoto et al., 2006). AEDA measures odours' thresholds, while Charm Analysis records the duration of the odours and generates chromatographic peaks that are proportional to the amount of compound in the extract and inversely proportional to the odour detection threshold (Delahunty et al., 2006). These techniques are therefore used to determine the key components related to each sensory impression (Steinhaus and Schieberle, 2000), but hop and beer flavours are the effect of coexisting compounds (Inui et al., 2013). For this reason, also the combination of GC or GC-MS and Quantitative Descriptive Analysis (QDA) could be effective in determining the key compounds of hop aroma (Kishimoto et al., 2006; Inui et al., 2013).

1.5. Hop cultivars

Hops are grown as a commercial crop for the brewing industry in many countries and there are hundreds varieties available. Hop cultivars are traditionally classified into three groups: aroma hops, high-bitter hops and intermediate bitter hops, depending on the concentration of hop acids. To the *Humulus* genus belong three species that are: *H. lupulus*, *H. japonicas* and *H. yunnanensis* (Neve, 1991). *H. lupulus* have a native distribution between 35° and 70° N latitudes (McAdam et al., 2014) and is mainly cultivated in the Northern hemisphere (between 35° and 55° N), but also in the Southern hemisphere in Australia, New Zealand and South Africa (**Table 4**) (Briggs et al., 2004). The largest hop growing areas are situated in the South-East and Mid-West of England, the Saaz and Auscha districts of Czechoslovakia, the Hallertau region of Germany, the Slovenian parts of Yugoslavia and in the states of Washington, Oregon and California in the USA (Verzele and De Keukeleire, 2013). Many hop cultivars are of European genetic origin, or are hybrids between European and North American germplasm (McAdam et al., 2014). Hop cultivation is instead not widespread in Italy, with the exception of few small farms where, however, hop is not the only source of profit. The characteristics of some commercial hop varieties are collected in **Table 5**.

Table 4 – Cultivars and growing regions of hops (*Humulus lupulus* L.) (Barth-Haas 2015; Hopunion 2015; Briggs et al., 2004)

Australia	Ella™, Enigma™, Galaxy™, Summer™, Super Pride, Sylva, Topaz™
China	Marco Polo, Tsingtao Flower
Czech Republic	Agnus, Bohemie, Bor, Harmonie, Kazbek, Premiant, Rubín, Saaz, Saaz Late, Sládek, Vital
France	Aramis, Strisselspalt, Triskel
Germany	Hallertau Blanc, Hallertau Magnum, Hallertau Mittelfrüh, Hallertau Perle, Hallertau Taurus, Hallertau Tradition, Herkules, Hersbrucker, Hüll Melon, Mandarina Bavaria, Monroe, Opal, Polaris, Relax, Saphir, Smaragd, Spalt Spalter, Spalter Select, Tettnanger
Japan	Sorachi Ace
Poland	Iunga, Limbus, Lomik, Lublin, Magnat, Marynka, Oktawia, Pulawski, Sybilla, Zbyszko, Zula
Slovenia	Aurora, Extra Styrian Dana, Styrian Gold, Styrian Golding (Bobek, Celeia), Styrian Savinjski Golding
South Africa	Southern Dawn, Southern Promise, Southern Star
UK	Admiral, Boadicea, Bramling Cross, Brewers Gold, East Kent Golding, Endeavour, First Gold, Fuggles, Northern Brewer, Pilgrim, Progress, Sovereign, Whitbread Golding, Wye Challenger, Wye Northdown, Wye Target, Yeoman
Ukraine	National
USA	Ahtanum, Amarillo®, Apollo, Azacca™, Bravo, Cascade, Cashmere, Centennial, Chelan, Chinook, Citra®, Cluster, Columbus, Comet, Crystal, CTZ, Equinox, Galena, Glacier, Millennium, Mosaic®, Mount Hood, Nugget, Palisade, Simcoe®, Summit®, Super Galena, Tahoma, Tomahawk, Triple Pearl, Ultra, Vanguard, Warrior®, Willamette, Yakima Gold, Zeus
New Zealand	Riwaka

Table 5 – Properties of different hop cultivars (Barth-Haas 2015; Hopunion 2015; Briggs et al., 2004)

Cultivar	Type	α-acids (%)	β-acids (%)	Oil (ml/100g)	Myrcene (%)	Humulene (%)	Caryophyllene (%)	Odour/aroma description
Brewers Gold	Dual Purpose	4.5-6.5	2.5-3.5	0.8-1.8	40-50	29-31	7-7.5	Spicy, fruity characteristics
Cascade	Aroma	4.5-7.0	4.8-7.0	0.7-1.4	45-60	10-16	3-6	Floral, citrus and grapefruit tones
Centennial	Aroma	9.5-11.5	3.4-4.5	1.5-2.5	45-55	10-18	4-8	Floral and lemon tones
Chinook	High Alpha	12.0-14.0	3.0-4.0	1.7-2.7	35-40	20-25	9-11	Spice and pine characteristics with grapefruit notes
Citra®	Aroma	11.0-13.0	3.5-4.5	2.2-2.8	60-65	11-13	6-8	Strong citrus and tropical tones
Columbus	High Alpha	15.0-17.0	4.5-5.0	2.5-3.5	50-60	12-18	9-11	Pungent, black pepper, liquorice, citrus tones
Fuggle	Aroma	3.0-5.6	2.0-3.0	0.7-1.4	24-28	30-38	13-13.5	Mint, grass and floral tones
H. Magnum	High Alpha	11.0-16.0	5.0-7.0	1.6-2.6	30-45	35-40	8-12	Mild flavour and low aromatic characteristics
H. Mittelfrüh	Aroma	3.0-5.5	3.0-5.0	0.7-1.3	20-28	50-55	15-17	Spicy, with floral and citrus tones
Hallertau Perle	Aroma	4.0-9.0	2.5-4.5	0.5-1.5	20-35	28-33	10-12	Spicy with herbal and floral characteristics
Mount Hood	Aroma	4.0-7.0	5.0-8.0	1.2-1.7	30-40	30-38	13-16	Herbal and pungent or spicy
Northern Brewer	Dual Purpose	8.0-10.0	3.0-5.0	1.5-2.0	50-60	20-30	5-10	Pine and mint characteristics
Nugget	High Alpha	11.5-14.0	3.0-5.0	0.9-1.3	27-42	16-19	7-9	Spicy, herbal tones
Riwaka	Aroma	4.5-6.5	4.0-5.5	1.4-1.6	60-68	9-10	3.5-4	Grapefruit and citrusy characters
Saaz	Aroma	3.0-6.0	4.5-8.0	0.4-1.0	25-40	35-40	9-11	Spice and earth tones

Table 5 – (continue)

Cultivar	Type	α-acids (%)	β-acids (%)	Oil (ml/100g)	Myrcene (%)	Humulene (%)	Caryophyllene (%)	Odour/aroma description
Hersbrucker Spät	Aroma	2.5-5.5	3.0-5.0	0.5-0.9	20-35	20-30	8-13	Herbal, with spicy, floral and fruit tones
Sterling	Aroma	6.0-9.0	4.0-6.0	1.3-1.9	44-48	19-23	5-7	Herbal and spicy, with floral and citrus notes
Willamette	Aroma	4.0-6.0	3.5-4.5	1.0-1.5	30-40	20-27	6-8	Spicy and floral tones
Wye Challenger	Dual Purpose	6.5-8.5	2.5-4.3	1.0-1.5	28-32	20-25	9-9.5	Green tea and floral characteristics
Wye Northdown	Dual Purpose	7.0-10.0	4.0-5.0	1.2-2.2	20-25	35-37	15-17	Spice and pine characteristics with floral tones
Yeoman	Dual Purpose	12.0-16.0	4.0-5.0	1.7-2.4	45-48	20-25	7-10	Mild flavour and low aromatic characteristics

Hop cultivars strongly differ in their secondary metabolite profiles, in terms of their presence, amount and relative proportions. For this reason, different hop cultivars are characterized by different levels of bitterness and a variety of flavours and aromas (McAdam et al., 2014). Aroma proprieties have a great importance for the brewer, who can benefit of particular hop varieties to add subtle tastes and flavours to beer. The development of new crop cultivars is becoming much more important for breeders that can choose among many potential selection criteria (e.g. yield per hectare, agronomic suitability or brewing quality and chemical characteristics) (McAdam et al., 2014). Methods based on DNA analysis reflect the genotype of the cultivar irrespective of the stage of plant development, the environmental or disease status (Briggs et al., 2004), however, making genetic improvements to these criteria is complex as many of the traits relevant to them are quantitative characters, likely controlled by a large number of genes (McAdam et al., 2014). Therefore, selection of parents for hop breeding depends nowadays mainly by agronomic and chemical characteristics (Field et al., 1996). The brewing industry relies to a great extent of morphological and organoleptic features for the evaluation and identification of hop varieties (De Cooman et al., 1998). Several types of hop secondary metabolites have been used for identification purposes, mostly volatile components of the essential oil (Likens and Nickerson, 1967; Peacock and McCarty, 1992; De Cooman et al., 1998; Perpete et al., 1998; Kovačevič and Kač, 2001; Lermusieau and Collin, 2001; Kovačevič and Kač, 2002; Shellie et al., 2009). Also hop polyphenols, including flavonoids, have been employed in establishing a chemical identification procedure (De Cooman et al., 1998). Despite this, there are no scientific studies that analyse the volatile profile of a high number of hop cultivars or evaluate key molecules for varietal characterization taking into consideration their variability among different cultivation years. Indeed, to analyse a high number of hop varieties allow us to clearly identify molecules that can be used as varietal markers and to highlight correlations between volatile molecules or between volatiles and hop sensory characteristics.

1.6. AIM

Chapter 2. The introduction of hop cultivation in Italy is interesting, but one of the first problems we have to deal with is drought stress. Indeed, in Mediterranean areas, the phenological cycle of hops coincides with factors that are supposed to affect hop crop and α-acid production: high air temperature and, in general, water stress. To counteract this problem, one of the first thing that can be done is to select drought tolerant varieties studying the physiological and molecular mechanisms activated in hop in response to water stress. However, the scientific knowledge in this field is still lacking and for this reason eleven hop cultivars were subjected to drought and physiological parameters were measured.

Chapter 3. In order to introduce hop cultivation in Italy, the achievable quality of hops cultivated in Northern-Italy and its variability among different cultivation years should also be verified and taken into account. To the best of our knowledge, no detailed information is available on hop cultivation in Italy and, while the effect of climatological conditions on α-acids and β-acids formation is well-known, there are no papers focused on the variability of hops' volatile profiles and their key molecules for varietal identification. For this reason, hop acids and volatile profiles of sixteen hop varieties cultivated in the same field in Parma in two different years were analysed. Moreover, beers flavoured with hop cones of eleven varieties cultivated in 2013 were sensory analysed by a trained panel to find relationships between hops' volatile profiles and sensory descriptors. To describe different kind of hop aroma impressions from a molecular point of view could indeed be useful for breeders in the developing of new hop cultivars or for brewers to find objective and reliable parameters able to describe hop aroma.

Chapter 4. To further investigate the relationship between the volatile profile and the sensory characteristics of hops, from January to November 2015, the research was carried out at the University of Leuven (KU Leuven), in the Laboratory of Enzyme, Fermentation and Brewing Technology (EFBT) in Ghent, Belgium. During this period the research was focused on the chemical and sensorial characterization of volatile compounds in different hop varieties to identify the odour-active molecules of particularly citrusy hops. Indeed, although different odour impressions such as green/grassy, floral/fruity, or spicy, have already been described, few attempts were made to clearly correlate hop 'citrus' aroma with the chemical profile of

hop oil. The essential oils of three hops with a pronounced citrusy flavour and of three hops with a spicy/herbal flavour were therefore analysed via gas-chromatography mass spectrometry/olfactometry, a specific technique that allow to individually identify odour-active compounds during a GC separation. The identification of key molecules for the characterization of ‘citrus’ sensory character increased our scientific knowledge on hop aroma and could be useful to hop breeders who aim to select new varieties with a specific citrusy character.

2. The response of eleven hop (*Humulus lupulus* L.) varieties to drought stress

2.1. INTRODUCTION

Drought is one of the major abiotic stresses affecting plants growth in Mediterranean climate areas. The increase of the temperature, the number of hot days and drought periods will have a direct impact on yield and quality of crops (Izaurralde et al., 2003). In his paper, Mozny et al., (2009) simulate the impact of weather conditions on yield and quality of Saaz hops, showing that the increase in temperature would cause diminished yields and will have a negative impact on the accumulation of α-acids in hop. Increasing water scarcity would therefore negatively affect hop cultivation, nevertheless, hop growers can potentially respond to the physiological impacts of climate change through cultivar selection and crop management practices. However, scarce information on mechanisms of drought tolerance in hop plants are available.

Generally, one of the first plant responses to decreasing soil water availability is the reduction of stomatal conductance and regulation of stomatal opening/closing, which is driven by chemical and hydraulic signals from the roots to the shoots (Lawson, 2009). As observed by Korovetska et al. (2014), most of the studies that demonstrated the importance of chemical signals in the regulation of stomatal movement during water stress were carried out on herbaceous plants (annuals), while only a few were conducted on woody plants. However, hop represents a particular case since it is herbaceous and annual, but it has very long stems (up to 8 m) and so it probably needs long distance signalling mechanisms between roots and leaves. Hop belong to anisohydric plants category that exhibit a less strict regulation of water use keeping their stomata open and photosynthetic rates high for longer periods, even in the presence of decreasing leaf water potential (Gloser et al., 2013). In two recent papers (Korovetska et al., 2014; Korovetska et al., 2015) the involvement of some chemical signals (e.g. abscisic acid, anions and phytohormones variation) on hop plant transpiration during water stress was outlined. However, few scientific papers focused on the effect of drought stress in hops and they all take into consideration a limited number of cultivars (only two-three varieties). Only Hejnák et al. (2015) captured some physiological parameters on a

higher number of hop cultivars subjected to water stress grown in a greenhouse. In particular they measured photosynthesis, transpiration, stomatal conductance and water use efficiency in 15 genotypes of young hop plants upon water stress, concluding that these parameters can be used for evaluation of genotypes in monitoring the impact of water deficit on hop. In our work, we first analyse the transpiration rate of eleven hop cultivars upon drought and then we pinpoint the effect of water stress on selected growth parameters. Our aim was therefore to determine the most tolerant/sensitive hop cultivars studying their response to prolonged water stress conditions.

2.2. MATERIALS AND METHODS

2.2.1. Plant material and growth conditions

Young plants of eleven hop cultivars (Brewers Gold, Cascade, Challenger, Chinook, Columbus, Fuggle, Hallertau Magnum, Hallertau Mittelfrüh, Hersbrucker Spät, Northern Brewer and Nugget) were obtained by direct rooting from 0.3 m scions. Scions were soaked in water for 24 h and transplanted into 3-L pots filled with organic Potgrond H substrate (Klasmann-Deilmann GmbH, Germany). Content of major nutrients and water holding capacity of substrate were: N 210 mg l^{-1}, P_2O_5 240 mg l^{-1}, K_2O 270 mg l^{-1}, total porosity 85% v/v. Plants were then adapted to soil and morphologically uniform plants at the 5–7 leaf stage were selected for the experiment. Drought stress experiments were conducted in an air conditioned greenhouse in Legnaro, Padua, Italy (45°21N; 11°56E; 15m above sea level) from the beginning of July to the end of August 2014, under natural light conditions. All plants were irrigated daily and supplemented with a micronutrient fertilizer (Ferty®3, Planta Düngemittel GmbH, Regenstauf, Germany) every 15 days until the start of the experiment. During this period plants were also managed by shoot removal to provide uniform material and at the time of sampling, plants were on average 80 cm long with 15-20 pairs of leaves.

8 plants for each cultivar were selected for the main drought experiment and were randomly divided to obtain two pools: 4 control plants and 4 plants exposed to drought stress. A total of 88 plants were considered in the study. The experiment was carried out using a complete randomized block design. Each replicate consisted of 22 plants, 11 controls (one for each variety) and 11 drought-stressed plants (one for each variety), giving a total of 44 plants per treatment. At the starting of the experiment all plants were watered to field capacity and then

each pot was sealed with a plastic cap and mastic to avoid evaporation. Control plants were watered every day to field capacity while stressed plants were left without irrigation till the end of the experiment to determine the permanent wilting point.

2.2.2. Estimation of the relative transpiration rate and the fraction of transpirable soil water

Plant transpiration was determined gravimetrically by weighing the plant pots on a daily basis (at the same time for well-watered and drought-stressed plants, in the morning between 9:00 and 10:00) for the entire duration of the experiment. Given that the pots were completely sealed, the amount of water consumed by plant transpiration was calculated just calculating the difference between the weight of the pot of one day and the day before. The water lost for transpiration in control plants was also determined daily and then immediately replaced to maintain the same level of drought (about the 95% of field capacity) for all the duration of the experiment. The transpiration data were analysed by the procedure previously described by Ray and Sinclair (1998). To evaluate the relative transpiration (RT) minimizing the influence of large variations in daily transpiration amongst days, the daily transpiration rates of the drought-stressed plants' pots were normalized against the daily transpiration rates of control plants (within plants belonging to the same variety) (1).

$$TR = \frac{transpiration\ of\ stressed\ plant}{average\ transpiration\ of\ control\ plants} \quad (1)$$

Then, to minimize the variation of TR amongst plants with different sizes, a second normalization was carried out. The transpiration rate of plants in the first three days of the experiment was considered as the maximum transpiration achievable by stressed-plants in an optimal condition. Given that the available water content of pots was substantially identical in the first days of experiment (almost 100%), differences in transpiration between plants can be considered as exclusively influenced by plant size/total leaves area. Therefore, the daily normalized transpiration rate (NTR) was finally calculated for each stressed-plant dividing its daily transpiration by the mean transpiration of the same plant in the first three days of the experiment (2).

$$(2) \qquad NTR\ of\ stressed\ plants = \frac{daily\ TR}{mean\ TR\ in\ the\ first\ 3\ days}$$

Soil water status was expressed as the fraction of transpirable soil water (FTSW). The use of transpirable soil water as the basis of comparing plant response to soil has been used in a number of studies (Verhoef and Egea, 2014; Catola et al., 2015; Hofmann and Schultz, 2015). To measure FTSW, three out of the seven plants consisting on stressed-plants replicates for each variety, were left without irrigation until they achieved constant pot's weight value and plant permanent wilting. Given that all the other weight factors (pots, soil and plants supports) were constant, and considering as negligible the increase of plant weight during the experiment, the transpirable soil water was estimated as the difference between the initial weight recorded once plants were over-irrigated and let to drain overnight (filed capacity) and the final weight recorded at wilting point. Daily values of FTSW where then calculated for each pot by dividing the daily pot weight minus the final pot weight by the transpirable soil water of that pot (3).

$$(3) \qquad daily\ FTSW = \frac{daily\ pot\ weight - pot\ weight\ at\ permanent\ wilting\ point}{pot\ weight\ at\ field\ capacity - pot\ weight\ at\ permanent\ wiltingt\ point}$$

2.2.3. Plant growth parameters

Leaves axes, shoots length, and non-destructive SPAD (Minolta) chlorophyll meter values were measured after 0, 3, 7, and 10 days of drought stress. Two fully expanded leaves at the third or fourth positions from the apex of each plant were used to conduct leaves axes (**Figure 5** shows which axes were measured) and SPAD measurements, and the third internode (starting from the principal shoot) was also measured for each plant. The phenological parameters' growth percentage was then calculated for each variety (both in control and stressed plants), between one time point and the previous one and means and standard errors were reported.

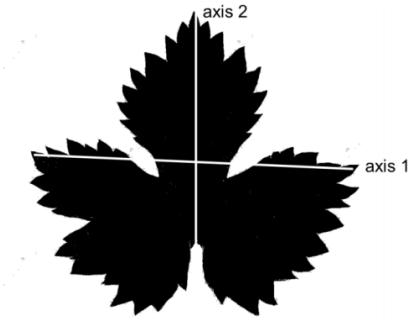


Figure 5 – Leaves axes measured

2.2.4. Statistical analysis

T test was carried out to highlight significant differences between control and water-stressed plants by using 't.test' function of R programming language (version 3.2.2.; https://www.r-project.org).

2.3. RESULTS AND DISCUSSION

2.3.1. Response of relative transpiration rate to drought stress

The behaviour of each variety in terms of normalized relative transpiration as a function of transpirable soil water content was outlined. To outline the best-fit equation that describe the relationship between FTSW and NTR for each cultivar, data were analysed using CurvExpert Professional version 2.3.0. (http://www.curveexpert.net/). The behaviour of hop cultivars transpiration in relation to soil drying, resulted to be described by a Chapman-Richards model (3) and is reported, for each variety, in **Figure 6**.

(3) y=a*(1-exp(-b*x))^c

Curves' correlation coefficients (r), curves' parameters (a, b and c) and standard deviations are reported in **Supplementary Table S1**, and curves used to estimate the average trends of FTSW and NRT across different cultivars are gather all together in **Figure 7**.

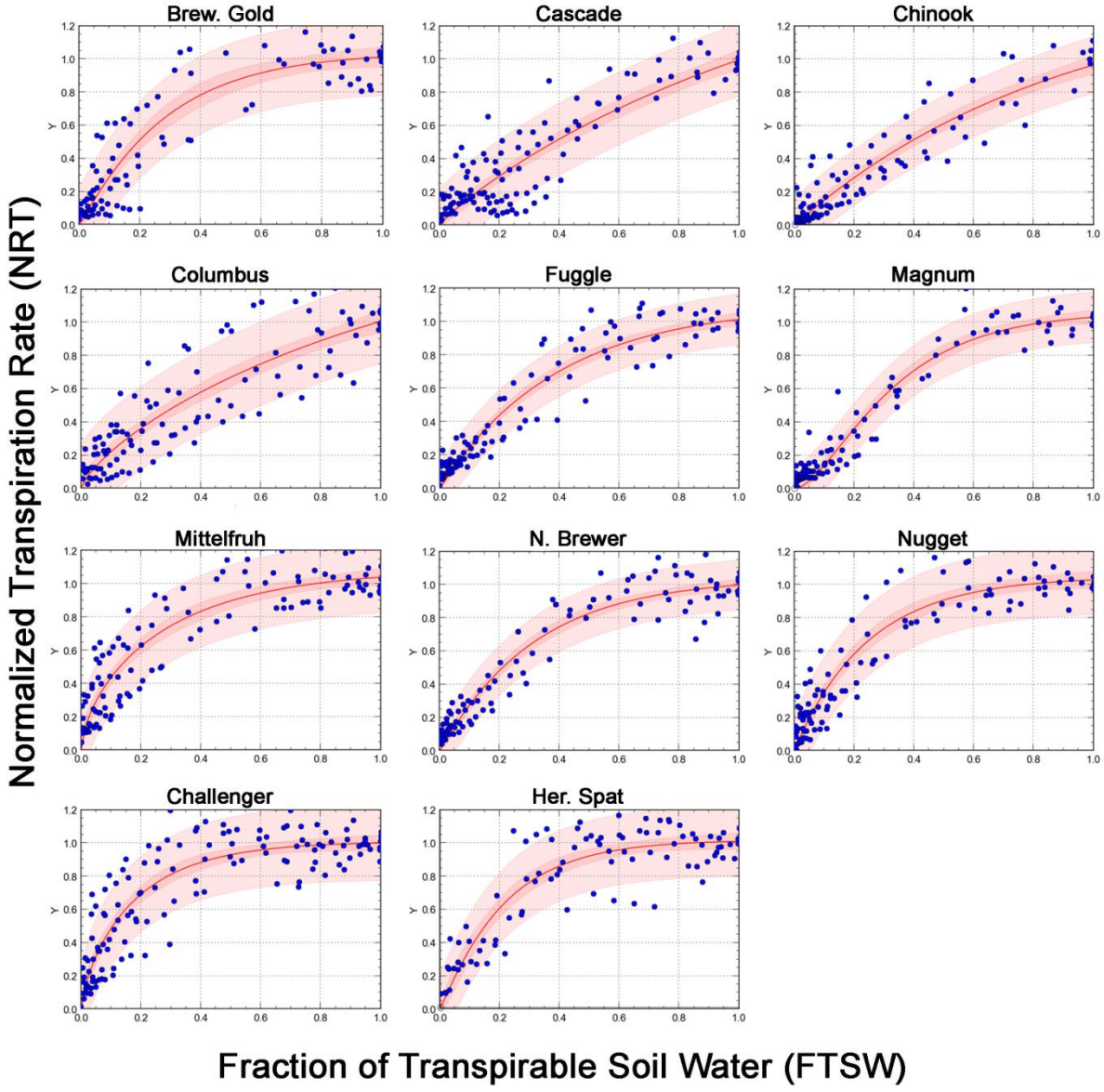


Figure 6 – Changes in transpiration rate (NTR) during soil drying determined experimentally for eleven hop cultivars. Each point represents an independent measurement on one plant and the average trend across plants replicates was also reported for each variety (continuous red line) together with its confidence band (red bands) and prediction band (light red bands) calculated at 95% probability level using CurvExpert Professional 2.3.0.. Curves' correlation coefficients (r) and parameters (a, b and c) are reported in **Supplementary Table S1**.

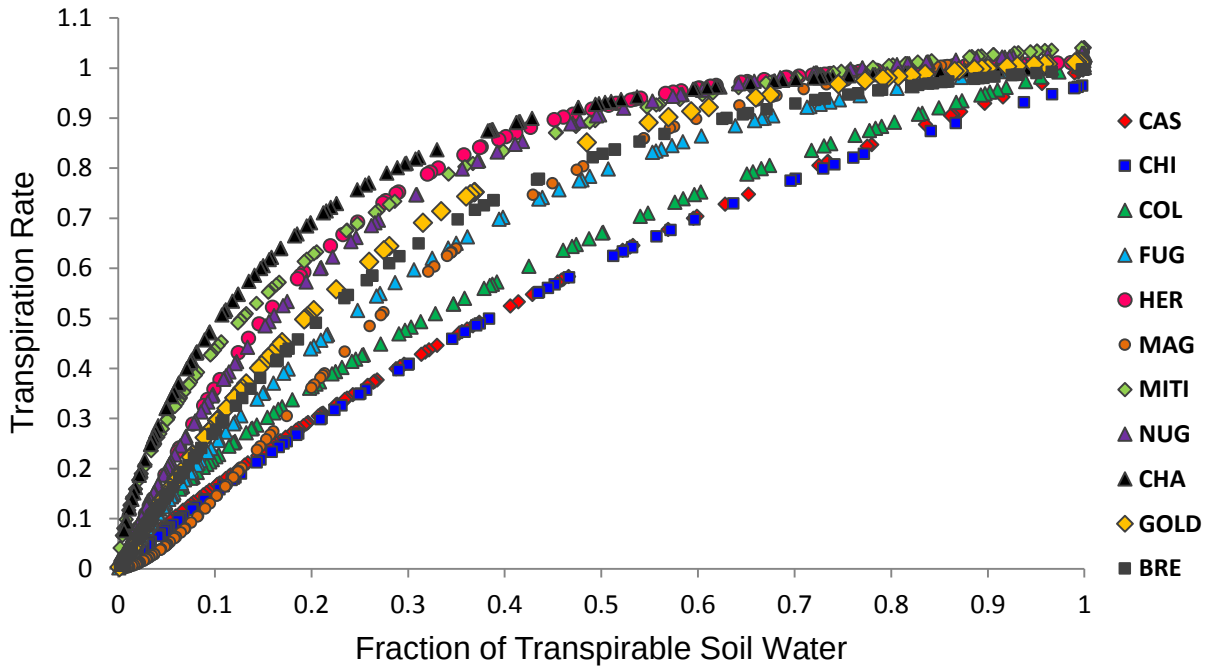


Figure 7 – Average trends of changes in transpiration rate (TR) during soil drying determined experimentally for eleven hop cultivars.

Most curves in **Figure 6** (gathered together in **Figure 7**) display a similar shape. Hop plants seems to experience no or little reduction in water availability until FTSW≈ 0.7, below which a decline in NTR takes place. This agrees with the behaviour highlighted in different other species as grapevines (Bindi et al., 2005), maize and sorghum (Verhoef and Egea, 2014), where plant transpiration remains generally unchanged until a fraction of the available water is lost, and then decrease until available soil water is exhausted. Hops seems therefore to belong to anisohydric plants, as hypothesised by Gloser et al. (2013), however, the big number of hop cultivars subjected to water stress in our experiment allowed us to observe different responses to water stress among different hop cultivars. Interesting exceptions are indeed the cultivars Cascade, Chinook and Columbus, that showed a more linear decline in NTR during water stress compared to the others cultivars. Another exception seems to be Magnum cv., which have a linear decline too, but only at higher stress levels (between FTSW = 0.5-0.1). Brewers Gold, Fuggle and Northern Brewer showed instead rather similar anisohydric behaviours, reaching a 0.5-0.55 of NTR at about 0.3-0.4 of FTSW, while at the

same FTSW percentage, cultivars that seems to have an 'intermediate' behaviour (as Nugget, Hersbrucker Spät, Mittelfrüh and Challenger) still have a NTR=0.8.

Summarizing, looking at the transpiration rate of different hop varieties in respect to transpirable soil water fraction, big differences were highlighted. Columbus, Chinook, and Cascade resulted to be the most drought-sensible varieties, showing a fast decrease in NRT already at high levels of FTSW (e.g. mild drought stress). Fuggle, Magnum, Northern Brewer and Brewers Gold cv. also showed a higher decrease in transpiration in comparison to the more tolerant varieties, but at higher levels of drought stress respect to Columbus, Chinook, and Cascade. Finally, the most tolerant varieties for what concerns NRT were Challenger, Hersbrucker Spat, Hallertau Mittelfrüh and Nugget. Therefore, it would be preferable to grow hop cultivars characterized by a higher resistant to drought stress since the selection of plants that express a more anisohydric behaviour would confer an advantage to hop growers in areas subjected to short periods of drought. On the other hand an isohydric behaviour could induce plants to save water even if associated with a reduction in yield.

2.3.2. Effect of drought stress on plant growth parameters

Internodes growth (**Figure 8**) and SPAD (**Figure 9**) values in control and drought-stressed plants are reported for each hop variety in respect to the number of days of drought stress.

After seven days of drought, big differences can be observed amongst cultivars. Brewers Gold, Chinook, Cascade, Columbus, Northern Brewer and Hersbrucker Spat cultivars resulted to be the most susceptible varieties. Indeed, in these varieties the internodes' growth of stressed plants resulted to be from two to three times lower than that of well-watered plants. Interestingly, in most cases the varieties that have a fast decrease of the growth of their internodes show also a fast decrease in transpiration during soil drying (see **Figure 7**). Internode length is already been suggested as one of the most drought sensitive factor of maize (NeSmith and Ritchie, 1992), soybean (Desclaux et al., 2000), lemon balm (Farahani et al., 2009) and rice (Todaka et al., 2012) and this is the first report for hop which, as described in the introduction, represents a particular case since it is an annual climbing plant with long stems. A further confirmation was given by the analysis of SPAD values, used to estimate chlorophyll content, while leaves axes' growth did not show significant differences between control and drought-stressed plants (data not showed). **Figure 9** shows indeed how SPAD

values generally decrease with increasing drought stress. It is well known that during drought stress, the earliest response is the accumulation of Abscissic Acid that led to stomatal closure. This mechanism is rapidly activated to prevent water loss and this cause a decrease in CO2 absorption, a decline in photosynthesis and an accumulation of Reactive Oxygen Species (ROS) that induced the programmed cell death (Apel and Hirt, 2004; Asada, 2006; Sun et al., 2013). The decrease of photosynthesis rate in hop water stressed plants was already been highlighted by Hejnák et al. (2015). In our experiment, the difference between control and stressed plants can be generally observed already between three and seven days after the starting of the experiment. Chinook, Cascade, Columbus and Northern Brewer were again the more susceptible cultivars, while Challenger, Fuggle, Magnum and Mittelfrüh showed no significant differences between control and stressed plants. It can be summarized that internodes' length growth and SPAD values show in general a different behaviour between control and stressed plants. Furthermore, the variation of these parameters is higher in cultivars that show a fast decrease of transpiration rate also at low levels of water stress. Internodes' length growth and SPAD values can therefore be used for the evaluation of hop cultivars in respect to the impact of water deficit on their transpiration.

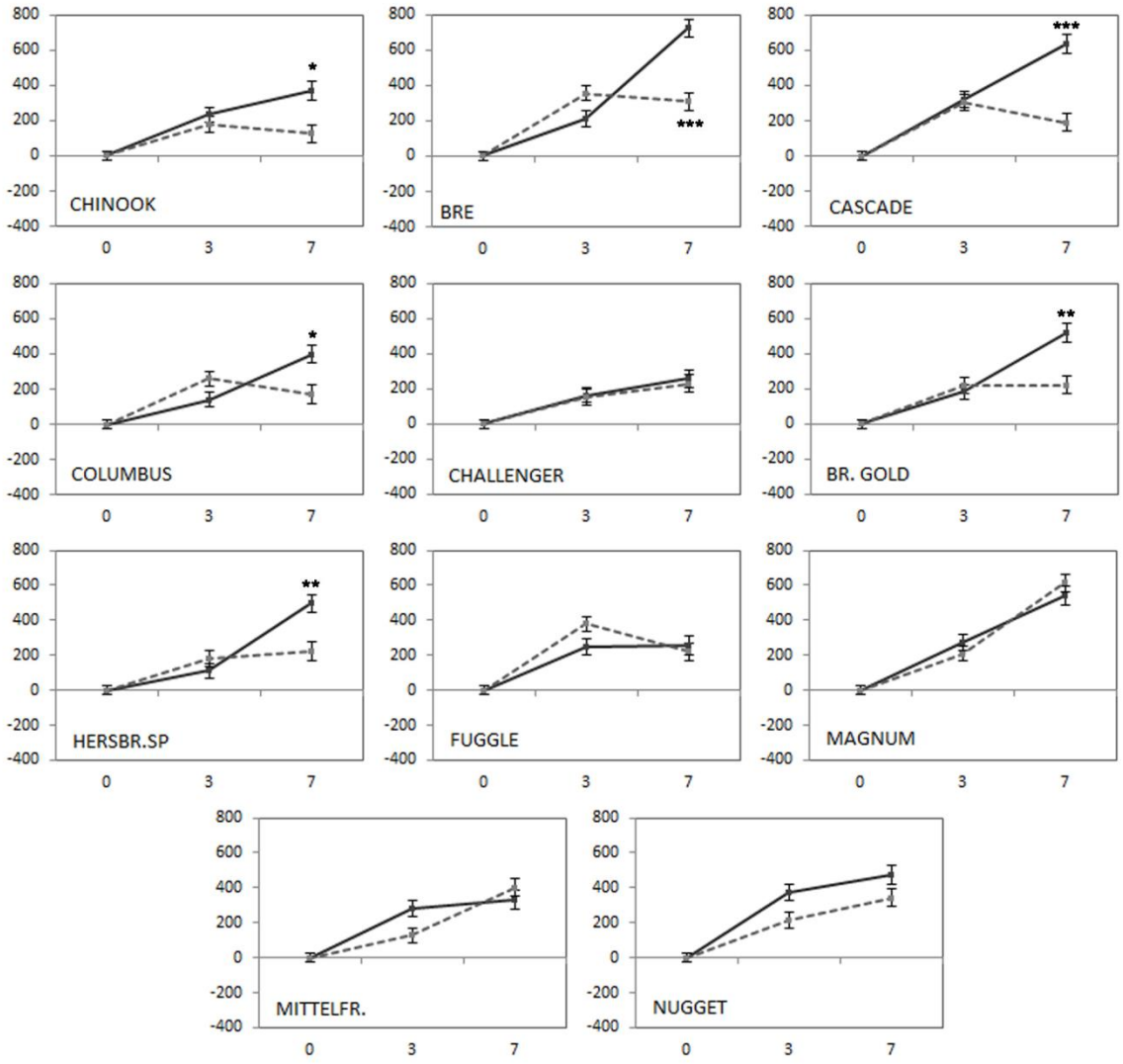


Figure 8 – Internodes length growth (difference between one time point and the previous one) during stress in well-watered (*solid line*) and stressed plants (*dashed line*) in different hop cultivars. Vertical lines show standard error and significant differences are indicated with asterisk (t test; *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$).

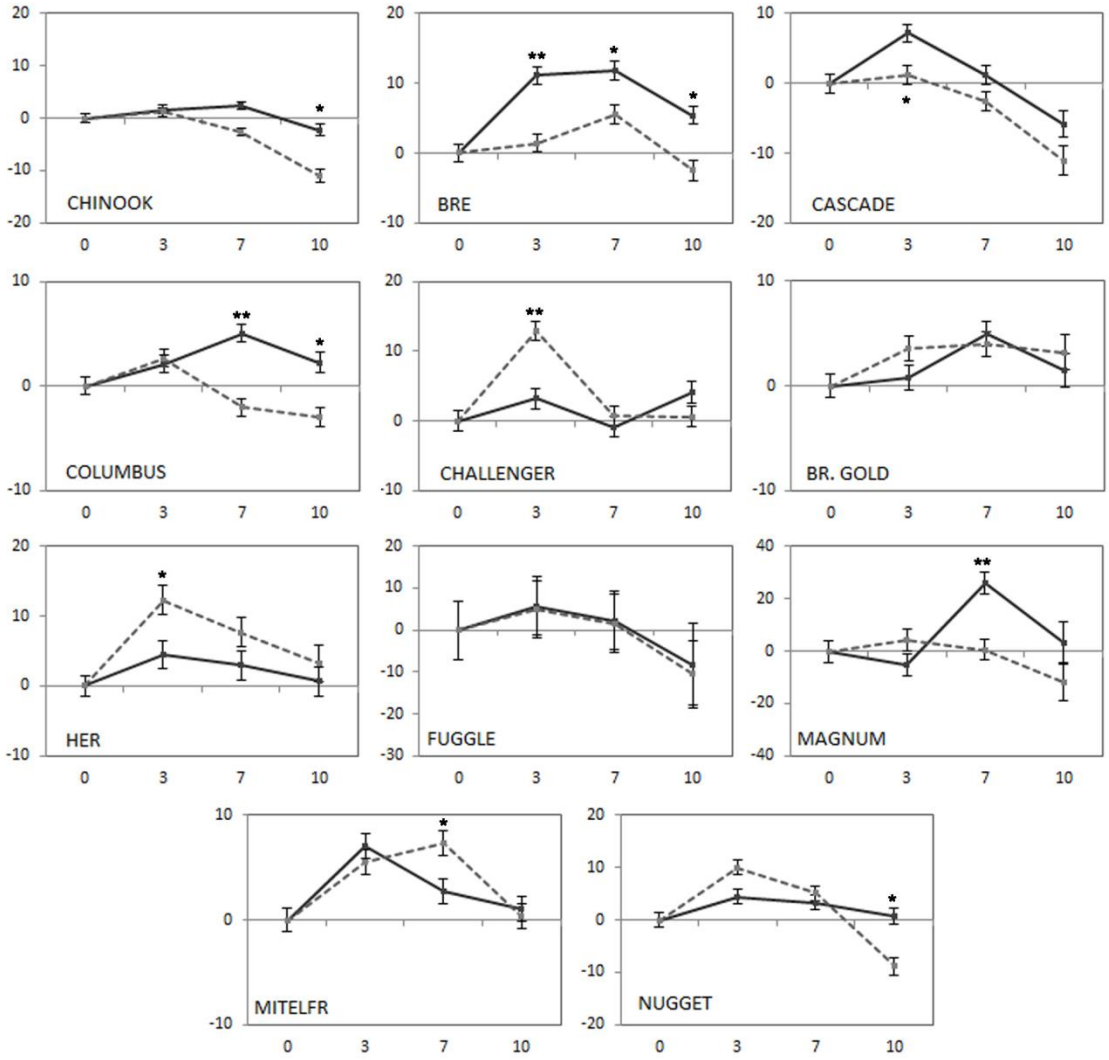


Figure 9 – SPAD growth percentage (difference between one time point and the previous one) during stress in well-watered (*solid line*) and stressed plants (*dashed line*) in different hop cultivars. Vertical lines show standard error and significant differences are indicated with asterisk (*= $p<0.05$; **= $p<0.01$).

2.4. CONCLUSION

The response to drought stress of eleven hop cultivars was outlined in this study. The behaviour of different cultivars in terms of relative transpiration as a function of transpirable soil water content was discussed and growth parameters were measured. Big differences were highlighted on hop plants transpiration amongst cultivars upon water stress. Generally this behaviour can be described by Chapman-Richards equation, where hop plants experience little reduction in transpiration, until the fraction of transpirable soil water (FTSW) equals approximately 70%. Even though most cultivars show a similar behaviour, Columbus, Chinook and Cascade were clearly the most drought-sensible cultivars, showing a fast decrease of transpiration also at high levels of FTSW, while Mittelfrüh, Challenger, Nugget and Hersbrucker Spät were the more drought-tolerant cultivars. Columbus, Chinook and Cascade cultivars showed also a fast decrease in internodes' growth. Indeed, after seven days of drought stress, the internodes' growth of stressed plants was from two to three times lower than that of well-watered plants. As internodes, also SPAD values (positively correlated with chlorophyll content) quickly decrease during soil drying. In our experiment, the difference between control and stressed plants can be generally observed already between three and seven days after the starting of the experiment, and once again the most important differences were highlight for almost all the drought-sensible cultivars.

Summarizing, a high number of hops were subjected to drought stress and the response of transpiration, plant growth and chlorophyll to soil drying was outlined, allowing us to pinpoint the most sensible and tolerant hop cultivars. Although water scarcity would affect hop cultivation, cultivar selection can therefore potentially respond to the physiological impacts of climate change and, beside the selection of hops with specific chemical and sensory characteristics, their response to drought stress should be taken into consideration. Aiming at the introduction of hop cultivation in Italy, it is therefore necessary to choose an adequate irrigation system depending on the cultivar. In this light, drought-sensitive cultivars (Cascade, Chinook or Columbus) showed a diminished growth than other cultivars and resulted able to save water at earlier stages of drought showing a faster reaction to stress.

3. The variability of hop acids and key aroma compounds of different *Humulus lupulus* genotypes cultivated in Italy

3.1. INTRODUCTION

Hop has a determining impact on the aroma properties of beer, although it represent only a minor ingredient in beer brewing. In last years, the number of Italian micro-breweries considerably increased and most of them are located in Northern-Italy. Despite this, hop cultivation is nowadays not widespread in Italy. For these reasons it would be interesting to determine the achievable quality of hops cultivated in Northern-Italy and its variability amongst different cultivation years. Indeed, researchers try to characterize hop varieties also on the basis of the presence and amount of key oil components, but the analytical method has to be reliable and reproducible. The climatic pattern is generally considered as an important factor that affect hop resins biosynthesis and an important factor influencing hop yield (Mozny et al., 2009). Despite this, few papers focused on the variation of the chemical composition of hops in respect to the cultivation year (Kralj et al., 1991; Hanke et al., 2008). In most cases authors were focused on the effect of climatological conditions on the formation of α-acids and β-acids (De Keukeleire et al., 2003; Krofta et al., 2007; Kučera and Krofta, 2010). To dissect qualitative and quantitative differences among commercial hops and to shed light into the effect of the growing season on those parameters, sixteen varieties cultivated in the same experimental field in 2013 and 2014 were used for the analyses. Hop cones were harvested at commercial ripening and subjected to chemical (hop acids and volatile components) evaluation. To fully characterize the selected hops, cones coming from 2013 cultivation year were also sensory analysed. Volatiles were extracted from dried hop cones by ultrasonic extraction and the resulting extracts were added to a *Blond Ale* beer and then sensory analysed by a trained panel using Quantitative Descriptive Analysis (QDA) in order to investigate the relationship between sensory and chemical characteristics.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and reagents

Bitter acids content was determined using the international calibration extract ICE-3 (Labor Veritas Co., Zürich, Switzerland). Volatile compounds were determined using reference compounds from Sigma-Aldrich (Milan, Italy). The reference compounds were of analytical grades: α-humulene (≥96 %), α-pinene (≥98 %), α-terpinene (≥95%), α-terpineol (≥90 %), β-caryophyllene (≥98.5 %), β-myrcene (≥95 %), β-ocimene (≥90 %), β-pinene (≥99 %), α,β-thujone (≥80 %), 2 undecanone (≥99.0%), 2-methylbutyl isobutyrate (≥98.0%), camphor (≥97 %), carvacrol (≥98 %), caryophyllene oxide (≥99 %), ρ-cymene (≥99.5 %), eugenol (≥98 %), geraniol (≥99 %), limonene (≥99 %), linalool (≥99 %), linalyl acetate (≥97 %), methyl nonanoate (≥99.8 %), nerol (≥97 %), nonanal (≥95.0%), γ-terpinene (≥98.5 %), trans-β-farnesene (≥90 %). N-hexane was obtained from CarloErba Reagents (Milan, Italy).

3.2.2. Hop samples

Sixteen commercial hop varieties (Brewers Gold, Cascade, Centennial, Challenger, Chinook, Columbus, Fuggle, Magnum, Hallertau Mittelfrüh, Hersbrucker Spät, Mount Hood, North Down, Northern Brewer, Sterling, Willamette and Yeoman) were cultivated in Sissa, Parma, Italy (44°57'N; 10°15'E; 32m above sea level; loam soil) in 2013 and 2014. From 8 to 10 years old plants were used for the experiment. Cattle farmyard manure was used to fertilize soil in April and July (10 tons/hectare each time) whereas only so-called Bordeaux pap was applied at regular time intervals as a natural fungicide (usually every 2 weeks from the beginning of May until July). The varieties were grown in the same experimental field, allowing us to compare the secondary metabolites concentration irrespective of field characteristics (as soil composition, longitude and latitude, altitude and exposure) and agricultural practices. Hop cones were collected at optimal ripeness (Briggs et al., 2004), from August 25th till September 15th in function of the earliness of the cultivar. Then, hop cones were air-dried at room temperature, pressed, vacuum-packed and stored at -18°C. It has been decide to study aroma hops (8 varieties), dual purpose hops (5 varieties) and bittering hops (3 varieties). The selected varieties also differ by origin: Germany (3 varieties), UK (6 varieties) and US (7 varieties) (see Chapter 1.5).

3.2.3. Analysis of hop bitter acids

Hop resins were analysed by HPLC according to EBC 7.7 method. The hop cones were ground and 2 g of ground sample were placed into a 50 mL flask and methanol (4 mL), diethyl ether (20 mL) and 0.1M HCL (8 mL) were added. The mixture was intensively shaken for 40 min and after phase separation, 1 mL of the supernatant phase was collected and added to 10 mL of methanol, stirred, filtered using a 0.45 µm micro-filter and injected into the HPLC system. The hop acids were separated and quantified using HPLC (model X-LC, Jasco, Japan). The liquid chromatography apparatus consisted of a MD-2015 diode array detector, an AS-2055 Autosampler, and Chrom-NAV chromatography software was used for chromatographic data analysis. The separation of compounds was obtained on a Tracer Extrasil ODS2 (5 mm, 250 mm, Teknokroma, Spain) operating at 40°C. The mobile phase consisted of a mixture of methanol/water/phosphoric acid (775:210:9 v/v/v) and the flow rate was 1 mL min^{-1}. The wavelength of 314 nm was used for the detection. Compounds were identified in the basis of the international calibration extract ICE-3 and the content of hop acids was expressed as percentage (w/w d.w.).

3.2.4. Volatile molecules analysis

Hop cones were soaked in n-hexane (8:1 [v/w], hexane to plant material) containing toluene as internal standard and the extracts were stored at –4 °C for 3 months (Wang et al., 2008). After filtration, the clear hexane extract of different hop cultivars was transferred into vials and volatile components were separated by gas chromatography (GCXGC-FID). Gas chromatographic analysis were performed in triplicate by injecting 1.4 µL of sample in the split mode (split ratio = 1:200) into a Agilent 7890A gas chromatograph (Agilent Technologies) equipped with an Agilent G3486A CFT Modulator, (Wilmington, DE, USA) and a flame ionization detector. Molecules were separated by non-polar and polar columns; the polar column was an Agilent 19091N-113 HP-INNOWax (5 m X 250 µm, 0.15 µm). while the non-polar column was a Varian CP5860 CP-SIL 8 CB LOW BLEED/MS (30 m X 250 µm, film thickness 0.25 µm). The carrier gas was hydrogen at a flow rate of 0.6 ml min^{-1} for the first column and 25 ml min^{-1} for the second column. The analysis was performed using the following temperature program: oven temps isotherm at 60 °C for 1 min, from 60 to 250 °C at the rate of 4 °C min^{-1} and isotherm at 250 °C during 4 min. The Split-Splitless injector

and the FID were kept at 250 °C. The analysis was done in three replicates for each sample and the peak volume and the relative percentage of the volatile molecules based on the total area of each sample were determined. Data were evaluated using GC Image software version 2.2b0 (Zoex, Houston) and molecules were identified by authentic reference compounds. As an example, **Figure 10** shows the GCXGC chromatogram of the hop hexane extract cv. Columbus.

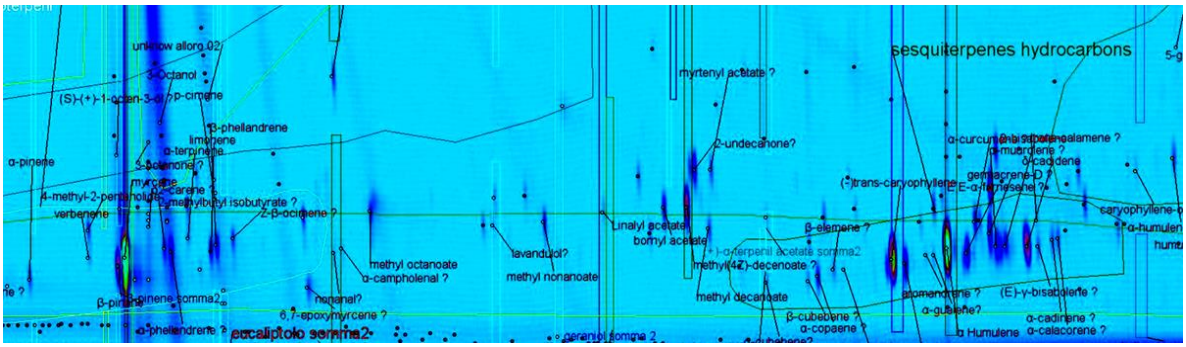


Figure 10 – GCXGC-FID profile of hop hexane extract cv. Columbus

3.2.5. Beer brewing

A *Blonde ale* beer (4.9 % v/v alcohol) was used as 'base beer' for the sensory analyses of ten hop varieties cultivated in 2013. Pilot-scale fermentation was carried out in a stainless steel fermenter (Braumaister, Speidel). Tap water (pH 7.4) was used for wort production, and pH was decreased at 5.3-5.5 and continuously controlled. Cooling of wort was performed using tap water. During mashing, wort was continuously mixed. Pilsner and Vienna malts (9 kg and 3 kg respectively) were used (Ireks, Kulmbach, Germany). Brew ground malt was mashed with 50 L of brewing water. The temperature was raised to 49°C and held constant for 20 min, and then the mixture was warmed at 65°C for 40 min and at 78°C for 10 min. Then, spent grains were separated from the sweet-wort and sparged with hot water (78°C) and the boiling of the sweet-wort took 90 min. Hops were added at the beginning of the boiling process (15 g of Perle and 20 g of Saaz hops for at least 60 min. and 50 g of Saaz hop for 30 min., to reach a total IBU-International Bittering Units of 18). About 10% of the total volume evaporated during boiling. After boiling wort was transferred into a disinfected stainless steel vessel, the clarified wort was cooled with tap water to 15°C, vigorously shaken, added with 22 g of *Saccharomyces cerevisiae* (Safale US-05, Fermentis) suspended in 220 ml of sterile

water and oxygenated with sterilized oxygen. The fermentation vessel was closed and kept at 21°C. After six days lees was removed and after seven days 5 g l^{-1} of sucrose were added to beer for the beer re-fermentation. Then, beer was transferred in 750 ml bottles and stored at 20°C for 15 days and at 4°C for two months. Cascade, Challenger, Chinook, Columbus, Fuggle, Hallertau Mittelfrüh, Hersbrucker Spät, Mount Hood, Sterling and Willamette hops were selected for sensory evaluation. The selection of these varieties was mainly made on the base of their use in brewing as 'aroma' hops. Hop hydro-alcoholic extracts were obtained by Ultrasonic-assisted Solvent Extraction performed in an ultrasonic bath (E0746, Albrigi Luigi), with input power 150 W, internal dimension; 240 mm x 300 mm x 770 mm, by the mode of direct sonication at the frequency of 25 KHz. 30 g of dried cones were added to 450 ml of a hydro-alcoholic solution (90/10 ethanol/water v/v) and sonication was held for 45 minutes at room temperature (Alissandrakis et al., 2003), (Cabredo-Pinillos et al., 2006). Then the extract was filtered and 15 ml l^{-1} of each hop extract were added to the base beer and sensory testing took place within 3 h.

3.2.6. Sensory Analysis of pilot reference beer aromatized with different hop extracts

For sensory evaluation, hop extracts were added to a pilot beer (see Chapter 3.2.5). It has been decided to use a Quantitative Descriptive Analysis (QDA). Sensory tests on hopped beers were carried out using a trained panel of 11 members. Panelists were trained to detect and identify specific flavours using a set of wine aromas (Pulltex SL, Barcelona, Spain) and to evaluate the intensity of typical beer descriptors (bitterness, astringency, sweetness, alcohol, hop, malt). At the time of the experiment panellists had already received 20 h of training. A common vocabulary was settled out based on 11 descriptors; hoppy, grassy, balsamic, fruity, fresh fruit, stewed fruit, citrusy, floral, spicy, biological/chemical and odour intensity. Each descriptor was evaluated by the panellists using a ten-point scale ranging from 0 (not perceptible) to 9 (very high intensity). Beers were served in TEKU glasses (version 2.0), encoded with random codes and served at 12°C. Four beers were randomly presented in each session to the panellists: the external control (the *Blonde Ale* beer without hop extracts addition) and three hopped beers. Each panellist was provided with mineral water and unsalted crackers as palate cleaner between samples. To calibrate the panel, in each sensory

session the external control was presented as first sample, sensory analysed and then the median scores reached for each descriptor by this beer were calculated and used by the panelists as reference before tasting other samples.

3.2.7. Statistical analysis

One-way ANOVA (Tukey HSD, $p< 0.05$) was conducted using Statgraphics Centurion software XVI version 16.2.04 (Statpoint Technologies, USA). Pearson correlation, Principal Component Analysis (PCA) and t test were carried out by using 'cor', 'prcomp' (scale = TRUE) and 't.test' functions of R programming language (version 3.2.2.; https://www.r-project.org), respectively. Visualization of t test by heat map was achieved using ggplot2 R package.

3.3. RESULTS

3.3.1. Climatological data

Seasonal fluctuations in hop acids level are associated with variations in temperature and rainfalls from the end of May to the end of August, when flowering, cone forming and ripening occur (Krofta et al., 2007). For this reason, climatological data were collected in Zibello (Parma, Italy) and meteorological parameters registered from June to August in 2013 and 2014 are reported in **Table 6**.

Overall, the climatological conditions of the harvest seasons chosen for this study were substantially different, given that June and July were hot and dry in 2013 and rainy in 2014, while August was rainy in 2013 and dry in 2014.

Table 6 – Climatological Conditions during the months of June, July and August 2013 and 2014 measured in Zibello, Parma, Italy (Source: Regional Environmental Protection Agency-ARPA-Emilia Romagna)

	June		July		August	
	2013	2014	2013	2014	2013	2014
min Relative Humidity (%)	31.13	32.40	30.87	44.42	32.68	44.87
max Relative Humidity (%)	88.93	89.37	88.97	93.58	92.45	92.48
min T (°C)	15.22	17.28	18.45	17.85	16.78	18.02
max T (°C)	29.04	29.94	32.81	29.17	31.31	28.82
precipitation (mm)	14.6	36.8	8.2	30.6	40	9.6
mean T (°C)	22.13	23.61	25.63	23.51	24.04	23.42

3.3.2. Qualitative and quantitative analysis of hop acids

A first step in chemical analysis was to investigate about α- (**Figure 11A**) and β- (**Figure 11B**) acids content amongst hop varieties.

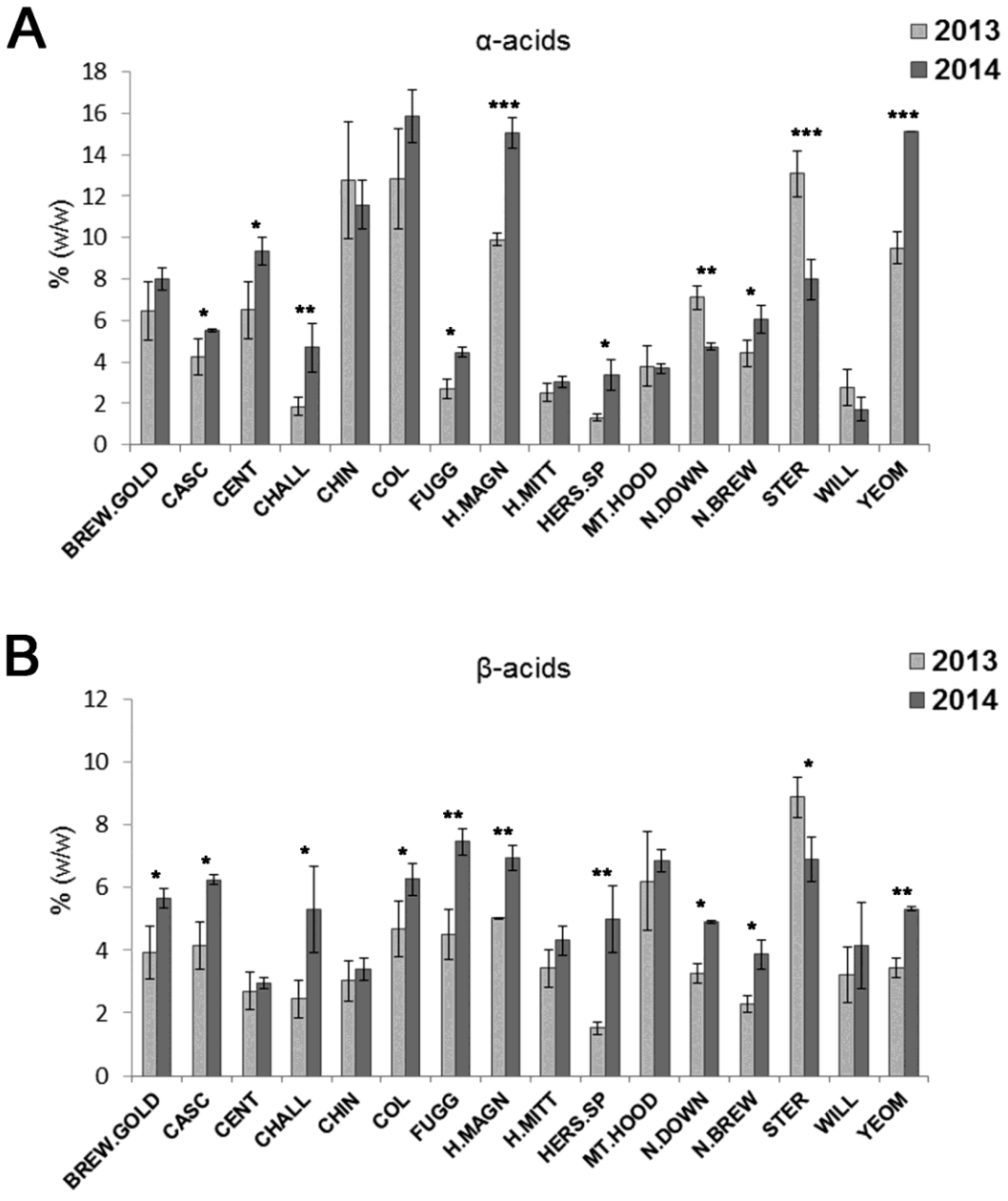


Figure 11 – α-acids **(A)** and β-acids **(B)** concentration, measured in two different cultivation years (2013 and 2014) in different hop cultivars: cv. Brewers Gold (BREWGOLD), Cascade

(CASC), Centennial (CENT), Challenger (CHALL), Chinook (CHIN), Columbus (COL), Fuggle (FUGG), Magnum (H.MAGN), Hallertau Mittelfrüh (H.MITT), Hersbrucker Spät (HERS.SP), Mount Hood (MT.HOOD), North Down (N.DOWN), Northern Brewer (N.BREW), Sterling (STER), Willamette (WILL) and Yeoman (YEOM). Values are expressed as w/w d.w., vertical lines show standard error and significant differences between 2013 and 2014 are indicated with asterisks (t test; *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$).

The total α-acids content varied from 15% in Hallertau Magnum and Yeoman to 1.3% of Hersbrucker Spät, while the β-acids content varied from 8.90% in Sterlyng to 1.50% in Hersbrucker Spät. The concentrations of α- and β-acids largely differ among growing seasons, with most varieties having higher values in 2014 compared to 2013. Nevertheless, among the sixteen varieties, the hop acids content of Chinook, Columbus, Hallertau Mittelfrüh, Mount Hood and Willamette did not showed significant differences between cultivation years, and only Sterlyng was found to be richer in α- and β-acids in 2013 than in 2014.
Cohumulone, adhumulone + humulene, colupulone and adlupulone + lupulone contents were also determined (see **Supplementary Table S3**) and the comparison between α- and β-acid concentrations of our samples and concentrations reported by the literature for the selected varieties is reported in **Table 7**. The α-acids levels of hops cultivates in Parma and their α/β-acids ratio were generally similar to those reported by the literature. Indeed, Why Challenger, Mount Hood, Northern Brewer and Willamette cultivars were the only cultivars that expressed lower levels of α-acids then those ones reported in literature.

Table 7 – α-acids contents and α/β-acids ratio of selected hops measured in 2013 and 2014 compared to levels found in literature *(Barth-Haas, 2015), **(Briggs et al., 2004)

	α-acids (w/w d.w)			α/β ratio		
Variety	2013	2014	literature range	2013	2014	literature range
BREW.GOLD	6.44	8.01	4.5 - 6.5 *	1.63	1.42	1.9 *
CASC	4.23	5.50	4.5 - 7.0 **	1.02	0.88	1.0 **
CENT	6.50	9.34	9.5 - 11.5 *	2.40	3.18	2.65 *
CHALL	1.85	4.70	6.5 - 8.5 **	0.76	0.89	1.8 **
CHIN	12.75	11.58	12.0 - 14.0 **	4.21	3.42	3.9 **
COL	12.82	15.86	15.0 – 17.0 **	2.74	2.53	3.1 **
FUGG	2.69	4.46	3.0 - 5.6 **	0.59	0.60	1.5 - 2.2 **
H.MAGN	9.90	15.03	11.0 - 16.0 **	1.97	2.16	2.6 **
H.MITT	2.52	3.04	3.0 - 5.5 **	0.73	0.71	1.0 **
HERS.SP	1.30	3.39	2.5 - 5.5 **	0.85	0.68	0.9 **
MT.HOOD	3.80	3.69	4.0 - 7.0 *	0.61	0.54	1.1 *
N.DOWN	7.11	4.75	7.0 – 10.0 **	2.17	0.97	1.5 - 2.2 **
N.BREW	4.42	6.06	8.0 - 10.0 **	1.93	1.57	2.0 **
STER	13.09	7.99	6.0 - 9.0 **	1.47	1.16	1.5 **
WILL	2.77	1.72	4.0 - 6.0 *	0.86	0.41	1.6 *
YEOM	9.50	15.10	12.0 - 16.0 *	2.76	2.84	3.1 *

3.3.3. Identification of hop key volatile molecules and their variability between 2013 and 2014

The volatile profile of the selected hop varieties expressed in %/total volume for the cultivation years 2013 and 2014 is reported in **Supplementary Table S4**. A total of 29 major volatile compounds were detected, among which 18 monoterpenes and monoterpenoids, 5 esters, 4 sesquiterpenes, 1 ketone and 1 aldehyde were identified. As expected, high levels were observed in all varieties for the monoterpene β-myrcene and the sesquiterpenes α-humulene and β-caryophyllene. Display data in percentage in respect to the total volume of volatiles is surely useful to compare the volatile profiles of hop varieties. Despite this, showing data as molecules' chromatographic peak volumes (**Supplementary Table S5**) allow us to determine the amount of each component irrespective of the total amount of hop oil produced by each singular variety and help us to establish connections between volatile molecules concentration and sensory parameters.

To investigate the variability of the volatile profile of hops amongst cultivation years, a t test was performed for each molecule and variety comparing 2013 and 2014 values (**Figure 12**).

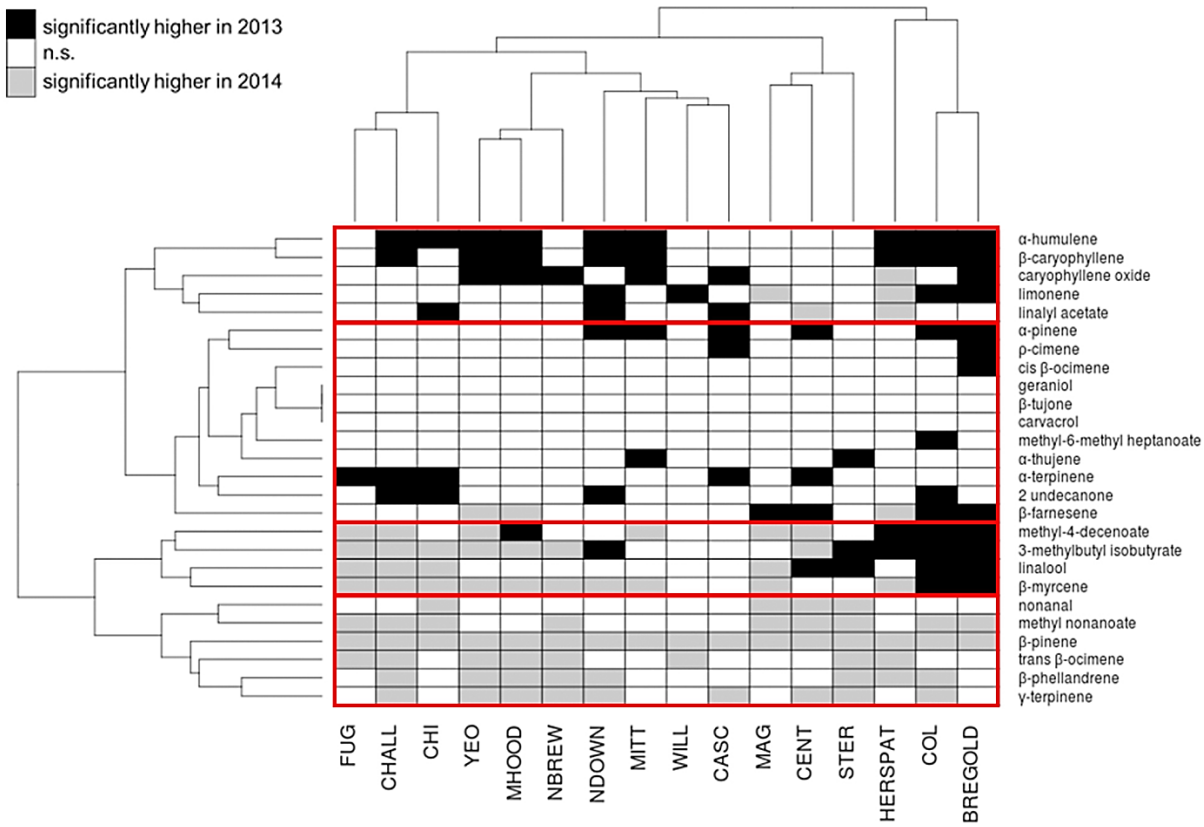


Figure 12 – Dendrogram and heat map obtained by ggplot2 R package. The variation of volatile molecules concentration among 2013 and 2014 is graphically represented for different hops cv. Brewers Gold (BREGOLD), Cascade (CASC), Centennial (CENT), Challenger (CHALL), Chinook (CHI), Columbus (COL), Fuggle (FUG), Magnum (MAG), Hallertau Mittelfrüh (MITT), Hersbrucker Spät (HERSPAT), Mount Hood (MHOOD), North Down (NDOWN), Northern Brewer (NBREW), Sterling (STER), Willamette (WILL) and Yeoman (YEO). Black boxes indicate a significantly higher concentration in 2013 cultivation year (t test; $p<0.05$), grey boxes indicate a significantly higher concentration in 2014 cultivation year (t test; $p<0.05$) and white boxes indicate no significant differences. Variables are in accordance with **Supplementary Table S5**.

Depending on the variety and the volatile compound, the behaviour of volatile components amongst different cultivation years was different. Columbus and Brewers Gold resulted to be the varieties with the most variable volatile profile, where more than the 50% of molecules significantly vary from one year to another, showing generally a higher content in 2013. Also Hersbrucker Spät, Challenger and Yeoman volatile profiles were particularly variable, but showed higher concentrations of volatiles in 2014. For almost all the varieties about 30% of molecules was subjected to a significant change, while Willamette and Cascade seems to be the most stable varieties. Focusing on the variability amongst different cultivation years in dependence of volatile molecules four groups can be distinguished. The first one (**Fig. 12, group A**) is characterized by highly-variable volatiles and consist mainly on sesquiterpenes (β-caryophyllene and α-humulene) that are generally more concentrated in 2013. These sesquiterpenes were highly correlated ($r=0.908$; $P<0.001$) among varieties and cultivation year and were positively correlated also with the oxygenated sesquiterpene caryophyllene oxide ($r=0.694$; $P<0.05$ and $r=0.766$; $P<0.01$). The other three groups are composed mainly by monoterpenes and esters; the first one (**Fig. 12, group B**) comprise stable compounds between cultivation years, while the second and the third (**Fig. 12, group C and group D**) comprise highly-variable molecules, generally more concentrated in 2014. Some important hop volatiles belong to these last two groups (**C and D**), such as trans-β-ocimene and linalool. Also the monoterpenes β-pinene and β-myrcene resulted to be particularly variable, showing significantly higher concentrations in 2014 for almost all the selected cultivars.

Looking at the volatile profiles of different hop cultivars (see **Supplementary Table S5**), in both cultivation years, the content of β-myrcene was high in almost all bittering or dual-purpose varieties, while was low in Hersbrucker Spät, Willamette and Challenger. As β-myrcene, α- and β-pinene were also found to be high in almost all high α-acids varieties. Trans β-ocimene is another monoterpene that was detected in high concentrations only in Columbus and Sterling for both cultivation years and could be a possible varietal marker for these varieties. High concentrations of β-farnesene were observed in Willamette and Cascade varieties in both cultivation years and was found to be present in quite high concentrations also in Mt. Hood, North Down, and Sterlyng and in low concentration in most of the high α-acid cultivars. The monoterpenes geraniol, limonene and linalool were present in high concentrations in Brewers Gold, Centennial, Columbus and Chinook. Limonene was present

in high levels also in Magnum, North Down, Northern Brewer and Sterling, and linalool in Fuggle, North Down, Hersbrucker Spät and Mittelfrüh. As for some monoterpenes, in our study high amounts of the esters methyl-4-decenoate and 3-methyl butyl isobutyrate were found in almost all high α-acid cultivars for both cultivation years. β-caryophyllene and α-humulene were generally present in high concentrations in Magnum, Columbus and Willamette and in low concentrations in Cascade, Hersbrucker Spät and Challenger while another sesquiterpene, caryophyllene oxide, was found to be high in Columbus, Fuggle, Sterling and Willamette. Finally, eugenol was previously found only in Hallertau Hersbrucker cv. (Eyres et al., 2007), but in our study was found only in Columbus hops.

To establish if hop oil composition is sufficiently constant to serve as a reliable basis for a system of varietal identification, the data matrix of volatile molecules content was pre-processed by auto-scaling (scale = T) and subjected to Principal Component Analysis (PCA) for 2013 (**Fig. 13A**) and 2014 (**Fig. 13B**) separately.

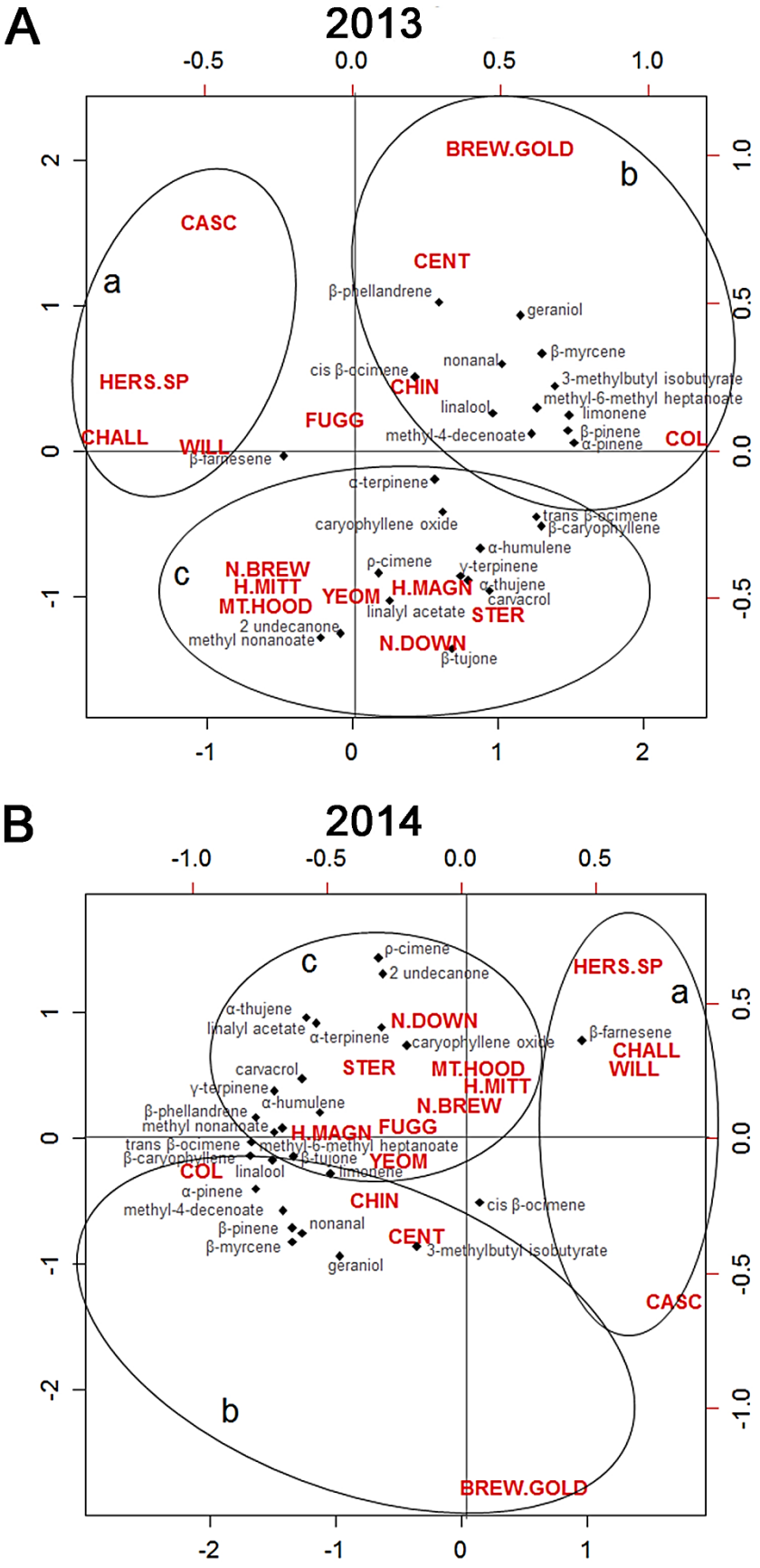

A
2013
B
2014
a
b
c
BREW.GOLD
CASC
CENT
HERS.SP
CHALL
WILL
FUGG
CHIN
COL
N.BREW
H.MITT
MT.HOOD
YEOM
H.MAGN
STER
N.DOWN
β-phellandrene
geraniol
β-myrcene
nonanal
cis β-ocimene
3-methylbutyl isobutyrate
linalool
methyl-6-methyl heptanoate
limonene
methyl-4-decenoate
β-pinene
α-pinene
β-farnesene
α-terpinene
caryophyllene oxide
trans β-ocimene
β-caryophyllene
α-humulene
ρ-cimene
γ-terpinene
α-thujene
carvacrol
linalyl acetate
2 undecanone
methyl nonanoate
β-tujone

Figure 13 – Biplots obtained by PCA of volatiles detected in the selected hop varieties for 2013 and 2014 cultivation year (resp. graph **A** and **B**) (scaled data). Hop varieties are represented as scores, and volatiles determined by GCXGC-FID as loadings. Variables are in accordance with **Supplementary Table S5**.

The first two principal components explain 62% (PC1= 44%, PC2= 18%) and 59% (PC1= 46%, PC2= 13%) of total variance in 2013 and 2014, respectively. Interestingly, the two PCA plots performed on 2013 and 2014 data matrix highlight similar molecules and varieties distribution. Indeed, in both years, Cascade, Willamette, Challenger and Hersbrucker Spät were the varieties with lowest contents of volatiles (**Fig. 13, cluster a**). Cascade and Willamette resulted to be characterized only by the presence of β-farnesene, in both cultivation years. Columbus, Chinook, Centennial and Brewers Gold were instead characterized by a higher content of specific monoterpenes as β-myrcene, limonene and α- and β-pinene, by specific esters as methyl-4-decenoate and methyl-6-methyl isobutyrate and by geraniol and linalool, two important odour-active molecules (**Fig. 13, cluster b**). Magnum, Sterlyng and North Down were instead characterized by high contents of sesquiterpenes and oxygenated sesquiterpenes as β-caryophyllene, α-humulene and caryophyllene oxide, and by p-cymene, carvacrol and 2-undecanone (**Fig. 13, cluster c**). The PCA plots show also that Northern Brewer, Mittelfrüh and Mount Hood resulted in very similar volatile profiles in both years.

3.3.4. The sensory profiles of beer flavoured with different hop varieties and correlations between sensory and chemical characters

To characterize the selected hops also from a sensory point of view, in 2013 the volatile components of ten hop varieties were extracted via ultrasonic solvent extraction and used to flavour a *Blond Ale* beer. Although trace amounts of the volatile constituents of malt and hop may survive after wort boiling, the major hop oil compounds undergo heavy losses in the boiling kettle and are therefore not detected in the final beer (Siebert, 1994; Kishimoto et al., 2005). For this reason, and because of the base beer used for the sensory test was the same for all the flavoured samples, the potential aroma given by the addition of hops during the initial stages of wort boiling can be considered negligible. Beers flavoured with different hop varieties exhibited significant sensory differences (Tukey HSD; $p<0.05$), especially for grassy, dried grass, fruity, citrusy and spicy sensory descriptors (**Supplementary Table S6**). Beers flavoured with Columbus, Hersbrucker Spät, Fuggle and Chinook reached higher scores for grassy odours compared to the other varieties. On the other hand, the highest scores for fruity odour were observed in Chinook, Cascade and Hersbrucker Spät. Beers flavoured with Chinook and Cascade hops exhibited a citrus odour too, while Hersbrucker Spät and Fuggle hops were characterised by a pronounced spicy odour.

A PCA was performed on the sensory data matrix to better understand the impact of each sensory impression on the sensory profile of the selected hops. PCA results (PC1 versus PC2) of sensory data are showed in **Figure 14**.

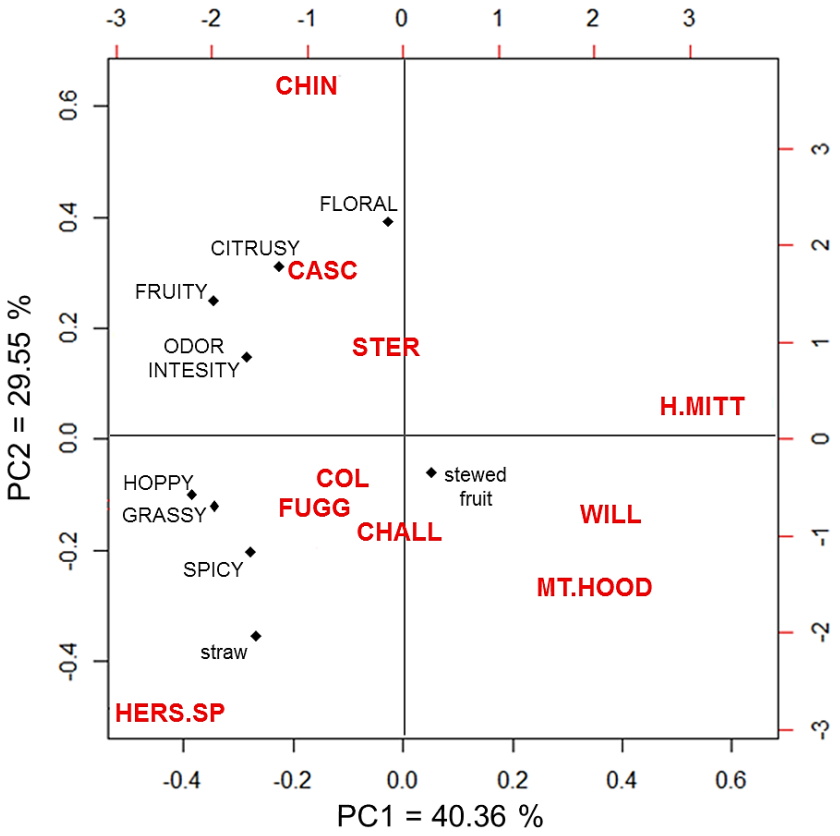


Figure 14 – Biplot obtained by principal component analysis of sensory descriptors' scores in beers flavoured with different hop cultivars. Hop varieties are represented as scores and sensory descriptors as loadings. Variables are in accordance with **Supplementary Table S6**.

PC1 accounted for the 40.36 % and PC2 for 29.55 % of variance. Three groups can be clearly distinguished in the plot, to the first one belong Columbus, Fuggle, Challenger and Hersbrucker Spät that were mainly associated to spicy and green odours, whereas Chinook, Cascade and Sterlyng cluster together and were characterized by floral, fruity and citrusy odours. The third group is composed by Mittelfrüh, Willamette and Mount Hood, which were instead characterized by low scores of almost all the odour descriptors. The presence of a fruity/citrusy scent in Cascade and Chinook varieties was already observed, as well as the herbal and spicy flavour with a hint of floral and lemon/pineapple' for Sterling (Hopunion, 2015). Columbus, Hersbrucker Spät, Challenger and Fuggle have instead been described mainly as 'earthy', 'spicy', 'herbal', 'woody' or 'earthy' (Hopunion, 2015) and the odour of

Willamette, Mount Hood and Mittelfrüh is often described as ‘mild’, ‘slightly spicy and floral’(Hopunion, 2015).

Pearson correlations between sensory descriptors, and between sensory descriptors and the volatile profiles of hops (expressed as peak volumes) were also carried out. As expected, the descriptor ‘hoppy’ was highly positively correlated with the descriptors ‘grassy’ ($r=0.914$; $P<0.001$), ‘dried grass’ ($r=0.969$; $P<0.001$) and ‘spicy’ ($r=0.898$; $P<0.001$). Another strong positive correlation was highlighted between the descriptors ‘fruity’ and ‘citrusy’ ($r=0.867$; $P=0.001$) and ‘citrusy’ and ‘floral’ ($r=0.716$; $P<0.019$).

It is noteworthy that hoppy, grassy and spicy descriptors were all positively correlated to p-cymene and carvacrol, that have been mainly described as ‘woody, spicy and herbal’ (The Good Scents Company, 2015) and with caryophyllene oxide. Another positive correlation was highlighted between the sesquiterpene α-humulene and vegetal descriptors, while hoppy, grassy and spicy terms were all negatively correlated with geraniol. β-farnesene was positively correlated with floral odour and could be one of the molecule responsible for the floral odour of Cascade hops. Negative correlations were found between ‘fruity’ odour and β-tujone ($r = -0.726$; $P<0.05$), trans-β-ocymene ($r = -0.688$; $P<0.05$) and methyl-6-methylheptanoate ($r = -0.607$; $P<0.05$), all molecules known for their herbal flavour (The Good Scents Company, 2015). Particularly methyl-6-methylheptanoate was found to be negatively correlated with ‘citrus’ ($r = -0.667$; $P<0.05$) and ‘floral’ ($r = -0.663$; $P<0.05$) odours too. It is remarkable that some important odorants quantified, such as 2-undecanone, were not correlated with any of the odour impressions generated in the flavoured beers.

3.4. DISCUSSION

In last years, the increasing spread of the craft beer markets is paralleled by an increase in hop production and acreage and the diffusion of hop cultivation in other countries than hop typical production regions (see Chapter 1.1.1). As concern the introduction of hop cultivation in Northern-Italy, the achievable quality of different types of hop cultivars (bitter, dual purpose and aroma hops) cultivated in this area and its variability among different cultivation years are two main parameters to take into account. With this in mind, sixteen different hop cultivars were cultivated in Parma (Italy) in 2013 and 2014 and their hop acid concentration, volatile profiles and variability were determined. Indeed, cultivation years considered in this study

were characterized by different climatological conditions (**see Table 6**) and where therefore considered suitable for our purpose.

Nowadays, α-and β-acids are the most widely used quality parameter for hop analytical evaluation, therefore it has been decided to first analyse the hop acids concentration of the selected cultivars and its variability among 2013 and 2014. As a general observation, hop acids levels obtained by hop cones cultivated in Parma, were generally similar to those reported in literature (Barth-Haas 2015, Briggs et al., 2004) for the selected cultivars (**see Table 7**). Despite this, hop acids highlight significant differences between cultivation years, being their concentration higher in 2014 compared to 2013 (**see Figure 11**). This trend could be partially associated by observed differences in temperature trends and rainfall regimes between sampling years. Indeed, 2013 was characterized by high temperature in July and a rainy August, while in 2014 summer was moist and particularly rainy. Our results can be partially explained by Kučera and Krofta (2010), who hypothesize that the intensive rains in August after a water stress period, promote hop growth cones but negatively affect hop resins biosynthesis that undergo a sort of "dilution" of hop acids. Furthermore, De Keukeleire et al. (2007) observed that the abnormally high temperatures occurred during the spring and early summer led to unusually low final levels of hop secondary metabolites throughout Europe. Even though our study was conducted for two years in one single location, the high number of hop varieties evaluated allowed us to determine that, although most of the varieties followed the behaviour previous suggested by other authors, different climatological conditions do not always affect the concentration of hop acids. The concentration of α- and β-acids of some of the selected cultivars was indeed constant between 2013 and 2014.

Nevertheless, while the effect of climatological conditions on α-acids and β-acids formation has been partially explained, the variability of hops' volatile profiles in different cultivation years and on a wide range of hop cultivars has not yet been analysed and is poorly understood. This is the first study that evaluate the volatile profile of different hop cultivars grown in Northern-Italy and their variability between two cultivation years.

Volatile molecules profiles were rather variable amongst cultivation years (**see Figure 12**). Indeed, depending on the cultivar, from 30 to 50% of the molecules showed significant differences between 2013 and 2014, being their concentration lower or higher depending on the cultivation year.

Amongst molecules, it is worth noting that β-caryophyllene and α-humulene showed an almost identical behaviour between cultivation years and varieties, showing higher concentrations in 2013 compared to 2014. This could be due to the fact that the two sesquiterpenes shared the same biosynthetic pathway, indeed *SESQUITERPENE-SYNTHASE1 (HlSTS1)* gene codify for the enzyme that catalyse the reaction that led to both β-caryophyllene and α-humulene biosynthesis from farnesyl pyrophosphate in hop trichomes (Wang et al., 2008). Other important volatiles, mainly monoterpenes, as β-myrcene, β-pinene, trans-β-ocymene, β-phellandrene and γ-terpinene were instead present in higher concentrations in 2014.

Therefore, the behaviour of volatile molecules depends on the cultivar and the volatile compound itself. This could be due to the complexity of hop volatile profiles. Hops essential oils are, indeed, composed by several molecules that belong to a wide range of different chemical classes, each one originated by different biosynthetic pathways which activation could be related to climatological conditions. Indeed, even though it is well known that plants' essential oil composition strongly depends to the genotype, growing and environmental conditions (for example temperature, daylenght, soil, nutrients levels and air moisture) may exert some direct or indirect influence on it (Carrubba and Catalano, 2009). Indeed, biosynthesis of plants' secondary metabolites is accomplished by several biochemical steps, each one characterized by different optimal temperature. Moreover, considering that secondary metabolites synthesis is strongly dependent on plant photosynthetic activity, it may be expected that variation in climatological conditions play a pivotal role in their accumulation (Carrubba and Catalano, 2009). Some terpenic compounds as pinene, ρ-cymene, α-terpinene, linalool and β-phellandrene, seem to be the most sensitive components to environment and grow condition modifications in coriander (Carrubba et al. 2002) and fennel (Carrubba et al. 2005) essential oils.

To shed light on the complexity of hop volatile profile, also in respect to its variability among different cultivation years, it is important to analyse hops' volatile profiles and their variability from a qualitative point of view, analysing the differences between the chemical profiles of different hop varieties. This can, indeed, be useful to identify stable potential selection criteria for the development of new cultivars and for the evaluation and identification of hop varieties. Several papers, indeed, focused on the identification of key

molecules for varietal characterization of hops (Peacock and McCarty, 1992; De Cooman et al., 1998; Perpete et al., 1998; Eri et al., 2000; Lermusieau and Collin, 2001; Shellie et al., 2009). In our study, key molecules for varietal characterization previously highlighted by other authors were confirmed on a higher number of varieties and, most remarkably, other potential markers were determined for the first time.

High α-acid cultivars (i.e. Centennial, Chinook, Columbus, Magnum and Yeoman) were characterized by high concentrations of specific esters and monoterpenes. Methyl-4-decenoate and 3-methyl butyl isobutyrate, resulted to be possible markers to distinguish bitter hops from aromatic cultivars, as proposed by Perpete et al. (1998). High α-acid cultivars were also characterized by high levels of β-myrcene and α- and β-pinene. These molecules were found in almost all high α-acid cultivars for both years, so they can probably be used as markers for bitter hops. The similar behaviour of some of these monoterpene hydrocarbons was previously highlighted by Dresel et al. (2015) that showed a positive correlation between the concentration of β-myrcene and β-pinene. Our study highlighted the tight bond between these two monoterpenes also for what concern their behaviour among different cultivation years, probably due to the fact that these two monoterpenes shared the same biosynthesis pathway (Thomas and Fallis, 1976, Baser and Buchbauer, 2009).

Other molecules were as well identified as possible varietal markers: for example eugenol, that was found to be present only in Columbus cv., in both cultivation years, and trans β-ocimene, that was detected at high concentrations only in Columbus and Sterling cultivars. Also β-farnesene resulted to be an interesting molecule for varietal characterization. It has already been used as key component for the identification of Willamette (Eri et al., 2000) and Cascade (Nance and Setzer, 2011) varieties, but in our study, it was found to be present in quite high concentrations also in Mt. Hood, North Down, and Sterlyng. At the same time, β-farnesene was present in low concentration in most of the high α-acid cultivars, according to what proposed by Peacock and McCarty (1992) and Kishimoto et al. (2006).

The high number of considered hop varieties, allowed us to identify new potential key molecules for varietal identification and to solidly confirm the presence of these molecules as markers of particular hop varieties. Once the presence of hop varietal markers has been pinpointed, it should be determined whether or not these molecules are sufficiently constant to

serve as a reliable basis for a system of varietal identification. To achieve this goal, the two data sets (hops volatile profiles in 2013 and 2014) were subjected to separate PCAs.

Volatile molecules concentration (quantitative analysis) of different varieties were highly variable between cultivation years (**see Chapter 3.3.3**). Despite this, PCA analysis highlighted that the volatile profile of hop cones obtained by two different cultivation years (and climatological conditions) showed a constant pattern (qualitative analysis) comparing 2013 and 2014 results (**see Chapter 3.3.4**). Indeed, high α-acids varieties (as Centennial, Chinook, Columbus cv.) cluster together and results to be always characterized by higher content of some important monoterpenes and esters (as β-myrcene, α- and β-pinene, methyl-4-decenoate, methyl-6-methyl isobutyrate), while, other varieties (as Magnum, Steling cv.) are always characterized by high contents of sesquiterpenes. Also the presence of varietal markers (as for example β-farnesene in Cascade and Willamette cv.) appears to be confirmed, irrespective of cultivation year.

Since some compounds for varietal characterization of hops' volatile profiles are stable, a further step in the analysis is aimed at the identification of relationships between these volatiles and their sensory character. Brewers still select hops by sensory evaluation and breeders need potential selection criteria for the selection of parents for new cultivars development, therefore, is important to characterize hop varieties also form a sensory point of view and to find out correlations between hops chemical profile and its sensory character. To determine the sensory profiles of hops, cones of ten varieties (mainly 'aroma hops') were used to flavour a *Blond Ale* beer and were sensory analysed by a trained panel.

In our study, few odorants positively correlate with the sensory descriptors 'citrusy', 'fruity' and 'floral', while high correlation coefficients were find between several volatile compounds and the aromatic descriptors 'hoppy', 'grassy' and 'spicy'. In this regard, the most remarkable result was given by the positive correlation of hoppy, grassy and spicy descriptors with p-cymene, carvacrol and caryophyllene oxide. Although in previous studies it was already shown that oxidized sesquiterpenes contribute to the spicy/herbal flavour of hop and beer (Goiris et al., 2002; Kishimoto et al., 2006; Eyres et al., 2007), p-cymene and carvacrol are generally described as 'spicy, woody, with cumin, oregano and thyme notes' (The Good Scents Company, 2015) and are constituent of a number of essential oils, most commonly thyme (Senatore, 1996) and oregano (Kordali et al., 2008) essential oil. In this work, these

two molecules were therefore reported as potential contributors to the spicy flavour of hops too. A high content of caryophyllene oxide, associate with a high intensity of vegetal and spicy odours was highlighted in Hersbrucker Spät, according to Van Opstaele et al. (2012b) and another important positive correlation was showed between the sesquiterpene α-humulene and vegetal descriptors. α-humulene has already been proposed as one of the most important odour-active volatiles of hops showing 'green/woody' odours (Van Opstaele et al., 2012a). Another interesting observation is that hoppy, grassy and spicy terms were all negatively correlated with geraniol, which is known for its contribution to floral and citrus flavours of beers (Takoi et al., 2010).

3.5. CONCLUSION

A high number of hops cultivated in the same experimental field in Northern-Italy for two years were subjected to chemical and sensory analyses. The hop acids and volatile compounds profiles allowed us to study their variability among two different cultivation years. In general, hop acids showed a typical basic pattern among different cultivation years: high α- and β-acids could be associated with a moist summer, and low contents with dry summers followed by a rainy August. Hops' volatiles behaviour seems instead to be more complex and strongly influences by variety and volatile component class. In both years Cascade, Willamette, Challenger and Hersbrucker Spät turned out to be the varieties with lowest contents of volatiles, almost all high-α-acids varieties were characterized by the presence of specific monoterpenes, while other varieties, especially Magnum, Sterlyng and North Down, highlighted higher content of sesquiterpenes. Although a strong variability in dependence of hop variety and chemical component was outlined among different cultivation years, a typical basic pattern of hops profile was highlighted. To fully characterize the selected hops, the extracts of eleven hop varieties cultivated in 2013 were sensory analysed and correlations between sensory impressions and volatile compounds were pinpointed. High correlation coefficients were find between sesquiterpenes and 'hoppy', 'grassy' and 'spicy' descriptors, while few odorants correlated with the 'citrusy', 'fruity' and 'floral' descriptors. For this reason in the next chapter we will focus on the fruity/citrusy character of hops, with the aim to describe this particular odour perceptions form a molecular point of view.

4. Characterization of the citrus character of hops via Gas Chromatography−Mass Spectrometry/Olfactometry

4.1. INTRODUCTION

Previous studies used GC-O technique to identify key aroma compounds in hops. With the current revival of very hoppy beers in Europe, the need to understand hop aroma is a priority in brewing research (Schönberger and Kostelecky, 2011). To describe different kind of hop aroma impressions from a molecular point of view could is indeed useful for hop breeders who have to develop new cultivars with particular organoleptic characteristics aromas and could represent an objective way to describe hop aroma for brewers who want to impart particular aromas to beer by adding hops or hop pellets at particular stages of beer production. As mentioned by Schönberger and Kostelecky (2011), green and grassy notes are due to aldehydes (Kishimoto et al., 2006), floral and fruity impressions are derived from linalool, geraniol, β-ionon, citronellol, and a variety of ketones, epoxides and esters (Marriott, 2001; Kishimoto et al., 2006; Van Opstaele et al., 2006) and spicy/herbal flavours can be attributed to oxidized sesquiterpenes (Goiris et al., 2002; Eyres et al., 2007). Although different odour impressions were described, few attempts were made to clearly correlate 'citrusy' aroma impressions of hop essential oil with its chemical profile. It has been suggested that 'citrus' flavour can be attributed to esters, nerol and linalool (Kishimoto et al., 2006), but ketones, monoterpenes and aldehydes (e.g. nonanal) seem to be important for 'citrus' connotations as well (Van Opstaele et al., 2012a). Furthermore, very recent papers hypothesised that also particular sulphur compounds can contribute to this particular odour impression (Kishimoto et al., 2008; Kankolongo Cibaka et al., 2015; Ochiai et al., 2015). However, the analytical protocols used for extraction and subsequent detection and quantification of particular sulphurs are not straightforward and the 'citrusy' character of hops is still far from being fully understood.

The objective of the present study was therefore (1) to characterise the volatile composition of particular hop oil fractions derived from citrusy (cv. Citra, cv. Cascade, cv. Riwaka) and non-citrusy hop varieties (cv. Saaz, cv. Perle, cv. Magnum) via automated extraction using SPME in combination with GC-MS, and (2) to detect the respective odour-active molecules in the

hop oil fractions via GC-olfactometry, thereby aiming at finding correlations between the chemical composition and, in particular, the citrusy aroma characteristics.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

Volatile compounds were determined using reference compounds purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade: 2-decanone (99.5%), 2-undecanone (99.0%), 3-methylbutyl isobutanoate (≥98%), camphene (95.0%), limonene (97.0%), linalool (98.5%), methyl 3-nonenoate (99.8%), methyl geranate, ocimene (≥90.0%, mixture of isomers), methyl heptanoate (≥99%), methyl octanoate (99.8%), nonanal (95.0%), p-cymene (≥99.0%), terpinolene (≥90.0%), α-humulene (≥98.0%), α-pinene (98.0%), β-caryophyllene (98.5%), β-myrcene (≥95.0%), β-pinene (99.0%), and γ-terpinene (≥97.0%). The water used was purified by a Synergy Water Purification System (Merck Millipore Co., Darmstadt, Germany) while ethanol absolute was obtained from VWR International Ltd (Lutterworth, UK).

4.2.2. Hop varieties

Cascade, Citra and Riwaka hop essential oils were kindly provided by Totally Natural Solutions (East Peckham, UK) and Saaz, Magnum and Perle hop essential oils were obtained in our lab by steam distillation. The sensory profile of this hop varieties is well-known; Cascade, Citra and Riwaka are characterized by a citrus flavour and are described as ‘floral, citrusy, with grapefruit fragrance’, ‘very citrusy with tropical tones of grapefruit’ and ‘powerful grapefruit and citrus’ respectively (Hopunion, 2015), (New Zealand Hops, 2015). On the other hand Magnum, Perle and Saaz are generally described as ‘spicy’, ‘green’ or ‘earthy’ (Hopunion, 2015). The selected varieties can therefore be divided in two groups; ‘citrusy’ and ‘not-citrusy’ hops.

4.2.3. Fractionation of the hop oil via Solid Phase Extraction (SPE)

We used Solid Phase Extraction (SPE) to fractionate hop essential oil under vacuum system in order to facilitate the separation of volatile molecules via gas-chromatographic analysis. A vacuum port with gauge was used to control the vacuum applied to the chamber. As stationary phase a C18 column (Varian Bond Elut C18 cartridges: 500 mg, 6 ml, Agilent Technologies, Lake Forest, USA) was used. Before the extraction the column was conditioned with MQ-

water, ethanol and a mixture 1/1 v/v ethanol/MQ-water, respectively. Then, 100 µl of hop oil were suspended in 5900 µl of a 1/1 v/v % ethanol/water solution (final dilution: 1:60) and this solution was pipetted on the C18 column and eluted to allow hop oil compounds to be adsorbed into the stationary phase. Next, hop compounds were selectively desorbed pipetting respectively into the column 3 ml of a 50, 60, 70, 80, 90 and finally a 100 v/v % ethanol/MQ-water solution and the effluents were collected each time in different vials (20 ml, clear glass, Chromacol, Welwyn Garden City, UK). Vials were then stored in a freezer at − 18 °C. Each variety was fractionated by SPE two times to obtain two extraction replicates.

4.2.4. HS-SPME-GC-MS-O analysis

Hop oil fractions were analysed by headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS).

100 µl of each hop oil fraction was added to 4850 µl of MQ-water and to 50 µl of internal standard (2-heptanol) in a HS-SPME vial (20 ml, clear glass, Chromacol). Vials were then closed with magnetic caps with silicon septum (Supelco, Bellefonte, USA). HS-SPME was performed using a CombiPal auto-sampler (CTC Analytics, Zwingen, Switzerland). During the extraction the fiber was exposed into the headspace of the vial (22 mm) for 30 min at 40°C.

Samples were analysed with an Ultra Trace gas chromatograph (Thermo Fisher Scientific, Austin, USA) and volatile molecules' separation was performed using a 40 m x 0.18 mm i.d. x 0.2 µm (film thickness) RTX-1 capillary column (Restek Corporation, Bellefonte, PA, USA). The carrier gas was pure helium and the flow rate was set at 0.8 mL min^{-1}. Injection was performed in the split mode (constant flow: 10 ml min^{-1}, split ratio: 12) and the injector temperature was set to 250 °C. The oven temperature was programmed to rise from 40 to 170 °C at 3°C min^{-1} and then to 250°C at 15°C min^{-1}.

Mass spectrometric detection of volatile molecules was obtained using a dual stage quadrupole MS (DSQ I, Thermo Fisher Scientific, Austin, TX) operating in the electron ionisation mode (EI, 70 eV). The ion source temperature was set at 240 °C and the electron multiplier voltage was 1,824 V. The mass spectra were scanned in the full scan mode (m/z: 40-270) and the MS detected positive ions with a total scan time of 0.29 s (3.5088 scans s^{-1}, scan rate: 873.2 amu s^{-1}).

Gas chromatography/olfactometry analysis (GC-O) was used to characterize odour-active compounds and was conducted on a GC interfaced to a Sniffer 9000 system sniffing device (Brechbühler, Switzerland). The gas chromatography effluents were split 1/1 (v/v) between the sniffing port and the MS detector and the MS transfer line was set at 260 °C. The capillary column outlet was connected to a line of humidified air. The sniffing was performed for all the samples immediately after HS-SPME by two trained assessors and, at the time of aroma perception, panellists verbally described it. 6 replicates of GC-O analysis were finally obtained for each variety. Volatile compounds were identified using reference compounds purchased from Sigma-Aldrich (St. Louis, MO, USA) and by mass spectral comparison via the Xcalibur software (v.1.4 SR1, Thermo Fisher Scientific, Austin, TX), using the 'NIST98' and 'Flavour MS library for Xcalibur 2003' spectral libraries (Interscience, Louvain la Neuve, Belgium). To give a further confirmation, also Kovàts indices (KI) were calculated using a series of normal alkanes (C10–C18; Sigma-Aldrich, St. Louis, MO, USA). The levels of hop oil compounds were standardized taking into account the area of the internal standard (2-heptanol).

4.2.5. Statistical analysis

Cluster analysis (Ward's Method, Squared Euclidean) and Principal Component Analysis (PCA) were carried out with 'hclust' and 'prcomp' functions of R programming language (version 3.2.2.; https://www.r-project.org) respectively.

4.3. RESULTS AND DISCUSSION

4.3.1. Descriptive sensory analysis pinpointed the citrusy character in 70/30 (v/v % ethanol/MQ-water) SPE fraction

A preliminary descriptive sensory analysis of Cascade, Citra and Riwaka hop oil fractions let us to pinpoint the 'citrusy' odour impression in 50/50, 60/40 and 70/30 (v/v % ethanol/MQ-water solution) SPE fractions, while 80/20, 90/10 and 100/0 fractions were mainly described as 'resinous', 'spicy' and 'woody' by panelists (**Table 8**). Moreover, both sensory and chromatographic analyses, highlighted that 50/50 and 60/40 fractions expressed a waxy 'citrusy' odour and were less rich in volatiles than 70/30 fraction (**Figure 15**). Chromatographic analysis of SPE fractions, also showed that 80/20, 90/10 and 100/0 fractions were characterized by high levels of sesquiterpenes and oxygenated sesquiterpenes, known for their spicy/herbal odours. Taking into account these information, these fractions were not considered in this paper, and, thanks to a further confirmation given by Van Opstaele et al. (2012b) that generally characterize the 70/30 SPE hop fraction as 'floral, fruity, with citrus and hoppy scents', it has been decided to focus on this fraction. Due to the importance given by the literature to linalool (Steinhaus and Schieberle, 2000; Kishimoto et al., 2006; Steinhaus et al., 2007; Hanke, 2009) and n-nonanal (Steinhaus et al., 2007; Van Opstaele et al., 2012a) it has been decided to include in the data matrix also these two compounds' peaks coming from the 60/40 SPE fraction.

Table 8 – Results of preliminary descriptive sensory analysis of Cascade, Citra and Riwaka. SPE fractions are indicated according to eluent composition (v/v % ethanol/MQ-water)

SPE fraction	**CITRA**	**CASCADE**	**RIWAKA**
50/50	weak citrus	weak resinous and citrus	weak citrus
60/40	weak citrus	weak resinous and citrus	weak resinous and fruity
70/30	**strong citrus (grapefruit)**	**strong citrus (orange), resinous**	**strong citrus, weak hoppy**
80/20	resinous, orange leaf	resinous, orange peel	resinous, waxy citrus
90/10	resinous, spicy, woody	weak resinous, woody, orange peel	resinous, citrus peel
100/0	weak resinous and spicy	weak resinous	weak resinous

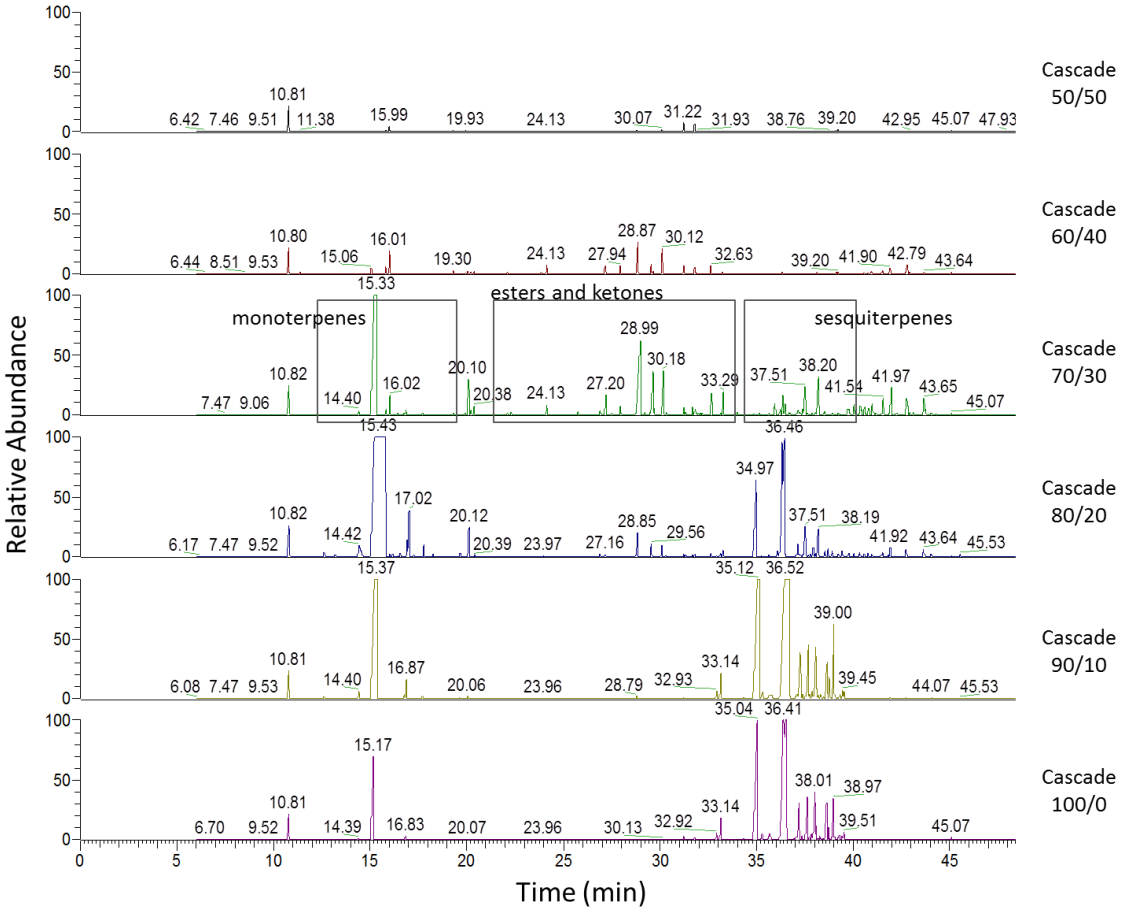


Figure 15 – HS-SPME-GC-MS profile of different hop oil SPE fractions (50/50, 60/40, 70/30, 80/20, 90/10 and 100/0 v/v % ethanol/MQ-water solution) cv. Cascade

4.3.2. The volatile profile of hop's citrusy fraction analysed via HS-SPME-GC-MS-O highlighted varietal differences between 'citrusy' and 'not citrusy' hops

The 70/30 SPE fraction of Cascade, Citra, Riwaka, Saaz, Magnum and Perle hop essential oils was analysed by HS-SPME-GC-MS and GC-O. Identified volatile compounds, together with their odour descriptions, are listed in **Supplementary Table S7**, and the relative composition (% of total peak area) of the selected SPE fraction based on different chemical classes is reported in **Supplementary Figure S1**. Considerable differences in the qualitative and quantitative composition between hop varieties were found. A total of 59 molecules were identified, mainly esters (16 molecules), monoterpenes (10 molecules), ketones (6 molecules), sesquiterpenes (2 molecules) and aldehydes (2 molecules). As expected the predominant compound of 70/30 SPE fraction was β-myrcene, accounting for 92-97 % of monoterpenes

and 34-58 % of the total volume. Methyl-trans-4-decenoate was found to be the major constituent of the esters group, accounting for 12-61 % of esters and for 3-18 % of the total volume, followed by trans-methyl-geranate, methyl caprate and geranyl butyrate. For what concern ketones, 2-Undecanone was found to be the predominant component accounting for 52-73 % of ketones and 2-19 % of the total peak area, followed by 2 dodecanone and 2 tridecanone. The selected hop oil fraction resulted to be lacking in sesquiterpenes; indeed the only two sesquiterpenes founded were α-humulene (0.1-25 %) and β-caryophyllene (0.05-9.5 %). 35 odour-active molecules were detected by the assessors during GC-O analyses. A number of vegetal odours contributed to the odour character of the fraction, resulting in 'hoppy', 'green' or 'spicy' impressions (for example β-pinene, β-myrcene, 2-methylbutyl 2-methylpropanoate, α-humulene, β-caryophyllene, p-cymene and cis-β-ocymene). A number of esters and ketones were instead perceived as 'fruity' (for example methyl octanoate, methyl nonanoate, methyl decanoate, methyl geranate, geranyl butyrate, 2 decanone and 2-undecanone) or 'floral' (for example 2 dodecanone, neryl acetate and geranyl propionate).

A hierarchical cluster analysis carried out on GC-MS profile of 70/30 SPE fraction allowed us to clearly observe the separation between 'citrusy' and 'not-citrusy' hops. Indeed, cluster analysis based on the identified volatiles, divided our samples into two clusters: Cascade, Citra and Riwaka from one side and Magnum, Perle and Saaz form the other (**Figure 16**).

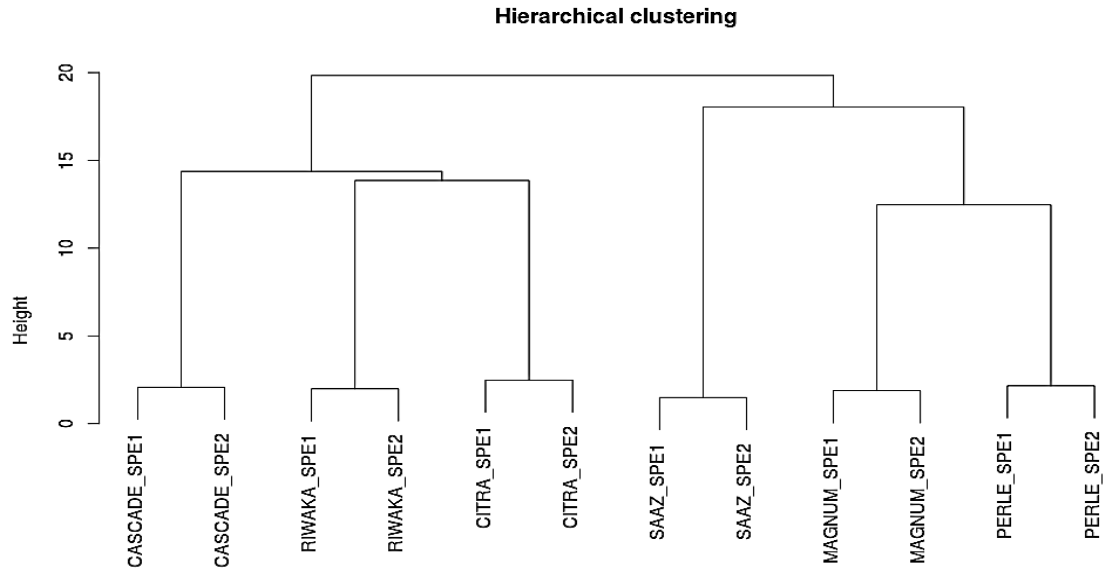


Figure 16 – Dendrogram obtained by hierarchical agglomerative clustering (Ward's Method, Squared Euclidean) of volatiles detected in the 70/30 (v/v % ethanol/MQ-water solution) SPE fractions of the selected hop varieties (scaled data). The dendrogram divide the selected varieties in two main clusters dividing the so-called 'citrusy' (Cascade, Citra and Riwaka) and 'not-citrusy' (Magnum, Perle and Saaz) varieties. Variables are in accordance with **Supplementary Table S7**.

To better understand the impact of each molecule on the observed separation amongst varieties, data matrix was subjected to Principal Component Analysis (PCA). First (PC1) and second (PC2) principal components accounted for 33% and 31% of the variance, respectively (**Figure 17**).

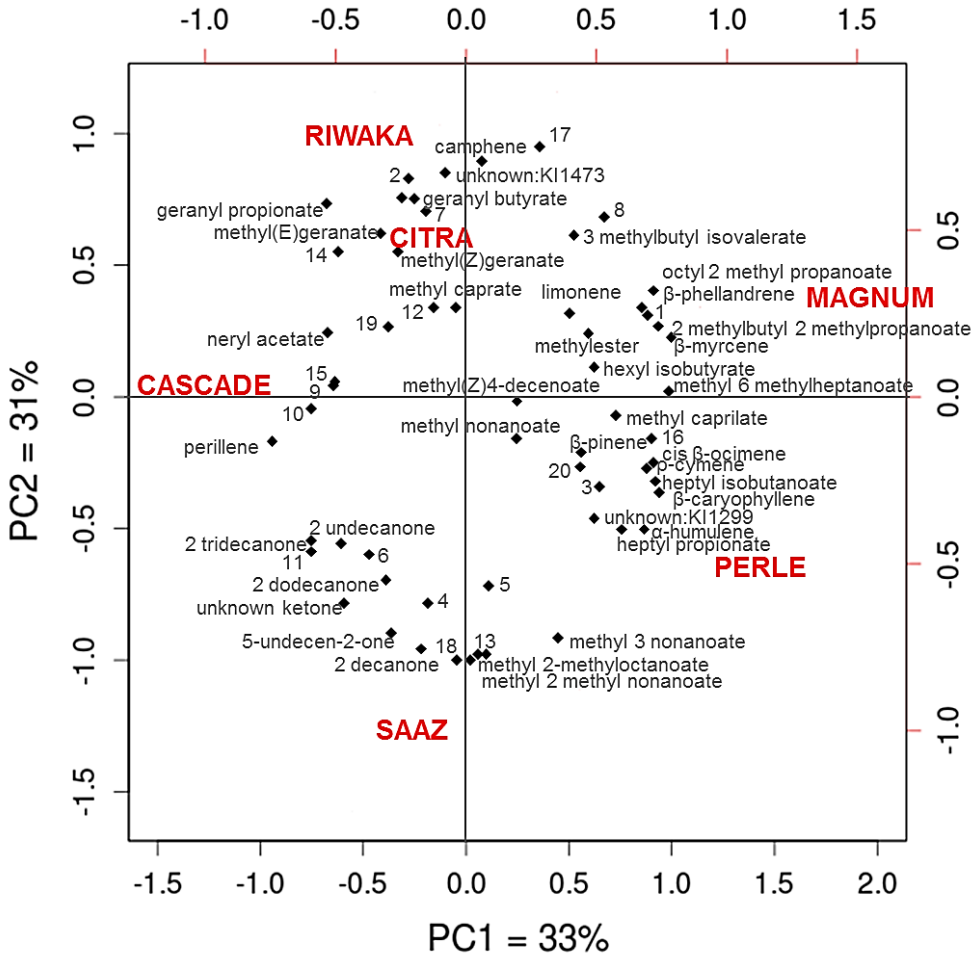


Figure 17 – Biplot obtained by principal component analysis of volatiles detected in the 70/30 (v/v % ethanol/MQ-water solution) SPE fractions of the selected hop varieties (scaled data). Hop varieties are represented as scores, and volatiles determined by HS-SPME-GC-MS as loadings. Variables are in accordance with **Supplementary Table S7**. Molecules not perceived as odorants by the assessors are reported as codes: α-pinene (1), 3 methylbutyl 2 methylpropanoate (2), methyl heptanoate (3), γ-terpinene (4), terpinolene (5), n-nonanal (6), linalool (7), 2 methylbutyl 3 methylbutanoate (8), valeric acid (9), decanal (10), thmyol/carvacrol methyl ether (11), ethyl citronellate (12), methyl 4,6-dimethyl octanoate (13), neryl formate (14), 2 undecanol (15), unknow KI: 1291 (16), isoamyl n-heptanoate (17), methyl 10 undecenoate (18), methyl 9-cyclopropylnonanoate (19), unknown KI: 1489 (20).

On the base of PCA, three groups can be identified: one belonging to Citra, Cascade and Riwaka hops, and two belonging to Saaz from one side and Magnum and Perle from the other. The sesquiterpenes α-humulene and β-caryophyllene were described as 'spicy' by the two assessors and were present in very high concentrations in Magnum, Perle and Saaz. The positive correlation between the sesquiterpenes α-humulene and β-caryophyllene and spicy/vegetal descriptors was highlighted in chapter 3 and further confirmed in this study. Magnum and Perle are often described as 'spicy' or 'green' and they were characterized by high concentration of β-myrcene (described as 'hoppy'), 2-methylbutyl 2-methylpropanoate and cis β-ocymene (both described as 'herbal and spicy') and methyl-6-methyl heptanoate (described as 'waxy hoppy'). The negative correlation between 'citrus'/'floral' odours and methyl-6-methyl heptanoate and β-ocymene highlighted in chapter 3 was therefore again confirmed in this study. All the not-citrusy hops were also found to be rich in methyl 3 nonanoate (described as 'grassy and citrusy') and β-pinene (described as 'waxy hoppy'). Magnum has sometimes a 'citrus scent' (Hopsteiner, 2015) and GC-MS analysis highlighted in this cultivar a high concentration of limonene, the principal component of the essential oil of different *Citrus* species (Espina et al., 2011) and a well know odorant of hop oil (Steinhaus et al., 2007; Van Opstaele et al., 2012a). This molecule could therefore be linked to the 'citrusy' impression that characterize Magnum variety, but no clear correlations were found between its concentration and the 'citrus' character of the selected varieties. Saaz was found to be particularly rich in ketones as 2 decanone (described as 'orange' and 'citrusy'), 5 undecen 2 one (described as 'floral' and 'fruity') and 2 tridecanone (described as 'hoppy' and 'floral'). Some ketones, as 2 undecanone (described as 'citrusy') were found to be particularly high in Cascade and Citra too. Cascade has been described as 'floral, citrusy and spicy, with grapefruit fragrance' (Hopunion, 2015) and in our study was characterized by high concentrations of trans methyl geranate, geranyl propionate, geranyl butyrate and neryl acetate. All these compounds were found to be odour-active and were described as 'citrusy' and 'floral' by the assessors and geranyl acetate and neryl acetate have already been characterized as chemical compounds typical of tangerine essential oil (Chutia et al., 2009). Neryl acetate concentration was particularly high in Cascade (five times higher than in other hop varieties) and could maybe be involved in the floral tones typical of this variety. Cascade was also found to be rich in perillene, which was found to be an odorant by our GC-O

analysis. In spite of this evidence, the assessors were not able to assign perillene odour to a category, although in literature it has been described as ‘citrusy’ (Van Opstaele et al., 2012a). It can therefore be summarized that floral and citrusy characters of Cascade are likely due to geranyl esters as proposed by Nance and Setzer (2011) but, for the first time, these esters were pinpointed and the role of neryl acetate and perillene in such character was outlined. As Cascade, also Riwaka was found to be rich in trans methyl geranate, geranyl propionate and geranyl butyrate. Moreover, Riwaka resulted to be rich in linalool and limonene too. Although these molecules has not been identified as odorants by the assessors, numerous studies highlighted their impact in the fruity/flowery flavour of hops (Hanke, 2009; Takoi et al., 2010; Van Opstaele et al., 2012a). The third ‘citrusy’ variety selected for the study, Citra, was instead characterized by a high concentration of methyl-nonanoate, cis methyl geranate, trans methyl geranate, methyl-trans-4-decenoate and methyl caprate. All these esters were described as ‘citrusy’ by the assessors, therefore they likely contribute to the very citrusy and fruity aroma typical of this variety. Especially, the level of methyl caprate was found to be particularly high in Citra, reaching a concentration twenty times higher than in the other varieties.

Aiming at the identification of key molecules markers for citrus impression in hops, some interesting molecules were therefore highlighted in this study. Trans methyl geranate shows a clear difference between so called ‘citrusy’ and ‘not-citrusy’ hops; its concentrations are almost triple in Cascade, Citra and Riwaka in respect to Magnum, Perle and Saaz. High concentrations of methyl geranate in Citra and Cascade were previously highlighted by Dresel et al. (2015) that in his study found high concentrations of this molecule also in Centennial, another hop variety described as ‘floral and citrusy’ (Hopunion, 2015). Another interesting molecule seems to be geranyl propionate, that was detected only in Citra, Cascade and Riwaka and was described as ‘floral and citrusy’ by the assessors. This molecules could be maybe used as a marker to distinguish citrusy and not-citrusy hops, and was found by Nance and Setzer (2011) in Cascade hop oil too, however, to confirm this, further investigations are needed and a higher number of hop varieties has to be taken into account. Also neryl formate could be involved in citrus aroma aspect, because it was found only in Cascade and Riwaka hops. This molecule has been described as ‘green, floral, fruity, citrus and tropical’ (The Good Scents Company, 2015) but in our study it has not been identified as an odorant.

Summarizing, citrusy hops were described both from the PC1 and the PC2 and were clearly characterized by a higher presence of saturated and unsaturated esters. Citrusy hops seem also to be characterized by the presence of ketones with 'floral' or 'citrus' flavours that, however, were present in high concentrations in Saaz too. Most of the molecules that influence Magnum and Perle PCA distribution were instead described by the assessors as 'spicy', 'green', 'hoppy' or 'woody'. GC-MS analysis showed, indeed, that these varieties are characterized by sesquiterpenes and by the presence of esters (as hexyl isobutyrate, methyl octanoate and heptyl isobutanoate) described mainly as 'green' and 'grassy'.

A further confirmation of the relevance of some of the selected molecules as markers for citrus character, was given by the analysis of lemon (*Citrus lemon* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) essential oils. The essential oils of these *Citrus* species were fractionated via SPE and 70/30 (v/v % ethanol/MQ-water solution) and SPE fraction was analysed via HS-SPME-GC-MS, as done for the hop essential oils used for the experiment. **Table 9** report all the volatile molecules founded to be present both in citrusy hops and in lemon, grapefruit and orange essential oils. The essential oil of *Citrus* species was characterized by a high concentration of limonene and orange essential oil was particularly rich in linalool. It is noteworthy that orange essential oil was found to be very rich in neryl acetate, a molecule found to be particularly high in Cascade. This results further confirmed that this molecule is likely linked to the 'citrus/orange' flavour of Cascade.

Table 9 – Volatile molecules of 70/30 SPE fraction detected both in citrusy hop varieties (Citra, Cascade and Riwaka) and lemon, grapefruit or orange essential oils analysed by HS-SPME-GC-MS. The identified compounds are presented together with their Kovàts retention indices (RI) and retention times (RT). Mean values of the GC analyses of duplicate SPE were normalized on the area of the internal standard.

Component	KI	RT	Citra	Cascade	Riwaka	LEMON	GRAPEFR.	ORANGE
α-pinene	< 1000	15.23	3.74	0.89	6.36	58.95	30.63	3.89
camphene	< 1000	15.84	3.84	0.91	4.88	7.15	0.33	1.50
β-pinene	< 1000	17.16	15.09	8.84	21.18	687.25	17.69	66.06
β-myrcene	< 1000	18.00	2031.41	911.35	1973.78	115.30	240.49	360.27
ρ-cymene	1013	19.23	6.17	3.19	14.23	155.32	5.98	18.28
limonene	1019	19.65	6.69	2.07	34.12	2839.36	5033.61	120.79
β-phellandrene	1020	19.66	15.28	6.23	10.53	-	-	2.70
cis β-ocimene	1039	20.50	8.51	2.74	2.71	10.42	11.46	295.28
γ-terpinene	1049	21.06	1.10	0.56	5.57	595.98	8.19	6.32
terpinolene	1079	22.47	1.61	0.50	1.53	24.21	1.38	56.19
n-nonanal	1084	22.52	0.52	1.63	2.42	8.26	1.46	-
linalool	1085	22.59	2.90	1.86	8.29	1.47	-	233.25
perillene	1089	22.88	50.83	87.24	36.82	-	-	3.62
decanal	1186	27.45	-	2.36	0.32	22.75	46.71	-
neryl formate	1284	31.80	-	5.12	7.06	-	-	6.57
methyl trans geranate	1309	32.80	330.01	134.40	112.27	-	-	1.51
neryl acetate	1363	35.43	3.91	53.73	12.87	163.07	5.36	835.26
β-caryophyllene	1415	37.73	4.09	0.84	47.82	2.53	5.85	0.35
α-humulene	1448	39.09	7.62	2.24	117.79	1.75	1.30	-
geranyl propionate	1454	39.20	18.57	30.60	42.30	0.83	-	-

4.4. CONCLUSION

The essential oils of six hop varieties (Citra, Cascade, Riwaka, Saaz, Magnum and Perle) were fractionated via SPE and the fractions of Citra, Cascade and Riwaka, all hops with a strong citrus character, were sensory analysed. Descriptive sensory analysis pinpointed 'citrus' impression in 70/30 (v/v % ethanol/MQ-water solution) SPE fraction and the volatile compositions of this particular fractions for all the selected hops were analysed via HS-SPME-GC-MS and GC-O. Cascade resulted to be characterized by high concentrations of perillene and neryl acetate, Citra by high concentrations of methyl nonanoate and methyl decanoate, trans methyl geranate and methyl trans-4-decenoate, and Riwaka by high concentrations of geranyl butyrate. Data were subjected to hierarchical cluster analysis and PCA that highlighted that hops that expressed a typical citrus aroma are characterized by high levels of esters and low levels of sesquiterpenes. From one side, not-citrusy hops were characterized by high concentrations of spicy molecules as α-humulene and β-caryophyllene and by herbal/grassy monoterpenes and esters as methyl-6-methyl heptanoate, β-ocymene, methyl 3 nonanoate and β-pinene. From the other side, hops that expressed a typical citrus aroma were characterized by high levels of specific esters as methyl (E)-4-decenoate, methyl nonanoate and methyl caprate and by high levels of esters of geraniol and nerol, all component described as 'fruity', 'citrusy' or 'floral' by the assessors.

This project has increased our understanding of hop citrus character and identified initial practical benefits for the hop industry. Indeed, the contribution of specific volatile molecules to the citrus character of particularly hop varieties was outlined, and will facilitate the screening for citrus character of new hop varieties with a specific citrusy character.

5. General conclusion

2014 global hop production and acreage increase of 12% and 4.3%, respectively, compared to 2013 (IHGC Market Report, 2015). Among European countries, Italy is the tenth for beer production and, although the number of Italian micro-breweries is increasing (Assobirra annual report, 2014), hop cultivation is not widespread in the county and hop is still imported in Italy as raw material (Table 1).

One objective of this PhD was to determine whether it is possible to cultivate hop in Italy and its achievable quality. In Mediterranean areas, the phenological cycle of hops coincides with high temperatures and drought that are supposed to affect hop yield and quality (Izaurralde et al., 2003; Mozny et al., 2009). However, as described in the second chapter of this thesis, the selection of hop cultivar with higher tolerance to drought, should be a successful strategy to overcome this problem. A high number of hop cultivars was therefore subjected to a long term drought stress, physiological (transpiration, plant growth and chlorophyll) analyses were recorded throughout stress kinetic. Taking into account results presented in this thesis, Challenger, Hersbrucker Spat, Mittelfrüh and Nugget hop cultivars showed a higher resistance to drought in terms of transpiration rate and growth, in comparison to Cascade, Chinook and Columbus more susceptible genotypes. Drought stress greatly influenced also hop internodes growth and chlorophyll content.

In order to extend the cultivation area of hop in non-traditional growing regions, such as Italy, and considering the ongoing climate change and variability, the aim of the second study was to investigate about the stability of hops' quality in relation to two different cultivation years. Sixteen hop cultivars were therefore cultivated in the same experimental field (Parma, Italy) in 2013 and 2014 and their hop acids concentrations and volatile profiles were analysed. The high number of hop varieties evaluated allowed us to conclude that, while α- and β-acids levels follow more or less the same behaviour among different varieties (being their concentration generally higher in 2014, a season characterized by a moist summer), the variability of hops' volatile profiles in respect to the cultivation year seems to be more complex. Climate is clearly a key factor that influence plants' essential oil (Carrubba and Catalano, 2009) and hop volatiles variability seems to be linked to the complexity of hop volatile profiles that derive from different biosynthetic pathways. Nevertheless, the large

number of hop cultivars considered, allowed us to identify key component for varietal identification and to observe how most hop cultivars maintain a similar key aroma pattern between cultivation years.

Organoleptic characteristics also play an important role in hop quality and for this reason they has to be considered in selecting hop varieties. The last part of this thesis was therefore focused on hop aroma characteristics. The chemical and sensory analysis of hops cultivated in Northern-Italy, highlighted correlations between sesquiterpenes and vegetal odours, and a further investigation conducted in EFBT laboratory (Ghent, Belgium) allowed us to pinpoint the specific odour-active molecules (mainly esters) responsible of the 'citrusy' aroma of specific hop cultivars.

Summarizing, in this work, the response of different hop cultivars to drought stress was outlined and the quality of hops cultivated in Northern-Italy and its variability were determined. Moreover, some hop aroma characters were characterized by a molecular point of view. This project has therefore increased our scientific knowledge of hop aroma and of hop response to drought stress and provided useful information for hop growers, breeders and in general, for the hop industry.

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Appendix – Supplementary Tables and Figures

Supplementary Table S1 – Curves' correlation coefficients (r), curves' parameters (a, b and c) and their standard deviations, and the integrals of different sections of the curves are reported in (from FTSW=1.0 to FTSW=0.8, from FTSW=0.8 to FTSW=0.6, from FTSW=0.6 to FTSW=0.4, from FTSW=0.4 to FTSW=0.2, from FTSW=0.2 to FTSW=0.0)

	CHA	**HER**	**MIT**	**NUG**	**GOLD**	**BRE**	**MAG**	**FUG**	**COL**	**CAS**	**CHI**
Corr. (r):	0.8892	0.8946	0.9147	0.9332	0.9214	0.9640	0.9688	0.9634	0.8862	0.9049	0.9432
St.Err.:	0.155	0.144	0.146	0.143	0.157	0.102	0.103	0.103	0.171	0.148	0.114
Model: y=a*(1-exp(-b*x))^c											
a	1.01 ± 0.01	1.02 ± 0.01	1.09 ± 0.03	1.05 ± 0.02	1.04 ± 0.02	1.04 ± 0.02	1.07 ± 0.02	1.1 ± 0.03	1.77 ± 0.52	1.9 ± 0.48	1.45 ± 0.15
b	4.42 ± 0.5	4.86 ± 0.52	2.66 ± 0.4	3.96 ± 0.4	3.65 ± 0.48	3.25 ± 0.27	4.05 ± 0.28	2.54 ± 0.24	0.63 ± 0.4	0.66 ± 0.32	1.1 ± 0.25
c	0.71 ± 0.05	1.08 ± 0.11	0.63 ± 0.05	0.97 ± 0.07	1.08 ± 0.1	1.04 ± 0.06	1.84 ± 0.13	0.99 ± 0.05	0.74 ± 0.07	0.89 ± 0.08	0.99 ± 0.07

Supplementary Table S2 – Anions and cations concentrations in leaves **(A)** and roots **(B)** of well-watered (treatment C) and water-stressed (treatment S) hop plants cv. Brewers Gold (GOLD), Cascade (CAS), Challenger (CHA), Chinook (CH), Columbus (COL), Fuggle (FUG), Hersbrucker Spät (HER), Hallertau Magnum (MAG), Hallertau Mittelfrüh (MITT), Northern Brewer (BRE) and Nugget (NUG). Means of control and drought-stressed plants are reported for chlorides (Cl^-), nitrates (NO_3^-), phosphates (PO_4^{3-}), sulphates (SO_4^{2-}), sodium (Na^+), ammonium (NH_4^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}). Data are expressed in mg/kg D.W..

A

		ANIONS				CATIONS				
variety	treatment	Cl^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	Na^+	NH_4^+	K^+	Mg^{2+}	Ca^{2+}
BRE	C	2555.0	27377.3	11872.1	2652.6	901.4	601.5	30429.6	9060.3	11385.2
	S	3175.7	28080.4	9215.0	3578.2	858.9	1402.8	30514.8	9747.1	12213.5
CAS	C	5075.8	21063.5	10015.9	2694.8	1180.6	247.8	30670.2	7581.9	10675.4
	S	6454.3	24259.9	6769.1	4419.6	821.6	273.4	30715.7	9535.4	11740.1
CH	C	4232.9	8924.2	9411.6	2829.8	1450.9	292.1	31164.5	6142.8	10024.8
	S	5129.6	5785.7	9812.8	4568.5	973.3	1385.3	32538.5	7244.2	11197.8
CHA	C	3810.3	24027.1	10291.9	2748.3	1009.6	674.0	33611.3	7265.6	11060.1
	S	5435.2	24770.8	10665.0	2620.0	1373.4	925.0	34696.4	7836.0	12404.8
COL	C	3151.4	9348.7	10092.1	2565.5	990.0	966.3	27166.5	7114.9	9067.7
	S	3371.5	11433.3	8758.0	3386.1	1050.3	1197.1	25091.0	9241.9	11229.4
FUG	C	4474.3	19068.8	14101.9	2973.1	869.3	130.9	39077.0	7837.9	10740.2
	S	4828.8	21978.0	14618.9	5236.4	967.0	534.2	41461.4	7956.2	10940.2
GOLD	C	3310.2	9422.9	9665.8	2442.8	1310.9	337.2	33743.7	8176.4	11777.6
	S	3355.0	20524.8	5732.4	2675.3	803.4	465.0	28462.0	8248.7	11963.0
HER	C	3776.0	23510.7	10199.8	2560.5	1518.1	549.6	34140.1	7785.7	10605.3
	S	4825.0	31640.2	7681.5	4233.2	1788.7	953.5	34221.8	10276.8	12435.7
MAG	C	2976.7	3856.3	10703.1	1851.8	1098.6	260.2	26823.5	7345.6	10331.9
	S	3498.4	5552.7	9523.3	2021.6	876.8	1180.1	30588.9	7460.8	10307.1
MITI	C	2798.1	31321.6	12445.2	4058.0	1043.7	1147.1	38706.9	9234.6	13057.2
	S	3068.1	33670.2	11761.2	5405.3	1039.3	2235.3	39254.1	9171.5	14841.8
NUG	C	1882.9	22171.8	12306.3	3453.4	696.5	250.0	37454.6	7020.5	10768.0
	S	2337.3	23895.2	10852.6	3817.1	1062.5	710.9	37942.6	7333.1	11148.9

B

		ANIONS				CATIONS				
variety	treatment	Cl^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	Na^+	NH_4^+	K^+	Mg^{2+}	Ca^{2+}
BRE	C	1442.5	11340.4	10124.3	3329.6	1691.6	1039.0	21017.0	5628.6	8286.6
	S	1928.7	14380.1	8264.8	3143.8	1552.3	1027.9	20292.3	5235.1	7748.8
CAS	C	2798.6	6492.0	9484.4	4516.6	1756.9	1184.7	18557.6	4908.5	8284.0
	S	1680.4	1384.7	5497.2	3243.5	1408.0	1353.5	12410.2	3960.8	7839.6
CH	C	1395.5	6415.9	8565.5	3323.7	1782.6	886.2	16815.8	4848.8	7780.4
	S	1843.9	4460.2	6710.6	2721.9	1652.8	926.1	15010.1	4146.6	8169.3
CHA	C	1795.5	9796.3	7644.7	2213.9	1189.6	1752.3	17609.3	3797.8	7345.7
	S	1218.5	8523.3	8159.4	2751.1	1368.4	1811.4	16222.1	3450.4	7492.6
COL	C	1607.0	6628.1	10268.3	3964.7	1361.3	439.8	21324.1	3501.3	7812.5
	S	1303.4	7226.7	7012.7	3347.8	1509.1	899.8	13923.2	3783.4	8087.8
FUG	C	2276.7	8513.0	7301.2	2003.6	1898.6	285.8	18809.4	4189.9	7595.1
	S	1928.6	5508.9	6553.3	2688.4	1879.6	398.9	16998.6	4067.4	8350.7
GOLD	C	1303.6	10029.9	9930.4	3696.4	1094.6	1418.3	19242.3	4820.6	7985.6
	S	1360.5	6957.2	6223.8	2426.0	1077.3	1896.2	16378.7	3829.5	7497.9
HER	C	1321.7	10862.4	8654.8	3018.6	1385.3	1424.2	18652.1	4571.3	7625.6
	S	1408.7	12195.2	7360.8	2967.0	1149.1	1004.1	20218.3	4525.0	9302.4
MAG	C	1751.3	8717.8	7384.5	3203.0	1624.2	367.3	19685.8	5227.5	8301.9
	S	1954.0	7879.4	6000.2	2050.6	1846.5	1286.9	17340.0	5409.6	8691.5
MITI	C	1611.7	9502.5	8867.4	2718.7	1186.9	546.5	18107.1	3879.0	7538.3
	S	1491.0	12386.2	11194.7	2822.6	1134.9	1953.7	17007.7	4269.3	8034.6
NUG	C	2015.5	13061.0	10326.4	3600.5	2500.0	319.6	21398.5	6224.9	8278.4
	S	2264.9	13907.3	7777.8	2644.6	2740.1	1723.2	22838.0	5736.6	8119.8

Supplementary Table S3 – Cohumulone (co-α), adhumulone + humulone (n+ad-α), colupulone (co-β) and adlupulone + lupulone (n+ad-β) contents (means ± standard deviations) of the selected hop varieties. Concentrations are expressed as W/W d.w..

	co-a		n+ ad-a		co-b		n+ ad-b	
variety	2013	2014	2013	2014	2013	2014	2013	2014
BREW.GOLD	2.39 ± 0.56	2.71 ± 0.21	4.04 ± 0.85	5.3 ± 0.34	2.39 ± 0.52	3.4 ± 0.19	1.55 ± 0.33	2.25 ± 0.12
CASC	1.52 ± 0.4	1.6 ± 0.01	2.71 ± 0.48	3.9 ± 0.09	2.02 ± 0.36	2.92 ± 0.07	2.14 ± 0.39	3.33 ± 0.08
CENT	1.74 ± 0.37	2.73 ± 0.21	4.76 ± 1.02	6.61 ± 0.49	1.25 ± 0.28	1.55 ± 0.1	1.45 ± 0.32	1.39 ± 0.09
CHALL	0.6 ± 0.15	1.33 ± 0.33	1.25 ± 0.27	3.37 ± 0.84	1.11 ± 0.27	2.36 ± 0.6	1.33 ± 0.32	2.93 ± 0.77
CHIN	3.73 ± 0.8	3.56 ± 0.36	9.01 ± 2.01	8.02 ± 0.81	1.54 ± 0.33	1.84 ± 0.21	1.49 ± 0.32	1.55 ± 0.15
COL	4.11 ± 0.81	4.81 ± 0.42	8.71 ± 1.59	11.05 ± 0.87	2.7 ± 0.52	3.5 ± 0.28	1.99 ± 0.36	2.77 ± 0.23
FUGG	0.55 ± 0.1	0.97 ± 0.05	2.14 ± 0.36	3.49 ± 0.19	1.77 ± 0.32	3.1 ± 0.17	2.75 ± 0.49	4.36 ± 0.26
H.MAGN	2.25 ± 0.3	3.37 ± 0.16	7.64 ± 0.01	11.66 ± 0.57	1.79 ± 0.01	2.63 ± 0.13	3.23 ± 0.02	4.32 ± 0.25
H.MITT	0.54 ± 0.09	0.62 ± 0.07	1.98 ± 0.34	2.42 ± 0.21	1.32 ± 0.23	1.71 ± 0.2	2.11 ± 0.37	2.6 ± 0.27
HERS.SP	0.26 ± 0.03	0.78 ± 0.17	1.04 ± 0.13	2.61 ± 0.57	0.59 ± 0.07	2.09 ± 0.45	0.93 ± 0.12	2.9 ± 0.62
MT.HOOD	0.82 ± 0.22	0.87 ± 0.05	2.99 ± 0.76	2.81 ± 0.2	2.42 ± 0.62	2.84 ± 0.12	3.78 ± 0.96	4.03 ± 0.23
N.DOWN	1.85 ± 0.004	1.28 ± 0.14	5.27 ± 0.56	3.47 ± 0.03	1.5 ± 0.15	2.26 ± 0.03	1.77 ± 0.17	2.65 ± 0.03
N.BREW	1.28 ± 0.26	1.65 ± 0.1	3.14 ± 0.38	4.41 ± 0.57	1.09 ± 0.14	1.85 ± 0.22	1.2 ± 0.14	2.02 ± 0.26
STER	4.5 ± 0.36	2.71 ± 0.33	8.59 ± 0.77	5.28 ± 0.65	4.85 ± 0.37	3.79 ± 0.41	4.03 ± 0.28	3.12 ± 0.31
WILL	1.03 ± 0.38	0.58 ± 0.18	1.75 ± 0.46	1.14 ± 0.39	1.65 ± 0.45	2.13 ± 0.69	1.57 ± 0.42	2.02 ± 0.67
YEOM	2.45 ± 0.17	3.75 ± 0.17	7.05 ± 0.6	11.36 ± 0.15	1.4 ± 0.12	2.3 ± 0.02	2.04 ± 0.18	3.01 ± 0.05

Supplementary Table S4 – Volatile Composition of hop hexane extracts cv. Brewer's Gold (BREW.GOLD), Cascade (CASC), Centennial (CENT), Challenger (CHALL), Chinook (CHIN), Columbus (COL), Fuggle (FUGG), Hersbrucker Spät (HERS.SP), Mount Hood (MT.HOOD), Hallertau Magnum (H.MAGN), Hallertau Mittelfrüh (H.MITT), Northern Brewer (N.BREW), North Down (N.DOWN), Sterling (STER), Willamette (WILL) and Yeoman (YEOM) expressed in %/total volume.

compound	BREW.GOLD		CASC		CENT		CHALL		CHIN		COL		FUGG		HERS.SP	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
α-thujene	0.02	-	-	-	0.04	0.02	0.08	0.07	0.06	0.06	0.03	0.04	0.05	0.03	0.07	0.05
α-pinene	0.14	0.10	0.16	0.11	0.20	0.11	0.07	0.10	0.09	0.10	0.10	0.11	0.11	0.09	0.14	0.12
β-pinene	0.09	0.76	0.08	0.83	0.04	0.75	-	0.45	0.14	0.50	0.24	0.65	0.04	0.60	-	0.58
β-myrcene	59.90	59.11	61.08	66.51	72.56	56.78	12.27	28.77	30.79	41.83	46.51	52.44	32.34	49.32	31.85	39.95
3-methylbutyl isobutyrate	0.70	0.72	0.43	0.51	0.25	1.15	0.14	2.34	0.60	1.76	1.69	0.30	0.10	0.55	0.08	-
α-terpinene	0.05	-	0.10	-	0.03	-	0.10	-	0.04	-	0.03	0.01	0.06	-	0.13	0.12
ρ-cimene	0.02	-	0.07	-	0.08	0.01	0.13	0.10	0.09	0.02	0.01	0.01	0.09	0.04	0.06	0.05
limonene	0.25	0.19	0.27	0.13	0.37	0.12	0.12	0.12	0.16	0.15	0.20	0.20	0.17	0.16	0.19	0.26
β-phellandrene	0.20	0.30	0.36	0.38	0.41	0.32	0.10	0.54	0.21	0.32	0.27	0.46	0.22	0.32	0.26	0.60
cis β-ocimene	0.08	0.08	-	-	-	-	-	0.06	-	-	-	-	-	0.03	-	-
trans β-ocimene	0.15	0.20	0.06	0.05	0.04	0.03	0.04	0.24	0.03	0.04	0.30	0.41	0.05	0.08	-	0.08
γ-terpinene	0.02	0.06	-	0.16	0.04	0.13	-	0.26	0.02	0.16	0.03	0.16	0.05	0.10	0.10	0.17
nonanal	0.05	0.04	-	-	0.03	0.07	-	-	0.05	0.12	0.19	0.24	-	-	-	-
methyl-6-methyl heptanoate	0.01	-	-	-	0.01	-	-	-	-	-	0.02	0.01	-	0.01	-	-
linalool	0.23	0.20	0.19	0.14	0.35	0.19	0.11	0.15	0.13	0.15	0.23	0.28	0.34	0.42	0.54	0.49
β-thujone	-	-	-	-	-	0.01	-	-	-	0.09	0.01	0.08	-	-	-	-
camphor	-	-	-	-	-	-	-	-	-	-	0.06	0.05	-	-	-	-
nerol	-	-	-	-	-	-	-	-	-	-	0.02	0.02	-	-	-	-
methyl nonanoate	-	0.04	-	-	-	0.09	-	0.20	-	0.08	-	0.13	-	0.07	0.03	-
geraniol	0.22	0.18	-	-	0.45	0.26	-	-	0.14	0.17	0.07	0.06	0.03	0.05	-	-
linalyl acetate	-	-	0.05	-	-	0.04	0.11	0.06	0.05	0.03	0.04	0.02	0.04	0.03	0.05	0.10
2 undecanone	-	-	0.12	0.12	0.04	0.07	0.61	0.51	0.13	0.09	0.18	0.16	0.19	0.17	0.43	0.41
methyl-4-decenoate	0.40	0.29	0.39	0.32	0.49	0.81	0.05	0.40	0.72	0.71	0.80	0.52	0.44	0.53	0.38	0.06
carvacrol	0.03	0.02	0.05	0.05	0.04	0.03	0.08	0.08	0.02	0.02	0.02	0.02	0.04	0.01	0.05	0.05
eugenol	-	-	-	-	-	-	-	-	-	-	0.02	0.02	-	-	-	-
β-caryophyllene	9.57	8.08	6.13	4.08	5.90	5.93	15.38	9.44	9.31	7.42	10.06	8.84	12.73	10.44	7.55	5.33
β-farnesene	0.06	0.02	5.06	4.92	0.16	0.02	0.76	0.79	0.07	0.32	0.02	0.02	0.07	0.21	0.10	13.84

α-humulene	19.46	14.13	14.48	9.66	11.41	11.69	35.30	21.49	19.28	15.00	11.92	10.46	27.70	23.48	16.55	11.80
caryophyllene oxide	0.10	0.07	0.16	0.08	0.17	0.06	0.30	0.28	0.06	0.05	0.07	0.09	0.23	0.24	0.12	0.15

compound	MT.HOOD		H.MAGN		H.MITT		N.BREW		N.DOWN		STER		WILL		YEOM	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
α-thujene	0.06	0.02	0.06	0.03	0.06	0.02	0.05	0.07	0.06	0.05	0.10	0.02	0.05	0.02	0.13	0.03
α-pinene	0.12	0.10	0.13	0.10	0.13	0.09	0.21	0.16	0.12	0.12	0.16	0.12	0.11	0.08	0.16	0.15
β-pinene	0.03	0.65	0.07	0.71	-	0.62	0.06	0.70	0.04	0.70	0.11	0.47	-	0.34	0.14	0.58
β-myrcene	31.00	48.90	32.06	53.60	25.24	45.41	19.29	38.91	26.74	43.25	29.35	37.76	12.33	19.92	9.09	39.45
3-methylbutyl isobutyrate	0.05	0.73	0.22	0.20	0.05	0.44	0.17	1.55	0.14	-	0.36	0.03	0.06	0.72	0.68	0.63
α-terpinene	0.11	0.02	0.09	0.05	0.16	0.04	-	-	0.19	0.10	0.09	0.02	-	-	0.04	0.06
ρ-cimene	0.12	0.04	0.03	0.03	0.08	0.06	0.09	0.06	0.06	0.04	0.06	0.04	0.05	0.03	0.15	0.04
limonene	0.17	0.17	0.21	0.22	0.19	0.18	0.32	0.28	0.23	0.26	0.21	0.14	0.13	-	0.16	0.30
β-phellandrene	-	0.50	0.16	0.38	-	0.32	-	0.53	-	0.51	0.15	0.44	0.13	0.30	0.13	0.54
cis β-ocimene	-	-	-	-	-	-	-	-	-	0.02	-	-	-	-	0.04	0.04
trans β-ocimene	0.06	0.11	0.17	0.16	0.05	0.03	0.28	0.55	0.11	0.12	0.73	1.29	0.06	0.10	0.15	0.12
γ-terpinene	0.07	0.16	0.07	0.14	0.09	0.14	0.08	0.26	0.03	0.19	0.05	0.25	0.06	0.08	0.19	0.26
nonanal	-	-	-	0.03	-	-	-	-	-	-	-	0.03	0.02	-	0.08	0.10
methyl-6-methyl heptanoate	-	-	-	-	-	-	-	-	0.01	-	0.01	-	-	-	-	-
linalool	0.22	0.20	0.11	0.12	0.50	0.58	0.14	0.14	0.32	0.31	0.15	0.12	0.19	0.15	0.08	0.19
β-thujone	0.04	0.03	0.03	0.02	0.02	-	0.04	-	0.04	0.03	0.07	0.04	-	-	0.04	0.03
camphor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
nerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
methyl nonanoate	0.05	0.05	0.02	0.15	0.02	0.07	0.05	0.13	0.04	0.09	0.07	0.20	0.05	0.03	0.09	0.08
geraniol	-	-	-	-	-	-	-	-	-	-	0.04	0.05	-	-	-	0.17
linalyl acetate	0.03	0.03	0.05	0.04	0.07	0.07	0.05	0.03	0.06	0.02	0.07	0.06	-	-	0.04	0.04
2 undecanone	0.18	0.16	0.22	0.21	0.47	0.40	0.32	0.26	0.35	0.17	0.18	0.15	0.06	0.07	0.32	0.34
methyl-4-decenoate	0.40	0.27	0.61	0.96	0.17	0.32	0.13	0.19	0.31	0.35	0.73	0.68	-	0.10	0.17	0.33
carvacrol	0.05	0.04	0.04	0.02	0.03	0.04	0.07	0.06	0.05	0.04	0.07	0.04	0.05	0.02	0.07	0.05
eugenol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-caryophyllene	12.48	8.27	9.37	6.56	10.50	8.53	12.76	10.75	12.19	10.35	10.31	10.52	14.94	14.53	12.69	8.58
β-farnesene	1.49	3.33	0.06	0.01	0.12	0.10	0.07	0.03	0.54	1.44	1.25	1.70	4.97	5.76	0.06	0.04
α-humulene	27.52	19.02	29.95	21.05	33.39	27.37	27.41	23.29	27.78	23.65	15.41	17.91	38.68	38.85	24.98	19.42
caryophyllene oxide	0.18	0.13	0.06	0.03	0.18	0.12	0.25	0.14	0.14	0.17	0.21	0.22	0.22	0.22	0.17	0.06

Supplementary Table S5 – Volatile Composition of Hop hexane extracts cv. (Brewer's Gold (BREW.GOLD), Cascade (CASC), Centennial (CENT), Challenger (CHALL), Chinook (CHIN), Columbus (COL), Fuggle (FUGG), Hersbrucker Spät (HERS.SP), Mount Hood (MT.HOOD), Hallertau Magnum (H.MAGN), Hallertau Mittelfrüh (H.MITT), Northern Brewer (N.BREW), North Down (N.DOWN), Sterling (STER), Willamette (WILL) and Yeoman (YEOM) expressed in peak volumes normalized on internal standard's peak.

compound	BREW.GOLD		CASC		CENT		CHALL		CHIN		COL		FUGG		HERS.SP	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
α-thujene	15.79	-	-	-	19.20	18.44	20.64	13.24	54.15	62.21	35.14	55.14	19.16	22.40	15.15	11.43
α-pinene	85.34	48.12	42.17	28.18	88.39	58.02	13.89	17.62	58.23	61.84	136.75	110.86	44.30	51.16	31.75	28.93
β-pinene	55.56	355.64	20.31	209.40	19.52	393.01	-	82.80	87.21	322.01	320.98	643.91	13.87	322.59	-	141.11
β-myrcene	36174.2	27642.6	16587.9	16785.7	32749.0	29985.0	2302.3	5301.7	19072.7	26743.6	62072.3	52109.7	12621.1	26735.0	7412.2	9760.0
3-methylbutyl isobutyrate	418.04	336.02	116.01	130.08	112.44	612.58	27.19	430.85	372.90	1126.51	2261.19	302.62	37.90	289.92	18.04	-
α-terpinene	33.08	-	26.57	-	15.72	-	19.70	-	24.75	-	38.64	16.49	22.10	-	29.98	29.01
ρ-cimene	11.59	-	17.72	-	36.21	11.31	24.52	18.08	51.75	16.43	25.49	12.03	36.23	21.58	20.74	16.74
limonene	151.69	89.22	74.31	47.41	168.17	89.80	22.46	32.21	99.04	93.99	265.06	197.25	67.86	88.91	44.50	63.31
β-phellandrene	179.57	138.32	98.25	94.84	185.24	172.18	19.18	99.04	128.81	205.84	366.55	453.02	84.41	173.83	60.17	146.49
cis β-ocimene	50.86	37.38	-	-	-	-	-	16.98	-	-	-	-	-	17.34	-	-
trans β-ocimene	89.02	94.54	16.35	12.97	18.20	17.89	12.45	43.41	18.57	25.23	396.47	411.79	18.85	43.11	-	18.39
γ-terpinene	12.78	30.06	-	40.77	17.41	68.35	-	47.65	12.23	101.52	43.01	158.59	17.95	54.44	24.41	41.37
nonanal	27.63	20.53	-	-	12.97	39.88	-	-	31.43	74.49	247.71	239.61	-	-	-	-
methyl-6-methyl heptanoate	13.51	-	-	-	11.91	-	-	-	-	-	26.52	11.72	-	10.79	-	-
linalool	137.42	95.69	51.36	36.72	157.82	100.52	20.40	27.15	83.18	97.68	310.58	276.53	132.53	226.97	126.49	120.98
β-thujone	-	-	-	-	-	12.44	-	-	-	88.29	13.60	82.38	-	-	-	-
camphor	-	-	-	-	-	-	-	-	-	-	78.79	46.08	-	-	-	-
nerol	-	-	-	-	-	-	-	-	-	-	23.95	21.51	-	-	-	-
methyl nonanoate	-	16.74	-	-	-	47.63	-	37.07	-	53.08	-	132.10	-	40.11	12.44	-
geraniol	129.82	85.29	-	-	201.29	137.33	-	-	86.59	105.99	93.75	57.11	19.29	28.40	-	-
linalyl acetate	-	-	12.33	-	-	19.37	20.54	16.32	30.65	20.77	54.63	49.43	13.70	15.91	12.39	25.26
2 undecanone	-	-	33.11	30.34	19.66	37.87	114.95	94.58	81.62	53.93	246.48	160.28	76.01	92.97	101.00	100.07
methyl-4-decenoate	237.93	135.03	104.81	79.17	222.68	427.51	9.19	74.26	443.50	457.74	1065.0	513.57	172.11	288.75	88.62	15.34

											7					
carvacrol	15.29	13.83	12.38	11.90	18.79	16.51	15.78	14.51	12.59	15.41	24.94	19.50	15.29	19.04	12.06	16.53
eugenol	-	-	-	-	-	-	-	-	-	-	17.04	32.21	-	-	-	-
β-caryophyllene	5789.9	3780.9	1664.1	1048.9	2672.7	3161.7	2882.6	1739.4	5768.2	4759.5	13425.5	8788.1	4966.4	5677.0	1757.5	1306.3
β-farnesene	37.23	11.73	1375.33	1261.71	73.95	13.62	141.93	146.09	41.15	223.40	32.86	18.61	25.55	125.80	22.60	3390.52
α-humulene	11750.3	6586.0	3931.7	2484.1	5168.1	6233.9	6618.4	3961.7	11946.1	9622.2	15913.4	10405.7	10807.2	12766.7	3855.1	2889.0
caryophyllene oxide	61.58	32.92	44.43	20.71	74.37	32.74	56.52	51.78	36.77	32.28	87.65	86.90	90.86	134.37	27.48	37.58

compound	MT.HOOD		H.MAGN		H.MITT		N.BREW		N.DOWN		STER		WILL		YEOM	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
α-thujene	23.79	12.10	37.90	28.61	21.24	10.26	15.33	21.44	31.56	26.86	47.60	11.91	16.80	10.44	45.66	14.02
α-pinene	43.87	40.07	81.59	75.68	48.23	32.45	60.99	52.71	65.87	47.96	76.58	56.31	39.05	30.23	56.17	67.37
β-pinene	12.70	249.87	39.99	525.09	-	232.25	15.88	232.27	22.77	276.85	54.52	218.56	-	135.87	50.45	264.87
β-myrcene	11581.9	18935.7	19421.6	39425.7	9693.7	17003.3	5524.1	12859.7	14439.1	17152.0	14400.1	17477.7	4318.0	7884.9	3266.9	18084.3
3-methylbutyl isobutyrate	18.48	283.83	133.65	148.88	19.17	162.23	48.39	508.34	77.27	-	174.98	22.73	20.64	285.15	244.84	293.05
α-terpinene	39.22	12.58	54.44	34.08	95.33	16.54	-	-	145.79	40.20	43.12	13.98	-	-	18.96	37.91
ρ-cimene	43.20	24.43	24.03	19.39	30.03	21.95	26.88	18.68	30.20	16.49	30.98	18.23	19.87	19.20	52.81	21.14
limonene	63.58	66.33	127.54	157.89	74.17	66.99	92.02	91.89	126.67	102.71	101.57	97.29	45.07	-	59.94	136.76
β-phellandrene	-	192.16	139.75	275.74	-	121.98	-	174.27	-	201.35	101.84	203.86	44.50	120.18	45.99	250.43
cis β-ocimene	-	-	-	-	-	-	-	-	-	12.68	-	-	-	-	20.77	18.50
trans β-ocimene	22.69	43.73	102.82	114.70	18.49	12.54	78.92	182.60	60.22	49.13	356.89	596.66	21.24	40.56	54.19	55.66
γ-terpinene	27.50	62.94	45.31	105.32	34.15	52.29	23.26	86.31	18.11	77.26	23.58	115.43	32.80	30.40	69.80	120.61
nonanal	-	-	-	19.68	-	-	-	-	-	-	-	15.85	10.76	-	28.63	47.90
methyl-6-methyl heptanoate	-	-	-	-	-	-	-	-	13.70	-	13.12	-	-	-	-	-
linalool	84.16	77.72	69.68	90.86	193.15	219.88	40.99	47.19	172.80	121.67	72.60	56.03	66.63	61.52	27.17	86.50
β-thujone	15.56	12.18	19.80	19.56	12.98	-	15.44	-	22.11	13.38	31.78	16.27	-	-	14.79	18.66
camphor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
nerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
methyl nonanoate	19.03	20.12	12.22	107.41	11.09	26.66	13.41	42.72	20.54	34.37	33.46	92.92	17.12	16.22	32.09	36.05
geraniol	-	-	-	-	-	-	-	-	-	-	18.09	22.97	-	-	-	77.21

linalyl acetate	12.48	12.53	29.88	30.24	28.57	24.46	14.88	14.72	34.73	11.27	34.26	27.24	-	-	14.25	18.41
2 undecanone	68.03	61.28	134.80	151.14	179.54	150.92	92.17	86.22	187.57	68.19	87.01	70.35	30.74	26.25	113.31	153.82
methyl-4-decenoate	151.26	104.03	367.41	705.30	63.98	121.48	36.24	61.82	168.59	138.48	359.79	316.05	-	37.82	62.85	150.97
carvacrol	19.71	15.66	24.13	17.21	12.78	13.79	19.23	18.60	26.50	14.80	32.00	18.02	16.84	14.29	27.02	21.64
eugenol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-caryophyllene	4683.9	3215.9	5676.4	4836.5	4035.0	3182.1	3652.6	3550.2	6574.7	4105.2	5084.9	4872.9	5236.8	5755.0	4573.8	3960.0
β-farnesene	551.97	1296.15	33.51	12.58	45.10	35.48	19.73	10.97	288.34	575.24	579.68	791.10	1741.63	2284.14	23.21	26.13
α-humulene	10330.5	7394.3	18149.1	15522.3	12834.0	10213.7	7846.9	7690.7	14970.3	9380.7	7567.4	8301.0	13551.9	15373.1	8997.5	8959.0
caryophyllene oxide	67.72	51.33	37.02	25.20	67.47	45.89	72.08	46.33	77.45	66.23	100.37	99.80	78.08	85.39	61.88	28.34

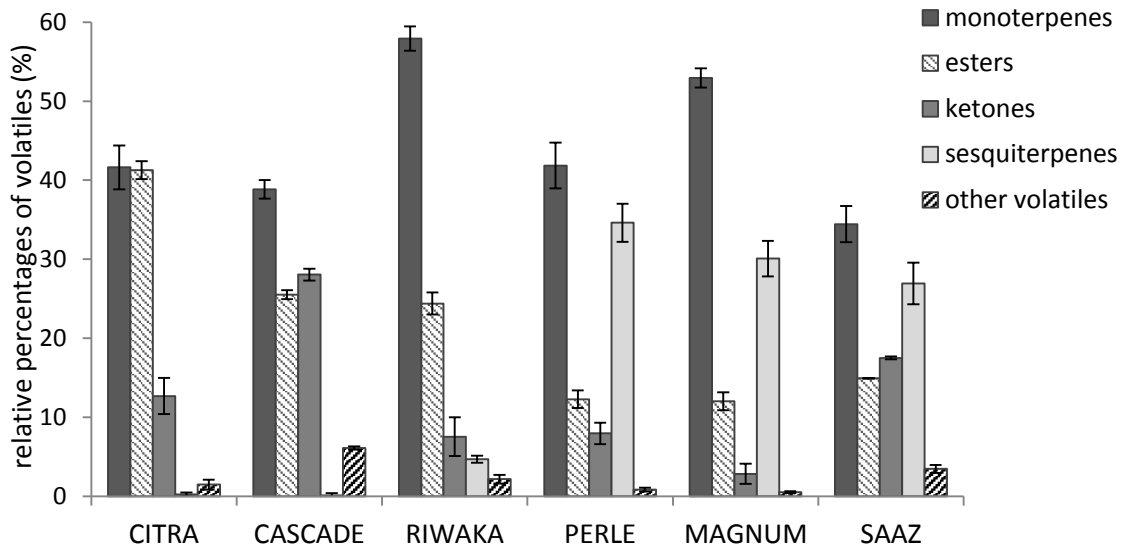
Supplementary Table S6 – Impact of aromatisation of a reference Blond ale beer with hop hydro-alcoholic extracts prepared from different hop cultivars on olfactory descriptors. Asterisks indicate significance at *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; n.s. not significant. Column values with no letter in common differ significantly at P ≤ 0.05 (Tukey's HSD test).

Variety	HOPPY	GRASSY	DRIED GRASS	FRUITY	CITRUSY	FLORAL	SPICY	ODOUR INTENSITY
Cascade	4.00 a	3.14 bc	2.86 bcde	3.43 ab	2.57 ab	2.00 a	2.14 ab	4.63 a
Fuggle	4.43 a	3.43 b	3.86 abc	2.43 bcde	2.86 a	1.57 ab	2.71 b	3.71 ab
Willamette	3.14 ab	2.86 bc	3.25 bcde	1.63 de	1.13 c	1.25 ab	1.00 c	3.86 ab
Chinook	4.00 ab	3.50 b	2.50 de	3.86 a	3.33 a	2.29 a	1.14 c	4.75 a
Hers. Sp	4.50 a	5.14 a	5.00 a	3.14 abc	1.29 c	0.86 b	2.71 ab	4.57 a
Sterlyng	3.50 ab	3.25 bc	2.71 cde	3.00 abcd	2.13 abc	1.50 ab	2.00 abc	4.86 a
Challenger	4.00 a	3.13 bc	3.83 ab	2.29 cde	1.71 bc	1.29 ab	1.75 bc	4.57 a
H. Mittelfrüh	2.71 b	2.00 c	2.14 e	1.71 cde	1.13 bc	1.57 ab	1.29 c	3.57 ab
Columbus	4.57 a	3.86 ab	3.71 bcd	2.43 bcd	1.38 abc	1.75 ab	1.50 c	4.25 a
Mount Hood	3.88 a	2.00 c	3.13 bcde	1.14 e	0.71 c	1.29 ab	1.75 bc	4.00 a
singnificance (ANOVA)	n.s.	***	**	**	**	n.s.	*	n.s.

Supplementary Table S7 – Volatile composition of 70/30 SPE fraction of Citra, Cascade, Riwaka, Perle, Magnum and Saaz essential oils analysed by HS-SPME-GC-MS and GC-O. The identified compounds are presented together with their Kovàts retention indices (RI), retention times (RT) and odour descriptions. Mean values of the GC analyses of duplicate SPE were normalized on the area of the internal standard. **Bold**= if detected by the assessors during GC-O analysis at least 3 times out of 6.

component	KI	RT	CITRA	CASCADE	RIWAKA	PERLE	MAGNUM	SAAZ	odour description
α-pinene	< 1000	15.23	3.74	0.89	6.36	4.92	11.58	3.36	
camphene	< 1000	15.84	3.84	0.91	4.88	1.06	**2.72**	0.71	woody, green
β-pinene	< 1000	17.16	**15.09**	8.84	21.18	46.21	75.90	41.42	waxy hoppy
β-myrcene	< 1000	18.00	**2031.41**	**911.35**	**1973.78**	**2643.87**	**3709.15**	**1432.04**	hoppy
3 methylbutyl 2 methylpropanoate	1001	18.57	10.44	12.61	10.31	8.55	21.53	2.97	-
2 methylbutyl 2 methylpropanoate	1005	18.77	**42.08**	**38.19**	**60.22**	**95.35**	**113.25**	**19.04**	hoppy, spicy
methyl heptanoate	1008	18.95	3.05	0.92	2.36	8.40	1.63	1.72	-
ρ-cymene	1013	19.23	6.17	3.19	**14.23**	1.65	2.19	1.80	hoppy, spicy, grassy
limonene	1019	19.65	6.69	2.07	34.12	14.29	32.17	17.03	-
β-phellandrene	1020	19.66	15.28	6.23	10.53	**16.18**	**39.61**	5.43	-
cis β-ocimene	1039	20.50	8.51	2.74	2.71	**96.43**	61.32	**7.62**	herbal, spicy
γ-terpinene	1049	21.06	1.10	0.56	5.57	5.01	3.78	32.23	-
methyl 6 methylheptanoate	1072	22.09	8.59	3.58	3.12	**26.00**	**29.52**	0.95	sweet, waxy hoppy
terpinolene	1079	22.47	1.61	0.50	1.53	2.04	4.75	9.82	-
n-nonanal (from 60/40 fraction)	1084	22.52	0.52	1.63	2.42	1.96	0.30	3.35	-
linalool (from 60/40 fraction)	1085	22.59	2.90	1.86	8.29	1.79	2.08	1.58	-
perillene	1089	22.88	50.83	**87.24**	36.82	16.49	18.85	63.95	-
2 methylbutyl 3 methylbutanoate	1091	22.99	12.93	10.39	22.31	16.06	26.29	6.44	-
3 methylbutyl isovalerate	1095	23.14	18.24	17.11	**12.93**	6.73	12.65	3.99	citrusy
methyl caprilate	1109	23.82	**28.59**	2.09	4.66	**27.90**	**19.17**	9.99	fruity, citrusy, orange peel
valeric acid	1131	24.90	-	4.00	-	-	-	-	-
hexyl isobutyrate	1135	25.07	8.87	6.08	2.26	11.02	**7.93**	1.24	fruity, grapefruit, waxy green
methyl 2-methyloctanoate	1148	26.60	0.62	0.35	0.08	1.83	0.29	**2.32**	off flavour
2 decanone	1173	26.97	**12.44**	**21.21**	3.29	**26.52**	**12.69**	**72.38**	orange, fresh, citrusy
unknown methylester	1178	27.14	**9.19**	1.45	1.02	2.35	10.97	3.51	-
decanal	1186	27.45	-	2.36	0.32	-	-	0.48	-
heptyl propionate	1189	27.68	1.83	1.16	0.15	**10.77**	4.73	2.96	-

methyl 3 nonanoate	1194	28.00	1.71	1.68	0.66	20.47	9.56	**20.01**	citrusy, grassy
thymol/carvacrol methyl ether	1203	28.50	1.27	2.54	1.76	1.44	1.30	2.82	-
methyl nonanoate	1210	28.63	**143.12**	**7.91**	4.74	**70.57**	**36.35**	**61.97**	fruity, citrusy, floral
heptyl isobutanoate	1233	29.67	12.17	**10.80**	1.69	65.00	17.80	5.11	fruity, citrusy, floral
unknown ketone	1239	29.98	22.24	53.65	34.97	36.66	17.88	**85.29**	citrusy
ethyl citronellate	1244	30.25	1.64	0.80	-	0.38	0.30	-	-
methyl 2 methyl nonanoate	1247	30.33	4.50	4.30	0.48	30.94	2.43	**42.01**	floral, fruity
5 undecen 2 one	1256	30.79	10.74	21.88	7.47	15.46	12.59	**50.64**	floral, fruity
methyl cis geranate	1262	31.04	**4.18**	1.69	4.95	1.36	1.01	2.00	citrusy, floral
methyl 4,6-dimethyl octanoate	1265	31.06	1.34	2.09	0.31	10.34	1.03	15.26	-
2 undecanone	1280	31.73	**474.97**	**473.73**	**151.05**	**335.69**	**121.17**	**493.30**	citrusy
neryl formate	1284	31.80	-	5.12	7.06	-	-	-	-
2 undecanol	1291	31.96	-	5.72	-	-	-	-	-
unknown	1291	31.99	-	-	2.46	5.44	4.66	1.64	-
methyl 4 decenoate	1298	32.49	**939.52**	**138.03**	**110.48**	**365.71**	**386.61**	**427.84**	citrusy
unknown	1299	32.61	**55.54**	**9.34**	16.43	**117.33**	**36.26**	**44.44**	citrusy
methyl trans geranate	1309	32.80	**330.01**	**134.40**	**112.27**	**41.68**	**48.06**	**32.19**	citrusy
methyl caprate	1314	33.27	**435.30**	7.50	6.29	9.49	25.86	16.24	citrusy
octyl 2 methyl propanoate	1333	34.10	16.21	4.66	8.80	**16.79**	**20.59**	1.93	hoppy, herbal, floral
isoamyl n-heptanoate	1337	34.29	3.49	2.35	4.39	2.68	3.89	1.43	-
neryl acetate	1363	35.43	3.91	**53.73**	**12.87**	-	6.72	1.36	floral, citrusy
2 dodecanone	1378	36.04	**57.07**	**73.84**	**14.12**	69.00	17.06	**66.48**	floral, citrusy
methyl 10 undecenoate	1386	36.65	2.05	2.87	0.75	3.55	2.37	5.51	-
β-caryophyllene	1415	37.73	4.09	0.84	47.82	**653.65**	594.08	219.12	spicy
α-humulene	1448	39.09	7.62	2.24	117.79	**1701.47**	1648.43	**994.81**	sweet, spicy
geranyl propionate	1454	39.20	18.57	**30.60**	**42.30**	-	-	-	floral, citrusy
unknown	1473	39.97	**21.44**	7.96	30.72	10.78	7.95	1.25	grassy
2 tridecanone	1479	40.24	**64.31**	**81.54**	**77.38**	74.98	23.17	**103.17**	hoppy, floral
methyl 9-cyclopropylnonanoate	1483	40.39	12.29	-	8.27	-	-	7.00	-
unknown	1489	40.63	34.95	2.75	21.68	20.02	59.87	50.84	-
geranyl butyrate	1496	40.90	52.16	**125.51**	**436.56**	5.09	114.17	3.74	citrusy



Supplementary Figure S1 – Relative composition (% of total peak area) of 70/30 (v/v % ethanol/MQ-water solution) SPE fractions of different hop varieties based on different chemical classes: monoterpenes, esters, ketones, sesquiterpenes and 'other volatiles' (aldehydes, furans and unknowns). Vertical lines show standard deviation of two separate SPE extractions.

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