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Growing hops in Portugal: A strategy for sustainability

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“*Temos, sobretudo, de aprender duas coisas: aprender o extraordinário que é o mundo e aprender a ser bastante largo por dentro, para o mundo todo poder entrar*. “

Agostinho da Silva

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Resumo

O lúpulo comum (*Humulus lupulus* L.) é uma planta trepadeira, dioica, perene, com cinco variedades taxonómicas selvagens reconhecidas, incluindo uma nativa da Europa. A variedade selvagem europeia é a principal ancestral das plantas cultivadas, embora outras contribuições parentais também sejam reconhecidas. As plantas femininas são as únicas cultivadas para a obtenção de inflorescências, denominadas 'cones'. Os cones são a parte comercial mais relevante da planta, destinada principalmente à indústria cervejeira.

Em Portugal, o lúpulo cresce de forma espontânea ao longo das margens dos rios, em particular no norte do país, o que evidencia boas condições ecológicas para a produção de lúpulo. No passado recente, o lúpulo foi uma das principais culturas do Norte de Portugal, com um contributo significativo para a economia nacional. Vários fatores contribuíram para o declínio desta cultura desafiante, que nunca recebeu atenção relevante da comunidade científica nacional. Atualmente, a capacidade produtiva instalada representa menos de 10% da procura nacional da indústria cervejeira e está localizada no Nordeste de Portugal. Um restrito número de agricultores cultiva aproximadamente um total de 13 ha de lúpulo. Os dois produtores principais estão instalados em Pinela (Bragança) e em Vinhas (Macedo de Cavaleiros). Ambos cultivam a cultivar amarga 'Nugget', que está bem estabelecida nas condições mediterrâneas desta região.

Apesar da boa adaptação da cultivar Nugget, o desempenho produtivo dos atuais campos de lúpulo é afetado por diversos fatores. A heterogeneidade dos campos de lúpulo é um dos fatores sem um diagnóstico de causas conhecidas. A rega "à manta" e questões nutricionais podem ser alguns dos fatores que influenciam a resposta produtiva da cultura e que precisam ser esclarecidos. O pH do solo é outro fator considerado relevante na produção de lúpulo. Normalmente a aplicação de calcário é recomendada em solos ácidos, embora poucos estudos tenham abordado este assunto na cultura do lúpulo e nenhum em condições mediterrânicas. As interações entre irrigação, acidez do solo e distúrbios nutricionais são normalmente encontradas na cultura do arroz irrigado por inundação e parecem ser suficientemente relevantes para serem estudadas também na cultura do lúpulo.

A fertilização do lúpulo é outro fator relevante numa cultura com grande exigência nutricional. A fertilização do lúpulo na região tem-se baseado principalmente na aplicação de fertilizantes convencionais ao solo. No entanto, os agricultores locais estão a começar a utilizar fertilizantes aplicados por via foliar em complemento aos seus programas de fertilização. Formulações comerciais com nutrientes minerais ou compostos bioestimulantes para as plantas, como extratos de algas marinhas ou aminoácidos, são algumas das alternativas. A aplicação foliar de potássio (K) durante a floração também tem sido uma prática comum entre os agricultores. No entanto, pouco se sabe sobre o efeito da aplicação destes produtos na cultura do lúpulo, principalmente em condições mediterrânicas.

Estratégias para gerir e valorizar os resíduos orgânicos produzidos nas explorações de lúpulo devem também ser consideradas. Após a colheita, uma grande quantidade de folhas e caules permanecem como resíduos orgânicos sem um aproveitamento específico. As folhas são muito ricas em nutrientes e poderão ser adequadas para usar em misturas para compostagem, agregando valor aos demais recursos orgânicos produzidos nas explorações.

Apesar dos desafios e constrangimentos que os produtores de lúpulo atuais enfrentam, existem também novas oportunidades a surgir devido às mudanças no mercado. O mercado mundial de lúpulo tem sido dominado por um pequeno grupo de grandes produtores e cervejarias multinacionais que dão preferência a cultivares de amargo, com elevado teor de alfa (α)-ácidos. Contudo, nos últimos anos o mercado de lúpulo passou por uma revolução com a expansão do mercado de cerveja artesanal. Os cervejeiros artesanais estão mais interessados em cultivares com aroma forte, sabores diferentes e menor teor de α-ácidos. Consequentemente, em alguns países a procura alterou-se desde as cultivares de amargo destinadas a um grupo restrito de grandes cervejeiros, para cultivares aromáticas vendidas em pequenas quantidades para um número maior de pequenos cervejeiros. Portugal também acompanha este movimento, o que tem originado um interesse renovado por esta cultura. Novos produtores, em pequena escala, estão atualmente a iniciar o cultivo de lúpulo e a testar diferentes cultivares. As necessidades de lúpulo deste nicho de mercado, em termos absolutos, são menores, mas podem ser mais adequadas à estrutura fundiária do norte e centro de Portugal. É, assim, estratégico testar o desempenho agronómico de cultivares de aroma de lúpulo e compará-lo com o da cultivar ‘Nugget’, estabelecida em Portugal desde o início da década de 1990.

Tendo em conta os desafios e oportunidades atuais, esta tese centra-se em dois objetivos principais: i) avaliar a situação dos atuais campos de lúpulo, nomeadamente os constrangimentos ao desempenho produtivo das plantas, e avaliar alternativas para melhorar a produtividade da cultura e gerir os resíduos orgânicos produzidos nas explorações de lúpulo; e ii) analisar as decisões e práticas agronómicas para o sector do lúpulo em Portugal, tendo em conta a evolução do mercado do lúpulo e o crescente interesse por cultivares aromáticas. Para cumprir os objetivos principais, foram estabelecidas linhas de trabalho específicas que incluíram: a avaliação da performance

produtiva e constrangimentos dos atuais campos de lúpulo (capítulo 3); o efeito da fertilização foliar na cultura (capítulo 4); a reciclagem dos resíduos da colheita do lúpulo através da compostagem (capítulo 5); o desempenho agronómico e químico de cultivares de aroma de lúpulo recém-introduzidas (capítulo 6); e a composição fenólica dos cones de lúpulo em resposta a vários fatores de variabilidade agroambiental (capítulo 7).

Na primeira linha de trabalho foram investigadas as causas da heterogeneidade dos campos de lúpulo que afetaram a produtividade através da avaliação de várias propriedades do solo e do estado nutricional das plantas (capítulo 3). Os resultados indicaram que o fraco desenvolvimento das plantas provavelmente resultou dos efeitos do alagamento, devido à privação de oxigênio na zona de enraizamento e ao aumento do efeito potencialmente fitotóxico do manganês (Mn) e do ferro (Fe). Os elevados níveis de Mn no solo e nos tecidos foram associados às parcelas de plantas de vigor fraco e os níveis elevados de Fe foram associados a plantas de vigor moderado. A deficiente drenagem dos solos e a deficiente distribuição de água causada pelo sistema de irrigação à manta parecem ter contribuído para a variabilidade no estado de oxidação-redução do solo, afetando os teores de Mn e Fe.

O efeito do alagamento em gradientes nas propriedades do solo e nas plantas ao longo das linhas de irrigação de aproximadamente 150 a 180 m de comprimento também foi avaliado. Adicionalmente, foi considerada a hipótese de que a aplicação de calcário poderia atenuar potenciais gradientes gerados pela irrigação. Foram registadas variações significativas nas propriedades do solo, na composição elementar das plantas e na produção de matéria seca total e de cones em diferentes pontos de amostragem ao longo das linhas, mas não em gradiente contínuo. Assim, as diferenças não puderam ser atribuídas ao efeito da irrigação, mas sim a uma variação errática de alguns dos constituintes do solo, como areia, limo e argila. A rega por inundação e as mobilizações do solo parecem ter afetado negativamente a porosidade e a densidade do solo, mas não em gradiente ao longo da linha. No entanto, a porosidade e a densidade aparente do solo demonstraram estar associadas, de forma positiva e negativa, respetivamente, à produtividade da cultura. A aplicação de calcário aumentou ligeiramente o pH do solo, mas não teve um efeito relevante em outras propriedades do solo e nas plantas, talvez devido à reduzida quantidade aplicada.

A aplicação de fertilizantes foliares nos campos de lúpulo foi avaliada conforme descrito no capítulo 4. Foram testados dois fertilizantes foliares aplicados através de pulverização, um com base em extratos da alga *Ascophyllum nodosum* (L.) e outro rico em nutrientes, com o objetivo de melhorar a uniformidade dos campos de lúpulo e restaurar a produtividade das parcelas com fraco desenvolvimento. Os fertilizantes

foliares foram aplicados em complemento ao plano de fertilização do agricultor. O uso desses fertilizantes foliares não demonstrou efeito significativo sobre as variáveis relacionadas com o desempenho fotossintético das plantas e concentração de α- e beta (β)- ácidos nos cones. A composição elementar dos tecidos vegetais foi menos influenciada pelos fertilizantes foliares do que pelo efeito do campo e do ano. O uso de fertilizantes foliares à base de extratos de algas apenas aumentou significativamente a produtividade nas parcelas com fraco desenvolvimento.

O uso de fertilizantes foliares ricos em aminoácidos e em K foi avaliado numa das explorações de lúpulo. Quatro aplicações do fertilizante foliar rico em aminoácidos foram realizadas em substituição de uma aplicação de azoto (N) de cobertura de ~70 kg N ha^{-1}, que é normalmente efetuada pelo agricultor. O fertilizante foliar rico em K foi aplicado uma vez, na fase de desenvolvimento dos cones, como um suplemento ao plano de fertilização do agricultor. O fertilizante foliar enriquecido com aminoácidos manteve a produção de matéria seca da cultura nos níveis do tratamento testemunha e aumentou a concentração de ácidos amargos dos cones com menor uso de N. O fertilizante foliar de K não aumentou a produção de matéria seca dos cones, o tamanho dos cones ou a concentração de ácidos amargos. A concentração de K nos tecidos não foi significativamente afetada pelos tratamentos foliares, enquanto a aplicação de K pareceu aumentar a absorção de N, tendo folhas e caules sido os tecidos de alocação predominante do nutriente. Os resultados parecem enfatizar a importância dos aminoácidos na biossíntese dos ácidos amargos, enquanto o K parece ter um importante papel secundário, talvez relacionado ao metabolismo do N e à sua incorporação em aminoácidos.

A utilização de folhas de lúpulo para produzir compostados de qualidade, adequados para aplicar como fertilizantes no crescimento de plantas, foi também avaliada conforme descrito no capítulo 5. As folhas de lúpulo foram misturadas com estrume de vaca e palha de trigo em várias combinações. A cinza de lúpulo, resultante do procedimento usual do agricultor de queimar os caules após a colheita, também foi utilizada para avaliar o seu efeito no processo de compostagem. Teve-se por objetivo estabelecer diretrizes para os agricultores poderem gerir e usar melhor esses valiosos recursos orgânicos. A qualidade dos compostos foi avaliada em relação aos efeitos sobre alface (*Lactuca sativa* L.) cultivada em vasos durante dois ciclos vegetativos consecutivos. A mistura de folhas de lúpulo com estrume de vaca produziu um composto maturado após nove meses de compostagem que pode ser utilizado em culturas hortícolas, independentemente da proporção de matéria-prima, devido à sua baixa e semelhante relação carbono (C)/N. As misturas de folhas e palha em proporções inferiores a 2:1 resultaram em compostos que não maturaram adequadamente, apresentando elevada relação C/N. Assim, para reduzir o tempo de compostagem e aumentar a qualidade do composto, a proporção folhas/palha deve ser a mais elevada possível, no mínimo 2:1. Alternativamente, o processo de compostagem pode demorar mais tempo, ou o composto com deficiente maturação pode ser aplicado com antecipação suficiente à sementeira para que os processos biológicos complementares possam ocorrer no solo. A cinza dos caules de lúpulo não beneficiou o processo de compostagem e demonstrou não justificar a sua inclusão nas misturas, podendo continuar a ser usado isoladamente como fertilizante.

O desempenho agronómico e o perfil químico de quatro cultivares de aroma de lúpulo foram também avaliados conforme descrito no capítulo 6. As cultivares recentemente introduzidas (‘Columbus’, ‘Cascade’ e ‘Comet’) foram comparadas com a cultivar Nugget, para produção de biomassa, composição elementar dos tecidos e concentração de ácidos amargos e nitratos (NO_3^-) nos cones. A cultivar Comet foi a mais produtiva, com maior produção de matéria seca total, produção de cones e peso seco de cones individuais. O ano influenciou os valores médios da produção de matéria seca e da concentração de α- e β- ácidos nos cones, com Cascade a apresentar a maior sensibilidade entre as cultivares. Colombus exibiu os teores mais elevados de α-ácidos, seguida de Nugget, Comet e Cascade, com valores próximos aos intervalos normais internacionalmente aceites para todas as cultivares. Os critérios de acumulação de nutrientes nos tecidos dos cones e das folhas parecem ser um fator de diferenciação entre cultivares com influência na biossíntese dos ácidos amargos e na produção de biomassa. De maneira geral, as quatro cultivares apresentaram desempenho notável em termos de produtividade de matéria seca e concentração de ácidos amargos nos cones quando comparadas aos padrões internacionais.

Foram instalados quatro ensaios experimentais que permitiram avaliar o efeito de importantes fatores de variação sobre o teor de fenóis e a composição fenólica dos cones de lúpulo e a relação com a composição elementar dos cones de lúpulo (capítulo 7). Os fatores considerados foram as manchas de diferente vigor das plantas, fertilizantes foliares ricos em algas e nutrientes, efeito da calagem, cultivar e ano. As concentrações de fenóis totais nos cones de lúpulo foram influenciadas significativamente pela maioria dos fatores experimentais. Os fertilizantes foliares e a calagem estiveram entre os fatores que menos influenciaram tiveram.

Em resumo, os resultados da avaliação da situação nos campos de lúpulo atuais destacaram os efeitos negativos do sistema de rega à manta nas propriedades do solo e na produtividade da cultura. As alternativas testadas para melhorar a produtividade da cultura e gerir os resíduos orgânicos forneceram diretrizes para os agricultores e permitem delinear futuras linhas de investigação. Atendendo à evolução do mercado de lúpulo, a introdução de cultivares aroma pelos atuais e novos produtores parece ser uma inevitabilidade. As cultivares de aroma testadas apresentaram bom desempenho em comparação com a cultivar Nugget, tradicionalmente cultivada na região, mas mais estudos são ainda necessários para aumentar a informação disponível sobre estas e outras cultivares com potencial para os cervejeiros artesanais.

Palavras-chave: *Humulus lupulus*, produtividade do lúpulo, propriedades do solo, nutrição das plantas, crescimento irregular, rega à manta, calagem, fertilização foliar, resíduos orgânicos, compostagem, cultivares, qualidade dos cones, composição fenólica.

Abstract

The common hops (*Humulus lupulus* L.) is a dioecious, perennial climbing plant, with five wild taxonomic varieties recognised, including one native to Europe. The European wild variety is the main ancestor of the cultivated plants, though other parental contributions are also recognized. The female plants are the ones cultivated for their inflorescences, known as ‘cones’. The cones are the most relevant commercial part of the plant, mainly destined to the brewing industry.

In Portugal, hop plants grow spontaneously along riverbanks, particularly in the north of the country, which evidences the good ecological conditions for hop production. In the recent past, the hop was one of the main crops in the North of Portugal, contributing to the national economy. Several factors contributed to the decline of this challenging crop, that have never received relevant attention from the national scientific community. At present, hop production in Portugal represents less than 10% of the national demand for the brewing industry and is located in the North-eastern (NE) Portugal. Few farmers grow approximately 13 ha of hops, the two most important located in Pinela (Bragança) and Vinhas (Macedo de Cavaleiros). Both grow the bitter cultivar Nugget that is well-establish in the Mediterranean conditions of this region.

Despite the good adaptation of the Nugget cultivar, the productive performance of the current hop fields is negatively affected by several factors. The heterogeneity of the hop fields is one factor without a diagnostic of the causes being known. Flooding irrigation and nutritional issues might be factors influencing crop response that need to be clarified. Soil pH is another factor that is considered to be relevant in hop production. Usually, lime is recommended in acidic soils, though few research have addressed this issue in hop crop, and not under Mediterranean conditions. The interactions between flooding, soil acidity and nutritional disorders are usually found in rice crop under flooding conditions and seem to be relevant enough to be also studied in the case of hops.

The fertilization of hops is another relevant factor in a crop that has high nutritional requirements. The fertilization of hops in the region has been mainly based on applying conventional solid fertilizers to the soil. However, local farmers are currently starting to use foliar sprays to complement their fertilization programmes. Commercial formulations with mineral nutrients or plant biostimulant compounds such as seaweed extracts or amino acids are alternatives. Foliar application of potassium (K) at early flowering has also been common in the region. However, little is known about the effect of applying these products in hop crop, particularly in the Mediterranean conditions.

Strategies for managing and valuing organic wastes produced on hop farms should also be considered. After the harvest, many leaves and stems remain as organic waste without a specific utilization. The leaves are very rich in nutrients and may be suitable for composting mixtures, adding value to other organic resources produced in the farm.

Besides the challenges and constraints that current hop farmers are experiencing new opportunities arise due to the market changes. The world hop market has been dominated by a small group of large producers and multinational brewers that have favoured bittering cultivars, with a high content of alpha (α)-acids. However, in the last years, the hop market has undergone a revolution with expanding the craft beer market. Craft brewers are more interested in cultivars with a strong aroma, different flavours and fewer α-acids. Hence, the demand in some countries has changed from bittering cultivars destined to a few macrobrewers, to aroma cultivars sold in small quantities to a larger number of brewers. Portugal is no exception to this movement, and currently, there is a renewed interest in this crop. On a small scale, new producers are at the present starting to grow hop plants and testing different hop cultivars. The hop needs of this niche market, in absolute terms, are smaller, but they may be more adjusted to the productive Portuguese structure. Therefore, it is strategic to test the agronomic performance of hop aroma cultivars and compare them with the well-established Nugget.

Considering the present challenges and opportunities, this thesis focus on two main purposes: i) to assess the situation of the current hop fields, namely the constraints to the productive performance of the plants, and to evaluate alternatives to improve crop productivity and to manage the organic wastes generated in hop farms; and ii) to analyse agronomic decisions and practices for the hop sector in Portugal, considering the evolution of hop market and the growing interest in aroma cultivars. To meet the main purposes, specific lines were established, and they included: the evaluation of the productive performance and constraints of the current hop fields (chapter 3); the effect of foliar fertilization on hop plants (chapter 4); the recycling of hop harvest wastes through composting (chapter 5); the agronomic and chemical performance of newly introduced hop aroma cultivars (chapter 6); and the phenolic composition of hops as a response to several agro-environmental variation factors (chapter 7).

In the first working line, the causes behind the heterogeneity of hop cultivated fields affecting crop yield were investigated by determining selected soil properties and plant nutritional status (chapter 3). The results indicated that the poor development of the plants was probably the result of the effects of flooding, giving rise to oxygen (O_2) deprivation in the rooting zone and increasing the potential toxic effects of manganese (Mn) and iron (Fe). The high levels in soil and plant tissues of Mn were associated with the plots of weaker vigour plants, and the high Fe levels were related to plants of fair vigour. The poor drainage of the soils and deficient water distribution caused by the surface irrigation system seems to have contributed to the variability in the soil redox potential, affecting the availability of Mn and Fe.

The effect of flooding on the creation of gradients in soil and plant variables along the irrigation rows of 150 to 180 m in length was also evaluated. Moreover, it was considered the hypothesis that the application of lime could attenuate the main gradients created by irrigation. Significant variations were found on soil properties, plant elemental composition and total and cone dry matter yield (DMY) at different sampling points along the rows, but not in a continuous gradient. Hence, the differences cannot be attributed to the effect of irrigation but rather to an erratic variation in some of the soil constituents, such as sand, silt and clay. Flooding irrigation and soil tillage affect soil porosity and bulk density negatively, but not in a gradient along the row. However, porosity and bulk density were found to be, respectively, positively and negatively associated with crop productivity. Lime application raised slightly the soil pH but did not have a relevant effect on soil and plants, perhaps because of the small amount applied.

The application of foliar sprays was evaluated as described in chapter 4. Two foliar sprays, one based in extracts of the algae *Ascophyllum nodosum* (L.) and the other particularly rich in nutrients, were tested to improve the homogeneity of hop fields and restore the productivity of the poorly developed patches. The foliar sprays were applied as a complement to the farmer's fertilization plan. The results of the use of these foliar sprays did not show any significant effect on variables related to the photosynthetic performance of the plants and cone α- and beta (β)-acid concentrations. Foliar sprays little influenced the elemental composition of plant tissues compared to the effects of the plot and year. The use of foliar sprays based on seaweed extracts only increased significantly crop yield in the plot of weak plant vigour.

The use of amino acid and K-rich foliar sprays was also evaluated in a hop field. Four applications of an amino acid-rich foliar spray were tested in place of a second side dress N application of ~70 kg N ha^{-1}, the farmer usually does. The K-rich foliar spray was applied once at the cone developing stage as a supplement to the farmer's fertilization plan. The amino acid-enriched foliar spray maintained crop DMY at the levels of the control treatment and increased cone bitter acid concentration using a reduced rate of N. Foliar K did not increase cone DMY, cone size or bitter acid concentration. Tissue K concentration was not significantly affected by foliar treatments, whereas the application of K seemed to increase N uptake, with leaves and stems being the predominant allocation tissues. The results seem to emphasize the importance of amino acids in the biosynthesis of bitter acids, while K appeared to play an important secondary role, maybe related to N metabolism and its reduction into amino acids.

The use of hop leaves to produced quality compost, suitable to be applied as a soil amendment, was evaluated as described in chapter 5. The hop leaves were mixed with cow manure and wheat straw in several combinations. The hop ash resulting from the usual farmer's procedure of burning the stems after harvest was also used to evaluate the potential effect in the composting process. The aim was to establish guidelines on how farmers can manage the raw materials and better use these valuable organic resources. The quality of the composts was evaluated concerning the effects on lettuce (*Lactuca sativa* L.) grown in pots over two consecutive cycles. The mixture of hop leaves with cow manure produced a stable compost after nine months of composting, which may be used in horticultural crops, irrespective of the proportion of raw materials, due to their low and similar carbon (C)/N ratios. The mixtures of leaves and straw in proportions of less than 2:1 resulted in composts that did not mature correctly, showing a high C/N ratio. Thus, to reduce composting time and increase the quality of the compost, the ratio leaves/straw should be as high as possible, at least 2:1. Alternatively, either the composting process should take longer, or the poorly-matured compost can be applied far in advance of sowing a crop so that complementary biological processes can take place in the soil. Ash from hop stems did not benefit the composting process and proved itself not worth using in mixtures.

The agronomic performance and chemical profile of four hop aroma cultivars were assessed under the Mediterranean conditions of NE Portugal, as described in chapter 6. The newly introduced cultivars (Columbus, Cascade and Comet) were compared with the well-established Nugget cultivar for biomass production, elemental tissue composition and bitter acid and NO_3^- concentration in the cones. Comet was the most productive cultivar with the highest total DMY, cone yield and dry weight of individual cones. The year affected the average values of DMY and the concentration of α- and β-acids in the cones, with Cascade showing the highest sensitivity between cultivars. Columbus exhibited the highest levels of α-acids, followed by Nugget, Comet and Cascade, with values close to the normal ranges internationally accepted for all cultivars. The nutrient accumulation criteria in cone and leaf tissues seem to be a differentiating factor between cultivars with influence on bitter acid biosynthesis and biomass production. Overall, the four cultivars showed notable performance in terms of DMY and bitter acid concentration in the cones compared to international standards.

Four field trials allowed evaluating the effect of important variation factors on hop phenol and phenolic composition and the relationship with the elemental composition of hop cone (chapter 7). The factors considered in the field trials were the patches of different plant vigour, algae- and nutrient-rich foliar sprays, liming, cultivar and year. The concentration of total phenols in hops were significantly influenced by most experimental factors. Foliar sprays and liming were among the factors that least influenced the measured variables.

In summary, the results of the evaluation of the situation in the current hop fields highlighted the negative effect of the surface irrigation system on soil properties and crop productivity. The alternatives tested to improve crop productivity and manage organic waste provided guidelines for farmers and outlined perspectives for future research. Regarding the evolution of the hop market, the introduction of aroma cultivars by current and new hop farmers seems to be an inevitability. The aroma cultivars tested showed good performance compared to the well-establish Nugget, but research should continue in Portugal to obtain data from more cultivars.

Keywords: *Humulus lupulus*, hop productivity, soil properties, plant nutrition, irregular growth, flooding irrigation, liming, foliar fertilization, organic wastes, composting, cultivars, cone quality, phenolic composition.

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Contents

Figures

Chapter 6.1.

Chapter 7.1.

Tables

Chapter 2.

Chapter 3.1.

Chapter 3.2.

Abbreviations

α – Alpha

β – Beta

δ – Delta

ε – Epsilon

3-CQA – 3-*O*-Caffeoylquimic acid

4-CQA – 4-*O*-Caffeoylquimic acid

5-CQA – 5-*O*-Caffeoylquimic acid

+K – Potassium-rich foliar spray

$[M-H]^-$ – negative mode of quasi-molecular ion

A

AA – Amino acid-based foliar spray

Al – Aluminium

Algae – Algae-rich foliar spray

ANOVA – Analysis of variance

B

B – Boron

C

C – Carbon

C (Section 4.2. and 5.1) – Control

C (Section 7.1.) – Cultivar

C (Section 7.1.) – Not limed treatment

Ca – Calcium

CaO – Calcium oxide

$Ca(NO_3)_2$ – Calcium nitrate

Cas – Cascade

CEC – Cation-exchange capacity

cis 3-*p*-CoQA – *cis* 3-*p*-Coumaroylquimic acid

Cl^- – Chloride ion

CO_2 – Carbon dioxide

CO_3^{2-} – Carbonate ion

Col – Columbus

Com – Comet

Cu – Copper

D

D1 – Single rate of compost corresponding to 20 t/ha dry weigh

D2 – Double rate of compost corresponding to 40 t/ha dry weigh

DM – Dry matter

DMY – Dry matter yield

E

EBC – European Brewery Convention

EC – Electrical conductivity

ER – Egner-Rhiem

EDTA – Ethylenediamine tetraacetic acid

F

F (Section 3.1, 4.2., and 7.1.) – Fair

F (Section 3.2 and 7.1.) – Field

F (Section 6.1.) – Function

Fe – Iron

F_M – Maximal fluorescence

Fnut – Nutrient-rich foliar spray

F_0 – Minimal fluorescence

FT – Foliar treatment

F_V – Variable fluorescence

F_V/F_M – Maximum efficiency of the photosystem II

F_V/F_0 – Activity of photosystem II

G

G – Good

GAE – Gallic acid equivalents

H

H_2O – Water

HIPT-1 – *Humulus lupulus prenyltransferase-1*

HM – Hops leaves and cow manure

HPLC – High performance liquid chromatography

HS – Hop leaves and wheat straw

HSA – Hop leaves, wheat straw, and hop stems ash

HSD – Honest significant difference

I

I – Fluorescence at 30 ms

IBM – International Business Machines

J

J – Fluorescence at 2 ms

K

K – Potassium

K_2O – Potassium oxide

K-3-G – Kaempferol-3-*O*-glucoside

K-3-2Rh-Ru – Kaempferol-3-*O*-(2-rhamnosyl)-rutinoside

K-3-6M-G – Kaempferol-3-*O*-(6 -O-malonyl)-glucoside

KCL – Potassium chloride

K-Ru – Kaempferol-3-*O*-rutinoside

L

L – Limed treatment

LQARS – Laboratório Químico Agrícola Rebelo da Silva

M

Mg – Magnesium

MgO – Magnesium oxide

Mn – Manganese

MS^2 – Tandem mass spectrometry

m/z – Mass-to-charge ratio

N

N – Nitrogen

N_2 – Dinitrogen

Na – Sodium

Na^+ – Sodium ion

NDVI – Normalized Difference Vegetation Index

NE - North-eastern

NH_4^+ – Ammonium

NH_4NO_3 – Ammonium nitrate

NO_3^- – Nitrate

NO_x – Nitrous oxides

Nug – Nugget

O

O – Origin fluorescence at 20 μs

O_2 – Oxygen

OL – Olsen

OM – Organic matter

P

P – Phosphorus

P (Section 4.1. and 4.2.) – Maximum fluorescence

P_2O_5 – Phosphorus pentoxide

PC – Principal component

PCA – Principal component analysis

PCM – Protein competition model

PV – Plant vigour

Q

Q-3-2Rh-Ru – Quercetin-3-*O*-(2-rhamnosyl)-rutinoside

Q-3-6M-G – Quercetin-3-*O*-(6 -O-malonyl)-glucoside

Q-3-H – Quercetin-3-*O*-hexoside

Q-3-Ru – Quercetin-3-*O*-rutinoside

S

SO_4^{2+} – Sulphate ion

SPAD – Soil and Plant Analysis Development

SPSS – Statistical Package for the Social Sciences

T

TOC – Total organic carbon

trans 3-*p*-CoAD – *trans* 3-*p*-Coumaroylquimic acid

U

UV – Ultraviolet

UV-Vis – Ultraviolet visible

V

VG – Very good

W

W – Weak

Y

Y – Year

Z

Z – Zinc

Chapter 1:

General introduction

The common hops (*Humulus lupulus* L.) is a dioecious, perennial, climbing plant, native from Europe and Western Asia, belonging to the Cannabaceae family (Small, 2016). Several uses have been given to hop plants throughout the ages, including to female inflorescences (cones), leaves and stems, although the main prevalent use to date has been for beer production (Delyser and Kasper, 1994; Sirrine et al., 2010). The cones of the female hop plants are the most important part for brewing purposes. They contain resins and essential oils, synthesized in the lupulin glands, which are the compounds responsible for beer bitterness and flavour (Almaguer et al., 2014). Hop cones also contain other important compounds such as polyphenols, which contribute to beer flavour, colour, taste and haze formation and strong antioxidant power (Almaguer et al., 2014; De Keukeleire, 2000). Other alternative uses for hops are also considered since hops contain many bioactive substances and compounds with interest in improving health, medicinal applications, and even being used as a pesticide (Chadwick et al., 2006; Karabín et al., 2016; Teghtmeyer, 2018). The taxonomy, chemistry and uses of hops and their importance in beer production were reviewed in **Section 2.1.**

Hop plants grow spontaneously in Portugal, an evidence of the good ecological conditions to produce hop cones in quantity and quality, the crop has already been highly relevant to the national and local economy (Rodrigues et al., 2015). Currently, hops production represents less than 10% of the demand of the national brewing industry and is located in the NE of Portugal, in the Bragança region. It is also relevant that, in addition to the national beer industry demand, the craft beer market is expanding (Euromonitor, 2020; Martins, 2015), which represents an opportunity for farmers. However, hop is a very demanding and challenging crop for new growers, who should take advantage of the knowledge of the current producers. Hence, it is of great interest that this crop continues to be grown in Portugal and that the new and actual producers can take advantage of the potential opportunities of an expanding market.

The expanding of a challenging crop, such as hops, in countries that are today relevant producers in the world market, would certainly not have been possible without investment in key areas, as occurred in genetic improvement (Darby, 2005; Jeske, 2007; Seigner et al., 2009), crop protection (Gent et al., 2014; Henning et al., 2016; Pethybridge et al., 2002), soil fertility management (Bavec et al., 2003; Čeh, 2014) and morphological, genetic and chemical characterization (Kavalier et al., 2011; Killeen et al., 2014; Ligor et al., 2014; Natsume et al., 2014).

In Portugal, in more than 50 years of history of cultivation of hops, the first 20 years of great success, with the national production ensuring the demand of the brewing industry, hops have never received relevant attention from the national scientific community. The current fields under production were installed for more than 20 years, producing the bitter cultivar ‘Nugget’ sold in the form of extract (produced in Germany) to the national beer industry (Rodrigues and Morais, 2015). At a global scale, the preference for bitter cultivars, with a high α-acids percentage, has dominated the world market, hop acreage and breeding efforts (Hieronymus, 2012; Seigner et al., 2009). However, in recent years, a turnaround has occurred due to the exponential growth of the craft beer market, changing the demand from bittering to aroma cultivars (Brown, 2015; Garavaglia and Swinnen, 2017; Teghtmeyer, 2018). Expanding the craft beer market opens new opportunities for small-scale growers to produce and supply desirable cultivars at more favourable prices (Small, 2016). Portugal is following this trend and, not only the current producers are starting to introduce aroma cultivars, but there is a rising interest in the crop by new potential producers. Therefore, it is strategic to test the agronomic performance of hop aroma cultivars and compare them with the well-established Nugget. In hop aroma cultivars, the market is not as monopolized as for bitter hop cultivars, being possible to sell hops in the form of dry cones or pellets. The hop needs of this niche market, in absolute terms, are smaller, but they may be more adjusted to the productive Portuguese structure. From the beginning until now, the cultivation of hops in Portugal, the cropping techniques in use, and the challenges and opportunities of this market sector were reviewed in **Section 2.2**.

The fertilization of hops is an issue of great relevance and has been mostly based on applying conventional fertilizers to the soil. However, due to the widespread of innovative fertilizing products for foliar application, local farmers are currently starting to use foliar sprays to complement their fertilization programmes. The formulations for foliar application are currently huge, and the application frequency is increasing (Dean, 2019). Commercial formulations with macro and micronutrients or plant biostimulant compounds such as seaweed extracts are some alternatives. The potential beneficial effects of such products in crop productivity and fruit quality have been studied in several crops (Battacharyya et al., 2015; Bindraban et al., 2015; Smoleń, 2012). However, little is known about applying these products in hop crop, particularly in the Mediterranean conditions (Procházka et al., 2018).

Amino acid-based fertilizers are also having an increasing use among hop producers in Portugal. Amino acids are also plant biostimulants, which can increase plant growth and development and reduce the need for fertilizers (du Jardin, 2015; Halpern et al., 2015). The use of K based foliar sprays is also increasing at a global scale, mainly due to their potential positive effects on crop quality (Dean, 2019). Positive effects of K on the growth phase of flowers and fruits have been observed in previous studies

(Hasanuzzaman et al., 2018; Hawkesford et al., 2012), including in the development of hop cones (Gingrich et al., 1994). In the region of Bragança, the use of K-based foliar sprays, late in the growing season, is a current practice among hop farmers, without the results ever being monitored by experimental work.

The application of foliar fertilizers can be a complementary alternative to improve the production and the quality of the cones and eventually for the restoration of the areas of poor productivity, being necessary to evaluate the effectiveness of these treatments. An overview of the effect of foliar fertilization in crop growth and yield with emphasis on plant biostimulants was presented in **Section 2.3**.

Besides fertilization, other factors are affecting the productive performance of the current hop fields. They should be identified, and at the same time alternatives to improve crop productivity must be evaluated. The heterogeneity of hop fields is one reason for the current reduced performance of the plants, and it may be related to nutritional issues that need to be clarified. Different gradients of soil fertility and plant nutritional status may occur due to flooding irrigation and soil acidity. In Portugal, hop farmers usually irrigate the crop by flooding the space between rows, which negatively impacts the soils and demands frequent tillage, with consequences for soil properties, plant nutritional status and plant health (Rodrigues and Morais, 2015). Hop to soil pH response is also an important factor in hop production (Čeh and Čremožnik, 2015; Sirrine et al., 2010). Hence, it is relevant to better understand the implication of the irrigation systems and soil pH on hop production.

Another relevant issue is the utilization of the whole biomass produced in the hop fields. Hop is a climbing plant with high nutrient demand that can reach nearly 7 m in height, producing large biomass. The hop cones (female inflorescences) represent a small part of the total biomass of the canopy. At the end of the growing season, large quantities of stems and leaves remain. The stems are usually burned along with the polyester strings (not biodegradable). This practice is legally disapproved, but producers justify it since burning prevents the spread of diseases. In the case of leaves, producers currently spread them as a fertilizer in other crops. Alternatively, these resources should be better-valued by composting with other farm organic materials, seeking to promote a more sustainable and circular production system for hop farms. Composting is one of the most effective processes to recycle organic waste, which reduces environmental problems and increases the fertilizer value of the composted materials (Antil et al., 2014; Pergola et al., 2018).

In summary, this thesis focuses on two main purposes:

1) To assess the situation of the current hop fields, namely the constraints to the productive performance of the plants, and to evaluate alternatives to improve crop

productivity and to manage the organic wastes generated in hop farms. The study of constraints includes assessing the heterogeneity of productive performance of the hop fields, the effect of soil acidity alleviation, and the effect of flooding irrigation in the creation of soil fertility and plant nutrition gradients. The alternatives include the evaluation of the efficacy of foliar fertilization to improve crop yield, cone quality and to restore areas of poor productivity, and the composting of the organic waste from hop harvest (hop leaves) with other farm materials (wheat straw, farmyard manure and ash from hop stems) to obtain suitable composts for agronomic use. This part of the study is based on the current productive sector, installed with the Nugget cultivar. At present, 13 ha of hops are in production, managed by few farmers. One is installed in Pinela (Bragança) with three fields with about 2 ha each. Another producer is installed in Vinhas (Macedo de Cavaleiros) and manages a field of ~ 6 ha.

2) To analyse agronomic decisions and practices for the hop sector in Portugal, considering the evolution of the hop market and the growing interest in aroma cultivars. This work line aims to study the agronomic performance and chemical profile of hop aroma cultivars (Cascade, Columbus and Comet) grown under the ecological conditions of NE Portugal.

To accomplish with the main purposes, specific working lines were established:

- Evaluation of the productive performance and constraints of the current hop fields (**Chapter 3**);
- Foliar fertilization effect on hop productivity and cone quality (**Chapter 4**);
- Recycling of organic waste from hop plants through composting and compost quality evaluation (**Chapter 5**);
- Agronomic and chemical evaluation of hop aroma cultivars grown under Mediterranean conditions (**Chapter 6**); and
- Phenolic composition of hop cones as a response to several agro-environmental variation factors (**Chapter 7**).

The general conclusions and the perspectives for future research are present in **Chapter 8**. The present thesis is organised into eight chapters, and the thesis outline is summarised in Figure 1.1.

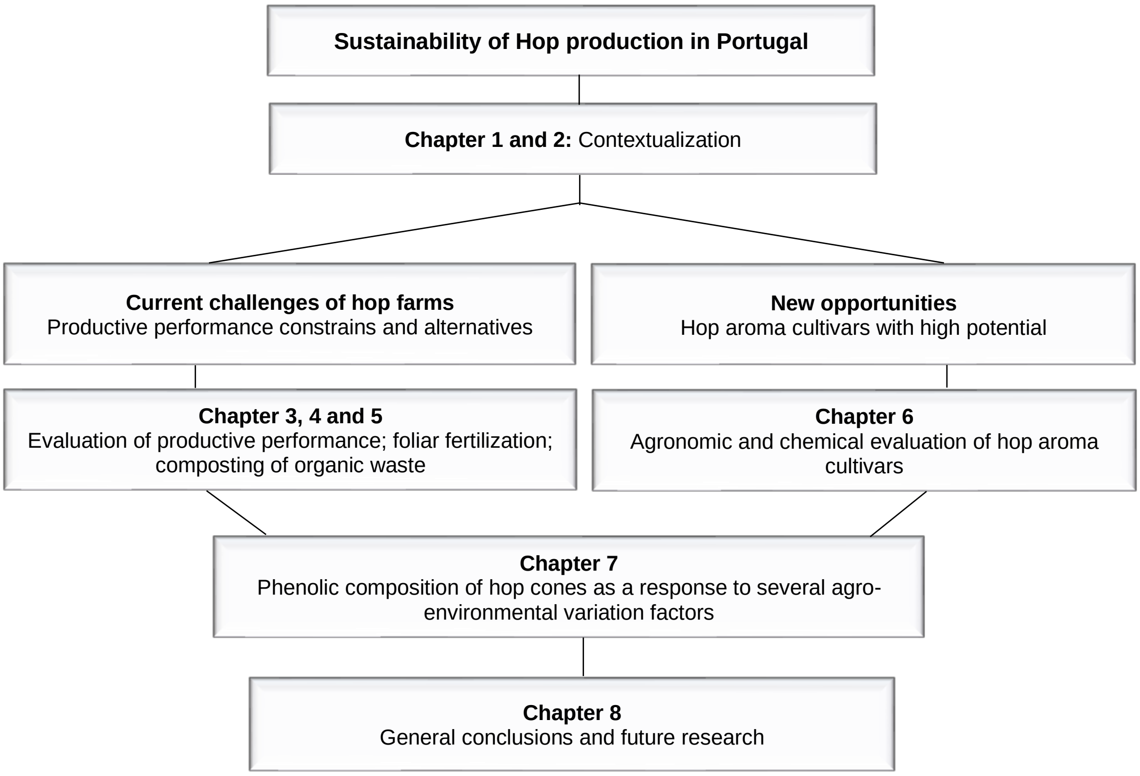

Figure 1.1. The sustainability of hop production in Portugal: thesis outline.

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Chapter 2:

State of the art

2.1. Hop (*Humulus lupulus* L.)

2.1.1. Hop botany

Hop plants belong to the Cannabaceae family, which include three species: (1) *Humulus japonicus* Zieb et Zucc., native from Japan, China, and adjacent islands; (2) *Humulus yunnanensis* Hu, currently confined to the Chinese province of Yunnan; (3) and *Humulus lupulus* L., the most common and the only used in beer production (Neve, 1991; Small, 2016). The common hops (*Humulus lupulus* L.) is a native plant in Europe, Western Asia and North America, and there exist references to hop gardens dating from the 9th century (Chapman, 1905; Small, 2016).

Common hop is a perennial climbing plant that grows every spring from the rhizomes of the underground rootstock (Zanoli and Zavatti, 2008). The name *lupulus*, derived from the Latin term *lupus*, is based on the habit of climbing on other plants (Koetter and Biendl, 2010; Zanoli and Zavatti, 2008). Hop plants produce annual shoots that can reach up to 6-15 m in length (Sirrine et al., 2010; Small, 2016; Zanoli and Zavatti, 2008). The shoots, also called bines, emerge every spring from a perennial rootstock (also named as a crown) and rhizomes (horizontal underground stems) and grow in a clockwise direction (Sirrine et al., 2010; Small, 2016). The perennial root system can live up to 50 years, and it is an extensive system that can reach more than 4 m deep and up to 5 m laterally (Small, 2016; Turner et al., 2011). The plant needs a certain length of the day to blossom, and this explains why hops grow better between the latitudes of approximately 35º and 70º N in the northern hemisphere (Koetter and Biendl, 2010; Neve, 1991). It is a dioecious species, and male and female plants produce, respectively, male and female inflorescences, with marked morphological differences, being the flowers the part of the plant that allows differentiating its gender (Zanoli and Zavatti, 2008). The female flowers occur in inflorescences that mature and originate the strobiles or 'cones', the relevant commercial part of the plant because it contains the lupulin glands, where the most important compounds are produced (Neve, 1991; Olsovska and Kolar, 2016).

The species of wild common hop have been classified into five taxonomic varieties, including one native from Europe. The European wild variety is the main ancestor of the cultivated plants, though there is also recognized the parental contribution of wild plants from Japan and North America (Small, 2016). The long-time cultivation of hops and the need to select specific organoleptic properties that would improve the flavour, aroma, and bitterness of beer resulted in many commercial cultivars and recognized chemotypes (Zanoli and Zavatti, 2008).

More recently, some researchers have characterized wild hops native to the Mediterranean region and have shown interest in using them to improve the varieties grown in the region (Martins et al., 2020; Mongelli et al., 2016; Mongelli et al., 2015).

2.1.2. Hop uses

Since ancient times, hop plants and their parts (inflorescences, leaves and stems) have been used for different purposes, namely as an ornamental plant, hair dye, fabric manufacture, paper fiber and use in packaging material (Delyser and Kasper, 1994; Sirrine et al., 2010; Zanoli and Zavatti, 2008). In the early 20th century, Clinch (1919) reported the use of hop bines for fodder, cattle litter, and as a basis for ricks of hay or corn. As an edible plant, it is well known to use young hop shoots cooked and eaten like asparagus according to traditional recipes (Rossini et al., 2020; Ruggeri et al., 2018). Hop utilization in cosmetics is also known in perfumes, skin creams and lotions (Zanoli and Zavatti, 2008).

Hop plants have an ancestral use in Europe and in North America by indigenous tribes to treat several diseases, including ear and headaches, sleep disorders, coughs, colds, treatment of wounds, bowel disorders, cancer, hematomas, delirium, fever, hysteria, diarrhea, jaundice, neuralgia, rheumatism, worms and scurvy (Biendl, 2009; Clinch, 1919; Small, 2016). In Ayurveda, the Indian medicine, the hop was used as a calming agent to cure inflammation and stomach disorders. In China and Japan, hop uses include treating insomnia and stomach problems and increasing urine flow (Biendl, 2009; Small, 2016; Zanoli and Zavatti, 2008).

Scientific research on hops started during the 19th and early 20th centuries and has confirmed many of the traditional uses, namely the effects of tranquillizer and sleep inducer, but also antitumor, antimicrobial, antioxidant, anti-diabetic, and estrogenic effect (Biendl, 2009; Delmulle et al., 2006; Gerhauser, 2005; Kac et al., 2008; Milligan et al., 1999; Small, 2016; Zolnierczyk et al., 2015).

Although spontaneous hops have been subjected to several uses in the past, currently, the main purpose of hop cultivation is to be used in beer production (Delyser and Kasper, 1994). It is not known, for certain, the date of the beginning of the use of hops in the manufacture of beer and its cultivation. However, records exist of hops in beer production in Europe at the beginning of the 10th century and North America at the beginning of the 17th century (Delyser and Kasper, 1994; Olsovska and Kolar, 2016). The use of hops in beer is essentially due to the action of resins as a bacteriostatic preservative while adding the characteristic bitter taste and the essential oils that provide aroma to beer (Almaguer et al., 2014; Ting and Ryder, 2017).

Despite the main use of hops in the beer industry, a low rate of hop production is also being used in sugar processing and as a preservative in ethanol production (Turner et al., 2011). The potential utilization in other areas is also in evaluation. Lower α-acid cultivars are being tested in the tea industry (Teghtmeyer, 2018). Several compounds are also being studied for health and medicinal applications (Bottner et al., 2008; Brunelli et al., 2009; Chadwick et al., 2006; Delmulle et al., 2006; Krause et al., 2014; Kyrou et al., 2017; Legette et al., 2013) or to be used as anti-adherent and antibiofilm (Bogdanova et al., 2018; Różalski et al., 2013). Other alternatives include its utilization in the manufacture of pesticides and repellents (Aydin et al., 2017; Bedini et al., 2015) and animal feed to reduce the use of antibiotics (Narvaez et al., 2011; Teghtmeyer, 2018). Hop waste from the brewing industry is also being studied to obtain high-added-value bioproducts (Hrnčič et al., 2019) or composted and applied to soil as organic amendments (Kopeć et al., 2020).

2.1.3. Chemical composition of hop

Hop is a dioecious plant (with male and female individuals), still only female inflorescences, called cones or strobiles, are of interest since they have glands where the lupulin granules, which resemble a yellow powder, are produced (Neve, 1991; Okada and Ito, 2001). The main constituents of lupulin are resins and essential oils with interest for the beer industry (Almaguer et al., 2014). The whole cones integrate into their composition several chemical compounds in addition to resins and essential oils, namely proteins, lipids, monosaccharides and polyphenols (Table 2.1).

Table 2.1. Chemical composition of dried hop cones (Verzele e De Keukeleire, 1991; Almaguer et al., 2014).

Compounds	Concentration (%)
Cellulose	40 – 50
Proteins	15
Alpha acids	2 – 12
Beta acids	1 – 10
Moisture	8 – 12
Amino acids	0.1
Polyphenols (tannins)	2-5
Oils and fatty acids	traces-25
Essential oil	0.5 – 3
Monosaccharides	2
Pectins	2

2.1.3.1. Hop resins

The total resins in hop cones correspond to about 15-30% of the total weight of the cones (Figure 2.1) and are subdivided into soft resins (soluble in hexane) and hard resins (insoluble in hexane) (Almaguer et al., 2014). The soft resins include α-acids (humulones, cohumulonas and adhumulones), β-acids (lupulones, colupulones and adlupolones) and non-characterized soft resins (Almaguer et al., 2014). Hard resins result from soft resins oxidation and include hard-β, -α, -delta (δ), -epsilon (ε) and uncharacterized resins (Almaguer et al., 2014). The α- fraction is considered to be more efficient than the β- fraction in terms of the amount of bitterness, responsible for a more pronounced bitter taste of beer (Rybáček, 1991; Ting and Ryder, 2017). Compared to α-acids, β-acids are less soluble in water resulting in higher losses during the brewing process, which are considered less relevant (Olsovska and Kolar, 2016). The iso-α-acids derived from α-acids, particularly the isohumulone analogue, contribute significantly to stabilize the foam of beer (Almaguer et al., 2014; Ting and Ryder, 2017).

The bitter quality of cohumolones is usually considered by brewers to be of low quality, though this is a controversial issue. Recent studies contradict this assumption based on just one study (Schönberger and Kostelecky, 2011; Ting and Ryder, 2017). Similarly, β-acids are commonly considered a negative factor in beer production, although they possibly contribute to preventing the oxidation of compounds in beer (De Keukeleire, 2000).

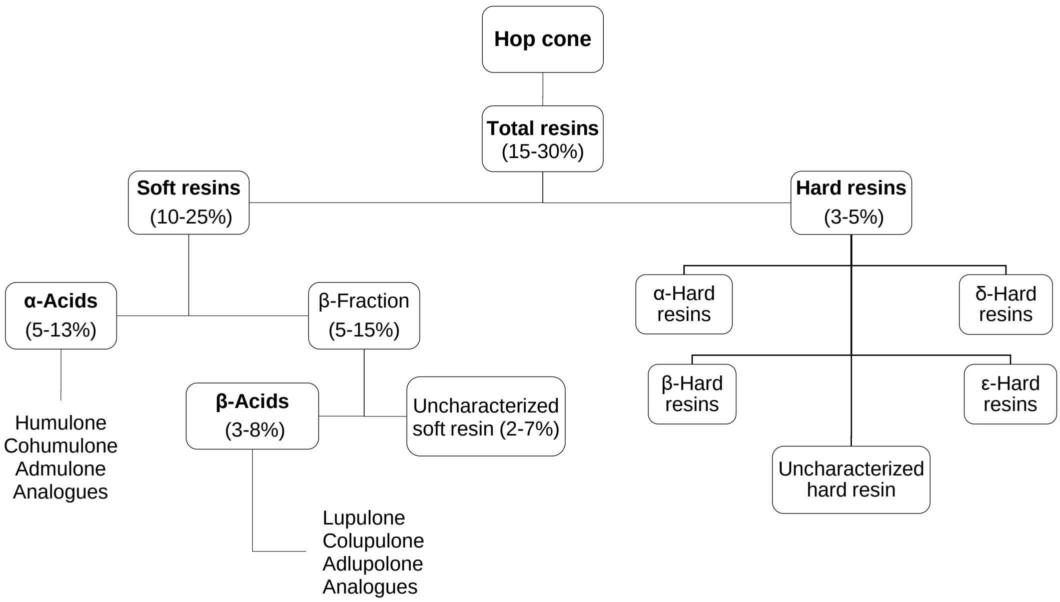

Figure 2.1. Classification of hop resins (adapted from Almaguer et al., 2014).

2.1.3.2. Essential oils of hops

The total content of essential oils in the hop cones may vary depending on several factors such as cultivar, growing conditions, drying, harvesting and storage (Almaguer et al., 2014; Inui et al., 2013; Kishimoto et al., 2006; Rettberg et al., 2018; Rodolfi et al., 2019). The essential oils represent less than 3.0% in dried hop cones, but they are a complex mixture of many volatile components (Almaguer et al., 2014). The concentration of hop oil volatiles and aroma precursors are of great relevance due to the impact on final beer aroma and flavour. To obtain beers with an intense hop aroma, brewers use multiple hop dosages added late and at the end of the process since much of the aroma volatiles are lost during the boiling process (Lafontaine and Shellhammer, 2019; Rettberg et al., 2018).

The hop oil constituents have been conventionally classified under three main groups (Almaguer et al., 2014; Schönberger and Kostelecky, 2011; Ting and Ryder, 2017): hydrocarbons; oxygenated compounds; and sulfur-containing compounds. Hydrocarbons represent about 70% and are classified into monoterpenes, sesquiterpenes and aliphatics. They have low solubility in water or beer and are usually lost during the boiling process (Almaguer et al., 2014; Ting and Ryder, 2017). The fraction of the oxygenated compounds represents about 30% of the total oil, including terpene alcohols, sesquiterpene alcohols, aliphatic and aromatic alcohols, aldehydes, ketones, esters, acids, terpene epoxides and others (Ting and Ryder, 2017). The sulfur compounds include thioesters, sulfides, and terpenoid sulfides, they are present in trace amounts but have potent aromas that usually impair undesirable flavours to beer (Almaguer et al., 2014; Ting and Ryder, 2017).

Many brewers select hops taking into account the visual and sensory evaluation according to the aroma characteristics they want for the beer (Lafontaine and Shellhammer, 2019). However, hop oils are subject to continuous change due to the secondary reactions, such as oxidation and hydrolysis, that occur through the brewing process, resulting in derived odorants different from the original (Rettberg et al., 2018). The identification of compounds that influence the aroma and their contribution has not been consensual, despite the available resources that allow the identification of numerous compounds in hops oil and their destination in the beer production process (Schönberger and Kostelecky, 2011). Between the most important compounds, myrcene (β-myrcene) is the most abundant monoterpene, representing approximately 30% of the total oil, and is responsible for the pungent odour of fresh hops (Almaguer et al., 2014). Linalool, the most abundant terpenoid alcohol, is widely accepted to contribute substantially to beer's aroma, being used by researchers as an indicator to measure the aroma of hops in beer (Peacock, 2010; Schönberger and Kostelecky, 2011). Besides linalool, it is of general agreement that also geraniol, b-damascenone, b-citronellol, esters (e.g., ethyl 4-methylpentanoate, (Z)-4-decenoate, 3-methylbutyl, 2-methylpropanoate), and organic acids (2- and 3-methylbutanoicacid) are significant contributors to beer flavour (Machado et al., 2020; Rettberg et al., 2018).

2.1.3.3. Polyphenols

Polyphenols represent about 4% of the total weight of dry cones and are found mainly in the petals and string of the cones, except for prenylflavonoids and, more specifically xanthohumol, which is also present in the lupulin glands (Almaguer et al., 2014). Biendl (2009) classified hop polyphenols into four groups (Figure 2.2): flavonols (e.g., quercetin and kaempferol); flavan-3-ol (e.g., catechins, epicatechins and tannins); phenolic acids (e.g., ferulic acid); and other polyphenols (prenylflavonoids, multifidol glycosides and resveratrol). The main compounds of hops are high molecular flavan-3-ols compounds (more than 80%), such as catechin, epicatechin, gallocatechin and tannins (Almaguer et al., 2014; Karabín et al., 2016; Olsovska and Kolar, 2016). The group of phenolic acids can be divided into derivatives of benzoic acid and derivatives of cinnamic acid. The most abundant compounds in this group are gallic, vanillic, protocatechuic, 4-hydroxybenzoic, ferulic, p-coumaric, caffeic and synaptic acids (Karabín et al., 2016). The glycoside multifidol and some prenylflavonoids (xanthohumol, desmethylxanthohumol, 6- and 8-prenylnaringenin) are only known in hops (Biendl, 2009; Karabín et al., 2016).

Hop polyphenols also contribute to beer flavour, colour, taste and haze formation and have a strong antioxidant power (Almaguer et al., 2014; De Keukeleire, 2000; Oladokun et al., 2016; Schönberger and Kostelecky, 2011). Between the polyphenols found in beer, the flavan-3-ol monomers and proanthocyanidin oligomers have received more attention due to their importance in beer quality parameters (Aron and Shellhammer, 2010). Hop polyphenols also have shown antimicrobial activity, particularly flavan-3-ols, flavonols, and tannins (Karabín et al., 2016). Besides, hop polyphenols also provide health benefits to consumers, particularly their anti-mutagenic and anti-carcinogenic activities (Biendl, 2009; Chen et al., 2014; Machado et al., 2017; Stevens and Page, 2004). The content of polyphenols in hops depends on the cultivar, and the quercetin and kaempferol glycosides are considered to be more specific of each cultivar (Almaguer et al., 2014). However, other compounds such as the prenylflavonoids seem to be suitable to distinguish between cultivars (Dresel et al., 2016; Krofta and Patzak, 2011).

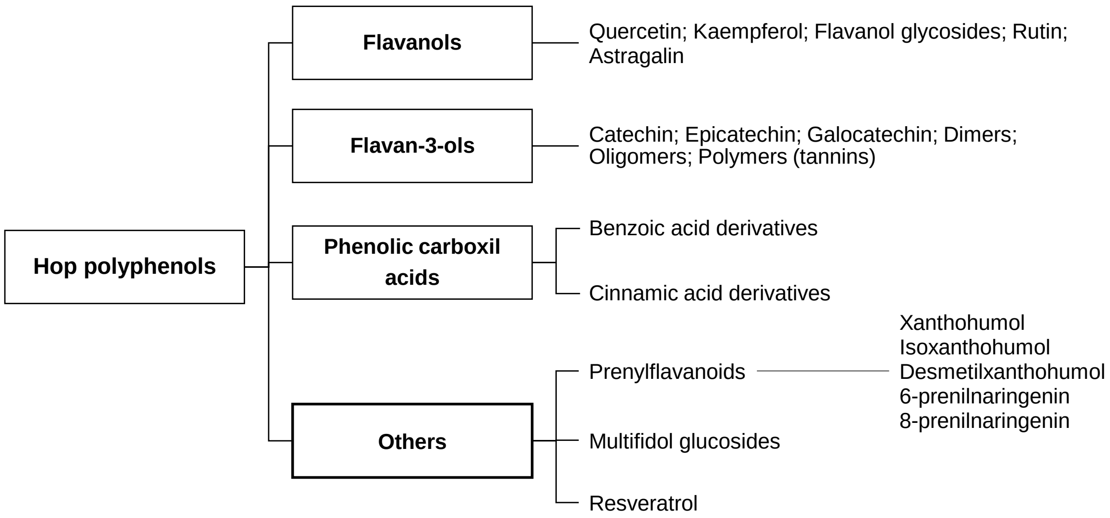

Figure 2.2. Constituents of the hop polyphenol fraction (adapted from Almaguer et al., 2014; Biendl, 2009; Karabín et al., 2016).

2.2. Hop cultivation in Portugal

2.2.1. From the 'golden age' to the decline

In Portugal, hop grows spontaneously along riverbanks, particularly in the north of the country, which indicates a high ecological potential for hop production (Cunha and Roque, 2008; Rodrigues et al., 2015a). The cultivation in the Iberian Peninsula goes back to the Philippine Dynasty, but it was abandoned and replaced by the vine crop (Carmona, 1982). More recently, records exist of a first trial carried out in the 1940s, with cultivars destined for beer production, but little is known about its results, except that crop cultivation did not continue (Almeida, 1981). The introduction of the crop began in 1962 due to some Portuguese farmer's curiosity about the crop at that time in production in Spain and expanding growth (Almeida, 1981). The vegetative material and the cropping technique were imported from Spain. The installation of the crop, despite the challenges, was fast, with the results in the first years exceeding what was expected (Pereira, 1981). From a starting point of an almost unknown crop in Portugal, in the following years, the enthusiasm was so great that hop became one of the main crops in the North of Portugal in ten years. It was only produced in two regions (Bragança and Braga), considered by a legal determination to meet the necessary ecological conditions to produce quality hops. Despite this, production was in enough quantity and quality to assure national demands and export (Gonçalves, 1982). The Portuguese Society for hops growing, named Lupulex, was created in 1963 with the capital of the national brewing industry to promote the cultivation of hops and ensure the reception and distribution of hop production (Almeida, 1981).

However, the 'golden age' of the crop was brief, and the growing stage's enthusiasm was followed by the disappointment of the decadence period that came next. Several years of decreasing yields, phytosanitary problems, nutritional disorders, cultivar inadaptation, unappropriated cropping practices, and the scarce knowledge about this crop, were some of the agronomic issues that lead to the abandoned of hop farmers (Rodrigues et al., 2015a). Economic reasons have also contributed to the difficult period that followed, namely the shortage of labour, the lack of investment incentives, the stagnation of prices in the international markets, and the quick rise in production costs (Pereira, 1981). The transformation of hops was also a high additional cost since hop cones were sent to Germany, transformed in extract, and later sent back to Portugal, a system that continues today. The possibility of installing a national unit for the extraction of hops was studied, considering a minimum cultivation area of 500 ha, a value that was never reached (Rodrigues and Morais, 2015).

In 1990, the Lupulex was extinguished due to the difficulties in selling the cultivar under production, the Brewer´s Gold. To succeed Lupulex and continue to support hop production, in 1991 it was created the Association of Hop Producers of Bragança and Braga, the Bralúpulo (Patrício, 1995). Bralúpulo carried out the reconversion of the hop fields using a new cultivar after a set of field experiments. The Nugget cultivar, which presented the best results in yield and α-acid content, was chosen and continues to be grown until today in NE (Rodrigues et al., 2015b). The farmers who have maintained production until today often obtain hop yields above 2000 kg ha^{-1}, values very competitive to those achieved in the main hop producing countries (Rodrigues and Morais, 2015).

Despite the challenging complexity that this crop presents, there are also favourable aspects to underline at this point, such as:

- the high ecological potential of the country, and in particular of the north, to produce hops with high yields and quality;
- the significant past contribution to the national economy by ensuring the needs of the national brewing industry, allowing to reduce imports;
- the economic relevance that it had in the past in the north region;
- the practicability of the crop in small family farms, allowing to obtain profitability of small plots of land, hence suitable for the NE region;
- the acquired knowledge of the hop farmers that continued until today is relevant to new producers who want to install.

2.2.2. Current context: hop farms and cropping technique

Currently, what remains from the more effusive period of hop crop in Portugal is two farms located in the district of Bragança, in Vinhas and Pinela, with a total cultivated area of approximately 12 ha. The production represents less than 10% of the hop needs of the national beer industry. The cultivar in production at both farms is the bitter cultivar Nugget destined for the national brewing industry. However, both producers are also currently testing aroma cultivars, namely Cascade, Columbus and Comet.

The cultivation of hops requires a dormant period of 5 to 6 weeks under temperatures near freezing and long daylengths to blossom and produce adequate cone yields (Sirrine et al., 2010; Turner et al., 2011). The minimum number of sunlight hours to ripen fully depends on the cultivar (Teghtmeyer, 2018). The average solar irradiation is considered adequate between 1800 to 2000 hours per year. The most favourable average annual temperature should be between 8 to 10 ºC, though some cultivars of hops are grown in regions with higher temperatures (Rybáček, 1991). In Portugal, a good relationship between the daily length and the productivity of hops was also found (Rodrigues et al., 2015b).

When introduced the first time, hop plants need a minimum of two years to produce a harvestable crop (Teghtmeyer, 2018). Once established, the replacement of the plants in the commercial fields is usually done at intervals of 15 to 25 years if deemed necessary due to the reduction of yields or accomplishing market demands (Small, 2016; Turner et al., 2011). The plants are often propagated from the cuttings or rhizomes of female plants, ensuring the production of clones from the same parent variety (Sirrine et al., 2010; Turner et al., 2011).

The vegetative cycle in the NE region begins in March with the breakdown of the dormancy of the perennial structure. The reproductive period starts in June with the formation and development of the cones and the ripening period takes place from the beginning of August until the end of September (Patrício, 1995).

The current cropping technique in Portugal has undergone little change compared to the past and is described in detail in Rodrigues et al. (2015b). Hop, as a climbing plant producing annual shoots that can reach near 7 meters high, needs a structure able to support the entire weight of the plant. In Vinhas and Pinela farms, hops are grown in a 7 meters high trellis system, the most commonly used for hop growth. The system is supported by concrete poles connected at the top with steel cables, to which strings are attached in a “V” design system. Despite being an expensive and challenging system, that needs to be rebuilt annually, it allows to obtain higher productivity levels.

However, it is a demanding system in terms of annual labour requirements, particularly in the spring. At the beginning of this season, it is necessary to attach the tutoring strings in the trellis structured and then hop shoots have to be chosen and trained up to grow in the strings. Labour is costly and scarce, and the need for training the new workers in the technical specificities of the crop is an additional constrain and common in other producing countries (Darby, 2004).

Hops produce better in well-drained soils with a pH between 5.7 and 7.5 (Sirrine et al., 2010). The crop has a high nutrient demand and nutritional unbalances were previously reported in the region. Because of the low pH of the soils in the north of Portugal, liming is usually recommended (Rodrigues et al., 2015b). The application of farmyard manure is usual and recommended considering soil aeration and frequent tillage.

The fertilization program includes applying a compound NPK fertilizer early in the spring, totalling between 180-200, 50-100 and 100-200 kg ha^{-1} of N, P pentoxide (P_2O_5) and K oxide (K_2O), respectively. During the growing season, two applications of N are usually performed, the first with nitromagnesium (27% NH_4NO_3 + 3.5% MgO + 3.5% CaO) and the second with calcium nitrate (15.5% NO_3^- + 27% CaO) (Rodrigues et al., 2015b).

Hops are usually planted in rows, and ridges are formed to facilitate surface irrigation and reduce soil compaction near the plants. The pruning of the stems is performed from the end of March to mid-April, using cutting machines. Firstly, the soil should be removed around the rootstock, the old stems cut away, and the ridges made up again. The ridges are usually prepared after pruning and before watering begins to protect the rootstock and enhance the uptake of nutrients and water (Rodrigues et al., 2015b).

When the plants reach between 60 to 80 cm in height, nylon strings are fixed from the soil to the top of the structure. The excess of shoots is removed and two or three of the most vigorous shoots are chosen and trained manually to climb the string in a clockwise direction. During August, the lowest leaves, up to approximately 1 meter in height, are removed (stripping). This procedure increases the airflow and reduces disease incidence, and it can be done manually or chemically (Rodrigues et al., 2015b; Sirrine et al., 2010).

The irrigation system presently in use consists of flooding the space between rows, which requires the levelling off the ground. This irrigation system may also contribute to soil compaction. The water needs exceed 5,000 m^3 per hectare and usually farmers alternate the watering lines. The irrigation system has high costs associated with several negative implications in many aspects of the cropping technique. As a result of soil compaction, several tillage passes must be performed every year to remove the crusts, allow water infiltration, and control weeds (Rodrigues and Morais, 2015).

Pest and disease control includes about 14 spray applications during the growing season. There are few products authorized to hop crop, so the farmers usually use fungicides and pesticides recommended for the vineyard (Rodrigues et al., 2015b). The crop can be damaged by several pests and diseases that require careful and routinely monitoring (Gent et al., 2015a). Between the main diseases that affect hops and require regular application of fungicides are downy mildew (*Pseudonospora humuli*) and powdery mildew (*Podosphaera macularis*). Among the pests that attack the aerial part, the hop aphid (*Phorodon humuli*) and the twospotted spider mite (*Tetranychus urticae*) are the more predominant (Gent et al., 2015a; Sirrine et al., 2010).

Harvesting occurs when the cones reach the ideal degree of maturity, that is, when they start to open, and the golden yellow lupulin is observed (Patrício, 1995). Hops are harvested usually between the end of August and the beginning of September in a 10- to 15-day period. In the past, hops were harvested by hand in the region, just as in other producing countries (Darby, 2004; Pereira, 1981; Teghtmeyer, 2018).

Currently, mechanization greatly facilitates the harvesting process and reduces the costs and the considerable labour required. In the field, the bines are cut close to the grown directly to the trailer in which are transported to the picking machine that separates the cones from leaves and stems (Figure 2.3). Subsequently, the cones are dried in the oven at 60 ºC until the moisture content stabilizes between 10 and 12%.

The hops can be used to produce beer in different forms, as fresh or dry cones, pelletized or as distilled extracts (Teghtmeyer, 2018). In the current hop farms, the dry cones are bagged and stored in cold rooms at 0 ºC to be sent to Germany to prepare the hop extract destined for the national brewing industry.

Figure 2.3. Pinela hop farm in 2017: A) hop field before harvest; B) cutting of the bines for the trailer at the harvest; and C) separation of cones, leaves and stems performed in the picking machine at harvest.

2.2.3. Hop market: challenges and opportunities

A small group of large producers has dominated the hop market and multinational brewers favouring cultivars with a high content of α-acids (Brown, 2015; Small, 2016). The world's largest producers are Germany and the USA followed by the Czech Republic and China. According to the latest data available from the Food and Agriculture Organization of the United Nations (FAOSAT, 2021), Germany and the USA produced in 2019, respectively, 50,820 and 48,500 t, and the Czech Republic and China produced, respectively, 7,150 and 7,036 t.

Conventionally, hop cultivars for brewing have been classified according to their chemical composition as 'bittering' or 'aroma', though a more recent designation of 'flavour' is being used to designate cultivars that suit a dual purpose of imparting aroma and bitterness to the beer (Almaguer et al., 2014). Usually, when multinational brewers buy hops, they are interested in the α-acids content they pay for. Hence, hop production of bittering cultivars has been encouraged (Brown, 2015). Craft brewers are more interested in cultivars with high aroma, different flavours and fewer α-acids (Hieronymus, 2012). The American Cascade cultivar is one of the most popular aroma hop cultivars, while Nugget is an important bittering cultivar (Almaguer et al., 2014; Brown, 2015).

In the last years, the hop market has undergone a revolution with expanding the craft beer market. The American craft beer industry experienced a huge increase of 273% in the last decade (Teghtmeyer, 2018). However, the craft beer revolution is a global phenomenon (Garavaglia and Swinnen, 2017). The change in consumers' preferences has forced growers to redirect production in response to the new demands from bitter to aroma cultivars. This trend has been more markedly in the USA. In Yakima Valley, the prominent hops producing region in the USA, 70% of the hop production in 2015 respected the aroma cultivars (Brown, 2015). Overall, the bitter cultivar dominance in the world hop production falls in 2016. The market share of aroma cultivars achieved approximately 60% and maintained, with a slight decrease, until 2020 (Hopsteiner, 2017, 2020). This movement opened opportunities for small growers to produce and sell especially desired cultivars in smaller quantities to many brewers at higher prices (Brown, 2015; Small, 2016). The increased interest in organic production and purchasing products at the local level also encouraged this trend (Sirrine et al., 2010).

Portugal is no exception to this movement, though it is more recent than in the other European countries (Machado, 2019). The Euromonitor report of June 2018 about “Beer in Portugal” stated that dozens of craft brewers were launching their products, especially through the local market. This new trend is encouraging the interest of Portuguese hop producers in aroma cultivars. At the same time, it is attracting new investors to this niche market, such as the Portuguese company ‘Esporão’, producer of wine and olive oils (Diário de Notícias, May 2018). Although the craft beer market in Portugal is still insignificant, the tendency points to exponential growth in the coming years, which is also encouraging the appearance of new hop producers, as was testified in the II Journeys of Hop and Beer in 2019 (Bedford, 2019; Macieira and Pereira, 2019).

Other possible future developments in the hop market may be related to the interest in the many alternative uses of hops, namely in the pharmaceutical industry, sugar and ethanol industries and animal feed (Teghtmeyer, 2018; Turner et al., 2011).

2.3. Foliar fertilization

2.3.1. Overview

The most common way to meet plant nutritional requirements is through the application of fertilizers to the soil. However, nutrient uptake can also take place from the leaf surface, stomata and other specialized cells, these routes being explored in the context of foliar fertilization (Bindraban et al., 2015; Oosterhuis and Weir, 2010). Foliar applications are more orientated to the target organs, with lesser amounts being supplied, which reduces the environmental impact usually associated with soil fertilization (Fernández and Eichert, 2009). The possibility of acting quickly and accurately over time is also advantageous for foliar fertilization, especially in agriculture where fertigation is not available (Oosterhuis and Weir, 2010). The response of the crop to foliar applications is often very rapid, allowing a faster correction of deficiencies than through soil applications (Fageria et al., 2009; Haytova, 2013). On the other hand, foliar applications often provide only a temporary response since nutrients can easily be washed out by rain. Leaf sprays can also damage leaf tissues when excessively concentrated (de Valença et al., 2017; Fageria et al., 2009).

A wide range of fertilizer formulations for foliar application is currently available, and its role in crop fertilization is increasing. The market demand for foliar fertilizer is expected to increase at a compound annual growth rate of 4% until 2028 (Dean, 2019). The most common commercial formulations combine macro and micronutrients or formulations aimed at specific crop or territory problems. The use of raw materials in the manufacture of foliar sprays that, in addition to mineral nutrients, contain compounds that may have a biostimulant effect on plants has also been increasing (Marketsandmarkets, 2019).

The potential beneficial effects of such products have been widely reported, and they include improving the nutritional status of plants, increasing crop yield and quality, inducing resistance to diseases and pests and improving tolerance to abiotic stresses (du Jardin, 2015; Haytova, 2013; Smoleń, 2012). Despite the many beneficial effects of foliar fertilization, the response of plants is not always positive and is highly variable, depending on the crop and nutrients applied (Fageria et al., 2009; Mallarino et al., 2001; Oosterhuis and Weir, 2010). Cereal crops, for instance, appear to respond less effectively to foliar applications than leafy vegetables. This is more evident when the nutrients applied are less mobile and less translocated to the grains (Bindraban et al., 2015). Regardless of the contradictory results, foliar fertilization is an agronomic approach with benefits and limitations that need to be well understood to suit each case better. Despite extensive research in different crops, the effects of foliar fertilization on hops are not so well known since few studies have focused on this field (Procházka et al., 2018).

2.3.2. Nutrient-rich foliar sprays

Crop fertilization is mainly based on soil application of fertilizers rich in macronutrients, in particular N, P and K. The demand of crops for these macronutrients is high, and usually agricultural soils do not contain them in amounts able to continuously sustain high plant performance (Havlin et al., 2014; Hawkesford et al., 2012). However, the exclusive application of NPK fertilizers over the years may result in soil micronutrients mining with negative implications not only to crop yields but also to food quality and micronutrient deficiencies in human nutrition (Bindraban et al., 2015; de Valença et al., 2017). Micronutrients, although needed in small quantities, are essential to several biochemical processes and, in case of deficiency, they can also negatively affect the performance of the plants (Broadley et al., 2012; Smoleń, 2012). Balancing the application of macronutrients with micronutrients may be important to avoid deficiencies (Bindraban et al., 2015).

Despite the restricted amounts of nutrients that can be supplied through the leaf, in general, both macro and micronutrients can be applied as foliar sprays. The macronutrient requirement in high-yielding crops is elevated and unlikely to be met with foliar sprays (Fageria et al., 2009). Instead, the partial replacement of conventional fertilization may benefit crop yield while reducing production costs and environmental damage (Haytova, 2013; Kolota and Osinska, 1999; Tandon and Dubey, 2015). Foliar sprays can also be applied in a complementary fertilization approach, for instance, late in the growing season when soil applied fertilizers would not be effective (Fageria et al., 2009). Later foliar application of N seems to be effective to enhance wheat grain quality (Rossmann et al., 2019; Woolfolk et al., 2002), and cotton yield when applied during the boll development period (Oosterhuis and Weir, 2010). The demand for N in the hop growth is little at the initial stage, but large and fast from mid-June to mid-July, as cone begins to develop (Gingrich et al., 1994; Sullivan et al., 2009). The application of N fertilizers to the soil has to be done previously, and high rates are usually applied (Gingrich et al., 1994), increasing potential losses. The supplementation with foliar N-containing fertilizers may benefit the hop crop, allowing more efficient management of N according to the needs of the plants.

In the case of K, the effect on the growth phase of flowers and fruits is well known (Hasanuzzaman et al., 2018; Hawkesford et al., 2012). This probably explains the good response of fruit crops to foliar applications of K, resulting in increased yields and improved fruit quality (Pathania et al., 2018; Smoleń, 2012; Valentinuzzi et al., 2018). K is also considered an important nutrient in the development of hop cones since nearly 25% of the nutrient is found in the cones (Gingrich et al., 1994). However, the potential effect of foliar application of K during the flowering phase is not yet confirmed by experimental research.

Foliar fertilization is often effective for supplying micronutrients, due to the small amounts required, and especially when the application to the soil is not practical, as occurs with Fe in calcareous soils (de Valença et al., 2017; Fageria et al., 2009; Fernández et al., 2008). The use of micronutrient based foliar sprays has also proven to be a successful approach to improve fruit development and quality associated with micronutrient deficiencies (Kaur et al., 2015; Khorsandi et al., 2009; Razzaq et al., 2013). Another interesting alternative that seems effective in micronutrients is crop biofortification, thus enhancing the nutritional value of food (de Valença et al., 2017; Prasad and Shivay, 2020; Wei et al., 2012). It is known that hop plants are especially sensitive to the deficiencies of the micronutrients boron (B) and zinc (Zn), required for optimum growth and cone yield (Gent et al., 2015b; Gingrich et al., 1994). The application of foliar sprays rich in macro and micronutrients may positively affect hops, a crop with high nutrient demand.

2.3.3. Plant biostimulants applied as foliar sprays

The production of fertilizers has been based on non-renewable resources such as mineral deposits and fossil fuels. However, the current trend is to transition to a bio-based economy through the substitution of mineral fertilizers by bio-based alternatives (Chojnacka et al., 2019). Biostimulants are one of the bio-based alternatives with increasing worldwide interest. The growing awareness of the need to promote sustainable agriculture, to improve the efficiency in the use of resources, and the investment in research, are driving factors that can explain the interest in plant biostimulants (du Jardin, 2015). The market of plant biostimulants is also being driven by the need to support crop growth under abiotic stress due to changing climatic conditions (Marketsandmarkets, 2019).

The word biostimulant was first used by horticulture specialists to describe substances other than nutrients, soil improvers, or pesticides, that could promote plant growth (du Jardin, 2015). The definition of biostimulant includes any substance or microorganisms other than a fertilizer applied to plants that can enhance nutrition efficiency, induce abiotic/biotic stress tolerance, and/or improve crop yield and quality (du Jardin, 2015; Hawrylak-Nowak et al., 2019). Seaweeds, for instance, are considered a plant biostimulant included in the category of natural substances (Hawrylak-Nowak et al., 2019). The biostimulant effect on plants is mainly attributed to the hormonal enhancing effect, stimulating plant growth and plant response to stress conditions

(Battacharyya et al., 2015; du Jardin, 2015). Seaweeds were used in ancient agriculture as a fertilizer and more recently have been adopted by the fertilizer industry to produced commercial algae-based products that can be applied to soil or plants (du Jardin, 2015).

The brown seaweed *Ascophyllum nodosum* (L.) is widely used in agronomy, and a source of complex polysaccharides, cytokinins, auxins, indole acetic acid, betaines, sterols, polyuronides and macro and micronutrients (Khan et al., 2009; Sharma et al., 2014). Beneficial effects of the application of *A. nodosum* based foliar sprays were reported on the growth of *Solanum quitoense* Lam. (Díaz-Leguizamón et al., 2016) and the yield of wheat (*Triticum aestivum* L.) (Stamatiadis et al., 2015), olive (*Olea europaea* L.) (Ali et al., 2016) and chili (*Capsicum annuum* L.) (Manna et al., 2012).

Amino acids are biological compounds and have a biostimulant effect on plants, increase their growth and development, and protect them against biotic and abiotic stresses (Halpern et al., 2015). Besides, some specific amino acids have chelating properties, contributing to an increase in the availability and uptake of some nutrients (du Jardin, 2015). Amino acid-based fertilizers may be applied to the soil or as foliar sprays, and positive effects in increasing growth and yield have been commonly reported in horticultural crops (Drobek et al., 2019). In fruit crops, the application of amino acids as foliar sprays can also improve yield and fruit quality (Abd El-Razek et al., 2018; Ilie et al., 2018). Amino acids are a reduced form of N, and their direct uptake by plants has a lower biosynthetic cost than other predominant uptake forms such as NO_3^- and ammonium (NH_4^+) (Lambers et al., 2008; McAllister et al., 2012). In an experiment with maize, amino acids application increased the yield even with an half reduction in N fertilization rate (Halpern et al., 2015). Amino acids may be used to reduce the amount of inorganic-N fertilizers applied to the soil, which are usually associated with a reduced N use efficiency and a high risk of environmental contamination (Dimkpa et al., 2020).

Regarding hops, there is little data on the use of plant biostimulants. Procházka et al. (2018) tested a set of biologically active substances in hops, one of which based on a seaweed (*A. nodosum*) extract containing macro and micronutrients and amino acids. However, this treatment did not produce relevant results in terms of hop yield and quality compared to the other fertilizing materials used in the trial.

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Chapter 3:

Evaluation of the productive performance and constraints of the current hop fields

Briefing note

This chapter is the starting point of this thesis focused on screening the current situation of the hop fields located in Bragança district. The productive performance and constraints of these fields were evaluated.

Chapter 3.1. addresses the issue of the irregular productive performance of the hop fields, presenting patches of poorly developing vegetation without being clear what were the causes affecting plant growth and yield. This chapter is an adaptation of Afonso, S., Arrobas, M., Rodrigues, M.Â., 2020. Soil and plant analyses to diagnose hop fields irregular growth. J. Soil Sci. Plant Nutr. https://doi.org/10.1007/s42729-020-00270-6.

Chapter 3.2. focus on flooding irrigation effect on soil properties and plant performance. This study evaluated the variation in soil properties and nutritional status and the productivity of hop plants created along the rows by flooding irrigation. It was also studied the effect of the application of lime to attenuate the variability created by the irrigation system. This chapter is an adaptation of the following article: Afonso, S., Arrobas, M., Rodrigues, M.Â., 2021.Twenty-years of hop irrigation by flooding the inter-row did not cause a gradient along the row in soil properties, plant elemental composition and dry matter yield. Horticulturae, *7*(7), 194. https://doi.org/10.3390/horticulturae7070194.

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3.1. Soil and plant analyses to diagnose hop fields irregular growth

Abstract

Purpose: In cultivated fields, patches of poorly developed vegetation often appear without a clear indication of what is affecting the growth of the plants. The purpose of this study was to investigate the causes behind these irregularities in the hop (*Humulus lupulus* L.) fields of NE Portugal which are greatly reducing crop yield and farmers' profits, and to provide guidance to farmers as to appropriate remedial action.

Methods: Patches of different levels of plant development were selected within hop fields and categorized according to plant vigour (weak, fair, and good). Several soil properties were determined and related to the plant nutritional status and DMY of different parts of the plant (hops, leaves, stems). Data was subjected to analysis of variance and principal component analysis.

Results: The results suggest that crop yield is reduced mostly due to poor soil aeration and excessive soil and tissue Mn and Fe levels. The plants from the plots of weaker vigour seem to be particularly affected by toxic levels of Mn, and the plants from the plots of fair vigour by Fe levels. pH, texture (clay content), cation exchange capacity (CEC) and organic C seem to be other soil properties with some degree of influence on plant performance.

Conclusions: From these results farmers are advised to increase soil aeration by implementing a drainage system and converting to a drip irrigation system, in addition to increasing soil pH by liming to reduce Mn toxicity.

***Keywords*:** *Humulus lupulus*; soil properties; nutritional disorders; waterlogging; plant growth and yield

1. Introduction

Hop (*Humulus lupulus* L.) is a perennial species that has a productive life of more than 20 years. In the NE of Portugal, cultivated hop fields sometimes display patches of heterogeneous plant vigour, even when managed by the same farmer and subjected to similar cropping techniques. This is an important issue since the loss of production is progressively reducing the farmers' profit. The causes are often not easily identified, but they may be related to soil properties and plant nutritional disorders.

Soil compaction and soil water content are spatially and temporarily variable factors which greatly influence soil available nutrients (Weil and Brady, 2017). In Portugal, hop farmers usually irrigate the crop by flooding the space between rows (Rodrigues et al., 2016). This system negatively impacts the soils, causing the appearance of areas with excessive or insufficient water content, and increasing soil compaction, which demands frequent tillage, with consequences for soil properties, plant nutritional status and plant health (Rodrigues and Morais, 2015).

In Mediterranean climates, most of the rain falls during the winter months (mainly between December and March), which in poorly drained soils may be a serious problem for winter and perennial crops. Flooding, caused by rain and summer surface irrigation, greatly influences the soil redox potential and consequently its chemical and physical properties (Favre et al., 2002). The first measurable effect induced by flooding is the disappearance of O_2 (Irfan et al., 2010; Parent et al., 2008). Plant roots need a regular O_2 supply for mitochondrial respiration. The availability of O_2 also regulates oxidation-reduction reactions, with a major influence on the bioavailability of several essential nutrients (Weil and Brady, 2017). Reduced levels of O_2 in the soil favours the activity of anaerobic microorganisms which increases denitrification, with the emission of important N gases, such as dinitrogen (N_2) and/or nitrous oxides (NO_x), into the atmosphere (Xia et al., 2019). Furthermore, flooding stress may reduce plant DMY (Gao et al., 2020).

During the thermodynamic sequence of the reduction process, NO_3^- reduction is followed by the reduction of Mn, Fe and sulphate (SO_4^{2+}) (Osman, 2013). The decrease in redox potential is followed by the increase in pH and soluble Fe, Mn, NH_4^+, Ca, magnesium (Mg), K and sodium (Na) (Pezeshki and DeLaune, 2012; Phillips and Greenway, 1998). Toxic products, such as ethanol, lactic acid, acetaldehyde, aliphatic acids and cyanoganic compounds are also produced under reduction conditions (Parent et al., 2008; Pezeshki and DeLaune, 2012). The accumulation of these compounds along with the increased available forms of Fe and Mn can have deleterious effects on plants (Millaleo et al., 2010; 2019; Pezeshki and DeLaune, 2012). The effects of flooding on plants can be diverse and may include deficiency of some and/or toxicity of other nutrients (Pezeshki and DeLaune, 2012; Stieger and Feller, 1994) and reduction in photosynthesis and biomass accumulation (George et al., 2012; Sparrow and Uren, 2014).

Organic matter (OM) is a key component of soil fertility and an important factor in soil structure formation (Osman, 2013). In general, OM favourably influences the physical, biological, and chemical properties of the soil. Among many other favourable effects on soil, OM reduces compaction, favours aeration and water holding capacity, enhances nutrient cycling and releases valuable nutrients for root uptake (Singer and Munns, 2002; Tan, 2011). The OM content of a soil depends on the annual input of organic debris (crop residues or manure), soil properties (such as texture), climate (precipitation and temperature) and agricultural practices, such as the intensity of soil tillage (Weil and Brady, 2017).

Soil pH is another major factor affecting nutrient availability and plant growth (Bindraban et al., 2015). Depending on soil pH, several essential nutrients become more or less available for root uptake. The bioavailability of the vast majority of essential nutrients increases with pH close to neutrality. As pH moves away from neutrality, the risk of deficiency or toxicity of certain elements increases. In alkaline soils, for instance, there is a high risk of Fe deficiency, while in acidic soils the risk of aluminium (Al) and Mn toxicity increases (George et al., 2012). Plant species differ in their preferences for soil pH. Some species thrive in acidic soils while others prefer alkaline soils. Most, however, thrive best in near neutral or slightly acidic soils (Tan, 2011). The pH range considered to be the most adequate for hop is between 6.0 and 6.7 (Čeh and Čremožnik, 2015; Gingrich et al., 1994).

The availability of essential nutrients, both macro and micronutrients, is of utmost importance for plant performance. Soil nutrient availability depends on natural factors such as soil texture and mineralogy. Clay soils, for example, may fix and/or adsorb high quantities of some nutrients that are progressively released to plants (Osman, 2013). Some agricultural practices, such as crop residues management and manure application, also have long-term effects on soil nutrient availability (Lipecki and Berbeć, 1997; Samson et al., 2019).

The main goal of this study is to try to establish the causes for the appearance of heterogeneous areas of poorly developed plants in commercial hop fields in NE of Portugal. The hypothesis set for this work is that anoxia, and its multiple relationships with other soil properties, and the availability of plant nutrients is responsible for the poor vigour of plants. Thus, the objectives of the study are: i) to relate several soil properties, such as soil pH, texture, organic C, electrical conductivity (EC), exchangeable bases and extractable macro and micronutrients to elemental plant composition (leaves, stems and flowers); ii) to relate all those soil and plant variables to aboveground DMY and in particular to hop cones DMY; and iii) to seek a diagnosis that will guide farmers to intervene in the fields to make them more uniform, eliminating the patches of low vigour plants which are responsible for reduced DMY. To this aim, there were selected for the study plots previously classified by farmers as producing plants of weak, fair or good vigour.

2. Materials and methods

2.1. Characterization of field plots and sample collection

The hop fields used for this study are located in Bragança, NE of Portugal, a region that benefits from a Mediterranean-type climate (average annual air temperature of 12.7 ºC; annual precipitation of 772.8 mm) (IPMA, 2020). The soil and plant samples were collected during the growing season of 2016, in two hop farms located in Pinela (41°40'33.6"N 6°44'32.7"W) and Vinhas (41°33'34.8"N 6°48'42.9"W). Each farmer grows approximately a total of 6 ha of hop of the cultivar Nugget. The fields are grown in a 7 m conventional high trellis system, with concrete poles, connected with cable, in a "V" design system. In Vinhas hop farm there are several plots showing a markedly different vegetative pattern in terms of the vigour of hop plants, previously classified by the farmers as weak, fair and good. In Pinela farm, hop plants generally present a fair or good vigour. Soil and plant samples were separately collected in the different vigour plots. Seven plots were selected corresponding to weak (Plot 1, Plot 2, Plot 3), fair (Plot 4, Plot 5) and good (Plot 6, Plot 7) plant vigour. Plots 1, 2 3 and 4 were marked in Vinhas farm and plots 5, 6 and 7 in Pinela farm.

Both farmers manage their fields by a surface irrigation system consisting of flooding the space between rows. Several tillage passes (3 to 4) are performed every year to remove the crusts and allowing water infiltration. Both farmers used to apply a compound NPK (7:14:14) fertilizer early in the spring, followed by two application of N as sidedress, totalling ~150, 44 (100) and 83 (100) kg N, P (P_2O_5) and K (K_2O) ha^{-1}. The farmer of Pinela also uses to apply 40 t ha^{-1} of farmyard manure. Both farmers follow a phytosanitary program to protect the crop from pests and diseases.

Composite soil samples (15 sampling points per composite sample) were randomly collected at 0-20 cm depth, between rows, in June 2nd, with three replicates per plot. The soil of a composite sample was homogenized in a bucket and separated from the larger stones. A fraction of ~500 g soil was placed in a labelled bag and sent to the laboratory. Three samples of 30 leaves (blades plus petioles) were collected per plot in three dates evenly spaced (June 2nd, July 1st, and July 27th) during the growing season. In the first sampling date the leaves were collected at ~1 m in height. In the second and third sampling dates, leaves were collected at ~1 m and ~2 m in height. At the harvest (September 1st), the aboveground biomass of six random plants per plot was separated in two different leaf samples [basal leaves (to 2 m in height) and top leaves (last 2 m from the top)], stems and hop cones. By analysing several times, different tissues and tissues at different position in the canopy, a more complete diagnosis of the nutritional status of the plants is potentially obtained. The samples of the aboveground biomass taken at harvest were weighed in fresh, separated into stems, leaves and cones. Subsamples of each plant part were weighed again and subsequently oven dried at 65 ºC and weighed dry for the estimation of the DMY of the original field samples. Thereafter, three subsamples of each plant tissue were ground (1-mm^2 sieve) and analysed for elemental composition.

2.2. Laboratory analysis

Soil samples were oven-dried at 40 ºC and sieved in a mesh of 2 mm. Samples were analysed for texture (pippete method), pH (H_2O and KCl 1M), EC of soil extract (soil:solution, 1:2.5), exchangeable complex (ammonium acetate, pH 7.0) and organic C (Walkley-Black method). The readily soluble forms of P and K were extracted by a combination of ammonium lactate and acetic acid buffered at pH 3.7 (Egner-Riehm). Extractable P was also determined by the Olsen method. Phosphate in the extract was determined calorimetrically with the blue ammonium molybdate method using ascorbic acid as reducing agent. Soil B was extracted by hot water and the extracts analysed by azomethine-H method. For more details of these analytical procedures the reader is referred to Van Reeuwijk (2002). The availability of other micronutrients in soil (Cu, Fe, Zn, and Mn) was determined by atomic absorption spectrometry after extraction with ammonium acetate and Ethylenediamine tetraacetic acid (EDTA), according to the method described by Lakanen and Erviö (1971). Soil acid phosphatase activity was determined as proposed by Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1977).

Elemental analyses of leaf, stem and cone samples were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K) and atomic absorption spectrophotometry [Ca, Mg, copper (Cu), Fe, Zn and Mn] methods after nitric digestion of the samples (Walinga et al., 1989). Additionally, NO_3^- concentration in hop cones was determined by ultraviolet (UV)-visible (Vis) spectrophotometry in a water extract (dry tissue:solution, 2.5:50) (Clescerl et al., 1998).

2.3. Data analysis

Data from soil and plant samples was subjected to one-way (plots of different plant vigour) analysis of variance (ANOVA), by using IBM SPSS v.25.0 program. When significant differences occurred, the means were separated by Tukey–Kramer HSD test ($\alpha = 0.05$). A correlation analysis was performed for soil data with Pearson coefficient when the assumption of normality and linearity was accomplished, and not being the case, with Spearman coefficient. The principal component analysis (PCA) was also applied to soil and plant data by symmetrical method. The components were retained considering the eigenvalues superior to 1 and the *Scree-plot*. Internal consistency was measured with Cronbach's alpha. Subsequently the differences between plots in the components extracted were subjected to analysis of variance (ANOVA) and Tukey–Kramer HSD test (α = 0.05).

3. Results

3.1. Dry matter

Plants classified as having higher vigour (good) in function of their development in the previous years produced higher average total DM (Figure 3.1.1). Values of the plot 6 were significantly higher than those of the plot 5 and plots of weaker plants (1, 2 and 3). Total DMY reached the highest average values in the plot 6 (2587 g plant^{-1}) and the lowest average value in the plot 1 (513 g plant^{-1}). The difference in cone production was even higher when comparing the plots of the plants of good vigour with those of fair and weak vigour. The highest (1193 g plant^{-1}) and the lowest (75 g plant^{-1}) values were respectively found in the plots 6 and 1. In turn, the differences in leaf and stem production were less marked between fair and good plots, though a significant difference had persisted.

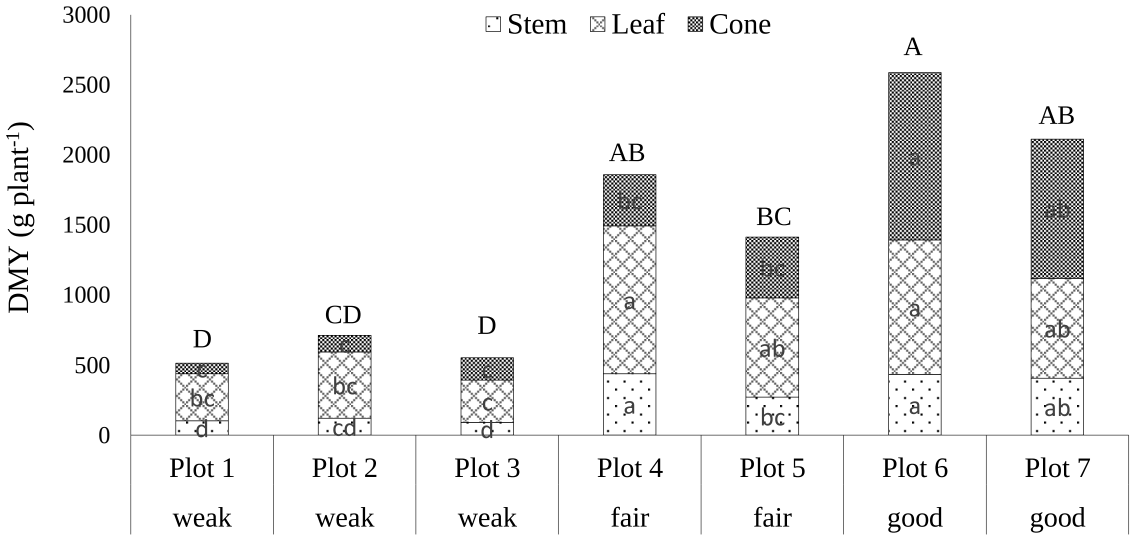

Figure 3.1.1. Average dry matter yield (DMY) (n=6) separated by plant parts (leaves, stems and cones) in the seven plots of plants with different vigour (weak, fair and good). For each plant part, means followed by different letters are significantly different by Tukey HSD test (α = 0.05).

3.2. Soil properties

Soil texture in the Plots 1, 2 and 3 (weak plant vigour) varied between sandy clay loam, loam and silty clay loam (Figure 3.1.2). The soils of the plots with fair plant vigour

(Plots 4 and 5) were clay loam. The soil of plots 6 and 7 (good plant vigour) varied between sandy clay loam to loam.

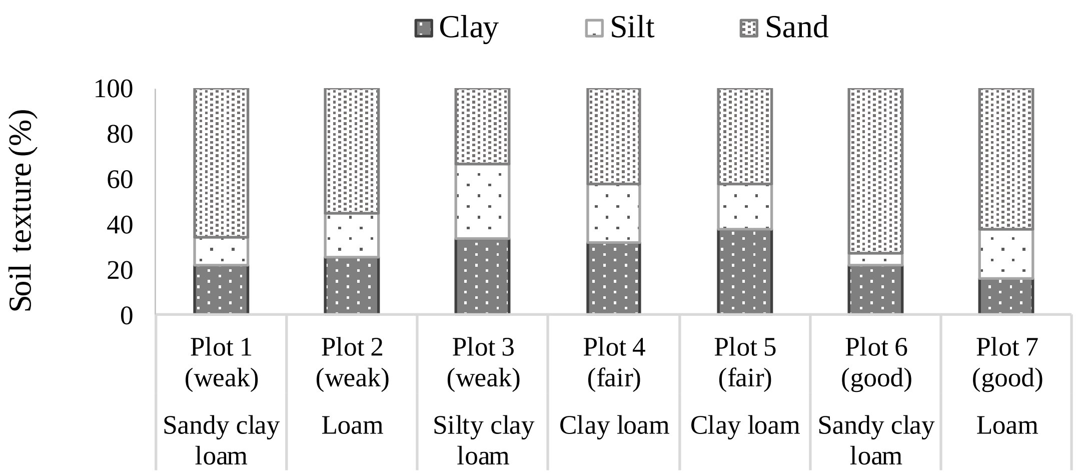

Figure 3.1.2. Soil texture of the samples (n=3) collected between rows at 0–20 cm depth in hop plots with plants of weak, fair and good vigour.

Soil pH (H_2O) significantly varied among experimental plots (Table 3.1.1). The lower (5.1) and higher (6.2) values were respectively found in the plots 7 and 2. Conductivity and acid phosphatase activity also significantly varied among plots. Conductivity ranged from 51.7 µs/m in plot 6 and 99 µs/m in plot 3. The higher values of acid phosphatase activity occurred in the plots 5, 4 and 3 which correspond to plants of fair (plots 4 and 5) and weak (plot 3) vigour. Organic C, for instance, did not significantly varied among experimental plots. Average vales varied from 7.3 g kg^{-1} (plot 6) to 16.7 g kg^{-1} (plot 4). Extractable P significantly varied among experimental plots as determined by both the analytical methods. The higher average values were found in the plot 5 and the lower values in the plot 3. Extractable K varied from 286 mg kg^{-1} (plot 7) to 93.0 mg kg^{-1} (plot 3), the differences being statistically significant. Exchangeable bases (Ca, Mg, K, and Na) and exchangeable acidity significantly varied among experimental plots. The higher average values of exchangeable bases were found in plots 4 and 5. Exchangeable acidity was higher in Plot 7 and lower in plots 1, 4 and 6. Fe, Mn, Zn, Cu and B average values were higher, respectively in the plots 2, 1, 2, 3 and 5. Fe, Mn, Zn and Cu values were lower in the plot 7 and B values in the plot 3.

Table 3.1.1. Soil properties (mean ± standard deviation) from the samples collected between rows at 0-20 cm depth.

Soil properties	Plot 1 (weak)	Plot 2 (weak)	Plot 3 (weak)	Plot 4 (fair)	Plot 5 (fair)	Plot 6 (good)	Plot 7 (good)	*Prob*
pH_{H2O}	5.4±0.09 cd	6.2±0.14 a	5.9±0.08 ab	5.8±0.02 ab	5.7±0.14 bc	5.5±0.17 bc	5.1±0.22 d	<0.0001
pH_{KCl}	4.5±0.04 bc	5.3±0.21 a	4.8±0.07 b	4.8±0.03 b	4.7±0.11 b	4.4±0.15 bc	4.3±0.23 c	<0.0001
Conductivity (µs/m)	65.6±14.03 bc	79.4±16.31 ab	99.0±15.12 a	75.6±11.18 ab	88.1±8.97 ab	51.7±4.67 c	88.3±2.67 ab	0.0035
Organic C (g kg^{-1})	9.2±0.25 b	15.4±0.18 a	16.3±0.11 a	16.7±0.05 a	14.7±0.26 a	7.3±0.06 b	14.5±0.34 a	<0.0001
Acid phosphatase activity (p-Nitrophenol ug g^{-1} dwt h^{-1})	99.0±29.27 d	173.1±31.31 bcd	197.4±22.10 abc	210.7±19.33 ab	265.2±21.56 a	131.1±42.78 cd	128±12.36 cd	<0.0001
Extract. P (mg P_2O_5 kg^{-1})[a]	422.9±72.83 ab	279.5±94.95 bc	146.6±23.24 c	410.6±93.89 ab	492.9±53.96 a	191.1±48.39 c	212.6±48.77 bc	0.0001
Extract. P (mg kg^{-1})[b]	33.0±1.33 ab	31.6±2.66 ab	16.5±2.41 c	34.7±2.27 a	37.2±1.10 a	27.3±3.75 b	20.4±2.63 c	<0.0001
Extract. K (mg K_2O kg^{-1})[a]	142.7±3.21 c	112.0±16.52 cd	93.0±1.00 c	198.3±22.14 b	187.7±23.25 b	111.0±10.15 cd	286.0±8.72 a	<0.0001
Exchan. Ca (cmolc kg^{-1})[c]	8.1±0.40 c	16.5±2.12 b	19.9±2.45 b	20.4±0.96 b	26.2±1.53 a	10.7±0.30 c	2.7±0.79 d	<0.0001
Exchan. Mg (cmolc kg^{-1})[c]	1.6±0.14 cd	5.7±0.54 b	7.0±0.81 b	6.8±0.87 b	12.1±0.79 a	2.7±0.13 c	0.5±0.06 d	<0.0001
Exchan. K ($cmol_c$ kg^{-1})[c]	0.3±0.02 abcd	0.3±0.05 bcd	0.2±0.01 d	0.5±0.12 a	0.5±0.07 ab	0.2±0.02 cd	0.5±0.14 abc	0.0005
Exchan. Na ($cmol_c$ kg^{-1})[c]	0.3±0.27 bc	0.2±0.04 c	0.1±0.04 c	0.7±0.15 a	0.5±0.03 ab	0.1±0.01 c	0.3±0.10 bc	0.0002
Exchan. acidity ($cmol_c$ kg^{-1})[c]	0.2±0.06 b	0.4±0.10 ab	0.3±0.06 ab	0.2±0.01 b	0.3±0.01 ab	0.2±0.06 b	0.6±0.23 a	0.0045
CEC ($cmol_c$ kg^{-1})[c]	10.7±0.89 d	23.5±2.51 c	27.9±1.97 b	28.8±2.05 b	39.9±1.08 a	14.0±0.37 d	5.1±0.64 e	<0.0001
Extract. B (mg kg^{-1})[d]	1.0±0.05 ab	0.9±0.43 ab	0.2±0.19 c	1.1±0.17 ab	1.3±0.27 a	0.8±0.23 abc	0.6±0.16 bc	0.0014
Extract. Fe (mg kg^{-1})[e]	239.4±25.27 c	408.9±48.37 a	232.2±22.81 c	336.4±18.66 b	208.9±12.64 c	114.2±11.70 d	105.7±7.65 d	<0.0001
Extract. Mn (mg kg^{-1})[e]	331.8±21.88 a	278.3±22.71 ab	141.9±20.94 e	206.4±17.55 cd	153.5±23.23 de	224.1±17.74 bc	57.4±13.66 f	<0.0001
Extract. Zn (mg kg^{-1})[e]	9.1±1.66 ab	12.0±1.42 a	8.4±0.15 ab	11.8±0.62 a	11.6±3.02 a	7.2±0.33 cd	3.9±0.79 d	0.0001
Extract. Cu (mg kg^{-1})[e]	11.7±1.62 cd	16.5±2.63 abc	20.8±1.45 a	18.7±0.71 ab	15.1±2.75 bc	10.1±0.67 d	4.0±1.33 e	<0.0001

[a]Egner-Rhiem; [b]Olsen; [c]ammonium acetate, pH 7; [d]azomethine-H; [e]ammonium acetate and EDTA. For each variable the significance level (*Prob*) is provided. Means followed by different letter in rows are statistically different by Tukey HSD test (α = 0.05).

The results for significant correlation coefficients between soil properties are shown in Table 3.1.2. No significant correlations were found for extractable P, K, B and exchangeable K and Na, hence results are not shown. Correlations were significant and positive between pH (H_2O and KCl) and organic C, CEC, exchangeable Ca and Mg and acid phosphatase activity, indicating that these variables increase to higher pH values. Significant and positive correlations were found between pH and the micronutrients Fe, Cu and Zn. For Mn a moderated and negative correlation with conductivity was found, suggesting that when conductivity is high, Mn is moderately low. Exchangeable acidity presented a moderated positive correlation with conductivity. Texture, CEC, exchangeable Ca, exchangeable Mg and acid phosphatase activity were strong and positively correlated to clay and negatively correlated to sand.

Table 3.1.2. Significant correlation coefficients of conductivity, pH (H_2O and KCl) and texture to soil properties.

	Conduct.	pH_{H2O}	pH_{KCl}	Clay	Silt	Sand
Organic C	†0.75**	‡0.49*	‡0.58**	‡0.52*	†0.87**	‡-0.84**
Cation-exchange capacity		‡0.61**	‡0.63**	‡0.95**	†0.44*	‡-0.71**
Exchangeable Ca		‡0.61**	‡0.61**	‡0.95**		‡-0.71**
Exchangeable Mg		‡0.61**	‡0.61**	‡0.95**		‡-0.70**
Exchangeable acidity	‡0.47*					
Acid phosphatase activity	†0.50*	‡0.56*	‡0.59**	‡0.83**	‡0.50*	‡-0.72**
Conductivity					‡0.63*	‡-0.61**
Fe		†0.77**	†0.81**			‡-0.46*
Mn	‡-0.51*				‡-0.63**	
Zn		†0.68**	†0.67**	‡0.62**		‡-0.46*
Cu		†0.77**	†0.66**	‡0.76**	‡0.54*	‡-0.80**

*, **Significant at 0.05 and 0.01 levels, respectively; †Pearson coefficient; ‡Spearman coefficient.

3.3. Nutrient concentration in plant tissues

In the first sampling date, leaf N concentrations were higher in the plots corresponding to plants of higher vigour (Table 3.1.3). The average values varied from 40.0 (Plot 1, weak) to 46.1 (Plot 7, good) g kg^{-1}. In the next sampling dates, there was observed and opposite trend, in particular in the lower strata of the plants, probably due to a dilution effect by the increase in plant biomass in the most productive plots. Plot 7 sometimes appeared as an exception with values similar to those of the plot 1. At harvest, the plots of plants of lower vigour also tended to present higher leaf N concentration than those of higher vigour (see plot 6) and the same is true for stems and cones.

Table 3.1.3. Tissue nitrogen concentrations (g kg^{-1}) (mean ± standard deviation) for different sampling dates and tissue position in the plant from the different experimental plots.

Date	Tissue (plant position)	Plot 1 (weak)	Plot 2 (weak)	Plot 3 (weak)	Plot 4 (fair)	Plot 5 (fair)	Plot 6 (good)	Plot 7 (good)	*Prob*
Jun 2nd	Leaf (1 m)	40.0±1.90 b	40.2±1.12 b	45.8±0.86 a	45.0±2.20 a	43.3±1.63 ab	45.3±2.46 a	46.1±0.44 a	0.0012
Jul 1st	Leaf (1 m)	39.5±0.92 ab	38.4±2.01 ab	35.9±1.50 bc	33.8±1.27 cd	36.0±0.44 bc	32.0±2.40 d	40.1±1.26 a	0.0001
	Leaf (2 m)	40.6±1.17 a	42.5±0.81 a	38.2±2.81 ab	32.3±1.22 c	34.7±1.36 bc	30.4±2.95 c	39.5±1.56 a	<0.0001
Jul 27th	Leaf (1 m)	38.1±1.88 a	36.5±0.75 ab	32.6±0.55 c	30.9±2.55 c	33.8±0.10 bc	33.2±1.25 bc	36.2±1.05 ab	0.0002
	Leaf (2 m)	44.7±0.69 a	42.0±0.44 b	36.1±1.48 c	38.3±0.40 c	37.7±1.36 c	37.7±0.85 c	38.6±1.25 c	<0.0001
Sep 1st (harvest)	Leaf (basal)	32.0±1.90 a	29.1±1.16 abc	26.5±1.05 bc	25.8±3.52 c	27.0±2.83 abc	24.4±1.21 c	31.5±1.03 ab	0.0026
	Leaf (top)	35.8±1.29 a	32.6±0.57 ab	29.8±0.45 b	32.0±2.10 ab	32.4±2.08 ab	30.4±1.61 b	35.0±2.06 a	0.0034
	Stem (total)	12.5±0.15 a	10.8±0.61 b	10.4±1.11 b	9.3±0.70 bc	8.6±0.92 c	8.3±0.29 c	8.1±0.15 c	<0.0001
	Cone (total)	31.9±2.79 a	29.0±0.76 a	29.4±3.25 a	31.8±2.00 a	29.5±1.00 a	29.3±2.14 a	22.5±0.76 b	0.0013

For each variable the significance level (*Prob*) is provided. Means followed by different letter in rows are significantly different by Tukey HSD test (α = 0.05).

Leaf P concentrations varied from the first to the last sampling date (Table 3.1.4). The highest average value (3.5 g kg^{-1}) was found in plot 1 in the sampling of June 2nd and the lowest average value (1.0 g kg^{-1}) in plot 6 in September 1st. In spite of significant differences among plots had been found for some sampling dates, there was not observed a coherent trend related to plant vigour. Cone and stem P concentrations also varied among plots but a relation with plant vigour was also not observed.

Tissue K concentrations significantly varied in all sampling dates. Plants with good vigour (Plot 7) clearly exhibit the higher values in all tissues, except in stems were weak plants in Plot 1 presented the higher K concentration (Table 3.1.5). The lower K values were recorded for plants with weak vigour in Plot 3. Leaf K concentrations ranged from 3.6 g kg^{-1} for weak plants in Plot 3 at harvest to 29.2 g kg^{-1} for good vigour plants in Plot 7 at July 1st and leaves taken at 2 m. In the cones, the higher and lower average leaf K values were 18.9 and 10.7 g kg^{-1}, which were respectively found in the plots 7 and 3.

For the other macronutrients analysed (Ca and Mg), full tables were not provided to reduce the length of the texts. However, leaf Ca levels significantly increased throughout the growing season. In June 2nd, the values varied from 4.6 (Plot 7) to 10.5 (Plot 4) g kg^{-1} and in September 1st from 15.5 (Plot 5) to 49.2 (Plot 1) g kg^{-1}. Leaf Ca concentrations significantly varied among experimental plots for all sampling dates. However, a coherent trend regarding plots or plant vigour was not found. Stems and cones presented Ca concentrations lower than the leaves collected at harvest. In spite of there had been found significant differences among plots, a coherent trend regarding plant vigour was also not observed.

Leaf Mg concentrations followed the trend observed for Ca. The results showed an increase of leaf Mg over the growing season. The lower average value (2.1 g kg^{-1}) was recorded in plot 1 on June 2nd and the higher average value (15.3 g kg^{-1}) was found in plot 5 on September 1st. Also, in spite of significant differences in leaf Mg concentration had been found in all sampling dates, a coherent trend among experimental plot with different plant vigour was not observed. As for Ca, the levels of Mg in stems and cones were lower than in the leaves taken at harvest.

Table 3.1.4. Tissue phosphorus concentrations (g kg^{-1}) (mean ± standard deviation) at different sampling dates and tissue position in the plant from the different experimental plots.

Date	Tissue (plant position)	Plot 1 (weak)	Plot 2 (weak)	Plot 3 (weak)	Plot 4 (fair)	Plot 5 (fair)	Plot 6 (good)	Plot 7 (good)	*Prob*
Jun 2nd	Leaf (1 m)	3.5±0.0 a	2.9±0.26 b	3.0±0.06 b	2.9±0.20 b	3.0±0.12 b	3.1±0.17 b	3.1±0.06 b	0.0013
Jul 1st	Leaf (1 m)	2.0±0.20 ab	1.9±0.21 b	1.9±0.5220 b	2.2±0.06 ab	2.0±0.06 ab	2.0±0.21 ab	2.4±0.15 a	0.0251
	Leaf (2 m)	2.5±0.06 a	2.6±0.10 a	2.8±0.25 a	2.8±0.12 a	2.8±0.15 a	2.6±0.25 a	2.6±0.12 a	0.1720
Jul 27th	Leaf (1 m)	1.4±0.06 a	1.4±0.06 a	1.6±0.06 a	1.5±0.20 a	1.5±0.06 a	1.4±0.20 a	1.4±0.06 a	0.0002
	Leaf (2 m)	1.6±0.15 a	1.8±0.06 a	1.9±0.06 a	1.7±0.15 a	1.7±0.06 a	1.9±0.36 a	1.5±0.10 a	0.0904
Sep 1st (harvest)	Leaf (basal)	1.2±0.12 abc	1.1±0.10 bc	1.4±0.01 abc	1.5±0.36 a	1.5±0.06 ab	1.0±0.01 c	1.4±0.012 abc	0.0065
	Leaf (top)	1.5±0.20 a	1.5±0.06 a	1.6±0.06 a	1.9±0.40 a	1.7±0.35 a	1.4±0.12 a	1.5±0.10 a	0.2423
	Stem (total)	1.2±0.15 b	1.0±0.12 bc	1.1±0.15 bc	1.7±0.32 a	1.7±0.17 a	1.0±0.10 bc	0.8±0.06 c	0.0001
	Cone (total)	2.5±0.21 bc	2.7±0.29 abc	3.2±0.21 ab	3.4±0.38 a	3.2±0.38 ab	2.9±0.15 abc	2.2±0.40 c	0.0032

For each variable the significance level (*Prob*) is provided. Means followed by different letter in rows are significantly different by Tukey HSD test (α = 0.05).

Table 3.1.5. Tissue potassium concentrations (g kg^{-1}) (mean ± standard deviation) at different sampling dates and tissue position in the plant from the different experimental plots.

Date	Tissue (plant position)	Plot 1 (weak)	Plot 2 (weak)	Plot 3 (weak)	Plot 4 (fair)	Plot 5 (fair)	Plot 6 (good)	Plot 7 (good)	*Prob*
Jun 2nd	Leaf (1 m)	13.9±1.01 ab	12.8±3.15 abc	7.9±1.35 c	15.8±2.46 ab	10.8±1.46 bc	10.7±1.25 bc	16.5±2.00 a	0.0011
Jul 1st	Leaf (1 m)	15.5±0.85 b	10.1±1.21 cd	6.7±1.02 e	13.3±1.08 bc	10.4±1.71 cd	9.5±1.85 de	23.6±1.17 a	<0.0001
	Leaf (2 m)	16.3±0.81 b	10.8±0.45 cd	7.5±1.15 d	16.0±0.67 b	13.2±2.03 bc	10.5±2.16 cd	29.2±1.99 a	<0.0001
Jul 27th	Leaf (1 m)	10.9±1.48 bc	7.1±0.49 cd	5.0±0.44 d	12.9±2.65 b	7.0±3.87 cd	7.6±2.10b cd	22.0±2.06 a	<0.0001
	Leaf (2 m)	15.1±0.25 b	8.5±0.64 cd	6.0±0.93 d	16.3±1.27 bc	13.9±2.82 bc	11.9±3.93 bcd	25.2±4.39 a	<0.0001
Sep 1st (harvest)	Leaf (basal)	10.6±0.25 b	5.1±0.56 cd	3.6±0.20 d	8.1±3.64 bc	7.3±2.04 bcd	4.8±0.78 cd	15.5±0.79 a	<0.0001
	Leaf (top)	9.9±1.15 b	5.4±0.36 b	4.7±0.90 b	8.1±3.55 b	7.4±2.73 b	6.9±2.76 b	15.1±0.83 a	0.0006
	Stem (total)	11.4±1.70 a	4.2±0.64 bc	2.7±0.38 c	8.4±1.33 ab	8.3±3.30 ab	5.1±1.30 bc	10.1±2.05 a	0.0002
	Cone (total)	14.8±2.46 ab	10.9±0.56 b	10.7±2.21 b	14.1±1.06 ab	16.2±2.33 ab	13.1±2.49 ab	18.9±4.35 a	0.0130

For each variable the significance level (*Prob*) is provided. Means followed by different letter in rows are significantly different by Tukey HSD test (α = 0.05).

The concentration of Fe in the leaves increased throughout the growing season (Table 3.1.6). On June 1st, leaf Fe levels varied from 91.3 (Plot 1) to 153.4 (Plot 2) mg kg^{-1} and on September 1st from 169 (Plot 7) to 691.2 (Plot 4) mg kg^{-1}. In most sampling dates, there were found significant differences in leaf Fe concentrations among the experimental plots. The plants with fair vigour in Plot 4 present the highest Fe leaf concentration from Jul 1st until the harvest date in all leaf position and in cones. The cones showed average values higher than the stems and lower than the leaves.

Leaf Mn concentrations ranged from a minimum of 36.1 mg kg^{-1} to a maximum of 542.3 mg kg^{-1}, respectively for plants of plot 3 and plot 1, both the records being from the harvest date (Table 3.1.7). Weak plants of the plot 1 usually showed higher leaf Mn values than the plants of good vigour of the plot 7. Stems and cones presented lower average Mn values than the leaves.

The tissue concentrations in Cu, Zn and B were also not presented in full tables. Leaf Cu concentrations significantly varied among experimental plot for most of the sampling dates. However, a clear relationship between leaf Cu levels and plant vigour was not observed. The lower (2.1 mg kg^{-1}) and higher (6.7 mg kg^{-1}) average values were found in the sampling of July 1st, respectively in the plots 6 and 2. Stems and cones showed leaf Cu levels not much dissimilar than those of the leaves. However, cone Cu levels appeared generally higher than those of the other tissues.

In most of the sampling dates, leaf Zn concentrations significantly varied among the experimental plots. Plants of higher vigour (Plots 6 and 7) usually showed lower leaf Zn levels. Between the plots of weak and fair plant vigour no consistency in results was found, with the lower and higher values interchanging among sampling dates. Leaf Zn levels varied from 18.4 mg kg^{-1} (Plot 6, July 1st sampling) to 448.2 mg kg^{-1} (Plot 5, July 27th sampling). At harvest, leaf Zn levels tended to be lower than those of the previous sampling dates. Cones exhibited higher Zn levels than the leaves and stems.

Leaf B levels significantly varied among the experimental plots for all sampling dates. Plots 7, 6, 5, but also Plot 1 showed a dominance of ‘a’ letter from the Tukey test, meaning that the average values tended to be higher in these plots. Leaf B levels early in the growing season (June 2nd) were usually lower than those found in the samplings performed later. Considering all the sampling dates, leaf B levels varied from 20.1 mg kg^{-1} (Plot 2, June 2nd sampling) to 97.5 mg kg^{-1} (Plot 7, September 1st sampling). Cones, and particularly stems, showed B levels lower than those found in the leaves.

Table 3.1.6. Tissue iron concentrations (mg kg^{-1}) (mean ± standard deviation) at different sampling dates and tissue position in the plant from the different experimental plots.

Date	Tissue (plant position)	Plot 1 (weak)	Plot 2 (weak)	Plot 3 (weak)	Plot 4 (fair)	Plot 5 (fair)	Plot 6 (good)	Plot 7 (good)	*Prob*
Jun 2nd	Leaf (1 m)	91.3±4.6 a	153.4±120.9 a	144.9±55.7 a	126.4±4.5 a	93.4±4.0 a	93.7±15.9 a	118.8±30.4 a	0.6336
Jul 1st	Leaf (1 m)	170.5±9.3 b	171.8±21.1 b	153.5±11.1 b	264.0±28.8 a	148.1±22.9 b	168.0±8.8 b	156.6±15.2 b	<0.0001
	Leaf (2 m)	155.2±10.5 bc	134.6±11.5 bc	109.9±10.2 bc	304.8±75.2 a	198.2±38.9 b	98.6±16.3 c	109.9±13.8 bc	<0.0001
Jul 27th	Leaf (1 m)	197.9±17.1 b	187.2±14.5 bc	173.7±5.9 bc	266.4±17.8 a	180.5±8.2 bc	126.1±20.7 d	155.8±14.1 cd	<0.0001
	Leaf (2 m)	174.6±17.7 b	178.6±21.5 b	141.1±4.9 bc	224.5±25.1 a	178.2±28.8 b	106.8±1.0 c	122.8±5.9 c	<0.0001
Sep 1st (harvest)	Leaf (basal)	258.4±25.1 b	256.1±12.9 b	373.4±33.5 b	691.2±136.1 a	313.5±24.0 b	233.3±34.0 b	294.7±12.1 b	<0.0001
	Leaf (top)	250.9±55.1 c	233.6±44.3 c	239.7±25.2 c	606.1±44.6 a	464.3±97.1 b	237.8±51.1 c	169.4±7.4 c	<0.0001
	Stem (total)	56.4±11.2 ab	65.0±9.9 ab	80.2±15.4 a	62.9±18.7 ab	40.8±4.5 b	40.8±1.1 b	67.2±1.1 ab	0.0051
	Cone (total)	180.1±49.9 a	151.2±17.2 a	183.7±26.9 a	315.0±30.2 a	242.9±178.1 a	239.5±36.8 a	138.8±21.4 a	0.1151

For each variable the significance level (*Prob*) is provided. Means followed by different letter in rows are significantly different by Tukey HSD test (α = 0.05).

Table 3.1.7. Tissue manganese concentrations (mg kg^{-1}) (mean ± standard deviation) at different sampling dates and tissue position in the plant from the different experimental plots.

Date	Tissue (plant position)	Plot 1 (weak)	Plot 2 (weak)	Plot 3 (weak)	Plot 4 (fair)	Plot 5 (fair)	Plot 6 (good)	Plot 7 (good)	*Prob*
Jun 2nd	Leaf (1 m)	320.2±33.2 a	253.4±50.6 ab	215.7±44.2 bc	143.5±5.9 c	176.1±11.3 bc	225.5±29.1 b	68.5±12.6 d	<0.0001
Jul 1st	Leaf (1 m)	296.0±33.7 a	288.0±45.7 a	115.0±12.1 bc	153.7±32.5 b	165.9±15.6 b	63.0±12.9 c	125.6±14.4 bc	<0.0001
	Leaf (2 m)	230.3±40.8 a	175.3±45.9 a	52.2±17.9 b	50.6±14.9 b	56.2±8.0 b	45.7±6.7 b	94.4±11.9 b	<0.0001
Jul 27th	Leaf (1 m)	459.2±170.4 a	194.9±26.6 b	63.4±7.5 b	111.8±4.2 b	143.8±25.8 b	92.1±9.5 b	181.0±11.5 b	0.0001
	Leaf (2 m)	420.7±18.3 a	129.5±27.5 bc	37.4±5.0 d	46.9±7.1 d	58.7±12.4 d	95.8±7.6 c	158.1±13.5 b	<0.0001
Sep 1st (harvest)	Leaf (basal)	536.5±130.3 a	113.1±1.7 b	56.8±16.8 b	136.2±25.0 b	147.6±52.1 b	131.6±20.7 b	129.0±29.3 b	<0.0001
	Leaf (top)	542.3±61.6 a	41.6±6.3 b	36.1±13.8 b	51.7±6.1 b	86.4±42.2 b	113.3±14.3 b	81.8±38.8 b	<0.0001
	Stem (total)	257.1±89.7 a	39.8±6.1 b	27.1±8.9 b	24.9±5.7 b	27.4±5.5 b	37.7±5.8 b	63.7±23.9 b	<0.0001
	Cone (total)	175.6±37.1 a	47.0±3.6 b	54.2±7.1 b	54.8±3.2 b	43.5±15.5 b	84.2±18.6 b	57.0±19.2 b	<0.0001

For each variable the significance level (*Prob*) is provided. Means followed by different letter in rows are significantly different by Tukey HSD test (α = 0.05).

3.4. Principal component analysis

3.4.1 Soil PCA analysis

A PCA was applied to soil properties in order to assess the possible relationships between them and the experimental plots of different plant vigour. Exchangeable acidity and pH_{KCl} were not included in the analysis, since both presented very low eigenvalues and do not contributing to explained variance. Three components were retained from PCA that explained 80.3% of total variance. The first component C1, explained 41.9% of the total variance, with a high alpha Cronbach (0.91). The CEC had the highest positive loading in this component (1.1127), indicating soil high CEC values, and therefore C1 was denominated as "High CEC" (Table 3.1.8). The other variables associated with this dimension presented also positive and high loadings, with a predominance of exchangeable bases (Ca > Mg) and cationic micronutrients (Zn > Cu > Fe), but also pH_{H2O}. The second component C2, explained 20.7% of total variance, with an acceptable alpha Cronbach of 0.76. The two variables with higher positive loadings in component C2 were extractable K_{ER} and exchangeable K, indicating soil with high K concentrations, and so C2 component was named "High K". Na, B and extractable P_{ER} also presented high positive loadings for this component. The third component C3, explained 17.7% of total variance, also with an acceptable alpha Cronbach of 0.71. This component was named of "High Mn", because of strong and positive association of Mn. Conductivity and organic C were strong and negatively associated with C3, indicating high soil Mn concentration associated with lower values for electrical conductivity and organic C.

Table 3.1.8. Components extracted with PCA analysis applied to soil properties (C1, C2 and C3), and respective eigenvalues, explained variance, Cronbach's alpha values and component loadings of each soil parameter in each component.

	Components		
	C1 - High CEC	C2 - High K	C3 - High Mn
Eigenvalue	7.120	3.514	3.011
Explained variance	41.9%	20.7%	17.7%
Cronbach's Alpha	0.913	0.760	0.710
Component Loadings			
Cation-exchange capacity	**1.127**	-0.173	-0.325
Exchangeable Ca	**1.107**	-0.371	-0.230
Extractable Zn	**1.087**	-0.098	0.455
Exchangeable Mg	**1.078**	-0.113	-0.315
Acid phosphatase	**1.033**	-0.050	-0.482
Extractable Cu	**0.948**	-0.745	0.047
pH_{H2O}	**0.864**	-0.809	0.178
Extractable Fe	**0.857**	-0.350	0.379
Extractable K_{ER}	-0.200	**1.239**	-0.708
Exchangeable K	0.440	**1.192**	-0.340
Exchangeable Na	0.687	**0.992**	-0.241
Extractable B	0.592	**0.885**	0.672
Extractable P_{ER}	0.696	**0.874**	0.613
Extractable Mn	0.200	-0.152	**1.412**
Conductivity	0.367	0.008	**-1.085**
Organic C	0.738	-0.050	**-0.924**
Extractable P_{OL}	0.678	0.832	**0.869**

ER, Egner-Riehm method; OL, Olsen method; values in bold correspond to the highest loading of each soil property in the respective component.

Considering that high scores in C1 indicate higher soil CEC values, high scores in C2 indicate higher soil K concentrations and high scores in C3 indicate higher Mn soil concentrations; and comparing the scores of each one of the plots in each one of the PCA components (Figure 3.1.3), results suggest that:

a) The plots with fair plant vigour presented the highest scores in C1 and thus the highest CEC values. The plots with good plant vigour presented the lowest CEC values and the plots with weak plant vigour settle in the middle, with higher scores for Plots 2 and 3. In C1 component the plots with the same vigour were more similar in between and dissimilar from the others;
b) The plots with good (Plot 7), fair (Plot 5 and 4) and weak (Plot 1) plant vigour presented the highest scores in C2 and thus the highest K soil concentrations, in decreasing order. C2 component did not explained variances between plant vigour, since plots with different plant vigour (weak, good and fair) did not differed statistically; and
c) The highest scores in C3, corresponding to the highest Mn soil concentration, were registered for Plot 1 followed by Plot 6 and Plot 2. For C3 similarity between plots with same plant vigour only occurred for plant with fair vigour that registered middle scores.

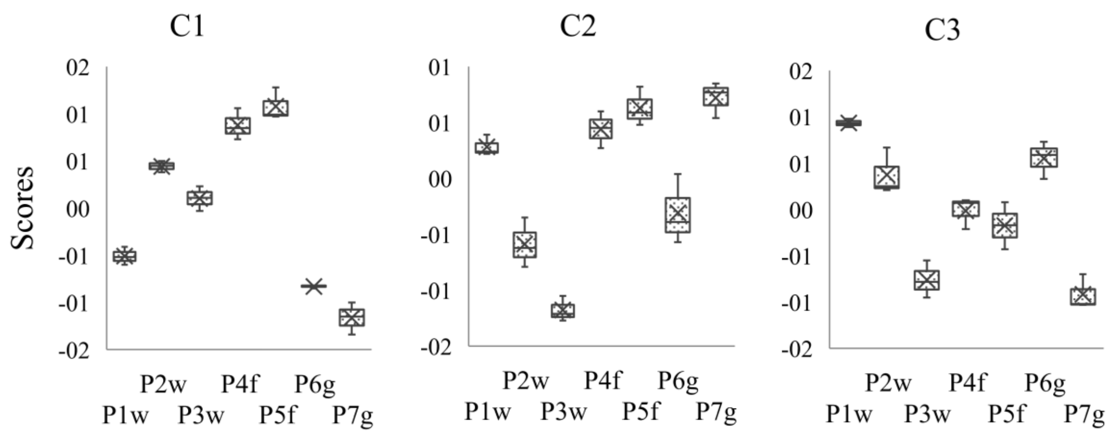

Figure 3.1.3. Scores distribution and means in each one of the components obtained in PCA analysis (C1, High cation-exchange capacity; C2, High K; C3, High Mn) for the soil collected in the plots (P) of weak (w), fair (f) and good (g) plant vigour. Means followed by different letter are statistically different by Tukey HSD test (α = 0.05).

3.4.2. Plant PCA analysis

PCA analysis was also performed from plant data and specifically for all sampling dates and analysed tissues. Three components were extracted in both PCA that explained a total variance of 70.3% for PCA in leaves data and 79.1% for PCA in harvest data. Table 3.1.9 provides an overview of both PCA's.

Table 3.1.9. PCA overview for the analysis performed in plant data with explained variance, Cronbach's alpha values and components summary.

		Components		
		C1	C2	C3
PCA in leaves data from June 2nd to July 27th	Explained variance	38.4%	16.7%	15.2%
	Cronbach's Alpha	0.822	0.446	0.380
	Components summary	Low P associated with high Ca, Mg, B, Fe	High Mn, K, N	High Cu, Zn
PCA in harvest data	Explained variance	36.7%	30.5%	11.9%
	Cronbach's Alpha	0.809	0.747	0.175
	Components summary	High Ca, B, Mg, Fe	High Zn, P, N, Cu	High Mn, K

For PCA results in leaves collected until July 27th, scores mean in each one of the PCA components and Tuckey HSD test were calculated for all plots, regarding the three sampling dates and leaf positions (Figure 3.1.4). The information was summarized as below:

a) High scores in C1 component indicate, in the comparison between plots, lower P leaf concentrations associated with higher leaf concentrations of Ca, Mg, B and Fe. The higher scores in C1 were recorded in the sample date of July 27th, and the lower in June 2nd, both for leaves collected at 1 m. The plots with fair vigour 5 and 4 generally presented higher scores in C1 and plots with weak vigour (Plot 1) and with good vigour (Plot 7) mostly presented the lower scores;
b) High scores in C2 component indicate higher Mn leaf concentrations, followed by higher K and N concentrations. The Plot 1 (weak) registered the highest scores in all dates and leaf position, related with highest Mn leaf concentrations. Plot 7 (good) was also high in July 1st and 27th, related with highest K and N leaf concentrations. All the other plots presented low scores; and
c) High scores in C3 component indicate higher Cu and Zn leaves concentration. In the first date (June 2nd) scores in C3 were positive and Plot 3 (weak) presented the higher scores. Plot 7 mostly presented the lower scores, related with lower leaf concentration in Cu and Zn.

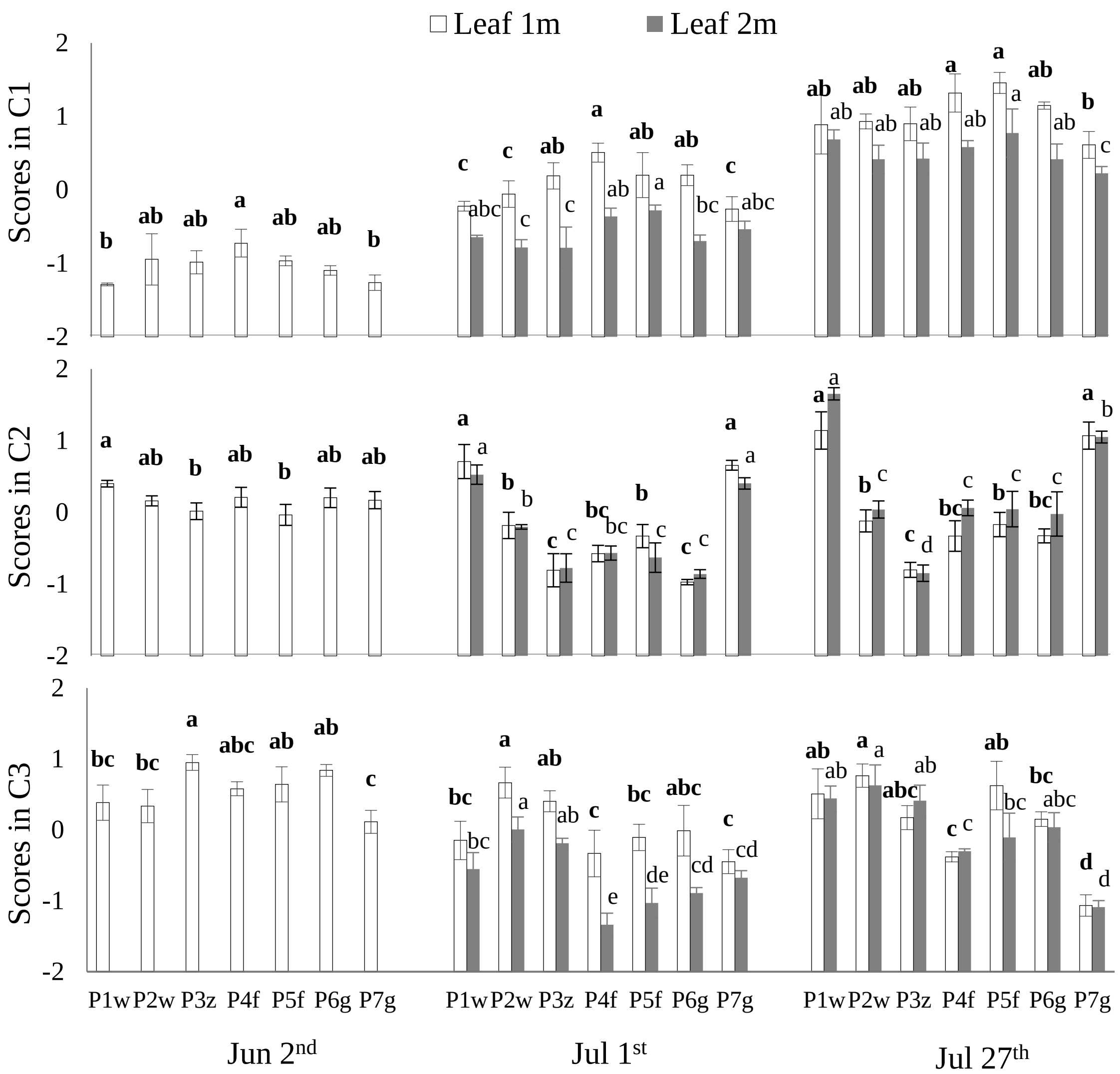

Figure 3.1.4. Scores means and standard deviations of the plots (P) of weak (w), fair (f) and good (g) plant vigour in each one of the components obtained in PCA leaves data (C1 to C3) for the leaves collected at different tissue position (leaf 1m and leaf 2m) and at the sampling dates of June 2nd, July 1st and July 27th. Means followed by the same letter (leaf 1m are in bold) are not statistically different by Tukey HSD test (α = 0.05).

For PCA results in plants collected at harvest date, scores mean in each one of the PCA components and Tuckey HSD test were calculated for all plots, considering the different sample tissues (leaf basal, leaf top, stems and cones) (data not shown due to its extensity). The information may be summarized as below:

a) High scores in C1 component indicate, in the comparison between plots, higher tissue concentrations in Ca followed by B, Mg and Fe tissue concentrations. The highest scores were registered in leaves and for Plot 1 (weak), followed by Plot 6 (good), explained mainly by Ca and B leaf concentrations. Scores were all negative for stems and cones;

b) High scores in C2 component indicate higher tissue concentrations in Zn followed by P, N, and Cu. The highest scores for C2 were registered for cones, and for plots with fair vigour (Plots 4 and 5) which differed significantly from Plot 7 (good) with the lowest scores. These differences were related mainly with Zn, P and N cone concentrations. The plots of weak vigour (Plot 1) and fair vigour (Plots 4 and 5) presented the highest scores in basal and top leaves, explained mostly by higher leaf concentration in Zn, P and Cu and also N for Plot 1. Scores were negative for stems; and
c) High scores in C3 component indicate higher Mn tissue concentrations followed by higher K. For basal and top leaves, Plot 1 (weak) presented the highest scores, followed Plot 7 (good) and both differed significantly from all the others. For cones and stems, Plot 1 (weak) also presented the highest scores and differ significantly from all the others, followed by Plot 7 (good). The higher scores for Plot 1 were associated with high Mn concentrations and for Plot 7 were associated with high K concentrations.

The three components indicated by each one of the PCA performed in plant data were similar but, except for the component associated with high Mn and K concentrations, a common trend was not found. Mn and Fe seems to be the nutrients which differentiated more between plant vigour and also K at some extend.

4. Discussion

Soil analysis did not reveal particular problems for most of the soil properties analysed. Although low values sometimes appeared for exchangeable Ca, Mg and Na and extractable B, as compared with the Portuguese standard classification of soils (LQARS, 2006), they were often associated with plots of plants with good vigour and not so much with plots of weak plants, which reduces their meaning in relation to plant nutritional status. The extractable P and K were at relatively high levels in the soil and usually within the sufficiency ranges in the leaves, as reported by Bryson et al. (2014), which probably also excludes them from the list of soil properties that are negatively influencing plant growth, even though hop is considered to be a highly demanding K species (Gingrich et al., 1994). In turn, extractable Mn and Fe were at very high levels in the soils and appeared also at very high levels in plant tissues, frequently above the upper limit of the sufficiency range (Bryson et al., 2014), which gives them a great centrality in this study in the search for the causes affecting plant growth.

Excessive Mn levels were predominantly associated with plots of weak vigour plants and excessive Fe levels with plots of fair vigour plants. Reduction conditions may increase Fe and Mn soluble forms in the soil due to the dissolution of Fe and Mn oxides, which can result in a strong uptake by plants (Phillips and Greenway, 1998; Singer and Munns, 2002). Stieger and Feller (1994) reported a considerable increase in Mn and Fe content in wheat plants growing in large pots under conditions of flooding. Pot experiments are often associated with high levels of Fe and Mn in plant tissues (Afonso et al., 2018; Rodrigues et al., 2018), probably due to the greater regularity of water supply and poor drainage than occur with plants grown in the field.

Leaf Mn concentrations can vary greatly depending on the availability of Mn in the soil. In turn, the sufficiency ranges for Mn, or the critical Mn concentrations vary greatly among plant species (Bryson et al., 2014; El-Jaoual and Cox, 1998). Symptoms of Mn toxicity can be observed at leaf Mn levels that range from 200 mg kg^{-1} in Mn sensitive plants to over 5000 mg kg^{-1} in tolerant plants (Weil and Brady, 2017). Hop is considered a sensitive species to high levels of Mn, and a negative influence on plant growth has been reported mostly on acid soils (Gingrich et al., 1994).

Mn toxicity can manifest in plants by the reduction of biomass and early leaf senescence through biochemical disorders and in particular by damage of the photosynthetic system (Millaleo et al., 2010; 2019). In this study, leaf Mn concentrations above 200 mg kg^{-1} were often recorded, mostly on plots of weak plant vigour (> 500 mg kg^{-1} at harvest in Plot 1). The sufficiency ranges established for hop in vegetative and blossom stages, after Bryson et al. (2014), are respectively 45 – 125 mg kg^{-1} and 50 – 150 mg kg^{-1}. This is a strong evidence of the negative effect of Mn in crop growth and yield.

Excessive leaf Fe levels were also observed but particularly in plants of fair vigour. Maximum average values were recorded at 691.2 mg kg^{-1} in Plot 4 from leaves sampled at harvest. The sufficiency range after Bryson et al. (2014) for leaf Fe in hop is between 35 and 151 mg kg^{-1}. As far as we know, a description of a specific symptomology for Fe toxicity in hop does not exist, but the common symptom of Fe toxicity in crops is ‘bronzing’ (Broadley et al., 2012). The plots of fair vigour plants, those probably with toxic Fe levels in tissues, showed not only a reduction in total DMY, but also a notable reduction in cone yield, whose differences were not statistically significant in comparison to the plots of weak plants. Fe toxicity is a serious nutritional disorder in crop production on waterlogged soils; it is the second-most severe yield-limiting factor in wetland rice (Broadley et al., 2012). Fe toxicity is usual in rice because of reduction processes associated with soil flooding (Sahrawat, 2005). Fe toxicity in rice plants usually occurs to leaf Fe concentrations above 300 mg kg^{-1} (Fageria, 2001). Plants with fair vigour in Plot 5 and Plot 4 registered leaf Fe concentrations at harvest higher than the values reported by Fageria (2001), which increases the evidence of the responsibility of Fe on the reduction in plant growth.

It seems that Mn will have been the more serious problem in the plots of weak plants and Fe in the plots of fair vigour plants. Antagonism between Mn and Fe has been widely reported (El-Jaoual and Cox, 1998; Fageria, 2001; Rietra et al., 2017) and, in spite of the physiological mechanisms not yet being clearly understood, it is currently accepted as occurring (Fageria, 2001; Havlin et al., 2014). Also, an early report oh hop from Thompson et al. (1950) showed that Fe deficiency was induced by high levels of Mn. This may mean that in weak vigour plants, the higher levels of Mn in plant tissue is the main reason for the reduced growth, and for the relatively low Fe levels in tissue due to an antagonistic effect on Fe uptake. The lower levels of Mn in the plants of fair vigour plots, may have allowed the increase in tissue Fe concentration, Fe being mainly responsible for the reduction of vigour in these plots.

One of the weaknesses of the surface irrigation methods is the heterogeneity in the spatial distribution of water (Walker, 1989). Thus, winter flooding and the irrigation system seem to be the major causes for the appearance of patches of poorly developed plants and for the increased levels of Mn and Fe in soil and plants. Mn redox reactions are very sensitive to the potential redox of the soil (Sparrow and Uren, 2014). In the thermodynamic sequence of the reduction process that takes place under flooding, Mn reduction occurs first, being followed by Fe reduction (Osman, 2013). In most plots, leaf Mn levels were higher on the first sampling dates. In spite of irrigation, drier summer conditions seem to reduce Mn availability. This may mean that reduced soil drainage during winter has been more damaging to the development of plants than even the deficient water distribution associated with the surface irrigation system in summer. Leaf Fe levels tended to evolve in a different way with comparatively low values on the first sampling dates, probably due to antagonism by Mn.

The risk of Mn toxicity increases in acidic soils, particularly under reduction conditions (Millaleo et al., 2010; Weil and Brady, 2017). When soils are flooded, the pH of acidic soils tends to increase (Parent et al., 2008). However, the pH of the soils sampled on June 2nd ranged from acidic (pH_{H2O} = 5.1) to slightly acidic (pH_{H2O} = 6.2), conditions that are still favourable for Mn toxicity to occur.

The presence of decomposable OM is one of the requirements for soil reduction (Pezeshki and DeLaune, 2012). In submerged soils, the decomposition of OM is slower because it is overtaken mainly by anaerobic microorganisms, and large amounts of partially decomposed OM tend to accumulate (Weil and Brady, 2017), influencing the degree of the reduction processes. Phillips and Greenway (1998) conducted an experiment under alternating waterlogged and drying conditions and concluded that in waterlogged soils the magnitudes of changes, which included the increasing of soluble Fe, Mn, Ca and Mg, were greatest in soils with the higher levels of OM. PCA analysis indicated that high soil levels in Mn were strong and negatively associated with organic C and conductivity.

It is considered that Mn is closely related to the transformation of organic materials in soils and Mn availability under flooding is usually greater when soils contain high levels of OM (El-Jaoual and Cox, 1998; Singer and Munns, 2002). However, although Mn is released under reduction conditions, it is also removed by cation exchange reactions, precipitations and the formation of insoluble complexes (Millaleo et al., 2010). Ponnamperuma (1972) concluded that under reduction conditions when soils are high in Mn and low in OM there can be high Mn peaks that will be late and broad; in contrast, in the case of high OM, the Mn peaks will be quicker and also quicker to decline. This seems to be the case in the present data: OM may play a role in the extension of high Mn concentrations during the growing season.

Conductivity was positively and strongly associated with organic C and negatively associated with Mn. Soil electrical conductivity measures soil salinity in terms of the total concentration of the soluble salts (Lund et al., 1999). Usually the electrical conductivity increases after flooding due to the increase in soluble Fe, Mn and other cations (Camargo et al., 1999). The explanation for the negative correlation found with Mn, might be related to Fe reduction. The first component extracted with PCA analysis of the soil data indicated that higher values of CEC were associated with higher concentrations of exchangeable bases (Ca and Mg) and cationic micronutrients (Zn, Cu and Fe). Thus, the plots with higher Fe concentrations were also higher in CEC and other cationic micronutrient influencing conductivity values.

Soil texture varied among plots of the same plant vigour. Positive and strong correlations were found between CEC, exchangeable Ca and Mg with the clay content. PCA analysis also indicated high CEC levels associated with high Fe soil concentrations. According to Singer and Munns (2002), in the majority of soils, the soluble Fe and Mn are mostly supplied by the dissolution of hydroxyoxides of the clay fraction. Favre et al. (2002), in a field experiment with rice, also observed a strong increase in CEC upon flooding. They attribute a CEC increase to changes in clay minerals as a result of octahedral Fe reduction and Fe oxyhydroxide coating solubilisation. They also concluded that CEC increases were reversible even in long term flooding but may substantially modify soil chemical and even physical properties. Kostka et al. (1999) also suggested that structural Fe reduction is catalysed by soil anaerobic bacteria through the decrease of clay swelling and surface area, and also through the increase of the clay surface charge density as a function of CEC. These findings are in agreement with the present data; the clay fraction seems to explain the predominance of Fe and CEC higher levels, particularly in the plots with fair plant vigour.

The availability of P and K in soil solution increases under reduction conditions (Phillips and Greenway, 1998). In the present study, K and P concentrations in the soil were generally high but leaf concentrations were often below the sufficiency ranges, particularly for P. Stieger and Feller (1994) reported decreased levels of K and P in wheat shoots and grain under waterlogged conditions. In addition, the negative influence of high levels of Mn in soil solutions with P uptake has also been reported (El-Jaoual and Cox, 1998).

The results also indicate variations in leaf K concentrations, with Plot 7 (good) presenting the higher values and the plots of weak plant vigour generally lower values, though in Plot 6 (good) the leaf K levels were lower than in Plot 1 (weak). This seems to be a contradictory result since K is usually associated with increasing plant growth (Hawkesford et al., 2012). However, K tends to vary greatly between cropping conditions and years (Rodrigues et al., 2020). Otherwise, probably this is due to the variability in soil reduction conditions along the fields and the subsequent nutrient interaction that takes place. Cationic antagonisms are predominant (K, Fe, Mn, Cu and Zn), but synergic interactions can also occur, as reported for Mn and K (Fageria, 2001; Rietra et al., 2017). Plot 1 registered the higher Mn concentration in plant tissues and, among the plots of weak plant vigour, it also showed the higher tissue K concentration, suggesting the occurrence of a synergetic effect between K and Mn, which is supported by PCA results.

Nutrient interactions are complex. They can influence plant growth by inducing deficiencies or toxicities and they are particularly relevant under reduction conditions (Fageria, 2001; Rietra et al., 2017). Mn toxicity, for instance, is highly dependent on environmental conditions and plant nutritional status, and can be lowered by the increase of Fe, Ca, Mg, P and K (Auda et al., 2002; El-Jaoual and Cox, 1998). Mn enters the root cells through a specific transporter protein with little competition by other cations, although high concentrations of Ca and Mg adsorbed to the aploplastic cell wall of the root, particularly at high pH, can reduce Mn adsorption and transport (Havlin et al., 2014). The results of PCA analysis for soil data indicated pH, Ca, Mg and Fe with the higher and positive scores in the same component (C1-High CEC) and with low or negative scores in the other components (C2 - High K; C2-High Mn). These associations were also expose in PCA results for plant data, underlining Fe, Ca and Mg antagonism in relation with Mn and K.

There are several factors that affect plant grown under stress flooding which cannot probably be fully elucidated through soil and plant analysis. When plant roots are submerged under water for short-term, the O_2 levels decrease below optimal levels (hypoxia), but under long-term flooding a complete lack of O_2 can occur (anoxia) (Sairam et al., 2008). The O_2 deprivation that takes place readily after soil flooding along with the increase of C dioxide (CO_2) and the decrease of the redox potential can reduce root elongation and penetration depth (Irfan et al., 2010; Pezeshki and DeLaune, 2012). Flooding per se changes also the physical properties of the soil which may increase the mechanical resistance to root penetration (Blom and Voesenek, 1996). As a result of reduction conditions, root dysfunction, death and blockages due to phytotoxin damage can lead to inhibition of nutrient uptake and transport, nutrient toxicity and reduced water uptake (Pezeshki and DeLaune, 2012). Mn and Fe seem to accumulate in roots in flooded soils, although Mn is less effectively retained (Millaleo et al., 2010; Alhdad et al., 2015). It has been reported that Mn toxicity can result in the shortening of the root and shoot length, with particular severity in the former (Auda et al., 2002; El-Jaoual and Cox, 1998). In the conditions of this fields, it seems clear that some patches experienced anoxia during winter months.

5. Conclusions

The results indicate high soil and tissue Mn levels associated with the plots of weak vigour plants and high Fe levels associated with fair vigour plants. The toxic levels of Mn and Fe seem to result from soil reduction conditions due to the poor drainage of the soils and deficient water distribution caused by the surface irrigation system. Thus, the poor development of the plants is probably the result of the effects of flooding, giving rise to O_2 deprivation in the rooting zone, and the consequent toxicity of Mn and Fe. Several factors, in particular the redox potential, pH, clay content, organic C, and nutrient interactions, seem to play a role in the prevalence of the effects of Mn over Fe, and vice-versa in different plots. In the light of the results, farmers should consider implementing a drainage system to improve soil aeration, to change the irrigation system to introduce drip irrigation, and to increase soil pH by liming. Taken together, these factors can reduce the bioavailability of Mn and Fe and favour plant growth. If these changes are made, OM could also be favourably applied.

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3.2. Twenty-years of hop irrigation by flooding the inter-row did not cause a gradient along the row in soil properties, plant elemental composition and dry matter yield

Abstract

In hops (*Humulus lupulus* L.), irrigation by flooding the inter-row can carry away suspended particles and minerals causing gradients in soil fertility. The effect of more than 20 years of flooding irrigation on soil and plants was evaluated in two hop fields by measuring soil and plant variables in multiple points along the rows. In a second experiment 1,000 kg ha^{-1} of lime was applied and incorporated into the soil to assess whether liming could moderate any gradient created by the irrigation. At different sampling points along the rows, significant differences were recorded in soil properties, plant elemental composition and dry matter yield, but this was not found to exist over a continuous gradient. The variations in cone yield were over 50% when different sampling points were compared. However, this difference cannot be attributed to the effect of irrigation, but rather to an erratic spatial variation in some of the soil constituents, such as sand, silt and clay. Flooding irrigation and frequent soil tillage resulted in lower porosity and higher soil bulk density in the 0.0-0.10 m soil layer in comparison to the 0.10-0.20 m layer. In turn, porosity and bulk density were respectively positively and negatively associated with crop productivity. Thus, irrigation and soil tillage may have damaged the soil condition but did not create any gradient along the row. The ridge appeared to provide an important pool of nutrients probably caused by mass flow due to the evaporation from it and a regular supply of irrigation water to the inter-row. Liming raised slightly the soil pH, but had a relevant effect on neither soil nor plants, perhaps because of the small amounts of lime applied.

Keywords: *Humulus Lupulus* L.; Soil porosity; Soil bulk density; Liming; Hop ridges

1. Introduction

Hop plants (*Humulus lupulus* L.) require an adequate supply of water during the growing season to sustain its huge canopy (Turner et al., 2011). In most of the hop producing regions of the world, the crop needs to be irrigated, particularly in lower latitudes of reduced precipitation in summer. Although hop fields have started to be drip irrigated all over the world, there is a long tradition of surface watering of this crop, by flooding the space between rows (Rossini et al., 2021; Turner et al., 2011). In this kind of surface or furrow irrigation system, water is applied at the top end of each furrow (in hops to the inter-row space) and flows down the field under the influence of gravity (Hoque, 2018). This is still the most commonly used irrigation method of hops in northern Portugal (Afonso et al., 2020). The water use efficiency with this irrigation technique is highly dependent on the field gradient and water infiltration rate, which can vary considerably, inducing spatial and temporal variability in the main soil properties (Hedley et al., 2014). In addition, flood irrigation can affect the spatial distribution of soil physico-chemical properties which may exacerbate the spatial variability in crop growth and yield (Simmonds et al., 2013).

Flood irrigation can have a major impact on soil properties by varying salinity, redox potential, compaction and/or porosity (Batey, 2009; Cerdà et al., 2021; Cox et al., 2018; González-Méndez et al., 2017). Furthermore, hop fields which are flood-irrigated need to be frequently tilled to control summer weeds and to reduce soil compaction and superficial crusts in the short term. This allows a better infiltration of water, but that can also have a negative impact on the soil in the long term (Arriaga et al., 2017; Shapiro and Elmore, 2017). Soil compaction, increased by furrow irrigation, may also reduce soil drainage and aeration, contributing to the reduction of soil redox potential which influences soil chemistry and plant nutrient availability (Batey, 2009; Nawaz et al., 2013). The degree of compaction of a soil can be assessed by measuring some physical properties, such as bulk density and porosity (Arriaga et al., 2017; Nawaz et al., 2013). As the soil becomes more compact, bulk density increases and soil porosity decreases, which reduces water and air diffusion into the soil (Indoria et al., 2020; Shapiro and Elmore, 2017). In some hop fields in northern Portugal it was found that the decrease in soil redox potential, associated with excess of water and/or poor drainage, was the main cause of the spatial variability found in crop growth and yield (Afonso et al., 2020).

Soil pH is another relevant issue in hop production. The range of pH most suited for growing hops is considered to be between 5.7 and 7.5 (Gent et al., 2015; Sirrine et al., 2010). The application of lime is recommended for acidic soils, and a positive relationship has been found between the increase in soil pH and hop yield (Čeh and Čremožnik, 2015; Sirrine et al., 2010). However, the effect of liming on crops can also vary with the irrigation system. Some researchers have studied the influence of liming in rice under flooding conditions, since great interactions between flooding, soil acidity and nutritional disorders are usually found (Sadiq and Babagana, 2012; Seng et al., 2006; Shi et al., 2019). In hops, these interactions are less well known, as well as the response to liming, but it is believed that it may be relevant enough to be studied, since the crop continues to be irrigated by flooding in several parts of the world.

This study evaluated the variation in soil properties and nutritional status and productivity of hop plants created along the rows by flooding irrigation. As a second line of study, the effect of the application of lime on soil properties and on hop nutritional status, growth and yield was evaluated, to ascertain if the application of lime could compensate for the variability created by the irrigation system. Both lines of study were carried out in commercial hop fields which had been flood-irrigated for over 20 years.

2. Materials and methods

2.1. General experimental conditions

The field experiments were carried out during two growing seasons (2017 and 2018) on a commercial farm located in Pinela (41°40'33.6"N; 6°44'32.7"W), Bragança, NE Portugal. A detailed location of field experiments is shown in Figure 3.2.1.

Figure 3.2.1. Map of Portugal indicating Pinela (left) and the two hop fields identified in this study as field 1 and field 2 (right). Images from https://www.google.com/maps/place/Pinela.

The region benefits from a Mediterranean-type climate, with an annual average temperature and accumulated precipitation of 12.7 ºC and 772.8 mm, respectively. Average monthly temperature and precipitation recorded during the experimental period are shown in Figure 3.2.2.

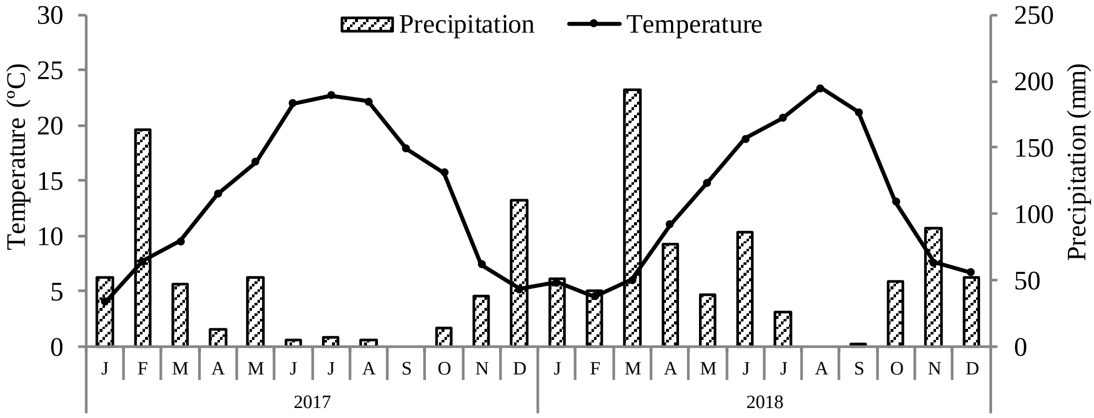

Figure 3.2.2. Average monthly temperature and precipitation recorded during the experimental period at a weather station located in Bragança, north-eastern Portugal.

The hop plots where the study was undertaken are ~2 ha in size each, with the rows having a length ranging from 150 to 180 m, and established with the cultivar Nugget. The fields are arranged in a 7 m conventional high trellis system, with concrete poles connected with steel cables, in a “V” design system. The farmer has managed the fields by flooding irrigation since the hop crop was installed more than 20 years ago. Several tillage passes (3 to 4) are performed every year to remove the crusts and facilitate water infiltration. The fertilization programme includes the application of a compound nitrogen (N): phosphorus (P): potassium (K) fertilizer (7:14:14, 7% N, 14% P2O5, 14% K2O) early in the spring, followed by two applications of N fertilizer (NH_4NO_3, 27% N) as a side-dressing, totalling ~150, 44 and 83 kg ha^{-1} of N, P and K, respectively. The farmer also follows a phytosanitary programme for crop protection against pests and diseases.

2.2. Field experiments and soil and plant sampling

The first experiment (Experiment 1) was carried out during the growing season of 2017 in two hop fields. It consisted of the evaluation of soil properties, plant nutritional status and crop yield, searching for any gradient along the rows created by the irrigation system. The rows used in this experiment were divided into nine segments of equivalent length, creating nine positions (P1, P2, …, P9) for soil and plant sampling. The soil was sampled between rows and on the ridges to a depth of 0.0 to 0.2 m. Three rows and inter-rows of hops were used to create three replicates for each position. Each soil sample for analysis resulted from six sampling points (composite samples). The soil was sampled by using an open-face auger.

For the determination of soil bulk density and porosity, a different approach to soil sampling was followed. It was found unnecessary to sample in the ridges since no compaction was expected in this part. Instead, the soil was sampled at two different depths, 0.0 to 0.10 m and 0.10 to 0.20 m. Due to the increased difficulty of sampling, particularly in the 0.10 to 0.20 m layer, only five positions were considered (P1, P3, P5, P7 and P9) and sampled in three replicates. For these analyses, undisturbed soil cores were taken by using appropriate cylinders of 100 cm^3. Soil samplings were carried out on March 10th 2017.

The plants used in this experiment for the evaluation of their nutritional status and crop productivity were randomly selected and marked when plant height was close to 3 m (to avoid using very atypical plants) and close to each of the positions used for soil sampling. Leaf sampling for crop nutritional status assessment was done at ~2 m in height, on July 17th 2017. At harvest (September 1st 2017), plant biomass was cut at ground level. Subsequently, the aboveground biomass was separated into leaves, stems and cones and weighed fresh. Subsamples of each plant part were weighed fresh again and then oven-dried at 70 ºC and weighed dry for determination of DMY.

The second experiment (Experiment 2) consisted of the application of 1,000 kg ha^{-1} of lime (55% $CaCO_3$, 28% CaO and 20% MgO) in February 2017, to assess the liming effects on soil properties and plants in comparison to the untreated control. This experiment was also carried out in two hop plots. The general methodology for soil and plant sampling was similar to that reported for Experiment 1, consisting of marking nine positions along the rows. The soil was sampled on January 4th 2019, only between the rows, at 0.0-0.20 m soil depth, using an open-face auger. Leaf samples were taken at ~2 m in height, on July 17th 2017 and July 18th 2018. At harvest (September 1st 2017 and August 31st 2018), plant biomass was cut at ground level and treated as reported for Experiment 1.

2.3. Laboratory analyses

The undisturbed soil samples from Experiment 1 were oven-dried at 105 ºC and weighed. Soil bulk density was estimated from the weight of dry soil divided by the volume of the cylinder. Soil porosity was determined as the ratio of nonsolid volume (soil particle density – bulk density) to the total volume of soil (soil particle density) [21]. The other soil samples from Experiments 1 and 2 were oven-dried at 40 ºC and sieved in a mesh of 2 mm. The samples were analysed for pH (H_2O and KCl), electrical conductivity (soil:solution, 1:2.5), exchangeable complex (ammonium acetate, pH 7.0) and organic carbon (C) (Walkley-Black method). Extractable P and K were determined by a combination of ammonium lactate and acetic acid buffered at pH 3.7. Soil boron (B) was extracted by hot water and the extracts analysed by the azomethine-H method. More details of these analytical procedures are given in Van Reeuwijk (2002). Other micronutrients [copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn)] were determined by atomic absorption spectrometry after extraction with ammonium acetate and EDTA, following the methodology reported by Lakanen and Erviö (1971). Tissue samples (leaves, stems and cones) from both experiments were oven-dried at 70 ºC and ground. Elemental tissue analyses were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K) and atomic absorption spectrophotometry [calcium (Ca), magnesium (Mg), Cu, Fe, Zn and Mn] methods after nitric digestion of the samples (Walinga et al., 1989) .

2.4. Data analysis

Data was subjected to analysis of variance, according to the experimental designs, using SPSS program v. 25. When significant differences were found between the experimental treatments, the means were separated by the Tukey HSD (Sampling position) and Student's-t (Field, Sampling site, Lime treatment) tests (α = 0.05). Linear regression analysis was performed to understand the effects of gradient on soil properties and plant nutritional status and productivity in Experiment 1 and the relationship between soil pH and plant variables in Experiment 2. The relation between the variables was obtained through correlation analysis with the Pearson coefficient, when the assumption of normality and linearity was accomplished; when this was not the case, the Spearman coefficient was used.

3. Results

3.1. Gradients in soil and plants along the rows

3.1.1. Soil properties

Silt and sand contents varied significantly between sampling positions (Table 3.2.1). The two fields also differed significantly in clay and sand content. Soil bulk density and soil porosity varied significantly between sampling positions and fields but in the opposite way. The interaction between sampling position and field was significant for soil porosity, which means that the effect of the irrigation on this variable depended on the field.

Table 3.2.1. Soil separates and soil bulk density and porosity from samples collected at 0.0-0.20 m depth, in March 2017, as a function of sampling position (1, …, 9), and field. Means followed by the same letter are not significantly different by Tukey HSD (Sampling position) or Student's-t (Field) tests (α = 0.05).

	Clay	Silt	Sand	Bulk density	Porosity
	---------------------(%)---------------------			(kg dm^{-3})	(%)
Sampling position (P)					
Lowest value	15.6 a	34.5 a	59.7 a	1.26 a	52.1 a
Highest value	11.8 a	28.5 b	49.9 b	1.18 b	48.0 b
Field (F)					
Field 1	16.0 a	33.2 a	50.8 b	1.25 a	49.1 b
Field 2	11.5 b	32.1 a	56.4 a	1.21 b	50.8 a
Prob (P)	0.2770	0.0386	0.0307	0.0143	0.0020
Prob (F)	0.0005	0.3741	0.0072	0.0260	0.0259
Prob (P×F)	0.8998	0.0432	0.1221	0.0874	0.0256

Soil bulk density was higher in the soil surface (0.0-0.1 m) when compared to the deeper (0.1-0.2 m) layer (Figure 3.2.3). Soil bulk density did not vary significantly along the rows for both soil depths. Soil porosity, in turn, was lower in the surface layer, and the gradient found along the rows was not significant for any of the soil layers. Soil bulk density and porosity varied significantly between the two fields, but the gradients found along the rows were not statistically significant.

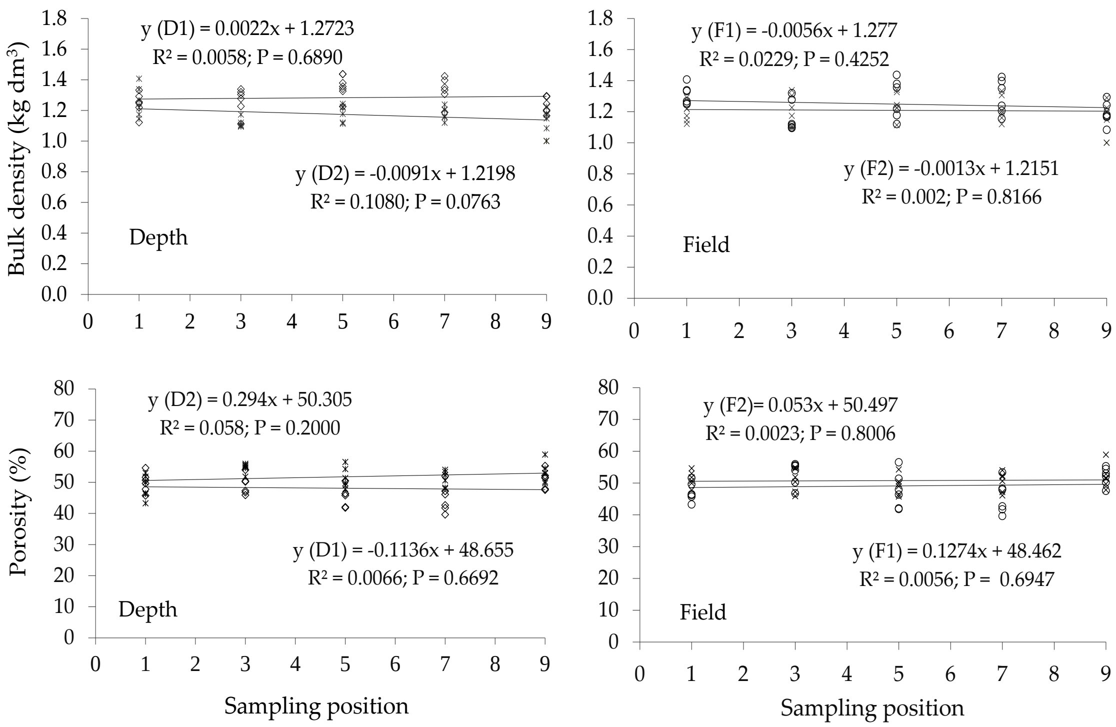

Figure 3.2.3. Soil bulk density and porosity from soil samples taken at different sampling positions along the gradient of irrigation (1, …, 9), as a function of depth (D1, 0.0-0.10 m; D2, 0.10-0.20 m) and field (F1, field 1; F2, field 2).

Some other soil properties determined from samples collected at 0.0-0.20 m depth varied significantly between sampling sites, sampling positions and fields (Table 3.2.2). Extractable P and K, conductivity, organic C, CEC and extractable Zn and B showed significantly higher values in the samples collected in the ridges. However, soil pH (H_2O and KCl), base saturation and extractable Mn were significantly higher in the samples collected in the inter-rows.

Most of the soil properties varied significantly between the sampling positions, the exceptions being soil pH, conductivity and extractable K. Soil properties also differed significantly between fields, except for soil conductivity. Significant interaction between sampling site and field was found for extractable P, conductivity, exchangeable Ca and extractable Fe. Significant interaction between sampling position and field was found for organic C and extractable Fe, Mn, Zn, Cu and B. No significant interaction was found between the three factors of this experiment.

Table 3.2.2. Selected soil properties from samples collected at 0.0-0.20 m depth, in March 2017, as a function of sampling site, sampling position (1, …, 9), and field. Means followed by the same letter are not statistically different by Tukey HSD (Sampling position) or Student's-t (Sampling site and Field) tests (α = 0.05).

	Extract. K	Extract. P	Conductivity	pH_{H2O}	pH_{KCl}	Organic C	Exchan. Ca	CEC	Base saturation	Extract. Fe	Extract. Mn	Extract. Zn	Extract. Cu	Extract. B
	(mg K_2O kg^{-1})[a]	(mg P_2O_5 kg^{-1})[a]	(µs/m)			(g kg^{-1})	(cmolc kg^{-1})[b]		(%)	(mg kg^{-1})[c]				(mg kg^{-1})[d]
Sampling site (S)														
Ridge	310.4 a	349.7 a	78.9 a	5.42 b	4.42 b	20.9 a	4.94 a	7.34 a	87.7 b	213.4 a	166.8 b	5.03 a	7.86 a	1.16 a
Inter-row	246.5 b	292.7 b	54.7 b	5.53 a	4.52 a	18.3 b	4.77 a	6.63 b	89.8 a	222.1 a	194.2 a	4.28 b	7.84 a	0.79 b
Sampling position (P)														
Lowest value	228.3 a	195.0 c	60.2 a	5.42 a	4.35 a	17.8 b	3.80 c	6.21 b	86.0 b	178.1 c	136.0 d	3.47 d	6.64 d	0.79 c
Highest value	313.6 a	399.7 a	70.0 a	5.60 a	4.64 a	21.3 a	5.73 a	7.68 a	92.1 a	262.2 a	217.3 a	5.64 a	9.22 a	1.17 a
Field (F)														
Field 1	361.8 a	286.3 b	65.9 a	5.75 a	4.67 a	18.4 b	5.22 a	7.31 a	93.6 a	207.2 b	134.1 b	4.96 a	10.35 a	0.91 b
Field 2	195.1 b	356.2 a	67.6 a	5.21 b	4.27 b	20.8 a	4.49 b	6.66 b	84.0 b	228.4 a	226.8 a	4.35 b	5.36 b	1.04 a
Prob (S)	<0.0001	0.0022	<0.0001	0.0028	0.0236	<0.0001	0.4028	0.0069	0.0179	0.2836	0.0003	0.0009	0.9386	<0.0001
Prob (P)	0.0758	<0.0001	0.9345	0.3221	0.0710	0.0058	<0.0001	0.0268	0.0012	<0.0001	<0.0001	<0.0001	0.0003	0.0007
Prob (F)	<0.0001	0.0002	0.5991	<0.0001	<0.0001	<0.0001	0.0004	0.0130	<0.0001	0.0100	<0.0001	0.0070	<0.0001	0.0075
Prob (S×P)	0.6500	0.9648	0.9341	0.9859	0.7781	0.8954	0.7956	0.7826	0.0938	0.9836	0.0644	0.8658	0.2274	0.5881
Prob (S×F)	0.8057	0.0001	0.0013	0.8860	0.7597	0.7527	0.0287	0.1220	0.0901	0.0053	0.6578	0.0738	0.1982	0.0810
Prob (P×F)	0.0904	0.0374	0.8663	0.1846	0.2467	0.0199	0.8269	0.6486	0.1179	<0.0001	<0.0001	0.0018	<0.0001	0.0497
Prob (S×P×F)	0.4096	0.9991	0.9791	0.5470	0.9433	0.5569	0.9503	0.9365	0.2005	0.9005	0.5541	0.6590	0.6793	0.4975

[a]Egner-Rhiem; [b]ammonium acetate, pH 7; [c]ammonium acetate and EDTA; [d]azomethine-H.

3.1.2. Hop dry mater yield and leaf nutrient concentration

Aboveground dry biomass (stems, leaves, cones and total) in Field 1 showed a clear tendency for a decrease along the rows (Figure 3.2.4). However, the decrease was only statistically significant for stem DMY. For all plant parts, the coefficients of determination (R^2) were not particularly high, which helps to explain the lack of significant correlation between the two variables. In Field 2, no clear tendency was found in aboveground DMY.

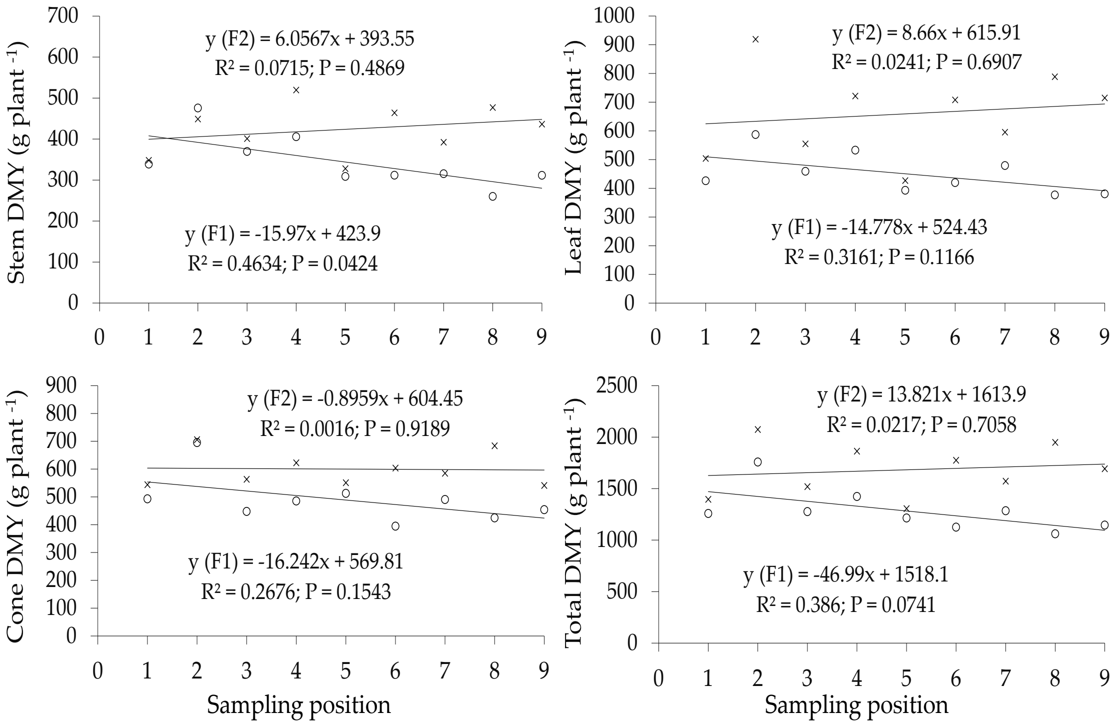

Figure 3.2.4. Dry matter yield (DMY) from plants collected at harvest in September 2017, at different sampling positions along the gradient of irrigation (1, …, 9), and as a function of field (F1, field 1; F2, field 2).

N concentration in the leaves taken at 2 m height did no vary significantly along the rows in any of the fields (Figure 3.2.5). Leaf P also did no vary significantly along the rows but the values in Field 1 were lower than in Field 2. Leaf K levels did not vary significantly along the rows in Field 1 but increased significantly in Field 2. Leaf Ca and Mg levels showed a slight tendency to increase in both fields but without statistical significance. In general, the micronutrients showed even more erratic tendencies when the values of the two fields were compared and only the values of leaf Cu showed a significant decrease along the rows in Field 1.

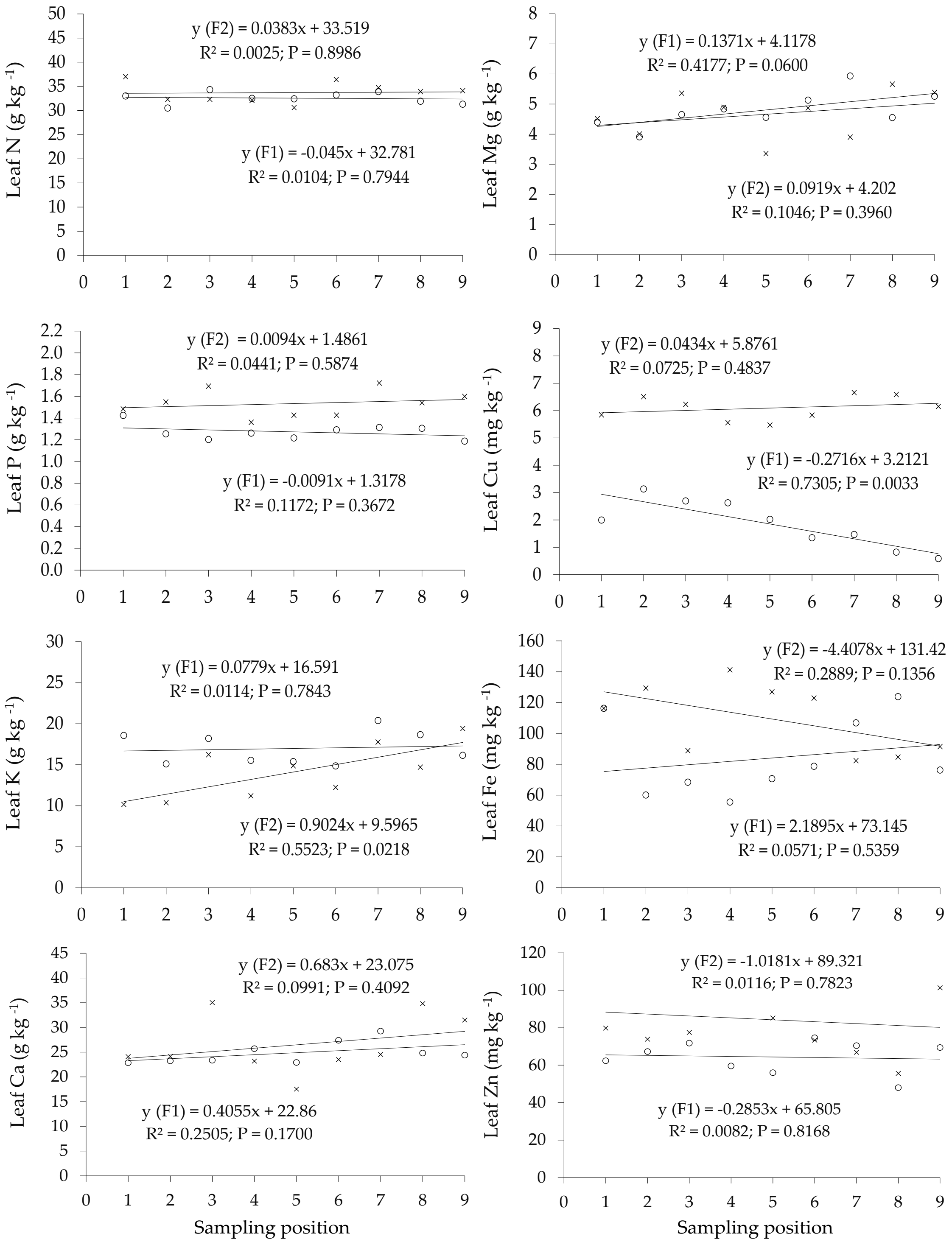

Figure 3.2.5. Leaf nutrient concentration from samples taken at 2 m height and at different sampling positions along the gradient of irrigation (1, …, 9), as a function of field (F1, field 1; F2, field 2).

3.1.3. Correlation analysis between soil properties and plant dry matter yield

Soil bulk density and porosity correlated in a different way with soil pH (H_2O and KCl), leaf P and total DMY (Table 3.2.3).

Table 3.2.3. Correlation coefficients of soil bulk density and soil porosity of samples collected in the inter-rows, at different depths (D1, 0-0.10 m; and D2, 0.10-0.20 m), with soil pH (H_2O and KCl) and leaf nutrient concentrations from samples taken at 2 m height in July 2017, and total and cone dry matter yield (DMY) from plants collected in September 2017.

	Soil†		Leaf nutrient†										DMY	
	pH_{H2O}	pH_{KCl}	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B	Total‡	Cone†
			------------(g kg^{-1})------------					------------(mg kg^{-1})------------					---(g $plant^{-1}$)---	
Soil bulk density														
D1 (0.0-0.10 m depth)	**0.422***	**0.440***	-0.442	-0.190	0.043	-0.209	-0.130	0.128	-0.067	-0.322	-0.515	-0.333	-0.243	0.139
D2 (0.10-0.20 m depth)	0.087	0.062	-0.239	**-0.690***	-0.046	-0.512	-0.249	0.626	-0.220	-0.525	-0.312	-0.459	**-0.706***	-0.128
Soil porosity														
D1 (0.0-0.10 cm depth)	**-0.396***	**-0.400***	0.418	-0.038	-0.110	0.055	0.075	-0.055	0.139	0.097	0.370	0.285	0.168	-0.261
D2 (0.10-0.20 m depth)	-0.020	0.015	0.248	**0.646***	<0.0001	0.406	0.185	**-0.632***	0.273	0.535	0.309	0.418	**0.714***	0.127
Soil separates														
Clay	**0.806****	**0.542***	-0.241	**-0.563***	**0.639****	0.038	0.057	-0.389	-0.197	**-0.773****	-0.459	**-0.707****	**-0.676****	**-0.666****
Silt	0.387	0.129	-0.220	0.066	0.323	-0.049	-0.084	0.042	-0.292	-0.179	-0.042	**-0.503***	-0.117	-0.005
Sand	**-0.703****	-0.391	0.276	0.339	**-0.562***	0.034	0.046	0.247	0.300	**0.571***	0.307	**0.639****	0.427	0.410

Significant correlations at the correspondent levels of *0.05 and **0.01; †Spearman and ‡Pearson correlation coefficients.

That is, the correlations of soil pH were positive for soil bulk density and negative for soil porosity at 0.0-0.10 m depth. Leaf P concentration was significantly and negatively correlated with soil bulk density at 0.10-0.20 m depth, in contrast to the positive correlation found with soil porosity. Leaf Fe concentration was found significant and negatively correlated only with soil porosity at 0.10-0.20 m depth. The strongest correlations were found for total DMY with soil bulk density (r = -0.706) and soil porosity (r = 0.714), both at 0.10-0.20 m depth. Significant correlations were found for soil clay content, positive for soil pH and leaf K, and negative for leaf P, leaf Cu, total DMY and cone DMY. In contrast, soil sand content correlated significantly and negatively with soil pH (H_2O) and leaf K, and positively with leaf Cu and B. Soil silt content correlated significantly and negatively with leaf B.

3.2. Liming experiment

3.2.1 Soil properties

Most soil properties, such as extractable K, P, Mn, Zn, Cu, B, conductivity and pH presented significantly higher values in the limed plot in comparison to the untreated control (Table 3.2.4). Exchangeable Ca and CEC showed higher values in the limed plot but not significantly different to those observed in the control. Significant differences between the two fields used in this experiment were also found for most of the soil properties, the values of extractable K, P, Zn, Cu and B, conductivity, pH, exchangeable Ca, CEC and base saturation being significantly higher in Field 1. Only extractable Fe was significantly higher in Field 2. The interaction between liming and field was significant for extractable K, conductivity, and pH.

3.2.2. Plant response to liming

The concentration of nutrients in the leaves taken at 2 m height showed significant differences between treatments for leaf P in 2017 and for leaf Fe and B in 2018 (Table 3.2.5). The values reported for P and Fe were significantly higher in the limed plots, and those reported for B were significantly higher in the control. Total and cone DMY were significantly lower in the limed plots with the exception of total DMY in 2017, whose differences between treatments were not statistically significant. When comparing fields, significant differences were found for some nutrients and total and cone DMY. However, only leaf concentrations of K, Cu and B, and total and cone DMY, maintained the same trend in both years and fields. In 2017, significant interaction between liming treatment and field was only found for leaf N and Mn and in 2018 for leaf P and total DMY.

Table 3.2.4. Soil properties from samples collected at 0.0-0.2 m depth, in January 2019, in the inter-rows, as a function of liming treatment and field. Means followed by the same letter are not statistically different by Student's-t test (α = 0.05).

	Extract. K	Extract. P	Conductivity	pH_{H2O}	pH_{KCl}	Organic C	Exchan. Ca	CEC	Base saturation	Extract. Fe	Extract. Mn	Extract. Zn	Extract. Cu	Extract. B
	(mg K_2O kg^{-1})[a]	(mg P_2O_5 kg^{-1})[a]	(µs/m)			(g kg^{-1})	---(cmolc kg^{-1})[b]---		(%)	------------------(mg kg^{-1})[c]-------------------				(mg kg^{-1})[d]
Treatment (T)														
Control	82.5 b	162.2 b	69.8 b	5.20 b	4.09 b	14.3 a	3.76 a	7.38 a	82.7 a	160.4 a	96.1 b	2.54 b	4.74 b	0.82 b
Lime	100.8 a	216.7 a	85.5 a	5.40 a	4.35 a	14.6 a	3.90 a	7.64 a	79.5 a	165.8 a	123.7 a	3.49 a	5.74 a	1.11 a
Field (F)														
Field 1	126.5 a	244.6 a	82.6 a	5.54 a	4.53 a	14.7 a	4.47 a	8.21 a	91.2 a	153.2 b	105.8 a	3.32 a	7.23 a	1.11 a
Field 2	56.8 b	134.3 b	72.6 b	5.06 b	3.91 b	14.2 a	3.19 b	6.81 b	71.1 b	173.0 a	114.0 a	2.71 b	3.25 b	0.82 b
Prob (T)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.6084	0.4582	0.1793	0.0638	0.5629	0.0009	<0.0001	0.0001	0.0002
Prob (F)	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	0.4112	<0.0001	<0.0001	<0.0001	0.0357	0.3074	0.0052	<0.0001	0.0003
Prob (T×F)	0.0009	0.1647	<0.0001	<0.0001	<0.0001	0.8899	0.1608	0.4658	0.0098	0.8719	0.4696	0.4311	0.0661	0.0079

[a]Egner-Rhiem; [b]ammonium acetate, pH 7; [c]ammonium acetate and EDTA; [d]azomethine-H

Table 3.2.5. Leaf concentration of macro and micronutrients in July 2017 and 2018, from samples collected at 2 m height, and total and cone dry matter yield (DMY) from plants collected in August 2017 and September 2018, as a function of liming treatment and field. Means followed by the same letter are not statistically different by Student's-t test (α = 0.05).

		N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B	Total DMY	Cone DMY
		(g kg^{-1})					(mg kg^{-1})					(g plant^{-1})	
2017	Treatment (T)												
	Control	3.31 a	0.14 b	1.56 a	2.57 a	0.47 a	96.7 a	374.1 a	3.97 a	74.3 a	71.7 a	1483 a	544.3 a
	Lime	3.39 a	0.15 a	1.66 a	2.72 a	0.52 a	95.3 a	316.9 a	4.59 a	82.9 a	69.3 a	1379 a	441.2 b
	Field (F)												
	Field 1	3.39 a	0.13 b	1.76 a	2.56 a	0.49 a	87.9 b	355.9 a	2.54 b	64.35 b	63.29 b	1271 b	446.2 b
	Field 2	3.31 a	0.16 a	1.46 b	2.73 a	0.50 a	104.2 a	335.1 a	6.03 a	92.88 a	77.70 a	1591 a	539.3 a
	Prob. (T)	0.2043	0.0440	0.3130	0.3597	0.1445	0.8597	0.0703	0.2111	0.2636	0.2542	0.2139	0.0033
	Prob. (F)	0.2180	<0.0001	0.0049	0.2909	0.7214	0.0447	0.5024	<0.0001	0.0007	<0.0001	0.0005	0.0073
	Prob. (T×F)	0.0024	0.7290	0.8820	0.9293	0.4216	0.2588	0.0358	0.1327	0.2608	0.0918	0.3402	0.5728
2018	Treatment (T)												
	Control	3.48 a	0.19 a	3.26 a	1.53 a	0.56 a	94.6 b	513.0 a	6.93 a	21.0 a	57.5 a	1681 a	475.2 a
	Lime	3.56 a	0.19 a	3.19 a	1.57 a	0.61 a	109.3 a	495.2 a	7.07 a	19.5 a	51.6 b	1407 b	380.2 b
	Field (F)												
	Field 1	3.55 a	0.20 a	3.83 a	1.43 b	0.62 a	91.0 b	408.9 b	6.26 b	20.51 a	52.37 b	1421 b	428.9 a
	Field 2	3.49 a	0.17 b	2.63 b	1.67 a	0.55 b	112.9 a	599.4 a	7.73 a	19.96 a	56.70 a	1666 a	426.4 a
	Prob. (T)	0.2155	0.8374	0.6215	0.4655	0.0550	0.0035	0.7162	0.5532	0.1170	0.0024	0.0016	0.0021
	Prob. (F)	0.3461	<0.0001	<0.0001	0.0001	0.0279	<0.0001	0.0004	<0.0001	0.5648	0.0218	0.0043	0.9304
	Prob. (T×F)	0.6601	0.0185	0.2051	0.0983	0.2205	0.9394	0.8796	0.6927	0.1228	0.4463	0.0057	0.3068

3.2.2. Correlation analysis between soil pH and plant variables

Significant correlations between soil pH (H_2O and KCl) and leaf nutrient concentration were found for several nutrients, but a similar trend over the two years was found only for leaf Cu and B, both presenting negative correlations with soil pH (Table 3.2.6). Soil pH and leaf P, for instance, showed a negative correlation in 2017 and a positive correlation in 2018. Significant and negative relations between soil pH and total and cone DMY were also found for the first year of plant sampling.

Table 3.2.6. Spearman correlation coefficients for soil pH (H_2O and KCl) from samples collected in January 2019, at 0.0-0.20 m depth, in the inter-rows, with leaf nutrient concentration from samples collected at 2 m height in July (2017 and 2018), and total and cone dry matter yield (DMY) from plant samples collected in August 2017 and September 2018.

	Leaf nutrient concentration										DMY	
Soil	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B	Total	Cone
	($g\ kg^{-1}$)					($mg\ kg^{-1}$)					($g\ plant^{-1}$)	
						2017						
pH_{H2O}	0.492*	-0.648**	0.323	-0.131	-0.088	-0.157	0.324	-0.531**	-0.503*	-0.788**	-0.635**	-0.657**
pH_{KCl}	0.519**	-0.651**	0.318	-0.068	-0.076	-0.137	0.315	-0.524**	-0.538**	-0.787**	-0.563**	-0.590**
						2018						
pH_{H2O}	0.315	0.544**	0.606**	-0.289	0.492*	-0.290	-0.714**	-0.611**	-0.144	-0.477*	0.093	-0.104
pH_{KCl}	0.269	0.526**	0.585**	-0.321	0.436*	-0.317	-0.717**	-0.670**	-0.127	-0.477*	0.188	-0.066

Significant correlations at the correspondent levels of *0.05 and **0.01.

4. Discussion

The results of Experiment 1 showed significant differences in some soil properties at different positions along the rows, but not over a continuous gradient. Thus, the results cannot be attributed to the flooding irrigation, but they were probably caused by heterogeneity in spatial variability of important soil constituents such as clay, sand and silt, since it is well-known that soil texture determines many other soil physical and chemical properties (Delgado and Gómez, 2016). Variations in soil properties were also found when comparing different soil layers. Soil bulk density was higher in the soil surface layer (0.0-0.1 m), and porosity was found to be higher in the deeper (0.1-0.2 m), layer. Soil bulk density and porosity in agricultural fields are influenced not only by soil texture but also by external loads which cause soil compaction (Alaoui et al., 2018; Hamza et al., 2011; Nawaz et al., 2013). In this particular case, it seems that the effects of frequent irrigation and soil tillage prevailed, which may have prevented a proper soil aggregation, leading to an increase in soil bulk density and a reduction in soil porosity on the surface layer which was directly impacted by the cultivator. The variation in soil properties was also significant when comparing fields. The field higher in clay and lower in sand presented significantly higher soil bulk density. Usually, clayey soils tend to have lower bulk density and higher porosity than sandy soils (Chaudhari et al., 2013). However, these results indicate an opposite trend, probably because of the negative effect on soil aggregation and compaction caused by frequent soil tillage. Other studies have also found spatial variability in bulk density and water infiltration on flooded fields caused mainly by tillage practices, particularly when heavy machinery is used (Cerdà et al., 2021; Green et al., 2003).

The soil samples collected from the ridges showed significantly higher values of extractable P and K. In the ridges, the conditions for nutrient uptake were poor since they are created every year by soil pushed from the inter-rows, which means that they contain nutrients barely taken up by the plant due to the limited expansion of roots in this position. In addition, in this irrigation system, the water flows from the inter-row to the ridge due to the gradient of water potential caused by the evapotranspiration from the latter and the continuous water supply to the inter-row. This means that nutrients tend to accumulate in the ridge, carried by mass flow, in contrast to what happens in the inter-row, from which nutrients tend to be leached out. Mass flow is the main driving force causing the movement of most nutrients in the soil (Comerford, 2005; Lambers et al., 2008; Weil and Brady, 2017). Thus, soil conductivity was higher in the ridge, due to the increased presence of salts as demonstrated by the increase in CEC. Organic C also appeared higher in the ridge, probably because this zone is not tilled so frequently, which reduces the exposure of OM to the heterotrophic microorganisms that cause its oxidation (Liu et al., 2006). This zone also contains the remaining bines (those that do not climb) and weeds, which are incorporated into the soil when the ridge is created, which usually represent more debris than that incorporated in the inter-row. B also increased in the ridge, perhaps due to higher levels of organic C, which have the ability of retaining B in the soil (Das and Purkait, 2020; Goldberg, 1997).

Soil pH (H_2O and KCl), base saturation and extractable Mn were significantly higher in the samples collected in the inter-rows. These results are probably related to the decrease in the potential redox, which may have increased the pH of the soil (Husson, 2013). The increase in soil pH in the inter-rows was probably also related to the increase in the concentration of cation ions such as Ca and Fe (Osman, 2013). Base saturation increased in the inter-rows probably due to the presence of the divalent cations, less available to move into the ridge by mass flow. The higher concentration of Mn in the inter-rows might have also been due to the reduction of Mn that occurred at the beginning of the reduction process. This can occur when the redox potential is still positive (George et al., 2012).

A clear gradient along the rows was not observed for total and cone DMY. These results did not corroborate the hypothesis that flood irrigation is creating a spatial variation in plant performance along the rows. The differences detected in the plants seem to be due to spatial variability in the soil constituents, namely the soil separates which, in turn, influence soil bulk density and porosity. The results from the correlation analysis showed significant and negative relations between total DMY and soil bulk density (r = -0.706) and between total DMY and clay content (r = -0.676). In contrast, total DMY and soil porosity at 0.10-0.20 m correlated significantly and positively (r = 0.714). The soil surface layer presented higher bulk density, which has already been explained by the effect of irrigation and frequent soil tillage, which reduces the stability of soil aggregates, increasing bulk density and decreasing porosity (Batey, 2009; Hamza et al., 2011). On the other hand, it seems that the higher porosity in the 0.10-0.20 m layer was an important factor affecting DMY, likely because in the surface layer the diffusion of O_2 to ensure the biological processes of the soil is always easier. Soils with higher clay content tend to retain more water, decreasing soil aeration which negatively affects the function of root and plant metabolism (Nawaz et al., 2013; Pezeshki and DeLaune, 2012). Under the conditions of this experiment, the clay content in the soil seemed to be negatively associated with hop DMY, mainly because clay is a determinant factor of soil bulk density and porosity, which were identified in this study as determinant factors in crop productivity.

Irrigation also did not cause any relevant gradient in tissue nutrient concentration as detected by the analysis of variance. However, correlation analysis provided some data that deserves to be commented on. Leaf P was significantly and positively correlated with soil porosity at 0.10-0.20 m, but was negatively correlated with soil bulk density at 0.10-0.20 m and clay content. Leaf P did not show any consistent gradient along the rows, but was lower in the field presenting higher soil bulk density and clay content. This reveals that P uptake was enhanced by the increased porosity of the soil at the deeper layer and by the lower clay content. Similarly, on barley (*Hordeum vulgare* L.) there was reported a reduction in P uptake and yield associated with heavy soil compaction (Nawaz et al., 2013). The higher porosity of soil may have facilitated P root uptake from the deeper layer, which is richer in P, probably due to the increase in the vertical movement of P as the result of fertilization and flooding as reported by Tian et al., 2017. In turn, the higher clay content may have resulted in higher P adsorption and lower P availability. In contrast, leaf Fe was significantly and negatively correlated with soil porosity at 0.10-0.20 m depth. Leaf Fe also presented an opposite tendency between fields, decreasing along the rows in the field with a lower clay content and higher soil porosity. This result is probably related to soil reduction conditions since the availability of Fe decreases when soil O_2 and redox potential increases (Osman, 2013). Leaf K showed a significant and positive correlation with soil clay content and a negative one with sand content. The availability of K in the soil is not directly affected by redox potential, but its fixation in 2:1 clay minerals is facilitated by the increase in soil pH (Husson, 2013). Also, there has been reported an antagonistic effect between Fe and K in paddy fields (Chen et al., 1997; Kundu et al., 2001), an aspect that may also have influenced these results.

In Experiment 2, the application of lime increased several variables of soil fertility, including pH, but did not significantly increase exchangeable Ca and CEC. In fact, the rate of lime applied in this experiment was too low to cause important changes to soil properties, as is usually achieved when using high rates of lime (Weil and Brady, 2017). In a previous study, Čeh and Čremožnik (2015) applied 2.3 t lime ha^{-1} and reported similar results, that is, a reduced effect on soil properties due to the application of lime.

The main effect on the elemental composition of the leaves resulting from the application of lime would have been the significant increase of leaf P in the first growing season after the lime application. This raised the soil pH contributing to a reduction in P fixation, which in acidic soils is due to reactions with Al and Fe oxides, which precipitate P as $AlPO_4$ and $FePO_4$ (Havlin et al., 2014).

Total and cone DMY did not increase with the application of lime, but rather showed a decreasing trend. It is generally considered that the optimal pH for hop growth is between 5.7 and 7.5 (Gingrich et al., 1994; Sirrine et al., 2010). In this study, soil pH was below the lowest value of the reported range, which would have favoured a positive effect on the vegetation. However, the lime application influenced some soil properties, but not enough to have a high impact on the elemental composition of the leaves. In general, the nutrient content of the leaves was found to be within the sufficiency ranges established for hops (Bryson et al., 2014), both in the limed and in the control treatments. Regarding total DMY, a significant interaction between lime treatment and field was recorded, which may also have contributed to difficulties in the interpretation of these results.

Correlation analysis, in turn, also did not show coherent trends over the two years of the study. Perhaps the most relevant result was the negative correlation between soil pH and biomass production in the first year, which again refers to diverse interactions which may have occurred between environmental variables (year) and factors under study (field and liming). The effect of environmental variables on the performance of the hop plant is well known (MacKinnon et al., 2020; Marceddu et al., 2020; Rossini et al., 2020), although in this study it was not possible to clarify the isolated effects of any of them.

5. Conclusions

Irrigation by flooding the space between rows over more than 20 years was not responsible for any gradient in soil properties, plant elemental composition and plant performance, although variations in those variables were found at different positions in the row caused by erratic spatial variability of some constituents of soil, such as sand, silt and clay. However, irrigation followed by soil tillage on repeated occasions during the growing season seems to have reduced soil porosity and increased soil bulk density in the surface 0.0-0.1 m soil layer. These variables were found to be related to crop productivity in positive and negative ways, respectively.

This study also showed that the ridge is a point of nutrient accumulation, particularly for those that move more easily in the soil by mass flow, thereby showing also higher conductivity and CEC. The reduced water potential in the ridge created by evapotranspiration is the driving force causing the water flow from the inter-row. Organic C was also higher in the ridge in comparison with the inter-row, probably due to the annual incorporation of weeds and weaker hop bines (those that did not climb) when the ridge is created in early spring.

Although the original soil was acidic, and the application of 1,000 kg ha^{-1} of lime caused a small increase in pH, this did not lead to other relevant changes in soil properties, nor in plant nutrition status or total and cone DMY. The liming effect might not have been enough to nullify the effects of the interaction between factors that always occur in field experiments.

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Chapter 4:

Foliar fertilization effect on hop productivity and cone quality

Briefing note

The objective of this chapter was to evaluate the effect of fertilizers applied by foliar spray on hop productivity and cone quality variables such as bitter acids, NO_3^- and mineral composition. Foliar sprays were used to supplement the farmer's fertilization plan and partially replace soil side-dressing fertilization.

Chapter 4.1. approaches the potential of two foliar sprays, one based in the algae *Ascophyllum nodosum* (L.) and the other rich in nutrients, to improve the homogeneity of hop fields and restore the productivity of the poorly developed patches.

This chapter is an adaptation of the following paper: Afonso, S., Arrobas, M., Rodrigues, M.Â. Response of hops to algae-based and nutrient-rich foliar sprays. Agriculture, 11, 798. https://doi.org/10.3390/agriculture11080798.

Chapter 4.2. it was focused on two main purposes: i) to evaluate the application of a foliar spray rich in amino acids as an alternative to reduce the proportion of the N applied to the soil as a side dressing; and ii) to evaluate the application of a foliar spray rich in K at the cone developing stage, as a supplement to the farmer's fertilization plan. This chapter is an adaptation of the following article: Afonso, S., Arrobas, M., Morais, J.S., Rodrigues, M.Â., 2021. Hop dry matter yield and cone quality responses to amino acid and potassium-rich foliar spray applications. J. Plant Nutr. 44(4): 2042-2056. https://doi.org/10.1080/01904167.2021.1889597.

4.1. Response of hops to algae-based and nutrient-rich foliar sprays

Abstract

Over recent years, some hopyards of northeast Portugal have presented poorly developed plants and reduced productivity. In this study, an attempt was made to improve the homogeneity of hop fields and restore their productivity by using plant biostimulants as foliar sprays. The experimental apparatus included four field trials carried out in four plots of different plant vigour, as evaluated by farmers over previous years (weak, fair, good and very good). The experiments were arranged as a factorial of foliar treatment (two plant biostimulants containing extracts of seaweed algae and an untreated control) and year (2017 and 2018). The plot and the year influenced greatly almost all the measured variables related to tissue nutrient concentration and crop performance. In the control plots, cone DMY varied from 83.3 to 394.4 g plant^{-1} from the weak to the very good plots. In 2018, cone DMY was significantly higher than in 2017. The use of foliar sprays influenced less the elemental composition of plant tissue than the plot or the year. The use of foliar sprays only increased significantly crop yield in the plot of weak plant vigour. The foliar treatments did not increase α- and β-acid concentration in the cones; in the control treatment of the most productive plot, the values were, respectively, 11.2 and 3.9%. Although seaweed extracts tend to help plants cope with several abiotic and biotic stresses, they showed to be effective in mitigating the stress that is affecting these plants, which probably is poor soil drainage caused by the flooding irrigation system, only under conditions of severe stress.

Keywords: *Humulus lupulus*; plant biostimulants; *Ascophyllum nodosum*; tissue nutrient concentration; chlorophyll fluorescence; cone α- and β-acids.

1. Introduction

A common way to meet crop nutritional requirements and improve crop productivity is through the application of conventional fertilizers to the soil. However, nutrient uptake can also take place via leaf surface, stomata and other specialized cells, which allows the use of foliar sprays as fertilizing materials (Weil and Brady, 2017). In general, both macro and micronutrients can be applied as foliar sprays. However, the restricted amounts of nutrients that can be supplied as foliar sprays make this strategy more attractive for the application of micronutrients, especially when the application to the soil is of little effect, such as in acidic or alkaline soils (Havlin et al., 2014).

The range of fertilizer formulations for foliar application is currently huge, and their use is expected to increase at a compound annual growth rate of 4% until 2028 (Dean, 2019). In addition to macro and micronutrients essential to plants, many products for foliar application contain substances of differing natures with the potential to have a biostimulating effect on plants. A plant biostimulant has been defined as a substance or microorganism applied to the soil, seeds or plants with the aim of enhancing nutritional efficiency, abiotic stress tolerance and/or crop quality, regardless of its nutrient content (Colla and Rouphael, 2015; du Jardin, 2015). Some commercial products can, however, be formulated as mixtures of more than one plant biostimulant substance (du Jardin et al., 2020; Rouphael and Colla, 2020). The European Union has recently recognized plant biostimulants as a distinct category within fertilizer products, in a regulation published on 25 June 2019 in the Official Journal of the European Union [Regulation (EU) 2019/1009]. Several substances have been recognized as having a plant biostimulant effect, namely humic and fulvic acids, seaweed and plant extracts, chitosan and other biopolymers, various inorganic compounds, such as phosphite and silicon, and beneficial microorganisms (du Jardin, 2015; du Jardin et al., 2020; Rouphael and Colla, 2020). Among these groups, seaweed extracts, in particular those obtained from *Ascophyllum nodosum* (L.) are the most studied and widespread in agriculture (Battacharyya et al., 2015; De Saeger et al., 2020; Shukla et al., 2019).

Seaweed algae extracts are complex products, containing plant hormones, brassinosteroids, betaines, polyamines, polymers and also macro and micronutrients (Stirk et al, 2020). Although the complexity of their composition tends to make it difficult to clarify their mode of action (De Saeger, et al. 2020; du Jardin, et al. 2020; Wozniak et al., 2020), several studies have shown beneficial effects from the use of seaweed extracts for the alleviation of abiotic (Al-Ghamdi and Elansary, 2018; Carmody et al., 2020; Goñi et al., 2018) and biotic (Gunupuru et al., 2019; Patel et al., 2020) stresses, and in increasing crop productivity and/or product quality (Procházka et al., 2018; Taskos et al., 2019; Viencz et al., 2020), even if they provide minute quantities of nutrients (du Jardin, 2015).

Hop is an important crop in several European countries, such as Germany and the Czech Republic, and also in the United States of America (FAOSTAT, 2020). In southern Europe, hop is less popular, but still important in countries like Spain, Italy and Portugal (FAOSTAT, 2020). Studies in southern Europe have been mainly focused on the difficulties imposed by the Mediterranean climate on hop cultivation (Marceddu et al., 2020; Rossini et al., 2020; Ruggeri et al., 2018) and on the adaptation of hop cultivars to new areas under cultivation (Forteschi et al., 2019, Rossini et al., 2016; Ruggeri et al., 2018). In Portugal, hop is currently only grown in the Northeast (Rodrigues et al., 2015), and crop production can vary greatly from field to field. Some farmers in this region have found that their fields have heterogeneous plant development, some with well-developed plants and others with obvious productivity problems, although the cropping techniques are similar. In a previous study, Afonso et al. (2020) reported that the heterogeneity in hop fields is mainly due to poor soil drainage and aeration, caused by the irrigation system, which consists of flooding the space between the rows. Farmers, however, perhaps because it is easier than changing the expensive irrigation system, are using conventional micronutrient-rich foliar sprays to try to mitigate the problem.

Thus, and in view of the increasing range of innovative products for foliar applications that are being used in the region on several other crops, it is hypothesized for this study that foliar sprays containing seaweed extracts of *A. nodosum* algae, which are known for their biostimulating effect on plants, could have a beneficial effect on hop productivity, in particular on the yield restoration of plots that have shown poor development over recent years. To evaluate the formulated hypothesis, four field trials were installed in plots of different yield potential. Based on the productivity of the last years, plots classified by farmers as having weak, fair, good and very good vigour plants were chosen. From the experimental apparatus, specific objectives were set to assess the effect of treatments on the nutritional status and photosynthetic performance of plants, on total and cone dry matter yield, and on α- and β-acid content in the cones.

2. Materials and methods

2.1. Field experiments characterization

A field trial was conducted during two growing seasons (2017 and 2018), in hop plots of cultivar Nugget, located in Bragança, NE Portugal. The region benefits from a Mediterranean-type climate, with average annual air temperature of 12.7 ºC and annual precipitation of 772.8 mm. Meteorological data recorded during the experimental period at the weather station of Sta Apolónia farm in Bragança is shown in Figure 4.1.1.

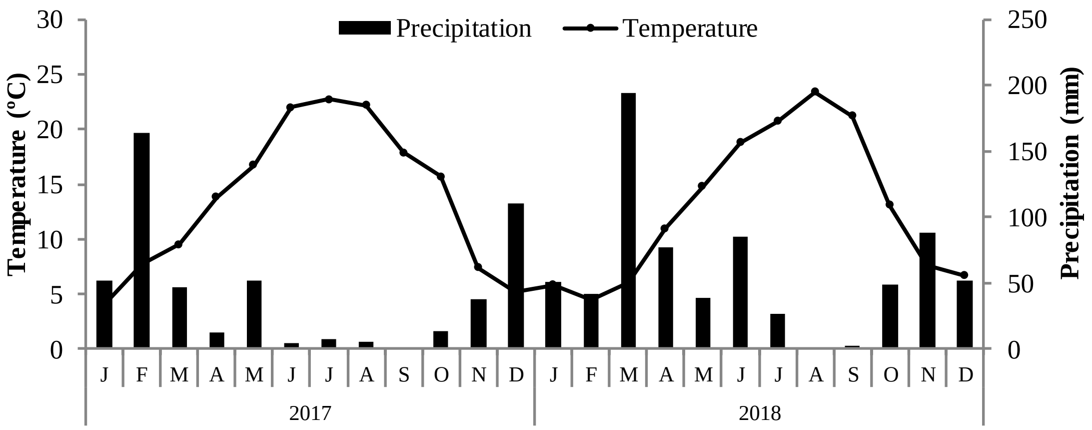

Figure 4.1.1. Average monthly temperature and precipitation during the experimental period.

The hop plots are arranged in a 7 m conventional high trellis system, with concrete poles, connected with cables, in a “V” design system. The plantations were installed about 20 years ago. In the original plantation, the rhizomes were spaced 2.8 m × 1.6 m between rows and within rows, respectively. From each position of an original rhizome, a double tutor thread was placed in “V” connecting the plants (groups of 3 to 4 stems) to the upper wire structure, which sets up a density of ~2.232 plants ha^{-1}.

The hop plots were selected according to their yield potential as recorded in the previous seasons by the farmers. Four extreme situations were considered adequate for this study, from the poorer to the better plots found in the region. With the help of the farmers the plots of different yield potential were named as weak (Plot 1), fair (Plot 2), good (Plot 3) and very good (Plot 4). The soils of the different plots used in this experiment were sampled before the trial started for characterization of the growing conditions. Three composite soil samples (15 sampling points) were taken between the rows on 2 June 2016, at 0–20 cm depth in each one of the plots. The soil textures varied from clay loam (Plots 1 and 2), to sandy clay loam (Plot 3) and sandy loam (Plot 4). More details on chemical soil properties determined from these samples are provided in Table 4.1.1.

Table 4.1.1. Soil properties (average ± standard deviation) determined from soil samples collected between rows at 0–20 cm depth on 2 June 2016.

		Plot 1	Plot 2	Plot 3	Plot 4
Soil Properties	Plant Vigour	Weak	Fair	Good	Very Good
pH_{H2O}		5.8 ± 0.12	5.8 ± 0.04	5.5 ± 0.10	5.1 ± 0.13
pH_{KCl}		4.8 ± 0.12	4.7 ± 0.04	4.4 ± 0.08	4.3 ± 0.13
Organic C (g kg^{-1}) [a]		13.4 ± 0.20	15.7 ± 0.10	7.6 ± 0.04	14.5 ± 0.20
Extract. P (mg P_2O_5 kg^{-1}) [b]		283.0 ± 44.7	451.8 ± 33.5	191.1 ± 27.9	212.6 ± 28.2
Extract. K (mg K_2O kg^{-1}) [b]		115.9 ± 7.8	193.0 ± 8.6	111.0 ± 5.9	286.0 ± 5.0
Exchan. Ca (cmolc kg^{-1}) [c]		14.8 ± 1.84	23.3 ± 1.39	10.7 ± 0.17	2.7 ± 0.46
Exchan. Mg (cmolc kg^{-1}) [c]		4.8 ± 0.84	9.5 ± 1.22	2.7 ± 0.07	0.5 ± 0.04
Exchan. K ($cmol_c$ kg^{-1}) [c]		0.3 ± 0.02	0.5 ± 0.04	0.2 ± 0.01	0.5 ± 0.08
Exchan. Na ($cmol_c$ kg^{-1}) [c]		0.2 ± 0.05	0.6 ± 0.05	0.1 ± 0.01	0.3 ± 0.06
Exchan. acidity ($cmol_c$ kg^{-1}) [c]		0.3 ± 0.03	0.3 ± 0.02	0.2 ± 0.03	0.6 ± 0.13
Cation-exch. capacity ($cmol_c$ kg^{-1})		20.7 ± 2.64	34.4 ± 2.56	14.0 ± 0.21	5.1 ± 0.37
Extract. B (mg kg^{-1}) [d]		0.7 ± 0.14	1.2 ± 0.10	0.8 ± 0.13	0.6 ± 0.09
Extract. Fe (mg kg^{-1}) [e]		293.5 ± 30.50	272.6 ± 29.11	114.2 ± 6.75	105.7 ± 4.41
Exctract. Mn (mg kg^{-1}) [e]		250.7 ± 28.95	179.9 ± 14.02	224.1 ± 10.24	57.4 ± 7.89
Extract. Zn (mg kg^{-1}) [e]		9.8 ± 0.66	11.7 ± 0.80	7.2 ± 0.19	3.9 ± 0.46
Extract. Cu (mg kg^{-1}) [e]		16.3 ± 1.43	16.9 ± 1.09	10.1 ± 0.39	4.3 ± 0.77

[a]Wet oxidation (Walkley-Black); [b]Egner-Riehm; [c]Ammonium acetate, pH 7; [d]Hot water, azomethine-H; [e]Ammonium acetate and ethylenediaminetetraacetic acid (EDTA).

2.2. Experimental design and treatment application

Four similar and independent field trials corresponding to the plots of weak, fair, good and very good vigour plants were arranged as a factorial design, to accommodate two experimental factors, foliar fertilization (three levels) and year (two levels, 2017 and 2018), in six replicates. As foliar fertilization, two commercial plant biostimulants were used as foliar sprays to which a non-fertilized control was added.

One of the plant biostimulants is particularly rich in nutrients (Folivex Crescimento®) and was named in this study as Fnut. It combines several macro and micronutrients and a small portion of an extract of the algae A. nodosum (1.4% w/w). Fnut contains (w/w) 12% N, 6% P2O5, 4% K2O, 0.025% B, 0.1% Fe-Ethylenediaminetetraacetic acid (EDTA), 0.05% Cu-EDTA, 0.05% Zn-EDTA, and 0.05% Mn-EDTA. The second plant biostimulant (Fitoalgas Green®) was selected due to its high content (15% w/w) of A. nodosum, and the treatment was named 'Algae'. The foliar sprays were applied at the rates recommended by the manufacturers. Fnut was applied at a rate of 3.5 L ha^{-1}, diluted in 1500 L water, three times during the growing season (20 June, 10 July and 27 July 2017, and 20 June, 8 July and 24 July 2018). The first foliar treatment was done when the plants of Plot 4 had reached 80% of the top wire height, the second at the end of bine growth, and the third during the enlargement of inflorescence buds. Algae was applied at a rate of 2 L ha^{-1}, also diluted in 1500 L of water, on the same dates mentioned for Fnut.

Each replication consisted of six twin canopies of three plants (in the "V" design system). These plants were marked when those in Plot 4 (very good) were ~3 m tall. In the other plots, plants with height within the plot pattern were selected.

All the plots received the basal fertilization plan usually used in the region, consisting of a compound NPK (7:14:14) fertilizer applied late in winter at a rate of ~500 kg ha^{-1}, and two side dress N applications performed during the growing season, the first with ~200 kg ha^{-1} of nitromagnesium (27% NH_4NO_3 + 3.5% MgO + 3.5% CaO) and the second with ~450 kg ha^{-1} of $Ca(NO_3)_2$ (15.5% NO_3^- + 27% CaO). The farmers manage their fields by a surface irrigation system, consisting of flooding regularly the space between rows. Several tillage passes (3 to 4) were performed every year to remove the crusts and allowing water infiltration.

2.3. Data acquisition in the field and tissue sampling

Leaf greenness was measured by using the SPAD (Soil and Plant Analysis Development)-502 Plus chlorophyll meter (Spectrum Technologies, Inc., Aurora, IL, USA). For each sampling date, treatment and replicate thirty readings were taken (to create the average values), from the distal lobe of young, fully expanded leaves. The readings were performed on 17 July 2017 and 16 July 2018. A Normalized Difference

Vegetation Index (NDVI) was determined by the hand held FieldScout CM 1000 (Spectrum Technologies, Inc). The measurements were taken from the same leaf parts and dates as SPAD readings. Chlorophyll *a* fluorescence and OJIP transient was determined by using the dark adaptation protocols F_V/F_M, F_V/F_0 and the advanced OJIP test by using the OS-30p+ fluorometer (Opti-sciences, Inc.). F_M, F_0 and F_V are, respectively, maximum, minimum and variable fluorescence from dark adapted leaves, and $F_V/F_M = (F_M-F_0)/F_M$ and $F_V/F_0 = (F_M-F_0)/F_0$. The OJIP test provides origin fluorescence at 20 μs (O), fluorescence at 2 ms (J), fluorescence at 30 ms (I) and maximum fluorescence (P, or F_M). Measurements were taken from the distal lobe of fully expanded young leaves, after a period of dark adaptation greater than 35 min.

In the middle of the growing season (15 July 2017 and 16 July 2018), samples of 20 leaves per replication were taken at ~2 m height for elemental analysis. At hop harvest (28 to 31 August 2017 and 27 to 31 August 2018), the aboveground biomass was cut at ground level and separated into two samples of leaves (bottom and top halves), stems, and cones, and weighed fresh. Subsamples of each plant part were weighed again, oven dried at 70 °C and weighed dry for determination of DMY of the different plant parts. Additionally, a subsample of 30 dried cones was randomly selected for determination of the dry mass of individual cones.

2.4. Laboratory analyses

The soil samples were firstly oven-dried (40 ºC) and sieved (2 mm). Thereafter, they were analysed for pH (H_2O and KCl) (soil: solution, 1:2.5), CEC (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley-Black method) and extractable P and K (Egner-Rhiem method). Extractable P was also determined by the Olsen method. Soil B was extracted by hot water and the extracts analysed by the azomethine-H method. For more details on these analytical procedures, the reader is referred to Van Reeuwijk (2002). The availability of other micronutrients (Cu, Fe, Zn, and Mn) in the soil was determined by atomic absorption spectrometry after extraction with ammonium acetate and EDTA, according to the method described by Lakanen and Erviö (1971).

Elemental tissue analyses were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K) and atomic absorption spectrophotometry (Ca, Mg, Cu, Fe, Zn and Mn) methods after nitric digestion of the samples (Walinga et al., 1989). Bitter acids (α and β) in hop cones were extracted with methanol and diethyl ether by high performance liquid chromatography (HPLC), according to the Analytica European Brewery Convention (EBC) 7.7. method (EBC Analysis committee, 1998).

2.5. Data analysis

Data was analysed for normality and homogeneity of variances using the Shapiro-Wilk and Bartlett's test, respectively. The analysis of variance was performed according to the experimental design as a two-way ANOVA. When significant differences were found among experimental treatments, the means were separated by the Tukey HSD test [for the factor of three levels (Foliar treatment)] or Student's t-test [for the factor of two levels (Year)] (α = 0.05).

3. Results

3.1. Plant dry matter yield

The total aboveground DMY, and DMY of the different plant parts (stems, leaves or cones) varied significantly between plots of different plant vigour the former from ~300 (weak) to ~1200 (very good) g plant^{-1} (Figure 4.1.2). The result was expected since the plot selection took into account the vigour of the plants in previous years. The nutrient-rich foliar spray did not significantly influence the total DMY or any of its components, including the DMY of the cones. Algae gave significantly higher values of cone and total aboveground biomass in comparison to the other treatments only in the plots of weak vigour plants. In 2018, the DMY was significantly higher for all plant parts in comparison to 2017. Significant interaction between the two factors was not usually observed, thus not deserving particular attention.

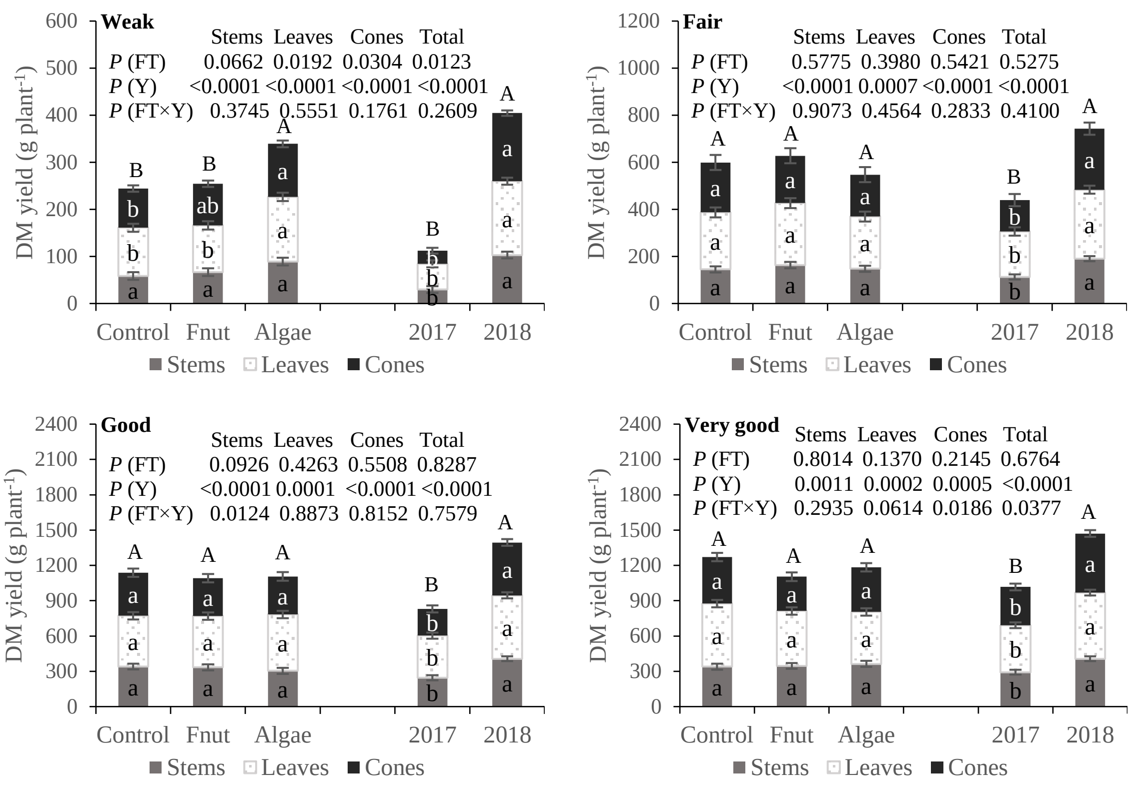

Figure 4.1.2. Dry matter (DM) yield of hop plant parts (average ± standard error) in the different plant vigour plots (Weak Fair, Good and Very good), as a function of foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray;

and Control) and year. Within each plant part (lowercase letters) or total DM yield (uppercase letters), means followed by the same letter are not statistically different (α = 0.05) by Tukey HSD test (Foliar treatment) or Student's t-test (Year).

Plant vigour had little influence on the size of the cones (Figure 4.1.3). The foliar treatment also did not significantly influence the size of the cones in most of the plots. However, in the plot of good vigour plants, cone dry weight was found to be significantly lower in the Fnut treatment. The year had a great influence on the size of the cones. Significant differences were found in the plots of weak, fair and good vigour plants, with 2018 showing the higher values.

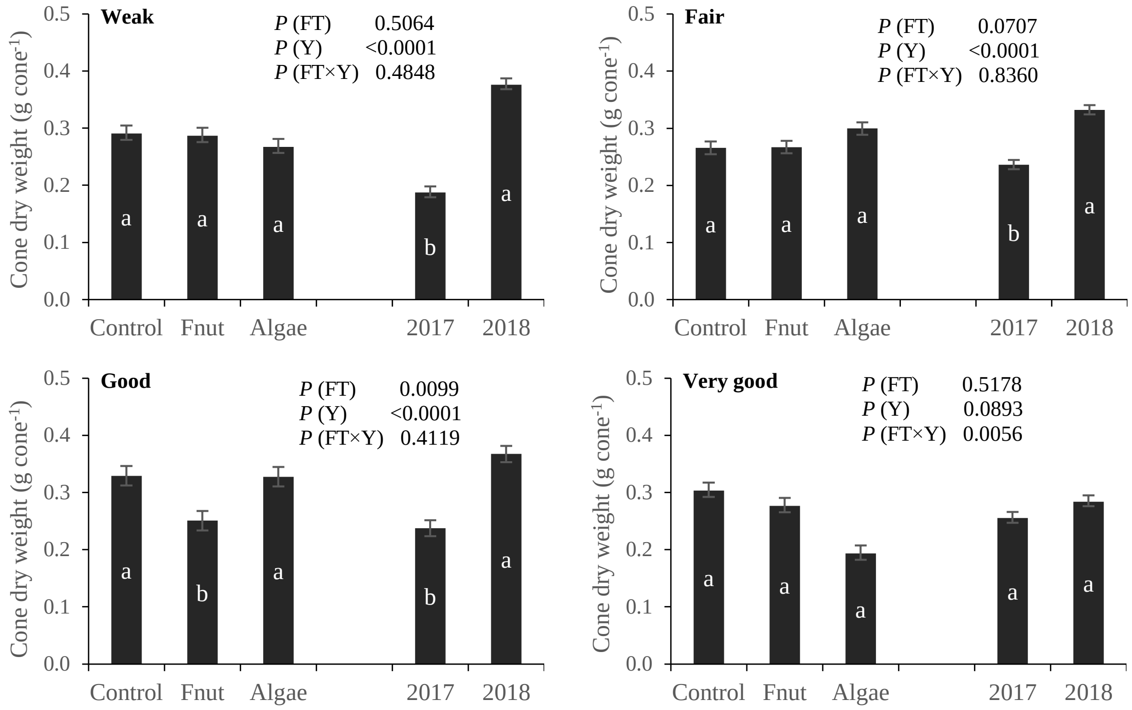

Figure 4.1.3. Dry weight of individual cones (average ± standard error) in the different plant vigour plots (Weak, Fair, Good and Very good) as a function of foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control) and year. Means followed by the same letter are not statistically different (α = 0.05) by Tukey HSD (Foliar treatment) or t-Student (Year) tests.

3.2. Tissue nutrient concentrations

The concentration of nutrients in the leaves varied greatly between plant vigour plots in the leaf samples taken at ~2 m height in July (Table 4.1.2). The leaf concentrations of N, Ca, and particularly K, were remarkably higher in the plot of very good plant vigour. In contrast, average leaf P and Mg concentrations were significantly lower in the plot of high vigour plants. The results of the plots of fair and good plant vigour were very close to those recorded in the plot of weak plant vigour. The effect of the foliar treatments was smaller for macronutrients, in spite of significant differences being found for P and Mg in the plot of weak vigour plants and for Ca in the plot of very good vigour plants.

Table 4.1.2. Leaf concentrations of macro and micronutrients (average ± standard error) in July from samples taken at 2 m height in the plots of weak and very good vigour plants as function of foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control), and year. Means followed by the same letter are not statistically different (α = 0.05) by Tukey HSD (Foliar treatment) or t-Student t (Year) tests.

	Nitrogen	Phosphorus	Potassium	Calcium	Magnesium	Iron	Manganese	Copper	Zinc	Boron
	(g kg^{-1})					(mg kg^{-1})				
Foliar treatment (FT)	Weak									
Control	30.6 ± 2.15 a	1.6 ± 0.10 a	4.3 ± 1.00 a	12.2 ± 1.70 a	11.8 ± 3.95 b	181.7 ± 91.6 a	49.4 ± 4.4 b	5.6 ± 0.88 a	18.5 ± 1.49 a	34.9 ± 11.86 b
Fnut	29.1 ± 2.09 a	1.4 ± 0.14 b	4.0 ± 0.63 a	12.4 ± 3.18 a	10.1 ± 1.81 b	169.0 ± 41.2 a	43.2 ± 12.5 b	5.1 ± 0.46 a	17.2 ± 1.61 a	30.4 ± 9.86 b
Algae	31.5 ± 1.96 a	1.6 ± 0.14 a	4.5 ± 0.85 a	14.0 ± 1.75 a	14.8 ± 4.68 a	127.5 ± 18.9 a	58.8 ± 8.7 a	4.8 ± 0.47 a	17.9 ± 3.11 a	44.7 ± 7.97 a
Year (Y)										
2017	30.9 ± 1.84 a	1.6 ± 0.14 a	3.6 ± 1.51 b	14.2 ± 2.01 a	9.2 ± 1.54 b	195.1 ± 76.3 a	53.2 ± 3.8 a	4.9 ± 0.47 a	16.3 ± 1.85 b	29.1 ± 9.19 b
2018	29.8 ± 2.47 a	1.5 ± 0.11 b	4.9 ± 0.43 a	11.5 ± 1.81 b	15.2 ± 3.35 a	123.8 ± 16.0 b	47.7 ± 14.8 b	5.5 ± 0.79 a	19.4 ± 1.01 a	44.2 ± 7.39 a
Prob. (FT)	0.1282	0.0165	0.0934	0.1362	0.0001	0.1919	0.0008	0.0872	0.3389	0.0030
Prob. (Y)	0.2555	0.0078	<0.0001	0.0028	<0.0001	0.0110	0.0424	0.0665	0.0009	0.0001
Prob. (FT×Y)	0.1167	0.5443	0.2154	0.0402	0.0060	0.2289	0.0005	0.4787	0.3977	0.3045
Foliar treatment (FT)	Very good									
Control	33.9 ± 2.17 a	1.4 ± 0.06 a	24.3 ± 9.48 a	21.5 ± 7.43 a	1.19 ± 0.63 a	100.9 ± 15.2 a	356.0 ± 88.9 ab	4.3 ± 1.29 a	76.7 ± 7.82 a	70.5 ± 9.50 a
Fnut	35.4 ± 2.43 a	1.5 ± 0.18 a	26.1 ± 12.05 a	17.6 ± 4.54 b	4.6 ± 1.06 a	113.2 ± 18.7 a	367.5 ± 36.3 a	4.7 ± 1.86 a	78.9 ± 40.29 a	64.4 ± 10.20 b
Algae	35.4 ± 1.60 a	1.6 ± 0.19 a	24.4 ± 7.20 a	19.4 ± 6.22 ab	4.6 ± 0.66 a	96.6 ± 16.6 a	285.7 ± 49.4 b	5.5 ± 1.88 a	71.8 ± 23.60 a	62.5 ± 7.63 b
Year (Y)										
2017	33.3 ± 1.69 b	1.4 ± 0.21 a	16.5 ± 1.74 b	24.8 ± 3.73 a	4.1 ± 0.69 b	114.9 ± 18.6 a	309.4 ± 73.7 b	3.7 ± 1.61	64.8 ± 15.86 a	73.4 ± 6.34 a
2018	36.4 ± 1.04 a	1.5 ± 0.12 a	33.4 ± 4.12 a	14.2 ± 1.04 b	5.6 ± 0.58 a	92.2 ± 5.7 b	363.4 ± 51.2 a	6.0 ± 0.48	86.8 ± 29.97 a	58.2 ± 3.66 b
Prob. (FT)	0.0697	0.0570	0.4558	0.0275	0.1925	0.0899	0.0343	0.2364	0.8772	0.0082
Prob. (Y)	<0.0001	0.3468	<0.0001	<0.0001	0.0002	0.0020	0.0441	0.0009	0.0792	<0.0001
Prob. (FT×Y)	0.1172	0.0285	0.0337	0.2236	0.6847	0.3624	0.2002	0.4213	0.2258	0.1930
Sufficiency range	[32–56]	[2.7–5.4]	[16–34]	[10–26]	[2.9–6.7]	[44–98]	[45–125]	[8–29]	[23–108]	[18–63]

Significant differences between foliar treatments in fair and good plots were not common, nor did they show a consistent trend with the results of weak and very good plots (data not shown). The years showed a large variation in the concentration of the macronutrients in the leaves, particularly for K, Ca and Mg. Leaf K levels were particularly high in 2018 and Ca levels in 2017. Significant interactions between foliar treatment and year was neither frequent nor consistent between plots for a given nutrient. The micronutrients Mn, Zn and B were particularly high in the leaves of the plots of higher vigour plants in comparison to the others (Table 4.1.2). The foliar treatments had little effect on the concentration of micronutrients in the leaves. Only leaf B levels were found to be significantly higher in the Algae in comparison to the other treatments. The year significantly influenced the concentration of most of the micronutrients in the leaves, although some were higher in 2017 and others in 2018.

The plant tissues analysed at harvest (top and bottom leaves and stems) showed many differences in comparison to the leaves sampled in July, related to the date of sampling, the position in the canopy and type of tissue, but maintained the trend of the effect of the treatments of the samples taken in July (data not shown). In comparison to the July samples, the leaves at harvest from the bottom half of the plants showed low levels of N and P and higher levels of Ca, Mg and B. As observed for the July samples, leaf concentrations of all nutrients varied greatly between plant vigour plots. Leaf concentrations of some nutrients also varied significantly with the foliar treatments. However, in general terms, the control did not show lower values than the fertilized treatments. The year effect was statistically significant for most of the nutrients, some being higher in 2018 and others in 2017. In the top half leaves, the concentrations of N, K and B were markedly higher and P and Mg markedly lower in the very good vigour plants, in comparison to the weak vigour plants. Although significant differences between foliar treatments were found for some nutrients, a clear pattern distinguishing between the results of the fertilized and the non-fertilized plots was not observed. The year again showed a marked influence on leaf nutrient concentrations. Stem nutrient concentrations were markedly lower for the majority of macro and micronutrients in comparison to the values found in the leaves. Significant differences between foliar treatments were also often found for some nutrients, but the control did not display significantly lower values than the fertilized treatments. The effect of the year was statistically significant for most of the nutrients, as observed for leaf analysis.

The concentration of the majority of the macro and micronutrients in the cones was markedly different from leaves and stems (Table 4.1.3). The levels of P and K were markedly higher in the cones in comparison to those of leaves or stems. The levels of Ca, Mg and B, for instance, were lower in the cones in comparison to the leaves.

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Table 4.1.3. Cone concentrations of macro and micronutrients (average ± standard error) in the plots of weak and very good vigour plants as a function of foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control), and year. Means followed by the same letter are not statistically different (α = 0.05) by Tukey HSD tests (Foliar treatment) or Student's t-tests (Year).

	Nitrogen	Phosphorus	Potassium	Calcium	Magnesium	Iron	Manganese	Copper	Zinc	Boron
	---------- ($g\ kg^{-1}$) ----------					---------- ($mg\ kg^{-1}$) ----------				
Foliar treatment (FT)	Weak									
Control	30.41 ± 5.81 a	3.63 ± 0.38 a	9.36 ± 2.01 a	3.53 ± 1.11 a	3.49 ± 0.70 a	236.07 ± 137.4 a	42.26 ± 7.36 ab	8.86 ± 1.21 a	31.99 ± 4.91 b	18.91 ± 4.59 a
Fnut	27.61 ± 2.58 b	3.28 ± 0.32 a	10.73 ± 2.94 a	3.43 ± 1.23 a	3.28 ± 0.60 a	198.64 ± 20.6 a	36.34 ± 9.99 b	8.33 ± 1.52 a	34.53 ± 5.61 ab	18.62 ± 3.22 a
Algae	28.80 ± 3.98 ab	3.60 ± 0.32 a	9.73 ± 2.69 a	3.71 ± 1.61 a	3.43 ± 0.78 a	275.19 ± 132.7 a	49.99 ± 7.94 a	9.05 ± 1.54 a	38.50 ± 11.85 a	20.53 ± 3.90 a
Year (Y)										
2017	32.92 ± 3.27 a	3.64 ± 0.39 a	7.62 ± 1.26 b	4.89 ± 0.65 a	3.98 ± 0.49 a	168.96 ± 27.5 b	39.33 ± 12.04 a	8.66 ± 1.57 a	40.95 ± 8.27 a	19.10 ± 4.62 a
2018	25.96 ± 1.67 b	3.40 ± 0.31 a	11.68 ± 1.63 a	2.56 ± 0.29 b	2.97 ± 0.39 b	287.38 ± 122.0 a	45.51 ± 7.40 a	8.82 ± 1.32 a	30.55 ± 4.51 b	19.55 ± 3.61 a
Prob. (FT)	0.0103	0.0598	0.2794	0.4119	0.6423	0.4401	0.0040	0.5315	0.0475	0.3316
Prob. (Y)	<0.0001	0.0803	<0.0001	<0.0001	0.0001	0.0078	0.0619	0.8059	0.0004	0.7716
Prob. (FT×Y)	0.0062	0.0691	0.5585	0.1571	0.4437	0.1396	0.0424	0.2594	0.0407	0.0182
Foliar treatment (FT)	Very good									
Control	25.5 ± 1.33 ab	2.9 ± 0.35 a	20.3 ± 2.75 a	4.1 ± 1.93 a	2.0 ± 0.24 a	151.1 ± 29.6 a	77.3 ± 15.94 a	6.7 ± 0.57 a	33.7 ± 3.80 a	27.8 ± 2.53 a
Fnut	26.2 ± 1.15 a	2.8 ± 0.18 a	21.1 ± 3.24 a	4.0 ± 1.77 a	2.2 ± 0.26 a	144.0 ± 17.8 a	83.0 ± 12.07 a	6.4 ± 0.58 a	32.2 ± 2.51 a	25.8 ± 1.24 ab
Algae	24.3 ± 1.00 b	2.8 ± 0.35 a	19.4 ± 2.48 a	3.7 ± 1.60 a	2.0 ± 0.12 a	128.0 ± 9.3 a	61.8 ± 5.90 b	6.3 ± 0.89 a	30.6 ± 0.94 a	25.1 ± 1.76 b
Year (Y)										
2017	25.7 ± 1.28 a	3.0 ± 0.23 a	18.0 ± 1.47 b	5.6 ± 0.5 a	2.0 ± 0.17 b	138.6 ± 9.0 a	68.5 ± 11.81 b	6.5 ± 0.45 a	32.3 ± 3.21 a	27.2 ± 2.03 a
2018	25.0 ± 1.43 a	2.6 ± 0.12 b	22.5 ± 1.81 a	2.3 ± 0.39 b	2.1 ± 0.25 a	143.6 ± 30.4 a	79.5 ± 15.65 a	6.4 ± 0.89 a	32.0 ± 2.62 a	25.2 ± 1.92 b
Prob. (FT)	0.0112	0.4108	0.5595	0.2999	0.0768	0.1051	0.0040	0.5088	0.1033	0.0125
Prob. (Y)	0.1509	<0.0001	<0.0001	<0.0001	0.0456	0.5648	0.0269	0.7436	0.7442	0.0097
Prob. (FT×Y)	0.3497	0.0452	0.8345	0.3995	0.7158	0.3533	0.8485	0.3855	0.3827	0.6418

The cone levels of N were similar to that of the leaves. The range of variation for each nutrient seemed to be lower than that observed for leaves. However, as observed for leaves, great differences between plant vigour plots were found for all nutrients. Very high vigour plants showed lower levels of N, P, Mg and Cu in the cones in comparison to the weaker plants. K and B, for instance, were significantly higher in the very good vigour plants than in the weaker plants. The effect of the foliar treatments on cone nutrient concentration was small, and not consistent between the different plots. Once again, the year showed a marked influence on tissue plant composition. Significant differences were found for almost all the nutrients, with the concentration of some to be found higher in 2017 and others in 2018.

3.3. SPAD readings, NDVI and chlorophyll fluorescence

SPAD, NDVI and chlorophyll *a* fluorescence varied greatly among plant vigour plots (Table 4.1.4). A tendency for a significant increase was found in these tests from the weaker to the higher vigour plants. In turn, the foliar sprays did not have a great effect on these indices of nutritional status and photosynthetic performance of plants, the most important exception being the higher SPAD values in the Algae treatment in the plot of weak vigour plants. The year seemed to influence some of those variables, but with little consistency between plots and DMY. NDVI, however, showed higher values in 2018, the most productive year.

Table 4.1.4. SPAD (Soil and Plant Analysis Development) readings, NDVI (Normalized Difference Vegetation Index) and chlorophyll *a* fluorescence (average ± standard error) on July in the plots of weak and very good vigour plants as a function of foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control) and year. Means followed by the same letter are not statistically different (α = 0.05) by Tukey HSD tests (Foliar treatment) or Student's t-tests (Year).

	SPAD	NDVI	O	J	I	P	F_V/F_M	F_V/F_0
Foliar treatment (FT)	Weak							
Control	33.5 ± 2.81 b	0.72 ± 0.08 a	256 ± 20 a	362 ± 15 a	503 ± 56 a	631 ± 39 b	0.74 ± 0.02 a	2795 ± 238 a
Fnut	33.2 ± 3.06 b	0.73 ± 0.04 a	265 ± 37 a	389 ± 54 a	561 ± 63 a	714 ± 65 a	0.76 ± 0.02 a	3265 ± 295 a
Algae	36.4 ± 2.42 a	0.74 ± 0.02 a	256 ± 53 a	372 ± 81 a	532 ± 53 a	697 ± 46 a	0.77 ± 0.03 a	3338 ± 598 a
Year (Y)								
2017	35.1 ± 1.92 a	0.69 ± 0.03 b	273 ± 44 a	397 ± 71 a	566 ± 45 a	708 ± 54 a	0.75 ± 0.02 a	3035 ± 354 a
2018	33.6 ± 3.78 a	0.77 ± 0.03 a	244 ± 22 b	352 ± 14 b	498 ± 54 b	653 ± 57 b	0.76 ± 0.03 a	3230 ± 545 a
Prob. (FT)	0.0172	0.5864	0.9039	0.7045	0.1160	0.0099	0.0997	0.0840
Prob. (Y)	0.1029	<0.0001	0.1361	0.1113	0.0073	0.01338	0.4274	0.3344
Prob. (FT×Y)	0.0031	0.0031	0.7454	0.6558	0.3110	0.1990	0.4231	0.4405
Foliar treatment (FT)	Very good							
Control	42.6 ± 2.75 a	0.77 ± 0.05 ab	256 ± 11 a	402 ± 21 a	705 ± 64 a	877 ± 88 a	0.81 ± 0.02 a	4291 ± 536 a
Fnut	43.7 ± 1.99 a	0.77 ± 0.03 a	250 ± 16 a	378 ± 16 a	722 ± 67 a	900 ± 55 a	0.82 ± 0.01 a	4547 ± 272 a
Algae	43.1 ± 2.66 a	0.75 ± 0.04 b	252 ± 17 a	399 ± 25 a	694 ± 70 a	886 ± 72 a	0.82 ± 0.02 a	4484 ± 454 a
Year (Y)								
2017	45.1 ± 1.16 a	0.73 ± 0.02 b	245 ± 9 b	389 ± 18 a	729 ± 44 a	922 ± 65 a	0.82 ± 0.01 a	4650 ± 407 a
2018	41.2 ± 1.51 b	0.80 ± 0.01 a	259 ± 15 a	396 ± 26 a	691 ± 74 a	862 ± 64 a	0.81 ± 0.02 a	4284 ± 386 b
Prob. (FT)	0.3879	0.0188	0.7242	0.0941	0.8514	0.8281	0.4669	0.5198
Prob. (Y)	<0.0001	<0.0001	0.0376	0.4652	0.2004	0.0558	0.0591	0.0488
Prob. (FT×Y)	0.4289	0.1555	0.7781	0.6826	0.3536	0.2864	0.2727	0.2393

3.4. Concentration of bitter acids in the cones

The concentrations of α- and β-acids in the cones were higher in the plants of very good vigour than in the plants of lower vigour (Figure 4.1.4). Foliar sprays did not significantly increase the concentrations of α- and β-acids in the cones in comparison to the control. The year had a marked effect on the cone composition; the values of α- and β-acids were significantly higher in 2018 in comparison to 2017. Significant interaction of foliar treatment × year was found to α-acid concentrations in the fair and good vigour plots and β-acid concentration in good vigour plot.

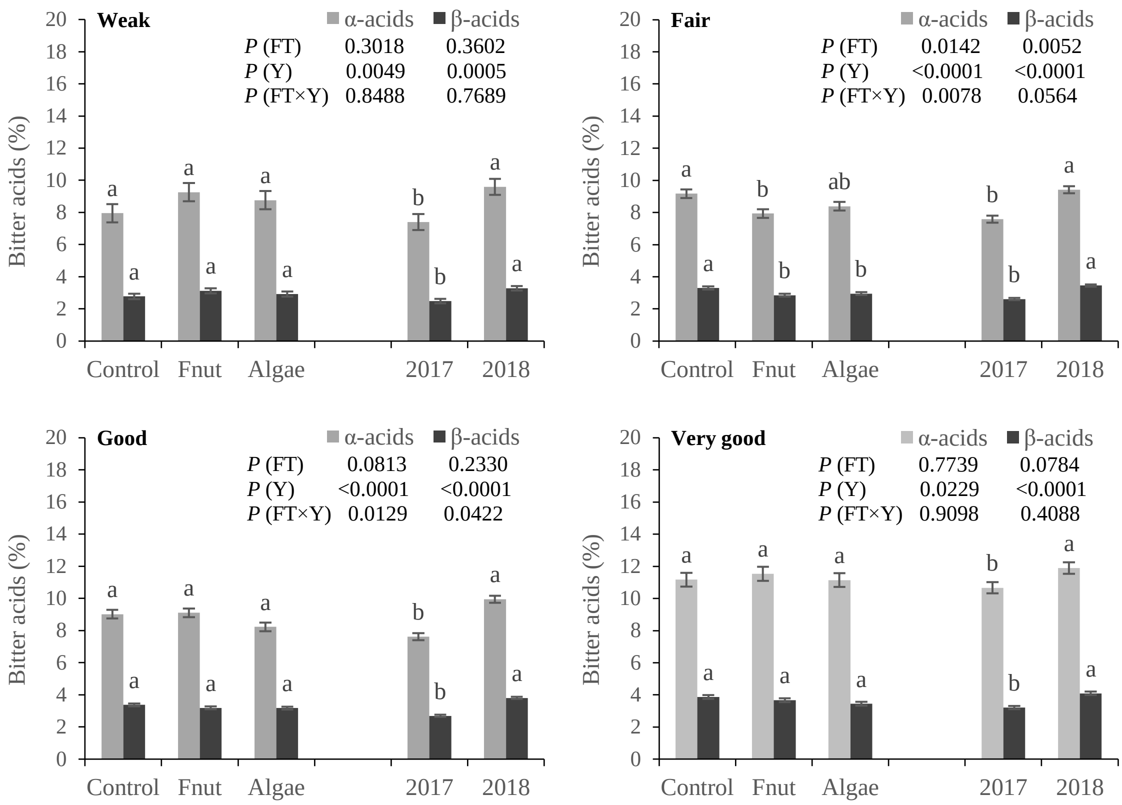

Figure 4.1.4. Cone α- and β-acid concentrations (average ± standard error) in the different plant vigour plots (Weak, Fair, Good and Very good), as a function of foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control) and year. Means followed by the same letter are not statistically different (α = 0.05) by Tukey HSD tests (Foliar treatment) or Student's t-tests (Year).

4. Discussion

The plots identified by farmers as the most productive in previous years (very good vigour plants) produced more biomass in all the plant parts (stems, leaves and cones) than the other plots. The cone size, however, did not differ between the different plots. The concentration of nutrients in plant tissues varied significantly with the vigour of the plants. Some nutrients were found at higher levels in the most productive plots, but the concentration in plant tissues of many other nutrients were higher in the less productive plots. However, no evidence was found that the nutritional status of plants had been an important factor influencing DMY, since most of the nutrients were found within or close to their sufficiency ranges as reported by Bryson et al. (2014). These variations in tissue nutrient concentrations were probably the result of dilution and concentration effects, which have been well-known for a long time (Jarrel and Beverly, 1981), and/or antagonism and synergism in plant nutrient uptake (Marschner and Rengel, 2012). SPAD and NDVI usually provide a good indication of the greenness of the leaves and general plant health (Afonso et al., 2018; Kalaji et al., 2014). In this study, however, only NDVI values were associated with the most productive plots. Chlorophyll fluorescence ratios (F_V/F_M and F_V/F_0) and OJIP transient are important tests to assess stresses that affect the function of photosystem II, and are also usually related to crop productivity (Dinis et al., 2016; Kahaji et al., 2016). In this study, these tests showed little sensitivity in discriminating between the different plant vigour plots.

In 2018, DMY of all plant parts was significantly higher than in 2017. The spring and summer of 2017 were warmer and drier than in 2018, accentuating the Mediterranean characteristics of the regional climate, which usually reduce the performance of hop crops (Marceddu et al., 2020; Rossini et al., 2020). The concentration of almost all the nutrients analysed varied significantly with the year, irrespective of plant tissue and sampling date. This great dynamic in tissue nutrient concentrations depends on diverse factors; one of the most relevant of these is the dilution/concentration effect related to nutrient uptake and C assimilation, and plant growth (Arrobas et al., 2018; Jarrel and Beverly, 1981), which does not justify further development here. SPAD readings and chlorophyll fluorescence and OJIP transient variables presented inconclusive results, they too are probably related to the concentration of nutrients in the tissues and dilution/concentration effects (Afonso et al., 2018). NDVI, in turn, showed consistently higher values in 2018, the most productive year. The most productive year displayed significantly higher α- and β-acid concentrations in the cones, showing that the conditions promoting plant growth also enhanced the accumulation of bitter acids in the hop cones. Hop-acids are soft resins produced by hop plants as secondary metabolites. The impact of the weather conditions during the two-week period before harvest have a strong influence on the accumulation of α-acids (MacKinnon et al., 2020). This is probably the reason explaining the greatest effect of the year in comparison to the plot or the foliar treatment.

The foliar sprays did not produce a significant effect on total DMY or on DMY of any of the different plant parts, including the cones in fair, good and very good vigour plots. However, in the plot of weak vigour plants, cone and total DM yields were significantly higher in the Algae in comparison to the other treatments. Due to the high amount of tissues analysed for their elemental composition, sometimes significant differences between treatments were observed but, in these cases, the control treatment never showed lower values. The fertilizer treatments also did not influence significantly the variables related to the photosynthetic performance of the plants, nor did they influence the levels of α- and β-acids in the cones in comparison to the control. Other studies can be found in the literature in which the application of algae extracts did not increase productivity or improve the quality of products (Amiri et al., 2008; Di Stasio et al., 2018; Mallarino et al., 2001). The efficacy of plant biostimulants can vary greatly depending on the conditions of application (concentration of active ingredients, phenological state of plant, etc.), including the competition for uptake by microorganisms in the phyllosphere (or rhizosphere, if the products are applied to the soil), an aspect that is recognized as needing further investigation (Colla and Rouphael, 2015; Yakhin et al., 2016). However, it is usually under stressful conditions that the use of algae extracts tend to give better results (Al-Ghamdi and Elansary, 2018; Carmody et al., 2020; Goñi et al., 2018), which is in agreement with the observations in this study. The subject has been under intense investigation during the last decades with numerous reviews updating the current knowledge (du Jardin, 2015; du Jardin et al., 2020; Rouphael and Colla, 2020). The use of seaweed extracts has been highlighted since these products are the most commonly used and those that usually present favourable results on crop growth (Battacharyya et al., 2015; De Saeger et al., 2020; Shukla et al., 2019).

Plant biostimulants have been shown to act as elicitors, enhancing plant growth and triggering stress responses by activating molecular and biochemical pathways (du Jardin, 2015; Stirk et al., 2020), so it should not be emphasized that, in this study, significant effects on the elemental composition of plants by the application of foliar sprays were not observed. Extensive research on the topic has shown that the beneficial role of plant biostimulants and, in particular, those made from algae extracts, has been observed mainly under harsh environmental conditions, such as drought (Frioni et al., 2021; Goñi, et al., 2018; Xu and Leskovar, 2015), heat (Carmody et al., 2020) or saline (Al-Ghamdi and Elansary, 2018) stress. In a first analysis, none of these stresses can be identified in these hop fields, which may help to explain the absence of a significant effect by the application of the plant biostimulants on crop productivity in the plots of fair, good and very good vigour plants. A previous study in this region reported poor soil drainage and aeration as the main reason for the heterogeneity observed in hop fields, which is exacerbated by the irrigation method, consisting of flooding the spaces between the rows (Afonso et al., 2020). Thus, under these conditions, the algae extract was only beneficial on highly stressed plants.

5. Conclusions

Under the conditions of this experiment, two plant biostimulants containing seaweed algae extracts did not positively influence the mineral composition of hop plants, indices of crop nutritional status and photosynthetic efficiency or the α- and β-acid concentrations in the cones. Crop yield was only significantly increased with the use of algae extract in the plot of weak plant vigour. However, almost all those variables changed greatly with the effect of plot and year. This poor effect on crop performance might have been due to the type of stress that limits plant growth, which is probably poor drainage and deficient soil aeration. Although the effectiveness of seaweed extracts in improving plant performance under conditions of drought, heat and salt stress is well known, they had little effect under the conditions of this experiment. Only on highly stressed plants did the positive effect of the algae extract prove to be significant. Thus, to help farmers to overcome the problem, other avenues should be explored, such as the use of different plant biostimulants and/or testing different conditions of application.

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4.2. Hop dry matter yield and cone quality responses to amino acid and potassium-rich foliar spray applications

Abstract

The use of amino acid and K-rich foliar sprays was evaluated in a commercial hop field in NE Portugal. Four applications of an amino acid-rich foliar spray were performed in place of a second side dress N application of ~70 kg N ha^{-1}, which is usually applied by the farmer. The K-rich foliar spray was applied once at the cone developing stage as a supplement to the farmer's fertilization plan. The amino acid-enriched foliar spray maintained crop DMY at the levels of the control treatment and increased cone α-acids concentration (41.8% in 2018 and 9.3% in 2019). Foliar K did not increase cone DMY, cone size or bitter acid concentration. Tissue K concentration was not significantly affected by foliar treatments whereas the application of K seemed to increase N uptake, with leaves and stems being the predominant allocation tissues. Both foliar treatments increased leaf and stem Mg concentrations. The results seem to emphasize the importance of amino acids in the biosynthesis of bitter acids, while K and Zn seemed to play an important secondary role, maybe related to N metabolism and its reduction into amino acids. The concentrations of total phenols in cones and leaves were lower in the foliar treatments in comparison to the control, and the higher values registered in leaves. In this study, the use of amino acids as a foliar spray provided an interesting result, since they maintained cone DMY and increased cone bitter acid concentration with reduced N use.

Keywords: *Humulus lupulus*; Foliar fertilization; Hop DMY; Hop quality; α and β-acids

1. Introduction

Commercial hop (*Humulus lupulus* L.) fields of NE Portugal are grown under the European Union norms of integrated crop management which allow the use of conventional mineral fertilizers. Hop is a climbing plant, exceeding 7 m in height. The biomass that the plant produces is huge, which makes it a highly demanding crop for nutrients, in particular for N, the nutrient required most for plant growth (Kraiser et al., 2011; Turner et al., 2011). Hop is a crop of high operational costs, which causes farmers to search constantly for more economical and/or effective alternatives to increase crop yield and quality.

N is taken up by plant roots mainly as NO_3^- and NH_4^+ ions, and to a lesser extent in the form of amino acids (Lambers et al., 2008; McAllister et al., 2012). NO_3^- is the predominant form of N taken up by plants in agricultural well aerated soils, NH_4^+ in non-fertilized soils or waterlogged conditions, and amino acids in areas of reduced ammonification and nitrification such as in boreal forests (Hawkesford et al., 2012; Schulze et al., 2019). When NO_3^- is the N form taken up by the plant, it needs to be reduced to NH_3/NH_4^+ to be assimilated into amino acids, which has a high energy cost to the plant. NH_4^+ resulting from NO_3^- reduction, or taken up directly from the soil, needs to be quickly assimilated since it is toxic to the plant (Lal, 2018a; Schulze et al., 2019). Thus, the uptake of a more reduced form of N, such as amino acids, may have several benefits to the plant which firstly include the lower biosynthetic cost (Lambers, et al. 2008). Moreover, soil inorganic N fertilization is usually associated with reduced N use efficiency and a high risk of environmental contamination, mainly due to NO_3^- leaching, denitrification and ammonia volatilization (Dimkpa et al., 2020). Amino acids can also have a biostimulant effect on plants, which increases their growth and development, and can reduce the need for fertilizers (Halpern et al., 2015).

Amino acid-based fertilizers may be applied to the soil or as foliar sprays (Drobek et al., 2019). Foliar applications are more orientated to the target organs, with lesser amounts being supplied. Although macronutrient requirement in high-yielding crops is unlikely to be met with foliar sprays (Fageria et al., 2009), partial replacement of conventional fertilization may benefit crop yield while reducing production costs and environmental damage. Positive effects of amino acid-containing biostimulants in the increasing of growth and yield have been commonly reported in horticultural crops (Drobek et al., 2019). In fruit crops, the application of amino acids as foliar sprays can also be effective in improving yield and fruit quality (Abd El-Razek et al., 2018; Ilie et al., 2018).

Regarding hops, there is little data on the use of biostimulants or amino acid-based formulations. Procházka et al. (2018) tested a set of biologically active substances in hop, one of which was based on a seaweed (*Ascophyllum nodosum*) extract, containing macro and micronutrients and amino acids. This treatment, however, did not produce relevant results in terms of hop yield and quality in comparison to the many other fertilizing materials.

Hop cones are the most valuable plant part, usually used in the brewing industry (Almaguer et al., 2014). It is in the lupulin glands of the hop cones that the bitter α- and β-acids are synthetized, which contribute to the bitter flavour of beer, its preservation, and foam stability (Almaguer et al., 2014; Ting and Ryder, 2017). The contribution of α-acids to beer bitterness is much more important than β-acids and the content of both in hop cones has to be measured to determine the addition ratio to the beer brew (Small, 2016). Consequently, the income of hop farmers depends greatly on the bitter acid content in hop cones. In this regard, factors promoting the size and quality of hop cones are of ultimate importance. The effect of K on the growth phase of flowers and fruits is well known (Hasanuzzaman et al., 2018; Hawkesford et al., 2012), including its role in the development of hop cones (Gingrich et al., 1994).

In plants, K does not form part of organic structures, although it has important roles in several biological processes such as photosynthesis, respiration, osmoregulation, phloem transport and biosynthesis of protein, sugar and starch, which ultimately contribute to plant growth and fruit quality (Kathpalia and Bhatla, 2018; Schulze et al., 2019). The use of K-based foliar sprays is currently increasing mainly due to their potential positive effects on crop quality (Dean, 2019). Valentinuzzi et al. (2018) found that foliar applications of K did not affect strawberry (*Fragaria* × *ananassa*) fruit yield but improved several quality parameters. In a review of the subject, Ahmad et al. (2018) reported that the positive effects of K application are usually more consistent under drought conditions.

Amino acid-based fertilizers are having an increasing use among hop producers in Portugal. In this region, hop farmers often apply foliar K sprays late in the growing season to increase cone size and quality, but without the results ever being monitored by experimental work. Thus, there is a scarcity of data on the effects of amino acid or K-rich foliar sprays in hop fields in Portugal as well as in other hop producing countries.

In order to reduce the gap in the data on such an important subject to hop farmers, the present research has two main objectives: i) to evaluate the effects on plant nutritional status, hop yield and quality of an amino acid-enriched foliar spray, in place of a proportion of the N applied to the soil as a side dressing; and ii) to evaluate the effects on cone yield and quality of a K-rich foliar spray applied at cone developing stage, as a supplement to soil fertilization.

2. Materials and methods

2.1. Field experiments characterization

The field trial was conducted during two growing seasons, from 2018 to 2019, in a hop farm in Pinela (41°40'33.6"N 6°44'32.7"W), NE Portugal. The region benefits from a Mediterranean-type climate (average annual air temperature of 12.7 ºC; annual precipitation of 772.8 mm). Some of physical and chemical soil properties, from composite soil samples collected at 0-30 cm depth, in the rows and between rows, previously to the beginning of the experiment, are presented in Table 4.2.1.

The cultivar Nugget is grown in a 7 m conventional high trellis system, with concrete poles, connected with cable, in a “V” design system. The plants are mechanically pruned in late winter. In summer, the hop is irrigated by flooding the space between rows. Several tillage passes are performed every year to prepare the ridges for irrigation, control weeds and remove soil crusts to allow water infiltration during summer. The farmer follows a crop health protection programme against downy mildew (*Pseudoperonospora humuli*), powdery mildew (*Podosphaera macularis*), several aphids and mites, which he applies as needed.

The experiment was arranged as a fully randomized design with three fertilizer treatments and four replications (four random plants of equivalent size selected before the application of the fertilizers). The fertilization treatments were: 1) Control, as the fertilization plan performed by the farmer, consisting of the application of 500 kg ha^{-1} of a compound NPK fertilizer (7:14:14) late in winter, followed by two side dress N applications performed during the growing season, the first with ~250 kg ha^{-1} of nitromagnesium (27% N as NH_4NO_3 + 3.5% MgO + 3.5% CaO) and the second with ~450 kg ha^{-1} of $Ca(NO_3)_2$ (15.5% N as NO_3^- + 27% CaO); 2) +K, consisting of the same fertilization plan referred to the control treatment with an additional application of a K-rich foliar spray at cone development stage; and 3) AA, where the second side dress N application of the control treatment was replaced by four applications of an amino acid-based foliar spray.

The K-rich foliar spray contained (w/w) 31% K_2O, 3% N and 1% EDTA and was applied at a rate of 4 l ha^{-1} at the end of July (July 30th 2018 and July 29th 2019). The amino acid-enriched foliar spray contained (w/w) 53% of total amino acids (8.6% free amino acids), 9% total N (8.6% organic N) and 54% total organic carbon (TOC) (27.2% organic C) and was applied at a rate of 2.5 l ha^{-1}, four times during the growing season (June 20th, July 8th, July 24th and July 30th 2018, and June 13th, June 28th, July 15th and July 29th 2019).

Table 4.2.1. Selected soil properties (average ± standard deviation) from composite samples collected in the rows and between rows at 0-30 cm depth.

Soil properties	In rows	Between rows
pH_{H2O}	5.7±0.29	5.8±0.15
Organic C (g kg^{-1})[a]	20.3±0.54	16.9±0.45
Extractable P (mg P_2O_5 kg^{-1})[b]	268.4±123.8	276.6±122.1
Extractable K (mg K_2O kg^{-1})[b]	376.8±116.8	326.5±53.5
Exchangeable Ca (cmolc kg^{-1})[c]	5.2±1.56	4.2±1.11
Exchangeable Mg (cmolc kg^{-1})[c]	1.3±1.79	0.6±0.11
Exchangeable K ($cmol_c$ kg^{-1})[c]	0.7±0.23	0.6±0.20
Exchangeable Na ($cmol_c$ kg^{-1})[c]	0.04±0.01	0.1±0.03
Exchangeable acidity ($cmol_c$ kg^{-1})[c]	0.3±0.14	0.2±0.10
Cation-exchange capacity ($cmol_c$ kg^{-1})	7.8±2.13	6.0±1.33
Extractable B (mg kg^{-1})[d]	1.0±0.19	0.5±0.17
Extractable Fe (mg kg^{-1})[e]	219.6±57.2	253.8±51.8
Extractable Mn (mg kg^{-1})[e]	144.7±28.2	194.5±19.3
Extractable Zn (mg kg^{-1})[e]	4.9±1.91	4.6±1.57
Extractable Cu (mg kg^{-1})[e]	11.7±2.59	12.4±1.68

[a]Wet oxidation (Walkley-Black); [b]Egner-Riehm; [c]Ammonium acetate, pH 7; [d]Hot water, azomethine-H; [e]Ammonium acetate and EDTA.

2.2. Data acquisition in the field and tissue sampling

Estimates of the leaf greenness were measured by using the portable SPAD-502 Plus chlorophyll meter, which provide adimensional values, proportional to the chlorophyll content of the leaves. The device measures the transmittance of light through the leaves in two wavelengths, at 650 nm (red light absorbed by chlorophyll) and 940 nm (infrared light, non-absorbed by chlorophyll). Thirty readings for each measurement were taken from the distal lobe of young fully expanded leaves on July 16th 2018 and July 5th 2019 in the Control and AA treatments, seven days after the second application of amino acids. SPAD readings were taken a second time on August 7th 2019 for all treatments, after the application of the K-rich foliar spray and the last application of the amino acids.

The NDVI was determined by using the hand-held FieldScout CM 1000. The meter senses and measures the ambient light at the wavelength of 660 nm and the reflected light (non-absorbed by leaf chlorophyll) at 840 nm wavelength. The NDVI values (between -1 and 1) are calculated from the equation [(%Near Infrared - %Red) / (%Near Infrared + %Red)]. The measurements were taken in the same leaf part and dates as SPAD readings.

Chlorophyll *a* fluorescence and OJIP transient was determined by using the OS-30p+ chlorophyll meter through the dark adaptation protocols F_V/F_M, F_V/F_0 and the advanced OJIP test. F_M, F_0 and F_V are, respectively, maximum, minimum and variable fluorescence from dark adapted leaves, and $F_V/F_M = (F_M-F_0)/F_M$ and $F_V/F_0 = (F_M-F_0)/F_0$.

The OJIP test provides origin fluorescence at 20 μs (O), fluorescence at 2 ms (J), fluorescence at 30 ms (I) and maximum fluorescence (P, or F_M). Measurements were taken from the distal lobe of fully expanded young leaves, after a period of dark adaptation longer than 35 min in the same dates as SPAD readings.

In the first date of field measurements, at the middle of the growing season (July 16th 2018 and July 5th 2019), leaf samples were taken at ~2 m height for elemental analysis. At harvest (August 27th to 31st 2018 and August 29th to 31st 2019), the aboveground biomass was cut at ground level and separated into two samples of leaves (bottom and upper halves), stems and cones and weighed in fresh. Simultaneously, subsamples of each plant part were weighed again in fresh, oven dried at 70 ºC and weighed dry for determination of DMY of the different plant parts. Additionally, subsamples of 30 dried cones for each replication were randomly selected for determination of the dry mass of individual cones. Thereafter, subsamples of all tissues were ground and analysed for elemental composition.

2.3. Laboratory analyses

The soil samples were oven-dried at 40 ºC and sieved in a mesh of 2 mm. The samples were analysed for pH (H_2O) (soil: solution, 1:2.5), CEC (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley-Black method) and extractable P and K (Egner-Riehm method). Soil B was extracted by hot water and the extracts analysed by the azomethine-H method. The analytical procedures for these analyses were performed according to Van Reeuwijk (2002). The availability of other micronutrients, Cu, Fe, Zn and Mn, in the soil was determined by atomic absorption spectrometry after extraction with ammonium acetate and EDTA, according to the method described by Lakanen and Erviö (1971).

Elemental tissue analyses were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K) and atomic absorption spectrophotometry (Ca, Mg, Cu, Fe, Zn and Mn) methods after nitric digestion of the samples (Temminghoff and, Houba 2004). NO_3^- concentration in hop cones was determined according to Clescerl et al. (1998), by UV-Vis spectrophotometry in a water extract (dry cone:solution, 2.5:50). Bitter acids (α- and β-acids) in hop cones were extracted with methanol and diethyl ether by HPLC, according with Analytica EBC 7.7. method (EBC Analysis committee 1998).

2.4. Data analysis

Data was subject to one-way analysis of variance, according to the experimental design, to check for significant differences between fertilizer treatments, using Statistical

Package for the Social Sciences (SPSS) program v. 25. When significant differences were found, the means were separated by Tukey HSD test (α = 0.05). A correlation analysis was also performed for cones data with Spearman coefficient.

3. Results

3.1. Plant dry matter yield

DMY of total aboveground biomass or plant part (stems, leaves and cones) did not significantly differ among treatments in both years (Figure 4.2.1). Total aboveground DMY varied from 1330 and 1494 g plant^{-1} in 2018 and from 1287 and 1524 g plant^{-1} in 2019. The DM of cones was found between 397 and 454 g plant^{-1} and 395 and 485 g plant^{-1}, respectively in 2018 and 2019.

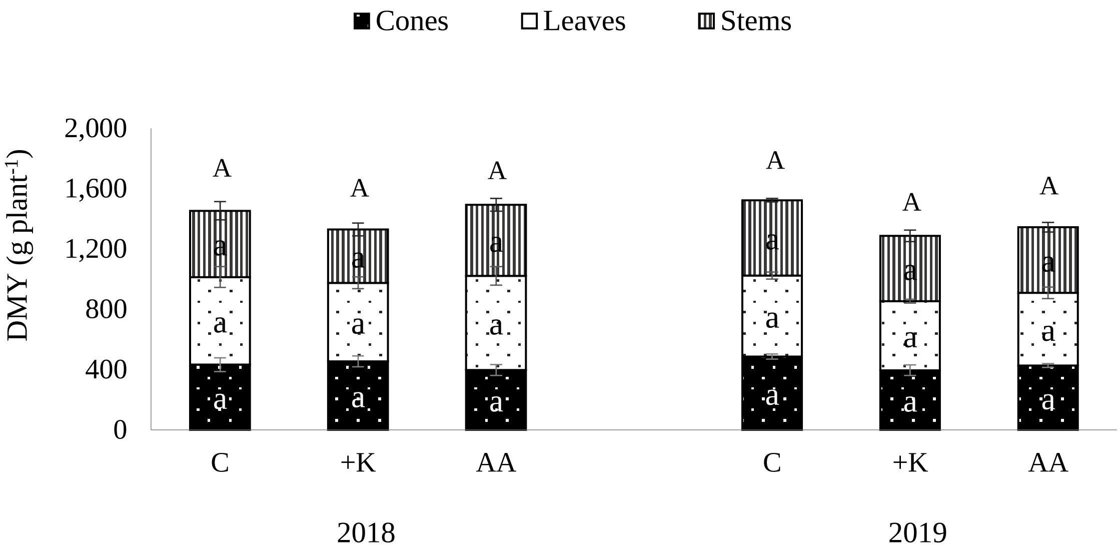

Figure 4.2.1. Dry matter yield (DMY) of hop plant parts and total, in 2018 and 2019, as a function of fertilizer treatment (C, control; +K, potassium-rich foliar spray; AA, amino acid-based foliar spray). Within each year, means followed by the same letter (lower case for each plant tissue and uppercase for total) are not statistically different by Tukey HSD test (α = 0.05). Error bars are the confidence intervals of the means (α = 0.05).

The size of the cones also did not significantly vary between treatments (Data not shown). Average values varied between 0.22 and 0.26 g cone^{-1} in 2018 and 0.20 and 0.22 g cone^{-1} in 2019.

3.2. Tissue nutrient concentration and removal

For the majority of the nutrients, the concentration in the leaves taken at 2 m height during the growing season, significantly varied between control and AA treatments (Table 4.2.2). The control treatment tended to show higher leaf concentration of N, P, Mn, Zn (significant differences in 2018) and K (significant differences in both years). The

AA treatment showed significantly higher values of Mg (in 2018), Fe (in 2018) and B (in 2019). Ca and Cu did not significantly vary between the control and AA treatments. Comparing the average leaf nutrient concentrations to the sufficiency ranges reported for hop mature leaves, several nutrients were found below or above, respectively to the lower and upper limits of the adequate range. Leaf P and Mn levels, consistently lower and higher than the sufficiency ranges, were probably the most noticeable cases. Average P levels were always below 2.2 g kg^{-1}, whereas the sufficiency range is set at 2.7-5.4 g kg^{-1}. Average Mn values were higher than 300 mg kg^{-1} in both treatments and years, whereas the upper limit of the sufficiency range is 125 mg kg^{-1}. Fe also tended to appear out of the sufficiency range. In 2019, for instance, the AA treatment registered a leaf Fe concentration of 253 mg kg^{-1}, a value highly above the upper limit of the sufficiency range set at 98 mg kg^{-1}.

At harvest, there were also found significant differences between fertilizer treatments for all the nutrients, at least for one of the years or leaf position in the canopy. The results of the bottom half leaves were also shown in Table 4.2.2. Those of the top half leaves were not present, due to their similarities. The most relevant trends probably are: the higher leaf K levels in the control and +K treatments in comparison to AA treatment; the higher levels of leaf N in the +K treatment; the lower leaf Mg levels in the control treatment; and the higher leaf Fe levels in the AA treatment. No consistency was found for punctual significant differences found for the other treatments. Leaf Mn and Fe levels appeared particularly higher than the values reported as sufficiency ranges.

The concentration of most of the nutrients in the stems significantly varied between fertilizer treatments, at least in one of the years, the exceptions being K and Fe (data not shown). However, average K concentration in stems was higher in control and +K treatments, following the trend observed in the leaves. Average stem N levels tended to be higher in the +K treatment and P, Mg and Zn tended to be lower in the control treatment. A consistent trend was not observed for the other elements even for those in which significant differences were found in a single year.

Nutrient concentration in cones varied less than in the other plant tissues (Table 4.2.3).

Table 4.2.2. Leaf nutrient concentration (average ± standard deviation) in July sampling (leaves taken at 2 m height) and at harvest (bottom half leaves) from the control (C), amino acid-based (AA) and K-rick (+K) foliar treatments. In July sampling the +K treatment was not yet applied and leaves were not taken. In columns, within each year means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05). For each variable the significance level *(Prob)* is provided.

Year	Treatment	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
		($g\ kg^{-1}$)					($mg\ kg^{-1}$)				
July sampling											
2018	C	34.7±0.57a	2.2±0.15a	41.8±4.60a	14.2±1.69a	5.8±0.60b	81.3±10.6b	494.4±60.4a	5.9±1.52a	23.9±2.61a	53.2±2.52a
	AA	31.9±0.26b	1.6±0.11b	26.7±1.92b	14.6±1.46a	8.5±0.58a	109.7±12.4a	340.5±17.0b	6.4±0.12a	15.1±1.20b	48.7±2.80a
2019	C	34.0±0.69a	1.4±0.07a	19.0±0.53a	11.2±2.06a	4.0±0.62b	192.3±32.7a	320.5±64.9a	8.4±1.32a	27.7±2.67a	54.2±4.83b
	AA	33.6±0.67a	1.5±0.10a	13.0±1.13b	12.9±0.97a	5.4±0.49a	252.9±52.8a	338.4±90.13a	10.7±2.69a	46.8±33.6a	61.1±1.99a
Prob (2018)		0.0001	0.0016	0.0009	0.7157	0.0006	0.0121	0.0027	0.5619	0.0009	0.0544
Prob (2019)		0.4749	0.6217	0.0004	0.2067	0.0187	0.1441	0.7836	0.2348	0.3817	0.0465
Sufficiency range		[32-56]	[2.7-5.4]	[16-34]	[10.3-25.7]	[2.9-6.7]	[44-98]	[45-125]	[8-29]	[23-108]	[18-63]
Harvest sampling (bottom half leaves)											
2018	C	27.4±2.78ab	1.3±0.05ab	29.5±4.53a	15.6±1.49ab	4.8±0.45b	262.5±8.47b	452.5±74.2a	7.1±0.57a	17.7±2.19a	62.3±6.64b
	+K	31.3±2.16a	1.3±0.06b	31.9±2.57a	16.4±0.64a	7.0±0.85a	249.8±21.3b	374.4±91.9a	6.0±0.38a	15.7±1.96ab	91.0±8.19a
	AA	26.2±2.12b	1.4±0.08a	24.9±4.53a	13.7±1.63b	7.4±0.69a	339.6±27.8a	428.0±94.6a	6.9±1.29a	12.8±1.87b	60.4±2.40b
2019	C	30.3±0.38ab	1.0±0.12b	24.6±2.32a	16.1±2.92a	6.4±1.16a	213.4±5.12b	346.3±15.3b	5.7±0.05b	14.3±0.71a	63.4±2.29b
	+K	32.4±2.07a	1.3±0.07a	21.6±6.47a	15.9±1.58a	6.1±1.12a	261.9±50.6ab	275.3±9.7c	6.3±0.48b	21.2±4.69a	73.7±2.85a
	AA	27.9±0.84b	1.2±0.05ab	17.0±3.21a	14.9±1.69a	6.2±0.99a	320.3±37.3a	387.2±20.4a	7.3±0.46a	22.5±4.33a	71.4±6.57ab
Prob (2018)		0.0345	0.0236	0.0876	0.0450	0.0009	0.0004	0.5149	0.1850	0.0234	0.0001
Prob (2019)		0.0053	0.0093	0.1467	0.7214	0.9426	0.0194	<0.0001	0.0018	0.0569	0.0441

Table 4.2.3. Cone nutrient concentration (average ± standard deviation) in August, at harvest, as a function of year and fertilizer treatment (C, control; +K, potassium-rich foliar spray; AA, amino acid-based foliar spray). Within each year, means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05). For each variable the significance level *(Prob)* is provided.

Year	Treatment	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
		(g kg^{-1})					(mg kg^{-1})				
2018	C	24.6±1.20a	2.6±0.17a	21.4±1.30a	3.0±0.11a	2.3±0.12a	229.9±75.2a	84.1±9.79a	12.1±1.02a	31.4±0.56a	25.5±0.97a
	+K	25.0±0.70a	2.5±0.19a	21.9±1.85a	2.6±0.30ab	2.0±0.24a	143.8±5.75a	66.8±8.55ab	6.0±0.96b	34.5±6.16a	27.9±3.99a
	AA	21.2±0.59b	2.9±0.16a	23.4±1.54a	2.2±0.38b	2.1±0.31a	185.2±33.4a	64.9±9.34b	6.5±0.30b	37.5±5.29a	27.3±1.19a
2019	C	27.3±0.60a	2.5±0.07ab	16.0±1.83a	2.6±0.41a	2.0±0.20a	111.5±10.9a	92.8±12.4a	6.3±0.39b	30.0±3.07a	26.7±1.57a
	+K	27.3±0.43a	2.8±0.21a	18.1±4.50a	3.0±0.28a	2.2±0.14a	193.5±80.1a	75.1±8.72b	7.5±0.59a	31.1±5.10a	26.6±2.05a
	AA	25.9±1.63a	2.4±0.15b	13.8±1.87a	2.6±0.28a	2.1±0.17a	174.0±18.8a	91.7±4.98a	6.8±0.52ab	30.0±3.11a	26.7±2.18a
Prob (2018)		0.0003	0.0550	0.2221	0.0095	0.3051	0.0857	0.0308	<0.0001	0.2309	0.3893
Prob (2019)		0.1206	0.0341	0.1779	0.1396	0.1894	0.0892	0.0221	0.0278	0.7372	0.9146

Significant differences among fertilizer treatments occurred only for five (N, P, Ca, Mn and Cu) of the ten nutrients analysed at least in one year. The concentration of K, Mg, Fe, Zn and B in the cones did not significantly vary among fertilizer treatments. The AA treatment showed the lower average cone N concentrations.

Plant nutrient removal did not significantly vary for N, P, Ca and B when appreciated by each plant tissue within each year or even when the sum of the total aboveground DM of the two years were analysed (data not shown due to its extensity).

For the majority of the other nutrients, significant differences between fertilizer treatments were found for a particular tissue and/or year, but when appreciated as the sum of all tissue parts and years, significant differences were only found for Mn. In this particular case, the plants of the control treatment recovered more Mn than those of the +K treatment.

3.3. SPAD, NDVI, chlorophyll fluorescence

In 2018, SPAD readings, NDVI and chlorophyll fluorescence variables did not significantly vary with fertilizer treatments (data not shown). In 2019, SPAD values also did not differ significantly between treatments in any of the measuring dates (Table 4.2.4). Average values varied from 40.2 (July 2019, C treatment) to 43.4 (August 2019, AA treatment).

NDVI significantly varied among the fertilizer treatments in the measurements of August 2019. In this date, the +K treatment gave significantly higher values than the control and AA treatments. Chlorophyll fluorescence variables showed also little sensitivity to the fertilizer treatments. Only in the O (origin), the values significantly differed among treatments in the measurement taken in august 2019.

3.4. Bitter acids and nitrate concentration in the cones

The concentration of α-acids in the cones significantly varied between the fertilizer treatments only in 2018 (Figure 4.2.2). The concentration of α-acids ranged from 10.4 to 14.8%, respectively in the control and AA treatments. The concentration of β-acids, in turn, significantly varied between the fertilizer treatments in both years. The higher β-acids concentrations were also found in the AA treatment (4.60 and 3.96 %, respectively in 2018 and 2019), whereas the lower values were found in the control treatment (3.57 % in 2018 and 3.59% in 2019). The NO_3^- concentration in hop cones did not significantly vary between treatments in any of the years (data not shown). Even so, it can be noted that the average values were consistently higher in the +K treatment.

Table 4.2.4. SPAD (Soil and Plant Analysis Development) readings, NDVI (Normalized Difference Vegetation Index) and chlorophyll *a* fluorescence in July (before the application of +K treatment) and August 2019 as a function of fertilizer treatment (C, control; +K, potassium-rich foliar spray; AA, amino acid-based foliar spray) and year. Within each measurement date, means followed by the same letter are not statistically different by t-Student (2018) and Tukey HSD (2019) tests (α = 0.05). For each variable the significance level *(Prob)* is provided.

Date	Treatment	SPAD	NDVI	O	J	I	P	F_V/F_M	F_V/F_0
July	C	40.2±1.31a	0.778±0.01a	232.3±10.05a	362.8±21.0a	553.3±41.8a	742.3±27.3a	0.797±0.01a	3936.8±226.4a
	AA	41.0±1.62a	0.770±0.01a	221.3±21.5a	334.8±15.3a	526.5±48.0a	725.8±56.1a	0.803±0.01a	4098.0±232.5a
August	C	42.7±1.56a	0.793±0.01b	221.5±15.9b	374.3±12.3a	699.3±28.2a	858.3±52.4a	0.837±0.01a	5169.5±190.4a
	+K	42.3±0.53a	0.810±0.01a	234.5±15.7ab	400.5±37.3a	768.8±54.9a	931.8±82.5a	0.842±0.02a	5405.8±602.5a
	AA	43.4±1.37a	0.778±0.01b	254.3±9.03a	404.3±13.7a	786.0±68.5a	950.5±60.1a	0.837±0.02a	5187.0±686.7a
Prob (July)		0.4719	0.2782	0.3891	0.0744	0.4330	0.6161	0.3634	0.3587
Prob (August)		0.4700	0.0008	0.0263	0.2116	0.2859	0.1705	0.8233	0.7924

O – origin fluorescence values at 20 µs; J – fluorescence values at 2 ms; I – fluorescence values at 30 ms; P – maximum fluorescence; F_V/F_M - ratio of variable fluorescence to maximum fluorescence; F_V/F_0 - ratio of variable fluorescence to minimum fluorescence.

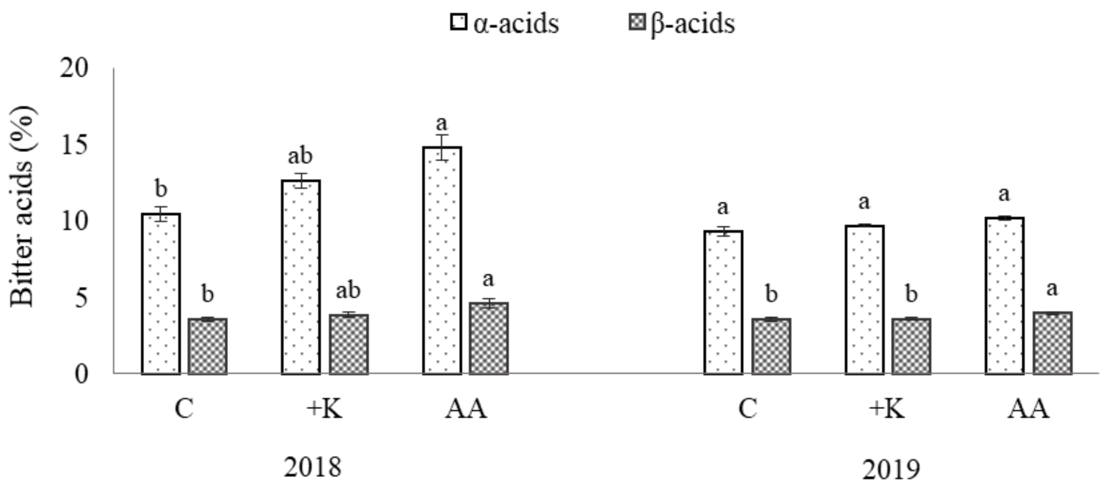

Figure 4.2.2. Cone α- and β-acids concentration in 2018 and 2019 as a function of fertilizer treatment (C, control; +K, potassium-rich foliar spray; AA, amino acid-based foliar spray). Within each year, means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05). Error bars are the confidence intervals of the means (α = 0.05).

3.5. Total phenol concentration in the cones and leaves

Total phenol concentration in cones and leaves in the samples collected at harvest in 2018 differed significantly between treatments for all tissues (Figure 4.2.3). In the cones, total phenol concentrations were significantly lower in +K in comparison to the other treatments. In the leaves, the higher values were found in the control treatment. The control treatment exhibited the higher average values in the cones and in the leaves. The effect of the treatments was more pronounced in the leaves than in the cones, the highest peaks being recorded in leaves in particular in those from the bottom of the canopy. Total phenol concentrations in cones ranged from 13.1 (+K treatment) to 19.9 mg g^{-1} extract (control treatment) and in leaves ranged from 40.5 (control treatment) to 9.6 mg g^{-1} extract (AA treatment).

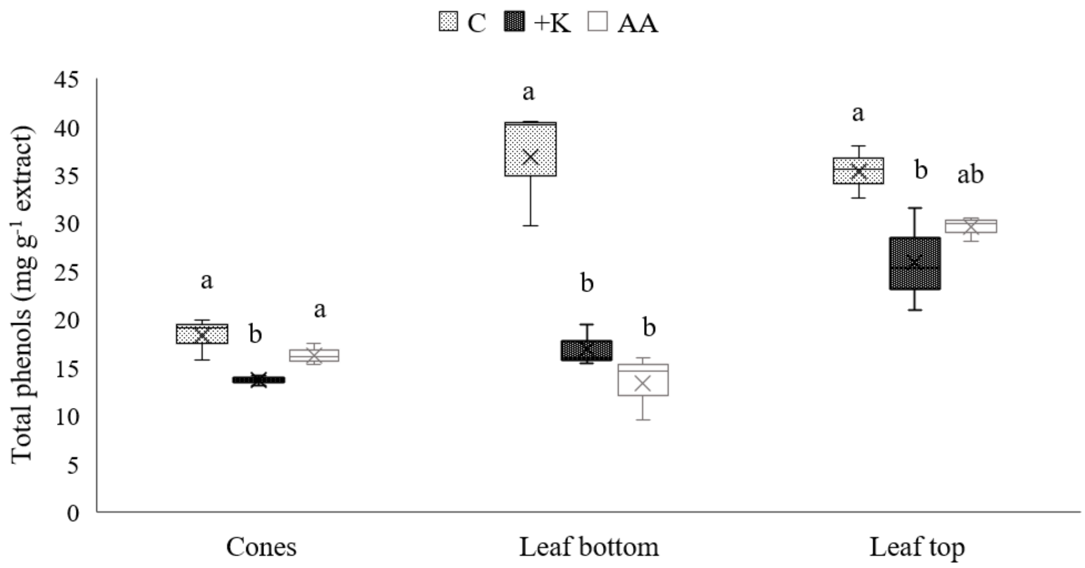

Figure 4.2.3. Total phenol concentration in the cones and leaves (from the bottom half and top half of the plants) in 2018, at harvest, as a function of fertilizer treatment (C, control; +K, potassium-rich foliar spray; AA, amino acid-based foliar spray). Means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05). Error bars are the confidence intervals of the means (α = 0.05).

3.6. Correlation analysis in cone data

Correlation analysis for cone data indicates some significant (and negative) correlations coefficients for bitter acids and NO_3^- in relation with cone nutrient concentrations (Table 4.2.5). The α-acids seems to be significant and negatively correlated with N, Mn and Ca and β-acids with Ca, Cu, N and Mg. For NO_3^-, correlations were significant and negative with K and Zn.

Table 4.2.5. Correlation matrix for bitter acids (α- and β-acids) and nitrate (NO_3^-) with nutrient in hop cones, with Spearman correlation coefficients.

	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
α-acid	-0.611**	-0.316	0.295	-0.478*	-0.380	0.231	-0.558**	-0.343	0.054	-0.250
β-acid	-0.462*	-0.403	-0.223	-0.580**	-0.456*	0.076	-0.251	-0.463*	-0.129	-0.337
NO_3^-	0.191	-0.221	-0.445*	0.124	-0.047	-0.317	-0.004	-0.275	-0.414*	-0.027

Significant correlations according to selected significance levels: * = 0.05, ** = 0.01.

4. Discussion

The application of amino acids produced similar DMY, including in the cones, as the control (the conventional fertilization plan). The result seems to mean that the fertilization programme of the farmer can undergo significant changes without affecting crop yield. In this experiment, amino acids were applied instead of the second side dress N application (~70 kg N ha^{-1}). Amino acids are a source of N in a reduced form, with less biosynthetic cost in relation to mineral N (Lambers et al., 2008), which can help to explain the result. Late application of K (+K treatment) did not increase DMY or cone size. Despite the high K requirements of hops (Gingrich et al., 1994), data on the effects of foliar K fertilizers in hops has not been reported. In agreement with our findings, Valentinuzzi et al. (2018) also did not find any increase in fruit yield in strawberry, after the application of a K-rich foliar spray.

Leaf and stem N levels were consistently higher in the +K treatment in comparison with the AA and control treatments. The effect of K applications on the increase in tissue N levels was often recorded in other species (Abdelwanise et al., 2017; Shehata et al., 2019). The AA treatment presented tissue N concentrations quite similar to the control, indicating the effectiveness of the amino acids applied as a foliar spray in sustaining the N nutritional status of the plants at the same level of the second side dress N application of 70 kg ha^{-1}. It was also found from previous studies an increase in N levels in plant tissues with the application of amino acids as foliar sprays (Abd El-Razek et al., 2018; Noroozlo et al., 2019; Shehata et al., 2011).

The fertilizer treatments had little effect on tissue K levels. However, the values of the control and +K treatments were consistently higher than those of the AA treatment. The results of the present study may be related to soil N fertilization which in the control and +K treatments consisted of two side dress N applications, while in the AA treatment just one. The transport of NO_3^- from the roots to the shoots takes place via xylem along with K^+ as a counter-ion (Schulze et al., 2019), which may justify the increased tissue K levels found in those treatments.

Both foliar treatments (AA and +K) increased Mg concentrations in leaves and stems in comparison to the control. Leaf and stem Fe levels were consistently higher in the AA treatment in both years, and were significantly higher in bottom leaves. Noroozlo et al. (2019) found that glutamine and glycine amino acids, sprayed at 250 and 500 mg L^{-1}, increased leaf Mg and Fe concentrations in basil (*Ocimum basilicum*), which is in agreement with the findings of the present study. The AA treatment association with higher Fe tissue levels could be due to the role of amino acids in nutrient transport. It is known that amino acids can chelate metals such as Fe, thereby increasing their assimilation through specific transporters (Halpern et al., 2015; Lal, 2018b).

Tissue Mn concentrations were generally higher in the control and lower in the +K treatment. Leaf Mn concentrations were above the sufficiency range and Mg concentrations below, in particular in the control treatment. Although hops seem somewhat sensitive to Mn toxicity (Afonso et al., 2020), applications of K may be able to alleviate the potential damage caused by excess Mn (Yu et al., 2020).

The concentration of nutrients in the cones varied less than in the other tissues, the results not being significant for most nutrients. However, the +K treatment presented slightly higher K levels in the cones in comparison to the control in both years, though differences were not statistically significant. The soil and the fertilization programme (the control treatment) seem to provide enough K to plants, as indicated by leaf K status in the control, generally being close to the upper limit of the sufficiency range (Bryson et al., 2014), which may have contributed to the lower efficacy of the +K treatment in increasing cone K levels.

Correlation analysis for cone data may help to clarify the trends of nutrient accumulation found in tissues. Results indicated NO_3^- strong and negatively correlated with K and Zn. The negative association with K could be the result of NO_3^- reduction into amino acids, since NO_3^- is transported together with K as an accompanying cation being released with the NO_3^- reduction (Schulze et al., 2019). Zn is also involved in N metabolism (Kathpalia and Bhatla, 2018), which can explain the negative interaction with NO_3^-. Therefore, higher N levels in the +K treatment may be related with a stimulated N uptake, in the presence of higher K levels, due to the increased reduction into amino acids. K and Zn followed the same trend of accumulation in cones, between treatments, corroborating a synergism between both. Some nutrients presented significant and negative correlations with α- and β-acids. N and Ca were negatively correlated with both α- and β-acids, Mn negatively correlated with α-acids, and Mg+Cu negatively correlated with β-acids. Since the hop bitter acid pathway begins with the amino acid precursor leucine (Champagne and Boutry, 2017), a higher production of bitter acids will demand a higher supply of amino acids normally through NO_3^- reduction. Ca is required in the reduction of NO_3^- namely by NO_3^- reductase kinase (Lal, 2018a). The results might indicate that higher concentrations of NO_3^- and Ca in the cones means lower NO_3^- reduction, and consequently lower bitter acid production. Regarding Mn, this nutrient competes with Mg for uptake and also for binding sites in some enzymes (Kathpalia and Bhatla, 2018). The *Humulus lupulus prenyltransferase-1* (HlPT-1) enzyme that is involved in the first step of bitter acid biosynthesis and the last step of β-acids production (Champagne and Boutry, 2017), exclusively requires Mg as a divalent cation for its activity and cannot be replaced by other divalent cations (Tsurumaru et al., 2012). Considering the leaf concentration of Mn and Mg, respectively above and below the sufficiency ranges, perhaps Mn decreased α-acid biosynthesis through Mg inhibition. The control treatment presented higher levels of Mn and also the lower rates of bitter acids, which seems to be in accordance. Mg negative correlation with β-acids may be related with the plant priorities in cone compound production since Mg is required in many metabolic functions (Lal, 2018a; Schulze et al., 2019). The increasing Cu concentration in hop cones associated with decreasing concentration of bitter acids might indicate a response to a stressful condition such as a disease. Cu acts in plant protection against pathogens (Kathpalia and Bhatla, 2018) and hop diseases can significantly reduce bitter acid content in cones (Jelínek et al., 2012).

SPAD readings, NDVI and chlorophyll fluorescence transients did not reveal clear differences between treatments. Only in August 2019, was the NDVI from the +K treatment significantly higher than that of the AA and control treatments. On the same date, the origin (O) fluorescence transient was significantly higher in the AA than in the control treatment. K is an important activator of enzymes associated with photosynthesis (Kathpalia and Bhatla, 2018) which might suggest that foliar K sprays (+K treatment) could have a positive effect on parameters related to the photosynthetic process.

Bitter acids are the most important parameters in hop cone quality for brewing purposes, particularly α-acids (Almaguer et al., 2014), and they seem to increase consistently with the application of amino acids as a foliar spray. Significant differences were found for α-acids in 2018 (AA treatment higher than control) and for β-acids in 2018 (AA treatment higher than control) and 2019 (AA treatment higher than +K and control treatments). Branched-chain amino acid derived compounds are building blocks for the biosynthesis of hop bitter acids, which are produced in large amounts in the lupulin glands of hop cones (Clark et al., 2013), which can explain the positive effect of the AA treatment on the increase of α- and β-acids.

The application of amino acids and K as foliar sprays seems to reduce the concentration of total phenols. Jelinek et al. (2012) found a significant increase in total phenols in hop plants as a response of a stressful situation caused by a virus infection.

Perhaps less stressed plants, in better nutritional conditions, displayed a reduced concentration in total phenols. On the other hand, results suggest that hop leaves can also be a useful source of polyphenols since the higher values of total phenols were observed in leaves (40.5 mg g^{-1} extract). However, Abram et al. (2015) and Ceh et al. (2007) found opposite results, with higher levels of phenols to be found in cones. Probably this is the result of the cultivars used in the different studies.

5. Conclusions

The amino acid-enriched foliar spray seems to be a competitive alternative to the second side dress N application carried out by the farmer. The plant biomass production was not affected, and nor was the N concentration in plant tissues and NO_3^- concentration in hop cones. Furthermore, the results show a consistent increase of cone bitter acids with the applications of amino acids as a foliar spray, which seems to be relevant to improve crop yield, while reducing the risk of environmental contamination. On the other hand, foliar K supplementation at cone developing stage did not display a positive result. The yield and quality of hop cones, particularly the size of the cones and the concentration of bitter acids, were not significantly improved by K fertilization in comparison to the control or AA treatments. However, the application of the K-rich foliar spray seemed to increase N uptake by the roots, with leaves and stems being the predominant allocation tissues. The results also emphasize the importance of amino acids in the biosynthesis of bitter acids in which K and Zn also seem to play an important role, perhaps because both are involved in N metabolism and in its reduction into amino acids.

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Declaration of interest statement

The authors declare that they have no conflict of interest.

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Chapter 5:

Recycling of organic wastes from hop harvest through composting and composts fertilizing value

Briefing note

This chapter addresses a relevant environmental issue that is the recycling of hop production wastes. These wastes include the leaves and stems that are generated in large amounts during the harvest season and remain on the farms without any treatment.

Chapter 5.1. describes the work undertaken to evaluate the potential use of hop leaves to produced quality compost suitable to be applied as fertilizers in plant growth. Besides, it was evaluated the effect on the composting process of the addition of ash resulting from the usual farmer's procedure of burning the stems after harvest.

This chapter is an adaptation of the following article: Afonso, S., Arrobas, M., Pereira, E.L., Rodrigues, M.Â., 2021. Recycling nutrient-rich hop leaves by composting with wheat straw and farmyard manure in suitable mixtures. J. Environ. Manag. 284, 112105. https://doi.org/https://doi.org/10.1016/j.jenvman.2021.112105.

5.1. Recycling nutrient-rich hop leaves by composting with wheat straw and farmyard manure in suitable mixtures

Abstract

The harvesting of hops (*Humulus lupulus* L.) generates large amounts of nutrient-rich leaves that can be used in composting mixtures to add value to other organic resources on the farm. In this study, hop leaves were mixed with cow manure and wheat straw in several combinations with the aim of establishing guidelines on how farmers can manage the raw materials and better use these valuable organic resources. The composting process was monitored and the quality of the composts evaluated in relation to the effects on lettuce (*Lactuca sativa* L.) grown in pots over two consecutive cycles. The mixture of hop leaves with cow manure produced a stable compost after nine months of composting which may be used in horticultural crops, irrespective of the proportion of raw materials, due to their low and similar C/N ratios. However, when using mixtures of leaves and straw in proportions of less than 2:1, the composts did not mature properly, showing high C/N ratios. Their application to the soil led to a strong reduction in plant tissue N concentrations, due to biological N immobilization, which significantly reduced lettuce DMY. Thus, to reduce composting time and increase the quality of the compost, the ratio leaves/straw should be as high as possible, at least 2:1. Alternatively, either the composting process should take longer, or the poorly-matured compost be applied far in advance of sowing a crop so that complementary biological processes can take place in the soil, as recorded in the second cycle of lettuce. Ash from hop stems did not benefit the composting process and proved itself not to be worth using in mixtures.

Key words: *Humulus lupulus*; *Lactuca sativa*; Compost maturation; TOC; C/N ratio; N immobilization

1. Introduction

Hops (*Humulus lupulus* L.) is a dioecious perennial and climbing plant which can reach nearly 7 m high, producing a large amount of biomass. In commercial fields, only female plants are grown, whose flowers are mainly used for brewing purposes (Almaguer et al., 2014). During the harvesting process, the flowers, usually known as cones, are separated from the stems and leaves which are frequently seen as waste. Leaves and stems represent a huge amount of biomass given that world hop production reached

131,173 t in 2020 (International Hop Growers' Convention, 2020). Hop cones are also a form of waste in themselves, after either extraction of α and β-acids or their direct use in the brewing process. In recent years, hop waste from the brewing industry has received increasing attention. It can be composted and applied to the soil as organic amendments (Kopeć et al., 2020) or be used to obtain high added-value bioproducts (Hrnčič et al., 2019). In contrast, the leaves and stems resulting from the harvesting process continue to remain on farms and have not yet received the same attention.

The European Union (2012) has been encouraging the use of bio-waste in agriculture, since it can improve the condition of the soil and provide valuable nutrients to plants. Recycling bio-waste into organic-based fertilizers also forms part of the action plan of the European Commission (2017) to support the transition towards a C-neutral economy. Composting is considered one of the most effective processes to recycle organic waste, which can be applied to soils as organic amendments (Antil et al., 2014; Pergola et al., 2018). Composting is a biological process of degradation of fresh organic residues under controlled aerobic conditions with the purpose of obtaining a compost of stabilized OM, rich in organic C and free from pathogens and weed seeds (Cesaro et al., 2015; Pergola et al., 2018).

To ensure the quality of the final compost, several variables should be considered during the composting process. Microorganisms are the primary driving agents in the decomposition of biodegradable materials. They use organic C as a source of energy, while N is used for growth and reproduction (Azim et al., 2018; Osman, 2013). Therefore, the initial C/N ratio is of great importance to favour microbial activity and to optimize the composting process (Bernal et al., 2009; Wong et al., 2017). Temperature during composting is a critical variable that should be monitored to ensure the reduction of pathogens and a quick decomposition and humification (Azim et al., 2018). The nutrient content of the final compost, and particularly the N content, is a quality parameter determining the value of the compost to be used in agriculture (Raviv, 2005; Wong et al., 2017).

The stability and maturity of a compost are also requirements for its safe use in agricultural fields. Compost stability is related to the level of microbial activity, while maturity depends on the level of humification and implies the absence of pathogens and phytotoxic threats to plant health (Azim et al., 2018; Cesaro et al., 2015; Prasad et al., 2010). A wide range of variables can be used to evaluate the degree of stability and maturity of a compost, such as physical properties (i.e. temperature, odour and colour), the C/N ratio, inorganic-N species (NH_4^+, NO_3^-, NH_4^+/NO_3^- ratio), content of OM and humic acids, salinity and seed germination, and plants growing in biological tests (Antil et al., 2014; Azim et al., 2018; Bernal et al., 2009).

Hop is a dioecious perennial species as mentioned above. In commercial fields only female plants are grown. This means that plants do not form seeds, which greatly reduces the remobilization of nutrients from the leaves. At harvest, hop leaves can be particularly rich in N, with values reaching 40 g/kg (Afonso et al., 2020), which makes them an excellent material for composting with farmyard manures or to add value to poorer quality materials such as cereal straw through suitable mixtures. Thus, the aim of this study was to monitor the composting process and assess the quality of the final products resulting from composting hop leaves mixed with wheat straw and farmyard manure at different rates. The quality of the composted materials was assessed by their physicochemical properties and through a biological assay, consisting of growing lettuce (*Lactuca sativa* L.) in a pot experiment over two cropping cycles in the same growing medium. It was hoped to establish guidelines for making the best use of these materials, particularly the proportions in which they should be mixed.

2. Materials and methods

2.1. Composting experiment

The leaves used in this study came from the hop harvest of 2017. All the other resouces used to prepare the mixtures for composting (cow manure, wheat straw and ash) came from the same farm, in which are raised animals and where the hop stems are usually burnt. The properties of the raw materials used in this experiment as composting materials are presented in Table 5.1.1.

Table 5.1.1. Carbon (C)/nitrogen (N) ratio, dry matter percentage and concentration of macro and micronutrients (average±standard deviation) in the raw materials used in the composting process.

	Cow manure	Wheat straw	Hop leaves	Hop stem ash
C/N	19.95	295.7	19.21	9.63
Dry matter (%)	93.9±1.14	93.1±0.03	92.1±0.05	99.1±0.13
Carbon (g/kg)	307.2±70.62	557.8±3.44	472.0±3.73	20.8±0.28
Nitrogen (g/kg)	15.4±3.22	1.9±0.07	24.6±0.91	2.2±0.29
Potassium (g/kg)	19.8±3.97	7.5±0.01	15.3±1.03	12.8±0.25
Phosphorus (g/kg)	3.3±0.43	1.0±0.05	1.2±0.07	5.7±0.78
Calcium (g/kg)	8.7±0.46	1.0±0.06	46.5±3.59	95.0±5.38
Magnesium (g/kg)	14.3±5.33	0.7±0.01	4.4±0.27	8.6±0.37
Iron (mg/kg)	10843±6 201	26.5±2.12	555±323.4	14403±2 230
Manganese (mg/kg)	269.4±47.5	37.9±1.20	317.7±24.97	898.6±62.7
Copper (mg/kg)	49.0±11.3	5.1±0.35	6.5±0.57	39.6±1.35
Zinc (mg/kg)	87.8±15.4	5.8±0.10	18.8±1.52	101.7±2.55
Boron (mg/kg)	18.6±2.96	3.0±0.26	62.0±1.66	56.3±1.58

The raw materials were placed in the composter in thin layers according to the ratios shown in Table 5.1.2. The mixtures were turned out manually at 14, 56, 147 and 210 days after the beginning of the composting process. The temperature in the composters was monitored daily during the first 10 days and thereafter at 15, 30, 60, 120 and 240 days. At the same time, the temperature was also recorded outside the composters. The moisture of the mixtures was controlled by regular observation, and water added as required to maintain biological activity. Six months after the start (March 21st 2018) and at the end (June 28th 2018) of the composting process, the mixtures were sampled in triplicate for analytical determinations.

Table 5.1.2. Proportion of raw materials and carbon (C)/nitrogen (N) ratios of the mixtures for composting.

Compostable mixtures	Ratio	Abbreviation	C/N
Hop leaves + cow manure	1:5	HM 1:5	19.97
Hop leaves + cow manure	1:3	HM 1:3	18.21
Hop leaves + cow manure	1:1	HM 1:1	14.56
Hop leaves + wheat straw	1:2	HS 1:2	41.30
Hop leaves + wheat straw	1:1	HS 1:1	29.10
Hop leaves + wheat straw	2:1	HS 2:1	21.00
Hop leaves + wheat straw + hop stem ash	1:1:0.04	HSA 1:1:0.04	29.10

2.2. Pot experiment

A pot experiment was conducted with lettuce (cultivar Wonder of Summer) in two growing cycles (June 28th to August 21st 2018, and April 30th to July 1st 2019), using the composted materials as treatments and mixed with soil to create the growing medium. Additionally, the composts of higher (HS1:2) and lower (HS2:1) C/N ratios were used at single (D1) and double rates (D2).

The experiment included also an untreated control (C), totalling 10 treatments and four replicates (four pots). Each pot received 3 kg of dried and sieved (2 mm) soil. The composts were used at rates equivalent to 20 (D1) and 40 (D2) t/ha dry weigh, giving that the dry mass of the < 2 mm soil fraction of the arable layer (0.2 m) in a hectare is 2240 t. The soil used in this experiment showed pH of 6.51, organic C of 5.2 g/kg, total N of 1.28 g/kg and extractable P and K of 47.8 mg P_2O_5/kg and 53.3 mg K_2O/kg, respectively.

Lettuce seedlings were prepared in micropots of 4 cm^3, by using a commercial germination substrate. They were transplanted at the 3rd true leaf unfolded growth stage, in June 28th and April 30th in the first and second growing cycles. Plants were harvested 54 and 60 days after transplanting in the first and second growing cycles. They were cut at ground level, oven-dried at 70 ºC and ground for elemental analysis.

2.3. Analysis of soil, raw materials, composts and plant tissues

Soil was analysed for pH (H_2O) (soil: solution, 1:2.5), CEC (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley-Black method), total N (Kjeldahl) and extractable P and K (Egner-Riehm method) (Van Reeuwijk, 2002).

The EC was determined in the composted materials from fresh samples, in a water:compost extract of 5:1 (v/v). All the other determinations were performed in dried tissue. TOC was determined by dry combustion, total N by kjeldahl, B and P by colorimetry, K by flame emission spectrometry and Ca, Mg, Cu, Fe, Zn and Mn by atomic absorption spectrophotometry (Walinga et al., 1989). NH_4^+ and NO_3^- concentrations in the composts were determined by UV-Vis spectrophotometry in a 2M KCl (3:50, compost:solution) extract.

2.4. Data analysis

Data was subject to analysis of variance in SPSS v. 25.0 program to check for significant differences between treatments (one-way ANOVA, according to the experimental design). When significant differences were found the means were separated by Tukey–Kramer HSD test (α = 0.05). A PCA was applied to the results of composting experiment to evaluate the differentiation of composts groups according to their physicochemical properties.

3. Results

3.1. Temperature during the composting process

The temperatures were high during the first five days of composting, followed by a gradual decrease to the levels observed outside the composters (Figure 5.1.1). Thereafter, the temperatures inside the composters changed in accordance with air temperature and on some dates during the autumn and winter they were lower inside than outside the composters.

The highest temperature (68.5 °C) was recorded on the first day in the HM1:1 mixture. Differences between composting mixtures were only evident in the first five days with the manure-based mixtures reaching the highest values. In some straw-based mixtures, the temperature did not reach 55 ºC.

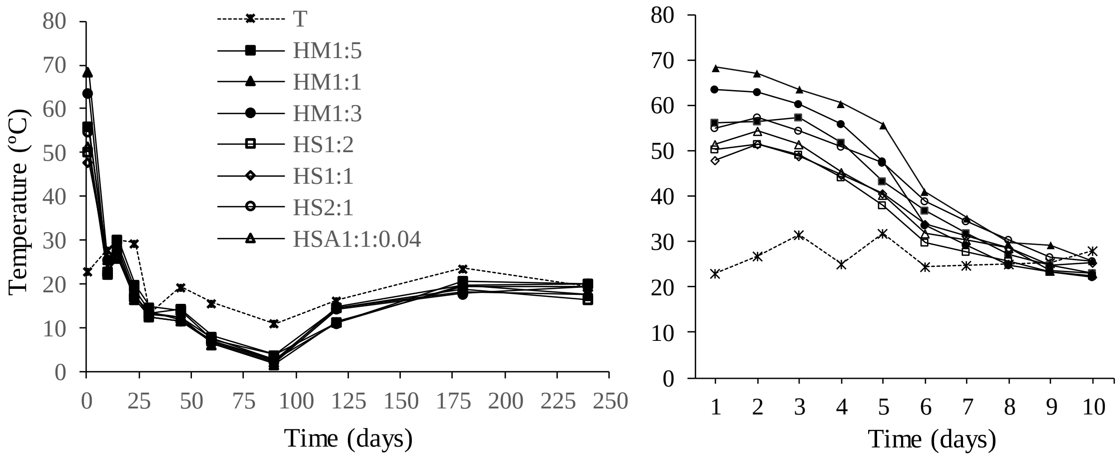

Figure 5.1.1. Temperatures (left) on days 1 and 10 and subsequent measurements, throughout the composting process, and (right) daily for the first 10 days for more detailed observation of the beginning of the process: HM, hop leaves+cow manure at the ratios 1:5, 1:3 and 1:1; HS, hop leaves+wheat straw at the ratios 1:2, 1:1 and 2:1; and HSA, HS+ash from hop stems at the ratio 1:1:0.04); T, air temperature.

3.2. Physical and chemical properties of composts

The concentration of nutrients in composted materials usually increased with time from the sixth to the ninth month of composting (Table 5.1.3). In general, the manured-based composts showed significantly higher levels of P, K, Mg, Fe, Mn, Cu and Zn than the straw-based composts. Regarding N, Ca and B, high nutrient concentrations were also found in the treatment of the highest proportion of leaves (HS2:1).

Comparing the treatments containing straw, the HS2:1 treatment presented the highest concentration of the majority of nutrients due to the presence of a higher proportion of leaves. In an overall comparison of composts, the treatment containing ash (HSA1:1:0.04) showed relatively low levels of N and K and high levels of Ca, but the results of this treatment were not the highest or the lowest for any of the nutrients.

Table 5.1.3. Nutrient concentration in composted materials after six and nine months of composting (HM, hop leaves+cow manure at the ratios 1:5, 1:3 and 1:1; HS, hop leaves+wheat straw at the ratios 1:2, 1:1 and 2:1; and HSA, HS+ash from hop stems at the ratios 1:1:0.04).

Compost	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
	(g/kg)					(mg/kg)				
sixth month										
HM 1:5	18.3 bc	4.0 a	23.6 a	12.1 a	23.5 a	14750 a	426.2 a	57.2 a	115.5 a	29.2 b
HM 1:3	18.1 bc	4.0 a	25.5 a	13.0 a	19.4 a	12078 ab	416.6 a	54.8 ab	110.4 a	33.4 ab
HM 1:1	23.6 a	3.9 a	25.6 a	24.3 a	15.2 a	8959 b	453.3 a	48.4 b	91.4 a	47.9 a
HS 1:2	11.6 d	1.4 b	12.2 c	8.0 a	2.2 b	191 c	130.2 c	8.49 c	69.7 a	25.1 b
HS 1:1	12.5 d	1.6 b	15.1 bc	8.8 a	2.7 b	194 c	137.3 c	9.01 c	102.4 a	27.2 b
HS 2:1	19.5 ab	1.4 b	17.3 b	11.7 a	3.3 b	270 c	214.1 bc	12.0 c	147.3 a	42.1 ab
HSA 1:1:0.04	14.2 cd	1.8 b	15.4 bc	11.8 a	3.0 b	1493 c	280.2 b	9.85 c	70.9 a	35.4 ab
Prob. > F	<0.0001	<0.0001	<0.0001	0.1075	<0.0001	<0.0001	<0.0001	<0.0001	0.8498	0.0068
Stand. error	0.93	0.27	1.19	1.60	2.02	1328	29.37	4.89	14.31	2.08
Ninth month										
HM 1:5	20.9 d	4.5 a	26.3 ab	9.7 cd	17.0 ab	12507 ab	495.6 ab	52.0 a	104.5 a	31.2 e
HM 1:3	20.5 d	4.3 ab	28.9 a	10.5 cd	18.3 a	13776 a	485.1 ab	47.3 b	90.7 ab	36.4 d
HM 1:1	25.5 b	4.1 b	28.0 a	12.8 bc	15.6 b	11226 b	544.9 a	49.3 b	81.1 b	46.5 c
HS 1:2	17.5 e	2.1 c	21.0 c	8.5 d	3.8 d	330 d	229.3 d	7.4 d	23.3 d	40.5 d
HS 1:1	21.9 c	1.8 d	21.9 c	7.9 d	3.0 d	392 d	300.3 d	7.5 d	28.4 d	56.4 b
HS 2:1	29.2 a	1.8 d	22.7 bc	16.5 a	5.1 c	521 d	412.7 c	8.9 d	35.7 cd	82.3a
HSA 1:1:0.04	16.8 f	2.1 c	20.1 c	14.2 ab	4.6 cd	3205 c	460.1 bc	14.0c	48.2 c	52.9 b
Prob. > F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Stand. error	0.90	0.26	0.77	0.70	1.44	1290	24.05	4.48	6.84	3.53

Means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05).

TOC and C/N ratios appeared divided into two groups, one of the mixtures containing manure and the other of the mixtures containing straw (Figure 5.1.2). The groups of manure-based composts showed significantly lower TOC and C/N ratios than the straw-based composts. Of the manure-containing composts, the one receiving the higher proportion of leaves (HM1:1) showed significantly higher TOC and a lower C/N ratio. Between the straw-containing composts, the one receiving the higher proportion of straw (HS1:2) showed the higher average TOC. Ash seems to have contributed to reducing TOC, when comparing treatments HS1:1 with HSA1:1:0.04. Of the treatments receiving straw, the one receiving the higher proportion of leaves (HS2:1) displayed the lowest C/N ratio. During the composting process, by comparing the results of months six and nine, C/N ratios decreased more markedly than TOC.

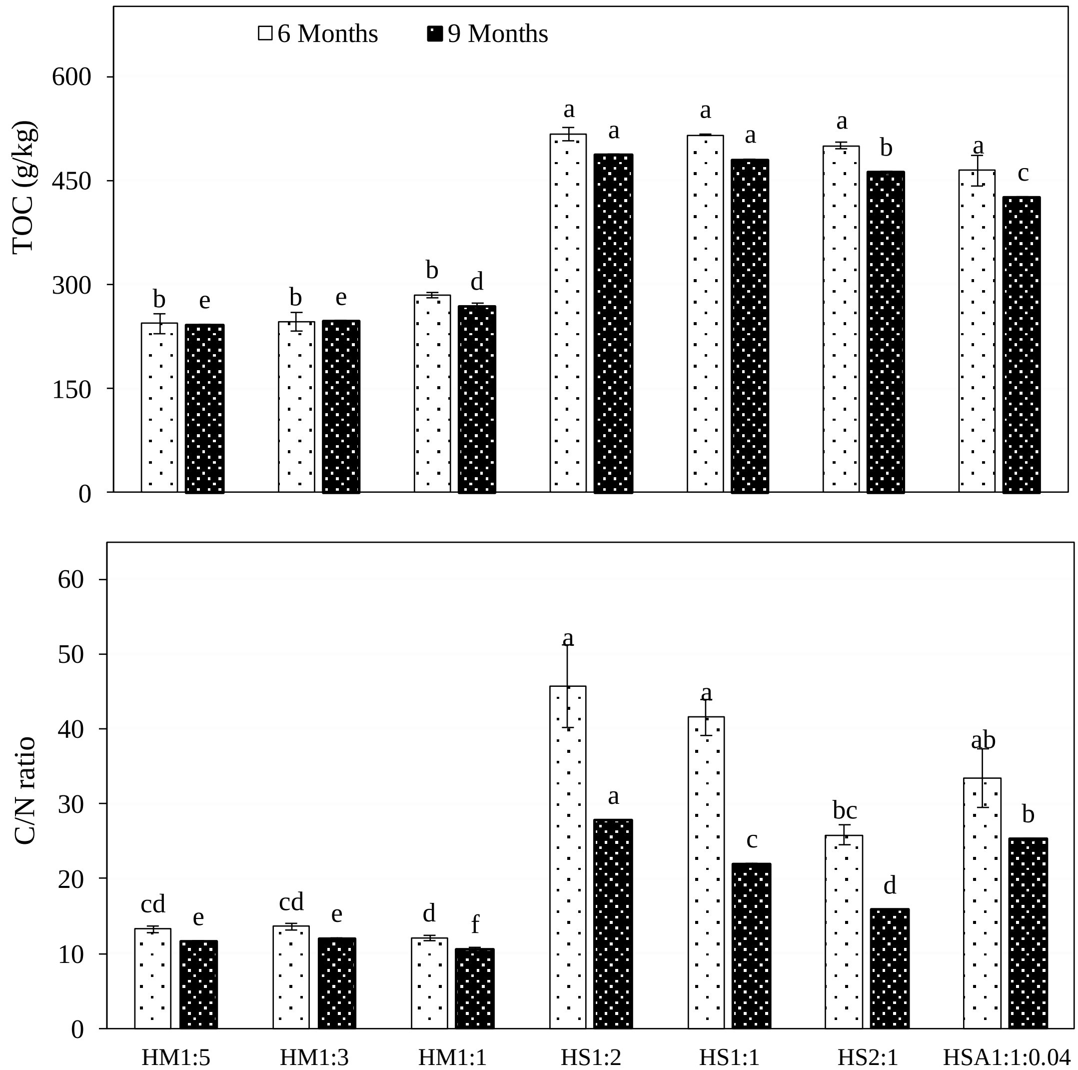

Figure 5.1.2. Total organic carbon (TOC) and carbon (C)/nitrogen (N) ratio after six and nine months (the end) of composting (HM, hop leaves+cow manure at the ratios 1:5, 1:3 and 1:1; HS, hop leaves+wheat straw at the ratios 1:2, 1:1 and 2:1; and HSA, HS+ash from hop stems at the ratios 1:1:0.04). Within each period, means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05). Error bars are the standard errors.

During the composting process, from the sixth to the ninth month, EC and NO_3^- values generally increased while NH_4^+ levels and NH_4^+/NO_3^- ratios decreased (Table 5.1.4). The final composts prepared with straw presented EC values significantly and noticeably higher than the composts which included manure. The final composts containing manure presented a trend to higher NO_3^- levels in comparison to those containing straw, although the values also depended on the proportion of leaves in the mixtures. In contrast, the straw-based composts showed increased values of NH_4^+ and higher NH_4^+/ NO_3^- ratios.

Table 5.1.4. Electrical conductivity (EC), nitrate (NO_3^-) and ammonium (NH_4^+) levels and NH_4^+/ NO_3^- ratio in in composted materials after six and nine months of composting (HM, hop leaves+cow manure at the ratios 1:5, 1:3 and 1:1; HS, hop leaves+wheat straw at the ratios 1:2, 1:1 and 2:1; and HSA, HS+ash from hop stems at the ratios 1:1:0.04).

Compost	EC (mS/cm)	NO_3^- (mg/kg)	NH_4^+ (mg/kg)	NH_4^+/ NO_3^-
		Sixth month		
HM 1:5	0.0027 c	7031 b	38.0 c	0.0055 c
HM 1:3	0.0031 c	8111 ab	33.4 c	0.0042 c
HM 1:1	0.0031 c	9791 a	41.8 c	0.0043 c
HS 1:2	541 b	7763 ab	80.2 b	0.0103 b
HS 1:1	605 b	6726 b	74.0 b	0.0111 b
HS 2:1	910 a	7075 b	94.7 a	0.0134 a
HSA 1:1:0.04	708 ab	7188 b	75.1 b	0.0105 b
Prob. > F	<0.0001	0.0031	<0.0001	<0.0001
Stand. error	81.42	256.62	5.07	0.0008
		Ninth month		
HM 1:5	0.0038 e	11014 b	25.2 b	0.0023 c
HM 1:3	0.0043 e	11251 b	26.2 b	0.0023 c
HM 1:1	0.0044 e	14310 a	29.2 b	0.0020 c
HS 1:2	909 d	7803 c	40.2 a	0.0052 a
HS 1:1	1285 b	10401 b	43.0 a	0.0041 b
HS 2:1	1776 a	9935 b	40.3 a	0.0040 b
HSA 1:1:0.04	966 c	6316 c	38.7 a	0.0061 a
Prob. > F	<0.0001	<0.0001	<0.0001	<0.0001
Stand. error	148.42	545.53	1.74	0.0003

Means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05).

3.3. Principal component analysis

The results of PCA on compost physicochemical properties indicated a clear differentiation into different groups of composts (Figure 5.1.3). The first two principal components (PC) explained 86.7% of total variance. The PC1 explained most of the variance (69.3%) and was positively affected by the levels of Cu, Fe, Mg, P, Zn, K and Mn and negatively affected by TOC, EC, NH_4^+ and C/N ratio. The manure-based composts (HM) showed higher scores in PC1 in contrast to the straw-based composts (HS), mainly due to the higher levels of Mn, K, Mg, Cu, Fe, Zn, and P but also due to the lower levels of EC, NH_4^+, TOC and C/N ratio. Within the HM group, HM1:1 diverged from the others due to the higher levels of NO_3^-. Within the HS group, HS2:1 was the most differentiated of all, displaying the higher scores in PC2, and was associated with higher levels of B, Ca and N.

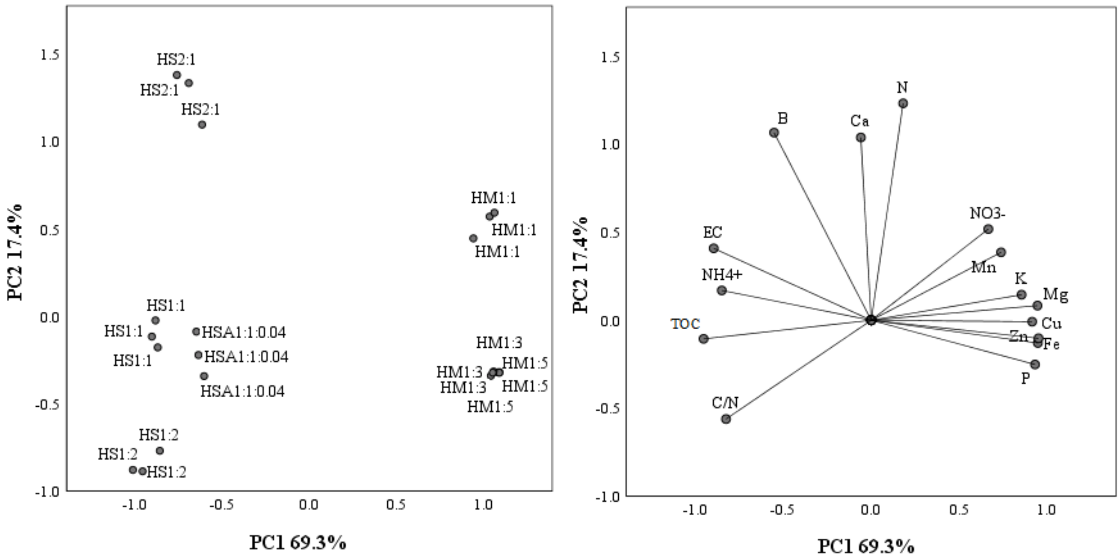

Figure 5.1.3. PCA scores plot (left) of the final composts (HM, hop leaves+cow manure at the ratios 1:5, 1:3 and 1:1; HS, hop leaves+wheat straw at the ratios 1:2, 1:1 and 2:1; and HSA, HS+ash from hop stems at the ratios 1:1:0.04) and PCA loadings plot (right) of physicochemical properties of composts.

3.4. Lettuce dry matter yield

A comparison of DMY between the different treatments gave rise to a completely different pattern between the two lettuce cycles, with marked differences between treatments in the first growing cycle and more stable values in the second cycle (Figure 5.1.4). In the first year, the higher average DMY was recorded in the HS2:1D2 treatment (8.71 g/plant), which was significantly different to the DMY recorded in the control and in several other treatments. In the first growing cycle, the manure-based composts gave, in general, significantly higher lettuce DMY's than the straw-based composts. The lower value was found in treatment HS1:2D2 (0.48 g/plant). Treatment HS1:2D1 also gave a significantly lower value (1.21 g/plant) than the untreated control (3.67 g/plant). The higher the proportion of straw in the mixtures, the lower the productive performance of the plant. The compost containing ash in the initial mixture (HSA1:1:0.04D1) did not perform better than the treatment with a similar proportion of leaves and straw without ash (HS1:1D1).

In the second year, the marked differences between the treatments containing manure or straw narrowed. Although significant differences were not usually found, the average values of lettuce DMY's were generally higher in the straw-based composts. It was also clear that the treatments producing less in the first growing cycle were those displaying the higher average DMY's in the second growing cycle. For instance, the treatment producing the lowest average value in the first growing cycle, HS1:2D2 (0.48

g/plant), gave the highest average value (11.8 g/plant) in the second growing cycle of lettuce.

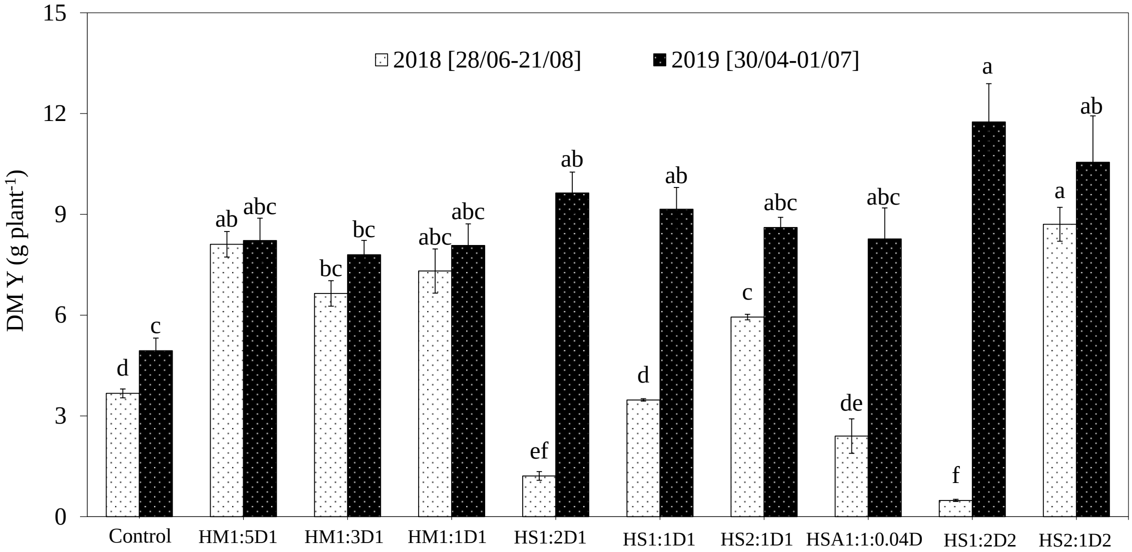

Figure 5.1.4. Lettuce dry matter yield (DMY) in 2018 and 2019 as a function of fertilizer treatments (HM, hop leaves+cow manure at the ratios 1:5, 1:3 and 1:1; HS, hop leaves+wheat straw at the ratios 1:2, 1:1 and 2:1; and HSA, HS+ash from hop stems at the ratios 1:1:0.04; D1 and D2, 20 and 40 kg dw ha^{-1}). Within each growing cycle of lettuce, means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05). Error bars are the standard errors.

3.5. Nutrient concentration in lettuce tissues

In the first growing cycle, the N concentrations in lettuce tissues showed a close relationship to lettuce productivity (Table 5.1.5). The HS1:2D2 treatment, which gave a minute productivity, showed a particularly low tissue N concentration (13.2 g/kg). Other treatments that led to very low lettuce yields, such as HS1:2D1, HSA1:1:0.04, HS1:1 and the control, showed tissue N levels below 26 g/kg, while all of the more productive treatments showed levels of N in the tissues higher than 28 g/kg. The concentrations of many other nutrients in lettuce tissues also varied significantly between treatments. However, no coherent relationship with lettuce yield was observed. The values probably varied according to the mineral composition of the mixtures, but they should not have had a relevant effect on lettuce productivity. In the 2019 growing cycle, the pattern of significant differences between treatments for some nutrients, including N, persisted, but it was not possible to establish a clear relationship between their concentrations in plant tissues and the performance of lettuce. For N, however, the concentration of the nutrient in plant tissues in the HS1:2D2 treatment increased from 13.2 g/kg in the first growing cycle to 24.6 g/kg in the second growing cycle which, in this case, was consistent with a great increase in lettuce DMY.

Table 5.1.5. Tissue nutrient concentration in the lettuces of the first and second growing cycles (HM, hop leaves+cow manure at the ratios 1:5, 1:3 and 1:1; HS, hop leaves+wheat straw at the ratios 1:2, 1:1 and 2:1; and HSA, HS+ash from hop stems at the ratios 1:1:0.04; D1 and D2, 20 and 40 kg dw ha^{-1}).

Treatment	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
	(g/kg)					(mg/kg)				
First growing cycle										
Control	24.7 bc	2.2 bc	101.4 a	7.1 bc	4.8 abc	1183 a	84.2 a	13.7 a	85.8 ab	43.1 ab
HM1:5D1	29.5 abc	2.6 b	93.4 ab	5.3 d	3.7 c	1013 a	82.1 a	18.2 a	82.4 ab	36.3 c
HM1:3D1	30.3 a	2.6 b	94.8 ab	5.1 d	3.6 c	772 a	84.5 a	14.7 a	146.5 ab	36.5 c
HM1:1D1	30.7 a	2.5 b	100.8 a	5.3 d	3.6 c	676 a	81.2 a	13.5 a	154.9 ab	36.6 c
HS1:2D1	24.8 bc	3.9 a	86.7 abc	8.0 ab	4.9 abc	1285 a	93.0 a	16.4 a	72.5 b	47.7 a
HS1:1D1	24.3 c	2.8 b	105.7 a	7.3 abc	4.3 bc	1369 a	96.6 a	16.4 a	57.0 b	47.5 a
HS2:1D1	28.9 abc	2.4 bc	91.5 abc	5.9 cd	4.1 bc	1297 a	102.6 a	20.5 a	172.9 a	43.3 b
HSA1:1:0.04D1	25.6 abc	2.0 bc	72.3 abc	5.2 d	4.0 bc	1639 a	100.2 a	22.2 a	125.9 ab	48.0 a
HS1:2D2	13.2 d	2.0 bc	53.3 c	8.8 a	6.3 a	1162 a	103.3 a	16.4 a	142 ab	nd
HS2:1D2	29.6 ab	1.6 c	59.4 bc	6.4 cd	5.2 ab	1251 a	96.5 a	13.8 a	91.7 ab	39.8 bc
Prob. > F	<0.0001	<0.0001	0.0004	<0.0001	<0.0001	0.4181	0.6624	0.1136	0.0026	<0.0001
Standard error	0.85	0.11	3.57	0.22	0.16	87.61	3.08	0.76	8.30	0.78
Second growing cycle										
Control	27.1 cd	2.8 ab	57.3 b	6.1 bc	5.8 a	1846 a	118.3 a	22.3 a	155.8 a	38.9 a
HM1:5D1	23.6 d	3.0 a	102.8 ab	7.8 a	4.8 b	753 b	67.2 b	11.8 b	91.7 b	40.9 a
HM1:3D1	26.9 cd	2.0 b	98.5 ab	6.0 c	4.5 b	1157 ab	93.1 ab	15.8 ab	130.4 ab	40.5 a
HM1:1D1	27.5 bcd	2.2 ab	105.7 ab	7.4 ab	4.8 b	1111 ab	91.1 ab	15.0 b	94.4 b	41.5 a
HS1:2D1	26.1 cd	1.9 b	77.9 ab	5.9 c	4.7 b	1117 ab	85.3 b	15.6 ab	110.6 ab	43.0 a
HS1:1D1	29.8 bc	2.2 ab	101.5 ab	6.3 bc	4.9 ab	1034 ab	80.7 b	17.1 ab	114.2 ab	45.3 a
HS2:1D1	32.0 ab	2.7 ab	81.5 ab	6.1 bc	5.3 ab	1104 ab	86.9 b	18.6 ab	116.3 ab	42.6 a
HSA1:1:0.04D1	26.9 cd	2.6 ab	77.4 ab	6.5 abc	4.8 b	1133 ab	90.2 ab	17.0 ab	144.1 ab	45.4 a
HS1:2D2	24.6 d	2.7 ab	115.4 a	6.41 abc	4.5 b	963 ab	91.1 ab	14.9 b	96.1 b	42.8 a
HS2:1D2	35.0 a	2.41 ab	105.7 ab	7.2 abc	5.0 ab	1040 ab	96.7 ab	16.8 ab	98.2 b	39.6 a
Prob. > F	<0.0001	0.0106	0.0093	0.0003	0.0006	0.1106	0.0013	0.0045	0.0051	0.0329
Standard error	0.58	0.07	3.93	0.13	0.08	71.68	2.62	0.58	4.71	0.52

nd, not determined (insufficient sample).
Means followed by the same letter are not significantly different by Tukey HSD test (α = 0.05).

4. Discussion

Temperature in the first days of composting was high, as is convenient in a composting process (Azim et al., 2018). The highest temperatures were reached in the manure-based mixtures, in particular in HM1:1 (68.5 ºC). The manure had probably already started the decomposition process in the cowshed, and combined with fresh leaves, they formed an easily degradable substrate, providing conditions for the increase in temperature and rapid OM degradation (Diaz et al., 2011). In a composting process, it is important that temperatures can reach 55 °C to destroy pathogenic microorganisms, but they should not increase above 70 °C to avoid inhibiting the growth of beneficial microorganisms (Wong et al., 2017). In the straw-based mixtures, excluding HS2:1, temperatures did not reach 55 °C, indicating low microbial activity. The higher C/N ratio of these mixtures, coupled with a large particle size of straw (it was not chopped), are reasons usually given to explain low temperatures during composting and reduced rates of substrate decomposition (Bernal et al., 2009). The temperatures within the composters decreased after the first 10 days of composting to values close to outside air temperature. This rapid decrease in temperature of the mixtures being composted was probably due to the exhaustion of the easily degradable substrates but also to the poor thermal inertia of the composters whose volume was only ~ 0.5 m^3.

The nutrient concentrations in a compost, and particularly of N, greatly determine its quality (Bernal et al., 2009). The final composts prepared with manure presented significantly higher levels of the majority of nutrients (P, K, Mg, Fe, Mn, Cu and Zn) in comparison to the composts based on straw. This is because uncomposted manure has higher levels of those nutrients than straw. The N concentrations in the composts greatly depended on the proportion of leaves in the initial mixtures. Between manure-based and straw-based composts, the higher compost N concentrations were found in the former, if mixtures of the same rates of manure and straw were compared. Thus, the compost showing the higher N concentration was HS2:1, followed by HM1:1. In addition, the manure-based materials might have lost N during composting due to ammonia volatilization, whereas the materials with higher C content, such as straw, might have immobilized more N (Bernal et al., 2009; Lim et al., 2017; Wong et al., 2017). Thus, the C/N ratio depended on the initial mixtures, the values being higher in those containing straw. The higher proportion of leaves, however, reduced the C/N ratio of the mixtures. It is well known that the initial C/N ratio and N content of a mixture are the main drivers of the rate and the level of decomposition, and values of a C/N ratio between 15 and 35 are considered the most adequate for a fast and effective decomposition (Azim et al., 2018; Bernal et al., 2009; Lambers et al., 2008). Organic C represents the source of energy for heterotrophic microorganisms and N is the raw material for protein synthesis (Weil and Brady, 2017). Hence, the mixtures HS2:1 and HM1:1 had an initial C/N ratio in the optimal range for composting and presented a final N concentration higher than 18 g/kg (Raviv, 2005), which should prevent net immobilization when the compost is applied to the soil. PCA results also highlighted the higher concentrations of several nutrients (P, K, Mg, Fe, Mn, Cu and Zn) in the manure-based composts and the higher concentrations of N, B and Ca in the compost prepared with a double rate of leaves (HS2:1).

The addition of ash (HSA1:1:0.04) significantly reduced the N concentration in the final compost but increased the concentrations of several other nutrients in comparison to the same mixture without ash (HSA1:1). The increase in minerals in the compost containing ash was a direct effect of their content in the ash as reported in previous studies (Bougnom et al., 2009; Juarez et al., 2015). The decrease in N concentration was probably due to the increase in the pH of the mixture, which favoured ammonia volatilization (Azim et al., 2018).

The quality of a compost depends also on its biological stability, which determines its behaviour in the soil. TOC, the C/N ratio, inorganic-N and EC are important indicators of the stability of compost (Azim et al., 2018; Bernal et al., 2009). TOC and the C/N ratio decreased during composting from the sixth to the ninth month as expected. The group of HM composts showed low C/N ratios particularly in the ninth month (10.5 to 12.0). The C/N ratios in the straw-based composts were higher and more dependent on the proportion of leaves in the mixtures, varying between 15.8 (HS2:1) and 27.8 (HS1:2). A well-matured compost should present a C/N ratio of below 20, and preferably below 12, and values above 25 indicate poor maturity (Antil et al., 2014; Bernal et al., 2009). The high C/N ratio of some straw-based mixtures may have slowed the process of decomposition, and nine months would not have been enough for a complete biological stabilisation. In addition, if straw had been chopped, the attack points for the microorganisms would have increased, which usually accelerates the decomposition process (Adhikari et al., 2009; Calabi-Floody et al., 2019). Following mineralization, nitrification is an important step in the composting process, reducing the levels of NH_4^+ that can be toxic to plants, mainly during the germination phase (Cáceres et al., 2018). The ratio NH_4^+/NO_3^- decreased during composting and was significantly lower in HM composts, though below 0.16 for all composts, which is a safe value for agricultural use (Bernal et al., 2009). Major differences were found in EC values between the HM and HS groups of composts. EC tends to increase during composting due to the increased concentration of soluble ions resulting from OM mineralization (Alavi et al., 2017; Petric et al., 2009). Overall, straw-based mixtures presented lower initial concentrations of most of the minerals determined. Thus, their higher EC values were probably due to the presence of non-determined ions, such as Na^+, chloride (Cl^-), carbonate (CO_3^{2-}) or SO_4^{2+}, which are usually responsible for an increase of EC in composted materials (Gondek et al., 2020). The PCA results also highlighted the higher maturity of the HM in comparison to the HS composts, the most stable being HM1:1. The higher levels of EC, NH_4^+, TOC and the C/N ratio, and the lower levels of NO_3^-, clearly differentiate the HM group from the HS group of composts. The addition of ash led to higher NH_4^+/ NO_3^- and C/N ratios and lower EC values in the final compost in comparison to the compost resulting from the same mixture without ash. Juarez et al. (2015) reported similar results for C/N ratio and EC values with wood-ash amended compost.

In the first growing cycle of lettuce, the manure-based composts gave higher average DMY than the straw-based composts, with the exception of the compost HS2:1D2, applied at a double rate. Within the group of straw-based composts, those treatments prepared with a higher proportion of straw, and particularly those applied at a double rate (HS1:2D2), gave the lowest DMY's of lettuce. These low producing composts showed lower maturity after nine months of composting, with higher C/N ratios and higher NH_4^+ and EC levels than the better producing composts. High levels of NH_4^+ and EC could have a detrimental effect on crop productivity (Weil and Brady, 2017). However, all the straw-based composts did not show very dissimilar values of NH_4^+ and EC, although productivity was significantly different, which may mean that those variables did not have a relevant effect on the DMY of lettuce. HS2:1, for instance, was the compost with higher EC and also that showing the higher DMY's of lettuce among the straw-based composts. Gondek et al. (2020) also showed that composts with EC values within the range found in this study have enhanced plant growth and yield.

The less productive treatments in the first growing cycle of lettuce were those presenting higher C/N ratios, and particularly the one applied at a double rate (HS1:2D2) as above mentioned. These immature composts, as indicated by the high C/N ratios, can support intense microbial activity when applied to the soil, in the continuing decomposition process, which can have phytotoxic effects by releasing volatile organic compounds and competing for O_2 with plants (Cesaro et al., 2015; Chefetz et al., 1996). However, the treatments associated with low lettuce DMY also showed low tissue N concentration. The HS2:1D2 treatment gave particularly low lettuce DMY (0.48 g/plant) and showed particularly low tissue N concentrations (13.2 g/kg), whereas the highest productive treatment (HS2:1D1) gave an 18-fold higher DMY of lettuce (8.71 g/plant) and a much higher tissue N concentration (29.6 g/kg). Thus, it seems that the real problem of the reduced growth of lettuce was N deprivation, caused by biological immobilization, which is common when organic amendments with high C/N ratios are applied to the soil (Weil and Brady, 2017).

The treatment displaying the lowest lettuce DMY in the first growing cycle (HS1:2D2) gave the highest value of all treatments in the second cycle. This means that after a period of N immobilization (first growing cycle) a period of net mineralization occurred (second growing cycle), benefiting the plants of the treatments with higher levels of total N in the soil. This is in contrast to the other treatments, in particular the manure-based ones, in which part of the N was taken up by the lettuce during the first growing cycle. This was clear from the result of the control, which showed the lowest

DMY of lettuce of all the treatments in the second growing cycle. N is a key component of proteins, nucleic acids and several enzymes and phytohormones (Hawkesford et al., 2012). Thus, in the present study, and in many others using organic amendments, increased plant growth is usually associated with increased N uptake (Erdal and Ekinci, 2020; Solaiman et al., 2019; Woldetsadik et al., 2017; Zandvakili et al., 2019;).

5. Conclusions

The evaluation of the physicochemical properties of the composted materials, and the results of the biological assay with lettuce, provided interesting guidelines for farmers that are summarized below. The mixture of hop leaves with cow manure produced a stable compost after nine months of composting that can be used in horticultural crops, irrespective of the proportion of raw materials, probably due to the low and similar C/N ratios of both materials. When hop leaves were mixed with wheat straw, in proportions of less than 2:1, the compost did not mature properly in nine months, which means that the farmer is unable to use the compost in horticultural crops in the following growing season. The mixtures containing straw with less than a double rate of leaves gave high total organic C and C/N ratios in the composts, causing N deprivation due to biological N immobilization, and greatly reducing lettuce DMY. Thus, to reduce composting time and increase the quality of the compost, the ratio leaves/straw should be as high as possible, at least 2:1. Alternatively, the poorly-matured compost must be applied far in advance of crop planting so that complementary biological processes can take place in the soil, as recorded in the second cycle of lettuce. Ash from the stems did not add any benefit to the composting process. It only increased the content of some nutrients contained within it, so it should be applied directly to agricultural soils, thereby avoiding the work of adding it to composting mixtures. Thus, as stated in the objectives for this study, farmers who follow these guidelines can take better advantage of the use of these important organic resources to fertilize their crops.

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Chapter 6:

Agronomic and chemical evaluation of hop aroma cultivars grown under Mediterranean conditions

Briefing note

The main purpose of this chapter was to evaluate the agronomic performance and chemical profile of hop aroma cultivars grown under the Mediterranean conditions of NE of Portugal.

Chapter 6.1. describes the results of a field experiment with the newly introduced cultivars (Columbus, Cascade and Comet) compared to the well-established Nugget cultivar.

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6.1. Agronomic and chemical evaluation of hop cultivars grown under Mediterranean conditions

Abstract

Aim of study: Evaluation of the agronomic performance and chemical profile of four hop cultivars grown under Mediterranean conditions.

Area of study: The study was undertaken in Bragança, north-eastern Portugal.

Material and methods: The newly introduced cultivars (Columbus, Cascade and Comet) were compared with the well-stablished Nugget. The field experiment was carried out between 2017 and 2019. Dry matter (DM) yield (plant and cones), tissue elemental composition and bitter acid and nitrate (NO_3^-) concentrations in the cones were assessed.

Main results: Comet was the most productive cultivar with the highest total DM yield (1,624 to 1,634 g plant^{-1}), cone yield (572 to 633 g plant^{-1}), and dry weight of individual cones (0.28 to 0.79 g cone^{-1}). Cascade showed the lowest average total DM yield (723 to 1,045 g plant^{-1}). The year affected the average values of DM yield and the concentration of bitter acids in the cones, with Cascade showing the highest sensitivity between cultivars. The concentrations of α and β-acids in the cones were within or close to the normal ranges internationally accepted for all cultivars. Columbus exhibited the highest levels of α-acids, ranging between 12.04 % and 12.23%, followed by Nugget (10.17–11.90%), Comet (9.32–10.69%) and Cascade (4.46–8.72%). The nutrient accumulation criteria in cone and leaf tissues seem to be a differentiating factor between cultivars with influence on bitter acid biosynthesis and biomass production.

Research highlights: All cultivars showed notable performance in terms of DM yield and bitter acid concentration in the cones when compared to international standards.

Keywords: *Humulus lupulus*; aroma; bitter acids; cone yield; cone quality.

1. Introduction

The cones of hop (*Humulus lupulus* L.) female plants are a primary ingredient in beer production and, although other substitutes can be used, the supremacy of hops has always prevailed. It is in the lupulin glands of the cones of female plants that a yellow resinous powder (lupulin) is synthetized. It contains the most valued compounds for brewing purposes such as the resins and aromatic compounds (Almaguer et al., 2014). Hop soft resins include the so-called bitter acids, the α-acids (cohumulone, humulone, adhumulone), which are the most valued constituents of the hop resins, and the β-acids (colupulone, lupulone, adlupulone) (Almaguer et al., 2014; De Keukeleire, 2000). The thermal isomerization of α-acids accounts for most of the bitterness of beer (Ting and Ryder, 2017), though β-acids can also make some contribution (Schönberger and Kostelecky, 2011).

Hop oils are considered to be the main source of beer aroma, consisting of three groups of compounds: hydrocarbons, oxygenated compounds, and sulphur-containing compounds (Schönberger and Kostelecky, 2011). Most of the hop aroma volatiles are lost when hops are added to the beer at the beginning of boiling, though derived compounds such as oxygenated terpenoids and oxygenated sesquiterpenes can impart aromas described as 'floral' or 'spicy' (Lafontaine and Shellhammer, 2019). To produce beers with intense hop aromas, brewers add aroma varieties at the end of boiling (late hopping) and/or to cold beer (dry hopping) and, in this case, the concentrations of hop oil volatiles and aroma precursors becomes of great relevance due to the impact on final beer aroma and flavour (Lafontaine and Shellhammer, 2019; Rettberg et al., 2018). The volatile composition of hop essential oils can present great variability between different hop cultivars (Inui et al., 2013; Kishimoto et al., 2006). The concentrations in cones of essential oils and bitter acids are commonly used to differentiate between cultivars (Ocvirk et al., 2016; Shellie et al., 2009; Štěrba et al., 2015).

Hop cultivars can be classified conventionally as 'bittering' or 'aroma' based on their chemical composition, though a more recent classification of 'flavour' hops is being used for cultivars that suit a dual purpose of imparting both aroma and bitterness (Almaguer et al., 2014). The breeding efforts to improve hop cultivars have allowed the development of a great variety of cultivars with specific traits, such as higher yielding, more resistant to diseases, and with increased bitterness or specific aroma, to meet the demands of the world market (Hieronymus, 2012; Seigner et al., 2009).

The increasing popularity of craft beer has changed the market demand from bittering cultivars, destined for a few macrobrewers, to aroma cultivars, sold in small quantities to a larger number of brewers. Although the craft beer revolution is a global phenomenon (Garavaglia and Swinnen, 2017), it has experienced a huge increase in USA of 273% in the last decade (Teghtmeyer, 2018). Craft brewers are more interested in cultivars with a strong aroma, different flavours and fewer α-acids (Hieronymus, 2012). The American Cascade cultivar is one of the most popular aroma hop cultivars while Nugget is an important bittering cultivar (Almaguer et al., 2014).

The hop crop has specific requirements for optimal growth, which include long day lengths to flowering and winter temperatures below 4.4 ºC. Thus, the cultivation areas considered to be adequate for hop growth should be at latitudes between 35 and 50 degrees (Sirrine et al., 2010). In Europe, hops are mostly produced in the north, because of the favourable growing conditions. Germany is the main European country and a major world producer (32,527 t in 2018), while in the Mediterranean, Spain is the country with the largest production (915 t in 2018) (FAOSAT, 2020). Italy is beginning hop cultivation, and the Cascade cultivar has been one of the most studied for its adaptability to Mediterranean growing conditions (Forteschi et al., 2019; Mozzon et al., 2020; Rodolfi et al., 2019). However, there is scarce information available on hop growing under Mediterranean conditions.

In Portugal, the commercial production of hops began in the early sixties in the north of the country, with the Brewer´s Gold cultivar, and quickly spread to other areas with the production reaching sufficient levels to satisfy national demand and even for export. However, the downturn in production, which followed a promising start, was caused by years of reduced productivity and other constraints (Rodrigues et al., 2015). At present, only a restricted number of producers, located in the Bragança region, maintain the crop, and all of them are growing the hop bitter cultivar Nugget. This seems to be well adapted to the drier climate of the NE region, though some fields are currently showing a heterogeneous growth (Afonso et al., 2020). Recently, the increasing popularity of craft beer in Portugal (Euromonitor, 2019) has awakened the interest in hop aroma cultivars, motivating current producers to invest in these cultivars and opening up new opportunities for growers.

The present research was carried out on a hop farm in Pinela, in the district of Bragança, located in the NE of the country, where hop aroma cultivars have been introduced in recent years. The trial includes the well-established bittering Nugget cultivar and the recently introduced Columbus, Cascade and Comet aroma cultivars, all with North American germplasm provenance according to Patzak and Henychová (2018). It is recognized by the local hop farmers that American cultivars are more suited to the region because of its similar drier climate and these are therefore the farmers' preferred option. The present research purpose is to study the agronomic performance and chemical profile of hop aroma cultivars grown under the Mediterranean conditions of the region, in order to produce useful data for both current and future growers. The newly introduced aroma cultivars (Columbus, Cascade and Comet) were evaluated and compared with the Nugget cultivar for biomass production, tissue elemental composition and bitter acid and NO_3^- concentration in the cones. Additionally, cone attributes (concentration of nutrients, bitter acids and NO_3^-) and the concentration of nutrients in the leaves, were used for differentiation between cultivars, by performing a stepwise discriminant analysis.

2. Materials and methods

2.1. Site characterization

A three-year field trial was conducted from 2017 to 2019 on a hop farm located in Pinela (41°40'33.6"N 6°44'32.7"W, 850 m a.s.l.), Bragança, NE Portugal. The region benefits from a Mediterranean-type climate, influenced by the Atlantic regime, with an average annual air temperature of 12.7 ºC and annual precipitation of 772.8 mm (IPMA, 2020). Meteorological data recorded during the experimental period at the weather station of Sta Apolónia farm in Bragança is presented in Figure 6.1.S1 (suppl).

The soil of the experimental field is a loamy textured, eutric Cambisol. Other physicochemical properties determined from composite soil samples (three samples composed by mixing the soil from 15 random points), collected just before the trial started, are presented in Table 6.1.1.

Table 6.1.1. Selected soil properties (average ± standard deviation) from soil samples collected between rows at 0-20 cm, just before the beginning of the experiment.

Soil properties		Soil properties	
pH (H_2O)[a]	5.64±0.06	Extract. P (mg P_2O_5 kg^{-1})[d]	284.51±43.81
Organic C (g kg^{-1})[b]	21.39±4.76	Extract. K (mg K_2O kg^{-1})[d]	238.67±1.15
Exchan. Ca ($cmol_c$ kg^{-1})[c]	4.35±1.32	Extract. B (mg kg^{-1})[e]	0.76±0.09
Exchan. Mg ($cmol_c$ kg^{-1})[c]	0.59±0.17	Extract. Fe (mg kg^{-1})[f]	100.31±1.01
Exchan. K ($cmol_c$ kg^{-1})[c]	0.42±0.07	Extract. Mn (mg kg^{-1})[f]	73.62±15.04
Exchan. Na ($cmol_c$ kg^{-1})[c]	0.69±0.31	Extract. Zn (mg kg^{-1})[f]	6.06±1.50
Exchan. acidity ($cmol_c$ kg^{-1})[a]	0.53±0.06	Extract. Cu (mg kg^{-1})[f]	5.77±1.37

[a]Potentiometry; [b]Wet digestion (Walkley-Black); [c]Ammonium acetate, pH 7; [d]Ammonium lactate; [e]Hot water, azomethine-H; [f]Ammonium acetate and EDTA.

2.2. Field experiments

The field trial was arranged as a completely randomized design with four cultivars (three in 2017) and four replicates (four plants per treatment). In 2017, the experiment included the bitter cultivar Nugget and the aroma cultivars Columbus and Cascade. In the next two years (2018 and 2019) the aroma cultivar Comet was also included. All the cultivars were grown under similar agroecological conditions, on a standard 7 m high trellis system in a 'V' design. At planting, the rhizomes were placed at 3.0 m × 1.6 m between and within rows. Thereafter, a double tutor thread was placed in the position of each rhizome giving a planting density of 4,167 “plants” of three to four stems per hectare. The plots of Columbus, Cascade and Nugget were established in 2014 and the plots of Comet in 2015. Sample collection began in 2017 for the first three cultivars and 2018 for Comet, when all were three years old, the age from which a hop plant can display its full productive potential.

Irrigation was performed through a surface irrigation system consisting of flooding the space between rows. The annual fertilization plan included the application of a compound NPK fertilizer (7:14:14) early in the spring and two side dress N applications performed during the growing season, the first with ~250 kg ha^{-1} of nitromagnesium (27% N as NH_4NO_3 + 3.5% MgO + 3.5% CaO) and the second with ~450 kg ha^{-1} of $Ca(NO_3)_2$ (15.5% N as NO_3^- + 27% CaO). Additionally, the farmer applied 20 t ha^{-1} of farmyard manure late in the winter.

2.2. Hop tissue sampling

Hop tissue sampling for analysis was made at harvest (August 28th to 31st 2017; August 27th to 31st 2018; and August 29th to 31st 2019). The aboveground biomass was cut at ground level and separated into two samples of leaves (bottom and top half), stems and cones. The leaf samples included blade and petiole. Tissue samples were weighed fresh to obtain data on total dry matter (DM) yield. From each plant part, a subsample was taken and weighed again fresh. Thereafter, the subsamples were oven dried at 70 ºC and weighed dry for determination of DMY of the different plant parts. Additionally, subsamples of 20 dried cones from each replication were randomly selected for determination of dry mass of the individual cones. Then, the samples were ground and analysed for elemental composition.

2.3. Chemical analyses

The soil samples were oven dried at 40 °C and sieved in a mesh of 2 mm. Thereafter, the soil samples were analysed for pH in water (H_2O) (soil: solution, 1:2.5), organic C (wet digestion, Walkley-Black method), exchangeable complex (ammonium acetate, pH 7.0), extractable P and K (Ammonium lactate) and extractable B (hot water and azomethine-H method) (Van Reeuwijk (2002). The availability of other micronutrients (Cu, Fe, Zn, and Mn) in the soil was determined by atomic absorption spectrometry after extraction with ammonium acetate and EDTA, according to Lakanen & Erviö (1971).

Elemental tissue analyses were performed by Kjeldahl (N), colorimetry (B and P), flame emission spectrometry (K) and atomic absorption spectrophotometry (Ca, Mg, Cu, Fe, Zn and Mn) methods after nitric digestion of the samples (Temminghoff and Houba, 2004). The NO_3^- concentration in hop cones was determined according with Clescerl et al. (1998), by UV-Vis spectrophotometry in a water extract (dry cone:solution, 2.5:50). Bitter acids (α and β-acids) in hop cones were extracted with methanol and diethyl ether by HPLC, according to the Analytica EBC 7.7. method (EBC Analysis committee, 1998).

2.4. Data analysis

Data was firstly tested for normality and homogeneity of variance using the Shapiro-Wilk and Bartlett's test, respectively. Thereafter, data was subject to analysis of variance (one-way ANOVA). When significant differences were found ($p < 0.05$), the means were separated by Tukey HSD ($\alpha = 0.05$). A stepwise discriminant analysis was applied to understand if the cone attributes, namely the concentration of nutrients, bitter acids and NO_3^-, were different between hop cultivars. A similar analysis was also performed for nutrient concentrations in the bottom and top half leaves of the plants. The stepwise methods were used for this purpose and the de Wilks lambda method was chosen; the F value criteria were applied for the removal ($F < 2.71$) and inclusion ($F > 3.84$) of the variables in the discriminant functions. The cone attributes (concentration of nutrients, bitter acids and NO_3^-) and the concentrations of nutrients in the leaves were used as independent variables, with the cultivars (Nugget, Columbus, Cascade, and Comet) as dependent variables. Self-validation and cross-validation methods were used to test the accuracy of the model. The statistical analyses were performed with the SPSS v. 25.0 programme.

3. Results

3.1. Plant dry matter yield

The comparison of total DMY between cultivars provided a very consistent result over the years (Figure 6.1.S2 [suppl]). The aroma cultivar Comet gave the highest average values, followed in descending order by Nugget, Columbus and Cascade. The order of the last three was the same in 2017, when Comet was not included in the study. However, in 2018 significant differences between cultivars were not found. In the comparison of the three years, the lowest average values for total aboveground DMY were recorded in 2017 in the Cascade (723 g plant^{-1}) and the highest in 2018 in Comet (1,634 g plant^{-1}). DMY of cones ranged between 326 (Nugget) and 363 g plant^{-1} (Columbus) in 2017, 445 (Cascade) and 633 g plant^{-1} (Comet) in 2018 and 323 (Cascade) and 572 g plant^{-1} (Comet) in 2019. Differences in cone DMY were only significant in 2019, with Cascade showing significantly lower values than the other cultivars. Cascade and Columbus also registered significant lower average values for leaf and stem total DMY, in comparison to Nugget in 2017 and 2019 and to Comet in 2019.

In 2017, cone dry weight did not differ significantly between cultivars and the average values ranged from 0.26 (Nugget) to 0.30 g cone^{-1} (Columbus and Cascade) (Figure 6.1.1). However, in 2018 the differences between cultivars increased, with Comet registering significantly higher values than Cascade and Nugget (Figure 6.1.S2 [suppl]). The average values in 2018 ranged between 0.29 (Nugget) and 0.79 g cone^{-1} (Comet). In 2019, average values varied between 0.16 and 0.28 g cone^{-1}, with Cascade and Comet showing, respectively, significantly lower and higher values than the other cultivars.

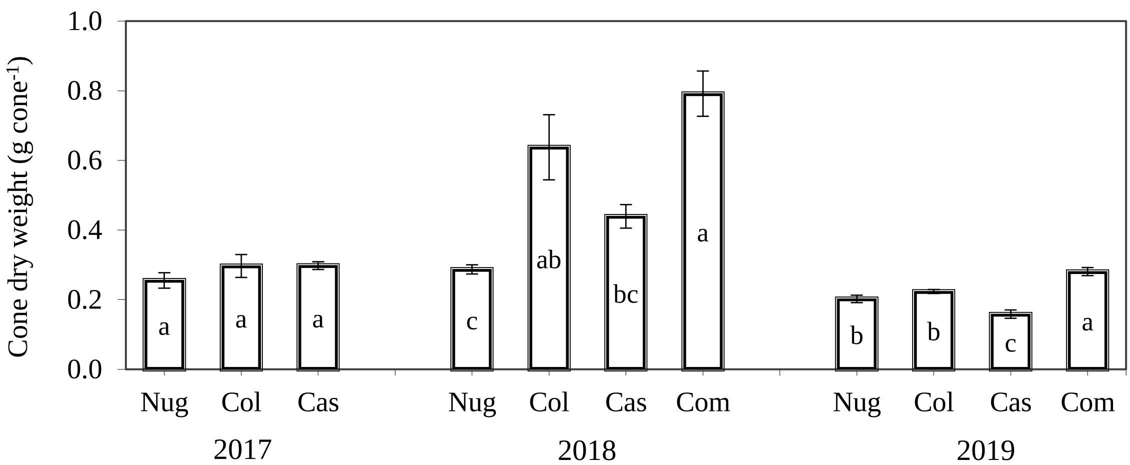

Figure 6.1.1. Dry weight of individual cones in 2017, 2018 and 2019, as a function of cultivar (Nug, Nugget; Col, Columbus; Cas, Cascade; and Com, Comet). Within each year, means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05). Error bars are the confidence intervals of the means (α = 0.05).

3.2. Tissue nutrient concentration

N concentrations in the bottom half leaves varied significantly between cultivars (Table 6.1.2). Cascade showed consistently higher leaf N concentration than the other cultivars. When present, Comet showed the lowest values. Regarding leaf P concentrations, Cascade showed consistently the lowest average values, in some years with significant differences to the other cultivars. The leaf K levels were consistently higher in Nugget, with significant differences for some of the other treatments. Cascade and Comet showed the lowest values. Regarding Ca and Mg, significant differences between cultivars were found for the majority of samplings, but a consistent pattern was not observed. Some cultivars displayed a high average value in a given year that was not maintained in the other years and vice-versa. The concentration of micronutrients in the leaves also did not show a consistent trend in spite of significant differences between cultivars being found for some samplings. However, Cascade showed consistently the lowest average values of B.

Cascade showed a tendency to display the highest average values of leaf N concentration when the results of the top half leaves were analysed (Table 6.1.2). Cascade also showed the lowest average P levels in the top half leaves. The top leaves also showed the highest K values in Nugget and the lowest values in the Cascade and Comet cultivars. In spite of significant differences between cultivars often being found, no consistent trends were observed in the levels of Ca, Mg, Fe, Mn, Cu and Zn from the top half leaves. The lowest average levels of B, however, were found in Cascade as observed in the bottom leaves. In general terms, N and P levels were found to be higher in the top leaves and many other nutrients such as Ca, Mg and B were found to be higher in the bottom leaves.

The concentration of the nutrients in the stems followed the most important trends observed in the leaves (Table 6.1.3). Cascade showed high and low levels of N and P respectively, and Nugget showed high levels of K. However, for the majority of the nutrients their levels in stems were lower than the values found in the leaves.

The patterns observed in the concentration of most of the nutrients in the leaves, when the different cultivars were compared, were not reflected in general terms in the cones (Table 6.1.3). However, Cascade showed consistently the lowest levels of P in the cones in comparison to the other cultivars as observed in the leaves. Several nutrients, such as N, Ca, Mg, Mn and B appeared to be less concentrated in the cones than in the leaves, whereas the concentration of P was higher in the cones than in the leaves. Several nutrients, and in particular K, showed a narrower range of variation between cultivars and years in the cones than in the leaves.

Table 6.1.2. Leaf nutrient concentration (average ± standard deviation) in August, at harvest, from the bottom and top half of the plants as a function of cultivar. For each year, means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05). For each variable the significance level *(Prob)* is provided.

Year	Treatment	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
		(g kg^{-1})					(mg kg^{-1})				
						bottom half					
2017	Nugget	24.8±0.58b	1.2±0.04ab	19.4±1.87a	26.9±6.05a	4.5±0.14b	319.9±59.5a	331.8±37.0a	11.8±1.85a	27.9±12.7a	87.4±5.84a
	Columbus	21.2±0.97b	1.3±0.15a	13.1±4.74ab	15.2±3.49b	4.6±1.09ab	312.0±105.8a	314.1±112.8a	12.3±2.56a	5.0±0.90b	79.4±8.32ab
	Cascade	40.9±6.93a	1.0±0.10b	10.7±2.32b	16.5±3.05b	6.8±1.59a	293.6±55.2a	198.7±37.1a	12.5±6.03a	6.4±1.16b	61.7±12.3b
2018	Nugget	31.2±0.65ab	1.2±0.04a	34.9±2.35a	14.9±1.45a	5.5±0.54b	262.7±10.1b	367.7±63.2ab	6.4±0.35b	16.4±0.58b	78.6±3.56a
	Columbus	23.3±1.69ab	1.2±0.06a	18.1±1.89b	17.0±3.68a	5.6±0.92b	238.3±47.4b	344.6±58.2ab	7.2±0.54ab	16.3±5.56b	58.0±5.33b
	Cascade	32.3±8.65a	0.7±0.07b	13.3±1.88c	16.4±0.52a	4.4±0.23b	384.2±66.0a	259.0±17.6b	7.0±1.03ab	7.1±1.48c	42.0±9.45c
	Comet	21.9±2.43b	1.1±0.07a	15.3±1.11bc	29.2±15.40a	9.8±1.45a	382.4±39.7a	410.1±65.3a	8.1±0.54a	29.1±4.10a	72.7±5.44a
2019	Nugget	27.9±0.84b	1.2±0.05a	17.0±3.21a	14.9±1.69a	6.2±0.99a	320.3±37.3a	387.2±20.4a	7.3±0.46a	22.5±4.33a	71.4±6.57a
	Columbus	28.0±1.22b	1.1±0.19a	12.3±1.72b	18.5±2.57a	5.2±1.18a	160.9±23.1c	396.5±72.6a	5.3±0.75b	16.4±4.83ab	42.3±1.40b
	Cascade	44.0±3.63a	1.0±0.08a	12.8±1.53ab	16.1±2.00a	5.5±0.21a	218.7±22.9b	380.1±97.2a	5.8±0.68b	9.7±1.68b	34.8±4.61b
	Comet	21.5±2.60c	1.1±0.09a	11.9±1.12b	10.0±1.26b	6.0±0.77a	168.1±4.33bc	342.6±37.3a	6.4±0.41ab	18.2±2.26a	41.6±2.89b
Prob (2017)		0.0002	0.0132	0.0112	0.0083	0.0306	0.8854	0.0557	0.9743	0.0028	0.0094
Prob (2018)		0.0130	<0.0001	<0.0001	0.0880	<0.0001	0.0007	0.0137	0.0229	<0.0001	<0.0001
Prob (2019)		<0.0001	0.0805	0.0142	0.0004	0.4317	<0.0001	0.6625	0.0023	0.0021	<0.0001
						top half					
2017	Nugget	29.0±0.86b	1.3±0.06a	16.4±1.51a	19.1±8.68a	2.8±0.76b	162.2±26.9a	231.2±47.9a	8.9±0.76a	20.0±11.9a	58.7±5.55a
	Columbus	26.4±1.69b	1.4±0.14a	12.8±3.96a	11.1±2.81a	3.0±0.30b	197.1±31.9a	220.4±72.3a	9.4±2.89a	4.7±0.83b	58.3±7.87a
	Cascade	47.4±3.99a	0.9±0.10b	11.3±3.21a	15.5±3.35a	6.7±2.30a	181.0±57.7a	164.5±17.0a	10.8±3.36a	5.5±0.59b	56.6±7.18a
2018	Nugget	34.1±1.53a	1.5±0.03a	21.2±4.33a	12.5±1.81ab	3.1±0.61b	225.9±16.5b	289.1±94.3a	7.0±0.45ab	12.7±1.75a	63.7±8.82a
	Columbus	26.0±1.88a	1.3±0.11ab	16.3±2.33ab	11.6±1.23b	3.1±0.57b	257.8±11.2ab	232.3±40.8a	6.5±0.43bc	10.5±1.32a	39.2±5.74b
	Cascade	36.8±10.97a	0.7±0.05c	11.4±1.76b	13.1±2.50ab	3.4±0.59b	343.7±38.7a	198.9±39.3a	6.2±0.12c	6.2±0.52b	35.0±10.7b
	Comet	26.4±1.97a	1.2±0.15b	10.4±2.14b	15.5±1.21a	6.5±0.72a	324.3±72.7a	309.1±39.4a	7.7±0.34a	12.7±3.16a	49.0±3.44ab
2019	Nugget	32.9±1.69b	1.4±0.24a	16.1±2.05a	11.0±1.82a	3.6±0.36a	244.4±29.5a	328.3±85.4a	6.9±0.93a	20.2±3.99a	65.0±9.95a
	Columbus	31.4±0.92b	1.1±0.20ab	12.0±3.29a	14.1±5.60a	4.0±1.38a	151.8±9.8b	302.0±102.7a	4.3±0.98c	10.1±3.04b	36.5±6.81b
	Cascade	47.3±5.51a	0.9±0.12b	11.0±1.94a	12.4±2.41a	4.5±1.54a	179.8±21.5b	276.7±67.9a	5.0±0.80bc	8.5±1.00b	27.3±5.56b
	Comet	23.4±2.41c	1.1±0.07b	10.5±3.71a	8.2±1.40a	5.2±1.56a	167.1±11.8b	250.1±30.4a	6.4±0.47ab	10.6±1.79b	30.4±4.02b
Prob (2017)		<0.0001	0.0003	0.1104	0.1807	0.0054	0.5121	0.1952	0.5766	0.0200	0.9020
Prob (2018)		0.0441	<0.0001	0.0005	0.0480	<0.0001	0.0061	0.0717	0.0004	0.0014	0.0009
Prob (2019)		<0.0001	0.0058	0.0657	0.1278	0.4030	0.0001	0.5326	0.0026	0.0002	<0.0001

Table 6.1.3. Stem and cone nutrient concentration (average ± standard deviation) in August, at harvest, as a function of cultivar. For each year, means followed by the same letter are not statistically different by Tukey HSD test (α = 0.05). For each variable the significance level *(Prob)* is provided.

Year	Treatment	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
		(g kg^{-1})					(mg kg^{-1})				
							Stem				
2017	Nugget	7.4±0.20b	1.0±0.07a	10.8±2.46a	8.4±1.27a	1.3±0.27a	26.9±6.11a	107.0±21.2a	3.9±0.87c	7.1±0.98a	12.8±0.64a
	Columbus	7.0±0.21b	1.0±0.18a	8.1±1.99a	6.6±0.76ab	1.1±0.29a	32.6±7.59a	86.9±45.9ab	5.3±2.25ab	6.3±2.02a	10.9±0.82b
	Cascade	13.1±2.62a	0.9±0.09a	8.1±1.38a	6.0±0.85b	1.0±0.16a	33.7±4.21a	44.3±3.93b	8.5±2.83a	7.2±1.02a	10.7±1.06b
2018	Nugget	8.0±0.14a	0.8±0.06a	15.3±1.24a	4.1±0.46ab	0.9±0.04b	45.3±5.43b	63.5±7.47b	6.4±0.42b	7.7±3.05a	12.2±0.56a
	Columbus	7.5±0.59a	0.8±0.06a	9.4±0.38b	5.3±0.90a	1.1±0.18a	40.1±2.62b	94.5±14.8a	9.6±0.49a	7.2±1.51ab	9.8±0.63a
	Cascade	8.7±1.50a	0.6±0.08b	5.7±1.20c	4.3±0.71ab	1.1±0.12a	55.7±5.97a	78.7±18.0ab	7.2±2.46ab	2.0±1.18c	10.6±1.75a
	Comet	7.2±0.72a	0.8±0.13a	8.0±1.82bc	3.4±0.18b	1.0±0.05ab	49.7±3.64ab	55.3±11.5b	7.0±0.73ab	3.3±1.14bc	10.0±2.17a
2019	Nugget	8.2±0.55c	0.8±0.06a	9.9±1.54a	6.2±0.49a	1.5±0.26a	70.8±8.14a	106.4±6.96b	8.6±0.36a	7.7±1.64a	15.0±0.81a
	Columbus	12.0±0.52b	0.6±0.03b	8.8±1.83a	3.6±0.80b	1.0±0.27bc	41.4±9.03b	155.8±17.6a	7.2±1.03b	5.4±2.81a	11.6±0.51bc
	Cascade	14.6±0.99a	0.6±0.09b	8.7±1.14a	3.1±0.32bc	1.2±0.07ab	56.9±5.24a	136.6±33.4ab	6.7±0.24bc	5.7±0.41a	13.0±1.85ab
	Comet	11.0±0.61b	0.6±0.04b	8.7±0.76a	2.3±0.17c	0.7±0.05c	39.7±5.62b	53.0±5.92c	5.6±0.13c	4.1±1.37a	9.6±0.26c
Prob (2017)		0.0005	0.9212	0.1341	0.0185	0.2597	0.2869	0.0385	0.0375	0.6372	0.0119
Prob (2018)		0.1216	0.0031	<0.0001	0.0107	0.0096	0.0031	0.0072	0.0251	0.0022	0.1366
Prob (2019)		<0.0001	0.0011	0.5418	<0.0001	0.0010	0.0001	<0.0001	0.0001	0.0834	0.0001
							Cone				
2017	Nugget	25.7±0.60b	3.0±0.13a	18.0±0.49b	5.6±0.26b	2.0±0.13b	138.6±3.51a	68.5±7.62a	6.5±0.36a	32.3±1.72a	27.2±1.36a
	Columbus	21.8±1.38b	2.8±0.63ab	20.5±0.80b	5.5±0.62b	2.1±0.27b	162.0±40.4a	63.3±19.02a	6.2±0.61a	28.5±3.86ab	27.6±0.41a
	Cascade	32.4±3.40a	2.1±0.06b	25.2±2.88a	9.0±1.15a	3.4±0.33a	177.1±30.5a	57.3±2.95a	6.2±0.28a	24.6±1.92b	27.3±2.83a
2018	Nugget	25.0±1.05a	2.6±0.07a	22.5±0.86a	2.3±0.08c	2.1±0.07c	143.6±15.9b	79.5±10.26a	6.4±0.12b	32.0±1.23a	25.2±1.02b
	Columbus	23.1±1.39ab	2.6±0.08a	17.5±1.32b	3.7±0.41b	2.2±0.18c	188.5±30.1b	79.4±7.02a	9.0±0.78a	26.2±3.29b	22.2±1.56b
	Cascade	20.2±2.96b	1.7±0.23b	18.7±1.14b	4.8±0.52a	2.5±0.07b	405.5±130.5a	81.3±5.48a	8.1±2.15ab	17.9±1.97c	21.8±3.56b
	Comet	21.7±1.41ab	2.4±0.20a	18.1±0.98b	3.7±0.30b	2.9±0.14a	276.8±42.8ab	88.0±8.23a	9.0±0.64a	20.9±1.86c	30.8±1.19a
2019	Nugget	25.9±1.63b	2.4±0.15b	13.8±1.87b	2.6±0.28ab	2.1±0.17c	174.0±18.8a	91.7±4.98a	6.8±0.52b	29.0±3.11a	26.9±2.18a
	Columbus	25.7±0.52bc	2.5±0.14ab	17.5±2.91ab	2.3±0.26b	2.0±0.09c	111.1±19.8b	95.5±12.57a	6.7±0.43b	21.5±1.43b	20.4±2.15b
	Cascade	30.5±1.48a	2.1±0.05c	18.1±0.60a	3.1±0.15a	2.7±0.06b	166.1±20.1a	105.3±9.91a	6.9±0.32b	23.0±2.13b	21.2±2.63b
	Comet	23.2±1.16c	2.8±0.18a	17.6±1.86ab	2.6±0.34ab	3.2±0.23a	160.6±15.7a	97.8±3.54a	8.1±0.43a	24.8±1.83ab	24.1±1.09ab
Prob (2017)		0.0002	0.0132	0.0008	0.0001	<0.0001	0.2269	0.4462	0.5743	0.0091	0.9447
Prob (2018)		0.0186	<0.0001	0.0001	<0.0001	<0.0001	0.0011	0.4157	0.0307	<0.0001	0.0002
Prob (2019)		<0.0001	0.0004	0.0357	0.0102	<0.0001	0.0019	0.2043	0.0014	0.0027	0.0035

3.3. Bitter acids and nitrate concentration in hop cones

Significant differences between cultivars in α-acids were not found in 2018 and 2019 (Figure 6.1.2). The highest average values were found in Columbus and the lowest in Cascade. The average values of α-acids ranged between 8.72% (Cascade) and 11.9% (Nugget) in 2018 and between 4.46% (Cascade) and 12.2% (Comet) in 2019. The concentrations of β-acids in cones only differed significantly between cultivars in 2018 with Cascade recording the highest concentrations. The average values of β-acids varied from 3.89% (Comet) to 8.00% (Cascade) in 2018, and from 3.87% (Comet) to 4.59% (Cascade) in 2019.

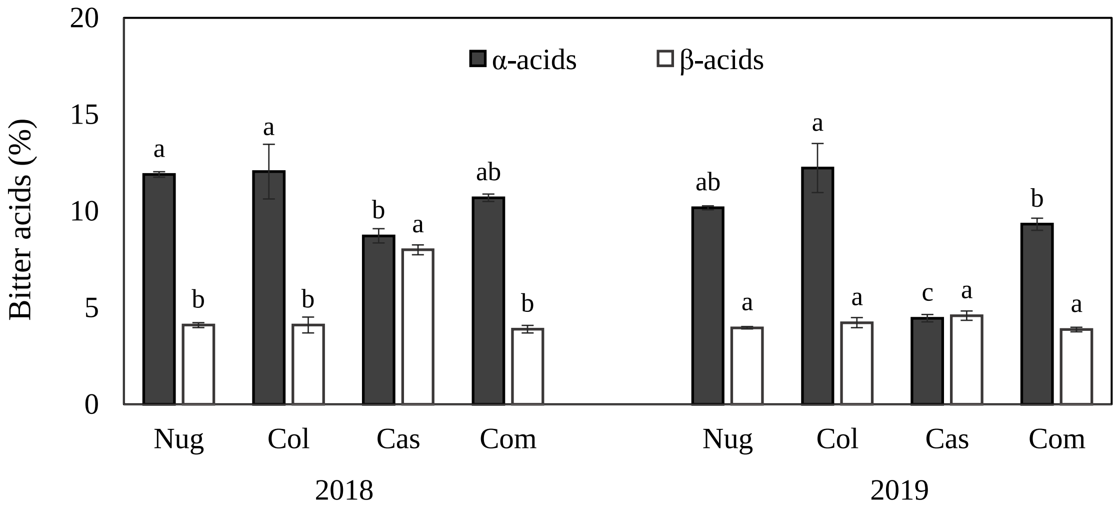

Figure 6.1.2. Cone α- and β-acid concentration in 2018 and 2019, as a function of cultivar (Nug, Nugget; Col, Columbus; Cas, Cascade; and Com, Comet). Within each year, same letters above the histogram bars indicate not significant differences by Tukey HSD test (α = 0.05). Error bars are the confidence intervals of the means (α = 0.05).

The concentrations of NO_3^- in the cones ranged from 2.57 (Comet) to 11.14 (Cascade) g kg^{-1} in 2018 and from 9.19 (Comet) to 14.56 (Cascade) g kg^{-1} in 2019 (Figure 6.1.3). The cultivars Comet and Cascade consistently displayed, respectively, the lowest and highest average values, which differed significantly between both years.

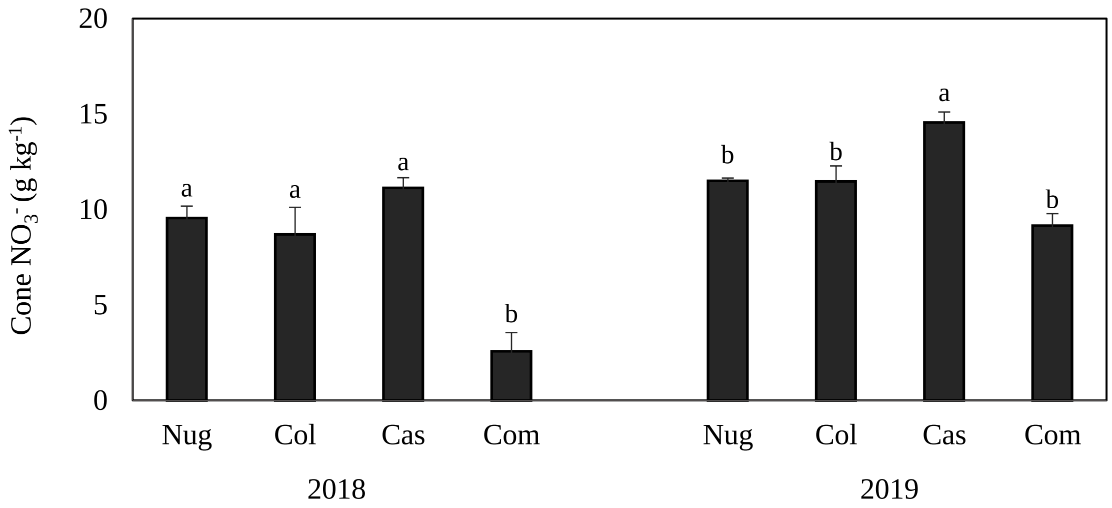

Figure 6.1.3. Cone nitrate (NO_3^-) concentration in 2018 and 2019 as a function of cultivar (Nug, Nugget; Col, Columbus; Cas, Cascade; and Com, Comet). Within each year, same letters above the histogram bars indicate not significant differences by Tukey HSD test (α = 0.05). Error bars are the confidence intervals of the means (α = 0.05).

3.4. Cone attributes in cultivar differentiation with stepwise discriminant analysis

According to the results produced by the stepwise discriminant analysis, using the cone attributes (concentration of nutrients, bitter acids and NO_3^-) as independent variables in the differentiation between cultivars (Nugget, Columbus, Cascade, Comet), three discriminant functions were constructed and four variables were selected with the stronger discriminant capacity (Mg, P, NO_3^- and Zn).

The first function (F1) with an eigenvalue of 13.34 explained the greater differences between the cultivar groups, corresponding to 69.7% of variance explained, and the second (F2) and third (F3) functions explained 25.4% and 4.9% of the variance, respectively. The Wilks' lambda indicated a high significance of the three functions (p < 0.001). The functions of the cultivar group centroid (Table 6.1.4) indicated that function F1 differentiated the Comet cultivar from the others and in particularly from the Nugget cultivar, F2 separated the Cascade cultivar and F3 separated the Nugget cultivar from the other cultivars.

The standardized canonical discriminant function coefficients (Table 6.1.4) reveal the more important variables in the construction of the functions, with Mg being more significant in function F1, P and NO_3^- more significant in function F2 and Zn more significant in function F3.

Table 6.1.4. Discriminant functions generated with the stepwise method applied to hop cone attributes (concentration of nutrients, bitter acids and NO_3^-) as discriminant variables between hop cultivars.

Functions at cultivar group centroid				Standardized canonical discriminant function coefficients			
Cultivar	Functions			Select variables	Functions		
	F1	F2	F3		F1	F2	F3
Nugget	-3.868	1.220	1.059	Magnesium	1.020	-0.324	0.339
Columbus	-1.387	1.291	-1.415	Phosphorus	0.803	1.024	-0.676
Cascade	-0.201	-3.572	-0.034	Nitrate	-0.452	-0.724	-0.369
Comet	5.456	1.061	0.390	Zinc	-1.047	0.054	1.155

The interpretation of these results seems to indicate that the Comet group cultivar was differentiated by the highest concentration of Mg in the cones, Cascade was differentiated by the lowest concentration of P along with the highest concentration of NO_3^- in the cones, and Nugget was differentiated by the highest concentration of Zn in the cones.

The self-validation classification results confirm that 96.9% of the original cases were classified correctly as Nugget, Columbus, Cascade and Comet. The cross-validation classification was of 90.6% and the misidentified cases belonged to Nugget (12.5% classified as Columbus) and to Columbus (25% classified as Nugget).

Therefore, the model based on the stepwise discriminant analysis was effective in the prediction of group membership. The classification of each cultivar group in the first two discriminant functions (F1 and F2), which explained most of the variance, showed that the centroids of Comet and Cascade were quite distant from each other and from Nugget and Columbus, which were both close (Figure 6.1.4).

Briefly, cone attributes such as the concentration of Mg, P, Zn and NO_3^-, helped to differentiate between the cultivars under analysis.

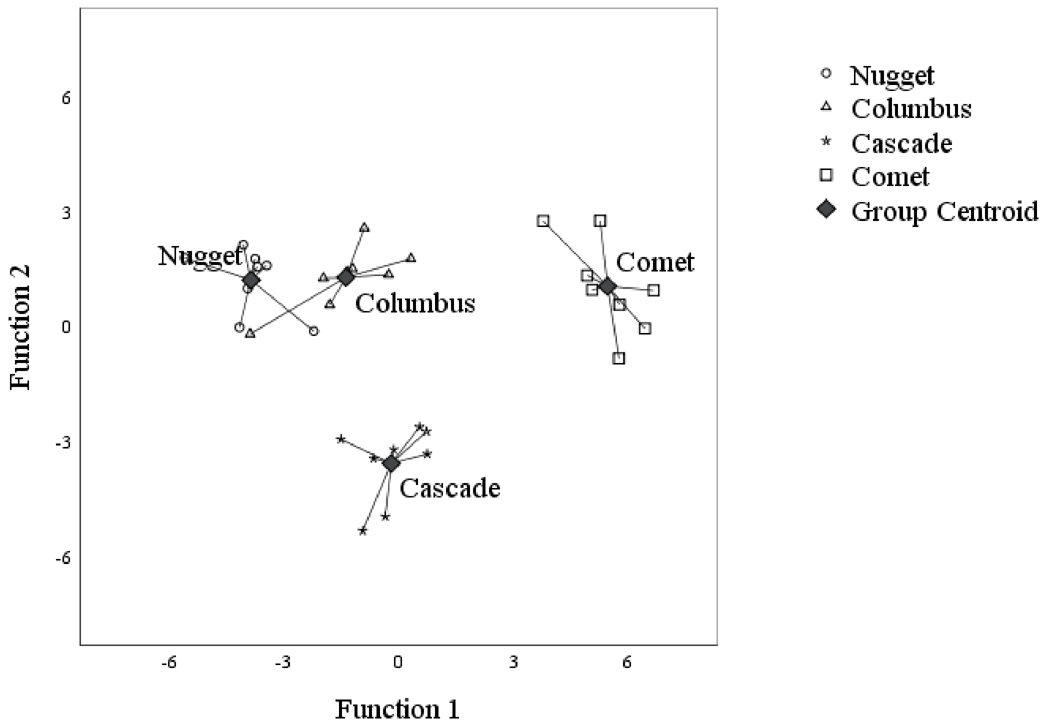

Figure 6.1.4. Distribution and centroid of each cultivar group in relation to the first two canonical discriminant functions generated with the cone attributes as independent variables (concentration of nutrients, bitter acids and NO_3^-).

3.5. Leaf nutrient concentration in cultivar differentiation with stepwise discriminant analysis

The stepwise discriminant analysis was also performed for the nutrient concentrations in the leaves of the bottom or top halves of the plants as independent variables in the differentiation between cultivars. For both analyses (leaf bottom and top halves) three discriminant functions were constructed with the selected variables for each case (Figure 6.1.5). Briefly, in relation to the results obtained for leaf nutrient concentration at the bottom half of the plant in the differentiation of cultivars, it can be noted that: i) Function 1 separated the Cascade group cultivar by the highest N and lowest P concentrations in relation to the remaining groups and particularly to the Comet group; ii) Function 2 separated the Nugget group cultivar by the lowest concentration of Mg and highest concentration of B in relation to the remaining groups, and in particularly to the Comet group; iii) Function 3 separated the Columbus group cultivar by the lowest concentration of Zn and Mg, particularly from the Comet group cultivar.

Regarding the results obtained for leaf nutrient concentration in the top half leaves, it can be noted that: i) Function 1 separated the Cascade group cultivar by the lowest concentrations of P and Zn in relation to the remaining groups and particularly to the Nugget group; ii) Function 2 separated the Comet group cultivar by the highest Mg and lowest N concentrations in relation to the Nugget and Cascade groups; iii) Function 3 separated the Columbus group cultivar by the lowest concentration of Zn and Mg particularly from the Comet group cultivar.

The cumulative variance explained by the first two canonical discriminant functions (F1 and F2) was of 94.2% (F1 with 71.3%) for the bottom half leaf analysis and of 95.8% (F1 with 56.3%) for the top half leaf analysis. In both cases, in relation to the classification in functions F1 and F2, the centroid of the Cascade group appeared as the most separated from the others, and the group separation was clearer from the top half leaf analysis (Figure 6.1.5). The self-validation classification results confirmed that 88.6% of the original cases were classified correctly in the bottom half leaf analysis and 90.9% in the top half leaf analysis. The cross-validation classification was 81.8% for the bottom half leaf analysis and 90.9% for top half leaf analysis. With the exception of the Comet group, all the others presented misidentified cases in the top half leaf analysis.

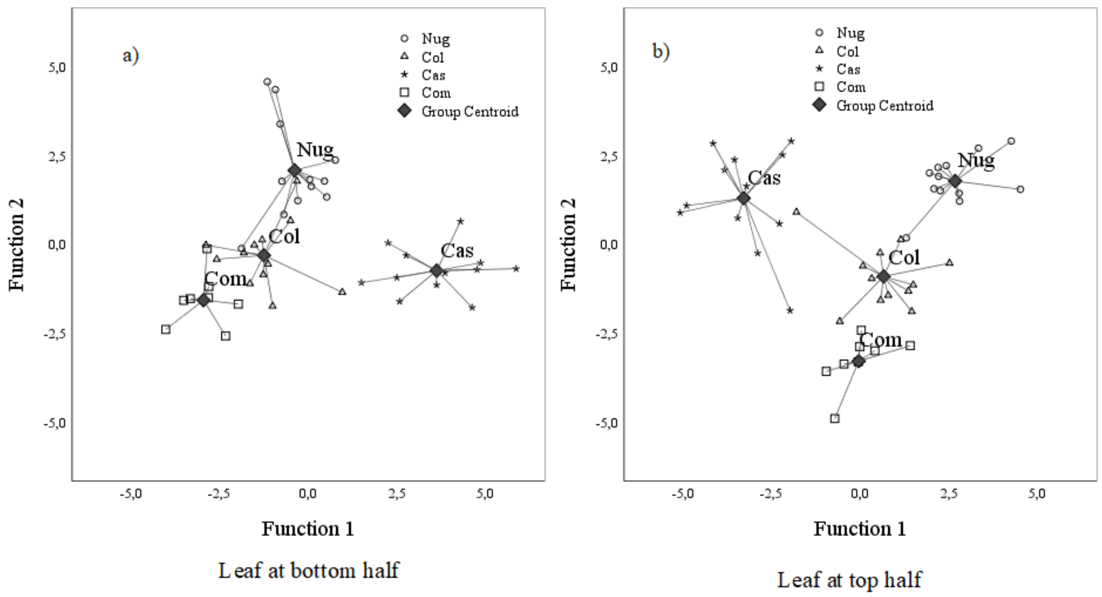

Figure 6.1.5. Distribution and centroid of each cultivar group (Nug, Nugget; Col, Columbus; Cas, Cascade; and Com, Comet) in relation to: a) the first two canonical discriminant functions generated in leaf bottom half analysis; b) the first two canonical discriminant functions generated in leaf top half analysis.

4. Discussion

The productivity of the tested cultivars stressed the good adaptation of Comet, which consistently recorded the highest average values of total DMY. The average DM yield of Columbus was slightly above that of Nugget, but without significant differences. Cascade exhibited the lowest values of total DMY. Cone yield only differed significantly between cultivars in the last year (2019). Comet registered the highest values in 2018 (633.5 g plant^{-1}, 2,640 kg ha^{-1}) and in 2019 (572.4 g plant^{-1}, 2,385 kg ha^{-1}), while the lowest average values were registered in the same period by Columbus in 2018 (479.9 g plant^{-1}, 2,000 kg ha^{-1}) and Cascade in 2019 (323.3 g plant^{-1}, 1,347 kg ha^{-1}). The reference values reported from Hopslist (2020) for cone yield indicate Comet (1,900 – 2,240 kg ha^{-1}) and Nugget (1,700 – 2,200 kg ha^{-}1) as having similar yield potential and Columbus (2,000 – 2,500 kg ha^{-1}) and Cascade (2,017 – 2,465 kg ha^{-1}) with slightly higher values. This is not in agreement with the present results, though the ranges of variation are similar.

Under Mediterranean conditions, Rossini et al. (2016) tested several hop cultivars including Cascade and Columbus and found that Cascade was one of the highest yielding cultivars while Columbus displayed a lower performance. Ruggeri et al. (2018) also found Cascade to be the highest yielding of the cultivars tested in a two-year experiment and reported an average yield of 470 g of cones per plant (248.87 and 691.20 g plant $^{-1}$ in the first and second years, respectively). Mongelli et al. (2016), in an experiment to test the adaptation of several hop cultivars in northern Italy, also observed higher yields for Cascade (952 kg ha^{-1}, dry weight) in comparison to Nugget (292 kg ha^{-1}, dry weight). These results seem to disagree with those presented in this work, in spite of Cascade showing good vegetative development during the growing seasons. Cone yield, in turn, was similar to that of the other cultivars and only in the last year was it significantly lower than that of Columbus and Comet, but similar to that of Nugget.

The year influenced the productivity of all cultivars. The highest average values of total biomass were found in 2018. Highest average cone yields were also achieved in the year 2018 for Nugget, Cascade and Comet and in 2019 for Columbus. The meteorological data for the year 2018 showed higher precipitation levels between March and July. 2019 showed lower average monthly temperatures in June and July and higher precipitation in August and September. The higher precipitation levels in the middle of the growing season in 2018 may have contributed to increasing biomass production. Rossini *et al*. (2016, 2020) also stated that in central Italy the growth and yield of hop cultivars were affected significantly by the weather conditions, particularly by reduced precipitation and high temperatures. Marceddu et al. (2020) reported a high variability of hop yield according to crop management in the region of Palermo in Italy.

The dry weight of individual cones differed significantly among cultivars in 2018 and 2019 and Comet exhibited once again the highest average values in both years (0.79 g cone^{-1} in 2018 and 0.28 g cone^{-1} in 2019). Nugget displayed the lowest average value in 2018 (0.29 g cone^{-1}) and Cascade in 2019 (0.16 g cone^{-1}). Between the years, the average weight of individual cones was higher in 2018 for Columbus, Cascade and Comet cultivars. Nugget displayed higher average cone DMY in 2018, though the values were lower than those of the other cultivars. Čeh et al. (2012) analysed the relationship between cone mass and length of the Slovenian cultivar Savinjski Golding and the weather conditions. They observed a significant effect of weather on cone traits which seems to be in accordance with the results presented in this study. The DM of 100 cones of the Slovenian cultivar Savinjski Golding varied between 10 and 16 g (Čeh et al., 2012), which is less than the values found in this study (0.16 to 0.79 g $cone^{-1}$). These results seem to be a positive indication of the good hop growing conditions in the north of Portugal.

Concerning tissue nutrient concentration, the most consistent trends between cultivars were observed in the leaves and to a lesser extent in the stems. Cascade showed high levels of N but low levels of P, K and B in plant tissues. Comet, which was the highest yielding cultivar, displayed lower values of N and K in leaf tissues, probably due to a dilution effect (Jarrel and Beverly, 1981). Nugget consistently presented the highest levels of K in the leaves. In general, the nutrient concentration in the cones did not follow the same trend reported for leaves. At this point, the results do not seem to suggest that the differences in the productivity between cultivars can be explained by the differences in tissue nutrient concentration.

The concentration of bitter acids in the cones differed significantly between cultivars. Columbus exhibited the highest levels of α-acids, ranging between 12.04 % and 12.23%, which are slightly below the general reference range of 14-18% (Hopslist, 2020), but higher than those reported by Mozzon et al. (2020) for Columbus (7.41%) grown in central Italy, also on a high trellis system. The average values of α-acids of Nugget (10.17 - 11.90%) and Comet (9.32 - 10.69%) were similar to those found in Hopslist (2020), as well as those of Cascade (4.46 - 8.72%), in spite of being lower than those of Nugget and Comet. Mozzon et al. (2020) reported similar α-acid levels for Nugget (10.61%) and Cascade (4.47%). Forteschi et al. (2019) also obtained α-acid levels between 5.00 and 9.05% for Cascade grown in Sardinia (Italy) on a low trellis system. Pearson and Smith (2018) reported α-acid levels slightly higher for Comet (11.2%) in the first-year of growth in Florida (USA), on a high trellis system.

Regarding β-acids, the values obtained for Nugget, Columbus and Cascade were similar to those reported by Mozzon et al. (2020) and Forteschi et al. (2019) for Cascade. The ratios obtained for β-acids were also generally in agreement with the reference values of the Hopslist (2020). The average values for α- and β-acids varied with the year, with the values of Cascade being the most affected. The values of 2019 were particularly low. Cascade seems to have good adaptation to high temperatures (Eriksen et al., 2020). Probably, the lower temperatures and the higher precipitation than usual, at the middle and end of the growing season of 2019, may have contributed to these negative results.

Despite the less favourable effect that the year may have had, all the cultivars displayed bitter acid contents close to the reference values.

The results of stepwise discriminant analysis performed with the cone attributes as discriminant variables presented a solution which differentiated mostly between Comet and Cascade and both these from the other cultivars (Nugget and Columbus). Comet seemed to display higher Mg concentration in the cones while Cascade presented lower P concentration and higher NO_3^- concentration than the other cultivars. The accumulation pattern of NO_3^- in the cones was markedly different between Comet and Cascade, which consistently exhibited the lower and higher average levels, respectively. Mg is involved in N metabolism and seems to be able to reverse NH_4^+ toxicity (Guo et al., 2016). Comet presented higher Mg concentrations which may be related to the lower levels of NO_3^-. Cascade and Columbus displayed respectively the lowest and highest values of α-acids, which is in accordance with the reference levels.

The variables that seem to differentiate better between these cultivars were the concentration in cones of P (higher in Columbus) and NO_3^- (higher in Cascade). The higher concentration of NO_3^- in the cones of Cascade, may mean that fewer amino acids were being synthetized via NO_3^- reduction (Lal, 2018). Consequently, the bitter acid biosynthesis was affected since branched-chain amino acids derived compounds are the essential building blocks for the biosynthesis of hop bitter acids (Xu *et al*., 2013). On the other hand, the reduction of NO_3^- to amino acids is an energy consuming process. Thus, it involves P as the main nutrient in energy metabolism and also in the phosphorylation and dephosphorylation of the NO_3^- reductase enzyme (Kathpalia and Bhatla, 2018; Lal, 2018). Moreover, P compounds are required in bitter acid biosynthesis (Champagne and Boutry, 2017). Hence, the lowest levels of P in Cascade may be related to a lower bitter acid biosynthesis, in contrast to Columbus which has higher levels of P and bitter acids. Cultivars may differ in nutrient uptake, which is probably related to the rate of production of important compounds such as bitter acids, which are very stable in each cultivar.

The results of stepwise discriminant analysis performed with the leaf nutrient concentration as discriminant variables presented a solution which differentiated mainly Cascade from the other cultivars. It also highlighted the lowest concentration of P in Cascade leaves and the highest concentration of Mg in Comet leaves. The higher uptake and accumulation of Mg by Comet may be related to higher biomass production. Comet stood out for its high biomass production compared with the other cultivars and interestingly presented the lowest concentrations of N and K in the leaves. These are macronutrients used in high amounts in plant growth (Hawkesford et al., 2012). Perhaps the higher uptake levels of Mg improved the efficient use of N and K in biomass production. Mg has a relevant role in photosynthesis and N and C metabolism, and it is probably more important in hop growth than is usually considered (Guo et al., 2016). The nutrient accumulation criteria in cone and leaf tissues seem to be a differentiating factor between cultivars with influence on bitter acid biosynthesis and biomass production.

In summary, Comet was the most productive cultivar, displaying the highest total DMY and cone production. Comet was followed by Nugget and Columbus, with similar values, with Cascade giving the poorest performance. The concentration of α- and β-acids in the cones, which is a very important quality parameter, was within or close to the range established as normal in Hopslist (2020) for all cultivars. However, Cascade showed high sensitivity to the year effect, which greatly influenced the average bitter acid yield. Cultivars greatly differed in leaf N, P, K and B concentrations. Cone attributes (concentration of nutrients, bitter acids and NO_3^-) and leaf nutrient concentrations were differentiating factors between cultivars. The results showed that the differences in the concentration of nutrients in the leaves and cones may be related to biomass and bitter acid production. Cascade was the least similar of the cultivars and was differentiated by the lowest concentrations of P in the leaves and cones and the highest NO_3^- concentrations in the cones. Columbus, in turn, was differentiated by the highest leaf and cone P concentrations, while Comet by the highest Mg concentrations in the leaves and cones.

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Supplementary material

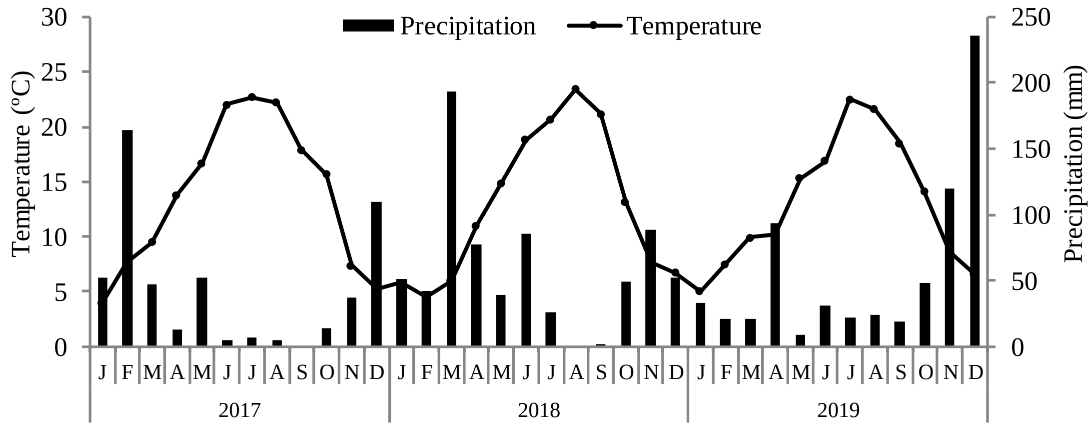

Figure 6.1.S1. Average monthly temperature and precipitation during the experimental period in the weather station of Sta Apolónia Farm in Bragança.

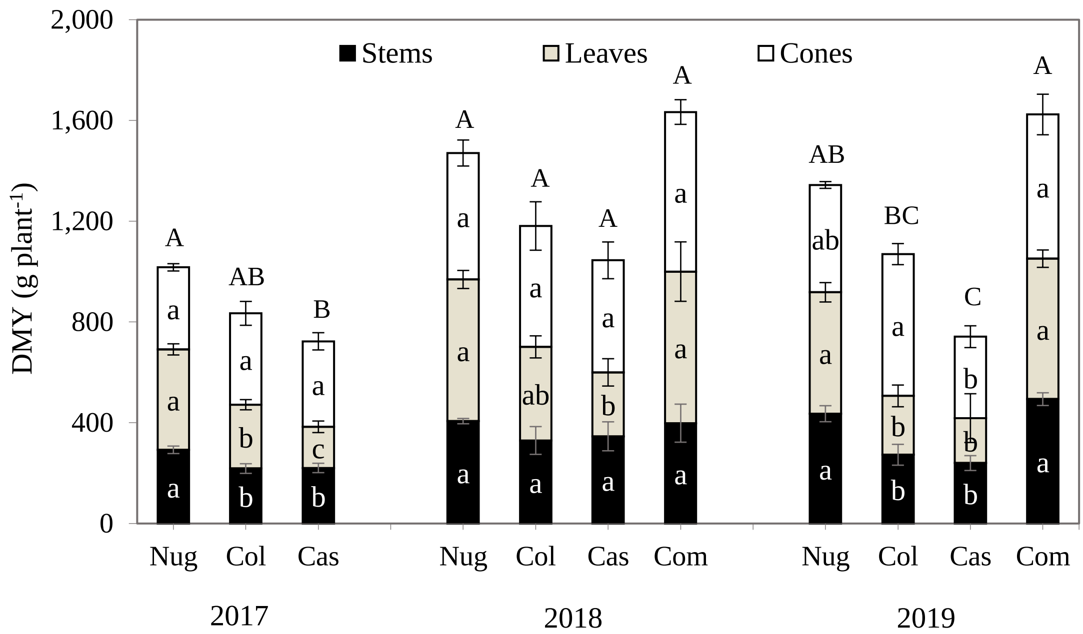

Figure 6.1.S2. Dry matter yield (DMY) of hop plant parts and total, in 2017, 2018 and 2019, as a function of cultivar (Nug, Nugget; Col, Columbus; Cas, Cascade; and Com, Comet). Within each year, means followed by the same letter (lower case for each plant tissue and uppercase for total) are not statistically different by Tukey HSD test (α = 0.05). Error bars are the confidence intervals of the means of the respective tissue (α = 0.05).

Chapter 7:

Phenolic composition of hops as a response to several agro-environmental variation factors

Briefing note

This chapter summarises the results obtained in four field trials to assess the effect of important variation factors on the phenolic composition of the hop cone and the relationship between hop phenol and the elemental composition of the hop cone.

In Chapter 7.1. the results obtained in the four experimental trials were discussed, and namely the influence of the several factors considered, which included the patches of different plant vigour, algae- and nutrient-rich foliar sprays, liming effect, cultivar and year effect.

This chapter is an adaptation of the following paper:

Afonso, S., Ferreira, I.C.F.R., Arrobas, M., Barros, L., Dias, M.I., Cunha, M., Rodrigues, M.Â. Phenolic composition of hops *(Humulus lupulus* L.) as a response to several agro-environmental variation factors (paper submitted to Agronomy Research in December 2021).

7.1. Phenolic composition of hops (*Humulus lupulus* L.) as a response to several agro-environmental variation factors

Abstract

Interest in expanding the production of hops outside the traditional cultivation regions, mainly motivated by the growth of the craft brewery business, justifies the intensification of studies into its adaptation to local conditions. In this study, four field trials were undertaken over periods of up to three years to assess the effect of important agro-environmental variation factors on hop phenol and phenolic composition and to establish its relationship with the elemental composition of hop cones. All the field trials were arranged as factorial designs exploring the combined effect of: 1) plots of different vigour plants × year; 2) plots of different vigour plants × algae- and nutrient-rich foliar sprays × year; 3) plot × liming × year; and 4) cultivars (Nugget, Cascade, Columbus) × year. Total phenols in hops, were significantly influenced by most of the experimental factors. Foliar spraying and liming were the factors that least influenced the measured variables. The year had the greatest effect on the accumulation of total phenols in hop cones in the different trials and may have contributed to interactions that often occurred between the factors under study. The year average for total phenol concentrations in hop cones ranged from 11.9 mg g^{-1} to 21.2 mg g^{-1}. The phenolic compounds identified were mainly flavonols (quercetin and kaempferol glycosides) and phenolic carboxylic acids (*p*-coumaric and caffeic acids).

***Keywords*:** Cultivars, foliar sprays, *Humulus lupulus*, liming, phenolic compounds, plant vigour.

1. Introduction

The most important hop (*Humulus lupulus* L.) compounds for brewing are resins and essential oils, which are responsible for beer bitterness and flavour. Both are synthesized in the lupulin glands of female cones (Bocquet et al., 2018; Korpelainen and Pietiläinen, 2021) . Hop cones also contain other important compounds, such as polyphenols, which contribute to beer flavour, colour, taste and haze formation and have a strong antioxidant power (Almaguer et al., 2014; Carvalho and Guido, 2022; De Keukeleire, 2000). Hop polyphenols include flavonols (e.g. quercertin and kaempferol), flavan-3-ol (e.g. catechins and epicatechins), phenolic acids (e.g. ferulic acid), prenylflavonoids (xanthohumol, isoxanthohumol, desmethylxanthohumol, 6- and 8-

prenylnaringenin), multifidus glycosides and resveratrol (Biendl, 2009; Bocquet et al., 2018; Olsovska and Kolar, 2016). The polyphenolic fraction of hops is so complex that researchers still continue to identify compounds (Bocquet et al., 2018; Tanaka et al., 2014).

In Portugal, hop plants occur spontaneously along riverbanks, in particular in the north of the country, a region that has been found to have some ecological potential for hop production (Rodrigues et al., 2015). The crop was introduced into the country in the early 1960s and currently the national production is located mainly in the Bragança district in the northeast of the country. Nugget, a bitter cultivar, is produced on all the local farms, being destined for a national brewing company. However, local farmers are currently interested in growing aroma cultivars, due to the recent growth of the craft beer industry both at home and abroad. According to the Euromonitor (2020) report "Beer in Portugal" dozens of craft brewers launched different craft beer products in 2019. Currently Cascade and Columbus are some of the aroma cultivars that regional farmers are starting to experiment with. With expanding the craft beer market, new opportunities arise for small-scale growers, producing and supplying desirable cultivars at more favourable prices (Small, 2016). The growing of aroma cultivars for the craft beer market is probably also more suited to the Portuguese production structure based on small-sized plots. To reduce the risk of failure, as occurred during recent years with the bitter cultivars grown for the conventional beer industry, greater knowledge should be applied to the production system. In other Mediterranean countries, hops also attracted the attention of producers and researchers, and recent work has shown good suitability of different cultivars both for the beer industry (Marceddu et al., 2020) and for the production of fresh edible shoots (Rossini et al., 2020; Ruggeri et al., 2018). Thus, an important step for Portuguese farmers is comparing the agronomic performance of other cultivars sought by the craft beer industry with the well-established Nugget. The response of hop to soil pH, for instance, is also important to be understood since pH is an important factor in hop production (Čeh and Čremožnik, 2015; Sirrine et al., 2010). Farmers are currently starting to use foliar sprays to complement their fertilization programmes, given the potential beneficial effects of such products in crop production and quality (Battacharyya et al., 2015; Bindraban et al., 2015; Ziogas et al., 2020). There is also growing interest in the use of biofertilizer formulations from readily available materials to improve soil conditions and plant yield (Balogun et al., 2021; Pascual et al., 2021). Over the years, some fields have been showing patches of poorly developed plants that reduce overall productivity and farmers’ incomes, without the cause being properly explained (Afonso et al., 2020). Thus, it is important to look at these patches of poorly developed plants and observe the effect of foliar sprays on hop cone quality.

As mentioned above, previous studies have shown that hops can be a promising crop for Mediterranean environments, although it is necessary to improve several aspects of the cropping technique (Marceddu et al., 2020). Thus, this study aims to carry out a set of experimental trials to test important factors (plant vigour, foliar sprays, liming, cultivar and year) that can influence the quality of the cones and particularly phenol concentration and phenolic composition. A high content of phenols is a positive trait of hop cones, due to their bioactive effect, which contributes to beer quality (Almaguer et al., 2014; Biendl, 2009; Olsovska and Kolar, 2016). The field trials included four factorial designs exploring the combined effects of 1) plots of different vigour plants and year, 2) plots of different vigour plants, algae- and nutrient-rich foliar sprays and year, 3) plots, liming and year and 4) cultivars and year. From these trials, the concentration of polyphenols in hop cones is reported. The samples presenting the higher polyphenolic content from each of the trials, were selected for phenolic characterization. Furthermore, it was also evaluated the relationship between total phenols and nutrient concentration in hop cones through a principal component analysis (PCA) and correlation analysis. The results of the elemental composition of hop cones, already reported in previous studies (Afonso et al., 2021a; Afonso et al., 2021b; Afonso et al., 2021c), were used to evaluate their relationship with hop phenols. Data on dry matter (DM) yield and hop-acids from these experiments have also been reported (Afonso et al., 2020; Afonso et al., 2021a; Afonso et al., 2021b; Afonso et al., 2021c) but the relevant information to understand the accumulation of phenols in plants in response to the different factors of variation was discussed here. In short, the ultimate objective of this research was to obtain useful data for both hop producers and the craft beer industry.

2. Materials and methods

2.1. Experimental conditions

The field trials were conducted on hop farms located in Bragança (41º41'33.6"N, 6º44'32.7"W, and 850 m above sea level), NE Portugal, from 2016 to 2018. The region benefits from a Mediterranean-type climate, with an average annual temperature and precipitation of 12.7 ºC and 772.8 mm, respectively (IPMA, 2020). Data on average monthly temperatures and precipitation during the experimental period is shown in Figure 7.1.1.

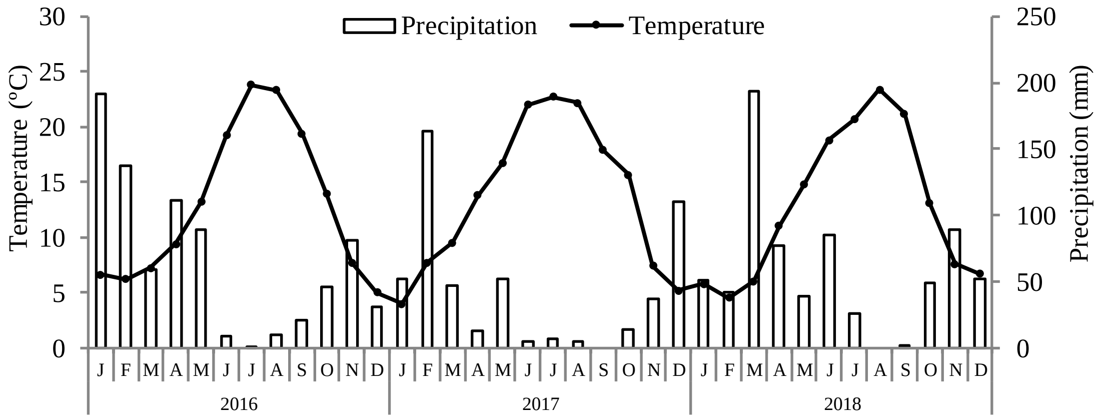

Figure 7.1.1. Average monthly temperature and precipitation during the three years of the study.

Before the installation of the trials, all the plots considered in the experimental protocols were analysed for soil properties. The soil samples were collected in three replicates at 0-0.20 m depth. Each replicate was a composite sample, prepared from soil collected from 15 random points. The samples were oven-dried at 40 ºC and sieved in a mesh of 2 mm. Thereafter, they were analysed for pH_{H2O} (soil: solution, 1:2.5), CEC (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley-Black method), extractable P and K (Egner-Riehm method) and soil separates (van Reeuwijk, 2002). The results of the soil analysis are presented in Table 7.1.1.

Table 7.1.1. Selected soil properties (average ± standard deviation) determined just before the start of the experiments from soil samples collected in plots of different plant vigour (weak, fair, good and very good) at 0–0.20 m depth.

Soil properties	Plot 1 (weak)	Plot 2 (fair)	Plot 3 (good)	Plot 4 (very good)	Plot 5 (very good)	Plot 6 (very good)
Clay (%)[a]	27.0 ± 5.8	35.0 ± 4.6	22.1 ± 2.0	18.1 ± 1.8	17.7 ± 2.1	16.8 ± 0.9
Silt (%)[a]	21.6 ± 10.7	22.8 ± 4.4	5.1 ± 1.6	35.5 ± 5.7	24.7 ± 3.2	24.3 ± 3.1
Sand (%)[a]	51.4 ± 16.5	42.2 ± 2.4	72.8 ± 18.4	46.4 ± 6.9	57.6 ± 4.8	58.9 ± 8.5
pH_{H2O}[b]	5.8 ± 0.12	5.8 ± 0.04	5.5 ± 0.10	5.1 ± 0.13	5.8 ± 0.03	5.3 ± 0.03
Organic carbon (g kg^{-1})[c]	13.4 ± 0.20	15.7 ± 0.10	7.6 ± 0.04	14.5 ± 0.20	17.2 ± 0.08	19.4 ± 0.07
Extract. P (mg P_2O_5 kg^{-1})[d]	283.0 ± 44.7	451.8 ± 33.5	191.1 ± 27.9	212.6 ± 28.2	296.1 ± 20.2	289.3 ± 16.1
Extract. K (mg K_2O kg^{-1})[d]	115.9 ± 7.8	193.0 ± 8.6	111.0 ± 5.9	286.0 ± 5.0	331.5 ± 8.7	161.7 ± 6.3
Exch. Ca (cmolc kg^{-1})[e]	14.8 ± 1.84	23.3 ± 1.39	10.7 ± 0.17	2.7 ± 0.46	4.9 ± 0.24	4.6 ± 0.18
Exch. Mg (cmolc kg^{-1})[e]	4.8 ± 0.84	9.5 ± 1.22	2.7 ± 0.07	0.5 ± 0.04	0.7 ± 0.03	0.6 ± 0.02
Exch. K ($cmol_c$ kg^{-1})[e]	0.3 ± 0.02	0.5 ± 0.04	0.2 ± 0.01	0.5 ± 0.08	0.6 ± 0.03	0.3 ± 0.01
Exch. Na ($cmol_c$ kg^{-1})[e]	0.2 ± 0.05	0.6 ± 0.05	0.1 ± 0.01	0.3 ± 0.06	0.06 ± 0.01	0.05 ± 0.01
Exch. acidity ($cmol_c$ kg^{-1})[e]	0.3 ± 0.03	0.3 ± 0.02	0.2 ± 0.03	0.6 ± 0.13	0.2 ± 0.02	0.4 ± 0.03
CEC ($cmol_c$ kg^{-1})[e]	20.7 ± 2.64	34.4 ± 2.56	14.0 ± 0.21	5.1 ± 0.37	6.7 ± 0.28	6.5 ± 0.17

[a]Pipette method; [b]Potentiometry; [c]Walkley-Black; [d]Egner-Riehm; [e]Ammonium acetate, pH 7

In all the plots where the experiments took place, hop plants were grown on a high trellis system supported by concrete poles and a network of steel cables placed at a height of 7 m. The hop bines were guided from the ground to the upper net with nylon threads. At planting, the rhizomes were spaced at 2.8 m × 1.6 m between and within rows. Two tutors emerged from each original place where the rhizomes were planted, giving rise to a density of 2,232 plants per hectare, which were trained in Spring into two twin canopies. The plots were irrigated by flooding the space between rows. The floor was managed by tillage (3 to 4 passes per year), which has a double function of controlling weeds and removing the superficial crust caused by this irrigation method allowing a better water infiltration at subsequent irrigation events. All the plots received an annual fertilization plan consisting of the application of a compound NPK (7:14:14) fertilizer late in winter at a rate of ~ 500 kg ha^{-1}. Thereafter, during the growing season, two side dress N applications were performed by using ~ 200 kg ha^{-1} of ammonium nitrate (27% N) followed by ~ 450 kg ha^{-1} of calcium nitrate (15.5% N).

2.2. Experimental designs

The experimental apparatus was divided into four field trials arranged as factorial designs with six replications (six twin canopies of three plants). The plants were randomly selected in the corresponding experimental plots when they reached 3 m in height in the plots of higher vigour plants.

Experiment 1 consisted of a factorial design including plots of plants of different vigour (weak, fair, good and very good) and years (2016, 2017 and 2018). The classification of the fields was made with the farmer's help and was based on the crop growth and yield in the previous years. In weak vigour plants, the hop bines did not reach 4 m in height; in fair, the plants did not reach the top of the pole (7 m); in good, the hop bines exceeded 7 m in height but the volume of the canopies, aboveground biomass and cone production were clearly below optimal; in the very good, the hop bines reached a full size and produced abundantly. Strictly speaking, the cause of these underdeveloped plant is not yet clearly known. There are no signs of phytosanitary problems. A previous study (Afonso et al., 2020) showed that underdeveloped plants have very high tissue levels of Fe and Mn, which indirectly may indicate poor aeration caused by the flooding irrigation and/or spatial variations in soil texture (Table 7.1.1). The plots were planted with the Nugget cultivar and were installed ~ 20 years ago.

Experiment 2 consisted of a factorial of plots of different plant vigour (weak, fair, good and very good), foliar sprays (algae- and nutrient-rich foliar sprays and control) and years (2017 and 2018). The algae-rich foliar spray (Algae) is a solution containing 15%

(w/w) the algae *Ascophyllum nodosum* (L.) Le Jolis, applied at a rate of 2 L ha^{-1} (diluted in 1,500 L of water) three times during the growing season, at the phenological stages of inflorescence emergence, flowering, and beginning of the development of cones (respectively on June 20th, July 10th and July 27th 2017, and June 20th, July 8th and July 24th 2018). The phenological stage of the plants, irrespectively of the vigour, was similar. The nutrient-rich foliar spray (Fnut) is a mixture of *A. nodosum* (1.4% w/w) enriched with macro- and micronutrients containing (w/w) 12% N, 6% P_2O_5, 4% K_2O, 0.025% B, 0.1% Fe-EDTA, 0.05% Cu-EDTA, 0.05% Zn-EDTA, and 0.05% Mn-EDTA. This fertilizer was applied at a rate of 3.5 L ha^{-1} (diluted in 1,500 L of water) on the dates reported for Algae. In each plot the foliar sprays were applied in four rows and the six twin canopies of each treatment were sampled in the two interior rows. The plots where this experiment was carried out were the same reported for experiment 1, although in a different part of the plots.

Experiment 3 was arranged as a factorial design and included hop plots (two) of good vigour plants (Plots 5 and 6), liming (limed and not limed) and years (2017 and 2018). The limestone (55% $CaCO_3$, 28% CaO and 20% MgO) was applied at a rate of 1,000 kg ha^{-1} in February 2017. Both fields, ~ 2 ha each, are of the Nugget cultivar and they are ~ 20 years old. Liming was done in a larger part of the area, with only four rows of ~ 150 m remaining for the control treatment, and the plants were sampled from the internal rows of the treated or untreated plots.

Experiment 4 was a factorial of cultivars (Nugget, Cascade and Columbus) and year (2017 and 2018). This experiment was carried out in Plot 4 which part of the plot was installed with several different cultivars, each one occupying a row of ~ 150 m. This hop field was planted in 2014.

An overview of the experimental apparatus is shown in Figure 7.1.2.

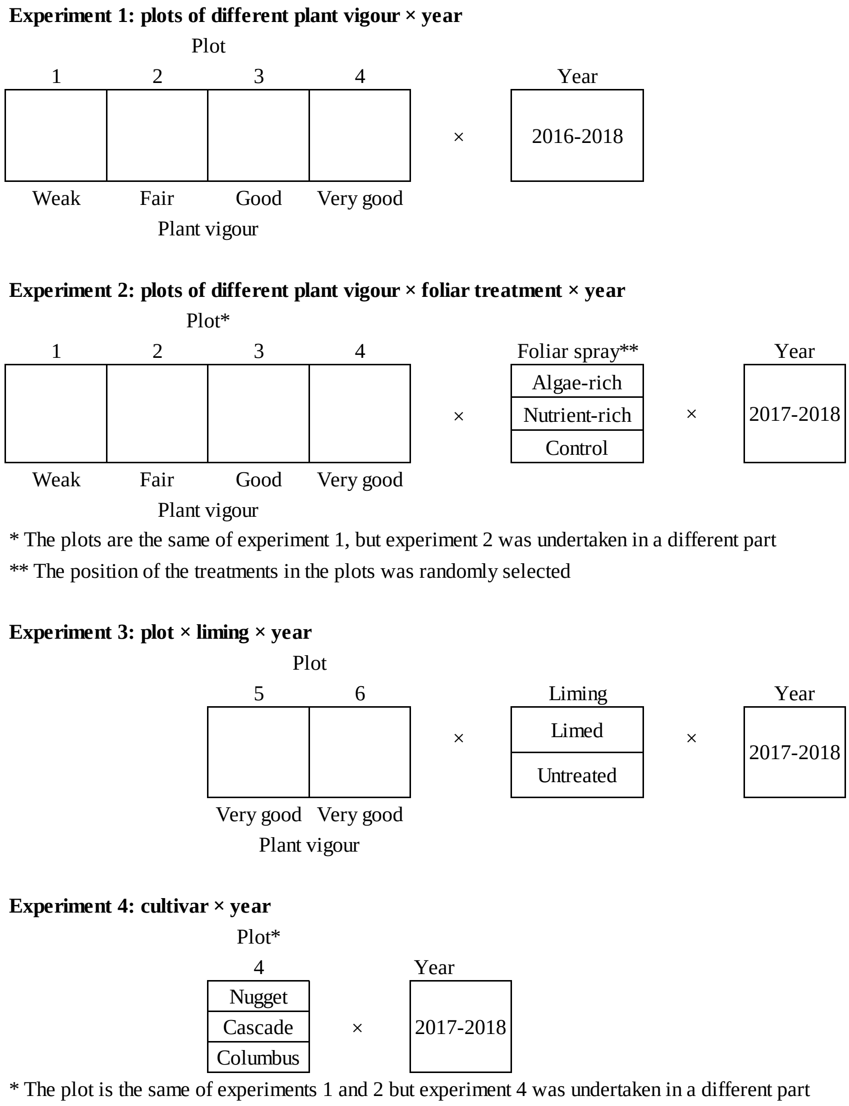

Figure 7.1.2. Schematic view of the experimental apparatus, including the four field trials reported in this study.

2.3. Plant sampling and tissue analysis for elemental composition

Plant material was collected at harvest and subsamples of fresh cones were carried to the laboratory, oven-dried at 60 ºC and thereafter ground for laboratory analysis. Tissue analysis for elemental composition was performed by Kjeldahl (N), colorimetry (P and B), flame emission spectrometry (K), and atomic absorption spectrometry (Ca, Mg, Cu, Fe, Zn, and Mn) methods (Walinga et al., 1989), after the samples were digested with nitric acid in a microwaves (MARSXpress CEM).

2.4. Analysis of total phenolics

Hop cone samples were ground in a Cyclotec mill, with a 1 mm mesh screen, to obtain a fine powdered sample. Infusion preparation was performed by using 1 g of fine powdered hop sample, which was added to 100 ml of boiling distilled water and left to stand at room temperature for 5 min, and then filtered. Total phenols were determined in a total of 204 samples (36 samples from Experiment 1, 72 samples from Experiment 2, 72 samples from Experiment 3 and 24 samples from Experiment 4). The extracts obtained were diluted 1:1. Folin Ciocalteu's assay, briefly, 0.5 ml of each diluted extract was mixed with the Folin-Ciocalteu reagent (2.5 ml). After 3 min, they were saturated with sodium carbonate solution (2 ml) and the reaction was kept in a water bath at 40 °C for 30 min. The absorbance was read at 765 nm (PG Instruments T80 UV/VIS Spectrophotometer, QLabo, Portugal). Gallic acid was used to prepare the standard curve and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract. The analysis of total phenols in each sample was carried out in triplicate.

2.5. HPLC analysis

The samples of phenolic extracts from each trial and from the years 2016 and 2017 were selected and analysed for their phenolic compound content in the directly infused extracts, and then filtered using 0.22 µm disposable filter disks. The operating conditions were followed according to that previously described by Bessada et al. (2016) using a HPLC system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) coupled with a diode-array detector (DAD, using 280 and 370 nm as preferred wavelengths) and a Linear Ion Trap (LTQ XL) mass spectrometer (MS, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The separation was made in a Waters Spherisorb S3 ODS-2 C18 column (3 µm, 4.6 mm × 150 mm; Waters, Milford, MA, USA). Tentative phenolic compound identification was made according to their UV and mass spectra and retention times compared with commercial standards when available, or using reported data from the literature. For the quantitative analysis of phenolic compounds, a 7-level calibration curve was obtained by injecting known concentrations. The results were expressed in mg per kg of fresh weight (fw), as mean ± standard deviation of three independent analyses.

2.6. Statistical analysis

Data was firstly tested for normality and homogeneity of variance using Shapiro-Wilk and Bartlett's tests, respectively. Thereafter, data was subjected to two- or three-way ANOVA according to the experimental design using SPSS v. 25.0 pro-gramme. When the means differed significantly, they were separated by the t-Student or Tukey HSD tests ($\alpha = 0.05$), when the factors were applied at two or more levels, respectively. A PCA was performed with the Object Principal Normalization method on data collected from 2016 to 2018 regarding total phenols and nutrient concentration in cones. The principal components were retained considering the eigenvalues superior to 1 and the scree-plot. Internal consistency was measured with Cronbach's alpha. In addition, the scores of each one of the PCA components were calculated as a function of plant vigour, foliar treatment, limestone treatment, cultivars and year, and subjected to analysis of variance, using the Tukey–Kramer HSD test (α = 0.05) to compare aver-ages for each trial and year. A correlation analysis was applied to the same data as the PCA analysis with the Spearman coefficient.

3. Results

3.1. Total phenols in cones

In the factorial experiment of plant vigour × year, a significant interaction was found for total phenols (Figure 7.1.3), meaning that the response of this variable to the field of plants of different vigour depended on the year and/or vice-versa. Observing the effect of each factor separately, significant differences were found between years but not between fields. In 2017 total phenols were particularly higher than in 2016 and 2018. The average values were 19.0, 11.9 and 15.1 mg g^{-1}, respectively in 2017, 2018 and 2016.

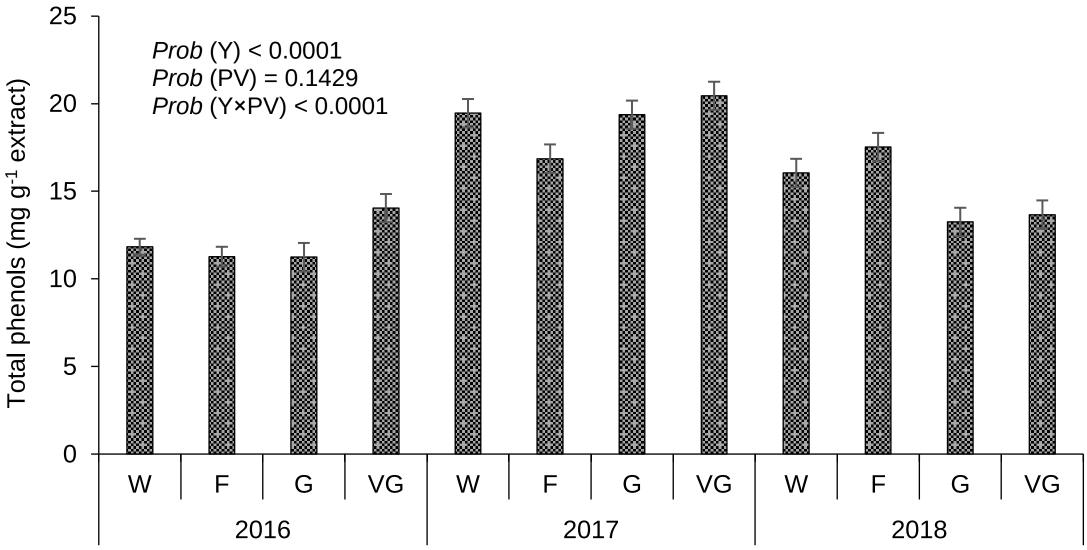

Figure 7.1.3. Total phenols as a function of year and hop plant vigour (weak-W, fair-F, good-G and very good-VG). Error bars are the standard errors (α = 0.05).

In the factorial experiment of plant vigour × foliar treatment × year, a significant interaction was found for total phenols for the combination of the three factors and for plant vigour × year and foliar treatment × year (Figure 7.1.4). Thus, the year seems to be the factor that adds more variability to the results, influencing the accumulation of total phenols in plants of different vigour and subject to different foliar treatment. By analysing the factors separately, differences in total phenols between plots were found, but without any relation to the vigour of the plants. Foliar sprays did not cause a significant effect on total phenols, but in 2017 the values were higher than in 2018. The average values were 21.2 and 14.7 mg g^{-1}, respectively in 2017 and 2018.

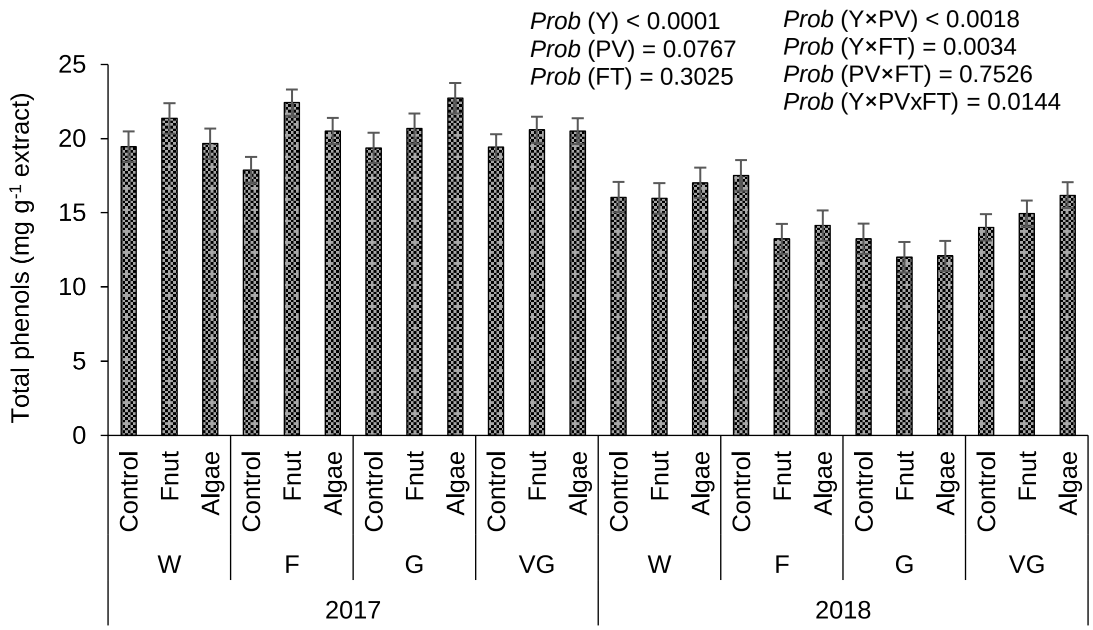

Figure 7.1.4. Total phenols as a function of year, plant vigour (weak-W, fair-F, good-G and very good-VG) and foliar treatment (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control). Error bars are the standard errors (α = 0.05).

In the factorial experiment of different fields × liming × year, significant interaction for total phenols only occurred between field × year (Figure 7.1.5), meaning that total phenol accumulation in plants from different plots was dependent on the year effect. In this experiment, the effect of the plot and year was not statistically significant, and lime's application significantly reduced the content of total phenols. The average values of total phenols were 17.8 and 16.5 mg $g^{-1,}$ respectively in control and limed plots.

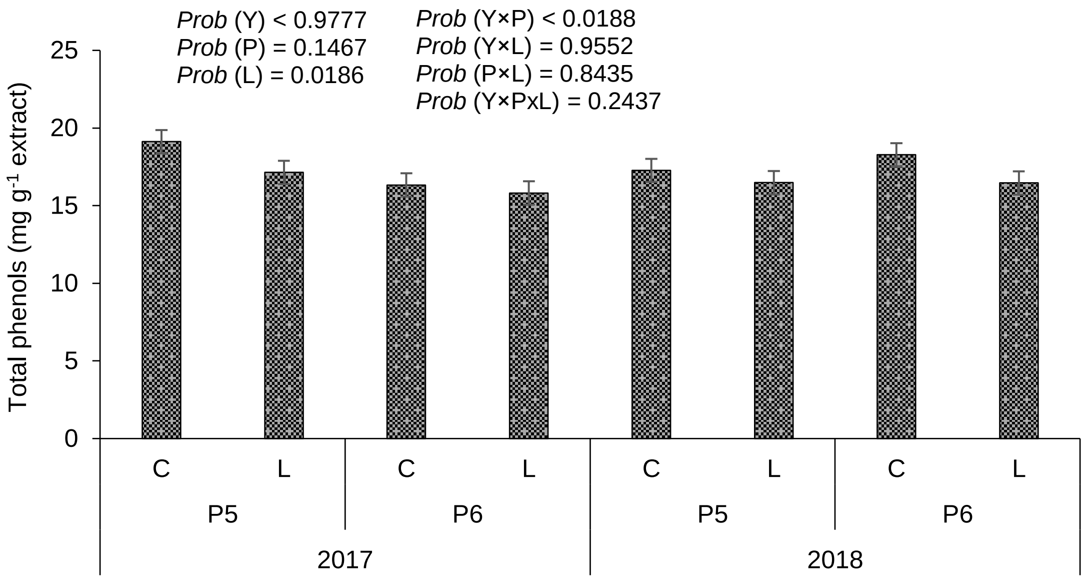

Figure 7.1.5. Total phenols as a function of year, plot (P5 and P6) and liming (L, limed; and C, not limed). Error bars are the standard errors (α = 0.05).

In the factorial experiment cultivars × year, significant interaction was found for total phenols which means that the response of the cultivars depended on the year (Figure 7.1.6). A separate observation of the effect of each of the factors indicated that Nugget showed significantly lower values than Columbus and Cascade, and the values of 2017 were significantly higher than those of 2018. The average values of Nugget, Columbus and Cascade were, respectively, 16.7, 19.9 and 19.6 mg g^{-1} and the average values of 2017 and 2018 were 19.9 and 17.6 mg g^{-1}, respectively.

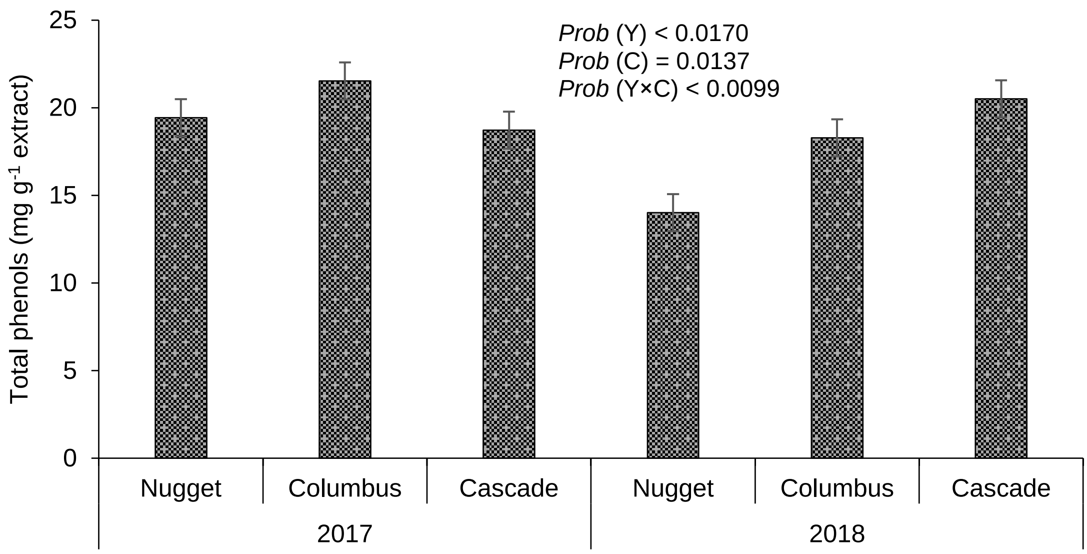

Figure 7.1.6. Total phenols as a function of year and cultivar. Error bars are the standard errors (α = 0.05).

3.2. Principal component analysis

The PCA applied to data collected from 2016 to 2018 concerning total phenols and nutrient concentration in hop cones resulted in four principal components (PC1 to PC4), which accounted for 70.02% of the variance explained. The main differences in the variance explained were between PC1 (23.35%) and PC4 (11.77%). All variables presented high scores for at least one, or more than one, PC (Table 7.1.2). The positive association with N, P, Mg and negative association with K seems to explain greater variance. The higher loading of total phenols was negative and registered in PC3 (-1.606), but scores were also high in PC4 (0.753) and in PC1 (- 0.730). These results seem to indicate a negative association of total phenols with Zn and B.

Table 7.1.2. PCA results for total phenols and nutrient concentrations on hop cones from 2016 to 2018.

	PC1	PC2	PC3	PC4
Eigenvalue	2.569	2.028	1.811	1.294
Cronbach's Alpha	0.672	0.558	0.492	0.250
Explained variance	23.35	18.44	16.46	11.77
Cumulative variance	23.35	41.79	58.25	70.02
Variable Loadings				
Total phenols	**-0.730**	-0.359	**-1.606**	**0.753**
Nitrogen	**1.391**	**-1.195**	0.369	0.618
Phosphorus	**1.516**	-0.091	-0.555	-0.074
Potassium	**-1.206**	-0.867	0.216	**1.134**
Calcium	0.272	**-1.982**	-0.014	0.311
Magnesium	**1.319**	-0.363	-0.584	**1.707**
Iron	0.054	**1.226**	0.822	**1.147**
Manganese	-0.868	-0.572	**1.136**	**1.238**
Copper	0.361	**1.265**	**1.071**	**1.363**
Zinc	**1.277**	-0.179	**1.409**	-0.823
Boron	-0.668	**-1.077**	**1.578**	-0.605

PC - principal component; values in bold correspond to the higher loadings of each variable in the respective PC.

Correlation analysis (Table 7.1.3) indicates total phenols significantly and negatively correlated with Zn followed by Cu, N and Fe in decreasing order. Positive and significant correlations of total phenols with other nutrients were not recorded. On the other hand, cone N concentration presented positive correlations with other nutrients and most significantly with Mg and P, while K was significant and positively correlated with Mn.

Table 7.1.3. Correlation matrix of total phenols (TPH) and nutrient in hop cones, with Spearman correlation coefficients.

	TPH	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B
TPH	1										
N	-0.223**	1									
P	-0.008	0.412**	1								
K	0.114	-0.052	-0.266**	1							
Ca	0.138	0.393**	0.103	0.078	1						
Mg	-0.051	0.513**	0.295**	0.032	0.147	1					
Fe	-0.186*	-0.077	-0.069	-0.126	-0.220**	0.232**	1				
Mn	0.074	-0.016	-0.298**	0.402**	0.019	-0.191*	0.096	1			
Cu	-0.295**	0.028	0.047	-0.139	-0.356**	0.294**	0.520**	0.116	1		
Zn	-0.456**	0.345**	0.371**	-0.269**	0.138	0.005	0.100	-0.071	0.197*	1	
B	-0.105	0.074	-0.176*	0.250**	0.348**	-0.273**	-0.058	0.311**	-0.070	0.223**	1

Significant correlations according to selected significance levels: * = 0.05, ** = 0.01.

3.3. Phenolic compounds identification and quantification

Data on the chromatographic characteristics (retention time, UV in the maximum absorption, molecular ion, and main MS^2 fragments) and tentative identification of the phenolic compounds found in the extracts of hop cones, are described in Table 7.1.4. Thirteen phenolic compounds were tentatively identified in the samples, being 5 phenolic acids (*p*-coumaroyl- and caffeoylquinic acid derivatives) and eight *O*-glycosylated flavonoids (quercetin and kaempferol derivatives).

Table 7.1.4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, and identification of the phenolic compounds present in hop cones extract.

Peak	Tentative identification	Abbreviation	R_t (min)	λ_{max} (nm)	[M-H] (*m/z*)	MS^2
1	3-*O*-Caffeoylquimic acid	3-CQA	4.80	340	353	191(100),179(47),173(3),135(7)
2	*cis* 3-*p*-Coumaroylquimic acid	*cis* 3-*p*-CoQA	5.46	310	337	191(10),163(100),119(10)
3	*trans* 3-*p*-Coumaroylquimic acid	*trans* 3-*p*-CoAD	6.31	310	337	191(53),163(100),119(12)
4	4-*O*-Caffeoylquimic acid	4-CQA	6.86	325	353	191(14),179(53),173(100),135(2)
5	5-*O*-Caffeoylquimic acid	5-CQA	7.25	323	353	191(100),179(15),173(5),135(2)
6	Quercetin-3-*O*-(2-rhamnosyl)-rutinoside	Q-3-2Rh-Ru	14.6	330	755	609(45),591(94),573(12),489(70),301(100)
7	Kaempferol-3-*O*-(2-rhamnosyl)-rutinoside	K-3-2Rh-Ru	16.59	330	739	593(26),575(100),393(8),285(38)
8	Quercetin-3-*O*-rutinoside	Q-3-Ru	17.86	353	609	301(100)
9	Quercetin-3-*O*-hexoside	Q-3-H	19.06	351	463	301(100)
10	Quercetin-3-*O*-(6 -O-malonyl)-glucoside	Q-3-6M-G	20.29	353	549	505(100),463(25),301(50)
11	Kaempferol-3-*O*-rutinoside	K-Ru	21.15	347	593	285(100)
12	Kaempferol-3-*O*-glucoside	K-3-G	22.52	345	447	285(100)
13	Kaempferol-3-*O*-(6 -O-malonyl)-glucoside	K-3-6M-G	24.72	347	533	489(100), 285(20)

Data on the quantification of phenolic compounds in the three different cultivars of hop cones are described in Tables 7.1.5 and 7.1.6. An example phenolic profile chromatogram of the Cascade cultivar is presented in Figure 7.1.7. The quantification of the individual phenolic compounds from the first trial (Plant Vigour × Year) revealed that some of the compounds were not detected in plants with good and very good vigour, in particular *O*-glycosylated kaempferol derivatives and caffeoylquinic acid derivatives (data not shown). Plants of weak vigour were generally higher in quercetin and kaempferol derivatives. The concentration of phenolic compounds in hop cones was very similar among foliar fertilizer treatments, although for most of the compounds the values were slightly higher in the control treatment and slightly lower in the algae treatment (Table 7.1.5). In comparison to control plants, limed presented a significantly higher concentration of kaempferol-3-*O*-(2-rhamnosyl)-rutinoside and also of 4-*O*-caffeoylquimic acid, though not very significantly.

Table 7.1.5. Phenolic compound quantification (mean ± standard deviation) in hop cone samples from 2017 as a function of foliar treatments (Fnut, nutrient-rich foliar spray; Algae, algae-rich foliar spray; and Control) and liming. Means followed by the same letter are not statistically different by Tukey HSD (Foliar Treatment) or t-Student (Limestone treatment) tests (α = 0.05). For each variable, within each treatment, the significance level *(Prob)* is provided.

Phenolic compounds (mg kg^{-1}, dw)	Foliar treatment				Limestone treatment		
	Fnut	Algae	Control	*Prob*	Limed	Control	*Prob*
3-CQA	37.1 ± 16.4a	39.8 ± 15.7a	40.5 ± 10.4a	0.9563	24.4 ± 7.0a	21.5 ± 6.4a	0.5619
cis 3-*p*-CoQA	27.9 ± 3.0a	23.8 ± 6.9a	28.0 ± 3.9a	0.5284	5.7 ± 3.6a	5.8 ± 7.1a	0.9793
trans 3-*p*-CoAD	23.6 ± 6.9a	16.0 ± 6.0a	19.0 ± 4.1a	0.3366	1.1 ± 2.2a	4.2 ± 7.3a	0.4504
4-CQA	24.4 ± 1.7a	23.2 ± 1.0a	25.3 ± 1.9a	0.3381	24.5 ± 4.8a	7.6 ± 15.2a	0.0783
5-CQA	31.1 ± 7.8a	26.1 ± 3.5a	28.7 ± 3.7a	0.5564	5.3 ± 10.7a	5.6 ± 11.2a	0.9731
Q-3-2Rh-Ru	47.5 ± 0.2a	47.0 ± 0.3a	47.2 ± 0.2a	0.0950	46.7 ± 0.4a	47.7 ± 1.4a	0.2389
K-3-2Rh-Ru	46.5 ± 0.1a	46.5 ± 0.2a	46.5 ± 0.1a	0.7156	46.5 ± 0.2a	11.6 ± 23.2b	0.0238
Q-3-Ru	56.1 ± 0.8a	54.5 ± 4.0a	58.5 ± 1.5a	0.2236	50.6 ± 1.1a	50.5 ± 2.1a	0.9754
Q-3-H	72.6 ± 5.1a	64.6 ± 4.6a	71.5 ± 1.5a	0.1010	54.6 ± 4.5a	56.6 ± 6.7a	0.6505
Q-3-6M-G	93.1 ± 4.8a	84.9 ± 9.0a	96.5 ± 6.4a	0.1924	70.2 ± 3.9a	66.1 ± 10.3a	0.4909
K-Ru	50.2 ± 1.3a	49.6 ± 1.5a	51.0 ± 0.3a	0.4124	47.5 ± 0.7a	46.9 ± 0.5a	0.1932
K-3-G	52.1 ± 0.6a	50.8 ± 1.1a	52.3 ± 0.6a	0.1233	48.0 ± 0.4a	36.2 ± 24.2a	0.4015
K-3-6M-G	59.1 ± 3.5a	57.3 ± 3.3a	62.3 ± 2.7a	0.2341	52.4 ± 1.2a	51.3 ± 3.1a	0.5659
Total phenolic compounds	621.4 ± 32.8a	584.1 ± 52.0a	627.2 ± 13.6a	0.3531	477.6 ± 25.4a	414.0 ± 81.0a	0.1845

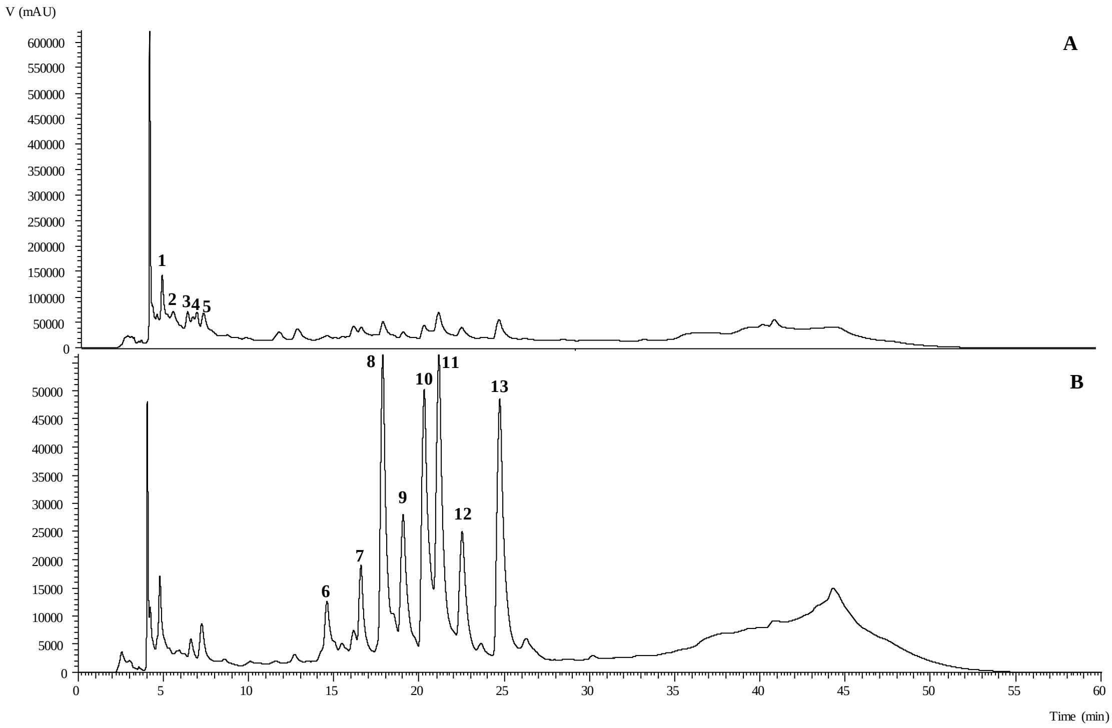

Figure 7.1.7. Chromatographic profile obtained at 280 nm (A) and 370 nm (B) of a hop cone extract (Cascade cultivar). The peaks 1 to 13 correspond to the phenolic compounds identified in Table 4.

Between cultivars, the differences in phenolic compound quantification were significant for most of the compounds, though not for the total sum of phenolic compounds. Cascade presented lower concentrations of *p*-coumaroylquinic acid (*p*-CoQA) and 4-*O*-caffeoylquimic acid (4-CQA), but was generally higher in quercetin and kaempferol derivatives (Table 7.1.6). Nugget and Columbus were overall very similar in their phenolic profile.

Table 7.1.6. Phenolic compound quantification (mean ± standard deviation) in hop cone samples from 2017 as a function of the cultivar. Means followed by the same letter are not statistically different by Tukey HSD tests (α = 0.05). For each variable the significance level *(Prob*) is provided.

Phenolic compounds (mg kg^{-1} dry matter)	Cultivar			
	Nugget	Columbus	Cascade	*Prob*
3-CQA	40.5 ± 10.4a	32.6 ± 5.9a	40.7 ± 9.9a	0.4928
cis 3-*p*-CoQA	28.0 ± 3.9a	24.6 ± 14.9a	11.6 ± 6.0a	0.1654
trans 3-*p*-CoAD	19.0 ± 4.1a	15.1 ± 4.9ab	9.0 ± 2.5b	0.0568
4-CQA	25.3 ± 1.9ab	28.2 ± 7.9a	14.7 ± 3.2b	0.0378
5-CQA	28.7 ± 3.7ab	22.1 ± 3.2b	32.1 ± 2.9a	0.0246
Q-3-2Rh-Ru	47.2 ± 0.2b	46.8 ± 0.2b	50.3 ± 0.6a	<0.0001
K-3-2Rh-Ru	46.5 ± 0.1b	46.6 ± 0.1b	54.5 ± 2.4a	0.0006
Q-3-Ru	58.5 ± 1.5b	52.3 ± 1.4b	72.4 ± 5.2a	0.0007
Q-3-H	71.5 ± 1.5a	62.3 ± 4.9b	56.9 ± 1.6b	0.0034
Q-3-6M-G	96.5 ± 6.4a	77.2 ± 11.4ab	71.5 ± 4.7b	0.0202
K-Ru	51.0 ± 0.3b	51.5 ± 1.4b	74.2 ± 3.1a	<0.0001
K-3-G	52.3 ± 0.6b	54.9 ± 2.9ab	58.5 ± 2.2a	0.0315
K-3-6M-G	62.3 ± 2.7a	71.3 ± 10.7a	76.1 ± 4.8a	0.1230
Total phenolic compounds	627.2 ± 13.6a	585.3 ± 61.8a	622.6 ± 35.7a	0.4547

4. Discussion

4.1. Total phenols in cones

In the four factorial experiments, a significant interaction was found between two or three factors of each experiment for several traits related to total phenols in the cones. This means that the effect of a factor on a given variable was dependent on the other(s) factor(s) under study, and the year was the factor with greatest influence. The accumulation of total phenols in plants of different vigour, in those subject to different foliar treatments and grown in different plots, and between different cultivars was dependent on the year. Abram et al. (2015) also reported that the year influenced the phenolic content of hop cones of different cultivars and of hop plants grown in different locations. The year effect results from the combination of important environmental variables, such as precipitation, temperature, solar radiation, …, which are able to influence physiological and biochemical processes in plants and also the efficiency of foliar nutrition (Smoleń, 2012). The year had a marked effect on total phenol content. Total phenols showed lower values in 2018 in most experiments in comparison with 2017. During important phases of the growing season, such as flowering and initial cone development (June, July), the temperature was lower in 2018 than in 2016 and 2017 and precipitation was higher (Figure 7.1.1). This region is at a low latitude, compared to Europe's major hop producing regions. In lower temperature years, plant growth conditions are closer to those observed at higher latitudes, where hops have better general growing conditions (Korpelainen and Pietiläinen, 2021; Rodrigues et al., 2015). In several studies it has been shown that the growing region, in general, has a great influence on the performance of hop plants (Čeh et al., 2012; MacKinnon et al., 2020; Marceddu et al., 2020; Matsui et al., 2016; Rossini et al., 2016; Rossini et al., 2020). It is also known that environmental variables can affect the secondary metabolism of plants and, therefore, the accumulation of phenolic compounds (Li et al., 2020; Treutter, 2010). The content of total phenols in hop cones did not vary significantly between the plots of different plant vigour. The stress affecting plant growth and yield in the low vigour plots did not influence total phenols in the cones. A previous study analysing these plots (Afonso et al., 2020) has shown that the plants appeared with excessive levels of Fe and Mn in the leaves, which may indicate poor soil aeration, probably caused by a deficient spatial water distribution along the rows by the flooding irrigation system. The soil texture in these plots did not seem to be different enough to create a gradient effect. Phenols significantly decreased with liming treatment. Likewise, Zu et al. (2020) found a decrease in the flavonoid content of *Panax notoginseng* with calcium and lime application under cadmium stress. Although calcium seems to have an inhibitory effect on important enzymes in the phenolic pathway, it seems that the greater amount of cadmium in the roots inhibited the absorption of calcium and influenced flavonoid content. Unfortunately, with the data collected, it was not possible to identify the stress factors that caused the reduction in the content of phenols in the limed plots.

The foliar sprays did not influence significantly the content of total phenols in hop cones. To the best of our knowledge, results from hop cones have not yet been reported from experiments using foliar sprays. Foliar sprays, including those containing seaweed extracts, usually tend to increase the content of total phenols in plant tissues (Battacharyya et al., 2015; Drobek et al., 2019; Salvi et al., 2019; Vasantharaja et al., 2019). However, some studies have also reported an absence of a significant response to the application of this kind of products (Chouliaras et al., 2009; Xu and Leskovar, 2015). Of the cultivars, Nugget showed lower average values of total phenols in comparison to Cascade or Columbus if the two years were taken into account. From the samples selected for phenolic characterization, Nugget presented slightly higher values of total phenolic compounds but, in this case, just the samples with higher phenol content from the first year were characterized. Previous studies have also shown significant differences in total phenols when different hop cultivars were compared (Abram et al., 2015; Arruda et al., 2021; Maliar et al., 2017). The phenols content seems to depend on the cultivar and, in general, low molecular weight phenols are found in greater amounts in aroma cultivars, as the increase in alpha acid content seems to be achieved at the expenses of the phenol content (Almaguer et al., 2014). This seems to be true for Cascade, which showed significantly lower levels of alpha acid content, but not for Colombus, which was similar to Nugget, both presenting significantly higher levels of alpha acid content in comparison to Cascade (Afonso et al., 2021a). Overall, the year average values found in this study ranged from 11.9 to 21.2 mg g^{-1} and were of similar magnitude to those reported by Kowalczyk et al. (2013), varying between 16.2 and 25.5 mg g^{-1} (water extraction, followed by the Folin-Ciocalteau method). Lower values of 7.12 ± 0.09 mg GAE g^{-1} were reported by Keskin et al. (2019) (methanol extraction, followed by the Folin-Ciocalteau method). These results emphasize the potential of the region to grow the cultivars Cascade and Columbus along with the well established Nugget.

4.2. Principal component analysis

PCA and correlation analysis indicate a significant and negative association between total phenols and Zn concentrations in the cones. The results also indicate a negative influence of Cu, N and Fe in the accumulation of total phenols in the cones. Hop is a species particularly sensitive to Zn deficiency, affecting plant growth and cone production (Gent et al., 2015). In this case, an association of Zn with plant vigour was not found, but higher concentrations of Zn, Cu and Fe were previously reported for these plots, and the result associated to poor soil aeration (Afonso et al., 2020). Enhance absorption of Zn and Cu was also notice in industrial hemp (*Cannabis sativa subsp. Sativa*) with higher irrigation level, with Zn showing higher mobility to aerial tissues (Golia et.al, 2021). The results of correlation analysis also showed significant and positive correlation between cone Cu and Fe, and cone Zn and Cu. Regarding Fe, the high levels previously reported in soil and plants (Afonso et al., 2020), may have contributed to lowering total phenol concentrations. Zn, Fe and Cu does not seem important nutrients in phenolic biosynthesis, and they may interfere negatively with other nutrients that provide co-factors for many enzymes of the flavonoid pathway (Treutter, 2010). Regarding N, its supplementation has been negatively associated with the phenolic composition of plant tissues in several crops (Elhanafi et al., 2019; Otalora et al., 2018), and associated with plant growth particularly in sensitive species to soil N availability (Rejmánková, 2016). In accordance with the protein competition model (PCM), since phenols and proteins compete for a common precursor, conditions that increase plant growth may reduce the concentration of total phenols (Rejmánková, 2016).

4.3. Phenolic compounds identification and quantification

The phenolic compounds identified were mainly flavonols (quercetin and kaempferol) and phenolic carboxylic acids (*p*-coumaric and caffeic acids), which represent a minor fraction of the polyphenols that can be found in hop cones (Biendl, 2009; Tanaka et al., 2014). The result might be due to the in-water extraction method, which while suitable for many applications, is less efficient than the hydroalcoholic extraction method, particularly on hop prenylated flavonoids detection, which are lipophilic compounds (Kowalczyk et al., 2013). The phenolic profile of *H. lupulus* is in accordance with those previously reported for bracts (Tanaka et al., 2014), leaves (Kite and Veitch, 2009) and cones (Magalhães et al., 2010; Sommella et al., 2017) and also for leaves, stem and roots of *H. japonicus* Siebold & Zucc (Choi et al., 2017). The identification of peaks 8 ($[M-H]^-$ at *m/z* 609), 9 ($[M-H]^-$ at *m/z* 463), 11 ($[M-H]^-$ at *m/z* 593), and 12 ($[M-H]^-$ at *m/z* 447), quercetin-3-*O*-rutinoside, quercetin-3-*O*-hexoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, respectively, was performed by comparison of their retention time, UV spectra, and mass fragmentation patterns with the available commercial standards. Three caffeoylquinic acid derivatives were tentatively identified regarding the phenolic acids groups, peaks 1, 4, and 5 (3-*O*-, 4-*O*-, and 5-*O*-caffeoylquinic acids, respectively). According to Clifford et al. (2003, 2005), peaks 1 and 5 present a major ion MS^2 fragment at *m/z* 191, while peak 4 presents at *m/z* 173 an abundance of 100%, indicating the connection 4-*O*- position in the molecule. The organization of the three peaks, besides the major abundant fragments, was performed according to the hierarchical keys developed by Clifford et al. (2003, 2005). The two 3-*p*-coumaroylquinic acids found (peaks 2 and 3, *cis* and *trans*, respectively) were also assigned using the same hierarchical keys developed by Clifford et al. (2003, 2005) the base peak at *m/z* 163 is for 3-*p*-coumaroylquinic acids. Since both peaks presented the same chromatographic characteristics, they were assigned as *cis* and *trans* isomers. Tanaka et al. (2014) have also reported the same phenolic acids in the bracts of hop plants and Choi et al. (2017) in the leaves, stem and roots of *Humulus japonicus* Siebold & Zucc. Finally, two *O*-glycosylated quercetin derivatives and two *O*-glycosylated kaempferol derivatives were also tentatively identified in the hop cones, peaks 6 and 10, and peaks 7 and 13, respectively. The tentative identification of these four peaks was performed based on those previously described in *H. lupulus* samples (Kite and Veitch, 2009; Tanaka et al., 2014).

The hop cones from the less vigorous plants were higher in quercetin and kaempferol while in the hops from the more vigorous plants, the kaempferol flavonoids and caffeic acids were found in small concentrations or were not even detectable. Environmental variables such as light exposure and temperature can significantly influence the accumulation of quercetin and kaempferol compounds in plant tissues (Treutter, 2010). Galieni et al. (2015) have also found an increase in caffeic acid and other phenolic compounds in *Latuca sativa* L. grown under drought stress and an increase in cell wall lignification as a tolerance response. In these experiments, the increased levels of phenolic compounds in less vigorous plants are probably a response to the environmental stress affecting plants' growth. The plants treated with foliar sprays presented slightly lower values of phenolic compounds. Similarly, Xu and Leskovar (2015) did not find any effect of applying a seaweed extract on flavonoid content in spinach. Hop cones from limed plants presented a significantly higher concentration of kaempferol-3-*O*-(2-rhamnosyl)-rutinoside and 4-*O*-caffeoylquimic acid though not significantly. Likewise, Ngadze et al. (2014) found an increase in caffeic acid content in potato (*Solanum tuberosum* L.) as a response to Ca applications. As far as we know, no studies have reported the phenolic composition of hop cones after liming. Cascade stood out from the other cultivars, showing higher concentrations in quercetin and kaempferol derivatives and lower in *p*-coumaric acids. Similarly, Almeida et al. (2020) reported isoquercitrin followed by quercetin as the major phenolic compounds found in extracts of Cascade hops grown in Brazil. Santagostini et al. (2020) identified quercetin-3-*O*-malonylglucoside and kaempferol-3-*O*-malonylglucoside compounds for the first time in Cascade hop. These compounds were also identified for the cultivars used in this study.

In agreement with the present results, other studies (Abram et al., 2015; Maliar et al., 2017) also showed significant differences in phenolic composition of different cultivars of hop, which probably was due to the potential influence of genetic factors on agronomic and biochemical traits (McAdam et al., 2014).

5. Conclusions

Total phenols in hop cones were influenced significantly by most of the experimental factors under study. However, in this study, foliar sprays and liming were among the factors that least influenced the measured variables. The year, which represents the joint action of several environmental variables (temperatures, rainfall, relative humidity, etc.) resulted as the most important factor for the phenols accumulation between plants of different vigour, subject to different foliar treatments and grown in different plots and between different cultivars. Nugget showed significantly lower average values of total phenols compared to Cascade or Colombus if the two years were taken into account. The high levels of Zn in hop cones seemed to be associated with the experimental plots showing lower concentrations of total phenol in the hop cones. The phenolic compounds identified were mainly flavonols (quercetin and kaempferol) and phenolic carboxylic acids (p-coumaric and caffeic acids). The less vigorous plants showed higher levels of quercetin and kaempferol in hop cones. The plants treated with foliar sprays presented slightly lower values of phenolic compounds, and limed plants were notably higher in kaempferol-3-*O*-(2-rhamnosyl)-rutinoside. Cascade stood out from the other cultivars, showing higher concentration in quercetin and kaempferol compounds and lower concentration in *p*-coumaric acids. The phenolic compounds quercetin-3-*O*-malonylglucoside and kaempferol-3-*O*-malonylglucoside, reported previously in other studies for the first time in Cascade, were present in this study in Cascade, Columbus and Nugget.

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Chapter 8:

General conclusions and future research

8.1. General conclusions

Present research included five working lines with the purposes of evaluate the productive performance and constrains of the current hop fields and to analyse agronomic decisions and practices considering the evolution of the hop market and the growing interest in aroma cultivars. The first working line, included in the Chapter 3, addressed the issues of the irregular productive performance of the hop fields, the effect of flooding irrigation on soil properties and plant performance, and the effect of the application of lime to attenuate the variability created by the irrigation system. The first field trial, included in chapter 3.1. was carried out in Pinela and Vinhas farms, located in Bragança region, during the growing season of 2016, with hop cultivar Nugget. The experimental design included patches of different plant vigour (weak, fair, and good) and several soil properties were determined and related with plant nutritional status and yield. The field trials, included in Chapter 3.2., were undertaken on Pinela farm, in two hop fields, during the growing season of 2017. Soil properties, plant nutritional status and crop yield were evaluated along the rows searching for any gradient created by the irrigation system and to assess the liming effects on soil properties and plants.

The second working line, included in chapter 4, addressed the effect of fertilizers applied by foliar spray on hop productivity and cone quality variables. The experimental trials were carried out with hop cultivar Nugget. The first trial, included in chapter 4.1, was undertaken on Pinela and Vinhas farm, during the growing seasons of 2017 and 2018. The purpose was to evaluate the potential of two foliar sprays, one based in the algae Ascophyllum nodosum (L.) and the other rich in nutrients, to improve the homogeneity of hop fields and restore the productivity of the poorly developed patches. The field trial was arranged in a factorial design including plots of different vigour (weak, fair, good and very good), three fertilizer treatments (control, algae foliar spray, and nutrient-rich foliar spray) and two year (2017 and 2018). The field trial included in chapter 4.2., was undertaken on Pinela farm, to evaluate the application of a foliar spray rich in amino acids as an alternative to reduce the proportion of the N applied to the soil as a side dressing, and to evaluate the application of a foliar spray rich in K at the cone developing stage, as a supplement to the farmer's fertilization plan. The field experiment was arranged as a fully randomized design with three fertilizer treatments (control, foliar spray rich in amino, and foliar spray rich in K), during two growing seasons, from 2018 to 2019.

The third working line, included in chapter 5, addressed the issue of recycling of hop production wastes. It was evaluated the potential use of hop leaves to produced quality compost suitable to be applied as fertilizers in plant growth. Besides, it was evaluated the effect of ash addition, resulting from the usual farmer's procedure of burning the stems after harvest, on the composting process. The trials included: i) a composting experiment with seven composting treatments, consisting of different compostable mixtures of the raw materials (hop leaves, cow manure, wheat straw and hop stem ash); ii) a pot experiment conducted with lettuce, in two growing cycles (2018 and 2019), with the composted materials as treatments, and two ratios of application, in a total of 10 treatments.

The four working line, included in chapter 6, focused on the evaluation of the agronomic performance and chemical profile of hop aroma cultivars grown under the Mediterranean conditions of NE of Portugal. The newly introduced cultivars (Columbus, Cascade and Comet) were compared to the well-established Nugget cultivar. The field experiment was conducted on Pinela farm from 2017 to 2019 and was arranged in a completely randomized design with the four cultivars.

The fifth working line, included in chapter 7, summarizes the results obtained in four field trials to assess the effect of important variation factors on the phenolic composition of the hop cone and the relationship between hop phenol and the elemental composition of the hop cone. The field trials were arranged as factorial design and were undertaken in Pinela and Vinhas farm, from 2016 and 2018. The factors included the patches of different plant vigour (weak, fair, good and very good), foliar treatments (control, algae foliar spray, and nutrient-rich foliar spray), liming effect (lime, no lime), cultivar (Nugget, Cascade and Columbus) and year (2016, 2017 and 2018).

The present research results have highlighted the negative effect on soil and plants of the surface irrigation, which is still the most used irrigation method of hops in northern Portugal. This irrigation system seems to be one of the major causes for the appearance of patches of poorly developed plants in the current hop fields due to the increased levels of Mn and Fe in soil and plants. On the other hand, the long-term irrigation of hop by flooding did not cause a gradient along the row on soil properties. Significant variations were found related to the erratic spatial variation in some soil constituents, such as sand, silt, and clay. Still, flooding probably aggravated the effects of the spatial variation on soil constituents. In addition, irrigation and frequent soil tillage seem to cause damage to soil properties such as porosity and bulk density which were found to be related to crop productivity. It was expected that the application of lime under these conditions could attenuate the effect of irrigation on soil properties. However, the lime application did not have a relevant effect on soil and plants, probably due to the small amount applied, which only slightly raised the soil pH. Hence, it seems of interest to establish reference values for the application of lime in the hop fields under Mediterranean conditions. Therefore, the farmers are advised to consider implementing a drainage system to improve soil aeration, to change the irrigation system to drip irrigation, and to increase soil pH above acidic levels by liming.

The field experiments with foliar sprays produced relevant information for farmers to consider in their fertilizer programmes. Foliar sprays of amino acid-based fertilizers showed an interesting alternative to reduce the amount of N applied to the soil, maintaining crop productivity and enhancing the biosynthesis of bitter acids. The results emphasize the importance of amino acids in the biosynthesis of bitter acids, in which K and Zn also seem to play an important role. Foliar sprays based on seaweed extracts of the *A. nodosum* algae showed to be effective in mitigating the stress affecting plant development, which probably is poor soil drainage caused by the flooding irrigation system, only under conditions of severe stress.

The application of K as a foliar spray, late in the growing season, also did not prove to increase cone quality. The lower efficacy of this treatment was probably related to the adequate levels of K in the soil. Data brought out through these experiments contributed to increasing the knowledge on this issue. Albeit the application of foliar sprays in hop fields is still an understudied issue, an effort should be made to develop further experimental work to produce reliable guidelines for farmers.

The adequate management of the waste generated in the hop farms can allow farmers to take advantage of resources usually undervalued. Hop leaves showed to be suitable to compost with other organic wastes usually available in farms such as cow manure and wheat straw. Guidelines are provided concerning the time of composting, the proportion of raw materials and soil application. The addition of ash from hop stems to the other raw materials did not benefit the composting process. Though it increased the concentrations of several nutrients, it significantly reduced N concentration in the final compost and slowed down the compost maturation. Thus, ash from hop stems should be applied directly to agricultural soils, or other alternatives should be considered.

The introduction of aroma cultivars by current and new hop farmers seems to be an inevitability in response to market demands. The aroma cultivars tested, Columbus, Cascade and Comet, showed good performance compared to the well-establish Nugget. Comet was the most productive cultivar, and Cascade showed the highest sensitivity to the year's environmental conditions. But all the cultivars displayed bitter acid contents close to the international reference values. The field experiment results seem to be a positive indication of the good hop growing conditions in the north of Portugal. Results also showed that cultivars seem to differ in nutrient uptake, which is probably related to the production rate of important compounds such as bitter acids, which are very stable in each cultivar. Comet seems to be more demanding on Mg uptake, while Columbus seems more demanding on P uptake. Hence, the nutrient accumulation criteria between cultivars may provide relevant information to adjust fertilization to specific cultivars.

From the results of the present work, suggestions and guidelines are provided to the current and new hop farmers:

i. The current hop farmers are advised to implement a drainage system to increase soil aeration and to convert to a drip irrigation system that should also be the preferential system to adopted by the new farmers;
ii. The application of lime in hop fields may be relevant to alleviate the toxic effects of high levels of Mn and Fe due to flooding. The application rate should be higher than 1,000 kg lime ha^{-1} and enough to increase pH above acidic levels. The results should be monitored to produce guidelines for the future;
iii. The used of foliar fertilizers with different plant biostimulants should be tested under different conditions of application. The application of amino acid-rich foliar sprays can be an alternative to reduce the application of N to the soil. Four applications of an amino acid-rich foliar spray, with at least 53% of total amino acids (w/w), can be applied in place of a second side dress N application of ~70 kg N ha^{-1}. However, farmers should be cautious in adopting the use of foliar sprays without criteria and without results being tested by experimental work;
iv. The hop leaf waste generated in the harvest can be valued through composting with other materials such as cow manure and wheat straw. The mixture of hop leaves with cow manure can produce a stable compost after nine months of composting, suitable for horticultural crops, irrespective of the proportion of raw materials. The proportion of the mixtures of leaves and straw should be at least 2:1 to produced compost able to mature in nine months. Alternatively, the composting process should take longer, or the poorly-matured compost can be applied far in advance of sowing;
v. The hop aroma cultivars Columbus, Cascade and Comet showed good agronomic and chemical performance under the Mediterranean conditions, compared to the well-established bittering Nugget cultivar. All cultivars presented bitter acid concentrations within or close to the normal ranges internationally accepted. Comet was the most productive cultivar, and Cascade showed the highest sensitivity to the year's environmental conditions. The fertilization programme of farmers may consider that Comet seems to be more demanding on Mg while Columbus seems more demanding on P uptake.

Hop continues to be a challenging crop, but the greater the challenges, the greater the potential that might have. Researchers and farmers should continue to work together to overcome the constraints and take advantage of the arising opportunities.

8.2. Future research

Humulus lupulus L. is an extensively studied plant, but there is still much to be understood. Agronomy is probably one of the research areas that less attention has received, and in Portugal, the knowledge produced until now is scarce and outdated. Therefore, this thesis provides background information to continue future research and establish reliable guidelines for current and future hop growers. Potential areas for future research are:

a) Reconversion of the surface irrigation system to drip irrigation. Information should be provided to support the reconversion of the surface irrigation system to drip irrigation in the current hop farms. A pilot study should be carried out with a partial reconversion on select zones, covering the areas that have shown different fertility gradients. The study should cover the evaluation of the reconversion costs, estimation of water requirement, estimation of irrigation volumes, evaluation of the efficiency of drip irrigation system, and evaluation of the effects on soil properties and plants.

b) Establish reference values for liming application on hop crop in Portugal. The application of higher rates of lime in the hop farms should be considered and monitored to establish reference values to increase soil pH to the levels accept as adequated for hop crop. If possible, experimental trials should be carried out in the current farms managed with the surface irrigation system and also in the recently implemented hop fields with drip irrigation system. The effect on soil properties and plant performance should be evaluated, considering the different irrigation systems.

c) Fertilization on hop crop. Further trials should continue to evaluate the adequacy of innovative products on hop plant nutrition. Plant biostimulants either in soil preparations or as a liquid foliar application may benefit hop plant performance and yield. But the effectiveness of these products and how they should be used in hop fertilization should be evaluated through experimental work. The results of this thesis highlighted the relevance of amino acids on bitter acids production, with K and Zn also playing an important role. The application of amino acid-rich foliar sprays and foliar sprays rich in K and Zn may be an interesting option for future research.

d) Cultivars and trellis system. The agronomic performance of other aroma cultivars with potential interest should continue to be evaluated under the Mediterranean conditions of the country. It would also be interesting to include dwarf cultivars suitable for growing in low trellis systems (2.5 - 4 m high). Hop plants are usually grown in high trellis systems to assure high yielding. The low trellis systems have economic, environmental, and agronomic benefits but typically are not considered as most cultivars perform better in the high conventional systems. However, there is little data available on cultivars yield under the low trellis systems. A future research line could include evaluating cultivar yield in both high and low trellis systems.

e) Valorisation of hop wastes. Hop leaves remaining from harvest proven to be appropriate to compost with other materials producing final composts suitable for agronomic purposes. The leaves from the bottom of the hop bines can be used as cattle fodder by grazing, a practice that also enhances the canopy's aeration. This practice simultaneously promotes the good health of plants and feeds the sheep. Hence, evaluating the potential of hop leaves for animal nutrition may be another option for future research on this field. Regarding hop stems, recycling through biochar production may also be an option with interest.