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The Expression of Genes Involved in Synthesis of Bitter Acids and Xanthohumol and the Content of These Compounds in Aroma and Bitter Hop under Reduced Nitrogen Fertilisation

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Abstract: Hop (*Humulus lupulus*) is cultivated for industrial purposes, primarily for brewing, but also for pharmaceutical and cosmetic applications. The hop cones are valued for their secondary metabolites, including bitter acids and prenylflavonoids. The objective of the presented study was to investigate the impact of reducing nitrogen fertilisation on the activity of genes involved in the production of bitter acids and xanthohumol in cultivars Lubelski (aroma) and Magnat (bitter) grown in the field. Furthermore, the content of these compounds was determined. The results demonstrated that the majority of studied genes were expressed at a similar level despite the reduction in the nitrogen dose by 25–30%. Nitrogen dose influenced the activity of genes *BCAT2*, *HS1*, and *PAL*. Moreover, the differences in gene expression were observed between cultivars and developmental stages. The reduction in nitrogen dose did not result in a reduction in secondary metabolite content. The content of alpha and beta acids in Magnat was approximately 14.8% and 3.2%, respectively, while in Lubelski it was approximately 7.7% and 5.8%, respectively. The content of xanthohumol in both cultivars was approximately 0.7%.

Keywords: hops; *Humulus lupulus*; nitrogen fertilisation; gene expression; bitter acids; xanthohumol

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1. Introduction

Hop (*Humulus lupulus* L.) is an industrial plant cultivated mainly for brewing, but it is also used in the cosmetology industry, improving skin elasticity and strengthening hair, as well as in the pharmaceutical industry for its sedative and sleeping properties, to relieve menopausal symptoms, and to slightly lower blood pressure. Hop is a rich source of valuable compounds that influence the bitterness, aroma, and preservation of beer and have health-promoting effects. Most important metabolites are produced in the lupulin glands present in the female inflorescences, i.e., the cones [1]. The main hop secondary metabolites are bitter acids, prenylflavonoids, and essential oils [2].

Bitter acids consist of alpha acids and beta acids, which are a mixture of homologues and analogues. Humulone, co-humulone, and ad-humulone are the major components of alpha acids, but pre-humulone and post-humulone are present in trace amounts [3]. The corresponding beta acids include similar analogues, i.e., lupulone, co-lupulone, ad-lupulone, pre-lupulone, and post-lupulone [4]. Bitter acids are derived from pyruvate precursors formed in the pathway leading to branched-chain amino acids (BCAAs), specifically, such as leucine, isoleucine, and valine. The final step in this pathway is catalysed by the enzyme BCAT2 (branched-chain amino transferase 2). Leucine is degraded in the mitochondrion by the enzyme BCAT1 (branched-chain amino transferase 1) and BCKDH (branched-chain keto-acid dehydrogenase) and then converted to phlorisovalerophenone

(PIVP) in the cytosol by valerophenone synthase. PIVP is transformed into deoxyhumulone by prenyltransferase (PT) and then to lupulone or humulone with humulone synthase (HS) [5].

Xanthohumol is the main prenylflavonoid found in hop cones. It accounts for nearly 1% by weight of the dried cones [1]. It is formed from phenylalanine, coming from the shikimate pathway. Phenylalanine is converted by phenylalanine ammonia lyase (PAL) to trans-cinnamate and then to 4-coumarate. Next, coumarate coenzyme A ligase (4CL) catalyses transformation of 4-coumarate into p-coumaroyl-CoA. In turn, this compound is converted to naringenin chalcone in a reaction catalysed by chalcone synthase (CHS). Naringenin chalcone, with the activity of the prenyltransferase PT1, is converted to demethylxanthohumol, which is methylated to xanthohumol by the O-methyltransferase, OMT1 [2,6–8].

Metabolic pathways are regulated by a number of genes encoding enzymes and transcription factors. Chalcone synthase is coded by gene *CHS_H1*, which was described by Matoušek et al. [9,10]. It belongs to the same family as *VPS* and shows homology to it. Gene *VPS*, encoding valerophenone synthase, was first described by Okada and Ito [11]. It belongs to the gene family *CHS*, which includes more than a dozen homologues [9]. Indeed, the VPS also has a weak activity of chalcone synthase (CHS) and is engaged in the phenylpropanoid pathway, but only to a small extent. *VPS* is active, especially in cones, while in other organs it is weakly expressed. Also, genes of enzymes such as BCAT1, CHS_H1, HS1 and HS2, PT1, PT2, and OMT1 are expressed much higher in cones than in other parts of a plant [7,12,13]. *VPS*, *PT1*, *PT2*, and *BCAT1* genes are 100 to 170 times more active in lupulin glands than in leaves [13].

The efficiency of the metabolic pathways leading to the production of bitter acids and xanthohumol is determined by the activity of genes encoding enzymes. However, their activity is influenced by transcription factors (TF) or the complexes they form. Many of these TFs belong to the families bHLH, MYB, WDR, and WRKY [7,14]. Transcripts of genes important in the regulation of prenylflavonoid production, such as bHLH2, WDR1, WRKY1, MYB1, MYB2, MYB3, and MYB7, were found not only in lupulin glands, but also in bracts and leaves, with the latter present in much lower amounts [7,15]. WRKY1 activates promoters of genes encoding chalcone synthase H1, valerophenone synthase, prenyltransferases, and O-methyltransferase 1 [16]. Moreover, WRKY1 has the ability to autoactivate by influencing its promoter and is regulated by post-transcriptional silencing (PTGS). Further, it stimulates the expression of gene *MYB3*, which is active, especially in lupulin glands [15]. The promoter of *CHS_H1* is regulated by MBW complexes (MYB3/bHLH2/WDR1, MYB2/bHLH2/WDR1, or bHLH2/WDR1) [5]. The binary complexes WRKY1/WDR1 and WDR1/MYB1 activate the *OMT1* promoter. Also, TF MYB8 activates gene *CHS_H1* but simultaneously represses the activity of genes *OMT1* and *PT1*, which are involved in biosynthesis of prenylflavonoids and bitter acids.

A number of factors influence the activity of genes, including the cultivar (genotype), organ, and developmental stage of the plant. Furthermore, the physiological condition of the plant is also a factor that can influence the activity of genes. This is connected with environmental conditions, including the nutritional status of the plant. Hop plants require relatively large amounts of nitrogen due to their rapid growth. The nitrogen requirements of hop are determined on the basis of the expected yield, typically ranging from 150 to 225 kg/ha per year [17,18]. Chemical fertilisers are primarily used to meet the nitrogen requirements of the crop. The overuse of fertilisers has a detrimental impact on human health and the environment, leading to a range of adverse effects on the soil [19,20]. In order to reinforce efforts to protect ecosystems and human health, the European Commission has devised the Farm to Fork strategy, which aspires to establish a healthy and environmentally friendly food system. One of the objectives of this strategy is to reduce the utilisation of chemical fertilisers by at least 20% by 2030, thereby mitigating the adverse effects on the environment [21]. Nitrogen is a crucial nutrient required for plant growth, playing a

fundamental role in numerous biochemical and physiological processes in plants, including the synthesis of proteins, nucleic acids, chlorophyll, hormones, and enzymes [20]. Therefore, in order to maintain plant productivity with reduced nitrogen supply, it is necessary to enhance the nitrogen use efficiency.

The nitrogen dose exerts a significant influence on both the yield and various aspects of hop cone quality [22]. In general, an increase in nitrogen dosage results in a higher yield of hops. However, as the nitrogen dose increases linearly, the alpha acid content shows a decreasing trend [22,23]. Furthermore, the nitrogen level affects the expression of genes in plants [24–26]. The successful application of real-time PCR and transcriptome analysis has enabled the identification of key genes involved in the biosynthesis of bitter acids and xanthohumol in hop plants [14,16,27,28]. Consequently, the study of gene expression could enhance the understanding of the impact of nitrogen reduction on secondary metabolite production in hops. The aim of this study was to examine the impact of a reduced nitrogen dosage on hop plants at the molecular level and assess its influence on the concentration of important secondary metabolites.

2. Materials and Methods

2.1. Field Experiment and Plant Material

A field experiment was conducted in three replicates on the hop fields at the Research Station of the Institute of Soil Science and Plant Cultivation in Jastków, Poland (Lublin province—51°18′9″ N, 22°25′1″ E). Two hop cultivars: Lubelski (aroma) and Magnat (bitter), were used in this study. Plants were grown with a spacing of 3.0 × 1.5 m on a 7 m trellis, with 2 strings per plant and 2 bines trained up each string. Both cultivars were treated with good agronomic practices, including disease and pest control. Two different nitrogen doses were applied. The first dose (N1) was in accordance with the recommended amount for production plantations, while the second dose (N2) was 25–30% lower. The basic nitrogen dose (N1) was determined for each cultivar separately based on the predicted yield of cones. Nitrogen doses of 140 kg/ha (N1) and 100 kg/ha (N2) in the form of ammonium nitrate were applied to the Lubelski cultivar, while 200 kg/ha (N1) and 150 kg/ha (N2) were applied to the Magnat cultivar. Each nitrogen dose was divided into three parts and applied at three different times: the first part after spring cutting (BBCH09), the second after training (BBCH36), and the third before flowering (BBCH51). A plot consisted of four rows running the length of the plantation, which was approximately 0.2 ha in size. Each nitrogen dose was replicated three times in a randomised complete block design.

The cone samples were collected from plants three times during the vegetation season, starting from the BBCH79 stage when nearly all cones reached maximum size but were still soft in texture, through to the BBCH85 stage of advanced maturity when 50% of cones were compact, up to the BBCH89 stage, i.e., technological maturity, when cones were ripe for picking. The cones were collected from the upper part of the plants, at approximately 6 m. The cones were stored at −80 °C until they underwent molecular and chemical analyses. For each date, the cones from 5–9 plants within each cultivar were collected as separate samples.

2.2. Gene Expression Analyses

2.2.1. RNA Extraction and Purification

The hop cones were ground in liquid nitrogen in mortars. RNA was extracted from 100 mg of tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the procedure recommended by the manufacturer. After extraction, the RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Then, RNA was purified, eliminating DNA. For this, the mixture containing 1 U DNase I Amp Grade (1 U/μg; Invitrogen, Carlsbad, USA) and 1 μL of 10x DNase I reaction buffer was added to 1 μg of RNA sample, completed with DEPC-

treated water up to the volume of 10 µL in 1.5 mL of RNase-free Eppendorf tubes on ice. The samples were incubated for 15 min at room temperature. Next, the DNase I was inactivated by the addition of 1 µL of 25 mM EDTA solution and heating at 65 °C for 10 min. Then, the RNA Integrity Number (RIN), RNA concentration, and 28S/18S ribosomal (rRNA) ratio of each purified RNA sample were measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waltbronn, Germany) with the use of RNA Nano Chips (Agilent Technologies, Waltbronn, Germany). The samples with a RIN value above 7 were classified as good-quality ones and were used as a template for cDNA synthesis.

2.2.2. Reverse Transcription

High-quality RNA was reverse transcribed. The reaction for first-strand cDNA synthesis was prepared in 0.2 mL tubes. The mixture contained 1 µg of RNA, 1 µL of Oligo(dT)$_{20}$ primer (50 µM; Invitrogen, Carlsbad, CA, USA), and 1 µL of 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP, and dTTP at neutral pH; Invitrogen, Carlsbad, CA, USA) and was increased to 13 µL with sterile Mili-Q water and heated to 65 °C for 5 min, and then incubated on ice for at least 1 min. Then, 4 µL of 5× first-strand buffer, 1 µL of 0.1 M DTT (Invitrogen, Carlsbad, USA), 1 µL of RNaseOUT™ Recombinant RNase Inhibitor (40 units/µL; Invitrogen, Carlsbad, USA), and 1 µL of SuperScript™ III RT (200 units/µL; Invitrogen, Carlsbad, USA) were added, and the mixture was incubated at 50 °C for 60 min, followed by inactivation at 70 °C for 15 min. The concentration of cDNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at a wavelength of 260 nm.

2.2.3. Real-Time PCR

The amount of cDNA was normalised for all samples to 5 ng/µL and it was used as a template in the real-time PCR reaction. The volume of the reaction mixture was 10 µL and contained the following: 4 µL of cDNA template, 0.15 µL each of 10 µM forward and reverse primers (Table 1), 5 µL of RT HS-PCR Mix SYBR (A&A Biotechnology, Gdańsk, Poland), which included 0.1 U/µL of Polimeraza DNA Taq, 4 mM of $MgCl_2$, dNTP mix (0.5 mM of dATP, dGTP, dCTP, and dTTP), and 2× reaction buffer with SYBR Green, as well as sterile water. The fluorescence acquisition was carried out at a wavelength of 510 nm.

The amplification was performed in Rotor-Disc 100 on a Rotor-Gene 6000 cycler (manufactured by Corbett Research, Australia). Each reaction started from an initial denaturation and polymerase activation step (95 °C for 3 min) and was followed by 40 cycles (95 °C for 5 s, 58 °C for 60 s, and 72 °C for 20 s). The amplicons were confirmed by melting, raising the temperature from 72 °C to 95 °C. Data were analysed with Rotor-Gene Q Series software 2.3.4.3. Each sample was replicated three times. The amplification of a gene of interest (GOI) and two reference genes (normalisers) was carried out in parallel for each sample. For all reactions, the same sample was used as the calibrator, and its expression equalled 1. The expression of the studied genes was determined as a multiple of the calibrator.

The expression of 15 genes of interest encoding enzymes or transcription factors involved in biosynthesis of bitter acids and xanthohumol was analysed relative to two reference genes (Table 1). The relative values of each GOI were calculated for each sample using the delta–delta method and corrected according to Pffafl [29], if needed.

Table 1. Analysed genes of interest, reference genes, and primers used for real-time PCR.

Gene	Encoded Protein	Primers (5′-3′)	Reference
	Bitter acid synthesis		
BCAT1	Branched-chain amino acid aminotransferase 1	ACGCTCCTCAACCTTGAAACC CGCCTGCTGCAAATGGTAC	[13]
VPS	Valerophenone synthase	GGGAAAATCAGCTTGGTTGA GGCGTCCGTAACTGTAGAGC	[27]

PT1	Prenyltransferase 1	ACAACGTATTCCGCAGAGAAGAG TCCAGGCTCGAGAGTTGGAT	[13]
HS1	Humulone synthase 1	GCGCAAGCTTTCTACCCAA CGCCTGGCATCCAATGAC	[13]
HS2	Humulone synthase 2	TGCGTGTGCAAGCTTTCTAC GGCATCCAATGACTCCCAATTTAG	[13]
	MEP pathway		
PAL	Phenylalanine ammonia lyase	CCGAAGTCTTGTCAGCCATT TGGGGTGATGTCCTAAGAGC	[30]
4CL2	Coumarate CoA ligase	CTCACCGGGCTCGTTATAGG TGTGGCACCGTAGTTAGGAA	[13]
CHS_H1	Chalcone synthase	ATCACTGCCGTCACTTTC AAATAAGCCCAGGAACATC	[30]
OMT1	O-methyltransferase 1	TAAAGGAACAGTGGTGGACGTTG ACCGCATCAGCACTAGGAATTGA	[30]
	Transcription factors		
bHLH2	Transcription factor bHLH2	AGCGGGTTGACTAGTGTGGAT ACTTGCACCGTTGTCTCTGG	[31]
WDR1	Transcription factor WDR1	TTGCTTAGATTGGCTTGGAATAA CCGGCTGAGCAGATATGTCTAT	[31]
WRKY1	Transcription factor WRKY1	CCCTTCGTCTTTCGTTAACCTC GGCCGTCAAGAACAACAAATTTCC	[13]
MYB7	Transcription factor MYB7	CCAACGCAGCCTACAACTCTTG GCTGGCGCTCACTACCTA	[13]
MYB8	Transcription factor MYB8	AGGTGGCGTGGCTTATTGAC GGAGCAATAAACGTGGGAGATTG	[13]
MYB78	Transcription factor MYB78	GGCGAAACCTGAGTCCCAAA TACACGCCGGAGAACTCTA	[13]
	Reference genes		
7SLRNA	7SL component of the signal recognition particle	TGTAACCCAAGTGGGGG GCACCGGCCCGTTATCC	[32]
YLS8	Yellow leaf specific protein 8	CGTACCTGCTTCCACATTTGC TCCCAGTCGTGGCCAAAA	[33]

2.3. *Determination of the Amounts of Bitter Acids and Xanthohumol*

Immediately after collection from the plant, the hop cones were placed in dry ice and transported to the laboratory. Then, they were frozen at −80 °C and freeze-dried. The samples were stored in a desiccator until analysis.

The content of bitter acids and xanthohumol was determined by the HPLC method using an Agilent Technologies 1200 Series chromatograph with a UV-VIS detector (Agilent Technologies, Santa Clara, CA, USA). The freeze-dried cones were ground and samples of about 10 ± 0.001 g were extracted for 40 min in 160 mL of toluene (HPLC, Avantor Performance Materials, Gliwice, Poland), 0.1 M HCl (AR, Avantor Performance Materials, Gliwice, Poland), and methanol (HPLC, Avantor Performance Materials, Gliwice, Poland) in a ratio of 100:40:20 *v*/*v*/*v*. Then, 5 mL of the extract was collected and diluted to 50 mL with methanol. The diluted extract was transferred to 2 mL chromatography vials. The compounds were separated on a Nucleodur 5-100 C18 ec, 125 × 4 mm HPLC column (Macherey-Nagel GmbH, Germany). A methanol, distilled water, and orthophosphoric acid mixture (HPLC, VWR Chemicals BDH, USA; 775:210:9 *v*/*v*/*v*) was used as the mobile phase. The following conditions were applied: the injection volume was 5 µL, the flow

rate of the mobile phase was 1 mL/min, and the column temperature was 40 °C. The determination of xanthohumol was carried out at 370 nm for the first 6 min, followed by the determination of alpha and beta acids at 314 nm. The total analysis time was 25 min. The identification and calculation of alpha acids, beta acids, and xanthohumol was based on the retention times of the International External Standard (ICE 3; Labor Veritas, Zürich, Switzerland), with a known composition of bitter acids and an external standard of xanthohumol (Applichem, Darmstad, Germany) with a purity of 99%. Retention times for individual compounds were as follows: xanthohumol t_R = 3.21 min; co-humulone t_R = 7.22 min; ad-humulone + humulone t_R = 9.0 min; co-lupulone t_R = 13.8 min; ad-lupulone + lupulone t_R = 17.9 min.

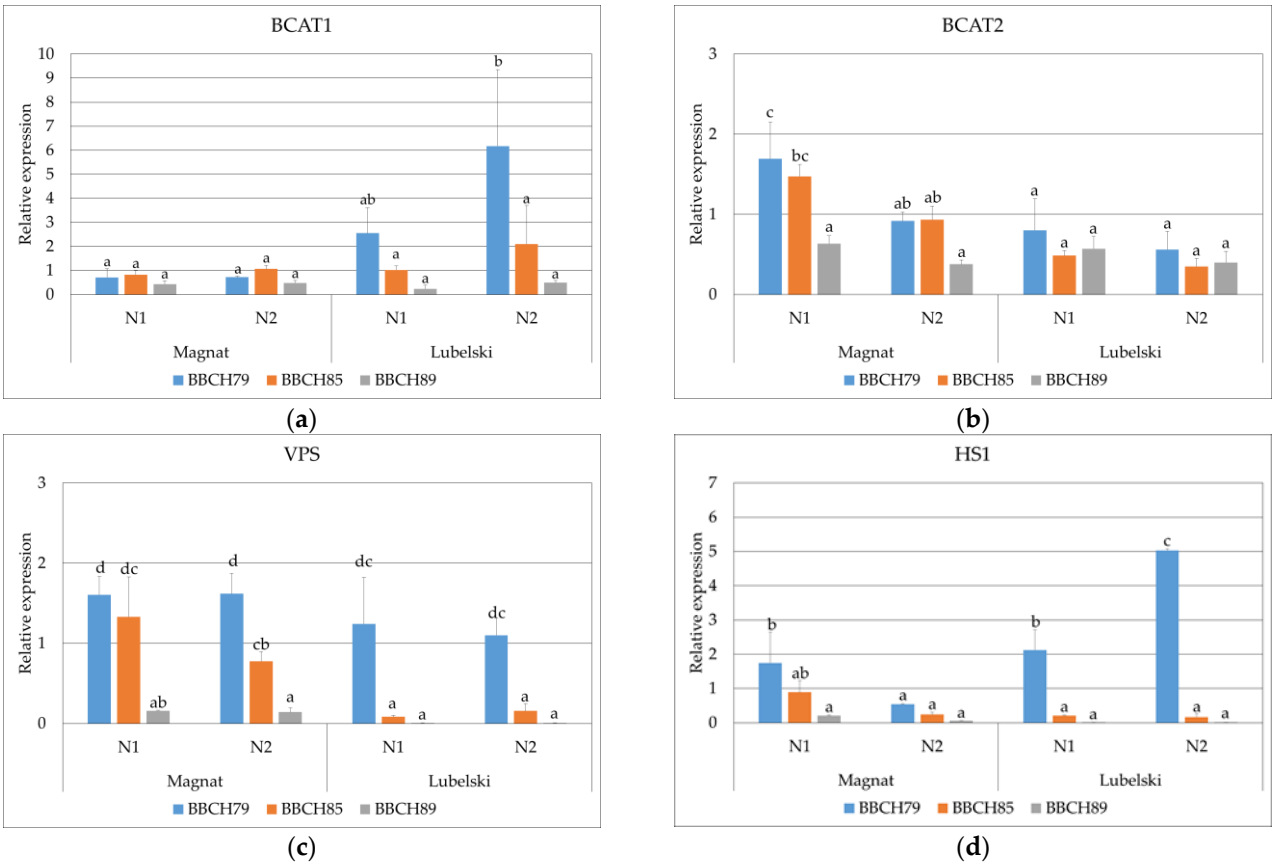
2.4. Statistical Analyses

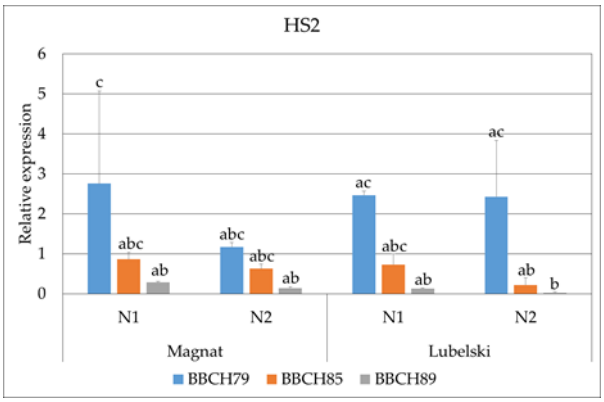
Statistical analysis was run with Statistica 13.3. The contents of bitter acids and xanthohumol, as well as values of relative expression calculated for each plant on three dates, were collated and subjected to analysis of variance (ANOVA) with repeated measures and Tukey's test, performed at a significance level of $p \leq 0.05$.

3. Results

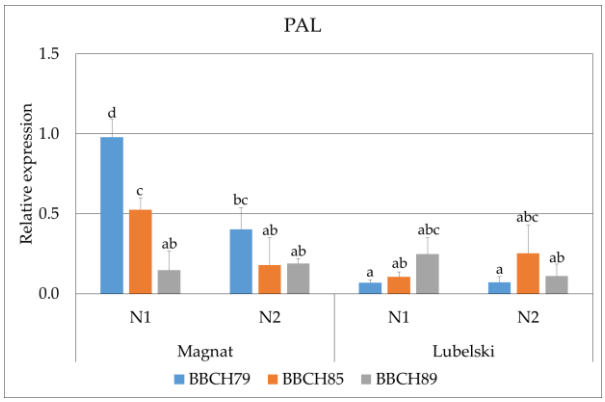
3.1. The Expression of Genes Involved in the Synthesis of Bitter Acids

The relative expression of genes coding enzymes involved in biosynthesis of bitter acids (BCAT1, BCAT2, VPS, PT1, PT2, HS1, and HS2) was determined in cones collected at various stages of plant development (Figure 1).





(**e**)



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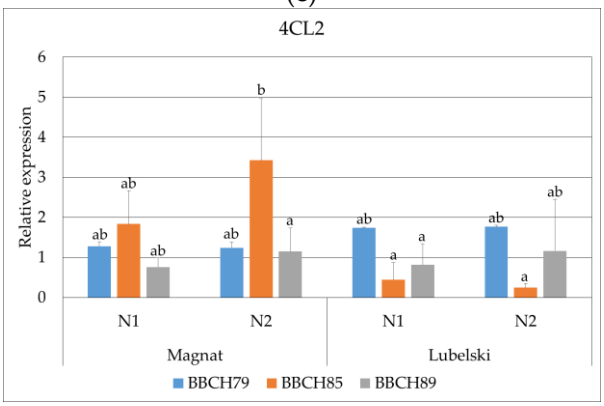

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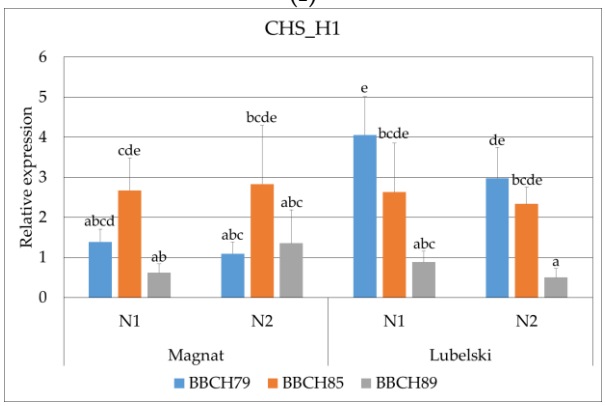

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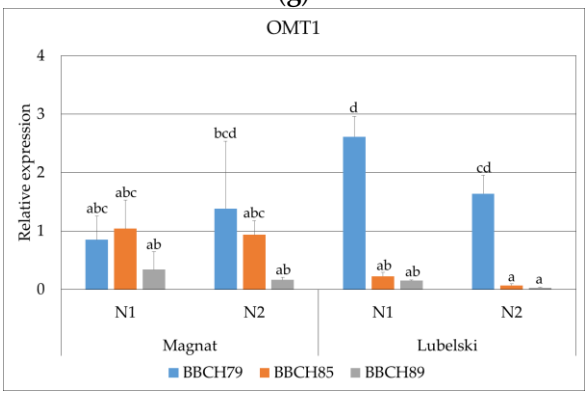

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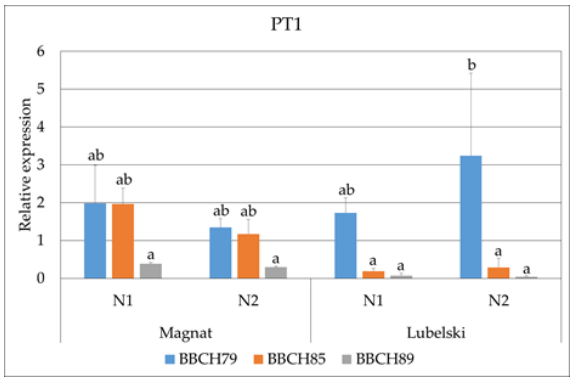

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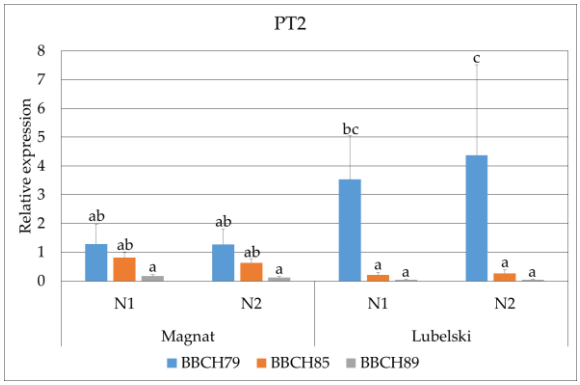


(**k**)

Figure 1. The relative expression of genes involved in the biosynthesis of bitter acids: *BCAT1* (**a**), *BCAT2* (**b**), *VPS* (**c**), *HS1* (**d**), and *HS2* (**e**), and of xanthohumol: *PAL* (**f**), *4CL2* (**g**), *CHS_H1* (**h**), and *OMT1* (**i**), as well as in both pathways: *PT1* (**j**) and *PT2* (**k**), in the cones of two hop cultivars (Magnat and Lubelski) in three BBCH phases, under fertilisation with a standard nitrogen dose (N1) and a dose reduced by 25–30% (N2). Developmental stages of plants: BBCH79 (nearly all cones reached

maximum size but were still soft in texture), BBCH85 (advanced maturity, 50% of cones were compact), and BBCH89 (technological maturity, cones were ripe for picking). Different letters denote statistical differences (according to Tukey's test at a probability level of $p \leq 0.05$).

The expression level of the BCAT1 gene in the bitter cultivar Magnat was low, regardless of the developmental stage and the level of nitrogen fertilisation (Figure 1a). In contrast, the aroma cultivar Lubelski showed higher expression of the *BCAT1* gene in cones coming from plants at BBCH79 compared to the later developmental stages. Under reduced fertilisation (N2), this expression was significantly higher (8.6 times) in the case of Lubelski compared to the bitter cv. Magnat. The *BCAT2* gene, which is responsible for the final step in the biosynthesis of branched-chain amino acids, remained consistently low in activity in cv. Lubelski (Figure 1b). In contrast, cv. Magnat exhibited high expression of this gene when plants were fertilised with a standard dose, but it gradually reduced by more than half in the technically mature cones. *VPS*, which is the key gene involved in bitter acid biosynthesis, showed the highest activity in the first developmental stage studied for both cultivars (Figure 1c). However, in the case of Magnat, its level decreased progressively with the maturity of the cones. In contrast, for Lubelski, the reduction in *VPS* activity was rapid. The decrease between the first and second terms was 15.5-fold and 7-fold for N1 and N2 fertilisation, respectively. The expression of *HS1* and *PT2* under reduced fertilisation was significantly higher on the first date for Lubelski than for Magnat (Figure 1d,k). In more developed Lubelski cones, a significant decrease of 11-, 17-, and 31-fold was noted in the expression of genes *PT1*, *PT2*, and *HS1*, respectively (Figure 1d,j,k). On the other hand, in the case of Magnat, although there were differences in gene expression at successive BBCH stages, they were not statistically significant. Both cultivars also showed a high expression level of gene *HS2* in the first studied developmental stage (Figure 1e). However, there were no significant differences between cultivars in subsequent stages.

Summing up, the reduction in the nitrogen dose resulted in a decrease in the expression of genes *BCAT2* and *HS1* in cv. Magnat. Conversely, in cv. Lubelski, the *HS1* gene demonstrated increased activity under reduced fertilisation. The genes *BCAT1*, *PT2*, and *HS1* were more active in Lubelski, while *BCAT2* was more active in Magnat. The genes *PT1* and *HS2* demonstrated no significant differences in activity between cultivars. In the youngest cones studied (BBCH79), the highest levels of activity were observed for most of the genes responsible for bitter acid production. The expression of *VPS* in Magnat declined at a slow rate, while in Lubelski it declined rapidly.

3.2. The Expression of Genes Involved in the Synthesis of Xanthohumol

Xanthohumol is produced in the phenylpropanoid pathway, which involves a number of enzymes and, consequently, a number of genes encoding them. The activity of certain genes was observed to change depending on the plant developmental stage and/or cultivar.

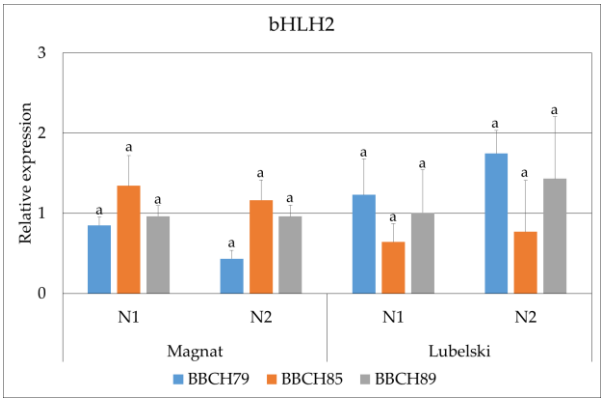
Specifically, changes were observed in the activity of the *PAL* gene encoding an enzyme involved in the first step of the pathway, but only in plants of the cultivar Magnat fertilised with the standard nitrogen dose. At the BBCH79 stage, the highest level of expression was recorded, which next decreased twice at the BBCH85 stage and then 3.5 times at the BBCH89 stage (Figure 1f). In contrast, for the same cultivar, the *4CL2* gene exhibited the highest expression at the BBCH85 stage and at the reduced nitrogen dose, which showed that it was significantly more active than in cv. Lubelski at the same developmental stage (Figure 1g). For Lubelski, there were no significant differences in *PAL* and *4CL2* gene expression between the cone-harvesting dates or between the fertilisation levels. The *CHS*_H1 gene showed the highest activity in cones of the Lubelski cultivar at the BBCH79 stage, and this was 2.9-fold and 2.7-fold higher than in Magnat at the same developmental stage when N1 and N2 fertilisation was applied, respectively (Figure 1h). For each cultivar, no significant differences were noted between nitrogen doses for the *OMT1*

gene. However, comparing cultivars, the expression of this gene was lower for Magnat in the youngest cones coming from plants fertilised with a standard dose (Figure 1i). The phenylpropanoid pathway leading to the synthesis of xanthohumol also involves *PT1* and *PT2* genes, whose activity was described above.

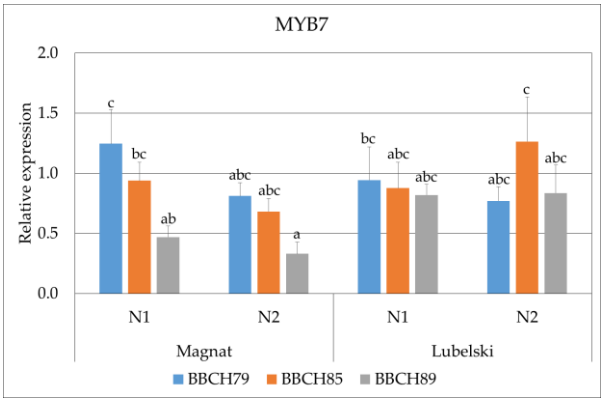
The activity of genes involved in the production of xanthohumol, such as *PT2*, *PAL*, *CHS_H1*, and *OMT1*, was different depending on the cultivar (Figure 1f,h,i,k). Reducing the nitrogen dose resulted in a decrease in activity of the gene *PAL*, but only in cv. Magnat (Figure 1f). Interestingly, the highest expression of the *CHS_H1* and *4CL2* genes was recorded in Lubelski at the BBCH79 stage, while in Magnat they reached the highest activity at a later stage, i.e., BBCH85 (Figure 1g,h).

3.3. The Expression of Genes Encoding Transcription Factors

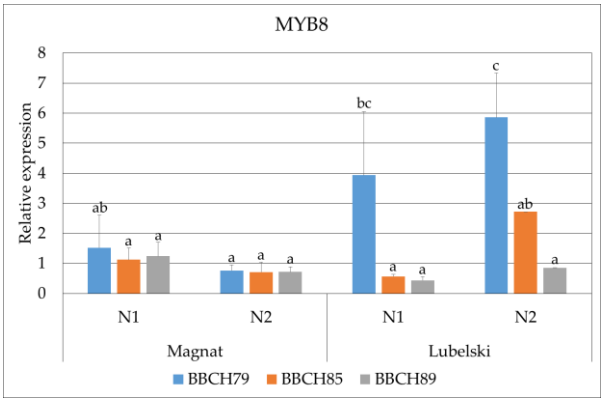
TFs regulate the expression of enzyme genes. Therefore, we studied the expression of some TF genes. No significant differences in TF gene expression were observed depending on the nitrogen dose (Figure 2a–f).



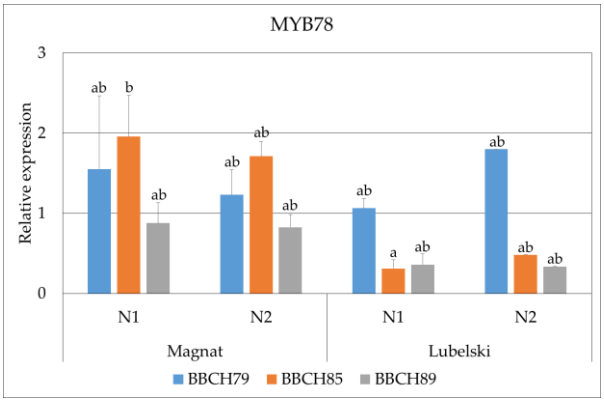
(**a**)



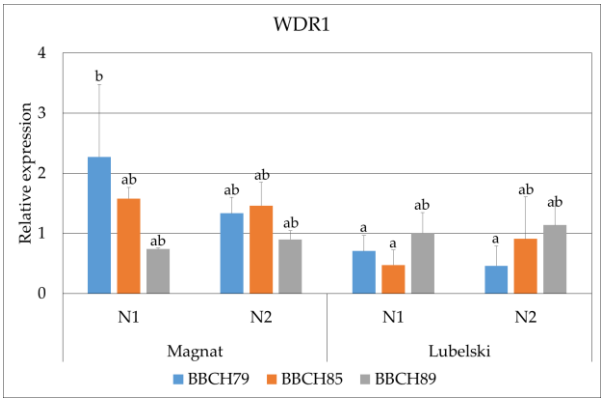
(**b**)



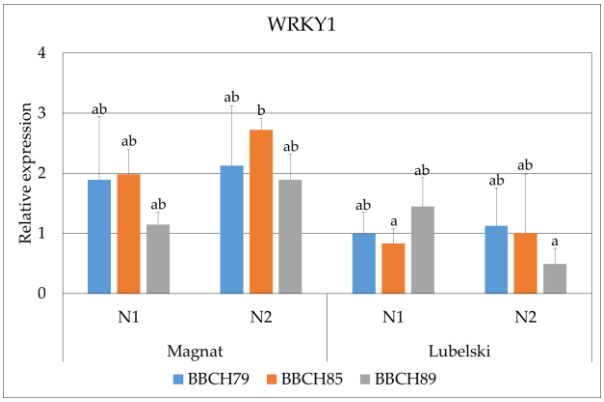
(**c**)



(**d**)



(**e**)



(**f**)

Figure 2. The relative expression of transcription factor genes involved in the biosynthesis of bitter acids and xanthohumol: *bHLH2* (**a**), *MYB7* (**b**), *MYB8* (**c**), *MYB78* (**d**), *WRD1* (**e**), and *WRKY1* (**f**), in the cones of two hop cultivars (Magnat and Lubelski) in three BBCH phases, under fertilisation with a standard nitrogen dose (N1) and a dose reduced by 25–30% (N2). Developmental stages of plants: BBCH79 (nearly all cones reached maximum size but were still soft in texture), BBCH85 (advanced maturity, 50% of cones were compact), and BBCH89 (technological maturity, cones were ripe for picking). Different letters denote statistical differences (according to Tukey's test at a probability level of $p \leq 0.05$).

However, a few differences in gene activity were observed between cultivars. At the BBCH79 stage and under N1 fertilisation, the *WDR1* gene was more strongly expressed in cones of Magnat than in those of Lubelski (Figure 2e). Also, the expression of the *MYB78* gene was higher in Magnat at standard fertilisation, but only in older cones at the BBCH85 stage (Figure 2d). Conversely, the *MYB8* gene was found to be significantly more active in cv. Lubelski than in cv. Magnat in cones at the BBCH79 stage when reduced nitrogen fertilisation was applied (Figure 2c). Furthermore, in Magnat, gene expression remained relatively stable throughout successive plant developmental stages, but in the case of cv. Lubelski, it changed. At the BBCH79 stage, *MYB8* gene expression was significantly higher compared to older cones, with a 7-fold and 2.15-fold increase under N1 and N2 fertilisation, respectively.

3.4. The Content of Secondary Metabolites

Hop cultivars Magnat and Lubelski used in this study represented bitter and aroma types, respectively. The cultivars differed significantly in their alpha and beta acid content. At the stage of technological maturity (BBCH89), the alpha acid content in the bitter cultivar Magnat was 14.64% and 14.89% for N1 and N2 nitrogen doses, respectively, while in the aroma cultivar Lubelski it was only 7.34% and 7.98%, depending on the nitrogen dose (Figure 3a).

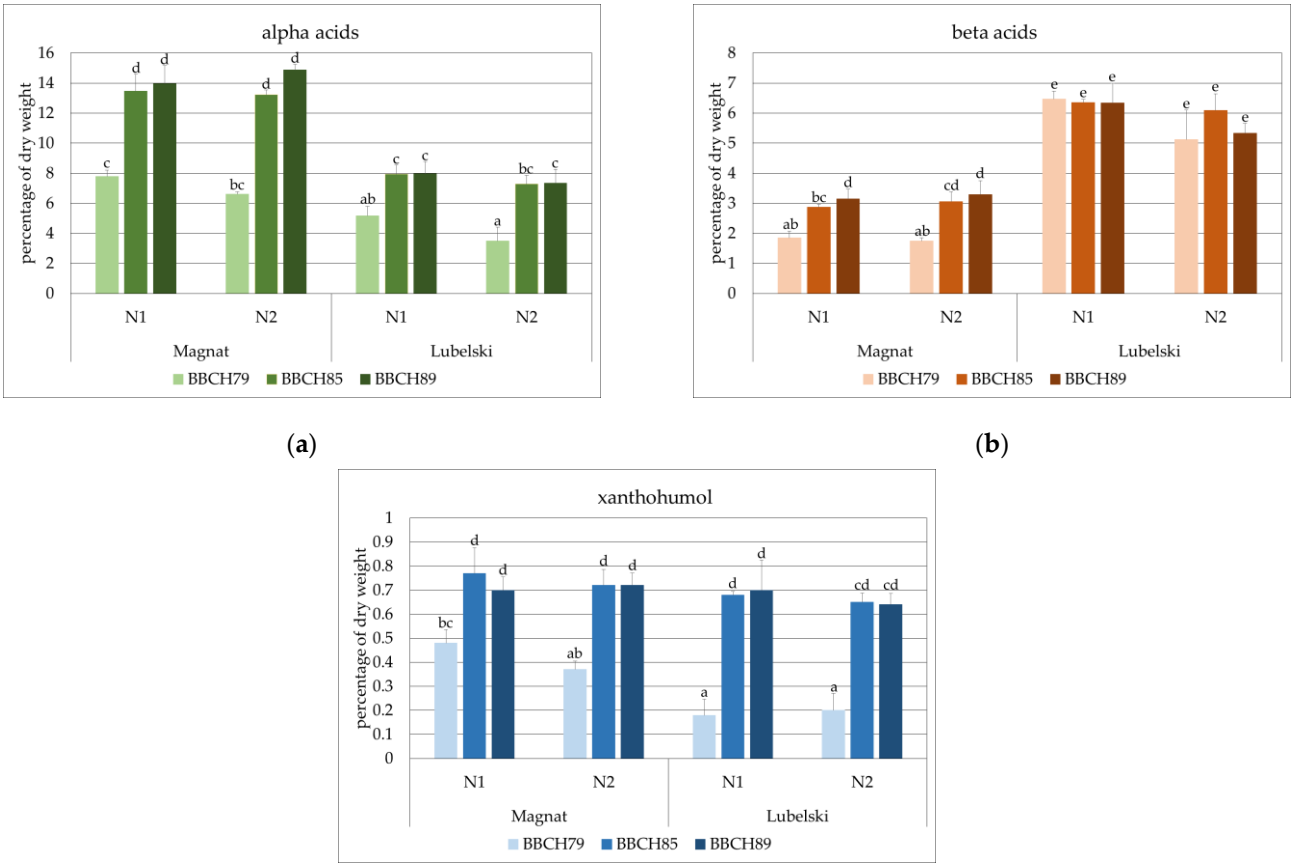


Figure 3. Changes in the content of secondary metabolites: alpha acids (**a**), beta acids (**b**), and xanthohumol (**c**), in hop cones of two cultivars (Magnat and Lubelski) depending on the plant developmental stage. Nitrogen fertilisation: N1—standard, and N2—reduced by 25–30%. Developmental stages of plants: BBCH79 (nearly all cones reached maximum size but were still soft in texture), BBCH85 (advanced maturity, 50% of cones were compact), and BBCH89 (technological maturity, cones were ripe for picking). Different letters denote statistical differences (according to Tukey's test at a probability level of $p \leq 0.05$).

The beta acid content ranged from 3.15% to 3.29% in the cones of Magnat and from 5.33% to 6.34% in the cones of Lubelski depending on the nitrogen dose (Figure 3b). The cultivars exhibited differences in their alpha to beta acid ratio. Magnat had a very high alpha/beta ratio of over 4, while Lubelski had a ratio close to 1. The investigated cultivars did not significantly differ in terms of xanthohumol content, which ranged from 0.64% to 0.72% at the stage BBCH89 (Figure 3c).

The nitrogen rate did not significantly affect the alpha acid content. Although the alpha acid content was lower at the reduced N rate (N2), particularly on the first sampling date, the differences were not statistically significant. As the cones matured, the differences in the alpha acid content between the N doses decreased. This trend was observed for both cultivars tested. Nitrogen dose also had no significant impact on the content of beta acids and xanthohumol.

The developmental stage of the cones significantly affected the alpha acid content of both cultivars. Regardless of the nitrogen dose applied, the lowest content of these compounds was observed in cones sampled at the first date (BBCH79). At this date, Magnat showed an average alpha acid content of 7.2%, while Lubelski had 4.3% (Figure 3a). At the next sampling date (BBCH85), the alpha acid content increased significantly, reaching 13.3% and 7.6% for Magnat and Lubelski, respectively. The third sampling date showed no significant change in alpha acid content compared to the second date. A similar trend

was observed for xanthohumol content in both cultivars tested (Figure 3c). Beta acid content tended to increase with maturity for Magnat, while for Lubelski the content remained consistent across all sampling dates (Figure 3b). The chromatogram obtained by HPLC analysis of secondary metabolites is presented in Figure S1.

4. Discussion

4.1. The Impact of Plant Developmental Stages on Gene Expression

A high level of gene expression preceded increased production of secondary metabolites. The highest expression levels of bitter acid metabolic pathway genes were observed in cones sampled on the first date, i.e., about four weeks before harvest. At subsequent developmental stages, the activity of these genes decreased, although the differences were not statistically significant in all cases. Differential expression of genes depending on the developmental stage of cones was also observed by Eriksen et al. [34]. These researchers found, based on transcriptome analysis of cones, that most of the genes involved in bitter acid biosynthesis were upregulated in mid-stage cones, i.e., about two weeks before harvest, and in late-stage cones they were strongly downregulated. Castro et al. [28] investigated the expression of *VPS* at various developmental stages, starting from the initial stage of cone development to the late stage, characterised by maximum size and the first signs of maturation. They found that mid-stage cones (which had a soft texture and a size of 1–3 cm) reached the highest level of gene expression, which remained stable in late-stage cones (showing initial signs of maturation). The mid- and late-stage cones corresponded to phases BBCH79 and BBCH85, the first two phases taken into account in our study. In contrast to Castro et al. [28], we observed a reduction in *VPS* gene expression as cones matured. In Lubelski, it occurred as early as the BBCH85 stage, while in Magnat, depending on the level of nitrogen fertilisation, it occurred at the BBCH85 stage (lower N dose) or the BBCH89 stage (higher N dose). We observed changes in the expression of genes involved in the phenylpropanoid pathway during the development of cones, but rarely were they statistically significant. Most of the genes showed the highest activity at the BBCH79 stage, with the exception of *4CL2* and *CHS_H1*. These genes showed higher expression levels at the BBCH85 stage, but only in Magnat, which was later compared to Lubelski. The *PT1* gene, which is involved not only in bitter acid synthesis but also in the phenylpropanoid pathway [35], also showed the highest expression at the BBCH79 stage, but a significant difference was observed only in the Lubelski cultivar under the reduced N dose. The last stage of xanthohumol production is methylation catalysed by enzyme OMT1. It was shown by Nagel et al. [36] that the *OMT1* gene had the highest expression in the mid-stage cones in lupulin glands. Similarly, in our study, significantly higher expression of this gene was observed in cv. Lubelski at the BBCH79 stage, which corresponds to the middle stage of cone development. At the next developmental stage, the activity of this gene declined rapidly. In cones of the Magnat cultivar, high *OMT1* activity was maintained for a longer time, up to the BBCH85 stage, after which a decrease in expression was observed. Considering that most of the studied genes involved in metabolic pathways of bitter acids and xanthohumol showed the highest level of expression at the BBCH79 stage, it should be assumed that this stage is crucial in terms of the biosynthesis of these metabolites. Significantly low levels of alpha acids and xanthohumol were observed at this stage of development, irrespective of the cultivar and the nitrogen dose. High gene expression at the same time as low metabolite levels suggests that metabolic pathways are just being activated. A decline in expression with cone maturation was observed in genes that were previously identified as specifically active in lupulin glands, including *BCAT1*, *VPS*, *PT1*, *PT2*, *HS1*, *HS2*, *CHS_H1*, and *OMT1* [7,13,27,36]. In contrast, other genes, including *BCAT2*, *PAL*, *4CL2*, and the majority of TF genes, exhibited increased expression in both lupulin glands and bracts [7,13,14,27]. Consequently, we observed minimal changes in the activity of these genes.

4.2. The Impact of the Cultivar Type on Gene Expression

The hop cultivars included in our study differed significantly in their alpha acid content, while they were similar in terms of xanthohumol content. Biosynthesis of both bitter acids and xanthohumol occurs in lupulin glands, the number and size of which affect the content of these compounds [37–39]. Variation in the expression of most studied genes between these cultivars was also observed, especially at lower levels of nitrogen fertilisation. *BCAT1*, *PT2*, *HS1*, and *CHS_H1* genes were more active in the cones of aroma Lubelski, whereas *BCAT2*, *VPS*, *PAL*, and *4CL2* genes were more active in the bitter Magnat. Patzak et al. [13] did not report differences in *VPS* and *PT2* gene activity between the bitter and aroma cultivars, while significant differences for *BCAT1* and *PT1* genes were observed. Moreover, the genes encoding humulone synthases HS1 and HS2 were 5.8 times more highly expressed in bitter cultivars. Our studies showed that *BCAT1*, *PT2*, and *HS1* were less active in bitter Magnat than in aroma Lubelski, particularly when plants were fertilised with a reduced N dose. *HS2* expression was similar in both cultivars. Novak et al. [40] observed higher activity of genes encoding chalcone synthase in hop cultivars with higher bitter acid content, while our study showed that the *CHS_H1* gene was more active in aroma Lubelski than in bitter Magnat. The higher activity of this gene may be due to the increased activity of the *MYB8* gene, which was also noticed in Lubelski. MYB8 does not form complexes with other TFs but it acts as a single TF [30]. Studies conducted on transgenic hop overexpressing the *MYB8* gene showed that the resulting transcription factor activated the expression of the *CHS_H1* gene and genes important in flavonoid biosynthesis. At the same time, MYB8 had an inhibitory effect on the expression of *OMT1* and *PT1* genes involved in the final steps of prenylflavonoid and bitter acid biosynthesis. Thus, the transcription factor has the ability to redirect the *CHS_H1* gene product towards the synthesis of flavonols while reducing the synthesis of desmethylxanthohumol, xanthohumol, as well as bitter acids. This action of MYB8 may explain the sudden significant decrease in the activity of the *OMT1* and *PT1* genes in early cones of the Lubelski cultivar observed in our studies. According to Patzak et al. [13], the genes for the transcription factors MYB7, MYB8, MYB78, and WRKY1 are expressed at similar levels in individual tissues regardless of whether the test material is an aromatic or bitter cultivar. In contrast, they showed that the *bHLH2* gene was significantly more active in the lupulin glands of aromatic cultivars than of bitter ones.

The activity of *OMT1* and *PT1* genes can also be regulated by transcriptional factors WRKY1 and WDR1. In addition, WRKY1 stimulates *MYB3* gene expression in lupulin glands [16]. Increased prenylflavonoids and bitter acids in transgenic hop with overexpression of *WRKY1* and *WDR1* genes indicate the involvement of WRKY1/WDR1 proteins in stimulating the MBW complex and the OMT1 enzyme [5]. Determination of gene expression showed significantly higher activity of *WRKY1* than *WDR1*. This is due to the ability of the transcription factor WRKY1 to activate the expression of its encoding gene [16]. This gene is controlled by a promoter that has a binding site for its product protein, and *WRKY1* gene autoactivation thus occurs. The WRKY1 and WDR1 factors, together or independently of each other, have an activating effect on the *OMT1* and *PT1* genes. They may also, in cooperation with the MBW complex, indirectly activate the *CHS_H1* gene [5]. However, our research showed only small differences in the expression of *WRKY1* and *WDR1* genes, which did not affect the activity of genes encoding enzymes.

Other genes, *PAL* (in the early stage) and *4CL2* (in the middle stage and N2 fertilisation), had higher activity in cv. Magnat than in Lubelski. Finally, the content of xanthohumol was similar in both cultivars.

4.3. The Impact of Nitrogen Fertilisation on Gene Expression

Our research showed that the gene expression studied in hop cones was generally not dependent on the level of N fertilisation. Zhao et al. [25], in a transcriptome analysis of Wels onion, showed that about 1665 genes were differentially regulated with different concentrations of nitrogen. The impact of N treatment on gene expression of the flavonoid pathway was studied in *Cyclocarya paliurus* under hydroponic conditions [41]. It was

found that the expression of genes *PAL*, *CHS*, and *4CL*, as well as *F3'H*, was significantly increased when the N dose was doubled. However, further increases in the nitrogen dose resulted in a gradual decrease in activity of the genes. In our study, the nitrogen dose was not decreased drastically, but by 25–30%. Reduced nitrogen availability might cause stress in plants. In hop, the effect of various stresses on the activity of genes responsible for the production of bitter acids has been studied, but the effect of reduced nitrogen fertilisation has not yet been investigated. The various stress conditions may affect the activity of genes. *HS1* and *HS2* were downregulated in cultivars Sládek, Saaz, Premiant, and Agnus infected with the viroid HLVd, and the content of bitter acids was reduced by more than 30% in comparison with healthy plants [42]. The infection with the hop stunt viroid also caused a decrease in the activity of the gene *CHS_H1*, as well as the gene encoding transcription factor *bHLH2*. Simultaneously, the expression of another gene, *MYB3*, increased [31]. These two TFs form, with WDR1, a ternary complex activating *CHS_H1*. As a result, the authors observed a decrease in the production of some metabolites. However, in our study, generally, we did not observe significant changes in the expression of TF genes between the levels of fertilisation. This may indicate that a reduction in the nitrogen dose by 25–30% was not a stress factor for the studied hop cultivars.

4.4. The Impact of Nitrogen Fertilisation and Plant Developmental Stages on the Content of Bitter Acids and Xanthohumol

Nitrogen is a crucial compound for plant growth. Its deficiency can have a negative impact on yield [20]. However, excessive N rates can reduce the product quality [43,44]. This adverse effect may be due to a shift in metabolism towards amino acid and protein synthesis at higher nitrogen doses, leading to increased competition for carbon during secondary metabolite synthesis [45]. Iskra et al. [22] found that increasing the nitrogen rate from 90 to 269 kg/ha resulted in a decreasing trend in alpha acid content, although the differences between the nitrogen rates were usually not significant. However, the amount of nitrogen fertiliser did not affect the beta acids. This finding is consistent with the results obtained by Senske [23]. In our study, we did not observe any statistically significant effect of the nitrogen dose on the alpha acid content. The maximum N doses were calculated separately for each cultivar based on its yield potential, determined by long-term observations. Therefore, these doses were not excessive and, as a result, no negative effect was observed on the alpha acid content. Similar to Iskra et al. [22] and Senske [23], the beta acid content did not show a consistent trend related to the N dose. This confirms that plants can compensate for lower fertiliser rates by more efficiently taking up and utilising available nitrogen [46].

The impact of the nitrogen dose on xanthohumol content has not been studied yet. We found that, similar to alpha and beta acids, the nitrogen dose did not have a significant effect on xanthohumol content. Hop bitter acids and xanthohumol are synthesised in lupulin glands (glandular trichomes) [37]. The metabolite content is positively correlated with the number and size of lupulin glands [37,38], and strongly dependent on their developmental stage [39,47]. It can be assumed that the cone maturity, as well as environmental and weather conditions, similarly affect the metabolite content.

The data indicated that the content of alpha acids and xanthohumol in both cultivars tested depended on the maturity of the cones. The greatest increase in the content of the tested metabolites occurred just after the cones reached full size. During the maturation of the cones, about two weeks before harvest, the content of these compounds did not change significantly. The results are in agreement with De Keukeleire et al. [48], who found that alpha acids and xanthohumol content increased significantly during the development from female inflorescences to cones. Eriksen et al. [34] concluded that the middle stage of cone development is the most critical period for the production of bitter acids, prenylated flavonoids, and volatile compounds. They also found a significant decrease in the expression levels of genes putatively involved in the final step in alpha acid synthesis in the period of close to harvest. This is in accordance with our findings, which indicated

that there were no significant changes in the concentration of both bitter acids and xanthohumol at the same stage of cone development.

The exception was the beta acid content of the Lubelski cultivar, which had already reached 99% of its maximum content at the earliest date of the study. In contrast, the alpha acids had only reached 56.5% of their maximum content at the same date. These results confirmed the findings of De Keukeleire et al. [48] that the formation of beta acids begins before alpha acids.

Our results showed that a reduction in nitrogen rates by 25–30% did not lead to a decreased result in hop quality. Furthermore, although comprehensive biometric assessments were not conducted, observations made throughout the growth period did not reveal differences in plant growth and development. This finding may encourage growers to consider using lower fertiliser rates.

5. Conclusions

Due to their intensive growth, hop plants have high fertiliser requirements. However, the heavy use of fertilisers poses a threat to ecosystems. It, therefore, seems beneficial to reduce the fertilisation rate to a level that still allows a high yield of good quality to be achieved. In the above study, the nitrogen dose was reduced by one-quarter compared with standard fertilisation. The reduction in nitrogen did not cause much disruption to the metabolic pathways leading to the formation of bitter acids and xanthohumol. This was evidenced by the relative expression of genes. Indeed, the majority of them demonstrated comparable activity regardless of the nitrogen dose applied. Under conditions of reduced N fertilisation, there was a significant decrease in the activity of several genes only in the young cones. However, this did not result in a reduction of secondary metabolites in mature cones. The majority of the observed differences in gene activity were between cultivars (bitter and aroma) and according to the maturity of the cones. This indicated that genetic factors and the developmental stage were of greater importance than nitrogen fertilisation. Consequently, a reduction in nitrogen fertilisation to approximately 75% of the standard dose may be recommended in hop cultivation, as it ensures an adequate content of industrially important compounds while reducing the risk of environmental pollution by excess nitrogen.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14081680/s1, Figure S1: HPLC profiles of secondary metabolites, alpha acids, beta acids, and xanthohumol, in hop cones.

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