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Dr. Elisabeth Seigner
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Contents

Foreword

León in Spain is – at least in Germany – a well-known city as a way-station for pilgrims on the Camino de Santiago (Way of St. James) leading to Santiago de Compostela. The sign-posts with the scallop as the typical symbol for this pilgrimage route lead along hop yards and quite a few pilgrims may have been wondering about this special crop or about the empty trellis systems in fall.

As chairman and secretary of the Scientific Commission of the International Hop Growers´ Convention we are happy to welcome you to this pilgrimage place and now we would like to draw your attention to our special crop “hop”. We are pleased to welcome again our colleagues from 14 nations every two years to this exchange of information. In addition to those hop growing nations that take part regularly we highly appreciate the attendance of our colleagues from Slovakia and Ukraine.

In 23 papers and 17 posters the latest results on hop breeding (classical cross-breeding and biotechnological methods as well), pest and disease management in hops, hop chemistry and the improvement of production techniques will be presented. Furthermore, we are looking forward to learning more about hop production in Spain.

We are happy that at least few experts from the brewing and hop industry are using this meeting to keep in touch with hop scientists from all fields to be informed about the latest research topics and results.

Our special thanks go to Mr. José Antonio Magadán, Technical Manager of S.A. Española de Fomento del Lúpulo, for all his efforts and all his time he spent in organizing this meeting here in León. We highly appreciate the support of the General Manager of S.A. Española de Fomento del Lúpulo Mr. Jacobo Olalla to hold this meeting here. We are grateful to Professor Dr. Mª Ángeles Revilla Bahillo for supporting Mr. Magadan in his work for the Scientific Commission and we thank all persons who have contributed their part to the successful outcome of this meeting.

We hope that this meeting will be a success, with a lot of interesting contributions and fruitful discussions.

Bernhard Engelhard — Chairman

Dr. Elisabeth Seigner — Secretary

I.H.G.C. Scientific Commission

Vorwort

Leon in Spanien ist, zumindest in Deutschland, eine bekannte Stadt am berühmten Jakobsweg, der nach Santiago de Compostela führt. Die ‚Muschel', mit der dieser Pilgerweg ausgezeichnet ist, führt direkt an Hopfengärten vorbei und mancher Pilger wird sich schon über die seltsame Kultur oder die leeren Hopfengerüste im Herbst gewundert haben.

Als Vorsitzender und Sekretärin der Wissenschaftlichen Kommission des Internationalen Hopfenbaubüros freuen wir uns, Sie an diesem Pilgerort begrüßen zu können und die Kultur Hopfen in den Mittelpunkt zu stellen. Wir freuen uns, wieder Kolleginnen und Kollegen aus 14 Nationen zu unserem alle zwei Jahre stattfindenden Erfahrungsaustausch willkommen heißen zu können. Neben den regelmäßig teilnehmenden Nationen mit Hopfenbau freut es uns, dass in diesem Jahr auch die Slowakei und die Ukraine vertreten sind.

In 23 Vorträgen und 17 Postern wird ausführlich über die neuesten Ergebnisse aus den Bereichen Hopfenzüchtung (konventionelle Züchtung und biotechnologische Methoden), Bekämpfung von Schadorganismen am Hopfen, Hopfenchemie und Verbesserung der Produktionstechnik berichtet. Wir sind auch gespannt, mehr über den Hopfenanbau in Spanien zu erfahren.

Wir sind froh, dass wenigstens einige Experten aus der Brau- und Hopfenwirtschaft durch ihre Teilnahme an diesem Meeting, die Möglichkeit nutzen, mit den Hopfenwissenschaftlern aus allen Fachbereichen in Kontakt zu bleiben und sich über die neusten Forschungsschwerpunkte und Ergebnisse zu informieren.

Besonderen Dank an Herrn José Antonio Magadán, Technischer Manager des S.A. Española de Fomento del Lúpulo, für all seine Mühen und seine Zeit, die er für die Organisation dieser Tagung aufgewendet hat. Wir danken dem Generalmanager des S.A. Española de Fomento del Lúpulo Herrn Jacobo Olalla für seine Unterstützung, dieses Meeting hier abzuhalten. Wir möchten auch Frau Prof. Mª Ángeles Revilla Bahillo für ihre Mithilfe danken, um Herrn Magadan bei seinen Arbeiten für die Wissenschaftliche Kommission zu entlasten. Schließlich sagen wir auch allen Danke, die ihren Teil zum Gelingen dieser Tagung beigetragen haben.

Wir wünschen, dass die Veranstaltung für Sie alle ein Erfolg wird, mit vielen interessanten Beiträgen und wertvollen Diskussionen.

Bernhard Engelhard
Vorsitzender

Dr. Elisabeth Seigner
Sekretärin

IHB, Wissenschaftliche Kommission

Lectures and Posters

I. Session:

Hop Breeding

THE INHERITANCE OF RESISTANCE TO APHIDS FROM THE NEW UK VARIETY 'BOADICEA'

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Abstract

The infestation by hop aphid, *Phorodon humuli*, of 508 seedling hops from 17 progeny families was assessed approx. 6 weeks after the start of the aphid migration in 2008. These progenies were from crosses made in 2005 and 2006 where one of the parents carried resistance to the hop aphid. Seedlings, including those in families derived from 'Boadicea', segregated for resistance. Results agreed well with the action of a single dominant major gene for resistance which has not been eroded during the development of 'Boadicea', or the other parents, from the original source material. Generally, alleles for resistance segregated independently in true Mendelian manner. Samples were harvested from female seedlings which combined resistance to aphids with the potential for an economic yield of hops. HPLC analyses indicated that resistance had successfully been combined with a wide range of analytical traits without any indications of negative genetic interactions.

Keywords: *Phorodon humuli*, resistance, Boadicea, breeding

Introduction

Since the discovery (Darby, 1994) of strong field resistance of hops to their main pest, damson-hop aphid, *Phorodon humuli*, much work has gone into incorporating the resistance into a commercial hop variety. The original source, INT101, was wild germplasm obtained from an uncultivated mountainous region of Japan and it lacked most of the attributes required for commerce. A suitable selection was not obtained until the third generation from the source. This selection was submitted for registration for EU Plant Variety Rights (PVR) under the name 'Boadicea' and was granted full PVR protection in 2008. Currently, it is the only commercial variety available with strong resistance to aphids.

However, the incorporation of such resistance is a feature of several of the primary objectives for the new NHA hop breeding programme in the UK (Darby, 2007). It is necessary to extend the range of germplasm with resistance to aphids to include the full range of analytical characteristics so as not to limit the progress which can be made towards incorporating resistance to aphids in new UK hop varieties.

Initial studies on the control of inheritance of aphid-resistance indicated the action of two dominant major genes (Darby, 1994). Subsequent breeding work and reanalysis of the original data has suggested that these are unequal in their effectiveness. Strong field resistance, as shown by 'Boadicea', is conferred by just one of these genes, the other conferring weaker resistance which is only effective in some seasons. This paper examines the hypothesis of a single major gene for resistance to aphids in 'Boadicea', comparing the pattern of inheritance from 'Boadicea' with other third and fourth generation parents from the original INT101 source.

Methods

Seedling hop plants from crosses made in 2005 and 2006 to incorporate resistance to aphids were planted in the field during spring 2007. Seedlings were planted in family blocks. These progeny, comprising 17 different families, all derived from crosses where one parent was known to carry resistance to aphids. Eight crosses were made with 'Boadicea', five crosses were made using three different aphid-resistant male parents, all the third generation from the original source, and a further four crosses were made with a single male parent from the fourth generation from the source material.

During 2008, all progeny received a full protective spray programme except that no insecticides were used. The migration of the winged form of the damson-hop aphid into the crop occurred during late May and early June and an exponential increase in numbers of the wingless forms on hop leaves followed. All 508 seedlings were individually assessed for the level of infestation approx. 6 weeks after the start of the migration and before the numbers started to decline due to the action of predatory species. Main bine leaves at 3m above ground, and lateral leaves at 3m and at the top of the plants were sampled and examined for the presence of aphids. In addition, six plants of both INT 101 and 'Boadicea' planted randomly within the trial area were scored "blind" for their resistance, as references. A plant was regarded as susceptible if any leaf was found to have more than three wingless aphids.
The sex of the seedlings was assessed and those female plants which had been recorded as resistant to aphids and which produced an economic yield of cones at harvest were selected and individually harvested. Cone samples, dried to 10% moisture, were analysed by HPLC for their resin characteristics.

Results and Discussion

All selections showing resistance to aphids within the UK hop breeding programme have been derived from a single source, INT 101, including its great-granddaughter 'Boadicea'. In this trial, no aphids were recorded on any of the plants of 'Boadicea', as well as on two plants of INT 101. The remaining four plants of INT 101 supported just one or two aphids on a leaf at the time of assessment. Thus, it would appear that the expression of resistance in 'Boadicea' has not been diluted during the several generations of crosses between it and the source material.
Seedlings segregated for the expression of resistance with adjacent plants in the field showing different responses. Given the undiluted expression of resistance between generations and the segregation of individuals within a family, the data was tested by chi-square analysis against a model for the action of a single dominant major gene for resistance (Table 1). Testing the segregation of resistance against an expectation of 50% resistance indicated, over 17 families, a highly significant deviation from this expectation. However, overall heterogeneity between families was not significant and, in almost all families, there appeared to be a surplus of susceptible phenotypes. The definition of resistance as supporting up to only three aphids per leaf is a much more severe definition than that used previously where up to 30 aphids per lateral was considered to indicate resistance. It is likely, therefore, that a small proportion of those individuals scored as susceptible could have been misclassified. The agreement between observed and expected improved as the level of misclassification increased until, at 41% resistance, there was no significant deviation from expectation. Thus, these data are consistent with the control of resistance to hop aphids being under the action of a single dominant major gene and where simple recognition of resistance phenotypes (as <4 aphids/leaf) captured >80% of the resistant population.
The absence of significant heterogeneity between families at this level of resistance indicates that there were no differences in the segregation of resistance between progenies derived from 'Boadicea' and those from the other third or fourth generation parents. This suggests that there has been no genetic erosion of resistance in the development of 'Boadicea' compared to the less commercial parents. Similarly, the action of the resistance allele has not been influenced by which parent carried it, or whether the parent was male or female. Thus, the trait is showing independent Mendelian segregation.
Families 20 – 27 were all derived from 'Boadicea' and families 44 – 47 were all derived from the same resistant male parent. There was, however, some variation between the families in the transmission of the trait. Not withstanding the independent segregation of alleles described above, this suggests that there may also be some very limited specific interactions occurring between parents and, therefore, some crosses may be more efficient than others for the transmission and recognition of resistance.

Table 1. The segregation of resistance to aphids in hop progenies

Cross	Origin	Family	observed		Chi-	Degrees	Prob
	of rest.	size	Rest	Susc	Square[#]	freedom	
20	Boadicea	24	7	17	4.17	1	0.04*
21	Boadicea	23	11	12	0.04	1	0.83
22	Boadicea	24	8	16	2.67	1	0.10
23	Boadicea	27	5	22	10.70	1	0.00***
24	Boadicea	28	12	16	0.57	1	0.45
25	Boadicea	29	17	12	0.86	1	0.35
26	Boadicea	29	8	21	5.83	1	0.02*
27	Boadicea	26	10	16	1.38	1	0.24
39	F3, parent 1	41	19	22	0.22	1	0.64
40	F3, parent 2	26	12	14	0.15	1	0.69
41	F3, parent 2	43	17	26	1.88	1	0.17
42	F3, parent 3	42	16	26	2.38	1	0.12
43	F3, parent 3	17	5	12	2.88	1	0.09
44	F4, parent 4	45	20	25	0.56	1	0.46
45	F4, parent 4	28	8	20	5.14	1	0.02*
46	F4, parent 4	12	7	5	0.33	1	0.56
47	F4, parent 4	44	8	36	17.82	1	0.00***
Totals			**190**	**318**			
	50% rest.	**Expect**	**254.00**	**254.00**	**57.60**	**17**	
		Deviations			**32.25**	**1**	**0.00*** **
		Hetero.			**25.34**	**16**	**0.06**
	41% rest.	**Expect**	**208.28**	**299.72**	**28.91**	**17**	
		Deviations			**2.72**	**1**	**0.10**
		Hetero.			**26.19**	**16**	**0.05**

assuming a single dominant major gene model.

* significant deviation from expectation at 5% probability.

*** significant deviation from expectation <1% probability.

A wide range of analytical values were recorded in the aphid-resistant progeny (Table 2). Clear family differences were apparent and the results from families 20 – 27 show that the aphid-resistant progeny of 'Boadicea' contain individuals which extend the range, for all parameters, beyond that found in 'Boadicea'. In general, the full commercial range of analytical characteristics has been found amongst aphid-resistant seedlings in this trial. There was no indication from these data of negative genetic interactions which would limit combination of resistance to aphids with desirable analytical traits. It will be possible, therefore, to develop putative parental hop lines with a broad range of analytical characteristics in combination with resistance to hop aphids.

It can be concluded that 'Boadicea' will be a useful parent for breeding for resistance to aphids; the resistance remains as strong as from the original source material and appears unlikely to be diminished by further breeding cycles to combine the trait with other desirable characteristics.

Table 2. Ranges of HPLC analysis values from aphid-resistant seedlings harvested during 2008.

Family	Alpha %	Cohum. %	Beta %	Alpha :beta	DMX* mg/100g	Xn** %
20	4.0 - 8.4	14 - 31	2.2 - 4.5	1.5 - 2.6	28 - 64	0.26 - 0.43
21	5.3 -11.0	25 - 30	3.2 - 5.3	1.4 - 3.1	61 -126	0.30 - 0.49
22	5.9 - 8.1	23 - 32	2.7 - 5.1	1.6 - 2.9	28 - 56	0.26 - 0.37
23	6.0 -10.4	29 - 35	3.0 - 5.2	1.2 - 2.7	83 -145	0.33 - 0.85
24	4.6 - 7.3	29 - 42	2.7 - 3.3	1.4 - 2.2	46 - 78	0.24 - 0.40
25	4.0 - 8.5	29 - 41	2.6 - 4.4	1.5 - 2.1	47 - 77	0.18 - 0.44
26	1.4 - 6.8	24 - 33	0.8 - 4.0	1.6 - 2.5	7 - 74	0.07 - 0.40
27	5.5 - 9.3	24 - 35	1.9 - 3.8	2.2 - 3.3	37 - 78	0.24 - 0.41
39	6.0 - 9.1	22 - 39	2.5 - 4.8	1.7 - 2.7	55 - 74	0.32 - 0.46
40	4.8 - 5.8	29 - 39	1.7 - 2.8	1.7 - 3.4	28 - 51	0.28 - 0.35
41	3.9 - 8.4	24 - 30	3.4 - 5.3	0.8 - 1.9	57 - 88	0.23 - 0.50
42	5.6 - 9.1	28 - 33	3.3 - 5.2	1.7 - 1.8	55 - 76	0.34 - 0.47
43	7.0	27	3.2	2.2	53	0.42
44	6.9 -10.7	28 - 30	3.2 - 5.5	1.9 - 2.2	53 -104	0.37 - 0.43
45	8.1 - 8.5	19 - 38	4.0 - 4.7	1.7 - 2.1	67 -135	0.43 - 0.51
46	10.0 -12.3	32 - 35	5.2 - 5.5	1.8 - 2.4	124 -192	0.30 - 0.40
47	10.5 -12.7	35 - 48	4.1 - 4.5	2.3 - 3.1	32 - 73	0.67
Boadicea	5.4 - 7.1	24 - 26	3.3 - 3.9	1.5 - 1.9	55 - 72	0.27 - 0.38

* DMX is desmethylxanthohumol

** Xn is xanthohumol

Acknowledgements

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VARIABILITY OF WILD HOPS

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Abstract

Hop Research Institute in Zatec has a collection consisting of nearly 220 wild hops. Chemical analyses show at considerable variability within the contents and structure of hop resins. Wild hops are typical of lower contents of alpha acids (average 2.18%) and higher contents of beta acids (3.33%). Wild hops from North America show higher contents of cohumulone and colupulone than wild hops from Europe and Caucasus. Generally, wild hops have low contents of essential oils (0.27%). Ratio of the individual compounds can be very variable. Higher contents of myrcene are typical for wild hops from North America. On the contrary, the highest quantity of caryophyllene was found out in wild hops originated from Caucasus. The highest contents of farnesene and selinene were determined in wild hops sampled within Europe and Caucasus. On the base of the analyses we can conclude that the tested wild hops belong to the individual groups according to their origin: European, Caucasian and North American.

Keywords: hop, *Humulus lupulus L.,* wild hops, hop resins, essential oils, DNA analyses

Introduction

Wild hops are very important within breeding programs. They are typical of wide genetic variability and thus they enrich the collection of hop genetic resources. Many wild hops have been selected by natural selection and in this way they have obtained some important characteristics (Nesvadba *et al.* 2007). These features are utilized in breeding aimed at tolerance and resistance to diseases, pests and drought. A great deal of contemporary breeding material is over-bred and depression is obvious. Wild hops used in breeding programs help to split progenies showing high vitality.

Wild hops show genetic, chemical and phenotype variability. It is necessary to transfer wild hops into field conditions to confirm that needed characteristics are based genetically and not influenced by environment. It is very difficult to assess tolerance and resistance, as the infection pressure in hop-yards is much higher than in natural habitats of wild hops. Hop Research Institute in Zatec takes wild hops collection trips every year. Its gene fond contains wild hops from Europe: Czech Republic, Austria, Belgium, France, Spain, Switzerland, etc. A great number of these plants have their origin in Caucasus as well as in North America (US, Canada). In 2008 we managed to get wild hops from Kirghizia in Asia.

Methods

Plant material

Monitoring of wild hops occurrence is carried out every year. In spring new localities are searched for and leaves sampled to do DNA analyses. Later since August to October these wild hops are evaluated (descriptions, occurrence of pests and diseases). Hop cones are sampled in perspective wild hops to carry out chemical analyses. After the assessment is finished perspective wild hops are selected and planted in a special hop-yard. Descriptions as well as chemical analyses are made in this stage as well. The aim is to confirm the characteristics, which were the plants sampled for in their natural biotopes. This assessment process was performed in the period 2005 - 2008. Totally 136 wild hops were researched.

Chemical analyses

Dry cones of the tested wild hops were used for chemical analyses aimed at hop resins and essential oils determination during four years. Hop resins were determined according to EBC 7.7. method by liquid chromatography (HPLC) on the column Nucleosil RP

C_{18} (Macherey-Nagel, Germany, 5 μm, 250 x 4 mm) using chromatograph SHIMADZU LC 20A (Shimadzu, Japan) with diode array detectors (DAD) according to Novak *et al.* (2006) and Krofta (2003). Hop essential oils were estimated from vacuum concentrated, water distilled samples by gas chromatography (GC) on capillary column DB 5 (Chromservis, CR, 30 m x 0.25 mm x 0.25 μm film thickness) using gas chromatograph Varian 3400 in the connection with mass detector Finnigan ITD 800 according to Krofta (2003). Compound identification was based on the comparison of GC retention indices and mass spectra with those of authentic compounds. Semi-quantitative evaluation of hop oils composition was performed on the basis of peak areas of individual components and expressed relatively to the total integrated area of all substances involved.

DNA analyses

DNA was isolated from young leaves of all samples according to Patzak (2001). For molecular analyses, we used nine SSR (Hadonou *et al.* 2004, Jakse *et al.* 2002) and three STS (Patzak *et al.* 2007) loci. In a typical PCR reaction (*Taq* PCR master mix kit, Qiagen, FRG) we used the following amplification conditions: 2 min at 94 °C, 35 cycles (30 s at 94 °C; 60 s at 54 °C, 90 s at 72 °C); 10 min at 72 °C. PCR was performed on TGradient thermocycler (Biometra, FRG). Amplification products were resolved via 5% denaturing (8M urea) polyacrylamide gel vertical electrophoresis and visualized by silver-staining (Patzak 2001).

Statistical and genetic diversity analyses

STATISTICA 8.0 CZ (StatSoft, USA) was used for evaluation of chemical analyses data by basic statistic functions, multi-factorial analysis of variance (ANOVA) and t-test module. NTSYS-pc v. 2.11V for WINDOWS (Exeter Software, New York, NY, USA) was used for cluster analysis. The presence or absence of PCR product was coded by 1 or 0, resp., to generate the raw data matrix. Genetic similarity was estimated using Euclidian distance or Jaccard's similarity coefficient The dendrograms were generated using the unweighed pair group method with arithmetic mean (UPGMA) clustering procedure. The correspondence between pairs of matrices was tested with the Mantel (1967) Z statistic and cophenetic correlation analysis.

Results

The average alpha acid content in the tested wild hops is 2.18%, whereas the average beta acid content amounts to 3.33%. Wild hops show higher contents of beta acids. The highest one was found out in wild hops originated from North America (Canada no. 35 = 8.23%) and Holland. The quantity of cohumulone depends on the origin of wild hops. European as well as Caucasian wild hops have cohumulone ratio under 40 % rel. On the contrary wild hops from North America show cohumulone ratio between 43 and 63 % rel.

The lowest essential oil content was determined in Caucasian wild hops (0.04%). On the contrary, the highest contents show wild hops from the USA (ten wild hops from North America have essential oil contents higher than 0.6%). The lowest ratio of myrcene was found out on a wild hop from France (no. 21) and it amounts to 8.7% rel. Only wild hops from North America reach the level of 40% rel. Myrcene ratio lower than 30% rel. is typical for European wild hops.

High variability in caryophyllene quantity is obvious in wild hops from Caucasus. Hop plant no. 93 shows the lowest caryophyllene ratio. Hops from this region are typical of caryophyllene ratio on the level of 20% rel. The lowest ration of humulone was found out in a wild hop from the USA (1.2% rel.). On the contrary the highest one (39.2% rel.) comes from CR. There is no influence of origin on the variability.

Wild hops from North America have also the lowest quantity of farnesene. Ratio of farnesene higher than 15% rel. show only hops from CR and Caucasus (Caucasus no. 34 = 27.4% rel.). Wide variability in selinene contents is typical for Caucasian wild hops. It is interesting that wild hops from North America have not selinene ratio higher than 14% rel.

Table 1: Variability in the contents and structure of hop resins

Parameter	Alpha acids (% w/w)	Beta acids (% w/w)	Ratio alpha/beta	Cohumulone (% rel.)	Colupulone (% rel.)
Min.	0,11	0,16	0,11	13,6	31,7
Max.	6,17	8,23	1,99	63,1	82,9
Mean	2,18	3,33	0,69	32,00	50,19
Standard deviation	1,261	1,340	0,352	14,216	15,852
Coef. variability (%)	57,9	40,3	51,4	44,4	31,6

Table 2: Variability in the contents and structure of hop essential oils

Parameter	Content (% w/w)	Myrcene (% rel.)	Caryophyllene (% rel.)	Humulone (% rel.)	Farnesene (% rel.)	Selinene (% rel.)
Min.	0,04	8,7	4,7	1,2	< 0,1	< 0,1
Max.	0,89	56,1	45,2	39,2	27,4	23,8
Mean	0,27	24,25	11,87	13,19	6,99	8,04
Standard deviat.	0,178	12,473	6,760	9,301	6,737	5,289
Coef. variab. (%)	66,6	51,4	57,0	70,5	96,4	65,8

In general, we have found that there is high molecular genetic variability within European wild hops. But it was necessary to evaluate its range in the context of world hop germplasm. Therefore, we carried out cluster analysis of all 136 hops (Fig. 1). It was evident from dendrogram that European wild hops formed bordered close group, which was grouped together with Euro-Asian wild hops (Caucasus region). Traditional cultivated hops (Osvald's clone 72, Fuggle) were clustered inside European germplasm. Cultivated hops with North American or Asian origin were clustered separately and closely to Euro-Asian germplasm. North American wild hops were clustered to discrete distant group, which was divided into two subgroups of *H. lupulus* var. *neomexicanus* and *H. lupulus* var. *lupuloides*. *H. japonicus* was separated from other hop genotypes.

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Fig. 1. Dendrogram of 136 individual wild hops and varieties revealed by UPGMA cluster analysis based on Jaccard's similarity coefficient determined using 23 STS and 46 SSR markers. CZ – Czech, SWITZ – Switzerland, FRA – France, US – United States, CA – Canada

HOP HYBRIDS ASSESSMENT THROUGH IMPLEMENTATION OF MULTICRITERIA DECISION MODEL

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Abstract

The article presents experimental hop breeding data from the Slovenian Institute of Hop Research and Brewing, which were used for creating a model of an expert system based on a multi-criteria decision modelling methodology DEX. Four different perspective Slovenian hop hybrids, A1/54, A2/104, A3/112, A4/122 and a reference variety with desired characteristics, »R« (Hallertauer Magnum) were used for testing the decision model. The results showed that A3/112 was assessed as the best of hybrids analysed. Test results validate the application of the model for further research.

Keywords: multi-criteria decision analysis, hop breeding, hybrids assessment, DEXi

Introduction

Production of hops (*Humulus lupulus* L.) in Slovenia has a tradition over 100 years. In 1952, the Slovenian Institute of Hop Research and Brewing was established to assist hop growers and to develop new hop varieties suitable for the Slovenian growing conditions and to follow the demands of the brewing industry. A general problem in developing new hop varieties is the long time required (at least ten years) especially because of the characteristics of a hop plant.

Expert systems application in agriculture based on decision methods has increasingly been influencing the decision-making process where computer models become a tool in hands of experts (Pavlovic et al., 2008). In this paper the DEXi multi criteria model for assessment of newly selected hop hybrids with respect to their biological, chemical and brewing characteristics is presented.

Material and methods

The model presents an integration of a knowledge base in a field of hop breeding and an inference engine for problems solving and generation of user oriented solutions. A particular knowledge base of the model therefore consists of a tree of attributes and utility functions. Furthermore, for this purpose a multi-criteria decision making methodology based on DEX is used (Rozman et al., 2006; Pazek et al., 2006). In the first stage of DEX-i decision model development, the possible alternatives are identified (hop hybrids A1/54, A2/104, A3/112, A4/122 and a reference variety »R« (Hallertauer Magnum) and the problem is dissected into individual less-complex problems (hierarchical tree of objectives - criteria). Basic attributes are organized hierarchically into sub attributes and represent farm decision sub problems.

There are four basic attributes / criteria. **Biology criteria** were put in two aggregate criteria. Those for a plant outlook (habitus) were descriptive, while others linked to a level of pest and disease resistance were numerical. **Criteria chemistry** was aggregated from three attributes such as essential oils, aging and bitter substances. In addition, the attribute of bitterness was

distributed amongst parameters like quantity of alpha-acids, beta-acids and cohumulon. All of these parameters possess a numeric set of values. **Morphology criteria** have a main effect on a production technology. Appropriate numeric values sets were given to preliminary qualitative descriptions. Within mechanical analysis characteristics of hop cones such as weight of 100 cones, length, weight and ratio of cone stems, number of nodes, cone density of superior hybrids were evaluated. **Brewing value** is a crucial criterion in assessment of a breeding material to be involved in next steps of the breeding process. In the model qualitative (sensory assessment) as well as quantitative criteria from beer analysis were integrated. This criterion was defined also as an eliminating one.

The corresponding utility functions are represented by defined decision rules. If - then decision rules can be interpreted as: if X1 = value 1 and X2 = value 2 and …and Xn = value n then Y = value (or value interval). In DEX-i, a utility function maps all the combinations of the lower-level attribute values into the values of Y. Utility functions are commonly defined using some from the weighted sum: f (X1, X2, …Xn) = w1 x X1 + w2 x X2 +...wn x Xn (where wn denote weights and Xn denote attributes (Bohanec et al., 2004)).

In the model 27 decision rules were defined. The two main criteria, chemistry and brewing value, had the most important role. Chemistry, as criteria, decides about the hop variety type (aroma, alpha, super-alpha). In the case where a new aroma variety is desired, a set of chemical values must also be defined. Furthermore, the brewing value criteria have an eliminating character. If sensory assessments did not meet minimal expectations of brewing experts, the hybrid was also not appropriate for further hop breeding. The 144 decision rules based on the breeding experiences were expressed by defining their minimum and maximum values. The hop hybrid with the lowest defined values in all parameters equated to the worst assessment result. The hop hybrid with the highest relative assessment result signified the hybrid with the highest optimal set of values.

Results and discussion

The main objectives of the multi-criteria model testing were analysis and evaluation of the hop hybrids included in the research. Thus four different perspective Slovenian hop hybrids A1/54, A2/104, A3/112, A4/122 and the reference variety »R« (Hallertauer Magnum) were used for testing the model. The hybrids involved in a research were analyzed and the results were additionally discussed. The developed decision model enabled the final assessment of hybrids based on defined criteria and decision rules within defined utility functions. For each hybrid involved, a set of values for all criteria was established (determined). The model has 18 criteria of which 13 are basic and five derived. Based on the model results and breeding experiences, the hybrids A3/112 and A4/122 were assessed as superior and prospective for further breeding procedure. On the contrary, A1/54 and A2/104 did not meet expectations related to reference variety R and were, therefore, assessed as being hybrids with little breeding potential.

Moreover, final results showed that A3/112 has been assessed as the best – even in comparison with the R. A3/112 showed its homogeny in its criteria characteristics. In biological criteria, it reached above average mark while in other criteria its marks related with the R were average (Figure 1).

Conclusions

Due to progress in science, cultivars of various agricultural plants have been developed that are better adaptable to the environment. Genetic improvement can be achieved by means of classical or molecular breeding techniques (Cerenak et al., 2005; Seigner et al., 2005). However, the aim of hop breeding is to satisfy the needs throughout the whole chain of hop industry. One-year experimental data obtained from the Slovenian Institute of Hop Research and Brewing have been used for creating a model of an expert system based on DEX which

is one of the multi-attribute decision modelling methodologies. Numerical as well as descriptive variables have been comprised in the data used for creating the model. It enables an estimate of individual hybrids, their ranging and a view of the tolerance limit of individual parameters. Four different perspective hop hybrids marked A1/54, A2/104, A3/112, A4/122 and a reference variety »R« (Hallertauer Magnum) have been used for the test model. Test results enable the application of the model for further research.

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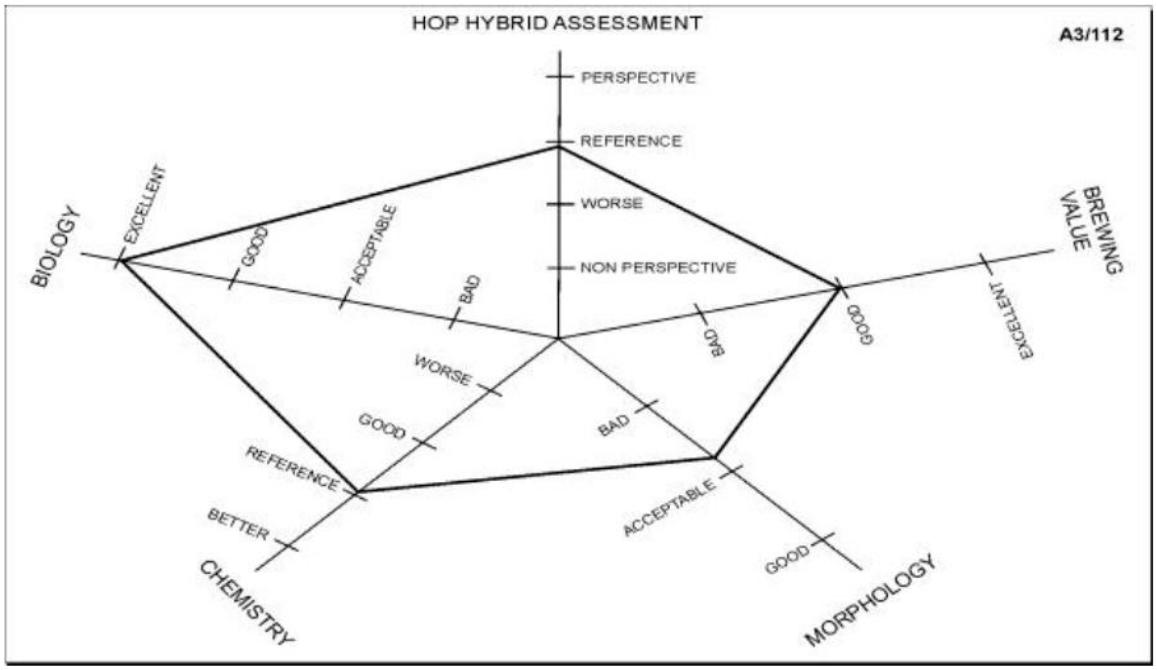

Figure 1. Model assessment of the superior hybrid A3/112

HOP BREEDING ON HIGH CONTENTS OF DESMETHYLXANTHOHUMOL

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Hop breeding had been aimed nearly entirely at the demands of brewing industry for a long time. Nevertheless, hops are becoming still more and more important within pharmaceutical industry recently. The objective is hop breeding on higher contents of DMX, which is known to be isomerized at 8-prenylnaringenin, the most potent phytoestrogen currently known. Hop crossing was carried out according to a methodology worked out by Hop Research Institute in Zatec. A German variety Taurus as well as a Czech variety Vital were chosen for model crossing. A male plant 00/13 originated from Agnus and the other male 00/15 originated from Magnum. The both plants were naturally pollinated – natural choice of pollen. Their seedlings were planted in field conditions. Samples of hops for chemical analyses were taken in the first growing year. DMX analyses were carried out by liquid chromatography on Shimadzu LC 10A and LC 20A instruments. The methodology issued from EBC 7.7 method.

It is evident from Table 1 that Taurus progenies showed lower DMX contents than Vital progenies. Only Vital x OP progenies show statistically conclusive difference within the contents of DMX.

Table 1: Variability in the DMX contents in the studied progenies

Parameter	Taurus x 00/13	Taurus x OP	Vital x 00/15	Vital x OP
Number	179	59	51	72
Min. (% w/w)	0,01	0,05	0,01	0,09
Max. (% w/w)	0,16	0,27	0,27	0,38
Average (% w/w)	0,08	0,11	0,15	0,19
Standard deviation	0,028	0,062	0,041	0,047
Coef. of variability (%)	35,2	32,1	37,4	31,0

A new Czech hop variety “Vital” was registered in 2008 with the help of selection within breeding material in Hop Research Institute in Zatec. Vital shows not only high contents of alpha (14-17%) and beta acids (8-11%) but very high contents of DMX (0.3-0.4%) as well.

Acknowledgement

This work was supported by Czech Ministry of Education within the Research Project no. 2B06011: “The Development of Hop Genotypes for Biomedical and Pharmaceutical Purposes.” Samples were obtained from the field collection of genetic resources, which is a part of “National Program of Conservation and Utilization of Genetic Resources in Plants and Biodiversity” (Mze 33083/03-300 6.2.1) issued by Czech Ministry of Agriculture.

HERKULES – THE NEW HÜLL HIGH ALPHA CULTIVAR

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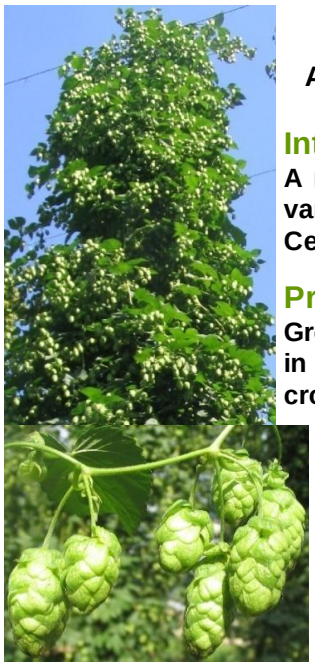
Bav. State Research Center for Agriculture

Institute for Crop Science and Plant Breeding
Hop Research Center Hüll



Herkules – the New Hüll High Alpha Cultivar

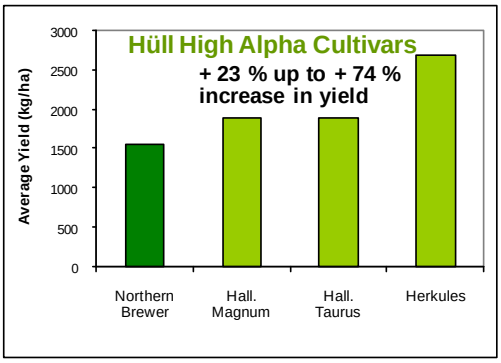
A. Lutz, J. Kneidl, H. Ehrmaier , K. Kammhuber, and E. Seigner

Introduction

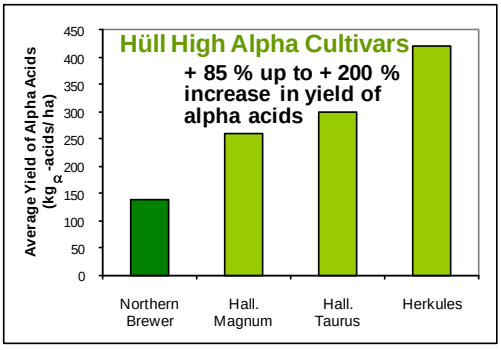
A new robust, high-yielding, high bittering hop cultivar tolerant to various diseases has been released in 2006 by the Hop Research Center Hüll.

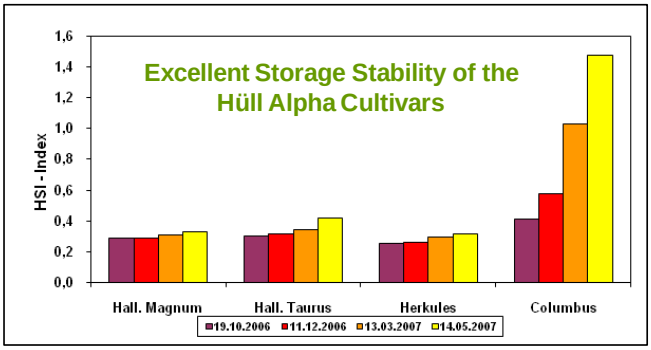
Progress in Breeding

Grown on 1,800 ha in 2008 Herkules proves its impressive progress in breeding for growers and brewers as well. Originating from a cross between "Hallertauer Taurus" x "PM-resistant Hüll male breeding line" the major advantages of Herkules are its stable high yield of 2,700 kg/ha on average and the very high alpha acid content of 15 - 17 % resulting in more than 400 kg alpha acids/ ha. In addition, it provides tolerance to downy mildew, *Verticillium* wilt and resistance to several powdery mildew strains as well as improved tolerance to crown rot. Excellent brewing quality combined with very good storage stability increases the value of Herkules for the brewing industry.



High-Alpha Cultivars	Aroma and Bittering Quality			
	Aroma Points	α-Acids (%)	Cohumulone (%)	kg α-Acids /ha
Northern Brewer	22	8-10	27-32	140
Hallert. Magnum	22	13-15	21-29	260
Hallert. Taurus	22	15-17	20-35	300
Herkules	22	15-17	32-38	420





Herkules has the potential to safeguard the stable supply of quality hops and increase the competitiveness of German hops on global markets in providing stable, very high yield and alpha acid contents together with broad disease tolerance and superior brewing value.

II. Session:

DNA-Based Studies in Hop Research

POWDERY MILDEW ON HOPS (*HUMULUS LUPULUS L.*): HISTOCHEMICAL STUDIES AND DEVELOPMENT OF A TRANSIENT TRANSFORMATION ASSAY

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Abstract

In order to study the pathosystem hop-powdery mildew at the single cell level, histochemical staining techniques have been established. The wheat germ agglutinin-tetramethylrhodamine (WGA-TMR) conjugate staining for chitin allows studying the spatio-temporal development of the fungus on the leaf surface. Staining of callose depositions makes defence mechanisms like the hypersensitive reaction or papilla formation visible. Currently these methods are used to study the cellular basis of resistance in different wild hops, cultivars and breeding lines. These findings may provide a new basis for an integrated breeding approach towards hop cultivars with improved powdery mildew resistance.

Another way to produce resistant plants is genetic engineering. Since the generation of transgenic plants is a time and cost intensive process, we aim at developing an assay for the transient expression of resistance associated genes in hop epidermal cells. Therefore, young leaves are bombarded with gold particles, coated with a reporter gene and the gene of interest. 24 hours after inoculation (hai) of the bombarded leaves with the the hop powdery mildew fungus *Podosphaera macularis* the interaction is evaluated by light microscopy. Subsequently the penetration rate of individual transformed cells points to gene function. Defence-associated genes from other plant species as well as genes associated with hop powdery mildew resistance are going to be checked for their function. Such genes may be candidates for a stable transformation or for marker assisted breeding.

Keywords: *Humulus lupulus,* powdery mildew, resistance, transient transformation, histochemical staining

Introduction

Powdery mildew resistance is one of the major objectives of hop breeding since fungal infections appeared to a much larger extent in the 1970s (Kohlmann and Kastner, 1975). Since resistance to powdery mildew is in most cases monogenetic (Royle, 1978) it can easily be overcome. In Germany currently the only remaining active resistance is based on the R2 resistance derived from the English cv. “Wye Target” (Seigner et al., 2006). Here pathogen growth is restricted by an early hypersensitive reaction (Godwin et al., 1987). Probably it is a matter of time until pathogen strains also overcome this type of resistance as it happened in England years ago (P. Darby, pers. comm). Therefore the German breeder began to integrate resistant wild hops from all over the world into breeding programs. Detailed knowledge about the different resistance mechanisms of the cultivars or wild hops currently used for breeding in Germany is missing. Unravelling the mechanisms leading to resistance in different wild hops or breeding lines may simplify breeding, as a combination of different resistance mechanisms might be promising. Beside the completely susceptible English cultivar “Northern Brewer” and the resistant cultivar “Wye Target” wild hops from six regions all over the world are going to be investigated. As each wild hop has another genetic background we expect to find differences in the spatio-temporal and quantitative development of the fungus.

Another approach leading to resistant plants is to have a detailed knowledge about resistance associated genes. The genetic features of the host in combination with those of the pathogen lead to resistance or susceptibility. Therefore knowledge about the function of those genes is the basis for transgenic approaches and also interesting for marker assisted breeding. The only way to assure the function of certain genes is to perform knock down or overexpressing studies. As the generation of transgenic hop plants is still a time and cost intensive procedure we want to work with the transient transformation of single epidermal cells which are subsequently inoculated with powdery mildew spores. Such systems have been described for example for barley and wheat (Shirasu et al., 1999; Schweizer et al., 1999). Here the transformation of the epidermal cells is achieved by biolistic bombardment. It is planed to prove the function of i) genes from other plant species, which proved to have an effect on resistance or susceptibility, ii) published ESTs from hop and iii) TDFs (transcript derived fragments) derived from cDNA AFLP studies (Seigner and Seefelder, in press; Seidenberger, in prep.).

Methods

Inoculation of leaves was carried out with an inoculation tower. Fungal structures were stained with the chitin specific fluorescence dye WGA-TMR (Deshmukh et al., 2006). Aniline blue was used for callose staining. H_2O_2, which accumulates in cell wall appositions and hypersensitive reacting cells was detected with diaminobenzidine (DAB), leading to brown precipitates (Thordal-Christensen et al., 1997).

The transient transformation assay works as follows (Schweizer et al., 1999): Gold particles are either coated with the β- glucuronidase (*GUS)* reporter gene alone or with the reporter gene and the gene of interest (overexpression or knockdown approaches). Subsequent bombardment of the young leaves leads to a transient transformation of single epidermal cells. Afterwards leaves are inoculated with the powdery mildew fungus. Staining for GUS enzymatic activity allows to identify the transformed cells. Under the microscope the cells expressing only the reporter gene are compared with the cells which are additionally characterized by the overexpression or knock down of the test gene. Finally differences in the penetration efficiency reveal the function of the test gene (Fig. 1). For establishment of the transient assay, cells are currently transformed only with the GUS reporter gene.

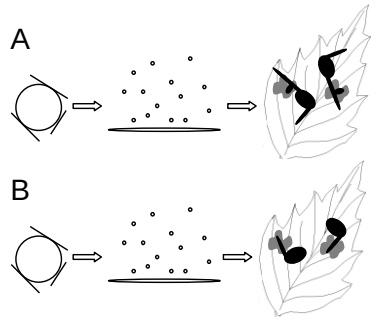

Fig. 1: Scheme of the transient transformation assay. First gold particles are either coated with the reporter gene alone (A) or with the reporter gene and the gene of interest (B). Then epidermal cells are transformed by particle bombardment (light grey). In this case the gene of interest confers resistance and leads to an incompatible interaction, characterized through restricted fungal growth (B).

Results and Discussion

Histochemical staining techniques have been successfully established. Staining of the fungus with WGA-TMR allows the investigation of the fungal development at different time points after the inoculation (Fig. 2). Staining of callose with aniline blue and detection of H_2O_2 with DAB makes defence reactions like cell wall appositions or hypersensitive reacting cells visible (Fig. 3). The development of the fungus on the English cv. “Northern Brewer” was studied to have a susceptible control for the resistant cultivars, breeding lines and wild hops, which are planed to be investigated. According to the results of Godwin et al. (1987) 24 hai a haustorial initial and a second germ-tube are formed. 72 hai up to five epicuticular hyphae can be observed and 6 days after inoculation (dai) the fungus sporulates. In further

experiments it will be interesting to find differences in the spatio-temporal and quantitative development of the fungus. Such different defence mechanisms might be papilla formation, a hypersensitive reaction in the early stages of the interaction or later restriction of fungal growth (Royle, 1978; Godwin, 1987; Kita et al., 1981).

The transient transformation assay has so far been established as 2,400 with the GUS reporter gene transformed cells per leaf can be counted leading to 20 interaction sites after subsequent inoculation (Fig. 4). For evaluation of one gene about 100 interaction sites have to be counted per biological repetition. After a few modifications within the inoculation rate and the leaf age (i.e. cell size) we plan to start with a well known resistance associated gene from another species as a potential positive control (e.g. *MLO*, Shirasu et al., 1999).

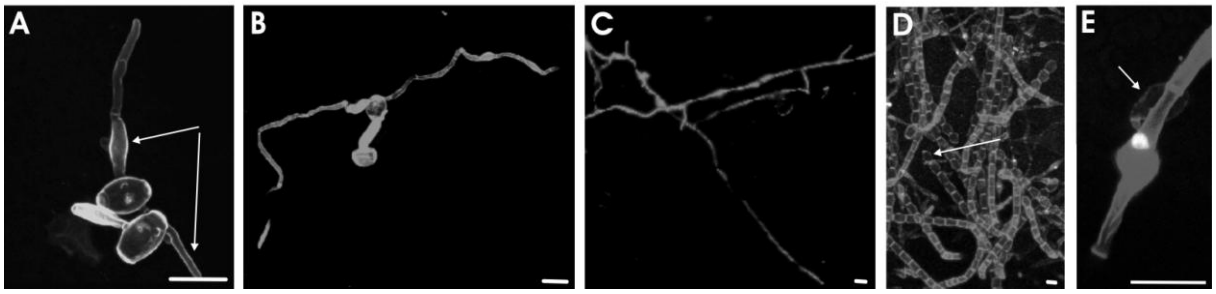

Fig. 2: Development of *Podosphaera macularis* on leaves of the susceptible English cultivar "Northern Brewer". Leaves were inoculated, then the fungus was stained at different time points with WGA-TMR. Scale bar = 20 µm; hai = hours after inoculation; dai = days after inoculation. **A**, 24 hai two germ tubes have emerged, (arrows). **B**, 48 hai elongated hyphae can be seen. **C,** hyphae are branched. **D,** 6 dai conidiophores can be seen, the fungus sporulates (arrow). **E,** beneath a swollen hyphae a haustorium can be seen (arrow).

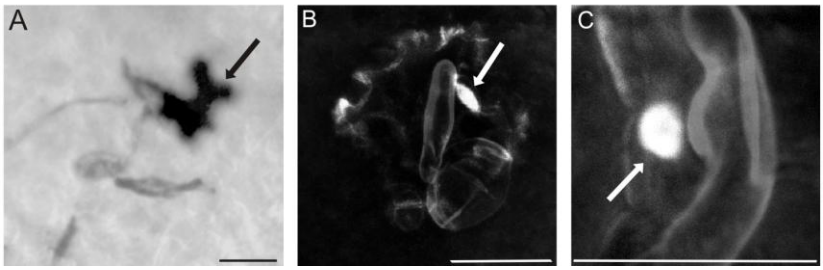

Fig. 3: Defense reactions of hop epidermal cells after attack of *Podosphaera macularis* spores. Scale bar = 20 µm. **A**, H_2O_2 accumulating in hypersensitive reacting cells was detected with DAB (arrow). **B**, aniline blue staining indicates callose depositions in a hypersensitive reacting cell. A small papilla containing callose can also be seen (arrow). **C**, failed penetration attempt, stopped through a papilla containing callose (arrow).

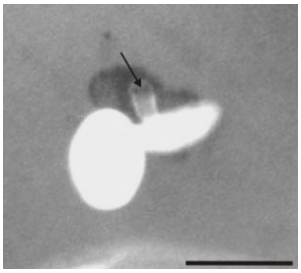

Fig. 4: Interaction of a transformed cell (blue, transformed with the GUS reporter gene) with the powdery mildew fungus. Scale bar = 20 µm. The cell contains a haustorium (arrow).

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assistance of Brigitte Forster is highly appreciated. In addition, thanks go to Dr. Stefan Seefelder and Rebecca Seidenberger for providing cDNA-based information. cDNA-AFLP analyses in hops have been financially supported by the EHRC (European Hop Research Council) and the Wifö (Scientific Fund of the German Brewing Industry).

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STUDIES OF WILT RESISTANCE IN HOP

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Abstract

Hop production is an export oriented agricultural branch in Slovenia. Slovenia produces well known aromatic varieties, all bred at the Slovenian hop institute and well adapted to the specific microclimatic conditions. Because of the outbreak of wilt, the development of cultivars with resistance to *Verticillium* spp. is currently one of the main focuses in breeding. The use of marker-assisted selection (MAS) would greatly accelerate the development of wilt-resistant hop cultivars, so a research program has been set up to study gene(s) conferring resistance *to V.albo-atrum* and to develop resistance linked markers applicable in MAS. A genomic approach has been applied for mapping and cloning of gene(s) and we have so far developed various molecular markers, established family segregating for Verticillium resistance and constructed a genetic map. The research will continue with the development of markers linked to resistance gene(s) and isolation of genomic and cDNA clones, with validation of their (gene) function. In parallel, proteomics has been used to study the pathogenicity of *V.albo-atrum* and plant-pathogen interactions. Proteomic study of two *V.albo-atrum* pathotypes revealed differences which may in part explain the differences in their virulence and in an artificial infection experiment, two induced plant defence proteins were identified in hop xylem sap the function of which is being examined. We intend to continue proteomic work by focusing on identifying a higher number of induced xylem as well as root proteins and on their functional and validational analysis using a transformation system. We expect to identify proteins (genes) related to pathogenesis, which will allow us to interpret the hop-*Verticillium* interaction and further develop linked markers applicable for breeding hop cultivars tolerant to wilt.

Keywords: fungi, plant diseases, *Humulus lupulus*,

Introduction

The problem of plant resistance to diseases and pests is extremely important in view of the high damage that various pathogens cause to crop yield. In managing pathogens, an understanding of the mechanisms of interaction between plants and pathogens is of central importance. Research in this area has intensified with the sequencing of the genomes of some pathogens and plants, the greater extent of available gene sequences of plants and pathogens, and with functional analyses of transcriptome, proteome and metabolome. Such data are still limited for the minor crops but, with the assistance of translational genomics tools, information already obtained on model and other organisms in dealing with a selected issue can be used.

Hop production is one of the important agricultural branches in Slovenia, representing 3.1% of total world hop growing areas. Slovenia produces well known aromatic varieties, all bred at the Slovenian Institute of Hop Research and Brewing and well adapted to the specific microclimatic conditions. Because of the outbreak of wilt, the development of cultivars with

resistance to *Verticillium* spp. is currently one of the main focuses in breeding. The outbreak and spread of the lethal *V.albo-atrum* pathotype has become a serious threat to Slovene hop production, in view of the high concentration of hop gardens in all production areas and the prevalence of susceptible hop varieties. Due to the lack of effective chemical control, crop rotation and phytosanitary measures are used to limit the spread of the disease but the most important and valuable control method is the planting of resistant (or tolerant) hop cultivars. The exploitation of resistance is restricted to that already available in existing genotypes, although there appear to be as yet undiscovered resistance resources in wild hops. A general problem in hop breeding is the long time (approx. ten years) required for the development of new cultivars but there are novel technologies to help overcome this difficulty. The use of marker-assisted selection (MAS) would greatly accelerate the development of wilt-resistant hop cultivars, so a research program has been set up to study the pathogenicity of the fungus, plant-pathogen interactions in vascular wilt disease, genome mapping and identification and characterization of gene(s) conferring resistance *to V.albo-atrum*. We have been applying two approaches:

A. Genomics

1. Mapping and cloning of Ve gene(s)
   a) Development of various genetic markers
   b) Establishment of the family segregating for Verticillium resistance
   c) Construction of a genetic map
   d) Development of markers linked to Ve(s)
   e) Isolation of genomic and cDNA clones
   f) Transformation of clones and validation of gene function
   g) Markers used for marker assisted selection

B. Proteomics

1. Study of proteomic differences of two *Verticillium albo-atrum* pathotypes
2. Study of differentially expressed proteins in xylem sap and roots after inoculation with lethal pathotype
   a) Identification of differentially expressed proteins followed by identification of genes
   b) Cloning and validation of genes

Results and discussion

Mapping and cloning of Ve gene(s)

Genetic mapping for hop wilt included crossing tolerant (Wye Target) and sensitive (2/1) parents to *Verticillium albo-atrum*, the lethal pathotype PV1. The family of crosses contained 152 progeny, which were maintained on a test field and phenotypically tested for resistance in growth chambers (14 clones of each individual, artificial inoculation with the pathotype PV1, assessment of symptoms, to date 120 progeny tested). AFLP and SSR markers were generated for mapping; 397 polymorphic AFLP markers were obtained with 12 primer combinations and 114 SSR markers segregated in the mapping population out of 314 developed SSR markers. We have produced a well characterized genetic linkage map with more than 100 microsatellites, several genes and 100 AFLP markers (A1, a-c) although we have not yet succeeded in locating the trait (tolerance to hop wilt) on the map (Jakše et al., 2006, 2007, 2008). In further research, we intend to seek the genes or QTLs for resistance, applying two approaches: a) further saturation of the gene map with larger numbers of newly developed SNP markers and b) discovering regions responsible for tolerance in hop with the aid of known genes for resistance to *Verticillium* in other crops and with the aid of resistance genes analogous.

Resistance genes analogous (RGA)

We used a PCR strategy to clone resistance gene analogs (RGAs) using degenerative primers designed at the conserved motif of cloned plant NBS-LRR *R* genes. Fifty-six sequenced PCR clones showed homologies to various *R* genes deposited in the GenBank database and these clones were further divided into 17 RGA groups. Phylogenetic analysis of hop RGA groups with cloned *R* genes revealed a separation of hop RGAs into TIR (7 RGA groups with 16 sequences) and non-TIR (10 RGA groups with 40 sequences) NBS-LRR protein classes. The amino acid identity of hop RGAs to various *R* genes ranged from 41 % (TIR-NBS-LRR) to 81 % (non-TIR-NBS-LRR). RGA primer sets, designed on 17 reference RGAs, amplified additional RGA sequences from two hop genotypes, which served for the development of 11 RGA markers, eight of which also amplified PCR products of expected sizes in various hop cultivars, indicating a high conservation level of RGA sequences within the hop genome and two RGA markers segregated in the mapping family. The structure of RGA sequences and similarity to other *R* gene or *R*-gene-like sequences is discussed. This is the first report on RGAs in hop and it provides useful data for further application for a targeted search of RGA markers for genetic mapping (Kozjak et al., 2009).

Differential display

An inoculation experiment with the high virulent pathotype of *V. albo-atrum* was set up to study differentially expressed mRNA and proteins in hop. After infection of resistant variety Wye Target and susceptible Celeia, mRNA was isolate to study the response reactions of these varieties. cDNA-AFLP was used as a method to display the expressed mRNAs differentially between the infected and control plants of resistant and susceptible cultivars. Up to 480 DNA fragments have so far been sequenced of which 103 were useable after initial bioinformatic processing and around 10 sequences are related to resistant genes. In further research, these sequences will be verified by qRT-PCR as well as transformed into SNP markers and mapped.

Study of proteomic differences of two Verticillium albo-atrum pathotypes

To study the differences between the mild and lethal *V. albo-atrum* hop pathotypes, we applied a proteomic approach with two-dimensional electrophoresis (2-DE). Four isolates were studied: mild (PG1) and lethal (PG2) isolates from Slovenia and mild (M) and lethal (PV1) isolates from England. A reference proteome map of *V. albo-atrum* was first established, resolving up to 650 protein spots on Coomassie stained gels, of which 268 matched spots showed the average coefficient of variance of 16 % and 15 %, respectively, for technical and biological variability. Principal component analysis (PCA) discriminated the geographic origin of the isolates and between the two pathotypes, and showed a closer relationship among English isolates than Slovene ones. The 2-DE patterns of mild (PG1 and M) and lethal (PG2 and PV1) isolates were compared and the major differentially expressed proteins were identified by MS. Our results indicate that the lethal pathotype is able to suppress plant defence better than the mild pathotype by increased expression of peroxiredoxine and ascorbate peroxidase. PG2 and PV1 also have increased levels of cytoskeleton components and regulators, suggesting a better infection ability since the cytoskeleton has an important role in root penetration, secretion of cell wall-degrading enzymes and conidiation at trapping sites. In addition, the rate of protein synthesis in general and energy metabolism is higher in PG2 and PV1. These results reveal differences on the protein expression level between the two pathotypes, which may explain in part the differences in their virulence (Mandelc et al., 2009).

2-D pattern of hop xylem proteins after inoculation by lethal Verticillium pathotype

Since *V. albo-atrum* is a pathogen growing in xylem vessels, we analyzed the xylem sap proteome of control and infected plants, artificially inoculated with lethal Verticillium pathotype, and compared the results from a susceptible (Celeia) and a tolerant (Wye Target)

cultivar. While some differentially expressed proteins between control and infected plants were observed in the tolerant cultivar, the difference was dramatic in the case of the susceptible cultivar. Two proteins were identified by mass spectrometry, both involved in plant defence. Pathogenesis-related protein 1 was up-regulated almost 5-fold in both cultivars after infection. A chitinase from glycoside hydrolase family 19 was identified in two spots with the same mass but different isoelectric point, which could be a result of different post-translational modifications, different isoforms or two different genes. Both spots showed up-regulation after infection although, surprisingly, up-regulation of both spots was stronger in the susceptible cultivar. Further investigation at the DNA level showed differences in the sequence of the chitinase gene between the cultivars (Mandelc et al., 2007). We intend to continue proteomic work by focusing on identifying a higher number of induced xylem as well as root proteins (2-D or DIGE; MS/MS) and on their functional and validational analysis using a transformation system. We expect to identify proteins (genes) related to pathogenesis (PR), which will allow us to interpret the hop-*Verticillium* interaction and further develop linked markers applicable for breeding hop cultivars tolerant to hop wilt.

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RESISTANCE GENE CANDIDATES IN HOPS

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Abstract

Cloning and sequence database search experiments on genes for resistance against pathogens in various plants have shown that many share conserved sequence motifs. Such knowledge makes it possible to isolate new sequences – tentative resistance genes - mainly by polymerase chain reaction (PCR) or by employing large insert libraries. Various PCR approaches have been employed in experiments on the isolation of resistance gene candidates (RGCs) from hop: 1) a degenerate oligonucleotide primers approach designed from motifs in the nucleotide binding site domains (NBS), 2) sequence similarity searches of current hop EST resources for transcripts containing domains known to be present in resistance genes (NBS, leucine rich repeat (LRR), Toll/interleukin-1 receptor-like domain (TIR) and transcription factors with conserved WRKY domains) and design of specific pairs of primers and 3) targeted gene approach with identification of specific sequences from hop with high similarity to the *Ve1* gene in tomato. The amplified products are further analyzed for the presence of open reading frames, with additional analysis of polymorphic sites, which could enable us to saturate the existing microsatellite-AFLP map of hop. Mapping and identifying disease resistance genes could facilitate marker assisted selection (MAS), which offers fast and reliable identification of resistant genotypes.

Keywords: resistant gene candidates, RGCs, resistant gene analogs, RGAs, NBS, LRR, TIR, WRKY

Introduction

The theory about how plants recognize and respond to the majority of diseases is known as “gene for gene” theory. Numerous genetic studies of plant and pathogen interactions have confirmed that this specificity is defined by a resistant gene (R gene) in a plant which has a corresponding gene for avirulence (Avr gene) in the pathogen.
Sequence comparisons of cloned plant resistant genes have shown that most of them contain similar sequences and structural motifs, whether they confer resistance to fungal, plant or viral disease causing agents (Kanazin et al., 1996). Their structural properties have a role in pathogen recognition and in cellular defense response signaling. The results show that they are very abundant in plant genomes and most of them belong to tightly linked gene families. Different genes within the same cluster may determine resistance to taxonomically diverse pathogens. Mutations and recombination events within a cluster, or even between different loci, drives their evolution. Further selection favors R genes that recognize specific pathogen products of avr genes (Hulberth et al., 2001).
Numerous cloning efforts of plant pathogen resistant genes in different plant taxa have been conducted in the last decade, using different approaches of R gene isolation, including map-based cloning, transposon tagging and, since the late 90’s, PCR amplifying and database searching. Structural and sequence analysis of R genes has revealed that many of them contain similar sequence motifs, even though they determine resistance to different pathogens, including viruses, fungi or bacteria. The majority of them encode leucine-rich repeats (LRRs) and share sequence motifs with nucleotide binding sites (NBS) of P-loop proteins (Hammond-Kosack and Jones, 1997). Additionally, they can also encode variable amino- and carboxy-terminal domains, such as the Toll/interleukin-1 receptor-like domain

(TIR), coiled-coil domain (CC) or zinc-finger transcription factor-related domain containing the WRKY sequence (WRKY) (McHale et al., 2006). The presence of these conserved domains in resistance genes offers the opportunity to clone additional resistance genes from different plant species by polymerase chain reaction (PCR) with degenerative primer sets complementary to the conserved motifs. A PCR-based strategy has proved to be an efficient approach for the isolation of R genes or resistance gene candidates (RGCs) in soybean (Kanazin et al., 1996), grapevine (Di Gaspero et al., 2002), common bean (Lopez et al., 2003), apple (Calenge et al., 2005) and many other plants.
Analysis of ESTs provides a powerful complement to genome sequencing for model plants and is the primary tool for gene discovery in many plant species of agronomic and economic interest. A high-quality cDNA library is a major tool for the discovery of useful genes by comparison of DNA sequences to those already characterized and registered in sequence databases. EST databases can be searched for similar sequences to already known R genes. Such an approach, using stringent BLAST search, has been successful in identifying 88 resistant gene analogs (RGAs) in sugarcane (Rossi et al., 2003). The recent contribution of hop EST sequencing projects with more than 24,000 EST sequences provides an invaluable source of information for the hop research community (Wang et al., 2008, Nagel et al., 2008).
In this paper, we report the results of PCR isolation of RGA sequences using degenerate primer sets and a search for RGAs using available hop EST resources. Additionally, attempts have been started to clone *Ve1*-like sequences from hop genome. We screened some isolated sequences for segregating polymorphisms to map RGAs on the available hop genetic map. Such an approach enables us to investigate their genomic distribution and their relationship with disease resistance loci in hop.

Methods

RGA PCR Cloning: Wye Target, a hop cultivar with resistance to hop wilt and powdery mildew, was chosen as a source genotype for the isolation of hop RGA sequences. Isolated RGA sequences were used to develop primers for the amplification of RGA markers and for further isolation of RGA sequences from hop breeding line 2/1, which shows good resistance to downy mildew. Developed RGA markers were tested for segregation in the F_1 family of a cross between cultivar Wye Target and male breeding line 2/1. Degenerate primers were designed at the conserved motifs of genes encoding the NBS–LRR protein. Three degenerate primer sets were used to amplify RGA sequences for the hop cultivar Wye Target: P-loop/Kin 2 rev, P-loop/Kin 2 TIR rev and P-loop/GLPL. Targeted bands of expected size were excised, purified, cloned and 114 colonies were sequenced. The obtained sequences were aligned and grouped into 17 different groups, from which RGA-STS markers were developed and further tested for polymorphism in the segregating family.

Hop EST database searching: Custom transcript assemblies of hop EST sequences (total 9789 sequences) were obtained from the Plant Genome Database (version 168a). Sequences were translated into 6 possible reading frames. Pfam motifs PF00560.25 (LRR1), PF07725.4 (LRR_3), PF00931.14 (NB-ARC – NBS), PF01582.12 (TIR) and PF03106.7 (WRKY) were used to build a local database, against which translated hop sequences were searched for the presence of conserved motifs of R genes using the hmpfam program. BLAST comparison was employed to find the most similar sequence from a plant containing the annotated gene, which enabled us to predict the intron-exon boundaries with the Wise2 tool. After intron site prediction, 5 primer pairs were designed with the Primer3 program and tested for amplification in 'Wye Target', male line 2/1 and 6 siblings originating from this cross.

Investigating the *Ve1*-like sequence in hop genome

The *Ve1* gene (AF272366) was amplified from the tomato genome (cv. 'Stupice') and sequence identity confirmed by sequencing (avoiding the LRR region of the gene). The PCR fragment was P^{32} end labelled and used as a probe in Southern hybridization to *Hind*III,

*EcoR*I and *EcoR*V digested hop DNA ('Wye Target', 'Wye Challanger', 'Savinjski Golding', 'Aurora', 'Celeia'. and 'Yeoman'). Blots were washed with 0.1XSSC and X-ray films exposed for two weeks.

Results

Three degenerate primer combinations targeting the region from the P-loop to the kinase-2 motif and from the P-loop and GLPL(A/T)L motif of the NBS region were used for PCR amplification of RGAs in the hop cultivar Wye Target. Major bands of approximately 250 bp and 500 bp were cloned and 114 clones were randomly chosen for restriction analysis. Sequences were identified based on unique restriction profiles and a total of 98 clones were sequenced. Similarity searches of hop nucleotide sequences in GenBank entries produced significant similarities only with deduced amino acid sequences for 65 clones (66%) to the NBS–LRR encoding R genes, RGAs or both, in various plant species using the blastx algorithm. In total, 63 hop RGA sequences had uninterrupted ORFs, of which 56 sequences showed differences at the nucleotide level and 49 at the amino acid level. Two RGA sequences were presumed pseudogenes, since stop codons were present. Based on the alignment of sequences, 17 groups of RGAs were defined and hop specific primers were developed. Two of them showed polymorphism in the hop mapping family.

Screening of 9789 hop EST sequences for motif specific sites yielded 24 sequences with the presence of LRR domains, 8 with the WRKY domain, 2 with the TIR and 1 with the NBS domain (total 35 sequences). BLAST comparison successfully revealed annotated genes in either *Arabidopsis* or *Populus* for all 5 analyzed sequences for successful identification of intron-exon boundaries. The developed primer pairs amplified a single PCR fragment in hop samples and will be further analyzed for sequence based polymorphisms.

Southern hybridization confirmed the presence of a *Ve1*-like sequence in hop. The hybridization pattern suggested a low copy number of the particular sequence in the hop genome.

Discussion

The aim of this study was to test different approaches for the isolation of RGAs from hop genome. Three degenerate primer combinations designed at the conserved motifs of plant R genes were used for the PCR amplification of RGA sequences in hop and 98 PCR clones were sequenced. Altogether, 56 clones with various nucleotide sequences with uninterrupted ORF were further analyzed. The alignment of deduced amino acid RGA sequences with R genes from other plants at the NBS domain revealed common features, but the level of similarity of amino acid hop RGA sequences to cloned R genes was mainly restricted to the presumed functional NBS domain, which may be a side effect of such an approach, as pointed out by Yaish et al. (2004). According to the alignment of the 56 nucleotide different RGA sequences, 17 RGA groups were defined and for each group a consensus sequence was constructed. These consensus sequences served for primer design aimed at identifying additional potential RGA markers. Twelve RGA markers were tested for segregation in the F_1 family obtained by crossing Wye Target X 2/1. Six RGA markers amplified monomorphic bands of identical size and sequences in Wye Target and line 2/1, while four failed to amplify PCR products. Only two RGA markers were found to be polymorphic and showed SNP and indel polymorphisms. A low acquisition of polymorphic markers was also detected in tetraploid cotton (Hinchliffe et al., 2005), in which nine polymorphic markers were found among 61 RGA-STS tested. Similar results were reported for siblings of two apple cultivars by Calenge et al. (2005).

A database mining approach showed a low level of R gene specific motifs in a set of 9789 hop ESTs, in which only 35 such sequences were identified (0.4%). This can be attributed to the fact that the majority of today's available ESTs were developed from glandular tissue of hop cones and not from different hop plant tissues. Nevertheless, such an approach seems to be good candidate given all the available resources of comparative genomics. Genic sequences are also easy to amplify in a PCR reaction compared to higher copy anonymous

sequences. The developed 5 primer pairs successfully amplified PCR fragments in the first round of optimization from hop genome. Further sequence analysis of PCR fragments also confirmed the correct identity of the sequence.
Hybridization analysis of the hop genome with the *Ve1* gene from tomato showed that a *Ve1*-like sequence might be present in the hop genome. The Ve1 gene from tomato is the first cloned wilt resistance gene. BLAST searching of hop ESTs did not reveal any expressed sequences with even low similarities with the *Ve1* gene. On the basis of our hybridization experiment, we can assume that such a sequence might be present in the hop genome.
Efforts at mapping and identifying disease resistance genes in hop could facilitate marker assisted selection (MAS), which offers fast and reliable identification of resistant genotypes and also the discovery of new allelic variants of hop R-genes in wild germplasm.

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DECIPHERING MORPHOGENESIS FOR HOP IMPROVEMENT. A TRANSCRIPTOMIC AND METABOLOMIC PERSPECTIVE

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Abstract

In hop, large scale regeneration can be achieved by induction of organogenic nodules derived from petioles and internodes. Organogenic nodules and somatic embryos have been used for definition of reliable protocols to further genetic engineering either of herbaceous or woody species. Although these two morphogenic processes have been obtained for a great number of plants, most of them have been achieved by tentative and error and are not completely reliable.

Hop is a sensitive plant does not flourish everywhere. Commercial plantations are frequently threatened by virus, fungi and pests. At present, unfortunately, there are no hop varieties known to present resistance to the main disease agents. So far, the production levels have to rely on the use of high amounts of fungicides and pesticides. With this idea in mind we have developed a reliable regeneration protocol for induction of organogenic nodules in hop based on a functional genomics approach, as a way to improve important traits in hops. New advances in genomics, metabolomics and proteomics are opening doors for improvement of plant protection strategies against pests and pathogens. Harnessing these technologies and focusing them on plant protection is critical to deliver a sustainable, and ecologically sound, agriculture for the future. It is worldwide known that the ongoing shortage and high costs of hop, a key beer component, have made brewers around Minnesota very unhappy. Several factors such as poor weather-related global harvests, decrease of farmers number growing hops, accompanied by decrease of yield production due to diseases, have shot hop prices to record highs.

Organogenic nodules formation and development

Organogenic nodules are dense independent cell clusters which form a cohesive unit and display a consistent internal cell/tissue differentiation pattern and a high regenerative capacity (Mc Cown et al., 1988). They provide useful systems to achieve large scale clonal propagation, as well as a tool for genetic transformation and clonal propagation of transgenic plants.

Organogenic nodule formation is a morphogenic process sharing features with somatic embryogenesis. In hop, it is induced *in vitro* by mechanical wounding under particular conditions of light, humidity, temperature and specific combination of sugars and growth regulators in culture media. Plant Morphogenesis induction is far from being understood. It is absolutely determinant to unravel the regulatory connections between inductive stress (e.g. wounding, hormones) signalling and transduction pathways. To afford a full understanding of these mechanisms and be able to monitor their systematic induction it is necessary to control the crucial steps of this process. Development of single somatic cells into embryos or nodules in higher plants has been amply demonstrated but it is far from being highly reproducible.

A morphogenic process, being formation of a somatic/gametic embryo or an organogenic nodule such as those of *Humulus lupulus* (Fig. 1), consists on the creation of new organizational forms in response to Changing Environmental conditions and **r**esults from two crucial steps: 1-Induction of Morphogenic Competence; 2 - Expression of Morphogenic Competence (Hilbert et al., 1992). These events normally depend on inductive signals that induce quiescent or proliferating cells to undergo a different pattern of differentiation.

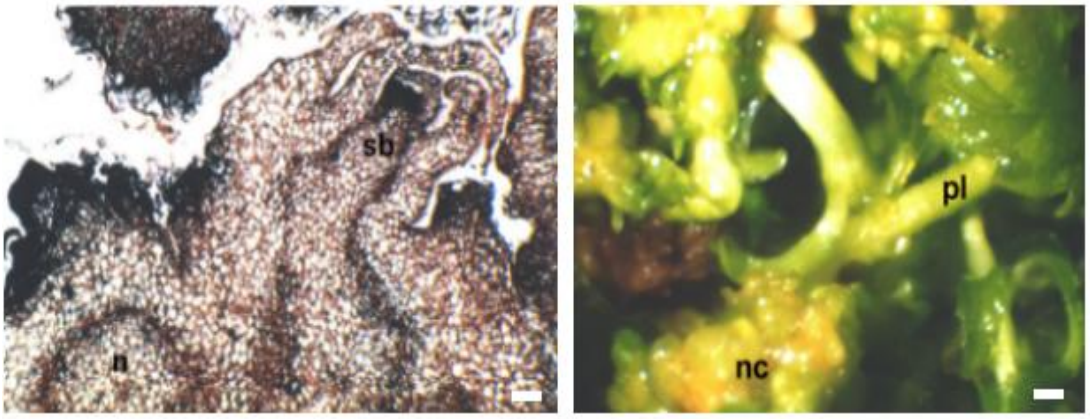

Fig.1- **Organogenic nodule formation in hop** (reproduced from *Ana M. Fortes et al. 2008; BMC Genomics)*
A Transversal section of a nodule (n) after 45 days in culture with IAA and BAP showing one shoot bud (sb) connected to the nodular vascular bundles;. **B**. Nodule cluster (nc) formed after 45 days in culture and showing several shoot buds and plantlets (pl). Bars in A = 150 µm, in B = 800 µm.

For studying these processes in hop internodes were morphogenically induced by a wounding stress (induction stimulus) followed by an auxin / cytokinin treatment and transfer to light conditions. Very soon after this induction stimulus the induced material produced H_2O_2, a Reactive Oxygen Species (ROS) molecule. ROS are ideally suited to be SIGNALLING MOLECULES since they are small and can diffuse into small distances, there are several mechanisms for their production, some of which are rapid and controllable and there are mechanisms for their rapid removal (Hancocket al., 2001). Under such conditions, cells must react quickly, creating intercellular signals, via transmembrane receptors and amplification of signals generating an appropriate response by modulation of enzymes activity and gene expression. A wide range of genes is regulated by H_2O_2. The consequences of oxidative stress upon cell behavior are diverse. Dividing cells stop division until DNA repair is complete. In animal cells in culture it has been shown that low-level oxidative stress may stimulate proliferation of many cell types. Low levels of H_2O_2 can activate signal transduction pathways by oxidizing –SH groups or raising Ca 2+ levels.
In hop organogenic nodule induction the raise of Ca^{2+} levels was concomitant with the formation of a callose layer around the proliferating cells, thus creating a special environment for morphogenesis to occur (Fortes et al., 2002). Cell competence seems to be associated with a particular metabolic cell-state most probably induced by growth regulators, and which enables, under stress conditions, to switch on defense mechanisms in a way that triggers morphogenesis. Thibaud-Nissen et al. (2003) suggested that arrangement of cells into organized structures (somatic embryos) might depend on a tight control between cell proliferation and cell death.

Transcriptomic analysis showed a clear up-regulation of genes related to stress response in organogenic nodules indicating that ROS are being produced but also a tight control of oxidative status is being achieved (Fortes et al., 2008). In fact, explants cultured on medium without growth regulators when compared to explants giving rise to organogenic nodules showed increased expression of a Senescence- Associated Gene and a gene coding for a putative gamma-thionin, and accumulation of glutamine, which gives strong support that

these tissues become extremely oxidized upon wounding and during *in vitro* culture. It seems that the lack of growth regulators impaired the signaling events leading to organogenic nodule formation to take place, instead cell death was widely occurring in these control samples.

Stress response, growth and development involve expression of lipoxygenases. Lipoxygenases catalyze the conversion of (1Z, 4Z)-pentadiene polyunsaturated fatty acids into their corresponding hydroperoxy derivatives (Rosahl, 1996). Several compounds in the linoleate cascade from LOX, e.g., jasmonic acid, methyljasmonate, and some volatile aldehydes, have physiological effects in plants (Creelman and Mullet, 1997).

Lipoxygenases were shown to be developmentally regulated throughout the morphogenic process, suggesting their involvement in the response of internodes to wounding, nodule formation, and plantlet regeneration from these nodules (Fortes et al., 2004). The authors have reported on a rapid increase on LOX activity parallel with elevated levels of lipid peroxides in response to wounding treatment; such increase may be involved in the synthesis of the wound-healing factor, traumatin and jasmonates that in turn can trigger the expression of wounding/ defense-related genes.

Recently, two ESTs coding for LOXs were found to be differentially expressed during organogenic nodule culture; they should correspond to enzymes located in different compartments and/or have different metabolic activities (Fortes et al., 2008). The localization of LOX preferentially in areas with high meristematic activity suggested an active role in growth and development during this morphogenic process (Fortes et al., 2004). Interestingly, also increased contents in α- linolenic acid, choline and a short chain fatty acid were found when comparing organogenic nodules to control explants cultured in medium without growth regulators (Fortes et al., 2008). *In vitro* morphogenesis seems to be induced due to an interaction of signals including growth regulators and sugars among others.

Allene oxide cyclase (AOC) is a key enzyme in jasmonates biosynthesis, catalysing the formation of cis-(+)-12-oxophytodienoic acid (OPDA) a stereoisomeric percursor of jasmonic acid (JA). It appears to be up-regulated in diverse developmental processes as well as in response to wounding and other abiotic and biotic stresses.According to Fortes *et al*. (2005) changes of AOC expression and localization during wound-induced organogenic nodule formation and plantlet regeneration. AOC transcripts and protein were shown to rapidly accumulate upon wounding of the explant indicating wound-induced *de novo* AOC synthesis**.** During formation of nodules AOC was immunolocalized to the amyloplasts present in the nodules and additionally in chloroplasts of differentiated nodular cells. This localization of AOC was related to JA generation involved in conditioning storage or mobilization of sugars occurring in growth and differentiation processes. Jasmonates are oxylipins (oxygenated compounds derived from polyunsaturated fatty acids) which have attracted interest as signaling molecules activating gene expression in different plant responses, such as to wounding (Léon and Sànchez–Serrano, 1999). Jasmonates include JA and its methyl ester, and octadecanoids comprising OPDA and its derivatives.

Fortes *et al*. (2005) reported on JA and OPDA levels during morphogenesis in hop. The increase in JA and OPDA levels within the first 24h after wound treatment of the cultured explants (internodes) was suggested to be involved in down-regulation of photosynthetic genes during inhibition of chlorophyll synthesis and amyloplasts differentiation observed in the early days after culture initiation. During plant regeneration from organogenic nodules, it was measured a new rise of those compounds, suggesting that these morphogenic processes might be affected by JA and OPDA as reported for somatic embryos (Blásquez *et al*., 2004).

Transcriptional profiling showed that several genes coding for proteins putatively related to photosynthesis such as Rubisco were down-regulated in prenodules and, to less extent, in nodular explants (Fortes et al. 2008). Down-regulation of proteins such as Rubisco may result from jasmonic acid action as well as the induction of proteinase inhibitors, thionins,

and enzymes involved in the biosynthesis of secondary metabolites (reviewed by Wasternack and Hause, 2002).

Hop morphogenic structures produce energy mainly anaerobically and according to the expression of genes coding for glycolytic enzymes this process is more active during the prenodular stage. The generated phosphoenolpyruvate provides carbon skeletons to the phenylpropanoid-flavonoid pathway. Since the increase in dihydrophenylpropanoids was observed after prenodule formation it is most probably related to development of prenodules than to stress response (Fortes et al. 2008). The knowledge on the involvement of phenylpropanoids and flavonoids in morphogenesis is to date very incipient.

Tremendous progress has been made in recent years regarding the genetic bases underlying both *in vitro* and *in situ* plant morphogenesis, stimulated by progress in functional genomics research. Nonetheless, the precise mechanisms controlling plant gene expression and the detailed steps by which these genes direct the specific process of morphogenesis remain far from being clearly understood. Thus, future trends involve characterization during morphogenesis of development-specific genes and subcellular localization of gene products to provide a deeper insight in the mechanisms involved during differentiation of competent cells and phenotypic expression of cellular totipotency in higher plants.

The data recently published on transcriptional and metabolic profiling of hop nodule culture have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO accession number GSE12339.
http://www.ncbi.nlm.nih.gov/geo/query/cc.cgi?acc=GSE12339.

Genetic Engineering using Hop organogenic nodules

Based on the highly productive nodule regeneration system developed for hop, two efficient transformation protocols were established for var. Eroica using petioles and green organogenic nodular clusters (GONCs) bombarded with *gusA* reporter and *hpt* selectable genes (pWRG1515, Paul Christou) (Batista *et al*., 2008). A total of 36 hygromycin B-resistant (hyg^r) plants obtained upon continuous selection were successfully transferred to the greenhouse, and a first generation group of transplanted plants was followed after spending a complete vegetative cycle. PCR analysis showed the presence of one of both transgenes in 25 plants, corresponding to an integration frequency of 69.4% and an overall transformation efficiency of 7.5%. Although GONCs seemed to be the ideal target material for transformation, bombarded petioles provided a higher number of transformed plants and of co-integration events (76.9%). Analysis of the same plants 6 months after transplantation revealed stability of integration in 54% of the petiole-derived positive plants. Real-time PCR (qRT-PCR) analysis with SYBR Green methodology confirmed co-integration in 86% of the plants tested and the stable presence of both transgenes for at least 2 years, until after the first vegetative generation, and identified positive plants amongst those previously assessed as *hpt*+ only by conventional PCR, which suggests that the initial transformation efficiencies may have been higher.

Using this system, the first successful hop transformation for the enhancement of disease resistance to fungal pathogens was accomplished with a chitinase (*chi*) and a β-1,3-glucanase (*glu*) genes from barley (pGJ40, Jach *et al*., 1995). Transgenic plants of two different varieties (Eroica and Osvald 72 mericlone 5223) were obtained proving that the method applies to other hop genotypes and potentially to hop in general. However, as opposed to the large number of transformants previously obtained with the reporter and selectable genes, only 3 kanamycin-resistant plants survived all selection phases (1 var. Osvald 72, plant g/c1 + 2 var. Eroica, plants g/c2 and g/c3) and were confirmedly positive for *glu* and *chi* presence, corresponding to an average transformation efficiency of 1.6%. The type and size of the genetic construct was probably the major constraint affecting the transformation success since pGJ40 is much larger and complex than pWRG1515 (14.5 kb vs 6.8 kb), and therefore more prone to fragmentation and integration problems. It is most

likely that the use of alternative simpler plasmids or linear cassettes would greatly improve the transformation rate.

A first qRT-PCR analysis confirmed the integration of *glu* and *chi* in all plants, although primer optimization is clearly needed to improve single amplification of the target fragments, especially for *chi* gene (Fig. 2). Testing of several different primers is currently underway to optimize the detection system and estimate copy numbers at this stage, as well as for revaluation of in vitro plants with 2 years old.

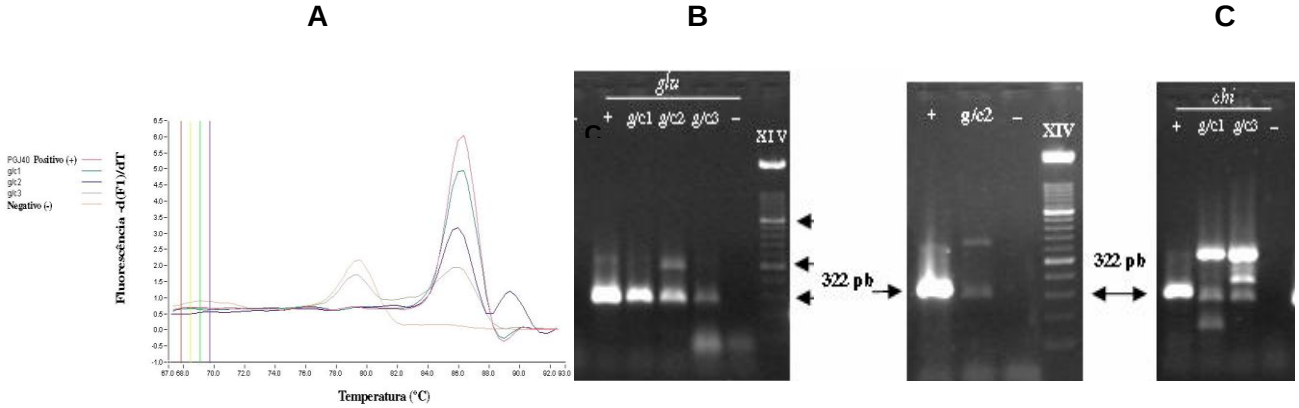

Fig 2. Real-time PCR amplification of *glu* (A,B) and *chi* (C) for all transformed hop plants: A melting curves depicting distinct signals from the desired product (glu gene fragment; highest peak – positive control) and the primer-dimers (*); B resolution of the same PCR products in agarose gel; C resolution of qRT-PCR products from the chi gene amplification in agarose gel. *g/c1*: transformed hop plant of var. Osvald 72 mericlone 5223; *g/c2 and g/c3*: transformed hop plants of var. Eroica; +: positive control (pGJ40); -: negative control (reaction mix).

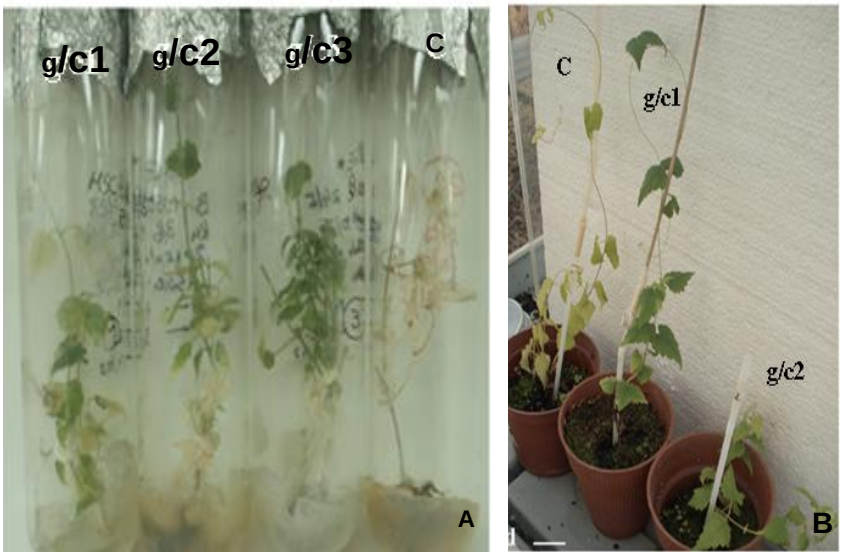

Fig. 3 Evaluation of *Verticillium* wilt resistance in hop transgenic lines: **A** *in vitro* response of hop transgenic (g/c1, g/c2 and g/c3) and untransformed control (C) plants 3 weeks after infection with *Verticillium albo-atrum* strain 157B; **B** Healthy transgenic hop plants (g/c1 and g/c2) compared to a slight wilted control plant (C) 3 months after additional soil infection

After a first acclimatation cycle under greenhouse conditions g/c1 and g/c2 plants were successfully established, but g/c3 plant failed to survive. In a first attempt to gather information on the response of hop transgenic lines to fungal infection, preliminary bioassays in greenhouse and in vitro were performed for wilt resistance testing by root-dip inoculations with *Verticillium albo-atrum* (strain 157B, kindly provided by Peter Darby). In vitro development of symptoms was very fast and a clear difference of response was observed between transgenic and untransformed control plants. While the control plant died in less than 2 weeks, all transgenic plants showed moderate symptoms, such as yellow leafs, among which g/c3 seemed to present higher tolerance (Fig. 3). Response to infection in

greenhouse plants was, on the contrary, slower and less evident, but, nevertheless, g/c1 and g/c2 plant development did not seem to be affected in anyway while some wilting and yellowing become apparent in the control hop plants 10 weeks after infection (Fig. 3). Even though no conclusions can be drawn for now, these results seem very promising as to a successful stable introduction of enhanced resistance against *Verticillium* wilt through the expression of barley chitinase and glucanase genes. After the development of new shoots this spring, these plants will complete their first vegetative cycle and a further assessment of transgene integration and levels of expression will be performed by qRT-PCR.

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PRELIMINARY ANALYSIS OF THE HOP (*HUMULUS LUPULUS* L.) PROTEOME

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Abstract

Hop breeding compels an understanding of the biosynthesis of commercially important secondary metabolites. The progression of this understanding will eventually call for a systems biology approach, examining all complex interactions of the many elements that make up the lupulin gland. This study outlines the first steps towards understanding the lupulin gland proteome. We are in the preliminary stages of developing a platform for the extraction and proteomic analysis of hop lupulin gland tissue, which will subsequently be used to generate a database of the protein complement of the lupulin gland. It is anticipated that this database will allow the identification of candidates for proteins involved in the biosynthesis of the commercially important secondary metabolites.

Keywords: Hop; *Humulus lupulus* L.; proteomics; lupulin gland; mass spectrometry

Introduction

Lupulin glands are highly complex and dynamic biological units within which an intricate spectrum of secondary metabolites form. Considerable variation exists in the composition of these secondary metabolites, with subsequent effects on flavour, biological activity and processing efficiency in their brewing and phytoceutical applications. The understanding of the biochemistry of these secondary metabolites, and the molecular mechanisms used by the hop plant to control and regulate their production, is essential to the breeding of a desirable chemical profile, and could be achieved using a systems biology approach. A systems biology approach is directed at systems-level understanding of biological organisation, encompassing genomics, transcriptomics, proteomics, lipidomics, glycomics, metabolomics, and interactomics (the study of the interactions that occur between them). The aims of this study will be to explore the potential of proteomics to better understand the protein complement of the hop lupulin gland. Proteomics is the systematic, large-scale study of the protein complement ("proteome") of an organism, tissue, cell or organelle (Pandey et al., 2000). It is a field that has recently evolved in plant research (Hochholdinger et al., 2006; Kersten et al., 2002), but has established a firm role in the research of microorganisms and mammals due to its speed, accuracy and high-throughput capabilities (Baginsky et al., 2006; Park, 2004; Phizicky et al., 2003; Rakwal et al., 2003; Roberts, 2002). The capacity of proteomics to penetrate the complex biochemical pathways and molecular mechanisms underlying organism growth, development and environmental response, makes it an ideal tool for the analysis of hop lupulin glands. An initial aim is to determine a suitable technique for the extraction and analysis of proteins from hop lupulin gland tissue. Once a suitable technique has been determined, a biologically meaningful proteomic database for hop lupulin glands can be established, which will allow the identification of candidate proteins involved in the biosynthesis of commercially important secondary metabolites from hop.

Methods

The key elements in the analysis of the plant proteome include the extraction, separation, analysis and identification of proteins (Sha Valli Khan et al., 2007). The technology employed for each of these elements has evolved rapidly, endeavouring to identify and characterise proteins more accurately, quickly and easily, and thus several different approaches can be taken, depending on the particular aims and requirements of the analysis. The preliminary aim of this study is to determine an appropriate approach for the analysis of the lupulin gland proteome. The requirements for this technique are that: (i) it produces an essentially complete protein complement of the lupulin gland, as opposed to only an 'extractome' (which may be difficult due to the resinous nature of the lupulin gland contents); (ii) it is reproducible across replicates, such that differences between cultivars, etc, can be recognised; (iii) the protein extraction is chemically compatible with the appropriate analysis technologies; and (iv) it is as safe, straightforward and time-efficient as possible.

Despite most developments in the methodology and technology of plant proteomics being aimed at increasing the resolution of protein separation and the speed of automated protein identification (Rose et al., 2004), high quality results from any proteomics study is most critically based on good sample preparation: the extraction of a maximum number of proteins with minimal contamination (Van Wijk, 2001). This extraction technique used in this study will involve the use of physical treatments, such as grinding or sonication, combined with chemical treatments, such as solvents and buffers. Traditionally, electrophoresis has been the primary means for the separation of plant protein extracts (Zhu et al., 2003); electrophoresis methods, however, are faced with many pitfalls: gels are cumbersome to run, relatively low-throughput, with no automation possible (Newton et al., 2004). This study will therefore use a mode of liquid chromatography to separate the complex mixture of proteins in the lupulin gland. Liquid chromatography overcomes many of the flaws of electrophoresis methods (Zolla et al., 2003), and can be directly interfaced with mass spectrometry (MS), the current technology used for the analysis and identification of proteins (Agrawal et al., 2005). Several different modes of MS currently exist; this study will examine the potential of these different modes for the analysis of proteins from the hop lupulin gland. Identification of extracted proteins is most commonly involves matching proteins to a protein sequence or nucleic acid sequence database. This study will match the proteins extracted from hop lupulin glands to multiple databases in order to maximise successful protein identification. Using the protein extraction technique developed in this research, the next objective is to establish a proteomic database of hop lupulin glands. This database will be generated from the lupulin glands of multiple hop cultivars (allowing various comparisons between the different cultivars). From this database, candidates for proteins of interest, for processes such as the biosynthesis of commercially important secondary metabolites, can be identified.

Results

As this research is only in the very preliminary stages of the development of an effective protein extraction and analysis platform, it is too early to give a full account of the outcomes and findings of the proteomic approach. Preliminary observations appear promising, with candidates for a number of proteins of interest clearly identified, as well as several potentially novel proteins.

Discussion

Although this study is only partially complete, the data generated thus far is already revealing the potential of proteomic analysis for the study of hop lupulin glands. The information revealed in this research will provide a sound basis for research on the lupulin gland proteome, and is a starting point for future research on the entire lupulin gland biological system. An integrated systems biology approach to the study of lupulin glands will better enable hop breeding to improve the chemical profile of hop cultivars for their various commercial applications.

Proteomics is commonly considered one of the most innovative and ground-breaking fields of study currently in development. However, the capabilities of identifying proteins, analysing their quantity, activity, structure and function, and relating them to genes has long preceded the emergence of the field of proteomics. Many of the technologies encompassed in the proteomics methodology (liquid chromatography, MS, etc) also have existed, in some form, long before proteomics was titled. There is rationale behind the reputation, though. Firstly, the recent boom in genomic sequence information has vastly reduced the amount of information required to definitively link a protein to a gene (Roberts, 2002). Secondly, the application of high throughput technologies (MS in particular) for the purpose of proteomics is only now being realised; the sensitivity and speed with which this technology enables matching between protein and gene information greatly exceeds that possible with previous methods used (such as Edman chemical sequencing). These two factors have allowed the fundamental difference between proteomics and previous approaches to protein research: the analysis of complex protein samples, as opposed to particular proteins one at a time. Through the study of the entire complement of proteins, a global view of all biochemical processes taking place can be achieved. It is this fact that is important in the study of the lupulin gland biochemical system, as all proteins involved in the biosynthesis of the commercially important secondary metabolites can be recognised, and in addition, novel mechanisms involved in the regulation of these biochemical processes may also be recognised.

Future investigations on the hop lupulin gland biochemical system, using the proteomic platform that will be developed in this research, could include the investigation of changes in the lupulin gland proteome in response to environmental and experimental variables, such as nutrient stress, increases in CO_2, disease resistance, water stress and temperature stress. These factors have been found to affect the seasonal yield of hop (Francke et al., 1982; Thomas, 1980; Thomas et al., 1969). By applying these variables to hop, the differentially expressed proteins can be examined to better understand the effects of environmental stresses on the biosynthesis of commercially important secondary metabolites. These types of studies have been successfully completed in other species (Bae et al., 2003; Bokhari et al., 2007; Bona et al., 2007; Sweetlove et al., 2002). This study was the first to be completed on the protein complement of hop lupulin glands, and the prospective possibilities extend to many different areas.

Aside from hop lupulin glands, this research may also have some implications for future proteomic analyses in other studies, particularly on plant species. Proteomics is a relatively recent area of research; while protoemics has established a firm role in the research of micro-organisms and mammals (Baginsky et al., 2006; Park, 2004; Phizicky et al., 2003; Rakwal et al., 2003; Roberts, 2002), its application to plants has been slower to evolve (Jorrin et al., 2007; Van Wijk, 2001). Currently, proteomics is generally restricted to the study of model plant species, with some broadening to globally important agricultural crop species. The results achieved in this proteomic analysis of hop may therefore provide a reference point to other proteomic analyses of plant species, particularly where there is limited genetic and protein sequence data.

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CHARACTERIZATION OF NEW GENES IN HOP *(HUMULUS LUPULUS* L.)

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Abstract

Microsatellite markers have been increasingly used in genetic studies of crop species for their high level of polymorphism and reliability. A new trend in molecular markers is to develop new molecular markers in specific sequences, near or within structural genes. In our work, we characterized some new genes and developed novel molecular markers close to specific structural genes for hop (*Humulus lupulus* L.). These genes belonged to transcription factors and genes involved in metabolic pathways of secondary metabolites. In EST database analysis, we found 28 EST-SSR markers, which represented 23 gene loci with total of 1263 EST sequences. We measured the level of polymorphism among 11 individual hop genotypes. These markers can be used for purity control of varieties, evaluation of genetic variability, marker-assisted selection (MAS), genetic linkage mapping and quantitative trait loci (QTLs) analysis.

Keywords: hop, *Humulus lupulus* L., sequence-tagged sites (STS), expressed sequence tag (EST), microsatellite, simple sequence repeat (SSR)

Introduction

Hop (*Humulus lupulus* L.) is a dioecious perennial climbing plant. Only female plants are cultivated for commercial use, mainly in the brewing industry and to a smaller extent for pharmaceutical purposes. Female inflorescences, referred to as cones, contain hop bitter resins, essential oils, polyphenols and tannins (Neve 1991). The characteristic profile of chemical components in hop cones varies in varieties and is used for identification. The development of DNA technology has provided a number of methods, which eliminate the influence of environmental factors, to detect differences at the sequence level for evaluation of hop genotypes. RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), STS (Sequence-Tagged Sites), SSR (Simple Sequence Repeat), ISSR (Inter-Simple Sequence Repeat) and AFLP (Amplified Fragment Length Polymorphism) were successfully used for DNA fingerprinting of hop genotypes (reviewed by Patzak, 2001). The microsatellite SSR markers have been the most used for studies of molecular variability of hops (e.g. Cerenak et al., 2004; Murakami et al., 2006; Stajner et al., 2008). The most of SSR markers occurs in non-coding regions of hop genome, but a new trend in molecular methods is to develop molecular markers in specific sequences, near or within structural genes. The possibility to use of polymorphism in structural genes was reported by Patzak et al. (2007), Bassil et al. (2008) and Castro et al. (2008). The increasing information about hop DNA gene sequences in GeneBank EST database provides for us possibility to look for new gene specific molecular markers. In this study, we reported about new characterized structural genes and possibility to use their sequence for STS markers. Additionally, we developed new efficient and reliable expressed sequence tag microsatellite repeats (EST-SSR) markers for genotyping of hop.

Materials and Methods

Plant material and DNA isolation

Eight world hop cultivars: Osvald's clone 72 – Czech Republic, Fuggle, Wye Target, Brewers Gold - UK, Southern Brewer – South Africa, Magnum, Taurus Germany, Columbus – USA, from the world hop collection of the Hop Research Institute in Žatec, three female individuals of wild hop from Russia – Caucasus region (*H. lupulus* var. *lupulus*), Canada (*H. lupulus* var. *lupuloides*) and USA (*H. lupulus* var. *neomexicanus*) were used for experiments. Additionally, a female individual of *Humulus japonicus*, from seed, were used for test of cross-species marker amplification. DNA was isolated from young leaves according to Patzak (2001).

Molecular analyses

In structural genes analysis, LASERGENE system v. 7.1 (DNAStar, Madison, WI, USA) was used for analyses of sequences, multiple alignments (MegAlign module) and design of primers (PrimerSelect module). DNA fragments and PCR amplified products were cloned in the vector pCR-Script Amp SK(+) (pCR-Script™ Amp Cloning Kit, Stratagene, La Jolla, CA, USA). Automatic sequencing was performed in sequencing laboratory of Charles University in Prague (Czech Republic).

GeneBank EST database of *Humulus lupulus* sequence were screened for di-, tri- and tetranucleotide microsatellite repeats using Advanced BLAST 2.0 (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Found EST sequences, with 20 base microsatellite repetition, were again screened by BLAST to GeneBank EST database with aim to find length polymorphism in similar sequences. BLAST analysis of GeneBank nucleotide sequences was used for homology study with known genes. Putative functions were assigned to the unigenes based on their homology to annotated sequences in GeneBank database (Table 1).

In a typical PCR reaction (*Taq* PCR master mix kit, Qiagen, Hilden, FRG) we used the following amplification conditions: 2 min at 94 °C, 35 cycles (30 s at 94 °C; 60 s at 54 °C, 90 s at 72 °C); 10 min at 72 °C. PCR was performed on TGradient thermocycler (Biometra, Goettingen, FRG). Amplification products were resolved via 5% denaturing (8M urea) polyacrylamide gel vertical electrophoresis and visualized by silver-staining (Patzak, 2001). The allele counts were scored for the presence or absence in each sample, based on allele size measured with 20 bp DNA Marker (Bio-Rad, Hercules, CA, USA).

Results

In our work, we characterized some new genes and developed novel molecular markers close to specific structural genes for hop (*Humulus lupulus* L.). Most of these genes consisted of structural genes involved in metabolic pathways of secondary metabolites. One of them is chalcone synthase gene, when we found there was the length polymorphisms in *chs*1, *chs*2, *chs*3 and *chs*4 genes (Table 1). Another key enzyme for prenylation is isopentenyl diphosphate isomerase (*idi*). We found two different *idi* alleles with promoters, which were composed of four exons and three introns. Next enzyme, which we characterized, was phenylalanine lyase (*pal*). We also found two different *pal* alleles with promoter and one intron. We found no polymorphism in both genes, only some single nucletide polymorphism (SNP) and indels in promoters. On the contrary, we found wide polymorphism in 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, which is involved in non-mevalonate (MVA) pathway of isopentenyl diphosphates synthesis (Table 1). Recently, four genes of terpene synthases were characterized. In our experiments, we found other analogue with promoter of monoterpene synthase (*mts*). Next we found polymorphism in intron 3 of leucoanthocyanidin synthase gene (*lar*). Transcription factors (TFs) are additional genes' group, which our research is focused. We found that there were two introns in *myb*1 (182 and 108 bp) and *myb*3 (209 and 119 bp) genes, respectively. We also partly

characterized one gene of WRKY and mitogen activated protein kinase (MAPK). We found no polymorphism in TFs, which indicated their conservative function in hop plant.

From search of GeneBank EST database of *Humulus lupulus* sequence, we selected 33 microsatellite SSR loci with total of 1434 EST sequences. Forty primer pairs were designed from these microsatellite SSR loci. Out of these primers designed, 34 amplified the expected products, four primer pairs had no products and two primer sets yielded larger products suggesting the existence of intron. For the primers with successful amplification, 28 pairs of primer revealed polymorphism among the tested 11 individuals of *H. lupulus* (Table 1). For the primers with successful polymorphism amplification, 18 pairs of primer revealed cross-species amplification in *H. japonicus* (Table 1).

All of the developed gene specific markers were useful for genotyping of hop genotypes. They can substitute and supplement known STS and SSR markers.

Discussion

This study provided characterization of structural genes and new gene specific markers for genotyping of hop (*Humulus lupulus* L.). Some of them were characterized previously (e.g. Novak et al., 2006; Matousek et al., 2005; 2007) and there are length and nucleotide polymorphisms in them. As gene specific markers were used chalcone synthase genes (Patzak et al., 2007; Bassil et al., 2008) and valerophenone synthase (*vps*) gene (Castro et al., 2008). Recently, Nagel et al. (2008) and Wang et al. (2008) characterized structural genes' background of secondary metabolic pathways was by ESTs analysis. Nagel et al. (2008) reported that methyl 4-erythritol phosphate pathway was ten times more preferable than mevalonate (MVA) pathway for isopentenyl diphosphate synthesis. Wang et al. (2008) reported about third allele of *mts* gene, which were not characterized, and possible three alleles of *pal* gene.

Nowadays, microsatellite markers are becoming the markers of choice due to the level of polymorphism, as well as higher reliability. The use of ESTs is a new trend for SSR markers in all main crops. Our search of GeneBank EST database produced 28 EST-SSR markers for hop (*Humulus lupulus* L.), which represented 23 gene loci with total of 1263 EST sequences (Table 1). Found EST-SSR markers were not homologous to previously reported SSR markers (e.g. Jakse et al., 2008; Hadonou et al., 2004; Stajner et al., 2005) by BLAST analysis.

We proved that developed gene specific markers could be successfully used for the analysis of genetic diversity in the hop collection. Therefore, they can be used as a powerful tool for the assessment of genetic variation within hop varieties, genetic resources and wild hop populations. Their presence in genes that can encode economically important traits might prove valuable for marker-assisted selection (MAS). They are readily applicable in breeding for genetic linkage mapping and quantitative trail loci (QTLs) analysis.

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Table 1. Characteristics of 28 polymorphic EST-derived SSRs for *Humulus lupulus* and cross-species amplification in *Humulus japonicus*.

						H. lupulus		*H. japonicus*	
Accession No.	*EST*	Gene	Region	Repeat motif	Primer sequence (5'–3')	PCR size range (bp)	N_A	N_A	PCR size (bp)
AB061020	81	chalcone synthase 2	intron 1	CT/AG, AT/TA	**F:** GGCCAATCCTGCCAACTACATCAC **R:** GTCACACATATTTGGGTTCTGCTTT	280-300	2	1	285
AB061022	0	chalcone synthase 3	intron 1	AT/TA	**F:** GGCCACTCCTGCCAACTTCATCAT **R:** TACGTCCAGGGACGGCGCCATGA	310-330	4	0	
AJ430353	20	chalcone synthase 4	intron 1	AT/TA	**F:** TGCATGTCCACCCAACTTCGTAAC **R:** GGTGCCGAGTACTCGCACATATTC	270-310	6	1	275*
AB053486	1	farnesyl pyrophosphate synthase	5'UTR	CT/AG, AT/TA	**F:** GTGGCCTCCAAAGCTCTATAA **R:** CCCATTGGCGAGAATCATCGGTGA	265-300	7	0	
EX515564	23	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	intron 1	T/A in/del(8)	**F:** CTCAAAGATTCTTCCTTTTCGGG **R:** TTGATGAAAACTGATGATGC	450-475	2	1	440
			intron 2 CDS	T/A GAA/TTC	**F:** AAAGCGAAAACTCATGAAAAGGTT **R:** GACACATGTAAGGTGACCTTTACA	230-255	5	1	240
EX519013	20	unknown hypothetical protein	CDS	AT/TA, in/del(10)	**F:** AGATCRGCCTGTCCACTTTG **R:** AGCCAGCTTACATTTATTCACTTA	230-260	6	3	225, 242, 262
ES653414	36	GAST-like gibberellin regulated protein	CDS	AT/TA	**F:** ACTTAAAACCATATGTCCATC **R:** CATATATTCTATCAGATAATAA	200-245	3	0	
ES655424	15	unknown hypothetical protein	CDS	CTT/AAG, T/A	**F:** TCCTTCCAACGCCGCTAC **R:** AAATCCAATAYTTAAACCAAATA	270-295	4	0	
				CTT/AAG, T/A	**F:** TCCAGATTCTTCGATATTTTC **R:** AAATCCAATAYTTAAACCAAATA	500-540	5	0	
EX518839	5	unknown hypothetical protein	CDS	CTT/AAG, in/del(110)	**F:** TGTGTTTATTAGTAGCTTCTTCAG **R:** CTTAGGGCCATCACAACACT	390-410	5	2	403*,410*
ES653576	5	complex 1 protein (LYR family)	CDS	GA/TC	**F:** GTGAAGAGAAGGCAGAAGAAG **R:** CTTGGGATTGGTAGAGTGAG	230-265	8	0	
EX520153	19	NADH-ubiquinone-like oxidoreductase	CDS	GA/TC	**F:** GAAGGTGTTGATTTCATCGAGAG **R:** TCGCGGCATCGAGACATACACT	120-150	4	0	
ES654506	4	unknown hypothetical protein	CDS	GA/TC, in/del(6)	**F:** CATGGCGGTTTTCGACTTTATCTA **R:** GTCCGTACACGATCCCTGAGA	360-370	4	1	385
EX519698	653	metallothionein-like protein	CDS	CA/GT	**F:** CTATGGGGCAGAGAATGATGG **R:** ATAGGACAGGAAATGGAGTTAGTA	300-310	4	2	360, 500

Accession No.	*EST*	Gene	Region	Repeat motif	Primer sequence (5'–3')	*H. lupulus*		*H. japonicus*	
						PCR size range (bp)	N_A	N_A	PCR size (bp)
EX516364	269	major allergen	CDS	CTT/AAG, in/del(4)	**F:** CCACTCCAAGTTCAAAGCCTTC **R:** GTTCCAGGCCCTCCATCTC	230-250	3	1	230*
				AT/TA, in/del(3)	**F:** GGAAAACCCTGACACTTACAAC **R:** CAAACATTACATATATAARCCAG	205-250	6	1	238
EX516827	12	non-photosynthetic ferredoxin	CDS	AT/TA, T/A	**F:** GGATTCATCTTACATCGCATATC **R:** CCCCTGAAAGATGACTCATTTC	220-235	4	4	220-235*
ES654004	14	unknown hypothetical protein	CDS	AT/TA, ATT/AAT	**F:** GGTGTCGATGGATATCAATGAAAG **R:** GGTTGTACATGTTAGTTATTGTTC	310-315	2	1	324
ES652842	14	unknown hypothetical protein	CDS	ACT/AGT, ACC/GGT, in/del	**F:** GAGTTTCCCGTCGTTTCAAG **R:** GTGTGCTCTGTGCCGCCTCATG	200-450	8	3	160, 200, 335
EX521346	35	histone H2B	CDS	ATG/CAT	**F:** GTACAACAAGAAGCCCACCAT **R:** TTATAAACAATCAATCCAAAACA	200-220	3	0	
EX520741	9	thioesterase family protein	CDS	ATG/CAT, in/del	**F:** TCTTGTTGAGCCAGGCCGTCTTCT **R:** CACAGCTGAGCCAACCAAATCCAC	160-330	7	1	192
ES437694	7	dormancy/auxin associated family protein	CDS	AT/TA, ATG/CAT	**F:** CAGCCCTCCTCCTCCTTACG **R:** CGATATAGCTACATCAAACAG	240-310	9	2	306*, 310*
ES654725	2	calcium-binding EF hand family protein	CDS	ATG/CAT	**F:** TTTAACAACTTTTCCAGGTC **R:** TAGTGTCATTAAGGCAAAAAC	200-215	4	0	
ES653423	13	dehydrin	CDS	ATG/CAT	**F:** GACCWCCACTACTGCTACTCATCA **R:** ACCACACCATAATCAAACGAAATA	210-220	3	2	196, 200
				ATG/CAT, in/del	**F:** AACTTTTCAGTTCTCACTCTCATC **R:** ACCGGTTCGTGTTCAGACAC	240-280	6	2	214, 220
				CTT/AAG, in/del	**F:** AAAAGCTCCACCGTTCTCACAG **R:** ACAGTCTCTGTTGTTGTGTAGC	320-360	4	1	365
EX517791	6	unknown hypothetical protein	CDS	ATG/CAT, in/del	**F:** ATAGCCATGGGAGCAACTTTA **R:** CACAACCGGCAGCACCCCTATTA	275-280	2	0	

EST, number of ESTs in GeneBank; N_A, number of alleles; *, same to *H. lupulus*

FUNCTIONAL ANALYSIS OF HOP *(HUMULUS LUPULUS* L.) REGULATORY FACTORS FROM BZIP AND BHLH FAMILIES IN TRANSIENT EXPRESSION SYSTEMS.

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Abstract

Functional analyses of cloned hop regulatory factors (TF) *HlbZIP1, HlbZIP2* and *HlbHLH1* were performed in transient expression system using *N. benthamiana* leaf co-infiltration with *A. tumefaciens* containing TF and three hop promoters fused to GUS reference gene. bZIPs and bHLH genes were combined with *HlMyb1*, l-*HlMyb3*, s-*HlMyb3* TFs to check possible interactions. Both *Hl*bZIPs and *Hl*BHLH1 activated mainly promoters of *chs*_H1 and *omt1* (P*omt1*) genes, while the response of *vps* promoter to cloned hop TFs was very low. It was found that *Hl*Myb1 activates strongly P*omt1* and could be thus useful in "myb biotechnology" to modify regulation of this gene in transgenic hop. HPLC analysis of secondary metabolites in infiltrated *Petunia hybrida* was performed. Whereas GUS analysis of TFs combinations revealed predominantly single additive activation effects on corresponding promoters, accumulation of some *P. hybrida* phenolic compounds occurred rather by more complex interactive mechanism.

Keywords: hop, *Humulus lupulus* L., transcription factors, transient expression assay, plant metabolome, prenylflavonoids, promoter elements, *P. hybrida*, *N. benthamiana*

Introduction

In natural evolution (e.g. Hanson et al., 1996) as well as in „evolutionary“ changes mediated by breeding and selection technology, the combinations (combinatorial action-see Singh et al. 1998) and interplay of transcription factors (TFs) provides new phenotypic variability that is a source of novel and genetically improved phenotypes. The same is true for signaling pathways regulating plant metabolome and TFs that influence transcriptome of metabolic enzymes. From various data obtained from research of *A. thaliana* (Riechmann and Ratcliffe, 2000) and other plant species, one can deduce that especially R2R3 MYBs, basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), zinc finger C_2H_2(Zn), MADS, and WRKY (Zn) can be involved in the development and production of plant secondary mebolome connected to flower organs and tissues like hop cones and lupulin glands.

bZIP TFs fulfill various regulatory functions in plants, for instance, from activation of genes responding to biotic or abiotic stress to genes connected to flower development. Jacoby et al. (2002) divided bZIP factors of Arabidopsis thaliana to 10 groups designated A-J and S, based on protein comparisons and analysis of conserved and functional domains. Previously**,** we selected two bZIP factors preferentially expressed in hop flowers and cones. These bZIPs were selected by the use of cDNA-AFLP technique (Bachem et al., 1996) and cloned from hop cDNA library using short ESTS as probes (Matoušek et al., 2008). Hop bZIP factors, *Hl*bZIP1 and *Hl*bZIP2 have predicted molecular masses of 27.4 and 34.2kDa, respectively. While *Hl*bZIP1 is rather neutral (pI 6.42), *Hl*bZIP2 is strongly basic (pI 8.51). Both these factors are partly similar to group A of G-box-like bZIPs. bHLH TFs comprise very heterologous group. Already Atchley and Fitch (1997) reviewed 242 bHLH proteins that were in arranged into five groups based on binding to DNA at the hexanucleotide E-box, the amino acid patterns in other components of the motif, and the presence or absence of a leucine zipper. The first bHLH from hop *Hl*bHLH1 has 33.6 kDa and predicted pI 6.08. This TF shows

maximal homology to sequence mapped to chromosome 2 of *Vitis vinifera* (EMBL:CU459228) and to *A. thaliana* factor bHLH71 of unknown function (EMBL:Q56XR0). bHLH factors are known for their potential to interact with MYB factors. These interactions are important for regulation of various developmental processes (e.g. Ramsay and Glower, 2005; Yamazaki et al., 2003, Hartmann et al., 2005).

Binding sites of bZIP and bHLH TFs were previously identified in promoters of the oligofamily of genes encoding "true" hop chalcone synthases-*chs*_H1 (EC 2.3.1.74) (Matoušek et al., 2002; Matoušek et al., 2006) expressed in glandular trichomes and presumably involved in production of prenylated chalcones (e.g. xanthohumol). These metabolites are of particular interest in view of their medicinal properties (e.g. Stevens and Page, 2004). Thus bZIP and bHLH motifs together with MYB binding sites seem to be important for regulation of this chalcone synthase.

In the present work we cloned promoter elements of two additional important lupulin-encoding genes 1. valerophenone synthase (*vps*) (Okada, 2001) and O-metyltransferase 1 (*omt1*) (Nagel et al., 2008) and compared interaction of these promoters with so-far cloned TFs from hop, especially from bZIP and bHLH families in transient expression system in Nicotiana benthamiana and Petunia hybrida plants.

Materials and Methods

TF genes *HlMyb1*, l-*HlMyb3*, s-*HlMyb3*, *HlbHLH1*, *HlbZIP1* and *HlbZIP2* were described previously (Matoušek et al., 2005, 2006, 2007 and 2008). These genes were cloned from cDNA library of hop clone Osvald 72 using probes generated from conserved motifs or based on cDNA ESTs selected using cDNA AFLP method (De Keukeleire et al., 2005; De Keukeleire, 2007). Materials were cultivated, *Agrobacterium tumefaciens* vectors were prepared and infiltration was performed essentially as described by Matoušek et al., 2006 and 2007. Promoter activity was evaluated using GUS assay (Jefferson 1987). Metabolite analyses were performed essentially as described by Matoušek et al., 2005, 2006 and 2007. *Omt1* promoter (P*omt1*) was cloned from Osvald's clone 72 using inverse PCR and includes sequence 570 bp upstream coding region cloned by others (Nagel et al., 2008); *vps* promoter (P*vps*) sequence (600 bp upstream *vps* coding region) was cloned from Osvald's 72 hop using sequence information (EMBL:AB047593) published by Okada et al. (2003).

Results

In our previous paper (Matoušek et al., 2008) 500 bp *chs*_H1 promoter (P*chs*_H1) was subjected to analysis for possible activation by cloned hop Myb TFs. We used system of infiltration of *A. tumefaciens* strains containing TF vector and GUS reporter/promoter containing vector into *N. benthamiana* leaves (Fig.1a and 1b), respectively mixed at 1:1 ratio. GUS activity was then measured in homogenates from leaves infiltrated with TFs and compared to controls (LBA 4404). In the present work we aimed to perform comparative functional assays of hop TFs using *chs*_H1, *vps* and *omt1* promoters. Therefore, plant vectors (Fig1a) bearing *HlZip1*, *HlZip2* and *HlbHLH1* genes were newly cloned and the reference vectors for P*vps* and P*omt1* of lengths similar to P*chs*_H1 were constructed. Unlike to P*chs*_H1 rich in putative bZIP, bHLH and MYB binding sites, in cloned P*vps* region only single G-boxA bZIP at position –(191-197) was identified. No MYB and bHLH (-like) boxes homologous to those identified in P*chs*_H1 (Matoušek et al., 2006) were identified in P*vps*. Nevertheless, using recent databases, putative Myb2 consensuat box YAACKG was found in P*vps* at position 459-465, MYC consensus box at position –(72-77) and HDZIPIII box at position –(62-72). Also in 600bp P*omt1* no classical bZIP G-boxes (CACGTG) were detected. Although quite unrelated promoters, there were several common binding TF elements in P*omt1* and P*chs*_H1, mainly GT1 consensus box, GTGA box, EBOX, CAAT and YACT motifs. MYB core box (GTGTTG) has been identified at position –(529-534) in P*omt1*. This box is quite different from MYB boxes characteristic for P*chs*_H1.

Transient expression system revealed that while very low response was detected for P*vps* after TF co-infiltration (the highest signal was observed for *Hl*bZIP2), there was much

stronger positive response (activation) observed for P*chs*_H1 and P*omt1* (Fig. 2). While P*chs*_H1 was activated more by *Hl*bZIP1 than *Hl*bZIP2 and significant activation was achieved by both *Hl*Myb3 variants, P*omt1* was mainly stimulated by *Hl*Myb3 variants than by *Hl*bZIPs (Fig.2). To assay possible combinatorial promoter responses to *HL*bHLH1, combinations of this TF with *Hl*MYB3 variants and *Hl*MYB1 TF (Matoušek et al., 2005) were analyzed (Fig. 3). It follows from these results that *Hl*bHLH activates mainly P*chs*_H1, while response of Pomt1 to this factor is significantly lower. P*vps* response to *Hl*bHLH was very low (Fig.3). It also follows from our results that *Hl*MYB1 TF activates all promoters with higher extent than variants of *Hl*MYB3 and that especially P*omt1* is strongly activated by this TF. Combinations of TFs show that there are rather additive than interactive effects of HlbHLH1 and cloned MYB TFs. Exception from this tendency was observed for *Hl*bHLH1/*Hl*MYB1 combination with P*omt1*, where inhibition was observed compared to independent stimulation of P*omt1* by *Hl*MYB1 (Fig.3).

It can be concluded that hop TFs activate selectively hop *chs*_H1, *vps* and *omt1* promoters. It was of interest to analyze possible selective action of cloned TFs on the level of metabolome. For this purpose we applied method of combinatorial infiltrations of *Petunia hybrida* developed previously (Matoušek et al. 2007, 2008). In infiltrated leaf sectors of *P. hybrida*, some phenolic acids and flavonol glycosides accumulated ~~to~~ much higher levels in comparison to the controls. For instance, peaks 1,3, representing phenolic acids (ferulate group) with retention times (Rt) of 9.3 and 13.4 min (not shown), were increased by *s-Hl*MYB3, similarly to our previous work (Matoušek et al., 2007). Combinatorial experiments showed that *Hl*bZIP1 TF co-activated accumulation of these compounds by an interactive manner if co-infiltrated with s-*Hl*MYB3 TF and that both TFs (*Hl*bZIP1 and *Hl*bZIP2) co-activated accumulation of Rt 9.3 and 13.4 compounds when co-infiltrated with l-*Hl*MYB3 (Fig.4A). All analyzed TFs, except for l-*Hl*MYB3, also co-activated the accumulation of flavonol glycoside (quercetin) with Rt 18.3, which is similar to flower anthocyanins. Combinations s-*Hl*MYB3+*Hl*bZIP1 and l-*Hl*MYB3+*Hl*bZIP2 lead to the strongest activations (Fig.4B). These results show that all analyzed TFs activate flavonol glycoside metabolome genes in *P. hybrida* supporting their involvement in flavonoid biosynthetic pathways in hop.

Discussion

We showed in this work that newly described hop TFs from bZIP and bHLH families are functional and activate promoters of hop true chalcone synthase, valerophenone synthase and O-metyltransferase with different selectivity. The most universal response was found for P*chs*_H1, where various general bZIP, MYB and bHLH binding elements were described previously (Matoušek et al., 2006). In general, the lowest response to TFs co-infiltration was found for P*vps*. These results are consistent with previous observations that VPS expression is very specific for hop glandular trichomes (Okada et al., 2003, Castro et al., 2008). It is probable that *VPS* regulation needs some tissue specific co-factor for general activation. *Hl*bZIP1 and *Hl*bZIP2 are able to activate both, P*chs*_H1 and Pomt1, suggesting that these G-box-like bZIPs are not strictly G-box specific. We found that *Hl*Myb1 TFs strongly activates Pomt1. Therefore, this MYB could be useful for “MYB biotechnology” to modify regulation of OMT1 and related network genes by means of hop transgenosis. Finally, we found specific activation of *P. hybrida* metabolome with hop TFs and some combinations of TFs lead to metabolite overaccumulation, obviously not by additive, but rather an interactive manner. This can be explained by independent co-activation of set of genes that interact within particular metabolical pathway, while TF action on single promoter(s) can have simple, additive character. We believe, these results are relevant to dissect hop metabolome network in the future.

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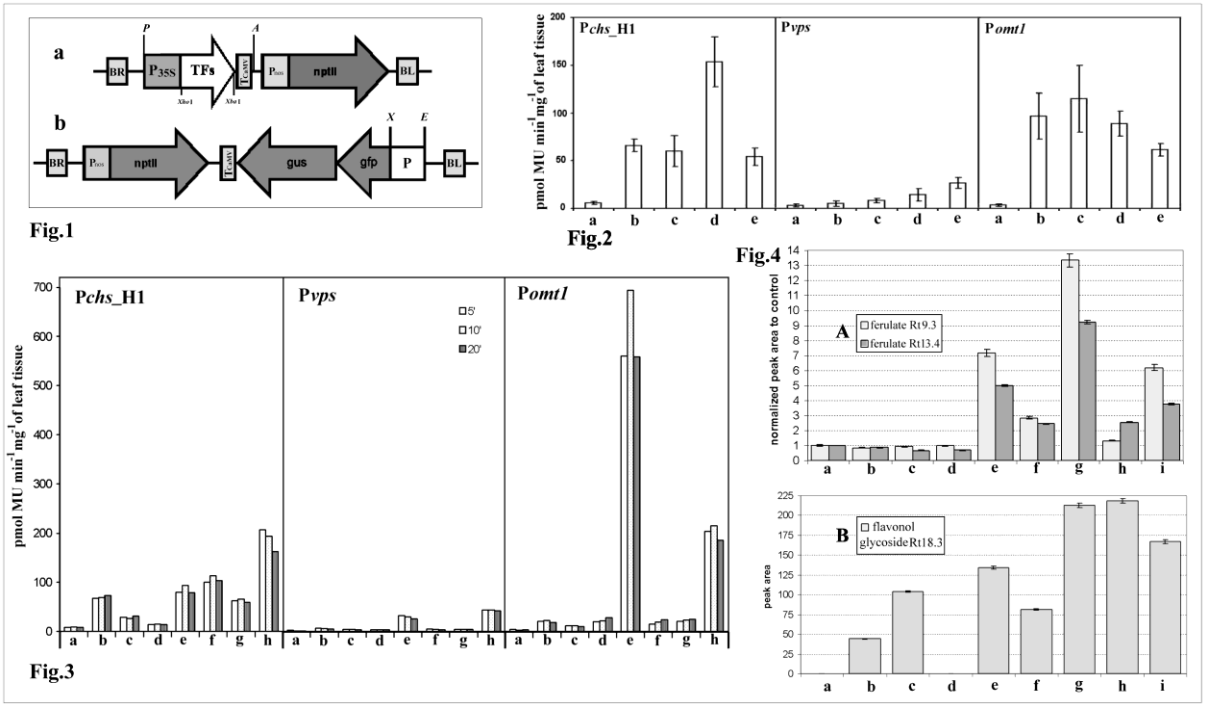

Fig. 1 Schematic drawing (not in the scale) of expression cassettes of TF (a) and GUS (b) vectors used for leaf infiltration. NptII-kanamycin resistance gene; BR and BL right and left T-DNA borders; P-promoters; integration with *Asc*1 (A), *Pac*1 (P), *Xho*I (X) and *Eco* R1 (E).

Fig. 2 Analysis of *chs*_H1, *vps* and *omt1* promoter activation by *Hl*bZIPs and by *Hl*Myb3 variants using GUS transient assay. Infiltrations-GUS vectors as indicated in the figure plus: a, LBA 4404-background control; b, l-*Hl*Myb3; c, s-*Hl*Myb3; d, *Hl*bZip1; e, *Hl*bZip2

Fig. 3 Analysis of *chs*_H1, *vps* and *omt1* promoter activation by *Hl*bHLH1 and *Hl*Myb factors using GUS transient assay. Infiltrations-GUS vectors as indicated in the figure plus: a, LBA 4404-background control; b, *Hl*bHLH1; c, l-*Hl*Myb3; d, s-*Hl*Myb3; e, *Hl*Myb1; f, *Hl*bHLH1+ l-*Hl*Myb3; *Hl*bHLH1+ s-*Hl*Myb3; *Hl*bHLH1+ *Hl*Myb1.

Fig. 4 Analysis of accumulation of ferulate group compounds (A) and quercetin-like compound (B) in *Petunia hybrida* leaves infiltrated with hop TFs. Vector combinations: a, LBA 4404-background control; b, *Hl*bZip1; c, *Hl*bZip2; d, l-*Hl*Myb3; e, s-*Hl*Myb3; f, *Hl*bZip1+l-*Hl*Myb3; g, *Hl*bZip1+s-*Hl*Myb3; h, *Hl*bZip2+l-*Hl*Myb3; i, *Hl*bZip2+s-*Hl*Myb3.

MODIFICATION OF THE SYNTHESIS OF BIOACTIVE FLAVONOIDS IN TRANSGENIC HOP *HUMULUS LUPULUS* L. BY PAP1/MYB75 FROM *ARABIDOPSIS THALIANA* L.

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Abstract

Biologically active flavonoids in plants, like xanthohumol, are produced in hop. Metabolic engineering by modifying biosynthetic pathways would increase the yield in hop. In order to determine in transgenic plants if and how the biosynthesis of flavonoids would be modified, hop plants (cv. Tettnanger) were genetically transformed with DNA of the regulatory factor PAP1/MYB75 (AT1G56650.1) from *Arabidopsis thaliana.* The gene was under the control of the 35S promoter. By molecular analysis of transgenic plants it was shown that PAP1/ATmyb75 was stably incorporated into the genome. Furthermore, the transgenic PAP1 was transcribed. By quantitative PCR it was demonstrated that in transgenic hop plants the rate of expression of chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3´hydroxylase (F3'H), valerophenone synthase (VPS), and desmethylxanthohumol 6'-O-methyltransferase (OMT1) was modified. Furthermore, the phenotype of plants was altered as well as their composition of flavonoids.

Keywords: Flavonoid biosynthesis**,** transgenic hop *Humulus lupulus* L. cv. Tettnanger, *Arabidopsis thaliana* L. PAP1/MYB75 regulatory gene, modulation of gene expression

Introduction

Production of high value compounds in plants, like biologically active secondary metabolites, constitute an attractive alternative to their *de novo* chemical synthesis. In hop (*Humulus lupulus* L.) flavonoids constitute such class of compounds. Some flavonoids like alpha and beta acids play an important role as flavoring compounds in beer brewing. Others like xanthohumol have cytostatic characteristics useful for cancer treatment. However, yield and/or extractability from the plants have often been poor. Metabolic engineering of plants by modifying biosynthetic pathways would overcome this problem. Biosynthesis of flavonoids is highly regulated. Therefore, constitutively over-expressing genes of this pathway may not be a successful approach. Unlike the former, regulating the expression of genes in transgenic hop by regulatory elements from *Arabidopsis thaliana* L. constitutes a more feasible approach. As it was shown that the PAP1/MYB75 gene transferred into heterologous systems enhanced gene expression in the phenylpropanoid and flavonoid pathways (Borewitz et al., 2000; Matousek et al., 2006). The aim of this study was the molecular characterization of transgenic hop plants containing the regulatory factor PAP1. It was tested if PAP1 from *A. thaliana* L. will be expressed in hop. Furthermore, by quantitative PCR it was determined to which extend the transcription of genes in the flavonoids biosynthetic pathway will be altered. Finally, it was analyzed if the chemical composition of flavonoids in transgenic hop had been altered.

Methods

Hop (*Humulus lupulus* cv. Tettnanger) was genetically transformed with *Agrobacterium tumefaciens* (Horlemann *et al.* 2003). The target gene in the binary vector was the regulatory factor PAP1/MYB75 (AT1G56650.1) from *Arabidopsis thaliana* L. under the control of the 35S promoter. The vector also harbored *nptII* for screening. Transgenic plants were

transferred from the greenhouse to an outdoor containment facility in 2008. The integration of the transgene and its transcription was verified by PCR (Horlemann 2003). Messenger RNA samples were isolated from leaves of plants grown in an outdoor containment facility. To prevent degradation of RNA, samples were frozen immediately in liquid nitrogen. Afterwards they were stored at -80°C until processed. The rate of transcription of genes was determined by quantitative PCR (Aldinger 2009, Alheit 2009).

Results

After transformation 20 regenerated plants were selected and the integration of the transgene (*nptII*) was verified by PCR (Fig. 1).

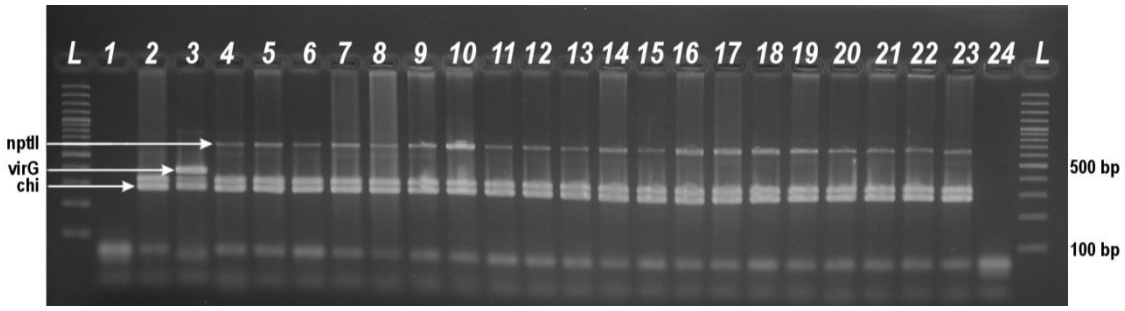

Figure 1: Triplex-PCR for identification of transgenic plants

L: DNA size 100 bp ladder, 1 and 24: PCR negative control, 2: non-transformed hop (WT), 3: plasmid DNA virG), 4 to 23: transgenic PAP1 hop plants (9, 10, 11, 14, 15, 16, 22, 24, 25, 27, 28, 29, 31, 32, 34, 41, 43, 54, 56, 203).

The integration of PAP1 into the genome was also verified by PCR.

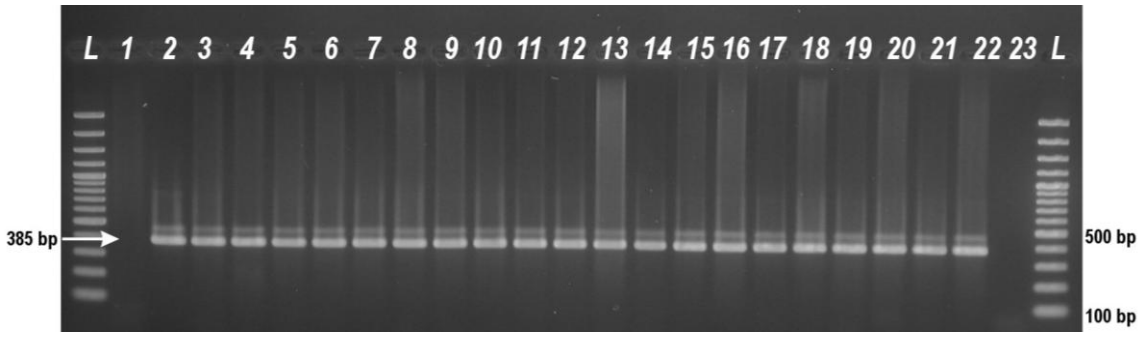

Figure 2: PCR analysis for the integration of the PAP1 transgenic plants

L: DNA size 100 bp ladder, 1: WT, 2: PAP1 plasmid DNA, 3-22: PAP1 transgenic hop (9, 10, 11, 14, 15, 16, 22, 24, 25, 27, 28, 29, 31, 32, 34, 41, 43, 54, 56, and 203), 23: PCR negative control.

From the transgenic plants 8 clones were selected for analyzing the expression of the regulatory gene using total mRNA. By RT PCR it was demonstrated that the gene PAP1 from *A. thaliana* was expressed in all plants. As an example the results of two transgenic clones are shown in Fig. 3.

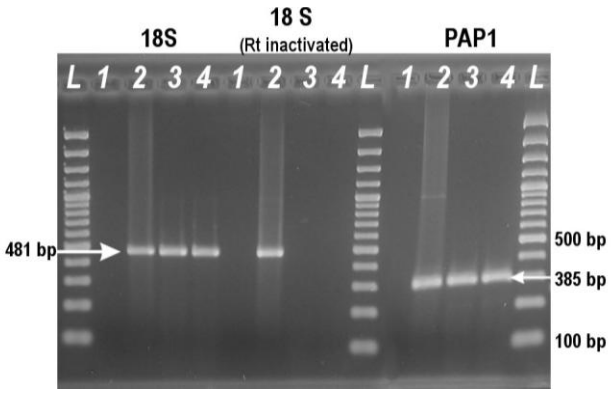

Figure 3: Expression of PAP1 by RT-PCR

L: 100 bp plus DNA ladder, 1: water (negative control), 2: PAP1 hop DNA (positive control), 3 and 4: PAP1 RNA hop samples (#10 and #14)

After verifying the expression of PAP1 in transgenic hop clones it was interesting to learn how the transcription rate of genes of the biosynthesis of flavonoids was affected. The biosynthetic pathways of anthocyanidins, prenylchalcones, and acylphloroglucinols as well as important enzymes are shown in Fig. 4.

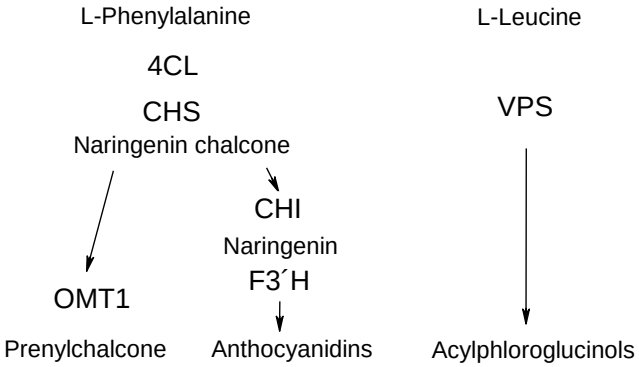

Figure 4: Schematic representation of flavonoid biosynthesis

4-coumarate CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3´hydroxylase (F3'H), valerophenone synthase (VPS), 6'-O-methyltransferase (OMT1)

For this purpose primer sequences for CHS (Matousek et al., 2002), CHI (Stahringer, 2008), valerophenone synthase (Pitsch 2006), and OMT1 (Nagel et al., 2008) were available. For F3'H new primers were designed and this gene was identified for the first time in hop (Aldinger *et al*., in preparation). The expression of all genes was verified by RT PCR in transgenic and non-transformed plants (WT).

In a next step primers were adapted to be employed for quantitative determination of transcripts by PCR. Messenger RNA from leaves was extracted and analyzed by quantitative PCR. Three transgenic clones were chosen to compare the transcription rate of their genes to that of non-transformed plants. It became evident that in transgenic plants constitutively expressed PAP1 had an significant influence on the expression of CHS, CHI, F3´H, VPS, and OMT1. Overall CHS, CHI, F3´H, and VPS were up-regulated. However, OMT1 was transcribed at a lower rate in transgenic clones compared to non-transformed plants.

Transgenic plants and non-transformed controls were grown in an outdoor containment facility during the summer of 2008 for the first time. Throughout the phase of vegetative growth neither the phenotype of transgenic plants nor their growth, respectively, was discernable from non-transgenic controls. However, a new phenotype became evident when the transgenic plants started to develop flowers as the bracts were distinctively colored.

Discussion

For the first time it was demonstrated in this work that a heterologous regulatory gene was expressed in transgenic hop. An important step towards metabolic engineering of the flavonoids biosynthesis was accomplished by genetic transformation with the regulatory gene PAP1/MYB75 from *A. thaliana* L. It was introduced into the hop genome by *Agrobacterium* mediated transformation. In transgenic plants the regulatory element was expressed constitutively. Furthermore, the rate of transcription of CHS, CHI, F3´H, VPS, and OMT1 was altered in transgenic plants. Currently, transgenic clones are chemically analyzed with respect to their composition of flavonoids and prenylated derivatives. Experiments are being performed to elucidate the effect of PAP1 on the expression of additional genes of the flavonoids biosynthetic pathway. The biosynthesis of flavonoids in *A. thaliana* is controlled by a number of regulatory elements (Stracke *et al.* 2007). Employing an efficient screening system, genes from heterologous sources, like *A. thaliana*, will be evaluated for their capability to modulate the biosynthesis of flavonoids in hop. Thus the enhanced production of pharmaceutically active compounds in hop, like the prenylchalcone xanthohumol, will be accomplished.

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GENE EXPRESSION, ISOLATION AND TRANSFORMATION OF HOP (*HUMULUS LUPULUS* L.) CANDIDATE GENES OF THE PRENYLFLAVONID PATHWAY

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Abstract

Hop (*Humulus lupulus* L.) has the property to synthesize bioactive compounds that accumulate in peltate trichomes. Compounds such as Desmethylxanthohumol and Xanthohumol, two of the products of the phenylpropanoid pathway, can have applications in human health. To better understand the working of this pathway, we isolated hop Cinnamic acid 4- hydroxylase (C4H) and 4- Coumarate:CoA ligase (4CL) homologs using Rapid Amplification cDNA-ends (RACE). We also demonstrate that the enzymes coded by the two cloned genes display the predicted activities, using a yeast heterologous system. The expression of these genes in vegetative and reproductive hop tissues, studied using Real Time-PCR, supported their implication in the synthesis of Desmethylxanthohumol and Xanthohumol. At this moment, overexpression approaches are being used to explore their function in hop.

SSR PCR ANALYSIS OF UKRAINIAN VARIETIES OF HOPS (*HUMULUS LUPULUS* L.) AND IN VITRO MULTIPLICATION AND FIELD TRIALS OF «NATIONAL» VARIETY

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Abstract

A set of nine SSR markers has been applied for the identification of thirteen Ukrainian hop varieties genotypes. All analyzed genotypes have unique combination of alleles. It allows us to identify each of them by using nine microsatellite loci. During 2005-2008 new aroma hop variety “National” has been in vitro multiplied. Since 2005 more then 200 ha of new hop gardens were created in several regions of Ukraine. The results were shown up to 0.8 t/ha yield and 5.2 % alpha acid productivity after first year of hop cultivating.

Keywords: Polymerase chain reaction, SSR-markers, analysis of genotype, hop (*Humulus lupulus L.*), in vitro multiplication, field trials, “National” hop variety.

Introduction

Hop varieties identification is an important aspect of breeding program, and plant breeders rights protection in Ukraine. Traditional methods of varieties identification are based on morphological and economic characteristic of hop plants. Expressions of both morphological and economic traits are sensitive to environmental conditions. Usage of DNA-markers for hop variety identification will allow avoiding these problems. SSR based methods of hop genotype identification and differentiation were previously developed (Jakse et al., 2001, Patzak J., 2002). SSR markers for identification genotypes of Ukrainian hop varieties have been applied.

Methods

Plant materials were collected from single certificated plant from origin varieties collection *in vitro* cultured plants of 13 Ukrainian varieties (Ruslan, Triumph, National, Slovianka, Kumir, Promin, Zlato Polissia, Obolons’kiy, Khmeleslav, Al’ta, Potiivs’kyi, Gaidamats’kyi, Zagrava). DNA extraction was performed as it’s described by Boom R et al. Ten hop microsatellite primer pairs, 11a-59, 3a-88, 7a-82, 5-2, HlG-T1, HlG-T2, HlG-T5, HlG-A3, HlG-A4 and HlG-A9 were synthesized by Applied Biosystems 6400. PCR reactions were carried out in a total volume of 25 µL, with 50 ng of genomic DNA, 1 U Taq DNA polymerase, 1× supplied PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, pH 8.3), 10 pmol of each primer, and 0.2 mM of each dNTP. After a first denaturation step at 94°C for 5 min, the reaction went through 36 cycles of 94°C for 45 s, 63°C for 1min, and 72°C for 30 s, followed by a final extension step of 10 min at 72°C, in a thermocycler 4700 (Applied Biosystems). Forward primers of all these loci were fluorescently labeled (6-FAM or JOE). Amplification products were analyzed with ABI PRISM 3110 Genetic Analyser (Applied Biosystems) using internal size standard GeneScan500 ROX (Applied Biosystems) and scored using GeneMapper software version 4.0 (Applied Biosystems).

Results and Discussion

In total, 45 alleles were identified with an average of 4.5 alleles per locus. Two markers 11a59 and 7a82 (data not shown) have minimum alleles among Ukrainian varieties. Only two alleles were identified for each locus 179 bp., 190 bp. and 196, 198, respectively. The most

number of alleles were detected for 3a88 and 5-2 SSR-markers. Numbers of alleles for both markers were seven. Results of allele identified for all of used markers are shown in Table 1.

Table 1: Profile of analyzed genotype

Markers	Allele	Genotypes												
		Ruslan	Triumph	National	Slovianka	Kumir	Promin	Zlato Polissia	Obolons'kiy	Khmeleslav	Al'ta	Potiivs'kyi	Haidamats'kyi	Zagrava
11a-59	A	179	179	179	179		179	179	179	179	179	179	179	179
	B	190	190	190		190	190	190	190	190	190	190	190	190
HlG-A3	A	147	147							147				
	B	150		150	150	150	150	150	150	150	150	150	150	150
	C	156	156											
HlG-T1	A	254	254	254										
	B												260	
	C	262	262	262		262	262	262	262			262	262	262
	D	264					264							
	E								266			266		266
HlG-T5	A					187								
	B	198	198	198	198	198	198	198	198	198	198	198	198	198
	C	201	201	201	201		201	201	201	201	201	201	201	201
	D							208				208	208	
	E								212					
3a-88	A							191	191					
	B	193	193	193	193	193	193	193		193			193	193
	C		196											
	D						198	198	198			198		198
	E											200		
	F	219				219				219				
	G												223	
5-2	A	149					149						149	
	B											168		
	C		176				176	176			176		176	
	D		179	179	179		179	179	179		179		179	179
	E	181												
	F									183				
	G	185		185	185				185			185	185	185
HlG-A4	A	225	225	225	225	225	225				225	225	225	225
	B								232	232			232	
	C	234	234	234	234	234	234	234	234	234	234	234	234	234
	D													
HlG-A9	A	194				194			194					
	B										216			
	C	218	218			218	218							218
	D		220	220			220							
	E			226										
HlG-T2	A	212	212			212		212		212	212	212	212	212
	B							214						
	C	218	218	218	218	218	218		218			218	218	218
	D										220			

Polymorphic identification content is high for most of all markers, excluding 7-a82. All varieties were heterozygous for two same alleles of this marker. Marker 5-2 had highest PIC

(0.77) marker HlG-A3 had the lowest one (0.46). Other markers have intermediate values (11a59/0.50, HlG-T1/0.64, HlG-T5/0.68, 3a88/0.72, HlG-A4/0.62, HlG-T2/0.59 and HlG-A9/0.73). All analyzed genotypes have unique combination of alleles. It allows identifying each of them using nine microsatellite loci. This set of nine polymorphic loci was applied for planting stocks genotype homogeneity estimation in the process of *in vitro* hop variety multiply.

During 2005-2008 new aroma hop variety “National” have been in vitro multiplied. Since 2005 more then 200 ha of new hop gardens were created in several regions of Ukraine. The results were shown 0.8 t/ha yield and 5.2 % alpha acid productivity after first year of hop cultivating. Virus-free hop gardens were formed by direct field adaptation of the in vitro plants without growing stage in hop nursery. It was shown high potential of leaves and roots formation for 98% of in vivo adapted hop plants. For the 2nd and 3rd year of cultivation of “National” hop variety were obtained 1.8 and 2.4 t/ha yield and 8,2-8,7 % alpha acid productivity. Dried hope cones were processed on minigranulators “ECO-BIO 100” and high quality hop granules have been obtained. Genomic analysis, biochemical and brewery tests were done in the leading German, Czech Republic and Ukrainian scientific laboratories and brewery test have shown good tasting facilities of excellent premium beer.

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GENETIC AND EPIGENETIC STABILITY OF *HUMULUS LUPULUS* AFTER *IN VITRO* CULTURE

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Abstract

Somaclonal variation could be a major problem during the *in vitro* procedures. The results presented here allow us concluding that epigenetic changes, most of them corresponding to demethylations, were produced during *in vitro* establishment, cryopreservation, cold storage, micropropagation, and, in adventitious regeneration of hop. The major amount of variation produced corresponded to the *in vitro* establishment step, needed for each of the techniques. A percentage of the variation, present in all treated samples, was attributed to each technique while some other was caused by singletons. Also, it was detected an increase of variation when the time of culture was increased in the micropropagation and adventitious regeneration techniques, measured as higher genetic distance to the control samples.

Keywords: AFLP (Amplified Fragment Length Polymorphism), MSAP (Methylation Sensitive Amplified Polymorphism), RAPD (Random Amplified Polymorphic DNA), REMAP (REtrotransposon Microsatellite Amplified Polymorphism), somaclonal variation.

Introduction

In recent literature, several biotechnological methods for hops had been described as micropropagation, virus elimination (Adams, 1975; Patzak *et al.*, 2001), cryopreservation (Martínez *et al.*, 1999; Reed *et al.*, 2003), cold storage (Reed *et al.*, 2003), tetraploid induction (Roy *et al.*, 2001), adventitious regeneration protocols (Gurriarán *et al.*, 1999; Batista *et al.*, 2000; Horlemann *et al.*, 2003, Okada *et al.*, 2003). Despite the amount of protocols available little has been published on the genetic stability of the recovered plants. Patzak (2003) detected genetic alterations in hop plants after thermotherapy treatments; however thermotherapy increased frequency of molecular differences similar as RNA sequence changes in hop latent viroid.

The aim of the present work is try to clarify whether somaclonal variation is produced and quantify the frequency of the events in hop plants after the application of biotechnological protocols as *in vitro* establishment, long-term micropropagation cryopreservation, cold storage, and callus regeneration. It was plausible to assume that different amounts of variation would be produced during each of the tissue-culture processes. The adventitious regeneration through callus phase would be the more likely to produce somaclonal variation as it has been broadly described as an unstable process due the des-differentiation tissue, uncontrolled growth, and re-differentiation steps needed to fulfil the regeneration. Cryopreservation is supposed to be a low-risk technique due the preservation of the tissues at ultralow temperature that would stop the metabolic activity but implies several steps as cooling-thawing periods and *in vitro* processes (i.e. tissue culture, pre-growth, cryoprotection, recovery, and plant regeneration) which can promote somaclonal variation. The expected somaclonal variation risk for the cold storage would be caused by two processes: cold and micropropagation. Micropropagation is a relative safe technique due it departs from an organized tissue but the stresses generated by the initial sterilization of the field tissues and the oxidative stress related to the wounding produced by subcultivation and the medium deficiencies could lead to genomic alterations.

Methods

Plant material: In vitro establishment and micropropagation: Field plants from cultivars 'Nugget' and 'Columbus' were introduced *in vitro* and subcultured during two years (12 cycles) in MS medium without growing regulators. 10 plants of each cultivar from the 1st, 3rd, 9th, and 12th were selected for the analysis. Adventitious regeneration: a total of 60 of hop plants (cv. Chinook) regenerated from organollenic calli according to Gurriarán *et al.*, 1999 and two control plants; field plant and *in vitro* plant. The regenerated plants corresponded to three sequential subcultures of the same calli. Cryopreservation and cold storage: 7 cryopreserved accessions, 36 cold stored and 4 accessions stored under both methods described in Reed *et al.*, 2003. Each accession consisted in a control greenhouse-grown plant and 2 or 4 treated samples. Among the analysed genotypes were included wild plants, breeding lines, cultivars, males, females, triploids, etc.

Molecular methods: Several molecular methods were selected to evaluate de genetic and epigenetic stability of the different protocols. At list two independent techniques were applied in each case, one evaluating the genetic stability and the other the epigenetic stability: RAPD (Pillay and Kenny, 1996); REMAP (Branco *et al.*, 2007); AFLP (Cervera *et al.*, 1998); MSAP (Cervera *et al.*, 2002)

Results and discussion

A total of 315 hop plants were analysed with unspecific molecular markers. 62 corresponded to callus-derived plants, 169 cold stored and/or cryopreserved, and 84 micropropagated plants. Up to 53 different genotypes were analysed, including cultivars, wild plants, females, males, etc. Although theoretical risks of variation were expected for each technique (high in adventitious regeneration; low in cryopreservation), no genetic variation was detected in any of the tested techniques. In the case of the micropropagated plants, nearly 8,000 DNA fragments were detected with RAPD corresponding to 95 *loci* for the cultivar 'Nugget' and 93 to 'Columbus'. For the regeneration protocol, 16 AFLP primer combinations were applied and a total of 876 *loci* detected (7,000 bands). As no genetic differences were observed when the time in culture was increased indicating the suitability of the protocols for long treatments. Regarding to the cryopreserved and cold stored accessions 51 genotypes were analysed with 11 RAPD primers 125 *loci* were scored (20,000 DNA fragments). An additional analysis with AFLP was performed on 5 cryopreserved and 5 cold stored accessions (6 primer combinations, 19,000 bands). The results presented here allow as to cautiously concluding that no major genetic variation is produced during the tissue culturing proceedings. Differences in the rate of somaclonal variation have been observed for different varieties or cultivars from the same species (Vázquez, 2001) but we didn't detected major differences in the behaviour of hop genotypes when treated.

Alterations of the methylation state were detected using MSAP in each of the treatments (Peredo *et al.*, 2006; Peredo *et al.*, 2008; Peredo *et al.*, 2009) (Resume in Table 1). Changes attributed to the *in vitro* establishment in 'Columbus' were detected in each analysed *in vitro* plant no matter the number of subcultures it had been through. We would like to point out that the percentage of variation attributed to this step was higher than the detected along the two years in culture (28.7% vs. 14.9%). It is also interesting that this amount of variation was similar to the variation shared by cryopreserved or cold stored plants of the same cultivar when compared to the greenhouse control material (23.7% 'Tardif de Bourgogne'; 26.2% 'USDA 21055'; 26.6% 'Calicross'). The changes detected in 'Columbus' suggested us that a major part of this variation detected in this cold stored or cryopreserved material might be produced also by the *in vitro* establishment step. A considerable percentage of variation can be attributed to the each applied technique, shared by all the plants of the same cultivar under the same treatment, so we strongly recommend taking into account the risk of epigenetic variation when applying any of these techniques. Up to date, no reports relate any phenotypic alteration attributed to the use of those techniques, e.g. micropropagated plants have the same field performance that the rhizome-propagated plants (J. Magadán personal

communication). However, the specific variation due to each was lower than the amount of variation related to singletons, changes in the expected methylation pattern present just in one treated plant, and in addition the grouping analysis (UPGMA, Neighbour joining) signalled an increase in the variation along time in culture, or subculturing cycles of the material, indicating an accumulation of mutations along time. In most of the cases, the variation detected corresponded to demethylation of the target sequence. As the demethylation of the genome has been proposed as one of the mechanisms influencing the activation of retrotransposons (Kato *et al.*, 2004) the stability of two retrotransposons, Ogre and Cyclops, was assessed using REMAP analysis in micropropagated plants ('Nugget' and 'Columbus') during a two year-period. No variation in the REMAP patterns was observed. Sixteen REMAP combinations were selected for the assay and up to 111 bands were scored for 'Columbus' and 114 for 'Nugget' indicating no activation of the tested transposons.

Table 1. Revision of the detected epigenetic variation* in several hop cultivar after different tratments with MSAP.

'Chinook'	'USDA 21055'		'Calicross'		'Tardif de Bourgogne'		'Columbus
Calli-reg	CS	CR	CS	CR	CS	CR	Microp.
Percentage of total loci polymorphic							
30.38	38.28		36.7		47.4		56.34
Percentage of loci related to *in vitro* establishment							
-	26.23[a]		26.59[a]		23.7[a]		28.73[b]
Percentage of polymorphism related to the technique							
4.06	4.29	5.07	2.66	2.66	8.67	9.82	6.99
(13.37)	(11.22)	(13.27)	(7.25)	(7.25)	(18.29)	(20.73)	(15.48)
Percentage of singletons							
13.78	2.73		4.78		5.19		13.24
(45.34)	(7.14)		(13.04)		(10.97)		(30.32)

*Between brackets the percentage related only to the polymorphic loci. [a] extrapolated data. [b] directly measured data.Calli-reg. Regenerated though calli phase; CS. 'cold stored'; CR. 'cryopreserved'; Microp. micropropagated.

As a conclusion, no genetic variation has been detected in hops during the *in vitro* establishment or when micropropagated, regenerated through callus-phase, cryopreserved or cold stored. However, epigenetic variations were detected in each treatment, usually related to demethylation of metilcytosines. Our data suggest that the step that causes most of the variation is the *in vitro* establishment; this result is not surprising as deep changes in the physiology of the plant are produced that might be methylation-mediated. However this variation should be considered as important as the variation caused by the biotechnological proceedings themselves as it is an essential step prior applying any other technique. On the other hand, the variation caused by singletons, which could be treated as random variation, was usually higher that the variation related to the protocol and present in all the treated plants. Further analysis of the methylation state of the treated plants, once fully established in the field, is needed to evaluate the amount of variation that is not reversible.

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METHODS OF *IN VITRO* STORAGE OF HOPS

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The cultivated hop (*Humulus lupulus* L.) is commonly used in brewing to add the bitter taste and the delicate hoppy flavour to beers. More recently, alternative uses of hop compounds and their effects on biological processes are becoming an area of strong interest. The increasing importance of hops leads to increased demand for hop germplasm that forms the basis of new breeding programmes, for example for non brewing applications. Traditionally, hop germplasm is preserved in the form of field collections in the areas of cultivation. However, *in vitro* techniques have been found to be useful in *ex situ* conservation of a number of plant species. Our objective was to establish an effective protocol for maintenance of meristem culture-derived virus-free hop stocks in *in vitro* conditions.

Nine cultivars of hops, Osvald's clone 31, Osvald's clone 72, Osvald's clone 114, Bor, Sládek, Sířem, Lučan, Aromat and Zlatan were used to initiate meristem cultures, and already virus-free plants of cultivar Premiant were used to establish an *in vitro* shoot culture for *in vitro* maintenance purposes. Several approaches have been tried to optimize the *in vitro* storage system based upon the slow growth method. These approaches were: the use of different culture vessel types (Baby Food jars, industrial glass, Magenta vessels, test tubes and VitroVent vessels), media modifications (mineral contents, sugar concentrations, type of solidifying agent and growth regulator content), cold treatment (25/20°C, 15/10°C and 5±2°C), use of plant growth retardants (ancymidol and paclobutrazol at 0, 1, 2.5, 5 and 10 µM, mannitol and sorbitol at 0, 10, 20, 30 and 40 $mg.l^{-1}$, respectively) and explant type (apical tip *vs*. nodal segment-derived explants with or without attached leaves). The genetic stability of *in vitro* stored plant material was studied in selected genotypes using analysis of microsatellite markers. Seven primer pairs, denoted FVIIex8, HBV5, 7a82, 3a88, 5-2, HlGA3 and HlGA4 (Brady et al., 1996) were used to compare the microsatellite profiles of plants derived from nodal cultures, meristem culture and *in vitro* thermotherapy treated mericlones stored in *in vitro* culture.

Comparison of the effects of different concentrations of minerals (full strength, ½ strength and ¼ strength) different sugars (glucose, sucrose) solidifying agents (agar, Phytagel) and growth regulators (added BAP+NAA or hormoneless) on different growth parameters, such as average shoot numbers, shoot length, leaf number, percentage of basal callusing, rooting frequency, average root number and root length, fresh weight of shoots, roots and leaves, respectively, resulted in the development of optimized maintenance medium containing full strength MS minerals, WS vitamins, 20 $g.l^{-1}$ glucose and 7 $g.l^{-1}$ agar, without growth regulators. Maintenance of shoot cultures at 5±2°C prolonged the subculture interval to 10 months at high survival rate (>90%) and quality parameters. Of the growth retardants used, sorbitol at 40 $g.l^{-1}$ concentration showed the best results regarding high survival (>91.7%), growth rate and acceptable physiological state of shoots. Microsatellite analyses showed that the lengths of amplified sequences were monomorphic, that means, neither the shoot culture, meristem culture and thermotherapy, nor the long-time (3 years) *in vitro* storage induced changes in studied regions (loci) of hop genome.

Keywords: *Humulus lupulus*, germplasm preservation, slow growth method, genetic stability

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III. Session:

Hop Diseases and Pests

NEW FUNGAL DISEASES ON HOPS IN SLOVENIA AND AUSTRIA

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Abstract

In 2005 and 2007, outbreaks of unidentified diseases on hop were observed in Slovenia and Austria, which induced leaf spots, flower decaying and browning of cones. The diseases spread and progressed rapidly to different varieties and premature harvesting of hop cones was performed in these years. Disease damage assessments revealed 7 to 35 % of infected cones, which is comparable with large outbreaks of hop downy mildew (*Pseudoperonospora humuli*), which is the most important disease on hops. On the basis of morphology, pathogenicity tests and molecular techniques, the fungi *Phoma exigua* and *Cercospora cantuariensis* were identified as the causal agents.

Keywords: fungi, plant diseases, *Humulus lupulus*, climate change

Introduction

From the very start, hop cultivation has been threatened by pests and diseases capable of causing high damage to yield or total decay of the plants. Until now, 35 different fungi, 2 oomicetes, 4 bacteria, 12 viruses, 3 viroids and one phytoplasma have been described as hop pathogens (APS, 2009). However, only a few, such as downy mildew (*Pseudoperonospora humuli* Miyabe & Takah.G.W.Wilson), powdery mildew (*Podosphaera macularis* Wallr. U. Braun & Takam.), verticillium wilt (*Verticillium albo-atrum* Reinke & Berthold; *V. dahliae* Klebahn) and diseases caused by viruses, have caused significant yield reduction in the majority of world hop growing areas. The remaining pathogens therefore present the potential for causing economic damage in hop growing in the future.
In 2005 and 2007, outbreaks of unidentified diseases on hop were observed in Slovenia and Austria, which induced leaf spots, flower decaying and browning of cones. Despite intensive spraying with copper fungicides, the diseases spread and progressed rapidly to different varieties and the premature harvesting of hop cones was performed in order to preserve the yield. The article presents the diagnostic techniques used in identification analysis, crop loss assessments, and directions for disease management.

Methods

Isolation and microscopic examination

Symptomatic leaves and cones from affected hop gardens were surface disinfected (1 min in 2% NaOCl). Small tissue pieces were excised from the margins of the lesion and placed on petri plates containing potato dextrose agar (PDA), and incubated at room temperature. To avoid bacterial contamination, each medium contained 50mg/l of streptomycin sulphate. For morphological identification, subcultures of selected isolates were transferred to oatmeal agar (OA), malt agar (MA), cherry-decoction agar (CA) and V-8 juice agar.

Molecular identification

Molecular analysis was done by PCR amplification of the ITS repeat regions of ribosomal

RNA genes by an ITS4/ITS5 pair of primers (White *et al*. 1990). The PCR reaction was performed in 25 ul of total volume with the following components: 20 ng of DNA, 1x supplied PCR buffer (100mM Tris-HCl (pH 8.8 at 25°C), 500mM KCl, 0.8% Nonidet P40), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 uM of ITS specific primers and 0.625U of *Taq* polymerase (Fermentas) and amplified in PE9700 thermal cycler with thermal profile: initial denaturation 2 min at 94°C, 30 cycles 1 min at 94°C, 1min at 58°C and 2 min at 72°C. Reaction products were resolved by electrophoresis on 1.6 % agarose gels in 0.5 x TBE buffer stained with ethidium bromide. Sequencing was performed by a commercial service (Macrogene, Korea), and sequence homologies were analysed with BLAST programs at the NCBI network service.

Pathogenicity tests

Pathogenicity tests were performed by spraying leaves and mature cones of detached lateral shoots with a pathogen inoculum. Controls were sprayed with sterile distilled water. Four bunches, each containing 2 lateral shots, were used for each treatment. Bunches were covered with plastic bags and incubated in a growth chamber (Kambič, RK-13300) with relative humidity at 80% under a 12-h photoperiod of fluorescent light (L 58W/77; Fluora, Osram). Temperatures were 20 ºC during the light period and 15 ºC during the dark period. Disease assessments were performed on a 0–5 scale (0 = no symptoms, 1 = 1 to 20 %; 2 = 21 to 40 %; 3 = 41 to 60 %; 4 = 61 to 80 %; and 5 = 81 to 100 %). Pathogen re-isolations were done from lesions on the leaves and cones.

Disease assessment in affected hop gardens

Disease assessments in affected hop gardens were done on cones during harvest. Samples (20 l bags of cones) were taken in the final line of the picking machine, from which 400 cones were randomly chosen for microscopic analysis and disease assessment. Cones were assessed on a 0-4 scale (0 = no symptoms, 1 = <1 %; 2 = 1 -5 %; 3 = 5-20 %; 4 = > 20%) and the disease index was calculated according to the Townsend-Heuberger formula.

Results

Analysis of outbreaks in 2005 in Slovenia

The outbreaks appeared in the middle of August 2005 on cultivars Bobek, Magnum and Merkur in the Koroška and Podravje hop regions. The symptoms on the leaves were initially small circular spots, which later increased in size (1-3 cm in diameter) and developed into oval greyish white lesions. Affected cones showed reddish-brown necrotic areas on the tips of the bracts and bracteoles, which in some cases progressed throughout the entire cone. Disease assessments of cones revealed 19 - 35% of infected cones and the disease index varied between 8 – 14 %.

Microscopic examination of the affected tissue revealed the presence of irregularly scattered globose pycnidia (80–200 µm wide) with a distinct ostiolum. Conidia were hyaline, ellipsoid, occasionally single septate, and measured 6.5 (4.5-8) × 2.5 (2-4) µm. Based on *in vivo* examinations of affected tissue and *in vitro* characters of isolated colonies on oatmeal agar (OA), malt agar (MA) and cherry-decoction agar (CA) (Boerema *et al*., 2004), the pathogen was identified as *Phoma exigua* Desm.

The identification was confirmed by sequencing of the ITS region spanning ITS 1, 5.8S rDNA and ITS 2 of two isolates (2PEX and 3PEX) and one reference *Phoma exigua* var. *exigua* isolate (CBS 431.74). All isolates yielded identical 523 bp long sequences and were identical (E=0.0) to *Phoma exigua* ITS sequences in GenBank. The nucleotide sequences have been assigned GenBank Accession No. EF136399 (2PEX) and EF136400 (CBS 431.74).

Pathogenicity tests were performed by spraying detached lateral shoots of cvs. Bobek and Merkur with a spore and mycelial suspension (10^6 CFU/ml). The first lesions developed on leaves and cones of both cultivars 6 days after inoculation. Controls sprayed with distilled water show no symptoms. The fungus *Phoma exigua* was reisolated from the lesions on leaves and cones.

Analysis of outbreaks in 2005 and 2007 in Austria and Slovenia

In August 2005, an outbreak was observed on hop varieties Celeia and Cicero in the Kärnten (Leutschach) region of Austria, caused by unidentified cercosporoid fungi. In 2006, the disease appeared to a minor extent, but in 2007 a new severe outbreak emerged, which also spread to a nearby (30 km air distance) hop growing area in the Koroška region (Radlje ob Dravi) of Slovenia. The most affected varieties were Celeia, Bobek and Aurora. Disease assessments of cones revealed 7 - 26% of infected cones and the disease index varied between 4 – 16 %.

The symptoms on the leaves were circular, irregular, purplish brown spots, 1-10 mm in diameter. The affected cones showed irregular brown necrotic areas on the bracts and bracteoles, which in some cases progressed throughout the entire cone. Microscopic examination of the affected tissue revealed the presence of pale olive brown conidiophores and conidia. Conidiophores were not branched, 1-7 septate, straight, measuring 10 (8-20) × 40 (25-200) µm, with a sinuous tip. Conidia were cylindrical, straight to mildly curved, 13 (10-21) × 250 (135-510) µm, 5-19 septate, with rounded to conical ends. On the basis of the morphological character, the fungus was identified as *Cercospora cantuariensis* Salmon & Wormald; synonym *Mycocentrospora cantuariensis* Deighton (Chupp, 1953).

Sequencing of the ITS regions of two representative isolates (1CCA and 2CCS) and one *C. cantuariensis* isolate obtained from CBS (CBS 112.24) yielded the same 566 bp sequence, which showed the highest identity (95%, 491/515) to the *Mycocentrospora acerina* isolate MA12 ITS sequence (AY266155) in GenBank. These sequencing data, together with previous reports (Goodwin *et al*. 2001), suggest that the name *Mycocentrospora cantuariensis* is more appropriate than *Cercospora cantuariensis.* The nucleotide sequences have been assigned GenBank Accession No. EU346862 (1CCS), EU346863 (2CCS) and EU346864 (CBS 112.24).
Pathogenicity tests were performed by spraying detached lateral shoots of cvs. Celeia with a spore suspension (10^5 conidia/ml). The first lesions developed on leaves and cones 3 days after inoculation. Controls sprayed with distilled water showed no symptoms. The fungus was re-isolated from the lesions on leaves and cones.

Discussion

The appearance of new diseases in plant production is a permanent process, because of constant adaptations of pathogens, changes in the environment or technology and introduction from already infected areas. Hop growing has historically already been faced with “new” devastating diseases, such as downy mildew. The disease was introduced from Japan in the 1920s to Europe and USA, where it caused dramatic yield reductions. Outbreaks of verticillium wilt since 1936 in England and 1997 in Slovenia have been causing significant economic damage as a consequence of the emergence of new highly virulent strains of *V. albo-atrum*.

Outbreaks of diseases caused by *P. exigua* and *C. cantuariensis* (Radišek et al., 2008; 2009) in such a short period with a high aggression level are uncommon and extremely rare. In the case of *P. exigua*, similar symptoms have been previously described in England in 1926 and the causal agent was described as *Ascochyta humuli* Kabát & Bubák infection (Wormald 1946). There is also a record from former republics of the Soviet Union (Pidopličko, 1978), although in both cases the disease infected only leaves. As well as *P. exigua*, previous reports describe *C. cantuariensis* as a minor leaf pathogen on hop, first recorded in 1922 near Canterbury in England, with minor fluctuating appearance in the following years (Wormald, 1946). It has been also detected in Germany, Russia, China and Korea where it has caused infections on Japanese hop (*Humulus japonicus*).

The first connections with outbreaks of new diseases in Slovenia and Austria can be found in intensive and frequent rainfall, with relatively high temperatures during August and September 2005 and 2007, significantly deviating from long-term averages. The average air

temperature in Slovenia in the last 30 years has increased by 1.5 °C, which affects atmosphere circulation and is reflected as a different distribution, intensity and quantity of rain. Mild winters in the last decade, with irregular rainfall, are ideal for the development of fungi from genuses such as *Phoma, Cercospora*, *Septoria, Erysiphe, Sphareotheca* and *Puccinia* (Patterson et al., 1999). Initial monitoring in 2008 showed that *P. exigua* is present in all Slovene hop growing areas, although *C. cantuariensis* has only been found in some areas and further spread is expected.

The first strategies of disease management are focused on sanitation measures, such as thermal composting of hop waste and use of plant protection products based on azoxystrobin, trifloxystrobin and miclobutanil.

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GENOTYPING OF *VERTICILLUM* PATHOTYPES IN THE HALLERTAU: BASIC FINDINGS TO ASSESS THE RISK OF *VERTICILLIUM* INFECTIONS

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Abstract

Hop wilt, caused by the *Verticillium* fungus, has been responsible for massive yield losses in some regions of the Hallertau since 2005. For the first time not only highly susceptible varieties such as “Hallertauer Mittelfrüher”, have been affected, but also previously wilt-tolerant cultivars, such as “Northern Brewer”. The intention is to assess the potential risk to the Hallertau by investigating the race spectrum of this fungal pathogen in the Hallertau. The occurrence of two forms of hop wilt (mild and lethal) caused by *V. albo-atrum* in Slovene hop growing areas, requires a characterisation of field isolates from inside the Hallertau.

Keywords: *Verticillium*, race spectrum, genotyping, risk assessment

Introduction

Hop wilt, caused by *Verticillium albo-atrum* Reinke & Berthold and *Verticillium dahliae* Klebahn is a very dangerous disease in hops. This soilborne pathogen enters the hop by root invasion and then colonizes the hop vascular system. Up to now no fungicides are effective to control this disease. Hop wilt was first reported in England in 1924 (Harris, 1927) were 1933 the lethal form was discovered (Keyworth, 1942). In the Hallertau bigger *Verticillium* damages occurred in 1952 (Kohlman and Kastner, 1975), but up to now no lethal *Verticillium* strains were discovered. In Slovenia hop wilt was first discovered in 1974, in the mild form, while from 1997 an outbreak of the lethal form was registered with *V. albo-atrum* as casual agent. Within the next 6 years nearly 180 ha of hop gardens have been affected by this dangerous lethal form (Radišek et al., 2004). From 2005, the *Verticillium* disease increased in some regions from the Hallertau (Fig. 1). All cultivated varieties, even the tolerant “Northern Brewer” and “Perle” were affected. What needs to be clarified is whether agronomic factors, such as excessive mineral fertilisation or the spreading of fresh bine material directly after harvesting, are responsible that hop wilt increases in special regions, whether lethal *Vericillium* strains from England or Slovenia have already appeared in the Hallertau growing region, or whether new highly virulent races have developed here. The primary aim is to investigate the *Verticillium* race spectrum in the Hallertau in order to develop way and means of preventing this fungal disease from spreading.

Methods

Isolation and microscopic examination

Differentiating the *Verticillium* strains collected first of all involved isolating and culturing the fungus from infected bines. To this end, symptomatic bines from affected hop gardens were surface disinfected. Small 2 cm^2 pieces from the interior of the bines were prepared under sterile conditions, transferred on plum-agar medium in Petri dishes, and incubated at 25 °C for two weeks in the dark. To avoid bacterial contamination, each medium contained 50 mg/l of streptomycin sulphate.

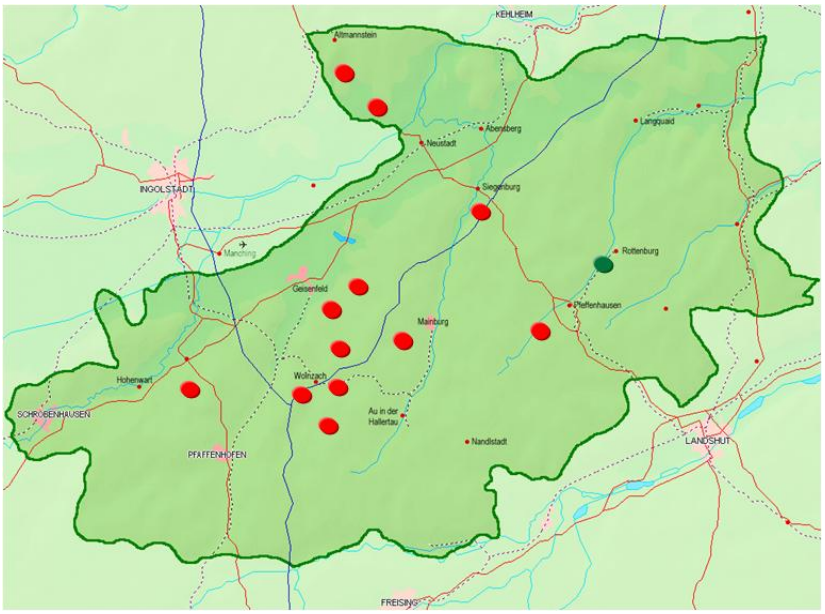

Fig. 1:
Increased appearance of hop wilt in several regions of the Hallertau with damage in several hop varieties

The cultures were then examined microscopically for any foreign fungi, such as *Fusarium* or *Alternaria*, and these Petri dishes were removed and discarded. After a further week of incubation, black structures had developed from the white fungal mycelium which permit to differentiate the two main *Verticillium* species in hops under the microscope. Whereas *V. albo-atrum* develops black hyphae as resting structure, *V. dahliae* forms black microsclerotia. Once the species had been clearly identified, single-spore mycelia obtained via a dilution-row in sterile water were incubated and grown on fresh medium.
One cm^2 pieces were cut out of the resultant single-spore mycelia and transferred to flasks containing 100 ml liquid glucose-peptone medium to allow further growth. Two weeks later, fungal-mycelium growth was sufficient to allow harvesting in a sterile filter by means of a water vacuum pump. The fungal material was freeze-dried, ground in a mill and the DNA isolated according to a modified protocol to Doyle and Doyle (1990).

Virulence assessment

Virulence of four *V. albo-atrum* hop isolates from which two were isolated from severe outbreaks (P10, P15) and two from mild outbreaks (P55, P83) were tested on five hop cultivars: “Northern Brewer”, “Hallertauer Tradition”, “Hallertauer Mittelfrüher”, “Hallertauer Magnum” and “Celeia”. Twelve plants of each hop cultivar were inoculated by root dipping technique using inoculum concentration 5 x 10^6 conidia per ml.
Control plants were similarly inoculated by sterile distilled water. As a reference Slovene isolates T6 characterised as lethal pathotype PV1 (genotype PG2) and Zup characterised as pathotype M (mild) were also included in testing. Inoculated plants were grown in an isolated test plot under field environmental conditions

Symptoms were evaluated four weeks after inoculation and then at weekly intervals for a further two weeks on a 0-5 scale according to the proportion of foliage affected by wilt symptoms. A score of 0 indicates no leaf symptoms, 1 = 1 to 20 % leaf area wilted, 2 = 21 to 40 % leaf area wilted, 3 = 41 to 60 % leaf area wilted, 4 = 61 to 80 % leaf area wilted and 5 = 81 to 100 % leaf area wilted. The level of disease severity for each isolate-cultivar combination was expressed by severity index which was calculated according to the Townsend-Heuberger formula (1943).

Molecular identification

AFLP-analysis and PCR-based studies using SCAR markers developed by Radišek et al. (2001, 2003, 2004) were conducted.

Results and Discussion

In the summer of 2008, work commenced at more than 30 locations with collecting bine sections from hop yards heavily infected with *Verticillium*. Sections were removed from 123 heavily diseased hop plants and from 28 phenotypically healthy ones in the immediate vicinity of the diseased plants. To start with, cultures of the *Verticillium* fungus were investigated. *Verticillium albo-atrum* infection was confirmed microscopically in all 151 samples from severely damaged and also from phenotypically healthy hop plants. Not a single sample exhibited microsclerotia, which are characteristic of *Verticillium dahliae*.

In addition, a qualitative *in planta*-test was performed on the first samples to determine the *Verticillium* species directly from the infected bines. For these studies the primers disclosed by the European and Mediterranean Plant Protection Organisation (OEPP/EPPO Bulletin, 2007) for detecting *V. albo-atrum* were used. *Verticillium albo-atrum* was identified in all samples collected from the Hallertau.

For further molecular analyses *Verticillium* isolates from Slovenia, England and Poland were taken as references using the method developed by Radišek as shown above. SCAR-markers as well as AFLP-markers showed no fragments describing lethal *Verticillium* strains from Slovenia.

Further investigations are needed, particularly comparison with hop isolates from other countries, to obtain more data on the genetic variability.

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A FORECASTING MODEL FOR THE CONTROL OF POWDERY MILDEW (*PODOSPHAERA MACULARIS*) IN HOPS (*HUMULUS LUPULUS*) UNDER CLIMATIC CONDITIONS IN THE HALLERTAU

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Abstract

To permit selective control of hop powdery mildew (HPM), a preliminary forecasting model has been under test in the Hallertau since 2003. This empirically deduced model is based exclusively on the evaluation of weather parameters (temperature, temperature difference between day and night, sunlight intensity and precipitation). The necessary data can be downloaded via the internet from the agro meteorological weather stations installed in the Hallertau. All the trials treated according to this model produced good results – healthier hops were obtained with fewer sprayings.

As part of a research project from 2007 to 2009, these findings have been investigated closely on a scientific basis in experimental plantings in hop yards and in laboratory and climatic-chamber tests. The results are being incorporated directly in a forecasting model – to be implemented in practice as early as 2009 – for powdery mildew control.

Keywords:

powdery mildew, *Podosphera macularis*, biological characteristics, forecast, hops

Introduction

Since the first major damage by powdery mildew was experienced in 1999, fungicides to the value of around 250 €/ha have been applied annually to control the disease. Trials with untreated plots show that too many prophylactic plant protection measures are implemented. However, these measures have been necessary so far in order to produce yields and quality in line with market needs.

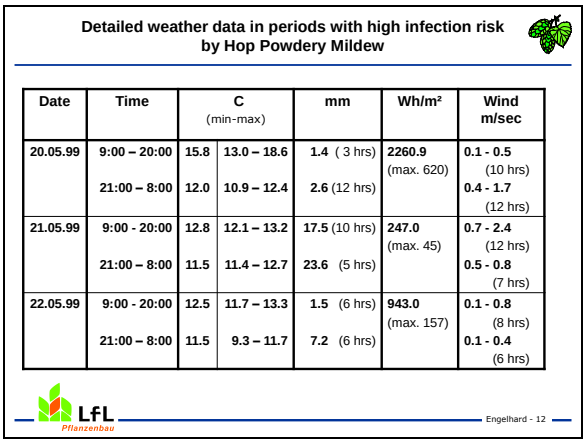
In light of the heavy PM infection in 1999 but its near absence at the same trial locations in 2000, research has been focused on the different weather conditions during those two years. Numerous comparisons have shown the existence of a logical connection between HPM infection and the following weather conditions:

- Mean day and night temperatures, recorded separately, above 10 °C in each case
- Little difference between day and night temperatures
- Low sunlight intensity; the upper limit was defined as 4 kilowatt-hours/m² (kWh/m²)
- Some rain is necessary
- These weather conditions must prevail for at least two and a half days.

The data from 20th – 22nd May, 1999 are listed by way of example in Fig. 1.

Fig. 1:

Weather conditions conducive to hop-powdery-mildew infection



Detailed weather data in periods with high infection risk by Hop Powdery Mildew

Date	Time	C (min-max)		mm	Wh/m²	Wind m/sec
20.05.99	9:00 – 20:00	15.8	13.0 – 18.6	1.4 (3 hrs)	2260.9 (max. 620)	0.1 - 0.5 (10 hrs)
	21:00 – 8:00	12.0	10.9 – 12.4	2.6 (12 hrs)		0.4 - 1.7 (12 hrs)
21.05.99	9:00 - 20:00	12.8	12.1 – 13.2	17.5 (10 hrs)	247.0 (max. 45)	0.7 - 2.4 (12 hrs)
	21:00 – 8:00	11.5	11.4 – 12.7	23.6 (5 hrs)		0.5 - 0.8 (7 hrs)
22.05.99	9:00 - 20:00	12.5	11.7 – 13.3	1.5 (6 hrs)	943.0 (max. 157)	0.1 - 0.8 (8 hrs)
	21:00 – 8:00	11.5	9.3 – 11.7	7.2 (6 hrs)		0.1 - 0.4 (6 hrs)

Similar weather conditions had prevailed from 10th – 12th May, 1999. Since nobody had considered any HPM control measures at that time, the fungus was able to spread. As of 2003, with this prior knowledge, trials were set up in hop yards in the Hallertau. Untreated experimental plots served as controls. There was no HPM infection on the trial plots where spraying was timed in accordance with the forecasting model.

Methods

In the course of a research project carried out during the period 2007 – 2009, the empirically deduced data was investigated closely on a scientific basis as part of a dissertation.

The following biological characteristics of HPM were reviewed in very extensive field trials and climatic-chamber experiments:

- Overwintering forms, primary infection and spore dispersal
- The susceptibility of various plant parts to infections and the variations in risk during the vegetation period
- The influence of various weather parameters (56 variants)
- The duration of effect of various fungicides

A “weather-based disease forecast” was derived from the information gathered from the results, and was tested for the first time under field conditions in 2008.

Results

Comprehensive disease surveys conducted in the vicinity of infection sources showed that spores are only transported over a very limited distance (Fig. 2). This is to advantage for a forecast.

It has been confirmed that, for the most part, only leaves in positions two to four can be infected. The risk of infection is particularly high, however, from May until the first week in June (Fig. 3). As from mid-June, it was no longer possible to induce an artificial infection. The enormous production of spores nevertheless poses a continued infection risk.

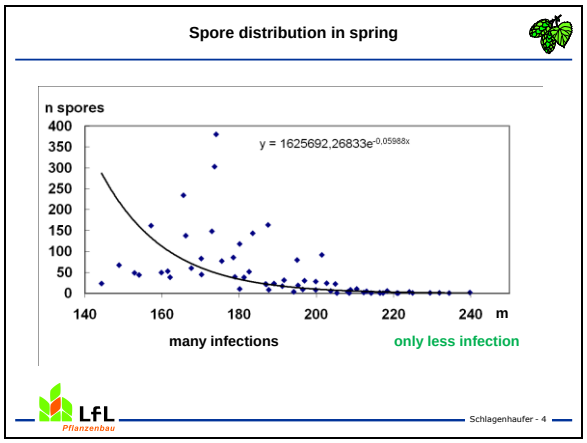


Fig. 2: Spore dispersal prior to flowering

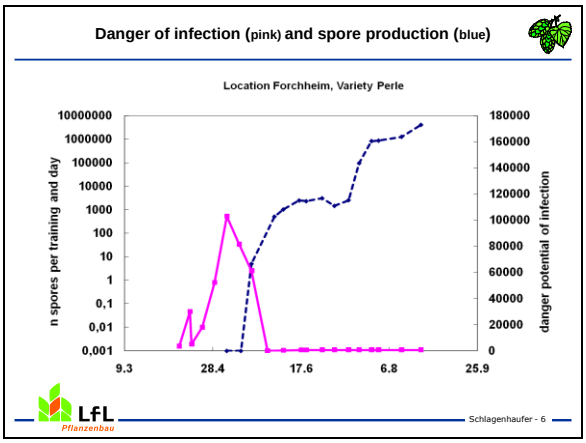


Fig. 3: Variation in infection risk during the vegetation period

It was verified in highly impressive trials that the currently registered plant protection agents have no prophylactic effect because new growth is not protected by a previous spraying. This means that if a forecast is to be effective, the exact time of a potential infection must be found in order to issue a spray warning. In a 2008 review of a "weather-based disease forecast", the results obtained in hop yards and in climatic chambers were included. The differently weighted parameters listed in Fig. 4 were evaluated to generate a daily forecast (Fig. 5).

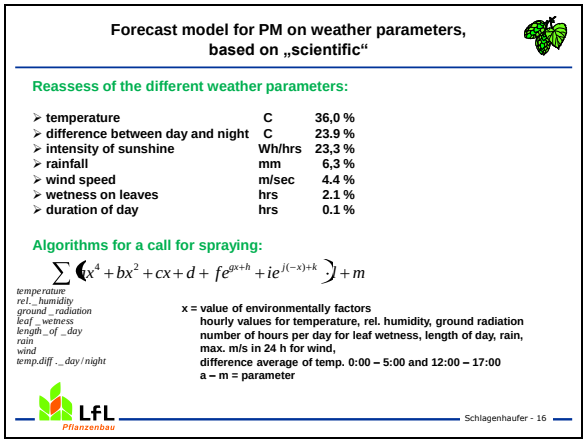


Fig. 4: Inclusion of weather parameters for a forecast

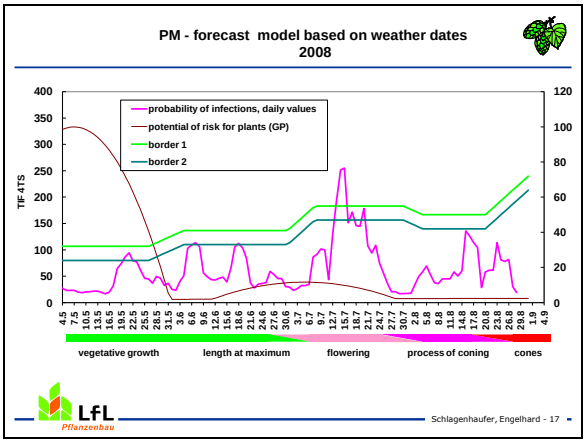


Fig. 5: Disease forecast for 2008, with preliminary control thresholds

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SYSTEMS APPROACHES TO MANGEMENT OF HOP DOWNY MILDEW

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Abstract

A multi-faceted, systems approach to downy mildew management continues to be developed in the U.S., and includes sporangia detection methods, epidemic modeling, and modified cultural practices to suppress the disease. During 9 location-years of validation of a PCR assay for detection of airborne *Pseudoperonospora humuli*, pathogen detection occurred no later than 8 days after the appearance of trace levels of disease or detection of airborne sporangia in a volumetric spore sampler. During evaluation in commercial yards, use of the PCR results to initiate the first fungicide application led to enhanced disease control or fungicide use reduction in four of six yards compared to growers' standard practices. We also have developed and evaluated a degree-day model that forecasts the first emergence of shoots systemically infected with *P. humuli* and a risk index for secondary spread of the disease. During four growing seasons in western Oregon, the degree-day model provided on average 4.9 days (median -0.5 days) of advanced warning before the first spike emerged. Surveys of 34 commercial hop yards in Oregon indicated the downy mildew severity during the previous season was negatively correlated with the degree-day emergence date of spikes the following year (r = -0.391). In experimental plots, treatments initiated using a degree-day threshold had disease severity similar to or less than that of plots that received routine fungicide applications during three seasons of evaluation. Model-aided treatments required four less fungicide applications compared to routine fungicide applications. Collectively these results indicate that the severity of downy mildew is influenced substantially by the efficacy and timing of control measures applied early in the growing season.

Introduction

Downy mildew of hop, caused by *Pseudoperonospora humuli*, is an important disease in many regions of hop production. Management of downy mildew is dependent on regular application of fungicides and sanitation practices such as spring crown pruning. The disease continues to threaten the economic viability of the U.S. hop industry because of widespread fungicide resistance in the pathogen population (Gent et al., 2008; Nelson et al., 2004) and lack of host resistance within most commercially acceptable cultivars.

Various disease forecasting systems have been developed to aid in timing fungicide applications for downy mildew in the U.S. and Europe (Johnson et al., 1994; Kremheller & Diercks, 1983; Royle, 1973). A limitation of many forecasting systems is the lack of an inoculum availability component or the necessity to enumerate sporangia with spore traps (Kremheller & Diercks, 1983; Royle, 1973). These limitations likely prevent more widespread adoption of disease forecasting, especially in the U.S. since hop farms tend to be large (average >250 hectares) and deployment of spore traps is impractical. Development of a species-specific PCR assay for *P. humuli* could allow for *in situ* detection of airborne inoculum at a broad scale if inexpensive approaches were available.

Alternatively, Johnson (1991) developed growing degree-day models (based upon air and soil temperature) that predicted the first emergence of basal spikes in the semi-arid hop production regions in Washington. Since downy mildew epidemics do not occur annually in Washington (Johnson et al., 1994), these models were promoted as tools to enhance

scouting efforts. For hop production regions where downy mildew is epidemic annually, such as the cool, maritime climate of western Oregon, these models may successfully predict when fungicide applications should be initiated to protect plants from early season spread of inoculum produced on newly-emerged basal spikes.

Our objectives for this research were to develop and validate approaches for downy mildew risk assessment. Information is presented on approaches to detect or predict inoculum and conditions favorable for secondary disease spread.

Methods

Pseudoperonospora humuli primer design and PCR sensitivity. DNA was extracted from sporangia of 59 isolates of *P*. *humuli* collected from the major hop production regions in the U.S. The rDNA internal transcribed spacer region was amplified with primers ITS1 and ITS4. A consensus sequence derived from 59 *P*. *humuli* isolates from the U.S. and related Peronsporale species (*P*. *celtidis*, *P*. *cannabina*, *P*. *cubensis*, *P. destructor*, *P*. *humuli*, *P*. *urticae*) available in Genbank was used to design primers HDM04 (5'AGCCAC-ACAACACATAGT3') and HDM07 (5' AGAATTGACTGCGAGTCC3'). These primers generated a 338 bp product specific to *P*. *humuli* and *P*. *cubensis*. The amplification program consisted of 94ºC for 2 min followed by 40 cycles of 94ºC for 20 s, 58.4ºC for 20 s, and 65ºC for 1min with a final extension at 70ºC for 10 min. The specificity of the PCR was verified by performing the PCR with DNA of 43 peronosporales species and other microorganisms associated with hop. Sensitivity was quantified by conducting 10 DNA extractions and amplifications on a dilution series of sporangia.

Epidemic modelling. An air temperature degree-day model similar to the model developed by Johnson (1991) was developed and evaluated in experimental plots of cultivar Nugget and 34 commercial hop yards over four seasons in western Oregon. The infection risk index developed by Royle (Royle, 1973; Royle and Kremheller, 1981) was evaluated in experimental plots of cultivar Nugget, and compared to fungicide applications made preventatively, fungicide applications timed according to the disease risk index coupled to a degree-day model to initiate applications, and non-treated hop plants.

Results

Pseudoperonospora humuli primer design and PCR sensitivity. Alignment of the ITS region revealed regions of sequence heterogeneity suitable for design of primers for detection of *P*. *humuli*. However, due to the close relatedness of *P*. *cubensis* and *P*. *humuli* (Choi et al., 2005), primers could not be designed in the ITS region that were specific to *P*. *humuli*. Primers HDM04 and HDM07 generated a 338 bp product for all isolates of *P*. *humuli* and *P*. *cubensis* tested. A high level of specificity to *P*. *humuli* and *P*. *cubensis* was observed when the primers were tested against other Peronsporales species and other organisms. The sensitivity of the PCR was 1 fg of genomic DNA of *P*. *humuli*. A single sporangium was detected on a silicon-grease-coated glass rod in 70% of PCR assays, and 100% of assays with 5 or more sporangia.

During nine location-years of validation, PCR detection of inoculum of the pathogen in air samples occurred no later than eight days after the appearance of trace levels of disease and/or airborne spores. Inoculum was detected on average 4.5 days before (range -8 to 14 days) the first appearance of basal spikes in six commercial hop yards, or 1.3 days after (range -5 to 1 days) sporangia were detected in a volumetric spore sampler in experiment plots. In two commercial yards in Oregon in 2007, use of PCR to initiate the first fungicide application enhanced disease control when PCR-aided applications were made 34 days before the growers' timing. Conversely, use of the PCR allowed the growers in Oregon in 2008 to delay their first fungicide application by 20 to 22 days, resulting in a savings of one fungicide application in both instances. The date of the first fungicide application was similar between the PCR and grower timing in Washington during 2007 since the growers' timing was within 3 days of inoculum detection.

Epidemic modelling. Predicted first spike emergence occurred on average 11.6 days (median 12 days) after spike emergence using a simple average degree-day model (base temperature 6.5ºC) developed for Washington State (Table 1). Predictions based on a single sine model (base temperature 6ºC) provided on average 4.9 days (median -0.5 days) of advanced warning before the first spike emerged. In surveys conducted in commercial hop yards, downy mildew severity in a previous season was negatively correlated with the degree-day emergence date of spikes the following year (r = -0.391).

In experimental plots, disease severity was significantly greater where fungicide applications were timed using a risk index compared to routine fungicide applications in 2005 and 2007, but statistically similar between these treatments during 2006 and 2008. However, for treatments initiated using a degree-day threshold disease severity was similar to or less than that of plots that received routine fungicide applications during three seasons of evaluation. Model-aided treatments required four less fungicide applications compared to routine fungicide applications.

Table 1. Characteristics of degree-day models for prediction of the first emergence of hop shoots systemically infected with *Pseudoperonospora humuli* in western Oregon

		Sine function[a]		
	Simple average[a]	**6.0ºC**	**5.5ºC**	**5.0ºC**
Degree-days				
Mean	90.7	142.2	157.9	179.6
Median	75.8	115.7	130.7	150.2
Range	165.9	199.5	214.3	233.6
Standard deviation	49.4	60.7	65.4	71.1
Relative standard deviation (%)[b]	0.55	0.43	0.41	0.40
Calendar days[c]				
Mean	-11.6	5.3	5.0	4.9
Median	-12.0	0	0	-0.5
Range	80.0	60.0	60.0	57.0
Standard deviation	20.9	16.7	16.6	16.3
Relative standard deviation (%)	1.80	3.2	3.3	3.3

[a] Simple average degree-days were calculated with a base of 6.5ºC from a biofix date of 1 February as reported by Johnson (1991), or using a single sine function. Data are from a total of 34 hop yards surveyed in western Oregon from 2005 to 2008.

[b] Relative standard deviation is the absolute value of the standard deviation divided by the mean.

[c] The median accumulated degree-days for spike emergence was used as the threshold for evaluation of the mean, median, range, standard deviation, and relative standard deviation of sine function models.

Discussion

The timing of initial fungicide applications for control of downy mildew has become more critical in the U.S. due to the emergence of insensitivity to phenylamide (metalaxyl and mefenoxam) and phosphonate fungicides (fosetyl-Al) in strains of *P. humuli.* Widespread insensitivity to both of these chemistries in certain production areas in the U.S. necessitate more intensive applications of other fungicides (e.g., cymoxanil, copper) with shorter residual activities. Current management recommendations are based on the assumption that inoculum is always present, since quantitative monitoring of sporangial density is not feasible for hop producers in the U.S. An inoculum detection approach for timing fungicide applications, as demonstrated in these studies, could help to reduce unnecessary early season applications under low disease pressure or enhance control under more moderate disease pressure.

In Washington State, the degree-day model developed by Johnson (1991) was designed to predict the first emergence of basal spikes on a regional basis as a conservative means to begin disease scouting and monitoring. The model was not designed necessarily to make predictions about when basal spikes might emerge within a specific yard because downy mildew outbreaks do not occur annually in Washington (Johnson et al., 1994). In this study, we designed a degree-day model to predict the emergence of basal spikes in individual yards, which appears to be related in part to the amount of inoculum overwintering from the previous season.

In summary, these studies indicate that downy mildew can be managed effectively with fewer fungicide applications than currently made by hop growers if fungicide applications are timed to coincide with the detection of airborne inoculum and/or emergence of basal spikes. Timing the first fungicide application to coincide with the predicted emergence of basal spikes coupled with later fungicide applications timed based on a risk index resulted in disease suppression similar or superior to that of a typical grower fungicide program (data not presented), but with four less fungicide applications. These findings have practical implications for disease management and suggest that control measures applied in early spring strongly influence the severity of the disease later in the season.

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PROGNOSIS OF DOWNY MILDEW *(PSEUDOPERONOSPORA HUMULI MIY ET TAK.)* WITHIN HOP PROTECTION MANAGEMENT IN CZECH REPUBLIC

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A method of short-time prognosis has been developed for **hop downy mildew** (***Pseudoperonospora humuli Miy et Tak***). It is based on daily meteorological records of temperature, air relative and total rainfall. The downy mildew index (i) is calculated from these data using alternative equations. The scheme for **Saaz aroma hops** has been in use since the eighties (**Petrlik & Stys, 1988**).

A/ for days with rain: $$i = 100 + 10\,(t - 15) + 2\,(R - 60) + r$$

B/ for days without rain: $$\frac{i = 100 + 10\,(t - 15) + 2\,(R - 60) + r}{S}$$

t = daily average temperature in °C, R = average relative air-humidity in %, r = total daily precipitations in mm, S = number of days without rain

Information additional to the prognosis is provided by the evaluation of the incidence of the disease on leaves, flowers and in cones at fifteen days intervals from the beginning of June until the period before harvest. A danger of mildew occurs, either if the downy mildew index reaches the value higher than 500, or at the incidence of the 100 blotches per 100 leaves or later whenever downy mildew infection is found in inflorescence or cones. During the period of vegetative growth, six separate sprays are scheduled between June 06 and August 25 for Saaz and Ustek Bohemian hop regions and seven between June 01 and August 25 for Trsice Moravian hop growing region.

Methodology of the protection within **hybrid hop varieties** against downy mildew has been developed recently. The main objective of the methodology consists in short-time prognosis of downy mildew for these varieties. As they are generally more susceptible to infection caused by downy mildew, it is impossible to use the traditional method recommended for aroma hops. Therefore, it was necessary to create a new strategy, which would be suitable also for the hybrid varieties; whose percentage has still had the progressive trend within Czech variety structure. It consists in the determination of different downy mildew indexes (i).

Thanks to this method it is possible not to carry out all the treatments if there is no or only low infection. In this way we not only save money to hop growers but we help to decrease the pollution of the environment in hop growing areas as well **(Vostrel et al., 2008).**

Up-to-date information on the occurrence of downy mildew and methodical recommendations on treatments against this disease are available on the following address: www.chizatec.cz. Czech Hop Grower's Association also sends them via e-mail.

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FEEDING PREFERENCE OF THE HOP FLEA BEETLE (*PSYLLIODES ATTENUATES* KOCH)

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Abstract

The hop flea beetle (*Psylliodes attenuatus* Koch) is an important pest of hop, which also infests hemp (*Cannabis sativa* L. ssp. *sativa* var. *sativa*) and the stinging nettle (*Urtica dioica* L.). Its feeding preference varies both to host plants and to hop cultivars. The intent of the study was to examine, under »*in vitro*« conditions, the preference to different hop cultivars and wild hop plants, to hemp and the stinging nettle in comparison with the hop cv. Aurora. The feeding preference of the beetle to hop and to the stinging nettle was found to be more common than to the hemp cv. Bialobrzeskie. The preference also differed with regard to hop cultivars. Compared with the hop cv. Aurora, the preferred host plant was the cv. Savinjski Golding, followed by the cv. Taurus, while the cv. Bobek was the least acceptable of all. The study aimed to determine the preference to different hop cultivars was conducted with the use of yellow sticky traps in a hop garden, in which 11 different hop cultivars were grown, in the years 2003, 2004 and 2005. The hops cv. Magnum and Celeia were found to be the most significant hosts of the beetle, whereas the cv. Buket was the least acceptable.

Keywords: hop, *Humulus lupulus,* hop flea beetle, *Psylliodes attenuatus,* preference, hemp, *Cannabis sativa* L. ssp. *sativa* var. *sativa*, stinging nettle, *Urtica dioica,* hop cultivars

Introduction

The hop flea beetle (*Psylliodes attenuatus* Koch) is an important pest that infests hop (*Humulus lupulus* L.) (Rak Cizej and Žolnir, 2003), feeding also on hemp (*Cannabis sativa* L. ssp. *sativa* var. *sativa*) and the stinging nettle *(Urtica dioica* L.) (Heikertinger, 1925). The host plant range of the beetle differs widely. Its preferred host plant is hop, followed by the stinging nettle, whereas hemp is the least acceptable. The preference to different hop cultivars also varies (Rak Cizej and Milevoj, 2006). The difference in the quality of a host plant does not depend only on the level of primary plant metabolites but also on the quantity and nature of secondary metabolites. The latter function similarly to toxins, feeding repelents or inhibitors of digestibility. Herbivores test, first of all, the level of nourishment, physical characteristics of a host plant and a possible presence of a natural enemy (carnivore) (Rosenthal and Berenbaum, 1991). The quality and quantity of chemical substances present in a plant have a considerable effect on the plant genotype and on the environment. The environment influences the quantity of secondary metabolites of a plant, which in turn affect noxious organisms (Speight et al., 1999). The taste and odour of some secondary metabolites have an evident stimulating effect on the feeding habits of phytophagous insects and so they determine the choice of a host plant and their behaviour (Visser, 1986).

Materials and methods

The intent of the study was to investigate the feeding preference of the hop flea beetle to its known host plants and non-host plants in an insectarium as well as in a hop garden.

»*In vitro*« tests of the feeding preference of the hop flea beetle

To study the feeding preference of the hop flea beetle to different host plants and non-host plants as well as to different hop cultivars, the hop cv. Aurora was used as a test plant in all trials. This is a Slovenian hop cultivar, which grows in over 60% of hop gardens in the country. Preliminary monitoring showed the feeding preference of the beetle to this cultivar. Compared with this preference, the feeding of the beetle was studied on all the hop cultivars registered in the Slovenian list of cultivars as well as on some common weeds in Slovenian hop gardens. Leaf material was supplied in the form of leaf discs (2.12 cm in diameter), cut from the plants with a metal blade. In all trials the hop cv. Aurora was used as a test plant. Five leaves of the cv. Aurora (standard) and 5 leaves of a test plant were placed in a plastic Petri dish. Then 30 hop flea beetles, 15 female and 15 male subjects, were added to each Petri dish. The Petri dishes were placed for 24 hours into an insectarium. Subsequently the area of discs consumed was measured using the image analyser Opto max. The preference index (PI) was calculated according to the following formula:

$$PI=2T/(T+K)$$

T=area of test disc eaten (in cm^2)
K=area of standard disc eaten

A rapid »*in vitro*« test of the feeding preference of the hop flea beetle to economically important hop cultivars and wild hop plants

In an insectarium a rapid test was carried out to study the feeding preference of the hop flea beetle to economically important hop cultivars (Aurora, Bobek, Celeia, Magnum, Taurus and Savinjski Golding) and to wild hop plants (Japanese and Slovenian). The plants were of the same age and grown under similar conditions. All the hop cultivars were placed at random in 4 insectaria along with 50 hop flea beetles, 25 female and 25 male subjects, collected in the hop garden a day prior to the start of the test. The beetles were allowed 12 hours to feed on the leaves, after which the plants were visually assessed as to which of them was their preferred host. The assessment was made as follows: (0) beetles did not feed on the plant at all; (1) they hardly fed on it; (2) they fed on it a little; (3) they readily fed on it; (4) they most eagerly fed on it.

Determination of the feeding preference of the hop flea beetle in a hop garden

The aim was to determine the feeding preference of the hop flea beetle in the field, that is in a hop garden with 11 different hop cultivars, 2 of which were German, while the others were Slovenian. In the hop garden under consideration individual hop cultivars were monitored to study the infestation using yellow sticky traps. The monitoring was conducted from April to the end of August in the years from 2003 to 2005. During this period, the same agricultural technology (cultivation, covering, additional fertilization, irrigation) was applied for all the cultivars, except for the time of cutting, which was carried out according to individual cultivars.

Statistical data analysis

The data on the feeding preference of the hop flea beetle obtained in the insectaria concerning different plant species and hop cultivars in comparison with the hop cv. Aurora were analysed with ANOVA. A linear mixed model was used to make a statistical comparison of the effect of different hop cultivars as to »*in vivo*« feeding preference of the flea beetle.

Results and Discussion

The »*in vitro*« tests of the diet of the hop flea beetle confirmed the observation that the beetle feeds on all host plants known so far (hop, hemp and the stinging nettle), whereas it does not feed on weeds such as the lambs quarters (*Chenopodium album* L.), the gallant soldier (*Galinsoga parviflora* Cav.) and the redroot pigweed (*Amaranthus retroflexus* L.). Thus it can be concluded that the hop flea beetle is oligophagous.

The value of the preference index (PI) was found to be between 0 and 2. If the value was PI=1, then the hop flea beetle had the same preference to the leaves of test plants as to the leaves of control plants, that is the leaves of the hop cv. Aurora. If PI>1, then the beetle had a greater preference to the leaves of test plants, and if PI<1, then the beetle had a greater preference to the leaves of control plants, that is the hop cv. Aurora. In comparison with the hop cv. Aurora, the preferred host plants of the beetle were the leaves of the hop cv. Savinjski Golding and the stinging nettle. The least acceptable food plants were the hop cv. Magnum and the hemp cv. Bialobrzeskie. Compared to the hop cv. Aurora, the beetle preferred feeding on the cvs. Bobek, Celeia and Taurus (Fig.1).

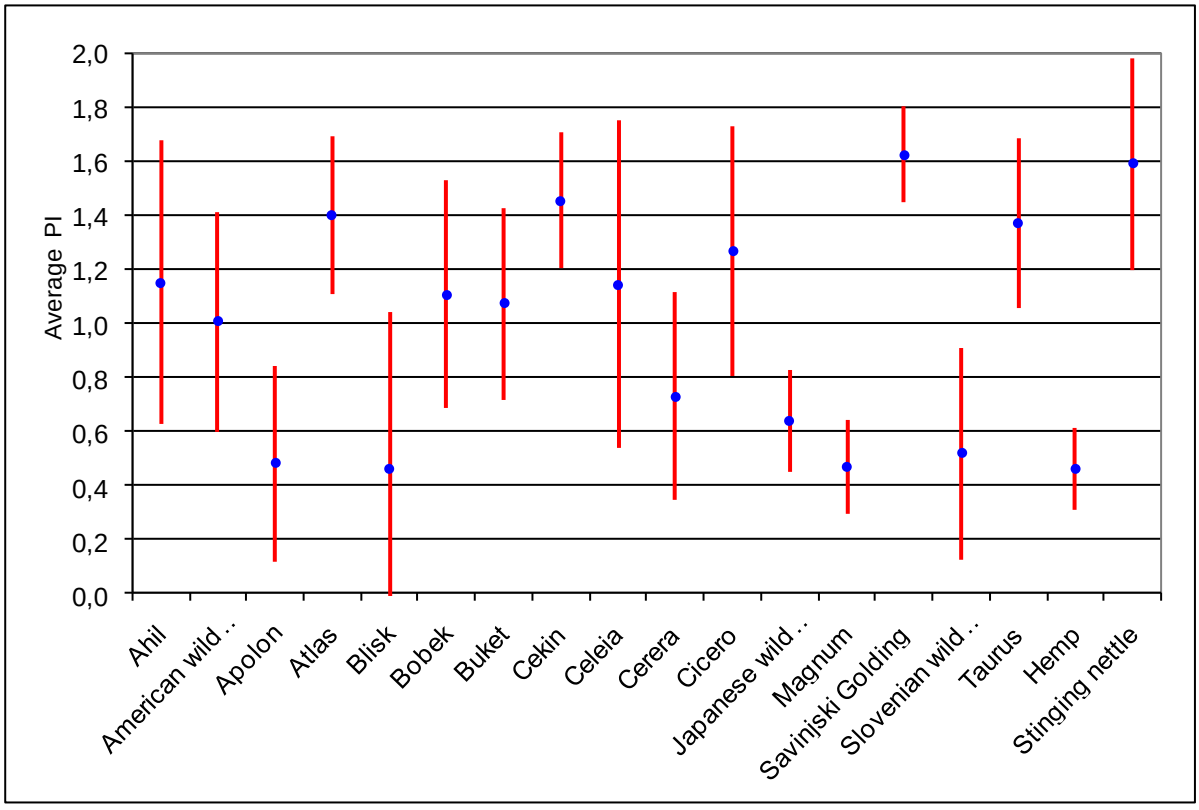

Fig. 1: The mean values and standard deviations of preference indexes (PI) of the hop flea beetle (*Psylliodes attenuatus*) for different cvs. of hop, hemp, the stinging nettle and wild hop plants in comparison with the hop cv. Aurora.

- If the beetles studied »*in vitro*« had a choice of feeding on all economically important hop cultivars as well as on wild hop plants, their preferred host plant was the hop cv. Savinjski Golding, followed by the cvs. Aurora and Celeia. The cvs.Taurus and Magnum were less acceptable as host plants. After 12 hours the beetles did not feed at all on wild hop (Japanese and Slovenian).
- During the whole 3-year period of study conducted in the hop garden, the beetles showed a statistically significant preference to the cvs. Celeia and Magnum, followed by the cvs.

Aurora and Savinjski Golding, whereas the cvs. Buket, Taurus and Bobek were the least acceptable as host plants (Table 1).

Table 1: The difference in the average number of hop flea beetles (*Psylliodes attenuatus*) captured with the use of sticky traps in a five-day period in the Žalec hop garden in the years 2003, 2004 and 2005.

Cultivar		BUK	BOB	BLI	CEK	TAU	SG	CIC	AU	CER	MAG	CEL
	Average	1.58	1.95	2.04	2.17	2.34	2.47	2.58	2.60	2.78	3.49	3.98
BUK	1.58							*	*	*	***	***
BOB	1.95										**	***
BLI	2.04										**	***
CEK	2.17										**	***
TAU	2.34										*	**
SG	2.47										*	**
CIC	2.58											*
AU	2.60											**
CER	2.78											*
MAG	3.49											
CEL	3.98											

Key: AU=Aurora, BLI=Blisk, BOB=Bobek, BUK=Buket, CEK=Cekin, CEL=Celeia, CER=Cerera, CIC=Cicero, MAG=Magnum, SG=Savinjski Golding, TAU=Taurus

*P≤0.05 significant effect; **P≤0.01 highly significant effect; ***P≤0.001 extremely highly significant effect

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FIRST STEPS TOWARDS A REVISED CONTROL THRESHOLD FOR THE DAMSON-HOP APHID *PHORODON HUMULI*

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Abstract

During the field season 2008, the first steps of a three year's study to revise the currently existing control threshold for *Phorodon humuli* were taken. In altogether 60 hop gardens of the Hallertau growing region (cvs HM, HS, HT, and SE), three plots were laid out with different insecticide uses, respectively (untreated/ one spraying/ two sprayings), and the effects on aphid infestation levels on both leaves and cones were assessed. Altogether 12 experimental harvests were conducted in three gardens of each cv. The aphid situation 2008 was extraordinary. At harvest time, 55 of the 60 untreated control plots appeared to be without any damage by aphids, although aphid migration and development during early summer was extremely high. The damage in the five affected control plots was not based on loss of yield, but on heavily infested, unmarketable cones. Four of the 12 experimental harvests revealed significant losses of yield or of alpha-acids in the practice plots that had been treated with insecticide twice, compared to untreated control plots. It can be concluded that unnecessary pesticide applications can cause definitely evitable loss of yield or of alpha-acids.

Keywords: *Phorodon humuli*, damson-hop aphid, control, control threshold, cone infestation, aphid damage, insecticide damage

Introduction

The damson-hop aphid *Phorodon humuli* (Schrank) is a major economic pest for hop growers in the northern hemisphere. The aphid can cause serious losses of yield up to the complete destruction of a crop, and even light infestations of the harvested cones can damage their quality and reduce their economic value (Barber et al., 2003). Farmers usually counter the economic threat with the prophylactic use of insecticides. However, the actual necessity and the timing of an insecticide application for the control of *P. humuli* currently cannot be foreseen by both hop growers and advisory services. In the Hallertau, a first spraying is hitherto officially recommended if a mean of 50 aphids per leaf is exceeded, or if single leaves with more than 200 aphids are found during aphid monitoring. Moreover, a first spraying is demanded in all cases until the flowering of hop, to keep the plants possibly free of aphids when cone formation starts. Under consideration of the aphid susceptibility of current high alpha cultivars and the efficacy of currently available insecticides, this postulation can be only hardly met.

To shed some light on the relationships between aphid infestation of hop leaves and cones, under consideration of recent varietal differences in aphid susceptibility, and the impact on yield, alpha-acids and quality of the harvested cones, an according research project was initiated in the Hallertau in 2008. Some results of the first year of this project, which is laid out for three years, can be presented as first steps towards a revised aphid control threshold.

Methods

Sixty hop gardens that were more or less evenly distributed within the area of the Hallertau growing region were chosen for the project. Four cultivars with 15 gardens, respectively, were considered: The susceptible high alpha cvs Hallertauer Magnum (HM) and Herkules (HS), the aroma cv. Hallertauer Tradition (HT) and the aphid-tolerant aroma cv. Spalter Select

(SE). In each garden three experimental plots (*ca* 400 m^2 each) were laid out: an insecticide-untreated control (P0), a plot with only one insecticide treatment (P1), and a plot that was treated according to the respective grower's practice – usually two insecticide applications (P2). All experimental gardens were monitored seven times for aphid leaf infestation in a two weeks' rhythm, from mid-May to late August. In addition, after the beginning of cone formation, 100 cones were taken randomly from each plot during each monitoring. The samples were returned to the laboratory on the same day, and all arthropods sitting in the cones were extracted by the use of modified Berlese funnels.

After the last monitoring, three gardens of each cv. were chosen for the execution of an experimental harvest. These harvests included the determination of yield and alpha-acids in all plots, and a final monitoring of leaf and cone infestation.

Results

Generally, the aphid situation in the Hallertau during 2008 was exceptional. Aphid migration started very early and at an extremely high level. An average number of 100 aphids per leaf was exceeded in some gardens already early in June, and those numbers sometimes increased to more than 500 aphids per leaf (Tab. 2a). However, during July the aphid populations collapsed in almost all control plots to values near zero. In the two aroma cvs, all 30 control plots then stayed almost without further infestation until harvest (e.g., Tab. 2b). In HM and HS, the aphid numbers on leaves were very low from July onwards, too, but increased in some cases again late in the season towards harvest (Tab. 1a, 1b, 2a). Those plots, in altogether five hop gardens (3 HM, 2 HS), also revealed an extreme infestation of cones at harvest. Moreover, the cone infestation of P1 plots did practically not differ from that of the control plots (Tab. 1a, 1b, 2a). However, even those plots with extremely high cone infestation were not significantly lower in both yield and alpha acids, compared to the P2 plots with usually only marginal infestation (Tab. 1a, 1b, 2a). On the other hand, in four of 12 experimental harvests (3 HS, 1 SE) the sprayed P2 plots revealed significant losses in either yield or alpha-acids, compared to untreated control plots (Tab. 2b).

The extraction from hop cones altogether yielded the following invertebrate taxa:

- Pests: Hop aphids *Phorodon humuli*; two-spotted spider mites *Tetranychus urticae*; flea-beetles (Coleoptera: Alticinae); thunder flies or thrips (Thysanoptera).
- Indifferent species: scavenging beetles (Coleoptera) and phorid flies (Diptera: Phoridae).
- Beneficials: flower bugs (Heteroptera: Anthocoridae, adults and larvae); ladybirds (Coleoptera: Coccinellidae, larvae); predaceous gall midges (Diptera: Cecidomyiidae, larvae); hoverflies (Diptera: Syrphidae, larvae); green lacewings (Neuroptera: Chrysopidae, larvae); brown lacewings (Neuroptera: Hemerobiidae, larvae); predatory mites (Acari: Gamasina).

The abundances of those invertebrate species or groups were sometimes extraordinarily high. The highest number was extracted from 100 cones of the heavily infested control plot at Buch, cv. HM, 8 September 2008 (cf. Tab. 1a), with 5047 aphids – i.e., an average of 50 aphids per cone! – plus 22 alate aphids, 17 larvae and 22 adults of Thysanoptera, 5 larvae and 2 adults of Anthocoridae, 16 larvae of Cecidomyiidae, 2 larvae of Coccinellidae, 18 larvae of Syrphidae and 4 larvae of Hemerobiidae.

Discussion

The aphid infestation situation of the year 2008 was exceptional. The early and heavy population buildup stimulated growers to an according early first application of insecticides in all cultivars. Therefore the abrupt aphid population breakdown in the control plots during July was totally unexpected and astonished even experienced growers – "where have all the aphids gone?" was a frequently heard question then. The main reason for this phenomenon was probably a combination of three circumstances: Firstly, the unusual early masses of aphids attracted a wide range of beneficials to hop gardens at an earlier stage than usual.

Table 1. Mean aphid leaf infestation (n = 50) in two Hallertau hop gardens during 2008, compared to the results of experimental harvests. Grey lines: insecticide application in the plots (f, flonicamid; i, imidacloprid; p, pymetrozine).

a) Buch 2008, cv. HM

leaf infestation	P0	P1	P2
26 May	22.2	12.0	16.1
11 June	40.5	0.5	0.5
24 June	17.3	0.2	0.2
08 July	44.6	0.8	0.8
22 July	78.8	7.2	8.0
12 August	8.2	3.1	0.5
25 August	11.1	16.3	1.1
08 September	169.6	66.7	5.6
cone infestation [%]	99.53	97.93	46.67
weighted average	3.681	3.319	1.83
yield [dt/ha]	22.43	25.98	23.85
alpha [%]	15.71	15.28	15.29
alpha/ha [kg]	352.5	397.2	364.3

b) Engelbrechtsmünster 2008, cv. HM

leaf infestation	P0	P1	P2
02 June	49.2	52.8	51.0
11 June	46.6	27.8	37.2
24 June	173.0	5.5	5.5
08 July	113.3	7.0	7.0
21 July	2.3	1.5	0.0
05 August	3.9	2.2	0.0
19 August	4.6	4.1	0.0
09 September	30.5	25.4	0.2
cone infestation [%]	81.7	84.95	7.00
weighted average	2.352	2.392	1.081
yield [dt/ha]	22.95	21.63	24.69
alpha [%]	15.3	14.64	15.52
alpha/ha [kg]	351.0	316.4	383.2

Table 2. Mean aphid leaf infestation (n = 50) in two Hallertau hop gardens during 2008, compared to the results of experimental harvests. Grey lines: insecticide application in the plots (f, flonicamid; i, imidacloprid; p, pymetrozine). Asterisk: Significant difference in yield (ANOVA, $p = 0.05$)

a) Kirchdorf 2008, cv. HS

leaf infestation	P0	P1	P2
02 June	116.9	86.7	101.8
13 June	46.2	1.6	1.6
27 June	157.8	0.9	0.9
09 July	529.5	1.6	1.8
24 July	509.3	5.3	6.9
12 August	1.3	0.5	0.0
21 August	0.4	0.1	0.0
18 September	6.7	2.9	0.1
cone infestation [%]	71.55	66.30	2.55
weighted average	2.156	1.925	1.03
yield [dt/ha]	28.75	29.16	30.22
alpha [%]	18.6	18.97	18.16
alpha/ha [kg]	533.89	553.2	548.6

b) Mantelkirchen 2008, cv. SE

leaf infestation	P0	P1	P2
30 May	24.3	23.3	23.8
13 June	56.0	0.3	0.5
27 June	20.3	0.3	0.5
09 July	2.8	0.0	0.0
24 July	1.5	0.0	0.0
04 August	0.9	0.1	0.0
21 August	0.2	0.0	0.0
12 September	0.0	0.0	0.0
cone infestation [%]	0.0	0.0	0.0
weighted average	1.000	1.000	1.000
yield [dt/ha]	24.21	23.69	20.97*
alpha [%]	5.81	6.28	5.73
alpha/ha [kg]	140.67	148.69	120.11*

Secondly, the climatic conditions in the Hallertau during July supported the development of an entomopathogenous fungus, which may lead to a widespread infection of aphids in the region, followed by a decrease of the pests – we have experienced that phenomenon probably every third year in our control plots. Finally, the introduced Harlequin ladybird *Harmonia axyridis*, an allochthonous Asian species, has reached the Hallertau in 2007 within his conquest of Europe and instantly became the dominant ladybird species in the region (Weihrauch, 2008). As a voracious aphid predator, the impact of *H. axyridis* on *P. humuli* is enormous, although in the long run this 'benefit' will probably be counteracted by its detrimental impact on indigenous ladybird species.

Another quite unexpected result of this first project year was the enormous infestation level of hop cones – up to >50 aphids per cone in average – without loss in yield or alpha-acids,

compared to double-sprayed, almost uninfested hops. Of course, during a 'neutral quality assessment' of harvested cones, as it is exercised in Germany, these hops will receive the highest penalty due to aphid infestation. However, this assessment is chiefly based on the mere appearance of cones and not on the actual value of their contents. These results demonstrate that concerning a possible reduction of pesticide use the end of the line has not yet been reached. Moreover, as one third of the experimental harvests proved a negative influence of two insecticide applications either on yield or alpha-acid contents, each preventative routine treatment exercised by growers must be seriously questioned – especially if mixtures of three or more compounds are applied. As can be clearly seen in Table 2b, the first treatment would not have been necessary, but is somehow justifiable under consideration of the aphid infestation situation during June. However, the second insecticide treatment, applied on 5 July during a routine spraying of a mixture with one acaricide and two fungicides, has obviously led to a yield reduction of approximately 13 %. Calculated roughly, in this particular case (3.1 ha, cv. SE, 2008 contract price € 3.85) the grower has suffered from a homemade damage of € 3850, which could have been easily avoided by an aphid monitoring in this garden prior to spraying.

The extraction of aphids and other invertebrates from hop cones by the use of Berlese funnels, as proposed by Lorenzana et al. (2004, 2007), proved to be a simple and highly effective method. The modified device that we constructed enabled the complete, synchronous extraction of 54 cone samples and hence yielded fully standardised results. These results prove that the invertebrate fauna of mature hop cones is rich in species and that many predators ar able to follow the aphids into the cones. Quite unexpected however was the coverage of thrips in the cones; these minor pests were present in practically all hop samples, with an overall average of 0.2 individuals per cone. Possibly the role of thrips as a hop pest has hitherto been underestimated.

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PROGNOSIS OF DAMSON-HOP APHID *(PHORODON HUMULI SCHRANK)* WITHIN HOP PROTECTION MANAGEMENT IN CZECH REPUBLIC

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For the monitoring of *Phorodon humuli* winged females migration from primary host plants (genus *Prunus*) we commonly use a method based on a sum of effective temperatures (SET). The principle of this method consists in counting biologically effective temperatures reaching the values over developmental threshold of *P. humuli* since that time when their number reaches the value SET, which is necessary for the development of a generation. SET for wingless females (virginoparae) that feed on the *Prunus* leaves amounts to 140 °C under the starting developmental threshold of 3 °C. This value was used as the basic one for the determination of the number of *P. humuli* generations on *Prunus.* The calculation of the expected number of generations (P_{GI}) on *Prunus spp* as well as on hop plants to each day is carried out with the help of the following formula:

$$P_{GI} = \frac{SETo i}{SET_{GM}}$$

SET_{oi} = sum of effective temperatures for the period from the occurrence of the wingless females on *Prunus spp*. or on hop plants to each day of assessment (i) is calculated by the following formula:

$$\sum = \frac{(t_{min} - t_{max})}{2} - SPV$$

t_{min} = minimal daily temperature, t_{max} = maximal daily temperature, SPV = starting threshold of *P. humuli* development, SET_{GM} = sum of effective temperatures for the development of a wingless female generation

The first occurrence of winged damson hop aphids is possible to observe when the SET = 345 °C, whereas the last one when the SET = 1200 – 1250.

Table1: The determination of migration time of the individual winged aphids (*migrantes alatae*) from *Prunus spp.* to hop plants

Number of generation on *Prunus spp.*	SET	Mean SET for development of a generation
1. generation	0 – 345	415
2. generation	345 – 485	431
3. generation	486 – 625	562
4. generation	626 – 765	675
5. generation	766 – 905	851
6. generation	906 – 1200	916

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THE RESISTANCE OF DAMSON-HOP APHID *(PHORODON HUMULI SCHRANK)* TO LAMBDA-CYHALOTHRINE IN CZECH REPUBLIC

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Abstract

Hop protection against damson-hop aphid *(Phorodon humuli Schrank)* in Czech Republic is based on the application of synthetic insecticides. Whereas imidacloprid and pymetrozine are the key ones, lambda-cyhalothrin is used in the years with higher occurrence of *P. humuli* to reduce aphids during their migration to hop plants and so to prevent damage caused by sucking aphids on young leaves. As a matter of fact lambda-cyhalothrin is the last pyrethroid used in hop protection against aphids. This group of insecticides used to be applied in the struggle against damson-hop aphid in the eighties of the last century. Later more efficient plant protection products due to resistance of *P. humuli* field strains substituted them at that time. Rare application of lambda-cyhalothrin to control aphids in the last two decades may have lead to its better efficiency in comparison with the eighties. To investigate its real efficacy on aphids and to determine resistance factors (RF), samples of *P. humuli* field strains were taken and subjected to laboratory tests in Potter tower. Values LC 50 of these strains were compared with the same ones of a susceptible strain.

The data show the resistance in damson-hop aphid feeding on Czech hops. Biological efficiency of lambda-cyhalothrin in the tested field strains in Trsice region is good enough to be recommended for practical hop protection against *P. humuli* for the purpose mentioned above. In other regions it is necessary to distinguish among the individual localities, as its efficiency is very different. Its efficacy is too low to control possible surviving aphids after treatment made by imidacloprid or pymetrozine. Moreover, this treatment kills predators, mainly aphidophagous coccinellids, which may help to control aphids feeding in inflorescence and cones and so to prevent economic damage.

Keywords: *damson-hop aphid*, lambda-cyhalothrin, pyrethroids, damage, field and susceptible strains, laboratory tests, Potter tower, LC 50 values, resistance factors (RF), biological efficiency, predators

Introduction

First damson-hop aphids *(Phorodon humuli Schrank)* resistant to organophosphorus insecticides (OPs) were found in Saaz hop-region in North Bohemia a few years after their use in practical hop protection (Hrdy, 1984). Shortly afterwards surviving aphids after application of OPs were announced from other hop growing countries as well (Kremheller & Kohlmann, 1979; Muir & Cranham, 1981). Less efficient insecticides had to be replaced by more effective ones gradually. At the turn of the 1970s and 1980s pyrethroid insecticides began to be used instead of less efficient OPs to control resistant strains of damson-hop aphids (Vostrel, 1996). Lambda-cyhalothrin, an insecticide belonging to this group, was introduced in hop protection against *P. humuli* in Czech Republic in the second half of the eighties. It is recommended in the time when it is necessary to prevent damage caused by sucking aphids before the key aphicide (imidaclopride, pymetrozine) application. Its efficacy varies among the individual hop regions in CR. Many surviving aphids were visible after its testing in laboratory conditions and field experiments at the beginning of the 1990s (Vostrel, 1995). Nevertheless, its rare use in hop protection against *P. humuli* in the last decade may have increased it efficacy. Unfortunately, in many tested strains lambda-cyhalothrin did not reach sufficient efficiency so as to be recommended for practical hop protection strategy (Vostrel, 2005). As we urgently need an insecticide to reduce aphids before the application of a key aphicide in the years with early and heavy intensity of *P. humuli* migration (e.g. in 2008) we carried out a screening on aphid samples taken in 2007 and 2008 in all hop

regions within Czech Republic. The results of these tests are reviewed and possible utilizations of lambda-cyhalothrin to control resistant field strains of *P. humuli* in Czech hop-yards are discussed.

Materials and methods

Samples of damson-hop aphids were taken from the selected hop-yards within the hop regions in Czech Republic in 2007 and 2008 (Zatec region: 6 samples from Louny district and 3 samples from Rakovnik district; 2 samples from Ustek and 2 samples from Trsice regions). Aphids were collected in the first decade of June before an insecticide treatment.

Field samples of *P. humuli* populations were transferred into breedings. Their offspring was used in laboratory tests. Aphids were placed in an air-conditioned room at a temperature of 20-22°C and 16-hours photoperiod. Relative humidity was kept at 60-70%.
As a host plant hop seedlings were used. These plants were grown in a glasshouse all over the year. Hop leaves with petioles were taken from untreated or residue-free hop plants.

Decapitated leaves were placed with their back side up on the bottom of a sedimentation tower (30 cm in diameter and 96 cm high) and sprayed with 1 ml of the solution of lambda-cyhalothrin with the help of Potter's nozzle under a pressure of 0,2 MPa. After the sedimentation time (10 minutes) treated leaves were removed from the tower.

The method (Hrdy, Kuldová, 1981), required glass cylinders (22 mm in diameter and 15 mm high) stuck on the inside of hop leaves with the help of paraffin and bee-wax mixture that was melted to 50 °C before they were used. Glass cylinders were coated with fluon to prevent escaping aphids. Then they were placed into panels with openings for vials containing water, into which leaf stalks of the treated leaves were inserted.

Two to three hours after spraying thirty-three aphids were transferred into each cylinder by a fine, slightly moist little brush in the following sequence: non-treated (control) leaves and treated leaves in order from the lowest to the highest tested concentration. Mortality of aphids was counted 48 hours after each treatment. The knocked down aphids and the ones, which were unable to crawl, were recorded as dead. The mortality of non-treated (control) leaves should not have been higher than 20% (if so, the experiment had to be repeated). Each test was carried out three times. That means 100 aphids were tested under each concentration of lambda-cyhalothrin in a geometric row.

As a standard reference susceptible strain from eastern Slovakia, sampled near Roznava in 1974, was used. Values of LC 50 for the resistant and susceptible strains were determined together with resistance factors (RF) as average values for individual regions.

Results and discussion

To find out resistance in field strains of damson hop aphid *(Phorodon humuli Schrank)* to lambda-cyhalothrin expressed by resistance factors (RF) we continued within the project mentioned in the acknowledgement in laboratory tests aimed at this phenomenon.

First reports on resistance phenomenon in field populations of *P. humuli* to pyrethroids were found out as early as in the middle of the eighties. Although the resistance of the tested field populations of *P. humuli* in Hallertau was comparatively lower than to OPs (Kremheller & Knan, 1984), the differences between a susceptible strain and the field ones had already been found out (RF 3-7 for cypermetrhin, deltamethrin and permethrin, resp.). In UK responses from a suspected deltamethrin-resistant field populations showed evidence of increased resistance do this pyrethroid in that time after its short using in practical hop protection against *P. humuli* (Furk & Buxter, 1987). Later during the eighties the resistance of damson-hop aphid to pyrethroids increased considerably and in Hallertau in 1986 it exceeded the level of RF = 10 for deltametrhin and reached nearly the value of RF = 20 for cypermetrhin (Kremheller, 1988).

Rare application of pyrethroids, including lambda-cyhalothrin, to control aphids in the last two decades may have led to its better efficiency in comparison with the end of the eighties when pyrethroids had to be replaced by diazinon and since 1993 by imidacloprid due

to their too low efficacy on resistant *P. humuli* in CR. It was the reason why pyrethroids could not be recommended for practical hop protection against this pest (Vostrel, 1995). Insufficient efficiency of pyrethroids was confirmed in laboratory tests carried out in the first half of the nineties (Vostrel, 1997). Nevertheless, at the end of the nineties lambda-cyhalothrin was recommended in the years with earlier and stronger migration of *migrantes alatae* from plums to hops before application of imidacloprid to prevent damage of young hop plants in a Moravian hop region at first, whereas diazion and carbosulfan were preferred in Saaz hop region in Bohemia (Vostrel, 1999).

Since 2004 we have been using a susceptible reference strain of *P. humuli* with its origin in east part of Slovakia (Roznava), which was sampled in 1974 and has been keeping at Institute of Organic Chemistry and Biochemistry in Prague.

If we compare LC 50 values of this *P. humuli* reference strain with field resistant strains (average from each hop growing region) we obtain resistant factors (RF) for the individual regions. From Tables 1 and 2 it is obvious that RFs are the highest for Rakovnik district (RF = 9.5 in 2007, resp. RF = 9.8 in 2008) and the lowest for Trsice region (RF = 6.8). Nevertheless, the differences between the individual regions and districts are not so clear as if imidacloprid was tested (Vostrel, 2007). If we compare the results from 2007 and 2008 we can conclude that approximately the same level of RF was determined in both years. On the contrary to imidacloprid RFs for the tested strains of *P. humuli* are rather lower but biological efficiency in registered concentration is much lower, which was caused by much slighter difference between C100M and registered concentration in the time of lambda-cyhalotrhin release in the second half of the eighties. As a matter of fact only *P. humuli* strains from Trsice region are “susceptible” enough so as lambda-cyhalothrin may be able to be recommended for practical hop protection against resistant populations of damson-hop aphid. In Bohemian hop-yards it is necessary to distinguish not only among the individual regions and districts but among the localities as well. Moreover, lambda-cyhalothrin can be recommended only for early season’s spray before the key aphicide application in the years with early and heavy migration of *migrantes alatae* so as to prevent the damage caused by sucking aphids. Later in the season its spraying can not be recommend in practical conditions due to its severe side effect on beneficials especially aphidophagous predators. Recent release of flonicamid and registration of spirotetramat in the near future should help to solve hop protection against this dangerous pest not only in Czech hop-yards but also in other hop-growing countries (Vostrel & Filkuka, 2008).

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Table 1: Biological efficiency of lambda-cyhalothrinu (Karate Zeon 5 CS) on damson-hop aphid (*Phorodon humuli Schrank*) in laboratory tests in 2007

Resistant strain (RS)	**Percentage of mortality**			
	Concentration in %			
Saaz region 1. Louny district	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Dubcany	94	62	41	15
Klucek	89	57	29	24
Lenesice	95	67	33	27
Markvarec	84	51	21	9
Pocedelice	90	59	39	21
Average	***90,4***	***59,2***	***32,6***	***19,2***
2. Rakovnik district	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Domousice	79	54	29	9
Knezeves	87	48	24	16
Nesuchyne	83	57	22	12
Average	***83***	***53***	***25***	***12,3***
Ustek region	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Libesice	89	54	22	10
Polepy	84	60	26	13
Average	***86,5***	***57***	***24***	***11,5***

Trsice region	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Doloplazy	96	63	44	29
Trsice	92	69	37	22
Average	***94***	***66***	***40,5***	***25,5***
Roznava – susceptible strain (SS): LC 50 SS = 0,002				
Saaz region Louny district	**LC 50 RS = 0,016**			**RF = 8,3**
Rakovnik district	**LC 50 RS = 0,0189**			**RF = 9,5**
Ustek region	**LC 50 RS = 0,0179**			**RF = 9,0**
Trsice region	**LC 50 RS = 0,0136**			**RF = 6,8**

Table 2: Biological efficiency of lambda-cyhalothrinu (Karate Zeon 5 CS) on damson-hop aphid (*Phorodon humuli Schrank*) in laboratory tests in 2008

Resistant strain (RS)	**Percentage of mortality**			
	Concentration in %			
Saaz region ***1. Louny district***	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Dubcany	96	59	39	13
Klucek	91	59	28	15
Lenesice	94	63	30	19
Markvarec	88	53	24	11
Pocedelice	87	52	37	19
Average	***91,2***	***57,2***	***31,6***	***15,4***
2. Rakovnik district	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Domousice	83	51	29	11
Knezeves	88	49	26	13
Nesuchyne	82	53	23	9
Average	***84,3***	***51,0***	***26,0***	***11,0***
Ustek region	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Libesice	87	50	20	8
Polepy	83	57	22	11
Average	***85,0***	***53,5***	***21,0***	***9,5***
Trsice region	**0,04%**	**0,02%**	**0,01%**	**0,005%**
Doloplazy	95	62	43	25
Trsice	94	65	40	24
Average	***94,5***	***63,5***	***41,5***	***24,5***
Roznava – susceptible strain (SS): LC 50 SS = 0,002				
Saaz region Louny district	**LC 50 RS = 0,0172**			**RF = 8,6**
Rakovnik district	**LC 50 RS = 0,0196**			**RF = 9,8**
Ustek region	**LC 50 RS = 0,0188**			**RF = 9,4**
Trsice region	**LC 50 RS = 0,0136**			**RF = 6,8**

PLANT PROTECTION IN ORGANIC HOPS

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Abstract

In the years 2007-2008 field studies were carried out to elaborate the plant protection against diseases and pests for organic hop production by using EM–Farming. Effective microorganisms (EM) protected hops against downy and powdery mildews and also against main pests i.e. aphids and spider mites, especially in year with low population of these pests. Good protective effects against hop aphids were obtained when two EM preparations in mixture with EM fermented common sow-thistle and common dandelion extracts were applied.

Keywords: hops, organic farming, diseases, pests, effective microorganisms

Introduction

For the control of pests and diseases on plants cultivated in organic farming, plant extracts have used very often. Some of the substances in extract involved in plant defense mechanisms may help to enhance the resistance of crops through structural strengthening of the plant, increasing its resistance to the penetration of fungal mycelia and sucking insects such as aphids, or through encouraging vigorous growth to overcome an attack, or by direct toxicity (Lampkin 2002). In the last years, Effective Microorganisms, also called EM Technology are used more and more often in organic farming. EM-Farming is based on EM technology that has been first developed by the Japanese agricultural scientist Prof. Teruo Higa. Mixture of microorganisms consisting mainly of lactic acid bacteria (*Lactobacillus plantarum*, *L. casei*, *Streptococcus lactis*), photosynthetic bacteria (*Rhodopseudomonas palustris, Rhodobacter sphaeroides*), yeast (*Saccharomyces cerevisiae*, *Candida utilis*) and *Actinomycetes* (*Streptomyces albus, S. griseus*) which co-exist for the benefit of whichever environment they are introduced. EM consists of many different kinds of effective, disease-suppressing micro-organisms. Each of these effective micro-organisms has a specific task. In addition, these micro-organisms enhance each other i.e. they act synergistically. The micro-organisms are naturally existing and are not modified or manipulated in any way and are cultured according to a specific method. Some of which are known to produce bioactive substances such as vitamins, hormones, enzymes, antioxidants and antibiotics that can directly, or indirectly enhance plant growth and protection The fermented plant extract is also used to enhance efficacy of EM in plant protection against diseases and pests (Daly, *et al.* 2000, Kyan *et al.* 1999).

The aim of this study was the arrangement of plant protection against diseases and pests for organic hop production by using EM–Farming.

Methods

The study on organic hop protection was carried out in the years 2007-2008 in a private, not certificated hop-garden at Jastków near Lublin. Efficacy of effective microorganisms (EM) in control of powdery and downy mildews, Damson-hop aphids and two spotted spider mites was evaluated on two hop cultivars: Marynka and Magnum. Area of experiment amounted 4,5 ha, i.e. 4 ha with cv. Marynka and 0,5 ha with cv. Magnum. Effective microorganisms applications were performed on the ground of two active preparation EMa and EMa5 obtained from EM-Faming mother material. Six following spraying were made in 2008:

1. end of May 20 l EMa in1000 l water/1 ha

2. beginning of June 20 l EMa + 3 l EMa5 in 1000 l of water/1 ha

3. mid of July 20 l EMa + 6 l EMa5 in 2000 l of water/1 ha

4. beginning of August 40 l EMa + 6 l EMa5 in 2000 l of water/1 ha

5. end of August 40 l EMa + 6 l EMa5 + 60 l of EM fermented common sow-thistle and common dandelion extracts in 2000 l of water/1 ha

6. beginning of September 40 l EMa + 6 l EMa5 + 60 l of EM fermented nettle and dandelion extracts in 2000 l water/1 ha (application only on cv. Magnum)

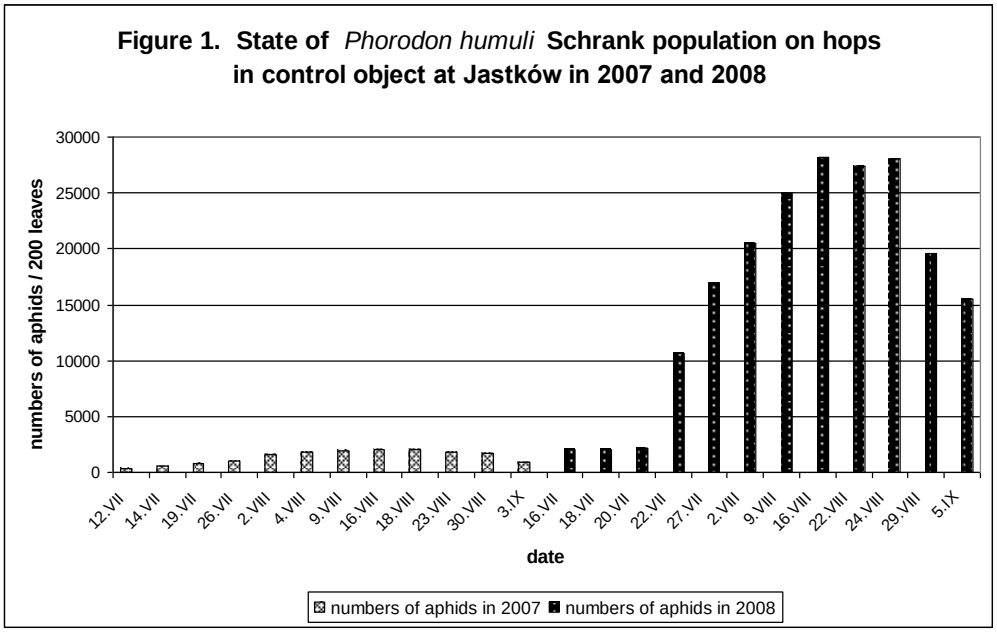
In 2007 the same applications were done, but without RM fermented plant extracts.

EM fermented plant extract was made from fresh weeds and EM according to procedure described by Kyan *et al*. (1999). Diseases incidence on plants was estimated according to method described by Solarska (1999). The numbers of Damson-hop aphids and two spotted spider mites were counted and efficacy was calculated according to Abbott formula.

Results

The applications with using EMa and EMa5 reduced incidence of downy mildew and powdery mildew effectively in both vegetation seasons. The symptoms of primary and secondary infection caused by fungus *Pseudoperonospora humuli* were not observed. The populations of aphids and spider mites in control objects differed between years of studies (fig. 1, 2). In 2007 the efficacy of Damson hop-aphids control by using EMa and EMa5 was very good (tab.1).First spraying was performed 12.07 when average number of exules amounted 392 on 200 estimated leaves. The aphids population was low during all vegetation period, its number in August not exceed 2075 individuals on 200 leaves (fig.1). Three applications with using EMa and EMa5 were performed and their efficacy amounted about 80% (tab. 1).

Figure 1. State of *Phorodon humuli* Schrank population on hops in control object at Jastków in 2007 and 2008



In 2008 Damson hop-aphids also appeared in July and its population increased rapidly and in August reached level of 28000 individuals on 200 leaves (fig.1). The protection spraying by using both preparations was performed at the end of second decade of July and reduced only ¼ of the pest population (tab.1). In order to prevent flowers against aphids, a decision was made that chemical pesticide - Confidor will be used before flowering of hops. The preparation was much better effective in aphid control than EM, but after its applications

also all exules not died and the pest population began to rebuild. At the end of August the application was done with using EMa and EMa5 in mixture with EM fermented common sow-thistle and common dandelion extracts. On the second day after application, the inhibition of aphid reproduction was observed, and on successive day, the increase of its size and change of its colour from green on brown and in such state the mortification of aphids in massive scale occurred. The next day the falling of died aphids from leaves was noted.

Figure 2. State of *Tetranychus urticae* Koch population on hops in control object at Jastków in 2007 and 2008

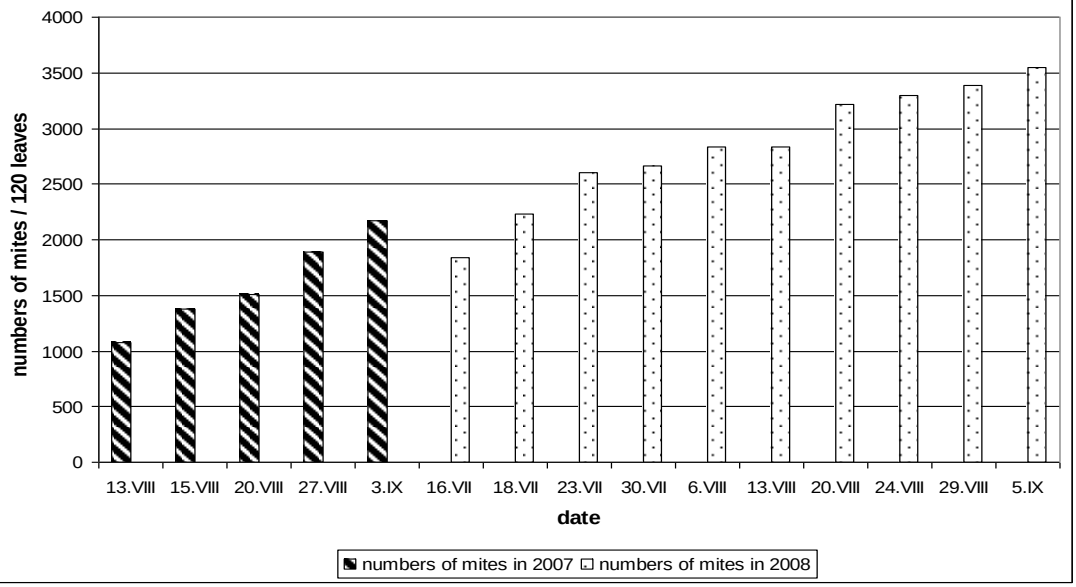


Table. 1. Efficacy of EM in aphids control on hop in 2007 and 2008, Jastków

Number of days after applications	Efficacy of EM in % 2007	Efficacy of EM in % 2008
7 days after I application	77	40
14 days after I application	80	-
21 days after I application	77	-
14 days after II application	75	87 (Confidor)
21 days after II application	-	82
28 days after II application	-	78
35 days after II application	-	70
14 days after III application	88	97
21 days after III application	80	-

Table. 2. Efficacy of EM in spider mites control on hop in 2007 and 2008, Jastków

Number of days after applications	Efficacy of EM in % 2007	Efficacy of EM in % 2008
14 days after I application	84	75
21 days after I application	82	76
28 days after I application	**80**	74
35 days after I application	78	59
7 days after II application	85	80
14 days after II application	82	69

The two spotted spider mites, second very dangerous pest of hops, were reduced to level not threatening to hops damage. In 2007 spider mite fed on hop plants from the mid of July but its number not exceed threshold value (fig. 2). In control object only just 13.08 1000 mites were found on 120 estimated leaves. Applications with using EMa and EMa5 and also EMa and EMa5 in mixture with EM plant extracts protected hops against the pest effectively (tab. 2). In 2008 spider mite occurred on hop plants in threshold number already in the mid of July and its population developed rapidly and was more numerous than in 2007 (fig. 2). Applications performed with using EMa and EMa5 and also in mixture with EM plant extracts kept in check the development of spider mites, but their efficacy was lower than in 2007 when population of mites was not such numerous (tab.2).

Discussion

The achieved results during studies show that protection of hops by organic methods such as use of effective microorganisms is possible. In other studies the application of EM gave also good effects in control of vine powdery mildew, winter wheat septoria and brown leaf blight (Boligłowa and Gleń 2008, Robotic *et al.* 1999). The use of EM in mixture with EM plant extracts defended some plants most effectively against diseases and pests (Kyan *et al.* 1999). With insect pest, the extract may be repellant as in the case of tansy and wormwood, or they may be directly toxic as in the case of pyrethrum, derris and quassia (Lampkin 2002). In the study, two preparation formulated on the ground EM-Farming mother material used in mixture with EM fermented common sow-thistle and common dandelion extracts were very effective against hop aphids. The studies on hop protection by organic methods will be continued.

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EVALUATION OF HEALTH STATES OF HOPS IN TRSCHITZ GROWING REGION

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Hop (*Humulus lupulus L.)* as a perennial crop propagated in a vegetative way is severely endangered by virus diseases, which cause economic damages due to decrease of alpha acid contents, i.e. a very important ingredient for beer production. Of course, the yield of dry hops declines as well. Many viruses and a viroid were determined in hop plants. Among the most important viruses belong the following ones: Apple mosaic virus (ApMV) from the genus Ilarvirus, Hop mosaic virus (HpMV), Hop latent virus (HLV) from the genus Carlavirus and Arabis mosaic virus (ArMV) from the genus Nepovirus. Hop latent viroid (HLVd) from the group Pospiviroid is the most frequent one found in hops.

State of health was assessed with the help of double antibody sandwich enzyme - linked immosorbent assay (DAS – ELISA) test with the plant material sampled in hop-gardens in Trschitz growing region. We evaluated samples from more than thirty hop-gardens planted with the following varieties: Saazer, Premiant, Magnum and Fuggle. We also checked presence of the above-motioned viruses and HLVd within a nursery planted with rootstocks. The presence of Apple mosaic virus (ApMV) as well as Hop mosaic virus (HpMV) and Hop Latent viroid (HLVd) were found out.

These results extend our knowledge on spread of these viruses in gene resources of hop. Assessment of health state in other hop growing regions (contamination by viruses and HLVd) will be studied within further research.

Keywords: hop, virus, viroid, ApMV, HpMV, ELISA,

Acknowledgement

Thanks for excellent laboratory work to Bc. Iva Malirova.

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POTENTIALLY DANGEROUS FUSARIOID MICROORGANISMS ASSOCIATED WITH ROT OF HOPS (*HUMULUS LUPULUS* L.) PLANTS IN FIELD CULTURE.

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Occasions of bines wilting and death of hop plant occur in spring period after training, the most frequently during May and June in all hop growing areas of Czech Republic. The incidence of hop canker in the field is usually sporadic, not every bine on hill is affected. But sometime there is an extensive occurrence and the whole plants can be damaged. The disease is described as "fusarium canker", fungus *Fusarium sambucinum* being usually considered to be a probable cause of it. This is not a specific problem of Czech hop cultures and information describing disease symptoms come also from Germany, Poland and England. Several fusarioid microorganisms were isolated as potential pathogens of hop (*Humulus lupulus* L.) but their virulence was not proved in inoculation trials in field conditions. Molecular search for other possible pathogens was performed. Using terminal restriction fragment length polymorphism (TRFLP), *Gibberella pulicaris* (anamorph: *Fusarium sambucinum*) was identified as probable cause of the hop wilting. The specific primer HLf1 was designed which can be used to detect the pathogen in soil and in damaged plant tissues. The primary cause of the disease is wounding of hop crowns by defect pruning, by feeding of rosy rustic moth (*Hydraecia micacea*) caterpillars or and other unfavourable circumstances. In June 2005 multiple wilt of hop plants was observed on farm of Hop Research Institute in Stekník. The death was observed mainly on margins of hop gardens which are usually weedier than internal rows. The presence of rosy rustic moth (*Hydraecia micacea*) was determined as primary reason of hop plants death that can survive there due to presence of couch grass (*Elytrigia repens L.)*. In early spring cutworms of rosy rustic moth start to eat young hop shoots, draw down to the crown and continue flattening of root system. Detrimental action of rosy rustic moth in the season terminates by turning to cocoon. Cocoons survive in soil and the process repeats the next spring.

Fig 1. Damaged root system by rosy rustic moth (*Hydraecia micacea*)

Eating of root system creates entrance gateway for action of pathogenic soil microorganisms. Damaged plants really showed typical „fusarium canker" symptoms. The affected bine wilted rapidly. They could be detached readily from the crown with a gentle tug. The point of bine attachment to the crown was tapered and rounded off so that only a few vascular elements connected the bine to the crown.

However, it is possible to minimize the opportunity of microbial attack of hop tissues by careful management (cutting) the plants, keeping hop gardens free of weed, avoiding crashing which produce large surface of the wounded tissue to soil microorganisms.

Acknowledgements

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IV. Session:

Chemical Analysis of Hop Compounds

IDENTIFICATION OF CZECH HOP VARIETIES BY ESSENTIAL OIL ANALYSIS

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Abstract

Until the beginning of the nineties the Czech hop growing industry only targeted growing fine aroma hops – specifically Saaz semi-early red bine variety (hereinafter referred to as Saaz). However in 1994 new varieties of hops were registered, which were to replace the importation of bitter hops from abroad. At present the range of registered varieties has been expanded to a total of 9 hop varieties – Saaz (4 627 ha), Agnus (58 ha), Bor (13 ha), Premiant (293), Sladek (275 ha), Harmonie, Rubin, Kazbek and Vital. Saaz remains the key variety however, with 87% of the area used for growing hops being used for this variety.

However the quality of grown hops cannot be assessed simply according to the contents and composition of hop bitter resins. Hop essential oils are an integral component of hops and are the reason hops are used in the brewing industry. The objective of this work is to characterise the composition of hop essential oils in individual varieties and to assess the possibility of differentiating them on the basis of this. The composition of hop essential oils is unique in Saaz but essential oil components in the other varieties have been found which are characteristic to the given variety and can be used to differentiate it from other varieties of hops.

Keywords: hops, *Humulus lupulus,* essential oils, identification of hop varieties, gas chromatography

GEOGRAPHICAL ORIGIN OF HOPS - DETERMINATION BY ISOTOPE RATIO MASS SPECTROMETRY (IRMS)

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Abstract

The isotope ratio mass spectrometry (IRMS) is a suitable method to determine the origin and the authenticity of agricultural products, foods and beverages. Examples for the application of this technique are asparagus, fruit juice, wine and ham. So the question came up if the IRMS method can be used for hops, too. The technique is applied to hops for the first time and it is shown that it is suitable. The method allows the discrimination between each individual growing area.

Keywords: Hops, origin of hops, agronomy

Introduction

By means of the IRMS technique the ratios of the stable isotopes are analysed. The growing regions with their climatic distinctions influence the stable isotope ratios of chemical elements in the biomass of plants. The environmental conditions have also an effect as well as the use of agricultural methods (e.g. fertilization). At work with hops the ratios of the following stable isotopes were investigated: hydrogen, carbon, nitrogen and sulfur.

Methods

On the one hand hops from the main German growing region (Hallertau) were studied to check the differences in the ratios of the stable isotopes in a limited area. Besides the location the influences of variety and crop year were regarded. On the other hand investigations were carried out on hops from different growing regions. The samples came from the other European and from important global growing regions.

Results

Over the ratio of 13C/12C and 2H/1H a differentiation in Hallertau hops is doubtful. But when additionally the ratios of 15N/14N and 34S/32S are taken into account the samples can be discriminated according the growing region. The influences of variety and crop year are little but nevertheless they must be regarded. The examination of all four stable isotope ratios allows the discrimination between each individual growing area.

Discussion

It is shown that the IRMS method can be used to differentiate the origin and the authenticity of hops. The bigger the distance between the growing areas the easier is the differentiation of the hops by means of the IRMS method. The use of it as a check instrument is imaginable.

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RELATIONSHIP BETWEEN XANTHOHUMOL, POLYPHENOLS AND FLAVONOIDS CONTENT IN HOP LEAVES WITH REGARD TO VEGETATION PERIOD

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Abstract

The aim of the study was testing of the content of some secondary metabolites of hop, polyphenol compounds, flavonoids, prenylated flavonoid xanthohumol during a vegetal period, which display a wide range of biological and pharmacological properties, including antimicrobial, and antioxidant, properties.
The content of secondary metabolites, phenol substances, flavonoids and especially xanthohumol in extracts from hop leaves was defined by standard analytical methods. The concentration of chemical substances was determined: phenol substances by Singleton's method, flavonoids by Rakotoarison's method and xanthohumol by HPLC.
We tested the samples of hop leaves taken from the Gene Bank of the Slovak Republic in the Institute of Plant Production, Piešťany (CVRV – VÚRV). The samples of different cultivars of hop were dried and homogenized. We used the following cultivars of hop: Osvald's clones 31, (K-31), and 72 (K-72), Bor, Sládek, Aromat, Zlatan, and Premiant. We collected the samples before flowering and at the end of vegetal period.

The experiments were carried out within the period of years 2007 and 2008.
All cultivars showed different contents of secondary metabolites during the vegetal period, their content in leaves decreased from June to September. Phenol substances in leaves were in the interval between 5.00 – 8.83 mg g^{-1} of dry matter in 2007 and 9.50 to 14.34 mg g^{1} of dry matter in 2008 before flowering, and from 0.90 to 2.25 mg g^{-1} of dry matter in 2007 and 3.03 – 4.68 mg g^{-1} of dry matter in 2008 at the end of the vegetal period. In the year 2008 there was less sunshine and more showery weather at the beginning of flowering period than in 2007. The quantity of phenol substances was higher in 2008 than in 2007. The highest content of polyphenols was detected in cultivar Bor at the beginning of vegetal period in the 2008.

Flavonoids content in leaves was similar, but different. We noted a decline during the vegetal period in both cases, so as in phenol substances. The quantity of flavonoids was higher in 2007. The content of flavonoids was within the range of 2.13 – 6.37 mg g^{-1} of dry matter in the leaves before flowering in 2007, and 0.14 to 1.43 mg g^{-1} of dry matter in 2008. The range of dry matter of flavonoids decreased to 0.48 – 0.76 mg g^{-1} at the end of vegetation in 2007, similar was the interval of flavonoids content, ranging from 0.10 to 0.20 mg g^{-1} of dry matter at the end of vegetal period in 2008.

Xanthohumol, prenylated flavonoid, was defined in extracts from leaves of hop. The highest content, similar to the content of phenol substances and flavonoids, was investigated before flowering, and lower at the end of vegetal period. The content of xanthohumol in leaves was higher in the 2007, so as in flavonoids. The quantity of xanthohumol was maximum 0.067 % of dry matter of the cultivar Bor at the beginning of vegetal period in 2007.
The content of the secondary metabolites in leaves depended mainly on the vegetal period. The influence of climatic conditions on the content of secondary metabolites of hop leaves was verified.

Keywords: polyphenols, flavonoids, xanthohumol, hop

POLYPHENOL AND FLAVONOID CONTENTS OF HOP CALLUS AND CELL SUSPENSION CULTURES

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It is well recognized that plants are a rich source of commercially important secondary metabolites. Hop (*Humulus lupulus* L.) is traditionally known as an essential ingredient in most beers. Secondary metabolites of hops important for the brewing of beer include α-acids and ß-acids, however, another group of compounds present in hops, such as prenylated chalcones, xanthohumol, and desmethylxanthohumol, were recently found to exhibit interesting bioactive properties. The increased demand for medicinally important secondary metabolites increases the pressure to produce these compounds via alternative ways, especially using cell/tissue cultures and transgenic plants, respectively. The aim of our study was to establish a convenient *in vitro* system, based on the induction of callogenesis and establishment of cell suspension culture in hops for chemical analyses of constituents of *in vitro* cultures and for potential production of interesting flavonoids in *in vitro* culture systems. For optimization of the *in vitro* system, we studied the effect of growth regulators (BAP + NAA or 2,4-D), culture conditions (continual dark *vs*. photoperiod of 16 h light/8 h dark), explant type (internodal segments *vs.* leaf segments) and genotype (K-31/3/7, K-70/4/1 and Lučan4/3) on callus culture of hops. Callus induction rate was independent of explant type and it was the highest on MS+2,4-D media in photoperiod and on MS+NAA in dark conditions. For maintenance of calli, culture in dark was more favourable, comparing to photoperiod, where higher frequency of necrosis of calli occured. Cell suspension cultures were established from stabilized callus cultures in liquid MS media containing 1.0 $mg.l^{-1}$ BAP with combination of 1.0 $mg.l^{-1}$ NAA or 1.0 $mg.l^{-1}$ 2,4-D. Cell suspension cultures derived from both the types of explants showed higher biomass accumulation (FW and DW) in conditions of photoperiod. Cell proliferation was higher in both culture conditions in cultures derived from internode-derived calli. Higher biomass accumulation was observed on media with NAA in comparison with media with 2,4-D. The viability of cells (assessed as % of TTC-positive cells) depended on the concentration of pectinase added to liquid media to liberate cells from cell clumps and ranged from 60.9-90.6 % in media without pectinase to 36.2-65.4 % in media with 1000 μl $pectinase.g^{-1}$ tissue FW. Content of total polyphenols depended on the type of *in vitro* culture and ranged 60.5-137.1 $mg.l^{-1}$ of gallic acid equivalent (GAE) in cell suspension cultures and 76.6-158.5 $mg.l^{-1}$ GAE in callus cultures in comparison to 121.4 $mg.l^{-1}$ GAE in the source shoot cultures of hops. Using HPLC analysis, we were able to detect also a production of xanthohumol in cell suspension cultures of hops. The highest production of xanthohumol was observed in cell suspension cultures established from leaf segment-derived calli in medium containing 1.0 $mg.l^{-1}$ BAP in combination with 1.0 $mg.l^{-1}$ 2,4-D without pectinase and cultured in dark conditions.

Keywords: *Humulus lupulus*, tissue culture, secondary metabolites, xanthohumol

INFLUENCE OF VEGETATION PERIOD ON ANTIOXIDAT AND BIOCIDE ACTIVITY OF EXTRACTS FROM HOP LEAVES

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Abstract

The aim of these experiments was testing influence of vegetation period on antioxidant and biocide activity of extracts from hop leaves.
We tested the samples of hop, taken from the Gene Bank of the Slovak Republic in the Institute of Plant Production, Piešťany (CVRV – VÚRV). We used the following hop cultivars: Osvald's clones 31, (K-31), and 72 (K-72), Bor, Sládek, Aromat, Zlatan, Sířem and Premiant. We collected the samples before flowering and at the end of vegetal period. Extracts were prepared by methanol extraction in ratio 1:20. Extracts were evaporated and retro dissolved in methanol.

We used DPPH method for determination of antioxidant capacity of the sample.

The antibacterial activity of extracts was determined *in vitro* against a variety of phytopathogene bacteria - Gram-positive bacteria (*Clavibacter michiganensis subsp. sepedonicus*), and Gram-negative bacteria (*Xanthomonas vesicatoria, Xanthomonas sp., Erwinia amylovora, Erwinia malotivora, Pectobacterium carotovorum subsp. carotovorum, Pseudomonas syringae pv. syringae*). Antimicrobial activities were tested by the standard plate diffusion method and zones of inhibition were measured in mm. A biocide effect was compared with the effect of 1.2% solution of TMTD (tetramethylthiuram disulfide), an active substance of commercial pesticides.

All cultivars showed different antioxidant activity during the vegetal period. The interesting changes in the leaves extract of cultivar Sířem were noted from the beginning until the end of vegetal period, when antioxidant activity decreased by 78%. On the contrary, antioxidant activities of the cultivar Bor and K-72 were climbing during vegetal period. Antioxidant activities in other cultivars, for example Aromat, remained relatively stable. These results show that antioxidant activity of the leaves extract depends mainly on the cultivar.
Both antibacterial and antioxidant activities were tested during the vegetal period of hop plants. The average size of inhibition zones of the extracts from leaves at the beginning was lower, than average size of the extracts before flowering time. The marked 89% of inhibition effect of the standard, TMTD, in the extract of Bor cultivar on *Erwinia mallotivora* was noticed. Extracts from cultivars K-31 a Lučan had similar effect on these bacteria - 67% of inhibition effect of standard TMTD. The effect of inhibition of the extracts from leaves of hop at the end vegetable period was minor.
The results of antibacterial activity approves, that this effect relates to sensibility of a particular bacterial strain and hop cultivar. We observed influence of the vegetal period, too.

Keywords: hop extracts, antioxidant activity, antibacterial effect

V. Session:

Hop Production

MATHEMATICAL MODEL FOR PREDICTION OF YIELD AND ALPHA ACID CONTENTS FROM METEOROLOGICAL DATA FOR SAAZ AROMA VARIETY

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Abstract

Mathematical models of the relationship between yield and alpha acid content versus meteorological parameters were worked out for Saaz aroma variety using data covering the years 1978-2007. Using multi-linear regression, a ten parameter model (alpha acids) and eleven parameter model (yield) have been outlined. Average temperatures from July and August, average air humidity from June, July and August, average daily sunshine duration from June, July and August and accumulated precipitation from August have been found as key input variables of the alpha model. Correlation coefficient of the relationship between predicted and actual alpha acid contents is r=0.914, coefficient of determinancy r^2=0.835. Yield model shows important role of rainfalls in the period May-July. Correlation coefficient of the relationship between predicted and actual yield of is r=0.923, coefficient of determinancy r^2=0.852.

Keywords: hops *(Humulus lupulus* L.), alpha acids, yield, weather conditions, mathematical modeling, correlation relationship

Introduction

Yield and alpha acid content in hop cones are the most important quality parameters of hops. Alpha acid content of a hop variety varies from year-to-year. The course of weather conditions is generally considered as an important factor of hop resins biosynthesis and an important factor influencing hop yield. Long-term monitoring of yield and alpha acid content in hops cultivated in the region of central Europe shows notable year to year variations (Krofta, 2007). In a study of hops grown in the Hallertau region of Germany over a period of 35 years (1926-1961) Zattler and Jehl (1962) concluded that high alpha was associated with a moist summer and below average temperatures but with an average amount of sunshine. Thompson and Neve (1972) concluded that seasonal fluctuations in alpha acid level were associated with variations in air temperature 40 to 60 days prior to harvest and thus not under the grower´s control. Multilinear mathematical model of the relationship between alpha acid content in hops and meteorological variables were worked out by Park (1988) for variety Hallertauer grown in South Korea in the period 1978-1986. This paper describes the estimation of a mathematical model for prediction of yield and alpha acid contents from meteorological data for Saaz aroma variety grown in Czech Republic. The model was outlined on the basis of more than 25 years analytical and weather experimental data.

Material and methods

Mathematical model of the relationship between yield, alpha acid contents and meteorological parameters was worked out for Saaz aroma variety on the basis of data analyses in the period of 1976-2007 in the locality Brozany, Czech Republic. Brozany is a traditional hop growing locality in the central part of Auscha growing region. Altitude of the site is 155 m above sea level. Hop is grown at the area of 50-60 hectares. Content of alpha acids were determined in individual lots of raw hops. Alpha acid content was measured by lead conductance method according to EBC 7.4 (Analytica EBC, 1998). Yield data were provided with grower. Meteorological data were provided with Czech Hydrometeorological

Institute observatory in Doksany located in 2 km distance from the farm. Average day temperatures, air humidity, daily sunshine duration and daily precipitation were used as input variables of the model.

The strategy of data analysis was based on the influence of weather conditions in different parts of the season on the alpha acid production. Therefore, the importance of different variables in different months relevant to plant growth and ripening was investigated. Twenty five years of time series data for all available environmental variables in daily resolution were put together into one file for data processing by Mini 32 software (Environmental Measuring Systems, Brno, CZ). Each environmental variable was split into eight sub-variables containing data values for a single month (January to August). Therefore, a file containing altogether thirty three monthly averages (four main environmental variables times eight months completed with the alpha acid percentage) in twenty five years was created for the next processing. We applied a simple “black box” approach based on the linear multiregression analysis. The multi-regression analysis was made with a “fit” module in Mini32 software package which uses an iterative method of finding regression parameters where the best-fitting line is obtained by the method of least squares. By using ordinary linear regression analysis the most important variables were pre-selected for next step of processing. During the analysis, the most relevant variables were selected according to standard error of parameter estimation.

Results and discussion

The average yield and alpha acid content in Saaz aroma variety grown in Brozany during the period 1976-2006 are summarized in Table 1.

Table 1: Input data (yield, alpha acid contents) for mathematical modeling

Year	Yield/*grower 1*	Alpha	Year	Yield/*grower 2*	Alpha
1976	869	-	1993	790	4,0
1977	1204	-	1994	830	2,1
1978	910	-	1995	1320	3,3
1979	1153	-	1996	1430	4,5
1980	759	-	1997	1430	3,9
1981	1408	5,2	1998	950	3,2
1982	1204	4,9	1999	1400	3,1
1983	1072	3,6	2000	1170	3,8
1984	704	5,1	2001	1540	4,1
1985	954	4,0	2002	1190	2,9
1986	1059	4,2	2003	1100	3,0
1987	1247	4,1	2004	1190	3,4
1988	910	4,6	2005	1530	3,5
1989	812	3,8	2006	hailstorm	2,6
1990	667	3,5	2007	hailstorm	3,0
1991	958	3,8	2008	1690	3,4
1992	729	3,2	2009	-	-

Yield = kg/ha alpha = % w/w

An assessment of the individual meteorological elements effect upon alpha acid content in Saaz aroma hops resulted in a ten-parameter multilinear mathematical model. The statistical analysis of weather elements showed that only June, July and August meteorological parameters had a significant effect on alpha acid content in Saaz aroma variety. The partial negative correlation between July temperatures and alpha acids significantly was observed

as having the greatest influence upon the model. (r_5=-0,236). It is interesting that precipitation is included only marginally in August (r_{10}= -0,004) and indirectly in air humidity and temperature. In some years it happens that intensive rains in the course of August, which come after water stress period, promote the growth of hop cones size but biosynthesis of hop resins falls behind. It results in"dilution" of alpha acids in cones. Thus, negative correlation coefficient $\underline{r_{10}}$ between alpha acid contents and August precipitations confirms empiric experience. The quality of outlined mathematical model confirms the relationship between actual and predicted alpha acid contents in Brozany in the period since 1981 till 2008 shown in Figure 1.

Alpha (model) = constant + parameter2*Variable2 +……+ parameter 10*Variable10

Order	**Parameter**	Variable	Std. error of parameter. estimation [%]
1	5,916	*Constant (offset)*	57
2	-0,220 (r_2)	mean day sunshine in June (hours)	44
3	0,117 (r_3)	mean day sunshine in v July (hours)	94
4	-0,218 (r_4)	mean day sunshine in August (hours)	81
5	-0,236 (r_5)	mean temperature in July (°C)	100
6	0,163 (r_6)	mean temperature in August (°C)	40
7	-0,092 (r_7)	mean air humidity in June (% rel.)	35
8	0,058 (r_8)	mean air humidity in July (% rel.)	49
9	0,061 (r_9)	mean air humidity in August (% rel.)	45
10	-0,004 (r_{10})	August precipitation (mm)	72

Crop harvests 2007, 2008 were the first testing period for the model. The predicted value of alpha acid according to the calculated model was 3,0 % (2007) and 3,4 % (2008). Actual contents were 2,9 % (2007) and 3,3 % (2008). It suggests very good agreement.

Similar ten parameter multilinear mathematical model was outlined for relationship between yield and meteorological variables in Brozany for the period 1976-2005.

Yield (model) = Constant + r_2 .parameter2 + r_3.parameter3 +…+ r_{10}.parameter 10

Order	**Parameter**	Variable	Std. error of parameter. estimation [%]
1	-1196	*Constant (offset*)	81
2	- 38,86 (r_2)	mean day sunshine in August (hours))	103
3	16,60 (r_3)	mean day temperature in June (°C)	118
4	22,14 (r_4)	mean air humidity in March (% rel.)	27
5	-13,86 (r_5)	mean air humidity in July (% rel.)	41
6	10,48 (r_6)	mean air humidity in August (% rel.)	73
7	1,092 (r_7)	May precipitation (mm)	74
8	0,722 (r_8)	June precipitation (mm)	113
9	2,455 (r_9)	July precipitation (mm)	32
10	409,32 (r_{10})	grower	15

Model shows that rainfalls in the period May-July play important role in yield of hops formation. Yield data (see Table 1) were collected from archive and provided with current grower. Data show that change in farm ownership resulted in significant increase of yield from 1993 till today. This relevant information had to be taken into consideration in model construction. Additional parameter "grower" was introduced. It takes the value "1" in the period 1976-1992, and the value "2" from 1993 till today. Actual and model yields of Saaz aroma variety in the period 1976-2005 are shown on the Fig. 2. Acuracy of the model reflects

high value of correlation coefficient 0,923. Year 2008 was the first testing season. Predicted value 1475 kg/ha is in good compliance with actual one 1690 kg/ha.

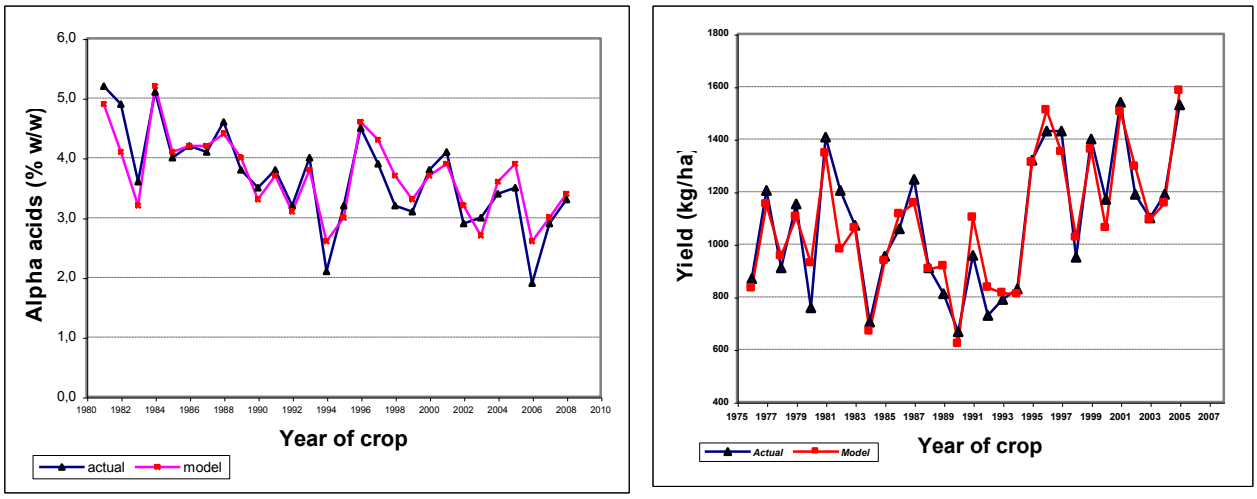

Fig. 1.2: Actual and model values of yield (right) and alpha acid contents (left) of Saaz aroma variety in Brozany region in the period 1976-2005 (yield) and 1981-2008 (alpha)

It is important to acknowledge that models only applies to Brozany growing region and one cultivar. Any extrapolation to other growing regions requires verification. Model was tested on one or two growing seasons. It is premature to state that the model is anything more than preliminary and further testing is inevitable.

Conclusions

The prediction of hop quantity and quality in terms of yield and alpha acid content specific to the Saaz hop cultivar grown in the Brozany, Czech Republic can be ascertained according to seasonal weather course based upon knowledge of the main climatic variables within the time period of vegetation months. Mathematical model for prediction of alpha acid contents from meteorological data showed that alpha acid contents in Saaz aroma hops was influenced by weather conditions in a relatively short period June-August. Yield model shows important role of rainfalls in the period May-July. Soil moisture values and non-linear fit could be used for increasing for the accuracy of modeling. The performed study might encourage next research work on this field that could lead in particular to more efficient irrigation management of hop gardens.

Acknowledgements

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UTILIZATION OF IRRIGATION SYSTEMS IN HOP PRODUCTION

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Abstract

Hop growing in the decisive hop regions within Czech Republic depends on precipitations. The fact, that irrigation is a very important factor under these conditions to cover rainfall deficits, is apparent at first in the recent years. The occurrence of the deficits in precipitations has a scholastic character within each locality and term.

Experimental as well as pilot trials show that new progressive irrigational systems (drop irrigation and micro-spraying) should be used for irrigation of hop gardens. Irrigational water becomes most effectively used under these systems. It is necessary to respect specific demands and characteristics of the individual kinds within the distribution of irrigational water in a hop garden.

Hop irrigation system placed at the ceiling of a wirework as well as micro spraying have a positive influence on the microclimate within a hop garden. It has a good effect on the growth of hop plants.

Drop irrigation placed in the space between rows 0.5 m under the ground does not provide uniform transmission of water inside a root system in a hop garden. Water loss by leakage is obvious under this type of irrigation. It depends on a soil type. Horizontal water spreading within the places between rows is another negative. Therefore, this type of irrigation is possible to use under its installation before planting of new hop plants, when the water piping with drop units is always placed in the axis of a planted row.

Micro-spraying placed on pole irrigates the whole area of hop gardens. Influence of wind at spraying has a negative share with uneven distribution of irrigation water. Increased requirement of water and hence a need of electrical energy insert to disadvantages of this irrigation system.

Hop irrigation means an important stabilization factor for effective hop growing. No statistically conclusive differences were found out between the compared variants. Important increase of hop yield (statistically conclusive) was revealed between the irrigational variants and a rain-fed plot. The average growth in hop yield reached 20-25 % (at fine aroma hops Saaz) and 18 % (at hybrid variety Agnus) in comparison with a rain-fed plot.

Slight increase in the contents of alpha acids was found out as well.

Expert design and realization of the irrigational system as well as good qualification of workers providing exploitation is necessary to reach above-mentioned effectiveness of modern irrigational systems in praxis.

Acknowledgement

The work was supported by the Czech Ministry of Education, Youth and Sports, project No. MŠMT 1486434701: Research and regulation of stressful factors of hops.

RECOVERING HOP CULTIVATION IN GALICIA (NW SPAIN)

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Abstract

Hop was harvested again in Galicia in 2006 in a small area of 1300 m^2, to commemorate the 100 anniversary of the local brewery "Hijos de Rivera S.A." (HdR) that made again beer with hop cultivated in this region. A 100% malt Christmas Beer was bottled as "Estrella de Navidad" and launched into the market, having a very good consumer´s acceptance. This led to the plantation of other 8700 m^2 in 2007 at the experimental farm and to 4 ha more at private farms in 2008.

Introduction

HdR, founded in 1906, decided to make beer in 2006 based again on hop cultivated in Galicia, as the main milestone to commemorate its 100 anniversary and to recover with it the role of hop cultivation promoter played by the brewery in the 30's of the 20th century. After a very active period from the 50´s to the 70's, the cultivation of hop stopped in the 80's. HdR contacted the Centro de Investigacións Agrarias de Mabegondo (CIAM) and the Sociedad Anónima Española para el Fomento del Lúpulo seeking for advise and collaboration in order to recover hop cultivation in Galicia.

Methods

An area of 1300 m^2 was planted in 2005 with cv Nugget at CIAM Research Farm, located in the heart of the area devoted to hop in the past. Due to the good results, in 2007 a new plot of 8700 m^2 was planted with 83% cv Nugget and 17% cv Columbus.

Results

In September 2006 a total of 145 kg of dry cones were harvested with a content of 12,4% in α-acids (Table 1). Yields and α and β acids content on years 2007 and 2008 are also shown on Table 1.

Table 1. Hop yields and α and β acids content for years 2006 to 2008.

Year / Variety	2006 Nugget	2007 Nugget	2008 Nugget	2008 Columbus
Pellets production (kg)	145	58	1250	125
Content in w/w%				
α-acids	12.4	11.4	13.3	12,3
Cohumulones (%α)	26.9	24.6	27.6	30.8
β-acids	4.5	4.4	5.3	4.4
Colupulones (%β)	52.2	51.9	53.5	57.3
Relation α/β	2.8	2.6	2.5	..2.7

Every December, since 2006, HdR bottled a 100 % malt Christmas Beer named "Estrella de Navidad", made with the hop cultivated at CIAM. The beer was launched into the market, with high acceptance of the customers.

Acknowledgement

The project is financed by the EAFRD Nº 2997-07 of the Consellería do Medio Rural.

THE RIGHT TIME TO HARVEST OPTIMAL YIELD AND QUALITY

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The Right Time to Harvest Optimal Yield and Quality

A. Lutz, J. Kneidl, E. Seigner, and K. Kammhuber

Introduction

Hop is harvested upon reaching the "technical ripeness" (highest brewing value), not at full or "physiological" maturity. Each variety has its own specific, genetically determined optimal time of harvest which is varied by the weather situation, location conditions and the cutting time.

Harvest time crucially affects:

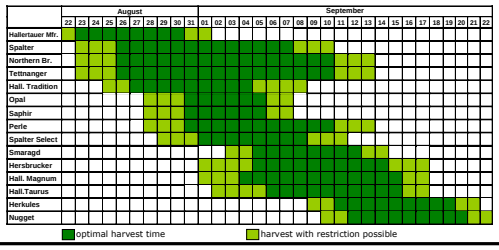
- α-acid contents
- yield
- outward quality (color and shine, infection with diseases and pests, shattering)
- aroma (aroma intensity, oil content and composition)
- vigor and vitality of the plant (in the next season)

↓

Economic interest of hop growers, traders and brewers

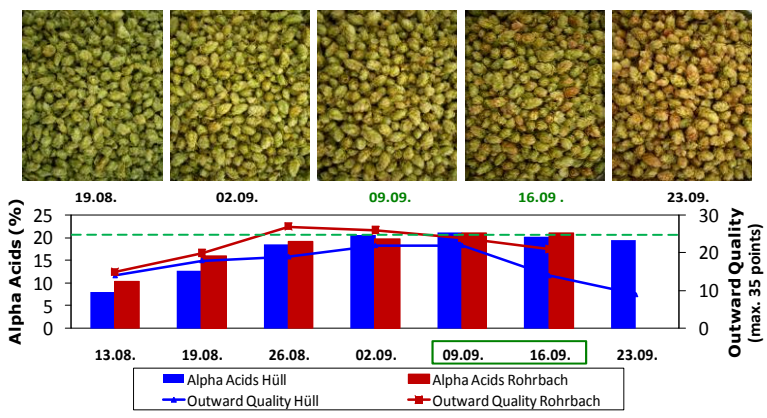
Results from harvest time studies

- 5 – 8 harvest times (2 dates / week), 4 replications with 20 bines each
- 3- 4-year-trials (climate, health and vitality)
- data for yield, α-acid contents, aroma, outward quality, shortcomings assessed



Biogenesis studies

Single bines of the most important hop cultivars are harvested at 2 different locations each week starting in mid August till late September. Cone samples are analysed at once and data concerning chemical compounds, aroma, outward quality and yield are assessed.



Improved harvest recommendations

Based on these results the optimal harvest period for each cultivar is adapted each year to the specific wheather conditions and recommendations concerning the best timing of picking are given to the growers and traders weekly.

HOP DRYING: TEMPERATURES INSIDE THE LAYERS OF HOP

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Abstract

During 2 years the University of León (Spain) has done 45 test hop drying research tests to determinate the evolution of the temperature inside the layers or stratums of the hop. In the hop drying, the hop stratum temperature varies with deepness and time. Initially, the lower stratums have more temperature while it is less in the higher ones. The length of this phenomenon takes about 3 hours from the beginning of the drying depending on speed and drying air conditions. The highest difference in temperature between the higher and the lower stratums occurs at the first hour of beginning the drying process. From the 3rd hour the temperature of the layer is homogeneous depending on speed and drying air conditions.

Keywords: hop, layer, temperature, time

Introduction

The University of León has researched about hop drying in an experimental drying kiln for 2 seasons. This research studied how different speed and drying air temperature conditions affect the following three aspect: the commercial quality (α-acids), the weight lost during the drying process and the temperatures inside the layers of the hop. Other characteristics were studied such as hop density and the humidity distribution in the fresh cone.

Methods

It was built a one layer experimental kiln, equipped with a control and data acquisition systems with similar functioning than the industrials kilns. The air warming was made through electrical elements. Drying air speed and air temperature were modified to obtain tests combinations in different conditions. The air speed was altered in the following values: 0,4, 0,5, 0,6, 0,7 and 0,8 $m \cdot s^{-1}$, while the temperature changed between 50, 60, 70 and 80 ºC.

On each test were registered 20 different signals which came from sensors of temperature transducers, air speed, relative humidity and weight.

The 45 drying tests were done with the Nugget hop variety for 2 consecutive seasons. The Nugget variety takes up almost the whole cultivated surface (around 700 hectares in the Province of León). From this tests were obtained results of hop density, humidity distribution in the green cone (fresh cone), quality and drying weight lost evolution.

The temperature was determined in different stratums or layers to study it in various depths inside the layers of the hop and inside the tray. This tray was filled in with green hop bed of 0,30 m deep. The temperature signals were of 0,05, 0,15 and 0,25 m from the bottom.

Results

The figures correspond to the test number 22 representing the 45 tests that were done with the Nugget variety in the second season.

The figure number 1 shows: the evolution of the air temperature in the kiln´s exit (INTOP) and the temperatures measured at 0,05 m (LBOT), 0,15 m (LMID) and at 0,25 m (LTOP) from the bottom of the tray.

All the figures show the same behavior pattern: a fast increase at the initial time that lasts three hours followed by a phase that finishes at the end of the drying process in which this

difference reduces until: air drying temperature, the temperature in the different stratums and the air temperature in the kiln´s exit become equals.

This is the reason why it has been shown only the 5 firsts hours of the drying process to emphasize the time where the difference in temperature between the lower stratum and the higher one is more evident. In the figure below it has not been shown what occurs after the fifth hour when the differences in temperature are smaller. From the hour number 5 until the end of the drying process, the temperature in the whole bed is similar.

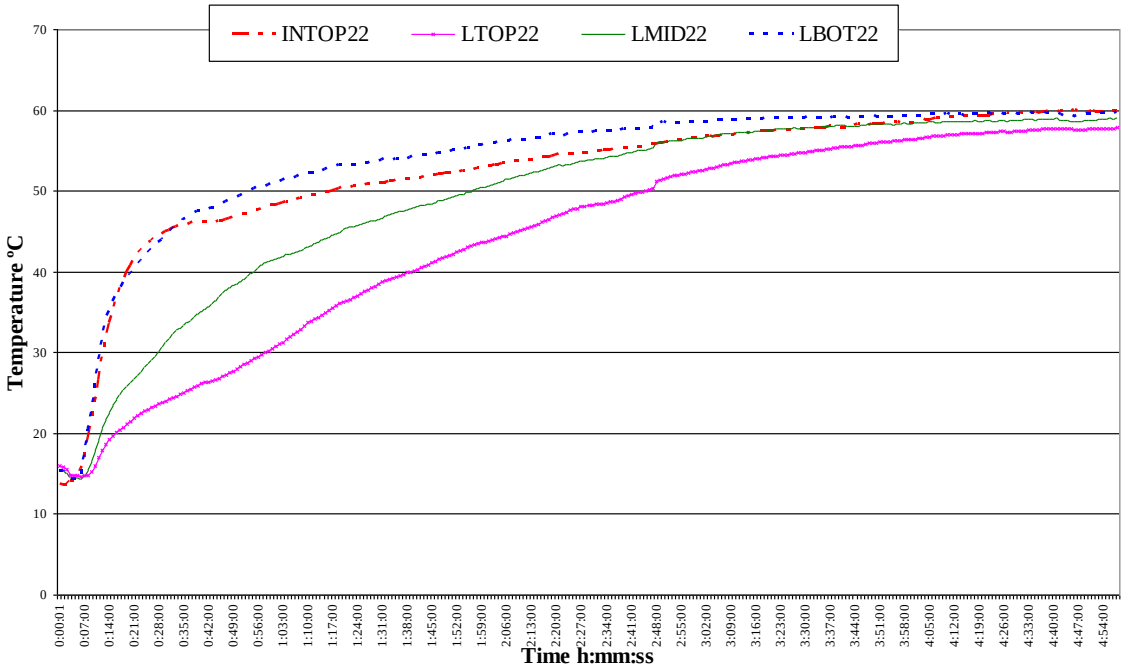

Figure 1. Test number: 22. Conditions: 60 ºC and 0,4 m·s^{-1}. Date: September 15th 2003.

Discussion

Examining the time where the difference in temperature is more evident inside the stratums of the hop, it is shown how the temperature is not homogeneous (varies with the depth). The higher stratums have more temperature, while the lower ones have less temperature. This concept is referenced in the bibliography consulted without giving specific details.

The most evident difference in temperature is produced in the first drying hour. This phenomenon takes about 3 hours. After the third hour the temperature of the whole bed is equal.

This phenomenon occurs at the beginning of the drying process, since it is produced a warming in the lower layer directly in contact with the hot air chamber. On that stratum is produced an initial drying process in which its evaporated water is swept by the hot upward air. In the higher stratums is produced the condensation of this water, which is led by the hot upward air causing four consequences:

- An increase in humidity in the higher stratums of the hop.
- A decrease in temperature in those higher stratums.
- The temperature in the lower stratum raises faster than in the higher ones.
- An overheating is produced in the lower stratums in order to get a complete drying in the higher one.

It has been created the figure number 2 from the information in the figure 1, which shows the differences in temperature expressed in Cº at 30 minutes drying intervals. 0.20 m is the distance between the lower layer (0.05 m from the bottom of the tray) and the higher one (0.25 m from the bottom of the tray). The bed is 0.30 m deep.

Time	LBOT-TOP22
0:01:00	-0,44
0:30:00	20,459
1:00:00	20,361
1:30:00	15,283
2:00:00	12,012
2:30:00	9,034
3:00:00	6,0546
3:30:00	4,3168
4:00:00	2,9396
4:30:00	2,1386

Table 1. Temperature differences (Cº) between the lower layer (0.05 m from the bottom of the tray) and the higher one 0.25 m (from the bottom of the tray).

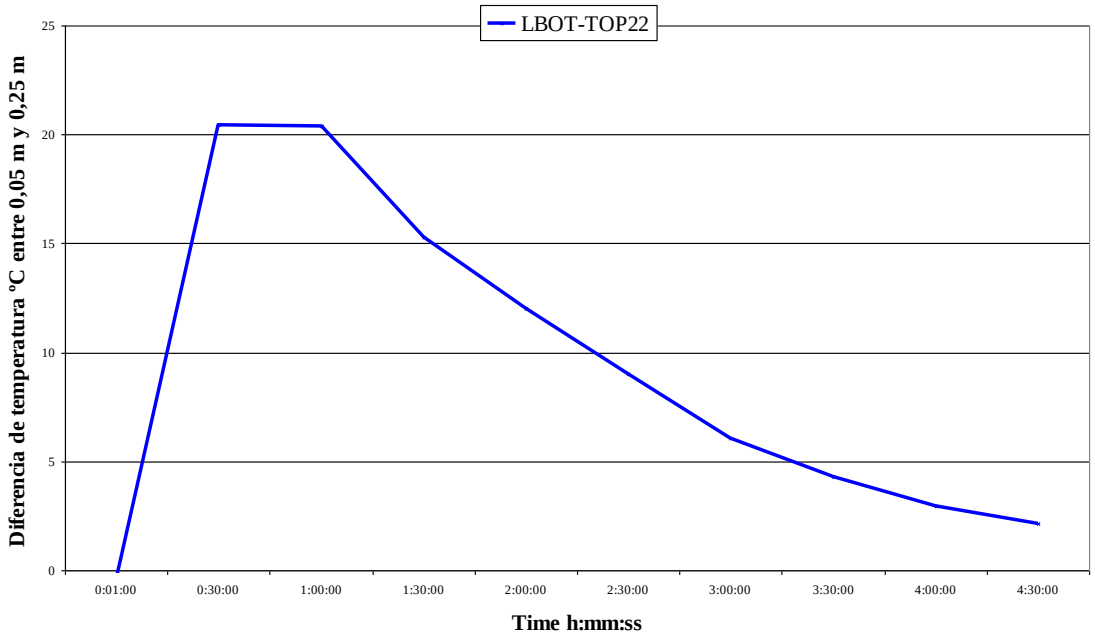

Figure 2. Differences in temperature in Cº between the lower layer and the higher one.

The figure number 2 shows the temperature gradient between the lower layer (0.05 m) and the higher one (0.25 m). This gradient varies from 10 to 20 Cº. This phenomenon takes 3 hours from the beginning of the process.

The references and the bibliographic revisions confirm the existence of this gradient of temperature but it was not possible to contrast it.

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DRYING HOPS BY MEANS OF THERMAL SOLAR POWER: OPTIMIZATION OF THE ENERGETIC EFFICIENCY OF THE PROCESS TO OBTAIN A QUALITY PRODUCT

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Abstract

It was used an experimental dryer at scale of the traditional dryers. It consists of a chamber at the bottom where the air is introduced through a fan and it is heated by a thermal heatsinks (radiator of aluminum) using like source of heat two solar thermal collectors *PPTT JEN-000-000* with 2,06 m^2 each. In the central part of the dryer there are two baskets where it placed the green hops to dry. The top of the dryer is open to allow the air with moisture go out after passing through the hops.

Monitoring of the experimental dryer was conducted with speed probes and temperature probes (data record with *454 Testo datalogger* with six channels). Tests at Vacuum were performed (to check the operation of the system) and then tests with hop were performed, to assess the quality of the final product.

in April 2009 it were measured radiation data with a solar radiation meter *SLM018 Mac Solar 2-c* while simultaneously it recorded the temperatures reached in the experimental dryer at vacuum, to establish the relationship between solar radiation and increased temperature in the dryer.

Keywords: Drying hops, thermal solar power.

Results and discussion

In the tests conducted at vacuum during 5 days in September 2008, it was obtained as average temperature drying at the entrance of the dryer 58.8ºC, however the extreme values ranging from 93.0ºC to 28.9ºC suggest that although it could obtain optimal temperatures for drying hops but obviously it is a system very sensitive to the presence or absence of solar radiation.

The tests with hops noted the importance of the deepness of the hop´s layer, when the deepness is 10 cm it reached 36.6ºC above ambient temperature, and of course the reduction of the moisture was from 70% to 15%, much higher than with larger deepness, with layer´s deepness from 20 to 25 cm the temperature increase compared to the ambient temperature was much lower (3.5ºC) and the decrease of the moisture was much lower (65% 43%).

It was noted the need to adjust the drying time to the solar time in order to maximize the solar radiation.

With the measures taken with the solar radiation meter during the month of April 2009, it was found that there is a high degree of correlation (0,9375) compared to the measures commonly used provided by the Ministry of Industry and Energy for Leon with an inclination of 45° (orientation south) so that they can take as valid, and with the data taken of the temperature reached in the dryer at vacuum during this period it can be concluded that the temperatures in the experimental dryer could get to 60ºC if the solar radiation is more than 812 Wh/m^2.