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June 2007

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Tettninger * Hopfen * Weltweit



T E T T N A N G

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Contents

	Page
Foreword	7
Vorwort	8
Lectures and Posters	9
I. Session: HOP BREEDING	9
The UK Hop Breeding Programme: A new Site and new Objectives P. Darby	10
Aspects of breeding aroma hops V. Nesvadba, K. Krofta	14
Breeding and development of triploid aroma cultivars R. Beatson, P. Alspach	18
Molecular marker development and use in the New Zealand hop breeding programme E. Buck, C. Wiedow, R.A. Beatson	22
Selection methods in hop disease resistance breeding in Slovenia S. Radišek, A. Čerenak, B. Javornik	26
Evaluation of some wild hops from Europe G. Probasco, C. Ocamb, S. Varnum, N. Miller	30
Wild hops – new sources for resistance to powdery mildew A. Lutz, J. Kneidl, E. Seigner	31
Variability of wild hops (<i>Humulus lupulus</i> L.) in Czech Republic V. Nesvadba, K. Krofta, J. Patzak	32
Wild Hops from Northern Parts of Caucasus V. Nesvadba	33
Cryoconservation of tissue cultures P. Svoboda, M. Faltus	34
II. Session: DEVELOPMENTAL PHYSIOLOGY	35
Phytohormones and phase change in hops M.A. Revilla, N. F. Villacorta, J.A. Magadán, H. Fernández	36
III. Session: HOP CHEMISTRY	40
Commercial hop extracts rich in Xanthohumol M. Biendl	41
Changes in antioxidant properties of hops in the course of drying, pelletizing and storage. K. Krofta, A. Mikyška, D. Hašková	45
Presenting, evaluating and comparing chemical composition of hop secondary metabolites at a glance M. Kač, R. Hrastar, I. J. Košir	49
The biosynthesis of the bitter acids in hops K. Kamhuber, M. Goese, S. Hecht	53

Low molecular polyphenols in beer influenced by different hop varieties R. Schmidt, B. Beck	54
IV. Session: DNA-BASED TECHNIQUES IN HOP RESEARCH	55
Molecular markers for alpha acids; study for practical application Y. Okada, K. Koie, A. Inaba, T. Kaneko and K. Ito	56
QTL Mapping of alpha acid content in hop A. Cerenak, Z. Satovic, J. Jakše, Z. Luthar, K. Carovic-Stanko and B. Javornik ...	60
Molecular methods in resistance hop research J. Patzak	63
cDNA-AFLP makers for powdery mildew resistance in hops (<i>Humulus lupulus</i> L.) R. Seidenberger, S. Mikolajewski, A. Lutz, E. Seigner, S. Seefelder, W.E. Weber	67
Genome analysis – an important tool to support classical hop breeding S. Seefelder, R. Seidenberger, A. Lutz, E. Seigner	71
Towards the genetic map for Verticillium wilt resistance J. Jakše, Z. Luthar, A. Čerenak, S. Radišek, B. Javornik	72
Use of Diversity Array Technology (DArT) for genotyping <i>Humulus lupulus</i> L. S. Whittock, J. Jakse, J. Carling, P. Matthews, G. Probasco, J. Henning, P. Darby, A. Cerenak, B. Javornik, A. Kilian, G. Leggett, A. Koutoulis	73
Constitutive Expression of a Grapevine Stilbene Synthase Gene in Transgenic Hop (<i>Humulus lupulus</i> L.) Yields Resveratrol and its Derivatives in Substantial Quantities G. Weber, M. Höhnle, N. Pitsch, K.R. Häntzschel, S. Stahringer, O. Spring, B. Pickel, A. Heyerick, D. de Keukeleire	74
Production of powdery mildew resistant hops via gene transfer H. Miehle, E. Seigner	78
Agrobacterium-mediated transformation of hop S. Škof, Z. Luthar	82
Some aspects of regulation hop chalcone synthase CHS_H1 in heterologous systems, cloning and analysis of hop regulatory factors J. Matoušek, J. Škopek, J. Patzak, T. Kocábek, L. Maloukh, J. De Keukeleire, A. Heyerick, Z. Fussy, I. Roldán-Ruiz, D. De Keukeleire	83
Molecular Analysis of Hop Chalcone Synthases J. Škopek, A. Heyerick, T. Kocábek, J. Matoušek	87
Optimisation of a Real Time RT-PCR protocol for the analysis of gene expression in hop tissues L. Maloukh, J. De Keukeleire, E. Van Bockstaele, I. Roldán-Ruiz	88
The propagation of HLVd in developing hop pollen and its elimination upon expression of apoptotic nuclease J. Matoušek, K. Pešina, J. Škopek, L. Orctová	89
V. Session: HOP DISEASES AND PESTS	90
Hop Flea Beetle (<i>Psylliodes attenuatus</i> Koch) in Slovenia M. Rak Cizej, L. Milevoj	91
Study on cause of <i>Fusarium</i> cone tip blight E. Solarska	95
Proteomic analysis of the fungus <i>Verticillium albo-atrum</i> S. Mandelc, S. Radišek, P. Jamnik, B. Javornik	99

Population dynamics of <i>Phorodon humuli</i> (Schrank, 1801) (Hemiptera: Aphididae) in hop cones A. Lorenzana, A. Hermoso de Mendoza, J.A. Magadán, M.V. Seco	100
Resistance to imidacloprid (Confidor 70 WG) in damson-hop aphid (<i>Phorodon humuli</i> Schrank) on Czech hops J. Vostrel	101
An ancient compound rediscovered: perspectives of aphid control in organic hop growing by the use of quassia products F. Weihrauch, M. Eckert, B. Engelhard	105
Contents of pesticide residues in hops K. Krofta, J. Vostřel	109
Standard ranges of the application of pesticides in hops – a proposal for the EPPO guide lines B. Engelhard, J. Schwarz, F. Weihrauch	110
Molecular Variability within the Coat Protein Gene of Hop Mosaic and Hop Latent Carlaviruses D. Crowle, S. Pethybridge, C. Wilson	114
Nematodes Associated with Hop Production in Tasmania, Australia S. Pethybridge, F. Hay	115
The Compendium of Hop Diseases, Arthropod Pests and Other Diseases S. Pethybridge, W. Mahaffee, D. Gent	116
VI. Session: HOP PRODUCTION	117
Influence of weather conditions and irrigation on yield and quality of hops J. Kopecký, J. Ježek	118
Hop wireworks in Czech Republic J. Kořen, V. Ciniburk, M. Eliášová, T. Vraný, J. Gregorik	119
VII. Session: HOP RESEARCH COUNCIL	120
Report on the Activities of the US Hop Research Council M. Nelson	121

Foreword

As Chairman and Secretary of the Scientific Commission of the International Hop Growers` Convention (I.H.G.C.) we would like to welcome our colleagues of the hop research and representatives of the hop and brewing industry from 14 countries all over the world in our home country.

This year the Scientific Commission holds its meeting in Tettngang. We have invited all persons interested and involved in hop research to join this meeting and learn much more about the hop production in the Tettngang region. Tettngang is one of the smaller hop growing regions in Germany with around 1,200 ha, but highly appreciated worldwide for its noble aroma hops produced here.

Special thanks are due to the organizers here in Tettngang – in particular to Dr. Willi Moosherrr and to the representatives of the Tettngang Hop Growers` Association Mr. Johann Heimpel, Mr. Wolfgang Ruther and Mr. Jürgen Weishaupt for their time and efforts they put into organizing this meeting. Mr. Heimpel and Mr. Ruther will present the special features of the Tettngang hop growing region during the poster-exhibition and the excursion on Tuesday.

We thank all delegates who are presenting papers and posters for their hard work in preparing their contributions and providing the written forms for the proceedings.

We are glad that there is still a considerable number of participants, who are taking the opportunity to join the post-conference tour and visit the most important hop research facilities in the Hallertau. It was our matter of concern to visit the state-run institutions in Freising, Hüll and Wolnzach as well as the private laboratories and facilities of the hop traders and processors.

Thank you very much to all the sponsors of the conference, their logos are shown on page 3. We wish a successful meeting with a lot of valuable information and fruitful discussions. Hopefully this conference initiates and supports a lot of joint projects for the sake of the hop and brewing industry.

Bernhard Engelhard
Chairman

Dr. Elisabeth Seigner
Secretary

Scientific Commission, I.H.G.C.

Vorwort

Als Vorsitzender und Sekretärin der Wissenschaftlichen Kommission im Internationalen Hopfenbaubüro (IHB) freuen wir uns, unsere Kolleginnen und Kollegen der Hopfenforschung und Vertreter der Hopfenwirtschaft aus 14 Ländern der Welt in unserem Heimatland begrüßen zu können.

Dieses Jahr ist die Wissenschaftliche Kommission mit ihrer Tagung in Tettngang. Wir haben alle Interessierten zu dieser Tagung eingeladen, um dabei auch mehr über den Hopfenanbau im Tettnganger Gebiet zu erfahren. Tettngang zählt mit rund 1.200 ha zu den kleineren Hopfenanbaugebieten in Deutschland, ist aber wegen seines hochfeinen Aromahopfens, der hier produziert wird, weltweit bekannt und geschätzt.

Ein herzlicher Dank gilt den Organisatoren hier in Tettngang, besonders Herrn Dr. Willi Moosherr und den Vertretern des Hopfenpflanzerverbandes Tettngang, Herrn Johann Heimpel, Herrn Wolfgang Ruther und Herrn Jürgen Weishaupt. Während der Posterausstellung und der Exkursion am Dienstag werden Herr Heimpel und Herr Ruther die Besonderheiten des Tettnganger Anbaugebietes vorstellen.

Wir danken allen Teilnehmern, die ihre Arbeiten in Vorträgen und Postern präsentieren, für ihre Mühen bei der Vorbereitung der Beiträge und bei der Zusammenstellung der geschriebenen Version für den Vortragsband.

Wir freuen uns, dass noch eine stattliche Zahl von Tagungsteilnehmern das Angebot des Anschlussprogramms nutzt und mit uns in die Hallertau fährt, um die wichtigsten Einrichtungen der Hopfenforschung zu besichtigen. Es war uns ein Anliegen, nicht nur die staatlichen Einrichtungen in Freising, Hüll und Wolnzach zu zeigen, sondern die privaten Labore und Anlagen der Hopfenhandels- und Hopfenveredelungsfirmen in das Programm aufzunehmen.

Ein herzlicher Dank gilt allen Sponsoren der Tagung; beachten Sie bitte die Firmenlogos auf der Seite 3. Allen Teilnehmern wünschen wir einen großen Wissenszuwachs und Zeit für einen umfangreichen Erfahrungsaustausch: Es bleibt zu hoffen, dass die Tagung gemeinsame Projekte initiiert und fördert - zum Wohle der Hopfen- und Brauwirtschaft.

Bernhard Engelhard
Vorsitzender

Dr. Elisabeth Seigner
Sekretärin

Wissenschaftliche Kommission, IHB

Lectures and Posters

I. Session:

HOP BREEDING

THE UK HOP BREEDING PROGRAMME: A NEW SITE AND NEW OBJECTIVES

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Abstract

The hop breeding programme founded in 1906 at Wye, England has been forced to close. The National Hop Association of England has set up a new company, Wye Hops Ltd., to continue hop breeding in the UK. A suitable site has been found on a commercial hop farm and planted with selected breeding materials from the Wye programme including germplasm, new selections and seedlings. The new programme will concentrate on four objectives, balancing applied work to develop new varieties with strategic development of hop germplasm. Variety development will seek to produce improved dwarf varieties based on pedigree breeding from 'First Gold', 'Boadicea' and 'Sovereign', and new aroma types bred from 'Fuggle'. Strategic research will address the enhancement of beta-acid content for non-brewing applications, and aspects of climate change in hops in south-east England.

Keywords: Breeding, variety, dwarf, aroma, beta-acid, dormancy

Introduction

The UK hop breeding programme started in 1906 when Prof. Salmon at Wye College, Kent extended the early hybridization experiments of Howard into a programme to develop commercial varieties (Darby, 2006). Although initially he used as parents plants available in England, his programme was distinguished by the then revolutionary approach of using wild plants as sources of new characteristics. He collected wild plants from North America, both the USA and Canada, and hybridized these with European genotypes. His programme was very successful and 'Brewers Gold', released in 1934, and 'Northern Brewer', released in 1944, have been the founding materials for hop breeding throughout the world. All bitter varieties developed and grown in the world today have one of Salmon's two varieties in their pedigree.

The programme at Wye, under the direction of Neve from 1953 and Darby since 1984, has remained at the forefront of hop breeding and has continued to innovate particularly in the development of resistance to pests and diseases in combination with good agronomic and brewing performance characteristics. For example, the world's first dwarf variety 'First Gold' was released in 1996 (Darby, 2004) and 'Boadicea' was released in 2004 as the world's first aphid-resistant hop variety.

Since 1948, the funding of the programme has been an equal partnership between industry and the UK Government. However, a realignment of government research policies away from crop production and single-sector commodities forced the closure of the Wye programme in March 2007 after a century of hop breeding. The British hop growers and brewing industry quickly determined that the future of the hop industry in Britain depends on retaining a breeding programme and the National Hops Association of England (NHA) has set up a new hop breeding company (Wye Hops Ltd) to run such a programme. A suitable site for the breeding field has been found on a commercial hop farm where there are

appropriate facilities and experience. A new hop garden, solely for hop breeding, has been constructed. This paper describes the renewed UK hop breeding programme; its materials and objectives.

Breeding materials

Many of the genotypes used by Salmon remained within the germplasm collection, held at Wye until 2007. The collection has gained accessions continuously throughout the century including wild plants from Japan and Italy, breeding lines from programmes throughout the world, commercial varieties from public programmes, and all the working parental materials from the Wye programme since Salmon's crosses. Such germplasm includes differential host series for major genes for powdery mildew disease, and distinct sources of resistance to downy mildew and wilt diseases. It also encompasses many novel characteristics and genotypes with chemical compositions well outside the normal commercial range. As such, it represents a collection with national and international importance.

The germplasm collection forms the basis of the breeding programme and, therefore, the relocation of selected parental materials from the site at Wye to the new site at a commercial farm underpinned the transfer of the breeding programme. At the start of 2007, the collection at Wye comprised 975 female genotypes and 558 male genotypes. This has been critically reviewed for duplications, redundancies and out-classed genotypes. As a result, 422 females and 358 males have been selected to form the basis of the new commercial programme, encompassing all the main breeding characteristics of hops and including wild accessions and historic varieties. All these genotypes were propagated during 2006 and were available from cold storage as small dormant perennial rootstocks for planting. Those that were weak in propagation were grown on as small pot plants for planting when strong enough. Where genotypes failed in propagation, the original stock plants in the field were lifted and moved from Wye to the new site. Much of this germplasm collection comprises genotypes susceptible to wilt disease (caused by *Verticillium albo-atrum*). It was essential, therefore, to choose a new breeding site where this disease is not present and where the management of the site reduces the likelihood of its occurrence.

In addition to the germplasm collection, the breeding programme at Wye also comprised genotypes that had been selected from segregating seedling progenies in recent years and that were being evaluated in further trials. Some of these genotypes were only identified as being of value following their assessment in the 2006 harvest and, therefore, were too late to be propagated using softwood cuttings. Thus, for 52 new selections (19 females and 33 males) the original stock plants from the field had to be lifted and moved.

Finally, seedling plants arising from crosses made in 2005 had been planted in the field during 2006 to develop perennial rootstocks. This entire population was individually labelled, lifted into cold storage, and planted in spring 2007 at the new site. Seeds from crosses made in 2006 were sown, and seedlings raised, under glasshouse conditions during spring 2007 ready for planting at the new site where the seedlings could be irrigated.

The new hop breeding site comprises a total of approx. 3.2ha of which 2.3ha will have been planted with materials originating from Wye by the end of 2007. The remainder of the area will be used for new seedlings and selections as the programme develops over the next few years.

New objectives

Although international merchants are now reporting a structural shortage of hops, in recent years there has been a world oversupply which has depressed market prices for bitter hops (Barth, 2006). Consequently, the area and value of such hops have declined markedly worldwide, including in the UK. In contrast, the market for English aroma and dual-purpose hops has recently improved, both for domestic and overseas customers. Against this background, the NHA have concentrated their resources to address four main objectives for the immediate future breeding programme.

Improved dwarf varieties

Dwarf hop varieties have been grown commercially in the UK since 1996 and, by 2005, occupied nearly 24% of the UK hop area giving UK growers a world lead in experience and knowledge of low-trellis hop husbandry. Dwarf varieties can be grown with markedly lower production costs allowing UK producers to be competitive in international markets. They also offer many environmental benefits such as reduced spray volumes, reduced spray drift, greater possibilities for integrated production and use of beneficial species (Gunn and Darby, 1987). The capital costs of wirework are markedly lower for dwarf hop husbandry and the NHA consider that any expansion of the area of hops in the UK will probably be of dwarf varieties. The most promising varieties developed to date have been 'First Gold', 'Boadicea' and the recently-released 'Sovereign'. The new programme will seek to improve the pest and disease resistances of these varieties, using them as parents to give further aroma dwarf selections. Additionally, crosses with "high resin" males will aim to give bitter or dual-purpose dwarf varieties with the habit, pest resistance and yield attributes of the maternal parents.

Aroma-type varieties

The traditional English variety 'Fuggle' has provided much of the world's aroma market either directly, or as the clone 'Styrian Golding', or through its seedlings such as 'Willamette' (USA) or 'Buket' (Slovenia). Although Salmon used both 'Fuggle' and 'Goldings' as parents in his first crosses (Darby, 2006), his success in increasing resin content came from the complementation of wild North American males with 'Goldings' as the maternal parent. Similarly, Neve developed resistance to wilt disease from a cross between Salmon's male OB79 and 'Eastwell Golding' clone (Neve, 1991). Thus, both breeders developed their hop improvement programmes based on 'Goldings' rather than 'Fuggle' and 'Fuggle' remains a germplasm that is hardly represented in the UK hop breeding programme. The variety is susceptible to all the main pests and fungal diseases found in the UK and a considerable amount of breeding is required to remedy such deficiencies. However, it is a variety which is greatly favoured by many of the UK regional and micro breweries. The new programme, therefore, will seek to develop varieties using 'Fuggle' as parent and, by incorporating improved pest and disease resistances, provide aroma varieties to be grown on conventional or dwarf wirework in the UK.

Increased beta-acid contents

Of the many compounds identified in hop oils and resins, the NHA consider that beta-acids have the most potential for exploitation by industries outside the brewing industry. Currently, use is made of the antimicrobial properties of beta-acids in the sugar extraction and animal feeds industries in USA and Canada (Haas and Barsoumian, 1994). Active research into other industrial applications is evidenced by the increasing number of patent applications for the use of hop beta-acids. Thus, the new breeding programme will focus on this non-brewing application alone. Diversification of the UK hop industry is considered to add to the sustainability of the industry and safeguard UK hop production for supply to the principal customers, the brewing industry. Preliminary selection work at Wye has identified a high-beta genotype (up to 12% beta-acid) with sufficient agronomic merit to plant on farm sites. It

should be in production from the 2007 harvest and will allow pilot extraction trials to develop the technologies associated with use of this compound. The selection will also be used as a parent in the breeding work to assess the potential to increase beta-acid content of hops, and to select new parental materials which can transmit high beta-acid content to their progeny. Genetic interactions of this new characteristic with existing traits are likely and studies have already indicated the possible presence of epistatic effects linked to the characteristic. For example, in progenies with high beta-acid parents, expression of alpha-acid content or powdery mildew resistance has not been as expected.

Reducing spring dormancy

In recent years, unusually warm and dry springs have caused developmental problems with some commercial varieties, notably 'Bramling Cross', some 'Golding' clones (Atkinson *et al*, 2006), and many dwarf selections within the breeding programme. This has been a feature of particular 'Golding' clones in East Kent in 2007 and has been seen as extended spring dormancy where the shoots fail to grow rapidly at the start of the season. If such weather is the result of climate change in the UK, then it is a problem which will become greater with time and could threaten hop growing in parts of England, especially the South East region. In this region, drier conditions now necessitate irrigation on all hop farms. The NHA consider that strategic work to develop elite parental germplasm which is able to tolerate less winter cold would be of value. Several genotypes within the germplasm collection are known to grow vigorously during spring regrowth irrespective of the temperatures and these will be used as the basis for this programme, together with germplasm from overseas. 'Pride of Kent', for example, consistently produces good regrowth and this genotype has been used to develop much germplasm growing in the southern hemisphere where winters are less cold (Neve, 1986), notably 'Pride of Ringwood'.

Thus, the new hop breeding programme in the UK will be more focussed and commercially orientated than the previous programme at Wye. It will encompass a balance of applied work to develop new hop varieties with strategic objectives to develop hop germplasm.

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ASPECTS OF BREEDING AROMA HOPS

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Abstract

Aroma hops must cope with all the same parameters as other hop varieties of lower quality (e.g. high yield, resistance, sensitivity to agricultural technology, stability of productivity). A high content of beta acids is of importance for aroma hops as well. Contents of cohumulone should not exceed the value of 30 % rel. as issues from demands of breweries. High contents of polyphenols as well as prenylflavonoids are very important for aroma hops too. Supplying such compounds to beer provides it with a natural character. Such beer has good qualitative parameters but unfortunately lower stability in comparison with beer brewed especially from CO₂ extract. With the help of chemical analysis it is possible to say, which breweries use aroma hops for production of their beers.

Keywords: hop, *Humulus lupulus* L., aroma hops, breeding, alpha and beta acids cohumulone, polyphenols, prenylflavonoids.

Introduction

Aroma hops breeding has a long history. Beginnings of this process can be found in the time when hops start to be used for beer brewing. The only criteria for selection of suitable hops were beer quality brewed from such hops. Later original wild hops were grown in the vicinity of towns and monasteries, which represent places where beer was brewed. Purposeful hop cultivation brought about other criteria and demands on hop quality parameters, such as good growth, resistance to mycosis and pests, yield of hops, etc. Original regional hop varieties had arisen at that time. When hops were intended to be exported abroad high quality hop varieties were preferred, which meant that a lot of original old varieties were not grown any more. As a beginning of breeding work we can mark hop growers' activity in choosing the best plants within their hop gardens for multiplication. The first breeding stations were established at the beginning of the 20th century. Clone selection in within original hop plants was carried out. To spread genetic variability hybridization was used. Chemical analyses served to determine contents of hop resins and hop breeding was thus aimed at increase of alpha acids. The process was influenced by economic demands of breweries, which needed cheaper hops to be competitive enough. Therefore acreage of aroma hops gradually decreased at the expense of bitter ones. At present many breweries do not use for production of some beers any aroma hops at all. Everything has been adapted to commercial and economic conditions. Quality of aroma hops has been neglected even though such hops are the base for production of good beer. It is necessary to understand that beer quality does not depend only on alpha acids but other important compounds as well. Results of research on this field show that very valuable compounds (polyphenols, xanthohumol, desmethoxyxanthohumol, etc.) are a part of hop cones too. It is the reason why pharmacy industry is interested in hops and enables hop breeders to aim at this field as well. It is necessary to show at hop quality from more views, not only from narrow economical commercial criteria.

Methods

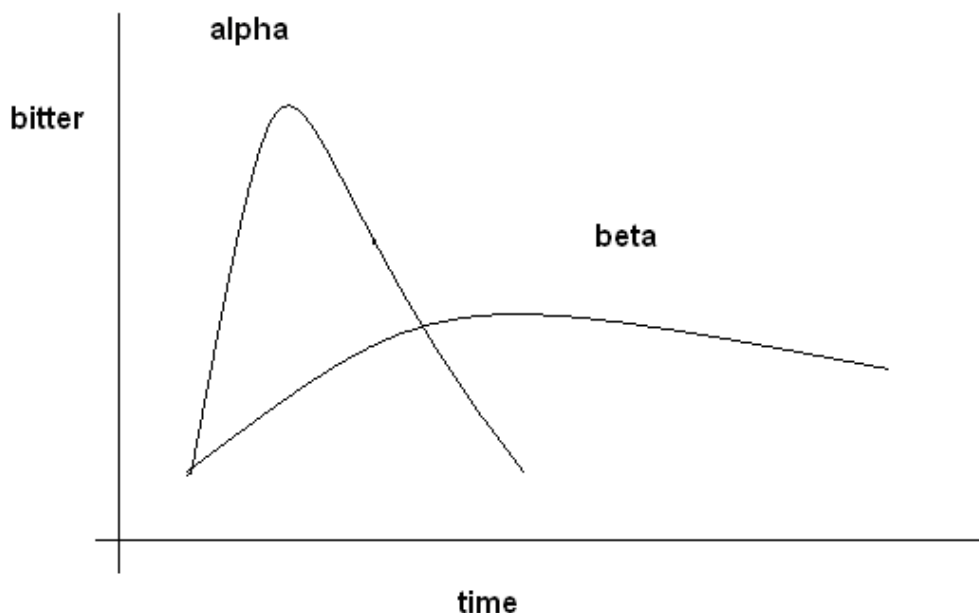
Obtained results were ensued from the assessment of hop genetic resources in Czech Republic (MZe 33083/03-300 6.2.1.). Experimental batches of beer were brewed in our experimental pilot brewery. To brew beer we used classical technology (brewing room – fermentation room – cellar). Produced beer was not pasteurized. Simple filtration with the help of paper sheets was made to be able to preserve natural character of the product. Experts from commercial breweries are invited to taste quality of beer. Hop resins as well as polyphenols were determined by liquid chromatography. Essential oils were determined with the help of gas chromatography.

Results

Selection criteria for aroma and super-bitter hops are in the basic demands the same (high yield, contents of hop resins and essential oils, resistance to mycosis, sensitivity to agricultural technology, yield stability for the whole time of its growing and stability of alpha acids contents during storing process). Importance of the individual varieties starting from aroma ones and finishing super-bitter ones is quite different. Super-bitter hops supply beer only the basic bitterness whereas aroma hops have distinctive influence on qualitative features of beer. What characteristics influence beer quality?

1. Beta acids

Beta acids have their bittering ability appr. ninetimes lower than alpha acids. Their character of bitterness is quite different. Whereas alpha acids are typical of their short bitter intensity, beta acids are typical of slower bitter efficiency accompanied by gentle bitterness (delightful taste) as obvious from Figure 1.



Beer brewed from hops with low contents of beta acids is typical of immediate but short bitterness. On the other hand, beer made from alpha acids is typical of rough bitterness. Beer brewed from hops with high contents of beta acids has a character of gentle bitterness. Beer made only from beta acids is typical of gradually increasing bitterness, which

is fading away for a longer time. Such bitterness is gentle without a rough effect. Therefore aroma hops with high contents of beta acids have good effect on quality and pleasantness of beer bitterness.

2. Cohumulone

Knowledge on cohumulone influence on quality of beer are very different. Many authors say that low ratio of cohumulone has a positive influence on gentle bitterness of beer. On the contrary others say that cohumulone has no distinctive effect on quality of beer bitterness. Our study shows that high ratio of cohumulone increases not only intensity of beer bitterness but sensoric bitterness as well. Beer with high ratio of cohumulone, brewed from the same weight of alpha acids, shows higher bitterness than beer brewed from hops with lower ratio of cohumulone (Table 1). It confirms higher rate of cohumulone utilisation during wort boiling.

Table 1: Influence of cohumulone on beer bitterness

Hop Variety	Alpha acids. (% of weight)	Cohumulone ratio (% rel.)	Grams of alpha/65 l of beer	Bitterness of beer (EBC)	Grams of alpha/65l of beer	Bitterness of beer (EBC)
Magnum	13,6	19,4	7	26,6	7	26,6
Columbus	14,6	30,6	7	27,9	6,7	26,4
Target	11,1	42,3	7	30,2	6,3	27,1

Tasting of beers with various content of cohumulone show that beers brewed from hops with lower cohumulone ratio (15-20% rel.) have non-distinctive neutral bitterness. If beers are made from hops with average ratio of cohumulone (20-30% rel.) higher sensoric and more pleasant bitterness is usually reached. Such bitterness has a delightful fading effect. Higher sensoric sticking bitterness is typical for beers made from hops with 30-40% rel. cohumulone ratio. Rough and unpleasant bitterness is characteristic for beers prepared from hops with cohumulone ratio over 40% rel. It is therefore clear that optimal cohumulone ration in aroma hops is 20-30% rel.

3. Polyphenols and prenylflavonoids

Polyphenols and prenylflavonoids contained in hop cones are very valuable compounds. They have distinctive influence on beer quality. Recently they have been used in pharmacy as well. A sample of Saaz semi-early red-bine hops contained 5,2% of weight of hop polyphenols. A sample taken from Agnus contained 3,1% of weight of these compounds. CO₂-extract contains no polyphenols at all. During preparation of experimental beer batches different contents of hop polyphenols in the forms of individual hop varieties were added into the process of beer production. A lot of other compounds are delivered into beer if a higher rate of hops is used (aroma hops). If a hop variety with high alpha acids content is used for hopping (Agnus) fivetimes lower quantity of polyphenols are given into beer in comparison with Saaz aroma hops. The results obtained from tasting unambiguously show on excellent quality of aroma hops. Nevertheless, two month later we organized another tasting and we found out opposite results (beer brewed from CO₂ extract was the best). We can ask a question: "Why?" And we can conclude: beer hopped by classic hops have higher natural value and therefore they taste better. Beer hopped only by hop resins (CO₂ extract) do not contain other accompanying compounds (mainly polyphenols), which influence beer quality in a positive way. Unfortunately, such beers have lower stability (their ageing is faster and tasting characteristics change gradually), which confirm natural character of such products. Beer made from CO₂ extracts show higher stability but it has nothing to do with an original natural beer.

Table 2: Results of tasting

Variety	Product	Product quantity	Quantity of polyphenols	1. tasting		2. tasting	
				∑ points	Order	∑ points	Order
Žatec	PE 90	210 g	10,9 g	65	1	32	3
Agnus	PE 45	58 g	1,8 g	44	2	46	2
Agnus	CO ₂ -extract	15 g	0 g	38	3	62	1

Content of prenylflavonoids has been observed not only from brewing but from the pharmaceutical point of view as well. Their good effect on human health is very important and therefore we investigate these compounds too. We are able to say which hops are used for brewing beer in the individual breweries on the base of isoxanthohumul analyses. Our analyses show whether the breweries use aroma hops or only CO₂ extracts. It is obvious from Table 3 that “A” brewery uses for hopping of lager beer aroma hops and CO₂ extracts for light beer production. “B” brewery uses just aroma hops as analyzed beers show high contents of polyphenols and prenylflavonoids. On the contrary “C” brewery uses only a small quantity of aroma hops for hopping of its beers.

Table 3: Chemical analyses of beers

Brewery	Type of beer	Bitterness (EBC)	Polyphenols (mg/L)	Prenylflavonoids (µg/l)	
				Isoxanthohumul	Xanthohumul
A	12° lager	37,2	231	1970	57
A	10°	15,2	141	145	25
B	12° lager	32,5	198	1720	120
B	12°lager – dark	24,2	348	2480	525
B	Free	29,7	94	580	64
C	12° lager	28,6	83,5	260	< 10
C	12° lager – dark	20,2	240	335	30

Discussion

Some parameters showing certain influence on beer quality are reviewed in the paper. It is impossible to determine strictly the individual compounds, their optimal contents and influence on beer quality. It is a complex of all the compound contained in hops, which have poly-factorial effects. Beer production preferred by an individual brewery is an important parameter. Some beers are typical for their rough bitterness and fine aroma hops would change their character. It cannot be forgotten that beer is a natural product and by supplying less hops into it we provide it with less valuable compounds. Extracts as well as total compensation in the form of tetra-hydro acids extend beer durability but beer is completely free of these precious natural products. It is necessary to separate the two trends of beer production. If we do not supply hops into beer we cannot speak about original natural type of beer. Optimal ratio of these valuable compounds supplying into beer in so one of the most important features of aroma hops.

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BREEDING AND DEVELOPMENT OF TRIPLOID AROMA CULTIVARS

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Abstract

'Old-line' (noble) aroma hop cultivars, from the traditional growing areas of Europe, have been considered the benchmark for brewing purposes. However, these cultivars are poorly adapted to New Zealand environmental conditions. A major breeding goal has been to produce seedless triploid aroma-type hop cultivars with essential oil fingerprints closely matching those of their 'old line' European parental cultivars, but which are adapted to the temperate oceanic climate and lower latitudes of the Nelson growing region in New Zealand. The main aroma breeding strategy is aimed at hybridising tetraploid ($2n = 4x = 40$) versions of 'noble aroma' (female) cultivars with diploid ($2n = 2x = 20$) male genotypes, to develop triploid ($2n = 3x = 30$) seedling populations. To date, four triploid aroma-type cultivars have been released from the programme. They currently make up about half of the New Zealand hop production, with c. 90% of total production exported to niche markets. Continuation of this programme has seen the development of several advanced aroma-type selections, which are currently undergoing extensive brewing trials.

Keywords: breeding value, genetic analysis, *Humulus lupulus*, plant breeding, seedlessness, triploids,

Introduction

Since the early 1960's, the focus of the New Zealand hop breeding effort has centred on the breeding of seedless triploids (Beatson *et al.*, 2003b). Triploid hops developed appear to perform consistently, being well suited to New Zealand's temperate-oceanic climate, and the lower latitudes of the growing region in the Nelson Province (41-42°S). While most of the releases have been 'high alpha' types, four of the 16 triploid cultivars released have been aroma cultivars, with their commercial importance increasing to the extent that about half of the New Zealand growing area is currently in aroma hop production, with c. 90% of the annual production exported to niche markets.

The most common way of creating triploid hop cultivars is to cross tetraploid ($2n = 4x = 40$) with diploid ($2n = 2x = 20$) parents. Usually the female parent, being a cultivar with a known commercial performance, is used as the tetraploid parent. The resulting triploid ($2n = 3x = 30$) seedlings thus derive two-thirds of their genetic constitution from the female parent. 'Old line', or 'noble aroma' cultivars from Europe have been used as a starting point in the New Zealand aroma breeding programme. The 'noble aroma' cultivars themselves are not suited for commercial production in New Zealand because they are agronomically weak.

Choosing male parents is also important. The performance of potential male parents for use in hop breeding can be examined in three ways: (1) progeny testing via some form of genetic design; (2) phenotypic performance of the chemistry components derived from resin glands contained in male flowering parts; and (3) marker assisted selection. Given the absence of suitable DNA markers and the expense of progeny testing, the New Zealand breeding programme has relied largely on chemistry testing of male parents.

This paper describes the current breeding and development of aroma-type hops in New Zealand.

Methods

a. Parental material

Tetraploid versions of European 'noble aroma' cultivars were crossed to two diploid males that were developed from the New Zealand breeding programme. Female parents were 'Fuggle', 'Hallertauer Mittelfrueh' and 'Saazer'. The male genotypes, designated as 97-68-14 and 93-12-34, were chosen for their chemistry and agronomic performance.

b. Triploid seedling populations

Seedlings were evaluated in the field over four seasons in three populations as follows.

1. The first population (designated as the '01 series') (c. 810 seedlings), used the male parent 97-68-14 crossed to the three 'noble aroma' tetraploid females (above), and the same seedlings were evaluated over two years, in 2003 and 2004.
2. The second series, (designated as the '03 series') (c. 785 seedlings), used the male parent 93-12-34 which was crossed to the same three 'noble aroma' tetraploid females. The seedlings were evaluated for a single season in 2005.
3. An additional population of the same crosses, as in (2) above, (designated as '04 series') (c. 550 seedlings) was grown and evaluated in 2006.

For each of the populations, the three crosses were arranged in a completely randomised design, with a plot size of six individually identifiable plants. Cultivar 'Pacific Hallertauer', used as a control, was randomised as single plants throughout the populations.

c. Measurements

For each of the seedling plants in the populations, gender determinations were made at flowering time. At harvest, each selected female seedling was measured for yield (kg green weight/plant), and a sample of cones was dried by standard procedures in a forced air kiln at 60°C. Each sample was stored in sealed plastic bags at -20°C for subsequent chemistry determination. Amount and type of alpha and beta acids were determined via HPLC (EBC, 1998), and cones from promising seedlings were steam distilled to obtain total essential oils (ASBC, 1996). GC profiles of the individual oil compounds were then obtained on those genotypes with better (c. 20%) agronomic performance.

d. Statistics

Breeding values for the parents were calculated by fitting the so-called 'animal model' to each variate separately (Lynch and Walsh, 1998). Year was fitted as a fixed effect and genotype as random, with the relationship matrix incorporated into the variance-covariance matrix of random effects, \mathbf{G} (i.e., $\mathbf{G} = \sigma_A^2 \mathbf{A}$, where σ_A^2 is the additive genetic variance and \mathbf{A} is the relationship matrix). The analyses were undertaken in GenStat (version 9.02), and residual plots were examined to check the validity of the analysis assumptions.

Results

Estimated breeding values (eBV) of the seedling populations are presented in Table 1. Because the populations were grown in different years, the statistical analysis involved adjustments for year effect. This was possible because the control cultivar (Pacific Hallertauer) was common to all populations examined. Also information from the ancestors, particularly the female parents, that were common to all populations was incorporated via the \mathbf{A} matrix. Most residual plots were satisfactory, although some showed deviations in the extreme high values and there were an excessive number of zero values for farnesene. Parental breeding values, which were essentially based on half sib family performances, showed that 'Hallertauer Mittelfrueh' was the best parent for yield and 97-68-14 was the better male. For amount of alpha acids, the best female parent was 'Fuggle', while of the two

males 97-68-14 was better. For amount of beta acids, 'Saazer' was the best. In terms of alpha: beta acids then, 'Fuggle' seedlings had the larger values (c. 2:1), while both 'Hallertauer Mittelfrueh' and 'Saazer' were lower (c. 1.4:1). 'Hallertauer Mittelfrueh' derived seedlings were generally lower in cohumulone, while the better male was 93-12-34. For amount of oils/g alpha, best parents were 'Fuggle', Hallertauer and male 93-12-34. Both 'Hallertauer Mittelfrueh' and male 93-12-34 were best parents for low myrcene content, and high humulene content. 'Hallertauer Mittelfrueh' was also clearly the best parent for use where no or low farnesene is considered desirable. Conversely, 'Saazer' would be best for breeding high farnesene genotypes. 'Saazer' and male 97-68-14 half sib performance for oxidation products (of humulene and caryophyllene) would indicate that they would be the best choice. For high floral-estery products, 'Fuggle', 'Hallertauer Mittelfrueh' and male 93-12-34 were all good parents, while for citrus-piney products, the same female parents had higher values than 'Saazer'. Male parent 97-68-14 had a higher value for citrus-piney products. Seedlings derived from the cultivar 'Saazer' had the highest amount of other compounds (i.e. the remainder of compounds left in the essential oil profile after the 4 main terpenes, oxidation products, floral-estery products and citrus-piney products were excluded). The parental performance for 'Hallertauer Mittelfrueh' and 93-12-34 would indicate that these would be the best choices for a high humulene: caryophyllene ratio. In summary, by appropriate choice of female and male parents it should be possible to derive aroma selections with essential oil fingerprints similar to the 'old line' European cultivars and good agronomic performance in New Zealand conditions.

Discussion

Under New Zealand conditions, noble aroma cultivars *per se* perform poorly for yield, hence the reason for the initiation of the breeding programme. Because New Zealand is free of the main disease and pest problems that affect hops in most of the major growing countries, selection has focussed almost entirely on agronomic, chemistry and flavour attributes. Selection amongst segregating seedling populations for promising aroma genotypes follows well established criteria. Of primary importance is the good agronomic (yield) performance. We routinely measure individual seedlings for yield which is subsequently confirmed in larger scale plots in future years. Also, the selection of diploid male parents that impart good agronomic performance to their progeny assumes importance. Once the better agronomic seedling performers have been identified, we then examine each of the chosen seedlings progressively for the amount and type of hop acids and their various essential oil components. It is well known that several essential oil compounds are positively correlated with European hoppy aroma and the aroma of beer (Kralj *et al.*, 1991; Deinzer and Yang, 1994; Moir, 1994). During our selection process at the seedling stage, consideration is given to these compounds. From the three populations described in this paper, a total of 76 selections have been made. Some of these are now in advanced selection replicated trials.

The most important property of any new selection is that it must have been tried and tested for its good brewing properties. Following on from our seedling selection work, 'pilot' and large scale brewing trials are conducted on all advanced selections in our breeding programme. This process has identified promising selections, some with unique brewing characteristics (Beatson *et al.*, 2003a, Graves *et al.*, 2002).

The goal of the New Zealand hop industry is for the continued production of triploid cultivars, as it has been demonstrated over the past 40 years that such cultivars perform well under New Zealand conditions. However, since triploids contain few if any viable seeds, they are difficult to use directly as breeding parents. In fact, we have previously demonstrated that triploid parents can be used in our hop breeding programme especially to produce tetraploid genotypes (Beatson *et al.*, 2003c).

In conclusion, breeding of triploid aroma hop cultivars for New Zealand growing conditions is now well established. It has been successful in delivering cultivars that have enabled the expansion of industry into export niche markets. The next generation of selections is nearing commercialisation, which will allow the New Zealand industry to meet changing market requirements.

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Table 1: Estimated breeding values (eBV) and their standard errors (s.e.) for the three females and two males.

	'Fuggle'		'Hallertauer'		'Saazer'		97-68-14		93-12-34	
	eBV	s.e.	eBV	s.e.	eBV	s.e.	eBV	s.e.	eBV	s.e.
Yield (kg GW/plant)	1.32	0.203	1.80	0.198	0.70	0.204	2.56	0.241	1.05	0.234
Alpha acids (%) ^a	6.75	0.451	5.71	0.439	6.47	0.453	8.76	0.546	6.57	0.538
Beta acids (%) ^a	3.33	0.239	4.00	0.231	4.41	0.240	3.85	0.299	2.81	0.293
Cohumulone (%) ^b	31.2	1.36	22.8	1.34	25.4	1.37	27.5	1.44	22.4	1.44
Oils (ml/100g) ^a	1.18	0.128	1.11	0.096	0.88	0.100	0.98	0.139	1.07	0.125
Oils (μ L/g alpha)	182	21.3	186	16.1	133	16.9	126	23.3	148	21.0
Myrcene (%) ^c	40.6	4.56	34.0	3.61	37.7	3.73	28.9	4.90	18.1	4.40
Caryophyllene (%) ^c	9.7	1.21	12.0	0.98	6.5	1.01	11.8	1.25	12.9	1.13
Farnesene (%) ^c	4.1	2.19	0.0	1.68	17.5	1.75	1.4	2.40	3.8	2.16
Humulene (%) ^c	26.3	4.03	37.0	3.28	18.0	3.38	39.4	4.02	43.5	3.65
Oxidation (%) ^{cd}	2.53	0.562	2.92	0.439	3.86	0.455	4.48	0.613	3.13	0.550
Floral-estery (%) ^{ce}	2.57	0.269	2.59	0.202	1.63	0.212	1.69	0.294	3.03	0.265
Citrus-piney (%) ^{cf}	5.38	0.387	5.30	0.301	3.37	0.313	5.65	0.423	4.72	0.379
Other (%) ^c	3.02	0.276	2.85	0.215	3.66	0.223	3.03	0.302	3.13	0.271
H:C ratio ^g	2.76	0.171	3.07	0.134	2.86	0.139	3.17	0.186	3.62	0.167

^a at c. 8% moisture; ^b percentage of total alpha acids; ^c percentage of total essential oils;

^d oxidation products of humulene and caryophyllene; ^e floral-estery products: linalool, geranyl acetate, geranyl isobutyrate, and geraniol; ^f citrus-piney products: limonene, δ -cadinene, γ -cadinene, muurolene, α -selinene and β -selinene; ^g humulene:caryophyllene ratio.

MOLECULAR MARKER DEVELOPMENT AND USE IN THE NEW ZEALAND HOP BREEDING PROGRAMME

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Abstract

The application of DNA marker technology to hop breeding holds the promise of increasing selection efficiency. HortResearch in New Zealand has developed eight RAPD based markers linked to sex specifically for the New Zealand hop breeding programme, using a high-throughput, automated bulked segregant analysis approach. One such marker, RAPD_A, is positioned 10cM from sex locus, and will be applied to the breeding programme. The current use of molecular markers to other plant breeding programmes in HortResearch is outlined and the future application of markers to the hop breeding is discussed.

Keywords *Humulus lupulus* L., RAPD, SCAR, marker assisted selection (MAS), sex locus

Introduction

Hops (*Humulus lupulus* L.) have been grown in New Zealand for over 150 years, with European immigrants bringing hop cultivars to New Zealand in the mid nineteenth century. A higher yielding hop cultivar was introduced from the USA in the early 20th century which proved to be better suited to the New Zealand climate. By the 1940's all of the commercially grown cultivars were of North American origin (Inglis, 2004).

The New Zealand Hop breeding programme at HortResearch began in the early 1950's, initially concentrating on black root rot resistance, but then shifting to the development of high yielding seedless triploid cultivars suitable for the New Zealand climate. The triploid breeding programme has successfully developed a suite of high alpha type cultivars and more recently the triploid aroma types have become a main focus (Frost, 1983; Beatson, 1993; Beatson and Inglis, 1999).

For future growth to the New Zealand hop industry, HortResearch is applying DNA technologies to accelerate traditional breeding of new plant cultivars using marker assisted selection (MAS). In 2004, HortResearch began developing hops molecular markers for MAS, and as a tool to help understand the genetic control of important hop characters. The usual strategy involves linking molecular markers with traits of interest, using a phenotyped, segregating population. This can be achieved based on candidate gene mapping, microsatellite markers spanning the genome, or bulked segregant analysis (BSA) (Hormazza *et al.*, 1994). Here we present a BSA approach to develop molecular markers for gender specifically for the New Zealand hops breeding programmes. Several of the published markers for sex have limited use within New Zealand hops, consequently the need to develop new markers for this material. We also describe three populations which will be the basis for further marker development, particularly for chemical characters. The molecular marker technologies HortResearch successfully applies to other plant breeding programmes are outlined, and their application to hops is discussed.

Methods

Three crosses were developed at the HortResearch, Nelson Research Centre; two developed in 2005, consisting of 500 seedlings each from a wide cross "Nugget" x "Yugoslavia 3/3M" (referred to as the Nugget population) and a "USDA21055" x "Yugoslavia

3/3M" cross (referred to as the USDA population), the third was an open pollination population (referred to as the OP population) of "USDA21055" developed in 2003 and consisted of 750 seedlings. "Yugoslavia 3/3M" was considered to be the major pollen donor (unpublished data), but other likely males included "USDA63015M", "Fuggle male" and "Cali male". Male germplasm samples used included: 10 male diploid accessions developed from the New Zealand breeding programme, diploid "Wye OB79" male and one male tetraploid accession ("Wye 169 male") imported from England. Female germplasm samples from New Zealand include the triploid cultivars; "Green Bullet", "Harleys Fulbright", "Sticklebract", "Super Alpha", "Alpharoma", "Pacific Gem", "NZ Hallertauer", "Southern Cross" and "Pacific Hallertauer". European diploid female germplasm included: "Bullion", "Bumford", "Cluster", "Fuggle", "Golding", "Perle", "Target", "Wye Northdown", "Eastwell Golding", "Tettanager", "Saaz" and "Hallertauer". American female germplasm included: diploid cultivars; "Chinook", "Cascade", "Comet", "Eroica", "Galena", "Hallertauer A-B", "Herbrucker A-B" and triploid cultivars; "Willamette", "Liberty" and "Mt Hood".

For the germplasm samples, parents of populations and 120 seedlings from each of the Nugget and USDA populations, total genomic DNA was extracted from one young, expanding leaf per plant using a modified version of the CTAB based Doyle and Doyle (1987) extraction method. Total genomic DNA was extracted from the OP population seedlings using a modified version of the Promega Magna-sil Kit with a custom built Theonyx DNA extraction robot. Four male (M1 to 4) and four female (F1 to 4) pooled DNA bulks were prepared consisting of four to 12 DNA samples from the germplasm and OP and Nugget populations. Samples in the male bulks consisted of M1: male American cultivars and the New Zealand diploid accessions; M2: the "OB79 male", "169 male", "Fuggle male" and "Yugoslavia 3/3M"; M3: five diploid males from the OP population; M4: 12 males from the Nugget populations. Samples in the female bulks consisted of F1: 12 females from the Nugget populations; F2: nine triploid New Zealand cultivars; F3: 12 diploid European female cultivars; F4: 12 American female cultivars. These eight bulks were screened using 587 randomly amplified polymorphic DNA (RAPD) primers (all Operon RAPD series AA to AZ and 67 primers from the A to Z series). The RAPD reactions were set up as outlined in Gardiner *et al.* (1996) in a total volume of 17 μ l and an annealing temperature of 37°C on a 384 well Hybaid MBS thermo-cycler machines. Selected primers, showing fragments potentially linked to sex, were subsequently screened, with a 96 well Hybaid MBS thermo-cycler machines, over 42 samples from the Nugget population: the parents, 20 male and 20 female seedlings. In addition to that six male individuals from the germplasm collection were also screened. Products were visualised using agarose gels, with reactions from bulked DNA, in 384 well format, loaded with a BioMek robot. For the marker co-segregating with gender the genetic distance was calculated by Joinmap® v.3.0.

The Nugget, USDA and OP population were also analysed for yield and chemical characters as described in Beatson *et al.* (2003).

Results

PCR products from the BSA varied from four to 12 fragments per primer ranging from 450 to 1600 bp. Twenty-one selected primers providing clear polymorphic fragments between male and female bulks and were screened over the 48 individuals. An example of a sex locus linked marker is presented in Figure 1, this marker was present in 10 out of 12 males from the Nugget population (recombinants indicated by arrows in Fig. 1), the diploid germplasm samples "OB 79 male", "Fuggle Male" and the tetraploid "169 male" and other male individuals from the New Zealand breeding programme. It was absent from 10 out of 12 females from the Nugget population, all of the triploid New Zealand bred females, the American and European female cultivars screened. This marker was located 10cM from the sex locus based on the Nugget population data. Along with seven others, this marker is currently being converted into the more user-friendly sequenced characterised amplified region (SCAR) markers.

Various chemical and yield measurements were obtained for the OP population in 2006 (Table 1). It was evident from this data that there was a wide range of values for the measured characters in this population. For example, yield varied from 0.15 to 6.05 kg/green weight per plant with an average of 1.67 kg/green weight per plant. Important chemical attributes also showed a wide variability, including xanthohumol which ranged from 0.16 to 1.23% and cohumulone content (25 to 62%). For the Nugget and USDA population (Table 2), yield ranged from 0.02 to 4.20 kg/green weight per plant and 0.05 to 2.28 kg/green weight per plant (respectively) for the 2007 harvest. The ranges in chemistry characteristics for the Nugget and USDA population are currently underway. The intention is to measure the phenotypic values for various agronomic and chemistry characters over a minimum of two seasons.

Discussion and Conclusions

Through the BSA approach, we identified eight RAPD fragments that could be used as markers for the sex locus in hops. One of these (Figure 1) is located 10cM from the sex locus. Published markers linked to the sex locus in hops (Polley *et al.*, 1997; Cerenak *et al.*, 2006) have been tested across similar germplasm at HortResearch. They could successfully identify only 50% of the males (unpublished data). However, our new markers can identify male cultivars from both Europe and America. These RAPD markers are currently being converted into the more transferable SCAR markers.

Besides yield, chemical data is now being obtained for the Nugget and USDA populations. This data will be used in the development of future molecular markers for key chemicals. In particular, high xanthohumol, amount of alpha acids low cohumulone content and various essential oil compounds are key characters for the HortResearch hops breeding programme. One of these populations is currently being used to construct a genetic map with microsatellite markers. Future DNA markers based on candidate genes and quantitative trait loci (QTLs) for chemical attributes will be placed on this map.

The development of molecular markers and their application to breeding programmes has been successful for several crops within HortResearch. For example, markers are being used to breed pest and disease resistant apple varieties (Gardiner *et al.*, 2006). Another example is the gender determination in kiwifruit (Gill *et al.*, 1998). This is possible by applying a high-throughput, fully automated DNA extraction system, which is successfully used to extract thousands apple and kiwifruit samples each year. For kiwifruit, 20,000 DNA samples were extracted and screened with the marker linked to sex. This task was completed within five months, saving orchard costs. A subset of the hops populations have been successfully extracted with the Theonyx DNA extraction system. In summary, the application of MAS will increase selection efficiency by reducing orchard space, time, labour, and therefore costs, for the New Zealand hops breeding programme.

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Figure and tables

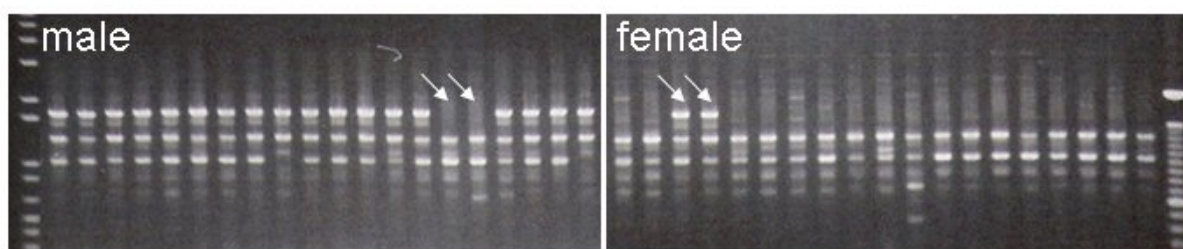


Figure 1. Amplification products from RAPD_ A in 20 male and 19 female samples from “Nugget” x “Yugoslavia 3/3M” population. Arrows indicate four recombinants.

Table 1. Chemistry analysis from the “USDA21055” open pollinated population from 2006.

Character	Mean	Max	Min
Plot wt [kg GW/plant]	1.67	6.05	0.15
Alpha acids	8.00	14.3	2.30
Beta acids	3.00	7.50	0.90
Cohumulone (%)	40	62	25
xanthohumol	0.65	1.23	0.16
ml oil/100g	1.30	2.70	0.20
myrcene	50.4	80.3	10.1
caryophyllene	7.30	16.6	0.50
farnesene	5.00	33.9	0.10
humulene	15.4	40.3	0.00
H/C ratio	2.04	3.45	0.00
CoL	63	94	45

Table 2. Yield from USDA and Nugget population 2007.

kg (green weight)/plant	Mean	Max	Min
USDA population (n=148)	0.56	2.78	0.05
Nugget population (n=244)	1.21	4.20	0.02

SELECTION METHODS IN HOP DISEASE RESISTANCE BREEDING IN SLOVENIA

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Abstract

The development of new hop varieties at the Slovenian Institute for Hop Research and Brewing is focused on improving the quantity and quality (bitterness, aroma) of the yield, resistance against diseases and pests. Methods for the assessment of resistance to the major pathogens (downy mildew, powdery mildew and Verticillium wilt) are outlined and discussed in the contribution.

Keywords: hop, breeding, diseases, resistance

Introduction

Disease resistance is an important component of integrated plant protection management and is one of the major challenges in plant breeding. In measuring resistance, the amount of tissue affected by the pathogen is in general a good indicator. However, it does not depend only on the level of resistance, since other factors (inoculum density, irregular distribution of inoculum, vigour of the plant, environmental factors...) may interfere. Suitable screening methods and an understanding of plant-pathogen interactions are therefore essential for developing plant material with improved resistance.

The hop breeding program at the Slovenian Institute of Hop Research and Brewing (SIHRB) has a tradition of more than 50 years, and 11 hop varieties have been released. Today, 95 % of Slovene hop fields are planted with Slovene varieties, of which Aurora represents more than 60 %. The disease resistance breeding program was initially orientated against two major diseases; downy mildew (*Pseudoperonospora humuli* (Miyabe & Takah.) G.W. Wils.) and powdery mildew caused by *Podosphaera macularis* [Braun] {formerly *Sphaerotheca macularis* [Wallr.:Fr] Lind. (synonym *S. humuli* [DC.] Burrill)}. Since 1997, when an outbreak of the lethal form of Verticillium wilt (*Verticillium albo-atrum* Reinke & Berthold) occurred in the Savinja valley (Radišek et al., 2006), breeding for resistance to wilt disease was included, and today this represents one of the most intensive parts of the breeding program. This paper describes screening and assessment methods used in breeding new hop varieties.

Powdery mildew

The plant pathogen interaction between *Podosphaera macularis* and hop has been shown to have a gene-for-gene relationship, in which different resistance genes (RB, R1, R2, R3, R4, R5, R6) and corresponding pathotypes have been described (Godwin, 1985; Darby, 2001; Seigner et al., 2001; Seefelder et al., 2005). In such a system, some major genes are effective against some pathotypes and ineffective against others. These latter genes may easily give an impression that partial resistance is present when the pathogen population consists of a mixture of different pathotypes. The deliberate use of pathotype mixtures in artificial inoculations should therefore be avoided. A single pathotype with as wide a virulence range as possible should be used instead (Parlevliet, 1983). In resistance screening, it is important to assess as many genotypes as possible. In the case of hop powdery mildew, this is possible on young seedlings, since major genes are expressed in juvenile tissue (Darby et al., 1989; 2001).

The initial selection is performed in a greenhouse. A single isolate is used as a source of inoculum, obtained in an infected hop field in the year before screening. The isolate is propagated on potted hop plants of susceptible varieties (Magnum, Northern Brewer, Southern Star...). After the dormant stage during the winter, infected "inoculator" plants are placed in the greenhouse one month before sowing hop seeds. Inoculator plants become well developed during this time and heavily colonized with powdery mildew. When the seedlings are 15-20 cm tall, inoculator plants are placed between them as a source of powdery mildew conidia. The inoculation process is encouraged by daily agitation of the inoculator plants. Inoculation by spore suspension has also been used in the last two years, which has the advantage of equal inoculum distribution and constant inoculum density. The inoculum is prepared by washing conidia from infected leaves of inoculator plants with a 0.01% (vol/vol) solution of Tween 20. The spore suspension is adjusted by Thoma counting chamber to a concentration of 50,000 conidia/ml. Seedlings are inoculated with a handheld atomizer. The selection and assessment of seedlings is performed approximately 2-3 weeks after inoculation. Families and individual seedlings are assessed on a scale from 0 to 4 based on the foliage affected with powdery mildew (0 = 0%, 1 = ≤ 10%, 2 = 11-30 %, 3 = 31-60%, 4 = > 60%). All plants with a score of 3-4 are discarded.

Downy mildew

Oomycete *Pseudoperonospora humuli* is one of the major pathogens of cultivated hop. It causes localized infections of leaves, flowers and cones, and systemic infections resulting in stunted chlorotic shoots ("spikes") and root rot. Various types of genetic resistance to oomycetes occur in plants and can be determined at the sub-specific or varietal level (race or cultivar specific resistance), or at the species or genus level (non-host resistance). Observations suggest that hop resistance is essentially quantitative and controlled polygenically rather than by major genes (Neve, 1991). Differential tissue expression of resistance is known in hop, since some varieties can be fairly resistant to systemic infection of rootstock but susceptible on cones and vice versa (Royle and Kremheller; 1981). In addition to resistance, the role of the sexual stage (oospores) and other genetic mechanisms in the physiological specialization of this pathogen is still unclear.

Resistance screening of seedlings to downy mildew is performed at SIHRB after initial powdery mildew selection. Primary and secondary spikes are collected in commercial hop gardens as a source of inoculum. To induce sporulation, spikes are sprayed with sterile distilled water (sdH₂O), enclosed in plastic bags, and incubated overnight at room temperature in the dark. The inoculum is prepared by washing sporangia from the leaves into sdH₂O, and the suspension of sporangia is adjusted by a Thoma counting chamber to a concentration of 50,000 sporangia per ml. Plants are inoculated by handheld atomizer and then enclosed overnight with plastic sheets to maintain high humidity. The first infections on leaves can be observed after 6-8 days, but selection is performed when terminal spikes are developed (14-21 days). All seedlings showing terminal spikes or high leaf infection are discarded and the resistance of the family is recorded as the percentage of plants selected.

Verticillium wilt

Verticillium wilt is a systemic disease caused by the soil-born fungi *V. albo-atrum* and *V. dahliae*. These fungi enter the roots by direct penetration in the region of elongation or through wounds that expose the vascular system. The accumulation of fungal biomass in the xylem disrupts the conduction of water and nutrients. Both fungi also produce phytotoxins and a variety of plant cell wall degrading enzymes, including pectinases, polysaccharidases and proteinases. Two conditions must be satisfied before disease can develop; (1) entry of the fungus to the vascular tissue and (2) fungal colonisation of the vascular tissue. It follows that any host mechanism which tends to exclude the pathogen from the vascular tissue or restrict its spread within it will contribute to wilt resistance (Talboys, 1972). Disease resistance mechanisms that affect the first condition are an accumulation of callose/lignin around the penetrating hyphae (formation of lignotubers) and the production of phytoalexins. Mechanisms observed after initial establishment of vascular infection are tylosis formation,

gel or coating formation, phytoalexin synthesis and oxidative reactions (Beckman, 1987; Talboys, 1972). The extra-vascular response is generally not specific and contributes to disease resistance at the species level, whereas inter-cultivar differences are determined in the vascular phase of infection (Talboys, 1958). Two disease syndromes are known in hop, lethal (progressive) and mild syndromes, to which pathogen virulence, the sensitivity of the hop cultivar and ecological factors contribute (Isaac and Keyworth, 1948; Sewell and Wilson, 1984; Talboys, 1972). Hop wilt due to *V. dahliae* is comparatively rare and is mild in character, whereas *V. albo-atrum* shows a higher preference for hop and causes the majority of outbreaks of both mild and lethal forms. Mild wilt arises from infections of sensitive hop cultivars by less virulent strains of the pathogen, or of tolerant cultivars by highly virulent strains, whereas the lethal disease form arises from infections of sensitive hop cultivars by highly virulent strains.

Resistance testing at SIHRB is performed in an isolated test plot under field environmental conditions and during the winter in growing chambers. The planting material consists of one year old potted plants derived from softwood stem cuttings. Each year 30-40 genotypes are tested, together with four reference cultivars: susceptible (Fuggle, Celeia), moderately resistant (Wye Challenger) and resistant (Wye Target). At least three highly virulent *V. albo-atrum* isolates (pathotype PV1; genotype PG2) which induce lethal wilt are used for plant inoculation. Inoculum is prepared by growing isolate cultures in liquid media (general fungal medium; Weising et al., 1995) by agitation on a rotary shaker (50 rpm) at room temperature for 5 days in the dark. Spores are separated from the biomass by filtration and adjusted to a concentration of 5×10^6 conidia per ml of sdH₂O. Twelve plants per genotype are inoculated by dipping their roots in inoculum for 10 min. Inoculated plants are transplanted to 4l pots, and single strings, suspended from overhead wirework (3 m high), are attached to each pot. A high quantity of nitrogen fertiliser is applied weekly to encourage infection levels and symptom development. The first symptoms appeared approximately 4 weeks after inoculation. Plants are assessed at weekly intervals 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. At the end of external symptoms assessment, the vascular tissues of plants are examined in cross-sections of stems and roots. For pathogen re-isolation and infection confirmation, the vascular tissue is plated on potato dextrose agar (PDA) and the species identification of isolate is checked by light microscopy. Final genotype assessment is expressed as the mean wilt score of the 12 replicate plants and, based on that, genotypes are classified into three resistance/tolerance categories: 0-1 resistant, 2-3 moderately resistant, 4-5 susceptible. Testing of resistant genotypes is repeated next season or during the winter in a growth chamber.

Field resistance (downey mildew and powdery mildew)

After initial division into powdery and downy mildew, seedlings are planted outside where further assessments of agronomic traits are performed. Susceptible varieties (Magnum for powdery mildew; Bobek or Styrian golding for downy mildew) are planted in the same field as a control. One year-old plants are treated according to good experimental practise, while two year-old and more plants are not sprayed against powdery mildew during the season. Treatment against downy mildew in the spring is applied later after signs of systemic infection, when the most susceptible plants are destroyed. Each plant is assessed according to the infected tissue on a scale from 0 – 2 and categorised as resistant (0 - no or low infection level), partially resistant (1 - medium infection level) and susceptible (2 - high infection level). The same procedure is used in five, ten or twenty plant blocks of the breeding line.

Future prospects

The reported selection methods have been used with the goal of releasing new hop varieties with durable and at least moderate resistance. Selection methods will be improved in the future using a molecular approach.

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EVALUATION OF SOME WILD HOPS FROM EUROPE

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Seeds were collected from wild hops in various locations of Europe. Collection areas are generally described as Bavaria, Berlin, Sachsen, Austria, and Italy. Seeds were germinated and seedlings were screened for powdery mildew resistance in the year 2001 at the German hop research station in Huell. In 2002, about 115 of these seedlings were sent to the U.S.A. for evaluation in the field on high trellis under commercial growing conditions. These plants were in quarantine for two seasons and finally released for planting in the field on high trellis in the spring of 2004.

During the second year in the field, the plants were evaluated for chemical and agronomic characteristics. In addition they were exposed to powdery mildew isolates containing the virulence genes Vb, V1, V2, V3, V4, V5, and V6. Many of these genotypes lacked vigor and only the most vigorous were evaluated. Evaluations were limited to those that reached the top or grew over the top of the 18' trellis. Of the original 115 starting number, 31 females and 9 males were evaluated.

Evaluations on both female and male genotypes were for alpha and beta acids contents, cohumulone content, storage stability of the alpha acids, and powdery mildew susceptibility. Further evaluations were made on the females to include total oil content, cone size and density, amount of lupulin in the cone, aroma of the cone, estimated number of cones, distribution of cones on the plant, lateral length and seed content.

Alpha acids contents (determined by ASBC Hops-6 spectrophotometric) in the females usually were less than 5.0% (with only one above) and many were less than 1.0% with the range being 0.3% to 11.4%. Beta acids contents were a little higher and ranged from 2.2% to 5.4%. Storage stability ranged from very good with only an 11% loss of alpha after 6 months at room temperature to very poor with a 56% loss. Cohumulone contents were generally low ranging from 10.0% to 27.0%. Total oil contents were low with all being below 1.0 mL/100 gram dried hop except the high alpha genotype which had total oil of 2.2 mLs/100 grams of dried hops. Cone density was more loose than compact with the one high alpha genotype having the only real dense compact cone. Cone size was medium to small with most being one inch or less in length. Lupulin content of the cones was low to medium and similar to that of low alpha aroma varieties, with the exception being the one female with the high alpha. Aroma was mild to nonexistent. Cone distribution was generally good with distribution over much of the length of the plant for many of the genotypes. Lateral length was ideally medium for most of the genotypes, not too long and not too short. There were numerous males shedding pollen near the females and seed content was good in all females with the exception of the female with high alpha, in which no seed developed in either of two years.

Hop powdery mildew isolates containing the above mentioned virulence genes were inoculated to all of the genotypes. This work is still in progress but to date evaluations are complete on the majority of these genotypes and only five have become infected with hop powdery mildew indicating that a large percentage of these wild genotypes contains new resistance to hop powdery mildew.

WILD HOPS – NEW SOURCES FOR RESISTANCE TO POWDERY MILDEW

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Wild Hops – New Sources for Resistance to Powdery Mildew

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Introduction

Hop powdery mildew (*Podosphaera macularis* ssp. *humuli* Braun) can cause heavy infections on leaves and in particular on cones associated with drastic loss of yield and quality on susceptible hop varieties. In 2002 for example in the Hallertau hop growing region 4.9 million € had to be applied for pesticides to control this disease. Thus, the main focus at the Hop Research Center Hüll is to improve resistance to hop powdery mildew (PM) in Hüll breeding lines and new cultivars.

Objective

Extensive studies on the virulence spectrum of powdery mildew populations from the Hallertau, England, France, and the USA had revealed that all hop resistance genes currently known (*R1-R6*, and *RB*) were overcome by virulent strains of PM (*v1-v6*, *vB*). Thus, for hop breeding it was urgently needed to look for new sources of resistance which were expected to be found in wild hops.



Results and Perspectives

Since 2001 more than 15,000 wild hops from throughout the world have been screened in the greenhouse for PM resistance. In this testing their reaction to PM strains of the *v3*, *v4*, *v6*, *vB* virulence type which already occur in the Hallertau region was assessed. Subsequently, resistant wild hops were retested in the laboratory using the detached leaf assay, where PM pathotypes from England and the USA carrying the *v1*, *v2* and *v5* virulence genes were deployed. Finally testing was continued in the fields under natural infection conditions. In this way 54 very promising wild hops were identified showing resistance to all virulent PM strains currently known.



Resistant individuals with other valuable traits will be used as crossing partners in our breeding programs to broaden the genetic basis for PM resistance in the Hüll hop germplasm.

Wild Hops - Origin		Number	Sex
Germany	Bavaria, Baltic Sea, Berlin, Eastern Germa Eifel, Saxony	14	female
		11	male
Turkey	Bursa	4 / 3	female/male
China/Japan	unknown	15 / 5	female/male
Sweden	Julita	1	female
New Zealand	unknown	1	female
USA	Nebraska	2*	female/male

* promising wild hops – after testing for 2 years



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VARIABILITY OF WILD HOPS (*HUMULUS LUPULUS* L.) IN CZECH REPUBLIC

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The aim of the wild hops study is to obtain some new genetic resources suitable for hop breeding, which will be resistant to mycosis and dryness. It is supposed that natural selection has selected resistant plants and susceptible ones did not go through this natural process. Wild hops can be divided into the following three groups with regard to their origin:

1. Original wild hops, which have not been used for commercial hop growing yet.
2. Escaped original domestic hops, which used to be cultivated in the vicinity of towns and monasteries where beer was brewed.
3. New genotypes of wild hops, which arise due to mutual pollination. New genotypes arise due to high degree of heterozygosity

Assessment of wild hops includes the following parts:

1. Spring investigation of the occurrence of wild hops. Each year we choose a part of Czech Republic territory where exploration of wild hops is carried out. In each found wild hop its GPS is determined and samples of young leaves are taken to a laboratory to do DNA analysis.
2. Summer assessment of wild hops. During its growth each genotype is evaluated in its own habitat. Attack by mycosis is monitored at first in this phase.
3. Autumn collection of hops. In female genotypes cones are sampled. After drying they are analyzed in a chemical laboratory and aroma is evaluated.

DNA analysis is used for evaluation of genetic diversity from Saaz semi-early red-bine hop and chemical analysis is used for evaluation of quality of hop resins content. We use six SSR and three STS loci for molecular analyses of Czech wild hops. We found that all genotypes were different from Oswald's clone 72, but very similar each other. Czech wild hops were separated into two groups around Oswald's clone 72 and Fuggle, respectively, differently from Caucasus and American wild hops.

Perspective genotypes are multiplied and planted in field conditions where characteristics, due to them each genotype was selected, are researched. Wild hops have been collected since 2003. Since the end of 2006 a considerable part of Czech Republic has been investigated as well. In table 1 you can see variability of hop resins contents.

Table 1: Variability of hop resins contents

Statistical value	Alpha acids (% w/w)	Beta acids (% w/w)	<i>alfa/beta</i> ratio	Cohumulone (%rel.)	Colupulone (%rel.)
Maximum value	6,77	7,72	2,01	31,9	53,6
Minimum value	0,73	1,21	0,33	18,8	38,5
Average	2,93	3,57	0,91	24,83	45,78
Statis. deviation	1,257	1,274	0,422	3,058	3,688
Variation coef. (%)	42,97	35,71	46,50	12,32	8,06

Acknowledgement

The research on wild hops is supported by Czech Ministry of Education within the project "Research and Regulation of Stress Factors of Hop" (MSM 1486434701) and project "The search of wild hop populations in North Osetia region ..." (ME 832) as well as National Program of Conservation and Utilization of Genetic Resources and Bio-diversity, (MZe 33083/03-300 6.2.1.), supported by Czech Ministry of Agriculture.

WILD HOPS FROM NORTHERN PARTS OF CAUCASUS

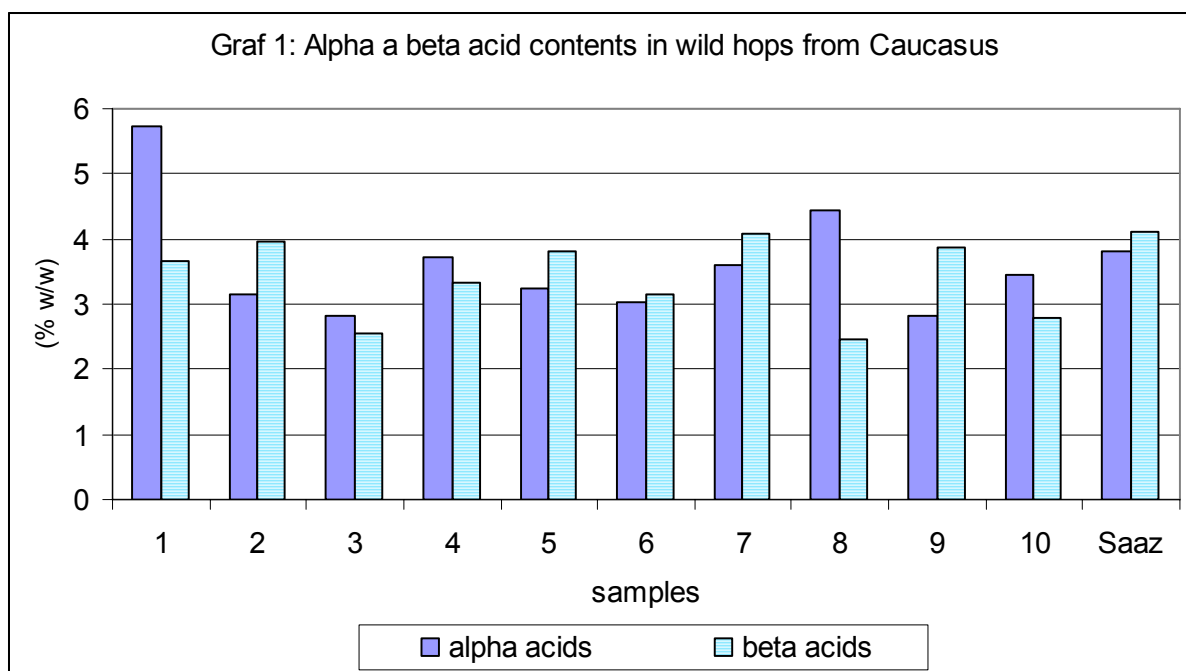
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Abstract

We have the new project „The search of wild hop populations in North Osetia region, determination of their genetic value with the aim at their utilisation as donors of demanded properties in the breeding process of Czech hops and assesment of local conditions for hop cultivation“. In 2006 an expedition do Northern Caucasus was organized. Its aim was to collect wild hops in this area, which is said to be the original one for *Humulus lupulus* L. Nine localities were found in all. Analyses of hop resins were carried out in ten samples of wild hops. The highest alpha acid content, which was determined, reached the value of 5,7 %. On the contrary the lowest one was only 2,8 %. Beta acid contents moved from 2,5 % to 4,1 %. Cohumulone content was in the range of 19 % rel. to 34% rel.

Key words: hop, *Humulus lupulus* L., wild hops, Caucasus, hop resins.



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The research on wild hops is supported by Czech Ministry of Education within the project (ME 832) “The search of wild hop populations in North Osetia region“.

CRYOCONSERVATION OF TISSUE CULTURES

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Cryoconservation is a process of plant conservation under ultra-low temperatures of 196 °C under zero in a liquid nitrogen. It is used for conservation of gene fond in plants, which are multiplied in a vegetative way. This method contributes to the conservation of genetic stability and prevent from ageing. It is used for conservation of genetic resources of cultural and wild plants in such virus free material, which is endangered by depreciation caused by biotic and non-biotic stresses if multiplied in *ex vitro* conditions. Plants transformed into *in vitro* cultivation serve as an original material for this purpose. Pollen for hybridization is conserved in this way as well. In 2003 the first cryobank in Czech Republic was established in Research Institute of Plan Production in Prague for plants multiplied in a vegetative way. In the first phase its activity has been aimed at the most important plants multiplied in this way: potato, hop, garlic, apple tree, pear tree, morello cherry tree, cherry tree, strawberry and grapes. Encapsulation methods (dehydration, controlled freezing and ultra-quick freezing) were tested in hops. *In vitro* cultures served as an initial material. They were multiplied to a needed number and tissues were sampled. Cryoconservation was carried out with the help of a pre-cultivation method of node segments. Desiccation of isolated tissues and ultra-quick freezing followed. Variety "Harmonie" had the best regeneration.

Nowadays 32 hop varieties are in cultivation *in vitro*, eight of them belongs to Czech ones. Fifteen hop varieties are conserved in liquid nitrogen. The objective of the cryobank is to conserve genetic resources of Czech origin hops and eventually some important foreign hop varieties as well.

Acknowledgement

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II. Session:

DEVELOPMENTAL PHYSIOLOGY

PHYTOHORMONES AND PHASE CHANGE IN HOPS

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Abstract

Hop flowering could be associated to a fall in the content of phytohormones as notable decreases were obtained when buds developed into flowers. The highest levels of phytohormones were reached in the third vegetative stage assayed (June 7th), when the plant had reached ripeness to flower, and very low contents were quantified in the next development stage (June 21st) when the plant had begun to flower. The gibberellin GA₄, and the cytokinins, free *t*-zeatin (*t*-Z) and free and conjugated iso-penteniladenine (iP) experimented a notable increase in buds collected from plants fully developed vegetatively and their levels fell dramatically with the onset of flowering. In general, higher contents of phytohormones were found in buds than in leaves. The auxin indole-3-acetic acid (IAA) was the most abundant phytohormone and none free cytokinin was found in leaves. IAA and the gibberellin GA₁ showed a gradual increase in buds along the vegetative growth and also lower values in the flowering stages. These results show the implication of phytohormones in the hop phase change.

Abbreviations: CKs, cytokinins; DW, dry weight; GAs, gibberellins; IAA, indole-3-acetic acid; iP, isopentenyl adenine; iPR, isopentenyl adenosine; iPR-P, isopentenyl adenosine ribotide; *t*-Z, *trans*-zeatin; *t*-ZR, *trans*-zeatin-N₉-riboside; *t*-ZR-P, *trans*-zeatin-N₉-ribotide.

Keywords: flowering, *Humulus lupulus*, mass spectrometry, phytohormones.

Introduction

It has been postulated that if the flowering process in hops is controlled by a balance of endogenous growth-retarding and growth-promoting substances, then the flowering process may be associated with a fall in the concentration of gibberellin-like substances in the plant (Thomas and Schwabe, 1969). Preliminary experiments made by those authors showed that exogenous application of GA₃ slightly delayed flowering time and increased the final number of flowers formed, whereas application of growth retardant sprays resulted in earlier flowering. Gibberellin A₁₉ along with zeatin, zeatin riboside and iP have been previously found in young shoots of hop but in the cones only were found the two former cytokinins (Watanabe and others 1978). Auxins have only been studied in vegetative development of hops (Galovic et al. 2001). Cultivars with an active vegetative growth showed a higher IAA content than cultivars showing a slower growth.

An extensive analysis of changes in phytohormones has not been reported previously in hops. Thus, our main objective was to characterise hormonal changes associated with flowering of hop and so that, elucidate the role of these phytohormones in the transition phase from vegetative growth to floral development.

Plant material and methods

Apical buds and the first pair of fully expanded leaves were collected every fifteen days from *Humulus lupulus* cv. 'Nugget' growing in the fields of S. A. E. de Fomento del Lúpulo (Villanueva de Carrizo, León, Spain). Samples taken on April and May (stages V1 & V2) would correspond to juvenile vegetative phases and the third sample taken in the beginning of June would correspond to the adult vegetative phase when the plant has reached "ripeness to flower" (stage V3). Samples taken on June and July (F1 & F2) would represent

two reproductive maturation stages corresponding respectively to the “f” stage -lupulin glands are formed on the bases of the bracts and the stigmas start to protrude from the bracts- and to the “h” stage -the bracteoles continue enveloping the developing gynoecium and the stigmas become highly papillated (Fig. 5 from Shephard and others, 2000). Upon harvest, plant material was immediately submerged in liquid nitrogen, crushed, lyophilised and stored at -20 °C until analysed. Every sample was analysed in duplicate. Upon harvest, plant material was immediately submerged in liquid nitrogen, crushed, lyophilised and stored at -20 °C until analysed. All the phytohormones were analysed in duplicate from the same sample. Gibberellins and indole-3-acetic acid were quantified by gas chromatography coupled mass spectrometry (gc-ms) and cytokinins were analysed by high performance liquid chromatography tandem mass spectrometry. Data statistical analyses were carried out using statistica® software (atsoft inc., oklahoma, usa). The kruskal-wallis non-parametric test was used to test differences for each phytohormone in buds and leaves along the studied development to detect differences among collections, group comparison was done for every studied phytohormone using the mann-whitney u test.

Results

Among the tested GAs (GA_1 , GA_3 , GA_4 , GA_7 , GA_9 and GA_{20}) just GA_1 and GA_4 were fully identified (Table 1). GA_3 content in the analysed sample was below detection limit, GA_7 , GA_9 and GA_{20} could not be identified due to the presence of co-eluting compounds at their retention index. Similar GA profiles were found for leaves and buds along the different stages of the vegetative and reproductive development of the hop plant, but much higher content was found in buds than in leaves and for GA_4 in respect to GA_1 . GA_1 content increased gradually with the vegetative growth, but decreased during floral development. Content of GA_4 was about one hundred times higher than that of GA_1 and a different pattern was obtained for this phytohormone, that shows a low content for the two first stages of vegetative growth (V1 & V2) and the two stages of floral development (F1 & F2) but a dramatic increase was obtained in samples taken at the third collection (V3) when plants were vegetatively growing but next to the flower development, which could be considered as the transition phase.

In general there was higher CKs content in buds than in leaves (Table 1), like is the case for GAs. Conjugated forms (ribosides or ribotides) were found for iP and *t*-Z in both leaves and buds. However free forms of CKs were only found in buds. *t*-Z increased gradually with the vegetative development reaching the maximum content in the transition phase and just a very low content in the flower development stage. Free and conjugated forms of iP also showed a maximum content in the transition phase and low content in the other studied stages. Conjugated forms of *t*-zeatin showed the highest content in samples collected on April when the plant begins to sprout (V1) or in the first vegetative development (V2), showing low content in the transition phase (V3) and during the floral development (F1 & F2).

The endogenous IAA content (Table 1) is remarkably higher than the GA and CK content, and also higher in buds than in leaves. They present a profile similar to that of GA_1 in buds, having the highest content along the vegetative development and declining during the following steps of floral development. In leaves, its content decreases during the studied development.

Table 1: Endogenous content gibberellins (pmol g⁻¹ dry weight), cytokinins, (pmol g⁻¹ dry weight) and IAA ((nmol g⁻¹ dry weight) in leaves and buds of *Humulus lupulus* L. (cv. 'Nugget') along vegetative growth (V1=April 29th, V2 = May 18th and V3 = June 7th) and along flowering development (F1 = June 21st and F2 = July 6th). Mean \pm standard error.

	V1	V2	V3	F1	F2
<i>Leaves</i>					
GA1	30.66 \pm 4	37.33 \pm 1.33	44.00 \pm 3.33	21.33 \pm 1.33	17.33 \pm 2.6
GA4	83.33 \pm 27.77	83.33 \pm 10.12	388.88 \pm 111	83.33 \pm 27.77	138.88 \pm 27.77
IAA	7.11 \pm 2.09	5.44 \pm 0.41	3.34 \pm 0.62	0.83 \pm 0.50	167 \pm 0.37
<i>t</i> -Z riboside	22.29 \pm 14.37	30.54 \pm 11.77	3.82 \pm 0.11	0.6 \pm 0.09	1.35 \pm 0.7
<i>t</i> -Z ribotide	31.56 \pm 0.05	350.08 \pm 149.5	1.39 \pm 0.1	3.69 \pm 1.72	3.49 \pm 1.51
iP- riboside	83.67 \pm 57.12	115.33 \pm 9.27	74.48 \pm 12.37	9.72 \pm 0.55	26.62 \pm 13.1
iP- ribotide	99.64 \pm 20.71	774.31 \pm 451.0	141.18 \pm 6.29	101.66 \pm 5.26	141.32 \pm 2.62
<i>Buds</i>					
GA1	34.66 \pm 1.33	66.65 \pm 3.99	82.64 \pm 1.33	39.99 \pm 9.99	14.66 \pm 3.99
GA4	416.66 \pm 55.55	11111 \pm 33.33	1666.6 \pm 111.1 6 1	500 \pm 40	138 \pm 27.77
IAA	11.30 \pm 1.25	9.20 \pm 2.51	14.23 \pm 1.04	4.18 \pm 3.76	4.18 \pm 2.51
<i>t</i> -Z	2.87 \pm 1.56	11.71 \pm 5.92	48.69 \pm 34.19	0.43 \pm 0.03	0.71 \pm 0.15
<i>t</i> -Z riboside	180.08 \pm 55.97	31.56 \pm 13.15	40.27 \pm 7.97	25.33 \pm 2.33	14.27 \pm 0.55
<i>t</i> -Z ribotide	326.17 \pm 97.99	139.97 \pm 71.41	25.67 \pm 8.3	29.45 \pm 5.61	56.4 \pm 6.13
IP	50.4 \pm 9.74	13.32 \pm 1.99	154.01 \pm 62.62	13.81 \pm 0.12	1.7 \pm 0.38
IP- riboside	388.99 \pm 47.09	81.85 \pm 1.21	1618.9 \pm 782.9 8	44.51 \pm 12.95	46.17 \pm 5.14
iP- ribotide	320.27 \pm 30.2	108.28 \pm 21.65	598.61 \pm 308.6	84.13 \pm 2.52	113.63 \pm 20.35

Discussion

The juvenile vegetative phase (V1 & V2), characterized by high rates of cell proliferation and shoot elongation, showed the highest content for GA₁ together with the auxin IAA and conjugates of cytokinins. Watanabe and others (1978a) showed the presence of iPR, Z and ZR in young shoots of hops. Contrarily to our results in buds, Galovic et al. (2001) presented data indicating that free IAA does not increase in apical buds of several hop cultivars during vegetative growth.

Dramatic increases in GA₄ content together with the highest content in *t*-Z and iP free forms and some conjugated forms of CKs obtained in buds belonging to the V3 collection samples, could be considered as indicators of a phase change in hop that would correspond to a maturation stage previous to flowering. Several papers connect the levels of GAs and CKs with the floral transition. Thus, Evans and Poethig (1995) established the influence of GA₁, GA₃ and GA₅ in floral development by means of analyses done in maize mutants. Indeed,

drastic changes in hormone levels have been observed and connected to floral development in *Arabidopsis thaliana* (Corbesier et al. 2003). However, Dewitte et al. (1999) observed decreasing levels of CKs in tobacco during the lapse between the arrest of leaf formation and the initiation of flower buds.

At the reproductive competent phase (F1 & F2), when buds start to develop into flowers, floral development coincides with lower content of CKs, despite the organogenic process observed. Contrarily, Chang et al. (1999) observed that in *Polianthes tuberosa*, CK levels increased after floral induction, suggesting that CKs have a role in flower bud development rather than in flower induction. Watanabe et al. (1978a, 1978b, 1978c and 1981) reported that mainly *c*-Z is involved in growth of hop mature cones whereas iPR content was below detection limit, which suggest a main role for iP-type CKs in early flower development. However, the floral development studied here does not investigate in such advanced developmental stages, therefore we cannot exclude that Z-type CKs can have a major role in inflorescence development at a later stage.

In conclusion, analyses of endogenous PGRs during hop development allow us to gain a better understanding of its role in this species. Certainly, our results support that GA₄, an active GA from the early non 13-hydroxylation pathway, seems to be the main GA involved in vegetative growth and flowering onset but not during the initial flower development. Also, iPR, the main CK present in the studied development, could play a role in the onset of reproductive growth like IAA that could promote floral bud growth and leaf development in this species.

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III. Session:

HOP CHEMISTRY

COMMERCIAL HOP EXTRACTS RICH IN XANTHOHUMOL

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Abstract

Pure resin extract resulting from large scale extraction of hops with ethanol can serve as a suitable starting material to produce hop extracts rich in xanthohumol and other prenylflavonoids. Such extracts could constitute innovative ingredients of functional food or food supplements. First nutraceuticals as well as a functional drink with hop extracts rich in xanthohumol have been introduced on the market recently.

Keywords: Hops, hop extracts, polyphenols, prenylflavonoids, xanthohumol

Introduction

Polyphenols occur ubiquitously in the plant world. They are regarded as so-called “secondary plant metabolites” as they are not participating in primary metabolism processes and are therefore not essential for the growth of the plants. From a chemical aspect polyphenols are substances which are built up from phenol- or phenol-similar units.

Since the 1980’s there have been more and more reports about positive properties which polyphenols may have on human health. The following effects have been ascertained:

- antioxidative (protecting from free radicals which damage the cells through oxidative effects)
- anti-carcinogenic (reducing the risk of cancer)
- anti-inflammatory (inhibiting inflammation)
- anti-microbial (protecting the body from fungal diseases, bacteria and viruses)
- modulating the immune reaction (strengthening the immune system)
- controlling blood pressure (prophylaxis for cardiovascular diseases)
- regulating blood sugar (prophylaxis for diabetes)

Due to these manifold positive health promising properties polyphenols can be found today in numerous food supplements (nutraceuticals) or as ingredients of functional food and functional drinks. For example “Resveroxan” rich in resveratrol (the well-known polyphenol of grapes and red wine) is marketed as “natural protection from ageing”. Also on sale is the red wine pill for “health benefits without intoxication”, which contains the “antioxidants from a glass of wine” in a concentrated form without any alcohol. Capsules with soy extract rich in genistein and other isoflavones are successfully being sold for “cancer prevention or reducing the risk of heart disease”. Another example is “Teavigo”, a green tea extract rich in epigallocatechin-gallat with “anti-obese effects”, sold as ingredient for foods or beverages.

For about 10 years there have been more and more reports on the beneficial properties of polyphenols in hops. Most of the hop polyphenols are made up of higher molecular compounds such as the flavanol type tannins. Only about 20% of the hop polyphenols consist of low molecular substances like catechin or proanthocyanidins, phenolic carbon acids (e.g. ferulic acid) and flavonols (quercetin, kaempferol) which are glycosidically bound to various sugars. All those components are wide-spread in the plant world. Even resveratrol could be detected in hops, but at very low concentrations (Jerkovic, 2005).

Furthermore there are some polyphenols being almost unique to hops. Each plant has a typical polyphenolic pattern and hops prove to be a rich source of prenylated polyphenols (prenylflavonoids). One of them is the prenylated chalcone xanthohumol showing an extraordinary broad spectrum of advantageous effects. Many research groups all over the world are currently investigating the according activities. In september 2005 the scientific journal "Molecular Nutrition and Food Research" has dedicated a complete issue to the "all-rounder" xanthohumol (Gerhaeuser and Frank, 2005). There are published many papers on the various anti-infective, anti-oxidative and cancer-preventive activities of xanthohumol. The cancer-preventive effects of xanthohumol are currently investigated by experiments with animals, amongst others at the German Cancer Research Centre. Very efficient inhibition of carcinogenesis was already demonstrated using an "ex vivo" model system with breast tissue removed from mice (Gerhaeuser, 2002). A first inhibitory effect on mammary carcinogenesis "in vivo" could be shown in mice after subcutaneous application (Gerhaeuser, 2005). In another "in vivo" experiment a positive influence of xanthohumol (oral application together with the daily diet) on fat metabolism was confirmed in a mouse model (Nozawa, 2005). Moreover xanthohumol was not estrogenic active in female rats (Gerhaeuser, 2005). The xanthohumol content of commercial hop varieties is rather high with "Hallertauer Taurus" from Germany showing the highest concentration (approx. 1 % in dried cones) of all hop varieties worldwide (Biendl, 2003).

Another prominent hop prenylflavonoid is 8-prenylnaringenin which has been shown to be one of the most potent phytoestrogens currently known. It is present in hops at a very low concentration (< 0.01 % in dried cones). The estrogenic activity of 8-prenylnaringenin proved considerably higher than that of other phytoestrogens such as genistein (present in soy) which is at least 100-fold less active. Phytoestrogens mimic the effects of the endogenous hormone 17-beta-oestradiol. Since hot flushes during menopause as well as osteoporosis are associated to the declining level of this hormone in the female body, intensive studies on the exact pharmacological profile of 8-prenylnaringenin are going on to find out whether it could be a suitable candidate for the so-called "hormone replacement therapy". A first pre-clinical trial with promising results has been carried out recently (Rad, 2006).

Methods

At least 30% of the hops are extracted by carbon dioxide (in the liquid or supercritical phase) or fermentation alcohol. These large scale extractions can be the basic processes for isolating hop polyphenols. With regard to the polyphenols there is a decisive difference between the two established extraction processes. Whereas after common extraction of hops with carbon dioxide (< 300 bar) all polyphenols inclusive of prenylflavonoids completely remain in the spent hops these components are extracted with ethanol.

The extraction of xanthohumol under very high pressure (up to 1000 bar) from the spent hops after common carbon dioxide extraction was already presented at the last IHGC-SC-meeting (Schmidt, 2005). Now in this paper the isolation of xanthohumol and other prenylflavonoids via large scale extraction of hops with ethanol is described.

Starting material for common ethanol extraction are whole hops which are extracted with a 90% ethanol and water solvent in a counter-current extractor. After the hops have passed through the extractor, they leave the extraction as spent material. The solution of ethanol and dissolved hop material leaves the extractor and is pumped to the evaporation stage. The solvent is gently removed in a multi-step vacuum evaporation system. The resulting raw extract can be separated into a so-called pure resin extract and a so-called tannin extract by means of a centrifuge. The tannin extract consists of approx. 50% water. The rest are water soluble components like mineral salts, carbohydrates, proteins and polyphenols. Hop essential oils and the complete spectrum of hop resins are recovered in the pure resin extract. Due to their chemical structure (the prenyl side chain is reducing the polarity) the

prenylflavonoids are the only class of polyphenols not transferred into the tannin extract but into the pure resin extract (e.g. the recovery of xanthohumol is > 95%).

For the enrichment of prenylflavonoids pure resin extract is at first re-extracted with supercritical carbon dioxide (50°C, 280 bar). Whereas alpha- and beta-acids are extracted with carbon dioxide together with some other (non-specific) soft resins, all the hard resins and the prenylflavonoids remain in the extraction residue. This extraction residue (“Xantho-Extract”) consists of (carbon dioxide non-soluble) resins and prenylflavonoids (5-20% xanthohumol depending on hop variety and extraction parameters). A selective separation of prenylflavonoids from resins is possible with the help of Polyvinylpyrrolidone (PVPP). When an ethanolic solution of “Xantho-Extract” passes through PVPP the prenylflavonoids are adsorbed and can later be eluted by ethylacetate. After gentle evaporation of the solvent under vacuum there results a yellow powder (“Xantho-Flav”) which only consists of prenylflavonoids with xanthohumol as main ingredient (65-85%). By a further re-crystallization step it is possible to get “Xantho-Pure” with a xanthohumol content above 85%. The production of “Xantho-Extract” is carried out using regular large scale ethanol and carbon dioxide extraction equipment. For production of “Xantho-Flav” a pilot plant has been installed. It is running since begin of 2007 and has a capacity of approx. 50 kg “Xantho-Flav” per year. “Xantho-Pure” is only produced in lab scale.

Results and Discussions

Table 1 shows an example for the compositions of the starting material and the various products rich in xanthohumol. “Xantho-Extract” consists of resins (mainly non-specific components of the hard resin fraction) and prenylflavonoids. Whereas alpha- and beta-acids are almost quantitatively separated it also contains the iso-alpha-acids present in ethanol pure resin extract at low concentrations (due to the technically unavoidable isomerisation during evaporation of the extraction solvent). With the help of PVPP a very efficient separation of prenylflavonoids from resins is possible resulting in “Xantho-Flav”, which is at the same time a suitable starting material to get rather pure xanthohumol.

Table 1: Compositions of starting material (pure resin extract produced by large scale ethanol extraction of the hop variety “Taurus”, crop 2005) and resulting extracts

Ingredient	Pure Resin Extract	Xantho-Extract	Xantho-Flav	Xantho-Pure
Essential oils	approx. 4 %	-	-	-
Total resins	approx. 90 %	approx. 85 %	-	-
Alpha-acids	45 %	1 %	-	-
Iso-alpha-acids	1.5 %	5 %	-	-
Beta-acids	18 %	< 0.5 %	-	-
Prenylflavonoids	approx. 4 %	approx. 12 %	> 90 %	> 98 %
Xanthohumol	3 %	10 %	80 %	95 %
8-Prenylnaringenin	0.02 %	0.06 %	0.2 %	< 0.02 %

The specifications of “Xantho-Extract” and “Xantho-Flav” are shown in Table 2. Both are dry powders which could constitute ingredients of solid functional foods. Alternatively they could also be processed to capsules and used in food supplements. First new nutraceuticals emphasizing xanthohumol are already on the market for a short while:

- “Xantho-C” – “A nutraceutical for highest ambitions with a hop extract rich in xanthohumol and vitamin C”.
- “Vitastar Complete” – “All-in-one nutraceutical with 12 vitamins, 10 minerals and 11 plant ingredients inclusive of a hop powder with xanthohumol”.

Table 2: Specifications of hop extracts rich in xanthohumol

Parameter	Xantho-Extract	Xantho-Flav
Appearance	green powder	yellow powder
Specific weight	approx. 0.75 kg /l	approx. 0.5 kg /l
Residual ethanol	< 0.5 %	< 0.5 %
Residual ethylacetate	-	< 0.5 %
Taste	typical hop bitterness	slight bitterness
Soluble in water	no	no

For applications in beverages the solubility of the extracts rich in xanthohumol has to be considered. They are hardly soluble in water but only in ethanol. Their fate during beer production has been already investigated since many years. In case of lager or pilsener type beers a maximum of approx. 1 mg/l xanthohumol was only achieved when an ethanolic solution of "Xantho-Flav" had been added to the green beer after main fermentation. During wort boiling xanthohumol is converted to isoxanthohumol. But roasted malt seems to influence the isomerisation rate and to improve the solubility of xanthohumol. When stout or porter type beers were produced with addition of "Xantho-Extract" (as the only hop product used) at begin of wort boiling a concentration of approx. 3 mg/l xanthohumol could be achieved in the final beers after filtration (Biendl, 2004).

To overcome the water insolubility of xanthohumol enriched hop products the addition of a food grade emulsifier is suggested. According basic formulations were developed by Doehler GmbH (Darmstadt, Germany). By using such basic formulations, the final beverages can show up to 10 mg/l xanthohumol or even more (Doehler GmbH, 2004). There is already a commercial beverage based on this development: "XAN" - a mixture of non-alcoholic beer with a fruit juice containing a hop extract rich in xanthohumol.

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CHANGES IN ANTIOXIDANT PROPERTIES OF HOPS IN THE COURSE OF DRYING, PELLETIZING AND STORAGE

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Abstract

Total antioxidant activity of hops was measured in the course of drying, pelletizing and storage by monitoring the loss of optical density of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in hot water hop extracts. Samples were withdrawn at different phases of green hops processing. A part of antioxidative activity of hops is irreversibly lost in the course of drying. The loss does not usually exceed 5 % of origin antioxidant activity value. Drying in belt and chamber kilns are comparable from the point of view of hops antioxidative properties conservation. Grinding and pelletizing has no conclusive effect on antioxidative status of hops. Antioxidative activities of hops declines in the course of long-term storage in dependence on storage temperature and hop form (raw hops, pellets). Storage temperature has no effect on antioxidative activity of hop pellets packed in multi-layer foil without air access.

Keywords: hops, polyphenols, antioxidants, DPPH, drying, grinding and pelletizing, hops ageing

Introduction

Interest is increasing in phenolic compounds, that may act as antioxidants with mechanism involving both free radical scavenging and metal chelation. Hop is a plant rich in polyphenols content. Polyphenols are released into wort in the course of beer production and form appr. 30 % of the total polyphenols in final beer. Its reducing activity can thus influence antioxidant status and sensorial stability of beer. Drying, grinding and pelletizing are processes when hops are heated up. There arising a question of how can heat burden effect antioxidant status of hops, important raw material for beer production. For example drying of hops is performed in two types of kilns, belt and chamber ones, at the temperature of 50-60 °C for 6-10 hours to final moisture content of 8-12 % w/w. Neither after processing composition of hops it remains stable. Ageing processes, that mean irreversible changes in the composition of hop secondary metabolites, start in dependance on storage conditions. Changes of antioxidant properties of hops in the course of drying, pelletizing and long-term storage have not been studied. In the lecture there are summarized the results of antioxidant properties assesment of fresh and dried hops, hops processed by pelletizing and aged hops stored under different conditions.

Material and methods

Antioxidant properties of raw hops and hop products were measured according to Kaneda method [1,2]. Principle of the determination is based on the reaction of stabile color radical of 1,1-diphenyl-2-picrylhydrazyl (DPPH) with antioxidants. Antioxidant activity of the samples RA_{DPPH} is expressed like relative decline of DPPH radical concentration under experimental conditions (Fig. 1). Immediate value of radical concentration is measured on UV-VIS spectrophotometer at the wave lenght of 525 nm. Reduction activity was measured in hot water extract of hops thoroughly filtered through membrane filter before analysis. Effect of drying to reducing capacity of hops was studied in 2005 and 2006 seasons taking samples of fresh and dried hops from picking facilities of several hop growers. Effect of pelletizing was monitored in samples of raw hops, ground hops and hop pellets taken from full-size production

line (Chmelařství Žatec). In October 2005 long-term storage test of raw hops and hop pellets concerning varieties Saaz aroma and Premiant was established. Samples were kept in a dark room at the temperatures of 20-24°C and 2-3°C for 12 months. Raw hops were wrapped into paper bags, pellets were sealed in multi-layer aluminium foil and vacuuated to avoid air access. Contents of moisture, alpha acids, HSI and antioxidative status of hops were measured at the start and at the end of the experiment. Contents of alpha acids were measured according to EBC 7.7 /3/. Hop storage index (HSI) was determined by spectrophotometric method according to ASBC /4/.

Results and discussion

Effect of drying on antioxidative activity of hops

In **Table 1** there are summarized the results of antioxidant activities determination in fresh (green) and dried hops from 2005 and 2006 crop harvests for Czech hop varieties. Antioxidant activities (RA_{DPPH}) of hot water extracts prepared from fresh and dried hops differ. Part of reducing activity of hops is irreversibly lost by drying, the loss is small and usually does not exceed 5 % of origin level RA_{DPPH} . Reducing activities of dried hops are systematically lower, in spite of differences being in the range of repeatability deviation /1/. Statistical evaluation by pair t-test proved statistical relevance of the differences. There is no difference between belt and chamber kilns from the point of view of effect on antioxidative status of dried hops. Big attention is devoted to maintain drying temperatures in practice because temperatures above 60°C lead to quick change colour of hops, lost of sensorial quality and hops thus become worth-less.

Effect of grinding and pelletizing on antioxidant activity of hops

Preliminary tests proved, that to secure technological link-up of the samples on full scale conditions (pelletizing line, Chmelařství Žatec), i.e. raw hops at the start, and pellets at the end of production line is speculative. The pelletizing process was therefore divided on two parts. Effect of grinding of hops on antioxidative capacity was evaluated in laboratory conditions. Distributions of the hops particle size on laboratory grinder (Retsch ZM1- sieve 1,5 mm, moisture of hops 7-9 %) and particles from dissolved pellets is practically identical. It could be supposed that extractability of hops in the course of sample preparation is comparable. The effect of pelletizing was investigated in pairs of samples taken from full-scale production line, ground hops from homogenisation silo and pellets sampled immediately from pelletizing machine. Results (data not shown) of antioxidative activity determination in hot water extracts prepared from raw hops and ground hops (crop harvests 2005, 2006) proved that differences of experimental data were very small and statistically not significant (pair t-test). The results of antioxidant activity measurements in ground hops and pellets for Saazer and Premiant cultivars are given in **Table 2**. (crop harvests 2005, 2006). Evaluation of experimental data were done in the same way like for raw hops. Differences of reducing activities between ground hops and pellets were not statistically significant. It can be stated that the whole pelletizing process has no significant effect on antioxidative status of hops.

Effect of ageing on antioxidative activity of hops

In **Table 3** there are summarized the results of antioxidative activities in all experimental variants at the beginning (October 2005) and at the end of hops aging experiment (September 2006). Other quality parameters of hops, content of alpha acids, hops moisture and hop storage index, are given in **Table 4**. Results of antioxidative activity show that reducing activity of hops declines in the course of ageing with different dynamics depending on storage temperature and form of hops. Storage temperature has no effect on antioxidant activity of hop pellets, packed in multi-layer aluminium foil without oxygen access. This is valid for both tested varieties, Saazer and Premiant. Simultaneously brewing quality got worse because alpha acid contents reduced in Premiant by 18,2 % (warm) and by 5,2 % (cold) and by 22,8 and 6,5 % in Saazer respectively. Deterioration of hops quality document increasing values

of hop storage indices. Different results were obtained for raw hops, especially for antioxidative activity values. Reducing activities of „cold“ raw hops were substantially worse compared to „warm“ hops. Brewing quality of „warm“ hops was very bad compared to „cold“ raw hops as documented by 30-60 % reduction in alpha acid contents. Values of hop storage index exceeded 1,0 limit. The cause of these apparently paradox findings can be high moisture contents of „cold“ hops stored in the conditions with high air humidity. As experimental samples of raw hops were of prism shape and of 10x10x4 cm size, moisture could be captured in the whole sample volume and thus accelerate reaction of polyphenol components of hops resulting in antioxidative status reduction. In practice hops are pressed to prisms of 50 x 60 x50 cm in size and 50 kg of weight. The phenomenon would be probably limited only on surface layer of pressed hops.

Conclusions

1. A part of antioxidative activity of hops is irreversibly lost in the course of drying. Drying in belt and chamber kilns are comparable from the point of view of hops antioxidative properties conservation.
2. Pelletizing process has no significant effect on antioxidative status of hops.
3. Reducing activity of hops declines in the course of ageing with different dynamics depending on storage temperature and form of hops. Storage temperature has no effect to antioxidant activity of hop pellets, packed in multilayer aluminium foil without oxygen access.

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Acknowledgement

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Table 1: Antioxidant activity of fresh and dried hops from 2005 and 2006 crop harvests

Variety	Locality	Kiln type	RA _{DPPH} (% rel.)		RA _{DPPH} (%) Difference
			Fresh	Dried	
2005 crop harvest					
Saazer	Stekník	Belt	78,7	75,8	- 2,9
Saazer	Vrbičany	Belt	78,5	75,3	- 3,2
Sládek	Stekník	Belt	54,6	50,9	- 3,7
Premiant	Stekník	Belt	56,3	53,5	- 2,8
Agnus	Stekník	Belt	33,0	32,0	- 1,0
2006 crop harvest					
Saazer	Sedčice	Belt	63,4	62,7	- 0,7
Sládek	Milostín	Belt	59,8	55,4	- 4,4
Sládek	Očihov	Chamber	47,6	44,2	- 3,4
Premiant	Stekník	Belt	50,1	48,0	- 2,1
Sládek	Stekník	Belt	47,9	45,1	- 2,8
Agnus	Stekník	Chamber	48,2	43,2	- 5,0

Table 3: Antioxidative activities of hops in the course of storage period RA_{DPPH} (% rel.)

Month	Cones		Pellets	
	warm	cold	warm	cold
PREMIANT				
October 2005	57,8	57,8	57,5	57,5
September 2006	53,2	45,6	50,5	50,2
SAAZER				
October 2005	67,1	67,1	64,7	64,7
September 2006	64,2	58,7	62,2	62,2

Table 2: Effect of pelletizing on antioxidant activity of hops

Variety	Specification	RA – DPPH (% rel.)		Diff. RA _{DPPH} (P – G, %)
		Ground hops (G)	Pellets (P)	
Crop harvest 2005				
Saazer	G 45 – NB	72,9	71,6	- 1,3
Saazer	G 45 – NB	70,9	71,9	1,0
Saazer	G 45 – NB	65,2	65,9	0,7
Premiant	G 90 – NB	45,6	45,7	0,1
Crop harvest 2006				
Saazer	G 90 – NB	76,5	75,0	- 1,5
Saazer	G 90 – NB	72,0	73,1	1,1
Saazer	G 90 – NB	70,2	70,9	0,7

Table 4: Contents of alpha acids, moisture and HSI of hops in the course of storage period

Month	Cold						Warm					
	Cones			Pellets			Cones			Pellets		
PREMIANT												
	alpha	moisture	HSI	alpha	moisture	HSI	alpha	moisture	HSI	alpha	moisture	HSI
October 2005	8,45	9,3	0,28	8,70	6,7	0,29	8,45	9,3	0,29	8,70	6,7	0,29
September 2006	6,17	12,6	0,48	8,25	6,6	0,34	4,09	6,2	1,19	7,12	6,4	0,43
SAAZER												
	alpha	moisture	HSI	alpha	moisture	HSI	alpha	moisture	HSI	alpha	moisture	HSI
October 2005	3,65	9,1	0,31	3,51	6,5	0,31	3,65	9,1	0,31	3,51	6,5	0,31
September 2006	2,52	15,2	0,65	3,28	6,4	0,39	1,58	7,4	1,40	2,71	6,6	0,46

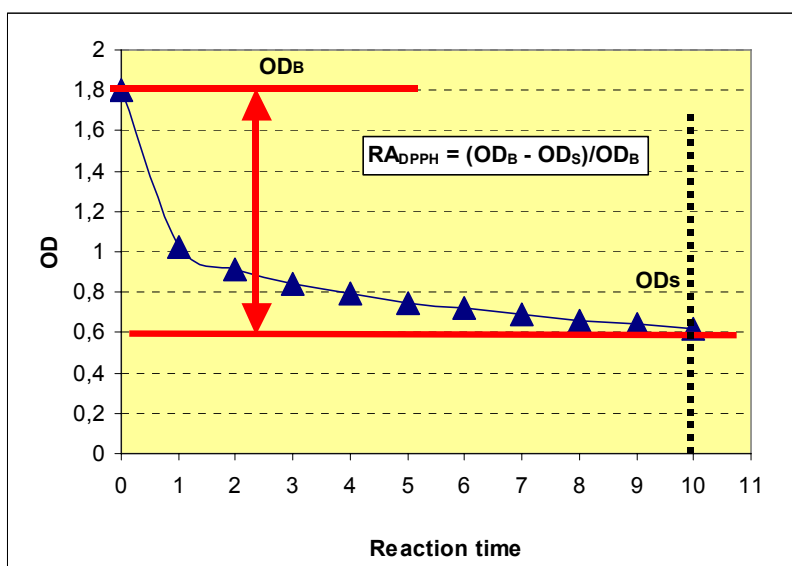


Fig 1: Decline of optical density (OD) in the course of determination of antioxidative activity of hops by DPPH radical

PRESENTING, EVALUATING AND COMPARING CHEMICAL COMPOSITION OF HOP SECONDARY METABOLITES AT A GLANCE CHEMOMETRICS OF BITTER SUBSTANCES AND XANTHOTHUMOL

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Abstract

Bitter substances and xanthohumol were analyzed in 27 hop samples, representing 5 different hop cultivars. Samples originated from four Slovenian hop growing areas. High performance liquid chromatography and lead conductance value were used, chemometrics was applied. Results confirm the cultivar influence on the xanthohumol (XAN) content, while influence of the growing area is hardly noticeable. Among Slovene cultivars Aurora (0.38 % in dry matter (DM)) is the one standing out for its high, and cultivar Celeia (0.16 % DM) for its low XAN content. The chemometric classification of hops according to their cultivar was obtained with a nearly 100 % degree of correct classification in the case of principal component analysis, hierarchical cluster analysis and regularised discriminant analysis. Relation between the content of XAN and other secondary metabolites was also analyzed.

Keywords: hop cultivars, growing area, xanthohumol, α -acids, β -acids, chemometrics

Introduction

As hops in the breweries are generally used on the basis of their α -acid content, the absolute content of XAN is not the only relevant value, its ratio to the α -acid content should also be taken into consideration. The content of XAN in hops as well as its ratio to the α -acids are known to vary according to the cultivar and growing area, while the crop year does not seem to have a significant influence; it seems that XAN preferably forms in Central Europe (Biendl, 2002/2003).

Good and comparable results concerning the characterization of hops authenticity and the classification of hops according to cultivar were obtained using the results of α -acid content and XAN content determined by high performance liquid chromatography (HPLC) (De Keukeleire et al., 2003, Stevens et al., 1999, Verzele, 1985, Stevens et al., 1997). Lead conductance value (LCV) is preferably used for the determination of bitter compounds such as α -acids. It is suitable for determination of α -acids in fresh dried hops, while for aged hops it does not give only α -acids but also some of the oxidation products. At the time of technological maturity of hops LCV method is a fast, simple and cheap way of determining the α -acid content (Košir, 1995). Comparison of results obtained by HPLC and LCV methods could be used to check the deterioration of stored hop cones (McMurrough et al., 1986).

Determination of many components in hops results in large amounts of data. The use and the evaluation of so many parameters are common problems in food chemistry. To deal with this problem, chemometrics have been applied to different chemical or physical variables. Principal component (PCA), hierarchical cluster (CA) and regularised discriminant (RDA) analyses were used on different variables to classify and to determine the authenticity of hop samples (Siebert, 2001, Brescia et al., 2003).

Slovenia has an important influence on the world hop market. With approximately 3 % of world production it is on the fifth place among the hop growing countries. The purpose of this work was to determine the influence of cultivar and growing area on the content of XAN and other secondary metabolites in hops grown in Slovenia using HPLC. LCV was also measured. The data obtained by analytical methods were treated with three chemometric methods: PCA, HCA, RDA. Possible indications for connections between the metabolic pathways of XAN and those of other secondary hop metabolites were studied. The data obtained could and should be included into various applications of the MIN-MAX model in order to make it work also for substances which play the most important role(s) in beer brewing.

Methods

Samples. Analyses were carried out on 27 hop samples. Characteristics of five Slovenian economically important cultivars were investigated: Aurora (AU, 7 samples), Bobek (BO, 5 samples), Celeia (CEL, 5 samples), Styrian golding (SG, 5 samples) and Magnum (MAG, 5 samples). Growing areas of these cultivars were given as four blocks, representing the entire Slovenian hop production: Žalec East (E) (Petrovče and Celje), Žalec West (W) (Braslovče, Vranksko, Šempeter, Prebold and Mozirje), Koroška (Radlje ob Dravi) and Ptuj. Hops were picked at the time of the technological maturity in the year 2005 and then dried. Hop cones were stored in a dark, dry place at ambient temperature until May 2006, when the analyses took place. Duplicates of all samples were stored as voucher specimens in the facilities of the Slovenian Institute of Hop Research and Brewing, where analyses took place.

All analyses were performed according to standard methods (Analytica-EBC 7.2, 7.7, 7.4). In the case of xanthohumol the Analytica-EBC 7.7 was slightly modified with the change of wavelength (370 nm).

Statistical Treatment of Data. Statistical elaboration of the data was done using SCANWIN software. The applied chemometric methods were PCA, HCA and RDA. PCA calculates orthogonal linear combinations of a starting set of variables on the basis of maximum variance criterion. Such linear combinations are called principal components (PC). The coefficients, by which the original variables must be multiplied to obtain the PCs, are referred to as loadings. The numerical value of a given variable loading on a principle component indicates how much the variable has in common with that component. Hence, the loadings can be interpreted as a correlation between the variables and the components (Massart, 1998). HCA classifies objects in clusters on the basis of inter-object distances in high dimensional space. The results are shown in a dendrogram, which may be used to detect groups of similar individuals (Frank, 1994). RDA obtains discriminant functions calculated to maximize distances between predefined groups (Massart, 1998). Its purpose is to calculate class models and boundaries, giving a rule of classification based on a set of known objects (training set). This rule can be applied to define the classification of unknown objects (test set), but it needs to be validated. In this work, the validation approach applied was the cross-validation test. For the cross-validation test, one individual at a time is removed from the initial population, and RDA is performed on the remaining individuals. This method gives an indication of the influence of each individual. The coordinates of the removed individual are calculated, and its group assignment is verified.

Results

The following parameters were determined: cohumulon (coh), n+adhumulon (n+adh), α -acids (α), colupulon (col), n+adlupulon (n+adl), β -acids (β), xanthohumol (xan), lead conductance value (LCV). Values were considered as percentage on dry weight. For statistical analyses they were used together with ratios cohumulone/ α -acids, colupulone/ β -acids, α -acids/ β -acids, all together 11 parameters.

The XAN content varies between 0.44 % and 0.14 %. The maximum average content was found in the cultivar AU (0.38 %), followed by MAG (0.32 %), BO (0.30 %), SG (0.23 %) and

CEL (0.16 %). Observing the amount of α -acids, the cultivar MAG is the one standing out (9.04 %), followed by AU (7.51 %), BO (3.47 %), CEL (2.97%) and SG (2.87 %). The ratio of XAN content to the α -acid content varies between 0.03 and 0.10. For the hop dosage in the brewing process this means that for example with a dosage of 10 g of α -acids per hectolitre the amount of XAN can vary between 0.3 g and 1.0 g depending on the variety. As to varieties grown in Slovenia clear differences are apparent for the high- α varieties, where MAG shows a relatively low XAN/ α -acids ratio (0.035). For all other varieties this value exceeds 0.05. Cultivars SG and BO exceed 0.08.

Discussion

PCA was applied to the data matrix formed by 11 statistical parameters (variables), obtained as described in the Methods section. On the score plot of the first two PCs, where this procedure accounts for 93.48 % of the total variance, a clustering of hops can be observed according to cultivars. Borders between clusters are clearly defined. The only exception is one sample of the cultivar BO, which is an outlier lying in the group of samples representing cultivar SG. Cultivars are divided into two groups: high- α varieties (MAG and AU) in the upper part and the so called aromatics (BO, CEL, SG) in the lower part of the plot. Loading values show the contribution of the variables to the more significant principal components. For example: variables such as percentage of cohumulone in α -acids (%coh/ α) and percentage of colupulone in β -acids (%col/ β) play a significant role in distinguishing cultivars MAG and BO from the others and at the same time they tell one from the other. Positions of variables XAN, α -acids components (n + adhumulon, cohumulon) and LCV show a similar influence on principal components and also an important correlation between the variables.

Further on, a hierarchical clustering analysis applying a Ward linkage method with Manhattan distances on the 11 PCs scores of the studied samples, considered as variables, was applied. Hop samples are clearly grouped into 5 different clusters corresponding to five cultivars used. The objects formed two evident groups: the first group includes MAG and AU cultivars, and the second BO, CEL and SG cultivars. This is in accordance with the statement that CEL, BO and SG cultivars are defined as typical aroma cultivars, while MAG and AU contain higher amounts of bitter substances. As in the case of PCA, also here one sample of cultivar BO could be defined as an outlier.

RDA was performed on the 5 PCs scores of the analyzed samples to discriminate the hop samples according to the cultivar. The model gave 100 % correct classification (Figure 1A). There are no outliers for individual samples inside each of the predefined groups. RDA hop merging gives us a restrictive model. This kind of restrictive model is very useful in the determination of cultivar purity to prevent marketing with unknown mixed hop cultivars. Cross validated assignments (Figure 1B) were 100 % correct.

With the use of simple regression, possible relationship between α -acid content and XAN content was observed. The equation of the fitted model is:

$$\text{XAN (\% DM)} = 0,159 + 0,0237 * \alpha\text{-acids (\% DM)}$$

Since the p-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between XAN content and α -acid content at the 99 % confidence level. The correlation coefficient equals 0,71, indicating a moderately strong relationship between the variables.

To summarize: studying the influence of cultivar and growing area on the content of XAN and other secondary metabolites in hops (*Humulus lupulus* L.) in Slovenia in combination with chemometric methods PCA, HCA and RDA it was shown that XAN content depends on the cultivar, while the influence of the growing area is hardly noticeable. Applied chemometric methods (PCA and HCA) showed clustering of samples according to the cultivars. RDA gave

a restrictive model for cultivar classification. There are obvious connections between the values referring to α -acids and those referring to XAN.

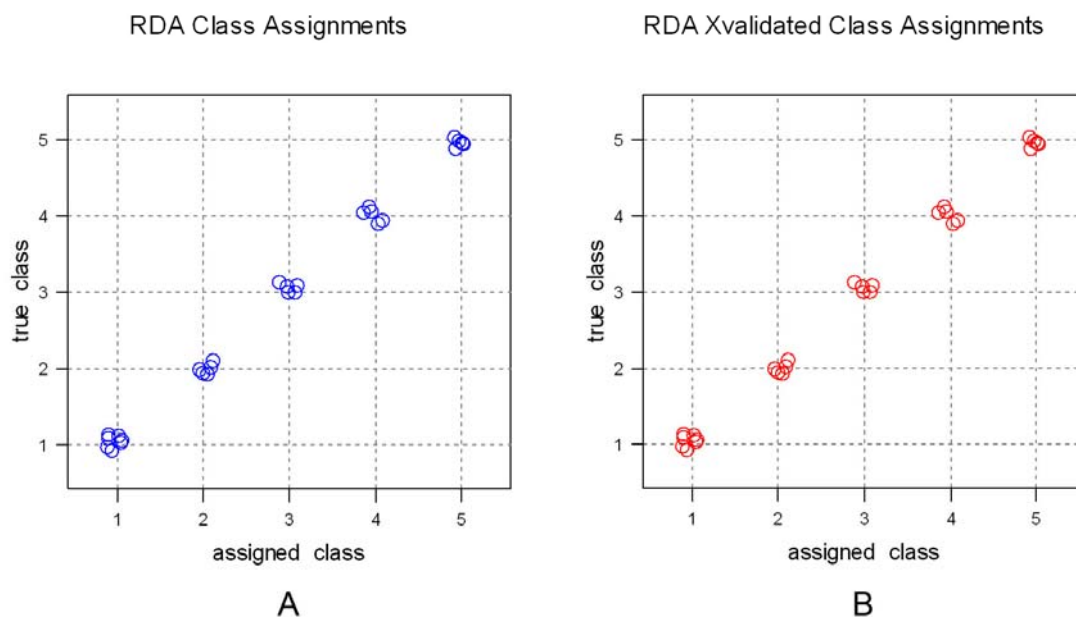


Figure 1. Classification of hops on the basis of calculated RDA model (A) and the results of validation model, using the cross-validation test (B).

Acknowledgement

We acknowledge hop growers for provided samples and personnel of Slovenian Institute for Hop Research and Brewing for technical assistance.

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THE BIOSYNTHESIS OF THE BITTER ACIDS IN HOPS

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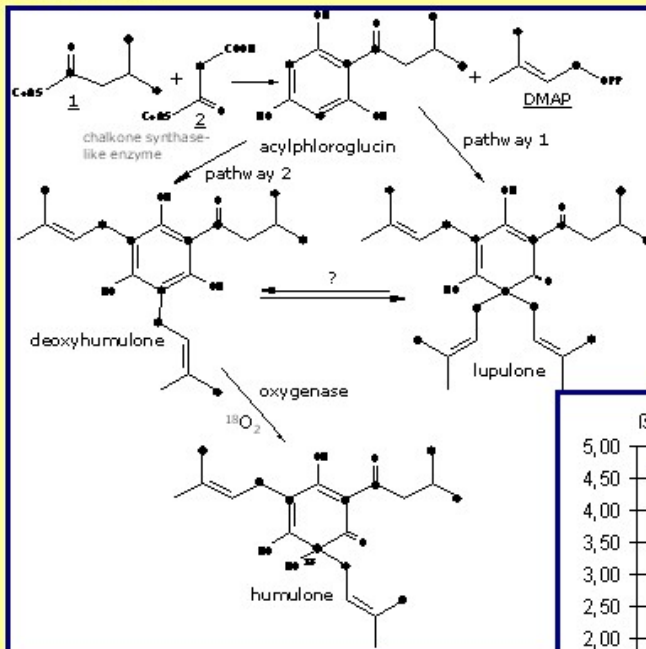


The biosynthesis of the bitter acids in hops

K. Kammhuber, M. Goese, S. Hecht

Introduction

The α -acids are considered to be the primary quality feature of hops, because they are a measure of the bitter potential. But also the β -acids are getting increasing importance for the food industry due to their antimicrobial and preservative properties. The following biosynthesis pathway is discussed in hops.



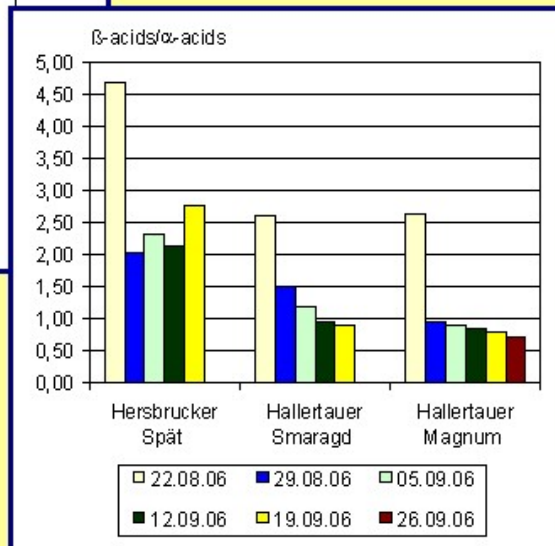
Method

Isotope incorporation experiments using [1- C^{13}]-glucose [1] and $^{18}O_2$ [2] revealed the shown labelling pattern. The isotope enrichment identify isovaleryl-CoA **1**, malonyl-CoA **2** and dimethylallyl pyrophosphate **DMAP** as precursors of humulone. The DMAP is generated via the deoxyxylulose pathway of the terpenoid biosynthesis. It is not clearly proved, whether the biosynthesis follows way 1 or 2.

Biosynthesis pathway of the α - and β -acids

Discussion

Investigations into the bitter substances of hop leaves [3] have shown that β -acids are formed in small amounts (up to 0,3 %). Alpha-acids are only found in traces. During the biosynthesis of the bitter substances in cones first a surplus of the β -acids compared to the alpha-acids is observed. These facts speak for pathway 1.



Ratio of the α -/ β -acids during the biosynthesis

Literature:

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LOW MOLECULAR POLYPHENOLS IN BEER INFLUENCED BY DIFFERENT HOP VARIETIES

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During the last years polyphenols from hops attracted more interest for the pharmaceutical industry. The most important reason is the antioxidative power of some compounds. Also for brewers the influence of hop polyphenols on the beers is still a field of concern. In this work the use of different hop varieties and their effects on the composition of the low molecular polyphenols in the beers were traced.

On behalf of the CMA (Central Marketing Association for German agricultural products) a portfolio was edited in 2005 giving splendid information about the German hop varieties. The quality of the hop varieties was assessed by judging their aroma. Also important analytical data for each variety are available.

Additionally brewing trials were carried out to show the influence of the different hop varieties on the beer. Trained taste panels assessed these beers. Furthermore the beers were analysed on their bitter and aroma compounds. The portfolio shows a good arrangement of the results and gives the brewer a helpful instrument to choose the right varieties for his beers.

The assessments in the hop variety portfolio are mainly based on the effects of the bitter and aroma compounds of the different varieties. The contents and the composition of the polyphenols in the beers are only concluded in the results of the beer tastings. Analytical data for this substance group were not available up to now. Thus, additionally to the published findings analyses on low molecular polyphenol compounds were carried out of four selected varieties. The results for the hops and the related beers complete the assessments of the hop variety portfolio.

The hopping rates for the beers vary between 190 and 1150 gram per brew due to the different alpha contents in the hops. The associated dosages of polyphenols range between 10 and 110 mg / liter. About 40 percent of the given low molecular polyphenols are found in the beers.

However the single compounds behave differently. The recovery of catechin and epicatechin lies over the average whereas procyanidins yields considerably less. Kaempferol-3-glucoside and of one of the caffeoyl quinic acids are found totally in the beers. Some low molecular polyphenols are characteristic for specific hop varieties. One of the kaempferol-diglycosides, typical for the Hersbruck variety, is detected in the related beer.

The results show that both, hopping rate and variety, influence the content and the composition of low molecular polyphenols in the beer considerably.

Keywords: Polyphenols, varieties, beer

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- CMA Hop portfolio „The spirit of beer“

IV. Session:

**DNA-BASED TECHNIQUES IN
HOP RESEARCH**

MOLECULAR MARKERS FOR ALPHA ACIDS; STUDY FOR PRACTICAL APPLICATION

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Abstract

The universal use of two QTL markers of “Chinook” for the content and composition of alpha acids in hop cones were verified. The relationship between the genotypes and the content or composition of the alpha acids were investigated on 7 cross lines by statistical analysis. As a result, it was suggested that “7H01” was located close to the gene which significantly affected the alpha acids content. It is highly possible that “3D03” was closely linked with the major gene of the cohumulone ratio. Considering the rate of genetic variance explained by the marker loci among the total variance, it is possible that they are valid for the practical breeding for the content or composition of the alpha acids.

Keywords: Marker assisted selection, alpha acids, cohumulone

Introduction

Hop breeding is one of the important ways to improve hop characteristics and it has contributed to the hop industry and breweries. However, conventional hop breeding has some inefficiencies which come from the nature of the hop. First, it is hard to estimate the potential of male hops, because they do not bare cones. Second, the contents of the chemical substances in the hop cones are not stable for 2-3 years after transplanting in a field. Third, the number of hop plants for investigation in a field is restricted because of its huge size. Marker assisted selection (MAS) is considered to have the power to solve these difficulties because this technique enable us to estimate the potential of hops by molecular genotypes regardless of their sex, and to select promising seedlings at the nursery stage.

The molecular markers for important traits have been developed. The qualitative traits such as sex (Polley et al., 1997) and powdery mildew resistance (Seefelder and Seigner, 2003) have been reported. Several studies about quantitative traits, such as cone chemical components, have also been reported (Koie et al., 2005, Čerenak et al., 2006). We developed 2 RFLP (Restricted Fragment Length Polymorphism) markers, named “7H01” and “3D03”, derived from lupulin-specific cDNA on QTLs for the alpha acids content and cohumulone ratio to the alpha acids (cohumulone ratio) using the maps of “Chinook” and “SaM” constructed in our previous report (Fig. 1, Koie et al., 2005).

In order to apply QTL markers to practical breeding, it is essential to know the availability of the markers to various cross lines. In this study, we investigated the availability of “7H01” and “3D03” for the alpha acids and cohumulone ratio, respectively, on 7 cross lines derived from 4 maternal and 5 paternal parents.

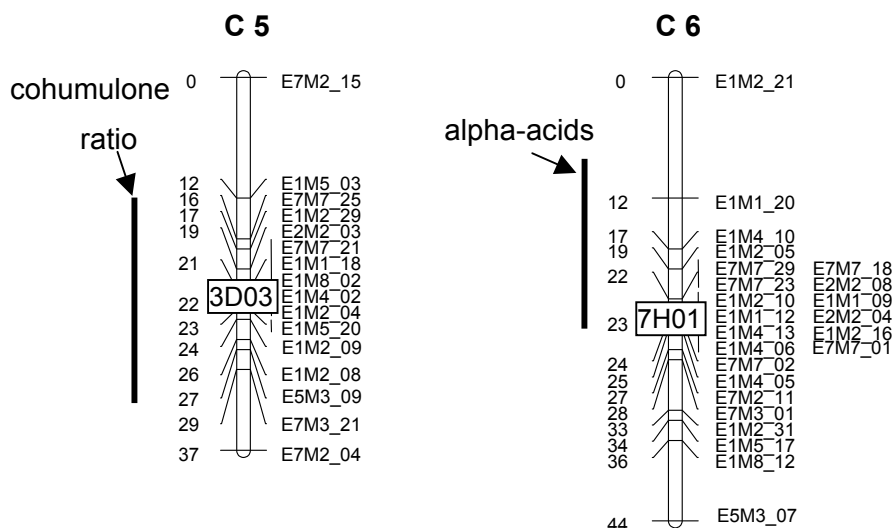


Fig. 1 “7H01” and “3D03” located on the linkage map of “Chinook” (Koie et al. 2004).

Methods

Seven lines from the crosses of “Daimanshu” x “M916001003”, “Daimanshu” x “980690”, “Hokusen Zairai” x “980690”, “0004B” x “971174”, “0004B” x “980573”, “Little Star” x “M843501246”, and “Little Star” x “980573” were used. In these cross lines, the genotypes of “7H01” and “3D03” were polymorphic.

The genome DNA was extracted using the CTAB extraction method with modifications. The genotypes of “7H01” and “3D03” were detected by Southern hybridization analysis. The alpha acids contents, cohumulone ratio were measured by HPLC using the method based on EBC 7.8.

Tests of significance for the mean value differences of the alpha acids contents and cohumulone ratio between the genotypes were performed. In the case of the test between 2 genotypes, the Student’s t test for equal variance or Welch’s t test for unequal variance was carried out according to variance similarities checked by the F test. In the case of the test between more than 2 genotypes, the combined method of the Kruskal-Wallis test and Scheffe’s test was performed. The rate of genetic variance explained by a locus among the total variance (%Exp) was calculated as follows,

$$\%Exp = [\text{Genetic variance explained by a locus } (V_{G_LOC}) / \text{Population variance } (V_P)] \times 100$$

V_{G_LOC} is calculated as

$$V_{G_LOC} = ((X_P - X_1)^2 \times N_1 + (X_P - X_2)^2 \times N_2 + \dots) / (N_1 + N_2 + \dots)$$

where X_P is the mean value of a population; X_1, X_2, \dots are the mean values of each genotype group; and N_1, N_2, \dots are the plant numbers of each group.

Results

Genotypes of “7H01” and “3D03”

There were 3 genotypes, “ll”, “lm” and “mm”, which consisted of 2 types of alleles, i.e., “l” and “m” for “7H01”, and 5 genotypes, i.e., “ee”, “ef”, “eg”, “fg”, and “gg”, which consisted of 3 types of alleles, i.e., “e”, “f”, and “g” for “3D03”, respectively, in the progenies of 7 crosses by Southern analysis (Tables 1, and 2). In the case of “3D03”, 2 to 4 types of 5 genotypes were detected in a single cross line.

Relationship between genotypes of “7H01” and alpha acids contents

In the cross lines of which the progenies segregated into genotypes of “II” and “Im”, the mean values of the alpha acids content of the groups of “Im” were significantly higher than those of “II” in 4 of 5 cross lines (Table 1). The values of %Exp in these 4 lines were 17-39%. On the other hand, there were no significant differences between the groups of “Im” and those of “mm” in 2 cross lines.

Table 1. The relationships between genotypes of “7H01” and alpha acids contents in hop cones.

Cross	Number of plants		Alpha acids (%)		Significance	%Exp (%)
	II	Im	II	Im		
Daimanshu (Im) x M916001003 (II)	17	8	4.89	7.00	*	20.86
0004B (Im) x 971174 (II)	16	23	8.76	9.11	N.S.	0.31
0004B (Im) x 980573 (II)	21	14	8.19	11.51	**	38.62
Little Star (Im) x M843501246 (II)	20	19	7.33	9.32	**	24.29
Little Star (Im) x 980573 (II)	27	23	9.15	10.91	**	17.25

Cross	Number of plants		Alpha acids (%)		Significance	%Exp (%)
	Im	mm	Im	mm		
Daimanshu (Im) x 980690 (mm)	18	12	4.84	4.41	N.S.	1.18
Hokusenzairai (Im) x 980690 (mm)	19	14	4.14	4.91	N.S.	3.08

*: $p < 0.05$, **: $p < 0.01$, N.S.: not significant

Relationship between genotypes of “3D03” and cohumulone ratio

The mean values of the cohumulone ratio were higher for the groups of the genotypes with the “f” allele than for those without the “f” allele. Especially the differences of the mean values were significant in the 3 cross lines, “Daimanshu” x “M916001003”, “0004B” x “971174”, and “0004B” x “980573” (Table 2). The %Exp values of 6 lines, in which the progenies segregated into the groups of the genotypes with and without the “f” allele, were 38-68%. On the other hand, there was no significant difference between the groups with the genotypes comprised only with the “g” and “e” alleles in the cross line of the “Little Star” x “M843501246

Table 2. The relationships between genotypes of “3D03” and cohumulone ratio to alpha acids in hop cones.

Cross	Number of plants					Cohumulone ratio (%)					%EXP (%)	
	ee	ef	eg	fg	gg	ee	ef	eg	fg	gg		
Daimanshu (ef) x 980690 (eg)	7	2	12	9		22.9 _a	36.6 _{ab}	24.4 _a	34.7 _b		61.49	
Daimanshu (ef) x M916001003 (gg)			17	8				22.7 _a	33.6 _b		64.38	
Hokusenzairai (ef) x 980690 (eg)	6	5	9	13		29.2 _{ab}	36.3 _{ab}	26.2 _a	39.0 _b		49.10	
0004B (gg) x 971174 (fg)				9	28				36.2 _a	26.8 _b	45.41	
0004B (gg) x 980573 (fg)				18	18				41.3 _a	26.8 _b	67.57	
Little Star (eg) x M843501246 (eg)	9		27	3		31.6		29.8		28.3	3.75	
Little Star (eg) x 980573 (fg)			12	13	18	7		38.7 _b	27.7 _a	39.8 _b	31.0 _{ab}	38.22

Significant differences of the mean values are shown as “a”, “ab”, and “b” on the right side of the mean values. The difference between “a” and “b” was significant at the significance level of $P < 0.05$, but the differences between “a” and “ab” and “b” and “ab” were not significant.

Discussion

Availability of “7H01” genotype on alpha acids content

It is reasonable that the gene affecting the alpha acids content located close to “7H01” would be a gene with a dominant effect. The reason is that there were no significant differences between the mean values of the alpha acids contents of the genotypes of “lm” and those of “mm”, while those of “lm” were significantly higher than those of “ll” in 4 of 5 cross lines. Besides, the values of %Exp in the 4 cross lines show that the genotypes of “7H01” contribute significantly to the alpha acids content (Table 1). Indeed the “7H01” genotypes of the high-alpha varieties, “Brewers Gold”, “Nugget” and “Columbus”, were “lm”, “lm”, and “mm”, respectively (data not shown). However, there was one cross line which did not show a significant difference between the groups of the genotypes of “lm” and those of “ll”. A further investigation is necessary to confirm the effect of the genotypes.

Availability of “3D03” genotype on cohumulone ratio

The groups of the genotypes with the “f” allele tended to have a higher cohumulone ratio than those without the “f” allele (Table 2). The high ratios of %Exp suggested that the gene close to “3D03” should have a notable contribution to the cohumulone ratio. Murakami (1999) mentioned about the existence of the major gene of the cohumulone ratio. It is highly possible that the gene close to “3D03” would be the gene mentioned by Murakami (1999).

Some differences in the mean values were not significant in the cross lines segregating into 4 genotypes. The reason for this should be the small plant number of one segregating group in these cross lines.

For practical application

The results in this study suggested that “7H01” and “3D03” could be powerful tools in breeding for the alpha acids content and cohumulone ratio, respectively. Investigating new genotypes on these loci would lead to finding new breeding materials in genetic resources. Furthermore, it is expected that there are still more unknown genetic factors for the alpha acids content or the cohumulone ratio. Therefore, it is necessary to search for new molecular markers derived from the other loci in order to improve MAS for hop breeding.

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QTL MAPPING OF ALPHA ACID CONTENT IN HOP

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Abstract

Effects of quantitative trait loci (QTLs) and the map locations were determined for alpha-acid content in hop (*Humulus lupulus* L.) using amplified fragment length polymorphism (AFLP) and microsatellite markers (SSRs). Genetic linkage maps were constructed from mapping population consisted of 111 progeny from a double pseudo-testcross. A total of 194 markers (150 AFLPs, 43 SSRs, 1 hypothetical sex marker) were located on the 20 linkage groups (LGs) of the maternal and paternal maps, covering total map lengths of 706 and 616 cM, respectively, due to the presence of 16 common biparental SSR markers the homology of seven LGs between parental maps could be inferred. The progeny segregated quantitatively for alpha-acid content determined in the years from 2002 - 2006. Thirteen putative QTLs were identified on both maps. Possible homologies between QTLs for alpha-acid content detected in different years as well as in both maps could be established in at least three chromosomal regions. Previously reported genetic map was successfully saturated with additional SSR markers providing a practical tool for the development of marker-assisted selection programme in hop.

Keywords: *Humulus lupulus* L., alpha acid content, genetic mapping, quantitative trait loci, quality

Introduction

Hop production is important agricultural branch in Slovenia, intensive for more than 100 years. At the moment there are 1467 ha hop fields in production (out of 1784 ha of hop fields together), more than 95 % are planted with Slovenian varieties. The most established is Aurora, presenting 62 % of hop acreage, following Savinjski golding with 13,2 %, Bobek with 9,7 %, Celeia with 7,5 %, Magnum with 4,5 %, Taurus with 0,2 % and on 4,9 % of hop fields other varieties are grown and hybrids.

Hop breeding programme at the Slovenian Institute of Hop Research and Brewing (SIHRB) has over 50 years long tradition. The main purpose is to develop hop cultivars with improved quantity and quality (bitterness, aroma) of the yield, resistance against fungal diseases and pests, combining classical and molecular approaches. Regarding to all aims involved in breeding process different selection criteria and methods are included during the season. A general problem in developing new hop varieties is the long time required (at least ten years) especially because of the characteristics of hop plant.

The aim of the research was to saturate existed genetic map and identify new QTLs involved in expressing alpha acid content in hop.

Methods

The F₁ full-sib family was obtained by crossing the German variety 'Magnum' with the Slovene breeding line '2/1'. The analysis included 111 full-sib genotypes, 97 female and 14 male plants and the two parents (female/male ratio \approx 6,9 : 1). Male hops were assigned as bearing the hypothetical male-determining locus *M* (Seefelder et al., 2000).

Total genomic DNA was extracted from young leaves using a modified CTAB method according to Kump and Javornik (1996). The AFLP protocol was carried out according to Vos et al. (1995). The progenies of the family were selectively amplified with 18 *EcoRI/MseI* and 18 *PstI/MseI* primer pair combinations. Forty-six microsatellites developed by Jakše and Javornik (2001), Jakše et al. (2001), Štajner et al. (2004) and new not-published SSRs were added to the analysis.

The content of alpha-acids was measured in years 2002, 2003, 2004, 2005 and 2006 with the lead conductance value (LCV) method (Analytica EBC 1998).

Linkage analysis was carried out using the same procedure as reported previous (Cerenak et al., 2006) with JoinMap Ver. 3.0 programme (Van Ooijen and Voorrips, 2001). The map was constructed using the LOD value of 5.0 for the grouping of markers.

MapQTL version 4.0 (Van Ooijen et al., 2002) was used to identify and locate QTLs associated with alpha-acid content, by performing the non-parametric test of Kruskal-Wallis as well as both interval mapping (Lander and Botstein 1989) and multiple-QTL mapping (MQM; Jansen and Stam 1994). In the regions of the putative QTLs, the markers with the highest LOD values were taken as co-factors. The individual parental effects and interaction effect of the each putative QTL were calculated according to Knott et al. (1997).

Results and Discussion

Both types of polymorphic markers (AFLPs, SSRs) were tested for their inheritance pattern in mapping population. Segregation ratios were tested using the chi-square test and as suspected great proportion of markers did not fit the expected Mendelian ratios ($p < 0.01$). Nevertheless, 43 out of 46 SSRs were successfully mapped.

A total of 116 markers (86 AFLPs, 30 SSRs) were placed on the 'Magnum' map forming 8 major linkage groups, 2 triplets and 4 doublets which are assumed to be part of the other groups, defining 706 cM of total map distance. A total of 94 markers (64 AFLPs, 30 SSRs) were placed on the '2/1' map, defining 616 cM of total map distance, with an average interval of 11 cM between adjacent markers. Nine 'major' linkage groups, 3 triplets and 2 doublets were detected which are also assumed to be part of 'major' linkage groups. Due to the presence of 16 common biparental SSR markers the homology of seven LGs between parental maps could be inferred.

QTL analyses were carried out using data of alpha acid contents obtained in the years 2002, 2003, 2004, 2005 and 2006. None of the 5 distributions deviated significantly from the expected normal distribution. A LOD score of 3.0 was used to declare the presence of a QTL linked to alpha acid content. A total of 13 putative QTLs were determined in all 5 years, 7 were detected on female map and 6 on male map. Two putative QTLs were identified in 2002 (one on female and the other on male map) and one QTL was associated with alpha acid content in 2003. Four QTLs were determined in 2004 and also in 2006 while 2 QTLs were localised on the map associated with 2005.

Possible homologies between QTLs for alpha-acid content detected in different years as well as in both maps could be established in at least three chromosomal regions. The regions containing temporally stable QTLs will be saturated with additional markers for further fine-mapping.

The existing hop map (Cerenak et al., 2006) has been saturated further with additional SSRs and the homology of seven LGs between parental maps has been established providing a practical tool for the development of marker-assisted selection programme in hop.

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MOLECULAR METHODS IN RESISTANCE HOP RESEARCH

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Abstract

Hop downy mildew (*Pseudoperonospora humuli*) and hop powdery mildew (*Podosphaera macularis*) are the most important fungal plant diseases of hop. Resistance breeding is the best chance to eliminate economical losses. Hop breeding is very time-consuming. The use of molecular methods is very effective tool for breeders. We used conserve regions of R genes as PCR primers in direct PCR reactions or in combination with AFLP method. In our experiments, we found some amplified polymorphic products, which partially correlated with hop resistance to fungal disease. These sequences can be transferred to marker-assisted selection (MAS) in the future. We also successfully used molecular methods for identification of individual isolates of fungal pathogens. We also analysed the distribution of hop powdery virulence genes in the Czech Republic.

Keywords: hop downy mildew (*Pseudoperonospora humuli*), hop powdery mildew (*Podosphaera macularis*), resistance to fungal diseases, molecular methods, resistance gene analog (RGA), marker-assisted selection (MAS), breeding.

Introduction

Hops (*Humulus lupulus* L.) are regularly subject to infection through filamentous fungi. The infection sometimes results in severe loss of production as the plants wither. From fungal diseases, hop downy mildew (*Pseudoperonospora humuli* [Miabe & Takah.] Wilson) and actually hop powdery mildew (*Podosphaera macularis* [Braun]) belong to the most important hop fungal diseases in the CR with considerable economic effects. *Pseudoperonospora humuli* infects young shoots, leaves, flowers and cones, causing “basal spikes”, angular black leaf spots and brown discolouration of cones (Neve, 1991). *Podosphaera macularis* infects leaves and cones, causing humps, blisters, pale spots, distortion of cones and white mould. The individual strains were described – 7 strains in Germany, 1 strain in France, 3 strains in England and 2 strains in the USA (Seigner et al., 2002).

A use of resistant plant materials is one of the best possibilities how to defend from enhanced occurrence of fungal pathogens. Hop breeding is a base for obtaining fungal resistant plant materials. Based on current knowledge, it was researched, that mechanism of resistance and architecture of individual plant resistance genes to different pathogens are very similar (Takken and Joosten, 2000). Plant resistance to different pathogens is mainly based on mechanism gene to gene. A specific resistance gene (R) recognizes a pathogenic virulence gene (Avr), wherewith starts a mechanism of hypersensitive response (HR) leading to systematic acquired resistance (SAR) and considerable reduction of infection (Takken and Joosten, 2000). Recently, it has been also researched, that majority of R genes contain exact conserve regions, multiple distributed in plant genomes. They are mainly nucleotide binding site (NBS) and leucine-rich repeat (LRR), also Toll/interleukin receptor (TIR) and leucine zip (LZ) in some cases. Within the NBS domain of resistance proteins there are several, small, presumably functional, conserved domains, such as the P-loop, kinase domains, MHD, and GLPL motifs (Meyers et al. 2003). These conserved motifs have been utilised in the design of PCR-based cloning and mapping strategies to characterise R genes. We have used these motifs directly for searching and detection of close sequences to fungal resistance genes in hop.

Genetic resources are one of suppositions for successful breeding process. The utilization of appropriate resistance donor is depend on occurrence of individual strains of fungal

pathogens in the Czech Republic, because an evolution of pathogens still has continued together with hop varieties. A use of molecular biology techniques is a novel and high effective method for determination of individual fungal pathogens (Patzak, 2005). We used these methods for identification of individual strains and isolates. Based on our results, we will set up hop breeding in the Czech Republic.

Methods

Sample sources and DNA extractions

In our experiments, we used female plants of resistance varieties Nugget, Target, Serebjanka, Zenith and Challenger and male plants 99/1, 99/11, 99/16, 01/2, 01/3 and 01/9 from genetic resources of Hop Research Institute Co. Ltd. in Žatec. Model crosses were realized and F₁ progenies were evaluated for resistance to fungal diseases in field conditions. DNAs were isolated from young leaves according to Saghai-Marooof et al. (1984) modified for hop Patzak (2001). Hop downy mildew (*Pseudoperonospora humuli*) and hop powdery mildew (*Podosphaera macularis*) were obtained as mycelia from infested hop plants from different regions. DNAs were isolated from samples with DNeasy Plant Mini kit (Qiagen, FRG) according to manufacturer's instructions.

Molecular analyses

Molecular genetic analysis by Hayes and Saghai Marooof (2000) was carried out on DNAs from selected hop genotypes. Twenty-eight PCR primers, derived from conserve regions of R genes, were used in direct PCR reactions or in combination with AFLP method. DNAs were digested with restriction enzymes *TaqI* and *Apal* or *EcoRI* and *MseI*. AFLP adaptors were ligated to digested fragments. Sixty-four combinations were totally used in PCR reactions. Taq PCR master mix kit (Qiagen, FRG) was used for PCR reactions according to manufacturer's instructions. PCR amplifications were carried out in T-Gradient (Biometra, FRG) or Genius thermocycler (Techne, UK). After denaturation step 95°C 3 min, 35 cycles of amplification (94°C 30s, 54°C 60s, 72°C 90s) and 10 min at 72°C were performed. The minimum of two amplifications was always performed in order to check consistency. Sequencing vertical polyacrylamide gel (5% gel, 8M urea) electrophoresis was used for separating PCR products at 45W. Polyacrylamide gels were stained according to Promega (USA) silver staining protocol. Stained and dried gels were duplicated to opaque daylight film (Promega, USA). Polymorphic amplified products were extracted from gels, reamplified, cloned to vector pSC-A (StrataClone™ PCR Cloning Kit, Stratagene, USA) and sequenced (Genomac International Co. Ltd., Prague, CR).

CAPS (Cleaved Amplified Polymorphism Sequence) analysis was used for evaluation of fungal isolate. The nuclear rDNA, including 18S, ITS1, 5.8S, ITS2 and 26S, were amplified according to Patzak (2005). Amplified products (10 µl) were digested with different restriction enzymes in 1x restriction buffer 2 hours at recommended temperature. Horizontal agarose gel (2% gel) electrophoresis was used for separating CAPS products in TBE buffer at 3 V/cm. Agarose gels were stained with ethidium bromide, visualised by UV and stored to PC via CCD camera (UVP Grab it system, UVP, UK). Virulence tests of hop powdery mildew were performed according to (Seigner et al., 2002).

Results and discussion

In our experiments, we used bulk segregate analysis (BSA) of F₁ progenies for molecular research of hop resistance to fungal diseases. Hop plants of realized model crosses were separated to two groups in dependency on resistance or susceptibility to fungal diseases, respectively. Parents' plants and bulked groups were used for molecular analyses. Amplified products were not found in all direct PCR reactions of primers, derived from conserve regions of R genes. Amplified polymorphic products in different resistance sources were found for two primer combinations: first for nucleotide binding site (NBS) domains (Deng et

al., 2000) and second for leucine-rich repeat (LRR) of *Xa21* gene, rice resistance to *Xanthomonas oryzae* (Chen et al., 1998). Both fragments were sequenced but any homology with known resistance gene sequences in GenBank was not found by BLAST analysis. The obtained sequences have not been tested for possibility to use them as molecular markers of resistance yet.

In next experiments, we used PCR primers, derived from conserve regions of R genes, in combination with AFLP method (Hayes and Saghai Maroof, 2000). Amplified polymorphic products were found in all primer combinations but mostly like single genotype differences. Amplified polymorphic products, partly correlated with fungal resistance, were found in *Apal* and *TaqI* digested samples for two primer combinations: RGA3+T-ACC a RGA5+A-AC. Both fragments were sequenced but also any homology with known resistance gene sequences in GenBank was not found by BLAST analysis. The obtained sequences were used for design of specific PCR primers. Unfortunately, the presence in all hop genotypes was proved by PCR reactions and therefore they cannot be used as molecular markers of resistance. One amplified polymorphic product, closely correlated with fungal resistance, was found in *EcoRI* and *MseI* digested samples for primer combination RGA3+M-CTC. Fragment was sequenced and partial homology with resistance cluster of potato (*Solanum tuberosum*) was found by BLAST analysis. The obtained sequence has not been tested for possibility to use them as molecular markers of resistance yet.

Our research is only on start, when we know that resistance to fungal diseases depends on many factors and genes. Resistance to hop powdery mildew is probably controlled only with one gene, but there are at least 6 different resistance genes (Darby, 2001). Resistance to hop downy mildew is probably under polygenic system control (Neve, 1991). Though, correlation between both resistances is high ($r = 0,8$) (Nesvadba et al., 1999). Molecular genetic methods are suitable for effective characterization of breeding materials and selection of possible resistance hybrid plants in breeding process. Our aim is to prepare effective marker assisted selection (MAS) system for hop breeders.

For effective breeding process is also necessary to know which individual strains of fungal pathogens occur in the Czech Republic. We used molecular methods for analyses of powdery and downy mildew isolates, respectively. Sequence rDNA differences were found between Czech and Slovak powdery mildew isolates by four restriction enzymes (Figure 1). Sequence rDNA differences were found for one (Želeč) downy mildew isolate by two restriction enzymes (not shown). This isolate was the most distant from others. We proved that CAPS method could be used for identification of individual fungal isolates. We also tested the virulence of hop powdery mildew Czech isolates by *in vitro* tests according to (Seigner et al., 2002). Results are summarized in Table 1. From results It is evident that Czech hop powdery mildew isolate successfully overcame the majority of resistance genes. Resistance gene Rb is at least effective. Czech hop powdery mildew isolate differed from other described world isolates. England isolate E8 and German isolate HU9 were the most similar, but E8 hasn't overcome resistance genes R4 and R6 and HU9 hasn't overcome resistance gene R1 (Seigner et al., 2002). Resistance genes R2 and R6, which has been overcome with some isolates in Germany, are the most effective in the Czech Republic and genetic resources with these genes are the most suitable for resistance hop breeding.

Acknowledgements

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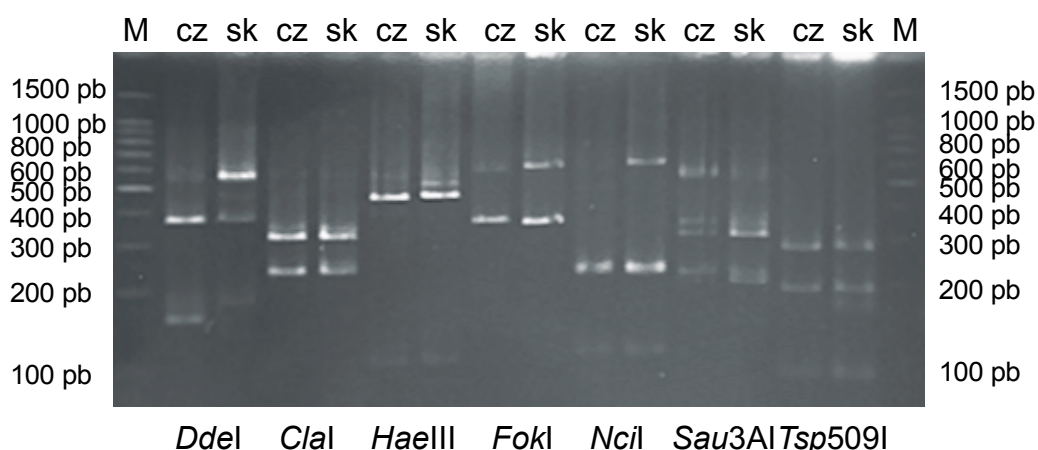
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Table 1: Percentage of hop powdery mildew infection on individual explants and plants after artificial infection.

Hop genotype	Resistance gene	<i>In vitro</i> 1 (% of infection)	<i>In vitro</i> 2 (% of infection)	Glasshouse (% of infection)	Total average
Northern Brewer	R0	80	80	50	70.0 %
Zenith	R1, (Rb)	20	60	80	53.3 %
Wye Target	R2	10	0	-	5.0 %
Wye Challenger	R3, (Rb)	80	80	-	80.0 %
Serebrjanka	R4	-	-	30	30.0 %
Nugget	R6	20	10	-	15.0 %
Buket	Rb	80	20	20	40.0 %
Yeoman	Rb	100	100	-	100.0 %

Figure 1: Analysis of CAPS fragments of hop powdery mildew rDNA in 2% agarose gel. cz – Czech isolate, sk – Slovak isolate, M – 100 bp Ladder (Promega, USA).



cDNA-AFLP MAKERS FOR POWDERY MILDEW RESISTANCE IN HOPS (*HUMULUS LUPULUS* L.)

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Abstract

Powdery mildew (PM) is one of the most important fungal diseases of hop (*Humulus lupulus* L.). The wild hop WH18 from the Eifel region revealing highly effective PM resistance was used to create a mapping population segregating for the WH18 resistance. Resistant and susceptible individuals from this population were used for the differential display approach in combination with the cDNA-AFLP analysis. For this purpose an efficient protocol for m-RNA isolation had been established. Subsequently, for the very first time in hop cDNA profiles of resistant or susceptible hops with or without contact to PM were screened for pathogen-induced gene expression. Transcript derived fragments (TDFs) are currently searched for sequence homologies to published resistance genes from other crops. In this way, resistance markers should be developed which are directly associated to genes responsible for the pathogen recognition and resistance reaction.

Keywords: hop powdery mildew, resistance, molecular markers, cDNA analysis

Introduction

Hop powdery mildew (PM) caused by *Podosphaera macularis* ssp. *humuli* [Braun] affects hop production in Europe and also in the USA. Infections on susceptible varieties result in significant loss of yield and quality. Thus, especially in years with highly conducive conditions for PM infections enormous amounts of pesticides and specific labor-intensive cultural practices are necessary to ensure high-yield and top quality hops. In order to reduce costs, hop growers are looking for PM resistant hop varieties which allow a cost-efficient, environmentally sound production of hop while being able to provide superior brewing quality with no harmful residues of pesticides to the brewing industry. Thus, in recent years, breeding for resistance to PM has been first priority at the Hop Research Center Huell.

The efficiency of developing PM resistant varieties can be increased significantly by using molecular markers. These markers based on the classical AFLP method have already being developed for various PM resistance genes (Seefelder et al., 2006). Only recently also AFLP markers have been identified in so far unexploited wild hop genotypes which confer broad resistance to all strains of PM currently known.

In this project differential display and the cDNA-AFLP technique is applied for the very first time in hop to identify resistance markers. The cDNA-AFLP method is an efficient and economical method to display whole transcript profiles of single tissues, particular developmental stages or other inducible characters (Bachem et al., 1996). cDNA based markers are highly informative and most reliable in giving information about DNA regions expressed after fungal attack to ward off pathogen infections.

Material and Methods

Since 2001 wild hops collected and maintained at the Hop Research Center Huell have been screened extensively for PM resistance in the greenhouse, the laboratory and later in the fields. The aim was to identify new resistances which cannot be overcome by pathotypes of *Podosphaera macularis* of the various virulence types (v1-v6, and vB) known so far. After

extensive screening over three years in the greenhouse and in the laboratory 56 very promising wild hops could be identified that proved resistance to all PM strains available so far. A wild hop from the Eifel region (WH18/97/003) showing resistance in all tests for more than four years was chosen as crossing partner in order to broaden the genetic basis for PM resistance. In this way five mapping populations were created which are suitable to develop molecular markers for the specific PM resistance gene/s. The mapping populations have been tested for their resistance in the laboratory using the detached leaf assay. Based on these resistance data two resistant and two susceptible genotypes from the progeny of Opal x WH18/97/003 were selected for the cDNA-AFLP analysis. These genotypes were propagated *in vitro* and after four weeks inoculated with a PM isolate of the v3, v4, v6, and vB virulence type (BU10, Seigner et al., 2002). Leaf samples for RNA isolation were taken at different points of time according to the developmental stages of the fungus during infection.

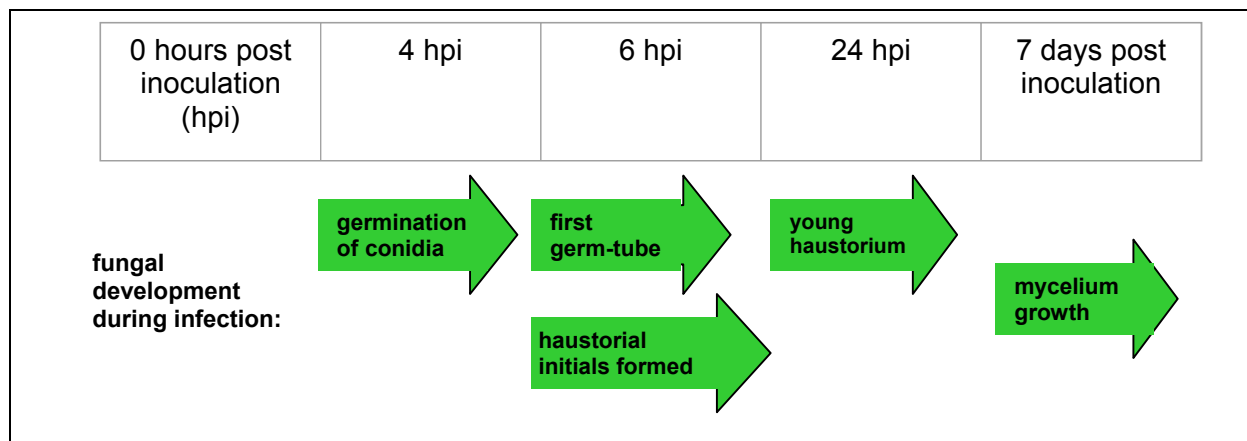


Fig. 1: Points of time for taking samples according to the developmental stages of the fungus (Godwin et al. 1987)

RNA isolation and cDNA analysis were performed using the protocol described by Herz et al. (2003) with minor modifications.

PCR products were cloned using the QUIAGEN PCR Cloning^{plus} Kit following the manufacturers protocol. Plasmids were purified using the NucleoSpin® Plasmid Kit (Macherey-Nagel) and transferred to a commercial sequencing service (MWG, Ebersberg, Germany). Sequence alignment and analysis were performed with the Vector NTI® Advance 10 software (InforMax, Bethesda, USA).

Results

Gene expression patterns of PM inoculated resistant and susceptible hop seedlings have been compared at different points of time using 50 AFLP primer combinations. On average, 15 bands were detected per primer combination. A total of approximately 750 expressed fragments could be identified. The pattern of expressed genes at the beginning of the PM inoculation test (Fig. 2; 0 hpi) represents the “normal” set of active genes in resistant and susceptible plants, respectively. Based on the assumption that disease resistance involves the early recognition of the invading pathogen and the activation of defense response genes, the cDNA-AFLP patterns of resistant plants were screened for newly expressed fragments which occur 4, 6, 24 and 168 hours after fungal attack and which are missing in susceptible ones. Fragments with similar intensity over the whole time scale and specifically expressed in only one genotype were detected, presumably representing genotype-specific fragments (Fig. 2). Some fragments only occurred in the resistant plants. Other TDFs were detected in the susceptible plants seven days after inoculation. These fragments are assumed to derive from the PM fungus. At different points of time fragments with increasing intensity in their expression could also be observed (Fig. 2). The time points for taking samples for the RNA isolation in the first experiments were chosen taking into account the findings of Goodwin et

al. (1987) on the resistance of the English cv. 'Wye Target' towards hop powdery mildew and the observations of Hückelhoven (2005) with barley powdery mildew. About 20 reproducible differences in the expression patterns occurred between the resistant and the susceptible plants. In the response reaction of the resistant plants towards PM, fragments could be detected which were early up-regulated at 4 and/or 6 hours respectively or late induced 24 hours after inoculation. Most of these expressed genes were down-regulated 7 days after inoculation.

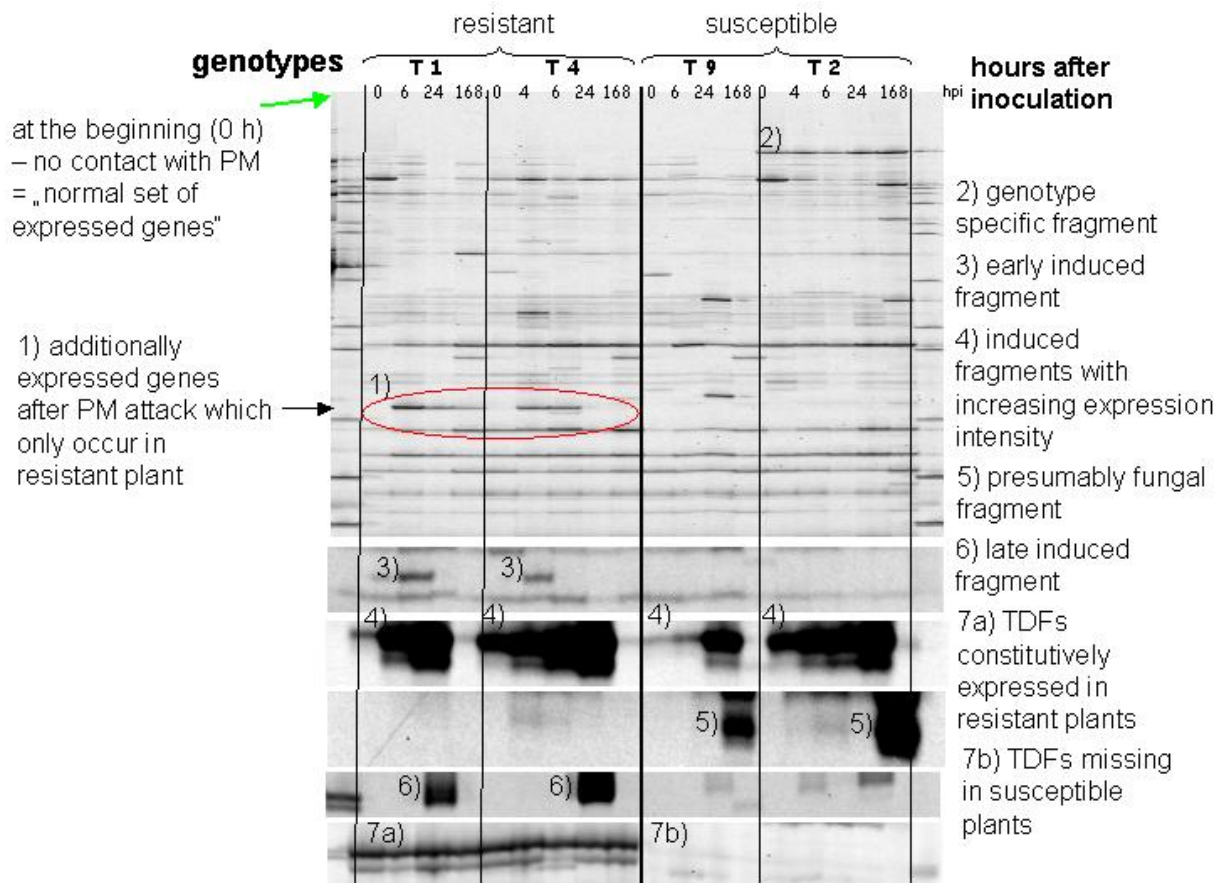


Fig. 2: cDNA-AFLP pattern of two resistant and two susceptible genotypes. Four to 24 hours after PM attack genes are newly expressed which only occur in the resistant plants.

Discussion

These studies presented were initiated to identify genes that are differentially regulated following the inoculation of PM resistant and susceptible hops with PM spores via cDNA-AFLP analysis. Based on the assumption that resistant in contrast to susceptible plants switch on genes involved in pathogen recognition and defense reaction, some of these activated genes are of great interest as reliable markers for selection and as a basis to understand the molecular background for PM resistance in hops. The cDNA-AFLP technique was successfully applied in similar investigations on barley and powdery mildew (Eckey et al., 2004). In this project for the first time in hop, differential display and the cDNA-AFLP technique are applied to identify resistance markers. In contrast to other fingerprinting techniques the cDNA-AFLP analysis offers the following advantages: cDNA-AFLP markers detect active regions of the genome and give information about genes expressed after fungal attack. Therefore it is possible to identify genes related to pathogen defense or resistance. By using the cDNA-AFLP, it was possible to study transcriptional changes with no prior assumptions about which genes are induced or repressed. That is especially important in these studies, because till now nothing is known about the host/pathogen interaction of the

wild hop WH18 and the PM fungus. The time points in this first study were chosen according to the investigations of Goodwin et al. (1987) and Hückelhoven (2005). In order to widen the knowledge of genes which are up or down regulated between 24 hours and 7 days after inoculation, an additional point of time at 48 hours will be examined in continuative studies. Furthermore, at every point of time a not inoculated hop leaf will be selected as reference to exclude other reactions like stress and wounding as inducing factors for newly expressed genes.

Out of the 50 primer combinations about 750 fragments could be observed. Most of the differentially expressed genes identified in this work were up-regulated after the inoculation with PM. The cDNA-AFLP technique also revealed differences in the expression pattern between resistant and susceptible hop at the different points of time. From the abundance of cDNA-AFLPs expressed after fungal attack the identification of genes related to pathogen defense, resistance or pathogen recognition is quite difficult, since it can be assumed that only about 1 to 2 % of all expressed sequences are associated with the resistance reaction (Fristensky et al., 1999).

Only recently, studies started with 10 promising TDFs induced by PM in resistant hop plants. These TDFs were cloned and BLAST searches were performed to look for similarities and homologies of the sequences of the clones to sequences of genes involved in host-pathogen-interactions or defense reactions in other crops in the EMBL and GenBank database. Sequence similarities of TDFs to a *Malus x domestica* cDNA clone, an *Ulmus americana* cDNA and to the *Vitis vinifera* genome sequence were identified, but so far among the TDFs investigated no genes related to pathogen defense or resistance could be found.

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GENOME ANALYSIS – AN IMPORTANT TOOL TO SUPPORT CLASSICAL HOP BREEDING



Bavarian State Research Center for Agriculture

Institute of Crop Science and Plant Breeding

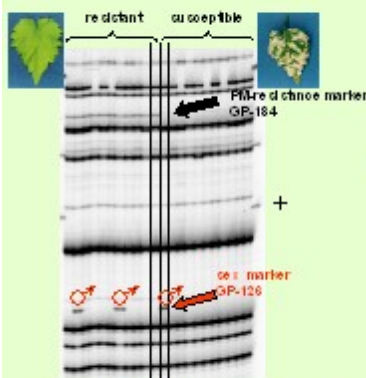


Genome Analysis - an Important Tool to Support Classical Hop Breeding

S. Seefelder, R. Seidenberger, A. Lutz, E. Seigner

Molecular markers for powdery mildew resistance and sex determination

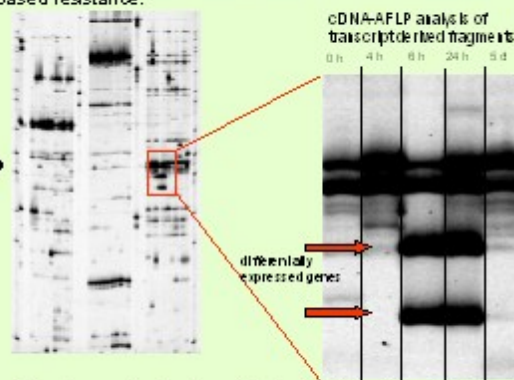
The main focus of the genome diagnostic work at the Hop Research Center Hüll is to identify molecular markers for known and new resistance genes for powdery mildew (PM). So far using the AFLP technique several markers in close linkage to the R2 gene from Wye Target and also sex differentiating markers have been identified. In addition to this efficient method, a differential display approach in combination with the cDNA-AFLP method was performed in order to detect markers for genes directly related to the resistance reaction. Moreover by applying this technique there is also the chance to get more information about the mechanism of the R2 gene based resistance.



One AFLP primer combination reveals a marker for PM resistance and a sex specific marker for males as well.



The detached leaf assay provides reliable resistance data as basis for the AFLP and cDNA-AFLP analysis.



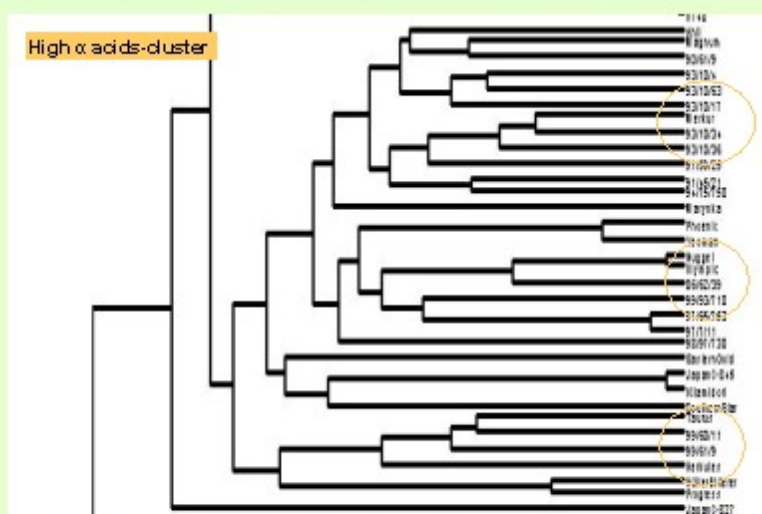
Wye Target was *in vitro* inoculated with the avirulent PM isolat BU10. For RNA isolation leaves were harvested after different points of time. Transcript derived fragments expressed 6-24 h after fungal attack are assumed to be involved in the defense reaction.

Estimation of the genetic diversity of important breeding lines and varieties

Using the AFLP technique the genetic relationship between the Hüll breeding lines and the world hop germplasm could be determined. Knowledge obtained from these studies allows a specific, more precise selection of crossing partners for special breeding strategies. In addition, the genetic fingerprint of hop varieties can help to ensure high quality which is crucial to the hop and brewing industry.

The figure shows a cluster with varieties and breeding lines characterized by high alpha acids content. The breeding lines 93/10/17, 93/10/34, 93/10/36 and 93/10/63 are closely related to their sister the Hüll variety Merkur. In addition, Herkules, the recently released Hüll high alpha variety, is grouped in the direct vicinity to its mother Taurus.

The extensive AFLP database built up during this work is also useful for a reliable and rapid identification of varieties.



Part of a dendrogram: Main cluster with hops containing high alpha acids content

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TOWARDS THE GENETIC MAP FOR VERTICILLIUM WILT RESISTANCE

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Verticillium wilt is an important disease of many worldwide plant species, including hop. After infection, hop plants exhibit mild or lethal disease syndromes, indicating the quantitative nature of the plant response (Isaac and Keyworth, 1948). The disease in its lethal form causes significant problems in hop production and field management (Radišek et al. 2006). Tagging genomic regions responsible for tolerance could facilitate the breeding of resistant cultivars and provide a deeper understanding of the molecular mechanisms of tolerance against this important hop disease.

Three genomic libraries enriched for microsatellite repeats were constructed using a mixture of hop genomic DNA digested with several restriction enzymes to obtain representative coverage of the genome (Jakše and Javornik, 2001). Sequencing of the microsatellite enriched clones revealed high degree of association of particular sequences with repetitive part of the hop genome. Two-hundred and fifty microsatellite specific primer pairs were developed and tested for amplification and segregation. Inheritance and linkage studies were conducted with developed genomic microsatellites, 12 AFLP primers combinations and SCAR markers in F₁ progeny, including 152 individuals of a cross between hop cultivar 'Wye Target' and male breeding line 2/1. This particular cross was chosen due to its distinct degree of tolerance to Verticillium wilt. Cultivar 'Wye Target' is one of the most tolerant cultivars against Verticillium wilt, while breeding line 2/1 shows severe symptoms soon after infestation. Linkage analysis was performed using the JoinMap Ver. 3.0 programme (Van Ooijen and Voorrips, 2001). The map was constructed using the LOD value of 4.0 for the grouping of markers. The Kosambi mapping function was used to convert recombination data to map distances.

Verticillium albo-atrum hop isolate, which causes the lethal form of Verticillium wilt on hop was used for phenotypical evaluation of plants. Twelve plants of each genotype were inoculated by dipping their root system into a suspension of conidia, and they were grown in a growth chamber. Symptoms were assessed three 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. Data for tolerance against wilt were obtained for 94 plants; other offspring are still in the testing phase. Currently, segregation data are available for 115 microsatellite and 3 SCAR markers, addition of 350 AFLP markers is in progress. The maternal map consisted of 100 markers mapped on 8 linkage groups defining 202 cM of total map distance. The paternal map included 59 markers assigned to 7 linkage groups covering 183 cM of hop genome. The two hop maps, after being completed, will be used for detection of QTLs involved in Verticillium wilt.

Keywords: mapping, microsatellites, enriched libraries, Verticillium wilt

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USE OF DIVERSITY ARRAY TECHNOLOGY (DART) FOR GENOTYPING *HUMULUS LUPULUS* L.

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Abstract

Understanding the distribution of genetic diversity among individuals, populations and gene pools is crucial for the efficient management of germplasm collections and breeding programs. Diversity Arrays Technology (DART - www.diversityarrays.com) is a micro-array based DNA marker technique for genome wide discovery and genotyping of genetic variation. DART potentially allows simultaneous scoring of thousands of restriction site polymorphisms between genotypes and does not require DNA sequence information or site specific oligonucleotides. An international consortium (Australia, USA, UK, Slovenia) was formed to screen 92 accessions of hop and outgroup (*Cannabis sativa*) using DART. Accessions were sourced from Europe, North America, Asia and Australia, and DNA was extracted from three tissue types (leaf, bud, and tissue culture). Sixty two cultivated varieties were included, while the remaining 30 accessions were wild hops from Europe and North America including examples of *Humulus lupulus* var. *lupuloides*, *H. lupulus* var. *pubescens* and *H. lupulus* var. *neomexicanus*. The sample set was dominated by female material, with only 8 male accessions (from Europe and North America) included. Most of the material was diploid with some triploid varieties were incorporated. The cultivars in the sample set included key current and historical, high and low α -acid, aroma, powdery and downy mildew resistant and susceptible varieties. From this sample set, DART revealed 730 polymorphic markers, with high reproducibility (approaching 100%) and an average call rate of 97.6%. There was little or no effect of source tissue on the results obtained. Approximately 80% of the markers reflect the differences between truly wild and cultivated material. Both the topology of a UPGMA dendrogram, and principle co-ordinates analysis showed close agreement with the current understanding of molecular phylogenetics and phylogeography in the genus *Humulus*. Clear distinction can be made both within and between material of European, North American and Asian origin. At a finer scale, samples known to be related (e.g. Brewers Gold, OB21 and Northern Brewer), and samples from a single variety known by several synonyms (e.g. Clusters and Ging Dao, or Saazer and Osvald's clone 72) cluster together. In conclusion, the reproducibility of the DART markers, the level of genetic variation detected in hop using this system and the resolution of known genetic affinities are strong positive signs that DART can provide the mechanism for future exploration of marker-trait associations in the interest of developing marker assisted selection technologies to improve the efficiency of genetic improvement in hop.

CONSTITUTIVE EXPRESSION OF A GRAPEVINE STILBENE SYNTHASE GENE IN TRANSGENIC HOP (*HUMULUS LUPULUS* L.) YIELDS RESVERATROL AND ITS DERIVATIVES IN SUBSTANTIAL QUANTITIES

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Abstract

We transformed hop (*Humulus lupulus* L.) plants of the Tettnang variety with a gene encoding for stilbene synthase (STS) from grapevine. Under the control of the constitutive 35S cauliflower mosaic virus promoter, expression of the transgene resulted in accumulation of resveratrol and high levels of its glycosylated derivatives in leaves and inflorescences. Piceid, the predominant derivative reached a concentration of up to 560 µg/g f.w. in hop cones, whereas no stilbenes were detected (threshold 0.5µg/g f.w.) in non-transformed controls (wildtype). In transgenic plants the amounts of alpha- and beta-acids, naringenin chalcone, and prenylated flavonoids did not change significantly when compared to non-transformed plants. Transgenic plants showed normal morphology and flower development as did the non-transformed controls. Our results clearly show that in hop constitutive expression of *sts* did neither interfere with plant development nor with the biosynthesis of secondary metabolites relevant for the brewing industry. Flavonoids like resveratrol could act as phytoalexin thus increasing enhanced pathogen resistance. Their function as antioxidant could provide beneficial properties for health, and open new venues for metabolic engineering of other flavonoids.

Keywords: Phytoalexin, antioxidant, prenylation, flavonoid, *Agrobacterium tumefaciens*

Introduction

Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin and antioxidant produced by a restricted number of plants such as grapes and peanuts (Douillet-Breuil et al. 1999, Chung et al. 2001). The various biological activities of stilbenes make it desirable to enhance their production by metabolic engineering. Additionally, the expression of only a single transgene is sufficient to produce resveratrol (and its derivatives) in plants (Hain et al. 1993). Interestingly, enhanced disease resistance of *sts*-transgenic plants against pathogens was observed (Hipskind et al. 2000, Yu et al. 2005). Furthermore, antioxidant levels increased in tomatoes transformed with the *sts* gene (Giovinazzo et al. 2005).

Here we report on the first transformation of hop (*Humulus lupulus* L.) with the *vst1* gene from grapevine expressed under control of the constitutive 35S cauliflower mosaic virus promoter (Horlemann et al. 2003, Schwekendiek et al. 2005). The impact of this transformation on the secondary metabolism of hop has been analyzed with particular focus on resveratrol derivatives and on important hop flavonoids like naringenin-chalcone, xanthohumol (X), desmethylxanthohumol (DMX), and 8-prenylnaringenin, as well as alpha- and beta-acids.

Methods

Transformation of inter-nodal stem segments of Tettnang hop was performed as described previously (Schwekendiek et al. 2005). Then shooting was induced by transferring them to standard growing conditions either in the greenhouse or transplanted to a containment facility outdoors in 2004. In spring and fall integration of the transgenes was verified by PCR. By Southern analysis 4 independent events were detected from 7 transgenic clones of 2 independent experiments. RNA was isolated from lupulin glands of wildtype and transgenic hop. RT-PCR of transgenic and non-transformed plants, was performed using *sts* specific primers. Flavonoids, hop acids, and stilbenes were analyzed from leaves and isolated inflorescences of transgenic plants and non-transformed Tettnanger grown outdoors in 2004 and 2005 (de Keukeleire et al. 2003). Resveratrol and piceid were used as standards allowing detection of both compounds (threshold approx. 1ppm in plant extracts). Identification of purified compound was done by NMR spectroscopy and APCI-LC-MS.

Results

In two independent experiments we transformed 1000 inter-nodal stem segments each of the hop genotype "Tettnanger" by co-cultivation with *Agrobacterium tumefaciens* carrying a binary vector with the *vst1* cDNA sequence from grapevine (Melchior and Kindl 1991) under control of the 35S promoter of cauliflower mosaic virus (CaMV). The transformation efficiency of the two experiments was 0.4% (explant to field stage). Southern analysis revealed 4 independent events with each harbouring a single copy of the transgene. These plants were numbered and had originally emerged from the following sources: 137, 371, 424 (experiment A; one cluster of regenerates from one stem segment), 138, 423 (experiment A; regenerates from stem segments, from a single petri dish different from the former); 160, 408 (experiment B; individual regenerates from one stem segment each, originating from 2 separate petri dishes). The expression of the *sts* transgene was analyzed by RT-PCR in ripe cones of transgenic plants. In all transgenic plants the expected amplicon size of 714 bp was detected. No signals were detected in the non-transformed controls.

Implications for other metabolic processes due to the expression of the *sts* gene in transgenic plants were analyzed by metabolic profiling of transgenic hop plants and non-transgenic controls grown in the outdoor plot during 2004 and 2005. Compared to non-transgenic plants, extracts from bracts of transgenic plants showed at least six additional peaks (A-F) in HPLC analyses (Fig. 1). Spectroscopic analysis identified trans- and cis-piceid (peak B, D) and trans- and cis-resveratrol (peak E, F). Peak A contained a diglycosylated stilbene with spectroscopic characteristics of astringin whereas the structural identity of an additional stilbene in peak C was not fully clarified due to low sample quantity. Trans-piceid quantities in dried bracts ranged from 2005 $\mu\text{g/g}$ d.w. - 2238 $\mu\text{g/g}$ d.w. being equivalent of 411 to 458 $\mu\text{g/g}$ f. w. of bracts, respectively. Cis-piceid was present in lower amounts reaching approx. 20% of the trans-isomer. The overall concentration of stilbenes in bracts reached 480 – 560 $\mu\text{g/g}$ f.w. None of the stilbene derivatives was detected in HPLC analysis of non-transformed wild-type plants of Tettnanger. Trans - piceid was also detected in true leaves of transgenic hop (average 195 $\mu\text{g/g}$ d.w.). Extracts from mechanically separated trichomes of leaves and bracts showed small amounts of trans-piceid besides the expected hop acids and prenylated flavonoids. These results indicate that the activity of stilbene synthase was constitutively expressed in all tissues under the regulation of the 35S promoter. Prenylated flavonoids were present in non-transformed and transgenic hop: desmethylxanthohumol (DMX) 0.4 mg/g - 0.9 mg/g d.w. and xanthohumol (X) 1.0 mg/g - 3.4 mg/g d.w. Except for the lower values in clone #160 there were no significant differences between transgenic and non-transformed plants. Likewise, hop acids showed 4 major peaks corresponding to the α -acids (cohumulone and n- and adhumulone) and the β -acids (colupulone and n- and adlupulone), respectively. Except for clone #160 no significant differences were found in the relative composition of α - and β -acids.

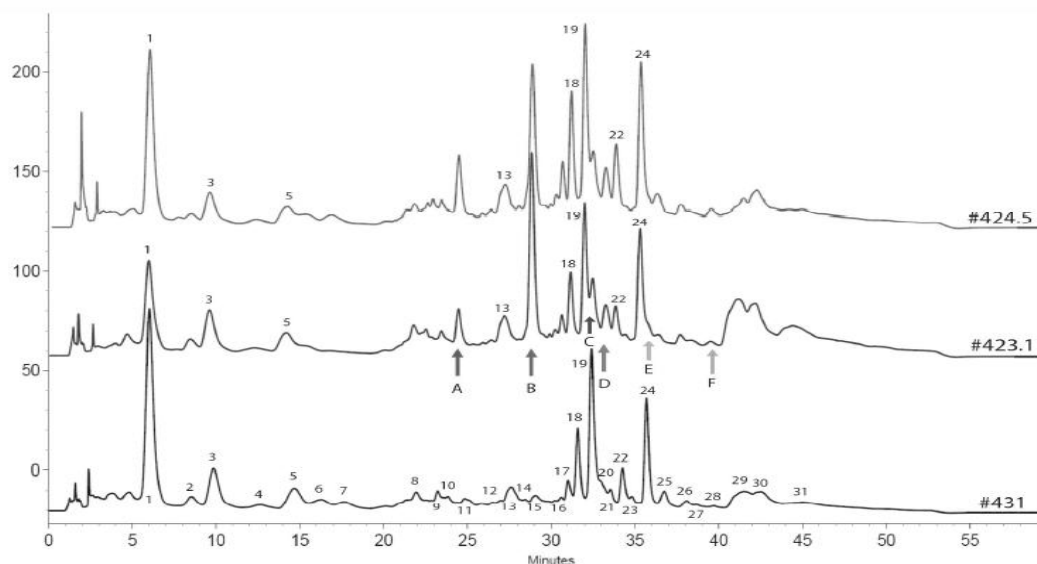


Figure 1. HPLC analysis of secondary metabolites in STS transgenic hop

Diagrams of HPLC (A320nm) with MeCN extracts from bracts of a non-transformed control (#431) and a transgenic clone #424 (event 1) and #423 (event 2). A-F indicate peaks present only in transgenic plants.

Table 1. Content (mg/g) of prenylated flavonoids and hop acids in dried hop cones from a non-transformed control and seven transgenic plants of 4 independent events.

Sample	DMX	X	Total α -acids	Total β -acids	Total flavonol glycosides (%)
Event 1 exp 1 (#137)	0.61 \pm 0.01	3.00 \pm 0.01	33.33 \pm 1.14	33.23 \pm 1.038	99 (+/- 28)
Event 1 exp 1 (#371)	0.70 \pm 0.01	3.00 \pm 0.010	40.65 \pm 0.94	30.00 \pm 0.565	131 (+/- 15)
Event 1 exp 1 (#424)	0.88 \pm 0.01	3.3 \pm 0.007	51.36 \pm 1.09	38.27 \pm 0.917	117 (+/- 20)
Event 4 exp 1 (#138)	0.86 \pm 0.02	3.4 \pm 0.024	47.33 \pm 1.41	40.35 \pm 0.850	114 (+/- 11)
Event 4 exp 1 (#423)	0.81 \pm 0.01	3.3 \pm 0.014	46.80 \pm 0.82	35.93 \pm 0.427	117 (+/- 17)
Event 2 exp 2 (#408)	0.73 \pm 0.01	2.24 \pm 0.008	25.43 \pm 0.21	35.10 \pm 0.457	133 (+/- 14)
Event 3 exp 2 (#160)	0.43 \pm 0.01 ^b	1.04 \pm 0.008 ^b	10.88 \pm 0.13 ^b	13.10 \pm 0.260 ^b	76 (+/- 12)
Non-transformed control	0.82 \pm 0.02	2.2 \pm 0.047	38.23 \pm 0.91	28.24 \pm 0.694	100 (+/- 25)

Discussion

In transgenic hop described here high levels of resveratrol were found which was quantitatively glycosylated (piceid). Piceid exceeded the un-glycosylated form by a factor of 100 or more. In spite of the constitutive expression of *sts* in hop, leaves contained only approx. 1/10 of the quantity of stilbenes present in mature bracts. Lupulin glands contained only trace amounts. No detrimental effects on transgenic plants were found as had been reported elsewhere (Fischer et al. 1997). Likewise, high stilbene content in bracts of cones of transgenic hop did not impede other agronomically important flavonoids and hop acids. Hop was not known to be a natural source for stilbenes until very low quantities were reported recently

from several North American hop cultivars (Jerkovic et al. 2005). Until now no stilbenes were found in European varieties like in non-transformed Tettnanger (detection limit 0.5 µg/g d.w.).

Currently we study if *sts* transformed plants display an increased resistance against pathogens. Furthermore, this study has shown that agronomically as well as pharmacologically important quantities of stilbenes in hop may be produced by genetic transformation enabling new venues for metabolic engineering of those secondary metabolites.

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PRODUCTION OF POWDERY MILDEW RESISTANT HOPS VIA GENE TRANSFER

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Abstract

The chitinase gene *HCH1* deriving from the powdery mildew resistant hop cultivar 'Zenith' was inserted into the genomes of two powdery mildew susceptible hop cultivars by *Agrobacterium*-mediated transformation. Transgenic hop plants were confirmed using PCR and RT-PCR. First tests for resistance to powdery mildew revealed that the transgenic hops carrying the chitinase gene showed enhanced levels of resistance.

Keywords: hop powdery mildew, resistance, chitinase, genetic engineering

Introduction

Genetic engineering is an innovative strategy to improve disease resistance. An approach most widely used was to over-express pathogen related (PR) proteins such as chitinases. Plants constitutively expressing different isoforms of chitinase genes showed enhanced resistance to various fungal diseases. For example increased resistance to powdery mildew could be achieved by the expression of a class I rice chitinase gene in strawberry and in grapevine plants (Asao, et al., 1997; Yamamoto et al., 2000).

A class I chitinase (*HCH1*) precursor deriving from the PM resistant English hop cultivar 'Zenith' was identified by Henning and Moore (1999) based on its homology to a pea endochitinase gene. Due to its specific sequence it was suggested that this *HCH1* gene may be involved in the resistance reaction of cv. 'Zenith' to powdery mildew.

Here we show the successful stable transformation of two different PM susceptible hop cultivars 'Hallertauer Mfr.' and 'Saazer' expressing the endochitinase gene *HCH1* deriving from the PM resistant hop variety 'Zenith' to enhance resistance to hop powdery mildew *Podosphaera macularis* ssp. *humuli* via genetic engineering.

Material and Methods

To induce the transcription of the hop endochitinase gene, plantlets of *in vitro* grown 'Zenith' have been infected with a specific strain of *Podosphaera macularis*. Seven to ten days after incubation at room temperature the *in vitro* infested plants were harvested, placed in liquid nitrogen and ground thoroughly with a mortar and pestle. RNA extraction followed with the RNeasy Plant Mini Kit (Qiagen) as described by the producer.

In total three PCR primer pairs were designed complementary to the sequence of the hop endochitinase *HCH1* gene (Henning and Moore, 1999) to meet specific requirements (Tab. 1). For the RT-PCR reaction from 'Zenith'-RNA primer pair A was deployed. 1 µg of total RNA was reversely transcribed and the cDNA was amplified through a one step RT-PCR reaction with Super Script Platinum Taq (Invitrogen) as described by the supplier using the following cycling conditions: RT 1 cycle - 50°C - 30 min; PCR 1 cycle - 94°C - 2 min, 40 cycles - 94°C - 30 sec - 55°C - 30 sec - 72°C - 1 min, 1 cycle 72°C - 7 min.

Re-amplification of the resulting PCR fragment was performed with a *Pwo* Polymerase (Roche) by adding an additional guanidine base to each of the 3'-ends of both primers and by raising the annealing temperature to 57°C (primer pair B). This amplicon was then sub-cloned into the pGEM-T vector (Promega) and suitable restriction sites were attached to the cloned *HCH1* cDNA by another PCR reaction deploying primer pair C (upper primer with 5'-*EcoRI* restriction site and lower primer with 5'-*BamHI* restriction site). The annealing temperature was finally raised to 66-68°C. This PCR reaction yielded 2-3 bands. The 948-bp-band was cut out of the agarose gel and cleaned up with QiaExII Gel Extraction Kit (Qiagen).

For a proper cleaving efficiency close to the termini of the PCR fragments a further sub-cloning step into a pGEM-T vector (Promega) was performed. To ensure an accurate cDNA sequence the amplicons were repeatedly sequenced (MWG Biotech).

Table 1: List of PCR primers for the amplification and detection of the HCH1 gene applied to RT-PCR of cv. 'Zenith' (primer pair A), re-amplification and detection of transgenes by PCR and RT-PCR (primer pair B) and attachment of restriction sites (primer pair C).

	upper primer	lower primer
primer pair A	5' atgaagaagtggtgtattattgt 3'	5' ctagccgaaaggagtttgatt 3'
primer pair B	5' atgaagaagtggtgtattattgt <u>g</u> 3'	5' ctagccgaaaggagtttgatt <u>g</u> 3'
primer pair C	5' <u>gaattc</u> atgaagaagtggtgtattattgt <u>g</u> 3'	5' <u>ggatcc</u> ctagccgaaaggagtttgatt <u>g</u> 3'

The resulting *HCH1* cDNA now including the appropriate restriction sites was finally cloned into a CaMV 35S promoter. Subsequently, the promoter-*HCH1*-construct was cleaved with *HindIII* and introduced into a binary vector. Indirect gene transfer into 'Saazer' and cv. 'Hallertauer Mittelfrüh' was obtained by co-cultivation with *Agrobacterium tumefaciens* for 2 to 3 days as described previously (Seigner et al., 2005). After two weeks selection for transgenic regenerating tissue was started using 25 mg/l kanamycin as medium additive.

The insertion of the *HCH1* cDNA into the hop genome and the presence or absence of *Agrobacteria* were confirmed by a duplex PCR in one single reaction with primer pair B (Tab. 1) and another primer pair specific for the agrobacterial sequence of *virG* (upper primer, 5' gccgacagcaccagttcac 3', and lower primer, 5' gccgtaagtttcacctcacc 3'; LAG, 1999). The PCR reaction was performed as follows: 1 cycle - 94°C - 4 min, 40 cycles - 94°C - 1 min - 57°C - 2 min - 72°C - 2 min, 1 cycle 72°C - 7 min with the HotStarTaq DNA Polymerase (Qiagen) yielding a fragment of 948 bp and 380 bp, respectively. As positive controls for the amplification of the *HCH1* cDNA and the *virG* sequence the cDNA/T-vector construct and the agrobacterial strain were deployed, respectively. The applied negative controls were: wild-type 'Saazer' DNA, wild-type 'Hallertauer Mfr.' DNA and the 'water control' (no template DNA).

Transgenic plants were tested for their resistance towards powdery mildew *in vitro* using the detached leaf assay (Seigner et al., 2002). Detached young leaves of *in vitro* grown transgenic plantlets were placed on water-agar plates and inoculated with conidia of two specific monosporic isolates (BU10 and BU13) of *Podosphaera macularis* deriving from the Hallertau hop growing region. These PM strains revealing virulence of the *v3*, *v4*, *v6*, and *vB* type can infect hop plants carrying the complementary *R* genes (*R3*, *R4*, *R6*, and *RB*) and susceptible plants with no resistance gene at all (Seigner et al., 2002). *In vitro* grown leaves of 'Zenith' (resistance based on *R1* gene) being resistant towards PM isolate BU10 and BU 13 and non-transgenic 'Saazer' being susceptible to all PM pathotypes were used as negative and positive control plants, respectively. Seven to ten days after inoculation under standardized incubation conditions growth of PM was assessed on the hop leaves.

Results

Putative transgenic plants from both cultivars were tested for the integrated chitinase gene using the duplex PCR. Based on strict PCR conditions the integration of the *HCH1* gene could be confirmed in fifteen 'Saazer' and three 'Hallertauer Mfr.' plants (Fig. 1), while non-transformed control plants from cv. 'Saazer' and 'Hallertauer Mfr.' did not show such a fragment. The simultaneous test for persisting *Agrobacteria* in the duplex PCR reaction by

adding *virG* specific primers yielded negative results in all tested cultivars (Fig. 1). Thus, it could be proved that the *HCH1* cDNA is stably integrated into the plant genome and that the duplex PCR amplicon does not result from plasmids of persisting *Agrobacteria*.

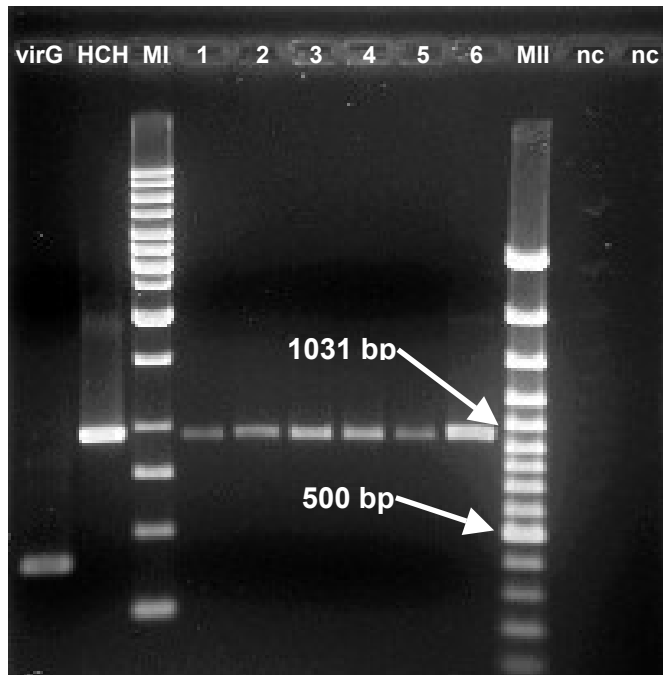


Fig. 1: Duplex PCR for the detection of *HCH1* and *virG* gene sequences in transformed 'Hallertauer Mfr.' and 'Saazer' plants

virG = *virG* *Agrobacterium* (pos. control)
380bp

HCH = *HCH1* construct (pos. control)
948bp

1-3 = transgenic 'Hallertauer Mfr.'

4-6 = transgenic 'Saazer'

nc = wildtype 'Hallertauer Mfr.' and
'Saazer' (neg. control)

MI = 1-kb-marker

MII = 100-bp-marker



Fig. 2: Detached leaf assay of three *HCH1* transgenic *in vitro* grown 'Saazer' plants performing various degrees of resistance towards powdery mildew.

Furthermore, a successful transcription of the *HCH1* gene has been confirmed in all of the transformed hop plantlets by RT-PCR as well (data not shown).

Finally the transgenic plants were tested for PM resistance using the detached leaf assay. Two different PM strains were used for the artificial inoculation. Seven to ten days after inoculation with PM spores vigorous growth of the mycelium could be observed on the leaves of non-transgenic plants of 'Saazer' and 'Hallertauer Mfr.', while no infection appeared on leaves of 'Zenith'. A more differentiated reaction towards PM could be seen on the leaves of the transgenic 'Saazer' and 'Hallertauer Mfr.' plants. Some plants showed the same susceptibility as the non-transgenic control plants, but on the leaves of two *HCH1* carrying hops reduced growth of the mycelium could be observed indicating an enhanced resistance to PM (Fig. 2).

Discussion

Regarding fungal resistance, Punja (2004) summarizes the various approaches to enhance resistance to fungal diseases in different crops. Besides the expression of pathogen-related proteins and antimicrobial compounds the most widely used approach was to over-express the various isoforms of chitinase, among the hydrolytic PR proteins. Thus, a class I hop endochitinase (*HCH1*) has been chosen and applied in this study as it is suggested to be involved in the resistance reaction of cv. 'Zenith' to powdery mildew.

Eighteen *HCH1* transgenic plants have been produced in total. The stable gene integration was proved by duplex PCR and the expression could be confirmed by RT-PCR. This is the first time that transgenic 'Hallertauer Mfr.' could be regenerated successfully. Reports published so far are restricted to the regeneration of transgenic plants of 'Tettlinger' (Horlemann et al., 2003; Schwekendiek et al., 2005), and 'Saazer'/'Oswald Clone' (Oriniakova and Matousek, 1996; Okada et al., 2003). Already performing tissue culture of the wild-type 'Hallertauer Mfr.' is challenging and its poor regeneration ability even declines after *Agrobacterium* infection and in the presence of antibiotics. Therefore, fully grown GUS expressing plants could not be obtained. In contrast to this, transgenic 'Hallertauer Mfr.' plants bearing the chitinase gene revealed increased vigor at a very early stage during the regeneration process. Thus, it may be assumed that this additional *HCH1* gene positively affected the vigor and regeneration ability of this cultivar.

Finally, using the detached leaf assay reduced growth of specific PM strains on the leaves of transgenic plants was observed. This is the first successful approach to enhance resistance to hop powdery mildew *Podosphaera macularis* via genetic engineering in hops.

Based on these very promising results further investigations on other genes conferring fungal resistance will follow.

Acknowledgements

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AGROBACTERIUM-MEDIATED TRANSFORMATION OF HOP

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Breeding in hops is lengthy and hindered by several obstacles, including difficulties in testing male plants, making breeding aims difficult to achieve. Gene transfer technologies are an attractive alternative to conventional breeding methods since they enable relatively rapid introduction of desirable characteristics into established hop cultivars without altering their quality profiles. Only two authors achieved stable *gus* reporter gene expression in two genetically closely related hop genotypes (Horlemann *et al.*, 2003; Okada *et al.*, 2003). Since a regeneration ability of hop is highly genotype dependent (Gurriarán *et al.*, 1999), a specific/modified regeneration and subsequently transformation protocol for each variety needs to be established. So far no successful transformation protocol in any Slovenian hop cultivar has been published.

Agrobacterium-mediated transformation of hop internodal explants was used for the introduction of a *gus* reporter gene and a *hptII* plant selection gene into Slovenian most widely grown hop cvs. Aurora and Savinjski golding. *A. tumefaciens* LBA4404 carrying plasmid pCAMBIA1301 (CAMBIA, Australia) was used for transformation. Approximately six months after transformation procedure emerging hop regenerants were tested for the *gus* gene expression by histochemical analysis of β -glucuronidase (GUS) activity as described by Jefferson *et al.*, 1987 and Hiei *et al.*, 1994. According to GUS test we obtained 10.5% transformation efficiency in cv. Aurora and 26.7% efficiency in cv. Savinjski golding. Molecular analysis by the PCR method of GUS positive regenerants was performed to check integration of the reporter and selection genes into hop genome. In cv. Savinjski golding PCR test demonstrated integration of both transgenes. In cv. Aurora we observed majority of shoots having both transgenes integrated (92.2%) and just a few shoots analyzed showed just *gus* (7.8%) gene presence.

Keywords: hop, transformation, *Agrobacterium tumefaciens*, *gus* gene, *hptII* gene, GUS-assay, PCR

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SOME ASPECTS OF REGULATION HOP CHALCONE SYNTHASE CHS_H1 IN HETEROLOGOUS SYSTEMS, CLONING AND ANALYSIS OF HOP REGULATORY FACTORS

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Abstract

Screening of a cDNA library of the hop cv. Osvald's 72 and genomic cloning were used to isolate members of oligofamily of *chs_H1* genes that co-determine the biosynthesis of prenylated chalcones known to be valuable medicinal compounds present in hop (*Humulus lupulus* L.). *chs_H1* oligofamily members show a high uniformity and a more than 99% and 98% identity on nucleotide and amino acid levels, respectively. *Chs_H1* genes within the oligofamily are very conserved in both, promoter and coding regions. In the promoter regions several binding motifs for regulatory factors including R2R3 Mybs were identified. Transactivation of *chs_H1* genes with Myb transcription factor PAP1 from *Arabidopsis thaliana* was found and the Myb biotechnology was proposed as valuable approach to modify hop metabolome. Based on PAP1 conserved motifs, two complete hop Myb factors *HIMyb1* and *HIMyb3* were cloned and analyzed. *HIMyb1*, that belong to plant family of P-like factors involved in pigment biosynthesis shows high expression in hop flowers. *HIMyb3* that is highly expressed in colored hop epidermis and in hop cones at the last stages of maturation, is quite unique and specific regulatory factor that exhibits two ORF variants acting antagonistically in heterologous plant transgenotes. Shorter variant is able to influence plant branching and accumulation of various secondary metabolites. Recent results support the idea about a combinatorial action of Myb factors with co-factors like bHLH or b-ZIP that remain to be isolated from hop and analyzed in the future. Artificial zinc finger factors were designed to modify activity of *chs_H1* genes.

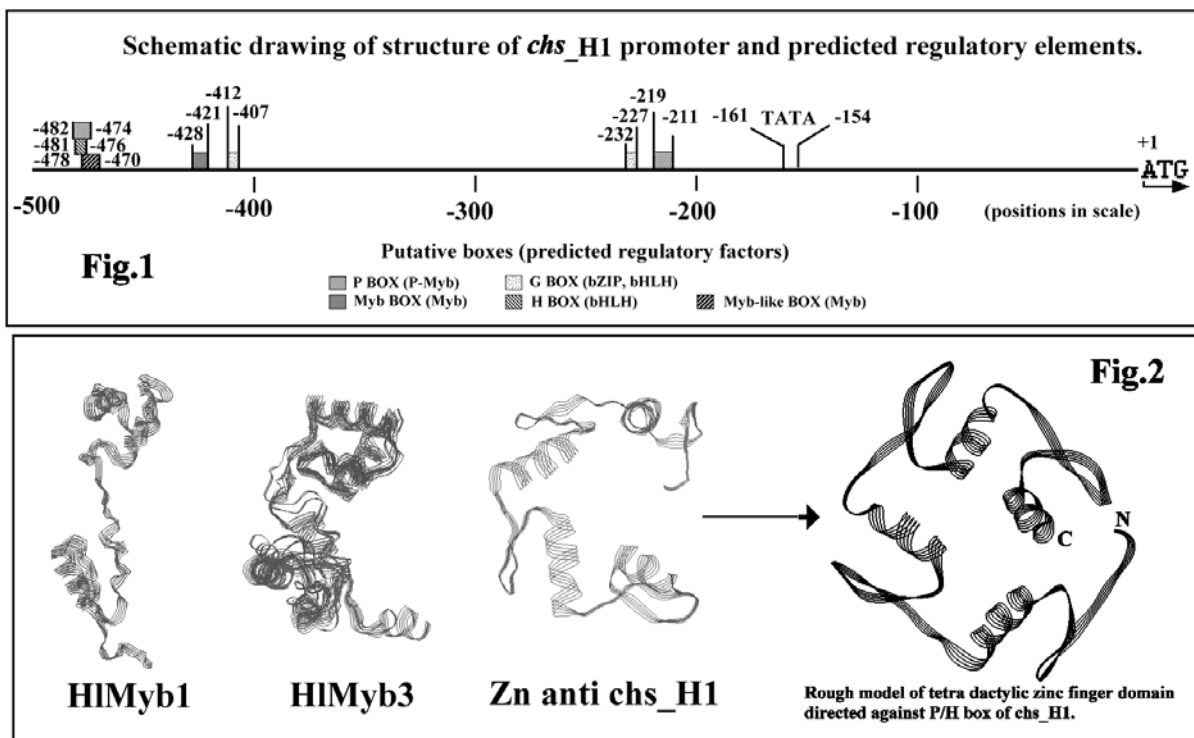
Keywords: transcription regulators, lupulin, hop metabolome, plant morphogenesis, *H. lupulus*, *P. hybrida*, *A. thaliana*, *N. benthamiana*, plant transformation.

Introduction

Hop (*Humulus lupulus* L.) is a dioecious perennial climbing plant cultivated for commercial use in brewing industry and known in traditional medicine from medieval times. Hop female inflorescences, referred to as cones, contain glandular trichomes (lupulin glands) that form a specific part of the hop metabolome containing bitter acids and having a relatively stable biochemical composition characteristic for individual hop genotypes (Menary and Doe, 1983, Burkhart, 1986, De Keukeleire et al., 2003). The main production trait that is evaluated for marketing is a α -bitter acid content. This content varies from 2 % (Saazer, CR) to 18 % (Columbus, USA) and divides hops to two groups: aroma and high alpha varieties. The changes of brewing technology have modified requirements on hops, preferentially towards high alpha varieties. Besides of α and β bitter acids valuable for the brewing process, several other compounds in the lupulin metabolome are of particular recent interest in view of their medicinal properties (Gerhäuser, 2005; Stevens and Page, 2004). In this respect, current research is focused on prenylated flavonoids that constitute a subclass of

polyphenols in the lupulin glands as a base for a fascinating cancer-chemopreventive (Gerhäuser et al., 2002; Miranda et al., 1999) and phytoestrogenic (Milligan et al., 1999) compounds.

Recent analyses, including our work, discovered several structural genes encoding for chalcone synthase (EC 2.3.1.74) (CHS) or CHS-like enzymes (Okada and Ito, 2001, Matoušek et al., 2002ab, Novák et al., 2003) that are involved in biosynthesis of lupulin metabolome. Especially the chalcone synthase CHS_H1 (Matoušek et al., 2002a,b) forming an oligofamily of identical members as to the catalytical core (Matoušek et al., 2006) has been shown to have 1000x higher expression in maturing cones than in somatic tissues and capability to catalyze both, chalcones as precursors of Xanthohumol, and bitter acids (Okada et al. 2004, Novák et al., 2006). Our studies (Matoušek et al., 2002ab, 2005, 2006), however, also document that the number of structural *chs* genes cannot simply explain differences among various cultivars, suggesting that the genotype-specific diversity is mediated rather by a combinatorial action of regulatory genes.



Results

Analysis of *chs_H1* promoter regions revealed sequence motifs specific for regulatory factors, like Myb-like boxes, H-box and G-boxes (Matoušek et al., 2002ab, 2005). In addition, recently we identified two P-boxes at positions $-(482-474)$ and $-(210-211)$. These boxes might be important for interaction with P-like factors such as isolated from *Zea mays* (e.g. Zhang et al., 2000) that are involved in the regulation of pigment biosynthesis.

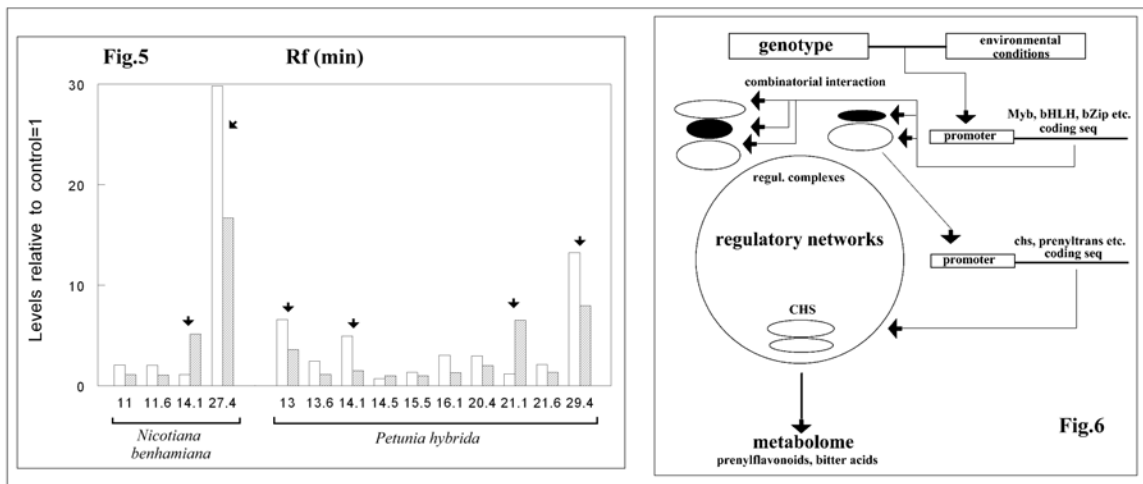
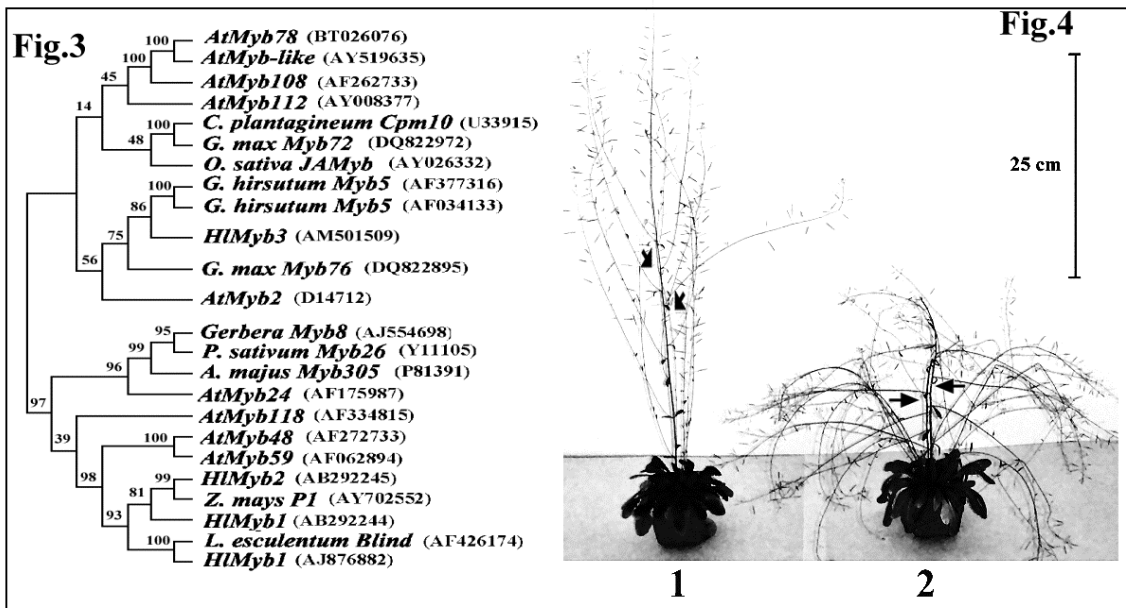
Chs_H1 activation in response to general heterologous activator of anthocyanin biosynthesis, PAP1 (R2R3 Myb) factor from *A. thaliana*, was proved experimentally (Matoušek et al., 2006), thus confirming the potential of Myb homologues to be involved in *chs_H1* regulation in hop. To analyze a possible interactions of selected transcription factors with *chs_H1* promoter region, we proposed to construct artificial zinc finger C2H2(Zn) domains targeted against suitable individual motifs in *chs_H1* promoter as binding competitor in plant infiltration experiments. As example, zinc tetra finger core structure targeted against 12bp site covering *chs_H1* P/H-boxes is seen in protein model on Fig. 2.

By means of cDNA library screening in combination with PCR methods, two full-length regulatory factors were isolated from hop (Fig.2). *H/Myb1* open reading frame encodes 272 amino acids (29.8 kDa), while *H/Myb3* exhibit two alternative open reading frames encoding for proteins having 269 and 265 amino acids with apparent molecular masses of 30.3 and 29.9 kDa, respectively. *H/Myb3* mRNA showed the lowest concentrations in hop flowers and high concentrations in maturing hop cones. High concentrations of *H/Myb3* mRNA were detected also in the colored epidermis of petioles indicating potential relevance of this factor in tissues, where flavonoid biosynthesis occurs. *H/Myb3* is quite unrelated to *H/Myb1* and occurs in a quite different cluster on the genealogy trees (Fig.3). In addition to the high degree of divergence, *H/Myb1* was most abundant in hop female and male flowers and is absent in cones as revealed by Northern blots, i.e. these two Myb factors show quite opposite pattern of expression in hop inflorescences, suggesting an involvement in different processes or function(s).

H/Myb3 is encoded by a unique gene in hop (not shown) and as a transgene it influences plant morphogenesis and pigment accumulation. For instance 29.9 kDa variant of *H/Myb3* changes branching morphogenesis of *A. thaliana* (Fig.4, compare control No.1 with transformed plant No.2) and composition of metabolites isolated in methanol/water-soluble fraction in *Nicotiana benthamiana* and *Petunia hybrida* transgenotes as shown in Fig.5 [by the arrows are indicated peaks showing significant differences in plants transformed shorter (empty columns) in comparison to longer (filled columns) variant of *H/Myb3*]. These results suggest that the *H/Myb3* gene has the potential to influence hop morphogenesis and possibly hop bine anatomy as an important genetic trait that may co-determine the yield of hop cones, as well as the metabolome composition during lupulin maturation.

Discussion

A term 'transcription factor (TF) means proteins with modular structure that recognize DNA in a sequence-specific manner and that regulate (activate, repress or both) the frequency of initiation of transcription upon binding to specific sites in the promoter of target genes. From various data obtained from research of *A. thaliana* (e.g. 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 C2H2(Zn), MADS, and WRKY (Zn) can be involved in the development and production of hop inflorescences, cones, lupulin glands and secondary metabolites of lupulin (see also De Keukeleire et al., 2007). It is obvious from recent studies that some transcription factors form more complicated complexes like Myb-bHLH-WD40 complex that mediate diversification of cell differentiation pathways (Ramsay and Glower, 2005) or activation of biochemical pathways like anthocyanin production (Yamazaki et al., 2003). In this view, it is important to analyze combinatorial interactions of TFs (Singh, 1998). In particular, analysis of interaction of R2R3 Mybs with corresponding bHLH factors is important to elucidate their role and pleiotropic effects often observed. Just because of this pleiotropic potential, it is important to analyze possible involvement of hop transcription factor homologues not only in metabolome production and changes, but also in morphogenetic processes e.g. apical dominancy, branching regulation, and trichome differentiation, because the total yield relates to the overall plant morphology. According to our recent knowledge about regulation of plant metabolism, there are TF complexes (Fig.6) that function on the basis of combinatorial action. These complexes enter the regulatory networks influenced by plant genotypes and environmental conditions (Fig.6). It is expected that new hop-specific TF homologues, Myb, bHLH and bZip factors with potential to regulate hop metabolome and yield of lupulin will be isolated and characterized. In future research such factors can be used for specific hop transformation to modify and improve hop metabolome within the prospect of TF-Mediated biotechnology (e.g. Broun, 2004). The work was supported by MŠMT 1-2006-01 and 01S00906 within a bilateral collaboration research project between the Czech Republic and Flanders.



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MOLECULAR ANALYSIS OF HOP CHALCONE SYNTHASES

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Abstract

The aim of our study is more complex quantification of expression of individual hop CHS homologues in hop tissues and organs. For this purpose we performed screening of phagemid cDNA library prepared from hop cones for CHS homologues by means of hybridization techniques and sequencing. As the result of this screening new sequence variants of *chs2* - *chs2*-1308 and *vps* - *vps*-1257 were identified. We suggested and successfully tested the system of primers for specific detection of individual hop CHS homologues isolated in our experiments and published earlier. At present we verify an applicability of this primer system for quantification of expression of the individual CHS homologues by means of real-time PCR.

Keywords: hop, bitter acids, prenylflavonoids, chalcone synthase, valerophenone synthase

Introduction

Hop cones are a source of bitter acids used in brewing industry and prenylflavonoids (especially xanthohumol and 8-prenylnaringenin) which are very attractive for pharmaceutical industry.

The crucial enzymes participating in biosynthesis of bitter acids and prenylflavonoids are chalcone synthases (*chs*). Nowadays 9 CHS and CHS-like genes - *chs_H1* (Matoušek et al., 2002^{a,b}), *chs_H1*-132, *chs_H1*-211, *chs_H1*-1539 (Matoušek et al., 2006), *chs2* (AB061020), *chs3* (AB061022), *chs4* (Novák et al., 2003), *chs4*-2 (Škopek et al., 2006) and *vps* (Okada and Ito, 2001) have been described in hop genome, nevertheless only *chs_H1* and *vps* recombinant proteins performed phloroisovalerophenone synthase and chalcone synthase (*vps* in limited extent) activities (Novák et al., 2006).

Acknowledgement

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OPTIMALISATION OF A REAL TIME RT-PCR PROTOCOL FOR THE ANALYSIS OF GENE EXPRESSION IN HOP TISSUES

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Hop (*Humulus lupulus* L.) is a dioecious, perennial, climbing plant, included in the family Cannabaceae. Female hop plants are cultivated in most temperate regions of the World, for the extraction of secondary metabolites. Important secondary metabolites produced by hop include α -acids and β -acids and essential oils, which are used in beer brewing, as well as the prenylated chalcones, Xanthohumol, and Desmethylxanthohumol, which exhibit interesting bioactive properties. All these compounds are synthesized in relatively high amounts during the development of the female inflorescences of hop into cones and are accumulated in the lupulin glands.

The most prominent hop prenylflavonoids are the prenylchalcones Xanthohumol and Desmethylxanthohumol. These prenylchalcones can be readily converted to isomeric prenylflavanones, whereby Xanthohumol gives rise to Isoxanthohumol and Desmethylxanthohumol renders a mixture of 8-prenylnaringenin and 6-prenylnaringenin. Xanthohumol displays a broad spectrum of inhibition mechanisms at the initiation, promotion and progression stages of carcinogenesis. 8-prenylnaringenin, which results from isomerization of Desmethylxanthohumol in the brew kettle, is the most potent phytoestrogen known to date. It is, therefore, of great interest to identify and characterize the genes that regulate the formation of Xanthohumol and Desmethylxanthohumol in female hop plants. In a previous study (De Keukeleire et al. submitted) we used the differential screening technique cDNA-AFLP to identify candidate genes involved in the biosynthetic pathway of prenylflavonoids in hop. However, up to now, only semi-quantitative expression profiles are available for these candidate genes. Therefore, a detailed expression analysis of the candidate genes in different hop tissues and at different developmental stages using Real-Time RT-PCR is essential to demonstrate the possible implication in these genes in the biosynthetic pathway of prenylchalcones in hop.

Our objective in this study was to develop a test for the analysis of the expression profiles of previously identified candidate genes in hop tissues. Here we report on the selection of suitable housekeeping genes and on the expression levels of a putative O-Methyltransferase homologue in different hop tissues from several genotypes, using Real Time RT-PCR.

THE PROPAGATION OF HLVD IN DEVELOPING HOP POLLEN AND ITS ELIMINATION UPON EXPRESSION OF APOPTOTIC NUCLEASE

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Abstract

We reported earlier that there is a low transmissibility of hop latent viroid (HLVd)-one of the most important hop pathogens that modifies a composition of lupulin metabolome (Patzak et al., 2001)-through a generative phase of hop (Matoušek et al., 2000). This fact is important for hop breeding programmes, because it leads practically to a complete elimination of HLVd from seeds and F1 hybrids. In the present study we analyzed process of development of hop pollen and HLVd elimination in detail. It was found that HLVd can still propagate in uninucleate immature pollen until phase of the first and the second mitotic divisions. Only traces of HLVd can be detected in the last stage of maturing pollen and no viroid was detected by RT PCR in *in vitro* germinating pollen grains. HLVd elimination correlates with the expression of apoptotic nuclease in developing pollen. The nuclease homologue was cloned in our experiments based on hop expression library described earlier (Matoušek et al., 2005). The nuclease has predicted molecular mass of 34.1 kD (300 amino acids), isoelectric point 5.1 and it shares 85% homology at amino acid level with previously described TBN1 nuclease from tomato (Matoušek et al., 2007). Our recent results are consistent with the measurements of nuclease activities in hop floral organs performed earlier (Matoušek et al., 2000). In addition, in the present work we developed the method of fractionation of hop anthers and isolation of immature hop pollen valuable for tissue culture experiments. This work was supported by GACR 521/06/1149, AS CR 1QS500510558 and AV0Z50510513 projects.

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V. Session:

HOP DISEASES AND PESTS

HOP FLEA BEETLE (*PSYLLIODES ATTENUATUS* KOCH) IN SLOVENIA

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Abstract

In addition to other typical pests that occur on hop (*Humulus lupulus* L.) every year, the hop flea beetle (*Psylliodes attenuatus* Koch) has become increasingly common in the last seven years. In Slovenia the beetle has a spring and a summer generation. In the past, hop growers observed its occurrence only in spring. Damage to leaves and shoots that appear at that time may have such an adverse effect on the yield that the application of a suitable insecticide is required. In recent years, however, damage to leaves and cones induced by a summer generation has also been noticed. The damage results in a decrease in the quantity and quality of hop yields. The reasons for this recent proliferation may be found particularly in the reduced use of contact insecticides and in climatic changes.

Keywords: hop flea beetle, *Psylliodes attenuatus*, hop, *Humulus lupulus*, pest, damage, spreading

Introduction

Hop (*Humulus lupulus* L.) is an important crop in Slovenia. Especially in the Savinja Valley, where it is grown in more than 1,000 ha of land, it has been one of the crops of most intensive agricultural production for a number of years. It is a host plant of many phytopathogenic and phytophagous organisms, which cause a considerable economic loss. Among the most important organisms are the following: the hop downy mildew (*Pseudoperonospora humuli* Miyabe & Takah.), the hop powdery mildew (*Sphaerotheca macularis* Wallr.), the hop wilt, caused by *Verticillium albo-atrum* (Reinke & Berthold) and *V. dahliae* (Klebahn), the hop damson aphid (*Phorodon humuli* Schrank), the spider mite (*Tetranychus urticae* Koch), and the European corn borer (*Ostrinia nubilalis* Hübner). In the last years the hop flea beetle (*Psylliodes attenuatus* Koch) has also gained in importance since it was first found to occur regularly in hop gardens (Rak Cizej, 2003).

The hop flea beetle (*Psylliodes attenuatus* Koch, Chrysomelidae, Coleoptera) has become a severe hop pest in Slovenia in the last seven years. The beetle is small, only 2 to 2.8 mm long, metallic green to bronze in colour, capable of jumping well. It is a typical oligophagous insect since it feeds on similar plants, that is the hop (*Humulus lupulus* L.), the hemp (*Cannabis sativa* L. ssp. *sativa* var. *sativa*), both belonging to the hemp family (Cannabaceae), and on the stinging nettle (*Urtica dioica* L.) from the Urticaceae family (Heikertinger, 1925). It has one generation in a year (Heikertinger, 1913; Rak Cizej, 2003).

Overwintered adult beetles usually occur in the early spring, that is in the middle of April, when hop starts growing. At that time they cause damage by feeding on leaves and sometimes on shoots as well. The new generation emerges in summer, in the middle of July, and attacks cones in particular. Larvae feed on plant roots, causing hardly any damage (Tölg, 1913). Therefore, the hop flea beetle management is usually directed only to adult stages in spring, when hop plants are small and highly susceptible to defoliation induced by the beetle. Contact insecticides are used. Hop growers have a problem here as there are not enough insecticides available, particularly for the summer application because of a long pre-harvesting interval. Current information about biological control of hop flea beetles is insufficient. Maybe some bird species feed on them.

Traps (coloured sticky plates) attract insects, in general, by the use of their physical or chemical stimuli, whereby the traps act passively as their collecting agent (Meyerdirk and Oldfield, 1985; Brødgsgaard, 1993). They can be used in order to monitor the presence of adult hop flea beetle populations in the field and to determine the relation between its occurrence on the one hand and weather conditions and a possible insecticide application on the other.

Coloured traps may very effectively control the activity of hop flea beetles, especially if they occur in large numbers. The colour of a trap is of importance. For example, yellow colour attracts many insects as it reflects light within the same range as the foliage but more intensively (Prokopy and Owens, 1983).

Materials and Methods

From April to November in the years 2001 and 2002, bionomy of the hop flea beetle was studied in four different locations in Slovenia, that is in Podvin, Miklavž, Šmartno near Slovenj Gradec, and in Trgovišče, all of which are situated in three different Slovenian hop growing regions. The hop cultivar Aurora was grown in all hop gardens under consideration. Imagoes were monitored by means of yellow sticky traps, hanging on bamboo sticks, at a height of 50, 100, 150, and 200 cm above the ground. In each hop garden there were 16 study sites, which were situated either in its central part or at the garden's edge (Fig.1). The traps were changed every fortnight.

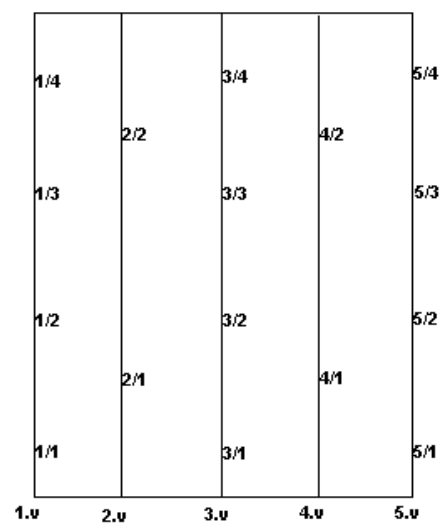


Figure 1: The arrangement scheme of study sites in a hop garden

When the traps had been collected, they were stored in a freezer until they were examined. The hop flea beetles were then determined and counted.

Results and Discussion

Hop flea beetles occur in all three Slovenian hop growing regions, but they are the most abundant in the Savinja Valley (Miklavž and Podvin) where most of the hop is grown (Fig.2). More beetles were caught in 2001. Hop flea beetles have one generation in a year, emerging in spring and in summer. Spring beetles are beetles that have overwintered, whereas summer beetles belong to the new generation emerging in July (Fig.3).

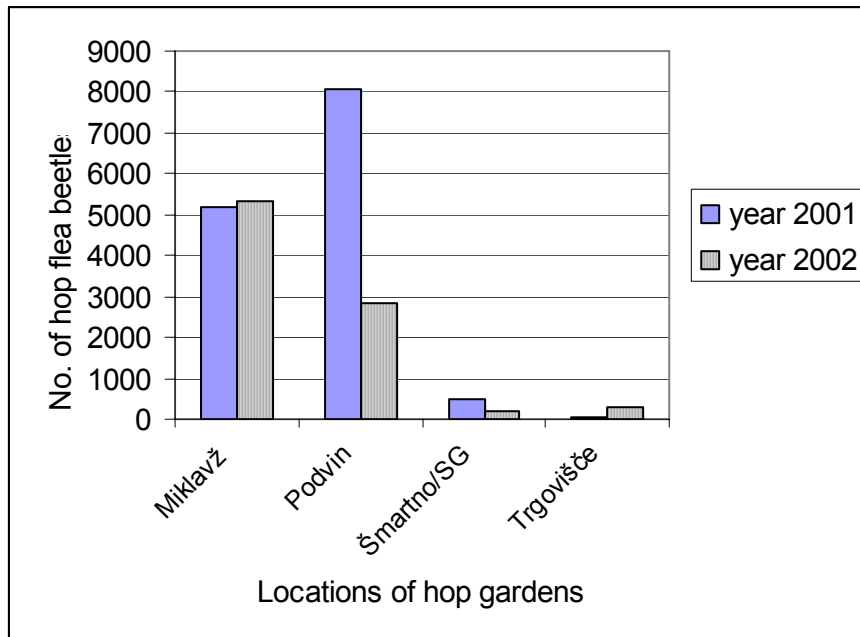


Figure 2: The number of hop flea beetles (*Psylliodes attenuatus*) caught by means of yellow sticky traps in different locations in Slovenia in 2001 and 2002

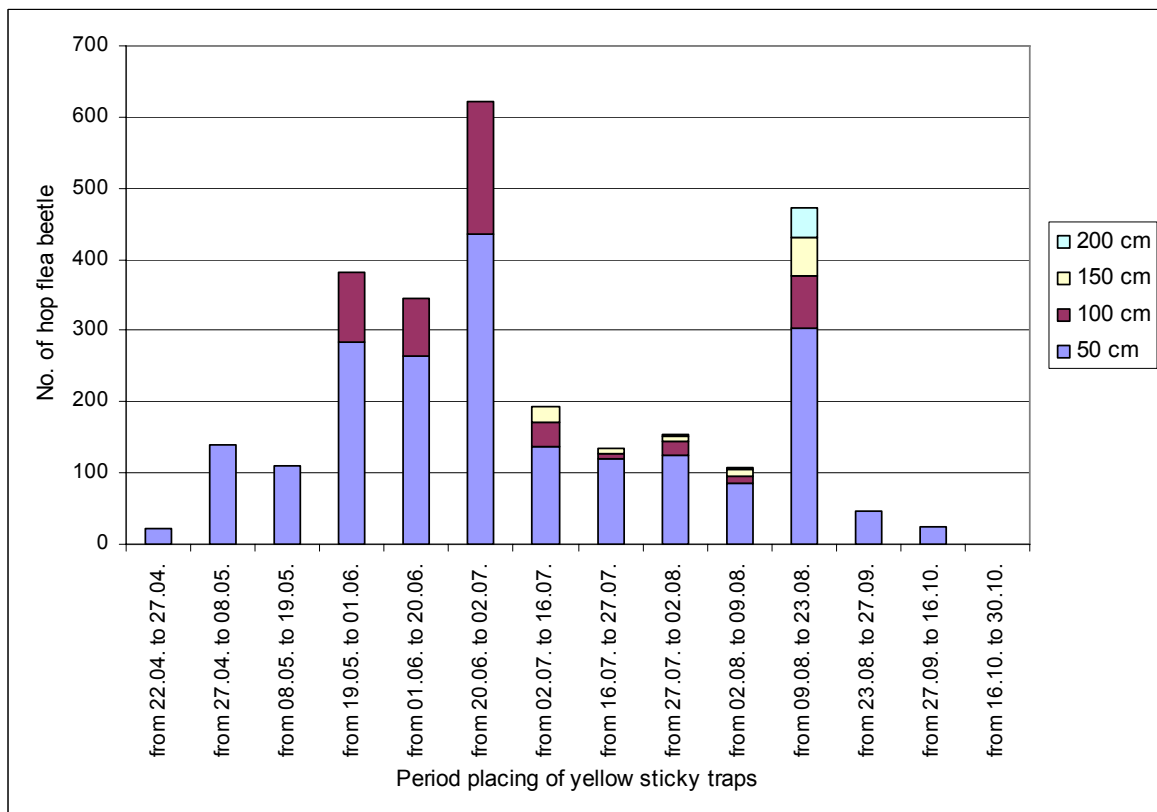


Figure 3: The number of hop flea beetles (*Psylliodes attenuatus*) caught by means of yellow sticky traps placed at different heights in Miklavž in 2001

The largest number of hop flea beetles were captured in all locations in traps placed at a height of 50 cm above the ground. Such a result was in accordance with the fact that the beetles rest during the night at the bottom of a hop plant or on the ground. The number of captured beetles was found to decrease in proportion to the height at which traps were placed. Hop flea beetles were the most abundant in the hop gardens of Miklavž and Podvin

in the Savinja Valley. In this area hop growers were compelled to apply foliar insecticides in order to control adult beetles.

During the monitoring period, other species of beetles were also caught with the use of yellow sticky traps as follows: *Psylliodes dulcamare*, *Phyllotreta atra*, *P. undulata*, *P. vittula*, *P. nemorum*, *P. ochripes*, *Crepidodera aurata*, *Longitarsus anchusae*, *L. succineus* and *L. reichei*. In the hop gardens of Miklavž, *Longitarsus anchusae* was found to be a very common species. It feeds on the common comfrey (*Symphytum officinale* L.) and on the tuberous comfrey (*Symphytum tuberosum* L.), both of which were present in the area. A test was carried out in the growing chambers in order to determine if the species *L. anchusae* was a hop pest. The results show that it did not feed on hop at all. Thus it can be concluded that only hop flea beetles (*Psylliodes attenuatus*) induce severe damage to hop crops.

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STUDY ON CAUSE OF *FUSARIUM* CONE TIP BLIGHT

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Abstract

In the years 2004-2005 study was performed on the cause of *Fusarium* cone tip blight. The pathogenicity of the fungi for hop cones and the susceptibility of hop cultivars on infection by *Fusarium* spp. were also determined. The disease assay based on PCR analysis showed that cause of cone tips blight were species *Fusarium avenaceum* and *Fusarium culmorum*. In result of artificial inoculation of hop plants by the fungi, the most cones with diseases symptoms were found on plants of Marynka cultivar. Infection of cones by *F. avenaceum* was reached in all stage of generative development i.e. during flowering, cones formation and cones development but the most cones with diseased symptoms occurred after inoculations during cone formation.

Keywords: *Fusarium* cone tips blight, .mycological analysis, PCR analysis, susceptibility of cultivars

Introduction

Fungi of the genus *Fusarium* acquire larger and larger significance as disease agents of many plant species in the last years (Logriego *et al.*, 2003). The increasing significance of the pathogens is observed also on hops, at the same time they are pathogenic for new organs of the plant. The new symptoms, a necrosis at the tip of cones, caused by *Fusarium* spp. were noted on hops in USA and Poland (Bienapfl *et al.* 2001, Solarska 2003). The disease was named *Fusarium* cone tip blight (Bienapfl *et al.* 2001). Till now fungi of the genus *Fusarium* were a cause cancer of hops. *Fusarium sambucinum* (Fr.) Sacc. and *Fusarium culmorum* W.G. Smith/Sacc. have been associated with the disease and also with hop bine rot above soil-level (Royle 1974, Solarska 1978, 2001).

Isolation of the pathogens employs the use of nutrient agar to culture pathogens and it requires long time to identify species level. Molecular-based techniques, such as polymerase chain reaction (PCR), can reduce the time needed for diagnosis of plant diseases when compared with classical isolation (Turner *et al.*, 1999).

The aim of the studies was to determine and estimate the occurrence of *Fusarium* pathogenic fungi on hop cones by means of microscopic, mycological analysis and molecular technique (PCR). Besides, the pathogenicity of the fungi for hop cones and the susceptibility of hop cultivars on infection by *Fusarium* spp. were determined.

Material and methods

The diseased cones of Marynka cultivar plants from production hop-garden were taken for mycological analysis in 2002. Six pieces of these organs from each plant were placed on mineral agar in 90 mm Petri dishes and after two-three days of incubation at 24°C transfers from colonies were made to PDA slants. Isolation and identification of fungi followed the method given by Solarska (1996). *Fusarium avenaceum* and *Fusarium sambucinum* determined in results of the mycological analysis were used in further study during two vegetation seasons 2004-2005. From sporulated mycelium of both fungi growing on PDA in Petri dishes, spores were harvested by shaking mycelium from each Petri dish in 15-20 ml ddH₂O and straining through several layers of cheesecloth. Spore suspensions of each fungus were used for inoculation of hop plants of four cultivars growing in field conditions.

The following hop cultivars were examined: Marynka, Junga, Lubelski and Magnum. Plants were inoculated by spraying of the spore suspensions in three growing stages: flowering, cone formation, cone development. Spore suspension of each fungus were inoculated onto three randomly chosen plants of each cultivar in each growing stage. There were two controls: non-treated and water sprayed. Observations of cones healthness were performed during growth of cones in the week intervals. Cones with diseased symptoms were taken to the laboratory. In the laboratory microscopic analysis of such cones was performed. In case of lack of *Fusarium* spores during microscopic analysis of cone, part of cone husks with disease symptoms were assigned to conventional mycological analysis (Solarska 1996) and part of them were frozen. In case of lack colonies of fungi from genus *Fusarium* around husks on PDA in Petri dishes, frozen cone husks were assigned for PCR analysis performed by means described by Solarska and Grudzińska (2005).

Results

Preliminary disease assay based on cultural and morphological features showed that cause of cone tips blight were species *Fusarium avenaceum* and *Fusarium sambucinum*. Species-specific primers used for PCR analysis of DNA isolated from mycelium of the fungi confirmed occurrence of only *Fusarium avenaceum* on cones with disease symptoms. *Fusarium sambucinum* specific primers were not given positive reaction for DNA isolated from mycelium of the second fungus. Use of *Fusarium culmorum* specific primers for PCR analysis of DNA from mycelium of the fungus was given visible product.

In result of artificial inoculation of hop plants by the fungi, the most cones with diseases symptoms were found on plants of Marynka cultivar in both study seasons and least of such cones on plants of Lubelski cultivar (table 1). Only the pathogenic fungus *Fusarium avenaceum* was recovered and the most such isolations was from cones of Marynka cultivar. Infection of cones by the pathogen was reached in all stage of generative development i.e. during flowering, cones formation and cones development but the most diseased cones occurred after inoculations during cone formation (tables 2, 3).

In result of artificial inoculation of hop plants by *Fusarium* spp., infected cones were observed on plants of all studied cultivars. (tables 2, 3).

Table 1 The occurrence of diseased cones on plants of different several hop plants

Cultivar	Number of cones with a necrosis at the tip	
	2004	2005
Marynka	20	11
Magnum	15	7
lunga	18	8
Lubelski	11	5

Discussion

Identification of *Fusarium* species based on cultural and morphological features is often complicated and confusing, even for expert taxonomists, because often the same species was identified as other species by different *Fusarium* taxonomists (Yoder, Christianson, 1997). In this study disease assay based on cultural and morphological features did not agree with the PCR pathogen diagnosis. Pathogens *Fusarium sambucinum* and *Fusarium culmorum* have very similar morphology, because belong to the same taxonomic group. *F. sambucinum* is known as a pathogen of hops causing cancer of the plants (Royle, 1974,) however in specific conditions i.e. during hot and dry weather *Fusarium culmorum* becomes

very dangerous cause of that disease (Solarska 1978). In some of the last years air temperature was very high and there were favourable conditions for development of the pathogen. High sensitive and very specific PCR technique verified the results of the conventional mycological analysis and permitted to prove that *Fusarium culmorum* is pathogenic for cones of hops. The main cause of a necrosis at the tip of cones was fungus *Fusarium avenaceum*. This result confirmed result obtained by Bienapfl *et al.* (2001) that *Fusarium avenaceum* is one from causes of *Fusarium* cone tip blight. The lack of *F.culmorum* recovery from diseased cones in seasons 2004-2005 probably was connected with not favourable weather conditions for infection of the organs by the pathogen in the periods in comparison with season 2002, when high moisture and temperature occurred during flowering and cone formation and then the fungus was isolated from cones with disease symptoms. Lower incidence of disease on hops after artificial inoculation in 2005 in comparison with 2004 also was influenced by weather conditions.

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Table 2: Identification of fungi from genus *Fusarium* from hop cones of different cultivars after artificial inoculation in 2004

Cultivar	microscopic observations						Conventional mycological analysis						PCR						
	flowering		Cone formation		Cone development		flowering		Cone formation		Cone development		flowering		Cone formation		Cone development		
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
Marynka				+						+									
Iunga		+																	
Lubelski												+							
Magnum																+			

1 – inoculation by *Fusarium culmorum*
 2 – inoculation by *Fusarium avenaceum*

Table 3: Identification of fungi from genus *Fusarium* from hop cones of different cultivars after artificial inoculation in 2005

cultivar	microscopic observations						Conventional mycological analysis						PCR						
	flowering		Cone formation		Cone development		flowering		Cone formation		Cone development		flowering		Cone formation		Cone development		
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
Marynka		+																	
Iunga																			
Lubelski																			
Magnum																			

1 – inoculation by *Fusarium culmorum*
 2 – inoculation by *Fusarium avenaceum*

PROTEOMIC ANALYSIS OF THE FUNGUS *VERTICILLIUM ALBO-ATRUM*

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Introduction

Verticillium wilt is a vascular disease caused by the soil borne pathogenic fungi *Verticillium albo-atrum* and *Verticillium dahliae*. Slovenian *V. albo-atrum* isolates infecting hop display different levels of virulence, resulting in mild (PG1) or lethal symptoms (PG2). The spread of the lethal form in hop gardens in the Savinja Valley is a serious threat to hop production as more than 180 ha of hop gardens were infected and almost 90 ha were destroyed in the past decade. We employed a proteomic approach to gaining a wide insight into the infection process and to identifying proteins related to infection.

Methods

Verticillium albo-atrum was grown in a general fungal medium for 1 week and mycelium was collected by filtration. Proteins were extracted according to Jamnik et al. 450 µg of proteins were precipitated, resuspended in rehydration solution and applied to 13 cm IPG strips (pH 4-7). After isoelectric focusing, strips were equilibrated, run on 12.5% polyacrylamide gels, stained with CBB G-250 and scanned. Dymension 2 software was used for image analysis and statistical tests. Four biological and three technical replicates were included in the experiment (24 gels).

Results

Separation of proteins was good and there were only a few spots with horizontal or vertical streaks. Reproducibility between technical replicates was excellent, while biological replicates displayed minor differences. On average, 660 spots were detected in PG1 and 721 spots in PG2. There were 173 spots present only in the PG1 pathotype and 234 spots were found only in the PG2 pathotype; 487 spots were observed in both pathotypes. A regulation factor of 2 resulted in 64 spots significantly more abundant in the PG1 pathotype and 90 spots more abundant in the PG2 pathotype ($p < 0,05$). Twenty-seven spots were chosen for MS analysis.

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POPULATION DYNAMICS OF *PHORODON HUMULI* (SCHRANK, 1801) (HEMIPTERA: APHIDIDAE) IN HOP CONES

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Abstract

Population dynamics of *Phorodon humuli* and its natural enemies in hop cones is analysed. The first aphid colonies were observed at the beginning of August. The number of aphids rose sharply from mid to late August onwards. Aphid contamination of cones reflected the numbers on foliage in July, with a greater population in the middle part of the plants than at the top. The hop aphid's main natural enemies found on the cones were anthocorids.

Keywords: *Phorodon humuli*, anthocorids, thrips, population dynamics.

Introduction

Cones of hops provide bitterness and aroma of the beer. Contamination of hop cones by the damson-hop aphid, *Phorodon humuli*, may impair their brewing value and, if visible, reduce the price achieved (Neve, 1991).

Methods

A field trial was performed on hop yards throughout 2002, 2003 and 2004 in order to analyse the population dynamics of *P. humuli* and its natural enemies in hop cones. Cones of nine plants without aphid treatments were sampled each year. A hundred cones were collected at different heights of the plants on several occasions until the harvest, extracting both the aphids and their natural enemies with a Berlese funnel.

Results and Discussion

Population dynamics of *P. humuli* in hop cones was similar during the three years. The infestation of cones began at the beginning of August. The initial increase of aphids in cones coincided with a fall in aphid population on leaves. This might be because some aphids emigrated from leaves to cones. From mid August onwards, it was observed that the population of aphids grew both on the leaves and in the cones. The distribution of aphids in cones by height corresponded with that of aphids on leaves during the month July, since the population was bigger at 2 and 3.25 m than at 6 m. The natural enemies most often found in cones were anthocorids, all belonging to the genus *Orius*. The anthocorid population in cones varied in a similar way to the aphid population. Aphids in cones can reduce the value of the crop and in some cases can lead to total loss (Campbell, 1978), so it is important to get that aphids do not gain access to cones.

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RESISTANCE TO IMIDACLOPRID (CONFIDOR 70 WG) IN DAMSON-HOP APHID (*PHORODON HUMULI* SCHRANK) ON CZECH HOPS

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Abstract

Contemporary hop protection against damson-hop aphid (*Phorodon humuli* Schrank) in Czech Republic has been for almost fifteen years nearly entirely based on the application of imidacloprid as a key aphicide. In the years with slighter to average intensity of migration one treatment by imidacloprid is usually sufficient enough to control this dangerous pest. In spite of its still good efficiency there have been some hop-gardens where aphids survive after imidacloprid application perhaps as a result of the previous under-dosing. To investigate the reason of their surviving, samples of field strains were taken and subjected to laboratory tests in Potter tower. Values LC 50 of field strains were compared with the same ones of a susceptible strain so as to be able to determine resistance factors (RF). Obtained data show the resistance in damson-hop aphid feeding on Czech hops. Nevertheless, biological efficiency of imidacloprid is in the most of the tested field strains good enough to be recommended for practical hop protection against *P. humuli* although there is an urgent need for a new efficient aphicide.

Keywords: *damson-hop aphid*, field and susceptible strains, laboratory tests, Potter tower, LC 50, resistance factors (RF), resistance, biological efficiency.

Introduction

Damson-hop aphid (*Phorodon humuli* Schrank) is besides two-spotted spider mite (*Tetranychus urticae* Koch) the most dangerous pest of hop in all the hop gardens to be found in the northern hemisphere. Nowadays, hop protection against these pests is based entirely on application of zoocides. Short development time gives birth many generations during a season, which increases the danger of resistance (Malais & Ravensberg, 1992). In damson-hop aphid first problems with surviving individuals after application of organophosphorous insecticides were found out in Bohemian hop-gardens as far back as ten years after their use in practical hop protection (Hrdy, Zeleny, 1968). Later similar cases were announced from other hop growing countries as well (Zohren, 1972; Muir, 1979). Less efficient insecticides had to be replaced by more efficient ones gradually. Since the middle of the eighties imidacloprid has been used to control resistant strains of damson-hop aphids (Vostrel, 1996). Nevertheless, its long use in practical protection against *P. humuli* has brought about logically problems with surviving aphids. Resistance of this species to imidacloprid was found out and discussed on the last meeting of the Scientific Commission (Vostrel, 2005). During the last two years new data have been obtained from laboratory tests.

Materials and methods

Samples of damson-hop aphid populations were taken from the selected hop gardens within the hop regions in Czech Republic in 2005 (Zatec region: 10 samples from Louny district and 6 samples from Rakovník district, 3 samples from Ustek and 3 samples from Trsice regions) and 2006 (Zatec region: 8 samples from Louny district and 5 samples from Rakovník district, 3 samples from Ustek and 5 samples from Trsice regions). Aphids were collected in the first decade of July (2005) and in the third decade of June (2006) just before a common treatment of Czech hop gardens with imidacloprid (Confidor 70 WG).

Field samples of *P. humuli* populations were transferred into breedings. Their offspring were used in laboratory tests. Aphids were placed in an air-conditioned room at a temperature of 20-22°C and 16-hours photoperiod. The 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 an insecticide 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 sedimentation tower. The method (Hrdý, Kuldová, 1981) required glass cylinders (22 mm in diameter and 15 mm high) stuck on the inside with the help of a paraffin and bee-wax mixture, which was melted to 50 °C before. 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 treatment. The knocked down aphids and the ones, which were unable to move, 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 experiment was carried out three times. That means 100 aphids were tested under each concentration of imidacloprid in a geometric row. As a standard reference susceptible strain from 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 increase of resistance in field strains of damson hop aphid (*Phorodon humuli* Schrank) to imidacloprid (Confidor 70 WG) expressed by resistance factors (RF) we continued, within the project mentioned in the acknowledgement, in laboratory tests aimed at this phenomenon.

Long-term utilization of imidacloprid in hop protection against resistant strains of damson-hop aphid connected with sublethal doses sometimes unreasonably used in practice to lower costs on hop protection are the two main factors of resistance development in *P. humuli* field populations to this compound.

First report on lower efficiency of imidacloprid after its treatment was known from Germany hop gardens (Engelhard, 2001). It was most likely due to a brush method of its application often used in this country, which enabled aphids to build up resistant populations more quickly in comparison with leaf spray of hop plants. In Czech Republic there were first surviving aphids after treatment with imidacloprid found four years ago in some hop-gardens in Rakovník district belonging to Zatec hop growing region (Vostrel, 2003).

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 by Dr. Hrdý at Institute of Organic Chemistry and Biochemistry in Prague. If we compare LC 50 values of this reference strain of *P. humuli* with field resistant strains (average from each hop growing region) we obtain resistant factors for the individual regions. From Tables 1 and 2 it is obvious that resistant factor (RF) are the highest for above-mentioned Rakovník district (13,1 in 2005, resp. 16,4 in 2006). Three years ago, as presented at the meeting of the Scientific Commission at George in South Africa, the average value for all the regions of CR was at the level of 7,5. Approximately the same level was determined in 2005 for Trsice hop region. In 2006 resistance considerably increased in Zatec hop region (Louny district) and it reached the values > 10 in all the regions within CR. In spite of this fact mortality of aphids is still 100% when 0,008% conc. of Confidor 70 WG (imidacloprid) is applied in laboratory conditions. On the other hand it is obvious still increasing percentage of surviving aphids if it is applied in the following, 0,004% conc., especially in the strains sampled from Rakovník district. From this reason it is necessary to find out a new high-effective aphicide to be able to replace replace imidacloprid in the next few years. Spirotetramat and flonicamid seem to be able to substitute it and in this way to help hop growers to cope with this difficult problem.

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Table 1: Biological efficiency of imidaclopride (Confidor 70 WG) on damson-hop aphid (*Phorodon humuli* Schrank) in laboratory tests in 2005

Tested strains	Concentration					
	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %
Zatec region						
Louny district						
Dubcany	100 %	100 %	85 %	70 %	61 %	44 %
Drahonice	100 %	100 %	80 %	73 %	60 %	39 %
Markvarec	100 %	100 %	91 %	80 %	69 %	50 %
Obora	100 %	100 %	89 %	72 %	59 %	44 %
Oploty	100 %	100 %	94 %	70 %	57 %	47 %
Rocov	100 %	94 %	88 %	68 %	64 %	51 %
Steknik I.	100 %	95 %	87 %	75 %	59 %	38 %
Steknik II.	100 %	96 %	89 %	77 %	60 %	47 %
Vinarice	100 %	100 %	93 %	66 %	53 %	38 %
Zatec	100 %	99 %	90 %	70 %	57 %	42 %
Average	100 %	98,4 %	88,6 %	72,1 %	59,9 %	44,0 %
Rakovnik district	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %
Domousice	100 %	93 %	77 %	69 %	55 %	40 %
Kounov	100 %	94 %	79 %	63 %	50 %	37 %
Kozojedy	100 %	94 %	83 %	68 %	53 %	39 %
Mutejovice	100 %	81 %	62 %	49 %	29 %	17 %
Nesuchyne I.	100 %	92 %	75 %	57 %	37 %	27 %
Nesuchyne II.	100 %	90 %	70 %	59 %	36 %	20 %
Ø	100 %	90,7 %	74,3 %	60,8 %	43,3 %	30,0 %
Ustek region	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %
Decany	100 %	99 %	88 %	73 %	49 %	31 %
Polepy	100 %	98 %	88 %	76 %	53 %	35 %
Sulejovice	100 %	98 %	85 %	75 %	57 %	39 %
Ø	100 %	98,3 %	87,0 %	75,7 %	53,0 %	35,0 %
Trsice region	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %

Doloplazy	100 %	98 %	90 %	69 %	60 %	40 %
Hnevotín	100 %	97 %	91 %	73 %	59 %	33 %
Trsice	100 %	93 %	89 %	71 %	61 %	38 %
Ø	100 %	96,0 %	90,0 %	71,0 %	60,0 %	37,0 %
Roznava susceptible strain	0,000312%	0,000156%	0,000078%	0,000039%	0,000019%	
	100 %	97 %	77 %	39 %	27 %	
Zatec region	LC 50 SS = 0,000053					
Louny district	LC 50 RS = 0,00034		RF = 6,5			
Rakovník district	LC 50 RS = 0,000692		RF = 13,1			
Ústek district	LC 50 RS = 0,000458		RF = 8,6			
Trsice district	LC 50 RS = 0,000391		RF = 7,4			

Table 2: Biological efficiency of imidaclopride (Confidor 70 WG) on damson-hop aphid (Phorodon humuli Schrank) in laboratory tests in 2006

Tested strains	Concentration					
Zatec region	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %
Louny district						
Dubčany	100 %	97 %	69 %	40 %	23 %	9 %
Kluček	100 %	99 %	73 %	49 %	30 %	16 %
Lenesice	100 %	100 %	90 %	75 %	49 %	21 %
Markvarec	100 %	99 %	80 %	63 %	38 %	10 %
Rocov	100 %	100 %	89 %	67 %	39 %	11 %
Stekník	100 %	95 %	79 %	59 %	34 %	12 %
Vinarice	100 %	99 %	90 %	69 %	50 %	24 %
Zatec	100 %	97 %	78 %	60 %	37 %	13 %
Average	100 %	98,3 %	81,0 %	60,3 %	37,5 %	13,3 %
Rakovník distr.	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %
Domoušice	100 %	95 %	78 %	60 %	35 %	21 %
Kneževy	100 %	91 %	74 %	55 %	30 %	12 %
Kounov	100 %	93 %	79 %	58 %	39 %	9 %
Mutejovice	100 %	87 %	72 %	53 %	28 %	8 %
Nesuchyně	100 %	90 %	74 %	55 %	26 %	11 %
Average	100 %	91,2 %	75,4 %	56,2 %	31,6 %	12,2 %
Ústek district	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %
Decany	100 %	100 %	78 %	65 %	42 %	25 %
Polepy	100 %	99 %	82 %	73 %	49 %	26 %
Sirejovice	100 %	100 %	89 %	78 %	55 %	29 %
Average	100 %	99,7 %	83,0 %	72,0 %	48,7 %	26,7 %
Trsice district	0,008 %	0,004 %	0,002 %	0,001 %	0,0005 %	0,00025 %
Čechovice	100 %	93 %	72 %	52 %	33 %	6 %
Doloplazy	100 %	96 %	81 %	70 %	45 %	22 %
Lipnany	100 %	95 %	79 %	65 %	40 %	18 %
Trsice	100 %	96 %	73 %	55 %	41 %	10 %
V. Bystrice	100 %	100 %	80 %	64 %	42 %	15 %
Average	100 %	96,0 %	77,0 %	61,2 %	40,2 %	14,2 %
Roznava susceptible strain	0,000312%	0,000156%	0,000078%	0,000039%	0,000019%	
	100 %	97 %	77 %	39 %	27 %	
Zatec region	LC 50 CK = 0,000053					
Louny district	LC 50 RK = 0,00077		IR = 14,5			
Rakovník dist.	LC 50 RK = 0,00087		IR = 16,4			
Ústek district	LC 50 RK = 0,00053		IR = 10,0			
Trsice district	LC 50 RK = 0,00073		IR = 13,8			

AN ANCIENT COMPOUND REDISCOVERED: PERSPECTIVES OF APHID CONTROL IN ORGANIC HOP GROWING BY THE USE OF QUASSIA PRODUCTS

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Abstract

In the first three decades of the 20th century, quassia extract was widely used in hop growing as a chemical agent to control *Phorodon humuli* and other insect pests. In the first years of the 21st century this compound was rediscovered by German organic hop growers. In nine efficacy trials conducted in five field seasons, quassia products proved to be effective control agents for *P. humuli* in organically grown aroma cultivars. As the best method of application a systemic variant was developed by painting a suspension of quassia extract to the bines. This method proved not only to be very effective but was also best from an environmental point of view as sprayed quassia extracts had side effects on non-target organisms. As an optimal systemic application rate 24 g/ha of the active ingredient quassine was determined.

Keywords: *Phorodon humuli*, damson-hop aphid, control, organic hop growing, quassia, *Quassia amara*

Introduction

The earliest materials used to control the damson-hop aphid *Phorodon humuli* (Schrank) by spraying were soft soap, quassia and nicotine. Only in the middle of the 20th century nicotine, the standard treatment of that time, was substituted by more effective compounds such as organo-phosphates (Neve 1991). Hence, especially quassia had the status of a sleeping beauty for more than 60 years, until it was rediscovered as an option for aphid control in organic hop growing in Germany in 2001. At that time, especially the pyrethrines registered for organic farming proved to be not satisfying in aphid control. As extracts made from the wood of the South American tree species *Quassia amara* were on the list of approved substances, German organic farmers started to spray quassia solutions they had extracted by homebrews from wood chips as an alternative. This option of aphid control was accompanied scientifically with efficacy trials from the first day onwards, and was advanced in the following years.

Methods

Altogether nine efficacy trials to test quassine, a pyrethrine ("Spruzit Neu") and an industrial neem extract ("NeemAzal T/S") were conducted in the five field seasons from 2002 to 2006 in two organic hop farms, one situated in Herpersdorf, Middle Franconia, Germany (former Hersbruck growing region), and one in Ursbach, Lower Bavaria, Germany (Hallertau growing region). The cultivar for trials was chiefly Perle, one trial each was run in cvs Hersbrucker Spät and Spalter Select. Quassine and NeemAzal were tested as spray and as systemic applications. Quassine in systemic application was tested in three different dosages of the active ingredient (12, 24 and 36 g/ha quassine). Each treatment was replicated in three or four plots. All trials included usually a weekly monitoring of aphids in each plot (50 leaves per plot, respectively) during the entire season, and an experimental harvest. In 2002 and 2003 the weekly monitoring included all beneficials and other non-target arthropods that were found on the assessed leaves.

Results

All trials we conducted during five years yielded good or satisfying aphid control in the quassia variants, both in systemic application of an industrial extract and spray application of a homemade extract from wood chips. Although never 100 % control was achieved, the average numbers of *P. humuli* remaining on the leaves never exceeded a level that might have been damaging to the plants. On the other hand, the control effects determined for Neem Azal – systemic or sprayed – and the pyrethrine were not satisfying or poor. Especially extremely high outliers of aphid numbers on single hop plants were not detectable in the quassia variants, contrary to the plots treated with the other compounds. Exemplary results for all trials are shown in Table 1 and Figure 1.

The aphid infestation was also mirrored by the results of the experimental harvests. As regards yield, the quassia variants always were within the significantly best group as shown exemplarily in Figure 2. It has to be noted however that the alpha acids had been reduced significantly in a systemic quassine variant in 2004, but this phenomenon was not confirmed later. The significantly lower alpha acids in systemic quassia plots, compared to sprayed variants, shown in Figure 2 are a result of clear soil differences within this hop garden.

Another problem became evident in the sprayed quassia plots during 2002: As all arthropods present on the monitored leaves were assessed that year, a significant decrease of small leafhoppers (*Cicadina*) was detected after a quassia spraying, compared to the other plots (cf. Engelhard & Weihrauch 2005). This means that non-target organisms are affected by quassia sprayings. Hence, due to the good control effects we evidenced, the systemic application of quassia is recommended as the currently best aphid control strategy in organic hop growing, as there are no negative environmental effects discernible. Probably the best amount of active ingredient will be 24 g/ha quassine.

Table 1. Aphid development in plots with different variants of aphid control. Means of three replications with 50 leaves, respectively. Herpersdorf, Germany, 2006, cv. Perle. Two applications, respectively, except ⁽¹⁾ one application only.

Variant	16 vi 2006 1 day before 1st application	20 vi 2006 4 days after 1st application	30 vi 2006 4 days after 2nd application	04 vii 2006 10 days after 2nd application	15 vii 2006 3 weeks after 2nd application
Control	50	70	73	111	120
Spray applications					
NeemAzal T/S	55	84	86	165	148
Pyrethrine	68	80	69	52	117
Quassia, homebrew	29	43	14	26	10
Quassia with soft soap	40	32	11	7	3
Systemic applications					
NeemAzal T/S	45	84	42	97	151
Quassine 12 g/ha ⁽¹⁾	44	76	24	49	23
Quassine 24 g/ha ⁽¹⁾	40	51	28	14	4
Quassine 36 g/ha ⁽¹⁾	32	43	42	8	10

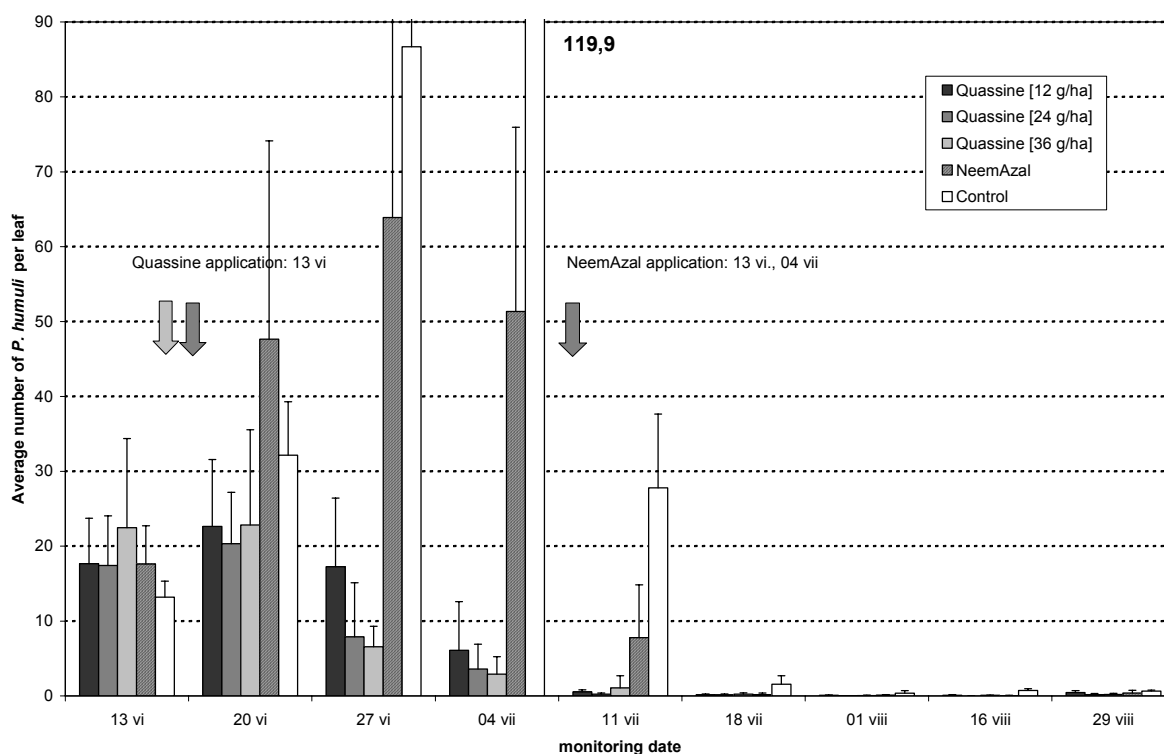


Figure 1: Aphid development in plots with different variants of systemic aphid control. Means and s.e. of four replications with 30 leaves, respectively. Ursbach, Germany, 2006, cv. Perle.

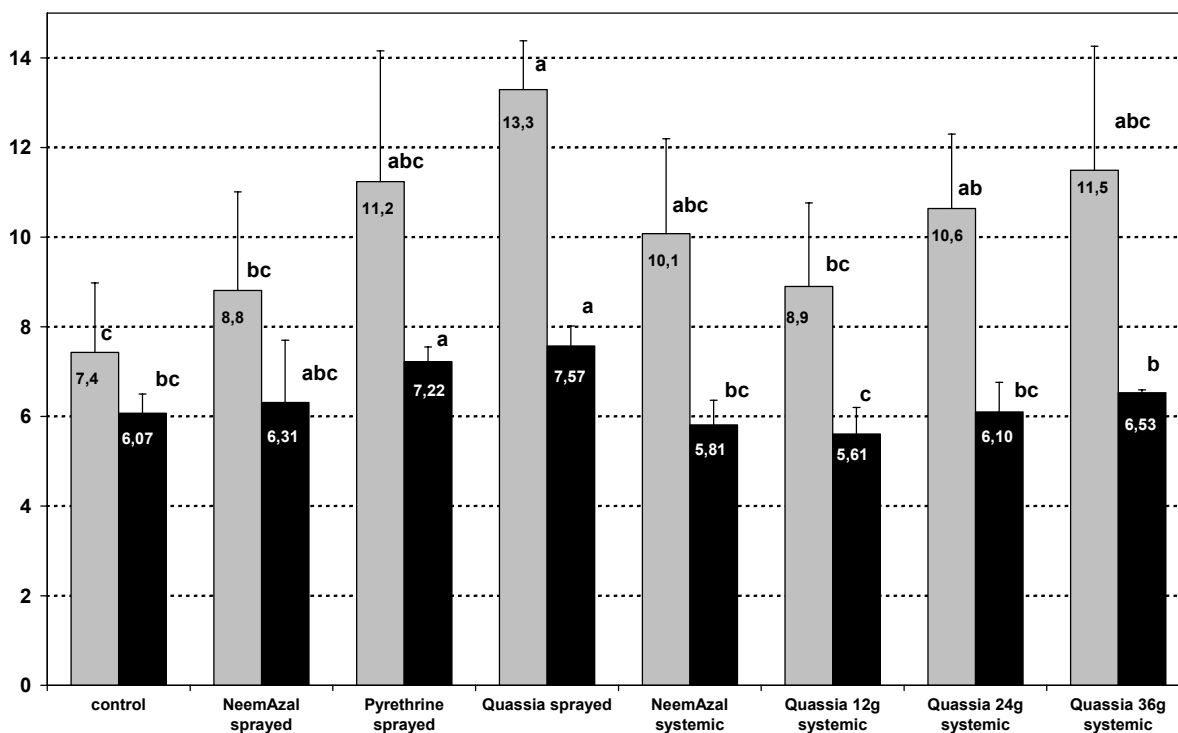


Figure 2: Experimental harvest, Herpersdorf, 05-ix-2006. Yield [dt/ha] (grey bars) and alpha acids [%] (black bars) of plots with different variants of aphid control. Bars with the same letter are not significantly different (ANOVA, $p < 0.05$)

Perspectives

The results we achieved during this study show that organic growers in Germany currently are dependent on quassia products in order to safeguard satisfying control of *Phorodon humuli*. Effective compounds or control strategies other than those we tested are not registered for organic farming in Germany, and those guidelines are very strict. At the moment no industrial quassia product is registered for aphid control in the EU, and the current modus operandi of organic growers, i.e. the use of homemade quassia brews, is situated legally within a “grey area”. Hence, it is most important to register an aphicide with a standardised content of quassine in Annex I of the EU Council Directive 91/414/EEC in order to make this compound available within the EU. Accordingly registration trials are performed in the Hallertau and Tettang growing areas in 2007, not only in organic but also in conventional farms. Of special interest will be what degree of aphid control can be achieved by a systemic quassine treatment in high alpha cultivars like Hallertauer Magnum. If these trials are similarly successful than those of the past years, quassine will not only be a benefit to organic growers, but probably may serve even as a “plan B” in conventional hop growing.

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CONTENTS OF PESTICIDE RESIDUES IN HOPS

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Application of pesticides against pests and diseases represents worldwide one of the most important factors of agricultural production intensification. Elimination or eradication of harmful organisms like insects, mites, fungal diseases or weeds enables to reach good yields, reduce the losses during storage and improve quality of the product. On the contrary residues of pesticide active ingredients (a.i.) can persist in the crop for a long time and can potentially endanger the health of consumers. Hops belong to the crops with intensive chemical protection. In spite of not being used for direct consumption, limit levels of residues in hops have to be established.

In the period 2004-2006 decline curves of several zoocide active ingredients were determined. Selected aphicides (imidacloprid, acetamiprid) and miticides (fenpyroximate, hexythiazox) were applied during season according to approved methodology. Samples of leaves and later cones as well were taken in regular intervals since treatment to harvest. Determination of residues in leaves is more accurate because the development of leaves is practically finished in the time of inflorescence and cones formation. On the contrary development of inflorescence and hop cones still goes on. Their growth has a considerable effect on residue contents.

Solvent extraction, liquid-liquid extraction, column chromatography and solid phase extraction (SPE) were used for purification of plant samples. Analytical determination of residues was performed by gas and liquid chromatography (GC/MS, HPLC/DAD).

The aphicides belonging to nitromethylene group, imidacloprid and acetamiprid, declines very rapidly in hop leaves. Owing to low application concentrations residue levels of imidacloprid and acetamiprid were far below MRL (Maximum Residue Level) immediately after treatment. Detectable residue levels were under limit values within 14 days after treatment. On the contrary acaricides fenpyroximate and hexythiazox were reliably detectable on hop leaves even one month after application. Nevertheless, no residues of all the tested zoocides were found in hop cones at the time of harvest. Potential risk of persistent pesticides findings in hops may threat from biological admixtures (leaves, stems, bine fragments) that are always a part of harvested hops.

STANDARD RANGES OF THE APPLICATION OF PESTICIDES IN HOPS – A PROPOSAL FOR THE EPPO GUIDELINES

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Abstract

In order to obtain a sufficient range of registered pesticides in hops also in future, the trial results have to be accepted mutually in the central European region (e.g. D, CZ, SK, PL, UK, B, A) and in comparable regions (e.g. F – Alsace). The high costs for registration of pesticides in hops can only be managed that way. The same applies to the southern European region.

Prerequisite for the mutual acceptance of trial results is

- an absolutely identical layout, execution and analysis of the trials and
- the agreement of the licensee of the pesticides (= plant protection companies)

For the execution of efficacy trials according to EPPO guidelines and for documentation according to GEP guidelines, standardised specifications are necessary with regard to application method and to dosage. A proposal is submitted for the crop hops.

Keywords: Hop, plant protection, EPPO guidelines, GEP guidelines, EU harmonisation during registration

Introduction

In order to facilitate the mutual acceptance of registrations between EU member states, it is useful to define standard declarations already in the national registrations (e.g. in the crop hops), which is foreseen in article 10 of the RL 91/414 (EEC).

The following “standard ranges of application” are suggested for the crop hops.

Results

1. Soil sprayings – surface application

Target organism / measure	Application	Instructions in the document of registration
<i>Otiorrhynchus ligustici</i> (Alfalfa snout weevil) <i>Agrotis segetum</i> (Turnip moth) <i>Agriotes segetis</i> (Wireworm)	single crown row application (band width 30 cm)	g/l per crown or concentration (e.g. 0,1%) and litre spray mixture per crown kg/l product per hectare
<i>Halticinae</i> gen. spp. (Flea-beetles)	row application (band width 100 cm)	kg/l product per hectare

<i>Cnephasia alticolana</i> (Tortricid moth) <i>Hydraecia micacae</i> (Rosy rustic moth) <i>Mamestra brassicae</i> (Cabbage moth)	row application (band width 100 cm)	kg/l product per hectare
Slugs	row application (band width 30 cm)	kg/l product per hectare
<i>Pseudoperonospora humuli</i> (Downy mildew) - primary infection –	single crown row application (band width 30 cm)	g/l per crown or concentration (e.g. 0,1%) and litre spray mixture per crown kg/l product per hectare
Hop stripping (Removal of lower shoots)	row application (= 1/3 of the total area)	kg/l product per hectare
Grass weeds e.g. <i>Agropyron repens</i> , <i>Poa annua</i>	row application (= 1/3 of the total area)	kg/l product per hectare
Weeds - annual, monocotyledonous weeds - perennial, root spreading weeds e.g. <i>Carduus nutans</i> , <i>Convulvulus arvensis</i>		
Hop grubbing - destroying of hop crowns for grubbing Deer repelling	row application (band width 100 cm) field margin application: various ways according to product	kg/l product per hectare

The amount of water usage can vary (except the indication “concentration in % and liter spray mixture per crown”). Recommendations (e.g. 400 l/ha) on behalf of the company or by advisory services are possible.

2. Misting / spraying, dependent on the growth stage according to BCCH-code

– Spatial application –

2.1 High trellis systems (6,0 – 7,5 m height)

By reaching full trellis height, the hop plant has developed a maximum of 50 % of biomass and hence of plant surface. Subsequent the development of laterals, flowerings and cones follows.

For the determination of the amount of product during the vegetation period, the necessary application rate for maximum plant development has to be determined, and then the proportionate amounts for various growth stadia have to be fixed.

2.1.1 Insecticides / acaricides

In previous registration tests and in practice it proved worthwhile to fix a definite product amount for insecticides and acaricides (l or kg/ha) from BBCH 39 onwards. Prior to BBCH 39, necessary applications are relatively rare.

Target organism / measure	Application	Instructions in the document of registration
<i>Phorodon humuli</i> (Damson-hop aphid) } <i>Tetranychus urticae</i> (Two-spotted spider mite)	until BBCH 39 = 67% from BBCH 39 onwards = 100 %	kg/l product per hectare
<i>Ostrinia nubilalis</i> (European corn borer)	from BBCH 39 onwards = 100 %	kg/l product per hectare

2.1.2 Fungicides / botryticides

The use of fungicides in spatial applications spans from BBCH 35 to BBCH 85. For the testing of the biological efficacy and in trials for the determination of maximum residue levels it makes sense

- to fix the concentration in % and
- to vary the amount of water usage according to **annex 1**

With these data it is possible to calculate the product usage in kg/l per hectare. For the document of registration it is sufficient to fix the amount of usage for three growth stadia.

Target organism / measure	Application	Instructions in the document of registration
<i>Pseudoperonospora humuli</i> (Downy mildew) - secondary infection - } <i>Podosphaera macularis</i> , formerly <i>Sphaerotheca humuli</i> (Powdery mildew)	until BBCH 37 = 45 % until BBCH 55 = 67 % from BBCH 55 onwards = 100 %	kg/l product per hectare kg/l product per hectare kg/l product per hectare
<i>Botrytis cinerea</i> (Grey mould)	until BBCH 55 = 67 % from BBCH 55 onwards = 100 %	kg/l product per hectare kg/l product per hectare

The amount of water usage can vary in practice.
 Recommendations on behalf of the company or of advisory services are possible.

2.2 Low trellis systems (2,5 – 3,0 m height)

For low trellis systems the same BBCH scale is valid as in high trellis systems. However, the foliage is more dense and reaches down to the ground (as a closed “foliage wall”). With similarly high yield, the quantity of cones is comparable to high trellis systems. Hence the amount of product can **not** be reduced in the same dimension, despite to the lowering of the system’s altitude by approximately 50 – 55 %.

According to experiences from

- the United Kingdom and
- research and development projects in Germany

in low trellis systems the amount in kg/l per hectare can be reduced **only by 25 %**, compared to high trellis systems.

MOLECULAR VARIABILITY WITHIN THE COAT PROTEIN GENE OF HOP MOSAIC AND HOP LATENT CARLAVIRUSES

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Australian hop production is free from the major fungal diseases and pests that cause significant losses in hop-growing areas around the world. The main pathological cause of yield loss is infection by viruses. Three viruses are commonly found in Australian hop gardens, *Hop latent carlavirus* (HpLV), *Hop mosaic carlavirus* (HpMV) and *Apple mosaic ilarvirus* (ApMV; Wilson *et al.* 2004). Two distinct strains of ApMV infecting hop have been identified through serological relationships and by sequence analysis of the coat protein gene (Crowle *et al.* 2003). Whilst possible symptom variants have been observed, there is no serological or molecular data to support variation within the common hop carlaviruses. Here we present partial coat protein sequence data for Australian isolates of HpMV and HpLV and show evidence for two distinct strains of HpMV.

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NEMATODES ASSOCIATED WITH HOP PRODUCTION IN TASMANIA, AUSTRALIA

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The abundance, diversity and temporal fluctuations in plant-parasitic nematodes associated with hop production, and their effect on cone yield and levels of alpha acids were assessed in two hop (cv. 'Pride of Ringwood') gardens in Tasmania, Australia at Forrester River and Gunns Plains.

The most abundant plant-parasitic nematode found at both sites was *Heterodera humuli* Filipjev 1934. Temporal fluctuations in the numbers of *H. humuli* suggested one generation occurred at both sites consisting of egg hatch and a subsequent peak in second stage juveniles (J2) early in the season in mid-October. Numbers of *H. humuli* J2 declined rapidly thereafter, most likely as J2 entered roots and established feeding sites. This is consistent with other studies, which have suggested only one generation of *H. humuli* per year (Duffield, 1927; von Mende and McNamara, 1995). However, at Gunns Plains there were two apparent peaks in the recovery of J2, one in mid-October and another in late November. The low numbers occurring between these dates may have resulted from fluctuations in soil temperature and lead to poor egg hatch. It is unlikely that two generations would have been possible during this short period of time.

At Forrester River, the numbers of *H. humuli* J2 in mid-October were significantly negatively correlated with cone yield. No significant correlations with cone yield and numbers of *H. humuli* J2 counted later in the season, or between *H. humuli* J2 at any sampling date and alpha acid content. Further work is needed to confirm the extent of the yield loss due to *H. humuli*. However, control of nematodes by nematicide is unlikely to be an economic or effective control strategy in hop gardens because of the extensive root systems and heavy clay soils. Studies in other countries have suggested that *H. humuli* can reduce growth in pot experiments but under field conditions hop has a great capacity to tolerate nematode feeding because of its perennial nature and extensive root system (Hafez *et al.* 1999).

Low numbers of *Helicotylenchus dihystra*, *Meloidogyne* spp., and *Pratylenchus* spp. were also found. The low numbers of these nematodes found in hop gardens during times of active hop growth suggests that hop was not their preferred host.

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THE COMPENDIUM OF HOP DISEASES, ARTHROPOD PESTS AND OTHER DISEASES

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The Compendium of Hop Diseases, Arthropod Pests and Other Disorders, is currently being prepared for publication by the American Phytopathological Society Press as part of their series of compendia. Publication is anticipated late 2007. The hop compendium includes introductory chapters on the taxonomy and distribution of *Humulus lupulus*, commercial and home production, hop use and chemistry, and a list of the world cultivars and their characters, in addition to sections on diseases, pests and disorders. In general, all sections are written by experts from at least two different countries. To ensure broad acceptance of this compendium each section has been reviewed by experts from alternative production regions. The authors represent researchers, brewers, private breeders, and private consultants from nine countries.

In general, for each disease, pest and disorder, information is presented on the symptoms, causal agent, disease cycle and epidemiology, management, and a small number of selected references. Color photos of excellent quality integrated within the text will be a feature of this publication. The images within the compendium will also be published as part of a companion image collection on a CD-Rom which will be sold together with the compendium or as separate items.

The compendium is being supported by brewers, various hop commissions in the United States and individual growers. Furthermore, commitments have already been made in advance from several companies for substantial purchases of the compendium. We hope this publication will become a highly regarded resource with broad appeal for growers, advisers, scientists, and home gardeners.

VI. Session:

HOP PRODUCTION

INFLUENCE OF WEATHER CONDITIONS AND IRRIGATION ON YIELD AND QUALITY OF HOPS

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Abstract

Production efficiency of economical system of hop irrigation has been statistically proved by a long-term field trial at a hop farm in Stekník. The farm is a part of Hop Research Institute. Žatec semi-early red-bine hop was used for this purpose. During twelve years of the trial yield of hops was higher by 22%. Nevertheless, typical quality features of Czech fine aroma hops were not influenced by this increase of hops production. Irrigation water supplied with the help of an irrigational system placed at the ceiling of a wire-work influenced microclimate in a positive way and was efficient as a thermo-regulatory factor. Irrigated air temperature was decreased by 4 °C. Under high day temperatures, typical for the last years, lower temperatures have pleasant influence on biochemical and physiological processes in hop plants. Yield stability is very dependant on weather conditions. Deficit of precipitations in the last years is more than 30% in comparison with a long-term average. High temperatures can enlarge the fact as well. The increase as against long-term values may be since 0,5 to 3,2 °C.

All above mentioned factors unfavorable for growth of hop plants is possible to a large extent eliminate with the help of irrigation, which can be characterized as an important stabilization factor with its help we are able to cope with precipitation deficit and it this way to decrease their negative effect on hop production. Repeated supplementary irrigation has a favorable effect on habitus of hop plants. Such plants are more vigorous, their assimilatory surface is larger and they are more densely put forth.

On the base of the obtained results we can conclude that irrigation has no negative influence on the contents of alpha acids in hop cones. Together with the stabilization of hop yield higher production of alpha acids per hectare is obtained. Progressive economical irrigation systems are recommended if we want to use an efficient irrigation system. Drop irrigation was placed on the ceiling of a wire-work (irrigation detail is placed above rows of hop plants) or underground irrigation system (irrigation detail is placed 60 cm under the ground level in the space between hop plants) were used. Drippers were used to provide uniform rate of irrigated water (2 l/hour).

A balanced method based on prognosis of irrigation water need in week intervals with the help of a graphic–analytic method was used to control irrigation regime nowadays. The method provides actual data under respect of regional local conditions. It issues from the humidity needs as well as weather conditions.

Acknowledgement

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HOP WIREWORKS IN CZECH REPUBLIC

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Growing yields of hops in new Czech varieties under existing types of wire-works with traditional wire elements increase requirements on maintenance and repair works are more often. In this way the risk of their breakdown becomes real. A new type of a wirework brings about higher stability under the yield of hops on the level of 3 t/ha. The costs keep on the same level even though quality is higher.

The following activities are included into the project:

- determination of data for carrying capacity of a wirework
- proposal of a wirework
- statistical calculation
- construction of an experimental wirework
- measurement of forces in anchorage rods
- design of a numeric model as well as testing of its characters
- economical assessment.

Space bar-shaped model of the wirework is in ANSYS program. The model was designed as geometric nonlinear. Severe deformations have been taken into account. Resilience plastic parameters of soil were lead in supports of the poles. Prestress was used in cable elements. Load state before harvest - normal forces (N) of crossbars, lengths, anchors.

The wirework was not fully loaded by vigorous hop plants in the first vegetation year. Nevertheless, deformation of soil under poles was obvious. Therefore the wirework had to be fastened after harvest. In 2007 measurements of forces in anchor connecting rods were repeated.

Acknowledgement

The work was supported by the Czech Ministry of Industry and Commerce, within the project no FI –IM2/180.

VII. Session:

HOP RESEARCH COUNCIL

REPORT ON THE ACTIVITIES OF THE US HOP RESEARCH COUNCIL

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Adapted from the Hop Research Council Website (www.hopresearchcouncil.org)

This report is included as a courtesy to enhance communication between the Hop Research Council, and the Scientific Commission of the International Hop Growers' Convention.

Mission of the Hop Research Council

The Hop Research Council was established in 1979 by a group of hop industry members that believed in the benefit of a united hop research effort. The Council is a non-profit organization that funds and directs hop research that benefits the United States hop industry. Members include domestic and international brewers, hop dealers and hop grower organizations.

The Hop Research Council's primary objective is to fund hop research. The Council strives to serve the needs of all segments of the hop industry by maintaining a membership that covers the entire industry and by supporting research that meets the needs of its members. Current priorities include improvement of agronomic and quality traits, lower cost of production and processing, and elimination or control of diseases and pests of U.S. hops. The Council has two project categories – Special and Ongoing. Special projects have a funding limit of three years. After the third year the Council has the opportunity to evaluate the project and determine if it should be continued as an HRC Ongoing project. Current Ongoing projects and two Courtesy projects are listed below. Courtesy projects do not receive funding from the Council, but the project leaders provide reports to the Council and participate in meetings. Detailed descriptions of Ongoing and Courtesy projects are available on the Hop Research Council Website.

Current Hop Research Council Projects

Breeding/Germplasm:

Hop Genetics and Breeding for Superior Germplasm:

U.S. Department of Agriculture – Dr. John A. Henning

Hop Cultivar Development, Physiology and Chemistry

Washington State University – Dr. Stephen T. Kenny

Chemistry/Brewing:

Chemistry of Hop Polyphenols

Oregon State University – Dr. Max L. Deinzer

Hop Chemistry

Oregon State University – Ms. Caitlin Prueitt

Influences of Hop Components on Beer Quality

Oregon State University – Dr. Thomas H. Shellhammer

Pathology:

Control of hop Diseases Caused by Viruses and Virus-Like Agents

Washington State University – Dr. Ken Eastwell

Biology, Epidemiology, and Management of Hop Diseases

U.S. Department of Agriculture – Dr. David H. Gent (Courtesy Report)

Fungal Diseases of Hop and Their Control

Washington State University – Dr. Gary Grove and Mr. Mark E. Nelson

Hop Disease Management

Oregon State University – Dr. Cynthia M. Ocamb

Entomology:

Development of Hop Integrated Pest Management Strategies

University of Idaho – Dr. James D. Barbour

Integrated Management of Insects and Mites on Hops

Washington State University – Dr. David G. James

Hop Quality:

Plant Protection Product Registration and International Harmonization

U.S. Hop Industry Plant Protection Committee – Ms. Ann E. George (Courtesy Report)

Current Membership of the Hop Research Council

Brewers: Anheuser-Busch inc., Heineken NV, Miller Brewing Company, Sierra Nevada Brewing Company, and Suntory Ltd.

Hop Dealers: Hop Union LLC, John I. Haas Inc., S.S. Steiner Inc., and Yakima Chief, Inc.

Hop Grower Organizations: Idaho Hop Commission, Oregon Hop Commission, and Washington Hop Commission.

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