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Dr. Elisabeth Seigner
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Foreword

This meeting of the Scientific Commission (SC) of the International Hop Growers Convention (IHGC) is an important forum for all scientists involved in hop research. Participants from all around the globe are interested in joining this meeting to exchange new ideas and information.

For the first time this meeting is held in a non-European country. When Gerrie Brits during the meeting at Canterbury proposed to hold the meeting of the Scientific Commission in South Africa in 2005, certainly there were sceptical views from all sides including me. This proposal clearly was beyond the principles of the SC: a meeting hold for the first time outside of Europe and being invited by a brewery.
Certainly, all participants were curious to get to know hop growing in South Africa for the first time – but could they arrange it to get the money for traveling.
Looking at the list of participants registered for this year´s meeting it was right to venture it. There are delegates from 10 countries all around the globe who are presenting their latest research works. Thank you to the large Czech delegation from the Zatec Hop Research Institute for attending this meeting. Representatives of hop trading and processing firms and of the brewing industry as well are here and we – the scientists rely on the information and the exchange of experience from these industry partners in order to adapt our research objectives on their needs and demands. Talks during this meeting are of utmost importance to make this meeting a great success.

- I would like to express my special thanks to Mr. Gerrie Brits who rendered outstanding services to this year`s meeting of the Scientific Commission.

- Gerrie Brits has organized everything concerning this meeting. Many thanks to Gerrie for guiding us through all SAB hop facilities and for showing us the special things of hop growing in South Africa. We are also interested in being informed about Gerrie´s breeding programs and his hop varieties. Moreover, we highly appreciate all his efforts in organizing a pre- and post conference tour.

- Thanks are also due to the board of directors from SABMiller. Only their willingness to provide spiritual and financial support for this meeting made it possible for us to come to South Africa.

- Thanks to the speakers who are presenting papers and posters for passing on their knowledge and experience to the hop industry.

- Thanks to the secretary Dr. Elisabeth Seigner for all her work before and during this meeting.

I wish this meeting much success so that this meeting of the Scientific Commission here in South Africa will remain in our minds for ever.

Bernhard Engelhard
Chairman of the Scientific Commission, I.H.G.C.

I. Session:

HOP BREEDING

THE ASSESSEMENT OF RESISTANCE TO DISEASES IN THE UK BREEDING PROGRAMME

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Abstract

The methods used at East Malling Research for the assessment of resistance to the major fungal hop diseases are outlined. Typical results are presented and discussed in relation to the host-pathogen interactions and the modes of inheritance of resistance to each disease.

Keywords : hop, breeding, diseases, resistance

Introduction

The incorporation of resistance to diseases is the stated aim of most of the hop breeding programmes throughout the world. Resistance is assessed from a direct challenge by a pathogen, either in the field or in a controlled environment such as a glasshouse. However, molecular markers for disease resistance are increasingly being sought as an alternative method of selection. In order to evaluate the correlation of molecular markers with the classification of disease phenotypes, it is important that the suitability and limitations of the methods used to assess resistance are understood. They must be appropriate for the biology of the host-pathogen interaction and for the mode of inheritance of the particular disease.

The breeding for resistance to fungal diseases is well established in the EMR programme and has successfully developed varieties providing strong, long-lasting resistance in commercial production. Examples include ‘Wye Target’ with resistance to powdery mildew and wilt diseases, and ‘Wye Challenger’ with resistance to downy mildew. The procedures developed during the selection of these varieties have been integrated with the selection procedures for other traits and now form a routine part of the breeding programme. This paper outlines these methods; interpretation of the results is illustrated with reference to those obtained in 2004.

Hop powdery mildew

Resistance to hop powdery mildew, caused by *Sphaerotheca humuli*, is expressed in the ability of haustoria to establish in epidermal cells. Haustoria are often limited by a host hypersensitive reaction (Godwin, Mansfield and Darby, 1987). There are pronounced age effects and the most susceptible tissues are young leaves and flowers (Darby, Godwin and Mansfield, 1989). Inheritance of resistance is controlled by a series of major genes with classic, differential, qualitative gene-for-gene interactions between host and pathotype (Royle, 1978), although modified by a polygenic system expressed as the number of infection sites and intensity of sporulation. Thus, with major gene effects expressed clearly in juvenile tissues, it is appropriate to assess the resistance of young seedlings as an indication of the resistance of the mature plant.

Seedlings are assessed in a shaded glasshouse when approx. 30 cm tall. Inoculum is generated on plants of cv. ‘Northern Brewer’. A single pathotype is used, selected as the most widespread pathotype in commercial production. An aggressive isolate of this strain, producing severe symptoms on ‘Northern Brewer’, is maintained and bulked-up for the

seedling screen to provide approx. one inoculator plant for every 70 test plants. Freely-sporulating inoculator plants are suspended 1.5 m above the test seedlings and spores descend on to the seedlings by natural air movements within the glasshouse, encouraged by daily agitation of the inoculator plants. Test plants are watered from below. Under these conditions, many of the major genes are seen to confer resistance rather than immunity, and sporulation is observed to some extent on all plants. Infection levels are assessed after 20 days and classified into five categories (Table 1). Included within the test glasshouse are at least two plants of genotypes known to give responses in each of these categories to the pathotype used. The amounts of sporulation on these plants are used as the reference for each category.

Table 1. Selected results from the 2004 powdery mildew screen at EMR-Wye showing the numbers of individuals in each disease category.

Family	Cross type	Expected ratio of R:S	Strong rest.	Rest.	? Rest.	?Partial rest.	Full susc.
11/03	S x S	0 : 1	0	0	0	0	39
32/03	S x ?S	0 : 1	0	0	0	22	37
21/03	S x ?R	1 : 1	3	19	0	3	25
51/03	S x R	1 : 1	20	9	4	0	30
50/03	R x R	3 : 1	14	26	4	0	13

This assessment allows identification and selection of individuals expressing resistance to the disease. It also indicates those individuals where polygenic resistance is enhancing the major gene effect, or providing partial resistance in a susceptible family. Furthermore, the balance of numbers of individuals between categories can give an indication of the resistance status of the parents at a more detailed level than that obtained from simple Mendelian analysis.

Molecular markers would seem to offer little advantage over this test which assesses large numbers of progeny at an early stage in development in a controlled environment where variability is small. However, the ability to identify genotypes carrying more than one effective major resistance gene would enhance breeding capability.

Hop downy mildew

Hop downy mildew, caused by *Pseudoperonospora humuli*, has a complex aetiology with systemic infection producing root rot, alteration of meristematic development resulting in growth abnormalities (known as “spikes”), and topical infection producing leaf lesions and cone discoloration (Royle, 1981). The expression of non-systemic resistance can be assessed from the intensity of sporulation, but the correlation between systemic and non-systemic symptom expression between genotypes is unclear. Hence, it is difficult to define resistance with a single measure. Inheritance of resistance appears to be polygenic and quantitative. Therefore, selection efficiency will be enhanced by assessment of as many individuals as possible.

The assessment of resistance to downy mildew at EMR is performed on very young seedlings to maximise numbers and the use of space. Seedlings are tested when they have produced two leaf pairs beyond the cotyledon stage, at approx. 6 cm tall. Seedlings in a frost-free glasshouse are contained within seed trays with plants only 3.25 cm apart. An aggressive strain of downy mildew, isolated from field plants, is maintained throughout the winter on detached leaves in a growth chamber to provide inoculum for the test. An aqueous

suspension of sporangia is generated sufficient to provide 30 mls of inoculum per 100 seedlings at 10,000 sporangia/ml. The suspension is atomised uniformly on to the seedlings which are then enclosed overnight with plastic sheets to maintain several hours of high humidity. Sporulation is apparent after 5 -7 days but the test is assessed after 15 - 20 days when terminal spikes have developed. Throughout the test period watering is from above to aid the spread of infection. Two reference progenies are always included in the test to indicate when symptoms have fully developed. These progenies are from the same crosses every year and have been devised to give a quantitative indication of resistance and susceptibility. All plants showing terminal spikes are discarded and resistance for the family is recorded as the percentage of plants without spikes.

This disease screen removes from the population the most susceptible individuals where meristematic infection has occurred. It provides no quantification of the resistance of the surviving individuals and additional records of the symptoms of natural infection of mature plants in the field under moderate disease pressure are required to distinguish levels of resistance between individuals. It does, however, provide quantitative information on resistance at the family level and, thus, on the ability of the parents to transmit resistance to their progeny (Table 2). This is particularly useful in identifying parents with marked resistance or susceptibility.

Table 2. Selected results from the 2004 downy mildew screen at EMR-Wye.

Family	% Resistance	Comment
1/03	86	Rest. reference family
2/03	44	Susc. reference family
38/03	98	Progeny of 73/98/54
39/03	93	Progeny of 73/98/20
40/03	92	Progeny of 73/98/20
41/03	54	Progeny of 44/84/20
42/03	83	Indicates a rest. parent
43/03	31	Indicates a susc. parent
44/03	56	Progeny of 44/84/20
211/03	18	Progeny of ‘Columbus’

Current work indicates that genotypes regarded as resistant to downy mildew in all its forms will be distinguished by high levels of progeny resistance in this seedling screen. The converse applies to highly susceptible genotypes.

Hop wilt

Hop wilt, caused by *Verticillium albo-atrum*, is controlled by complex genetics with the action of epistatic genes and both a major and polygenic system, reflecting different sources of resistance (Darby, 2001). Resistance is expressed in the endodermis in the root which determines the degree of colonisation of the vascular system and the restriction of the pathogen to the root tissues (Talboys, 1958). Symptom expression in the aerial parts of the host results from the action of toxins and spores carried in the xylem vessels out of the root system. Thus, assessment of resistance in an individual requires a mature, differentiated root system and the size of such a hop rootstock necessitates that assessment is in a field test.

Inoculum is derived from up to five isolates obtained from wilt outbreaks in commercial plantings of highly resistant varieties, ensuring that the most virulent strains available are included in the test. Aqueous conidial suspensions are prepared from agar-grown colonies of each isolate and used to seed sterilised straw, which is incubated until uniformly colonised. At planting time, 60 g of straw inoculum is placed in the planting hole immediately beneath the rootstock. The use of such artificial inoculum reduces the presence of other root infecting hop pathogens such as *Fusarium* spp. which can complicate assessment. The planting material is in the form of one-year old dormant rootstocks derived from softwood stem cuttings grown until senescence 20 cm apart in a field nursery. The trial is laid out as three blocks with plots, comprising 10 rootstocks of a genotype, randomised within each block. It has been found that this 30-plant test is the minimum to give consistent results, overcoming the high variability inherent in a field test. High quantities of nitrogen fertiliser, amounting to in excess of 300 Kg/ha, are applied as a split dose during the early spring to encourage infection levels above that experienced in commercial production (Sewell and Wilson, 1967). Plants are assessed for the presence of aerial symptoms, notably severe wilting causing death of the bine. At the end of the season, stems are examined in cross-section for staining of the vascular bundles indicating infection has occurred. Bine sections, 10 cm long, are excised 5 cm above ground from any plants remaining symptomless after these examinations. They are plated on to selective media to reveal the presence of *V.albo-atrum*.

Because it is a field test, there is considerable seasonal and environment x genotype variation (Table 3). Soil temperatures in particular greatly influence infection and symptom development with infection decreasing as soil temperatures increase (Talboys and Wilson, 1970).

Table 3 The incidence (%) of wilt disease in reference varieties in recent wilt tests at East Malling Research.

Variety	2000	2001	2002
Fuggle	100	100	100
Keyworth's Mid	67	80	66
Progress	70	80	90
Target	45	56	52
27/57/264	36	35	50

Therefore, results are interpreted relative to those obtained in the same test from reference varieties which have known relative resistances. A wilt score is determined arithmetically (usually a mean value) from a combination of data on the incidence of severe symptoms and total infection such that the reference varieties are ranked in the expected order (Table 4). The data from the individuals under test are used similarly to determine their wilt score. Wilt scores vary greatly for a given genotype between seasons, so results for test individuals are always expressed as a ratio of their score to that of a known resistant reference variety. Historically, at EMR-Wye, this has been 'Keyworth's Midseason' which has been in all tests since 1953, thereby providing continuity.

The wilt test described here requires 30 mature plants of each genotype, which limits the number of genotypes that can be assessed in any season. It also takes a total of two years to perform, including propagation, and results are subject to great environmental variability. In the EMR hop programme it is, therefore, limited to assessment of genotypes at the later stages of selection and cannot be used as a primary selection tool. The assessment of resistance to wilt disease would be a prime candidate for the use of molecular markers which would allow selection at the seedling stage from large numbers of progeny with no environmental confounding.

Table 4 The incidence of wilt disease in reference varieties in the 2004 wilt tests at East Malling Research.

Variety	% plant deaths	% infection	Wilt Score 2004
Fuggle	100	100	100
Keyworth's Mid	59	90	75
Progress	38	100	69
Target	26	67	47
Pilgrim	25	50	38
27/57/264	9	43	26

Concluding remarks

Other fungal diseases may become important in the future as the climate changes or cultural practices are modified, such as by the growth of dwarf varieties. The breeder needs to keep under constant review the methods and procedures used to incorporate disease resistance into new hop varieties.

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STABILITY OF THE PRODUCTIVITY OF WORLD HOP VARIETIES AS AN IMPORTANT FEATURE FOR THE SELECTION OF PARENTAL COMPONENTS

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Introduction

The base for hop breeding process with the help of crossing is the selection of convenient parental components. This selection is influenced by two viewpoints. First, mother and father are selected on the base of their qualitative parameters such as structure of hop resins essential oils, smell of hop cones, resistance and on the base their of quantitative parameters such as yield of hops and production of resins (Nesvadba, Krofta, 2002). Shape of hop plants (length of laterals, height and density of cones on plants, width of vines) is evaluated as well. Second, transfer of demanded features on the progeny is evaluated. This parameter is very important but its assessment is very difficult. A great number of features are polygenic and therefore it is difficult to say if these features will be transferred to progeny after their parents (Nesvadba et al., 2003). Combinational ability of selected parents is very important from this point of view. The base for selection of convenient parents is a wide genetic diversity of breeding material (Chloupek, 2000).
For hop breeding demanded parameters of breweries, market and pharmacy are very important. Hop breeder aims at first at resistance, yield, contents of hop resins and other features. Even after developing a new variety there may be some problems. A new variety is usually grown in a hop-garden at minimum for fifteen years and it is necessary for it to be successful to keep its parameters (Nesvadba, Krofta, 2003). This paper is about the stability of yield and alpha bitter acids contents. Long lasting results of original material evaluation for hop breeding show the both their productivity and last but not least their stability of productivity. It is known that productivity of many hop varieties declines in the time of their cultivation. It is important for hop breeding process not to use such non-stabile varieties as an origin material. It is necessary to say that obtained results are important at first for growing conditions of Czech Republic. Variability of many hop varieties is influenced by weather conditions in individual years (Krofta et al., 1997). On the other hand these conditions are the same for all grown varieties. Statistical analyses show that some varieties are typical for their decrease of productivity whereas some varieties have lower variability during their cultivation time. Stability is a very important feature of productivity. From this point of view it is necessary for a breeder to take into account stability of productivity and influence of natural conditions typical for a country where these varieties are grown.

Material and methods

Hop Research Institute in Žatec has a collection of hop genetic sources, which was newly established in 1976 and it is gradually enriched. Obtained results are only from one locality. All the cultivated hop varieties have therefore the same growing conditions. The collection is assessed regularly every year and therefore the results from many years are at disposal. Basic statistical parameters are used for their evaluation: arithmetic average (x), conclusive variation (s) and variation coefficient (Vk), which means extent of variability in % for comparison of the features on their productive level. In another part of the statistical evaluation we set out the differences among the collections. From the obtained results of the tested varieties with the help of the analysis of the dispersion of double crossing it is possible to determine the difference among them ($P=1-\alpha$). For the assessment of the influence of the age of evaluated plants function of linear regression was chosen. The tightness of dependence was determined with the help of correlation coefficient r. One hundred times higher value than determination coefficient (r^2) says from how many percent is the value of the shown feature at the posterity caused by the age of evaluated plants. Yield of hops is

shown in kg per fresh hops. A sampled picking machine Wolf harvested eight plants of each variety. Contents of alpha bitter acids are determined with the help of KH method (EBC 7.4) or HPLC (EBC 7.7).

Results and discusion

In Table 1 there is shown and average yield and KH value within hop varieties assessed since 1977 to 2004. It is obvious that the most productive are the varieties Brewers Gold, Late Cluster and Vojvodina. On the contrary, the lowest yields show varieties Saaz, Savinský Golding and Star. The highest variability shows Late Cluster and the lowest one Star. By the influence of the age an important decrease of yield in Neoplanta (r=-0,61) and Golding (r=-0,54) were determined. High decline is obvious in Apolon, Fuggle, Saaz and Vojvodina as well. The highest decrease of KH caused by the age of the evaluated plants is in Record, Vojvodina, Northern Brewer and Aurora. The results confirm our experience that in many genotypes and also in other generations Northern Brewer has genotypes with non-stabile contents of KH. The highest variability shows Record and the lowest one Atlas. It is obvious from the results that for a breeding process aimed at alpha bitter acids contents it is better to use e.g. varieties Atlas or Apollon than Aurora in spite of the fact that this variety has the highest contents of KH. With the help of analyses of dispersion it was determined that higher contents of KH have the following varieties: Ahil, Apollon, Atlas, Aurora and Northern Brewer and therefore a breeder should take into account a variety stability in its productivity when he is looking for a mother plant convenient for higher contents of alpha bitter acids. Our long time (28 years) of variety evaluation is very valuable as a breeder can compare productivity of varieties within the conditions of cultivation in his country.

Table 1: Productivity and stability of productivity of the hop varieties cultivated in Czech Republic since 1977 to 2004

Variety		Yield (kg/plant)				KH (% w/w)			
Name	Country	**x**	Vk (%)	r	r^2 x 100	**x**	Vk (%)	r	r^2 x 100
Ahil	Slovenia	**2,4**	27,3	0,15	2,3	**6,6**	29,3	-0,31	9,7
Apolon	Slovenia	**2,7**	30,1	-0,33	11,0	**6,3**	29,1	-0,19	3,6
Atlas	Slovenia	**2,4**	26,8	0,12	1,4	**6,3**	23,2	0,00	0,0
Aurora	Slovenia	**2,6**	26,4	0,02	0,1	**6,8**	29,1	-0,53	28,1
Brewers Gold	England	**3,7**	24,6	-0,08	0,6	**5,5**	21,7	-0,09	0,9
Cascade	USA	**2,8**	22,4	0,40	15,7	**4,0**	30,2	-0,16	2,4
Dunav	Yugosl,	**3,2**	25,7	-0,18	3,2	**4,4**	29,6	0,01	0,0
Fuggle	England	**2,7**	20,7	-0,33	11,2	**3,5**	33,7	-0,32	10,2
Golding	England	**2,5**	28,7	-0,54	28,8	**3,1**	27,3	-0,28	7,7
Hallertau	Germany	**2,6**	22,2	-0,24	5,8	**2,9**	38,3	-0,39	15,4
Hallertauer Gold	Germany	**2,3**	22,8	-0,24	5,6	**4,5**	25,7	-0,34	11,9
Hüller Bitterer	Germany	**2,9**	31,6	-0,19	3,7	**4,3**	26,1	0,05	0,3
Late Cluster	USA	**3,5**	37,6	0,37	14,0	**2,9**	27,6	0,20	4,1
Neoplanta	Yugosl	**3,0**	23,4	-0,62	37,9	**5,5**	28,8	-0,46	20,9
Northern Brewer	England	**2,8**	29,0	-0,22	4,8	**6,1**	30,5	-0,58	33,5
Os. Klon 72	Czech R	**1,7**	34,0	-0,47	22,2	**2,8**	30,1	-0,13	1,8
Record	Belgium	**2,4**	25,3	-0,61	37,6	**3,6**	42,8	-0,64	40,7
Savinský Golding	Slovenia	**2,1**	30,28	-0,02	0,1	**2,9**	31,9	-0,32	10,1
Star	Belguim	**2,2**	18,6	-0,08	0,7	**2,5**	38,6	-0,43	18,1
Vojvodina	Yug.	**3,5**	28,9	-0,44	19,7	**4,4**	39,3	-0,60	36,1

In Table 2 there is shown an average yield and KH value in the varieties evaluated since 1982 to 2004. The most productive varieties are the following ones: Talisman, K 692266 and Sládek. On the contrary the lowest yields were obtained in Wye Viking, Estera and H. Fortschritt. The highest variability shows Pride of Ringwood and the lowest one Bor. By the influence of age an important decline is observed in many varieties, the severest one in Perle (r=-0,77). The highest KH show Target, K 692266 and Viking. On the contrary the lowest KH have H. Fortschritt and Žitomirský 5. A lot of varieties show high variability of this group (the highest one Wye Northdown). The lowest variability was obtained in Wye Viking (Vk = 24,7%). High decline of KH values in the relation to the time of cultivation show Wye Northdown, Buket, Perle, Sládek, Bobek and Polesský. From the common view of productivity and stability of productivity (yield and KH) show the best parameters Columbia, H. Aroma and Wye Challenger. Of course, other evaluated varieties have a good stability in yield or KH as well.

Table 2: Productivity and stability of productivity in the hop varieties cultivated in Czech Republic since 1982 to 2004

Variety		Yield (kg/plant)				KH (% w/w)			
Name	Country	**x**	Vk (%)	r	r^2 x 100	**x**	Vk (%)	r	r^2 x 100
Blisk	Slov.	**2,6**	37,7	-0,37	13,5	**5,4**	42,9	0,00	0,0
Bobek	Slov.	**3,3**	24,5	-0,31	9,9	**4,1**	77,4	-0,51	26,5
Bor	Czech	**3,0**	22,7	-0,51	26,5	**5,9**	48,0	-0,40	15,7
Buket	Slov.	**3,1**	26,7	-0,60	35,7	**6,5**	74,8	-0,65	42,7
Columbia	USA	**3,0**	28,1	-0,26	6,7	**4,5**	59,5	-0,33	10,6
Estera (klon 18)	Poland	**1,7**	30,4	-0,41	16,9	**3,5**	40,3	-0,33	10,8
Fuggle	Eng.	**2,7**	29,8	-0,59	34,9	**2,9**	41,6	-0,22	4,7
Hüller Aroma	Ger.	**2,4**	27,5	0,34	11,4	**3,6**	93,8	0,23	5,3
Hüller Fortschritt	Ger.	**1,7**	30,9	-0,01	0,0	**2,7**	29,7	-0,05	0,2
K 692266	Japan	**4,1**	26,7	-0,55	30,0	**7,2**	70,8	-0,13	1,7
Lublinský	Poland	**2,2**	31,8	-0,51	26,3	**3,1**	48,9	-0,37	13,8
Perle	Ger.	**3,0**	35,0	-0,77	59,8	**5,2**	62,0	-0,61	37,0
Polesský	Ukraine	**2,6**	27,0	-0,45	19,9	**5,8**	61,9	-0,50	25,5
Pride of Ringwood	Austr.	**2,2**	43,9	0,41	17,1	**4,4**	78,7	-0,05	0,3
Ringwood Speciál	Austr.	**4,0**	30,3	-0,29	8,6	**3,1**	70,5	0,17	2,8
Silnyj	Ukraine	**3,5**	31,8	-0,63	39,7	**5,3**	47,5	0,01	0,0
Sládek	Czech	**4,0**	23,6	-0,62	38,0	**4,1**	45,2	-0,55	29,8
Talisman	USA	**4,5**	33,0	-0,48	23,2	**3,8**	43,3	-0,13	1,6
WyeChallenger	Eng.	**3,1**	25,6	-0,05	0,3	**5,0**	52,7	-0,31	9,5
Wye Northdown	Eng.	**2,2**	26,9	0,21	4,3	**4,3**	108,6	-0,68	46,8
Wye Target	Eng.	**3,9**	25,6	-0,06	0,4	**8,4**	51,1	-0,29	8,5
Wye Viking	Eng.	**1,4**	41,8	0,41	16,8	**7,0**	24,7	-0,05	0,3
Willamette	USA	**3,3**	26,0	-0,45	20,4	**3,1**	31,7	-0,16	2,5
Žitomirský 5	Ukraine	**1,9**	39,0	-0,42	17,4	**2,9**	29,8	-0,37	13,4

In Table 3 there is shown the average yield and KH values in the varieties evaluated since 1991 to 2004. The most productive are American varieties Galena, Chinook, Eroica and

besides them also Southern Brewer and Premiant. On the contrary the lowest yield show Orion, Hall, Tradition, Yeoman and Comet. The highest variability is obvious in Hall. Tradition, Galena and Eroica. The lowest variability show Zenith and Spal. Select. It seems to be very interesting that by the influence of the age a severe decline of yield was observed in German varieties (Spal. Select, Hal. Tradition and Hersb. Pure). All of them have r higher than - 0,6. It may be caused because these varieties had not been bred for conditions with low precipitations. Relatively high decline in yield shows Yeoman (r=-0,54). From our experience we can say that also the posterities of this variety show decrease in productivity with the time of their cultivation. The highest contents of alpha bitter acids show Olympic, Magnum, Yeoman and Galena. Of course, the lowest content of alpha bitter acids is obvious in aroma varieties Hersbr. Pure, Spal. Select and Hall. Tradition. We can conclude that conditions of Czech Republic are not suitable for Southern Brewer, which has a long vegetation period and therefore it does not finish its ripeness in our country. This variety show the highest variability, which correlates with the weather conditions of the individual years – a warm year means higher contents of alpha bitter acids. The following varieties show high variability as well: Spalt, Select and Eroica. Low variability in the contents of alpha bitter acids show Friský, Hersb. Pure and Olympic. The highest decrease in alpha bitter acids show Hersb. Pure (r=-0.8). It means that this decline is caused by the time of cultivation from 64%. High decrease was found out also in Hal. Tradition, Orion, Spal. Select, Premiant, Zenith and Galena. From the results it is obvious that the most convenient material for hop breeding process aimed at high alpha bitter contents are Magnum, Olympic and Nugget. For the group of the bitter hops it is possible to recommend Friský, Chinook and Omega.

Table 3: Productivity and stability of productivity of the varieties cultivated in Czech Republic since 1991 to 2004

Variety		Yield (kg/plant)				KH (% w/w)			
Name	Country	**x**	Vk (%)	r	r^2 x 100	**x**	Vk (%)	r	r^2 x 100
Comet	USA	**2,9**	36,2	-0,15	2,4	**7,7**	25,0	0,11	1,2
Eroica	USA	**3,9**	38,1	0,44	19,3	**5,6**	34,1	-0,36	13,0
Friský	Russia	**3,1**	30,5	-0,42	17,7	**7,9**	15,0	-0,34	11,6
Galena	USA	**4,0**	38,8	0,25	6,3	**10,0**	17,4	-0,49	24,0
Hal. Magnum	Germany	**3,2**	28,9	-0,32	10,2	**10,8**	21,5	-0,24	5,8
Hal. Tradition	Germany	**2,8**	39,0	-0,65	42,4	**4,3**	26,0	-0,61	37,2
Hersb. Pure	Germany	**3,5**	26,3	-0,62	38,0	**3,9**	15,9	-0,80	64,0
Chinook	USA	**4,0**	25,7	0,60	35,6	**8,6**	20,0	0,03	0,1
Nugget	USA	**3,7**	23,6	0,15	2,2	**9,9**	20,1	-0,11	1,2
Olympic	USA	**3,1**	25,4	-0,04	0,1	**11,0**	16,5	-0,16	2,6
Omega	Eng.	**3,1**	20,8	-0,16	2,7	**6,7**	24,5	-0,38	14,4
Orion	Germany	**2,5**	23,6	-0,02	0,0	**6,4**	24,3	-0,61	37,2
Premiant	Czech R.	**3,7**	30,9	-0,08	0,7	**7,9**	27,8	-0,58	33,6
Sout. Brewer	JAR	**3,8**	21,2	0,45	20,6	**4,7**	38,8	-0,15	2,3
Spal. Select	Germany	**3,3**	18,5	-0,69	47,2	**4,2**	35,4	-0,60	36,0
Yeoman	England	**2,8**	25,1	-0,54	28,7	**10,6**	18,8	-0,35	12,3
Zenith	England	**3,4**	17,9	0,04	0,1	**6,8**	23,7	-0,50	25,0

The following Figures show the stability of productivity of the tested varieties. From Figure 1 it is obvious that a high decline is usual in Hal. Tradition during eleven years of its cultivation in CR. High decrease in alpha bitter acids contents can be seen in Record during the time of 28 years (see Figure 2). A very good stability of alpha bitter contents was found in Nugget (variations are only in the eighth and the thirteenth tested year due to unfavourable weather

conditions), which is possible to see in Figure 3. High variability of alpha bitter contents is obvious from Figure 4 in the case of a South African variety Southern Brewer. It means that this variety is not suitable for a crossing process in Czech Republic because alpha bitter contents varies from 2,7 – to 8,6 of weight percentage.

Figure 1: Hal. Tradition

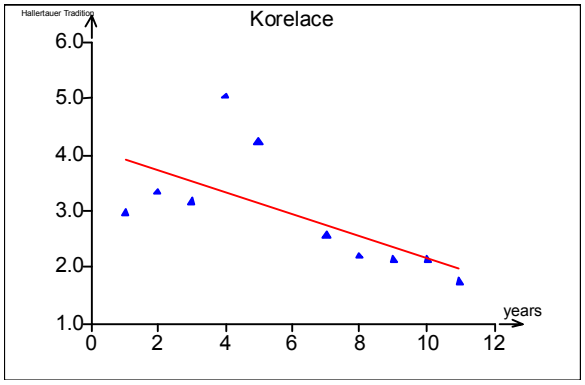


Figure 2: Record

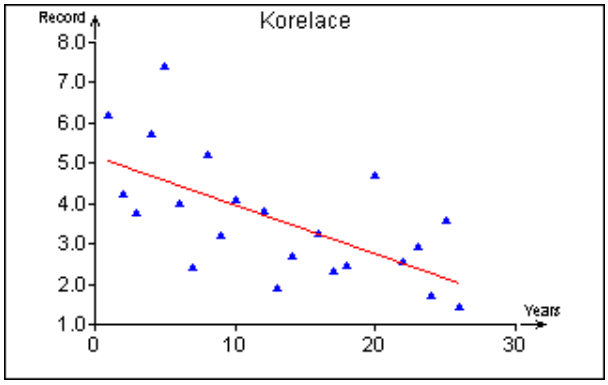


Figure 3: Nugget

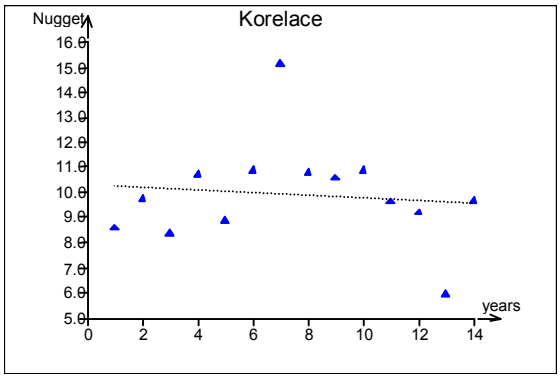
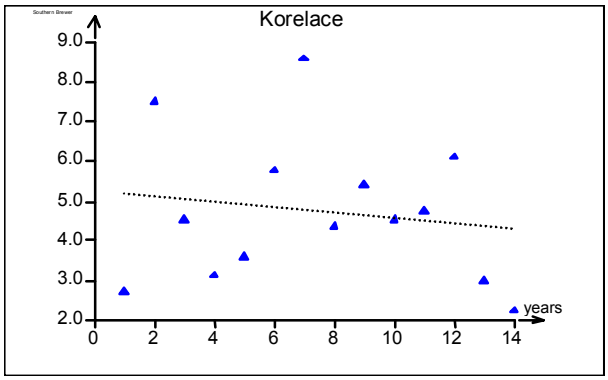


Figure 4: Southern Brewer



Conclusion

The results show that productivity parameters are not the only criterion for the selection of convenient parental partners within the process of hop crossing. Stability of productivity seems to be a very important feature as well. Every breeder is able to find out, which varieties are the most suitable for breeding in specific conditions. From the experience obtained in Czech Republic it was proved that new varieties with high productivity might not be the most convenient for hop crossing. Stability of productivity is more important as the posterities show high decline of productivity increasing with the time of their cultivation. This acknowledge show Northern Brewer, Vojvodina, Perle, Hal. Tradition, Galena and Yeoman during the last years of their cultivation and breeding in Czech Republic. It is necessary to say that the results concerning the tested varieties obtained in Czech Republic need not be in correlation with the productivity of these varieties if they are grown in the countries of their origin. These results can be used at first in the breeding process carried out in Czech Republic but on the other hand they show other breeders, which productivity tendency have some older varieties as well. We believe that some older varieties can sharply influence breeding research as the last registered varieties are over-bred and therefore they may be more sensitive to many factors. It may influence the fact that every variation from the common conditions can lead to the decline of their productivity or it can cause high variability within their productivity.

Acknowledgements

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BREEDING AND DEVELOPMENT OF HOP VARIETIES AT THE HOP RESEARCH CENTER HUELL

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Abstract

The development of new hop varieties at the Hop Research Center Huell is focused on the demands of growers, brewers and the hop industry as well. The new aroma varieties ´Smaragd` and ´Opal` and the high alpha variety ´Herkules` carry on the tradition of Huell cultivars in providing high yield, good to very good resistance or tolerance to various fungal diseases and in particular by showing excellent brewing characteristics. Continuing breeding efforts to improve and adapt hops to various changing needs and conditions are necessary. DNA based selection and gene transfer are new options to increase the efficiency of classical breeding programs.

Keywords: breeding, *Humulus lupulus*, brewing quality, disease resistance, hop varieties

1 Introduction

The Hop Research Center at Huell is one of the leading institutions in scientific and agronomic studies on hop worldwide. In close cooperation with the Society of Hop Research, our partner from the brewing industry, activities at our institute are focused on breeding, plant protection, hop quality, and on technical issues surrounding hop production. Breeding and the development of new varieties, which meet the changing needs of the market, are first priority at Huell. Based on a broad germplasm approximately 100 crosses are conducted each year to improve the field performance, the brewing quality and the resistance or at least tolerance toward diseases and pests. Nowadays Huell varieties account for almost 70% of the total hop acreage in Germany and are exported into over 100 countries all around the globe.

2 Achievements and current breeding objectives

The advantages and benefits of Huell varieties are quite obvious for growers and brewers as well. Crucial characteristics of Huell cultivars are given in table 1 and 2.

2.1 Brewing quality

All Huell aroma varieties show fine to very fine aroma. Especially the new cultivars ´Opal` and ´Smaragd` including ´Saphir` provide special aroma profiles for brewers with specific demands for their beers. In addition, these varieties are showing low contents of cohumulone below 20%. Concerning the high alpha varieties – especially ´Hallertauer Magnum` and ´Hallertauer Taurus` are highly esteemed for their excellent brewing quality showing high alpha acid contents with pleasant aroma. This also applies to the new high alpha varieties recently introduced into practical tests.

Hop Breeding - Aroma Varieties

	Yield	Brewing Quality			Resistance to				
	kg /ha	aroma points	α-acids (%)	cohumu-lone (%)	Vertic. wilt	downy mildew	powdery mildew	spider mite	D.hop aphid
Aroma Variety									
Hallert. Mittelfrüh	**1,250**	**26**	**3-5**	**18-28**	**- - -**	**- - -**	**+**	**-**	**-**
Hallert.Tradition	**1,850**	**26**	**4-7**	**24-30**	**+**	**+ +**	**+/-**	**- -**	**-**
Spalter Select	**1,900**	**26**	**3-6**	**21-27**	**+ +**	**+**	**-**	**-**	**+**
Perle	**1,800**	**24**	**4-9**	**29-35**	**+ +**	**+ +**	**-**	**-**	**-**
Saphir	**1,750**	**27**	**3-5**	**12-17**	**+ +**	**+**	**+**	**-**	**-**
New Aroma Var.									
Smaragd	**1,850**	**26**	**4-6**	**13-18**	**+ +**	**+ +**	**-**	**-**	**-**
Opal	**1,850**	**26**	**5-8**	**13-17**	**+ +**	**+ +**	**+/-**	**-**	**-**

Key: + + + very good, + + good to very good, + good; - - - very low, - - low to very low, - low

Fig. 1. Aroma varieties in Germany, Huell breeds depicted in bold letters

Hop Breeding - Bitter Varieties

	Yield	Brewing Quality			Resistance to				
	kg/ha	aroma points	α-acids (%)	cohum-ulone (%)	Vertic. wilt	downy mildew	powdery mildew	spider mite	D.hop aphid
Bitter Varieties									
Nugget	**2,200**	**19**	**9-13**	**24-30**	**-**	**- - -**	**- -**	**-**	**-**
Hallert. Magnum	**2,000**	**22**	**11-16**	**21-29**	**+ +**	**+**	**- - -**	**-**	**-**
Hallert.Taurus	**1,850**	**23**	**12-17**	**20-25**	**+**	**+**	**- -**	**-**	**-**
Hallert. Merkur	**2,000**	**22**	**10-14**	**17-22**	**+ +**	**+ +**	**+ + (+)**	**-**	**-**
New Bitter Var.									
93 / 10 / 34	**2,000**	**22**	**12-16**	**20-24**	**+ +**	**+ (+)**	**+ +**	**-**	**-**
93 / 10 / 36	**2,200**	**22**	**12-16**	**20-24**	**+ +**	**+**	**+ +**	**-**	**-**
93 / 10 / 63	**2,400**	**21**	**11-15**	**30-34**	**+ +**	**+**	**+ +**	**-**	**-**
Herkules (95 / 94 / 816)	**2,500**	**21**	**12-17**	**32-38**	**+**	**+**	**+**	**-**	**-**

Key: + + + very good, + + good to very good, + good; - - - very low, - - low to very low, - low

Fig. 2. Bitter varieties in Germany; Huell breeds depicted in bold letters

2.2 Increased productivity

Looking at the agronomic performance which is crucial for growers, productivity of all Huell varieties has been increased drastically over the years. ´Hallertauer Mittelfrueh` which was mainly grown in the Hallertau region till the 1970s reached only a yield of 1,200 kg per ha. Breeding efforts in Huell led to an impressive increase in yield per ha. ´Perle` and all subsequently bred aroma varieties provide yields of more than 1,800 kg / ha on average. In the bitter sector ´Hallertauer Magnum`, ´Hallertauer Taurus` and ´Merkur` even excel those yields with 1,900 kg/ha and 250 kg alpha-acids per ha. ´Herkules` revealing 2,500 kg/ha with alpha acids contents of 350 kg /ha has recently been registered as a new variety at the Community Plant Variety Office (France). These Huell varieties increase the competitiveness of German hops on the world market.

2.3 Enhanced tolerance or resistance to diseases

2.3.1 Downy mildew (*Pseudoperonospora humuli*) and V*erticillium*-wilt (*Verticillium albo-atrum, V. dahliae*)

For an environmentally-friendly and cost-efficient production of hops disease resistance is of utmost importance. Initially, in the 1920s the main aim was to breed for resistance to downy mildew. After the release of the first downy mildew tolerant cultivars in the early 60s a second phase started addressing the threat of *Verticillium* wilt in the Hallertau growing region. ´Hallertauer Mittelfrueh` being highly susceptible to downy mildew and *Verticillium* has been replaced by Huell aroma and bitter varieties proving good or excellent tolerance and resistance to downy mildew and to *Verticillium* wilt.

2.3.2 Powdery mildew (*Podosphaera macularis*)

In recent years, when conditions were highly conducive for infections with powdery mildew (PM) caused by *Podosphaera macularis* [Braun] (formerly called *Sphaerotheca humuli* [DC.] Burrill), especially high alpha varieties were prone to show increased susceptibility to powdery mildew – including the Huell cultivars ´Hallertauer Magnum` and ´Hallertauer Taurus`. Since these varieties are grown on more than 30% of the Hallertau acreage, in 2002 fungicides at a cost of 4.9 million Euro had to be applied in order to fight powdery mildew. Therefore, first priority was to increase PM resistance in the Huell varieties.
´Hallertauer Merkur` (released in 2000) was our first achievement in improving resistance to powdery mildew. Several other promising high alpha varieties including ´Herkules` followed. Resistance in all those varieties is based on the R2 gene deriving from the English cultivar ´Wye Target`.
Extensive studies revealed that from seven major resistance genes known so far, resistance based on the *R1, R2* and *R5* genes deriving from the English cultivars ´Zenith`, ´Wye Target` and ´Early Choice` still confer resistance to PM populations occurring in the Hallertau. However, virulent pathotypes overcoming all these resistances have recently appeared in powdery mildew population in England and the USA. Thus, it is vital to identify new genetic sources of resistance that are expected to be found in wild hops.

Wild hops as source of new PM resistances

A large collection of wild hops has been built up at Huell encompassing germplasm of wild hops of European, Asian and North American origin. Since 2001 more than 10,000 wild hops have been screened extensively for PM resistance in the greenhouse and the laboratory after artificial inoculation with powdery mildew spores.

In the greenhouse young seedlings were infected with fungal spores using highly infected plants which are placed between the seedlings. The seedlings were tested for their reaction towards PM strains already occurring in the Hallertau growing region.

Subsequently, the 180 wild hops, pre-tested in the greenhouse as being resistant, were screened for powdery mildew resistance using the detached leaf assay (Seigner et al., 2001) in the laboratory.
Out of a set of 14 single spore isolates from *Podosphaera macularis* deriving from various hop growing regions specific pathotypes from England and the USA representing the *v1, v2* virulence type were used for inoculation. Using the detached leaf assay resistance towards PM strains that are aggressive or that do not occur in the Hallertau region have been tested without risk for the nearby hop growing region. Tests for resistance to PM strains expressing the *v5* virulence gene are still in preparation.

In this way, 96 wild hops could be selected as being resistant so far. Wild hops revealing new effective PM resistances will be used as crossing partners in various breeding programs to broaden the genetic basis for resistance in the Huell hop germplasm.

DNA markers for PM resistance

All studies to identify closely liked molecular markers to various PM resistance genes are presented in another session of this meeting (Seefelder et al., 2005).

Genetic engineering to improve disease resistance

Enhanced resistance to fungal diseases using gene transfer is a perspective for the future.
The advantages of genetic engineering in hop breeding are quite obvious. In contrast to conventional breeding programs involving cross-pollination, PM resistance can be improved by inserting a single resistance gene with no other changes in the hop genome. In this way, well-established hop cultivars can be genetically improved without changing their agronomic performance and brewing quality. Moreover, by introducing resistance genes beyond the limits of cross compatibility, for instance from barley, grape or even from bacteria, a more durable form of resistance is expected .

For exploiting all these benefits first of all a method for stable transformation had to be established in our lab.

A specific strain of *Agrobacterium tumefaciens* harboring the GUS gene was used for the transformation process. During the co-cultivation of hop internodes and Agrobacteria the foreign gene was incorporated into the hop genome. From these cells green plants could be obtained. While plantlets of ´Hallertauer Mittelfrueh` showed poor growth, fully grown plants of ´Saazer´ were obtained and have been transferred from in vitro-culture in the greenhouse in spring 2003.

Leaves of putative transgenic plantlets were tested and confirmed as stable transformants using PCR and GUS staining.

The next objective was to insert a resistance gene which should improve resistance to fungal diseases. Based on the sequence information of a chitinase gene published by US researchers (Henning and Moore, 1999) work had been started to insert this resistance gene deriving from the English cv. ´Zenith` into our hop genotypes.

In a labor-intensive procedure - including RNA isolation, RT-PCR and PCR - cDNA of this specific chitinase gene from ´Zenith` has been produced and integrated into a transformation vector. The *Agrobacterium* strain with the integrated chitinase gene was used for co-cultivation and green plantlets of ´Hallertauer mittelfrueh` and ´Saazer` could be regenerated deriving from the co-cultivated internodes. Fully grown plantlets could be obtained from

´Hallertauer Mittelfrueh` and ´Saazer`. PCR analysis confirmed the stable transformation showing the typical DNA fragment of the chitinase gene. In addition, leaves of putative transgenic ´Saazer` plants were tested for resistance after inoculation with specific powdery mildew races in the laboratory using the detached leaf assay. Reduced growth of PM could be observed on the leaves of transgenic plants. Resistance test for ´Hallertauer` are in preparation.

Work is being continued to look for other genes that may as a single gene or especially in combination improve fungal resistance.

3 Conclusions

Breeding is a long-term procedure. New occurring disease pathotypes and pests, changing criteria for brewing quality and new agrotechnical procedures are some reasons why continuous efforts are necessary to develop hop varieties that are adapted to these changing conditions. Broadening the genetic variability via cross breeding including so far unexploited wild hop germplasm is one of the key issues for great achievements in classical breeding at Huell. During the last decade, DNA based screening for disease resistance and other characteristics has being introduced to increase selection efficiency. Gene transfer as a new tool is currently being investigated to enhance resistance to fungal diseases. Continuing efforts in breeding and research work at our institute are the basis to ensure international competitiveness of German hops in the future.

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TRANSFER OF A RESISTANCE GENE INTO HOPS

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Transfer of a resistance gene into hops

H. Radic-Miehle, E. Seigner

Resistance breeding

Hops – i.e. mainly the female cones - produce resins and essential oils which are used for bittering and flavoring of beer.

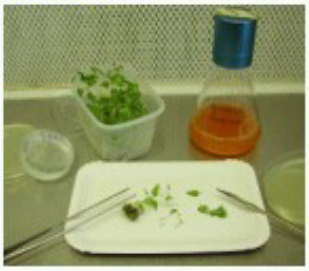
Some fungal pathogens cause severe damage in hop production. Thus, breeding for resistance is of great importance.

Classical breeding efforts are very time consuming and the gene-pool resources in hops - especially regarding resistance traits - are limited. Furthermore, crossbreeding leads not only to an improvement of resistance traits, but often also to unwanted changes in brewing quality.

Gene transfer may help to overcome such limitations.

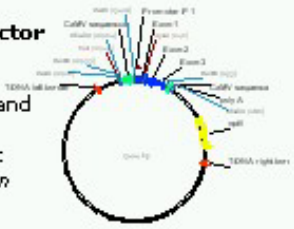
Indirect gene transfer

The gene transfer via Agrobacterial infection has been optimized and applied successfully. Well adjusted regeneration protocols were established to obtain completely transgenic plants deriving from just one cell.



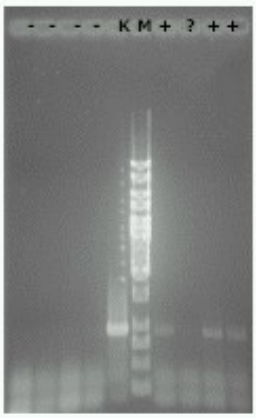
Construction of a chitinase gene vector

A chitinase gene was prepared by RT-PCR and cloned into a binary vector. This construct was transferred into *in vitro* grown hop internodes.

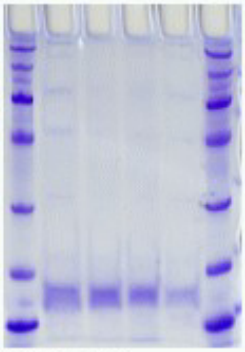


Detection of transgenes bearing and expressing the chitinase gene

After regeneration of plants, transgenes were distinguished from non-transgenic plants and examined regarding their genotype (DNA) and phenotype (protein, trait). PCR and SDS-PAGE allow a detection of the transferred chitinase gene. The expressed resistance towards powdery mildew is tested *in vitro* by standardized artificial inoculation with spores of characterized powdery mildew isolates.

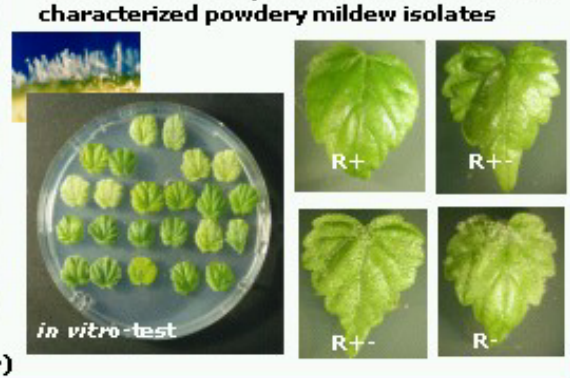


PCR



SDS-PAGE
(chitin-binding assay)

Resistance tests by artificial inoculation with characterized powdery mildew isolates



UTILISATION OF CHEMOTAXONOMY OF MALE HOPS FOR BREEDING

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Crossing is the most frequently used method for hop breeding at the present time. Hop is a dioecious plant. Inflorencence contains much less lupulin glands in comparison with female hops, therefore it makes their chemotaxonomy evaluation more difficult. First descriptive information about male hops evaluation are obtained during a vegetation season: flowering time, susceptibility or resistance against pests and diseases, plant habitus, lenght of laterals, flower density etc. These ones are complemented by chemotaxonomy parameters. Chemotaxonomy of male hops consists of determination of the contents and composition of bitter acids and hop oils. Inflorescences of male hops dried in a dark room at ambient temperature are used for analyses. Analyses of bitter acids are performed by HPLC according to modified EBC 7.7 method. Hops amount (10 g) and injection volume (20 μL) is doubled in comparison with female hops. Simultaneously prenylflavonoids xanthohumol and desmethylxanthohumol can be determined (detection at the wavelenght of 370 nm). Male hops contain usually 0,5 – 3,0 % w/w of alpha acids. Contents of beta acid are approximately within the same interval. Cohumulone ratio is very low, usually below 20 % rel., only rarely exceeds 25 % rel. Composition of male hops oils is determined by gas chromatography. Hop oils components are separated by solid-phase microextraction procedure (SPME). Small amount of male hops inflorescences (0,3 g) is conditioned at 50 °C for 75 minutes in a closed vial. Filament PDMS-DVB 65 μm is used for volatiles separation. When sorption period is finished, SPME filament is immediately desorbed in the injection port of gas chromatograph. The composition of male hop oils is typical by high contents of beta-caryophyllene (10 - 25 %), alpha-humulene (20 – 40 %) and low contents of esters (methylesters, isobutylesters, 2-methylbutylesters). Oxygen compounds are predominantly represented by a group of aliphatic methylketones (2-nonanone, 2-decanone, 2-undecanone). Terpenic alcohols linalool, nerol and geraniol are seldom present in the amounts higher than 0,5 %. Beta-farnesene is of rare occurence in larger amounts similarly to female hops. Myrcene, most abundant monoterpene in female hops, is found in wide interval of 1 – 30 %. Chemotaxonomy of male hops helps to make breeding process more effective. Selection of male parent plants gives higher probability that progenies will obtain demanded properties.

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GENETIC SOURCES OF HOPS IN CZECH REPUBLIC

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Czech Ministry of Agriculture approved "National program of conservation and utilization of genetic sources of plants, animals and micro organisms important for agriculture and nourishment" for the years 2004-2008. Within its frame it supports this activity with the help of Grant project 6.2.1. "Support of a unique field collection of hop genefond." Hop Research Institute, Co. Ltd. in Žatec is authorized and fully responsible for the control and preservation of the collection of hop genetic sources (GS). The collection is a part of plant GS within Czech Republic, which are a part of the team of eleven cooperating institutes – participants of National program (NP). Their work is controlled with the help of Frame methodology of NP. The coordinator is Research Institute of Plant Production in Prague together with advisory authority of NP, which is Council of Genetic Sources of Plants. Data concerning Czech collections of GS are a part of international databases. Unfortunately, hop, as an agricultural crop has not had an international working group and database so far.
Czech collection of hop GS is documented in an informational system EVIGEZ, which contains a passport and a describing part. Each item (GS) is recorded in a passport part, where each GS has its own indicator (national record number). Basic data as a name of GS, origin, donor, a breeding method leading to GS obtaining, ploidity and other features are included there. Describing data characterize each GS and they are obtained on the base of results of field and laboratory tests. New items (plants) are after their multiplication planted in four repetitions, each of them contains eight plants. They are evaluated minimally for the time of five years. After finishing the assessment features and obtained results are on the base of a classificatory transferred into a computer program EVIGEZ, which has point values on a scale 1-9. The classificatory contains 71 features, which are divided into four groups: morphological, biological, economical and additional ones. After finishing the evaluations three repetitions of hop plants are dug out and the field collection has then only one repetition as a deposit. With this deposit we continue in following the productivity only in important genotypes (important cultivated varieties, varieties as donors of important productive features, which can be used for further breeding work). All the evaluated GS (newly or long-term assessed) are a part of the active collection. Other GS are kept in field collection, which has a character of a gene bank (GS contained in it are not regularly assessed). In extreme years (too high or too low summer temperatures, extreme dry) some genotypes are evaluated also in this part of the collection if they show good parameters of their productivity. On the base of these results some genotypes can be used for crossing process concerning resistance of these varieties to dry, high temperatures and so on.
The collection of hop world genetic sources in CR contains 323 items counted to the end of 2004. The highest number among them belong to Czech genotypes (sixty items), 38 items have their origin in England, 31 items are from the USA and Ukraine, resp., 29 items are from Germany, 26 items are from Poland, 21 items are from Slovenia, 18 items are from Russia, 12 items are from Japan, 11 items are from Yugoslavia, nine items are from France, three items are from Bulgaria, two items are from Australia, New Zealand, Denmark and Austria and one item is from Hungary and Sweden, resp. Eight items are missing at present because of death of plants. We are dealing with foreign partners about supply of these items now. The whole collection is gradually evaluated. Nearly 50% of them is a part of a describing part of EVIGEZ.

The collection contains also wild hops. Up to now we have had 260 wild hops, which have their origin in CR (133). Canada (78), USA (17), Belgium (25) and Austria (7). After evaluation of wild hops those genotypes will be selected, which will be typical for a certain region or extend bio-diversity of our field collection. Wild hops are described, chemically

analysed and determination of DNA is provided as well. The first obtained results show that also in Czech Republic can be sampled wild hops not only genetically different from the contemporary cultivated varieties (at first Saaz hops) but also some perspective genotypes convenient for further breeding work (hops with their alpha bitter contents higher than 6% weight).

The poster is also an appeal to other research institutes, which have a collection of hop GS, for establishment of other mutual cooperation and databases of assessed genotypes of hops. It would be also possible to begin duplication of items, which are very valuable (original varieties, varieties that are not cultivated any more and so on).

Acknowledgements

This poster was carried out on the base of contribution of Czech Ministry of Agriculture, which supports plant GS within “National program of conversation and utilization of plant genetic sources and biodiversity.” (Grant 6.2.1.).

Genetic sources of hops in Czech Republic

CULTIVATION OF CZECH HOP VARIETIES IN A FARM OF HOP RESEARCH INSTITUTE, CO., LTD. IN ŽATEC, CZECH REPUBLIC

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There is a trend of higher utilization of new Czech hop varieties in growing conditions of Czech Republic recently. Good growing and economical characteristics of new Czech hop varieties have been confirmed in our farm in Stekník. Extension of growing area of these varieties has been supported by the interest of Czech and foreign breweries.

Average yields of individual hop varieties in t/ha of dry hops

Variety	Crop year 1999	Crop year 2000	Crop year 2001	Crop year 2002	Crop year 2003	Crop year 2004	Average yield of hops
Saaz hops	1,318	1,050	1,475	1,275	1,291	1,731	1,356
Premiant	1,880	2,716	2,730	2,218	2,069	2,580	2,368
Bor	1,992	2,150	2,200	2,150	2,001	1,714	2,035
Agnus	0	2,155	2,400	2,457	2,530	2,426	2,395
Sládek	2,139	2,750	2,260	2,502	2,083	2,394	2,354

Average contents of alpha bitter acids in percentage by EBC 7.4 method

Variety	Crop year 1999	Crop year 2000	Crop year 2001	Crop year 2002	Crop year 2003	Crop year 2004	Average contents o
Saaz hops	3,37	5,20	3,94	3,41	3,18	3,89	3,83
Premiant	9,32	9,65	9,99	8,39	6,58	9,63	8,93
Bor	7,65	8,00	8,45	7,90	5,69	7,75	7,57
Agnus	0	12,50	13,11	10,59	10,80	11,65	11,73
Sládek	5,56	7,24	6,24	5,61	4,29	5,76	5,78

Hop growing area of individual hop varieties in hectares in the farm

Variety	Crop year 1999	Crop year 2000	Crop year 2001	Crop year 2002	Crop year 2003	Crop year 2004
Saaz hops	40,25	34,70	45,60	47,35	39,69	39,69
Premiant	9,25	11,47	11,47	15,77	19,23	19,23
Bor	1,6	1,6	2,58	3,22	3,22	3,22
Agnus	0	0,98	1,92	1,92	2,95	11,46
Sládek	18,10	19,94	19,14	19,14	20,72	22,29

Czech hop growing technology is used in all cultivated varieties. It is supported by irrigational systems. The average age of hop plants is seven years. Planting in new hop gardens is carried out with the help of pre-planted rootstocks with higher weight. During the first crop year it is usually reached yield higher than one ton of dry hops per hectare. Nearly 100% fullness of growth is commonly reached during the first year of cultivation.

Basic growing parameters of Czech hop varieties

Variety	Type of hops	Length of vegetation period	Term of the beginning of the harvest	Time of harvest	Earliness
Saaz hops	Fine aroma	122 – 128 days	August, 18	10 days	Semi-early
Premiant	Bitter	128 – 134 days	August, 26	6 days	Early
Bor	Bitter	130 – 135 days	August, 30	2 days	Middle-early
Agnus	High-alpha	132 – 138 days	September, 01	7 days	Semi-late
Sládek	Aroma	133 – 140 days	September, 07	7 days	Semi-late

Sortiment of Czech hop varieties enables to spread seasonal demands during spring and harvest works if they are grown in a good balance. It also helps to increase utilization of machines and functional buildings.

IDENTIFICATION OF GIBBERELLINS AND INVOLVEMENT IN HOP FLOWERING

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Gibberellins (GAs) play a main role in internode spacing and induction and promotion of flowering in many plants. Techniques based on exogenous application of these hormones have been developed in hops [1] but with undesirable effects due to the lack of knowledge about the flowering mechanisms.

Analyses of gibberellins have been carried out using the variety Nugget growing in the fields of the *S.A.E. de Fomento del Lúpulo* sited in Villanueva de Carrizo (León, Spain). Six types of gibberellins have been so far identified in hop (GA_1, GA_3, GA_4, GA_7, GA_9, GA_{20},) and quantifications have been carried out from the first pair of fully expanded leaves, apical shoots, axillary buds and flowers along different times of the growth and flowering season:

Extraction and purification processes involved liquid and solid phase extraction, separation of interesting molecules by reverse phase HPLC and its derivatization prior to its quantification by GC-MS. Results are still in progress and will be presented in the congress.

Recent studies reported how auxin influences the metabolism of GAs: IAA synthesized in barley inflorescence is necessary for the synthesis of the active GA_1 and GA_3 in nodes and internodes acting in the latter part of the gibberellin biosynthesis pathway [2]. Particularly, it has been shown that IAA plays an important role in hop vegetative growth [3].

Our objective is to investigate the role of the gibberellins together with auxins and other plant regulators in vegetative and flower development of hops to set up a relationship between the flowering initiation and the endogenous hormone balance. So, we are developing a method which will allow us to analyse the main groups of growth regulators (gibberellins, auxins, cytokinins and abscisic acid) using the same plant extract, taking advantage of the mass spectrometry high sensitivity.

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II. Session:

DNA-BASED TECHNIQUES IN HOP RESEARCH

MAPPING OF A POWDERY MILDEW RESISTANCE GENE IN HOP (HUMULUS LUPULUS L.)

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Abstract

Breeding for resistance is first priority in controlling infections of powdery mildew (*Podosphaera macularis*) in hop. The development of powdery mildew resistant hop varieties can be facilitated by using molecular markers. Based on the double pseudotest-cross strategy 642 amplified fragment length polymorphism (AFLP) and 18 microsatellites were used to construct a female and male linkage map. Bulked segregant analysis strategy enabled us to identify and map 6 AFLP markers linked to the *R2* gene which confers resistance to powdery mildew in hop. Two tightly linked AFLPs flanking at a distance of 1.7 cM the resistance locus *R2* could be detected which will be the start of using these markers in practical breeding.

Keywords: molecular markers, marker assisted selection, *Podosphaera macularis,* disease resistance,

1 Introduction

Powdery mildew, caused by the fungus *Podosphaera macularis* [Braun] (formerly called *Sphaerotheca humuli* [DC.] Burrill), is a serious disease causing drastic losses of yield and quality in hops worldwide. In seasons with high infection potential a high number of fungicide treatments are necessary amounting to around 4.9 million Euro alone in the Hallertau region in 2002. Thus, breeding strategies to improve powdery mildew resistance in hop varieties are crucial. Using DNA based markers closely linked to various powdery mildew resistance genes is a promising way to increase selection efficiency in breeding programs. Moreover, molecular markers for various resistance genes are required for successful pyramiding of several genes in one individual. Extensive investigations of the virulence behavior of approximately 90 PM strains occurring in Germany, England, France and the USA revealed that from 7 major genes known so far only the *R1*, *R2* and *R5* gene deriving from the English cultivar ´Wye Target`, ´Zenith` and ´Early Choice` still confer resistance in Germany. So far the *R2* gene is mainly the basis for PM resistance in our breeding programs at the Hop Research Center Huell. Our objective was identify molecular markers closely associated to this gene and to map these markers.

2 Material and methods

2.1 Plant material and assessment of PM resistance

One hundred and twenty F1 individuals deriving from the cross of a powdery mildew resistant mother plant directly deriving from ´Wye Target`(84 /08 /24) and a highly susceptible male breeding line (98 /44 /49) were used in these studies. This F1 full sib progeny was assessed for powdery mildew resistance in the laboratory using the detached leaf assay (Seigner et al., 2002). For inoculation a specific PM strain prevalent in the Hallertau hop growing region which can not overcome the *R2* resistance was provided by EpiLogic, Freising, Germany. Epilogic also conducted the resistance assay. All seedlings

from the mapping population were scored for PM symptoms 8-10 days after inoculation. Seedlings were classed as resistant when scored as either 0 or 0.1 on a scale of 0-1. Since leaf age is a very critical parameter for infection, each test was replicated 2-3 times in an interval of 2-4 weeks

2.2 Extraction of Genomic DNA and AFLP Analysis

Genomic DNA was extracted from freeze-dried hop leaves by a modified CTAB method (Seefelder et al., 2000). AFLP markers were generated using the protocol of Vos et al. (1995) with minor modifications. PCR products were separated on 5% denaturing polyacrylamide gels under standard conditions and analyzed by the Fluorimager 595 (Molecular Dynamics) using the software package Fragment Analysis 1.1 (Molecular Dynamics, Krefeld, Germany).

2.3 Linkage Analysis and Mapping

All data analyses were performed using JoinMap version 3.0 (Van Ooijen and Voorrips, 2001). Recombination frequencies were converted into map distances using the Kosambi mapping function (Kosambi, 1944). Based on a LOD (logarithm of the odds) of 6, loci were assigned to linkage groups.

3 Results and Discussion

3.1 PM resistance linked to the R2 gene

One hundred and twenty individuals of a full-sib mapping population segregating for the powdery mildew resistance gene *R2* were screened for disease resistance in the laboratory using the detached leaf assay after the inoculation with a specific PM strain. Resistance data (53 resistant to 67 PM susceptible individuals) which did not deviate significantly from a 1:1 ratio of segregation (χ^2= 1.63, P>0.05, df = 1) confirmed that a single dominant gene *R2* is involved in the resistance behavior of the seedlings.

3.2 Data scoring

Using 91 AFLP primer combinations 620 polymorphic fragments could be produced. 93 markers showed severe distortion from the expected segregation ratios of 1:1 and 3:1 (15%). Considering the significant preponderance of female individuals over males (104 female : 16 male hops) in this mapping population which was observed in many studies, all markers associated to the sex should also reveal a clear deviation from the expected 1:1 Mendelian segregation. This is the reason why all markers even those showing strong distortion were used in linkage analysis. 549 fragments were classified as 1:1 segregating markers (ab x aa), 414 contributed by the female (84 /08/24) and 135 by the male parent (98 /44 /49). 97 markers were heterozygous in both parents (ab x ab) segregating 3:1.
Segregation of 17 microsatellites (Brady et al., 1996; Seefelder et al., 2002; Hadonou et al., 2004), was also investigated in our mapping population. They were scored as dominant markers because it was not possible to determine the SSR fragments as different loci or different alleles (more than 4 fragments). The assignment of the different fragments to loci or alleles can be performed after completion of the mapping procedure.

3.3 Bulk segregant analysis to identify PM resistance markers

Among all AFLP primer pairs tested 20 could differentiate between the DNA bulks of 10 full resistant and 10 highly susceptible hop plants. Chi-square analysis revealed that most of these AFLP markers followed a Mendelian segregation. AFLP markers were re-examined on all individuals of the progeny including the parents. Seven DNA fragments inherited from the resistant parent (markers in coupling phase) were identified as putative AFLP markers linked to the PM resistance locus *R2*.

3.4 Linkage analysis and mapping

Using 620 AFLPs (which include the above mentioned putative *R2* resistance markers) and 17 microsatellites, the *R2* resistance locus and the male sex locus *M* linkage analysis was performed and the male and female maps were constructed using JoinMap 3.0.
Based on a LOD of six, 303 AFLP markers and all 18 SSRs (single sequence repeats) were assigned to nine female linkage groups covering 320.6 cM. In the male map 150 AFLPs and 15 SSRs were placed on eight linkage groups spanning a distance of 237.4 cM.

Located on the largest female group Gr-F1 covering 101 cM the *R2* locus conferring PM resistance was flanked by two AFLP markers at a distance from 1.6 cM. This is for the first time that two markers adjacent to the R2-PM resistance locus could be detected. Additional markers for PM resistance could be identified at a distance from 1.7 to 2.6 cM. This is also the first time that PM resistant markers could be assigned to a genetic map in hops.

As reported in other studies the presented results reveal also dense clusters of AFLP markers (Saal and Wricke, 2002; Stromer et al., 2002).

4 Conclusions

Humulus lupulus has 20 chromosomes in the diploid genome. In this mapping almost all 20 linkage groups could be covered with molecular markers, only two female and one male group are still missing. Thus, these maps based on 648 molecular markers can be expected to be a reasonable starting point for new efforts in mapping, to fill in gaps and to add the missing male linkage group. Markers already mapped in a previous studies (Seefelder et al., 2000) will be integrated into these linkage maps. Especially codominant markers, being highly informative in outcrossing species like hop, should be exploited to a much higher extent. Comprehensive maps for both sexes saturated with markers are the key elements to identify markers for various traits based on simple major genes or on complex QTLs being the start for establishing marker assisted breeding in hop.

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HOP (*HUMULUS LUPULUS* L.) GENETIC MAP AND QTL ANALYSIS

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Abstract

An F_1 population from the cross between the German variety 'Magnum' showing high levels of alpha acids and a wild Slovene male hop '2/1' was employed to develop genetic maps of the parentals based on AFLP and SSR markers and to identify QTLs associated with alpha acid content. The variability of alpha acid content was evaluated in Žalec, Slovenia in the 2002, 2003, and 2004. The maternal map consisted of 90 markers mapped on 13 linkage groups and the paternal map included 70 markers assigned to 12 linkage groups. Four putative QTLs were identified on three linkage groups on the female map. The most promising QTL will be used in Marker-Assisted Selection (MAS) process. The saturation of the map, particularly in the regions of putative QTLs, will be carried out using additional SSR markers. The stability of the QTLs will be tested in next years.

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

Introduction

Growing and cultivation of hop (*Humulus lupulus* L.) for the brewing industry has a long tradition in Slovenia. In 1952, the Slovenian Institute of Hop Research and Brewing was established to assist hop growers and, in particular, to develop new hop cultivars suitable for the Slovenian growing conditions and to follow the demands of the brewing industry. Today, 1625 ha of hop fields are planted with 95% of Slovenian cultivars, the most established being ‘Aurora’, which is grown on over 60% of hop fields, followed by ‘Savinjski golding’ (15%), ‘Bobek’ (10%), ‘Celeia’ (5%) and others. The German introduced cultivar, Magnum, is grown on 5% of hop fields.

Hop is a perennial dioecious plant and only female plants bear hop cones. The resins produced in the lupulin glands of the cones are used in the brewing industry. There is an increased demand for hop cultivars exhibiting improved quality, namely higher levels of alpha acids. Moreover, alpha acid content in hops is polygenically based. Conventional breeding, which includes several crossing experiments and selections of plants with agronomically important traits, is a time consuming process and so molecular technology has an important role as a supplementary activity in this area. Molecular markers linked to agronomically important traits can singificantly increase the efficiency of breeding, as already performed in many important crops. Molecular markers can be used for tracking agronomic traits during selection and for construction of genetic maps based on recombination experiments. Thus, the aim of this research was to develop a genetic map in order to identify QTLs controlling alpha acid content in hop.

Materials and Methods

The 'pseudo-testcross' mapping population consisted of 111 F_1 indivudals from the cross between the German variety 'Magnum' and a wild Slovene male hop '2/1'. The German variety 'Magnum' shows high levels of alpha acids.
A total of 97 hops produced female flowers and 14 showed the male phenotype with typical anther-containing flowers (female/male ratio ≈ 6,9 : 1). Male hops were assigned as bearing the hypothetical male-determining locus *M* (Seefelder et al., 2000).
Genomic DNA from the F_1 individuals and both parents was extracted according to Kump and Javornik (1996). AFLP analyses were performed as described by Vos et al. (1995). A total of 36 AFLP primer combinations (using *Eco*RI, *Pst*I, and *Mse*I restriction enzymes) was used in screening of the entire mapping population. Eighteen microsatellites developed by Jakše and Javornik (2001) and Jakše et al. (2001) were also surveyed as described by Štajner et al. (2004).
The variability of alpha acid content was evaluated in Žalec, Slovenia in the 2002, 2003, and 2004. The distribution of alpha acid content in all 3 years was tested with the Kolmogorov-Smirnov test.
Linkage analysis was carried out using the 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. In each linkage group the order of the markers was infered using the pairwise data of only those loci that had a recombination frequency smaller than 0.35 and a LOD value higher than 2.0. The Kosambi mapping function was used to convert recombination data to map distances.
The computer programme MapQTL Ver. 4.0 (Van Ooijen et al., 2000) was used to identify and locate QTLs affecting 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

Polymorphic AFLP markers amplyfied by 36 primer combinations were tested for their inheritance pattern in mapping population. Segregation ratios were tested using the χ^2 test and a total 62.86% of AFLP markers did not fit the expected Mendelian ratios ($p<0.01$). Similary, significant levels of segregation distortion ($p<0.01$) were observed for 5 of the microsatellite loci out of 18 microsatellite markers surveyed in the segregating population.
For the maternal map, 137 AFLP markers and 14 microsatellite markers were included in linkage analysis. Groupings performed with a LOD threshold of 5.0 resulted in the assignment of 90 markers (79 AFLPs, 11 SSRs) to 9 major linkage groups. The 115 markers (104 AFLPs, 10 SSRs, and the the hypothetical male-determining locus *M*) segregating from the male parent produced 12 linkage groups, of which 7 contained more than two loci.
QTL analyses were carried out using data of alpha acid contents obtained in the years 2002, 2003, and 2004. None of the 3 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. One putative QTL, was identified in 2003 on one linkage group of the female map. Three QTLs associated with alpha acid content in 2004 were localized on three different linkage groups. The most promising QTL will be used in Marker-Assisted Selection (MAS) process. The saturation of the map, particularly in the regions of putative QTLs, will be carried out using additional SSR markers. The stability of the QTLs will be tested in next years.

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MOLECULAR DIVERSITY OF HOPS (*HUMULUS LUPULUS* L.)

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Abstract

We analyzed genetic structure and diversity among hop genotypes using 25 new microsatellite markers and a set of 67 wild and cultivated hop accessions, well representing the available diversity among currently and historically used lines. Model-based clustering (Structure) placed the accessions into five gene pools that correspond to major breeding groups and to accessions showing mixed origin. A phylogenetic clustering shows good agreement with pedigree information and five gene pools, and further assigned eight accessions omitted from Structure analysis into two additional gene pools. The seven defined gene pools were best represented by 50 accessions. The cultivated hops differ significantly from the wild ones, although most of the variability was found within groups. Molecular variances within groups of cultivated and wild hops were homogeneous, suggesting that a similar level of molecular variability is found in both groups of accessions. Core sets of cultivated hop accessions that capture maximum allelic richness were defined, generally including hop cultivars of 'mixed origin' that combine alleles originating from different gene pools and thus have the greatest allelic richness.

Keywords: *Humulus lupulus* L., microsatellites, genetic diversity

Introduction

Hop (*Humulus lupulus* L.) is a dioecious perennial climbing plant cultivated primarily for its use in the brewing industry. The commercial product of the hop plant is the female inflorescence (cone). Its lupulin glands produce resin for giving bitterness, and essential oils that contribute flavour and aroma to beer. Traditional recombination and selection procedure have been successful in the development of hop cultivars but there is an ongoing need to develop improved cultivars in terms of yield, high resin content and disease resistance, which are the principle aims of hop breeding programs. Broad-based germplasm collections (wild hops, breeding lines, diverse cultivars) in main hop breeding centres provide good starting material for breeding. Knowledge of the extent of the diversity present within a crop is very important since it enables its rational use in breeding via gene incorporation or introgression by means of genome mapping, marker-assisted selection and/or cloning and genetic transformation. The effective and rational use of genetic diversity has significantly improved since the introduction of DNA analysis which enables an improved understanding of functional genetic diversity and the processes that control this diversity.
Different molecular markers (RAPD, AFLP and SSR) have been applied in our hop genetic research. The assessment of world collection of hop cultivars by RAPD markers (Šuštar-Vozlič and Javornik, 1999), and subsequently by AFLP and four SSR markers (Jakše et al., 2001) showed the presence of two geographically distinct germplasms in hop cultivars – European (EU) germplasm characterised by its fine aroma and North American (NA) germplasm characterised by a distinct bitter taste. Within European germplasm, a distinct grouping of accessions was also established reflecting the history of past hop breeding practices adopted in different European hop growing regions. Work on hop genetic resources

has been extended to studies of wild hops and male and female breeding lines using four SSR markers (Jakše et al., 2004), since wild hops have been the source of many important genes. The geographic classification of hop germplasm into European and North American has been confirmed, showing different degree of variability present within each of the hop geographic groups.
Among molecular markers used so far in hop genetic research, we have found SSR markers to be the most informative. Here we present our most recent work on the assessment of genetic variability and relationship among 67 geographically diverse wild accessions and hop cultivars by 25 newly developed SSR markers.

Materials and Methods

Materials

Hop accessions were provided from the hop germplasm collection and hop genebank held at the Institute of Hop Research and Brewing, Zalec, Slovenia. Three North American wild male accessions were obtained from Oregon State University, Corvallis, OR., USA. A total of 67 hop accessions (34 cultivated and 33 wild hop accessions) were included in the analysis.

SSR analysis

Total genomic DNA was extracted from young leaves of analysed accessions using a modified CTAB method according to Kump and Javornik (1996) and SSR analysis was performed according to Štajner et al. (2004). Microsatellite amplification was performed in a 20-μl solution containing: 1x PCR buffer (10 m*M* Tris-HCl, 50 m*M* KCl, pH 8.3), 1.5 m*M* $MgCl_2$, 0.2 mM each of dNTPs, 10 μM of each primer (forward Cy5 labelled), 0.5 U of Taq polymerase and 40 ng of hop DNA. Amplification was performed in a PE9700 Thermal Cycler (Applied Biosystems) using the following thermal cycling protocol: 95°C for 5 min, followed by the appropriate number of cycles of 45 sec at 94°C, 30 sec at 54 – 57 °C and 1 min 30 sec at 72°C. The reactions were completed by incubating at 72°C for 8 min and held at 4°C. Microsatellites were separated and detected on an ALFexpress II automated sequencer (Amersham Biosciences). Alleles were sized against a 50-500 bp ladder (Amersham Biosciences), followed by analysis by 1.03 AlleleLocator software (Amersham Biosciences).

Data analysis

The log-transformed proportion-of-shared-alleles distance between pairs of hop accessions was calculated using MICROSAT (Minch *et al.*, 1997). An unrooted phylogenetic tree was constructed using Fitch-Margoliash least-squares algorithm (Fitch and Margoliash, 1967) implemented in FITCH programme of the PHYLIP ver. 3.6b software package (Felsenstein, 1993). Pairwise Nei's standard genetic distance (1972) was calculated between gene pools. A model-based clustering method was applied on multilocus microsatellite data to infer genetic structure and define the number of clusters (gene pools) in the dataset using the software STRUCTURE (Pritchard et al., 2000). The pairwise log-transformed proportion-of-shared-alleles distance matrix was analysed by the analysis of molecular variance (AMOVA) approach (Excoffier et al., 1992) using WINAMOVA 1.55 program (Excoffier, 1992).

Results and discussion

We analyzed genetic structure and diversity among hop genotypes using 25 new microsatellite markers and a set of 67 wild and cultivated hop accessions, well representing the available diversity among currently and historically used lines. Model-based clustering (Structure) placed the accessions into five gene pools that correspond to major breeding groups and to accessions showing mixed origin. A Fitch-Margoliash least-square tree shows good agreement with pedigree information and five gene pools, and further assigned eight

accessions omitted from Structure analysis into two additional gene pools. The seven defined gene pools were best represented by 50 accessions. The cultivated hops differ significantly from the wild ones, although most of the variability was found within groups. Molecular variances within groups of cultivated and wild hops were homogeneous, suggesting that a similar level of molecular variability is found in both groups of accessions. Core sets of cultivated hop accessions that capture maximum allelic richness were defined, generally including hop cultivars of 'mixed origin' that combine alleles originating from different gene pools and thus have the greatest allelic richness. The above results are consistent with two biogeographically separated germplasms and with the known history of accessions and have so far given a most detailed insight into the structure and extent of genetic diversity present in collections, which is of prime importance for any breeding programme, in order to secure and enlarge the variability and to make a more informed choice of the breeding material used in the development of new cultivars.

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NEW MOLECULAR MARKERS FOR HOP (*HUMULUS LUPULUS* L.)

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The development of DNA technology has provided a number of methods, which eliminate an influence of environmental factors, to detect the differences at DNA sequence level for evaluation of hop (*Humulus lupulus* L.) genotypes. RFLP, RAPD, STS, SSR, ISSR and AFLP are successfully used for DNA fingerprinting of hop genotypes. The use of molecular methods was introduced to hop breeding and management, to identification and determination of released varieties and new promising breeding materials, to control of authenticity and purity of varieties, to marker-assisted selection (MAS), to identification of somaclonal variability and genetic stability, to study of genetic diversity, to detection and diagnosis of different pathogens, etc. The most reliable methods of all are STS and SSR, which amplified specific, sequence characterised, DNA loci. These systems have been published by Tsuchiya et al. (1997), Araki et al. (1998), Murakami (1998), Jakše et al. (2002), Čerenak et al. (2004) and Hadonou et al. (2004). A new trend, in these methods, is to find new molecular markers in specific sequences, nearly or within structural genes.

In our laboratory, we have aimed on several groups of different genes. At the first, we have studied structural and functional aspects of genomic organization of genes and regulatory elements, which are predicted to be involved in biosynthesis of bitter acids and prenylated flavonoids (Matoušek et al., 2001, Novák et al., 2003). We prepared genomic and cDNA libraries of hop, Czech cultivar Osvalds'72 for fishing these genes. Genomic library had size $1x10^6$ clones. Average size of inserted DNA was 15kb. There is 99.87 % probability to isolate an individual sequence from established library. The cDNA library has contained $1.2x10^6$ pfu with average size of inserted DNA from 600 bp to 1900 bp. We suppose that there are differences between individual chalcone synthase homologues in different hop genotypes, which can be used for molecular marker identification. For example, we found microsatellite sequence in specific nucleic DNA binding protein (Fig. 1a). PCR analysis of 69 hop genotypes detected 4 different alleles in *H. lupulus* (Fig. 2b) and 2 other in *H. japonicus* (genotype 69).

Next sequences, which we have studied in our laboratory, are resistance genes. 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; Young, 2000; Pan et al., 2000). Plant resistance to different pathogens are 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 (Fig. 2). These gene mechanisms are very similar in insect and mammalia, and it can be supposed, that they are universal for all organisms (Pan et al., 2000). These motifs can be used directly for searching of resistance genes and their homologues (Yu et al., 1996) or associated regions (Hayes and Saghai Maroof, 2000). At present time, we use this method for the detection of close sequences to hop powdery mildew resistance genes

The last group of sequences, which we have started to study, is retrotransposons. Retrotransposons are mobile genetic elements that transpose through reverse transcription of an RNA intermediate. Retrotransposons are ubiquitous in plants and play a major role in plant gene and genome evolution. In many cases, retrotransposons comprise over 50% of nuclear DNA content, a situation that can arise in just a few million years (Kumar and

Bennetzen, 1999). Retrotransposons consist of the long terminal repeat (LTR) and the non-LTR retrotransposons (Figure 3). LTR retrotransposons are further sub-classified into the Ty1-*copia* and the Ty3-*gypsy* groups. Retrotransposons sequences have been used in sequence-specific amplification polymorphism (SSAP), retrotransposon-based insertion polymorphism (RBIP), retrotransposon-microsatellite amplified polymorphism (REMAP) and inter-retrotransposon amplified polymorphism (IRAP) (Kumar and Bennetzen, 1999). We used conserve regions of RT gene for PCR amplification of this sequence from hop. Sequence characterization and multiple alignment (Fig. 4a) approved that there are retrotransposons also in hop genome. Phylogenetic analysis classified this sequence (RTrevised1) to Ty3-*gypsy* groups from barley, maize and tobacco and to retrovirus elements from soya and *Solanum* (Fig. 4b).

Acknowgledgements

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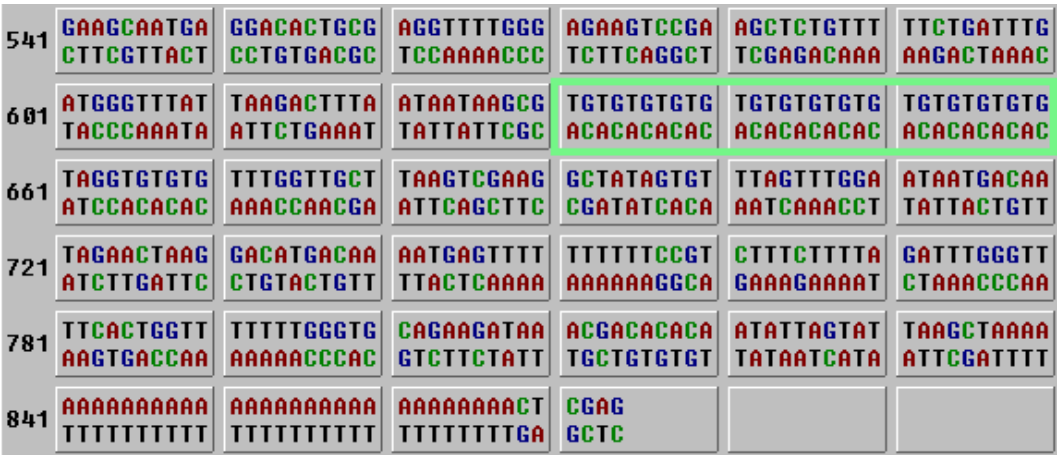
Figures

a)



b)

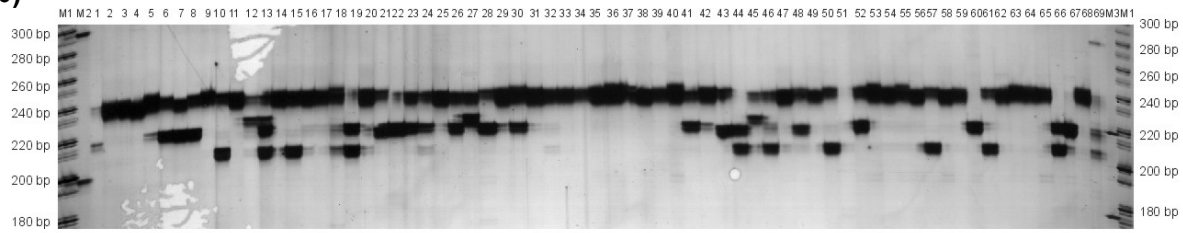


Figure 1: **a)** Sequence analysis of specific nucleic DNA binding protein **b)** Analysis of amplified PCR products of NDBP-derived primers in 5% polyacrylamide denaturing (8M urea) gel

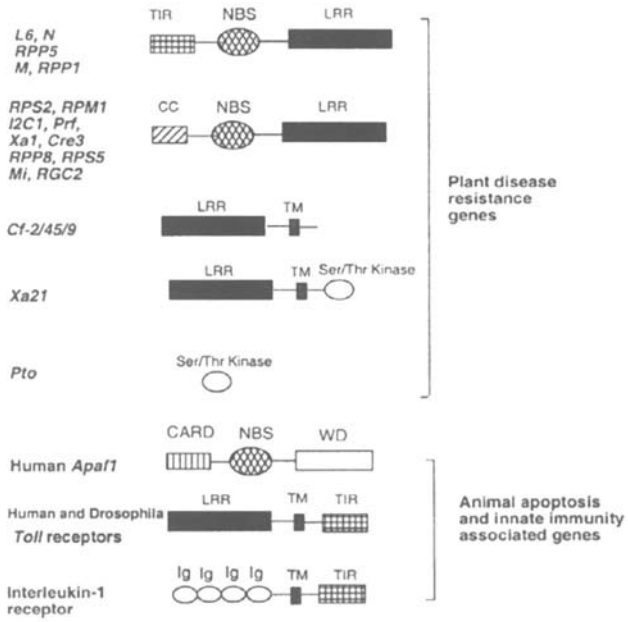


Figure 2: Conserve regions of different R genes (Young, 2000).

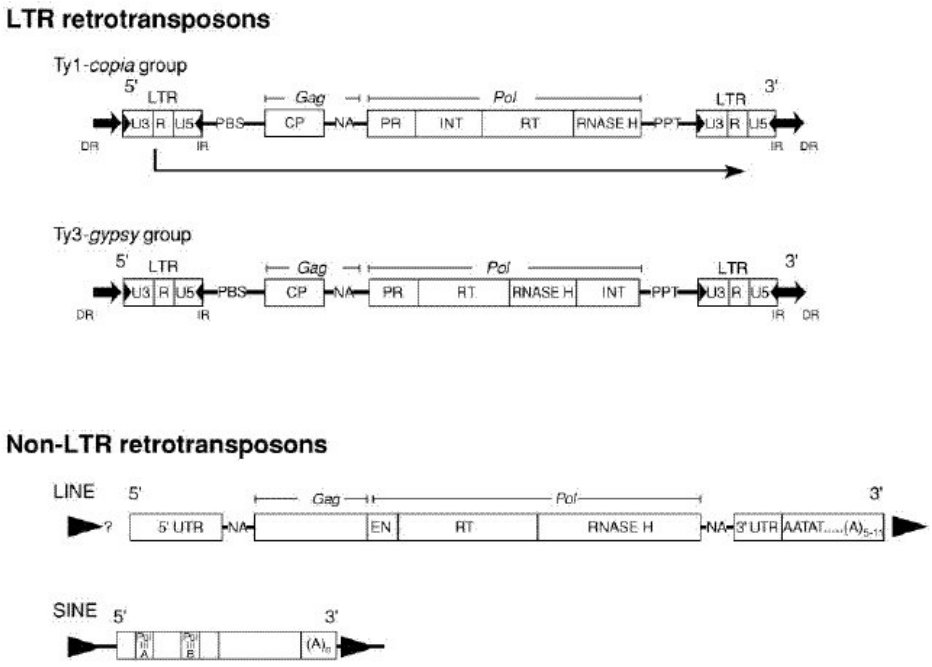
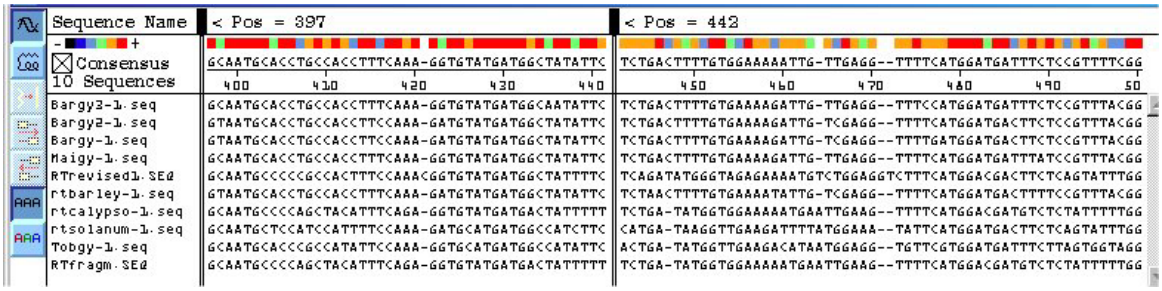


Figure 3: Structure of retrotransposons in plant (Kumar and Bennetzen, 1999).

a)



b)

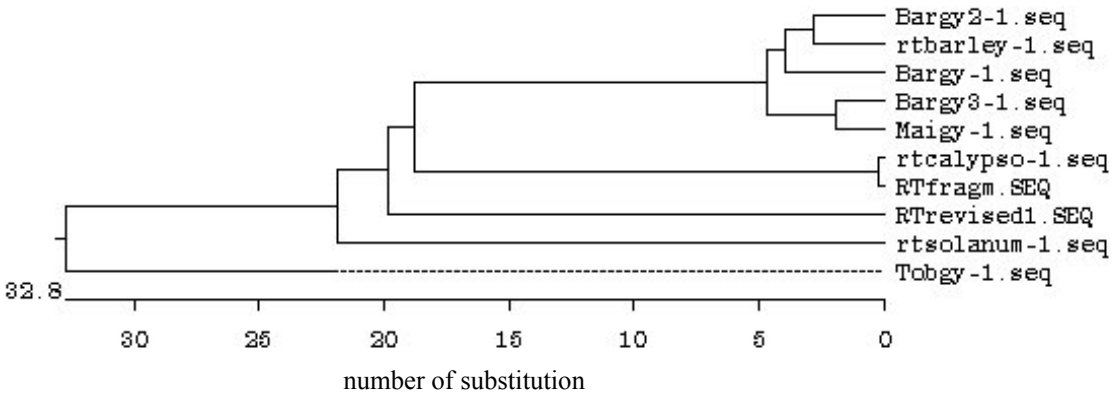


Figure 4: Multiple alignment **a)** and phylogenetic analysis **b)** of RT gene sequence from hop.

CHARACTERISATION OF *VERTICILLIUM ALBO-ATRUM* HOP ISOLATES BY MOLECULAR MARKERS

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Abstract

The appearance of different *V. albo-atrum* hop pathotypes required characterisation of isolates from various hop growing regions. Fifty-five *Verticillium albo-atrum* hop isolates collected from Slovenia, England, Germany and Poland, were therefore analysed for genetic variation by amplified fragment length polymorphism markers. Artificial inoculations of different hop cultivars were also employed to estimate the virulence of five isolates from England, and a comparison made with the Slovene isolate classified as pathotype PG2. The molecular analysis revealed two basic groups that correlated with the virulence. The first group consisted of subgroups of lethal isolates from England and Slovenia, respectively. The mild isolates formed the second group, with sub-clustering independent of geographical location.

Introduction

Verticillium albo-atrum Reinke & Berthold and *V. dahliae* Klebahn are important plant pathogenic fungi causing vascular wilts in many crop species (Pegg and Brady, 2002). In Europe, these two soil-borne fungi have caused considerable economic damage to hop (*Humulus lupulus* L.), with *V. albo-atrum* being the principal pathogen in most hop growing areas (Neve, 1991).

Verticillium wilt on hops has appeared in fluctuating (mild) or progressive (lethal) forms, depending on the pathogen virulence, the sensitivity of cultivars and ecological factors (Isaac and Keyworth, 1948, Talboys, 1972). The main difference between the forms is that the lethal one causes hop to wither, whereas plants continue to grow with the mild form. In England, three types of lethal *V. albo-atrum* isolates (PV1, PV2, PV3) and one mild (M) have been reported, which can be distinguished on the basis of pathogenicity tests on different sets of hop cultivars (Sewell and Wilson, 1984). There have also been reports of Verticillium wilt from Germany, Poland, Belgium and France, but none of these countries has had such serious problems as England (Data Sheets on Quarantine pests, 1990).

Verticillium wilt in Slovenia was discovered in 1974, when the mild form was detected caused by *V. albo-atrum* and *V. dahliae* (Dolinar, 1975). Until 1997, the disease appeared only sporadically in some hop gardens, but in that year, an outbreak of the lethal form of hop wilt was registered in the western part of the Savinja valley. On the basis of pathogenicity tests and molecular analysis, *V. albo-atrum* hop isolates from Slovenia have been classified as PG1 (mild) and PG2 (lethal) pathotypes (Radišek et al. 2003).

The appearance of different hop strains or pathotypes required characterisation of *V. albo-atrum* isolates. Our primary objective in this study was to assess genetic variability in populations of *V. albo-atrum* hop isolates from important hop growing regions in Europe. We also wanted to compare the virulence of hop isolates from England and Slovenia, since lethal hop isolates have only been identified in these countries. Such information could help in resistance breeding strategies, disease management and more detailed understanding of the evolutionary behaviour of this organism.

Material and Methods

Fungal isolates

A total of 55 *V. albo-atrum* hop isolates, 34 from Slovenia, 15 from England, 5 from Germany and one from Poland, were analysed. Of the 34 Slovene hop isolates, 27 had been previously characterised as PG1 or PG2 pathotypes by pathogenicity tests and SCAR markers (Radišek et al. 2004). The remaining seven hop isolates were considered to be lethal in view of the symptoms on the plants from which they were isolated. The virulence characterisation of nine English isolates were specified by the supplier as mild or lethal isolates. All isolates used in this study were maintained in the culture collection of the Slovenian Institute for Hop Research and Brewing, Slovenia, as monospore cultures on potato dextrose agar (PDA; Fluka, Buchs, Switzerland) at 4 ºC, or stored as cultures in general fungal medium in 20% glycerol at - 80 ºC.

Virulence assessment

The virulence of *V. albo-atrum* isolates was evaluated on four hop cultivars, susceptible Fuggle and Celeia, moderately resistant Wye Challenger and resistant Wye Target. Five plants of each hop cultivar were inoculated by stem – puncture inoculation in the basal part of the bine. Control plants were similarly inoculated by sterile distilled water. Inoculated plants were grown for 5 weeks in the growing chamber (Kambic, RK-13300) under 12-h photoperiod of flourescent light (L 58W/77; Fluora, Osram). Temperature and relative humidity, respectively, were 22 ºC and 65 % during the light period, and 20 ºC and 70 % during the dark period. Inoculum preparation and symptom assessment were carried out according to Radišek et al., 2003. For reisolation, xylem tissue above the base of the bine was plated on PDA, and the species identification of isolates was checked by light microscopy.

AFLP analysis

An optimised AFLP assay for analysis of *V. albo-atrum* and *V. dahliae* was used (Radišek et al. 2001). The primary template was prepared by digesting 500 ng of genomic DNA with 2.5 U each of *Eco*RI and *Msp*I restriction enzymes. The DNA fragments were ligated to *Eco*RI and *Msp*I adapters and such adapter-ligated fragments served as the template for primers in the PCR reaction. For preamplification, a 10-fold diluted ligation mixture was amplified for 20 cycles at 94 ºC for 30 s, 56 ºC for 60 s, 72 ºC for 60 s, using *Eco*RI and *Msp*I primers having no selective nucleotides. Selective amplification was conducted with 11 primer combinations having two selective bases (E-GA/M-AT, E-GA/M-TA, E-GA/M-AG, E-GA/M-CG, E-GA/M-GT, E-GT/M-GA, E-GT/M-CG, E-AC/M-TC, E-AC/M-TA, E-AC/M-GT and E-TC/M-TA). The *Eco*RI primer was end-labelled with Cy5. The PCR program for the selective amplification was 13 cycles of 30 s at 94 ºC, 60 s at 65 ºC, 60 s at 72 ºC, in which the annealing temperature was subsequently reduced by 0.7 ºC each cycle. This was followed by 23 cycles of 30 s at 94 ºC, 60 s at 56 ºC, 60s at 72ºC. The PCR products were separated and detected on an ALFexpress II automated sequencer (Amersham Biosciences). The AFLP fingerprints were scored manually for the presence and absence of bands. The genetic relationship among isolates was assessed by clustering by UPGMA on the basis of the Jaccard coefficient of similarity.

Results and Discusion

Molecular and virulence markers were used to elucidate the genetic variation in *V. albo-atrum* population of hop isolates from Slovenia, England, Germany and Poland. The virulence tests included six English isolates from different hop growing regions and one Slovene isolate previously characterised as lethal pathotype PG2, which present standard

isolate with known virulence. Measurements of the disease severity for each of test hop cultivars revealed differences in the virulence, which according to the Sewell and Wilson (1984) pathotype classification seated five English isolates into the pathotype PV1 and one isolate into the mild pathotype designated as M. The comparison of the disease severity caused by English PV1 isolates and Slovene PG2 standard isolate showed no significant differences. This virulence data may suggest that the Slovene lethal pathotype PG2 identified in 1997 has been introduced from England as pathotype PV1. However, virulence data alone may not reflect the true genetic variability and evolutionary history of the pathotypes. For example, genetically different isolates could have the similar or identical virulence patterns if they have been subjected to the same selection pressure by common set of hosts (Pongam et al. 1999).

In order to supplement the virulence data, molecular characterisation were employed. In all, fifty-five *V. albo-atrum* hop isolates were analysed by AFLP analysis using eleven primer combinations. The results of AFLP analysis revealed two distinct basic groups that correlated with the virulence of the isolates. The first group included all lethal isolates, which was further clustered into a subgroup of twenty-one Slovene isolates designated as pathotype PG2, together with six isolates obtained from hop plants expressing the lethal form of Verticillium wilt. These six isolates revealed the same AFLP banding patterns as PG2 isolates, and are therefore considered to be PG2 pathotype. Twelve isolates from England were clustered into other subgroup, in which five isolates were classified as PV1 pathotype, six as lethal isolates and one isolate classified as mild pathotype M according our virulence testing. Such clustering of lethal isolates suggests two different origins, one in England and the other in Slovenia, with confirmation that the recently identified Slovene pathotype PG2 has arisen *de novo*. The same AFLP banding pattern in all PG2 isolates also confirmed the homogenous clonal population of this pathotype in Slovenia.

The remaining 16 isolates with low virulence were clustered into the second group, within which one subgroup was composed of three mild English isolates (pathotype M), three isolates collected in Germany and one Slovene isolate classified as PG1. The other subgroup of mild isolates included two German isolates, six Slovene PG1 isolates and one isolate from Poland. Genetic variation observed among the mild isolates, irrespective of geographical location, suggests some common origins, within which England is the most likely since *V. albo-atrum* on hops was first identified there.

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CALLUS-DERIVED HOP PLANTS SHOW CORRELATION BETWEEN EPIGENETIC INSTABILITY AND TIME IN CULTURE

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Callus-derived hop plants may be different from the original due to DNA modifications produced along the tissue culture. The phenotype variation is determined by genetic and epigenetic changes, and it can be manifest as somatically or meiotically stable events. The somatically variation is often not transmitted to the progenies but it is important in clonally propagated cultivars as *Humulus lupulus* L. Well-known factors as the genetic background, explant source, culture-medium composition, and age of the culture affect the rates of somaclonal variation (1). Nowadays, molecular techniques are used to evaluate the genetic stability of cultures because they present greater precision than karyological and phenotypic analysis. Gurriarán et al. (1999) hop regeneration protocol departures from an organized tissue and it has a long-term callus-phase, both characteristics could be related to a high genetic instability risk. Although several regeneration and transgenic protocols have been published for hops, the genetic stability of the recovered plants had not been tested yet to the best of our knowledge.

Our studies with genetic markers indicate that the three first sequential subcultures of hop regenerated plants (cv. *Chinook*) are stable and true-type when comparing to control plants (field and *in vitro*). The stability was tested using 16 AFLP different primer combinations which produced a total of 876 loci. MSAP technique was applied to certify the epigenetic stability of the callus derived plants. A statistical study of the similarity between *in vitro* controls and the regenerates was carried out and it revealed that only a 13.37% of the total of variation was present in all the callus-derived plants contrasting with the high percentage of variation assigned to singletons. The third subculture pools presented a significant augment in number of exclusive changes and also they clustered apart from the controls and the first and second callus-derived pools in the UPGMA tree. Kaeppler et al. (2000) proposed a sequential accumulation of mutations over time, and Brar and Jain (1998) considered the age of culture as one of the main factors influencing somaclonal variation. The results presented here indicate that most of the epigenetic changes detected are produced by random modifications of the isoschizomeres recognition sites, which were significant increased when the culture period was prolonged. The analysis of the each epigenetic change detected in the pairwise comparison of the band patterns produced by the isoeschizomeres suggested us that the kind of process involved in the epigenetic changes is a demethylation of the DNA.

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Acknowledgements

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III. Session:

HOP CHEMISTRY

CONTENTS OF PRENYLFLAVONOIDS IN CZECH HOPS AND BEERS

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Abstract

The contents of prenylflavonoids (xanthohumol, desmethylxanthohumol, isoxantho-humol) were investigated in all Czech hop cultivars during the period 2000-2004. The concentrations of xanthohumol varied between 0.2 and 1.1% (w/w) with the highest amounts found in the new Czech high-alpha hop cultivar, Agnus. The highest ratio of xanthohumol/alpha acids, greater than 10^{-1}, was determined for Sladek. A positive correlation was observed for the xanthohumol and alpha acids. Fresh hops contain also small amount of isoxanthohumol (up to 2.10^{-2} %(w/w). Desmethylxanthohumol was present in Czech hop cultivars in the range of 0.05 to 0.20% (w/w). The contents of prenylflavonoids, predominantly isoxanthohumol, in Czech beers were usually between 0.3 and 1.9 mgL^{-1}. Low isoxanthohumol levels were found if hop CO_2 extracts were used. Prenylflavonoids were very minor constituents in draught or diet beers. Losses of isoxanthohumol on beer production were highest during wort cooling and trub removal.

Keywords: hops, *Humulus lupulus*, prenylflavonoids, xanthohumol, bitter acids, beer brands, isoxanthohumol, liquid chromatography

Introduction

Prenylflavonoids are plant secondary metabolites that are prominently present in hops (*Humulus lupulus* L.). The most important hop prenylflavonoids are xanthohumol (X), desmethylxanthohumol (DMX), isoxanthohumol (IX), 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN). Importance of prenylflavonoids consists in wide range of biological effects (Stevens et. al., 1997; Miranda, 1999), The composition of prenylflavonoids largely depends on the variety. The ripening stage and storage conditions constitutes up to 90 % of the mixture of prenylflavonoids (Biendl, 2002). During beer brewing X is isomerized to IX, hence this is the most prevalent prenylflavonoid in beer. Similarly DMX is converted to 6-PN and 8-PN. Beer is the most important source of prenylflavonoids in the human diet (Stevens et. al, 1999).

Material and methods

Czech hop cultivars from the harvest seasons 2000-2004 were collected for quantitative analyses of prenylflavonoids by liquid chromatography and with UV (LC/UV) and mass detection (LC/MS). Various types of Czech beers (lagers, draught beers, diet and special beers) were investigated during 2003-2004. Beers were bought at local store. X and DMX (detection at 370 nm) were determined according to the EBC (Analytica-EBC) 7.7 method simultaneously with alpha and beta acids. Mobile phase consisted of 900 ml of methanol with 190 ml of water containing 5 g of 85% phosphoric acid. It was mixed and filtered through 0,45-μm nylon filter. The analytical column was Nucleosil EC C18 Hop (4 x 250 mm). The column temperature was 40°C and the flow rate was 0,8 ml/min. Beers preconcentration was effected by solid-phase extraction (Supelclean LC-18, 500 mg/6 ml cartridges). Cartridges were conditioned with methanol (2 x 10 ml) and water (2 x 5 ml). Beer (50ml) with addition of 0,1 ml phosphoric acid. Fraction of interest was eluted with 2,5 ml of methanol. Eluate (10 μl) was injected in the HPLC instrument (detection at 290 nm). MS detection was performed in the positive chemical ionization mode at atmospheric pressure (LC-API-MS) using an ion trap FINNIGAN LCQ Deca mass detector. The HPLC eluent was introduced into mass

spectrometer via a heated nebulizer interface 450 °C. Ionisation of the analyte vapor mixture was initiated by a corona discharge needle at 6 kV and discharge current 5 μA. Prenylflavonoids were detected in single ion monitoring mode.
X was purchased from Phytochem, Germany, DMX was obtained from Dr. A. Heyerick (Ghent University, Belgium), standards of 6-PN and 8-PN were given by Dr. F. Stevens (Oregon State University, Corvallis,USA).

Results and discussion

The concentration ranges of X and alpha acids in Czech hop cultivars (2000-2004) are summarized (Table 1). X contents are in the interval 0.2 to 1.1 % w/w. The highest amount of xanthohumol was found in Agnus. Sládek showed remarkably high xanthohumol/alpha acids ratio. The ratio is an important parameter from brewer´s perspective. Positive correlation between X and alpha acid contents was demonstrated (Fig. 1). The relationships between X and alpha acid contents in German, American, Czech, English and Slovenian hop cultivars are shown (Fig. 2). As the content of alpha acids increases, levels of X also increase. Scattering of X levels in high-alpha hops suggests that X is influenced by environment and genetic factors. DMX contents in Czech hop varied between 0,05 and 0.20% (w/w) (Table 2) in agreement with published data (J. de Keukeleire, 2003). Also small amounts of IX were observed in raw hops (up to 0.02 % w/w). A direct correlation with the concentrations of X is evident (Table 3).

The summarised contents of prenylflavonoids for various brands of Czech beers are shown (Table 4). IX is the most abundant prenylflavonoid in beers. Widely varying contents reflect different hopping regimens. When hop extracts (carbon dioxide) or advanced hop products are used low or negligible amounts of prenylflavonoids are found in the final beers. The highest concentration of prenylflavonoids, higher than 2 mg/L, was determined in Pilsner Urquell 12 % lager. Czech lager beers contained significantly more prenylflavonoids than did draught beers. Traces of individual prenylflavonoids in a lager beer as obtained by LC/MS procedure are shown (Fig 3)

Significant losses of prenylflavonoids occur during brewing (Stevens, et al. 1999). The contents of IX were monitored at several stages in the course of lager production (Fig 4). IX was lost particularly during wort coolling and trub removal. IX was drastically removed while further losses downstream were less pronounced. The utilisation from hot wort to final beer was about 50 %.

Conclusions

1) The concentrations of X and DMX in Czech hop cultivars were in the ranges of 0.2 to 1.1 % (w/w) and 0,05 to 0.20 % w/w. Agnus is the richest in X, Sládek in DMX.
2) Total contents of prenylflavonoids in Czech beers may be as high as 2 mg/L. Czech lager beers contain more prenylflavonoids than draught or diet beers. The most important losses of IX in the course of brewing occur during wort coolling and trub removal.

Acknowledgements

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Table 1. Concentration ranges of xanthohumol (X) and alpha acid in Czech hop cultivars (2000-2004).

Cultivar	X (% w/w)	Alpha acids (% w/w)	Ratio X/Alpha.10^2
Saaz	0.25 – 0.45	3.0 - 4.0	9 – 13
Bor	0.40 – 0.60	7.0 – 10.0	4 – 7
Sládek	0.45 – 0.80	4.0 – 8,0	9 - 15
Premiant	0.30 – 0.50	7.0 – 11.0	3.5 – 4.5
Agnus	0.70 – 1.10	10.0 – 15.0	6 – 8

Table 2. Concentrations (mean values and ranges) of desmethylxanthohumol (DMX), xanthohumol (X) and alpha acids in Czech hops (2003-2004).

Cultivar	Year	DMX (% w/w)	X (% w/w)	Alpha acids (% w/w)
Saaz	2003	0.073 (0.053 – 0.120)*	0.35 (0.28 – 0.42)*	3.3 (2.3 – 4.4)*
	2004	0.111 (0.080 – 0.167)	0.40 (0.33 – 0.46)	3.8 (2.6 – 5.0)
Sládek	2003	0.120 (0.050 – 0.202)	0.48 (0.29 – 0.62)	4.1 (1.6 – 5.7)
	2004	0.176 (0.122 – 0.300)	0.75 (0.60 – 1.04)	6.8 (4.4 – 9.5)
Premiant	2003	0.081 (0.051 – 0.110)	0.29 (0.22 – 0.41)	6.7 (4.8 – 8.7)
	2004	0.116 (0.064 – 0.191)	0.43 (0.35 – 0.51)	10.6 (7.5 – 12.7)
Agnus	2003	0.111 (0.090 – 0.160)	0.77 (0.74 – 1.00)	11.1 (7.9 – 13.1)
	2004	0.150 (0.119 – 0.190)	1.00 (0.86 – 1.18)	13.0 (12.4 – 15.4)

*mean value/interval of experimental results

Table 3. Concentrations (%w/w) of xanthohumol (X) and isoxanthohumol (IX) in Czech hops (2002)

Cultivar	Locality	X (% w//w)	IX (% w/w)
Saaz	Drahonice	0.20	0.004
Saaz	Ročov	0.30	0.006
Sládek	Žatec	0.51	0.015
Premiant	Staňkovice	0.22	0.005
Premiant	Brozany	0.20	0.004
Bor	Nesuchyně	0.29	0.006
Agnus	Staňkovice	1.03	0.018

Table 4. Concentrations of prenylflavonoids in Czech beers (X = xanthohumol; IX = isoxanthohumol;
6-PN = 6-Prenylnaringenin; 8-PN 8-Prenylnaringenin).

Beer brand	Prenylflavonoids ($\mu g.L^{-1}$)			
	X	IX	6-PN	8-PN
Bráník, *draught*	9	206	5	< 2,0
Gambrinus,*draught*	34	450	21	9
Primus, *draught*	< 0,2	< 20	< 2,0	< 2,0
Měšťan, *diet beer*	1,9	250	7	5
Bernard, *12 % lager*	28	1350	31	15
Premium Žatec, *11 % lager*	28	1180	30	12
Pilsner Urquell, *12 % lager*	26	1910	29	22
Budweiser Czech, *12 % lager*	16	860	27	25

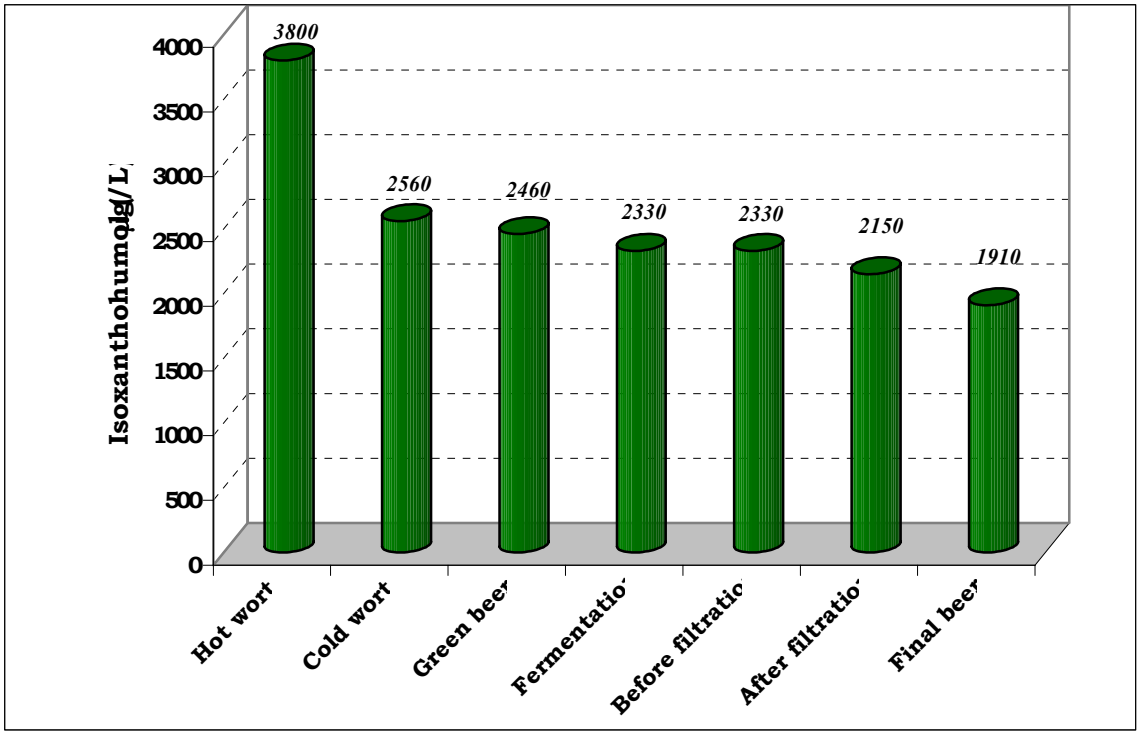

Fig. 4: Contents of isoxanthohumol in semi-products and final beer in the course of 12 % lager production *(Pilsner Urquell Brewery, Plzeň, Czech Republic)*

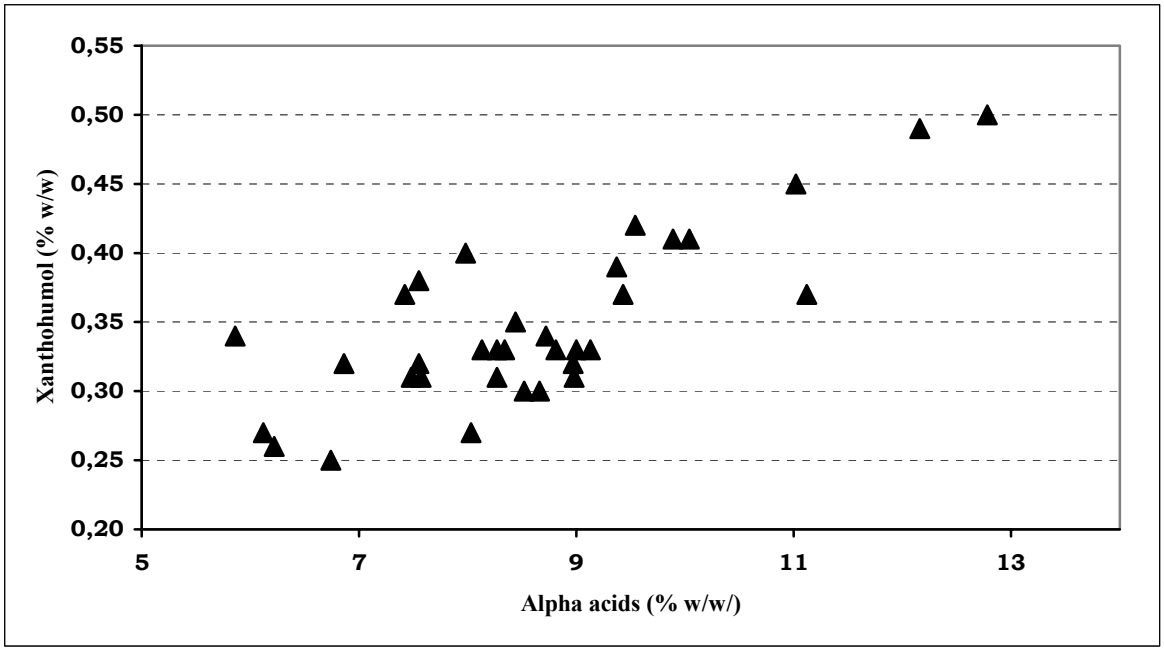

Fig. 1: Relationship between the concentrations of xanthohumol and alphạacids contents in Premiant cultivar, harvest season 2002. Triangles represent various localities in growing areas, r = 0,780.

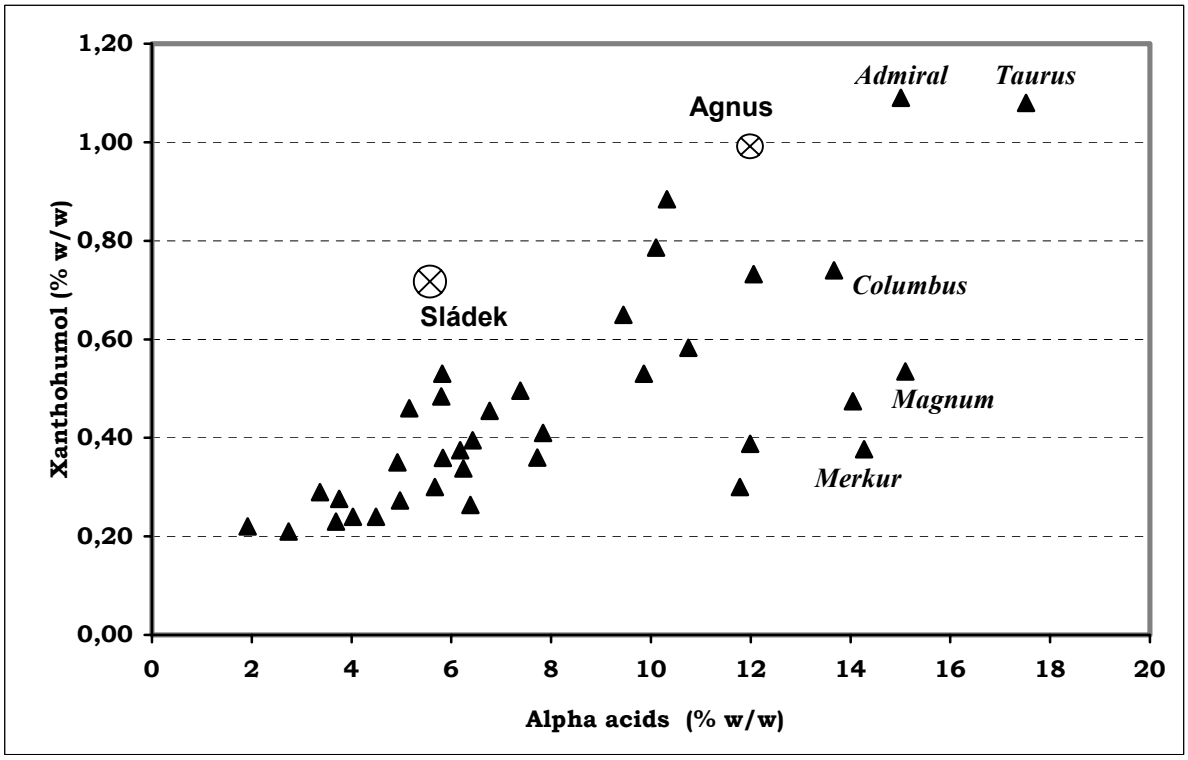

Fig. 2: Relationship between the concentrations of xanthohumol and alphạacids contents in various hop cultivars, harvest season 2002. Triangles represent hop cultivars from USA, Germany, England, Slovenia and Czech Republic.

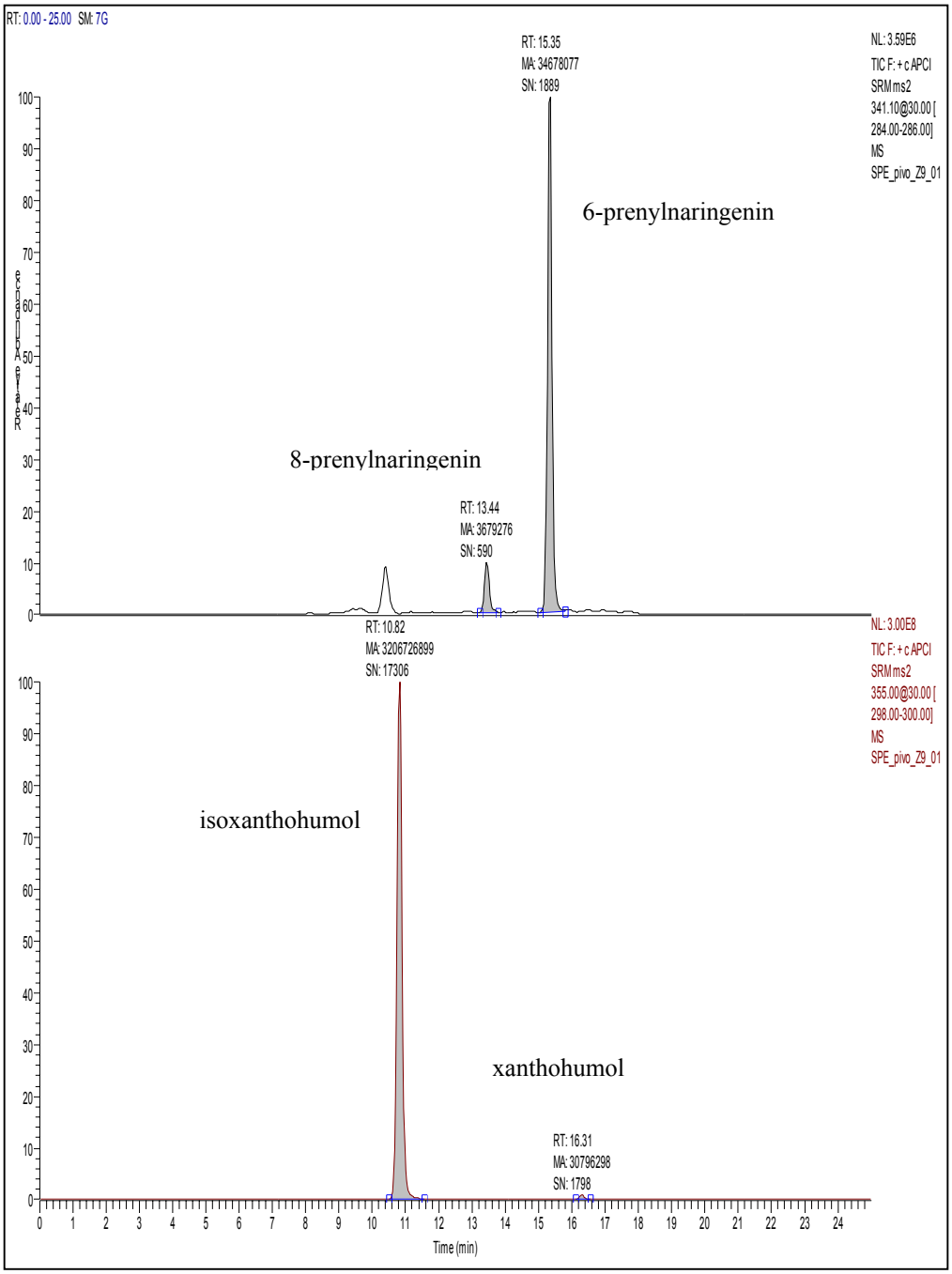

Fig. 3: Chromatogram of individual prenylflavonoids in a lager beer (LC/MS, single ion monitoring)

PRODUCTION OF XANTHOHUMOL ENRICHED HOP EXTRACT USING CARBON DIOXIDE AS SOLVENT AT PRESSURES UP TO 1000 BARS

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Hop polyphenols cannot be extracted with carbon dioxide under the conditions used for common hop extracts destined for the application in the brew house. Also the prenylated flavonoid xanthohumol belongs to this substance group and it is not extractable at 250 bars. Xanthohumol has shown a significant anticarcinogenic potential in in-vitro tests and at present clinical trials are carried out to try to confirm this in-vivo(1).
Two possibilities using solvents with different polarities for the enrichment of xanthohumol are described (2). Either an ethanol hop extract is extracted with carbon dioxide or the spent hops of the extraction with carbon dioxide are extracted by means of an ethanol/water mixture to separate xanthohumol from the hop bitter acids. In both cases at least two solvents are needed to utilise their different polarities. And furthermore the first method requires a carrier material as an aid. Moreover an alternative method is suggested using only carbon dioxide as solvent.
Solubility of substances in supercritical carbon dioxide depends on pressure and temperature of the solvent. Higher pressure and therefore higher density often improves the solubility of substances. Beyond this changes in interactions allow to extract molecules which are difficult to dissolve at lower pressures. This fact is utilised to get a xanthohumol enriched extract only using carbon dioxide as solvent.
Spent hops of the conventional extraction with carbon dioxide are extracted a second time by means of carbon dioxide but now at pressures up to 1000 bars (3). The product is a dry dark green extract containing between 10 and about 30 % xanthohumol. The wide area of the xanthohumol content depends on the separation conditions chosen. With this technique about the half of the xanthohumol put in is extractable. Besides xanthohumol hard resin compounds such as humulinic acids and hulupones make up the essential parts of the extract.
The xanthohumol enriched extract is totally soluble in ethanol and in this form it can be dosed to wort or beer whereas national regulations have to been followed. Well known is the loss of xanthohumol during the brewing process when it is given to the wort. Producing cloudy beers with 2.5 mg/l xanthohumol yet according the German purity law is possible. The technique of that procedure has been described earlier (3).

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IV. Session:

HOP DISEASES AND PESTS

DEVELOPMENT AND TESTING OF A FORECASTING MODEL FOR POWDERY MILDEW (PODOSPHAERA MACULARIS) IN BAVARIAN HOPS

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Abstract

With the introduction of cultivars susceptible to Hop Powdery Mildew (HPM) in the Hallertau, this disease became gradually unpredictable in the growing region. As hitherto no prediction on the epidemiology of HPM was possible, there was no alternative to preventive fungicide applications even if the risks of infection were low. Infestation and damage by HPM are very variable from year to year, most likely due to varying weather conditions.

We compared weather parameters in varying sequences with the actual situation of HPM infection in the years 1997 to 2004, and empirically deduced a preliminary forecasting model for HPM in Bavarian hop growing regions.

Introduction

Infection and economic damage of Hallertau hops by Hop Powdery Mildew *Sphaerotheca humuli* (De Candolle) Burrill (HPM) are varying from year to year clearly recognizable (Figs 1 and 2).

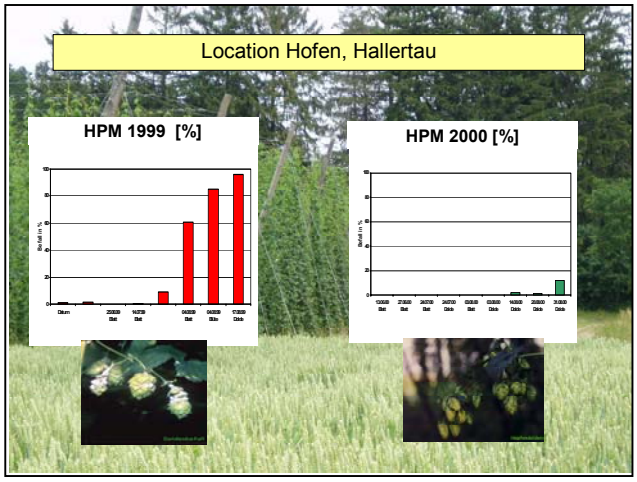
Fig. 1.

Situation of Hop Powdery Mildew (HPM) infection in hops of the Hallertau growing region

Year	Extent of Infection	Costs € / ha
1997	Heavy disease pressure and first late infection	160,-€
1998	No problems	180,-€
1999	Hitherto earliest and heaviest disease pressure and damage	250,-€
2000	Only very little infection	270,-€
2001	In some lacations big problems (June)	280,-€
2002	Strong infection and damage	350,-€
2003	No infection	220,-€
2004	Very little infection	260,-€

Engelhard-IPZ5b-Pflanzenschutz-1

Fig. 2



In the years 2000, 2003 and 2004 an enormous amount of fungicides was applied, although many sprayings would not have been necessary from a later point of view. The differences between 1999 and 2000 were especially grave: Although in a trial in 1999 all HPM-infested hop plants had been chopped and left in the field, in 2000 the plants of the same field showed no infection at all. This was the starting point for the consideration that the key for a control strategy had to be searched in the differing weather parameters of those two years.

Material and methods

Under consideration of different data on the epidemiology, weather parameters of the months from April to July of the years 1999 and 2000 were layered according to a great variety of distinctive marks, and compared with data on HPM infection recorded in the trials.

Fig. 3:

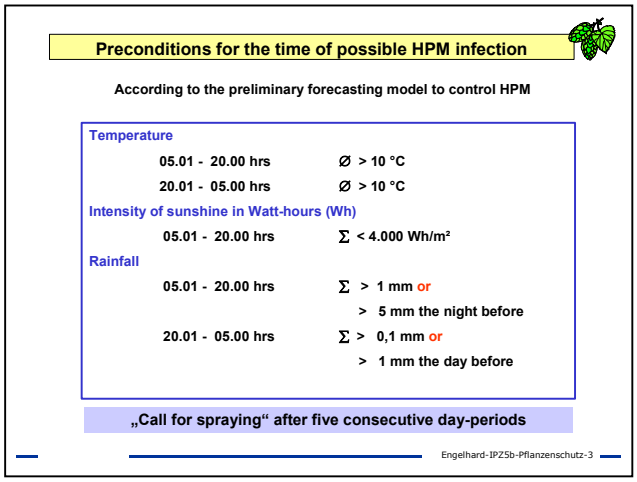
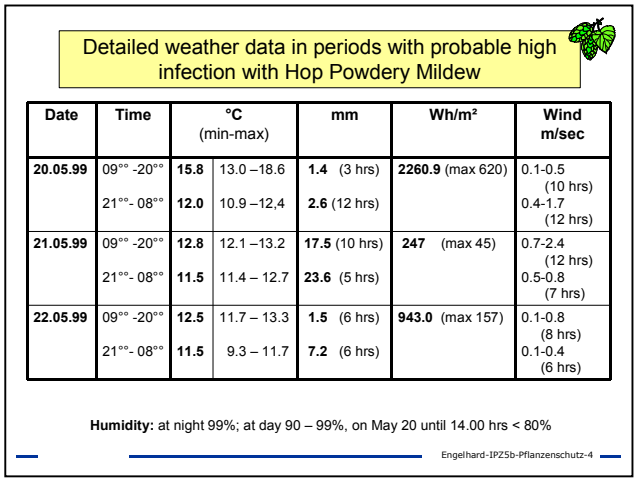


Fig. 4:



After numerous attempts with varying handicaps, the values shown in Figure 3 were determined, which are in good accordance with the HPM infection level in the Hallertau. The following handicaps were essential for the determination of those intermediate stages:

- Differentiation between average values during daytime and night;
- Consideration of the intensity of sunshine.

As a comparison to the analysis of meteorological handicaps (Fig. 3), the assessed infection levels in official trials for pesticide registrations and the overall infestation of cones in the “Neutral Quality Assessment” procedure was used.

The detailed analysis of weather data in periods with probable infection of hops by HPM resulted regularly in values as shown in Figure 4.

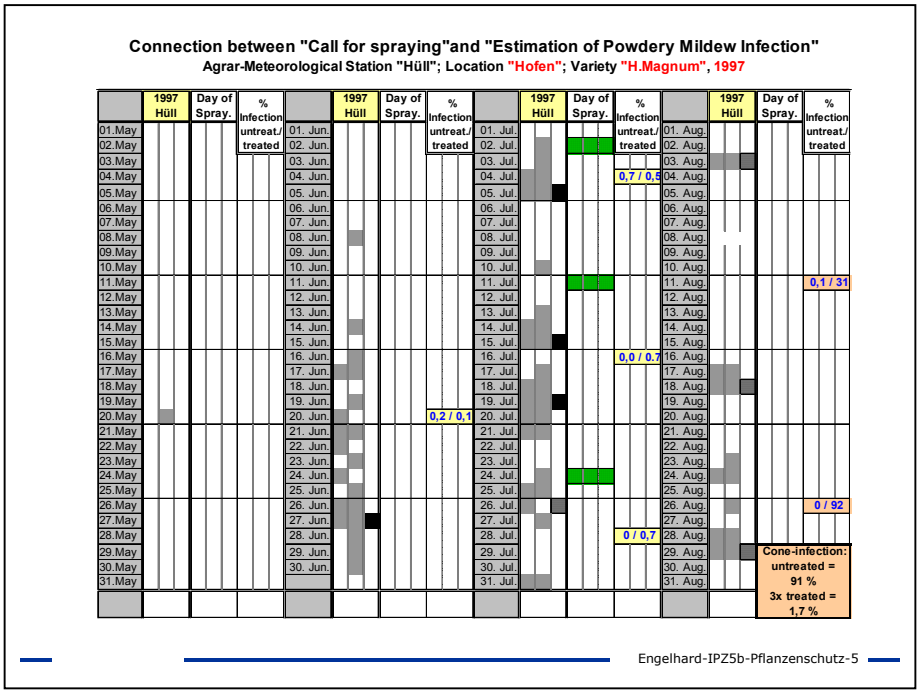
As a basis for the analyses serve weather stations, which are able to put hourly average values for various parameters at one’s disposal. Within Bavaria, a network of 123 according weather stations exists. Seven of those weather stations can be used for the model in the hop growing region.

Results

With the beginning of the examination of the model in 2004, a “call for spraying” was sent out as soon as the assumed weather conditions for HPM infection had been met at two consequent days. Under consideration of modified handicaps regarding intensity of sunshine and rainfall by night after the 2004 field season, a “call for spraying“ in future will be sent out as soon as: assumed conditions for HPM infection are met at five consequent day periods.

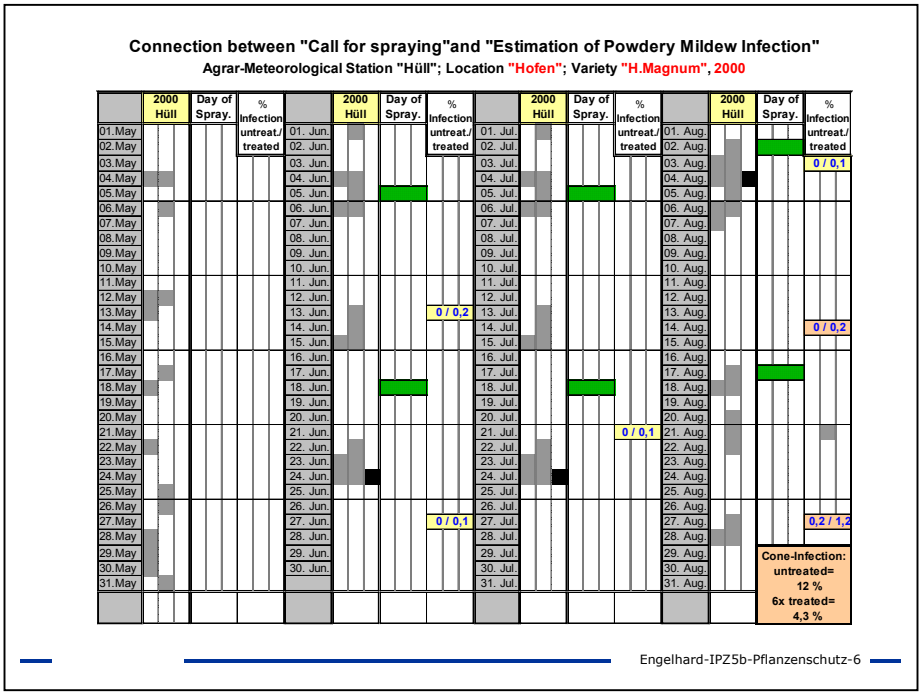
Comparisons of the model were made at different locations in the years 1997 – 2004. Three especially extreme examples are presented:

Fig. 5:



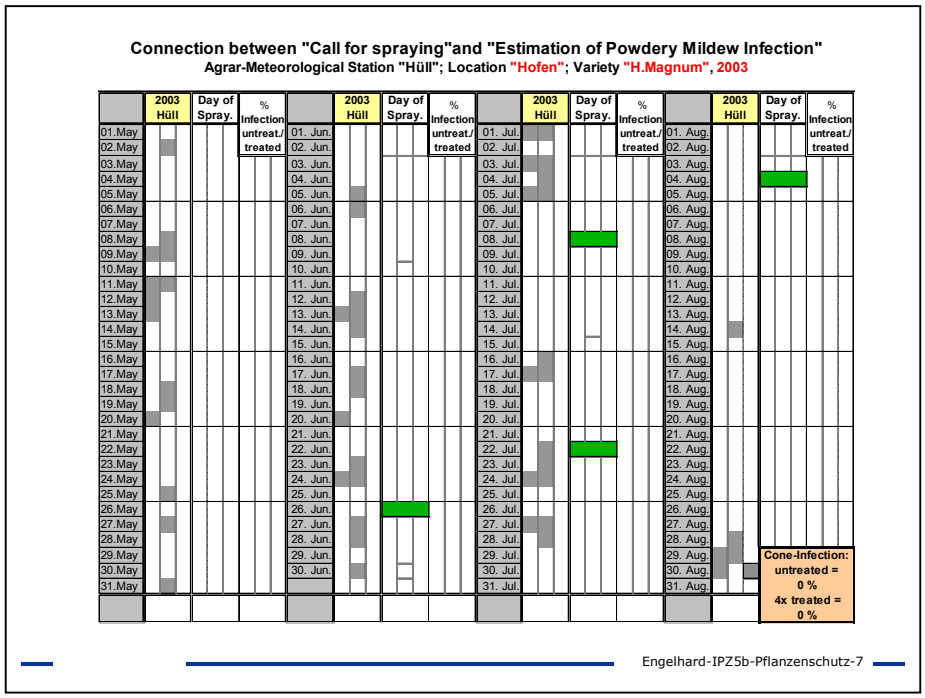
Despite heavy disease pressure, hops were kept uninfested with only three fungicide applications. The timings of “calls for spraying“ were shortly before the applications (purely by chance)!

Fig. 6:



Despite little infection at harvest time (12 % cone infection in untreated plots), six fungicide applications did not lead to a substantial success in controlling HPM. The sprayings were in all cases timed in a way that they had been carried out clearly outside the periods calculated as infection periods by the preliminary forecasting model.

Fig. 7:



In the year 2003, untreated experimental plots showed no HPM infection at all. Four fungicide applications that had been carried out would not have been necessary. The preliminary forecasting model did not trigger any “call for spraying“.

Discussion

The empirically determined weather conditions as handicaps for a forecasting model for HPM are in the years from 1997 to 2004 in good accordance with the numbers of “calls for spraying” and the actual HPM infectation situation in the field. The analyses from different weather stations indicate that the preliminary model can also be used on a large scale, e.g., for the entire Hallertau growing region.

The preliminary forecasting model is pending further evaluation in additional tests in the field. Especially the period of time that may lie between a “call for spraying” and the actual fungicide application needs further checking. Additionally, in experimental plots it has to be examined what group of pesticides can be used at certain periods of time.

Acknowledgements

We wish to thank all colleagues of the Hop Research Center and the plant protection industry for constructive discussions, and the 35 hop growers, who tested the model in the year 2004 in their fields at their own risk. Thanks go also to Christian Goldbrunner and Stefan Fuß for their diploma theses. We are most grateful to Anheuser-Busch, Inc., and to Dow AgroSciences for financial support of the project.

THE RESISTANCE PHENOMENON IN DAMSON-HOP APHID (PHORODON HUMULI SCHRANK) IN CZECH REPUBLIC

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Abstract

Damson-hop aphid (Phorodon humuli Schrank) is the most important pest of hops not only in Czech Republic but in the entire hop growing countries on the Northern Hemisphere as well. Till the beginning of the 20th century natural enemies were the only regulating factor of its population density. Tobacco extract was in the middle of the fifties changed by organophosporous insecticides and the problem seemed to be solved. Nevertheless, a decade later numerous aphids survived after treatment with these insecticides. Since that time research work has been aimed at this problem and new groups of insecticides were gradually used to be able to cope with the resistant aphids phenomenon. Pyrethroids had to be sprayed instead of organophosphorous and carbamate insecticides in the eighties but their efficiency was early too low as well. Since that time hop protection against resistant strains of damson-hop aphid has been practically based on imidacloprid, which has become the key aphicide in P. humuli control. Lambda-cyhalothrin is recommended in the time when it is necessary to protect hop plants against damage before imidacloprid application. Resistance of field strains of damson-hop aphid to these compounds is discussed.

Keywords: damson-hop aphid, resistance, biological efficiency, insecticides, imidacloprid, lambda-cyhalothrin, pyrethroids.

Introduction

Hop (Humulus lupulus L.) is a very demanding plant not only from cultivation point of view but from protection point of view as well. Damson-hop aphid (Phorodon humuli Schrank) is the most important pest (Neve, 1996).
In the past aphids severely influenced hop crop and both its quantity and quality depended on the activity of their natural enemies (Růžička, Vostřel, Zelený, 1988).
Organophosporous insecticides were highly efficient for more than ten years and the problem of P. humuli control seemed to be solved forever. But a few years later there appeared first aphids surviving after treatment with thiometon (Hrdý, Kříž, 1981). The efficiency of pesticides from this group decreased very fast and until 1975, according to data published by FAO and WHO, resistance to insecticides was found in 364 species of agricultural importance (Georghiou, Taylor, 1976). Therefore on the initiative of the West Palearctic Regional Section of IOBC a sub-group “Integrated Control in Hops” was established in the framework of the Working Group “Integrated Control in Orchards”, where a standardized spray residue method for measuring, and a dip-test for monitoring resistance in aphids were presented (Hrdý, Kuldová, 1981).
Results of laboratory bio-assays with aphids collected from sites in Kent and West Midlands together with a susceptible stock obtained from wild hops remote from commercial hop growing areas, which were tested in a Potter tower with chosen insecticides, were presented by Muir (1979). Increasing difficulties in controlling P. humuli in German hop gardens led to examination of the insecticide resistance of this pest. These investigations, which were initiated in 1980, indicated that aphids from Hallertau region were resistant against many organophosphates (Kremheller and Knan, 1984).
From the beginning of the eighties more attention was devoted to a new group of insecticides, pyrethroids due to resistance of P. humuli to numerous organophosphates and carbamates. Responses from a suspected deltamethrin-resistant field population showed

evidence of increased resistance to this compound compared with other tested populations (Furk and Baxter, 1988). Also other European hop-growing countries were involved into this research: Žolnir (1988) in former Yugoslavia and La Messeliere (1986) in France.
The group of pyrethroids used almost exclusively in the eighties did not reach such a high efficiency to be further recommended for practical hop protection against resistant P. humuli at the beginning of the nineties. The same holds true in the case of diazion, an insecticide from the group of thiophosphates, which used to be a decisive aphicide at the turn of the eighties and nineties. Since that time imidacloprid (Confidor 70 WG) has become the key aphicide within P. humuli control strategy (Vostřel and Veselý, 1995).
The survey of results obtained from laboratory tests with the most important zoocides describing their biological effectiveness on resistant P. humuli and T. urticae since the beginning till the middle of the nineties was presented by Vostřel (1995, 1996).
Imidacloprid was very efficient on damson hop aphid resistant to organophosphates, carbamates and pyrethroids almost for ten years. Nevertheless, in the last years there were reported some surviving aphids after treatment by this compound. First reports about lower efficiency of imidacloprid came from the neighbouring Germany (Engelhard, 2001). In Czech Republic there were first surviving aphids found after a treatment with imidacloprid two years ago (Vostřel, 2003).
Besides problems with resistance of P. humuli to this insecticide, it was found that it also stimulated fecundity of T. urticae that ingested imidacloprid (James, Price, 2002). On the other hand it was proved that imidacloprid also stimulated egg production in a predatory mite Amblyseius (=Euseius) victoriensis Womersley (James, 1997).

Materials and methods

To be able to compare recent results of laboratory tests with resistant aphids with older ones a standardized spray-residue method for monitoring of resistance in Phorodon humuli developed by Hrdý and Kuldová (1981) was used. Samples of damson-hop aphids populations were taken from the selected hop-gardens within the hop regions in Czech Republic at the beginning of June 2004 before application of imidacloprid. These samples were transferred into laboratory 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%. Hop seedlings were used as a host plant. 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 (imidacloprid and lambda cyhalothrin, resp.) with the help of Potter's nozzle under a pressure of 0,2 Mpa. After the sedimentation time (10 minutes) the treated leaves were removed from the sedimentation tower. The method requires glass cylinders (22 mm in diameter and 15 mm high) stuck on the inside of treated leaves 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 crawl, were recorded as dead. The mortality on non-treated (control) leaves should not have been higher than 10% (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 a tested insecticide. As a standard reference susceptible strain a sensitive strain from Slovakia, sampled near Rožňava in 1974 was used. Values of LC 50 and for the resistant and sensitive strains were determined together with index of resistance.

Results and discussion

The results of laboratory tests with Confidor 70 WG (imidacloprid) carried out in 2004 are reviewed in Table 1. Ten "resistant" field strains taken within Saaz (Žatec) hop region were tested in the following geometric row of concentrations: 0,01%, 0,005%, 0,0025%, 0,00125%, 0,000625% and 0,000312%. No surviving aphids were observed only if the highest concentration of this insecticide (0,01%) was sprayed. If aphids were exposed to another concentration (0,005%), one hundred per cent mortality was observed only in 70% of the tested field strains. LC 50 for these populations was slightly above the level of the lowest tested concentration (0,000312%) on average. LC 50 for a sensitive strain "Rožňava" was much lower, approximately on the level of 0,000053%. If we compare these two obtained values we can conclude that index of resistance for imidacloprid is on average of about 7,5 Table 1).
Unfortunately, no laboratory tests with a sensitive strain were carried out in CR up to 2004 and so any data concerning LC 50 for such a strain are not available. Therefore it is impossible to compare present values of IR for imidacloprid. On the other hand we have some results with biological efficiency of this compound from laboratory tests, which were made in the middle of the nineties. Even if three times lower concentration (0,002%)than the recommended one (0,008%) was tested, no surviving aphids were observed (Vostřel, 1997). Similar results were obtained two years later (Vostřel, et al., 1999). The average mortality of field populations sampled within Saaz hop region if 0,001% concentration of Confidor 70 WG was sprayed was 99,5%, which is approximately the same level as five times higher concentration (0,005%) in 2004. At that time less than 5% of tested aphids survived the lowest tested concentration (0,000312%) where now more than 50% survived. The increase of C 100 M was during six years from 0,0005 – 0,001% to 0,005 to 0,01%, that means ten-times.
Resistance of P. humuli to pyrethroids of the first generation, such as cypermethrin, deltamethrin and permethrin began shortly after their establishment into practical hop protection, although values of IR were still low at that time (Kremheller and Knan, 1984). The increase of P. humuli resistance to above-mentioned pyrethroids was 2-20 times higher if we compare 1981 and 1986 field populations in Germany (Kremheller, 1987).
During the eighties biological efficiency of pyrethroid insecticides quickly decreased. The most severe fall was obvious in fluvalinate and s-fenvalerate. Lambda-cyhalothrin (Karate 2,5 WG) also showed low efficacy not only in field trials but also in laboratory tests carried out on 1992-1993 in Czech Republic. If applied in 0,1% concentration only 57% mortality of tested aphids, which had their origin in Czech hop gardens was observed (Vostřel, 1995). From this reason it had not been recommended for practical hop protection against aphids resistant to pyrethroids in the nineties any more
Nevertheless, in the end of the millennium it was tested again as there was a need of an insecticide, which would be possible to use before application of imidacloprid, if necessary. Since there was no difficulty from MRL point of view, we decided to try it again after these years. Probably because of this long pause in practical hop protection strategy in CR its efficiency increased. If applied in 0,04% concentration, only seven percent of tested field strains of aphids survived in our laboratory tests (Vostřel et al., 1999).

In 2004 six field strains were tested in a geometric low of concentrations (Table 2). Unfortunately, the average mortality was much lower in comparison with the results obtained five years ago. Even if the highest tested concentration (0,2%) was used 100% mortality of tested aphids was not observed. In 0,05% concentration, which is the closest one to the concentration tested in 1999 (0,04%) the mortality of aphids was slightly under 70%, that means nearly by 25% lower than in the above mentioned year. It may have been caused by the fact that lambda-cyhalothrin has been used within this period in field conditions. The average LC 50 for resistant strains was 0,025% and for a sensitive strain "Rožňava" nearly ten times lower (0,0026%). IR is therefore 9,6.

We can conclude that on the contrary to imidacloprid, lambda-cyhalothrin does not reach a sufficient biological efficiency to be recommended for practical hop protection strategy. In the case of imidacloprid is biological efficiency still high enough to control resistant strains of P. humuli in field conditions in spite of the fact that there was found out a sharp increase in C 100 M within a geometric row of the tested concentrations. Fortunately, originally registered concentration was high enough and it may also be the reason why imidacloprid has kept sufficiently high efficiency for more ten years. Nevertheless, registration of new biologically efficient aphicides is necessary for us to be able to control resistant P. humuli in future.

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Table 1: Biological efficiency of imidacloprid in laboratory tests carried out in 2004

Locality	**0,01 %**	**0,005 %**	**0,0025 %**	**0,00125 %**	**0,000625 %**	**0,000312 %**
Dubčany	100 %	100 %	100 %	97 %	73 %	49 %
Kadaň	100 %	100 %	100 %	100 %	90 %	63 %
Klůček	100 %	100 %	100 %	97 %	70 %	43 %
Kozojedy	100 %	100 %	100 %	97 %	87 %	55 %
Markvarec	100 %	100 %	99 %	85 %	75 %	48 %
Obora	100 %	99 %	97 %	71 %	63 %	37 %
Ročov	100 %	100 %	94 %	86 %	82 %	50 %
Smolnice	100 %	98 %	90 %	70 %	59 %	29 %
Stekník	100 %	100 %	98 %	89 %	67 %	40 %
Šlapanice	100 %	99 %	94 %	87 %	72 %	45 %
average	**100 %**	**99,6 %**	**97,1 %**	**88,0 %**	**73,8 %**	**45,9 %**
		0,0312 %	**0,00156 %**	**0,00078 %**	**0,000039 %**	**0,000019 %**
Rožnava		100 %	97 %	77 %	39 %	27 %
Average LC 50 for RS = 0,0004 %, LC 50 for SS = 0,000053 %, IR = 7,5						

Table 2: Biological efficiency of lambda-cyhalothrin in laboratory tests carried out in 2004

	0,2 %	**0,1 %**	**0,05 %**	**0,025 %**	**0,0125 %**	**0,00625 %**
Dubčany	100 %	100 %	79 %	55 %	40 %	28 %
Kadaň	98 %	82 %	61 %	50 %	37 %	27 %
Klůček	97 %	83 %	55 %	40 %	28 %	19 %
Obora	100 %	91 %	75 %	45 %	19 %	11 %
Stekník	100 %	95 %	59 %	45 %	34 %	15 %
Šlapanice	98 %	88 %	71 %	67 %	45 %	23 %
average	**98,8 %**	**89,8 %**	**69,3 %**	**50,3 %**	**33,8 %**	**20,0 %**
		0,0125 %	**0,00625 %**	**0,00312 %**	**0,00156 %**	**0,00078 %**
Rožnava		100 %	96 %	74 %	39 %	19 %
Average LC 50 for RS = 0,025 %, LC 50 for SS = 0,0026 %, IR = 9,6						

ECONOMIC THRESHOLD OF INTERVENTION AGAINST *PHORODON HUMULI* (SCHRANK, 1801) (*HEMIPTERA: APHIDIDAE*) IN LEON (SPAIN): HOP PARAMETERS PRIOR TO CALCULATING THEM

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The hop aphid, *Phorodon humuli* (Schrank), is one of the main hop (*Humulus lupulus* L.) pests in the world. The extent of the damage caused has resulted in the use of aphicides, but only when pest density exceeds the thresholds of economic intervention when working within a system of integrated pest management. These thresholds have always been calculated in an approximate way and vary considerably according to different authors. Such variability indicates that economic thresholds must be calculated methodologically.

Although the number of aphids per leaf has normally been used to express the pest density corresponding to these thresholds in hops, other parameters, such as the number of aphids per m² of plant surface (Hermoso de Mendoza *et al*., 2001, for *Aphis gossypii* in citrus), per dm² of leaf (Campbell, 1978) or per a certain volume of plant can also be used. With this last parameter as a basis the total number of aphids on each hop plant at any given time can be calculated. In order to express pest density according to some of these parameters certain plant measurements, which are not easily obtained in a simple sampling, such as the area of the hop leaf or the number of leaves in a certain volume of plant, are necessary. They could probably be obtained from more simple measurements, for example, the length of the leaf, which would give us its area, or the section of the string (product of its width multiplied by its depth), which would give us the number of leaves in a determined volume (product of that section by a certain height of the plant) as well as in the whole plant (knowing its whole height).

The experiments were carried out in León (Spain) during 2002 on a Nugget hop plot with two aims: firstly, to calculate the area of the hop leaf, for which the function relating length (x) to the area (y) of the leaf was determined ($y = 1.13 x^2 - 3.55 x + 3.64$). Secondly, to determine the number of leaves in a volume of plant, for which the function relating the area (x) of the string section of the hop plant to the number of leaves (y) in the volume of plant formed by this area as a basis and a height of 20 cm was obtained ($y = 0.029 x$).

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THE OCCURRENCE OF PHYTOPLASMA ON HOPS IN POLAND

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Hop plants (*Humulus lupulus* L.) with symptoms characteristic of phytoplasmal infection were observed in a commercial hop garden with cvs Magnum and Marynka during the period 2003. Association of phytoplasmas with the disease was confirmed by polymerase chain reaction using three different universal primer pairs and a group specific primer pair: R16(I)F1/R16(I)R1. Restriction fragment length polymorphism analysis of nested PCR products showed that the phytoplasma infecting hop plants belonged to the aster yellows phytoplasma group (16SrI), subgroup B.

The symptoms were observed mainly on plants of cv. Magnum. Shoots of diseased plants were leafless, pale green and shortened, with a maximum length of approximately 7 cm, whilst normally growing hop plants were above 50 cm at that time and were trained. Disease symptoms of severe shoot proliferation resembling phytoplasmal disease symptoms were also observed. In the summer 2003 the hop plants with stunted growth, chlorotic leaves and deformations were observed in the same hop garden in an area of 80 ares. In the remaining part of the hop garden the plants showed normal growth without disease symptoms on leaves, flowers or cones. It is also interesting that the strongest disease symptoms were observed in hop plants which were planted near the raspberry plantation. We suspected that plantation may have been the potential source of infection but because all raspberry plants had been eradicated the verification of this hypothesis was impossible. However, in 2004, at a distance of 200 m from the hop garden with diseased hop plants, raspberry plants showing symptoms of shoot proliferation, typical of phytoplasmal disease were found, which confirms our hypothesis.

The preliminary results of the study reporting the first appearance of phytoplasma infection in hop plants have been published (Solarska et al., 2004). It seems that detection of phytoplasma in hop plants is an isolated event and that the observed symptoms of infection were connected to the special growth conditions of hop seedlings in 2002 when plants trailed on the ground all season instead of being trained and also probably due to the presence of infected raspberry plants growing in the vicinity. It is likely that such an untypical cultivation system increased the susceptibility of plants to insect vectors feeding on neighbouring raspberry plants resulting in the occurrence of severe disease symptoms. Hop plants cultivated in the traditional way did not show such disease symptoms, although they were infected by phytoplasma.

Phytoplasmas, normally thought to be associated with hot climates are increasingly being seen as important pathogens of many vegetable crops, ornamentals and trees (Berges et al., 2000). The fast spread of phytoplasma in 2002-2003 could be connected with the high temperature during summer 2002. Our studies indicate that the hop plant as a new natural host of phytoplasma can play a role in the spread of phytoplasmal diseases especially at high temperatures.

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INFECTION OF HLVD IN HOP GARDENS IN CZECH REPUBLIC

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Viroids are the smallest autonomously replicating pathogenic agents yet describe. They are responsible for significant losses of agricultural crops e.g. potato, avocado, coconut palm and hop. In hop plant are describe two viroids: hop stunt viroid (HSVd) and hop latent viroid (HLVd) with worldwide distributions. In our experiments we for identification and control of HLVd used isolation pathogenic RNA from cones with and Dot – blot molecular hybridisation with radioactively designated probes and from tissues and leaves by Concert™ Plant RNA reagent (Invitrogen, USA) for RT- PCR. Molecular sampling from hop garden was performed. We controlled *in vitro* plants in the Gene bank of hop, mother plant in greenhouse, experimental hop garden, hop garden under health control, nursery, young and old hop gardens. No morphological symptoms were observed on infected plants. In our experiments we have found HLVd and infection is practically 100% and spreading in hop garden is very rapid. We discuss these results in this contribution. Results are important for elimination of HLVd and production virus and viroid free planting material of hop in Czech Republic.
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SECOND THOUGHTS ABOUT DIFFERENT WAYS OF EVALUATION OF SPRAYING TECHNIQUES

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As the title tries to suggest, these remarks should be considered more as reminders not as well established and with solid figures supported facts. My sincere hope is that they will provoque questions, objections and remarks. If they fail to do so, they may still do their job by raising some further second thoughts.
The data about the deposit of phytopharmaceutical preparations on treated plants, namely their dependence on the way of application, on weather and other conditions are of great importance when one wants to evaluate different kinds of pesticide application.
The deposit itself, namely its amount as well as its distribution depends on various variables. Only numerous determinations under various conditions combined with biotic tests and practical experiences can result in a more comprehensive picture about various ways of application of phytopharmaceutical preparations.
On the Slovenian Institute for Hop Research and Brewing in Žalec we have beed involved in such testing for more than two decades, during this long period various techniques have been used in hop gardens as well as in other cultures and many practical plant protection problems have been at least better understood if not solved.
The most frequently used methods used for the determination of the deposit were:

– semiquantitative evaluation of deposit (copper ions) after spraying the pieces of chromatographic paper with a rubean-hydrogen solution,

– quantitative determination of deposit (copper ions) by atomic absorption spectrometry,

– quantitative determination of deposit by tracer (helios) by fluorophotometry,

– evaluation of spraying techniques by water sensitive papers (image processing technique).

The details of the experimental procedures are explained in detail in the references cited, so here only a very brief description of some more complex procedures is given:

Semiquantitative evaluation of deposit (copper ions) after spraying the pieces of chromatographic paper with a rubean-hydrogen solution

Pieces of paper of suitable size (chromatographic paper which can get soaked with liquid) were fixed on the upper and on the lower leaf sides of the treated plants. After application the pieces of paper were dried and sprayed with a 0.5 % ethanol solution of rubean-hydrogen. The deposit was semi-quantitatively evaluated according to the scale after Kohimann.

Quantitative determination of deposit (copper ions) by atomic absorption spectrometry

The treated plants were equipped with pieces of paper as for the semi-quantitative evaluation (see above). After application the pieces of paper were dried, than the copper ions were quantitatively extracted into 0.05 molar solution of Na-EDTA (sodium salt of

ethylenediaminetetraacetic acid) and their concentration was quantitatively determined by atomic absorption spectrometry.

Mutatis mutandis this method can also be used with plant leaves as collectors.

Quantitative determination of deposit by tracer (helios) by fluorophotometry

Uvitex (helios) was added to the application broth. After the application, dried leaves were sampled and kept in light tight bags till the measurements could be performed. The tracer was quantitatively rinsed from the leaves using a suitable solvent and helios was fluorophotometrically determined in the extract thus obtained.Either pieces of filter paper (of known surface) or plant leaves (green leaves directly) were used as collectors and in the case of green leaves their surface was determined from their mass. The surface of a given mass of leaves was determined by leaf contours on a representative sample of appropriate size. For hop plants it was established that reproducible results can be obtained only with samples which include 500 or more leaves.If the deposit is determined directly from the leaves and no chromatographic paper is used, we enjoy the advantage of less time consuming preparation work. Additionally, it is possible that pieces of paper retain more or less broth compared to an equal surface of green leaves (depending primarily on the weather conditions and on the amount of the broth used but also on other physical and chemical parameters).The methods where deposit is determined from pieces of paper have another advantage. The surface from which the deposit is being determined is well defined and known. If the method on the other hand involves a determination of the leaf surface via its mass it is very possible that one can not always quantitatively consider the fact that the surface of a definite mass of leaves depends strongly on their stage od development. Following observations were made for hops: The ratio between the surface and the mass of the leaves is constant on the main stem (± 4 %) and does not change with the mass of single leaf, while on the side shoots it is basically dependent on the mass of a single leaf If the sampling is done correctly and precisely one can determine the leaf surface with a precision of ± 4 %. The sample used to determine the surface of the leaves should correspond as much as possible to the sample which is used to determine the deposit.If the deposit on the top of the hop plant is determined using an average leaf sample for the surface determination (or even a leaf sample from the main stem), the result obtained is usually too high, as the sample from the top usually contains a great deal of leaves from the side shoots. In this case i. e. when determining the deposit on the top of the hop plant) the results using pieces of chromatographic paper would very probably give better results.The semi-quantitative evaluation of the deposit on the chromatographic papers after spraying with a solution of rubean-hydrogen is very simple and handy as far as the laboratory part of the analyses is concerned. But one should be careful as the evaluation is somewhat subjective and it can not always be relied upon - especially if the persons evaluating the results are not experienced enough. This method is very suitable for approximate evaluation of deposit, especially if the application has been performed in such a way, that one can be sure, no broth dripped from the treated plants. It is very useful if one wants a quick answer to the question if the treatment has reached the distant parts of the plants. Because the differences in the colouring are not very distinct in the cases when the entire surface is coloured, the results in these cases are usually higher compared to those obtained by atomic absorption spectroscopy.If the deposit is determined using pieces of chromatographic paper, differences between the deposit on the upper and on the lower leaf side can be determined. This can be very important, especially in the cases when an application of contact preparations has to be evaluated. As a rule the deposit on the lower side

of the leaf is only somewhat lower, but the differences can become significant. These differences would have escaped unnoticed if the deposit had been determined directly from the green leaves. In spite of time consuming and troublesome work with the pieces of paper it is - in some cases - wise to consider if the use of this method – often preferably combined with a fluorophotometric determination of the tracer - is not indicated.Further on the fluorescent tracer helios has another important advantage: it can be used with all the plants in every stage of development, while the preparations containing copper compounds, can in some cases be harmful to the plants.Nevertheless some difficulties arising from the fact that use of some otherwise very practical but toxic organic solvents is now prohibited (tetrachloromethane is not allowed any more) must be taken into account.Further on, it was found out that errors committed in some phases of work can surpass those in other phases for some orders of magnitude. The results supporting this statement were obtained analyzing spraying of hop plants. For other plantations (lower plants, uniform and thin leafage etc.) other factors may be decisive for the accuracy of the deposit determination. A critical evaluation of errors committed in individual phases of the determination process is nevertheless advisable. It enables a well justified conclusion about the significance of the differences between results. In this way many a troublesome procedure can possibly be avoided if it does not contribute to the accuracy of the final result. Performing time consuming operations to avoid minor errors becomes meaningless if greater errors in other phases cannot be avoided.All the estimations which include the leaf surface of the treated plants (e.g. the recovery vs. the drift of the pesticides) have to be critically considered: the approximate evaluation of the leaf surface can seriously influence the accuracy of the final estimation.We want to point out the importance serious faults committed when determining very small deposits. Usually "low results" are considered favourable in the environmental science as they usually mean that the pollutant(s) in question have not been detected. If the result determined is too high, we are "on the safe side". In plant protection the opposite is true, so extreme precautions are needed when speaking about "low results".

Some additional “chemical technicalities”

Reproducibility, of individual phases of the fluorophotometrical determination of pesticidal deposit

Accuracy and reproducibility of fluorophotometrical determination of pesticidal deposit on hop plants were determined for individual phases of the entire analysis under field conditions.

The reproducibility of the portable fluorophotometer was determined by measuring standard solutions of the same concentration several times and was found to be ± 2,5% under field conditions.

The (non)linearitv of the relationship between the tracer concentration and the signal measured can be a serious source of error, when the concentration of the solution measured is less than 30 μg/dm^3. The results in this concentration region are usually much too high and the real deposit is lower than the determined value.

Determining the reproducibility of the method when analyzinq a homogenous sample several times and its reproducibility when analyzinq samples along a hop row revealed several other sources of error. One has to take care of the "saturation" of the solvent if the mass of the extracted leaves is too high, but on the other hand the sample has to be big enough to be representative. The uncertainty of the result can amount up to ±25 % if no special precautions are taken but even with most careful work and with a great number of repetitions one has to reckon with an uncertainty of 10-15 %.

Another remark on determination of the deposit stability: as helios is a compound which is very sensitive to light, fluorofotometrical evaluation of deposit cannot be used in studies dealing with the so called “persistence of the deposit or staying of the phytopharmaceutical preparations on the plants needing protection”. In this cases analytical procedures involving determination of the active substance in question are *conditio sine qua non*. This opens a set of new or at least additional questions, specific in each case.

Conclusion

The deposit determination and evaluation includes time consuming and troublesome work in the field as well as in the laboratory. In order to get the best possible results one has to analyze a lot of samples in a relatively short time. So it is very important to select a method that will give the most reliable and meaningful results in the shortest time and to avoid the troublesome and time consuming work if it doesn't mean considerably better final results.

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