ISSN 1814-2206

International Hop Growers` Convention
I.H.G.C.

Proceedings of the Scientific Commission

Canterbury, Kent, England
5 – 7 August 2001

Edited for the Scientific Commission
by

Dr. Elisabeth Seigner
Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Hüll

Contents

Session III: DISEASES AND PESTS

Session III: HOP BREEDING

Session V: HOP CHEMISTRY AND QUALITY

Vorwort

Die Wissenschaftler in der Hopfenforschung treffen sich zu ihrem Erfahrungsaustausch an für die Hopfenzüchtung historischer Stätte.
„Wye“ ist ein Synonym für erfolgreiche Selektionen neuer Sorten.
Die verantwortlichen Wissenschaftler in Wye haben schon vor 100 Jahren erkannt, dass neben vielen anderen Merkmalen die Resistenz gegen Krankheiten und Schädlinge wichtige Zuchtziele sind. Weltweit führend ist Wye heute in der Züchtung von „dwarf-hops“.

Die Tagung der Wissenschaftlichen Kommission im Internationalen Hopfenbaubüro (IHB) bietet ein wichtiges Forum zum Informationsaustausch für die Wissenschaftler und findet erfreulicherweise weltweites Interesse – es haben sich Teilnehmer aus 18 Ländern angemeldet. Die Zahl der angemeldeten Beiträge machte es erforderlich, erstmals in Vortragsveranstaltung und Posterpräsentation zu trennen. Erstmals treffen sich auch Spezialisten des Pflanzenschutzes und der Züchtung zu getrennten Sitzungen. In fünf Sektionen werden dann Themen vorgetragen, die grundsätzliche Bedeutung haben.

Im Namen aller Teilnehmer möchte ich herzlichen Dank sagen:

- Dank für die Einladung nach England
- Dank der „Association of Growers of the New Varieties of Hops (AGNVH)“ für die Übernahme der Kosten für die Festveranstaltung
- Dank dem „Imperial College at Wye“ und der Universität von London für die finanzielle Unterstützung
- Dank der „Horticulture Research International-HRI“ für die Übernahme der finanziellen Abwicklung
- Dank an Dr. Peter Darby für die Gesamtorganisation;
  lieber Peter, Du hast alles unternommen, diese Tagung erfolgreich zu gestalten!
- Dank an die Mitarbeiter der Abteilung Hopfenforschung, HRI-Wye, für ihre Mithilfe während dieser Konferenztage

Erstmals finden die Tagung der WK und der Hopfenkongress zeitgleich im gleichen Land statt.

- Dank an Dr. Colin Campbell, der die Verbindung zum Hopfenkongress herstellt und betreut.
- Dank an die Redner und die Aussteller von Postern, die ihr Wissen an die Hopfenwirtschaft weitergeben
- Dank an Frau Dr. Elisabeth Seigner, die in ihrer Aufgabe als Sekretärin mehr als ihre Pflicht tut.

Als Vorsitzender der WK hoffe ich, dass es nicht nur bei einem Erfahrungsaustausch bleibt, sondern Grundlagen für Kooperationen geschaffen werden. Das Geld ist weltweit in der vergleichsweise kleinen Hopfenbranche zu knapp, um sich Doppelarbeiten leisten zu können; auch die Brauereien sollten mit in das Boot einsteigen.

Bernhard Engelhard
Vorsitzender der Wissenschaftlichen Kommission des IHB

Foreword

Hop researchers meet this time for their exchange of experience at a historical location of hop breeding. “Wye“ is a synonym for successful selection of new varieties.
The scientists in charge have recognized even 100 years ago, that besides many other traits resistance to diseases and pests are important breeding objectives. Today “Wye” leads the world in breeding dwarf-hops.
This meeting of the Scientific Commission of the International Hop Growers` Convention provides a valuable forum for the exchange of information and we are glad that scientists from around the world take great interest in this meeting – participants from 18 countries are registered. Due to the great number of announced contributions for the first time it was necessary to separate the presentation of papers and posters. Also for the first time experts of pest management and breeding meet in two concurrent sessions. In five sessions topics of fundamental nature are presented.

On the behalf of all participants I would like to say thank you very much:

- Thanks for the kind invitation to England
- Thanks to the „Association of Growers of the New Varieties of Hops” (AGNVH) who have contributed to the costs of the conference dinner
- Thanks to the „Imperial College at Wye” and the “University of London” for their financial support
- Thanks to „Horticulture Research International-HRI“ for handling the financial arrangements
- Many thanks to Dr. Peter Darby for his hard work in organizing this conference:

Dear Peter, you have done everything to make this meeting a great success!

- Thanks to the staff of the Department of Hop Research, HRI-Wye, for their help during these days of the meeting

For the first time the meeting of the Scientific Commission and the main congress take place at the same time in the same country.

- Thanks to Dr. Colin Campbell, who is organizing the IHGC main congress and gets us in contact with the delegates from the main congress.
- Thanks to all delegates presenting papers and posters. I am grateful to all of you for passing on your knowledge to the hop business.
- Thanks to Dr. Elisabeth Seigner who - in her task as secretary -does a lot more than her duty.

As chairman of the Scientific Commission I hope that this contact is not only an exchange of experience, but a starting point for creating cooperation. Money is too tight worldwide in the comparatively small hop business, and thus we cannot afford to spend money for parallel work, also the brewing industry should join us.

Bernhard Engelhard
Chairman of the Scientific Commission of the IHGC

RAISING INTERNATIONAL COORDINATION TO ITS NEXT LEVEL

Joint Presentation:
Mr. Bill Bryant, Bryant Christie Inc.
Ann E. George, U.S. Hop Industry Plant Protection Committee

This presentation will review recent efforts to harmonize international hop chemical residue standards, to discuss the effect the European Union's 2003 initiative has on that project, and to propose longer term solutions to facilitate compatible standards first between the US and the EU, and then between other trading partners.

Over the past seven years, US, UK and German industry representatives and researchers have exchanged information that has facilitated research on new substances, which has coordinated the timing of registration and setting of residue levels in the US and Europe. As a result, there has not been a major chemical residue trade problem reported in recent years. Clearly, even in the absence of any cooperative regulatory agreement, the coordination of research priorities and the exchange of data for registration purposes has helped prevent unnecessary impediments to trade. The manufacturers and the US IR-4 program are major contributors to this success. Their willingness to supply data, and to assist in ensuring the data is presented in a format consistent with the requirements of different agencies, has moved our harmonization project forward.

Over the next twenty-four months continued cooperation between manufacturers, regulatory and research agencies, and industry representatives in both the United States and Europe will be critical. The European Union is scheduled to complete its review of active substances by July 2003. Of the 600 substances that are set to expire in 2003, the EU has only been notified on about 240. Those for which a notification will not be received will no longer be approved for use in the EU member states after July 2003. Fortunately, for the most part, the US and EU hop industries international harmonization priorities are on the notification list. However, this means it is incumbent upon industry representatives to ensure that all required data is supplied to the rapporteurs. Because of existing registrations and research projects, this burden primarily falls on US and German officials and representatives. Otherwise, even though the substance might be approved for use, hops may not be included among the crops for which an EU residue level is established.

For this reason, an aggressive twenty-four month program is needed to coordinate the research and regulatory efforts of US, German and UK industries, agencies in those governments, manufacturers of all the substances under review, and the end users-brewers that manufacture and sell their beer in the US and Europe. This effort could be even more effective if additional European hop producers could be formally represented. This campaign's objective will be to ensure that hops are included in the reviews being conducted by the European Commission, and that the Commission has a single contact it can reach whenever additional data is required or there are questions that need to be answered.

In addition, as developing countries begin the come into compliance with WTO standards, they are increasingly deferring to Codex standards. A few years ago this would not have been so commercially important. Codex was a sleepy organization in Rome. The signing of the World Trade Organization agreement and the rising influence of developing countries has changed that. Regulatory agencies in many countries are now deferring to Codex standards, and regulatory agencies in all countries, including the US have an obligation to take Codex standards in account when setting their own maximum residue levels.

The hop industry has been rather passive when it has come to securing Codex standards. If a chemical were already on the list for review, the industry has encouraged the manufacturer to provide data for the inclusion of hops. This has been done successfully with a number of chemicals. However, we have not worked to shape the agenda so that chemicals we use were

reviewed, nor have we worked to coordinate the priorities of the Codex delegations from our respective countries. If we did that, we could be a very influential force. While a passive approach might have made sense in the 1990's, it probably is not an acceptable commercial policy in this decade.

Over the next twenty-four months, not only should we launch a coordinated effort to secure the necessary EU tolerances, but also we should implement a strategy to secure the tolerances we need within the Codex structure. That would be an important first step in establishing the international regulatory framework we will need throughout the decade.

Finally, we need to shift our focus away from solving individual problems, and aggressively work to develop a regulatory environment that minimizes the need to address every registration/residue inconsistency between trading partners. The EU-2003 review process heightens the need for such a regulatory framework. A critical part of this twenty-four month campaign that I am proposing is the negotiation of a US-EU mutual recognition agreement that will facilitate the acceptance of a chemical residue when a product is registered for use on hops in the exporting country but not the importing one.

Our emphasis in recent years has been on preventing future problems. Our success has proven this a prudent approach. However, just as we recognized seven years ago that our efforts were insufficient and that we needed to increase our coordination, so it is today. What we have done has worked well, but the next decade presents new challenges, and we need a new approach to address the issues we will confront.

The U.S. Hop Industry Plant Protection Committee has coordinated the current international harmonization activities for the benefit of the world's hop and brewing industries. However, we are only able to formally represent the U.S. industry in our contacts with EU and Codex. To address the challenges of the next 24 months, it will be necessary for other hop producing countries to also be aggressively represented in these deliberations.

We understand the difficultly for countries that do not have the resources or personnel to become actively involved in these issues. However, during the past several years, the firm of Bryant Christie Inc. has shown its ability to effectively intervene in these regulatory proceedings. They understand the regulatory systems and have the necessary contacts within governments, chemical companies, and the hop industry to undertake this aggressive 24-month campaign within EU and Codex. Therefore, the USHIPPC invites members of the International Hop Growers Congress to formally join us in this campaign, and endorse Bryant Christie Inc. to represent of the needs of all IHGC member countries in the development of an international regulatory framework that will eliminate pesticide-related barriers in the trade of hops.

Attachment: Current list of international harmonization priorities.

International Harmonization Priorities (U.S., U.K. & Germany)

* U.S. residue study underway or complete
# U.S. efficacy study only

Downy Mildew

Dimethomorph (Acrobat)*
Cymoxanil (Curzate)*
Famoxate (Tanos)*
Folpet*
Azoxystrobin (Abound/Quadris)# – see Footnote #1
Tolylfluanid (U.S. import tolerance only)
Dithianon (U.S. import tolerance only)

Powdery Mildew

Trifloxystrobin (Flint)*
Myclobutanil (Rally/Systhane)*
Quinoxyfen (Quintec/Fortress)*
Spiroxamine (Impulse/Neon)*
Tebuconazole (Folicur)*

Insecticides

Pymetrozine (Fulfill/Plenum)*
Imidacloprid (Provado/Admire/Confidor)*
Spinosad - *tentative*

Miticides

Hexythiazox (Savey/Ordovol)*
Cyhexatin/Pennstyl* (German import tolerance only)
Abamectin (Agri-Mek, Vertimec)*
Amitraz (U.S. import tolerance only)
Fenpyroximate (Kiron)*

Herbicides/Dessicants

Carfentrazone-ethyl (Aim)* - *tentative (to be considered for addition to the list after efficacy studies in Germany and UK are evaluated).*

Footnotes:
#1 – Azoxystrobin: Zeneca will not allow registration of this product on U.S. hops, due to potential for phytotoxicity to MacIntosh-type apples (concerns over potential drift). However, they intend to seek a U.S. import tolerance for hops.

THE IR-4 PROJECT - PROVIDING PEST MANAGEMENT SOLUTIONS TO GROWERS OF HOPS, FRUITS, VEGETABLES AND OTHER MINOR CROPS.

D. L. Kunkel and R. E. Holm
Interregional Project Number 4 (IR-4), Cook College, Rutgers University, New Brunswick, New Jersey, 08902-3390, USA

ABSTRACT

In 1962, the State Agricultural Experiment Station Directors recognized the needs of growers and requested the U.S. Department of Agriculture's (USDA) Cooperative State Research Service to initiate an interregional research project to coordinate the agricultural community's efforts and assist growers in obtaining registrations of agricultural pesticides for their minor use needs. The Interregional Research Project Number 4 (IR-4) was established in 1963. The IR-4 Minor Use Program has grown in scope since its beginning. In 1975, Regional Leader Laboratories were established at SAES to provide regional coordination and analytical services. In 1976, the USDA-ARS established a minor use program to provide further support for IR-4. The objectives of IR-4 were expanded in 1977 to include registration of pesticides needed for the protection of crops from nursery and floral crops to forestry seedlings and turfgrass. The program was further expanded in 1982 to include the registration of biological pest control agents such as microbials and biochemicals.

IR-4 has supported more than 5,500 food use clearances since 1963. In 2000 alone, IR-4 data help to establish one hundred and sixty two tolerances (Maximum Residue Limits) and these MRLs should support a total of 450 new minor uses being added to pesticide labels. These clearances comprise over 40% of the total granted by EPA. Many of the new approvals are a direct result of the partnerships that IR-4 has built with the U.S. Environmental Protection Agency as well as with those companies that produce pest control products. In recent years IR-4 has expanded its partnerships across its U.S. boarders to Canada and even with Germany to assist in resolving various trade irritants and to pursue global registration for minor crops.

THE IR-4 MISSION

The IR-4 Mission is to provide pest management solutions to growers of fruits, vegetables and other minor crops. People who benefit from IR-4 are minor crop growers, food processors and consumers.

IR-4 develops data for submission to EPA to support registrations of new crop protection chemicals on minor food and ornamental crops and assists in the maintenance of existing product registrations. IR-4 provides help in the development and registration of biopesticides and expedites new pest control technologies for minor crops.

ORGANIZATION

An IR-4 organization has developed to accomplish this mission. The organization involves coordination of both state and federal components. The national headquarters coordinates the U.S. and International programs and is located at Rutgers University/Cook College in New Jersey. The Executive Director and Study Directors are located here. Four regional offices are located at the University of California at Davis, Michigan State University, University of Florida, and Cornell University at the Experiment Station in Geneva, New York. Each region has a

Regional Director, a Leader Laboratory Coordinator, and a Field Coordinator. A Quality Assurance Office is located in each region and at IR-4 headquarters. The Project Management Committee provides strategic focus and policies to the program and is comprised of the four Regional Directors, ARS and CSREES Representatives, the Commodity Liaison Committee Chair, the Administrative Advisory Committee Chair and IR-4's Executive Director and Executive Secretary. The Administrative Advisory Committee provides liaison between State Agricultural Experiment Stations, the USDA, and IR-4 and is comprised of one representative from each region, CSREES and ARS. The Commodity Liaison Committee is a stakeholder organizational link between IR-4 and minor food and ornamental crop growers and provides guidance and advice on how the program can best serve minor crop producers.

The four regional offices of IR-4 and ARS oversee approximately 27 field research centers located throughout the U.S. Each field research center employs one or two individuals whose sole responsibility is to conduct IR-4 residue trials. This has allowed these individuals to focus their full attention on conducting the trials and completing the field notebooks. Each field research center conducts from 15 to 40 field trials. Residue analysis is conducted at the four regional leader laboratories, three ARS laboratories, as well as several satellite laboratories and a number of private and chemical company laboratories. All of the laboratories conduct their work for IR-4 using Good Laboratory Practices.

HOW IR-4 OPERATES

IR-4 is a grass roots organization. Pest management needs are identified and requested of IR-4 by individual growers, grower organizations, nurserymen, agricultural scientists and extension personnel. In addition, there is a network of state and federal IR-4 liaison representatives throughout the U.S. and its Territories.

Pest control needs are made known to IR-4 in a number of ways. Requests can be submitted through the regional offices, through the IR-4 liaison at state land-grant universities and more recently through the IR-4 web site: http://www.cook.rutgers.edu/~ir4. Requests are submitted in the form of a Project Clearance Request (PCR). Once a PCR is submitted, it is shared with the regional offices and sent to the pesticide registrant. The registrant must give IR-4 approval to proceed and be willing to register the use before IR-4 will consider a project. IR-4 limits its scope to testing for effectiveness against the target pest, crop safety (phytotoxicity), and magnitude of residue studies on food crops. Therefore, IR-4 must check with the registrant to ensure that all the necessary core data requirements, such as chemistry, toxicology and environmental fate, have been completed and accepted by EPA.

IR-4 sponsors a Food Use Workshop, in September, to review and prioritize all of the approved requests. State and Federal minor-crop pest control experts, growers, commodity organizations and representatives from EPA and industry attend the workshop. Industry representatives are provided time prior to each session to make presentations on new chemistry. Priorities are set for the upcoming year's research based on the importance of the pest problem, the availability of alternatives, the existence of data gaps, and the value to integrated pest management programs. Only high priority projects (priority A and B) are slated for research. Following the Food Use workshop, the Regional Field Coordinators seek additional advice from minor-crop pest control experts, growers, and commodity organizations regarding specific needs of their region. Planning for the upcoming year takes place at a National Planning meeting October. At this meeting, field trials are assigned to specific Field Research Directors by the Regional Field Coordinators and ARS leadership. The location and number of research trials is predetermined by EPA, and relates to the major growing regions of a crop. Laboratory analyses are assigned to specific laboratories. In recent years, the average workload has been 130 residue studies with over 600 field trials (over 700 field trials in 2000). In addition, approximately 35 biopesticide projects, and 600 ornamental field trials are conducted each year.

All of the data generated during the field and laboratory phases of the research are sent to IR-4 Headquarters. The data are reviewed by scientists and written in final format for submission to EPA. For food crops, the final format is a petition that requests either the establishment of a tolerance (MRL) or an exemption from the tolerance requirement.

EPA carefully reviews the petitions and data packages. When EPA approves a petition, a Notice, followed by a Final Rule is published n the Federal Register. Registration follows after the registrant requests EPA's approval of the specific directions for use, which will appear on the label. The product may be made available for national use, be confined to a limited geographical region, or be identified for a Special Local Need (24c) in a specific state or states.

FOOD QUALITY PROTECTION ACT

The 1996 Food Quality Protection Act (FQPA) has posed federal legislative challenges for all crops by establishing new health based standards for crop protection chemicals in food. The FQPA requires that all of the nearly 10,000 chemical tolerances in effect in 1996 be reassessed over a 10-year period. The ultimate impact of the FQPA on existing crop protection tools for minor crop growers is uncertain, but minor crops will be impacted by this act.

STRATEGIC PLAN

In the last decade, the agrochemical industry has developed a range of new, safer products. These newer crop protection tools are much more selective against target pests, exhibit low human toxicity and have minimal impact on the environment. The EPA recognized this trend and created a classification of Reduced Risk for compounds that meet strict criteria. IR-4 recognized these trends, and in its 1995 strategic plan focused on the new, Reduced Risk chemistries. When FQPA was enacted in August 1996, IR-4's strategy was already being implemented and in the subsequent four years IR-4 has increased the number of these projects to over 75% of IR-4's 2000 program focused on Reduced Risk active ingredients.

BIOPESTICIDE RESEARCH

IR-4 supported the registration of the Bt products in the early 1970's which is used today by many organic growers and other producers. In 1982, IR-4 started a dedicated biopesticide program. The program was expanded in 1994 to provide competitive grant funds to land-grant university and government researchers to support studies on early stage development biopesticides for minor crop uses. Beginning in 1999, funding was also provided to develop efficacy and performance data on biopesticides currently being commercialized to speed these newer technologies to minor crop agriculture. These newer products can compete with traditional chemistries as part of total integrated pest management programs and will provide resistance management alternatives. Organic growers will benefit from these new biopesticides. Organic agriculture contributed $4 billion to the agricultural economy in 1999 and sales are growing at a rate of 20% per year. In 1999, IR-4 received 58 biopesticide clearances for food crops. IR-4 was recently added as an Ad Hoc member of Biopesticide Industry Alliance (new trade association).

EPA PARTNERSHIP

Both the EPA and IR-4 recognized the potential serious impacts FQPA could have on minor crop growers and acted in 1998 to strengthen and expand its current working relationships through the Minor Use Team to optimize IR-4's Reduced Risk chemical strategy. The result has been the formation of the EPA/IR-4 Technical Working Group, which meets quarterly to develop procedures to facilitate minor crop registrations. The initiatives have resulted in IR-4 submitting a three-year work plan enabling more efficient petition scheduling and bundling of product submissions for risk assessments. The Agency and IR-4 continue making extensive use of residue data by using crop groupings which allows for representative minor crops in various crop and sub-crop groupings to be tested for residues and a tolerance established on

the entire crop group or sub-group. For a limited number of Reduced Risk compounds, EPA has accepted arguments from IR-4 to extend uses by means of surrogate data to other crops. This has allowed the new Reduced Risk products to be registered more rapidly for use on many more minor and ultra minor crops without generating residue data.

CURRENT FUNDING AND CAPABILITIES

IR-4 is fortunate to have financial support from several sources to fund the minor use program. These include direct funding by Congress through USDA, from the land-grant university system, commercial registrants and commodity groups and from in-kind support from the land-grant university system and federal research institutions. In 2000, direct funding from USDA-CSREES was 8.99 million. The minor use program in USDA-ARS is presently funded at $3.1 million and additional Federal Hatch funding of around $0.5 million brings the total federal budget support to around $12.6 million. Funding support from private sector sources varies year by year, but has averaged between $0.5 and $0.8 million in recent years.

HOP RESEARCH

IR-4 conducts a number of studies with Hops each year. Registrations are expected for several new pest control products in the near future. New fungicide registrations expected in the next year include: cymoxanil, dimethomorph, fosetyl-Al, myclobutanil, Quinoxyfen, and tebuconazole. Other new products include the insecticide pyridaben and the herbicide clopyralid. Although submissions have been made for pirimicarb and folpet on hops, it is not known at this time if they will be registered in 2002 or not. Many of these new hop uses are important to the U. S. Hop growers and are also important in resolving various trade irritants.

INTERNATIONAL COOPERATION

IR-4 has worked cooperatively with Canada and Germany in the development and exchange of food crop residue and efficacy data over the last few years. Residue field trials have been conducted in Canada and overseen by IR-4 as a regular part of one of IR-4's studies. These trials have been used to supplement and to replace U.S trials of similar growing regions. Data packages developed for U.S. production have been provided to Pest Management Regulatory Agency (PMRA) of Canada in support of tolerances for production in Canada or for import tolerances of U.S. agricultural products. Canadian and Mexican representatives have attended the IR-4 Food Use Workshop. The German BBA and IR-4 have shared lists of projects to try to identify areas of common interest. The BBA conducts many efficacy trials and IR-4 conducts many residue trials. Sharing data on similar uses has provided each group with information that extends the understanding even though they may not be directly applicable. A cooperative residue project was initiated in 1999 with the German State Office of Plant Production and Plant Protection to study residues of dimethomorph on melons. The cooperation will continue to grow between IR-4 and Germany. The number of cooperative projects has increased considerably since 1999 with projects on quinoxyfen and pymetrazine. German scientist have also attended the IR-4 Food Use Workshops. Continuous interaction will be necessary to ensure that global registrations can be realized.

SENSITIVITY OF HOP DOWNY MILDEW FROM WASHINGTON, IDAHO, AND OREGON TO FOSETYL-AL (ALIETTE)

M. E. Nelson, J. D. Barbour, C. M. Ocamb, R. Klein and K. C. Eastwell

First and fifth authors: Washington State University, Irrigated Agriculture Research and Extension Center, Prosser, WA, USA. Second Author: University of Idaho, Parma, ID, USA. Third author: Oregon State University, Corvallis, OR, USA. Fourth Author: California Pistachio Commission-Fresno, CA, USA.

Abstract:

There have been recent failures of fosetyl-Al (Aliette) to control hop (*Humulus lupulus*) downy mildew in Idaho and Oregon. To determine if resistance of the pathogen (*Pseudoperonospora humuli*) to the fungicide has occurred, leaf disk assays for sporulation were performed with serial dilutions of Aliette. Dose response curves of transformed data were linear. The Aliette concentration effective against 50% of the *P. humuli* population (ED_{50}) from each State was estimated from the linearized data. The ED_{50} values indicate that Aliette was approximately one-half as effective against *P. humuli* isolates from northern Idaho and Oregon compared to isolates that had never been exposed to the fungicide.

Key words: fosetyl-Al, Aliette, hop, *Humulus lupulus*, downy mildew, *Pseudoperonospora humuli*

Introduction:

Downy mildew of hop, caused by *Pseudoperonospora humuli* (Miyabe & Takah.) Wilson, is a serious disease of hop in the Pacific Northwest region of the United States. The fungus overwinters in the infected perennial hop rootstock from which systemically infected shoots (primary basal spikes) emerge in the spring (Skotland, 1961; Romanko *et al.*, 1964). Zoosporangia, which develop on these spikes, serve as the primary inoculum. Foliar lesions can occur in the spring, but soon dry and are of minor importance to the epidemic (Skotland & Romanko, 1964). However, secondary spikes develop when growing tips become infected, and these perpetuate the disease through the growing season (Skotland & Romanko, 1964). Significant yield losses can occur when cones or cone bearing sidearms (branches) become infected. In addition, crown rot and subsequent plant death can be a consequence of infection of highly susceptible cultivars (Skotland & Romanko, 1964; Skotland & Johnson, 1983).

Control of hop downy mildew has relied on sanitation, copper sprays, and other fungicides (Hunger & Horner, 1982; Skotland & Johnson, 1983; Klein, 1994). In the early 1980's, a single application of metalaxyl provided season long control of hop downy mildew (Hunger & Horner, 1982). By the early 1990's, strains of *P. humuli* resistant to metalaxyl had developed in Oregon and northern Idaho (Klein, 1994). Since that time, control of this pathogen has primarily relied on fosetyl-Al (Aliette). In recent years, growers have observed that much higher rates of fosetyl-Al are required for satisfactory control of hop downy mildew.

The objective of this study is to determine if populations of *P. humuli* have developed resistance or tolerance to fosetyl-Al. In leaf disk assays with fosetyl-Al, diminished sensitivity of *P. humuli* populations previously exposed to the fungicide relative to unexposed populations would suggest the development of field tolerance to this fungicide. Leaf disk assays have been shown to be reliable indicators of the sensitivity of isolates of other fungi to fungicides (Sozzi & Staub, 1987). Furthermore, leaf disk assays were previously utilized to detect strains of *P. humuli* resistant to metalaxyl (Klein, 1994).

Materials and Methods:

Stock suspensions of fosetyl-Al (pH = 6.5) were prepared in double-distilled H_2O (ddH_2O), added to molten 1% Bacto-agar (Difco Laboratories, Detroit, MI) that had been cooled to 45C, and the resulting suspension poured into 60 mm petri dishes. Fosetyl-Al concentrations of 0, 200, 400, 800, and 1600 ppm active ingredient were added as Aliette 80 % WDG (Aventis, Research Triangle Park, NC). One day prior to inoculation, leaf disks 1 cm in diameter were cut from fully expanded hop leaves (cv. Symphony) with a No. 6 cork borer, and disks were placed in each dish with the abaxial surface up. Plates containing leaf disks were placed under fluorescent lights in an incubator (18C; 16-hr photoperiod).

Hop shoots systemically infected with downy mildew (spikes) were collected from commercial yards in Oregon and Idaho experiencing control problems, and from a research yard at Washington State University-Prosser that has never been exposed to fosetyl-Al. To induce sporulation, spikes were sprayed with ddH_2O, enclosed in a plastic bag with stems in a beaker of ddH_2O, and incubated overnight at room temperature (approximately 20C) in the dark. Zoospores were harvested by shaking each spike in 15-20 ml ddH_2O and straining through several layers of cheesecloth.

Leaf disks were inoculated by pipetting 12 µl of the zoosporangial suspension onto each of three spots on each disk. Zoosporangia from each spike were inoculated onto three sets of seven leaf disks for each fosetyl-Al concentration. There were a total of 63 inoculation sites for each fungicide concentration and the non-treated control. Inoculated leaf disks were returned to the incubator (18C; 16 hr photoperiod). After 16-24 hr incubation, the remaining inoculum suspension was removed from the inoculation sites by aspiration. After five to seven days of additional incubation, leaf disks were examined and the number of inoculation sites with sporulating *P. humuli* colonies were counted. Five and 15 spikes from Washington and Idaho, respectively, were evaluated in 2000. Fifteen spikes from both Washington and Oregon were evaluated in 1999.

Data were analyzed using Probit analysis (Finney, 1971) to determine if differences existed in the effectiveness of fosetyl-Al against *P. humuli* collected from the different growing regions. In this analysis, dose-response curves were linearized by plotting the log of the dose against Probit values and the linearity of the transformed data was statistically confirmed or rejected. The data were then analyzed to determine if the transformed dose response curves from the three growing regions were parallel (the same slope with different intercepts). The dose effective against 50% of the *P. humuli* population (ED_{50}) in each of the States was estimated from the transformed data.

Results:

Analysis indicated that the transformed data were linear, and observed (actual data) values were very close to those expected (theoretical data). Dose response curves from each of the States over the two-year period were parallel (had the similar slopes), with the intercepts for Washington differing from those for Oregon and Idaho (Figure 1). The data were analyzed using the common slope in order to estimate the ED_{50} values for fosetyl-Al from each state (Table 1). ED_{50} values from Oregon (176.4) and northern Idaho (149.4) were about double those from Washington (81.8 in 1999 and 63.1 in 2000). Equations describing curves from the different States are presented in Table 1.

Figure 1. Dose response lines for hop downy mildew isolates from WA, OR, and northern ID tested against fosetyl-Al in 1999 and 2000.

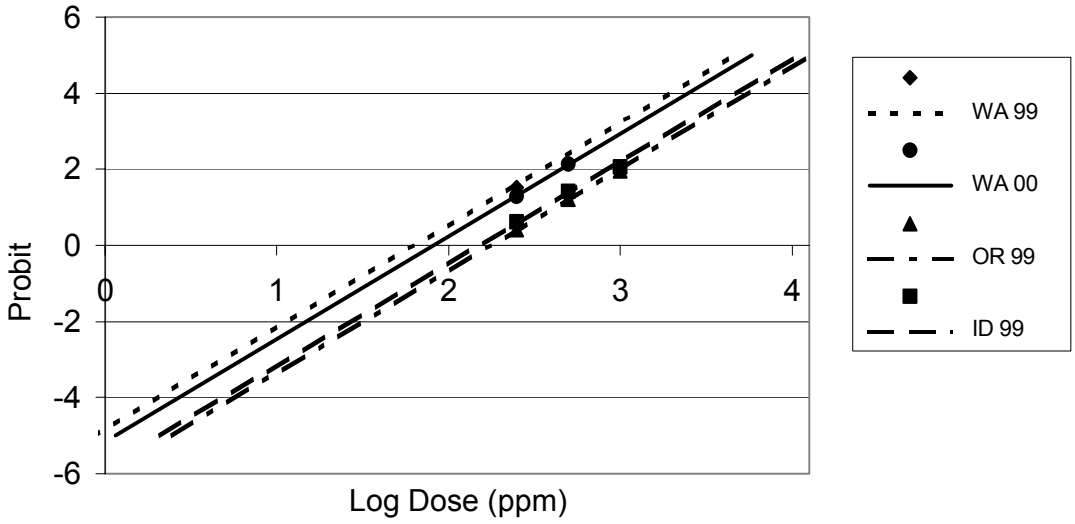


Table 1. ED_{50} estimates for hop powdery mildew isolates from WA, OR, and northern ID tested against fosetyl-Al in 1999 and 2000. For equations, Y=probit value; X=log of dose in ppm.

State	Year of Analysis	ED_{50} Estimate (ppm)	Dose Response Equation
WA	2000	63.1	Y=-4.856 + 2.698X
WA	1999	81.8	Y=-5.161 + 2.698X
OR	1999	176.4	Y=-6.061 + 2.698X
ID	2000	149.4	Y=-5.866 + 2.698X

Discussion:

Sozzi and Staub (1987) demonstrated that assays utilizing potato leaf disks, detached leaves or whole plants are reliable to monitor phenylamide sensitivity, are useful predictors of fungicide resistance in the field, and that differences in fitness of resistant and susceptible isolates are unlikely to influence test results. Hop leaf disk assays have also been effective in previous fungicide sensitivity evaluations of *P. humuli* populations (Klein, 1994). In the present case, leaf disk assays revealed *P. humuli* population differences regarding tolerance to fosetyl-Al. Development of *P. humuli* populations tolerant to fosetyl-Al in Oregon and northern Idaho is indicated by ED_{50} values approximately double those observed for isolates from Washington.

Selection pressure for fungicide-tolerant strains of *P. humuli* in Oregon and northern Idaho is probably due to the exclusive use of a single fungicide combined with environmental conditions conducive to disease development. Resistance of *P. humuli* to metalaxyl has been reported in Oregon and northern Idaho (Klein, 1994) as well as Germany (Hellwig *et al.*, 1991). In the Yakima Valley of Washington, conditions conducive to downy mildew are less frequent, with severe epidemics occurring only in 9 of 28 years (Johnson *et al.*, 1983). Over time, this should result in fewer life cycles and less opportunity for the fungi to develop tolerance. Consequently,

fungicide resistant populations of *P. humuli* appear to emerge less rapidly in the Yakima Valley (Klein, 1994).

No data exist regarding sensitivity of *P. humuli* populations to fosetyl-Al prior to commercial use of the fungicide. However, isolates from hop yards in Oregon and northern Idaho where fosetyl-Al fails to adequately control downy mildew have been compared to isolates from a research yard in Washington that has not been exposed to the fungicide. This evaluation has provided the best opportunity available to investigate the loss of disease control by fosetyl-Al in some hop yards. Data obtained in this study indicate that *P. humuli* has acquired tolerance to fosetyl-Al after several years of reliance on this single chemical for control.

Acknowledgements:

This research was supported in part by grants from the Hop Research Council, Washington Hop Commission, Idaho Hop Commission, Oregon Hop Commission, Busch Agricultural Resources, Inc., and Miller Brewing Company.

Literature Cited:

Finney, D. J. 1971. Probit Analysis, 3rd Ed. Cambridge University Press, Cambridge, 333 pp.

Hunger, R. M. and Horner, C. E. 1982. Control of hop downy mildew with systemic fungicides. Plant Disease. 66:1157-1159.

Hellwig, K., Kremheller, H. T. and Agerer, R. 1991. Untersuchen zur resistenz von *Pseudoperonospora humuli* (Miy. & Tak.) Wilson gegenuber Metalaxyl. Gesunde Pflanz. 43:400-404.

Johnson, D. A., Skotland, C. B. and Alldredge, J. R. 1983. Weather factors affecting downy mildew epidemics of hops in the Yakima Valley of Washington. Phytopathology 73:490-493.

Klein, R. E. 1994. Occurrence and incidence of metalaxyl resistance in *Pseudoperonospora humuli* in the Pacific Northwest. Plant Disease. 78:161-163.

Romanko, R. R., Ogawa, J. M., Skotland, C. B., Horner, C. E., and Brooks, S. N. 1964. Hop Downy mildew: A symposium. Modern. Brewery. Age 66:45-52.

Skotland, C. B. 1961. Infection of hop crowns and roots by *Pseudoperonospora humuli* and its relation to crown and root rot and overwintering of the pathogen. Phytopathology 51:241-244.

Skotland, C. B., and Johnson, D. A. 1983. Control of downy mildew of hops. Plant Disease. 67:1183-1185.

Skotland, C. B. and Romanko, R. R. 1964. Life history of the hop downy mildew fungus. Wash. Agric. Exp. Station. Circ. 433.

Sozzi, D., and Staub, T. 1987. Accuracy of methods to monitor sensitivity of *Phytophthora infestans* to phenylamide fungicides. Plant Disease. 71:422-425.

TOWARDS THE REGENERATION AND TRANSFORMATION OF HOP

Christoph Horlemann and Gerd Weber

Department of Plant Breeding and Biotechnology,
Institute of Plant Breeding, Seed Science, and Population Genetics,
University of Hohenheim, D-70599 Stuttgart, Germany,
Tel.: +49-711-4592341, Fax: +49-711-4592343,
horleman@uni-hohenheim.de, weberg@uni-hohenheim.de

Abstract

Here we present data aimed at efficiently regenerating hop varieties like “Tettnanger” and “Hallertauer mittelfrüh”. Protocols for regeneration of different hop genotypes in tissue culture were optimised for several culture conditions as well as media formulations. Using optimised conditions, we obtained more than 50% regenerated plants from shoot internodes. At least from each of those one shoot was formed.

Furthermore, transformation by *Agrobacterium tumefaciens* of shoot internodes with a reporter gene (GUS) was performed. After consecutive selection cycles, stable expression of transgenes was verified in at least 40% of all calli.

Introduction

Hop is damaged by several pathogens. It would be interesting to generate hop varieties with increased resistance without changing its quality characteristics. One approach would be transformation of hop with gene constructs known to enhance resistance against pathogens.

The objective of our work is the transformation of the hop variety “Tettnanger” without changing its quality characteristics in order to provide it with a higher degree of resistance against the fungus *Pseudoperonospora humuli* (Miy. et Tak.) Wilson. An important milestone towards transformation of hop was to establish a procedure for efficient regeneration of several hop varieties. Few papers have addressed conditions for regenerating hop in tissue culture (e.g. Motegi 1979, Heale et al. 1989, Rakousky and Matousek 1994, Batista et al. 1996, Gurriarán et al. 1999, Becker 2000). Furthermore, no experimental procedures for transferring DNA into the hop varieties “Tettnanger” and “Hallertauer mittelfrüh” have been developed so far.

Regeneration

Starting tissue for regeneration of hop

We investigated the following explants from *in vitro* cultures for their ability to regenerate: shoot internodes, section of leaves, and petiols (Tab. 1). Callus growth was observed regardless of the source of tissue. The most prominent growth was observed when shoot internodes were cultured. To a lesser degree petiols grew callus. No regeneration was observed when sections of leaves were used.

In a second set of experiments, the regeneration of shoot internodes from the greenhouse without an intervening tissue culture step was investigated. Shoot internodes from the greenhouse were more vital than those from tissue culture. But in contrast to tissue culture grown shoot internodes, they have not regenerated.

Media and culture conditions for regeneration of hop

Several different basal media compositions were tested (e. g. Adams 1975, Eppler 1984, Becker 2000). No major differences were observed. The most suitable medium formulation was based on a Murashige and Skoog basal media combined with Gamborg's B5 vitamins. As carbon source only glucose in concentrations from 2 to 3% was used. Others reported that sucrose inhibited the growth of hop tissue culture (Adams 1975, Eppler 1984). Suitable conditions were found to be 2% to 3% glucose.

The most important factor for a medium promoting regeneration is its composition of growth regulators. During normal *in vitro* propagation of explants a spontaneous regeneration frequency of approximately 0.055 % was observed. Known phytohormone compositions as well as several modifications thereof were investigated. The most suitable combination for "Tettnanger" was found to be a medium supplemented with thidiazuron (2 mg/l) and indole-3-acetic acid (0.25 mg/l).

Using this medium, at least one shoot regenerated from 50% of all internodes. Often more than one shoot regenerated from those explants.

Transformation

A dose response of the antibiotics kanamycin and cefotaxim was performed prior to transformation. (Fig. 1). The optimal concentration of kanamycin suppressing the growth of the genotype "Tettnanger" was determined to be 25 mg/l. At this concentration only 8% growth compared to the controls was recorded (Fig. 1). To suppress the growth of *Agrobacterium tumefaciens* the antibiotic cefotaxim was used. The most favourable concentration of cefotaxim was determined to be 500 mg/l. At this concentration a growth of 112 % compared to the control plants (100%) was observed. This surprising finding has been described (Mathias and Boyd 1986, Lin et al. 1995).

Agrobacterium tumefaciens was used for transformation of hop. A plasmid construct carrying genes for kanamycin resistance and a GUS reporter was used for the first set of experiments. Several transformation experiments were performed. The results of one of those experiments are shown in table 2. After consecutive selection cycles covering a period of at least 30 days 40% of all growing calli expressed the transgene.

Outlook

We are using this efficient regenerating process and transformation protocol to introduce genes for fungal resistance into hop varieties.

Acknowledgements

This work was funded by "Ministerium Ländlicher Raum, Baden-Württemberg" and "Hopfenpflanzerverband Tettnang e.V." whose supports are gratefully acknowledged. We would like to thank Molecular Plant & Protein Biotechnology Cologne GmbH, Cologne for making several *Agrobacterium tumefaciens* strains and gene constructs available and Azupharma GmbH, Gerlingen for donating cefotaxim.

References

Adams, A.N. 1975. Elimination of viruses from the hop (*Humulus lupulus*) by heat therapy and meristem culture. J: Hort. Sci. 50: 151-160.

Batista, D., Sousa, M.J: Pais, M.S. 1996. Plant regeneration from stem and petiole-derived of *Humulus lupuls* L. (hop) clone Brangança and var. Brewers's Gold. In Vitro Cell. Dev. Biol. – Plant. 32: 37-41.

Becker, J. 2000. Untersuchungen zur *in vitro* Regeneration und genetischen Transformation von Hopfen (*Humulus lupulus* L.). Ph. D. thesis. University of Kaiserslautern, Germany.
Eppler, A. 1984. Hl-16, ein Kulturmedium zur Ein-Schritt-Regeneration von Hopfen-Pflanzen aus Meristemkulturen. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft. 223: 173-174.
Gurriarán, M.J., Revilla, M.A., Tamés, R.S. 1999. Adventitious shoot regeneration in cultures of *Humulus lupulus* L. (hop) cvs. Brewers Gold and Nugget. Plant Cell Rep. 18: 1007-1011.
Heale, J.B., Legg, T., Grar, J., Fabb, A., Bainbridge, B. 1989. Application of plant tissue culture and molecular biology techniques to progressive wilt of hop caused by Terticillum albo-artrum. Proceedings of the European Brewery Convention Congress, Symposium on Plant Biotechnology, Helsinki. pp. 70-82.
Lin, J.J., Assad-Garcia, N. and Kuo, J. 1995. Plant hormone effect of antibiotics on the transformation effiency of plant tissues by *Agrobacterium tumefaciens.* Plant Science, 109: 171-177.
Mathias, R.J. and Boyd, L.A. 1986. Cefotaxime stimulates callus growth, embryogenesis and regneration in hexaploid breeding wheat (*Triticum aestivum* L. Em. Thell.). Plant Science, 46: 217-223.
Motegi, T. 1976. Induction of redifferentiated plants from hop leaf callus culture. Proc. Crop Sci. Soc. Jap. 45: 175-176.
Rakousky, S., Matousek, J. 1994. Direct organogenesis in hop: a prerequisite for an application of *A. tumefaciens*-mediated transformation. Biologia Plant. 36: 191-200.

Tables and Figures

Table 1:

Growth and regeneration from hop explants in tissue culture.

starting material	**shoot internodes**	**petiols**	**leaves**	**shoot internodes**
source	*in vitro*	*in vitro*	*in vitro*	greenhouse
callus growth	++	+	-	+++
regeneration	++	n.d.	n.d.	-

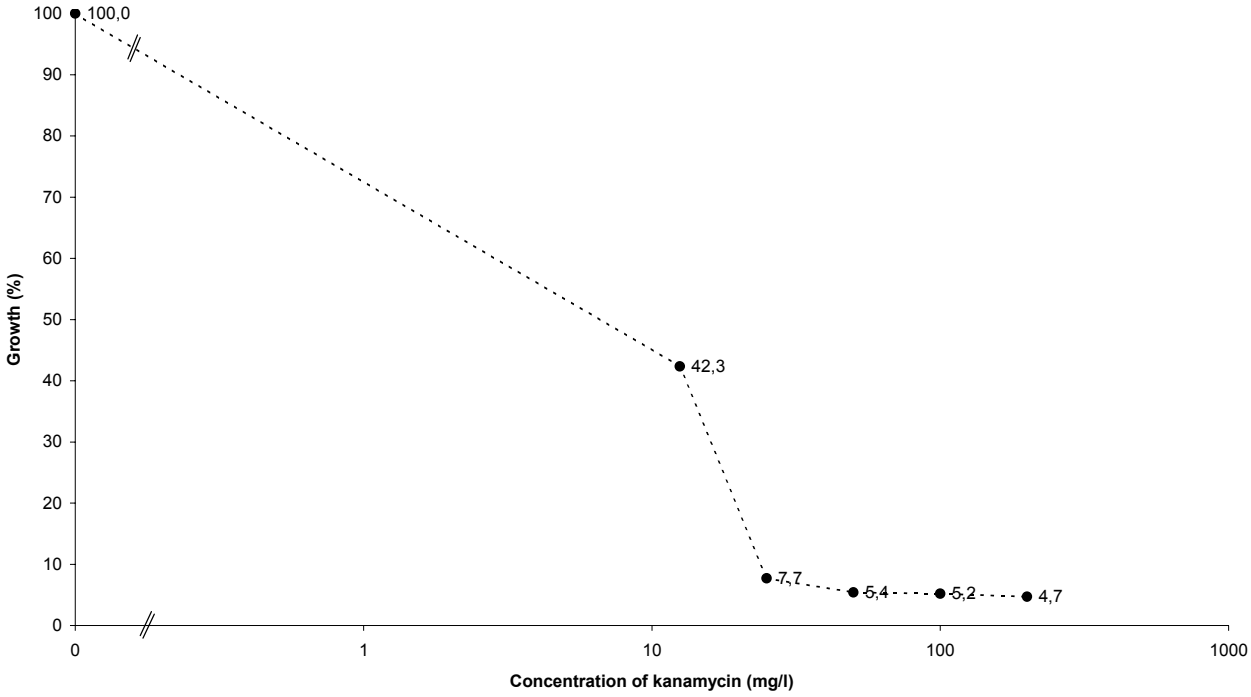
Table 2:

Transformation of hop with *Agrobacterium tumefaciens*. The expression of GUS was used to verify transgenic tissue after four weeks on kanamycin containing medium.

time	**number of explants**	**number of grown calli**	**number of transgenic calli**
day 0	250 (100%)	-	-
day 30	210 (84%)	99 (40 %)	70 (28 %)

Figure 1:

Growth of hop in tissue culture on kanamycin. The length of grown shoots and their number were recorded after 5 weeks of culture.



IMPROVING HOP (*Humulus lupulus* L) FOR RESISTANCE AGAINST FUNGI.

Batista, D., M.J. Sousa, M.J. and M.S. Pais.
Laboratory for Plant Biotechnology-ICAT, Ed. ICAT, Campo Grande, 1749-016 Lisboa, Portugal.

For centuries fungal diseases, in particular powdery mildew and verticillium wilt, have been the scourge of European hop producers. In the last years American hop production has also been seriously affected by these diseases. Cross-breeding new resistant varieties and application of fungicides does not constitute, until now, an efficient solution. Attempts to overcome this situation were made in our laboratory using a hop spontaneous clone and the hop variety Eroica. Cultures of both plants were transformed by particle bombardment using the selectable *hpt* (hygromicin) and *nptII* genes and the reporter *uidA* (GUS) gene. Selective agents are compared regarding their influence on plant regeneration and development. Regenerated plantlets were successfully analysed by PCR for the presence of these genes. The introduction of a β-1-3-glucanase encoding gene is now being performed. Our results open good perspectives towards the achievement of important improvements in hop breeding.

This work was supported by the Foundation for Science and Technology (Programme Praxis XXI-BD 3112 and BD 3110).

STUDY ON THE BIOSYNTHESIS GENES FOR RESIN AND ESSENTIAL OIL IN HOP

Y. Okada & K. Ito
Plant Bioengineering Research Laboratories, Sapporo Breweries Ltd., 37-1, Kizaki, Nitta, Gumma, 370-0393 Japan

Abstract

Two genes responsible for resin and essential oil biosynthesis were isolated. One is the valerophenone synthase (VPS) gene and the other is the farnesyl pyrophosphate synthase (FPS) gene. The translation product of the VPS gene had both activities of VPS, which catalyzes the biosynthesis of phlorisovalerophenone (precursor of resin), and chalcone synthase (CHS), which catalyzes the biosynthesis of naringenin-chalcone (precursor of flavonoid such as xanthohumol). Northern analysis showed that the VPS gene was specifically expressed in the lupulin gland. On the other hand, the translation product of the FPS gene catalyzed the biosynthesis of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP), which are the precursors of essential oils. Northern analysis revealed that the FPS gene was expressed in the lupulin gland, bract, leaf, and stem. It was expressed most strongly in the lupulin gland than in the others.

1 Introduction

Recently, Marker Assisted Selection (MAS) and transformation have been developed in many plant species. These new techniques are very useful for increasing the breeding efficiency. In order to use new breeding methods such as MAS or transformation for improvement in the content and quality of the resin and essential oil accumulated in the lupulin gland, identification of the genes responsible for their biosynthesis is important. However, the molecular studies of these compounds of hop have not been reported. In this study, we have tried to isolate the genes responsible for the biosynthesis of the resin and essential oil in the hop lupulin gland and analyze the function of these genes.

Firstly, we hypothesized that the genes responsible for the biosynthesis of the resin and essential oil should be specifically expressed in the lupulin gland, because these compounds specifically accumulate in the lupulin gland. As a result, we isolated the valerophenone synthase (VPS) gene, which is responsible for the biosynthesis of resin and prenylflavonoids, by screening of the genes specifically expressed in the lupulin gland.

Secondly, we tried to isolate the genes responsible for the biosynthesis of the resin and essential oil from hop based on the amino acid sequences of other plants' enzymes. As a result, we isolated the farnesyl pyrophosphate (FPS) gene, which is resposible for the biosynthesis of the essential oils.

2 Cloning and analysis of VPS gene

Based on our hypothesis, we prepared a lupulin fraction and a non-lupulin fraction. The lupulin fraction mainly consists of the lupulin glands and broken pieces of bracteole. The non-lupulin fraction mainly consists of bracts with the lupulin glands removed as much as possible. The big difference between these fractions is the content of the lupulin glands. Therefore, there is a high possibility that the genes obtained by subtracting the genes expressed in the non-lupulin fraction from those in the lupulin fraction are specifically expressed in the lupulin gland.

In order to isolate the cDNA clones specifically expressed in the lupulin gland, the cDNA library constructed from the lupulin fraction was screened using lupulin-specific probes prepared by the subtraction. About 1,000 plaques were screened and 3 positive plaques were obtained. We analyzed one clone, which had the longest insert among these 3 clones. The nucleotide sequence of this gene is registered on the DDBJ/EMBL/GenBank (Accession No. AB015430).

This gene has a high similarity with the chalcone synthase (CHS) (EC 2.3.1.74) gene, which is well known in many plants.
The northern analysis of the total RNA from the leaf, stem, the non-lupulin fraction, and the lupulin fraction using this chalcone synthase-like (CHSL) gene as a probe indicated that the CHSL gene seemed to be specifically expressed in the lupulin fraction. Furthermore, a much weaker signal at the same position as the signal of the lupulin fraction was detected in the non-lupulin fraction by an extended exposure of the X-ray firm. Although the slight presence of lupulin glands in the non-lupulin fraction may cause this signal, there is the possibility that the gene is also expressed in the bract and the bracteole more weakly than in the lupulin gland.

CHS is the key enzyme in the biosynthetic pathway of flavonoids, which contribute to flower pigments, protection against UV light, and phytoalexins against pathogens. Recently, Fung et al. reported that valerophenone synthase (VPS) catalyzed the synthesis of phlorisovalerophenone (PIVP), which is the precursor of resin (Fig.1) (Fung et al., 1997). These reactions resemble the reaction catalyzed by CHS. Paniego et al. purified VPS and analyzed the partial amino acid sequence (Paniego et al., 1999). The deduced amino acid sequence of the CHSL protein described in this report includes the partial amino acid sequence given by Paniego et al. On the other hand, one of the prenylflavonoids, xantho-humol, has received much attention from hop researchers for its anticancer activity. Prenylflavonoids including xanthohumol also accumulate in the lupulin gland, and they are presumed to be synthesized from naringenin-chalcone (Fig.1). However, Paniego et al. reported that CHS activity was not detected from the purified VPS in their experiment (Paniego et al., 1999). Therefore, we investigated whether the translation product of CHSL gene (CHSL protein) has both VPS activity and CHS activity.

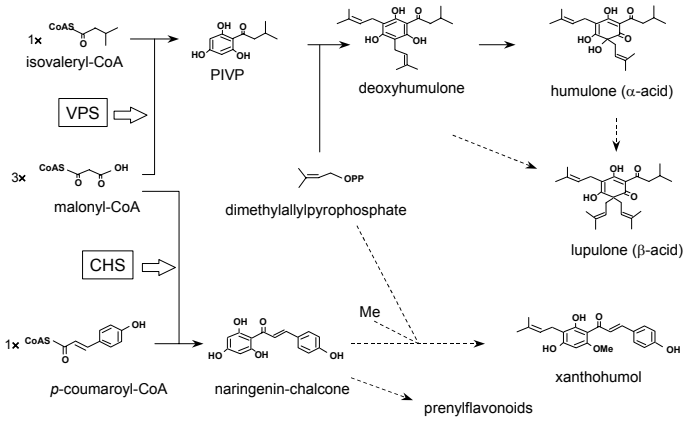


Fig.1 Presumed biosynthesis pathway of resin and xanthohumol

Table 1. VPS and CHS activities of CHSL protein

	isovaleryl-CoA (VPS activity)	*p*-coumaroyl-CoA (CHS activity)
specific activity (pkat/mg)	35.76	6.05

The CHSL protein was produced in *E. coli* and purified using the "QIAexpress System" (QIAGEN). [2-^{14}C]Malonyl-CoA and isovaleryl-CoA were used for the VPS assay, and [2-^{14}C]malonyl-CoA and p-coumaroyl-CoA were used for the CHS assay as the substrates, respectively. Table 1 shows the specific activities of the CHSL protein as VPS and CHS, respectively. This result indicates that the CHSL protein has both VPS and CHS activities. However, the specific activity as CHS (6.05 pkat/mg) was much lower than that as VPS (35.76 pkat/mg). It seems that the CHSL protein contributes to both the biosynthesis of the resin and prenylflavonoids including xanthohumol in the lupulin gland, but the major activity of the CHSL protein is the VPS activity. Therefore, we call this CHSL gene the VPS gene, and call CHSL protein the VPS, respectively. The resin content in the cone is much greater than that of the prenylflavonoids. This difference might be due to the difference between the VPS activity and CHS activity of VPS.
We also did southern analysis using the VPS gene as a probe. Four types of restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Xba*I) were selected for the southern analysis. Because there is no restriction site of these restriction enzymes on the gDNA of the VPS gene, the number of

signals derived from the cloned VPS gene in the southern analysis should be only one. The result of the high stringency analysis, in which two signals were detected, suggests the existence of two types of VPS genes. On the other hand, more than two signals were detected besides the signals derived from the VPS genes in the low stringency analysis. This suggests there are multiple CHSL genes besides the VPS gene in hop. Matousek et al. isolated the CHS gene which seems to be one of these genes (DDBJ/EMBL/GenBank: Accession No. AJ304877). We have also cloned two more CHSL genes different from both the VPS gene and the gene isolated by Matousek et al. (DDBJ/EMBL/GenBank: Accession No. AB061020, AB061022). We are interested in what function these genes have.

3 Cloning and analysis of FPS gene

Many of the essential oils belong to the terpenoids. Terpenoids function not only as essential oils but also as membrane constituents, photosynthetic pigments, electron transport carriers, growth substances, plant hormones and so on. Therefore, many of the biosynthetic pathways of terpenoids have been clarified by the studies of many plant species. Farnesyl pyrophosphate synthase (FPS) [EC 2.5.1.1, EC 2.5.1.10] catalyzes part of the biosynthetic pathway of terpenoids. It functions both as a dimethylallyl transferase (EC 2.5.1.1) forming geranyl pyrophosphate (GPP) from dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) and as a geranyltransferase (EC 2.5.1.10) forming farnesyl pyrophosphate (FPP) from GPP and IPP. In the hop cone, the major essential oils (myrcene, humulene caryophyllen, farnesen) seem to be synthesized from GPP or FPP (Fig. 2).

Upon the cloning of the hop FPS gene, the DNA primers were synthesized based on the conserved amino acid sequences of the other plants' FPS. A partial DNA fragment of the gene was cloned by PCR using these primers, and furthermore, the upstream and downstream regions of the DNA fragment were isolated by Inverse PCR and Cassette-mediated PCR. The gDNA sequence of the hop FPS gene is registered on the DDBJ/GenBank/ EMBL (Accession No. AB053486). The cDNA of the cloned gene was also isolated from the total RNA of the lupulin fraction by RT-PCR based on the sequence information of the gDNA (DDBJ/GenBank/ EMBL: Accession No. AB053487).

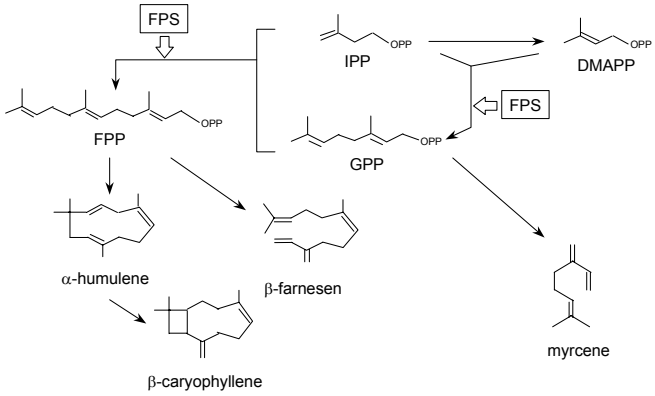


Fig.2 Presumed biosynthesis pathway of essential oil

Figure 3 shows the northern analysis of the FPS gene. Although the signals were detected in all tissue fractions, the FPS gene is strongly expressed in the lupulin gland, and this strong expression might cause the specific accumulation of essential oils in the lupulin gland. For the VPS gene, it is not expressed in the leaf or stem, while the FPS gene was expressed not only in the lupulin gland but also in the leaf and stem. This should be because FPS is a key enzyme of not only essential oil biosynthesis but also the biosynthesis of other terpenoids as membrane constituents, photosynthetic pigments, electron transport carriers, growth substances, plant hormones, and so on.

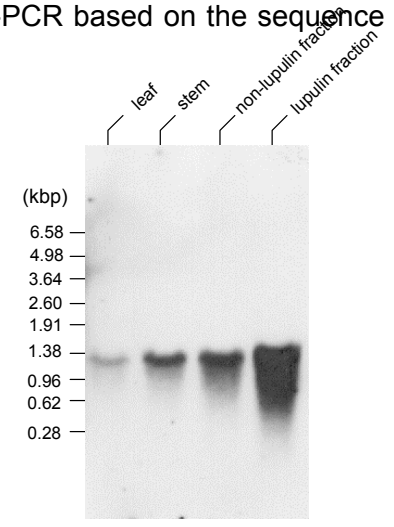


Fig.3 Northern analysis of FPS gene

The cloned cDNA of the hop FPS gene was expressed in *E. coli* and the translation product was purified using the "QIAexpress System" (QIAGEN). We confirmed the FPS

activity of the translation product in this experiment. On the other hand, we suspected that the FPS gene might also be responsible for the resin biosynthesis. It is reported that prenyltransferase transfers the dimethylallyl of DMAPP to PIVP to catalyze the biosynthesis reaction of the resin (Zuurbier et al. 1998). However, this enzyme has not yet been purified, and the amino acid sequence of this enzyme is unclear. The FPS also catalyzes the transfer reaction of DMAPP in the biosynthetic pathway of the essential oil. Therefore, we investigated whether the FPS has prenyltransferase activity. The translation products of the FPS gene were incubated with DMAPP and PIVP; however, the prenyltransferase activity was not detected in this experiment. We are currently planning further investigations to determine if the prenyltransferase in the resin biosynthesis really differs structurally from FPS.

4 Supplements

The details of the VPS gene are described in Biosci. Biotechnol. Biochem. 65, 150-155 (2001). The details of the CHS activity of the VPS have been submitted to J. Am. Soc. Brew. Chem., and the details of the FPS gene is in press on J. Plant Phys.

5 References

Fung,S.Y., Zuurbier,K.W.M., Paniego,N.B., Schffer,J.J.C., and Verpoorte,R., Enzymes from the biosynthesis of hop α- and β-acids. European Brewery Convention, Proceeding of The 26th Congress, 215-221 (1997).

Paniego,N.B., Zuurbier,K.W.M., Fung,S.Y., Heijiden,R., Scheffer,J.J.C., and Verpoorte,R., Phlorisovalerophenone synthase, a novel poluketide synthase from hop (*Humulus lupulus* L.) cones. Eur. J. Biochem., 262, 612-616 (1999).

Zuurbier K.W.M., Fung S.Y., Scheffer J.J.C., Verpoorte R., In-vitro prenylation of aromatic intermediates in the biosynthesis of bitter acids in *Humulus lupulus*. Phytochemistry, 49, 2315-2322 (1998).

IDENTIFICATION AND VARIABILITY OF HOP CHALCONE SYNTHASE GENES

J. Matoušek[a*] and P. Novák[a,b]

[a]Institute of Plant Molecular Biology AS CR, Branišovská 31, 37005 České Budějovice, Czech Republic;[b]South Bohemian University, Biological Faculty, Branišovská 31, 37005 České Budějovice, Czech Republic.

Abstract

Chalcone synthase-encoding hop gene was identified and cloned. The gene designated *chs*_H1 (AC AJ304877) consists of two exons and one 187 bp intron. Conserved motifs characteristic for the light regulated genes were identified within the promoter. CHS_H1 is 42.5 kDa protein that retains important features including 28 conserved residues characteristic for CHS enzymes (EC 2.3.1.74). 5 additional *chs*-like genes were predicted from Southern blot analyses previously; in this study at least two *chs*_H1 sequence homologues were partially characterised. The genetic polymorphism and variability of hop chs-like sequences suggests the specific re-arrangements of hop *chs* genes during breeding and selection processes and can be used to create new genetic markers. Sequence analysis of new *chs* homologues from hop is in progress.

Introduction

Chalcone sythase is a member of the CHS superfamily of polyketide synthases (for review see Schröder, 1997) that play an essential role in the biosynthesis of a wide spectrum of biologically active compounds including antimicrobial phytoalexins, flavonoid inducers and cancer chemopreventive phenylpropanoids (for review see Schröder, 1999). Recently, prenylated flavonoids (Stevens et al., 1997) and in particular, prenylated chalcones were isolated from hop extracts (Etteldorf et al., 1999). These compounds were investigated as potential antiproliferative and anti-cancer drugs (Miranda et al., 1999). The presence of prenylflavonoids in hop cones suggests the participation of an enzyme with true chalcone synthase (EC 2.3.1.74) specificity. CHS activity was detected in protein extracts from hop cones by Zuurbier et al. (1995); however, corresponding gene(s) encoding hop CHS have not yet been cloned and characterised.

In the present study we characterise a complete gene *chs*_H1 encoding a chalcone synthase type of enzyme from hop, a crop of great importance and a source of biologically active compounds potentially valuable for human health. Furthermore, we describe the evidence for other CHS homologues and an existence of the polymorphism of *chs*-specific sequences in hop genome. Our sequential, structural and genomic analyses suggest the presence of a multifamily of *chs*-related genes in hops, which clearly differ from the previously described (Paniego et al., 1999) *chs* homologue encoding VPS.

Materials and methods

Osvald's clone 72 was used to characterise *chs*-homologues. The method used for isolation of genomic DNA was essentially as recommended for Qiagen DNA extraction. The preparation of probe for analysis of phlorisovalerophenone synthase (vps) was based on the sequence published in GeneBank (AC: AB047593). For PCR amplification of the chalcone synthase-specific DNA fragment H1 from the hop genome, we used primers designated as "statistic primers" CHSJ3 and CHSJ4 (Matoušek et al., submitted), and for the amplification of *chs*_H1 coding region to express recombinant protein H1 in *E.coli*, primers CHS_H1Nde (5' AGGACATATGGTTACCGTCGAGGAA 3') and CHS_H1Bam (5' CTAGGATCC CACACTGTGAAGCAC 3') were used. Inversion PCR reaction was performed in order to amplify the *chs*_H1 promoter sequence. The whole cloned sequence *chs*_H1 from our experiments has AC AJ304877 (see GeneBank database [Benson et al., 1993]). The theoretical 3-D structures of CHS_H1 and VPS were portrayed against the template of CHS2 of *Medicago sativa* (PDB ID 1BI5), determined by Ferrer et al. (1999).

Results and Discussion

A complete chs-specific gene was isolated from hop (Fig.1) using a combination of PCR, RT PCR and inverse PCR methods (Matoušek et al., submitted). It was predicted that cDNA of *chs*_H1 encodes for 399 aa protein having 42.5 kDa and characteristic CHS/STS signature box (WGVLFGFGPGLT), located close to the C terminus. The coding region (exon 1 plus exon 2) of chs_H1 gene is 74, 75 and 77% identical to *Arabidopsis*, alfalfa *chs*2 and petunia *chs*A chalcone synthase genes at the nt level, respectively. This identity was calculated to be 85, 84 and 87% for *Arabidopsis*, alfalfa CHS2 and petunia CHSA chalcone synthase at the aa level, respectively. Various conserved regulatory motifs reviewed by Martin (1993) and Rushton and Somssich (1998) were identified within the promoter region of *chs*_H1 gene (ref. for AC AJ304877). For instance, upstream from the TATA signal (5' TATAAATA 3'), two G-boxes (5' CACGTG 3') were found, which are characteristic for *chs* genes responding to UV irradiation or pathogen attack. A CHS-like box (5' TACCACTACCAACAT 3' was identified by 25 nucleotides downstream from the TATA signal. In addition, a motif for tissue-specific expression (5' TACTAT 3') having general consensus TACPyAT, was identified within the promoter region. A polyadenylation signal-like element (5' AATAATA 3') was identified at position 2055-2061 on the *chs*_H1 gene (Fig. 1), 34 nucleotides upstream from the polyadenylation site.

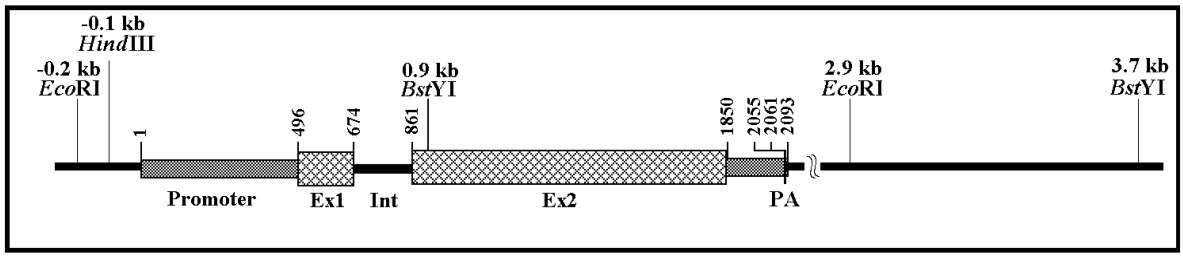


Fig. 1. Schematic drawing of genomic organisation of *chs*_H1 gene. The scheme of cloned *chs*_H1 gene (position 1-2093) is depicted within suppositious DNA fragment. Positions of the restriction sites are given in kb relative to the first nucleotide of *chs*_H1. Ex1, Int, Ex2 and PA designate exon 1, intron, exon 2, and polyadenylation signal, respectively.

A comparative modelling approach was used to calculate the theoretical structure for CHS_H1 (Fig. 2). The structural model of CHS_H1 was compared with the structure of with alfalfa chalcone synthase, as well as with 3-D model, which we predicted for the second known chalcone synthase homologue from hop, VPS having different substrate specificity than CHS (Fig. 2B). We found some important differences between CHS_H1 and VPS including three structural loops I, II and III in Fig 2. Also differences in amino acid region forming the cyclisation pocket and surrounding the catalytical center. For instance, T197, which contributes to the coumaroyl binding site in chalcone synthases, has no homologous position in VPS; it follows from our comparisons that there are some non-homologous amino acid changes at such CHS/VPS positions as D255/A258, R259/T262 and V261/A264 that surround conserved CHS/VPS residues I254/I257, G256/G259 and F265/F268, respectively, and form part of the cyclisation pocket that dictates the specificity of the condensation reaction (see Ferrer et al. ,1999). It can be concluded from our analyses that the characterised *chs*_H1 gene encodes for a protein which retains sequential and structural features characteristic to chalcone synthases and clearly differs from the VPS homologue which was isolated from hop (Paniego et al., 1999). We assayed the specificity of *chs*_H1 expression previously (Matoušek at al.,2000, Matoušek et al., submitted) and found that there is some level of tissue specific expression of *chs*_H1. The high expression of this gene in hop female inflorescences suggest its involvement in formation of secondary metabolites, such as prenylated chalcones. In this study we aimed to express hop chalcone sythase in *E. coli* to develop polyclonal antibody (Fig. 3). This antibody be used in future experiments to characterise expression of *chs*-specific genes in different hop genotypes.

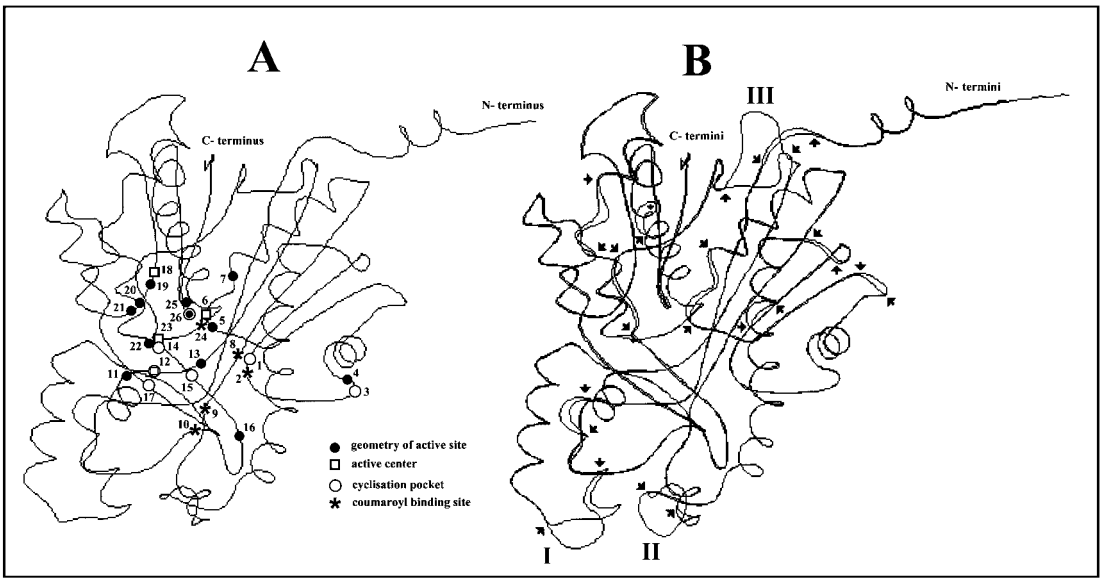


Fig. 2. Structure model of CHS_H1 presented as a single-line ribbon monomer (A) and alignment of two monomer structures, CHS_H1 and phlorisovalerophenone synthase (B). Both views are oriented from the same angle in order to give optimal visualisation on calculated structural differences. These differences are indicated by the arrows on the alignment (B). The largest loops are marked by Roman numerals I, II and III. Essential residues forming geometry of the active site, the active center, the cyclisation pocket, and the coumaroyl binding site were identified by their analogy with the alfalfa chalcone sythase crystallography (Ferrer at al., 1999) and mapped on the structure A, using swiss viewers. The following residues are numbered in sequence from N to C terminus: 1- T132; 2- S133; 3- M137; 4- P138; 5- G163; 6- C164; 7- G167; 8- E192; 9- T194; 10- T197; 11- L214; 12- F215; 13- D217; 14- I254; 15- G256; 16- G262; 17- F265; 18- H303; 19- P304; 20- G305; 21- G306; 22- G335; 23- N336; 24- S338; 25- G374; 26- P375.

It is known that *chs* genes are usually organised in small multigene families. According to our Southern blot analyses performed previously (Matoušek et al. submitted), there are at least 5 additional *chs*-like genes related to *chs*_H1 in the hop genome. A genomic arrangement different from phlorisovalerophenone synthase sequences was found for these genes and RFLP analyses using DNA from fifteen hop cultivars and *H. neomexicanus* species revealed several distinct dendrogram clusters, suggesting specific re-arrangements of hop *chs*-like genes during evolution and/or during the breeding and selection processes. In this study we present oligopeptides (Fig.4), homologues of CHS_H1, which we predicted from DNA fragments obtained by genomic PCR using degenerated primers based on CHS_H1 amino acid sequence (Fig.4). Although it can be assumed that there are genetic differences in the expression and activity of CHS, VPS or other CHS-like homologues that could be involved in the biosynthesis of valuable secondary metabolites in hop, another study is necessary to analyse this possibility.

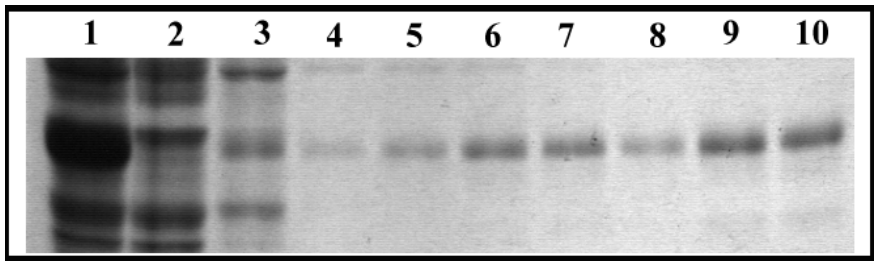


Fig.3. CHS expression and purification under denaturing conditions on Ni-NTA agarose.1, crude cell lysate; 2, flow-through fraction; 3-4, wash (pH 6.3); 4-7, eluates (pH 5.9); 8-9, eluates (pH 4.5).

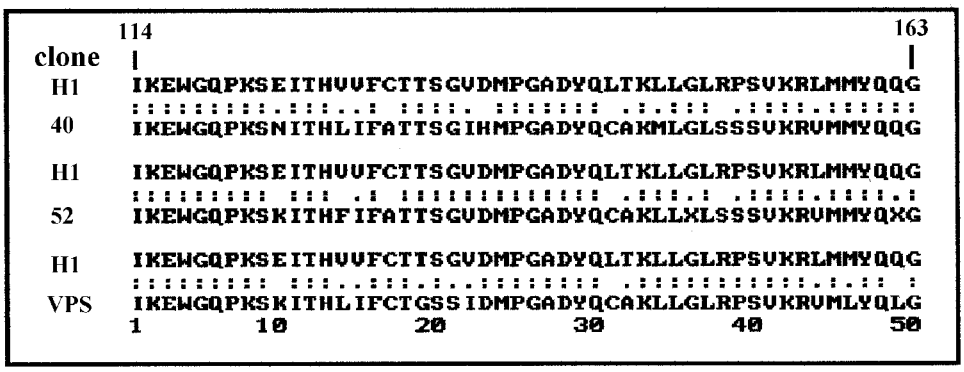


Fig.4. Amino acid sequence analysis of hop CHS_H1 homologues No 40 and 52.

The work was supported by grant 521/99/1591 from the Grant Agency of Czech Republic.

References

Benson, D., Lipman, D. J., Ostell, J., 1993. Nucleic Acids Res. 21, 2963-2965.

Ettendorf, N., Ettendorf, N., Becker, H., 1999. Z. Naturforsch. 54c, 610-612.

Ferrer, J.L., Jez, J.M., Bowman, M.E., Dixon, R.A., Noel, J.P., 1999. Nat. Struct. Biol. 6, 775-784.

Martin, C.R., 1993. Int. Rev. Cytol. 147, 233-284.

Matoušek, J., Bříza, J., Patzak, J., Novák, P., Niedermeierová, H., Orctová, L.: Vortr. Pflanzenzuchtg., 47: 29, 2000.

Matoušek, J. Novák, P. Bříza, J. Patzak, J. Niedermeierová H. Cloning and characterization of *chs*-specific DNA and cDNA sequences from hop (*Humulus lupulus* L.).Gene, submitted.

Miranda, C.L., Stevens, J.F., Helmrich, A., Henderson, M.C., Rodriguez, R.J., Yang, Y.H., Deinzer, M.L., Barnes, D.W., Buhler, D.R., 1999. Food Chem Toxicol. 37, 271-285.

Paniego, N.B., Zuurbier, K.W.M., Fung, S.Y., van der Heijden, R., Scheffer, J.J.C., Verpoorte, R., 1999. Eur. J. Biochem. 262, 612-616.

Rushton, P.J., Somssich, I.E., 1998. Curr. Opin. Plant Biol. 1, 311-315.

Schröder, J., 1997. Trends Plant Sci. 2, 373-378.

Schröder., J., 1999. Nature Struct. Biology 6, 714-716.

Stevens, J.F., Ivancic, M., Hsu, V.L., Deinzer, M.L., 1997. Phytochemistry 44, 1575-1585.

Zuurbier, K.W.M., Fung., S.-Y., Johannes, J., Scheffer, C., Verpoorte, R., 1998. Phytochemistry 49, 2315-2322.

ANALYSIS OF MOLECULAR MARKERS CORRELATED WITH RESISTANCE OF HOP (*Humulus lupulus* L.) TO DAMSON-HOP APHID (*Phorodon humuli* Schrank)

Andreja Čerenak[1], Branka Javornik[1,2]
[1] Institute of Hop Research and Brewing Žalec, Žalec, Slovenia
[2] University of Ljubljana, Biotechnical Faculty, Centre for Biotechnology and Plant Breeding, Ljubljana, Slovenia

Abstract

Marker assisted selection has been widely applied in plant breeding programs in crops with well developed genetic maps and it has been shown to be an efficient approach to improvement of plant cultivars. A program for development of marker(s) of hop resistance to damson-hop aphid (*Phorodon humuli* Schrank) was initiated at the Institute of Hop Research and Brewing Žalec. Several controlled crossings of selected accessions at different levels of resistance were carried out. Parental accessions and progenies were analysed with molecular markers (RAPDs, AFLPs) and phenotypic observations were conducted under field conditions.

1 Introduction

Hop breeding is a lengthy process due to the dioecious nature of hop, which produces highly heterozygous offspring and only female plants are of commercial interest. Although hop has a long tradition, with well documented botany, classical breeding and cultivation, there are still no well established markers for particular traits, or genetic maps which could be used to improve the breeding process. Molecular marker technology, with the ability to generate a large number of polymorphisms independent of environmental factors, has found numerous applications in breeding programs of different crops, and recent hop research in this field indicates potential for hop breeding. One highly applicable field of marker technology is the identification of markers linked to loci or genes of agronomically important traits. Marker assisted selection can be invaluable in breeding programs and markers linked to genes that encode resistance to different diseases and pests are among the most important applications. Different molecular methods are used for generation of polymorphic markers, among which RAPD (Random Amplified Polymorphic DNA) (Rafalski et al., 1991) and AFLP (Amplified Fragment Length Polymorphism) (Vos et al., 1995) are very common.

Damson-hop aphid (*Phorodon humuli* Schrank) is a very important pest in hop fields and effects over 90 % of world hop growing areas. Hop fields form a large proportion of agricultural areas which, together with numerous generations of aphids during a season, enables relatively fast development of resistance to aphicides. Only a few registered insecticides are used for suppression of pests, which makes anti-resistance strategies ineffective. Hop breeding has been relatively successful in the development of commercial cultivars resistant to fungal diseases, while breeding hop for resistance to damson-hop aphid has so far been limited. The majority of work has been done in England, where progenies with a high level of resistance to aphid development and reproduction were obtained using a wild Japanese male accession (Darby, 1999). In relation to aphid resistance, there have been studies on hop secondary metabolites. Kralj et al. (1998) introduced identification of hop resistance to damson-hop aphid based on the amount and ratios among three biochemical markers, alpha-pinene, beta-pinene and unindentified chromatographic peak 92.

The aim of our work was to study the association of molecular markers of hop resistance to damson-hop aphid. A Japanese male accession with well documented resistance to pest was

used as a source of resistance in controlled crosses with selected female genotypes (resistant, semi-susceptible, susceptible) in which we obtained five F_1 families. So far, the parents have been analysed with RAPD and AFLP methods for potential molecular markers.

2 Material and methods

2.1 Material

Parental genotypes were selected on the basis of long standing phenotypical observations and from the results of classification by essential oils (Kralj et al., 1998). Seven crossing combinations were done – resistant male genotype No3-38 was crossed with 3 susceptible cultivars 'Cascade', 'Ahil' and 'Nordgard 1478', three semi-susceptible cultivars 'Apolon', 'Aurora' and 'Savinjski golding' and with one resistant Slovene breeding line 205/108. All plant material was from the hop collection held at the Institute for Hop Research and Brewing Žalec. Families of crossings included in the research are presented in Table 1.

Table 1: Families of crossings achieved in years 1999 in 2000.

No.	Family of crossing
1	'Cascade' x No3-38
2	'Nordgard 1478' x No3-38
3	'Apolon' x No3-38
4	'Savinjski golding' x No3-38
5	'Aurora' x No3-38
6	'Ahil' x No3-38
7	205/108 x No3-38

Molecular analysis included all parental genotypes except cultivars 'Ahil' and 'Apolon' because of the insignificant number of seedlings obtained from both crosses. Two breeding lines, 200/22 and 200/69 (progenies of resistant Japanese male No3-38) with well documented resistance to pest, Slovene wild male 3/3 and 'Žateški polurani červenjak', the parents of resistant line 205/108, were added.

2.2 Methods

DNA isolation

Total genomic DNA was extracted by the procedure described by Kump et al. (1996). Fresh leaves were first homogenised with CTAB extraction buffer. The mixtures were treated by repeated extraction with chlorophorm:isoamylalcohol 24:1. After centrifugation, the DNA was precipitated by the addition of sodium acetate and ice-cold isopropanol. The pellet was washed with cold ethanol, dried and resuspended in TE buffer. The DNA concentration was estimated by mini DNA fluorimeter (Hoefer, TKO 100) and dilutions of 20 ng/μl and 100 ng/μl were made.

RAPD analysis

PCR was carried out in a 25 μl reaction volume containing 2 μl genomic DNA (conc. 20 ng/μl), 1 x PCR buffer, 3,5 mM $MgCl_2$, 200 μM each dATP, dGTP, dCTP and dTTP, 0.2 μM of RAPD primer and 0.5 units of Taq polymerase. The PCR reaction was run in a Perkin-Elmer/Cetus Thermal Cycler 480 with the following temperature profile: preliminary denaturation of DNA for 4 min at 95 °C followed by 40 cycles of 30 sec at 94 °C, 30 sec at 38°C and 1 min 30 sec at 72 °C. There was a final extension step of 7 min at 72°C, followed by a 4°C soak until recovery.

Preselected 85 RAPD primers from commercial kits (OPA, OPB, OPD, OPE, OPM and OPX, Operon Technologies) were used in the analysis. Amplified fragments were resolved by 1.4% agarose (Seakem LE Agarose, FMC BioProducts) in 0.5 x TBE buffer and detected by EtBr staining under UV.

AFLP analysis

The AFLP method established by Vos et al. (1995) was slightly modified in the detection techniques, whereby silver staining was used instead of radioactivity. Total genomic DNA was digested with 2.5 U *Eco*RI and 2.5 U *Mse*I enzymes. Digested DNA fragments and *Eco*RI (5 pmol) and *Mse*I (50 pmol) adapters were ligated with T4 DNA ligase. The resulting DNA (50 ng) was used as primary template DNA in pre-amplification using *Eco*RI and *Mse*I primers (50 ng), both with one selective nucleotide. Selective amplification was performed with eight primer pairs (15 ng) having three additional selective nucleotides, chosen from the 64 primer pair combinations reported by Jakše et al. (2001): E-AGG/M-CTA, E-ACC/M-CAG, E-ACG/M-CTG, E-ACC/M-CTG, E-ACG/M-CAT, E-ACT/M-CTA, E-ACT/M-CAA, E-ACG/M-CAC.
The AFLP products were amplified in a Gene Amp PCR System 9700 Thermocycler with the following temperature profile: preamplification was prepared in a total volume of 50 μl and amplified using 20 cycles of 30 sec at 94 °C, 1 min at 56 °C and 1 min at 72 °C. The following touch down protocol was used for selective amplification in 10 μl: 13 cycles of 30 sec at 94 °C, 30 sec at 65 °C with decreasing –0,7 °C per cycle and 1 min at 72 °C, followed by 23 cycles at the annealing temperature of 56 °C.

Amplified fragments from each primer combination were separated on 5 % denaturating polyacrylamide gels in 1 x TBE buffer and detected with silver staining using the Promega Silver Sequence™ protocol with some modifications (Jakše et al, 2001).

DATA analysis

Banding patterns of both marker systems were scored according to the presence or absence of intensive and repeatable DNA fragments.

3 Results

In our molecular analysis, we focused on polymorphic fragments that were present in three resistant genotypes, No3-38, 200/22 and 200/69 but absent in the other genotypes susceptible or semi-susceptible to damson-hop aphid. Eighty-five primers used in the RAPD analysis generated 220 polymorphic RAPD markers (43.6 %) of which three (primers OPA-9, OPM-12 and OPX-16) were specific between resistant and susceptible genotypes.
A similar level of polymorphism was found with amplification of *Eco*RI and *Mse*I restriction fragments of genomic DNA with eight primer combinations, using three selective nucleotides. Seven specific AFLP markers (primer combinations E-AGG/M-CTA, E-ACC/M-CAG, E-ACG/M-CTG, E-ACC/M-CTG, E-ACG/M-CAT, E-ACT/M-CTA, E-ACT/M-CAA) for resistant parents were found among the polymorphic fragments.

The determined specific fragments represent potential markers for hop resistance to damson-hop aphid. We were not able to test them on the progenies due to weak infestation of hop gardens with damson-hop aphid in 1999/2000 in Slovenia and poor pest development due to extreme weather conditions. Only artificial infestation of two-year seedlings was possible for the estimation of resistance, and the obtained results are only indicative.

Nevertheless, the development of five F_1 families (249 progenies) and the determination of potential specific molecular markers are a solid basis for segregation analysis and linkage of potential marker(s) of hop resistance to damson-hop aphid. We assume that in future research

a reliable estimation of progeny resistance to damson-hop aphid based on observation over a number of years can be obtained and segregation analysis of phenotypic and molecular markers can provide results with practical value for efficient hop breeding.

References:

Darby P. 1999. New selection criteria in hop breeding. In: Proceedings of the Scientific commission. Pulawy, Poland, 27-30 July 1999. Seigner E. (ed.). Pulawy, International Hop Growers' convention: 3-6

Jakše J., Kindlhofer K., Javornik B. 2001. Assesment of genetic variation and differentiation of hop (*Humulus lupulus* L.) genotypes by microsatellite and AFLP markers. – accepted in Genome

Kralj D., Kač M., Dolinar M., Žolnir M., Kralj S. 1998. Marker-assisted hop (*Humulus lupulus* L.) breeding. Monatsschrift für Brauwissenschaft, 7/8: 111-119

Kump, B., Svetek, S., Javornik, B. 1992. Isolation of the high-molecular DNK from plant tissue. Research Reports, Biotechnical Faculty, University of Ljubljana, 59: 63-66

Rafalski J.A., Tingey S.V., Williams J.G.K. 1991. RAPD markers – a new technology for genetic mapping and plant breeding. AgBiotech News and Informations, 3, 4: 645-648

Vos P., Hogers R., Bleeker M., Reijans M., van den Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, 23, 21: 4407-4414

HIGH THROUGHPUT DEVELOPMENT OF SIMPLE SEQUENCE REPEAT MARKERS IN HOP (*Humulus lupulus* L.)

J. Jakše & B. Javornik
Centre for Plant Biotechnology and Breeding, Biotechnical Faculty, Agronomy Dept., Jamnikarjeva 101, 1000 Ljubljana, Slovenia

Abstract

Four different hop genomic DNA libraries, enriched for $(GA)_n$, $(GT)_n$, $(AT)_n$ and $(TTA)_n$ types of SSR motifs, were constructed using an enrichment procedure based on a restriction digest using a cocktail of several restriction enzymes, which greatly improves representation of nearly all genome sequences, rather than using size selection. The capture of microsatellites containing sequences, using small membranes with attached microsatellite probes, provides a cost effective alternative to the widely used magnetic bead capture.

Introduction

Simple sequence repeats (SSR), or microsatellites, consist of tandem arrays of short nucleotide repeats from 2-6 bases per unit, densely distributed throughout eucaryotic genomes. The loci are highly polymorphic, with heterozygosities exceeding 0.5, as a result of very high mutation rates of repeat sequences. Slippage during DNA replicaton is the most likely mechanism for adding or substracting units of repeats, although studies in various organisms have provided evidence of more complex changes. The structure and length of microsatellite repeats are considered to be the major factor affecting their variability and it is important to account for these factors when using SSR markers.

Plant microsatellites have been studied much less than those in animals and humans, and most research has focused mainly on economically important species. Plant SSR have been isolated either from genomic libraries or derived from the GeneBank database, the latter showing lower variability. These results suggest that SSR are less frequent in plant than in mammal genomes and that relative frequencies of the different repeats differ from those in mammals. For example, $(AT)_n$ and $(GA)_n$ are so far the most abundant di-nucleotide motifs in plants and $(CA)_n$, most common in mammals, is less frequent. The highest number of SSR markers used so far in plants has been in rice, wheat, soybean, maize and barley, followed by other crops, and an increasing number of reports on new SSR isolations have shown their successful application in germplasm management, genetic diversity studies, genetic mapping and marker-assisted selection. In addition to these practical applications, information about the distribution and variability of SSR sequences in the genome of a given species provides a means of elucidating the genetic history of the species from the standpoint of evolution and artificial selection.

The establishment of a microsatellite analysis system for a new species represents a considerable financial and technical challenge, but this does not prevent their use. On the contrary, there are many new reports on plant microsatellites using enrichment procedures for isolation and developing hundreds of microsatellites in different crop species (Huang et al., 1998; Rivera et al., 1999; Cordeiro et al., 2000; Jones et al., 2001). In hop, only four polymorphic microsatellites are known to date (Brady et al., 1996), although their suitability has been shown in hop mapping (Seefelder et al., 2000) and in study of hop genetic diversity and cultivar identification (Jakše et al., 2001). Allele size variation of highly polymorphic loci not only unambiguously fingerprinted different genotypes but also showed the specific distribution of two geographically different hop germplasms, as deduced from pedigree data of the hop cultivars. More hop microsatellite markers could provide a good marker system for genetic mapping, linkages of important agronomic traits and for establishing an easily exchangeable database of variety fingerprints. Here we report on the isolation of a sample of microsatellites from a genomic library, developed by a modified enrichment method.

Materials and methods

The enrichment procedure for microsatellite isolation was based on digestions of DNA with a cocktail of restriction enzymes described by Hamilton et al. (1999) and capture of microsatellites containing DNA by long probes attached to nylon membranes (Edwards et al. 1996) with some modifications (Jakše & Javornik, in preparation).
H. lupulus L., var. Savinjski Golding plants were obtained from in vitro cultures propagated at the Institute for Hop Research & Brewing, Žalec. Leaves were harvested and genomic DNA was extracted according to Kump et al. (1992) to obtain clean, high molecular weight genomic DNA.
Processing of insert DNA was based on restriction digest with a set of enzymes (*Nhe*I, *Alu*I, *Hae*III and *Rsa*I) that cut the genomic DNA such that a large proportion of fragments is in the 1000 to 200 base pair range. After digestion, the restricted fragments were purified, dephosphorylated and ligated to double stranded (ds) phosphorylated SNX linkers (Hamilton et al., 1999).
Long microsatellite probes were made by PCR reaction in the case of $(GA)_n$ and $(GT)_n$ repeats, in which two complementary primers with core microsatellite repeat were used as starting DNA. In the case of $(AT)_n$ and $(TAA)_n$ microsatellites, probe extension with a slippage reaction by Klenow enzyme was used according to Schlötterer & Tautz (1992). Probes were spotted on small pieces of nylon membrane, air dried, linked to it on an UV source, washed for two days in formamide buffer and stored until use at –20°C.
For capturing of DNA fragments containing microsatellites, a modified procedure of Edwards et al. (1996) was used. PCR amplified ligated hops DNA was added to membranes in buffer [3x SSC, Na-phosphate pH 7.0, 0.5% SDS, 50% formamide] prewarmed to 70°C. The temperature was gradually cooled to 37°C and kept overnight at this temperature. The next day, the membranes were washed, boiled in 200 μl of sterile water, quickly pipeted out and stored at –20°C. The enrichment rate was determined with dot spots of 50 ng of restricted hop DNA and 50 ng enriched fragments. Hybridisation with biotin labelled microsatellite probes was performed according to Westneat et al. (1988) and detected by a Phototope Star Detection Kit. Enriched fragments were ligated into the *Xba*I site of dephosphorylated pBlueScript plasmid, transformed to competent XL-10 Gold cells (Inoue et al., 1990) and selected on LB agar plates with IPTG, X-gal and carbenicillin. White colonies were arrayed on new agar plates, lifted to the nylon membranes and screened for positive clones containing microsatellites. In the case of AT clones, the colony screening step was omitted because of the self-complementary nature of AT fragments. Instead, 10 randomly selected white colonies were sequenced.
Positive clones were sequenced using Cy-5 labelled M13 primers, using a Thermo sequenase cycle sequencing kit (Amersham Pharmacia).

Results & discussion

Restriction digest and processing of hop genomic DNA

A commonly employed procedure is to cut genomic DNA with one frequent cutter and to select fragments in the desirable range from agarose gels. It was shown that using only a portion of the genome for microsatellite cloning gives significantly different numbers of positives for the same repeat sequence, and that using multiple restriction enzymes when constructing DNA libraries may increase the diversity of microsatellites available (Hamilton & Fleischer, 1999).
In our analysis, we used a cocktail of restriction enzymes (Hamilton et al., 1999) for hop DNA digestion to obtain a majority of fragments smaller than 1000 bp., which is very important for further ligation and sequencing. Several combinations of restriction enzymes were tested: (*Nhe*I+*Alu*I+*Hae*II+*Hae*III), (*Nhe*I+*Alu*I+*Hae*II+*Rsa*I), (*Nhe*I+*Hae*II+*Hae*III+*Rsa*I) and (*Nhe*I+*Alu*I+*Hae*III+*Rsa*I). With the last combination, the majority of fragments were below 1000 bp and it was chosen for hop DNA digestion. A simplification was also made to the original procedure in the purification of restricted fragments by using phenol extractions and ethanol precipitation instead of commercially available spin columns.

Capturing of microsatellites

In our modified protocol, we used long microsatellite probes for capturing DNA fragments instead of the commonly used short primers (~ 30 bp) attached to small nylon membranes (Edwards et al., 1996). With this modification, twice as many microsatellites containing fragments were obtained in comparison to membranes with attached $(XY)_{15}$ oligos.
Before the enrichment step, fragments with ligated dsSNX linkers were amplified to increase the amount of DNA needed for hybridisation and to select fragments with ligated linkers. Up to 10 μg of amplified DNA was obtained from the 500 ng of fragments used in ligation.
After the enrichment step, we employed a simple dot spot procedure to check the enrichment of PCR fragments. In the case of successful enrichment, a strong signal was observed compared to unenriched DNA.

Library construction, colony screening & sequencing

Enriched libraries were made for 4 different microsatellites motifs GA, GT, AT, ATT (Table 1). In case of the GA and GT librariess, a 37% and 35% enrichment level was achieved, which is slightly lower than data in some literature, but it was achieved by a single enrichment step. If we compared our results with unenriched library data (Brady et al., 1996) our results showed twice as many GA clones in screening 70 times fewer colonies. In the case of GT microsatellites, the comparison is even more dramatic: 16 times more positives were obtained in screening 78 times fewer colonies.
In the case of AT and ATT repeats, we were unable to detect any positive clones. Less stringent conditions in hybridisation would probably solve the problem with the ATT repeat. These two repeats are the most common in the plant kingdom among di- and tri- nucleotide repeats (Morgante & Olivieri, 1993). The AT motif is normally excluded from enrichment libraries due to its self-complementary nature.

Table 1: microsatellite discovery in 4 different enriched hop libraries.

Item	Library				Total
	GA	GT	AT	ATT	
Colonies screened	2790[a]	1020[a]	/	744	4554
No. of positives	1038	358	0[b]	0	1396
Level of enrichment	37%	35%	0	0	36%[c]
No. of clones sequenced	51	21	10	/	82
Clones with microsatellite loci	51 (100%)	21 (100%)	0	/	72 (100%)[c]
Duplicate clones	33 (65%)	11 (55%)	/	/	44 (61%)
Unique loci	28 (55%)	14 (67%)	/	/	42 (58%)
Loci not suitable for primer development	8 (16%)	1 (5%)	/	/	9 (13%)
Loci suitable for primer development	20 (39%)	13 (62%)	/	/	33 (46%)

[a]This number represents only 10% of library, which could be obtained from enrichment step.
[b]Ten clones were random sequenced and no AT repeats were present in any of them.
[c]Total only for GA and GT results.

DNA sequences of GA and GT libraries have so far been obtained from a total of 72 clones, and all of them contained microsatellite loci (Table 1). A high degree of redundancy was found in both libraries, which may be attributable to clone duplications or to locus duplications. However, redundancy limits the number of effective loci that can be developed, but it seems that such results are frequent (Rossetto et al., 1999; Jones et al., 2001). In addition to redundant clones, truncated clones with less than 30 nt present on either side of the microsatellite motif are quite common. Such clones are unsuitable for primer development and in comparison to literature data, our results showed a very low level of such clones. In the GA library, predominantly interrupted perfect and complex microsatellites were found with a longest perfect repeat of $(AG)_{23}$. In the case of the GT library, longer perfect or compound repeats were obtained, the longest having 35 dinucleotide repeats.

This paper reports on efforts to isolate a large number of positive microsatellite containing clones. In the continuation of the work, the majority of these clones will be transformed into microsatellite markers which will provide a valuable marker system for various hop genetic studies and a potential tool for an inter-laboratory exchangeable database of variety fingerprints.

References

Brady, J.L., Scott, N.S., and Thomas, M.R. 1996. DNA typing of hops (Humulus lupulus) through application of RAPD and microsatellite marker sequences converted to sequence tagged sites (STS). Euphytica, 91, p. 277-284.

Cordeiro, G.M., Taylor, G.O., and Henry, R.J. 2000. Characterisation of microsatellite markers from sugarcane (*Saccharum* sp.), a highly polyploid species. Plant science, 155, p. 161-168.

Edwards, K.J., Barker, J.H.A., Daly, A., Jones, C., and Karp, A. 1996. Microsatellite libraries enriched for several microsatellite sequences in plants. Biotechniques, 20, p. 758-760.

Hamilton, M.B., and Fleischer, R.C. 1999. Cloned microsatellite repeats differ between 4-base restriction endonucleases. The journal of heredity, 90, p. 561-563.

Hamilton, M.B., Pincus, E.L., Di Fiore, A., and Fleischer, R.C. 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. Biotechniques, 27, p. 500-507.

Huang, W.-G., Cipriani, G., Morgante, M., and Testolin, M. 1998. Microsatellite DNA in *Actinidia chinensis*: isolation, characterization, and homology in related species. Theoretical and applied genetics, 97, s. 1269-1278.

Inoue, H., Nojima, H., and Okayama, H. 1990. High efficiency transformation of Escherichia coli with plasmids. Gene, 96, p. 23-28.

Jakše, J., Kindlhofer, K., and Javornik, B. 2001. Assessment of genetic variation and differentiation of hop genotypes by microsatellite and AFLP markers. Genome, accepted.

Jones, E.S., Dupal, M.P., Kölliker, R., Drayton, M.C., and Forster, J.W. 2001. Development and characterisation of simple sequence repeat (SSR) markers for perennail rygrass (*Lolium perenne* L.). Theoretical and applied genetics, 102, p. 405-415.

Kump, B., Svetek, S., and Javornik, B. 1992. Izolacija visokomolekularne DNA iz rastlinskih tkiv. Zbornik Biotehniške fakultete Univerze v Ljubljani, Kmetijstvo, 59, s. 63-66.

Morgante, M., and Olivieri, A.M. 1993. PCR-amplified microsatellites as markers in plant genetics. The plant journal, 3, s. 175-182.

Rivera, R., Edwards, K.J., Barker, J.H.A., Arnold, G.M., Ayad, G., Hodgkin, T., and Karp, A. 1999. Isolation and characterization of polymorphic microsatellites in *Cococ nucifera* L. Genome, 42, p. 668-675.

Rossetto, M., McLauchlan, A., Harriss, F.C.L., Henry, R.J., Baverstock, P.R., Lee, L.S., Maguire, T.L., and Edwards, K.J. 1999. Abundance and polymorphism of microsatellite markers in the tea tree (*Melaleuca alternifolia*, Myrtaceae). Theoretical and applied genetics, 98, p. 1091-1098.

Schlötterer, C., and Tautz, D. 1992. Slippage synthesis of simple sequence DNA. Nucleic acids research, 20, 2, p. 211-215.

Seefelder, S., Ehrmaier, H., Schweizer, G., and Seigner E. 2000. Male and female genetic linkage map of hops, Humulus lupulus. Plant breeding, 119, p. 249-255.

Westneat, D.F., Noon, W.A., Reeve, H.K., and Aquadro C.F. 1988. Improved hybridization conditions for DNA 'fingerprints' probed with M13. Nucleic acids research, 16, p. 4161.

INVESTIGATIONS ON THE VIRULENCE SPECTRUM OF HOP POWDERY MILDEW (*SPHAEROTHECA HUMULI*)

E. Seigner[1], S. Seefelder[1], B. Haugg[1], H. Hesse[1], H. Rösch[2], and F. Felsenstein[2]

[1]Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Institut für Hopfenforschung, Hüll 5 1/3, D-85283 Wolnzach, Deutschland,
[2]EpiLogic GmbH Agrarbiologische Forschung und Beratung, Hohenbachernstraße 19-21, D-85354 Freising-Weihenstephan
Elisabeth.Seigner@lbp.bayern.de; Friedrich.Felsenstein@EpiLogic.de

Abstract

Hop powdery mildew, caused by the fungus *Sphaerotheca humuli* (Burr.), has become a serious problem in almost all hop growing regions of Europe and the USA. In order to prevent loss of yield and quality associated with heavy infection of cones with powdery mildew, in recent years great amounts of pesticides had to be applied which drastically increased production costs. In our efforts to control powdery mildew breeding for resistance is crucial. For specific incorporation and utilization of different genetic sources of resistance to powdery mildew it is vital to evaluate the effectiveness of these sources. For that purpose it is necessary to determine the spectrum of virulence genes of *S. humuli* populations occurring in various hop growing regions. Using an infection system with detached leaves the potential of 89 isolates of powdery mildew to infect thirteen differential hop cultivars and breeding lines has been tested under standardized conditions. Differential hop genotypes carried different resistance genes, partly characterized in literature. These investigations on the regional spectrum of virulence genes in powdery mildew strains from various Hallertau growing regions, from England, France and the USA revealed very interesting insights into the local infection potential of *Sphaerotheca humuli*. At the same time the effectiveness of race-specific resistances (R-gene) based on major genes that are currently utilized in breeding could be evaluated. Virulence analyses clearly showed that several resistance genes are already overcome by corresponding virulence genes in the powdery mildew populations or have at least increased frequencies in specific regions. In the Hallertau region the R1, R2, R5 and RW gene have been recognized as still effective sources of resistance. Furthermore, it became quite obvious that it is absolutely necessary to broaden the genetic basis for resistance in hop germplasm. New genetic sources of resistance are expected to be found in wild hops.

Keywords: Hop, *Humulus lupulus,* powdery mildew, *Sphaerotheca humuli*, virulence gene, resistance gene, breeding for resistance

1 Introduction

Hop powdery mildew, caused by the fungus *Sphaerotheca humuli* (Burr.), has become a serious problem in almost all hop growing regions of Europe and the USA. While in the UK infections of powdery mildew are noted almost 300 years ago, in Germany it was a disease of little significance until in the early 70s Northern Brewer was planted extensively. This variety is particularly susceptible and powdery mildew infections have increased as a consequence. In the 1997 growing season this disease occurred for the first time in the fields of the western USA.

In order to reduce dramatic losses of yield and quality associated with powdery mildew infections of cones large amounts of pesticides had to be applied which drastically increased production costs. The most effective and most environmental friendly way to control powdery mildew is the cultivation of hop varieties with a genetically fixed resistance.

For more than 2 decades main emphasis in our Hüll breeding programme has been put on the development of powdery mildew resistant hops. For that purpose each year around 60.000 seedlings deriving from specific crosses are tested for their resistance after artificial infection with powdery mildew spores. For specific incorporation and utilization of different genetic sources of resistance to powdery mildew it is vital to evaluate the effectiveness of those sources, which implies that fundamental knowledge on the spectrum of virulence genes occurring in *S. humuli* populations in various hop growing regions already exists.

Based on the classical gene-for-gene concept that has been described by Flor (1957) each resistance gene in the host corresponds to a specific virulence /avirulence gene in the pathogen. According to this gene-for-gene relationship a series of various powdery mildew strains with specific virulence genes has been characterized in England (Royle, 1978; Darby, personal comm.; Darby, 1998) due to their specific reaction and their capability to infect hop cultivars with known major resistance genes.

At the moment only very rough ideas exist on the variability and regional distribution of pathogenic strains of hop powdery mildew that already occur in the Hallertau and other hop growing regions. In cooperation with EpiLogic, Dr. Felsenstein, an infection test system using detached leaves has been established to screen for resistant hop plants. Using this screening system the variability of virulence genes of various pathogenic strains of *Sphaerotheca humuli* that occur in the Hallertau and in other important hop growing regions should be determined.

2 Leaf assay for screening for resistance to hop powdery mildew

An infection test system using detached leaves has been adapted to the specific requirements of the hop – *Sphaerotheca humuli* system. For the reliability of the virulence analyses with detached hop leaves it was of utmost importance to use only those leaves that fully expressed a specific powdery mildew resistance or susceptibility. Moreover, it had to be ensured that the observed reactions in this leaf assay corresponded with the behavior under natural infection conditions. Besides incubation temperature and light conditions for optimal germination of the spores and growth of the mycelium on detached hop leaves in particular the leaf age proved crucial: Under controlled inoculation conditions only the very young leaves at the first node from the terminal bud of a susceptible hop genotype could be easily infected by virulent strains, while on the other side leaves from the third node could not be infected.

3 Virulence analyses

During the summer of 1999 and 2000 powdery mildew infected hop leaves from various hop growing regions - from the Hallertau, from England, France and the USA - were collected and monosporic isolates were produced in the laboratory of EpiLogic and maintained on leaves of the highly susceptible cv. “Northern Brewer”. Just from the beginning on strict quarantine conditions were established in order to prevent “escapes” of any pathogenic strain into the hop growing regions. A total of 89 isolates of powdery mildew was provided for the virulence tests. Based on the mutual identification of virulence gene in the pathogen and corresponding resistance gene in the host 13 differential hop genotypes with different resistance genes – partly characterized by Royle (1978) and Darby (1998) – were used in these investigations. Detached young hop leaves were inoculated with the spores of various monosporic isolates of *Sphaerotheca humuli* using a simple “settling tower” and 8 days after inoculation growth of the mycelium on the leaves was macroscopically assessed:

Leaves showing the highest level of infection, in the general the leaves of the highly susceptible cultivar “Northern Brewer” (R0), were rated as 100% infected. Leaves of the differential genotypes on average showed less or no visible infection and were assessed in relation to the control leaves of “Northern Brewer” showing 100% - 10% or 0% of maximum infection (= 100%). In this way the sporulation intensity of each series of test could be compared. Three main classes of host-pathogen reactions could be distinguished: virulent strains with the corresponding virulence gene causing 100 – 50% of maximum infection; intermediate reaction meant 40-20% of maximum infection and avirulent strains could not overcome the resistance gene of the hop plant and therefore no or very few (0-10%) infection could be observed.

4 Spectrum of pathogenic strains and effectiveness of resistance genes

These first investigations of the regional spectrum of pathogenic strains from the Hallertau, England, France and the USA hop growing regions revealed very useful insights into the regional infection potential of *S. humuli* (Table 1). At the same time the effectiveness of currently used race-specific resistances (based on major genes) could be evaluated. The virulences analyses clearly showed that most of the resistance genes known so far have been overcome by corresponding virulence genes in the pathogen populations. Resistance of “Alpharoma” and “Yeoman” (RB) have been defeated by races with the matching virulence genes in the Hallertau and in all other hop growing regions - with the exception of France. In France, due to the extensive cultivation of “Strisselspalter” with no detectable resistance, there existed no pressure towards the selection and development of new virulent races. *Sphaerotheca humuli* is an obligate parasite in hop which means that this fungal is strictly dependent on the supply of nutrients from its host. In this case always strict coevolution between resistance gene in the host and virulence gene in the pathogen can be observed. According to these data (Tab.1) partial resistance (expressed as intermediate reaction of infection) can be assumed for the cultivar “Wye Challenger”. This may be explained as the result of an additional gene (RB) besides R3. Virulent races overcoming the R6- and R4-gene from “Nugget” and “Serebrianker” respectively have already occurred in the Hallertau region.

No virulence genes could be detected in the Hallertau regions that can break the resistance of “Zenith” (R1), “Wye Target” (R2) and “Early Choice (R5) and that of the three wildhops from Thuringia (RW?). At the moment it cannot be proven whether the source of resistance of all those wildhops is identical or different. In England all these resistances are already overcome by corresponding virulent strains within the *S. humuli* populations. In these investigations including 68 isolates no v5 virulence could be detected, but Darby (1998) already reported about the occurrence of virulent strains in England defeating the action of the R5 gene, even though at low frequencies.

According to these data (Tab. 1) resistances based on the R1, R2 and R5 gene from the English cultivars “Zenith”, “Wye Target” and “Early Choice” still confer protection against infections of powdery mildew, at least in the Hallertau regions. Also the resistances of the three wildhops from Thuringia are still effective.

Based on these investigations on the variability of virulence genes in various populations of *Sphaerotheca humuli* it seems to be reasonable to broaden the basis of resistance to powdery mildew by utilizing - in addition to „Wye Target“ which has been used for years as donor for resistance - “Early Choice”, “Zenith” and the wildhops as crossing partners to incorporate resistance genes into the Hüll germplasm. It is to be expected that the pyramiding of various resistance genes in one plant increases the durability of resistance to powdery mildew.

5 Outlook

Out of a total of 89 isolates of powdery mildew which have been characterized by EpiLogic during this project 15 isolates have been selected that are to be maintained and used for

Table 2: Virulence testing

Virulence reaction of 13 isolates of powdery mildew from England, France, the USA and the Hallertau with an assortment of differential hop genotypes consisting of 13 cultivars / breeding lines with various resistance genes (monosporic isolates from 1999 and 2000)

		Powdery Mildew Isolates												
Hops	**R-Gene**	E6	E8	E9	F1	USA2	USA3	HU2	HU9	BU10	BU13	ES1	HA1	PZ1
North. Brewer	R0	100	100	100	100	100	100	100	100	100	100	100	100	100
Zenith	R1, (RB)	0	100	100	0		0	0	0	0	0	0	0	0
Wye Target	R2	0	10	100	0	0	0	0	0	0	0	0	0	0
Wye Challenger	R3 (RB)	30	30	40	0	50	0	30	100	10	80	10	0	0
Serebrianker	R4	0	0	0	0	0	0	0	0	70	90	60	100	60
Early Choice	R5	0	0	0	0		0	0		20	10	10	0	0
Nugget	R6	0	0	0	0	0	0	0	0	100	100	50	80	60
Yeoman	RB	60	100	30	0	20	100	100	100	100	90	50	0	70
Alpharoma	R?	20	100	60	10	70	100	30	50	100	100	80	0	0
3/63/51	R?	100	100	80	0	0	100	100	100	100	100	80	0	100
Wildhopfen 25	RW?	0	100	100	0	0	0	0	0	0	0	0	0	0
Wildhopfen 30	RW?	0	100	100	0	0	0	0	0	0	0	0	0	0
Wildhopfen 49	RW?	0	60	30	0	0	0	0	0	0	0	0	0	0

Monosporic isolates: England: E6, E8, E9; France: F1; USA: USA2; USA 3; Hallertau: HU2; HU9; BU10; BU13; EB1; HA1; PZ1;

Intensity of infection : 100 - 50% of maximum infection ➔ pathogen virulent
40 - 20 % of maximum infection ➔ intermediate reaction
10-0 % of maximum infection ➔ pathogen avirulent

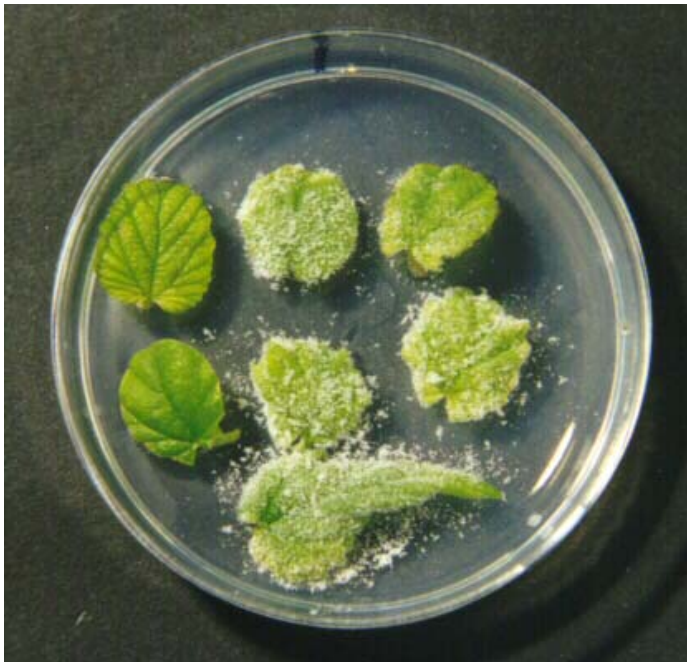


Figure 1:
Response of an isolate of *Sphaerotheca humuli* grown on "Wye Target" to 6 differential hop genotypes: „Early Choice" (R5), „Zenith" (R1), 3/63/51 (R?) [1. row from the left to the right side]; „Nugget" (R6), Wildhopfen W30 (RW?), „Wye Target" (R2) [2. row from the left to the right side]. Control: leaf of the highly susceptible cv. „Northern Brewer" (R0) [at the bottom]. Based on the capacity of this isolate (no. 9 from England) to infect specific host genotypes the virulences genes v1, v3/63/51 , v w und v2 could be identified.

further screening of powdery mildew resistance. With this set of virulence genes all currently known and used sources of resistance (R-gene) can be identified and distinguished. Furthermore this infection system established by EpiLogic guarantees a standardized test system in petri dishes and a safe handling even of hazardous pathogenic strains of *Sphaerotheca humuli*. Another advantage of this system is that for the first time specific *Sphaerotheca* strains with characterized virulence behavior can be used for the infection of hop seedlings. In this way the impact of a specific R-gene can be recognized, even for those genes that are already defeated. This effective, reliable screening of great numbers of seedlings is a crucial prerequisite for the identification of closely linked molecular markers for powdery mildew resistance genes.

6 References

Darby, P., 1998: The Symptoms and Biology of Hop Powdery Mildew. Amer. Soc. of Brew. Chemists NET. (www.scisoc.org)

Felsenstein, F.G., 1991: Virulenz und Fungizidsensitivität des Weizenmehltaus, *Erysiphe graminis DC. f. sp. tritici* Marchal, in Europa. Dissertation, TU-München

Flor, H.H., 1971: Current status of the gene-for-gene-concept. Ann. Rev. of Phytopathology **9**, 275-296.

Royle, D.J., 1978: Powdery Mildew of the Hop. In: Spencer D.M. (ed.), The powdery mildews. London, UK. Academic Press, 381-408.

Olmstead J.W. and G.A. Lang, 2000: A leaf disk assay for screening sweet cherry genotypes for susceptibility to powdery mildew. HortScience **35 (3)**, 274-277.

Acknowledgements

The authors wish to thank the "Wissenschaftsförderung der Deutschen Brauerwirtschaft

e.V." for funding this work.

THE EFFECT OF FUSARIUM SAMBUCINUM INFECTION ON HOPS

E.Solarska
Institute of Soil Science and Plant Cultivation, 24-100 Puławy, ul. Czartoryskich 8, Poland

Abstract

In the years 1999-2000 study was performed on the occurrence of pathogenic fungi on stem base of hops on two hop cultivars and two localities of Poland. The results of mycological analysis of diseased plants indicated that cause of hop cancer was Fusarium sambucinum. But different types of disease symptoms caused by this pathogen were observed depending on cultivar.

Introduction

The cancer of hop (Humulus lupulus L.) occurs every year in some hop gardens in Poland (Solarska, 1981). It is caused by fungi from genus Fusarium. Its causal fungus *Gibberella pulicaris* (Fr.) Sacc. spreads infection by two kinds of spores: 1. The common, summer, sickle-shaped, two to six-celled conidia named *Fusarium sambucinum* (Fr.) Sacc. and the rarer four-celled ascospores produced in bluish-violet over-wintering perithecia, named *Gibberella pulicaris* (Burgess, 1964). The first outward sign of Fusarium cancer is a wilting of one or more bines of the affected hill at any time in the growing season. Diseased tissues rot, and when the stem becomes girdled the bine above it will wilt, with the leaves becoming flaccid, dropping, and pale green and drying up with a puckered surface. A slight tug on such a bine causes it readily to break away at the cancer (Burgess, 1964; Solarska, 1981). *Fusarium culmorum* was found to cause hop cancer but with more severe symptoms than these caused by *Fusarium sambucinum* (Solarska, 1978; Solarska, 1981).

In the years 1999-2000 the composition of fungi on diseased stem bases of hop was determined in the end of June. Results obtained provided the information concerning a possible role of these fungi on two hop cultivars in conditions of Poland.

Material and methods

Field inspections were conducted during the last ten days of June in the hop-gardens localised at Rogów and Kłodnica.

Samples of bine bases and lower part of main bines of two hop cultivars: Lubelski and Marynka were collected. Isolations were made on mineral agar from small fragments of diseased tissue, disinfected for 1 minute in 1% $HgCl_2$ in 50% ethyl alcohol. After disinfection plant material was rinsed three times, for 3 minutes each in sterile water and planted on the medium. Plates were incubated at room temperature. Mycelium from fungal colonies that grew from the plant material was transferred to plates on PDA. Identification of the fungi was made from single spore cultures prepared by the multiple dilution method.

The temperature and rainfall were recorded in each year of studies.

Results and discussion

Within hop-gardens from 0,2 to 10% plants were diseased. In case of Lubelski cultivar, stem base rot was observed in each year at both sites and the most diseased plants of this cultivar was found in 1999. Similar symptoms but localised on lower part of main stem over the soil surface were observed on Marynka cultivar and only at one from sites and only in 2000 (table 1).

Table 1:

Incidence of hop cancer in the two localities of Lublin region

Site	Cultivar	Diseased plants (%) in 1999	Diseased plants (%) in 2000
Kłodnica	Lubelski	8	0,5
	Marynka	0	0
Rogów	Lubelski	4	0,2
	Marynka	0	10

As a result of mycological analysis of diseased plants in 1999-2000, 280 isolates belonging to 11 fungal species were obtained. Each year the most frequently recorded species was *Fusarium sambucinum* which comprised 93% of all the isolates obtained. Remaining fungi: *Fusarium oxysporum, Fusarium equiseti, Alternaria alternata, Gliocladium catenulatum, Cylindrocarpon destructans, Penicillium simplicissimum, Penicillium humuli, Rhizoctonia solani, Trichoderma koningii, Trichoderma viride* were isolated infrequently and not every year (table 2).

Table 2.

Fungi isolated from diseased hop plants

Fungus	Number isolates in:	
	1999	2000
Alternaria alternata /Fr./ Keissl.	1	2
Cylindrocarpon destructans /Zins./ Scholten	0	1
Fusarium equiseti/Corda/ Saccardo	0	1
Fusarium oxysporum Schl. Snyd. Et Hans	2	3
Fusarium sambucinum Fuck.	112	148
Gliocladium catenulatum Gilman et Abbott	2	1
Penicillium humuli van Beyma	0	1
Penicillium simplicissimum Oudemans/Thom	0	2
Rhizoctonia solani Kühn	1	0
Trichoderma koningii Oudemans	2	0
Trichoderma viride Pers.ex Fr.	0	1

Performed studies confirmed hitherto informations about large harmfulness of *Fusarium sambucinum* as pathogen damaged bine base (Burgess, 1964; Royle, 1974; Schmidt et al., 1969; Solarska, 1981) but new information is that it can to attack a places above soil-level on the bines as it has taken place in case of Marynka cultivar. The cultivar has very abundant lateral shoots and foliage and after too early suckering is possible that infection of such part of bine relies upon wounds. Fusarium sambucinum has been shown to be a weak pathogen, commonly encountered in hop garden soils. Fungus requires to enter wounds in hop rootstocks for diseases to became established but the extent of damage then caused seems to vary with the time of year when infection is initiated and with the conditions prevailing after inoculation

(Royle, 1974). Weather conditions occurred in June of 2000 could to favour development of infection (fig.2). According Schmidt et al. (1969) and earlier own studies (Solarska, 1981) hop cancer caused by Fusarium sambucinum occurs mainly on hops growing on moisture soils what explains particularly high incidence of disease on Lubelski cultivar in 1999 when large amount of rainfall was noted (fig.1). These studies confirmed also that hop cancer is an annual disease, those plants infected in one year not necessarily retaining the infection the next (Royle, 1974).

Conclusions

1. In the years 1999-2000 Fusarium sambucinum caused stem base rot of Lubelski cultivar and stem rot over the soil surface of Marynka cultivar.
2. The wet soil favours the occurrence of stem base rot caused by the pathogen.

Fig. 1. Day and night average temperature and sum of rainfall in Powiśle region in 1999.

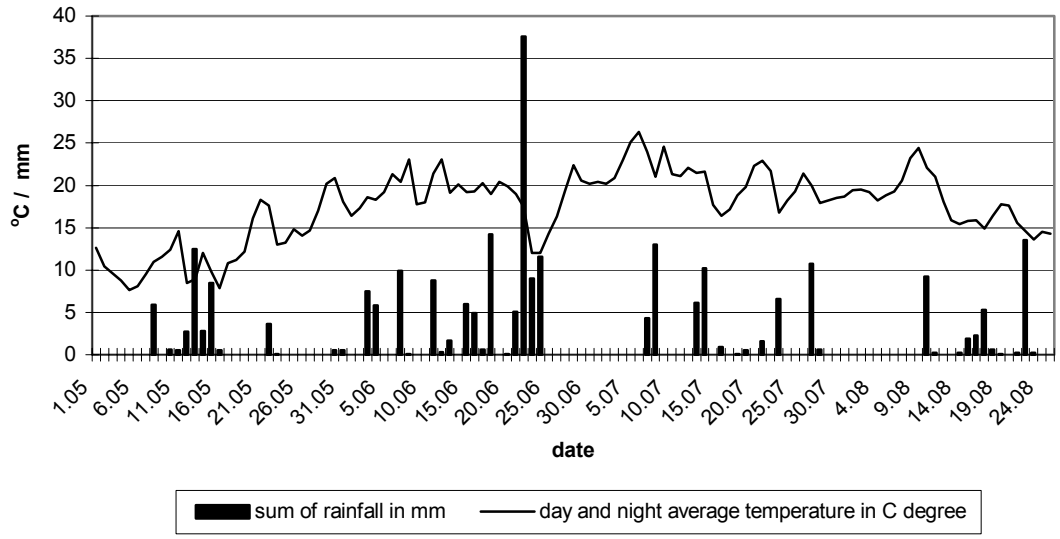
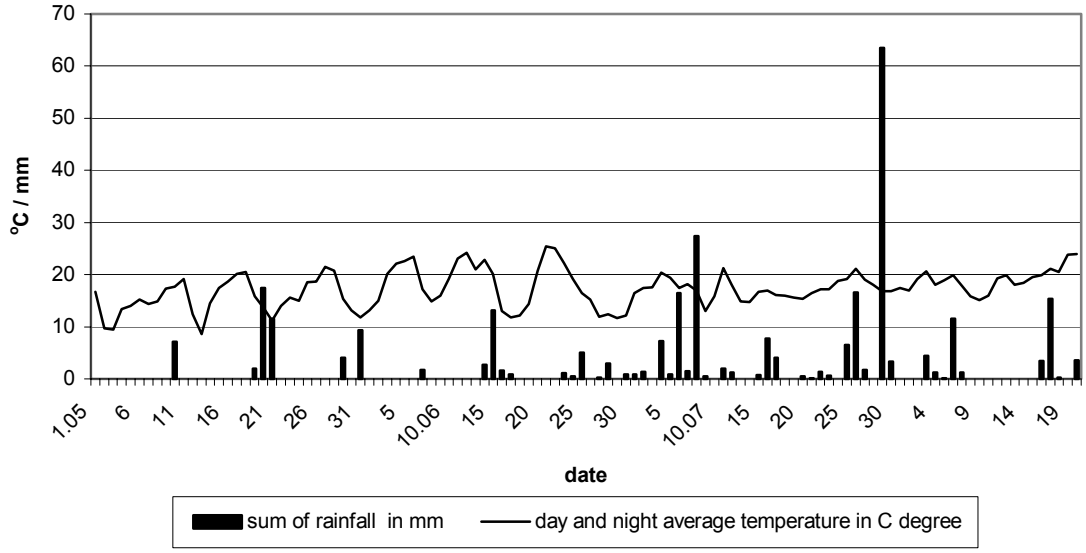


Fig. 2. Day and night average temperature and sum of rainfall in Powiśle region in 2000



References

1. Burgess A.H.1964.Hops. London. Leonard Hill;159-161.
2. Royle D.J.1974. Fusarium cancer . Plant pathology section, Annual Report. Wye College:26-27, 67-68.
3. Schmidt H.E., Naumann K., Holtz J. 1969. Welkeerscheinungen des Hopfens in Gebiet der Deutschen Demokratischen Republic, hervorgerufen durch Fusarium sambucinum Fuck. Nachrichtenbl. dtsch. PflschDieienst. 7:121-126
4. Solarska E. 1978. Attempts to isolate some fungi causing hop wilt in the Lublin region of Poland. Proceedings of the Scientific Commission of The International Hop Growers' Convention. Puławy, Poland:28-32
5. Solarska E. 1981. Więdnięcie infekcyjne chmielu (Humulus lupulus L.) uprawianego na Lubelszczyźnie. Puławy R(156).

HOP PRODUCTION IN SLOVENIA THREATENED BY *VERTICILLIUM SPP*.

Andrej Simončič, Sebastjan Radišek, Marta Dolinar, Milan Žolnir
Institute of Hop Research and Brewing Žalec, Žalec, Slovenia

Abstract

Hop wilt, caused by *Verticillium albo-atrum and Verticillium dahliae,* occurs in a non-lethal, fluctuating form and lethal or progressive form. Disease severity varies mainly due to pathogenicity of the strains and Hop cultivar's resistance. The fluctuating wilt was first found and recognised in Slovenia in 1974, while more pathogen, progressive wilt was first found in 1997 just before harvest. The symptoms of lethal strain were observed and correspond to the description of progressive form of wilt in England. In 1998 and 1999 over 32 ha oh Hop gardens on small area were found with progressive wilt and another 65 ha within four new areas in the year 2000. Almost half of the Hop growing area in the Savinja Valley had locations with infested Hop gardens. Since 1998 all required and recommended quarantine measures have been taken to prevent the Hop wilt from spreading. Different activities in the field of research and advisory service are described in the paper, including fungi research, detection and inspection methods, Hop breeding, phytosanitary measures and others. In the areas, where Hop wilt occurred in 1998 and 1999 and where all quarantine measures were used, no new outbreaks of the disease have been detected. Based on the recent experience in Slovenia, it can be expected that strict quarantine measures could eradicate, or at least prevent further wilt spreading and help to preserve the existent acreage of Hop gardens.

INTRODUCTION

Hop wilt is caused by a tracheomycosic fungi *Verticillium albo-atrum* (Reinke and Berthold), and *Verticillium dahliae* (Klebahn). There are two strains of it: mild and progressive, which depends on virulence of isolate, cultivar's sensitivity and ecological conditions. These two fungi belong to the group of the most dangerous quarantine pathogens in Slovenia and are therefore on EPPO A2 list of quarantine pathogens.
The mild, fluctuating wilt strain was first discovered in 1924 in England (Harris, 1927). In 1933 lethal strain was discovered, and it had affected around 2.400 hectares of hop gardens by the year 1960 (Jary, 1961). Second larger area infected with hop wilt was Bavaria, where in 1970s an area of around 800 ha size suffered great damage.
First diseased plants in Slovenia were found in 1974 (Dolinar 1974, 1975, 1976), when most of Aurora cultivar gardens were infected with mild hop wilt. This was also the year when both fungi *Verticillium albo-atrum* and *Verticillium dahliae* were first isolated from hops. In the next few years the infection ceased and it occurred only sporadically. In 1997 it appears again on a larger scale in Gomilsko region in the Savinja Valley but this time in a more pathogen, progressive form (Dolinar and Simončič, 1999). So what we did here was diagnosed and isolated mostly *Verticillium albo-atrum*. Identification was confirmed in 1999 when the infection appeared again and with re-isolation from the host plant and with the analysis made by Prof. dr. Zinkernagel from the Phytopatological Institute, Weihenstefan, Munich.

Hop wilt and its spreading from 1997 to 2000

The first symptoms were observed in Slovenia on 5 August 1977 in a 4-year old hop garden of Bobek cultivar in the western part of Savinja Valley shortly before the harvest. We assumed that hop wilt first appeared in this area in progressive strain already in 1995, that is on the far western part of the same complex, only some 10 to 20 metres away. The infection spread from here on. When infected hop plants from this garden were identified, our suspicions about hop wilt were confirmed. But the symptoms were not identical to the ones caused by a mild strain.

Since all this took place practically during the harvest, we decided to monitor the health status of these hop gardens in the next season.
In 1998 we noticed that a lot of plants started wilting in 2 hop gardens in Gomilsko. We attributed this to hop wilt. Since *Verticillium albo-atrum* and *Verticillium dahliae* are on A2 quarantine list, we immediately, that is on 7 April 1998, informed the Inspectorate of the Republic of Slovenia, Phytosanitary Inspection Service in Ljubljana and Žalec and Plant Protection Department at the same ministry. All further preventive measures and recommendations regarding hop wilt were then co-ordinated by these services at the Ministry of Agriculture, Forestry and Food (MAFF).
As from this point on we can say that we immediately announced a state of emergency. Everyday inspections of hop gardens from May and June on were practically the responsibility of all employees in the Department of plant protection at the Institute of Hop Research and Brewing Žalec. As soon as the symptoms of hop wilt were noticed on a larger scale outside of the two complexes where they were first detected, we started inspecting all hop gardens in the vicinity thoroughly as well as the infected ones which were the property of the same hop growers. At the same time we performed thorough inspections in all registered nurseries in Slovenia.
Throughout 1998 we additionally tried to identify and determine the causes of hop wilt in other hop gardens. We found out that hop wilt was largely present also in hop gardens of other hop growers in the above mentioned complex or in the vicinity, and where we discovered only some diseased plants. There were other locations not far from these where we discovered hop wilt later on in the season. We managed to establish that hop wilt did not attack only certain cultivars since it was detected practically on all other cultivars. The end of hop season recorded a total of 21 hectares of hop gardens where we found a large number of diseased plants and two hop gardens with only one or two diseased plants.
During the season a lot of samples were taken from infected hop gardens. These were examined thoroughly. The results confirmed the presence of fungi *Verticillium* spp. We finally got a proof when we isolated fungi and informed MAFF, Phytosanitary Inspection Service about it on 1 October 1998, when it was found that the examined samples were infected with *Verticillium albo-atrum*.
At the end of July and in August we paid a visit to all hop growers with infected hop gardens, and together with them we agreed on what actions to take to dispose of the infected harvest rests and on their thermic treatment. At the same time we also visited all hop growers who use a communal harvest machine, so that we could present the problem. In this regard we prepared Technical Instructions on what to do with the plants in case of infection and opinions on suitability of locations for deposit and thermic processing of infected harvest rests. Based on those two things hop growers received detailed instructions issued by the Phytosanitary Inspection Service on how to act in case of infection. Hop growers followed our instructions at harvesting, and hop rests were disposed of as agreed.
At the end of 1998 most hop growers with a larger number of diseased or dead plants in their hop gardens had already done preventive sprays and ploughed the gardens as it was instructed. Hop growers who were outrun by snow did this at the beginning of 1999. Here we refer to 21 ha of previously mentioned hop gardens.
Two hop growers in whose hop gardens we found only one or two diseased plants had to destroy these hop plants and the ones in the near vicinity because of the suspicion that they might be infected. These sites as well as whole fields were checked thoroughly in 1999.

The results showed that in 1999 hop wilt spread to extra 10,5 hectares of hop gardens owned by 11 hop growers. In most cases we are talking about areas in the neighbouring complex, which is why we suggested eradication to Phytosanitary Inspection Service. There were other two hop gardens outside the complex that were instructed to do the same.

At the beginning of 2000 we continued with regular and systematic inspections with the main objective to check and determine the health status of plants. Special attention was paid to hop gardens in particularly exposed areas and to nurseries where planting material is selected. In

Gomilsko, where hop growers had to destroy 33 hectares of hop gardens between 1998 and 1999 because of the progressive hop wilt, we detected only one field infected with progressive hop wilt, which indicates how successful our procedures for controlling disease in this area were.
On our second inspection we discovered totally new sites of infection in western part of the Savinja Valley (figure 1). We were surprised by the size of it – four large hop-growing areas stretched on 775 ha of hop gardens. There are 65,5 ha of hop gardens infected with hop wilt with different levels of infection. Besides these there are hop gardens in crop rotation, for which we can claim with certainty that they are already infected and they present a great potential for the rapid spread of disease.
During the year we regularly monitored the level of infection in the exposed areas and we immediately passed on this information to the Phytosanitary Inspection Service, who then acted against the progressive strain of hop wilt. We prepared detailed instructions and suggested ways of carrying out phytosanitary measures in the whole process of hop production, harvesting and handling hop rests. Various different mediums were used to inform the public, such as automatic answering machines, information given to hop growers, articles in professional journals, booklet on hop wilt etc. We also organised many meetings with hop growers whose hops were infected or were in danger of infection.

MATERIALS AND METHODS

Subject matter, time and place of inspections

Systematic control over hop gardens has been carried out in Slovenia since 1998. This helps us detect and determine the state of infection with hop wilt.
The subject matter here are all hop gardens in the time of vegetation with the focus on the following hop gardens:
- those where we discovered symptoms of hop wilt,
- those that are located near the infected fields,
- those in which the same equipment is used by different hop growers and
- in nurseries.

These inspections take place between 1 June, when first symptoms can be detected, and 15 September when the harvest is over. The inspections include:
- first inspection of exposed hop gardens (all hop growers' gardens in which we detected the symptoms of hop wilt, the ones in the vicinity and the ones where the same equipment in hop growing is used) between 1 and 15 June,
- first inspection of all nurseries in Slovenia between 15 and 30 June,
- second inspection of exposed hop gardens between 1 and 15 July,
- second inspection of all nurseries in Slovenia between 30 July and 15 August and
- third inspection of exposed hop gardens between 15 August and 15 September.

Activities in the time of inspection include visual inspections of hop gardens, sampling and laboratory identification.

Analysis method and sampling

The first samples for lab examination are taken between 1 and 15 July (120 samples). These samples are taken from plants we suspect might be infected and also other samples in exposed area up to number 60. The remaining 60 samples are taken from hop gardens where mild strain of hop wilt often occurs, and on randomly selected locations. This sampling is repeated between 30 July and 15 August.
The sample itself are 20 cm long bines which are cut from the plant at the height of 60 to 80 cm. To determine the pathogen in the lab we use classical method of fungi isolation. PDA

(potato dextrose agar) modified to 4.8 pH with streptomycin sulphate supplement serves as nursery. In sterile conditions we apply parts of vascular tissue. After the five-day incubation period and with the help of the luminous microscope we are able to identify fungi. Ten or fourteen days later, when fungi organs are fully developed, the type of fungi can be identified.

RESULTS

Between 1998 and 2000 approximately 300 lab analyses of different samples were performed annually. 300 analyses in the year 2000 show that 201 samples contained fungi *Verticillium albo-atrum.* One sample contained fungi *Verticillium* dahliae. All other samples were infected with other different fungi (for example *Fusarium oxysporum*), or pathogens were not detected at all. Phytosanitary Inspection Service and Plant Protection Department were regularly informed of the results, and they acted in accordance with the instructions.

Phytosanitary measures

Systematic hop gardens' inspections in which Institute of Hop Research and Brewing Žalec detected progressive hop wilt proved to be indispensable for hop growers. Therefore they were reported regularly to the Phytosanitary Inspection Service.
After that Phytosanitary Inspection Service started the necessary procedure to eradicate hop wilt. This procedure had to comply with the Plant Protection Act (Ur.l. RS, number 82/94). Phytosanitary Inspection used the Institute's reports on infection with hop wilt and also expert opinion of those who studied the cases of infection.
Taken all this into consideration, Phytosanitary Inspection Service carried out the procedure to eradicate whole hop gardens, or only certain diseased plants with the adhering plants in the protected zone and in the 3-metre radius circle. If the infection spread on a large scale from the first to the second inspection, Phytosanitary Inspection issued an order for the hop gardens to be completely destroyed. Where the infection from the first to the second inspection did not spread or only spread to the neighbouring plants, only certain plants in the adhering protected zone had to be removed and destroyed.
Phytosanitary Inspection Service performed a hearing of hop growers and field inspection, at which point it acquainted them with the infection and the necessary phytosanitary measures that would have to be considered. The infected area was marked with signs of warning and special tape.
Based on Institute's instructions, Phytosanitary Inspection Service instructed the following:
- that stated hygienic standards are met at harvesting, which means proper transport of hop plants so that they do not get strewn about during the transport, and special cloth-covers have to be fitted to the trailers,
- compulsory desinfection of mechanisation , equipment and footwear during the transport and after the harvest,
- compulsory deposit of hop rests after the harvest only to special locations,
- compulsory thermic treatment of hop rests by covering it with a PVC foil,
- transport of thermically treated hop rests only to selected locations (non-hop locations),
- destruction of certain diseased plants by spraying with non-selective herbicide, with digging out and burning in the hop garden,
- destruction of whole infected hop gardens by spraying with non-selective herbicide, ploughing and burning and
- compulsory conforming to 4-year rotation of grass-leafed crops such as cereals, grasses and maize and regular eradication of broad-leaved weeds.

During the harvest time and eradication Phytosanitary Inspection Service`s role was to supervise if hop growers complied with the issued regulations. A lot of hop growers made a claim on the insurance for the damage they had suffered. Hop growers were entitled to

indemnity, and the procedure was started to obtain these means. Diseased hop plants were eradicated with spraying, ploughing and burning. In some gardens the final burning can be done by the end of March, which means that the deadline is extended and the burning can take place in the presence of Phytosanitary Inspection Service.

CONCLUSIONS

Systematic control over hop gardens has been done in Slovenia since 1998. This helps us detect hop wilt and determine the level of infection. Since 1997 the progressive strain of hop wilt has been appearing in the small area of Gomilsko in Savinja Valley. In this area phytosanitary measures helped us prevent its further spreading.
On our second inspection of the main hop-growing areas in the western part of the Savinja Valley new areas with progressive hop wilt were discovered in the year 2000. Hop gardens varied in the level of intensity, which was from a few hop plants to whole hop gardens. This state led us into the conclusion that we were most probably dealing with an old infection which was not detected on time. There must be quite some infected hop gardens under crop rotation in this area for which we can also claim with certainty that they are infected, which gives further potential to hop wilt spreading in exposed areas and further on.
We have to emphasise, however, that in nurseries of this area the infection was not detected, which rules out the possibility of disease spreading with the planting material in the past years and today. Despite this we have issued a 2-year preventive prohibition order for the use of planting material from exposed locations.
The main vector of disease spreading presents infected hop rests which can be the source of infection not only with hop wilt but also other diseases and pests if mishandled. This is the reason why it is so important that hop growers comply with the regulations for the correct handling of hop rests, and with the basic phytosanitary measures in hop gardens.
Weather conditions in the year 2000 also contributed its share towards epiphytic infection since they lessened plant resistance to infection, which definitely resulted in better conditions for the disease to develop. We therefore think that we should start introducing more resistant cultivars in exposed areas since this in addition to suitable agrotechnics and phytosanitary measures proved to be one of the most efficient ways of controlling hop wilt.
In the Savinja Valley we have to consider that progressive strain occurs in restricted area, which justifies eradication. This approach for now has proved sensible since we have managed to prevent further spreading of hop wilt in the region, where it first occurred.

REFERENCES

Dolinar, M. (1975, 1976): Hmeljeva uvelost (*Verticillium albo-atrum* in *Verticillium dahliae*). Poročila za sklad B. K., IHP Žalec, Inv. št. 18 in 46.

Dolinar, M. (1974): Pojav uvelosti hmelja v Sloveniji. Priloga hmeljarja, 8, p. 26.

Dolinar, M. and Simončič, A. (1999): Hmeljeva uvelost (*Verticillium albo-atrum* Reinke at Berthold in *Verticillium dahliae* Klebahn) v Sloveniji. Lectures and papers, 4th Slovenian Conference on Plant Protection, Portorož, 1999, p. 201-208.

Harris, R. V. (1927): A wilt disease of hops. Rep. E. Malling Res. Stn. for 1925, II Suplement, 92-93.

Jary, C. L. (1961): A summary of the present position. Ass. Growers New Varieties of Hops, Ann. Booklet, p. 11.

EFFECT OF VIRUSES ON THE YIELD AND BREWING CHARACTERISTICS OF FOUR HOP (*HUMULUS LUPULUS* L.) CULTIVARS IN AUSTRALIA

S.J. Pethybridge[1], C.R. Wilson[1], L.J. Sherriff[2] & G.W. Leggett[2]
[1] University of Tasmania, PO Box 447, Burnie, Tasmania, 7320, Australia
[2] Australian Hop Marketers, GPO Box 104A, Hobart, Tasmania, 7001, Australia

Abstract

The effect of *Hop latent carlavirus* (HpLV), *Hop mosaic carlavirus* (HpMV), and *Prunus necrotic ringspot virus* (PNRSV (apple, A, and intermediate, I, serotypes)), on cone yield, and levels of brewing organic acids in mature plants, was assessed in four hop (*Humulus lupulus* L.) cultivars in Tasmania, Australia. In cv. 'Nugget', infection by the virus combinations studied was not associated with reductions in yield, however plants infected by PNRSV-I, in 2000, had 11 % lower alpha acids and 7 % lower beta acids than uninfected. In 'Opal', infection by HpLV, HpLV + HpMV + PNRSV-A and HpLV + HpMV + PNRSV-I was the most damaging to yield, resulting in reductions of 40 – 42 %, 40 – 58 %, and 47 – 50 %, respectively over the two years of study. Infection by HpLV + HpMV + PNRSV-A and HpLV + HpMV + PNRSV-I reduced the levels of alpha acids by 23 – 30 % and 33 – 43 %, respectively. In 'Pride of Ringwood', infection by HpMV had the greatest effect, reducing yield by 50 – 54 % and alpha acid levels 11 – 19 % compared to virus-free control plants. In 'Victoria', virus infection was not associated with any significant change in alpha and beta acid levels compared to virus-free control plants in either year.

Introduction

Three viruses, *Hop latent carlavirus* (HpLV), *Hop mosaic carlavirus* (HpMV), and *Prunus necrotic ringspot virus*, apple (PNRSV-A) and intermediate (PNRSV-I) serotypes, are found commonly infecting cultivars in Australian hop gardens (Pethybridge *et al.,* 2000). All three viruses have been reported to reduce production (yield and levels of brewing organic acids) in hop cultivars overseas, however their effects vary with cultivar, virus combinations, and virus strains.

This study quantified the effect of these viruses on production of newly developed cultivars, and aimed to determine whether infection poses a significant constraint to Australian hop production.

Materials and Methods

The trial was established on 15th October 1996 in a three hectare hop garden consisting of 151 plots. Plots contained six virus infected ('treatment') plants, arranged with three plants along two rows. These were surrounded by 24 virus free plants (those with no detectable virus infection by serological testing) of the same cultivar as the treatment plants to slow the rate of virus re-infection into the treatment plants. The plants were spaced 2.1 m both along and across rows, and all management operations were similar to those used in a commercial hop gardens in Australia.

Planting material was obtained from softwood cuttings from virus tested mother plants the previous season. For virus free plants, cuttings were taken from mother plants, surrounded by at least three virus free plants in each direction. Softwood cuttings (3 cm of stem below each node) were taken from mother plants in early spring and placed under intermittent mist until sufficient root development.

Treatment plants within each plot were tested for virus infection in the spring of 1997, 1998, and 1999, to ensure the virus status of each plant had not changed from the original planting material. Samples (0.1 g) from six randomly selected expanding leaves from individual plants were homogenised together using a leaf press in 1.0 ml of 0.01 M phosphate buffered saline (pH 7.4) containing polyvinyl pyrrolidone (MW = 40,000). Extracts were tested for the presence of viruses by double antibody sandwich enzyme-linked immunosorbent assay. Polyclonal antisera and dried infected controls to HpLV, HpMV, *chestnut mosaic ilarvirus*, and *rose mosaic virus 3* were obtained from D.J. Barbara (Horticultural Research International, UK). The latter two antisera were used to differentiate between serotypes of PNRSV (Pethybridge *et al.,* 2000)

The effect of virus infection on cone yield and levels of brewing organic acids was assessed in the second and third years of this study. Plants were removed and taken to an Allaeys picking machine. The cones from each plot were weighed, whilst sub-samples were taken for analysis of brewing organic acid composition. Brewing organic acids were extracted from 10 g of dry hops, through 30 minutes of pulverization in 100 ml of toluene. Brewing organic acid composition was analysed by high performance liquid chromatography (HPLC), on a liquid chromatograph equipped with a filter photometer at 314 nm. Sample values were compared to those on a standard curve, constructed using the ICE-2 standard. Cone yield and levels of brewing organic acids from virus infected and virus free hops were compared by analysis of variance (Genstat 5; Version 3.1).

Results

Infection by HpLV, HpMV, PNRSV-I, and HpLV + PNRSV-I in ‘Nugget’ in both years, and infection by HpLV, PNRSV-A, PNRSV-I, HpLV + PNRSV-A, HpLV + PNRSV-I, HpLV + HpMV, HpMV + PNRSV-A, HpLV + HpMV + PNRSV-A, and HpLV + HpMV + PNRSV-I in 'Victoria’ in 1999 had no significant effect on cone yield (Table 1). In ‘Opal’, infection by HpLV, HpLV + HpMV + PNRSV-A, and HpLV + HpMV + PNRSV-I significantly reduced cone yield compared to virus free plants in both years. In ‘Pride of Ringwood’, infection by HpMV was the most deleterious, reducing cone yield by 50 – 54 % in both years. Infection by PNRSV-I also reduced yield by 50 % in the first year of study, however yield was not significantly different from virus free plants in 2000 (Table 1).

Table 1. Effect of virus infection on cone yield (% loss in kg of green weight compared to virus free plants) in 'Opal', 'Pride of Ringwood', and 'Victoria' in 1999 and 2000. Figures in brackets are % loss compared to virus free plants. POR = Pride of Ringwood. NA = material not available. ns = not significantly different from virus free plants.

Virus	1999			2000		
	Opal	POR	Victoria	Opal	POR	Victoria
HpLV	- 42	- 23	ns	- 40	- 20	ns
HpMV	- 17	- 50	NA	- 27	- 54	NA
PNRSV-A	NA	- 34	ns	NA	ns	ns
PNRSV-I	ns	- 50	ns	ns	ns	ns
HpLV + PNRSV-A	NA	- 28	ns	NA	- 27	- 18
HpLV + PNRSV-I	NA	NA	ns	NA	NA	- 44
HpLV + HpMV	NA	- 34	ns	NA	- 27	- 35
HpMV + PNRSV-A	NA	NA	ns	NA	NA	ns
HpMV + PNRSV-I	ns	- 45	NA	ns	- 39	NA
HpLV + HpMV + PNRSV-A	- 58	- 39	ns	- 40	- 33	ns
HpLV + HpMV + PNRSV-I	- 50	NA	ns	- 47	NA	- 22
Virus free	1.2	1.8	1.7	1.5	1.5	2.3
df	23	39	45	23	39	45
LSD	0.4	0.7	---	0.4	0.3	0.3
P<	0.001	0.02	0.08 (ns)	0.001	0.001	0.002

Infection by HpLV, HpMV, PNRSV-I, and HpLV + PNRSV-I in 'Nugget', and infection by HpLV, PNRSV-A, PNRSV-I, HpLV + PNRSV-A, HpLV + PNRSV-I, HpLV + HpMV, HpMV + PNRSV-A, HpLV + HpMV + PNRSV-A, and HpLV + HpMV + PNRSV-I in 'Victoria' in both years had no significant effect on alpha acid levels (Table 2). Infection by all three viruses in combination had the most deleterious effect on alpha acid levels in 'Opal' in both years. In 'Pride of Ringwood', infection by HpMV reduced alpha acid levels by 18 % in 1999, but failed to significantly effect alpha acid levels in 2000. Whilst, infection by PNRSV-A significantly reduced alpha acid levels by 15 % in 1999 and 11 % in 2000 (Table 2).

Infection by HpLV, HpMV, PNRSV-I, and HpLV + PNRSV-I in 'Nugget' in 1999, and infection by HpLV, PNRSV-A, PNRSV-I, HpLV + PNRSV-A, HpLV + PNRSV-I, HpLV + HpMV, HpMV + PNRSV-A, HpLV + HpMV + PNRSV-A, and HpLV + HpMV + PNRSV-I in 'Victoria' in 1999 and 2000, and the virus combinations studied in 'Opal' and 'Pride of Ringwood' in 2000 had no significant effect on beta acid levels (Table 3). In 1999, infection by all three virus combinations in 'Opal' significantly increases beta acid levels compared to virus free plants. In 'Pride of Ringwood', infection by HpMV reduced beta acid levels in 1999 by 15 %. In 'Nugget', infection by PNRSV-I reduced beta acid levels by 7 % in 2000 (Table 3).

Table 2. Effect of virus infection on alpha acid levels (% loss compared to virus free plants) in 'Opal', 'Pride of Ringwood', and 'Victoria' in 1999 and 2000. POR = Pride of Ringwood. NA = material not available. ns = not significantly different from virus free plants.

Virus	1999			2000		
	Nugget	Opal	POR	Nugget	Opal	POR
HpLV	ns	ns	ns	ns	ns	ns
HpMV	ns	ns	-18	ns	ns	ns
PNRSV-A	NA	NA	-15	NA	NA	-11
PNRSV-I	ns	Ns	ns	-11	ns	-11
HpLV + PNRSV-A	NA	NA	ns	NA	NA	ns
HpLV + PNRSV-I	ns	NA	NA	ns	NA	NA
HpLV + HpMV	NA	NA	ns	NA	NA	ns
HpMV + PNRSV-A	NA	NA	NA	NA	NA	NA
HpMV + PNRSV-I	NA	ns	-12	NA	ns	ns
HpLV + HpMV + PNRSV-A	NA	-23	-12	NA	-29	ns
HpLV + HpMV + PNRSV-I	NA	-33	NA	NA	-43	NA
Virus free	12.4	12.6	10.3	13.4	13.3	10.9
df	17	23	39	17	23	39
LSD	---	1.2	1.2	0.7	1.4	1.1
P<	0.613 (ns)	0.001	0.001	0.002	0.001	0.013

Table 3. Effect of virus infection on beta acid levels (% loss or increase) in 'Opal', 'Pride of Ringwood', and 'Victoria' in 1999 and 2000. Figures in brackets are % loss compared to virus free plants. POR = Pride of Ringwood. NA = material not available.

Virus	1999		2000
	Opal	POR	Nugget
HpLV	ns	ns	ns
HpMV	ns	-15	ns
PNRSV-A	NA	ns	NA
PNRSV-I	ns	ns	-7
HpLV + PNRSV-A	NA	ns	NA
HpLV + PNRSV-I	NA	NA	ns
HpLV + HpMV	NA	ns	NA
HpMV + PNRSV-A	NA	NA	NA
HpMV + PNRSV-I	ns	ns	NA
HpLV + HpMV + PNRSV-A	+ 17	ns	NA
HpLV + HpMV + PNRSV-I	+ 15	NA	NA
Virus free	5.9	6.9	4.6
df	23	39	17
LSD	0.6	0.7	0.2
P<	0.001	0.001	0.008

Discussion

Virus infection was associated with significant reductions in cone yield and levels of brewing organic acids in hop cultivars grown in Australia. In 'Opal', infection by HpLV, HpMV, HpLV + HpMV + PNRSV-A, and HpLV + HpMV + PNRSV-I significantly reduced yield compared to virus free plants in both years. Furthermore, infection by all three viruses in combination (with either serotype of PNRSV) was associated with significant reductions in alpha acid levels, and in 1999, significant increases in beta acid levels, compared to virus free plants. The larger reduction in yield and alpha acid levels in plants infected by all three viruses in combination compared to plants infected by all three viruses alone, is suggestive of a synergistic effect between the viruses. In 'Pride of Ringwood', HpMV exerted the most damaging effect on cone yield and brewing organic acids levels in both years, and generally the carlaviruses were more damaging to yield than the ilarviruses. The consistently demonstrated low rate of virus re-infection in commercial 'Opal' and 'Pride of Ringwood' gardens (Pethybridge *et al.,* 2000), and low rate of virus infection in artificial challenges (Pethybridge, 2000) have demonstrated these cultivars have a low susceptibility to virus infection. This suggests that establishing 'Opal' and 'Pride of Ringwood' gardens with virus free material is useful for virus control. In 'Victoria', the significant effect of certain virus combinations on yield in 2000 suggests that as plants age, the effect of virus combinations may be more severe. A synergistic effect was also quantified in 'Victoria'. Infection by HpLV, PNRSV-A, and PNRSV-I alone had no effect on yield, but combinations of HpLV + PNRSV-A, and HpLV + PNRSV-I reduced yield by 18 % and 44 % respectively. Studies of the incidence of viruses in commercial 'Victoria' (Pethybridge *et al.,* 2000) gardens and artificial virus challenge studies (Pethybridge, 2000) have consistently demonstrated this cultivar is highly susceptible to infection by ilarviruses. However, the effect of virus infection on softwood cutting propagation (Pethybridge, *unpublished data*) demonstrates that if virus free mother plants are not chosen for softwood cuttings, this may severely affect the success rates in propagation. We therefore recommend the continuation of the virus certification scheme for the planting material of this cultivar, however the adaptation of management practices to reduce the rate of virus transmission in this cultivar is the subject of further study. In 'Nugget', infection by PNRSV-I was associated with significant reductions in brewing organic acid levels in 2000 suggested that ilarviruses are damaging to the quality of hop products from this cultivar, and their effect may increase with plant age. Unfortunately, no studies have been made to determine the rate of virus re-infection in 'Nugget' gardens in Australia, nor their susceptibility to artificial virus challenges, which limits our recommendations for this cultivar.

References

Pethybridge S.J., *et al.,* 2000. Virus incidence in Australian hop gardens and cultivar differences in susceptibility to infection. *AJAR,* **51**: 685-689.

Probasco E.G., Murphey, J.M. 1996. The effects of hop viruses on brewing and agronomic characteristics in the hop variety Chinook. *MBAA Tech. Quart.* **33**: 160-165.

PATHOGENIC EFFECTS OF HLVD

J. Patzak*, J. Matoušek**, K. Krofta* & P. Svoboda*
Hop Research Institute Co.Ltd., Kadaňská 2525, 438 46 Žatec, Czech Republic, *
Institute of Plant Molecular Biology AS CR, Branišovská 31, 370 05 České Budějovice, Czech Republic, **

Abstract

The viroid-free mericlones from Osvald's clone 31, 72, 114, and cv. Premiant were used to evaluate effect of viroid infection with hop latent viroid (HLVd) on the composition of hop resins and essential oils in the first production year. Healthy plants were compared with naturally re-infected individuals under field conditions. On average, viroid infection decreased content of α-bitter acids by 40 %. The content of β-bitter acids, as well as the ratios of humulone/cohumulone and lupulone/colupulone was not influenced by viroid infection. The content of all monoterpenes was for 29, 37.4 and 41.6 % higher for myrcene, α-and β-pinene, respectively, in infected plants compared with the healthy controls. The contents of sesquiterpenes like β-caryophyllene, α-humulene, α-copaene, γ-muurolene, β-bisabolene, γ-cadinene, and δ-cadinene decreased by 13.7, 13, 14, 18.5, 29, 21.7 and 18.5 %, respectively, due to viroid infection. The possible influence of some oxidative-reduction processes activated by viroid-caused pathogenesis was assumed to be involved in the accumulation of terpenes alcohols like geraniol and methylgeranate, and in the reduction of the contents of the majority of ketones detected in the spectra of essential oils.

Additional key words: hop (*Humulus lupulus* L.)**,** hop latent viroid (HLVd), pathogenetic effects, hop resins and essential oils.

Introduction

Hop latent viroid (HLVd) was characterised by Puchta *et al.* (1988) as world-wide distributed hop pathogen including the most countries in the Europe, Asia, Africa and America. It follows from our previous work that HLVd spreading and re-infection occurs in some materials with very high rate. For instance, the increase of HLVd incidence in Czech Osvald's clone 72 from zero to 65 % within three years was observed (Matoušek and Patzak 2000). In this respect two practical questions have to be solved. The first problem is dealing with the elimination of HLVd (Patzak and Matoušek, 1999) and the second question is dealing with the real assessment of "latency" or "pathogenicity" of HLVd infection in different hop cultivars and genotypes.
Despite the absence of characteristic morphological symptoms of infection, HLVd infection has been associated with changes in the composition of some secondary metabolites in lupulin glands (Barbara *et al.* 1990, Adams *et al.* 1991, 1992) suggesting great practical significance of HLVd. Adams *et al.* (1991) reported that viroid infection significantly decreased α-bitter acid contents approximately by 11 %, increased β-bitter acid contents approximately by 8 % and myrcene approximately by 38 %. In addition, the cones of viroid infected hops were by 11 % smaller than those from viroid-free plants. Adams *et al.* (1992) reported a decrease in α-bitter acid contents ranging from 20 - 50 % varying with cultivar. These results suggest genotype-dependent effects of HLVd infection, however, neither HLVd structure responsible for this reaction has been identified, nor detailed analysis of biochemical response of the HLVd infected host plants has been performed. Such study appeared to be rather problematic, because it required a complete elimination of virus infections, which could interfere with viroid pathogenesis.
In our previous work, we partly characterised horizontal (Matoušek *et al.* 1994) and vertical (Matoušek *et al.* 1995) distribution of HLVd in Czech hop Osvald's clones 31 and 72. However,

no information was available about the influence of HLVd infection on lupulin composition of these economically important clones. In this study we use viroid-free mericlones, which provided suitable genetically invariant materials for detailed study of the influence of viroid infection on chemical characteristics of lupulin.

Material and methods

One viroid-free mericlone of Osvald's clone 114, one of Osvald's clone 31, and one of cv. Premiant were selected for experiments. In addition also two viroid-free mericlones of Osvald's clone 72, which have been grown in *in vitro* conditions for 10 years, were used. Four re-infected and healthy plants of every meristem clone were analysed for their composition of hop resins and essential oils. HLVd infection was quantificated by sensitive method of RNA-RNA dot blot hybridisation using ^{32}P[UTP]-labelled HLVd riboprobe (Matoušek *et al.* 1995) and by means of *STORM PhosphorImager* device and *ImageQuant* software (*Molecular Dynamics*, Sunnyvale, USA). All plants were also tested by enzyme-linked immunosorbent assay (Clark and Adams 1977) for virus infections with 12 viruses. Lupulin compounds were analyzed in cones collected as mixed samples from either healthy or infected plants. Hop resins composition was determined by high pressure liquid chromatography (HPLC) according to *EBC 7.7* procedure (1997). Hop essential oils were isolated from hops by steam distillation method. Analyses were performed on gas chromatography–mass spectrophotometry system *Varian 3400* + *Finnigan ITD 800* mass detector (Walnut Creek, USA). Contents of various compounds in healthy and infected cones were compared using the *t*-test.

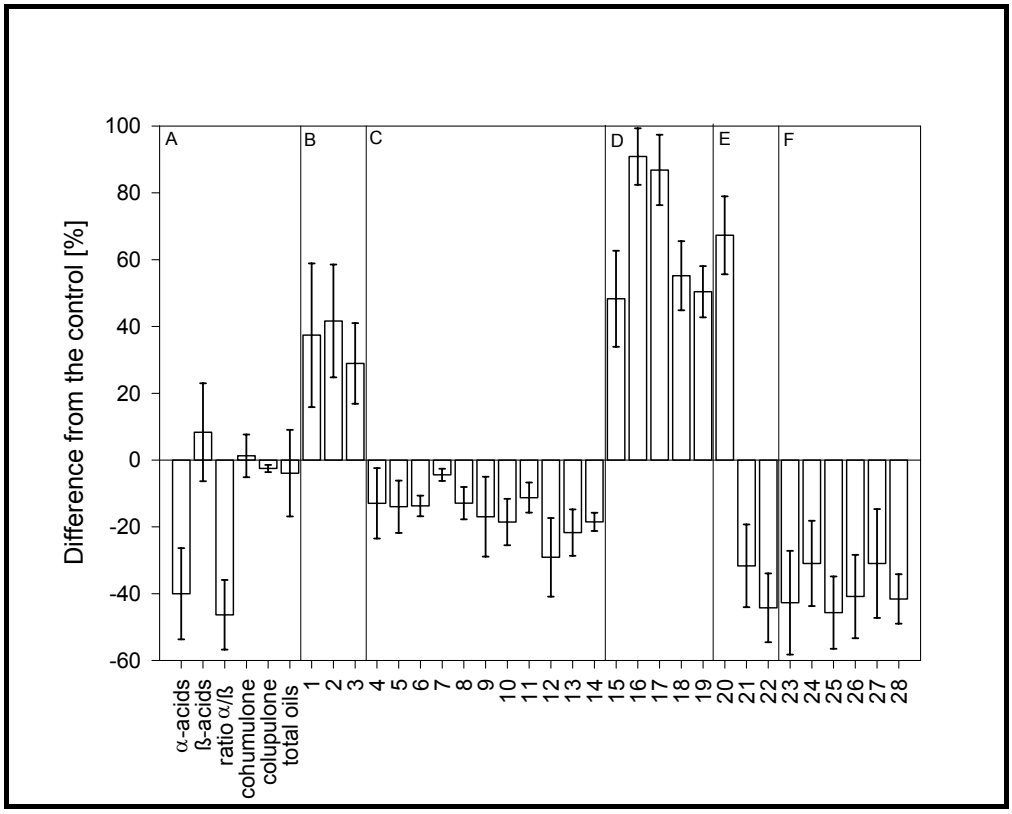
Results

The field grown viroid-free mericlones of Osvald's clone 31 and 114, cv. Premiant and two control viroid-free mericlones of Osvald's clone 72 were used for evaluation of the influence of HLVd on the contents of hop resins and essential oils in the first production year. In the first year of hop growing in the field conditions, on average 0.4 pg HLVd per mg (f. w.) was detected in approximately 25 % of naturally re-infected plants, which were then selected as positive controls. In the second year (i.e. in the first production year) the viroid content in these plants increased to an average of 1.8 pg (HLVd) mg^{-1} (f. w.).

Viroid infection led to a significant reduction of the content of α-bitter acids, 40 % in the average (Fig. 1a). While the decrease in the content of α-bitter acids in infected plants was the major effect for the most of the mericlones, there was also some tendency observed in the increase of β-bitter acid contents (Fig. 1a). The α/β ratio showed clear tendency to be lower in infected materials (Fig. 1a). It is interesting to note that contents of cohumulone and colupulone were very stable and independent of viroid infection. The second important class of chemical compounds in hop cones are essential oils. Although their content is relatively small, there are around 200 individual components. The total content of essential oils was not significantly lower in cones of viroid-infected plants than in healthy plants (Fig. 1a), but the contents of some components were significantly different. For instance, the content of myrcene increased about 29 % in infected plants compared with healthy controls (Fig. 1b). Moreover the contents of α-pinene and β-pinene also increased and were, on average, 37.4 % and 41.6 % higher, respectively, in infected plants than in healthy controls. The contents of limonene and ocimene varied independently upon infection (Fig. 1b). Another group of compounds forming essential oils are sesquiterpenes. The content of most sesquiterpenes was significantly lower in infected than in healthy plants (Fig. 1c). For instance, the sesquiterpenes α-copaene, γ-muurolene, β-bisabolene, γ-cadinene and δ-cadinene were lower by 14, 18.5, 29, 21.7, and 18.5 %, respectively, in infected plants than healthy controls (Fig. 1c). While contents of β-caryophyllene and α-humulene in the infected plants decreased, there was significant increase of their epoxides (Fig. 1d). Some terpenes alcohols, mainly geraniol and methylgeranate (Fig.

1d) which are often missing in healthy plants are present in infected materials. It is interesting to note that significant fraction of ketones is reduced due to infection (Fig. 1f). Some changes in composition of esters (Fig. 1e) were also observed. For instance, 1-octen-3-yl acetate and methyl dekanoate show clear tendency to decrease in infected mericlones, while the content of methyl non-6-enoate was enhanced in all infected materials. The contents of most esters identified show rather high variability independently on HLVd infection. The contents of limonene, ocimene, α-bergamotene, β-selinene, isobutyl isobutyrate, 2-methyl butyl-isobutyrate, methyl heptanoate, methyl 6-methylheptanoate, methyl oktanoate, methyl nonanoate, methyl deca-4-enoate, methyl deca-4,8-dienoate did not significantly changed.

Fig. 1. Influence of HLVd infection on contents of hop resins and essential oils. The graphs show differences in the contents of individual compounds in hop cones of infected plants *versus* healthy controls. The differences are expressed in percents: a) hop resins, b) monoterpenes - hydrocarbons, c) sesquiterpenes - hydrocarbons, d) terpenes - alcohols, e) esters and f) ketones. Individual compounds of essential oils: 1 - α-pinene, 2 - β-pinene, 3 – myrcene, 4 - α-ylangene, 5 - α-copaene, 6 - β-caryophyllene, 7 - β-cubenene, 8 - α-humulene, 9 - β-farnesene, 10 - γ-muurolene, 11 - α-selinene, 12 - β-bisabolene, 13 - γ-cadinene, 14 - δ-cadinene, 15 - linalool, 16 - geraniol, 17 - methylgeranate, 18 - caryophyllene epoxide, 19 - humulene epoxide-I, 20 - methyl non-6-enoate, 21 - 1-octen-3-yl acetate, 22 - methyl dekanoate, 23 - 2-nonanone, 24 - 2-decanone, 25 - 2-undecanone, 26 - 6-undecanone, 27 - 2-dekanone, 28 - 2-tridekanone. Confidence intervals are given at α=0.05.



Discussion

Biochemical analyses revealed that that viroid infection led to a significant reduction of the content of α-bitter acids, while contents of β-bitter acids slightly increased or remained unchanged in infected plants. Our finding agrees with the results of Adams *et al.* (1991, 1992). It is interesting to note, that contents of cohumulone and colupulone were very stable and independent of viroid infection. It is known that the ratio of the homologues of bitter acids is in

general a relatively stable genetic trait (Peacock and McCarty 1992). The stability of the ratio of humulone/cohumulone and lupulone/colupulone could mean that viroid infection has no significant influence on expression and catalytic activity of phlorisovalerophenone synthase, which was recently identified by Paniego *et al.* (1999). This enzyme is involved in the synthesis of basic precursors for both analogues. The humulone/lupulone and cohumulone/colupolone ratios could most probably be influenced *via* activity of some oxido-reductase, which could convert β- to α-bitter acids (Zuurbier *et al.* 1995). However, the putative enzyme has not been yet identified.

The second important class of chemical compounds in hop cones are essential oils. Every slight difference in essential oils can distinctly change aroma of hop and beer. The results of analysis of essential oils included 40 main components. The total content of essential oils was not significantly lower in cones of viroid infected plants than in healthy plants, but the contents of some components were significantly different. Adams *et al.* (1991) reported only about distinctive increase of myrcene. We found in addition that the contents of α-pinene, β-pinene also increased due to infection, while the contents of limonene and ocimene varied independently upon infection in spite of the fact that they are direct metabolites of myrcene (Neve 1991). According to published biochemical routes of terpene biosynthesis (Dieckmann and Palamand 1974) one can assume that myrcene was more rapidly converted in infected plants especially to linalool and geraniol than to limonene and ocimene. Another group of compounds forming essential oils are sesquiterpenes. The content most sesquiterpenes, which are involved in the same biosynthetic pathway (Tressl *et al.* 1987), was obviously lower in infected than in healthy plants. The decrease of content of sesquiterpenes could be correlated to the increasing content of monoterpenes, which are synthesised from the same precursor. The decrease of β-caryophyllene and α-humulene in the infected plants could be also correlated with the increase of their epoxides (Neve 1991). Very similar changes in the composition of essential oils, i.e. the increase of the content of myrcene and the general decrease of sesquiterpenes, was observed during hop maturation (Murphey and Probasco 1996, Menary and Doe 1983, Skinner *et al.* 1974). However, unlike to processes observed during hop maturation, viroid infection clearly caused the decrease of α-bitter acids. The possible influence of viroid infection on activation of some oxidative-reduction processes could lead to the increase of contents of terpenes alcohols mainly geraniol and methylgeranate, which are often missing in healthy plants. It is interesting to note that significant fraction of ketones is reduced due to infection. The conversion of ketones could be also caused by increasing activity of some oxidative pathway in viroid infected plants. The interpretation of changes in composition of esters is very problematic, because the information about their synthesis is still unknown. Some of them like 1-octen-3-yl acetate and methyl dekanoate show clear tendency to decrease in infected mericlones while the content of some like methyl non-6-enoate was enhanced in all infected materials.

Present observations suggest that HLVd pathogenesis probably promotes some disbalance in expression of host components involved in the synthesis of the secondary metabolites.

Acknowledgements

Authors would like to thank Prof. Detlev Riesner (Institut of Physical Biology, Heinrich-Heine University, Düsseldorf, Germany) for his help and fruitful discussion and to Dr. Rudra P. Singh (Potato Research Centre, Agriculture and Agri-Food Canada, Federicton, New Brunswick, Canada) for his critical review of the manuscript. We thank Ms. Ivana Malířová and Mrs. Světlana Brabcová from Hop Research Institute, Žatec, Ing. Lidmila Orctová, Mgr. Lukáš Vrba and Mrs. Helena Matoušková from Institute of Plant Molecular Biology AS CR, České Budějovice for their help and excellent technical assistance.
This work was supported by the National Agency for Agricultural Research of the Ministry of Agriculture of the Czech Republic, project No. EP 0960996299 and EP 1183, and by the Grant Agency of the Czech Republic grant No.521/99/1591.

References

Adams, A.N., Barbara, D.J., Morton, A.: Effects of hop latent viroid on weight and quality of the cones of the hop cultivar Wye challenger. - Ann. appl. Biol. **118** (Suppl.): 126-127, 1991.

Adams, A.N., Morton, A., Barbara, D.J., Ridout, M.S.: The distribution and spread of hop latent viroid within two commercial plantings of hop (*Humulus lupulus*). - Ann. appl. Biol. **121**: 585-592, 1992.

Barbara, D.J., Morton, A., Adams, A.N., Green, C.P.: Some effects of hop latent viroid on two cultivars of hop (Humulus lupulus) in the UK. - Ann. appl. Biol. **117**: 359-366, 1990.

Clark, M. F., Adams, A.N.: Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. - J. gen. Virol. **34**: 475-483, 1977.

Dieckmann, R.H., Palamand, S.R.: Autooxidation of some constituents of hops. I. The monoterpene hydrocarbon, myrcene. - J. agr. Food Chem. **22**: 498-503, 1974.

Matoušek, J., Patzak, J.: A low transmissibility of hop latent viroid through a generative phase of *Humulus lupulus* L. - Biol. Plant. **43**: 145-148, 2000.

Matoušek, J., Trněná, L., Svoboda, P., Růžková, P.: Analysis of hop latent viroid (HLVd) in commercial hop clones in Czech Republic.- Rost. Výroba (Praha) **40**: 973-983, 1994.

Matoušek, J., Trněná, L., Svoboda, P., Růžková, P., Lichtenstein, C.P.: The gradual reduction of viroid levels in hop mericlones following heat therapy: a possible role for a nuclease degrading dsRNA. - Biol. Chem. Hope-Seyler. **376**: 715-721, 1995.

Menary, R.C., Doe, P.E.: Some morphological and chemical changes in hops during maturation. - J. Sci. Food Agr. **34**: 921-929, 1983.

Murphey, J.M., Probasco, G.: The development of brewing quality characteristics in hops during maturation. - MBAA Tech. Quart. **33**: 149-159, 1996

Neve, R.A.: Hops. - Chapman and Hall, London 1991.

Paniego, N.B., Zuurbier, K.W.M., Fung, S.-Y., Heijden, R., Scheffer, J.J.C., Verpoorte, R.: Phlorisovalerophenone synthase, a novel polyketide synthase from hop (*Humulus lupulus* L.) cones. - Eur. J. Biochem. **262**: 612-616, 1999.

Patzak J., Matoušek J.: The elimination of hop latent viroid (HLVd) infection by meristem *in vitro* culture of hop (*Humulus lupulus* L.). - Proccedings of Scientific Commission of IHGC, Pulawy, Poland, July 27 - 30, p. 124 – 129, 1999.

Peacock, V.E., McCarty, P.: Varietal identification of hops and hop pellets. - MBAA Tech. Quart. **29**: 81-85, 1992.

Puchta, H., Ramm, K., Sanger, H.L.: The molecular structure of hop latent viroid (HLVd), a new viroid occurring worldwide in hops. - Nucl. Acids Res. **16**: 4197-4216, 1988.

Skinner, R.N., Kavanagh T.E., Hildebrand, R.P., Clarke, B.J.: Hop maturation and resin formation: Field trials. - J. Sci. Food Agr. **25**: 1121 - 1133, 1974

Svoboda, P.: [Clonal propagation of hop *in vitro*.] - Rost. Výroba (Praha) **37**: 705-708, 1991. [In Czech]

Tressl, R., Kossa, M., Köppler, H.: Changes of aroma components during processing of hop. – In: EBC monograph XIII - Symposium on Hops. Pp. 116-128. Elsevier Scientific Publishing Company, Amsterdam 1987.

Zuurbier, K.W.M., Fung, S.-Y., Scheffer, J.J.C., Verpoorte, R.: Formation of aromatic intermediates in the biosynthesis of bitter acids in *Humulus lupulus*. - Phytochemistry **38**: 77-82, 1995.

DEVELOPMENT OF A CONTROL THRESHOLD FOR THE TWO-SPOTTED SPIDER MITE (*Tetranychus urticae*) IN HOPS OF THE HALLERTAU GROWING REGION

Florian Weihrauch
Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Abschnitt Hopfen
Hüll 5 1/3, D-85283 Wolnzach, Germany

Abstract

A research project was conducted during three field seasons from 1998-2000 in order to develop a control threshold model for two-spotted spider mites (*Tetranychus urticae*) in hops of the Hallertau growing region. The project included the monitoring of over 100 hop gardens per year considering the cultivars Hallertauer Magnum, Hallertauer Tradition and Perle. In each hop garden an acaricide-untreated plot was established which served as a control for unrestricted spider mite population development. Spider mites were monitored at least twice a year, once in mid-June and shortly before harvest. The study included the development of a six-stage infestation index for spider mites in order to facilitate field monitoring. The results indicate that in the Hallertau 60% of the applications of acaricides are merely prophylactic and would not have been necessary. A dynamic control threshold model is introduced which is able to reduce the applications of acaricides by approximately 20% by two field censuses per hop garden. Practical problems in operating this model are listed and discussed.

Introduction

For the control of the two-spotted spider mite (*Tetranychus urticae* KOCH, 1835) in hops, farmers in Europe generally have no alternative other than applying acaricides in a preventative manner, usually on a very personal decision in line with their previous experience with the pest. In the Hallertau this has led to a prophylactic routine application by which today approximately two thirds to three fourths of the area under cultivation are treated regularly once a year with acaricides. Often, this is done without any preceding check for an actual infestation event. Due to this tendency, the annual costs for acaricides in the Hallertau hop growing region alone have reached a level of approximately 2.5 million € in the meantime. In the light of these facts, the development of a dynamic threshold model for the control of the spider mites seemed to be absolutely crucial. Preferably, such a control method would provide farmers with a tool enabling them to decide on objective and sound ground whether a prophylactic use of acaricides is actually necessary or not. Successful implementation of such an approach would reduce the overall amount of applied acaricides, thereby not only reducing the actual costs for farmers, but also providing another piece in the puzzle of environmentally more compatible integrated pest management measures.

The overall study is subject to a Ph.D. thesis which is due to be published in spring 2002 and should be used for more detailed information.

Project design

In the three field seasons from 1998 to 2000, a monitoring programme for *T. urticae* was run in more than 100 hop gardens which were distributed evenly over the Hallertau growing region. The hop gardens included the three cultivars Hallertauer Magnum (HM), Hallertauer Tradition (HT) and Perle (PE) in equal proportions. Each of these gardens had been regularly treated with acaricides in former years. An experimental plot of 84 hop plants with approximately 168 trainings was laid out in each garden at the beginning of the programme. This control plot was left acaricide-untreated throughout all three years, and was compared to the rest of the field. Pests and diseases other than *T. urticae* were controlled in the plots according to the respective farmer's routine, in the case of possible influences of other pesticides on spider mites with prior consultation. The treatment of the rest of the gardens was left completely to the farmer's decision.

In general, each garden was monitored shortly before the first application of an acaricide, and finally shortly before the harvest. The monitoring procedure was conducted separately for the control plot and the acaricide-treated rest of the garden. A number of plots was also monitored in between these two dates. The number of assessed leaves was 20 per date and sampling event, respectively. The leaves were taken randomly within the garden or the plot from the lower part of the bines in June, and from the middle and upper regions of the bines in July and August. During the entire project approximately 42.000 leaves were assessed and further analysed. In twelve gardens per year (four of each cultivar, respectively) an experimental harvest was carried out, comparing yield and quality of the acaricide-treated hops with the untreated plot in view of the infestation level at harvest date.

Development of an infestation index for *T. urticae* in hops

So far, the monitoring of spider mites in German hop research consisted of the exact counting of live animals up to a number of 100 per leaf and an estimation of higher numbers per leaf. Due to three reasons, this mode of action seemed not appropriate for the development of a practical forecasting system: First, an exact identification and counting of spider mites, especially younger stages, cannot necessarily be expected from growers and is very time consuming. Second, the infestation level of e.g. 60 and 85 spider mites per leaf is practically the same, and extremely high outliers are levelled out. Third and most important, the mere counting of spider mites does not consider their eggs which represent an important part within a pest forecasting system. Under consideration of these problems, a six-stage infestation index was developed. This index contains classified estimations of the numbers of spider mites and their eggs per leaf, assigning each monitored leaf a value from nil to five. The values of the leaves are summarised and divided through the number of leaves, thus receiving a value in the range from 0.00 to 5.00 which provides a handy value for the spider mite infestation level. Even inexperienced persons can cope with this method by only little training. The classifications of spider mite and egg numbers in this index are given in Table 1.

Table 1: Classifications of spider mites and their eggs in a six-stage infestation index

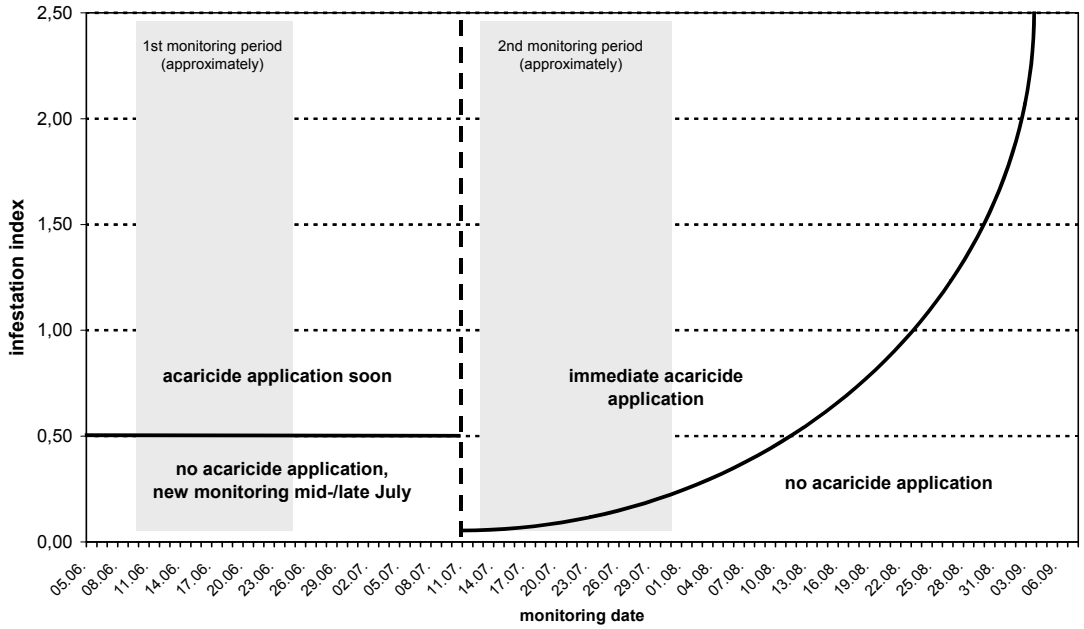
Infestation Index	Number of spider mites plus eggs per leaf (x = few/some eggs, xx = many eggs, xxx = lots of eggs)
0	0
1	0x, 1-9x
2	0xx, 1-9xx, 10-49x
3	0xxx, 1-9xxx, 10-49xx, 10-49xxx, 50-99x, 50-99xx
4	50-99xxx, 100-999x, 100-999xx
5	100-999xxx, 1000>

The control threshold model

Based on the results of the 36 experimental harvests in three years, an infestation index of 2.00 for cultivars PE and HT, and 2.20 for cultivar HM at harvest time was determined which are the values below which the crop is in absolutely no danger of any quantitative or qualitative damage. Possibly, even higher values must not necessarily lead to losses in yield or quality. Assuming that an exponential curve of spider mite population growth represents a "worst case scenario", the population has to start its build-up around mid-July in order to reach a maximum infestation index of 2.00 to 2.20 at harvest time. (Fig. 1). This means that any monitored infestation index of a hop garden in middle or late July being below the curve in Fig. 1 will not cause any economic damage. On the other hand, infestations indices of 0.00 in June under certain conditions may eventually reach a value at harvest high enough to bring about quantitative and/or qualitative losses. It must therefore be concluded that infestation indices of *T. urticae* obtained before mid-July do not allow an exact prediction of yield losses within the framework of the presented approach.

These considerations have led to a control threshold model which requires two monitoring-based decisions whether an application of acaricides is actually necessary or not, one in mid-June and another in mid- or late July. On the first occasion, all hop gardens with an infestation index of 0.50 or higher are recommended for acaricide treatment, as the project data indicate that such a value almost inevitably leads to a population development which can damage the crop. Hop gardens with infestation indices below this value are recommended to be left untreated until the second monitoring in July. On this second occasion, a final decision for an immediate or for no application has to be made, where the respective value can be taken from the curve in Fig. 1 according to the date - the later, the better. Of course, it must be considered that the date of this final decision has to be in accordance with a number of other factors such as cultivar, harvest time and the period of delay of the acaricide as well as weather conditions allowing a prompt application.

Figure 1:
Control threshold model for *Tetranychus urticae* in hops



Problems and perspectives in establishing the model

During winter 2000/2001, about 15 talks were given to growers and representatives of plant protection and agricultural commerce companies to introduce the model. The responses were generally positive and the acceptance from the growers´ side was very good. On the other hand, there are certainly a number of factors which will provide difficulties for the successful establishing of the model in practice:

- A successful converting of the model into practice includes that the necessary monitoring is carried out by the growers who, consequently, have to be trained to recognize spider mites and their eggs as well as to handle the monitoring pattern and the infestation index satisfactory. In the 2001 field season, it is planned to train a number of persons who later can serve as multipliers for this task (e.g. agricultural advisory officers, private advisory circles and advisers of commercial plant protection companies).
- The indispensable exact monitoring requires farmers who are willing to devote time to this task.
- Prophylactic acaricide applications in mid-June can be done with less amount of water and pesticide than in mid-July, due to the different growth stages of the hop plants. Some growers have to be convinced that the model still leads to a reduction of acaricides.
- The periods of delay of in Germany registrated acaricides range from 35 days for amitraz, which currently is most commonly used in the Hallertau, to 28 days for hexythiazox or abamectin and 21 days for fenpyroximate. This has to be taken into account with an application late in July, especially for early harvested cultivars.
- Growers in the Hallertau usually are in constant fear of *T. urticae*, as it generally is regarded as the most unpredictable pest organism. They have to be convinced that they are not taking any risks when they are doing without acaricides, provided that the model is used correctly.

Considering these problems, it becomes evident that the establishing of the model into practice cannot be done within a short time. The training of multipliers will take at least one field season, and probably a number of reliable, respected growers must use the model successfully for some time until more sceptical growers will be ready to accept it. On the other hand, the data of the described project have shown that in general 60% of the acaricide applications would not have been necessary and, by the use of the model, approximately 20% of the applications could have been spared. Regarding the sum of approximately 2.5 million € which currently is spent for acaricides in the Hallertau per year, this seems to be the most important argument for a successful converting of this model into practice within the next few years.

Acknowledgements

This work was funded by Deutsche Bundesstiftung Umwelt, Osnabrück, Germany. For three years of most valuable assistance in the field my thanks are due to Olga Ehrenstrasser, Maria Felsl, Maria Fischer, Silvia Hagl and Klaus Nöscher.

INTEGRATED MANAGEMENT OF DAMSON-HOP APHID ON DWARF HOPS.

C A M Campbell
Horticulture Research International, East Malling, Kent, ME19 6BJ, England

Abstract

A single soil application of imidacloprid provided acceptable control of aphids on First Gold in field experiments in 1998 and 1999. Predators regulated the numbers of aphids as the effects of the aphicide diminished. Yields of dried hops and their alpha acids content were unaffected by the numbers of aphids found on the crop in June and July. The numbers of aphids infesting cones reduced the value of untreated First Gold and plants treated with less than the full dosage of imidacloprid. Few aphids colonised an aphid-resistant genotype and no economic benefit was obtained from applying imidacloprid.

Introduction

All current hop cultivars are susceptible to attack by the damson-hop aphid (*Phorodon humuli* (Schrank)), the most serious pest of hops in England and an important pest in all other major hop-growing regions except Australasia. Feeding by *P. humuli* can debilitate hop plants and may reduce yields (Neve, 1991), sometimes totally (eg Campbell, 1978). Contamination of the harvested cones by aphids or their remains may also decrease the monetary and brewing values of the crop (Neve, 1991). In recognition of the threat posed by *P. humuli,* hops have been sprayed in England for more than 300 years (Meager, 1697). Spraying with aphicides became routine about 1865 (Whitehead, 1890). Owing to the high selection pressure applied, since the 1960's, *P. humuli* has developed resistance to successive groups of synthetic pesticides across Europe (see Campbell & Hrdy, 1988). In order that the pattern of pesticide usage which induces resistance may be broken, alternative strategies are needed to provide “an adaptable range of pest control methods which is cost effective whilst being environmentally acceptable and sustainable” (Perrin, 1997). Darby and Campbell (1996) argued that the lack of an aphid-resistant cultivar was the key factor inhibiting effective integrated management of pests on hops.

Breeding hop cultivars resistant to key fungal diseases has been routine in Europe for decades (Neve, 1991), but a similar programme for aphids begun in the 1960's was hindered by a lack of strong sources of genetic resistance (Campbell, 1983). A strong source of partial resistance was identified in a wild Japanese male hop in the 1980's, which has since proved heritable in female offspring (Darby & Campbell, 1996). In laboratory studies, aphids on the resistant genotype had poorer survival rates, slower nymphal development, lower reproductive rates, and lower fecundities than aphids on susceptible cultivars (Campbell et al., 1993).

The present study compares chemical, integrated and biological control of *P. humuli* on a representative aphid-susceptible dwarf hop cultivar ('First Gold') and an aphid resistant genotype '23/90/08'. Chemical control of aphids (and two-spotted spider mites) was by the application of one or more sprays of tebufenpyrad. For the integrated programmes the effects of single soil-applications of imidacloprid at three doses were compared. *Phytoseiulus persimilis* was released for the control of *T. urticae* (Campbell & Lilley, 1999).

Methods

Each plot consisted of a row of 66 plants spaced 0.5 m apart. Plots were separated by 2.5 m bare ground between rows and by not less than 10 m bare ground between experiments. The bines were supported on polypropylene netting (14 cm square mesh) suspended vertically between

parallel horizontal wires at 0.15 m and 2.5 m above ground level. The five treatments in each experiment were arranged in randomised complete block designs, replicated five-fold. Treatments were; single applications to the soil and stem bases of imidacloprid in 100 ml water per plant at (a) the full approved dose (178.5 g ha^{-1}), (b) half dose (89 g ha^{-1}), and (c) quarter dose (44.5 g ha^{-1}), (d) foliar spray(s) of tebufenpyrad at the manufacturer's recommended doses for control of damson-hop aphid (1500 g ha^{-1} in 1000 l water ha^{-1} at first and then 1200 g ha^{-1} in 500 l water ha^{-1} for a later application, if needed), and (e) untreated. The detection of any adult aphids on a leaf sampled from tebufenpyrad-treated plots would trigger further sprays of that pesticide. *Phytoseiulus persimilis* was released at 10 individuals per plant during May in all plots for the control of *T. urticae*. Tebufenpyrad is acaricidal at doses below those used against aphids on hops, so the sprays used for aphid control here would suppress *T. urticae* also.
Twenty fully expanded main-bine leaves from near the bine apices were sampled per plot per week for aphids during 1998, and ten per plot in 1999. The numbers of damson-hop aphids, spider mites and their natural enemies were recorded for each leaf. At harvest, a sample of 50 hop cones was collected from each plot. The cones were dissected in the laboratory under a stereomicroscope and the number of aphids in each cone was recorded. Plots were harvested during the first week of September using a mobile harvester and then cleaned. The weight of fresh hops was recorded and a weighed sub-sample was dried to 10% moisture content. The % alpha-acids in the sub-samples were measured by hplc. Yields of 'First Gold' were assessed in both years, but those of '23/90/08' were assessed in 1999 only.

Results

Imidacloprid was applied to the soil and base of bines on 13 May 1998 and on 21 June 1999. Foliar sprays of tebufenpyrad were applied on 28 May 1998 and on 15 June 1999.

'First Gold'

Aphid densities reached peaks in early June on all treatments (Figure 1). The lowest numbers of aphids were recorded on plots treated with tebufenpyrad and with imidacloprid at the full dose. There were no significant differences between these treatments post-application in any week during 1999, but in 1998 significantly fewer aphids were found on the tebufenpyrad plots (<20 aphids per leaf throughout the summer), than on full-dose imidacloprid ones during June. No significant differences in aphid numbers were recorded between untreated plots and those treated with either half- or quarter-dose imidacloprid in either year. The highest aphid densities were recorded in 1998 with means fluctuating around 100 aphids per leaf on untreated and the quarter and half dose imidacloprid plots until early July before falling to low levels in all treatments. In 1999 numbers barely exceeded ten per leaf in any treatment, and any differences between treatments were small. The numbers of aphids on untreated plots remained higher for longer than on other treatments, but every year they fell to low levels around mid- to late-July in all treatments. Individual aphids were still found occasionally up to when foliar sampling ended in mid-August, and in 1998 there were indications of weak population resurgence on untreated plots.
The patterns of infestations of cones from each treatment reflected that on foliage at the time of flowering in early July of that year (compare Table 1 with Figure 1a, b), nevertheless, the relationship was not straightforward. Less than a mean of one aphid per leaf was found on untreated plots in July 1999 and 50 per leaf in July 1998, yet cone infestations were similarly high in both years and the numbers of aphids in cones varied inversely with the dosage of imidacloprid. In 1999, the percentage of infested cones from untreated plots was over five times greater than that from the plants treated with the full dose of imidacloprid and the difference in numbers of aphids was more than 15-fold. However, unlike 1998, cones from plots sprayed with tebufenpyrad in 1999 were significantly more heavily infested than those from the full and the half doses of imidacloprid. Despite the absence of adult aphids from leaves sampled in tebufenpyrad plots after the initial treatment, a second spray might have reduced infestation of cones in 1999.

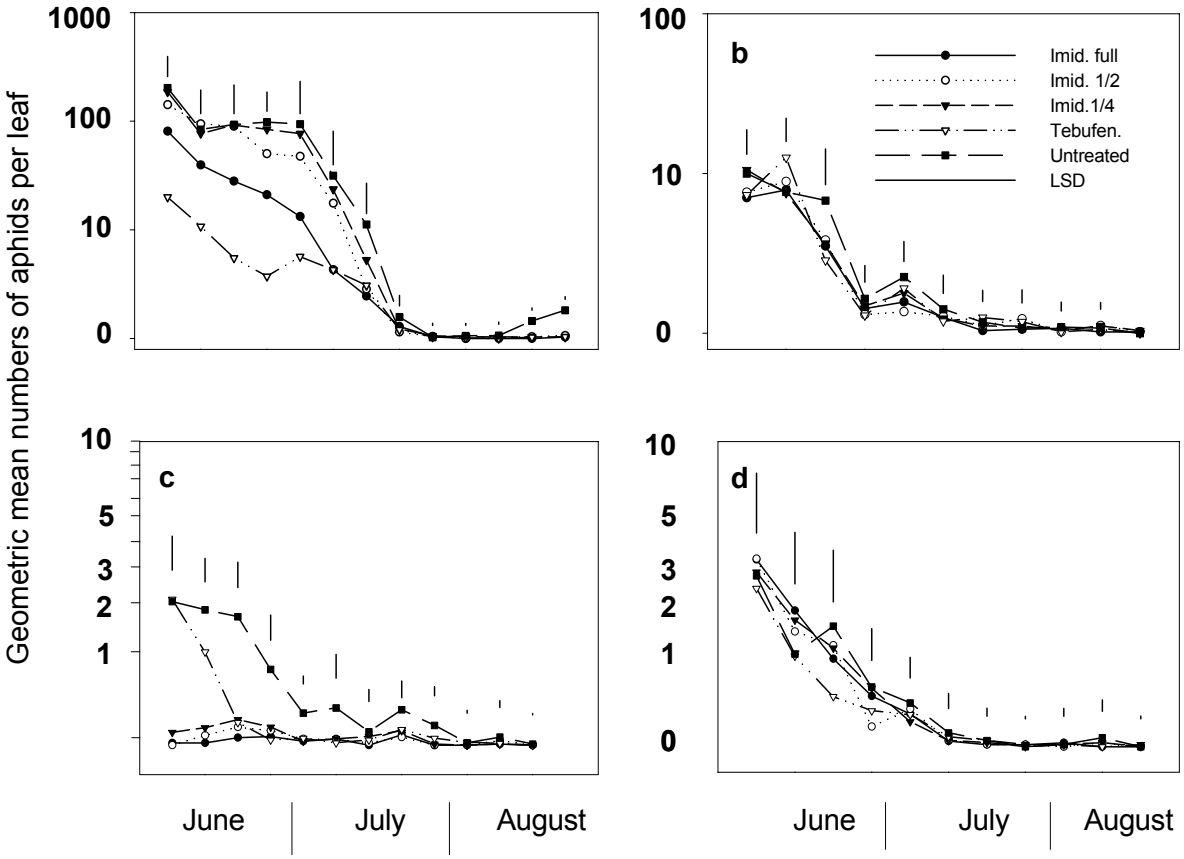


Figure 1. Population development of *Phorodon humuli* on 'First Gold' in a) 1998 and b) 1999 and on '23/90/08' in c) 1998 and d) 1999.

'23/90/08'

Numbers of aphids remained below a mean of two per leaf in 1998 and four per leaf in 1999 (Figure 1c, d). Although the mean numbers on untreated plants were significantly higher than those on other treatments on several sampling dates during June and early July 1998, the low numbers of aphids recorded on untreated plants were of no practical importance. In 1999, the numbers declined at similar rates on all treatments from a peak of just over four per leaf at the beginning of June to virtually nil by mid-July and remained low thereafter.

Despite the low numbers of aphids found on foliage throughout July 1998, some infestation by aphids of the cones was detected (Table 2). None was infested from plants treated with the full-dose imidacloprid and few aphids were found in cones from the half- and quarter-dose imidacloprid and tebufenpyrad treatments. Significantly more aphids were found, and a higher percentage of cones was infested, from untreated plants than other treatments. Nevertheless, the numbers of aphids in cones from the untreated plants were unlikely to be detectable during an unaided visual evaluation of the dried crop and would not affect the valuation. Only one cone was infested by aphids (a total of 12 aphids) of the 1250 examined in 1999 so a statistical analysis of the data was not justified. No significant differences were recorded between treatments in the weights of dry hops, nor in their content of alpha-acids. As expected from a non-commercial early selection, alpha-acids levels were low compared to those from 'First Gold'.

Table 1 Geometric mean infestations of cones by *P. humuli*, dry weight yields (kg ha^{-1}) and % alpha acids of 'First Gold' in 1998 and 1999.

Treatment	1998				1999			
	Aphids per 100 cones	Cones infest. %	Yield	Alpha %	Aphids per 100 cones	Cones infest. %	Yield	Alpha %
Imidacloprid full	46 b	32 b	780 a	9.8 a	16 c	12 d	787 c	8.0 a
Imidacloprid ½	66 b	39 b	633 a	9.6 a	44 c	27 c	780 c	8.0 a
Imidacloprid ¼	241 a	72 a	610 a	9.6 a	148 b	46 b	922 b	8.4 a
Tebufenpyrad	64 b	40 b	742 a	9.7 a	165 b	58 ab	970 ab	8.3 a
Untreated	216 a	70 a	688 a	9.8 a	278 a	67 a	1035 a	8.2 a
LSD (5%)		24.6	205.4	0.42		13.3	70.5	0.77

Means followed by the same letter in each column were not significantly different (P<0.05).

Table 2 Geometric mean infestations of cones by *P. humuli* in 1998 and 1999 on '23/90/08', and the dry weight yields and % alpha acids in 1999.

Treatment	1998		1999			
	Aphids per 100 cones	Cones infested %	Aphids per 100 cones	Cones infested %	Yield (kg ha^{-1})	Alpha %
Imidacloprid full	0	0	0	0	838.2 a	5.6 a
Imidacloprid ½	2.5 b	1.2 b	0	0	756.9 a	5.4 a
Imidacloprid ¼	2.3 b	2.0 b	0	0	755.7 a	5.9 a
Tebufenpyrad	8.9 b	5.6 b	0	0	687.1 a	5.7 a
Untreated	20.0 a	15.2 a	1.0	0.4	737.9 a	5.5 a
LSD (5%)		4.63			152.9	0.5

Discussion

Similar patterns of population development occurred each year. The annual influx of migrant aphids from *Prunus* spp started in May and aphid population densities on foliage reached a peak usually in June before declining around mid-July in all treatments. The formulation of imidacloprid as a soil drench for hops in England minimises any potential harm to naturally occurring predators of aphids and mites. As in previous studies (Campbell, 1983, Campbell & Cone 1994) aphids were virtually eliminated from the foliage by predators after the toxic effects of the pesticide diminished. Although the times of peak numbers of aphids on each treatment usually coincided within any year, the peak aphid population densities varied between years, between treatments, and between the two hop genotypes.

Despite the higher numbers of aphids recorded on untreated and quarter dose imidacloprid plots of 'First Gold' than in some other treatments, yields were not depressed by aphids. Indeed, although some of the lowest aphid numbers were recorded on plots treated with full dose imidacloprid, that treatment produced lower yields than the control on ‘First Gold’ in 1999. On neither cultivar was the alpha acids content of cones affected by aphids on leaves or cones. If the aim was simply to maximise production of alpha acids for extraction (Hall, 1971), then a quarter dose of imidacloprid might be adequate on 'First Gold', as at no time did the presence of aphids on the crop in that treatment reduce yields. However, for conventionally marketed crops, where aphid contamination affects the valuation, the increased financial risk from reducing the dosage of imidacloprid to less than the recommended is unlikely to be acceptable. Fewer aphids were found on untreated plants of the aphid-resistant genotype than on 'First

Gold' in both years. No significant differences were recorded in aphid numbers among any of the treatments applied to '23/90/08' during 1999. All of the pesticide treatments reduced the numbers of aphids on leaves below those on untreated plots during 1998, however, numbers on untreated plants never exceeded three per leaf throughout the summer, too few to affect yields detrimentally. The results indicate that the impact of the inherent resistance of '23/90/08' on *P. humuli* was equivalent in magnitude to that of a single application of either of the two aphicides. In neither year were aphids sufficiently numerous in any treatment to damage yields or quality, showing that a commercial cultivar possessing a similar level of aphid-resistance would not require treating with aphicides. For the first time in more than 400 years of hop growing in England, non-pesticidal control of damson-hop aphid, a pest described by Lance (1838) as 'a barometer of poverty' for growers of hops, is a realisable and realistic objective.

Acknowledgement

The work was funded by the Ministry of Agriculture, Fisheries and Food.

References

Campbell, C.A.M. (1978). Regulation of the damson-hop aphid (*Phorodon humuli* (Schrank)) on hops (*Humulus lupulus* L.) by predators. *Journal of Horticultural Science* **53**, 235-242.
Campbell, C.A.M. (1983). Antibiosis in hop (*Humulus lupulus*) to the damson-hop aphid, *Phorodon humuli. Entomologia experimentalis et applicata* **33**, 57-62.
Campbell, C.A.M. & Cone, W.W. (1994). Influence of predators on population development of *Phorodon humuli* (Homoptera: Aphididae) on hops. *Environmental Entomology* **23**, 1391-1396.
Campbell, C.A.M., Darby, P. & Morgan D. (1993). Dynamics of damson-hop aphid on aphid-resistant hop plants. *Proceedings of the Scientific Commission of the International Hop Growers' Convention, Wye College, 26-29 July 1993*, (Eds: R.E. Gunn & C.P. Green), pp 61-68.
Campbell, C.A.M. & Hrdy, I. (1988). Integrated pest and disease control on hops. *International Organisation for Biological Control/West Palaearctic Regional Section Bulletin* **11**, 99 pp.
Campbell, C.A.M. & Lilley, R. (1999). The effects of timing and rates of release of *Phytoseiulus persimilis* against two-spotted spider mite *Tetranychus urticae* on dwarf hops. *Biocontrol Science and Technology* **9**, 453-465.
Darby, P. & Campbell, C.A.M. (1996). Aphid-resistant hops – the key to integrated pest management in hops. *Proceedings of the Brighton Crop Protection Conference 1996*, 893-898.
Hall, R.D. (1971). Hop extract and the future of hop growing in the UK. *Hop Growers' Conference 1971, Geigy Agricultural Chemicals*, 25-37.
Lance, E.J. (1838). *The hop farmer.* Ridgway, London.
Meager, L. (1697). *Mystery of husbandry.* (cited from Parker, H.H. (1934) *The hop industry.*P.S. King & Son, London.)
Neve, R.A. (1991). *Hops.* Chapman & Hall, London.
Perrin, R.M. (1997). Crop protection: taking stock for the new millenium. *Crop Protection* **16**, 449-456.
Whitehead, C. (1890). Fifty years of hop farming. *Journal of the Royal Agricultural Society* **51**, 321-348.

THE CONTROL OF TETRANYCHUS URTICAE BY PREDATORY MITES PHYTOSEIULUS PERSIMILIS ATHIAS HENRIOT, TYPHLODROMUS PYRI SCHEUTEN AND AMBLYSEIUS CALIFORNICUS MC GREGOR (ACARI: TETRANYCHIDAE, PHYTOSEIIDAE) ON HOPS

J. Vostrel
Hop Research Institute, Co., Ltd., 2525 Kadanska, 438 46 Zatec, Czech Republic

Abstract

Biological control of Tetranychus urticae with the help of Phytoseiid mites Phytoseiulus persimilis Athias-Henriot, Typhlodromus pyri Scheuten and Amblyseius californicus Mc Gregor was studied on hops in Czech Republic. P. persimilis was able to control two-spotted spider mite on leaves of hop rootstocks planted in a nursery school in 1999. T. pyri and A. californicus succeeded to keep T. urticae under its economic injury level on a small experimental hop-garden in 2000, the year with suitable weather conditions for the development of this pest.

Key words: biological control, spider mites, predatory mites, Tetranychus urticae, Phytoseiulus persimilis, Typhlodromus pyri, Amblyseius californicus, experimental hop garden, nursery school, rootstock material.

Introduction

Damson hop aphid (Phorodon humuli Schrank) and two-spotted spider mite (Tetranychus urticae Koch) are two major pests of hop plants in many parts of the world where this crop is cultivated. Hop protection against these arthropod pests is based on application of insecticides (imidacloprid, pymetrozine, lambda-cyhalothrin) and acaricides (hexythiazox, propargite, abamectin, fenpyroximate), as was said by Vostrel (1997, 1999). Nevertheless, these pests can quickly develop resistance to pesticides (Hrdy, 1983). To be able to cope with this problem new possible ways of hop protection are to be searched for. An attempt to control populations of resistant P. humuli by its native predators was made in the eighties. It was found out that aphidophagous coccinellids were able under certain suitable conditions to control P. humuli after migration from neighbouring crops (Ruzicka et al, 1986, 1988). Ten years later promising results were obtained with an early introduction of the exotic ladybird, Harmonia axyridis Pallas in France (Weisenberger, 1999).

A lot of experimental work has been devoted to biological control of twospotted spider mites with the help of predatory mites in the USA. Relationships between Phytoseiulus persimilis and other enemies of T. urticae as well as biological observations of Typhlodromus occidentalis on hops were studied nearly thirty years ago by Pruszynski and Cone (1972, 1973) in Washington state. T. occidentalis appeared to be a better candidate than P. persimilis. In hop-gardens of Oregon state inoculative release of Phytoseiid mites and the important question of spatial aggregation and refugia of T. urticae a Neoseiulus fallacis were studied by Strong and Croft (1995, 1997). The most effective control was reached by N. fallacis and T. occidentalis or by a mixture of the both species. It was also found out that refugia might add to stability and persistence of predators on hops. In England Campbell and Lilley (1999) studied the effect of timing and rates of release of P. persimilis against two-spotted spider mite in Kent. In all treatments the numbers of spider mites decreased when the prey and predator ratio reached approximately 10:1. The earliest releases of the predator maintained spider mites at lower population densities than did those made later in the season. Population dynamics of P. humuli and T. urticae and beneficial organisms were studied in Hallertau hop growing region in

Germany. Two-spotted spider mite appeared to be more suitable pest for biological control than damson-hop aphid (Benker, 1997).

Material and methods

The field trial with P. persimilis was established in a nursery school for the production of hop planting material in a village of Rybnany (5 km from Saaz). Predatory mites were their origin in Biola Chelcice, which is the producer of bioagens in CR. Mites were released from transport containers. The experimental plot was 300 m long. Five rows were treated with 20000 adults and nymphs of P. persimilis on August 24, 1999. There are usually 5 rootstocks per 1 m. That means 7500 rootstocks were included into our experiment. On the average 2,7 predatory mites were released per one rootstock. Each plant had approximately four laterals, which were 30 cm long and each of them had on the average fifteen leaves. Nearly half a million (450 000) of hop leaves were controlled biologically with the help of P. persimilis. In the time of the release the occurrence of two-spotted spider mite was in foci and its intensity was low to medium. This fact was taken into account not only in the time of the release but during taking samples as well. The samples were brought into a laboratory and population densities of T. urticae and all the stages of P. persimilis were determined there. Totally six takings were carried out between August 31 and October 05, 1999. Samples containing 100 leaves were taken.

Predatory mites Amblyseius californicus and Typhlodromus pyri were released in a small pole hop-garden in a Franciscan monastery in Kaaden (20 km from Saaz) on April 21, 2000. The hop-garden was established there in 1999 together with two vineyards from the reason of preservation of the nature of the monastery from baroque period, the time when it was established. The both species were obtained for this reason from Biola Chelcice as well. T. pyri was released on the experimental plot with the help of felt belts which contained five adults each. A. californicus was delivered for this purpose in a transport container with 2000 predatory mites. The experimental plot was divided into four parts. In the first plot (T1) was put 1 felt belt (5 gravid females) per plant, in the second (T2) two belts (10 gravid females) per plant, in the third (A1) were released 10-20 adults of A. californicus per one plant tapping from a container with sawdust on the ground around hop shoots emerging from the soil. The last part was biologically non-treated. No pesticides were used during the experiment. Forty-two bines were included into each variant in six rows. Samples of 50 leaves were taken since May 18 till September 13, 2000. Totally thirteen takings were done. Only a very low density of T. urticae was in the time when predatory mites were released. The samples were brought into a laboratory where the quantity of pests and predators were checked.

Results

The population density of T. urticae increased at first. More than 7,7 mites per leaf were found during the check before the release of P. persimilis in a nursery school in Rybnany in 1999. The decrease of the pest abundance began since the second half of September and in the time when the last checking was carried out only 2,5 pests were determined per one leaf. This number is approximately seventeen times lower in comparison with the checking done three weeks after the release of P. persimilis. The high predatory activity is perceptible also from the percentage of leaves free from T. urticae (4% on September 15, resp. 95% on October 10, 1999). This trend is followed also by population dynamics of P. persimilis, which increased and decreased in accordance with the prey. Nevertheless, in the critical period (second half of September) the predator was present in all its stages nearly on every second leaf. The results are shown in Figure 1.

In the experimental pole hop-garden in the monastery in Kaaden in 2000 a faster increase if population density of T. urticae was perceptible during the first decade of June (approximately 20 eggs and 10 mobile stages per leaf) on a biologically non-treated plot. Later

during June the increase in the density of the pest was nearly doubled because of weather conditions suitable for the development of two-spotted spider mite. The last checking on a biologically non-treated plot (NT) was carried out on July 07, 2000, when nearly 140 eggs and 70 mobile stages were observed per one leaf there. Abamectin (Vertimec 1,8 EC) was used at this plot to prevent the injury of hop plants, which are grown in the monastery for ornamental purposes.

Predatory mites T. pyri and A. californicus survived nearly a month without their prey, T. urticae. In a low density they were present on hop plants treated with them since the middle of May (the first checking) till the middle of September (the last checking). Native species T. pyri, which is commonly used in the protection of orchards and vineyards against phytophagous mites, was observed in the lowest population density since May to the middle of June, then its abundance increased but it remained still on a low level. The fact that two-spotted spider mite was kept under economic threshold practically for the whole vegetation without acaricides seems to be promising. The low number of T. pyri on leaves may have been influenced by migration of these mites in the time between taking leaves and their checking in a laboratory. The same was observed in A. californicus, which also succeeded in keeping T. urticae under economic injury level in spite of the fact that the highest number represented only 2,1 mites per leaf (July 07, 2000). Later in the season the population density of T. urticae was rather higher. Within T1 and A1 plots resp. the numbers of T. urticae eggs exceeded the value of twenty whereas on T2 plot neither eggs nor mobile staged reached this density (Figure 2).

Conclusion

On the base of obtained results its is possible to conclude that P. persimilis is under favourite weather conditions able to control resistant populations of T. urticae in a nursery school of hop rootstocks. If we want to succeed it is necessary to carry out regular checkings of T. urticae and P. persimilis population dynamics and if the number of two-spotted spider mite extends beyond economic threshold, chemical treatment with a highly effective acaricide is recommended.

Predatory mites T. pyri and A. californicus were also highly effective in the struggle with two-spotted spider mite on our small experimental hop-garden in Kaaden. Nevertheless, we have had good results only from one year's experiment. Their biological effectiveness will have to be confirmed in the next years not only in the conditions of a small pole hop-garden but in the practise of commercial hop-yards as well.

References

Benker, U. (1997): Population dynamics of pests (damson-hop aphid Phorodon humuli Schrank and two-spotted spider mite Tetranychus urticae Koch) and beneficial organisms in hops. Proceeding of the Scientific Commission of the International Hop Growers' Convention, Zatec, Czech Republic, 21-27.

Campbell, C.A.M., Lilley, R. (1999): The effect of timing and rates of release of Phytoseiulus persimilis against two-spotted spider mite Tetranychus urticae on dwarf hops. Biocontrol Science and Technology 9: 4, 453-465.

Hrdy, I. (1984): Stability of resistance to insecticides and the spreading of hop aphid resistant biotypes in Czechoslovakia. WPRS Bulletin, Integrated pest and disease control, Freising, Germany, 2-10.

Pruszynski, S., Cone W.W. (1972): Relationship between Phytoseiulus persimilis and other enemies of the twospotted spider mite in hops. Environmental Entomology 1: 4, 431-433.
Pruszynski, S, Cone W.W. (1973): Biological observations of Typhlodromus occidentalis (Acarina: Phytoseiidae) on hops. Annals of the Entomological Society of America, 66: 1, 47-51.
Ruzicka, Z., Vostrel, J, Zeleny, J. (1986): The control of Phorodon humuli by aphidophagous Coccinelliade with the help of crop diversification. Proceedings of Symposia Ecology of Aphidophaga II. Academia, Prague, 435-439.
Ruzicka, Z., Vostrel, J., Zeleny, J. (1988): Interaction between Phorodon humuli and indigenous predators in a pesticide untreated hop garden. WPRS Bulletin, Integrated pest and disease control, East-Malling, England, 64-72.
Strong, W.B., Croft, B.A. (1995): Inoculative release of Phytoseiid mites (Acarina: Phytoseiidae) onto the rapidly expanding canopy of hops for control of Tetranychus urticae (Acarina: Tetranychidae). Entomological Society of America 24, 2, 446-453.
Strong, W.B., Croft, B.A. (1997): Spatial aggregation and refugia of the mites Tetranychus urticae and Neoseiulus fallacis (Acari: Tetranychidae, Phytoseiidae) on hop. Environmental Entomology 26, 3, 323-329.
Vostrel, J. (1997): The problem of damson-hop aphid and two-spotted spider mite resistance to pesticides in Czech Republic. Proceedings of the Scientific Commission of the International Hop Growers' Convention. Zatec, 41-45.
Vostrel, J. (1999): Hop protection against pests and diseases in Czech Republic (up-to-date information). Proceedings of the Scientific Commission of the International Hop Growers' Convention. Pulawy, Poland, 120-122.
Weissenberger, A. (1999): Biological control of the damson-hop aphid, Phorodon humuli (Schrank) with the ladybird Harmonia axyridis (Pallas) in Alsace: comparison between the natural and flightless strain. Proceedings of the Scientific Commission of the International Hop Growers' Convention. Pulawy, Poland, 99-103.

Figure 1

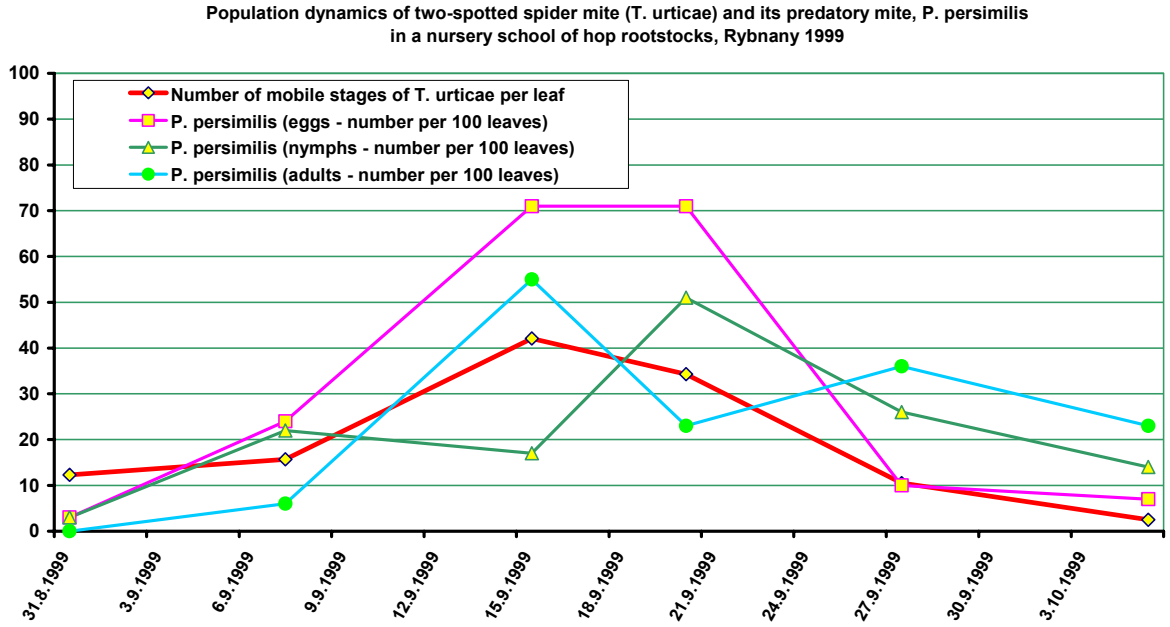
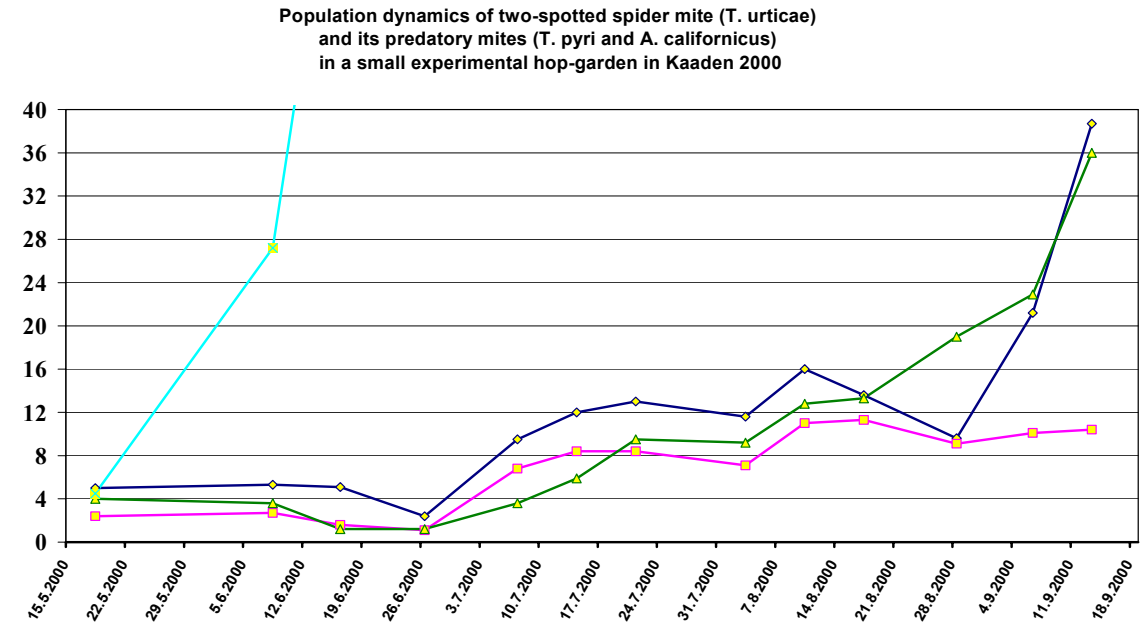


Figure 2



	18.5.2000	8.6.2000	16.6.2000	26.6.2000	7.7.2000	14.7.2000	21.7.2000	3.8.2000	10.8.2000	17.8.2000	28.8.2000	6.9.2000	13.9.2000
T1	5	5,3	5,1	2,4	9,5	12	13	11,6	16	13,6	9,6	21,2	38,7
T2	2,4	2,7	1,6	1,1	6,8	8,4	8,4	7,1	11	11,3	9,1	10,1	10,4
A1	4	3,6	1,2	1,2	3,6	5,9	9,5	9,2	12,8	13,3	19	22,9	36
NT	4,5	27,2	68,3	145,4	207,8								

BIOLOGICAL CONTROL OF MITES AND APHIDS ON WASHINGTON HOPS: AN ASSEMBLAGE APPROACH

David G. James, Tanya S. Price, Lawrence C. Wright and Joe Perez
Irrigated Agriculture Research and Extension Center, Washington State University, 24106 North Bunn Road, Prosser, Washington 99350, USA

Abstract

Studies on the abundance and phenology of spider mites (*Tetranychus urticae*) and aphids (*Phorodon humuli*) and their natural enemies on pesticide-free and pesticide-treated hops in Washington State during 1999/2000, provides a new perspective on biological control of these pests and its development as an effective commercial proposition.

Spider mite and aphid populations on pesticide-free hops were invariably low (< 10 mites or aphids/leaf) and non-damaging. Similarly low populations also occurred on commercial hops but these received 1-4 applications of miticide and 1-2 applications of aphicide. In some instances, miticides failed to provide control and extremely large populations of mites (> 300/leaf) developed. Abundance of predatory mites (phytoseiids) was similar in pesticide-free and pesticide-treated hops but the abundance of non-phytoseiid predators of mites was three times greater in pesticide-free hops.

Intensive monitoring of pest and natural enemy populations in a single pesticide-free hop yard showed that 'hot spot' populations of mites in spring were efficiently targeted and controlled by an assemblage of predatory insects, including *Stethorus picipes,*and *Orius tristicolor*. Consequently, 'hot spots' did not spread before phytoseiid (*Galendromus occidentalis/Neoseiulus fallacis*) populations developed during June-July, further augmenting and maintaining mite control. Mite numbers did not exceed a mean of 6 per leaf during the season and there was no economic damage to the cones. Hop aphid populations increased to sub-damaging levels during spring and declined during July due to the effects of natural enemies and high temperatures.

These data indicate conservation and utilization of an assemblage of natural enemies provides better biological control of mites and aphids, than conservation and use of a single group of predators (e.g. phytoseiids for spider mites). The challenge will be to provide a chemically benign environment in hop yards to preserve natural enemy assemblages by identifying and using only pesticides that are safe to natural enemies.

Introduction

Hop plants, *Humulus lupulus* L., are attacked by several arthropod pests, the most important being the hop aphid, *Phorodon humuli* (Schrank), and twospotted spider mite, *Tetranychus urticae* Koch (Campbell, 1985; Cranham, 1985; Neve, 1991). Currently, insecticides and miticides are routinely used to control these two pests on hops grown in Washington State. In many horticultural crops, *T. urticae* is often an economic problem on crops only when its natural enemies are removed by the use of broad-spectrum pesticides (Helle and Sabelis, 1985). The degree to which pesticides induce or exacerbate spider mite outbreaks in Washington hops has not been studied.

Insect and mite management in Washington hops is currently being re-evaluated due to increasing concerns over the cost-effectiveness, reliability and sustainability of pesticide inputs. Chemical control of mites in hops is often difficult due to the large canopy of the crop and problems with miticide resistance (James and Price, 2000). The development of partial or comprehensive biological control strategies for *T. urticae* and *P. humuli* is therefore of considerable interest to the hop industry in Washington. Research to date on the biological control of mites in hops has centered on the use of phytoseiid mites through conservation or augmentation of populations (Campbell and Lilley, 1999; Strong and Croft, 1993, 1995, 1996; Pruszynski and Cone, 1972), but has not shown much commercial promise, despite some partial successes. Whilst phytoseiid mites are undoubtedly important predators of *T. urticae* in hops, support from other mite predators may be necessary to provide levels of biological control acceptable to growers. The use of natural enemy assemblages in crop ecosystems, as opposed to single specialist type biological control agents, is receiving interest as a crop protection strategy (Murdoch *et al*., 1985; Ehler, 1992; Reichert and Lawrence, 1997). Campbell and Cone (1994) showed that *P. humuli* on Washington hops is regulated by an assemblage of predators with good potential for economic control.

To determine the importance of the endemic natural enemy community in regulating populations of *T. urticae* and *P. humuli* on hops in Washington State, we monitored the abundance and phenology of *T.urticae*, *P. humuli* and their natural enemies monthly on commercial (pesticide-treated) and escaped (pesticide-free) hops during two seasons. We also monitored *T. urticae, P. humuli* and natural enemy populations weekly in an experimental, pesticide-free hop yard at WSU-Prosser during March-October 2000.

Materials and Methods

Monitoring of *T. urticae , P. humuli* and natural enemies in pesticide-free and pesticide-treated hop yards

Hops at sixteen sites (commercial hop yards or escaped hops) were monitored in 1999. A pair of hop yard/escaped hop sites was established at eight locations in the Yakima Valley of Central Washington from Prosser to Yakima. Seven of the locations were used in 2000. Sites in each pair were located within 5 km of each other. Escaped hop sites usually consisted of a small number of plants climbing along a fence or up a pole, usually at the side of a road. Mite and aphid populations were sampled by collecting 30 leaves randomly from each site monthly during June-October 1999 and April-October 2000. Leaves were placed in plastic bags and kept cool until examined under a stereomicroscope. Numbers of motile *T. urticae*, and Phytoseiidae were recorded. Samples of adult phytoseiids were placed in alcohol for later identification using a compound microscope. Non-phytoseiid predators of *T. urticae* and *P. humuli* (mostly insects) present on leaves were also recorded and identified. Data were analyzed using analysis of variance and Student's t test.

Monitoring of T. urticae. P. humuli *and natural enemies in a pesticide-free, experimental hop yard*

A 2.7 acre hop yard containing a mixture of Galena, Columbus and Chinook hops at WSU-Prosser was monitored weekly from 23 March to 4 October 2000 for *T. urticae*, *P. humuli* and natural enemies. Populations were sampled by collecting 30 leaves randomly from the yard and examining them under the microscope. Searches were also made weekly for mite damaged and honeydew-covered leaves to locate mite and aphid 'hot spots'). Some sub-sampling of 'hot spots' was conducted.

Results

Monitoring of T. urticae , P. humuli *and natural enemies in pesticide-free and pesticide-treated hop yards*

All commercial sites received 1-4 applications of miticide each season (1999 mean: 2.3; 2000 mean: 2.1). Abamectin was used at all sites and was usually the first miticide applied in late June. In 1999, cyhexatin was generally the next miticide applied (July/August) at most sites with dicofol and hexythiazox used at single sites. Cyhexatin was not available in 2000 and growers either used abamectin more than once and/or applications of bifenthrin or propargite. At least one application of an aphicide (imidacloprid) was also applied to all sites (late June) with some receiving another application in late July/early August (1999 mean: 1.4; 2000 mean: 0.9).

The mean abundance of *T. urticae* and *P. humuli* in both years when analyzed over the season, did not differ significantly between commercial and escaped hops. In 1999, miticides at three hop yard sites failed to provide adequate mite control. This resulted in a mean population for the three yards of 300 mites/leaf in August, compared to means of .01 and 0.1 mites per leaf for the other hop yards and the escaped hop sites, respectively ($P < 0.05$). In 2000, good mite control was obtained in all hop yards with mean populations generally below 5 mites/leaf in all months except July (16.3/leaf). Numbers of mites at escaped hop sites did not exceed 5/leaf in any month. The mean abundance of phytoseiids in both years when compared on a seasonal basis, did not differ significantly between commercial and escaped hops. Peak aphid numbers (8-10/leaf) occurred in June but were not economically damaging.

At least six insect and one non-phytoseiid mite species known to be predators of spider mites were recorded from the leaf samples. Four of the species are also known to be aphid predators. The seven species (total number recorded from all sites during two seasons) were: *Stethorus picipes* Casey (Coleoptera: Coccinellidae) (195), *Chrysopa* spp. (Neuroptera: Chrysopidae) (81), *Hemerobius* sp. (Neuroptera: Hemerobiidae) (80), *Feltiella* sp. (Diptera: Cecidomyiidae) (53), *Orius tristicolor* (White) (44), *Scolothrips sexmaculatus* (Pergande) (Thysanoptera: Thripidae) (25) and *Anystis* sp. (Acari: Anystidae) (18). The incidence of non-phytoseiid predators in both seasons was more than three times greater at escaped hop sites (ratio, commercial: escaped hops: 1: 3.2 in 1999, 1: 3.6 in 2000).

Monitoring of T. urticae, P. humuli *and natural enemies in a pesticide-free, experimental hop yard*

T. urticae and *P. humuli* were first seen on May 3 but remained at low levels for the next two months. Numbers of *T. urticae* did not exceed 1 per leaf until July, although 'hot spots' were observed in the yard during June. These consisted of single bines where mite populations sometimes exceeded 10/leaf. However, in all instances significant populations of *Stethorus picipes* and *Orius tristicolor* were also present in 'hot spots' feeding on mites. Regular observations showed that mites on 'hot spot' bines were rapidly brought under control by *S. picipes* and *O. tristicolor*. These two predator species were also frequently recorded in leaf samples during June-July. Predatory mites, (primarily *Galendromus occidentalis* Nesbitt and *Neoseiulus fallacis* Garman), did not occur in large numbers until July when they became a significant source of mortality for *T. urticae.* Large numbers of phytoseiids (up to 8/leaf) occurred late in the season. Numbers of *T. urticae* peaked at 6 per leaf in mid July and remained below 5/leaf for the rest of the season.

Aphid populations increased to about 12 per leaf in late June before declining rapidly in early July to 1-2/leaf. Numbers increased to 5/leaf in September. Predators, particularly the ladybirds, *Hippodamia convergens* and *Harmonia axyridis* and the predatory bugs, *Geocoris* spp. and *O. tristicolor* appeared to be largely responsible for the generally low numbers of *P. humuli.*

Discussion

The results of this study on abundance and phenology of mites, aphids and their natural enemies on pesticide-free and pesticide-treated hops in Washington, indicate that biological control provided by an assemblage of natural enemies has the potential to provide effective management.

This study has for the first time demonstrated the importance of considering biological control of mites and aphids in hops from a whole community perspective. To date, all published research on biological control of mites in hops has focused on the conservation and use of phytoseiid mites. This study suggests phytoseiids are only part of the story.

A plethora of non-phytoseiid predators appears to play an important role in regulating mite populations on hops in Washington in unsprayed situations. We consider the abundance of non-phytoseiid predators of mites and aphids reported in this study to be an under-representation of the real situation. Leaf sampling does not provide accurate data for mobile, winged predators like adult *S. picipes, O. tristicolor*, etc.

One of the major drawbacks to the use of natural phytoseiid populations on hops is their slow development during spring (Strong and Croft, 1993). Non-phytoseiid predators may effectively fill this early season 'niche' as they did during the 2000 study at WSU-Prosser. In fact, containment of early season mite populations, preventing 'hot spots' from becoming wide area infestations, may be the key to effective biological control of mites on hops.

Where to from here? These results are clearly limited in that they were collected from only two seasons and the intensive study was conducted at one site in one season.
However, they do provide a sound basis and direction for future work on biological control of mites and aphids in Washington hops. The program has been expanded to cover three intensively monitored sites in 2001, including two commercial hop yards.

The challenge for the future will be to integrate community-based biological control into commercial hop production with all of its chemical inputs. A program has been established at WSU-Prosser that will determine the compatibility of all currently used hop chemicals with biological control. Toxicity profiles for most hop insecticides have been developed for Washington strains of *G. occidentalis, N.fallacis, S. picipes* and *H. axyridis* (James and Coyle, 2001), and it is clear that some currently used materails like abamectin and imidacloprid must be replaced by 'softer' alternatives for conservation biological control to work. Fortunately, there are alternatives available and the progressive introduction of these to Washington hop production should go hand in hand with biological control.

References

Campbell, C. A. M. 1985. Hops (*Humulus lupulus*), pp. 192-194. In N. W. Hussey and N. Scopes (eds.), Biological pest control: the glasshouse experience. Cornell University Press, Ithaca, NY.

Campbell, C. A. M. and R. Lilley. 1999. The effects of timing and rates of release of *Phytoseiulus persimilis* against two-spotted spider mite *Tetranychus urticae* on dwarf hops. Biocontrol Sci. & Tech. 9: 453-465.

Cranham, J. E. 1985. Hop. In W. Helle and M. W. Sabelis. Spider Mites: Their Biology, Natural Enemies and Control, Vol 1B, New York, Elsevier. Pp. 367-370.

Ehler, L. E. 1992. Guild analysis in biological control. Environ. Entomol. 21: 26-40.

Helle, W. and M. W. Sabelis. 1985. Spider Mites: Their Biology, Natural Enemies and Control, Vol 1B, New York, Elsevier.

James, D. G. and Price, T. C. 2000. Abamectin resistance in mites on hops. Agrichem. and Environ. News. 170: 4-5.

James, D. G. and Coyle, J. 2001. Which pesticides are safe to beneficial insects and mites? Agrichem. and Environ. News. 178: 12-14.

Murdoch, W. W., J. Chesson and P. L. Chesson. 1985. Biological control in theory and practice. Am. Nat. 125: 344-366.

Neve, R. A. 1991. Hops. Chapman and Hall, New York.

Pruszynski, S. and W. W. Cone. 1972. Relationships between *Phytoseiulus persimilis* and other enemies of the twospotted spider mite on hops. Environ. Entomol. 1: 431-433.

Riechert, S. E. and Lawrence, K. 1997. Test for predation effects of single versus multiple species of generalist predators: spiders and their insect prey. Entomol. Exp. et Applic. 84: 147-155.

Strong, W. B. and B. A. Croft. 1993. Phytoseiid mites associated with spider mites on hops in the Willamette Valley, Oregon. J. Entomol. Soc. B. C. 90: 45-52.

Strong, W. B. and B. A. Croft. 1995. Innoculative release of phytoseiid mites (Acarina: Phytoseiidae) into the rapidly expanding canopy of hop for control of *Tetranychus urticae* (Acarina: Tetranychidae). Environ. Entomol. 24: 446-453.

Strong, W. B. and B. A. Croft. 1996. Release strategies and cultural modifications for biological control of twospotted spider mite by *Neoseiulus fallacis* (Acarina: Tetranychidae, Phytoseiidae) on hops. Environ. Entomol. 25: 529-535.

SINGLE GENE TRAITS IN HOP BREEDING

P.Darby

Horticulture Research International, Imperial College, Wye, Kent TN25 5AH, England.

Abstract

During conventional hop breeding, many traits are observed which may be controlled by single genes. Such traits may be considered as the first targets for new breeding techniques and approaches. This paper reviews the phenotypic expression of these traits, and the segregation ratios which have been recorded for them within a commercial breeding programme. Examples of unambiguous expression and segregation will be sought to provide model systems for future research.

Introduction

Modern genetics stems from Mendel's work on the inheritance of single gene traits in *Pisum*. In this and many other species, the characterisation of such traits has allowed genetic linkage maps to be established to the great benefit of breeding programmes. However, similar work on hops has not occurred. This may be due to the highly heterozygous, dioecious nature of the hop preventing selfing to produce an F2 generation, and the lack of colourful flowers or seeds to aid phenotypic discrimination. With the recent advent of molecular markers, interest in mapping the hop genome has re-emerged and it would be of advantage to practical breeding programmes to be able to associate phenotypic characteristics, particularly those with economic importance, with the linkage maps being produced using molecular markers.

This paper reviews data from the HRI hop breeding programme from 1935 to the present to discern traits which are likely to be controlled by just one or two major genes. Such traits will be distinguished by a clear segregation of phenotypes into different classes, and the undiluted transmission of these phenotypic responses between generations.

Pests and diseases

From the gene-for-gene hypothesis for host-pathogen interactions, it might be expected that resistances to pests and diseases would be traits where major gene control could be found.

Between 1972 and 1984, a series of seven genes was postulated to explain the observed differential reactions between hop varieties and isolates of powdery mildew (*Spherotheca humuli*). To obtain genetic confirmation of this hypothesis, a series of crosses were made in 1985. Parents thought to be carrying the relevant resistance gene were crossed with susceptible parents all of the same genetic background (seedlings of 'Northern Brewer'). The progeny were screened against an appropriate isolate of the pathogen which lacked the corresponding virulence gene. The results (Table 1.) indicated that resistance is controlled by a single gene in each progeny.

Resistance to progressive Verticillium wilt in the HRI programme would appear to be from two distinct sources ; Y 90 and AA 7. The mean values for resistance in progeny derived from Y 90 indicate additive gene action and there is no evidence for discontinuity of individual values. Thus, it may be concluded that the control of resistance from this source is polygenic. However, progeny from crosses made in 1957 and 1959 with parents derived from AA 7 gave an

indication of segregation (Table 2.). Resistant seedlings survived challenge with the pathogen whilst susceptible seedlings were killed. Furthermore, in crosses made from 1935 to 1990 between resistant progeny and susceptible parents (encompassing up to four generations), the level of resistance in the 'resistant' class has remained remarkably constant despite increased virulence in the pathogen. Control by two dominant complementary genes is consistent with the segregation ratios observed and, in unselected progeny obtained more recently, the same pattern can still be seen.

Table 1. Segregation for resistance to powdery mildew (resistant x susceptible)

Cross no.	R gene	No. resistant	No. susceptible	Chi square value for 1:1
6/85	Blister	65	72	0.36
7/85	1	34	30	2.32
8/85	2	20	31	2.37
9/85	3	80	90	0.59
10/85	4	59	72	1.29
11/85	5	40	58	3.31
35/85	6	45	54	0.82

Table 2. Segregation for resistance to Verticillium wilt

Cross no.	Type	No. resistant	No. susceptible	Ratio tested	Chi square value
27/57	R x S	47	63	3 : 5	1.28
22/59	S x S	8	18	1 : 3	0.46
91/97	R x S	35	85	3 : 5	3.56
93/97	R x S	41	97	3 : 5	3.57

A source of strong resistance to the damson-hop aphid (*Phorodon humuli*) was discovered in accession INT 101 in the mid-1980s at HRI-Wye. On this accession, aphid reproductive rates were much reduced. In the absence of pesticide control, susceptible varieties would be defoliated by the pest but INT 101 retained all its leaves with only a few aphids surviving until the end of the season. INT 101 was crossed with a susceptible parent to give family 55/87 and it was clear that there was segregation for resistance to aphids in the field amongst the progeny; adjacent plants differed in the retention of foliage. It was suggested that the observed segregation ratio (Table 3.) could be explained by the action of two dominant genes. Thus, resistant seedlings could carry either or both of these genes, and their own progeny should segregate in a 1:1 or 3:1 ratio of resistant to susceptible types accordingly. Results from progeny derived from resistant members of family 55/87 support such an explanation (Table 3.).

Plant habit

Most plant habit characteristics of the hop show continuous variation and there have been no reports of discontinuity in such characteristics as lateral length, leaf shape or size, cone

number, cone weight etc. However, identification of dwarf plants in progeny in the Wye breeding programme in 1977 provided an example of a plant habit trait where clear segregation could be discerned. Dwarf plants produced a terminal inflorescence before the main bine had reached the wirework height of 4.5m and there was segregation for plant height in mature field plants. This segregation was also observed in later progeny derived from the original population (Table 4.). The ratio of tall to short plants indicates the action of a single dominant gene modified by an epistatic major gene.

Table 3. Segregation for resistance to damson-hop aphid

Cross no.	No. resistant	No. susceptible	Chi-square value for 1 : 1 or 3 : 1
Original family :			
55/87	184	70	0.89
Derived families :			
4/89	25	21	0.35
10/89	75	21	0.50
11/89	60	20	0.00
12/89	90	33	0.22
13/89	22	14	1.78

The development of dwarf hop varieties is a major objective of the current breeding programme at HRI-Wye and expression of the trait remains distinguishable in segregating progenies.

Table 4. Segregation for plant height (short x tall)

Cross no.	No. tall plants (>4.5m)	No. short plants (<4.5m)	Chi-square value for 5 : 3
38/77	27	18	0.12
11/84	85	44	0.63
22/86	86	48	0.16
26/86	12	7	0.00
Total	210	117	0.41

Chlorophyll deficiencies

In several of the families derived from 'Hallertau mf' or 'Saaz', albino seedlings arise; cotyledons and stems are white, lacking any chlorophyll. These seedlings have not survived beyond the emergence of the cotyledons, although there is now the possibility of maintaining such plants by rescue through tissue culture procedures. Segregation data and pedigree records (as illustrated in Table 5.) indicate that this trait is the result of a recessive single gene with phenotypic expression when homozygous.

Ornamental hops such as 'Aureus' have foliage with reduced chlorophyll levels, being yellow in bright sunlight and turning very pale green in shadier conditions. Seedlings from such yellow varieties segregate as either yellow or green leaves immediately after the cotyledon stage and

intermediates are not observed. Thus, the criteria for a single gene trait are met and the observed segregation ratios (Table 6.) can mostly be explained as the expression of a dominant major gene which is lethal in the homozygous state. However, whilst progeny from most 'yellow x green' crosses segregate as 1:1 as expected, the results from 'yellow x yellow' crosses are not always as predicted, indicating a more complex genetic explanation when homozygosity is apparent.

Table 5. Examples of segregation for albino cotyledon colour in hop seedlings

Female	Male	Cross no.	No. albino	No. green	Chi-square for 1 : 3
Hallertau	30/58/29	27 / 76	43	178	3.62
Hallertau	15/65/271	19 / 65	0	89	66.75 ***
Hallertau	18/67/6	12 / 69	15	44	0.01
Derived families :-					
27/76/8	19/65/29	50 / 85	72	254	1.48
12/69/15	19/65/29	34 / 81	236	697	0.04

Table 6. Segregation for yellow leaf colour in seedlings from 1997 crosses

Cross no.	Type	No. yellow seedlings	No. green seedlings	Proposed ratio	Chi-square value
39/97	Y x Y	18	9	2 : 1	0.00
40/97	G x Y	10	12	1 : 1	0.18
41/97	G x Y	22	30	1 : 1	1.23
42/97	Y x Y	42	28	2 : 1	1.40
43/97	Y x Y	10	17	2 : 1	10.67 ***

Resins and oils

Although there are conflicting reports on the inheritance of individual components of the resins, most published reports conclude that alpha-acid content is under polygenic control with continuous variation for the trait amongst progeny and improvement through selection for additive gene action. However, some male parents at HRI-Wye have been identified recently where there is an indication of segregation for alpha-acid content in their progeny (Table 7.). Such data alone are not evidence for major gene action but the possibility is under current investigation in studies at HRI-Wye.

Table 7. Segregation for alpha-acid content (by HPLC) in selected families

Female	Male	Cross no.	Seedling alpha-acid contents (%)
23/90/48	45/85/159	48 / 94	9.3, 8.9, 8.5, 8.0, 6.1, 8.7
15/85/35	45/85/159	24 / 91	6.5, 3.7, 3.9, 6.0, 5.7
15/85/35	32/85/61	25 / 91	**16.1**, 4.2, 3.9, 9.8, 4.3, **14.8**, 5.3

43/85/27	32/85/61	36 / 93	8.5, **13.1**, **12.0,** 6.2, 5.0, 8.7

It has been previously reported that the presence or absence of sesquiterpene oils, notably farnesene and selinene, in the resin glands of both male and female hops is under simple genetic control with segregation following Mendelian ratios. These findings (unpublished) were obtained from detailed analyses in 1966 and 1967 of progeny 1/63 (selinene present x selinene absent) where selinene was found in large quantities in the glands of 37 seedlings and was detectable in low amounts in the glands of 34 seedlings.

Because the relative amounts of the sesquiterpene oils alter during maturation of hop cones, the amount present is usually expressed as the ratio of the oil to caryophyllene. Such results from recent oil composition analyses of advanced breeding selections at Wye (Table 8.) confirm that farnesene is either present or absent (or in trace amounts), and that selinene is in relatively high or low quantities. Furthermore, analysis of related progeny confirm that there is segregation of progeny between these classes and transmission of the trait through several generations.

Table 8. Oil analysis results from 1999 harvest at HRI-Wye

Plot no.	F* : C *	S*: C	Plot no.	F : C	S : C
6	0.31	0.25	*Seedlings of female 33/82/22 :-*		
7	0.00	0.29	84	0.00	0.16
8	0.00	1.40	85	0.01	0.81
9	0.85	1.87	86	0.01	1.10
11	0.00	0.96	90	0.01	1.21
12	0.00	1.35	95	0.01	1.10
13	0.00	0.34			
14	0.00	1.53	*Seedlings derived from male INT 101 :-*		
16	0.00	2.25	*First Generation*		
			30A	0.96	2.27
Fuggle	0.79	0.27	30B	0.47	1.79
Challenger	0.17	1.73	*Second generation*		
Target	0.02	0.33	28A	0.29	0.29
			28B	0.50	2.01

*F = Farnesene, *C = Caryophyllene, *S = Selinene

Conclusion

Data have been presented which indicate that several measurable or observable traits in hops are under simple genetic control by single genes, with progeny segregating according to Mendelian principles. It is likely that some of these characteristics may show genetic linkage or association; the link between the ‘blister’ gene for powdery mildew resistance and the presence of selinene in the essential oil has been documented. The development of linkage maps for hops incorporating these traits and molecular markers obtained from AFLP and microsatelite studies is now awaited, and segregation for these traits should be a consideration in the generation of the large, unselected progeny from wide crosses that are necessary for such work.

THE HOP CULTIVATING MODELS IN CHINA

Zhang Tianhu
Gansu Yasheng Industrial (Group) Co., Ltd., 219 Zhangye Road, 730030 Lanzhou, Gansu, People´s Republic of China

The history for hop grown in large scale in China is very short. Tsing Tao is main variety in hop production. According to the data from China Hop Growers Committee, total areas for hop growing in 2000, is 4386 ha. Tsing Tao is 4002 ha, about 90% of the total areas.
On the beginning of the hop growing, the tall trellis was used. But the situation changed quickly. The lower-roof system has become main model in China hop production. The advantage of this model is of resistance to wind and well reception of solar radiation. Also, bumper harvest always obtained. But the field manage on the later period is very difficult. Some new systems have been developed.

NEW INFORMATION CONCERNING BREEDING PROCESS IN CR

Ing. Vladimír Nesvadba, Ing. Jiří Kořen
Hop Research Institute Co., Ltd., Žatec, CR

Abstract

Model crossing of female (Target, Magnum, Yeoman, Premiant) and male plants having their origin in our hybridization program were carried out in 1997 and 1998. Evaluation or progenies of the both generations was performed in 1998 – 2000. Transmission of important characteristics (yield, resistance to downy mildew, alpha-acids content, hop aroma and length of vegetation period) from parents to their progenies was taken into account above all. Perspective new hybrids for individual types of hops including choice of the most suitable varieties for different growing areas within CR are reviewed as well.

Introduction

Hop breeding in Czech Republic has a long tradition. It used to be based on clone selection of Saaz semi-early red-bine hop. Nowadays hybridization of suitable parents is exclusively used. Hybridization process is a very problematic one. Evaluation of heredity of important features in hops is complicated not only by heterozygous character of hop plants but by their dioeciousness as well. Particularly dioeciousness is the main problem within the process of hop breeding. Only female plants bear hop cones and it is the reason why male plants enter into this process as a nearly unknown pollinator. Content of hop resins, yield of hops and the sensitivity to hop downy mildew and hop powdery mildew are very difficult to determine within male plants. The main objective of our work has been appointed with regard to above-mentioned reasons and is the following: „Heredity of important characteristics in hop plants.“

Material and methods

Selection of material

Hop plants for model crossing were selected in 1997. Next year progeny from these plants was obtained. The objective of this activity was to choose plants, which were different in their genetic origin. Nearly 300 different female genotypes and 110 male genotypes are available in the collection of genetic sources in Hop Research Institute in Saaz.The following genotypes were selected:

female plants (varieties): Target, Magnum, Yeoman, Premiant
male plants: 82/6, 86/4, 87/3, and clone no. 72.

Hybrid progenies of F_1 and I_1 generation (obtaining)

Male and female plants were crossed within model crossing process (dialel crossing). Female plants had been isolated before flowering began and later during inflorescence selected female plants were pollinated. Seeds from these plants were sown into soil. Later the small plants were transplanted and in the middle of May they were planted into an experimental hop-garden.

Evaluation of progenies

During the first year of cultivation plants reach the height of 5 to 7m. Sex of obtained progenies, colour of bine and resistance to downy and powdery mildew are possible to determine but no productive characteristics can be evaluated in the first year. In the second half of September the bines are decapitated. Plants begin to grow in spring of the following year and they are able to reach the top of a wire-work (7 m). In this year plants are grown up and qualitative and quantitative parameters of the genotypes are possible to evaluate. It is the reason why progenies of F_1 and I_1 generations were apprised in the second year after their planting.

Evaluation of these progenies was carried out with the help of classification of Hop Research Institute in Saaz. Eight characteristics were appraised on the base of the following scale:

Productive features:	height of growth of fructiferous laterals
	length of laterals
	density of laterals (distance of internodes)
	density of cones
	size of cones
Hop resins:	content of alpha-bitter acids
Resistance:	resistance to hop downy mildew
	resistance to powdery mildew

Statistical methods

The data are evaluated with the help of basic statistical characteristics: average, coefficient of variability, statistic deviation and analysis of variance. For the determination of the differences between progenies of tested female and male plants and for the differences between individual generations parameter (t-test with equality of statistic deviations, pair t-test) and non-parameter tests (χ^2 test, Kolgomorov-Smirnov's test) were used. For the evaluation of the influence of manifestation of the features in parents and its transmission to the studied progenies, function of linear regression was used. Close of dependence was determined by correlation and determination coefficients, resp.

Coefficient of heritability (h^2) of the evaluated features was determined on the base of regress coefficient (b). This coefficient was also determined with the help of variability of clone material of several different genotypes on the base of a long-term evaluation.

Results and discussion

Acquirement of data for evaluation of results is very demanding from the time point of view in hops. Hybridization of selected parental components (female plants Target, Magnum, Yeoman, Premiant and male plants kept under denomination 82/6, 86/4, 87/3 and clone 72) was carried out in 1997. Progenies of F_1 generation were planted in field conditions in 1998.

Relative crossing for obtaining I_1 generation within progenies of F_1 generation was carried out in 1998 as well. Progenies of I_1 generation we planted in field conditions in 1999. From this schedule issues that evaluation of F_1 generation was performed in 1999 and the one of I_1 generation in 2000.

On the base of obtained results tested female and male plants, which are able to transmit studied features to the progeny of F_1 generation can be recommended for crossing within breeding program for issue of new perspective hop varieties.

Female plants

Target – it transmits long laterals, high density of laterals and at first high resistance to downy mildew and powdery mildew to a progeny of F_1 generation. Unfortunately, progenies of this variety bear too small hop cones.
Magnum – it transmits high alpha-acids' content to a progeny of F_1 generation, but progenies show rare structure of laterals and high sensitivity to downy mildew.
Yeoman – very low growth of fruitful laterals from the ground is transmitted to F_1 generation together with large cones and high alpha-acids' content. Unfortunately, progenies of this variety have very short laterals and they show the highest degree of sensitivity to downy and powdery mildew.
Premiant – the highest density of hop cones together with too high growth of fruitful laterals from the ground are transmitted to a progeny of F_1 generation.

Male plants

82/6 – low growth of fruitful long laterals and resistance to downy and powdery mildew are transmitted to a progeny of F_1 generation. Unfortunately, progenies of F_1 generation have a low density of hop cones.
86/4 – high alpha-acids' content and too short laterals are transmitted to a progeny of F_1 generation.
87/3 – the lowest growth of fruitful laterals and high alpha-acids' content are transmitted to a progeny of F_1 generation. On the other hand progenies of F_1 generation have too low density of laterals and last but not least they are sensitive to downy and powdery mildew.
Clone 72 – long laterals and high density of large hop cones are transmitted to a progeny of F_1 generation. On the contrary progenies have the highest growth of fruitful laterals and the lowest content of alpha-acids.

During our evaluation of characteristics of F_1 generation the dependences between appraised features were determined. The closest dependences were evaluated in the following way:

- between density of laterals and density of hop cones correlation ($r = 0,66$) was determined. A hundred higher value of coefficient of determination shows that density of laterals influences density of hop cones from 43,56%.
- between resistance to downy mildew and resistance to powdery mildew correlation coefficient 0,80 was determined. A hundred higher value of coefficient of determination shows that plants present the same resistance to both diseases from 64,7%.

Transmission of important features to a progeny of I_1 generation

Generation I_1 was acquired from a crossing of a daughter (selected in F_1 generation) and a son (selected from coincident crossing of F_1 generation). The origin parental components are:

P_1 – female plants of Target, Magnum, Yeoman and Premiant varieties
P_2 – male plants under denomination numbers 82/6, 86/4 and 87/3.

The objective of I_1 generation appraising was not only to compare average values with

F_1 generation but to determine the possibility concerning realization of the selection of perspective genotypes in relation to transmission of evaluated features as well. The following six characteristics were taken into account:

1. **Height of fruitful laterals' growth** – the progenies of I_1 generation show lower growth of laterals on bines from the ground level than progenies of F_1 generation but the difference is not statistically significant. The both progenies show the same variability, which means that it is possible to carry out the selection concerning this feature in both generations.
2. **Length of laterals** – with 90% of probability the progenies of I_1 generation show shorter length of laterals than the progenies of F_1 generation. It is the reason why we can recommend selections on this feature only in F_1 generation.
3. **Density of laterals** – the progenies of I_1 generation have lower density of laterals than the progenies of F_1 generation, but the difference is not statistically significant. The both progenies show nearly the same variability. Therefore, the selection on this feature is possible to do in I_1 and F_1 generations.
4. **Density of cones** – with 98% of probability the progenies of I_1 generation show higher density of hop cones than the ones of F_1 generation. The variability of the progenies of the both generations is nearly the same. We can conclude that suitability of the selection on this feature is better to do for I_1 generation.
5. **Size of hop cones** – the progenies of I_1 generation show smaller size of hop cones but without statistical significance. On the contrary they have higher variability than progenies of F_1 generation. The selection on this feature is possible to do in the both I_1 and F_1 generations.
6. **Content of alpha-acids –** with 99% of probability the progenies of I_1 generation show conclusively lower content of alpha-acids than the progenies of F_1 generation. The variability of this feature is nearly the same in the both generations. It is clear that the selection of genotypes concerning alpha-acids' content is possible to do only within F_1 generation.

Results obtained during the process of hybrid breeding carried out after zonation of new hybrid hop varieties cultivated in CR will be a part of our speech.

Conclusion

The obtained results include the transmission of important characteristics from parental components to the progenies of F_1 generation. The results of features' data including variability of the progenies of F_1 and I_1 generations show suitability of a choice for obtaining of those genotypes, which have the best parameters of evaluated characteristics. The methodological data will be fully applied within our breeding program. For evaluation of the progenies some statistical methods were chosen. They will find their assertion in the process of evaluation of origin breeding material in the following period of our hybridization program.

New experience will contribute to a higher effectiveness of the hybrid breeding program in Czech Republic.

IDENTIFICATION AND USE OF SEXUALLY DERIVED TETRAPLOIDS IN HOP (*HUMULUS LUPULUS* L.)

R.A. Beatson[1], A.R. Ferguson[2], I.E. Weir[2], L.T. Graham[1], K.A. Ansell[1] & H. Ding[2]

[1] HortResearch, Nelson Region, PO Box 220, Motueka, New Zealand
[2] HortResearch, Mt Albert Research Centre, Private Bag 92 169, Auckland, New Zealand

Abstract

The New Zealand hop breeding programme is based solely on the development of seedless triploid cultivars. This relies on the use of tetraploid parents. While asexually derived tetraploid parents have been used successfully, sexually derived tetraploids offer a useful alternative. They are less inbred and are easier to obtain. Methods for the identification of tetraploids from seedling populations by flow cytometry are described. Information on the ploidy variation and gender within various populations is presented. Sexually derived tetraploid parents have been used successfully in the breeding programme, from which several promising triploid selections have been made.

Introduction

The production of seedless triploid hop cultivars has been the cornerstone of the New Zealand hop breeding programme for four decades (Beatson, 1993). During that time, 12 cultivars of triploid chromosomal constitution have been released. Currently, seedless triploid cultivars make up 98% of the commercial hop plantings in New Zealand, and they remain the main focus of the hop breeding programme. All the cultivars released have resulted from crossing tetraploid female parents with diploid males. The tetraploid females were created asexually by the use of colchicine (Roborgh, 1969). While these procedures have been successful, other means of deriving tetraploids are being investigated. One is the production of sexually derived tetraploids. The main advantage of such sexually derived tetraploids is that they are less inbred than are colchicine-derived tetraploids (Watanabe *et al*, 1991).

The key to the successful production and use of sexually derived tetraploids is their identification amongst segregating seedling populations in relatively large numbers. Haunold (1970) described the chromosomal segregation of a triploid X diploid seedling population. In this population, about 30% of the seedlings were identified as tetraploid by cytological means. Haunold suggested that triploid X diploid crosses could produce tetraploids with different genetic backgrounds for use in a polyploid breeding programme. However, cytological verification of sexually derived tetraploids is relatively arduous and is a limiting factor to the large-scale production of sexually derived hop genotypes.

In the mid 1990's, we started to investigate the possibilities of using flow cytometry for the identification of tetraploids from seedling populations. This paper describes the practical use of flow cytometric techniques to identify sexually derived tetraploid hop genotypes, and the subsequent use of these tetraploids for hop breeding.

Methods

Plant material

All plant material used in the experiments was derived from open-pollinated triploid cultivars or

advanced seedling selections. The cultivars used were ‘Pacific Hallertauer’, ‘Alpharoma’, ‘Pacific Sunrise’, ‘Southern Cross’, ‘NZ Hallertauer’, ‘Nelson Sauvin’ and advanced selections 93-22-02, 93-27-23 and 95-15-24.

Experiment 1

This involved planting in the field, in September 1998, seedlings derived from triploid X open pollinated populations, that had been grown in a nursery the previous year. Samples for flow cytometry were taken from these young field-grown plants during October and November 1998. A total of 303 seedlings were screened for ploidy. Gender was recorded during flowering and female seedlings were harvested in March 1999. Each seedling harvested was measured for yield, and HPLC determination of alpha and beta acids. Selections were made based on ploidy and chemical characteristics.

Experiment 2

Samples were collected from 454 young seedling plants in a glasshouse during October-December 2000 for estimation of ploidy by flow cytometry. Selections were made based on ploidy. Also, representatives of each ploidy category were kept for later cytological verification.

Flow Cytometry

Young shoot tips, or, occasionally, a couple of small leaves, were harvested from each plant. Samples were collected in Nelson and kept cold in the field (or glasshouse) and during transport to Auckland.

Nuclei were extracted by chopping the tissue in a Petri dish using two single-edged razor blades into 2-3 ml ice-cold Galbraith's Buffer (Galbraith et al., 1983) containing 3% polyvinylpyrrolidone-10 to inhibit oxidation. The preparation was filtered through a 32 µm stainless steel mesh filter into a conical centrifuge tube and made up to about 15 ml with modified Galbraith’s Buffer before centrifugation at 300 x *g* for 4 minutes. The supernatant was removed and the pellet resuspended in 300 µl of 100 µg/ml propidium iodide in Galbraith's Buffer. Nuclei were left to equilibrate for at least 30 minutes on ice before analysis.

Nuclei were analysed using a Coulter Epics Elite *EPS* flow cytometer (Beckman Coulter, Hialeh, Fl., USA) fitted with a 140 µm quartz flow cell tip. An air-cooled argon laser emitting light at 488 nm was used with fluorescence measured using a 610 nm ± 10 nm bandpass filter.

Cytologically verified diploid, triploid and tetraploid genotypes were used as standards for flow cytometry.

Results

Experiment 1

A summary of flow cytometry results for 5 triploid X op field-grown populations, by ploidy and gender is presented in Table 1. Ploidy ranged from diploid to pentaploid, with 16% (49) of the 303 plants tested being tetraploid. It can be seen that for one of the families (‘Nelson Sauvin’ X op) 33 of the 53 plants tested (62%) were tetraploids and there were very few diploids. The other four families had a much lower percentage of tetraploids. Over all five families, about 40% of the seedlings were triploid, while there were slightly more (43%) diploids.

Table 1: Flow Cytometry Results: 1998-99 Field Trial, by Ploidy and Gender.

Cross	Ploidy	F[1]	H[1]	M[1]	N-F[1]	*Total*	Tetraploid (%)
Nelson Sauvin X op	**5x**	0	2	0	0	**2**	
	4x	14	16	1	2	**33**	**62.3**
	3x	10	2	1	1	**14**	
	2x	2	0	1	1	**4**	
Alpharoma X op	**5x**	0	0	0	0	**0**	
	4x	5	2	0	1	**8**	**6.1**
	3x	22	17	6	9	**54**	
	2x	31	2	18	19	**70**	
NZ Hallertauer X op	**5x**	0	0	0	0	**0**	
	4x	1	0	2	0	**3**	**4.1**
	3x	13	9	1	3	**26**	
	2x	27	1	5	11	**44**	
Pacific Hallertauer X op	**5x**	0	0	0	0	**0**	
	4x	1	0	2	1	**4**	**14.3**
	3x	6	6	0	4	**16**	
	2x	7	0	0	1	**8**	
Southern Cross X op	**5x**	0	0	0	0	**0**	
	4x	1	0	0	0	**1**	**5.9**
	3x	9	1	0	1	**11**	
	2x	4	0	1	0	**5**	
							Ploidy (%)
Summary	**5x**	0	2	0	0	**2**	**0.7**
	4x	22	18	5	4	**49**	**16.2**
	3x	60	35	8	18	**121**	**39.9**
	2x	71	3	25	32	**131**	**43.2**
Gender Total (#)		**153**	**58**	**38**	**54**	**303**	
Gender (%)		**50.5**	**19.1**	**12.5**	**17.8**		

Footnote [1]: Where F=female, H=hermaphrodite, M=male, and N-F= non-flowering.

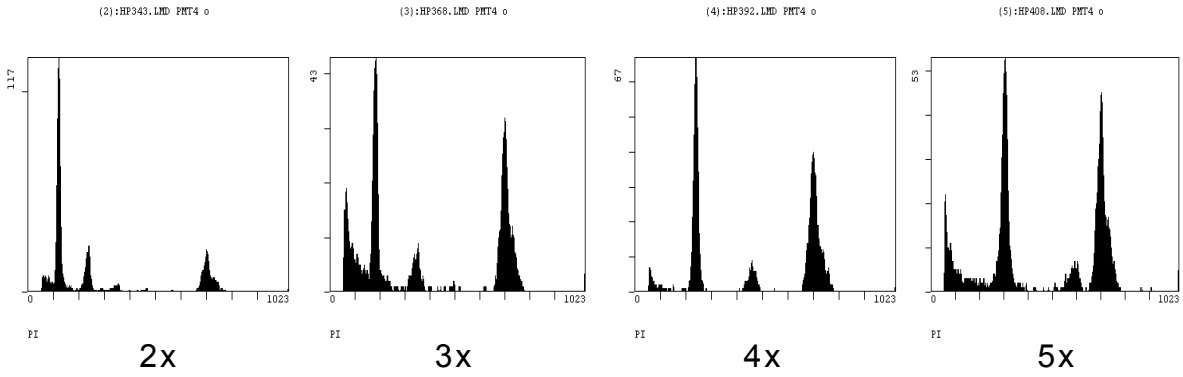
Table 2: Flow Cytometry Results: 2000-01 Season, by Ploidy.

Cross	1x	2x	2x-3x	3x	3x-4x	4x	4x-5x	5x	6x-7x	Cross Total	Tetraploid (%)
94-21-19 X op	1	42	7	103	0	37	0	1	0	**191**	**19.4**
93-27-23 X op	0	32	4	55	0	10	0	3	1	**105**	**9.5**
95-15-24 X op	0	10	2	19	0	8	0	3	0	**42**	**19.0**
93-22-02 X op	0	36	7	56	4	12	1	0	0	**116**	**10.3**
Ploidy Total	**1**	**120**	**20**	**233**	**4**	**67**	**1**	**7**	**1**	**454**	
Ploidy (%)	**0.2**	**26.4**	**4.4**	**51.3**	**0.9**	**14.8**	**0.2**	**1.5**	**0.2**		

Experiment 2

A summary of flow cytometry results for 4 triploid X op glasshouse-grown populations is presented in Table 2. About 15% were tetraploid, a little over 50% were designated as triploid, and a further 25% as diploid. One plant was found to be haploid and one was a 6*x*-7*x* aneuploid. The remaining plants were classified as aneuploid. Most of these putative aneuploids were rechecked and the results consistently indicated aneuploidy. Flow cytometry histograms for various ploidy levels are presented in Figure 1. The peak to the right in each histogram is an added standard.

Figure 1: Flow Cytometry Histograms of Hop Samples.



Discussion

The only similar cytogenetic study published on hops is that of Haunold (1970). He examined seedlings from a triploid X diploid cross cytologically and found an extensive aneuploid series, with a higher proportion of aneuploids than we found. However, flow cytometry is most effective in allocating plants into broad ploidy classes and, with 2*x* = 20 for hops, it is possible that we did not detect trisomics or tetrasomics or even double trisomics and tetrasomics, but only higher aneuploids. Haunold (1970) also identified a much lower proportion of triploids. These differences warrant further investigation, as do the differences we observed between families in this study. Our families were derived from open pollination and the particular sources of pollen may have caused some of the differences seen.

Flow cytometry used over two seasons allowed the ploidy of some 750 seedlings to be estimated. To check such numbers of plants by cytological techniques would be a daunting task, even if the finer detail revealed might be scientifically interesting. Instead, flow cytometry is now an essential part of the New Zealand hop breeding programme to aid the breeding of genetically improved triploid genotypes. The results presented show that sexually derived tetraploids can be readily obtained from triploid X op populations. Some of these sexually derived tetraploids are now being used as parents in the breeding programme. Also, as can be

seen from the results presented, a significant proportion of the seedlings from triploid X op populations are themselves triploid. These plants are now being screened for their commercial prospects alongside triploids derived from conventional tetraploid X diploid crosses. 'Pacific Sunrise', a new high alpha seedless cultivar with a good yield potential, was identified by flow cytometry as a triploid seedling from a 'Southern Cross' X op population. Also, several promising aroma-type triploids have been identified *via* flow cytometry from similar populations.

We now screen plants for ploidy at a younger age: seedling plants still in the glasshouse are analysed by flow cytometry rather than older plants growing in the field. The advantages are that the plants tested are under more controlled and uniform conditions, that the actual samples collected yield clearer results, and that significant cost savings in terms of eliminating certain ploidy genotypes can be achieved prior to field establishment.

One of the limiting factors of using asexually derived "noble aroma" tetraploids (*e.g.*, 'Saazer', 'Hallertauer Mittelfrueh', or 'Fuggle') in New Zealand is that they are very poor performers agronomically, and their progeny are usually weak growing as well. Also, asexually derived tetraploids are more inbred than are sexually derived tetraploids (Watanabe *et al*, 1991). The use of sexually derived tetraploids has already enabled the selection of promising triploid progeny that have good agronomic potential. For example, one of these seedless triploid selections with 'Hallertauer Mittelfrueh' in its genetic background has a chemistry profile closely aligned to internationally accepted aroma cultivars.

While the use of colchicine-derived tetraploids remains an important breeding strategy, flow cytometry has enabled a rapid expansion in the number of tetraploid genotypes that can be used for breeding. An important bonus for the breeding programme that has resulted from the use of flow cytometry has been the identification of some male tetraploid genotypes. As the supply and diversity of both male and female tetraploid genetic material expands, it is hoped that a broad-based tetraploid recurrent selection population can be used as the starting point from which tetraploid parents for the seedless triploid breeding programme can be selected.

Acknowledgements

Funding and travel for this project was received from NZ Foundation for Research Science & Technology and New Zealand Hop Research Committee.

We thank T. Jürgens, V. Horstmann, P. Harris-Virgin and S. Griffith for technical assistance.

References

Beatson, R.A. 1993. Breeding and development of hop cultivars in New Zealand. Proceedings of the Scientific Commission of the IHGC, Wye, England: pp 12-18.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., Firoozabady, E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science **220**: 1049-1051.

Haunold, A. 1970. Fertility studies and cytological analysis of the progeny of a triploid X diploid cross in hop, *Humulus lupulus* L. Can. J Genetics & Cytology **12**: 582-588.

Roborgh, R.H.J. 1969. The production of seedless varieties of hop (*Humulus lupulus*) with colchicine. NZ J Agr. Research **12**: 256-259.

Watanabe K., Peloquin S.J., Endo, M. 1991. Genetic significance of mode of polyploidization.

Genome 34: 28-34.

PLOIDY LEVEL OF COLCHICINE TREATED HOPS

Ashis T. Roy[1,2] , Leanne Sherriff[1], Anthony Koutoulis[2], and Grey Leggett[1],
[1]Australian Hop Marketers, GPO BOX 104A, Hobart, Tasmania, 7001, Australia
[2]Cell Biology Group, School of Plant Science, University of Tasmania, GPO Box 252-55, Hobart, Tasmania, 7001, Australia

Abstract

As part of a study into the *in vitro* induction of tetraploid hops flow cytometry has been extensively used to determine ploidy level. Interesting differences have become evident in the ploidy level of various tissues of an individual plant following colchicine treatment. Plants that are shown to be mixoploid after analysis of apical and leaf tissues can display very high levels of tetraploid cells in the root. The ploidy level of mixoploid progeny is also being investigated.

Introduction

Tetraploid hops play an important role in hop breeding in Australia as triploid progeny can be produced when they are crossed with diploid males. Triploid hops are superior to diploids under Australian growing conditions as they are more vigorous and higher yielding and are also relatively seedless.

This study used *in vitro* techniques to induce tetraploid hops from a range of diploid varieties using colchicine. Although *in vitro* induction of polyploidy permits the mass production of pure tetraploids, it can also generate mixoploids (i.e. chimeras consisting of diploid and tetraploid cells). Consequently, an effective method for ploidy screening is required to identify the ploidy level of individual plants. Classically, screening of potential tetraploids involves a cytological approach to count chromosomes (Hamill *et al.* 1992), which can be time-consuming. Although expensive, flow cytometry analysis of nuclear DNA content is being increasingly used for high-throughput ploidy screening (van Duren *et al.* 1996). The number of nuclei that can be assessed using this technique is also far greater than is possible using microscopy, and hence the results are more reliable.

This study reports on the effect of tissue type of an individual plant on ploidy level and the implications that this has for unequivocal identification of tetraploids, and, hence, breeding programs.

Materials and methods

The experimental, diploid hop variety H138 and *in vitro* colchicines-induced polyploids derived from it (Roy *et al*. 2001), were used for flow cytometry analysis in this study. When the mixoploid and tetraploid plantlets were tested they were approximately 1-3 cm high with 1-5 nodes expanded. Samples were also analysed from a putative tetraploid Galena plant imported to Australia from the USA.

The different tissue types tested were leaf, shoot apex, stem and root. After collection material was stored at 4°C until preparation. The youngest, expanded leaf was used, and the first 10 mm of enough root tips to give approximately 0.5 g root material. The shoot apex was considered to be just the unexpanded tip (approximately 0.5 g), and the stem tissue came from the internode above the youngest expanded leaf.

Nuclei were collected by chopping 1 g of plant tissue in Galbraith's buffer (Galbraith *et al.* 1983) on ice. To label DNA, nuclei were stained with 1 $mg.l^{-1}$ propidium iodide. Propidium iodide fluorescence was analysed using a Coulter Elite ESP flow cytometer equipped with a 100 W high-pressure mercury arc lamp (excitation using a 488 nm air cooled argon laser and flourescence collected using a 600 nm Dichroic LP and 575 nm BP). Routinely, $1x10^4$ nuclei were measured per sample and evaluated using the Elite 4.02 and CellQuest 3.2.1 (Beckton Dickinson, USA) software packages. For each analysis time, known diploid and/or tetraploid samples of the same variety were used as controls to set the diploid and tetraploid peak positions.

After flow cytometry analysis samples were classed into different ploidy types according to the peaks obtained (Figures 1-3). In addition to pure diploid (Figure 1) and tetraploid (Figure 3), there were three classes of mixoploid designated as follows: (i) grade 1 mixoploids have at least one and a half times as much diploid as tetraploid nuclei; (ii) grade 2 mixoploids have approximately equivalent amounts of diploid and tetraploid nuclei; (iii) grade 3 mixoploids have at least one and a half times as much tetraploid nuclei than diploid nuclei.

Results and Discussion

The output from the flow cytometer are histograms of DNA intensity (Figures 1-3). The DNA intensity is directly related to ploidy level (a higher DNA intensity indicates a higher ploidy level). Peak areas for the diploid, tetraploid and octaploid peaks are also calculated for each sample. The latter are the results that are used for classifying the grade of the sample. Figures 1, 2 and 3 demonstrate the flow cytometer output for diploid, grade 2 mixoploid and tetraploid samples, respectively.

After testing leaf material using flow cytometry, the Galena plant imported from the USA was classified as a grade 1 mixoploid (Table 1). This plant was identified in the USA as a tetraploid by chromosome counts of root tissue. When root tissue was analysed using flow cytometry the sample exhibited a tetraploid profile (Table 1). This casts doubt on the reliability of using root tissue for ploidy testing (including using roots for chromosome counts). There was also variation in ploidy level between the other tissue types tested.

Table 1: Ploidy type by tissue type of Galena (plant glasshouse grown)

Tissue	**Ploidy type (%)**		
	2n	**4n**	**8n**
Leaf	56	36	4
Shoot apex	59	35	2
Stem	50	42	4
Root	30*	27*	37

* root sample had debris associated with the diploid and tetraploid peaks. Consequently these values overestimate the percentage of diploid and tetraploid nuclei.

From previous work (Roy et al. 2001), there were H138-derived plantlets with a range of ploidy types available in the collection. It was decided to perform a detailed survey of the ploidy level of the tissue types of these different grade plantlets. For the purposes of this study the definition of the ploidy level of a plant is based on the result obtained from flow cytometry testing of the leaf material. The results obtained from testing other tissue types from an individual plant are listed below the results for the leaf material (Table 2). Leaf and shoot apex tissue always produced similar ploidy profiles (Table 2). However, results from stem and root tissue conferred higher ploidy levels than that from leaf and shoot apex tissue. For example, it is impossible to differentiate between a pure tetraploid and a grade 3 mixoploid using root tissue (Table 2).

Table 2: Ploidy type by tissue type and plant of H138-derived *in vitro* plantlets

Tissue	Ploidy level	Ploidy level of nuclei (%)				
		Diploid*	**Grade 1 mixoploid***	**Grade 2 mixoploid***	**Grade 3 mixoploid***	**Tetraploid***
Leaf	2n	80	53	43	40	11
	4n	9	30	39	45	58
	8n	1	3	3	3	5
Shoot apex	2n	80	50	50	38	9
	4n	11	30	46	45	60
	8n	1	2	1	1	13
Stem	2n	74	30	30	26	13
	4n	9	47	49	56	56
	8n	2	4	5	6	12
Root	2n	48	38	24	9	10
	4n	39	46	54	50	48
	8n	1	2	14	28	27

*Classification of individual plants was made using results obtained from analysis of leaf tissue (ie, a diploid plant has a diploid profile of leaf material but may or may not have a different ploidy profile in other tissues)

Although using root tissue may not be satisfactory for accurately determining ploidy level, it may not be necessary to accurately differentiate between mixoploids and tetraploids. Mixoploids may still be useful in breeding programs as long as they are able to produce triploid progeny (ie, the reproductive parts are tetraploid).

The relationship between ploidy level of the tissue types already tested and that of the reproductive tissue has not been determined. It is difficult to test the ploidy level of the reproductive tissue (ie, flower) in a meaningful way as only a very small part of the flower, the ovule, contributes to the ploidy level of the progeny. The only way to evaluate the ploidy of the female reproductive tissue is by assessing the seedlings produced. As yet, no data have been collected on the ploidy level of progeny produced from the mixoploid and tetraploid H138-derived females. The H138-derived plants used in this study have just completed their first season in the field and seed collected from the individual plants will be germinated and tested this coming spring (August/September).

The stability of the colchicine-induced chimeras and, indeed, of the pure tetraploids is not known. The field planted H138-derived plants will be tested annually to determine whether there are any changes to their ploidy level.

Importantly, it is known (data not presented) that triploid progeny have been produced from the grade 1 mixoploid Galena plant, both in Australia and the USA. This suggests that not only is it possible to produce triploid progeny from mixoploids, but that the amount of tetraploid nuclei in the mixoploid (ie, the mixoploid grade) is immaterial. This theory is supported by results from Roy *et al.* (2001), where there was no relationship found between the number of tetraploid plantlets regenerated from callus tissue of different mixoploid grades.

Although the division into grades of polyploids can be considered somewhat arbitrary due to a continuum of peak ratios, it is a useful way of classifying the ploidy of samples. The results from this study demonstrate that the type of tissue used for flow cytometry analysis can have an enormous impact on the results obtained. Where the ploidy level of a plant needs to be accurately determined the leaf would be the best source of testing material. However, it may be possible for mixoploids of any grade to be suitable for breeding triploid hops. This is a promising result as mixoploids are much more frequently produced after treatment with colchicine than pure tetraploids.

Acknowledgments

The authors wish to thank Mr Mark Cozens for assistance with flow cytometry. Funding from Australian Hop Marketers and the Horticultural Research and Development Corporation of Australia supported this work.

References

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049-1051

Hamill SD, Smith MK, Dodd WA (1992) *In vitro* induction of banana autotetraploidy by colchicine treatment of micropropagated diploids. Aust J Bot 40: 887-896

Roy AT, Leggett G, Koutoulis A (2001) *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). (submitted)

van Duren M, Mopurgo R, Dolezel J, Afza R (1996) Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. Euphytica 88: 25-34

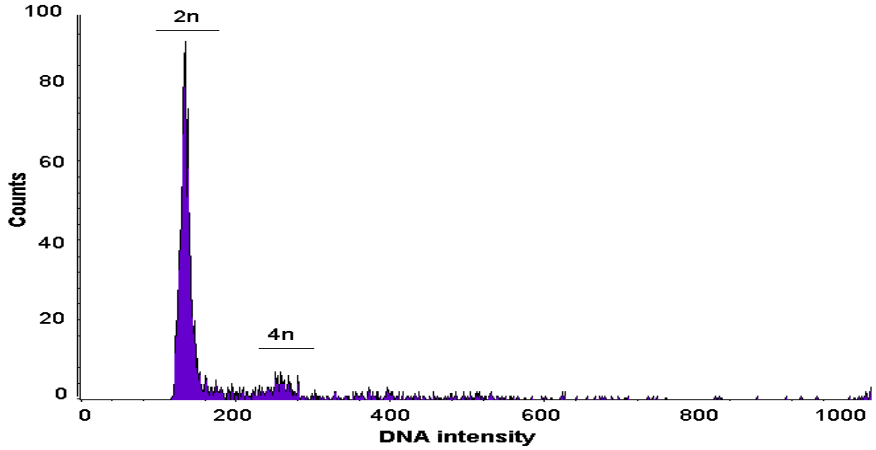


Figure 1: Typical flow cytometer histogram of a diploid profile showing diploid and tetraploid peaks.

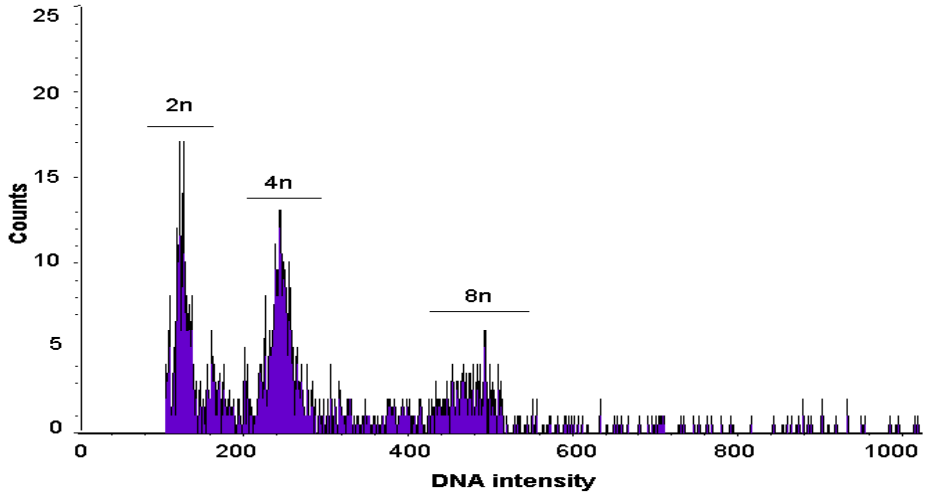


Figure 2: Typical flow cytometer histogram of a grade two mixoploid profile showing diploid, tetraploid and octaploid peaks.

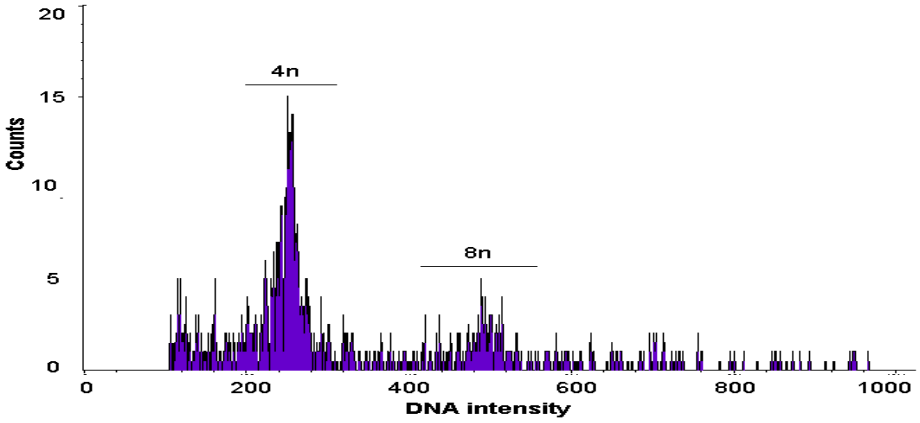


Figure 3: Typical flow cytometer histogram of a tetraploid profile showing tetraploid and octaploid peaks.

QUANTIFICATION OF INDOLE-3-ACETIC ACID IN HOPS CULTIVARS BY GC-MS ANALYSIS

Galovic, V.[1], Navalusic, J.[1] Baraldi, R. [2], and Ludwig-Muller, J. [3]

1 Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Yugoslavia
2 Instituto di Ecofisiologia delle Piante Arboree da Frutto, Area della Ricerca di Bologna, via Gobetti 101, 40129 Bologna, Italy
3 Jochan Wolfgang Goethe - Universitat, Botanisches Institut, Siesmayerstr. 70, 60054 Frankfurt, Germany

Abstract

Not much is known about the kind of indole-3-acetic acid (IAA) and their concentration in hops tissue. In this study we have isolated, characterized and quantified the IAA in different hops organs in the phenophase of intensive growth. That was performed by HPLC and subsequent GC-MS analysis. As an internal standard for quantitative mass spectral analysis indole-3-acetic-acid labeled with $^{13}C_6$ was used. Two forms of IAA was established and determined their endogenous concentration levels: minimum of free IAA in range of 2,65 ng/gFW, maximum of free IAA is 33,13 ng/gFW, minimum of conjugated IAA is 0,89 ng/gFW and maximum of conjugated IAA is 19,17 ng/gFW.

Introduction

The effects of IAA in plant are extremely varied. In particular, the hormone regulates growth processes with different sites of action showing qualitatively different competences. IAA plays an important role in plants in suppressing the formation of side shoots maintaining the apical dominance. Trewavas (1981) pointed out that hormone control is not by its concentration but by a change in sensitivity of the tissue to the compound.
Indole-3-acetic acid (IAA) is the main auxin in most plants.
IAA is synthesized from tryptofan or indole primarily in leaf primordia and young leaves, and in developing seeds. Recent research has shown that IAA is transported from cell to cell. Transport to the root probably also involves the phloem. IAA has several effects: cell enlargement, cell division, vascular tissue differentiation, root initiation, tropistic responses, apical dominance, leaf senescence, leaf and fruit abscission, fruit setting and growth, assimilate partitioning, fruit ripening, flowering, growth of flower parts, promotes femaleness in dioecious flowers (via ethylene).
After **Ludwig-Muller et al. (1996**) auxin conjugates are known to play a major role in the regulation of the content of free indole-3-cetic acid. IAA is known to be conjugated either to sugar moieties via an ester linkage, or to amino acids or peptides via an amide linkage. It was established for all cabbage plants that have been studied that most of the IAA was in conjugated form and that the conjugates, in some tissue, accounted for over 90% of the total IAA. Recently it was indicated that conjugates play an important role in auxin physiology and metabolism (**Kleczkowski and Schell, 1995**.). The putative roles include storage and protection against degradation, and the conjugates are thought to be a means of transport, possibly including tissue targeting (**Cohen and Bandurski, 1982**). The equilibrium between free and conjugated hormones is important for the homeostatic control of the concentration of the free hormone in the plant (**Bandurski, 1980**). In addition, conjugation has long been recognized as a way for plants to detoxify excess hormone (**Feung et al., 1974, 1975).**
While ester-linked forms of IAA are the predominant conjugates in maize and have been extensively studied (**Cohen and Bandurski 1982**), amide conjugates seem to be the bulk of

conjugated IAA in most dicotyledonous plants.
Hop is an intensive crop and climber plant which undoubtedly possess great emerge energy as much as apical domination.
One of the most important biological and economical factors in planting hops is the existence of torsia ability through the first third of vegetation. Once the plant reaches the top of the trellis the apical domination becomes weaker.
Torsial movements are very intensive at the stage of intensive growth and act mutually with the apical dominance. The ability of the torsial movements of the hop shoots depends exclusively on genotype. According to that all hops genotypes can be divided into three groups: weak torsial ability (shoots grow i.e. spread at an angle of 180 degrees), medium torsial ability (shoots grow at an angle of 45 degrees) and excellent torsial ability (shoots grow almost entirely up straight at an angle of 90 degrees).
During the phase of intensive growth it is hypothetically assumed that the free form would be predominated in hops in relation to the conjugated form of IAA. That the free IAA would be of greater concentration in the genotypes with pronounced growth in the phenophase of intensive growth. It is also assumed that the IAA concentration would appear in great amount at the apical meristem, as well as in the nodes and in the leaf buds.
There are no records about the endogenous concentration of IAA in hops, so the aim of this investigation was to determine the content of IAA in hops cultivars, the form of IAA (free or conjugated) that prevails in different cultivars and organs during the stage of intensive growth.

Material and methods

Plant material. Hops woodcuttings of 9 different hops genotypes (three replications) were potted at the end of december and after one month green shoots were harvested. They were collected at an intensive growth phenophase and cutted to parts of interest. Apical part of the shoot, nodes, internodes and leafs were used as a matrix for determination the content of IAA. Further on they were treated with liquid N_2 and kept at deep freeze until examined.
The quantification method consists of three steps: IAA extraction, component separation (by hydrolysis and purification) and IAA quantification.

Extraction, hydrolysis and purification. Approximately 1g of plant material (fresh basis) were extracted according to **Chen et al., 1988.** To determine the content of IAA that are present as ester or amid conjugates, it was necessary to hydrolyze plant samples using basic conditions to get free IAA from conjugated form. Treatment with 1N NaOH for 1h duration at room temperature resulted in quantitative hydrolysis of ester conjugates and a treatment with 7N NaOH for 3h duration at 100o C hydrolyzed the amide conjugates. As the direct injection of a crude biological sample onto the HPLC column could cause severe problems it is essential to undertake a cleaning step prior to analysis. Free IAA purification of diluted plant extract (1:10) was carried out using conditioned amino anion exchange column (Baker 10 SPE). A Hydrolysate for the determination of total IAA was passed through the conditioned SPE C_{18} column. Eluents were evaporated near to dryness, resuspend in 100 μl MeOH for HPLC.

HPLC purification. The HPLC method was used for extract component separation and isolation of the certain compound, in this case-IAA, and was performed through a Lichrosorb C18 ODS column (125 x 4.5 mm, 5 μm diameter and 10 mm pore size particles) diluted with 1% acetic acid (A) and 100% MeOH (B) four step gradient (20 min 25%B, 10 min 100%B, 5 min 25%B, 5 min equilibrium 25%B; flow rate 0.7 mL/min). UV detection at 280 nm. The radioactive fractions corresponding to the elution time of IAA was collected, pooled and purified resuspended in 100 μL MeOH, methylated using ethereal diazomethane according to **Cohen et al., 1987**.

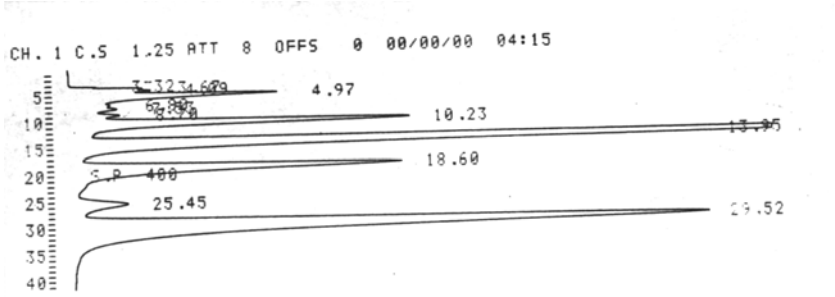


Fig. 1 HPLC spectrum of Chinook cultivar free IAA

Mass spectroscopy. GC-SIM MS was undertaken for quantification of endogenous IAA. The IAA containing fractions, after HPLC purification, were reduced to dryness. The dry sample was resuspend in 100 μL MeOH and methylated with diazomethane. Spectra were taken in selective ion monitoring mode. Quantification of endogenous IAA was performed by using the isotope dilution technique against $^{13}C_6$ IAA as internal standard. The ions at m/z 130 and 189 are from the quinolinium ion and molecular ion, respectively, of the methyl ester of the endogenous IAA. Ions at m/z 136 and 195 were monitored for the base peak and molecular ion, respectively of the $^{13}C_6$ IAA internal standard.

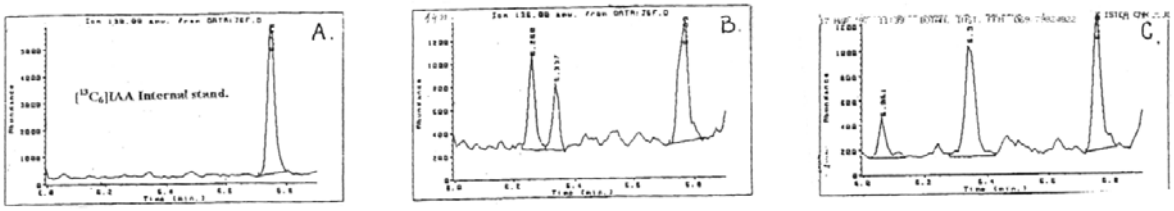


Fig. 2 Selected ion chromatogram of the methylated HPLC purified sample of free IAA from hops College Cluster cultivar (A-internal standard; B-quindinium ion of the endogenous IAA; C-molecular ion of the methyl ester of the endogenous IAA.

All analysis were conducted at three different laboratories: collecting of the material in the Institute of Field and Vegetable Crops Department of Hops, Yugoslavia, IAA extraction and preparation for HPLC and GC-MS analysis in JF Goethe-Universitat, Frankfurt, Germany and GC-MS analysis in Instituto di Ecofisiologia delle piante arboree da frutto, Bologna, Italy.

Results and discussion

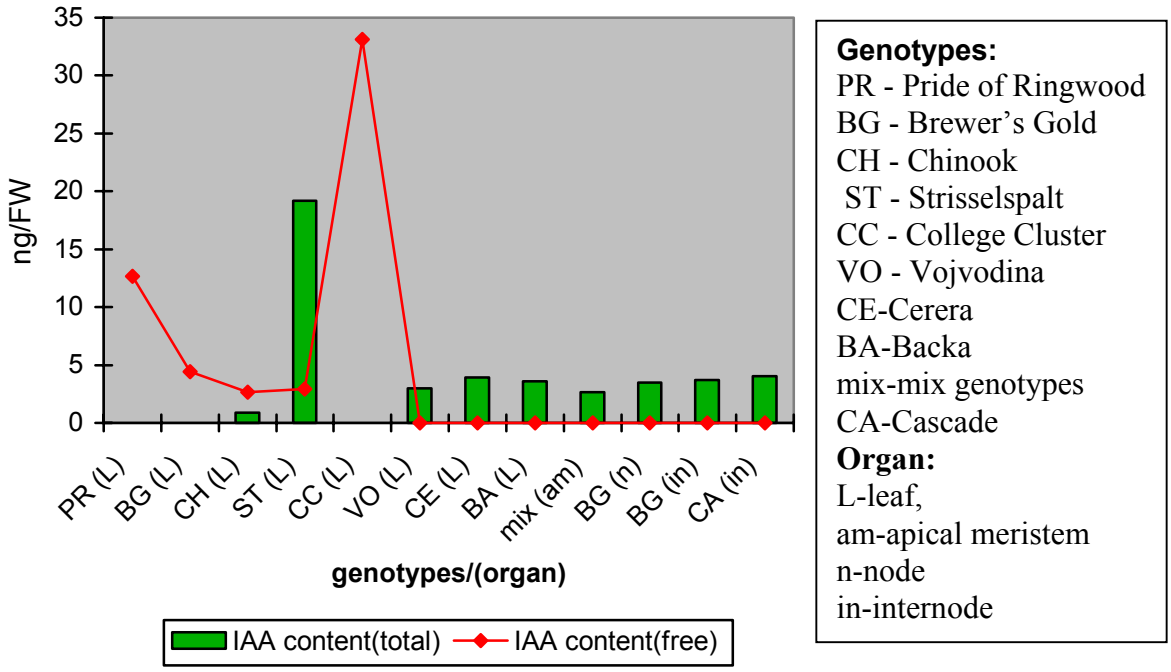
Isotope dilution equation. The amount of endogenous IAA in different hops cultivars and in selected parts were calculated according to modified isotope dilution equation:

$$\frac{area130 + area136}{area136} - 1x\frac{x}{R} / FW \text{ of sample}$$

X = amount of $^{13}C_6$ IAA internal standard (ng)
R = 1.13 (correction for natural abundance of $^{13}C_6$)

According to the results (Graf. 1) obtained by the equation above it could be assumed that both forms of IAA, free and conjugated, were appeared respectively in the leaf samples while in nodes, internodes and apical meristems were no traits of free form of IAA. On the contrary only

conjugated IAA form at low concentration were found. This result is in accordance with **Ludwig-Muller, 1996.**
It is very interesting to note that in Pride of Ringwood cultivar, a late cultivar under Vojvodina conditions, the free form of IAA was found in high concentration in young leaves. This could point out to very fast growth during the first third of the vegetation i.e. during the phase of intensive growth that was confirmed by phenological investigation under field conditions (**Galovic**, **unpublished**). Also in College Cluster cultivar we established only free IAA form in extremely high concentration. This cultivar is early under Vojvodina conditions meaning very intensive growth throughout the vegetation, phenotypic abundance, and short vegetation period. This behavior could be explained by extremly high concentration of free IAA form in young leaves. These results are in accordance with the statement of **Mohr and Schopfer, 1995**, that IAA produced in young leaves could be formed on de novo synthesis or on release from bound forms which could be the reason of free IAA domination in those cultivars.
The free IAA were detected in leaves of Brewer's Gold cultivar, however in its nodes and internodes prevails the conjugated IAA form, with somewhat higher content found in internodes.



Graph. 1 Content of conjugated and free IAA (ng/gFW) in hops

In leaves of Chinook cultivar the concentration of free was three times higher in comparison to conjugated IAA. The extremely high content of conjugated IAA was found in the leaves of Strisselpalt cultivar, and this form of IAA is the only one found in Backa cultivar. This could be explained by similar slow growth rates in both cultivars under field condition, and by the investigation concerning the origin of Backa variety that confirmed the close genetic connection of both cultivars (**Galovic**, **1996**).

Conclusion

Since these were the preliminary investigations, and basic by their nature, it can be concluded

that there is the accordance between results obtained on the basis of laboratory analyses and those recorded on the basis of phenotypic and phenological observations. The content of IAA varied according to the genotype, the organ under observation, and the form of IAA. The free form of IAA prevails in comparison to conjugated IAA form in young leaves of hops cultivars especially in College Cluster that showed the fast growth under the phenological investigation. Both forms of IAA were found in hops leaves however only conjugated one was established in other organs (nodes, internodes, apical meristem) under observation.
On the basis of these investigations the limits for estimation of the IAA concentration in the hops are: minimum of conjugated IAA = 0.89 ng/gFW, and maximum of conjugated IAA = 19.17 ng/gFW. Minimum of free IAA = 2.65 ng/gFW, and maximum of free IAA = 33.13 ng/gFW.
The above mentioned results only opened the way for raising many more questions concerning this physiological field of investigation and they should be continued.

References

Bandurski, R. S., 1980: Homeostatic Control of Concentrations of Indole-3-Acetic Acid. *In:* Plant Growth Substances (F. Skoog, ed.), . Springer-Verlag, Berlin. ISBN 3-540-10182-9, pp. 37-49.

Bandurski, R. S., Cohen, J. D., Slovin, J. and Reinecke, D. M., 1995: Auxin biosynthesis and metabolism, chapter B of plant hormones, 39-65: Physiology, Biochemistry and Molecular Biology, 2 nd Edition, Peter J. Davies editor, Kluwer Academic Publishers.

Chen, Kai-Hsien, Miller, N. A., Patterson, W. G., and Cohen, D. J., 1988: A Rapid and Simple Procedure for Purification of Indole-3-Acetic Acid Prior to GC-SIM-MS Analysis. Plant Physiology, 86: 822-825.

Cohen, J. D., Bausher, G. M., Bialek, K., Buta, J. G., Gocal, F. W., G., Janzen, M. L., Pharis, P. R., Reed, N. A., Slovin, P. J., 1987. Comparison of a Commercial ELISA Assay for Indole-3-Acetic Acid at Several Stages of Purification and Analysis by Gas Chromatography- Selected Ion Monitoring- Mass Spectrometry Using a 13C6-Labeled Internal Standard1. Plant Physiology, 84: 982-986.

Feung, C. S., Mumma, R. O., & Hamilton, R. H., 1974. Metabolism of 2,4-Dichlorophenoxyacetic Acid. VI. Biological Properties of Amino Acid Conjugates. – J. Agric. Food Chem. 22: 307-309.

-, Hamilton, R. H. & Mumma, R. O., 1975. Metabolism of 2,4-dichlorophenoxyacetic acid. VII. Comparison of Metabolites from Five Species of Plant Tissue Culture. J. Agric. Food Chem. 24: 1013-1015.

Galovic, V., 1996: Genetic Homogenity of Quantitative Characteristics of Hops (*Humulus lupulus* L.) Variety 'Backa'. M. Sc. thesis, University of Novi Sad, Faculty of Agriculture, Novi Sad, Yugoslavia.

Kleczkowski, K. and Schell, J., 1995: Phytohormone conjugates: Nature and Function. Crit. Rev. Plant Sci. 14: 283-298.

Ludwig-Muller, J., 1996: Auxin-Conjugate Hydrolysis in Chinese Cabbage: Characterization of an Amidohydrolase and its Role During Infection with Clubroot Disease. Physiologia Plantarum, 97: 627-634.

Mohr, H., Schopfer, P., 1995: 23. Physiology of Hormone Action. *In:* Plant Physiology, Sprnger-Verlag Berlin Heidelberg, pp. 383-408.

Trewavas, A., 1981: How does Plant Growth Substances Act? Plant Cell Environment 4: 203-228.

OPTIMISATION OF THE ANALYSIS OF HOP OILS BY SOLID-PHASE MICROEXTRACTION

Karel Krofta
Hop Research Institut Co., Ltd., Kadanska 2525, 438 46 Žatec, Czech Republic

Abstract

An analytical procedure based on headspace solid-phase microextraction (HS-SPME) has been used for isolation of hop oils from various hop matrices. Four SPME fiber coatings of different polarity have been evaluated.Several parameters such as exposure time and temperature, sample weight, desorption conditions have been optimised. Sorption of analytes was considerably influenced by temperature and exposure period. Sample weight in the range of 0,1 - 0,3 g did not influence sorption significantly. Factors such as hop variety, age of hops and type of hop matrix have to be taken into account for choice of a suitable SPME fiber. The PDMS 30 μm fiber proved to be most suitable for isolation of hop oils from female hops, PDMS/DVB 65 μm fiber is the best one for isolation of hop oils from male hops. Optimised sampling parameters include 0,2 g of sample weight, temperature 50 °C and exposure period 2 hours. The composition of hop oils isolated by HS-SPME method showed good complience with conventional steam-distillation method.

1. Introduction

Essential oils belong to an important hop secondary metabolites from brewing and chemotaxonomy point of view. Careful isolation of hop oils from matrix is necessary to prevent secondary changes of its composition. While gas chromatography is almost exclusively used for analysis of hop oil composition several methods can be used for preparation of hop oil sample from hops. Steam-distillation is a conventional method where hop oils components are released from hops by distillation with water steam. These methods are time consuming and require relatively big amount of hops (min. 50 g) if reliable results have to be obtained.

Principially different methods of hop oils isolation are based on direct sampling of gas phase above hops sample in close vial (”headspace” methods). In static ”head space” modification is sample of gas phase directly injected to analytical column of gas chromatograph. To attain a sufficient sensitivity of the method heating of the sample at elevated temperatur is necessary. Solid-phase microextraction (SPME) method is based on adsorption of analytes on a phase-coated fused silica fiber. Fiber can be immersed in the liquid or exposed to the headspace above the sample. SPME methods use no solvent for extraction and are relatively low cost. The compounds adsorbed to the fiber are thermally desorbed onto GC column by inserting SPME fiber into the injection port. It has been developed for extracting volatile and semi-volatile compounds from waste water samples but it can be used for analysis of less volatile compounds without phase equilibrium attainment if sufficient concentration on the fiber surface is achieved.

2. Experimental

2.1. Samples

Following hop varieties and breeding stock were used for experimental work: Saaz hops, crop 1999, 2000, Premiant (Czech hybrid variety), crop 2000, 4587 (high-alpha breeding stock), crop 2000, 4587, CO_2-extract, crop 1999, inflorencence of male hops. Hops were picked at optimum stage of maturity and dried at 55 °C for 12 hours. Samples were stored at + 4 °C in a dark place until analysis.

2.2. Isolation of hop oils by steam-distillation method and SPME procedure

The isolation of hop oils was performed by conventional steam-distillation method as a comparable procedure. Solid-phase microextraction procedures were performed with SPME manual holder (SUPELCO) and four types of SPME fibers of 10 mm in length (PDMS 100 μm, PDMS 30 μm, PDMS/DVB 65 μm, PA 85 μm) were used for extraction of hop oils components. Hop cones were ground just before the analysis. The sample of ground hops was placed into 4

ml amber vial, tightly closed and put on the heating block. SPME fiber was placed to the headspace immediately after piercing the vial septum. Different extraction periods from 30 minutes to 4 hours, various temperatures 25 °C, 40 °C, 50 °C and sample weight in the range of 0,1 - 0,3 g were tested for optimisation of extraction conditions for all fibres.

2.3. Instrumental analysis

All analyses were performed on Varian 3400 gas chromatograph in the connection with mass detector Finnigan ITD 800.Separation of hop oil components was performed on capillary column DB 5 (30 m x 0.25 mm x 0.25 μm film thickness). The initial oven temperature of 40 °C was held for 3 min. and then gradually elevated to 250 °C. Helium was used as a carrier gas with 1 ml/min. of column flow. Liquid samples (steam-distilled oils) were injected in split ratio 1:50. Mass detector based on ion-trap principle worked at ionisation regime electron impact (70 eV) at the scan frequence of 1 scan/sec.

3. Results and Discussion

3.1 Sample amount

The effect of sample weight on the detector response was examined at the range of 0,1 – 0,3 g of ground hops and sorption temperature of 50 °C for 60 minutes. It can be stated that dependence of sorption of hop oil components on sample amount has minor importance in comparison with other aspects (see below).

3.2. Influence of hop variety, age of hops and sample matrix

Hop variety, age od hops and form of hop (cones, pellets, extract) have the influence of principle to the sorption of hop oil components on the SPME fibers. The detector responses expressed in electronic area counts for seven hop oils components determined for Saaz, Premiant and 4587 varieties are summarized in Table 1. The results show, that raw hops is a very unstable matrix in time horizon of a few weeks and months. Hop oils content declines and changes its composition. Saaz hops from 1999 crop, that was analysed 7-8 monts after picking, has substantially lower responses compared to the ones for fresh cones of the same variety. Significant difference in detector responses (peak areas) were monitored between fresh cones of hybrid 4587 (crop 2000) and CO_2-exctract of the same variety too.

Table 1: The influence of hop variety to detector responses of some hop oil components (PDMS 30 μm, 60 minutes, 50 °C, 0,2 g)

Component	Detector response (electronic area counts)				
	Saaz, 1999	*Saaz, 2000*	*Premiant, 2000*	**4587, cones, crop 2000**	**4587, extract crop 1999**
Myrcene	5000	20000	50000	90000	17500
Methylheptanoate	21	160	340	180	31
Linalool	26	120	490	420	250
2-undecanone	85	330	440	480	650
β-caryophyllene	950	6000	10000	18000	17500
α-humulene	2600	15000	27000	21000	25800
β-farnesene	1800	11000	2000	< 10	< 10

3.3 Comparison of SPME fibers

In Table 2 there are summarized comparative data of sorption of hop oils components to all tested SPME fibres. Data are expressed relatively to the sorption of PDMS 100 μm fiber (100

%). Experiments were performed with Saaz variety, crop 2000, one hour exposition at 50 °C. Sorption capacity of PDMS 30 μm fiber is lower for all components and roughly balanced at the range of 30-40 % compared to PDMS 100 μm fiber. Polyacrylate (PA) fiber has higher afinity to polar compounds. This tendency is most evident for terpenic alcohols linalool, geraniol and in less extent for 2-undecanone a methylheptanoate. The highest sorption capacity was determined for PDMS/DVB 65 μm fiber for either terpenic and oxygen hop oils components.

Table 2: Relative detector responses for various SPME fibers

Component	Fiber coating			
	PDMS 100 μm	PDMS 30 μm	PS 85 μm	PDMS/DVB 65 μm
β-pinene	100	34	20	114
Myrcene	100	43	50	144
Methylheptanoate	100	35	57	184
Limonene	100	36	46	170
Linalool	100	31	95	231
Geraniol	100	34	151	306
2-undecanone	100	28	56	195
β-caryophyllene	100	30	18	111
α-humulene	100	35	20	118
β-farnesene	100	42	38	112
δ-cadinene	100	38	26	114

3.4. Influence of experimental temperature and time

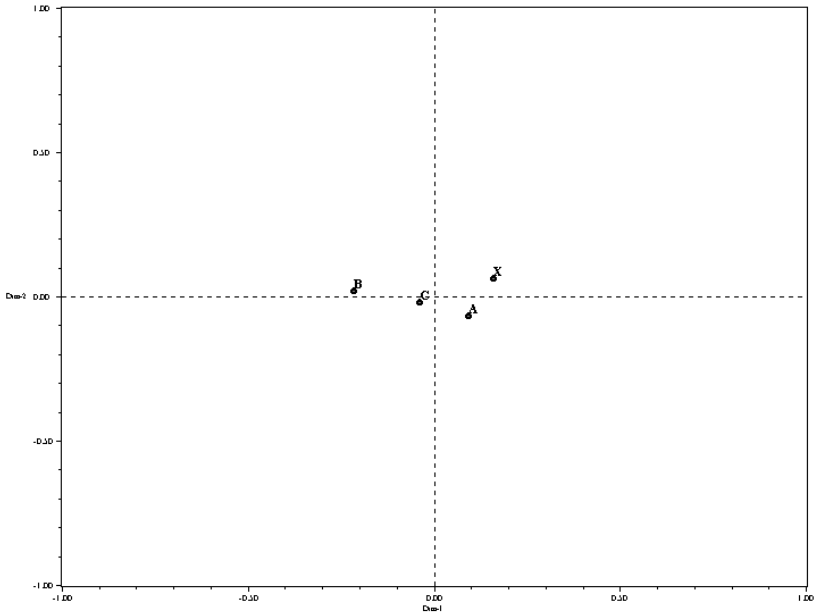
The sorption dynamics of hop oil components was investigated for exposure periods ranging from 30 minutes to 4 hours at the temperature of 25 °C, 40 °C, 50 °C (Saaz variety, 1999). Longer exposure periods at the temperature above 50 °C were not systematically tested due to possible oxidation of some hop oil components. Hop oils composition is most influenced by content of myrcene, β-caryophyllene, α-humulene and β-farnesene. While myrcene amount captured on fiber stationary phase slightly declines with time, the amount of less volatile terpenes has strongly progressive character. The equilibrium concentration of myrcene as a relatively more volatile hop oils component (b.p. 167 °C) is established within 2 hours at 25 °C on non-polar PDMS fiber and within 1-2 hours on slightly polar PDMS/DVB fiber at 25 °C. Increasing temperature and prolonged exposure period result in a slightly decline of myrcene concentration probably due to strong competitive sorption. On polyacrylate fiber myrcene reached equilibrium concentration at 50 °C and in two hours of exposure period. The dynamics of α-humulene sorption is very different from myrcene. It reaches equilibrium concentration neither after 4 hours of exposure period at 50 °C on PA 85 μm and PDMS 100 μm fibers. The equilibrium concentration was reached only on PDMD/DVB 65 fiber at 50 °C and 2 hours of exposure period. Similar data were obtained for caryophyllene. Terpenes with higher boiling point than α-humulene (β-farnesene, γ-muurolene, α-selinene, β-selinene, γ-cadinene, δ-cadinene) do not reach equilibrium concentration on any tested fibres either after 4 hours of exposure period at 50 °C. It was confirmed by a few analyses at 60 °C when detector responses of less volatile terpenes were higher than those of measured at 50 °C. The methylesters of fatty acids have different character of sorption. The amount captured on fiber coatings declines with elevated temparature regardless of the SPME fiber. The equilibrium concentration was reached after 2 hours of exposure time on PA 85 μm and PDMS 100 μm fibers, on PDMS/DVB 65 μm fiber captured amount slightly declines with prolonged exposure

period. Sorption of 2-undecanone depends on temperature and extraction period on all SPME fibres. Equilibriun concentration is reached on PA 85 μm fiber (50 °C, 2 hours) and PDMS/DVB fiber at the same conditions. The above mentioned data indicate that in a complex system of many hop oil components of various chemical compositions, volatilities and polarities only a few of them reach full equilibrium concentration on SPME fiber coatings. Competitive sorption have to be taken into consideration. In spite of this reproducible results can be obtained if all experimental conditions are strictly maintained. Consistent timing is more important than full equilibrium.

3.5 Comparison of head-space SPME hop oils fingerprints

Detailed examination of chromatograms showed, that spectrum of hop oils components captured on all tested SPME fibers was practically identical with those of conventional steam-distillation method. Differences exist in proportion of individual hop oils components. This finding with respect to big variation in physical and chemistry properties of individual hop oil components is very positive. Therefore HS-SPME method represents an excellent alternative method for isolation of essential oils from hops and subsequent fingerprinting of hop varieties. The composition of hop oils of Saaz variety (crop 1999) determined by steam-distillation method and by HS-SPME method on three SPME fibers is summarized in Table 3. Data show that composition of hop oils measured by HS-SPME procedure is similar to steam distillation analysis. It is difficult to distinguish the analysis with the highest similarity at the first glimps and subsequently to chose the most suitable fiber. Objective comparison of similarity resp. differences of individual analyses were performed by statistical methods of multivariate analysis, cluster analysis and analysis of principal components.Obtained results (principal components) are shown on Fig. 1. Head-space SPME analysis with PDMS/DVB 65 μm fiber were evaluated to be in statisticaly best accordance with steam-distillation procedure by both multivariate methods. All tested fibers can be principally used for isolation of esential oils from hops but PDMS and PDMS/DVB coatings are prefered.

Fig.1 **The evaluation of SPME fibers by method of principal components (X=steam-distillation, A=PDMS/DVB 65 μm, B=PA 85 μm, C=PDMS 100 μm)**



3.6 Choice of SPME fiber

The fiber PDMS/DVB 65 μm has limited utilisation for isolation hop oils components from various matrices. Excellent sorption capacity can result in lost of resolution of neighbouring eluated

components with close retention times if fresh hops or varieties with high oils content are analysed. First eluated peak is tailing into the second one. It is of real concern of 2-methylbutylpropanoate /β-pinene, methylgeranate/Methyl- decanoate and preferably α-humulene/β-farnesene resolution. This problem has been satisfactory solved by replacement of PDMS/DVB 65 μm fiber by PDMS 30 μm fiber. HS-SPME method showed to be very useful for oil analysis of male hops. The weight of 0,3 g of dried male inflorencesce was extracted with PDMS/DVB 65 μm at 50 °C for 2 hours without any additional sample treatment. SPME method showed to be of general purpose. It can be performed with fresh and old hops, hop pellets and hop extracts. It can be performed with green, undried hop cones as well. Only one cone is sufficient for the analysis. Conditions of hop oils analysis by headspace SPME method are always a compromise among intensity and character of oil compoments sorption, chromatography resolution and detection of low concentration analytes. The choice of used fiber is made with respect to the hop variety, age of hops and type of hop product. The fiber PDMS 30 μm is most suitable for fresh hops with high content of hop oils. Excellent sorption capacity of PDMS/DVB 65 μm and PDMS 100 μm can be utilised if old hops with low content of hop oils have to be analysed and for analysis of male hops.

Table 3: Comparison of hop oils composition - steam-distillation – SPME procedures (Saaz, 1999)

Component	Steam-distillation	PDMS/DVB 65 μm	PA 85 μm	PDMS 100 μm
	X	A	B	C
β-pinene	0,36	0,37	0,54	0,68
Myrcene	16,8	21,2	27,4	26,0
Methylheptanoate	0,18	0,19	0,21	0,17
Limonene	0,32	0,49	0,53	0,41
methyl 6-methylheptanoate	0,20	0,19	0,27	0,20
2-nonanone	0,25	0,26	0,46	0,19
Linalool	0,39	0,39	0,89	0,24
2-nonanol	0,18	0,10	0,17	0,08
Methyloctanoate	0,15	0,14	0,15	0,11
2-decanone	0,37	0,24	0,28	0,19
Methylnonanoate	0,19	0,16	0,09	0,12
Geraniol	0,14	0,15	0,28	0,04
not identified	0,55	0,34	0,46	0,24
methyl 8-methylnonanoate	0,08	0,08	0,09	0,07
2-undecanone	1,97	1,34	1,38	0,90
methyl-4-decenoate	1,86	1,52	1,81	1,27
methyl-4,8 decadienoate	0,66	0,69	0,79	0,40
methylgeranate	0,09	0,13	0,17	0,06
methyldecanoate	0,08	0,09	0,08	0,05
α-copaene	0,29	0,45	0,29	0,46
2-dodecanone	0,29	0,21	0,23	0,16
β-caryophyllene	7,77	8,79	6,33	8,07
trans-α-bergamotene	1,17	1,11	0,95	1,74
α-humulene	25,2	28,4	18,8	25,9
β-farnesene	17,5	15,5	13,8	16,7
γ-muurolene	0,86	1,19	0,69	1,08

β-selinene	0,52	0,68	0,48	0,57
α-selinene	0,53	0,72	0,48	0,62
2-tridecanone	1,69	1,34	1,30	1,04
γ-cadinene	1,07	1,54	1,01	1,06
not identified	0,16	0,21	0,15	0,12
δ-cadinene	1,54	2,35	1,32	1,62

INCLUDING DATA OBTAINED BY SOLID-PHASE MICROEXTRACTION IN THE MIN-MAX MODEL ON THE COMPOSITION OF HOP ESSENTIAL OIL

M. Kovačevič[1] & M. Kač[1,2]

[1]Institute of Hop Research and Brewing Žalec, Žalskega tabora 2, SI-3310 Žalec, Slovenia

[2]Universty of Ljubljana, Biotechnical Faculty, Department of Food Science, Jamnikarjeva 101, SI-1111 Ljubljana, Slovenia

Abstract

The MIN-MAX model is a handy tool for identification and quality evaluation of hop cultivars. The reliability of the method depends on a comprehensive data base. The data set currently used by the Institute of Hop Research and Brewing Žalec results from analysing essential oils obtained by steam distillation. Some 1999 and 2000 hop samples were analysed also by the much handier and much quicker solid-phase microextraction (SPME) method. In spite of differences regarding relative contents of some components, the model is robust enough for these data to be used in routine identification of hop cultivars and determination of aroma properties.

Introduction

The MIN-MAX model which has been developed on the Institute of Hop Research and Brewing in Žalec for presenting the chemical composition of hop essential oil is routinely used for identification of hop cultivars and for determination of aroma properties (Kač & Kralj, 1998; Kač & Kovačevič, 2000). The routine method for hop essential oil analysis is based on oil preparation by steam distillation followed by gas chromatographic separation. The sample preparation is relatively simple, but it is a time consuming procedure (3-4 hours of distillation).

Solid-phase microextraction is a relatively new sampling and concentration technique. A sampling device – a fused silica fibre coated with a polymer sorbent – concentrates analytes by adsorption and/or absorption and the extracted material is released into a GC system by means of thermal desorption in a GC injector (Pawliszyn, 1999). In the case of volatile analytes, such as essential oil in hop cones, it can be used as a solvent-free headspace sampling technique (Zang & Pawliszyn, 1993). The composition of extracted volatiles depends on sampling parameters like extraction temperature, extraction time and sample weight (Kovačevič & Kač, 2001) and on the type of SPME fibre used (Krofta & Čepička, 2000). Additionally, there are strong analyte – matrix interactions with less defined influence on extraction procedure. It is

therefore impossible to achieve results that are statistically equivalent to those obtained by steam distillation for each essential oil compound. Despite this limitation, headspace SPME has been used as a tool for hop characterisation by monitoring the ratio between alpha-humulene and beta-caryophyllene (Field *et al*., 1996).

Since MIN-MAX model is a very robust method for data presentation, it was tested as a tool for processing results obtained by the SPME method. The main concern was the compatibility of these with the reference data base obtained by steam distillation and routinely used for identification of cultivars.

Experimental

The MIN-MAX model was tested on 31 hop samples belonging to four cultivars commnly grown in Slovenia: Aurora, Celeia, Magnum and Savinjski golding. They originate from different Slovenian hop growing areas and are representative of the 1999 and 2000 crops.

SPME sample preparation was as follows: hop cones were ground in a coffee mill, 2 g of sample was weighed into a 40 mL headspace vial and closed with a screw cap. The headspace vial was conditioned in a thermostatic water bath at 70 °C for 45 minutes. The next step was the SPME extraction using a Supelco fibre with a 100 μm polydimethylsiloxane coating at extraction temperature of 70 °C for 30 min.

Extraction completed, the fibre was retracted and injected into the Hewlett Packard 5890 gas chromatograph using flow splitting of 1:50, at 0.5 mL/min carrier gas flow (N_2, 5.0), a HP-1 capillary column (25 m × 0.2 mm, 0.11 μm) and a flame ionisation detector. The temperatures of injector and detector were 240 °C and 280 °C, respectively. The temperature programme was 3 min at 60 °C, from 60 °C to 190 °C at a rate of 2.5 °C/min and 1 min at 190 °C. The chromatograms were recorded and integrated using a Hewlett Packard 3396A integrator. The results of chromatography were given as relative areas of all essential oil components.

Results and discussion

Since the results of the SPME analysis are expressed in the same way as those obtained by direct injection of essential oil prepared by steam distillation, the data manipulation was the same. From original relative peak areas, the so-called indexes were calculated as described elsewhere (Kač & Kovačevič, 2000). The indexes were introduced for various practical reasons, mostly to get the data about constituents of the essential oils which differ also for some orders of magnitude (Figure 1) on the same scale. The presentation of each cultivar as a "corridor" indicating minimal and maximal values of the components in question gives a fingerprint of the cultivar and it becomes obious that statistically different chromatograms still belong to the same cultivar as long as the shape of the pattern (corridor) remains the same. Samples of all four groups of tested cultivars were charted into the MIN-MAX model for each cultivar and the matching was observed. In practice the identity of a hop sample is confirmed if model of the sample in question fits into the MIN-MAX model (corridor) for that particular cultivar. The system works very well in the case where steam distillation data are used, since the model was constructed from such data. It has been shown (Kovačevič & Kač, 2001), that results obtained by both methods are similar, but not statistically equivalent. So, theoretically speaking, data obtained by SPME can not be compared with steam distillation data, which is a major drawback for introduction of the handy SPME method into a daily laboratory practice.

Since most of the essential oil data obtained in our laboratory are treated through the MIN-MAX model, we tested the ability of this model to use SPME data as well. It is clearly shown on Figures 2 and 3, that the current models constructed of steam distillation data can be used for SPME data as well. The differences between steam distillation and SPME are therefore reduced by robustness of the MIN-MAX model. Since hop aroma evaluation is also performed by the MIN-MAX model, SPME analysis can be used also for that purpose.

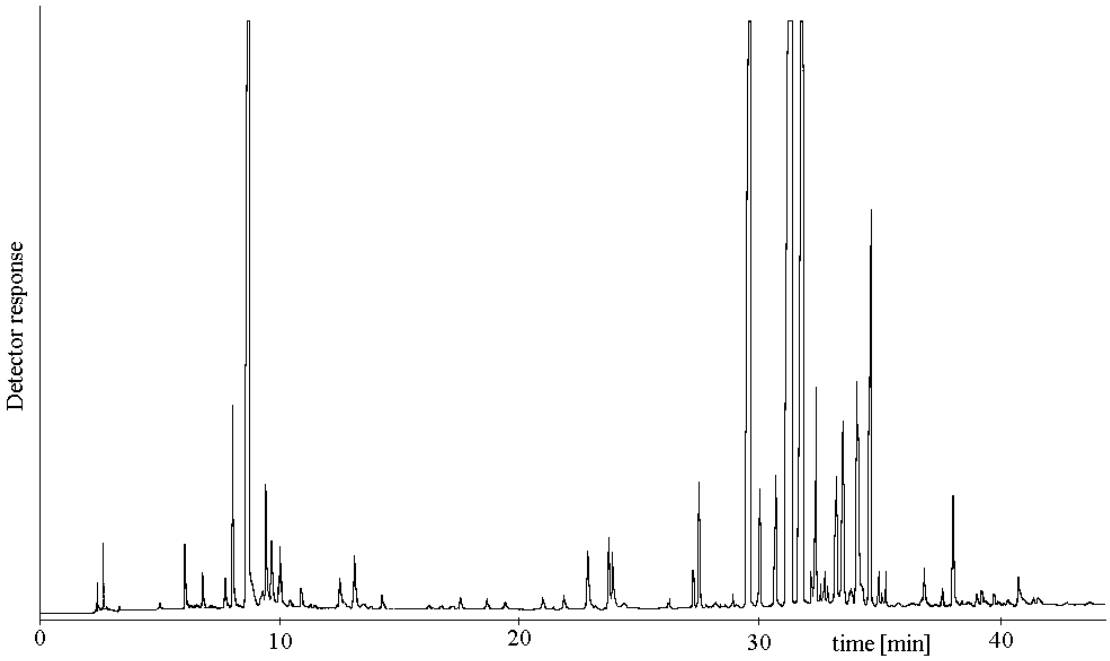


Figure 1: A typical GC chromatogram of the volatiles of the cultivar Aurora, SPME sampling

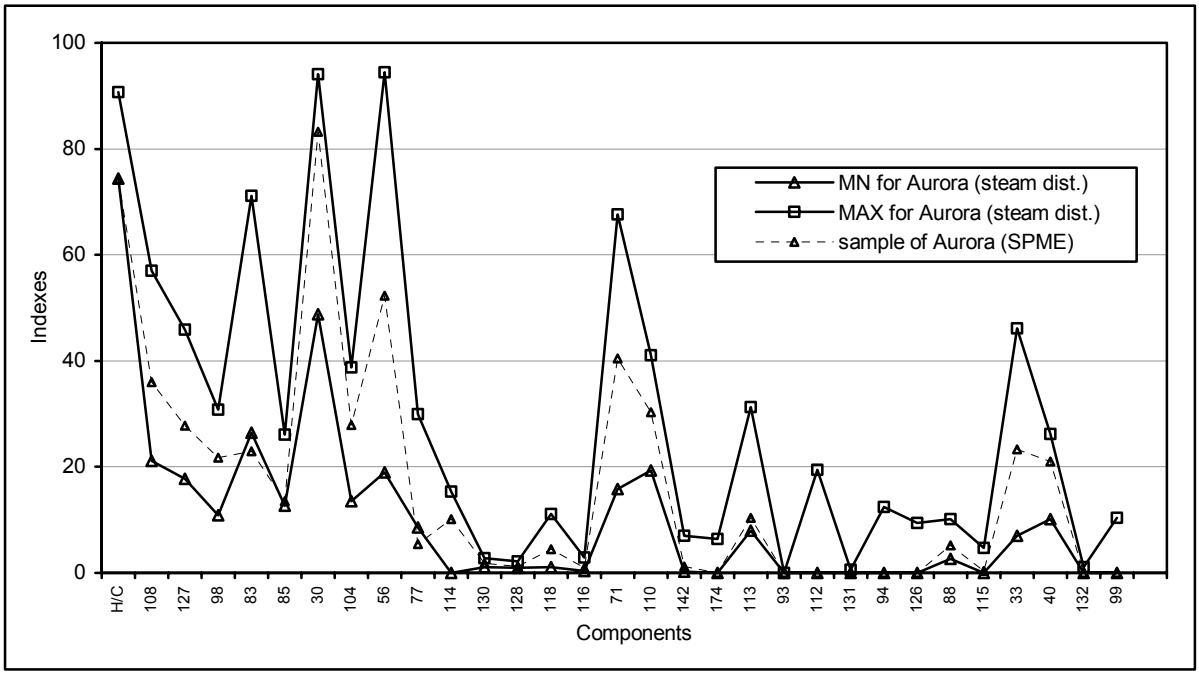


Figure 2: A typical fingerprint of a sample of cultivar Aurora analysed by SPME method charted into the corridor (MIN-MAX model) obtained from reference samples by steam distillation

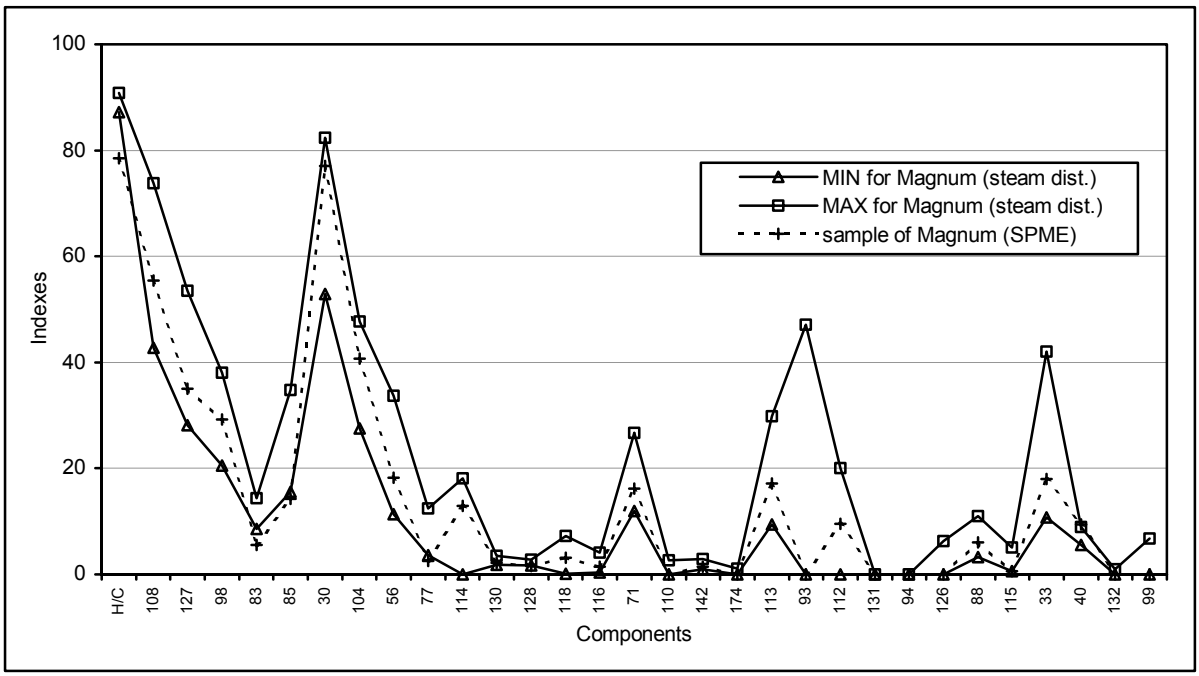


Figure 3: A typical fingerprint of a sample of cultivar Magnum analysed by SPME method charted into the corridor (MIN-MAX model) obtained from reference samples by steam distillation

Conclusions

The results obtained by SPME are similar but not statistically equivalent to those obtained by direct injection of essential oil prepared by steam distillation.

The robustness of MIN-MAX model overcomes that problem and enables the use of SPME data together with the routinely used reference database originally including only data obtained by steam distillation.

The SPME method together with the MIN-MAX model is therefore a satisfactory alternative procedure for hop essential oil analysis and will find its place in the field of cultivar identification and aroma evaluation.

References

Field J.A., Nickerson G., James D.D., Heider C. (1996): Determination of Essential Oils in Hops by Headspace Solid-Phase Microextraction, *J. Agric. Food Chem.*, **44**(7), 1768-1772.

Kač M., Kovačevič M. (2000): Presentation and Determination of Hop (*Humulus lupulus* L.) Cultivars by a Min-Max Model on Composition of Hop Essential Oil, *Monatsschr. Brauwiss.*, **53**(9/10), 180-184.

Kač M., Kralj D. (1998): Studying biodiversity of hop (*Humulus lupulus* L.) accessions from the composition of their essential oils, *Acta hortic.*, **476**, 313-319.

Kovačevič M., Kač M. (2001): Solid-phase Microextraction of Hop Volatiles: A potential Use for determination and Verification of Hop Varieties, *J. Chromatogr. A*, **918**(1), 159-167.

Krofta K., Čepička J. (2000): Determination of Hop Oils Using the Solid-Phase Microextraction Method (SPME), *Kvasny Prum.*, **46**(9), 235-241.

Pawliszyn J., in Pawliszyn J. (Editor): Applications of Solid phase microextraction, The Royal Society of Chemistry, Cambridge, 1999, 3-21.

Zang Z., Pawliszyn J. (1993): Headspace Solid-Phase Microextraction, *Anal. Chem.*, **64**(14), 1843-1852.

Acknowledgements

The authors thank Mrs. Irena Friškovec, B. Sc., from the Slovenian Agricultural Advisory Service for providing hop samples from different hop growing regions in Slovenia. The skillful technical assistance of Mr. Sebastjan Žvipelj, B. Sc. and Mrs. Mira Hrovat as well as funding from the Slovenian Ministry of Education, Science and Sport are gratefully acknowledged.

DEVELOPMENT OF A RISK INFECTION FORECASTING INDEX FOR HOP POWDERY MILDEW.

Walter F. Mahaffee[1], Carla Thomas[2], Cynthia M. Ocamb[3], and William W. Turechek[4]. [1] USDA-ARS-HCRL, Corvallis, OR; [2] FieldWise, Inc. Bodega Bay, CA; [3] Dept. Botany & Plant Pathology, Oregon State University;.[4] Dept. Plant Pathology, Cornell University.

In 1997, hop powdery mildew (*Sphaerotheca macularis* [Wallr.:Fr] Lind. (synonym *S. humuli* [DC.] Burrill)) was introduced into Yakima Valley and subsequently spread to the growing regions in Oregon and Northern and Southern Idaho. The disease now appears to be endemic in Oregon and Washington and intermittent in Idaho (i.e., not present some years). In an effort to develop economical control measures for the management of hop powdery mildew an infection risk forecaster has been developed.

Temperature was manipulated in three different ways in controlled environments to examine the effects on infection of leaf tissue by conidia. In the first, plants were inoculated with powdery mildew conidia and exposed to constant temperatures of 12, 15, 18, 21, 24, 27, and 30 C. Disease development was observed at all temperatures except 30C, with latent periods approximately 10 days at 12 and 15 C and 5 days at 18-27 C. Infection frequencies and lesion sizes were significantly reduced at temperatures above 24 C (Turechek, et al. 2001). In the second set, plants were inoculated and exposed to temperatures 30 C or greater for various time intervals and then placed at 18 C to allow disease development. This experiment was designed to determine the minimum exposure needed at these higher temperatures to inhibit disease development. Results indicated that exposure of conidia to temperatures above 30 C for 6 h reduced the infection frequency by half with no infection occurring when conidia were exposed to temperatures greater than 36 C for as little as 3 h.

For the third set, plants were inoculated then incubated at 18 C for a period of 8 - 48 before being exposed to temperatures above 30 C for 6h. Exposure of young colonies (i.e., 2 days old) to temperatures greater than 39 C for 6 h resulted in colony death.

These data, in conjunction with field data from Washington, Oregon, and Germany (provided Bernhard Englehard, Bayerische Landesanstalt für Bodenkultur und Pflanzenbau Abschnitt Hopfen, Huell, Germany), were used to adapt the Gubler/Thomas disease forecasting model for grape powdery mildew (Thomas, et al. 1994; http://www.scisoc.org/feature/pmildew/Top.html).

The start of the model is triggered by either 15-30 cm of growth from 50% of the hills at bud break or 15-30 cm of regrowth after spring pruning (assuming that prunning results in removal of all green plant material). The infection index increases by 20 points on days where: 1) a minimum of six continuous hours of temperatures between 16 C <= T <= 27 C occurs; 2) there are less than 6 hours with temperatures above 30 C; and 3) less than 2.5 mm of rainfall occurred on that day. On days when these three conditions are not met, 10 points are subtracted from the index. If after subtracting a day's points the index is less than zero, reset the index to zero or after adding a day's points the index is greater than 100, reset the index to 100. On any one day the index should not decline by more than 10 points or increase by more than 20 points.

The index value is used to determine the spray interval (number of days) between applications. An index of 30 or less indicates that disease pressure is low and that a spray interval can be stretched to the label maximum. An index of 40 to 50 indicates that a spray interval can be of intermediate length. An index of 60 to 100 indicates spray intervals should be shortened to the label minimum due high disease pressure. This model is currently being validated in Oregon, Washington, and German yards.

Thomas, C. S., Gubler, W. D., and Leavitt, G. 1994. Field testing of a powdery mildew disease forecast model on grapes in California. Phytopathology 84:1070 (abstr.).

Turechek, W.W., Mahaffee, W.F., and Ocamb, C.M. 2001. Development of Management strategies for hop powdery mildew in the Pacific Northwest. Plant Health Progress (Online. Plant Health Progress doi:10.1094/PHP-2001-0313-01-RS.

The Spatial Pattern of Hop Powdery Mildew in Pacific Northwestern United States

W. W. Turechek, Dept. of Plant Pathology, Cornell University, Geneva NY;
W.F. Mahaffee, USDA-ARS Hort. Crop Research Lab, Corvallis, OR;
C. M. Ocamb, Dept. of Botany and Plant Pathology, Corvallis, OR.

The spatial pattern of the incidence of hop powdery mildew (*Sphaerotheca macularis*) was characterized in commercial hop yards in the Pacific Northwest in 1999 and 2000. A total of 50 yards were selected for sampling and included the varieties Columbus, Tomahawk, Zeus, Perle, Willamette, and Galena. Yards were sampled regularly from shoot emergence through harvest. Sampling began by randomly selecting 2-9 rows in each yard. In each row, 10 leaves along the bottom 2.5-2.75 m of the hop bine from the first 75-100 hills were sampled and were assessed for the presence of HPM signs. Disease incidence in individual yards ranged from 0-0.57 over the two years. Disease incidence fit the beta-binomial distribution better than the binomial distributions indicating that HPM was distributed in an aggregated pattern. Aggregation in relation to disease incidence and sampling date was characterized by fitting variance data from individual yards to the binary power law. The slope parameter was slightly greater than 1 and varied relative to sampling date. This indicates that the degree of powdery mildew aggregation was dependent on disease incidence and sampling date.
Collectively, results suggest that the distribution of HPM in Pacific Northwest hop yards is slightly aggregated and aggregation is highest early in the season when incidence is low. The aggregated distribution has implications on the design of management action thresholds as well as the development of a disease forecaster.

FUSARIUM CONE TIP BLIGHT: A NEW DISEASE OF *HUMULUS LUPULUS*.

J.C. Bienapfl, C.M. Ocamb[1], R. Klein[2], and M. Nelson[3]
[1]Dept. of Botany and Plant Pathology, Corvallis, OR.,[2]California Pistachio Commission-Fresno, CA, USA, [3]Washington State University, Irrigated Agriculture Research and Extension Center, Prosser, WA, USA.

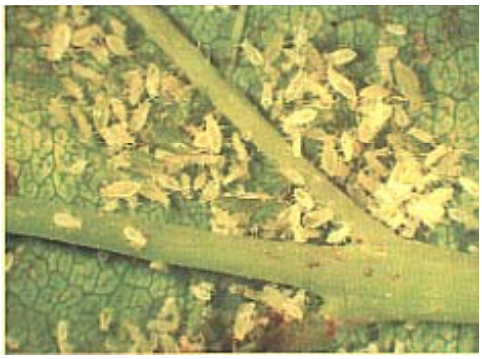
A necrosis at the tip of cones was observed on hop, cultivar 'Nugget', grown in Oregon. Isolations were made from symptomatic cones. *Fusarium sambucinum* and *F. avenaceum* were recovered. Cones were collected from different fields and placed in moist chambers. Spores of individual *Fusarium* species were sprayed onto cones and necrosis was noted. Inoculations with *F. sambucinum* yielded 60% and 65% necrotic cone incidence for cultivars 'Nugget' and 'Willamette', respectively. Inoculations with *F. avenaceum* yielded 65% and 61% necrotic cone incidence for 'Nugget' and 'Willamette', respectively. Cones sprayed with water had 32% (Nugget) and 34% (Willamette) necrotic cone incidence.

FIRST SURVEY OF BENEFICIAL ORGANISMS IN A NON TREATED HOP GARDEN IN ALSACE

A. WEISSENBERGER(1), V. TABOURIN(2), J. CASPAR (1), D. BAUDOIN(2)
(1) Service Régional de la Protection des Végétaux d 'Alsace, route de Saverne 67370 WIWERSHEIM
(2) FDGDEC du Bas-Rhin, route de Saverne 67370 WIWERSHEIM

Methods:

Weekly survey of the Damson hop aphid population on 80 bines of a non treated hop. The entomofauna of this hop garden is sampled every 15 days with a D-vac succion system. The groups which are able to contain some predatory or parasitoïd species are preserved for an exact identification.



Conclusion:

Without any insecticide or acaricide treatments, the hop gardens show a very numerous and various entomofauna. Some years , this rich population of beneficial organisms is able to control the Damson hop aphid population without any damages for the hop.
In the next years other technics must be used to complete this survey (particularly for diperta and hymenoptera…).

Results:

Coleoptera			
	Staphylinidae	*Oligota flavicornis* Boisduval & Lacordaire	Occasional
		Philonthus sp.	Rare
	Coccinellidae	*Adalia bipunctata* L.	Very frequent
		Adalia decempuctata (L.)	Occasional
		Calvia decemguttata (L.)	Rare
		Calvia quatuordecimpunctata (L.)	Occasional
		Coccinella septempunctata L.	Occasional
		Propylea quatuordecimpunctata L.	Frequent
		Synharmonia conglobata (L.)	Occasional
		Stethorus punctillum (Weise)	Rare
Dermaptera			
	Forficulidae	*Forficularia auricularia* L.	Occasional
Diptera			
	Syrphidae	Larva (no identified)	Frequent
	Cecidomyiidae	Larva (no identified)	Frequent
Heteroptera			
	Anthocoridae	*Orius minutus* (L)	Very frequent
		Orius sp.	
		Anthocoris nemoralis (Fabricius)	Frequent
		Anthocoris nemorum (L)	Frequent
		Anthocoris sp.	Frequent
	Miridae	*Deraeocoris lutescens* (Schill.)	Occasional
		Deraeocoris sp.	Occasional
		Heterotoma meriopterum (Scopoli)	Rare
		Phytocoris sp.	Rare
	Reduviidae	*Empicoris* sp.	Rare
Hymenoptera			
	Braconidae	*Aphidius matricariae* Hal.	Frequent
		Aphidius sp.	Frequent
		Ephedrus plagiator Nees	Frequent
		Praon volucre	Occasional
		Praon sp.	Occasional
		Lysiphlebus sp.	Rare
		Trioxys humuli Mack.	Rare
Nevroptera			
	Chrysopidae	*Chrysoperla carnea* (Steph)	Frequent
		Chrysopa sp.	Rare
	Hemerodidae	*Hemerobius humulinus* L.	Frequent
		Hemerobius micans Olivier	Rare
		Wesmaelius sp.	Rare
Thysanoptera			
	Aeolothripidae	*Aeolothrips intermedius* Bayral	Rare
		Aeolothrips sp.	Rare

Acknowledgements: *Authors would thank the entomologists who help them to identifie some species: J. BRUN, J.F. GERMAIN, J.P. LAFONT et P. REYNAUD*

10 years of virus free hop programme in Czech republic

P. Svoboda

Hop research institute Co., Ltd., Kadaňská 2525, 438 46 Žatec, Czech Republic,

e - mail: p.svoboda@telecom.cz

In Czech republic is 6123 ha of hop and 5908 ha (96%) is Saaz semi - early red - bine hop. From this reason is health program of czech fine aroma hop very important. This programme was stared in 1991 and up to day virus - free hops are grown at the area of 1 860 hectares, it represents 30 % of the total hop garden area - see table1. Are grown virus free varieties of Saaz semi - early red - bine hop - Osvald´s clone No. 31, 72 and 114. For obtaining virus - free material are used cultures of meristems tips and thermotherapy. Viruses are controled by ELISA for ApMV, PNRSV, ArMV, HMV and HLVd by molecular hybridization. Reinfection by ApMV has not been observe or detected yet in the hop gardens from 1991. Content of α-bitter acids is higher by 30 – 50 % and yield is higher by 20 to 30 % see table 2. The analyses of hop oils comfirm presence of farnesene as the most important marker of Saaz semi - early red - bine hops and composition of hop oils of virus free hop is identical with standard hops. System production of rootstocks under Certificate schema of EPPO PM4/16(1) is prepared.

Table 1: Varietal structure of hop in Czech republic

Varieties	**ha**	**%**
Saaz semi - early red - bine hop(traditional)	4048	66
Saaz - Semiearly redbine – virus free (VF)	1860	30
New varieties and anothers	215	4
Total	**6123**	**100**

Table 2: Comparison alpha acid and harvest of virus free (VF) and traditional hop

Year	**Alpha acid (%) E.BC.**				**Harvest (t/ha)**			
	Traditional control	Traditional Index %	VF hop	VF hop Index %	Traditional control	Traditional index %	VF hop	VF hop Index %
1992	3,3	100	5	151	0,33	100	0,58	175
1993	3,6	100	5,6	155	0,82	100	1,21	147
1994	2,3	100	4,9	213	0,92	100	1,18	128
1995	3,2	100	6,4	200	1,1	100	1,53	139
1996	4,8	100	6,7	125	1,33	100	1,78	133
1997	4,3	100	6,6	153	1,1	100	1,38	125
1998	3,4	100	4,7	138	1,04	100	1,22	117
1999	3,15	100	3,48	110	1,22	100	1,36	111
2000	3,56	100	4,69	128	0,75	100	0,8	107
Ø	**3,51**	**100**	**5,34**	**152,56**	**0,96**	**100**	**1,23**	**131,33**

NEW SEQUENCE VARIANTS OF "LOW LEVEL" HLVd APPEAR DUE TO HEAT STRESS OF HOP.

Matoušek, J., Orctová, L. and Matoušková, H.

Institute of Plant Molecular Biology Czech Academy of Sciences, Branišovská 31, 37005 České Budějovice

We have previously shown that heat-treatment of hop plants infected by hop latent viroid (HLVd) reduces viroid levels and sequence variability of HLVd (Matoušek et al.,2000; Matoušek et al. in press). A “hot spot” region was identified located in position known as a “pathogenicity domain” in the group representative, potato spindle tuber viroid (PSTVd) and most of the mutations were predicted to destabilise HLVd secondary structure. In the present study we found that all mutated cDNAs were infectious and initiated an evolution of complex progeny populations containing molecular variants maintained at low levels (about 1-10% of the wild type control) in infected hop. Some of cDNA variants had deletions in LCCR. Mutated pool of HLVd forms was possible to transfer to tomato, where it replicated to the level detectable by molecular hybridisation methods and showed the highest concentration in epical meristems like PSTVd. It was possible to easily transfer such HLVd mutants to *N. bentamiana,* where distinct HLVd sequence forms appeared after more than one year evolution (Fig). Our results show that replication of HLVd under stress conditions led to production of low-rate replicating quasispecies, potentially important for viroid evolution in so-called non host plants. These quasispecies could be the re-sources of viroid contaminations of hops.

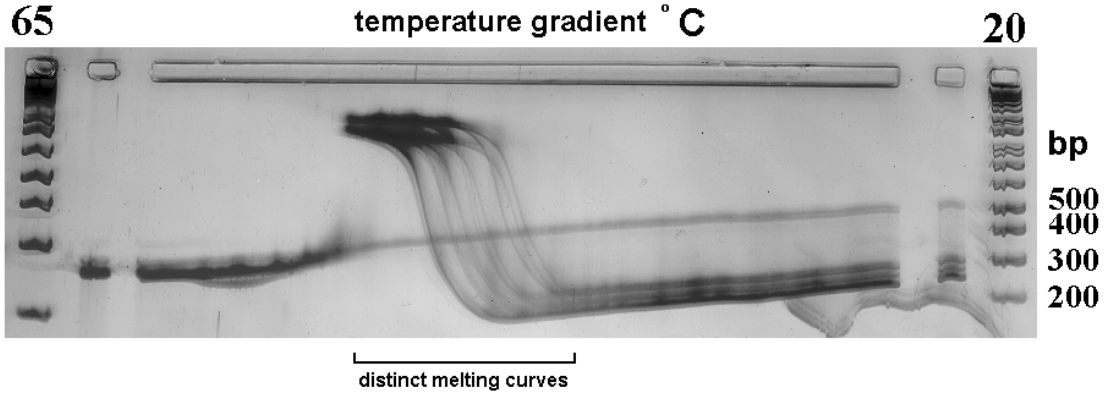


Fig. Temperature gradient-gel (TGGE) analysis of low-rate replicating HLVd quasispecies that were maintained for long time in "non host" species, *N. benthamiana*. An accumulation of individual HLVd mutants is judged from an appearance of distinct cDNA melting curves on TGGE.

This work was supported by the grant of GA AV ČR AA85051014 and NAZV AS ČR EP 1183 entitled: Complex diagnostics and distribution of viroid pathogens of hop, potato, grapevine, fruit and ornamental plants in CR.

References:

Matoušek, J., Patzak, J., Schubert, J., Steger, G., Riesner, D.: Replication of HLVd in thermotreated hop leads to enormously high level of viroid mutagenesis. - In: Book of Abstracts from EMBO Workshop "Plant Virus Invasion and Host Defence", Kolymbari, Crete, Greece, 28 May - 1 June 2000, p. 75, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology - Hellas, Heraklion, Crete, Greece, 2000.

Matoušek, J., Patzak, J., Orctová, L., Schubert, J., Vrba, L., Steger, G., Riesner, D.:The Variability of Hop Latent Viroid as Induced upon Heat-treatment.- Virology, in press

OPTIMIZATION OF HOP (*HUMULUS LUPULUS* L.) REGENERATION THROUGH CALLUS CULTURE

N. Ferant[1] & B. Javornik[1,2] & Z. Luthar[2]
[1] Institute of Hop Research and Brewing Žalec, Žalec, Slovenia
[2]University of Ljubljana, Biotechnical Faculty, Centre for Biotechnology and Plant Breeding, Ljubljana, Slovenia

Methods for plant genetic transformation generally require that single transformed cells can be regenerated into whole plants. Several species, such as *Nicotiana*, *Petunia*, *Lycopersicon*, *Solanum* readily regenerate into whole plants from callus cultures. Major crops such as maize, wheat, many forage legumes, and other plants, including hop, are more difficult to regenerate from callus tissue.
Methods for hop regeneration *in vitro* have been elaborated in several laboratories (Rakousky & Matoušek, 1994, Šuštar-Vozlič, 1997, Batista et al., 2000,) but most of these systems show too low regeneration frequency for successful use in genetic transformation.
Although we have been trying to improve hop regeneration for several years without noticable success, we have now started to study the effects of sucrose and glucose concentration, contents of auxin and cytokinin and effects of gelling agents on hop regeneration.
The regeneration capacity of Slovenian cultivars, Aurora, Savinjski golding and Bobek were tested. The explants used were strips of internodes, leaves, and petiols which were placed in 70 mm petri dishes containing 18 ml of medium. The petri dishes were sealed with Parafilm and exposed to a 16/8 photoperiod at 22-23 °C and illumination of 80 μmol m^{-2} s^{-1}. The basal medium consisted of MS macro- and microelements and vitamins (Murashige & Skoog, 1962), BAP 2 mg/l, 20 g/l glucose, 7 g/l agar, (this medium is a control medium), pH 5.8 and different concentrations of auxins, cytokinins and sugar.
Of the various media tested, MS media supplemented with 20 g/l glucose and 3.5 mg/l BAP were found to be suitable for callus induction and plant regeneration. The highest number of plants was obtained when we used explants of regenerants and when the embryogenic calli were separated from the underlyng translucent dark callus while subculturing on fresh medium. The best regeneration of shoots from callus was on MS medium with BAP 3,5 mg/l BAP, glucose 20 g/l and agar 7 g/l (10 %) compare to the control. Shoot regeneration may also be dependent on the genotype of the donor plants.

References

Batista, D. Ascensao, L. Sousa, M.J. Pais, M.S. (2000): Adventitious shoot mass production of hop (Humulus lupulus L.) var. Eroica in liquid medium from organogenic nodule cultures. Plant Science 151, p. 47-57.

Rakousky, S. Matoušek, J (1994): Direct organogenesis in hop - a prerequisite for an application of *A. tumefaciens*-mediated transformation. Biologia Plantarum 36 (2), p. 191-200

Šuštar-Vozlič, J. (1997): Evaluation of genetic variability among hop (*Humulus lupulus* L.) accessions and study of somaclonal variation of regenerants *in vitro*. PhD thesis. Ljubljana, University of Ljubljani, Biotechncal Faculty, 119p.

ASSESSMENT OF SOMACLONAL VARIABILITY IN HOP *IN VITRO* CULTURE BY MOLECULAR METHODS.

J. Patzak
Hop Research Institute Co.Ltd., Kadaňská 2525, 438 46 Žatec, Czech Republic

Abstract

Plant tissue culture is an enabling technology from which many novel tools have been developed to assist plant breeders. The utilization of plant tissue culture is associated with occurence of somaclonal variability, which first defined by Larkin and Scowcroft (1981). Molecular analysis of somaclonal variation provides an opportunity to eliminate influence of environmental factors and to provide a quantitative measure of somaclonal variability (Veilleux and Johnson, 1998). Different molecular techniques can be employed to detect somaclonal variability (Henry, 1998). In my experiments, I used RFLP, STS, RAPD, ISSR and AFLP (Patzak, 2001) for assessment of somaclonal variability in hop meristem tip culture of cv. Yeoman (UK), Southern Brewer (South Africa), Eroica (US), Premiant (CR), Galena (US) and Osvald's clone 72.
Any differences were not found between mother plants and meristem derived *in vitro* clones by RFLP and STS analysis. One different mericlone of Eroica was found by RAPD (primers OPE-03 and OPG-04) analysis and one different mericlone of Southern Brewer was found by ISSR (primer $(CGT)_5C$ and combination $(CGT)_5C+(TGTC)_4T$) analysis. The frequency of sequence changes of DNA was low and this variability (0.965 for RAPD and 0.913 for ISSR) did not exceed genetic dissimilarity between mother plant and other cultivars. All mericlones were different by AFLP analysis, but their variability (0.993 - 0.824) did not exceed genetic dissimilarity between mother plant and other cultivars.

Acknowlegments:

The authors wish to give special thanks to the National Agency for Agricultural Research of the Ministry of Agriculture of the Czech Republic for supporting this research in project No. EP 7254 and to the Grant Agency of the Czech Republic for supporting in project No. 521/99/1591.

References:

Henry, R.J.: Molecular and biochemical characterazition of somaclonal variation. In **Jain, S.M., Brar, D.S., Ahloowalia, B.S.:** Somaclonal variation and induced mutations in crop improvement. Kluwer Academic Publishers, Dordrecht, 485-499, 1998.
Larkin, P.J., Scowcroft, W.R.: Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* **60:** 197-214, 1981.
Patzak, J.: Comparison of RAPD, STS, ISSR and AFLP molecular methods used for assessment of genetic diversity in hop (*Humulus lupulus* L.). *Euphytica*, 2001, in press.
Veilleux, R.E., Johnson. A.A.T.: Somaclonal variation: Molecular analysis, transformation interaction and utilization. *Plant Breeding Reviews* **16:** 229-268, 1998.

EVALUATION OF SOMACLONAL VARIATION IN HOP (Humulus lupulus L. var Chinock) BY AFLPs MARKERS

Jose Antonio Magadán[1], David Martínez[2], Rosa Arroyo García[3] José Miguel García Zapater[3] and M. Angeles Revilla[2]

[1]S.A.E. Fomento del Lúpulo, Villanueva de Carrizo, León. Spain
[2] Dpto. Biología de Organismos y Sistemas. Universidad de Oviedo. Spain
[3] Centro Nacional de Biotecnología. Universidad Autónoma. Madrid. Spain

Plant regeneration from callus cultures may offer an interesting tool for hop breeding through the potential somaclonal variation produced by *in vitro* culture.

Hop complete plants have been regenerated from callus of the variety Chinock, following an efficient protocol previously described for the varieties Brewers Gold and Nugget (Gurriarán et al., 1999). Callus were initiated from internodal segments cultured on a solid medium (salts of Murashige and Skoog, 1962, vitamins of Wetmore and Soroking, 1955 and 3% glucose) supplemented with several phytohormones. Only calli initiated in presence of zeatin (4.4 μm) or 4.4 μm BAP plus 0.5 μm IBA showed shoot organogenesis after being transfered to medium with the last hormonal supplement.

The morphogenetic capacity of the calli showing some morphogenic pattern increased gradually through the successive subcultures. The highest regeneration rate (expressed as percentage of callus with shoots) was achieved on the third subculture (66%), followed by the second (35%) and the first (4.7%). There was also an increase in the number of shoots developed on each callus. However, after the fourth subculture a decrease in the morphogenetic capacity was observed.

Regenerated shoots were detached from the callus along the first three subcultures and transferred newly to fresh medium. Finally, they were grown under greenhouse conditions for two months. Fresh leaves taken from these plants will be used for nucleic acid extraction to perform AFLPs analysis in order to evaluate the influence of the longevity of the callus on the induction of somaclonal variation.

Gurriarán, MJ Revilla, MA Tames RS. (1999). Plant Cell Rep. 18:1007 - 1011.
Murashige T, Skoog F (1962). Physiol Plant 15: 473 - 497.
Wetmore RH , Soroking S (1955) J. Arnold Arb 36: 305-317.

This work is supported by a FEDER project: 1 FD97 – 0744

AFLP FINGERPRINTING OF FUNGI OF THE GENUS *VERTICILLIUM*

RADIŠEK S[1]., JAKŠE J[2]., JAVORNIK B[2].

[1]Institute of Hop Research and Brewing Žalec, Cesta Žalskega tabora 2, 3310 Žalec
[2]Centre for Plant Biotechnology & Breeding, Biotehnical Faculty, University of Ljubljana, Jamnikarjeva 101, Ljubljana 1000, Slovenija

Poster manuscript:

The genus *Verticillium* Nees ex Link contains a numerous group of asexual fungi, of which the plant pathogen species *Verticillium alboatrum* Reinke et Barthold and *Verticillium dahliae* Klehban are economically important. These two fungi appear to a major extent on hop, where they cause hop wilt disease in mild and lethal forms. In Slovenia, *Verticillium alboatrum* causes both forms of hop wilt, whereas *Verticillium dahliae* causes only the mild form of the disease. Traditional detection and diagnosis of *Verticillium* species is primarily based on the use of semi-selective media, vegetative compatibility analysis, differences in virulence, cultural differences and biochemical characteristics (Arora et al. 1996). These methods are lengthy and laborious, but necessary for the identification of species and pathotypes for appropriate phytosanitary measures, for disease resistance breeding and crop selection for a particular field (Koike and Fujita.1996). Wider use of molecular methods in plant pathogen diagnostics have shown that classical methods can be successfully complemented with such methods because molecular analysis provides an accurate, faster and less resource demanding means of pathogen detection. AFLP (amplified fragment length polymorphism) is a novel molecular technique with wide application in many different organisms, mainly due to the ability to detect a very high number of polymorphisms in a single assay, good repeatability and possibilities of automation (Vos et al. 1995). It is based on the selective PCR amplification of restriction fragments from total digestion of genomic DNA. We adapted the AFLP technique for the analysis of *Verticillium alboatrum* and *Verticillium dahliae.* Fungus isolates were initially screened with 40 AFLP primer combinations which produced an average of 35 to 65 bands in a range of 50 to 330 bp. All primer combinations used clearly distinguished *Verticillium alboatrum* and *Vericillium dahliae* isolates, while 8 primer combinations showed polymorphisms between mild and lethal forms of *Vericillium alboatrum* from hop. In further research, genetic variations among isolates will be assessed and potential diagnostic markers for two forms of *V. alboatrum* will be tested.

References:

1. Arora, D. K., Hirsch, P. R., Kerry, B. R. (1996): PCR- based molecular discrimination of *Verticillium chlamydosporium* islolates.- Mycological Research, 100: 801-809.
2. Koike, M., Fujita, M. (1996): Random Amplified Polymorphic DNA Analysis of Japanese Isolates of *Vericillium dahlie* and *V. albo-atrum*.- Plant Disease, 11: 1224-1227.
3. Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zebeau, M. (1995): AFLP: a new technique for DNA fingerprinting - Nucleic Acids Research, 21: 4407-4414.

THE INFLUENCE OF DIFFERENT TYPES OF WEEDINESS ON THE QUANTITY AND QUALITY OF HOP YIELD

Andrej Simončič
Institute of Hop Research and Brewing Žalec, Žalec, Slovenia

The article gives results of micro trials in the Hop garden in Žalec and one near Žalec in village Latkova vas. In the trials, carried out between 1997 and 1999, the effects of different types of weediness and its duration on the quantity and quality of yield were measured. Besides the alpha acid content and the weight and size of weeds were measured. The research included the most important weeds which are found in Slovenia. We compared different types of weediness with autochtonus weed flora in Hop gardens to that of manually weeded plots. The first group, in our opinion, consisted of low, harmless weeds such as *Stellaria media*, *Veronica* spp., *Capsella bursa-pastoris and Poa annua*. Second group, which in our opinion, can be a more serious threat to Hop, consisted of weeds such as *Chenopodium album* and *Amaranthus retroflexus*. Apart from annual weed species, the research also included perrenial weeds, *Convolvulus arvensis*, *Rumex obtusifolius* and *Symphytum officinale*. The results show that time and type of weediness affect mainly the quantity of yield while the quality seems to be less affected. Less exuberant, low growing weed species did not have unfavorable effect on the yield quantity or quality during the time of research work. In most cases statistically significant differences between hand-weeded plots and the ones overgrown by weeds were just in the case of *Chenopodium album* and *Amaranthus retroflexus* at the high density of the weeds and its longest duration. In comparison to annual weed species, bigger differences existed in the yield quantity where perennial *Rumex obtusifolius* and *Symphytum officinale* were present. Still, these differences were only noted in 1999, while in 1997 and 1998 they were not ststistically significant.

Key words: Hops, weeds, weediness, weed competition, Hop yield, Hop quality

ADVANCES IN BREEDING STRATEGIES OF THE SOUTH AFRICAN BREWERIES HOP FARMS IN GEORGE, SOUTH AFRICA

Gerry Brits
South African Breweries Hop Farms, P.O. Box 1498, George, South Africa 6530

Commercial hop growing in South Africa commenced In 1936 with hop varieties imported from England. Because of large climatic deficiencies (shorter day lengths and warm winters) all imported varieties fared poorly or failed.
The breeding program of the South African Breweries Hop Farms (Pty) Ltd (then Union Hop Growers), commenced in 1956 under the supervision of the General Manager, Barry Robinson. At the time the local hop industry was limited to four farms with a total size of less than 100ha and the main variety grown was “Golden Cluster”. Seed from Fuggle and Hallertauer crosses were imported from Wye, England and these formed the basis of the breeding program. The first variety released from the program was “Southern Brewer”, a “Fuggle” cross with an alpha acids content of 9 – 10%, which was commercially released in the early 1970’s and until recently became the mainstay of the local industry.
With the rapid expansion of the industry in the 1970’s and 80’s and the development of super alpha varieties internationally, the breeding program was accelerated to keep in line with international trends and breeding lines from the main hop regions were imported.
Progeny of these high alpha acids breeding lines with vigor and adaptability to local conditions are currently being used to replace ‘Southern Brewer’.
With current international emphasis falling on super alpha varieties with low cohumulone levels and good aromatic properties, low cohumulone breeding lines have been developed. These will be utilised in the high alpha breeding program. The local hop industry’s strategic importance disappeared dramatically in the 1990’s with South Africa’s re-entry into the “Global Village”
Without a strong breeding program, the poor performance of imported hop varieties in South Africa would therefore have resulted in the closure of the industry.
Although small in comparison to the American, German, Czech and Chinese hop industries, the local breeding program and associated agronomic research program has catapulted the industry to the forefront of international hop development.
Aromatic, dual purpose and super-alpha hop varieties have been developed which compete analytically and economically with the best in the world.
Outeniqua, a high alpha, Southern Star, a super alpha and Southern Promise, a dual purpose hop variety are being used for commercial brewing.
US4/78, an aroma hop variety, are at an advanced stage of brewing trials.
Highly adapted to the marginal hop growing conditions of the Southern Cape, these varieties grow free of hop diseases, resulting in an unsurpassed quality.
Low cohumulone (a harsh bittering alpha acid component) breeding lines have been identified and will continue to be used in the program.
The industry is poised to develop mutually beneficial supply agreements with all the breweries of Southern and Central Africa.

THE INFLUENCE OF THE CHEMICAL CHANGES IN HOPS ON THE BREWING VALUE

Majda Virant

Institute of Hop Research and Brewing Žalec, 3310 Žalec, Slovenia

INTRODUCTION

The brewing value of hops is determined by the content of bittering substances (alpha-acids), aromatic substances (essential oils) and tannins (polyphenols, antocyanogens, tannoids), further on by its storage stability (decrease of the alpha-acid content), alpha-acid utilisation, bittering quality and hop aroma (1, 2, 3, 5). The brewing value of hops depends also on technological ripeness of hops (4, 6).

MATERIALS AND METHODS

HOPS: The trial was performed in the 1995-2000 period with two cultivars belonging to the aroma hops group, namely Aurora and Bobek. Hops were picked in the time of technological ripeness (end of August, beginning of September) and in the time of physiological ripeness (end of November).

Pilot brewing: The necessary amount of hops was calculated according to its alpha-acid content in order to assure and addition of 8.0 g of alpha-acids per 100 L of wort in all the cases. All other parameters were standard and the same in all the cases.

Analyses: Hops, wort and beer were analysed according to routine methods Analytica-EBC, 1998, MEBAK Band II, 1993 and the ANOVA statistical method (the analysis of variance).

RESULTS AND CONCLUSIONS

- the average loss in total hop resins was 7.6 rel. % in physiologically ripe cultivar Aurora and 4.8 rel. % in cultivar Bobek (compared to technologically ripe hops)
- the average loss in alpha-acid content was 12.5 rel. % in physiologically ripe cultivar Aurora and 10.4 rel. % in cultivar Bobek (compared to technologically ripe hops)
- the average increase of the beta fraction was 10.9 rel. % in physiologically ripe cultivar Aurora and 9.8 rel. % in cultivar Bobek
- the content of the essential oil and its chemical composition were significantly different for physiologically ripe cultivar Bobek while no such differences were observed for cultivar Aurora (compared to technologically ripe hops)
- essential oil of physiologically ripe cultivar Bobek contained less myrcene, but more alpha-selinene, humulene epoxyde-1, humulene epoxyde-2, alpha-humulene and beta-caryophyllene (compared to technoloically ripe hops)
- the content of the xanthohumol showed no significant differences during the 3-year study (either for cultivar Aurora or cultivar Bobek)
- the brewing value of physiologically ripe hops was superior to that of technologically ripe hops for both cultivars (better utilisation of bittering substances, better physical stability, lower contents of total polyphenols and antocyanogens in beer)
- hop aroma (its intensity as well as its quality) is more pronounced in the case of physiologically ripe cultivar Bobek (compared to technologically ripe hops)
- physiologically ripe hops is very suitable for late hopping (end of boiling and whirlpool) and for dry hopping; for special beers with pronounced and agreeable hop aroma.

REFERENCES

1. Kaltner, D., Thum, b., Back, W., Brauwelt International 2001/I, 2001, 40-45.
2. Foster, A., Beck, B. and Schmidt, R. Hopfenrundschau International, 1999, 68-74.
3. Narziss,L., Die Technologie der Würzebereitung 6. Auflage, 1985, 63-77.
4. Kammhuber, K., Monatschrift für Brauwissenschaft Heft 7/8, 2000, 138-142.
5. Hops and Hop Products, EBC Manual of Good Practice,1997, 25-36.
6. Garden, D. S. J., Monograph XXII, E. B. C.-Symposium on Hops, 1994, 114-124.