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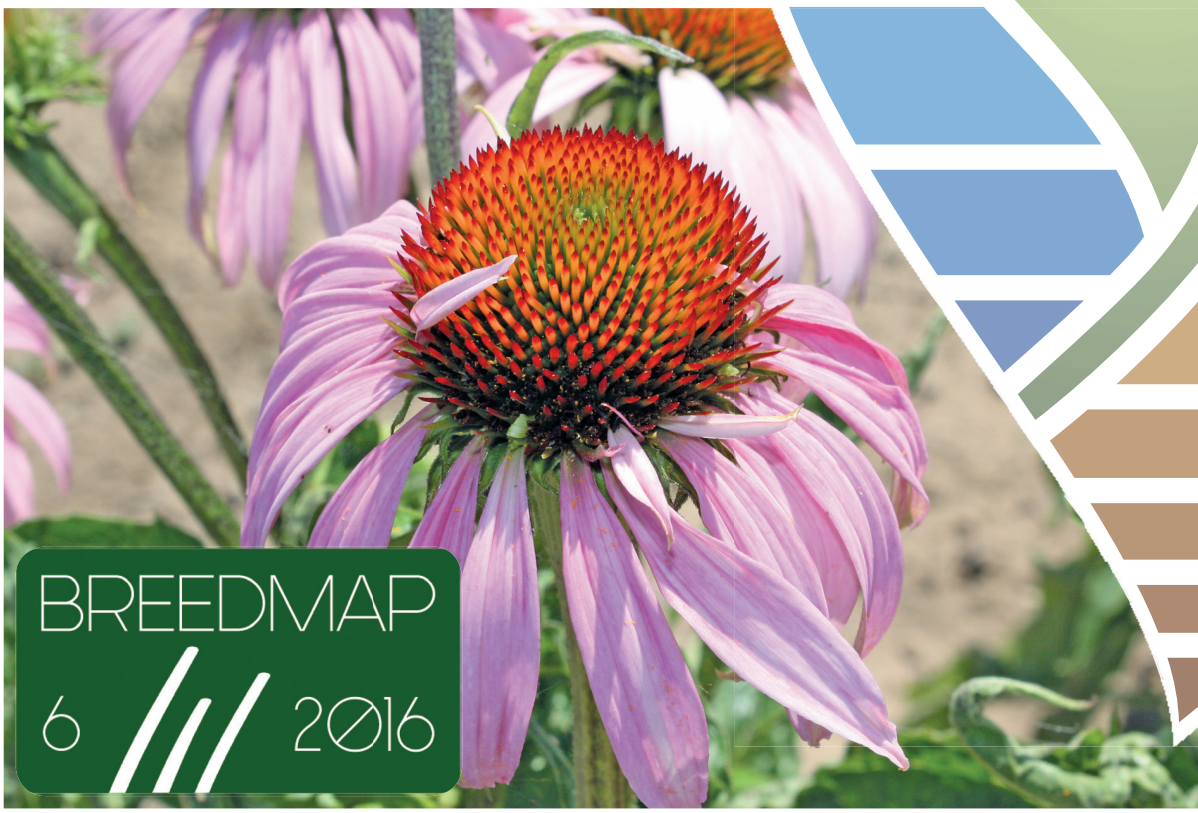
Frank Marthe, Heike Riegler

6th International Symposium Breeding Research on Medicinal and Aromatic Plants (BREEDMAP 6),

Quedlinburg, Germany
June 19 - 23, 2016

Abstracts of Oral Presentations and Posters



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Bundesforschungsinstitut für Kulturpflanzen

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Welcome to the 6th International Symposium Breeding Research on Medicinal and Aromatic Plants (BREEDMAP 6)

Frank Marthe

This meeting continues a series of international symposia for Breeding Research on Medicinal and Aromatic Plants, which started in 1996. Open to all aspects of basic and applied research in plant breeding, the conferences place emphasis clearly on medicinal and aromatic plants.

This BREEDMAP 6 Symposium is organized by the Julius Kuehn Institute, Federal Centre for Cultivated Plants (JKI) in collaboration with the Leibniz Institute for Plant Genetics and Crop Plant Research (IPK) and the Society for Medicinal Plant and Natural Product Research (GA).

Customers all over the world are interested in products based on natural sources. There is a permanent demand for high-quality products. Prerequisite are new varieties and lines of medicinal and aromatic plants with better resistance to biotic and abiotic stress, with adaptation to different conditions in cultivation, and finally with an increase of active principles. In many cases we do have a breakthrough but for a broad range of medicinal plants much can still be done in order to improve quality and quantity and to get constant high levels of active principles. The BREEDMAP 6 Symposium presents the international platform to share new results and techniques with an international audience to create new ideas and fruitful collaborations in this promising field.

The symposium focuses on the following main topics:

- **A.** Next generation methods and chances for medicinal and aromatic plants
- **B.** Cell, tissue and organ culture, cryopreservation, endophytes
- **C.** Resistance breeding and new phytopathogens
- **D.** Improvement of organisms for bioreactors and photobioreactors
- **E.** *Ex situ* and *in situ* genetic resources – protection and use by collecting practice – cultivation of new species
- **F.** Plant breeding and plant analytics
- **G.** CBD, Nagoya Protocol, EU regulations

Workshop: Pyrrolizidine alkaloids – a problem?

I look forward to an attractive program of sessions, lectures and workshops, and I hope that Quedlinburg 2016 will provide a place for stimulating discussions between scientists from different regions of the world. The scientific program is rounded off with excursions that provide an insight into MAP research institutes and modern production as well as the beautiful atmosphere and scenery of the historical towns and the surrounding landscape.

Dr. Frank Marthe

for the Scientific Committee of BREEDMAP 6

Aims and history of BREEDMAP - Welcoming address of the initiator of the BREEDMAP symposium series

Friedrich Pank

Journal of Applied Research on Medicinal and Aromatic Plants
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The international symposium series Breeding Research on Medicinal and Aromatic Plants has been launched 1996 in Quedlinburg in view of the fundamental importance of breeding research for the progress in medicinal and aromatic plant (MAP) breeding. There is already a vast number of MAP symposia round the globe but none of it is exclusively dedicated to breeding research.

Breeding is one of the most important factors for the promotion of the MAP branch. Breeding adapts genotypes to the particular requirements of the stakeholders in the supply chain: seed companies, farmers, processing industries, trading and – most important – to the consumers' demand. High performance new cultivars contribute considerably to the production of high quality products in a profitable and sustainable way without additional production expenses because the characteristics are controlled genetically. Breeding research paves the way and develops the tools to improve the effectiveness of breeding procedures.

The aim of BREEDMAP is to promote the special field of science of MAP breeding research by providing a platform for the mutual exchange of knowledge of the comparably few involved experts. The intensification of the exchange of information and the initiation of co-operation are even more important because there is a lack of scientific information on breeding methods, the available research capacities are insufficient, the number of MAP species as breeding objects is particular large, the breeding aims are very diverse often also depending on different uses of one and the same species, the costs of needed chemical analysis are particular high and only limited financial funds are available due to the small reflux of the breeders' expense.

The symposium brings together experts of several scientific disciplines related to all aspects of breeding research on MAP: e.g. botany, plant genetic resources, agronomy, phytpathology, genetics, selection theory, cytology, cytogenetics, biochemistry, biotechnology, molecular genetics, chemical analytics and intellectual property rights. The following examples of important goals demonstrate the need of multidisciplinary approaches: to gain initial breeding material as donors of important characters like content of essential compounds, resistance to pathogens and pests, agronomic traits; to explore the inheritance of characters, cytogenetics, reproduction biology, crossing techniques, mutagenesis, selection methods including the use of markers, fundamentals of hybrid breeding, cell and tissue cultures, somatic hybridization, creation of doubled haploids, genetic engineering, molecular markers, rapid and nondestructive analytical methods for selection on chemical compounds, cost-benefit-ratio of breeding methods and plant breeders' rights.

Thanks to the support of the German Federal Ministry of Food, Agriculture and Forestry and the scientific organizations Society for Medicinal Plant Research (GA), European Association for Research on Plant Breeding (EUCARPIA), International Society for Horticultural Science (ISHS) and German Society of Plant Breeding (GPZ) Friedrich Pank could start with the first BREEDMAP Symposium in 1996 at the Federal Centre for Breeding Research on Cultivated Plant in Quedlinburg (afterwards Julius Kuehn Institute) assisted by Frank Marthe who is now the convener of the sixth symposium of this series. The conveners of the second symposium 2000 in Chania, Greece were Chris Johnson and Chlodwig Franz. The third symposium 2004 in Campinas, Brazil was directed by Pedro Melillo de Magalhães. The fourth symposium was held in Lubljana, Slovenia in 2009 under the leadership of Dea Baričevič. Johannes Novak invited the experts to the fifth symposium at Vienna, Austria in 2012. Now in 2016, the symposium returned to its birthplace in Quedlinburg and we thank Frank Marthe and all involved colleagues for the efforts to organize BREEDMAP 6. That the BREEDMAP symposium series is still alive after a period of 20 years is the proof that the idea meets a real need of the concerned scientific community.

I wish BREEDMAP a successful future to continue bringing together the experts concerned with MAP breeding research for exchange of the most recent findings, fruitful discussions and launching new multidisciplinary co-operation for the progress in this important and exciting field of science.

PD Dr. Friedrich Pank

Session A: Next generation methods and chances for medicinal and aromatic plants

APL 1: Next-generation sequencing in MAP breeding – efficient and affordable?

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Abstract

'Next-generation sequencing' (NGS, syn. 'high-throughput sequencing', 'massively parallel sequencing') are terms comprising different new DNA sequencing technologies allowing sequencing DNA and RAN much more quickly and cheaply than the 'classical' Sanger sequencing (Metzker, 2010). The most widely used NGS technology is Illumina, which has short reading lengths of up to 2x 150 base pairs (bp) (Sanger sequencing: 600 – 1000 bp). However, in contrast to maximum 96 sequences per run in Sanger sequencing, Illumina is able to generate up to 3 billion sequences per run. Although the price per million base pair has decreased from 1.700.-€ (Sanger sequencing) to 0.07.-€ (Illumina sequencing), the price for one NGS run is high. Therefore, it depends on a clever strategy to include NGS affordably into MAP breeding. The most widely used strategy is 'sample barcoding'. The primers used in sequencing are extended by some base pairs unique for each sample ('barcode'). The samples are mixed and sequenced in one NGS run. Afterwards, the bulk of sequences are separated into sequences per sample by sorting them according to their barcode.

Molecular markers are valuable tools in plant breeding used to study genetic relationships, following crosses and finding markers linked to specific phenotypes in order to efficiently select for wanted traits as early as possibly, shortening breeding time ('marker assisted selection', MAS). NGS can be used to develop efficiently molecular markers. Instead of weeks and months necessary to develop around 10 microsatellites by the classical approach of cloning and sequencing, hundreds of microsatellites can be identified in one NGS run today (Takayama et al., 2011). In a similar approach, microsatellites were identified in the plastome of oregano, useful to follow specifically matrilineal relationships (Lukas and Novak, 2013). This fast development of a plethora of molecular markers creates the possibility to increase the number of molecular markers used in breeding. An approach to use NGS in both, SNP marker discovery and genotyping is called 'genotyping by sequencing' (GBS) (Kim et al., 2016).

NGS and the fast development of bioinformatics driven by NGS have revolutionized the fields of genomics and transcriptomics, thus increasing the number of genomes and transcriptomes publicly available as information resources. Posivitely for MAP breeding, genome and transcriptome data are not only limited to model organisms any more, but already extended to some MAP plants as well. Instead of indirect markers for specific traits, NGS offers the possibility to speed up identification of the mutations directly responsible for a specific phenotype. Especially metabolic pathway analysis of medicinal and aromatic plants by plant transcriptome analysis ('RNA-sequencing' RNA-seq) opens the way to functional plant breeding of MAPs (Hao et al., 2012).

Keywords: next generation sequencing, medicinal and aromatic plants, molecular markers, genomics, RNA sequencing

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ASL 1: Towards developing a genetic linkage map of isabgol (*Plantago ovata* Forsk.), a medicinal plant with potent laxative properties

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Abstract

Genetic linkage maps facilitate the genetic dissection of complex traits and comparative analyses of genome structure, as well as molecular breeding in species of economic importance. Isabgol [*Plantago ovata* (Forsk.)], a medicinal plant with potent laxative properties is used in several traditional systems of Medicines and cultivated in India. We explored the DNA sequences of Isabgol in the Genbank (NCBI) and developed over 1500 simple sequence repeats (SSR) markers. Some of them were validated through DNA amplification. Transferability of SSRs from wild *Plantago* species viz., *P. major*, *P. coronopus*, *P. lancelolata*, *P. maritina* and *P. intermida* into *Plantago ovata* was studied. We developed a genetic linkage map using recombinant inbred lines (RILs) population which comprises of 30 random amplified polymorphic DNA (RAPD) markers spreading across 11 linkage groups (PO-1 to PO-11) with a total map distance of 75.6 cM. The SSR markers developed will have applications in assessing the functional diversity, comparative mapping and other applications in isabgol.

Keywords: Isabgol, *Plantago*, Genetic linakge map, simple sequence repeats (SSR) markers, medicinal plant

Introduction

Isabgol also known as Blond psyllium [*Plantago ovata* (Forsk.)] belonging to the family-Plantaginaceae is an important medicinal plant cultivated in India. The husk and seeds are economically important parts used in traditional medicine. Enhancing the yield and quality are the important objectives of isabgol breeding. Attempts to increase the seed yield and husk quality through conventional breeding methods by exploiting natural variation were made. However, not much progress has been realized till today. The lack of clear-cut segregation and small undetectable effects of individual minor genes make it impossible to monitor these traits in traditional breeding programs. However, the advent of molecular markers has facilitated the identification of hidden genetic variability, which can be exploited for the construction of high-resolution genetic linkage maps and detection of QTL governing these traits (Collard et al., 2005). The DNA markers flanking the major QTL can be pyramided into the genetic backgrounds of adaptable and agriculturally desirable genotypes. Nonetheless, a few reports are available on the use of molecular markers including random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and inter-simple sequence repeat (ISSR) for assessment of genetic diversity and population stratification in *Plantago* species (Wolff and Richards, 1998; Squirrell and Wolff, 2001; Koorevaar et al., 2002; Marie and Wolff, 2003; Vahabi et al., 2008; Singh et al., 2009; Vala et al., 2011; Rohilla et al., 2012).

The main objectives of study are to (1) develop Simple sequence repeats (SSR) markers for genomics applications and (2) to develop genetic map using random amplified polymorphic DNA (RAPD) markers.

Materials and Methods

Development of simple sequence repeats (SSR) markers

The raw reads of Isabgol from NCBI-Short Read Archive deposited by Jensen et al. (2013) was used for SSR marker development. The high quality data was assembly using CLC genomics workbench

on default parameters. For identification of SSRs, all the Isabgol transcript contigs were searched with Perl script MISA (http://pgrc.ipk-gatersleben.de/misa/_) with a minimum repeat length of 12bp. Primers were designed using primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) software with the default parameters. The genomic DNA was extracted using the CTAB method as given in Wolff et al. (2009). PCR reactions were set up as described by Wolff et al. (2008). Further, 62 SSR markers reported by SQUIRRELL AND WOLFF (2001); KOOREVAAR et al. (2002); HALE AND WOLFF (2003); Nilsson et al. (2006) and WOLFF et al. (2008) for wild *Plantago* species viz., *P. major, P. coronopus, P. lancelolata, P. maritina* and *P. intermida* were tested for amplification in *Plantago ovata*.

Genetic mapping

The pure seed of DPO-185 and DPO-14 were grown at the research farm of the ICAR-Directorate of Medicinal and Aromatic Plants Research (DMAPR), Anand during the year 2010 and the F_1 hybrid seed of DPO-185 x DPO-14 was produced. The F_1 seeds were grown during the year 2011 and F_2 seeds were produced selfing and futher advanced to F_5 through single seed decent method (GOULDEN, 1941). The genomic DNA of parents and F_5 RILs were extracted as above and were amplified using RAPD markers (WOLFF AND MORGAN RICHARDS, 1998). PCR products were scored as describe by REDDY et al. (2014) and was used for linkage map construction. The Kosambi mapping function was used to convert recombination into genetic distance.

Results

Development of SSR markers

A total of 23,586 transcript contigs having more than 200bp size were obtained after the assembly. The details of the assembly statistics are given in Table 1. Analysis using MISA software identified 16,375 simple sequence repeats (SSRs) having more than 12bp motif length in 10,308 transcript contigs (43.7 % of 23,586 transcript contigs), indicating an average frequency of 1 SSR per 1.2 kb. This frequency is consistent to the frequency range of 2.65 to 16.82 % which has been reported in 49 dicot species (KUMPATLA AND MUKHOPADHYAY, 2005). SSR frequency is dependent on several factors such as genome structure, arithmetical method for SSR detection, and the parameters for exploration of microsatellites (TOTH et al., 2000). Of which 352 (2.15 %) were dimers, 2808 (17.15 %) were trimers, 1127 (6.88) were tetramers, 345 (2.11 %) were pentamers and 11743 (71.71 %) were hexamers. This is consistent with the SSRs distributions reported in the transcriptome of soybean (LI et al., 2010) and sorghum (REDDY et al., 2012). Primers were designed for one thousand five hundred SSR markers. Of the 300 markers tested, 280 (93.3 %) markers showed amplification. The majority of SSRs generated high-quality amplicons, suggesting that ESTs are suitable for specific primer design. These results suggest that the assembled transcripts were of high quality and that the SSRs identified in our dataset could be used in the future.

Tab1. Details of the assembly statistics of Isabgol transcriptome

Description	Plantago ovata
Number of transcript contigs	23586
Transcriptome length(bp)	19686649
Max transcript contigs size(bp)	9806
Min transcript contigs size(bp)	200
Mean transcript contigs size(bp)	835
N50 value (bases)	1075

Transferability SSR markers

SSRs derived from the expressed sequence tags (EST-SSRs) are popular markers due to their rapid *in silico* development and high cross-species and genera transferability (REDDY et al., 2011). Inter-

specific transferability of SSRs from wild *Plantago* species viz., *P. major, P. coronopus, P. lancelolata, P. maritina* and *P. intermida* in to *Plantago ovata* was studied. Of the 60 SSRs tested from the wild species, 43 (71.6 %) SSRs showed transferability into *Plantago ovata*. The markers with high Inter-specific transferability will be highly useful for assessing the functional diversity, comparative mapping and other applications.

Genetic mapping

One hundred RAPD markers (OPA1-OPA20, OPB1-OPB20, OPC1-OPC20, OPD1-OPD20 and OPE1-OPE19) were tested for parental polymorphism between mapping parents (DPO-185 and DPO-14). Eighteen RAPD markers were found polymorphic and subjected to genotyping of 160 F_5 DPO-185 and DPO-14 recombinemnet inbred lines. The genetic map comprises of 33 markers spreading across 12 linkage groups (PO-1 to PO-12) (Figure 1.) with a total map distance of 13.7 cM. The linkage groups PO-06 and PO-10 were having maximum distance. The staturation of present may with more marker is being carried out.The genetic linkage map will have the potential to facilitate the genetic dissection of complex traits and comparative analyses of genome structure, as well as molecular breeding efforts in species of agronomic importance in isabgol.

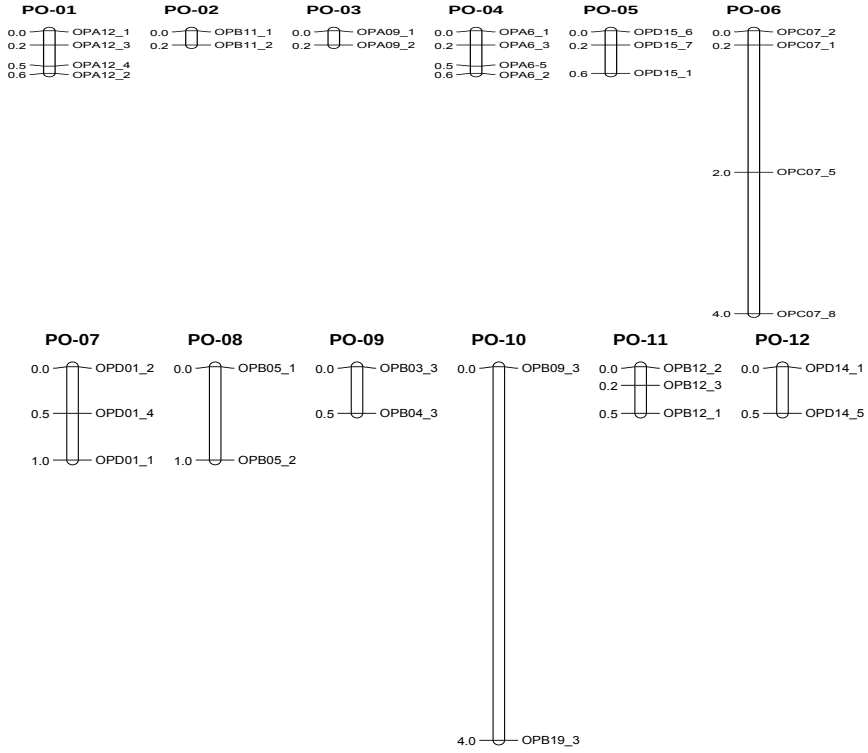

Fig1. Genetic linkage map of Isabgol (*Plantago ovata*) with 33 RAPD markers.

Acknowledgements

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ASL 2: *Artemisia annua* L.: Polyploidy and NIRS, two tools to improve breeding efficiency

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Abstract

Breeding a new cultivar needs 5 to 15 years according to the species and the breeding objectives from bioprospection up to cultivar registration. This is a very long time for companies developing and trading plant based products. To react more quickly to the requirements of the stakeholders, methods to accelerate the breeding procedures have to be taken into account. Among different possibilities, polyploidy induction and rapid methods to measure target traits with near infrared spectroscopy (NIRS) were tested on *Artemisia annua* L.

Tetraploid progenies were compared to the cultivar Apollon. These tests showed no significant differences between the tetraploid plants and the cultivar Apollon for the artemisinin content, as well for the leaf and the artemisin yield.

The determination of artemisinin in powder of *Artemisia annua* using a hand-held NIRS device showed accurate results in predicting artemininin contents. Root mean square error values of cross-validation and prediction of 0.1 % were calculated, in both cases.

Keywords: Artemisinin, *Artemisia annua*, breeding, NIRS, polyploidy

Introduction

Artemisia annua L. is an important medicinal plant for the production of antimalarial drugs based on artemisinin. Artemisinin, a sesquiterpene lactone endoperoxid isolated from the leaves of *A. annua*, is a highly potent antimalarial compound, which is also efficient against multidrug-resistant strains of *Plasmodium falciparum* (ALIN, 1997). With the support of the WHO the artemisinin-based combination therapies (ACTs) became the first line of treatment against malaria. Despite the research of new technologies, the extraction from *A. annua* leaves remains the only source of artemisinin. Only the distribution of cultivars with a high artemisinin production potential allows making this new culture attractive and this way answering to the increasing demand for low cost artemisinin (FERREIRA et al., 2005). The breeding work conducted by Mediplant since 1989 allowed the development of hybrids with artemisinin contents in the leaves with up to 2 % (SIMONNET et al., 2008). To increase the breeding efficiency, induction of polyploidy and the NIRS-method were tested on *Artemisia annua*.

Materials and Methods

Polyploidiy: The increase of chromosomes sets per cell can be artificially induced by applying the molecule colchicine, which leads to a doubling of the chromosome number. To improve the basis for breeding, such a polyploidy induction was made on in-vitro plantles and seeds of *A. annua*. Different concentration (0 to 2 % cholchicin) and different durations (6 to 48 h) were used. The surviving tetraploid plants were used as parental lines to get seeds. The tetraploid plants from this seed were compared in the field with 3 replications (RBC) and 18 plants per replication with the cultivar Apollon.

NIRS: This rapid, low-cost method was developed to determin artemisinin content in dry powder of *A. annua* leaves (Camps et al., 2011). A calibration set of 60 samples and a validation set of 40 samples of *A. annua* hybrids with artemisinin contents varying between 0.7 and 1.6 % were used. The NIRS device was a handheld Phazir 1018 (Thermo Scientific, Wilmington, MA, USA).

Results

Polyploidy: The use of colchizin at different concentration and durations allowed to get some tetraploid *A. annua* plants. The crossing of these tetraploide plants was not very successful. Only one combination of two parental lines gave seeds. The progenies of both paretal lines were tested individually in comparision with the cultivar Apollon. These tests showed no significant differences between the tetraploid plants and the cultivar Apollon of the artemisinin content in the leaves, as well as of the leaf and the artemisin yield (Tab. 1).

Tab. 1 Artemisinin content in the leaves and leaf yield of 3 hybrids of Artemisia annua. Values in brackets indicate the standard deviation (±sd).

Hybrids	**Artemisinin content in dried leaves** (%) (±sd)	**Dried leaf yield** (g per m^2) (±sd)
Cultivar Apollon	**1.33** (±0.07)	**4.46** (±0.03)
Tetraploide A	**1.37** (±0.03)	**4.56** (±0.29)
Tetraploide B	**1.50** (±0.08)	**4.89**(±0.41)

NIRS: The development of this method is crucial in order to analyse a large number of samples corresponding to hundreds of hybrids in a breeding program. The results obatained showed the feasibility of artemisinin quantification by uing NIRS (Fig. 1). Root mean square error values of cross-validation and of prediction of 0.1 % were calculated, in both cases. The accuracy of the prediction is about 0.1 % in a fully external validation. This threshold of precision has been judged as correct for application of the method in a breeding program.

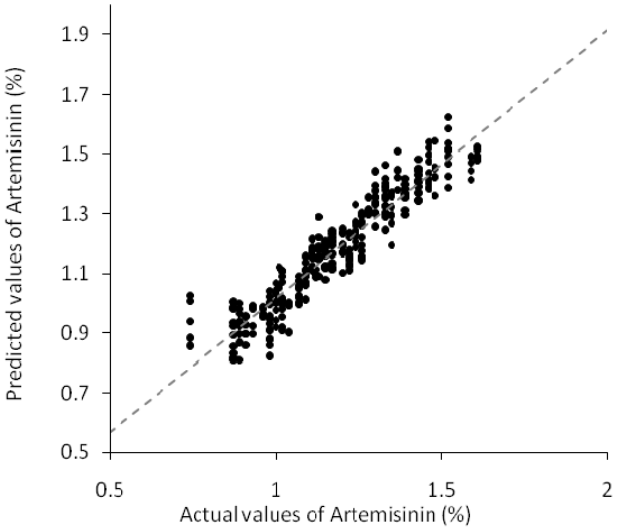

Fig. 1 Actual values (thin layer chromatography) versus predicted values (NIRS) with the partial least squares regressions (Camps et al., 2011).

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ASL 3: Use of genotyping by sequencing (GBS) in chamomile (*Matricaria recutita* L.) to enhance breeding

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Abstract

German chamomile (*Matricaria recutita* L.) is an important medicinal plant with a wide range of medical uses. Genotyping by Sequencing (GBS) was performed for this non-model organism on a panel of 33 different origins (varieties, populations, accessions; in total 91 plants with 2-4 plants per origin), and for 2 accessions of *M. discoidea* as outgroup. High-quality SNP identification (single nucleotide polymorphisms) was done using the pyRAD pipeline, and genetic diversity was analysed with STRUCTURE and CLC Genomics Workbench 8.5.

The analysis revealed one group with low genetic differentiation (14 origins with 39 plants), whereas clear differences could be identified between the remaining 19 origins (52 plants). Single plants of the same origin were mostly genetically similar, although elevated genetic diversity could be identified between single plants of 13 origins.

Phenotypic traits (flowering time, chemical compounds, powdery mildew infection) and ploidy were also measured. Ongoing work includes the identification of associations between genetic data (SNPs) and these traits. These data will be used to improve the exploitation of genetic resources in chamomile (e.g. utilization of heterosis, generation of cytoplasmic male sterility, facilitation of breeding of varieties with improved traits).

To summarize, we have applied next generation genomics methods to medicinal plants as a basis to improve the breeding process. The efforts to perform such an analysis are manageable, especially regarding the decreasing costs and increasing availability of bioinformatic pipelines for the GBS-analysis.

ASL 4: Next Generation Complex Genome Assembly

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Abstract

Whole genome assembly boosts the discovery of genes and pathways involved in the key metabolites produced in medicinal plants. Many medicinal plants possess large, polyploid and/or heterozygote genomes, thus *denovo* assembly of these genomes poses a significant challenge both algorithmically and economically. DeNovoMAGIC-2 assembler has successfully reconstructed some of the largest most repetitive, polyploid and heterozygote plant genomes. Using only high coverage of short Illumina reads, DeNovoMAGIC-2 has assembled over 90 % of the genome sequence of the 16 Gb, hexaploid wheat and the 1 Gb, tetraploid and heterozygote mango genome, with N50 of ~7 Mb and ~1 Mb respectively. Assemblies were completed in 14 and 2 days using 1 Tb and 0.512 Tb RAM computers, respectively. BUSCO analysis revealed full intact gene content for over 90 % of the genome, with clear phasing of allelic and paralog genes. Similar employment of DeNovoMAGIC-2 is expected to reconstruct the genome sequences of many medicinal plants, boosting our basic understanding of metabolite production and accumulation, towards industrializing medicine production from plants.

Materials and Methods

In brief, DenovoMAGIC-2 TM assembly has the following steps:

- Reads pre-processing and error correction:
  - PCR duplicates, Illumina adaptor are removed.
  - Use 2x250 450bp Paired-End (PE) libraries overlapping to create stitched reads (SR).
  - All reads that contain putative sequencing error (contain a sub-sequence that does not reappear several times in other reads) are filtered out.
- De Novo Assembly:
  - Build a De Bruijn graph of contigs from the overlapping SR.
  - SR are used to find reliable paths in the graph between contigs for repeat resolving and contigs extension.
  - Contigs are linked into scaffolds using the filtered SR and Mate-Pair (MP) information, estimating gaps between the contigs according to the distance of PE and MP links.
  - A final fill gap step use PE and MP links and De Bruijn graph information to close gaps.

Results

Assessing genome assembly and annotation completeness with single-copy orthologs. Felipe A. Simão, Robert M. Waterhouse, Panagiotis Ioannidis, Evgenia V. Kriventseva, and Evgeny M. Zdobnov B*ioinformatics*, published online June 9, 2015, doi: 10.1093/bioinformatics/btv351

TA Assembly QA - BUSCO Results (Benchmarking Universal Single-Copy Orthologs)*

Wheat Assembly QA - BUSCO Results (Benchmarking Universal Single-Copy Orthologs)*

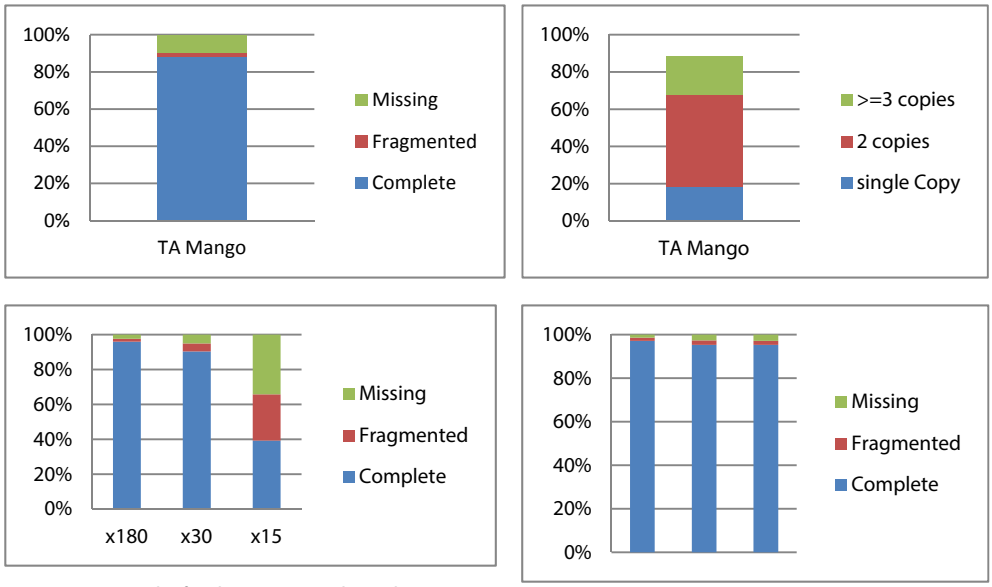

Fig. 1 Busco Results for the Mango and CS wheat

Tab. 1 Selected DeNovoMagic-2 results (DN2 results summary table)

Parameter	Diploid Wheat (Aegilops tauschii)	Tetraploid Wheat (Wild Emmer)	Hexaploid Wheat (Chinese Spring)	Maize	Wild Soybean	TA Mango
Fold coverage of short reads (PE & MP)	200X	180X	180X	180X	230X	205X
Run time- days	5	10	14	2	1	2
Contig N50	69 Kbp	57 Kbp	52 Kbp	73 Kbp	24 Kbp	28 Kbp
Scaffold assembly N50 (L50)	11.4 Mbp (106)	7.0 Mbp (414)	7.06 Mbp (566)	9.4 Mbp (68)	4.68 Mbp (57)	0.99Mbp (202)
Scaffold assembly N90 (L90)	2.27 Mbp (405)	1.15 Mbp (1827)	1.26 Mbp (2363)	1.95 Mbp (256)	0.72 Mbp (260)	0.024Mbp (2152)
Total assembly size	4.09 Gbp	10.50 Gbp	14.53 Gbp	2.18 Gbp	0.997 Gbp	0.81 Gbp
Unfilled gaps	1.39 %	1.63 %	1.80 %	1.88 %	3.50 %	6.13 %

References:

Prof. Jan Dvorak, Department of Plant Sciences, University of California, Davis
"Wild Emmer Wheat assembly by NRGene is unquestionably the best assembly of wheat genomic sequence to date."

Prof. Thomas P. Brutnell, Donald Danforth Plant Science
"The W22 Maize genome assembly is the best maize assembly that I have seen!"

Prof. Curtis Pozniak, the University of Saskatchewan, Canada
"The computational tools developed by NRGene, which use Illumina's sequence data, combined with the sequencing expertise of IWGSC has generated a version of the wheat genome sequence that is better ordered than anything we have seen to date. We are starting to get a better idea of the complex puzzle that is the wheat genome."

Prof. Nils Stein, of Germany's Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)
"Overall, the quality is breathtaking. NRGene's results are just amazing and will have a major impact."

DeNovoMAGIC wheat assemblies show high coverage of the genic regions

Using DeNovoMAGIC combined with GenoMagic power one can assemble high quality genomes from low coverage sequencing

Session B: Cell, tissue and organ culture, cryopreservation and endophytes

BPL 1: Cell, tissue and organ culture, cryopreservation, endophytes in relation to medicinal and aromatic plants

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Abstract

Plant cell, tissue, organ and protoplast culture methods offer a rich scope for creation, conservation and utilization of genetic variability for the improvement and production of elite planting material of medicinal and aromatic plants. Besides, tissue culture techniques are now increasingly being used for the production of bioactive compounds in vitro. Micro propagation ensures true to type, rapid and large scale multiplication under disease free conditions. In the absence of seasonal constraints, 10-30 cycles, depending upon the plant species, can be completed in one year, ensuring 5-50 times multiplication per cycle.

We have developed micro propagation systems for *Mentha* sps., *Aloe vera*, *Chlorophytum borivillianum, Stevia rebaundiana, Azadirachta indica, Bacopa monnieri* and *Capsicum annuum*. Meristem (0.2 - 0.4 mm) culturing, followed by disease indexing and micro propagation ensures disease free planting material that is otherwise difficult to obtain in the vegetatively propagated plant species. We have exploited this method for rejuvenation and large scale multiplication of three commercial mint varieties belonging to *Mentha piperita, M. spicata* and *M. cardiaca*. Biotization with endophytic fungus, *Piriformospora indica* and bacteria *Pseudomonas fluorescens*, improved survival rate, nutrient acquisition, field performance and saponin content of micro propagated *Chlorophytum borivilianum*. Biotization of micro propagated *Aloe vera* plantlets with *Piriformospora indica* and *Pseudomonas fluorescens* improved the plantlet survival rate in the field. We developed a cell line of *Capsicum annuum* that produced 7 times more capsaicin in cell suspension cultures.

Somaclonal variation is a potent emerging aspect for broadening the genetic base and thus obtaining incremental improvement in the commercial cultivars, more particularly, in the vegetatively propagated species. Production of haploids is being exploited for the early release of varieties. Embryo culture is the practical approach to obtain inter specific and inter generic hybrids among otherwise hard to cross parents. Somatic cell hybridization involving fusion of protoplasts from different species is considered an important approach to combine characteristics even from otherwise sexually incompatible species and to obtain cybrids (cytoplasmic hybrids) and organelle recombination not possible through conventional methods.

Tissue culture helps in international exchange of germplasm avoiding the risk of spreading pathogens and insects. In vitro freeze-storage and cryopreservation are very important techniques for germplasm conservation especially of the vegetatively propagated species. The role of endophytes in relation to micro propagated plants will be discussed.

BSL 1: Biotechnological tools for improvement of black nightshade (*Solanum nigrum* L. complex), valuable medicinal and vegetable plants in Kenya

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Abstract

Solanum nigrum complex is a group of plant species used as indigenous vegetables but also as traditional medicinal plants in Kenya and other parts of the world. In Kenya, just like in most African countries, both the unripe fruits and leaves are used to cure different ailments. This vegetable is said to improve the CD4 count in HIV patients and all HIV patients are encouraged to take it as part of their diet. Despite that high value, African nightshade is a neglected crop. Up to now the farmers exploit traditional landraces and accessions. For development of improved African *S. nigrum* varieties, knowledge is necessary about the genetic structure of the local African nightshade accessions. Amplified fragment length polymorphism (AFLP) technique was performed to discriminate accessions from the Western region of Kenya. Production of haploid plants of the *S. nigrum* complex and subsequent chromsome doubling is a promising tool to obtain pure inbred lines in a short time. Therefore, in this study, anthers of *S. nigrum* were cultivated in vitro. It was observed that the microspores underwent the first divisions and calluses were formed.

Keywords: AFLP, African nightshade, anther culture, flowcytometry, neglected crops

Introduction

There are a number of species in the *Solanum nigrum* complex which include *S. nigrum, S. villosum, S. scabrum, S. americana, S. burkankii,* and *S. schenopodioides* among others. The *S. nigrum* complex which refers to all of the dozens of black nightshade species around the world formally called *S. nigrum* comprises of both native and bred *Solanum* species used as vegetables and source of fruits in Kenya and other parts of the world. These plants are believed to have a high nutritional value (Schippers, 2000). It has been reported that the nutrient content of this vegetable can provide 100 % of the recommended daily allowance for an adult for calcium, iron, b-carotene, and ascorbic acid and 40 % of protein if 100 g of the fresh vegetable is consumed (Abukutsa-Onyango, 2003). The high nutritional value makes African nightshade especially important for poor people. Demand for the vegetable has increased in the urban areas of Kenya making it a cash crop (Ojiewo et al., 2013).

Medicinally, African nightshades are used for stomach upsets, duodenal ulcers, and swollen glands (Edmonds and Chweya, 1997; K'opondo et al., 2005). They are also squeezed on babies' gums to ease pain during teething. The HIV patients are given this vegetable together with anti-retroviral drugs at the AMPATH in Eldoret, Kenya (http://www.ampathkenya.org/). Studies on in vitro antiviral activity of *S. nigrum* against Hepatitis C Virus by Javed et al. (2011) showed that methanol and chloroform extracts of *S. nigrum* seeds exhibited 37 % and more than 50 % inhibition of Hepatitis C Virus replication, respectively.

In Western Kenya, three species have been identified namely *S. scabrum, S. nigrum* and *S. villosum*. These species have shown significant antimicrobial activities on different microbes of both humans and plants (Matasyoh et al., 2014). The work showed that the *S. villosum* had the best activity against numerous bacterial species, among others *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli* and *Bacillus subtilis.* These microbes are very important because they cause diseases like typhoid, pneumonia, ring worms, mouth ulcers, diarrhoea etc which affect most poor people who cannot afford nor access modern medication. *S. scabrum* showed best antifungal results against all the *Fusarium* species which affect crops.

The knowledge that the *S. nigrum* complex has high nutritional and medicinal benefits has led to an increase in their consumption, monetary value and demand in Africa. There is a high demand to improve *S. nigrum* cultivars. The production of doubled haploid plants ensures that homozygous lines can be obtained in a shorter period, unlike conventional breeding which takes 5 - 6 years of selfing to achieve this homozygosity level. Through development of haploids and later doubled haploids, cross breeding process can be initiated. Here we present the first results of anther culture of the Kenyan *S. nigrum* complex accessions. Furthermore, for development of new African *S. nigrum* varieties knowledge is necessary about the genetic structure of the local African nightshade accessions. For that purpose amplified fragment length polymorphism (AFLP) technique was used.

Materials and Methods

Plant material

Twelve accessions belonging to the *Solanum nigrum* complex (*S. scabrum, S. nigrum* and *S. villosum*) were used as anther donors and for DNA extraction. Seeds of the *S. nigrum* complex were obtained from the farmers from Kenya, and the Germany seeds were obtained from the garden at the Julius Kühn Institute, Quedlinburg (Table 1, Fig. 1). They were grown in pots in a greenhouse providing standard horticultural conditions from March to August.

Tab. 1 Used accessions of Solanum nigrum complex from Kenya and Quedlinburg, Germany.

Number	Species	Description, origin
1	*S. scabrum*	Indigenous species, Kabras, Kakamega
2	*S. scabrum*	Improved species, Bungoma
3	*S. scabrum*	Improved variety, Botsotso, Kakamega
4	*S. scabrum*	AMPATH seeds grown in Kakamega
5	*S. scabrum*	Species, Eldoret
6	*S. scabrum*	Species, Ahero, Kisumu
7	*S. villosum*	Indigenous species, Eldoret
8	*S. villosum*	Indigenous species, Bungoma
9	*S. nigrum*	Indigenous species, Kabras, Kakamega
10	*S. nigrum*	Indigenous species, Bungoma
11	*S. nigrum*	Quedlinburg, Germany
12	*S. nigrum*	Quedlinburg, Germany

Anther culture, flowcytometry, and AFLP analysis

Flower buds were surface sterilized by submerging them in a NaClO solution (3 % active chlor) for 15 min, prior to three washes with sterile water. The anthers were excised from the flower buds, and cultured on 35 mm Petri-dishes containing 3 ml liquid medium either K0 + S with 4 mg/l 2,4 D, 1 mg/l zeatin (KELLER et al., 1975), medium N6 or EPM + 0.2 mg/l 2,4 D (CHU, 1978, modified), respectively. Anthers were initially treated with 6 °C or 30 °C for three days, or 32 °C for one day and further cultivated at 25 °C in darkness. At least 50 anthers per variant were tested. Anther callus was transferred to solid regeneration medium (MS + 2.0 mg/l kinetin, 0.5 mg/l IAA, 5 % coconut water, 800 mg/l glutamine, 2 % sucrose) and cultivated under light conditions; 16 hrs light / 8 hrs dark at 25 °C.

Fig. 1 Plants of the S. nigrum complex in greenhouse.

For estimation of the DNA content of plants and callus material was cut with a sharp razor blade in Galbraith buffer (GALBRAITH, 1983). The filtered suspension of nuclei was stained with propidium iodide. Measurement was done using BD FACSCalibur™. AFLP analysis was described in MATASYOH et al. (2015).

Results

Anthers from various accessions of *S. nigrum* complex have shown a high responsiveness to all three induction media (Fig. 2a). It was impossible to decide if the callus was developed from somatic anther tissue or from microspospores. Although few anthers showed open thecae with callus (Fig. 2b) and first divisions of microspores were found (Fig. 2c). The highest callus induction (100 %) was observed after 30 °C treatment on medium EPM for accession 10 (*S. nigrum* species indigenious from Bungoma). The response to induction medium was genotype depending. While for *S. scabrum* accessions the highest callus induction frequency was noticed on K0 + S for *S. nigrum* and *S. villosum* EPM medium was the best (Table 2).

Androgenetic calluses were further cultivated on several media with the aim to induce shoots. Calluses are growing over two years but regeneration could not be induced.

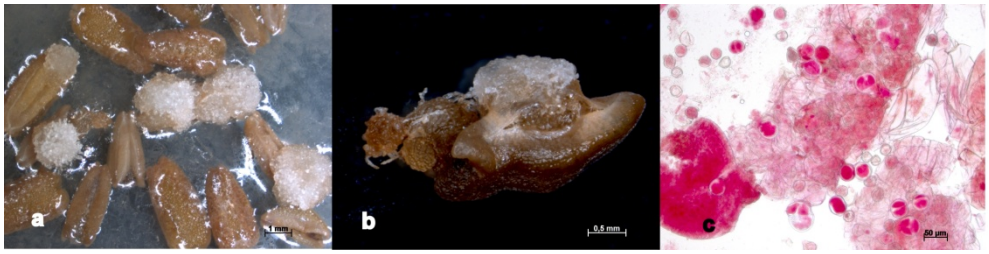

Fig. 2: Development of anthers in vitro, a: Callus on anthers Solanum scabrum on medium K0 + S after four weeks, b: Anther with open theca and callus, c: Divisions of microspores of S. nigrum, one week on EPM.

Tab. 2 Survey about anther culture

Genotype	Induction medium	number of cultivated anthers	Number of calluses	Regeneration frequency (%)
Solanum. scabrum	EPM	200	1	0.5
Accessions 1, 4, 5, 6	K0+S	200	34	17.0
	N6	200	6	3.0
S. nigrum	EPM	500	300	60.0
Accession 10	K0+S	300	28	9.3
	N6	350	85	24.3
S. villosum	EPM	200	62	31.0
Accessions 7, 8	K0+S	300	35	11.7
	N6	250	60	24.0

Generally flowcytometry showed the same DNA content of calluses as the donor plant. One callus from accession 10 revealed the haploid DNA content indicating that a further development from haploid cells is feasible.

By AFLP analyses the twelve *Solanum* accessions were clearly distinguishable (MATASYOH et al., 2015). *S. villosum* was the most divergent accession. *S. nigrum* and *S. scabrum* accessions were near up to intermixing in the cluster analysis. This underpins the difficulty to classify the accessions of the *S. nigrum* complex.

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BSL 2: Endophytes in commercial micropropagation - friend or foe?

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Abstract

Medicinal and aromatic plants are superorganisms like all plant species- naturally colonized by bacteria, fungi and protists. Micropropagated plants are facing different challenges under *in vitro* and *ex vitro* conditions: Mixotrophic growth under low light conditions on artificial nutrient media, poor gas exchange in small vessels, abiotic stress, bad rooting, transplanting stress, low survival rate during acclimatization in greenhouse. The use of endophytes in micropropagation can improve plant growth, yield, and health and induce tolerance to abiotic and biotic stress. A tool for the use of competent endophytes in micropropagation under *in vitro* and *ex vitro* conditions is "biotization" of plantlets with useful bacterial and fungal inocula. Fungal inocula which are used commercially are e.g. arbuscular mycorrhizal fungi) in form of spores and extraradical mycelium on different carrier materials like expanded clay, vermiculite, sand or peat. Furthermore representatives of the root fungal genus *Trichoderma* are applied as spores formulated in powder. Plant-growth promoting rhizobacteria of the important genera *Bacillus*, *Pseudomonas*, *Azospirillum* and *Azotobacter* in form of lyophilised endospores/bacterial cells in powder or liquid formulation are also available on the market.

Keywords: Endophytes, rhizobacteria, mycorrhiza, inoculation, micropropagation

Introduction

Herman (1996a; 1996b) was one of the first to think on using those microorganisms as stress eleviating factor. In 1998 Jerzy Nowak (Nowak, 1998) reviewed the benefits of *in vitro* "biotization" of plant tissue cultures with microbial inoculants, but the methods hardly found application in commercial tissue culture. First reports of microorganisms living in micropropagated plants (medicinal plant Grotkass et al., 2000, reviewed by Cassels, 1991 and Leifert et al., 1994) were seen as problem-causing inhabitants and contaminants. Evolving imaging and molecular techniques (-omics technologies) helped to discover the properties of endophytes, phytopathogens and other microorganisms from plant and soil habitats and will allow us to better understand mutualism and pathogenicity, culminating in Hardoim et al. (2015), who give ecological and evolutionary considerations for defining functioning of microbial endophytes.

But there are still challenges. It is not easy to discriminate between endo- and epiphytes, technically and scientifically. More than assumed 80 % of endophytes are ‚un-culturable', which only means that there still isn't enough knowledge on necessary culture conditions. Reports on tripartite systems, e.g. mycoviruses in fungal endophytes regulating the hypervirulence of pathogenic fungi (Ahn and Lee, 2001) or the interkingdom transfer of the acne causing agent from human to grapevine (Campisano et al., 2014), make the systems even more complex, thus difficult to understand - but understanding is the key for commercial use as commercial use of endophytes must be safe, predictable and cost-effective.

Results

Various fungi are used in micropropagation, such as arbuscular mycorrhizal fungi (Vestberg et al., 2004, Kapoor et al. 2008), ectomycorrhizal fungi (for review see Rai, 2001) and ericoid mycorrhizal fungi (Jansa and Vosatka, 2000), *Beauveria bassiana* (Akello et al., 2007), *Piriformospora indica* and other members of the Sebacinales (Sharma et al., 2014), *Fusarium oxysporum* (Ting et al., 2008), *Ophistoma* similar fungal species (Mucciarelli et al., 2003), *Phialocephala fortinii* (Vohnik et al., 2003) and *Trichoderma harzianum* (and other *Trichoderma* species) (Franken, 2012, Vestberg et al., 2004).

Examples for bacteria are *Acetobacter diazotrophicus* (AZLIN et al., 2007), *Achromobacter xylosoxidans* (BENSON et al., 2014), *Azospirillum brasilense* (CREUS et al., 1997; LARABURU and LLORENTE, 2015a), *Azotobacter chroococcum* (LARRABURU et al., 2007), *Bacillus subtilis* (WILHELM et al., 1997; VESTBERG et al., 2004), *B. megaterium* (TRIVEDI and PANDEY, 2007), *Burkholderia phytofirmans* (AIT BARKA et al., 2000), *B. vietnamiensis* (GOVINDARAJAN et al., 2006), *Enterobacter* sp. (MIRZA et al., 2001), *Klebsiella variicola* (WEI et al., 2014), *Microbacterium* sp.(QUAMBUSCH et al., 2014), *Pseudomonas fluorescens* (VESTBERG et al., 2004; THOMAS et al., 2010) and *P. putida* (LIFSHITZ et al., 1987).

Increased plant biomass was reported (shoot and root fresh weight and dry weight, plant height, leaf area, rhizom weight) (RAI et al., 2001; KAPOOR et al., 2008), ‚better' rooting in vitro (number, length) (LARRABURU and LLORENTE, 2015b), ‚better' acclimatization (survival rate, plant performance) (DUFFY et al., 1999; OVANDO-MEDINA et al., 2007), earlier flowering and increased flower number (VARMA and SCHUEPP, 1995), induction of stress resistance (NOWAK and SHULAEV, 2003), biocontrol effects (AIT BARKA et al., 2000, HARISH et al., 2008). Also, altered secondary metabolite profiles were reported (ZABETAKIS, 1997).

In the presentation, more information will be given on how microorganisms can be isolated, identified, cultured and inoculated. Furthermore, methods for characterization of endophytic microbes will be shown, esp. bacteria by different biochemical properties in order to determine a possible plant growth promotion potential especially for medicinal and aromatic plants.

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BSL 3: Alternative strategies to by-pass the plant-based Azadirachtin-A production

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Abstract

All parts of Neem (*Azadirachta indica* A. Juss) show a broad spectrum efficacy against insect pests including insecticidal, anti-feedant or insect repellent activities. Several studies have shown that plant cell cultures can produce azadirachtins. We induced more than 40 novel Neem cell lines in modified Murashige and Skoog (MS) media containing different concentrations of auxins and cytokinins. To enhance the Azadirachtin production from 1 mg/l, it was necessary to optimize the media composition separately for biomass and secondary metabolite production. In light of this complex challenge we used our novel fully automated high-throughput microbioreactor system that allows us a fast and controlled batch and fedbatch screening in 48-well microtiter plates. There is increasing evidence that plants like *Azadirachta indica* contain endophytes which are able to colonize internal plant tissue without causing visible disease symptoms. The estimated high species diversity of endophytes suggests a rich and almost untapped source of new secondary metabolites. We isolated more than 340 endophytes from various plant tissues and tested if they were able to produce Azadirachtin-A. Here, we present data on isolation of endophytes and induction of callus as well as first results of our microbioreactor system.

Keywords: Azadirchtin-A, endophytes, plant cell cultures, high-throughput microbioreactor

Introduction

The neem tree belongs to the Meliaceae family, it originated from Asia and is currently grown in all subtropical areas around the world (Rodrigues et al., 2014). Its use for therapeutical and agrochemical applications has enhanced its industrial value. The tree produces various secondary metabolites with different biological effects for commercial applications. The best known active compound is Azadirachtin-A, which is already used as broad-spectrum biopesticide. Common ways for the extraction of the active compounds are still expensive and very time consuming. In consideration of the fact that the interest on plant secondary metabolites for the pharmaceutical and agricultural application has increased rapidly in the last years new and complementary ways must be found to satisfy the demand of these active compounds. The challenge during the development of new production strategies is to preserve the production potential of the strain or culture. A multiplicity of studies have shown that the use of submersed plant cell cultures is a promising way to create a competitive production procedure compared to conventional manufacturing processes. Besides the plant cell cultures, endophytes seem to be involved or even responsible for the production of pharmaceutical compounds, such as Taxol (Stierle et al., 1993) and Camptothecin (Rehman et al., 2008) as well as Azadirachtin-A (Kusari et al., 2012)

The overall aim of a BMBF funded project is to develop a competitive process to produce high concentrations of bioinsecticidal compounds with Neem plant cell cultures or endophytes. The bioactivity of the produced compound is tested in a high-throughput bioassay based on Sf9 cells.

Materials and Methods

Induction of plant cell lines and isolation of endophytes

In a first step various plant tissues were surface sterilized as previously described (PRAKASH and SRIVASTAVA, 2008). In case of the isolation of endophytes the protocol was optimized related to the time and concentration of the used chemicals. Afterwards cut explants were incubated on appropriate media followed by a transfer into submersed cultivation in shake flasks.

Extraction and detection of secondary metabolites

The secondary metabolites of the submersed cultures were extracted using a commonly applied liquid liquid protocol as well as following a novel *in situ* product removal strategy based on resin beads. The received samples were analyzed via UHPLC-DAD-ESI-TOF-MS/MS measurement.

Results

We could successfully induce callus cultures from stem and leaf tissues. For this we analyzed more than 4200 explants incubated in the dark as well as with a dark/light rhythm of 8 to 16 hours. For example, figure one shows from how many stem explants we could finally induce callus material. Not surprisingly the MS media containing synthetic cytokinins and auxins showed the highest callus proliferation from more than 90 % of the explants (Figure 1).

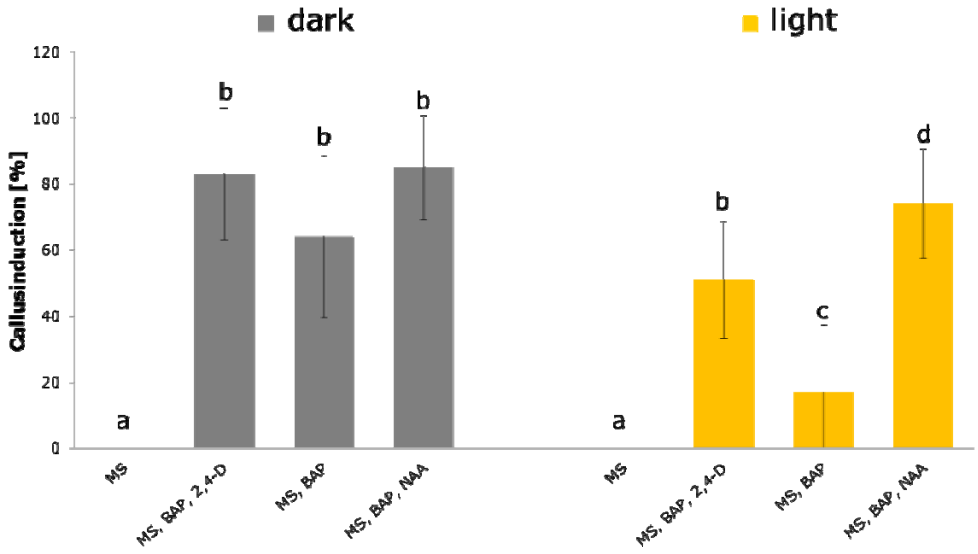

Fig. 1 Influence of medium compositions on callus induction mean (±SD) of stems; n=20. Different letters above bars indicate significant differences according to Kruskal-Wallis test with Mann-Whitney-U post hoc test at $P<0.05$.

The isolation of endophytes from different tissues and locations has led to 346 isolates so far (Figure 2). Most endophytes were isolated from leaf and stem tissues, 56 % of them were fungi and 44 % were bacteria.

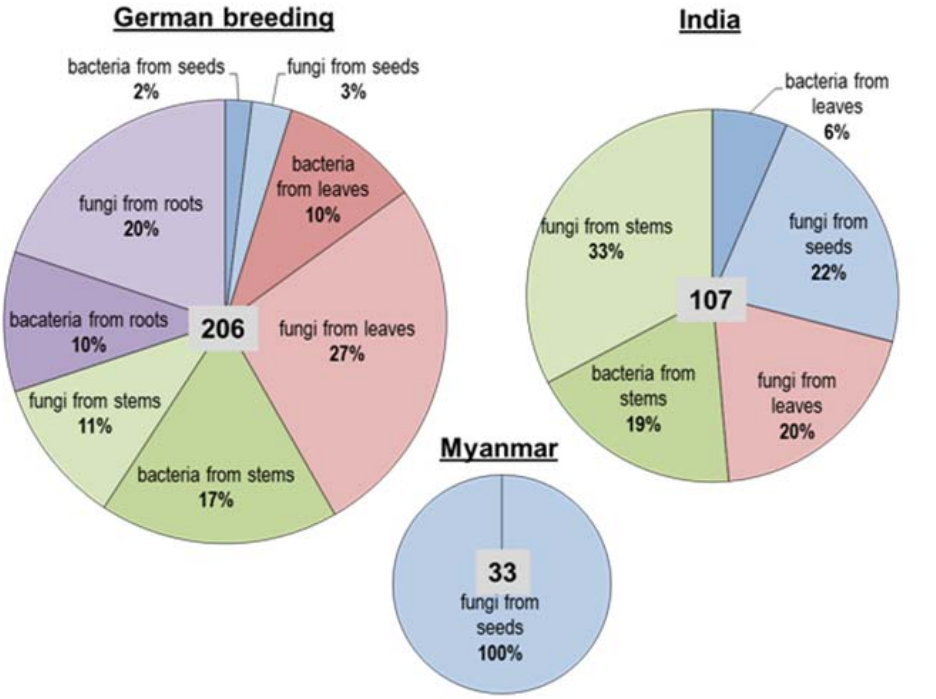

Fig. 2 Total number of isolated endophytes from various plant tissues and destinations.

The measurement via UHPLC-DAD-ESI-TOF-MS/MS allows a specific identification of Azadirachtin-A. We were able to detect the expected mass fragments of Azadirachtin-A in 25 % of our plant cell lines (Figure 3).

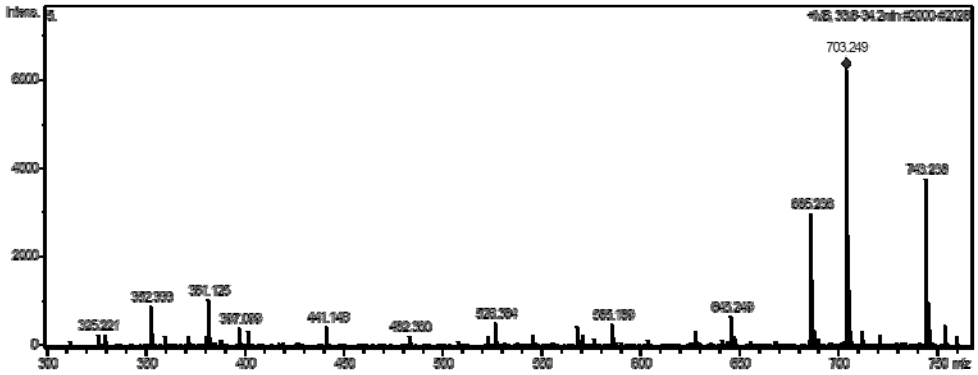

Fig 3: Mass spectrometry data of extracted Azadirachtin-A from plant cells including the expected fragment masses.

In on-going experiments we use the microbioreactor system to optimize the media composition to increase the Azadirachtin production. Furthermore we use the microbioreactor to develop an automated high-throughput screening, to test the efficacy of endophytic isolates against Sf9 cells.

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Session C: Resistance breeding and new phytopathogens

CPL 1: New, emerging and re-emerging fungal diseases on medicinal and aromatic plants in European domain

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Abstract

Plant diseases cause agricultural and economic loss and impact negatively on human and animal health through mycotoxins and allergens produced by them. They also have consequences for biodiversity conservation. The pathogens could be classified in five categories: new - detected within the last five years; emerging - have always been present in an area but have grown in importance over the years; re-emerging - have been previously controlled but are once more a major problem associated with chemical resistance or changes in management or cultivars; threatening - not reported or limited in distribution in Europe and chronic-spreading – known for longer than 20 years and causing increased concern. Diseases emerge or re-emerge due to changes in farming practices, development of new strains of the pathogen, climate change, introduction of the pathogen to new geographical locations, or introduction of more efficient pathogen vectors. During the last years emerging infectious diseases (EIDs) are of special concern to researchers. Among all pathogens fungi are responsible for the greatest damage to plants in both agricultural and natural ecosystems. They represent over 70 % of all plant pathogens and over 30 % of plant EIDs. Surveys on fungal diseases of medicinal and aromatic plants have been carried out in the framework of several research projects between Germany, Bulgaria, Lithuania and Poland in the last two decades. EIDs have been reported, either as novel pathogens or as familiar pathogens affecting new host species. The importance of the problem could be illustrated by such examples as some phytopathogenic fungi on Apiaceae and Lamiaceae hosts discussed in the present work.

Keywords: Apiaceae, Lamiaceae, fungal pathogens, asexual morphs

Introduction

Medicinal and aromatic plants (MAPs) are considered minor crops generally grown on limited area. There was a view that they had no serious diseases. The sources of information related to the diseases of MAPs were mostly limited to the areas in which their cultivation reached appreciable levels. During the last decades, mainly Europe and America have experienced an increase trend towards healthy diet and natural products, which led to a growing demand for MAPs, partly satisfied by collections of wild-growing plants, but to an enhanced extent by cultivation. The increased interest in the use of MAPs is also recognizable regarding the bigger diversity of genera processed in Europe. A negative consequence of the growing concentration in cultivation is an increase of pathogen occurrence (GABLER, 2002). The control techniques take a great relevance inside the recommended growing protocols for MAPs because the diseases can cause economically consid-

erable decrease in yield but also in the quality of production (CARRUBA et al., 2015). The pathogens could be classified in five categories: new - detected within the last five years; emerging - have always been present in an area but have grown in importance over the years; re-emerging - have been previously controlled but are once more a major problem associated with chemical resistance or changes in management or cultivars; threatening - not reported or limited in distribution in Europe and chronic-spreading – known for longer than 20 years and causing increased concern (DAMSTEEGT, 1999). Despite the fact that MAPs produce secondary metabolites with antimicrobial, including antifungal action, they are attacked by a number of pathogens including fungi. The information about the most commonly widespread fungal pathogens affecting MAPs is summarized by CARRUBA et al. (2015). During the last years emerging infectious diseases (EIDs) are of special concern to researchers (ANDERSEN et al., 2004; FLETCHER et al., 2010; FISHER et al., 2012).The purpose of present work is to share long-years' experience and knowledge about new, emerging and re-emerging pathogens mainly on representatives belonging to Apiaceae (caraway, coriander, dill, fennel,) and Lamiaceae (oregano, sage, summer savory), which are known as medicinal, spice and essential oil crops.

Materials and Methods

The investigations on fungal diseases of MAPs were conducted in the framework of several research projects between Germany, Bulgaria, Lithuania and Poland in the last two decades. Observations for disease incidence were made on plants in commercial fields and private gardens. Samples of diseased plants were taken for laboratory studies. The presence of fungi was established on the basis of etiological symptoms occurring on the infected above-ground and underground plant parts and mycological analysis. A complex approach was applied in pathogen diagnostics and their characterization including phytopathological, mycological, phytochemical, immunological, molecular and statistical methods. Pathogenicity of the isolates was proved using whole plants or detached plant parts. Toxigenic properties of fungal isolates were investigated by extraction and purification of secondary metabolites and application of a leaf puncture bioassay for the rapid determination of phytotoxic activity of the fractions and of pure compounds (EVIDENTE et al., 2011). The investigation on molecular identity and similarity of the fungal isolates was carried out by blast sequencing the internal transcribed spacer (ITS) region of rDNA. Total genomic DNA was extracted from pure cultures. The generated DNA sequences of ITS region were compared with other fungal sequences from the National Center for Biotechnology Information (NCBI, Bethesda, USA) database and our own accessions isolated from different host plants using BLASTn (Basic Local Alignment Search Tool) (KAČERGIUS et al., 2011). The phylogenetic analysis was performed applying the Neighbor-Joining method by MEGA5 (TAMURA et al., 2011). Searching for resistance to some fungal diseases was performed in field experiments including Bulgarian and German cultivars of fennel, dill and caraway. The evaluation of the cultivar reaction was made on the basis of disease incidence (% diseased plants), disease severity (disease index, DI) and disease progress (area under the disease progress curve, AUDPC).

Results

Apiaceae hosts

Besides the chronic-spreading and well known fungal pathogens on Apiaceae hosts, several asexual morphs of *Diaporthe* species have been reported as the causal agents of new diseases. *Phomopsis diachenii* Sacc. occurred on caraway (*Carum carvi* L.) as an emerging disease in Czech Republic and caused considerable yield losses (more than 50 %) (ONDŘEJ, 1997). Several years later the first detection of the pathogen in Germany was made (GABLER und EHRIG, 2000). *P. diachenii* showed very high aggressiveness in pathogenicity tests. Its temperature optimum was between 25 ^{0}C and 30 ^{0}C. There was a positive correlation between the disease progress in the field and some climate data, above all the air temperature and rainfall, which could be a possible explanation for high disease occurrence in hot showery weather (GABLER, 2002). Later, this caraway disease

has been reported in Bulgaria (RODEVA and GABLER, 2004), Poland (MACHOWICZ-STEFANIAK, 2009), Hungary (NAGY, 2009) and Lithuania (MAČKINAITĖ, 2012). At the end of the 1970s, an outbreak of new disease (umbel browning and stem necrosis) occurred on fennel (*Foeniculum vulgare* Mill.) in France and was described as a new species *P. foeniculi* Du Man. et Vegh (DU MANOIR and VEGH, 1981). In 1990 the presence of the pathogen was detected for the first time in Italy (MUGNAI and ANZIDEI, 1991). The fungus regarded as highly aggressive caused considerable losses in yield. In Germany *P. foeniculi* was detected for the first time in 1991 (PLESCHER, 1992). Lately, this fennel disease has been found in Bulgaria for the first time (RODEVA and GABLER, 2011). *Phomopsis* sp. was established as the causal agent of the same disease of dill, too (RODEVA and GABLER, 2006). Pathogenicity tests and cross-inoculations were performed with *P. diachenii*, *P. foeniculi* and *Phomopsis* sp. isolated from caraway, fennel and dill, respectively. On the basis of disease development and plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) using a polyclonal *Phomopsis*-genus-specific antiserum (IgG59/III) it was found that all isolates provoked the typical *Phomopsis* symptoms on all three host plants; however, the disease scores and ELISA values varied (RODEVA and GABLER, 2006; 2011). The pathogens were reisolated from inoculated plants. The sexual counterpart of *Phomopsis* spp. isolated from caraway, fennel and dill was not observed under field conditions. Its development was provoked on diseased stem fragments incubated in moist chamber or in vitro mainly on oatmeal and malt yeast extract agar and was assigned to *Diaporthe angelicae* (Berk.) D.F. Farr & Castl. (RODEVA and GABLER, 2004; 2011). Umbel browning and stem necrosis were also found as typical symptoms on wild-growing Apiaceae species and *Phomopsis* spp. were isolated as causal agents (RODEVA et al., 2006), which deserved great interest because these hosts could serve as a source of inoculum for cultivated ones. On the other hand, the negative impact of *Phomopsis* diseases on biodiversity might reduce the potential for the discovery of new pharmaceuticals or new crops. Morphological and molecular investigation was performed on 46 isolates obtained from cultivated and wild-growing Apiaceae hosts in Bulgaria, Lithuania and Germany. The phylogenetic analysis revealed a close relationship of *D. angelicae* and its asexual morphs like *P. diachenii*, *P. foeniculi* and some newly isolated *Phomopsis* spp. in Bulgaria (from coriander and parsley) and Lithuania (from coriander and dill) (KAČERGIUS et al., 2011). The diseases caused by *Phomopsis* spp. were highly devastating for studied umbelliferous plants and led to premature drying up of umbels and stems and even the plant death. Full destruction of affected umbels was observed thereby preventing seed set. The highest plant susceptibility was observed during flowering. It could be possible these new emerged warm-temperature diseases may become more important in the future years. The possibility of limiting the growth and development of *P. diachenii* was studied applying in vitro two biotechnical preparations (Biosept Active and Beta-chikol) and 12 fungicides from different chemical groups. The most promising compound in reducing the growth and development of *P. diachenii* was mancozeb (ZALEWSKA et al., 2013). The resistance evaluation of fennel, dill and caraway cultivars made on the basis of disease incidence, disease severity and disease progress showed that all cultivars under study were attacked but differences in their susceptibility were established, which is very promising in searching sources of resistance and their use in breeding (RODEVA and GABLER, 2007). Disease resistance is an important character of cultivars, especially MAPs as the application of pesticides is hardly accepted.

It is known that some *Phomopsis* species, including *P. foeniculi* produce in liquid culture phytotoxic metabolites, which could be involved in pathogenesis (EVIDENTE et al., 1994; CORSARO et al., 1998). Considering the economical importance of fennel fruits, the increased disease occurrence in Bulgaria and the fact that the causal agent *P. foeniculi* is a toxigenic fungus, research was undertaken to isolate and characterize the phytotoxins produced by Bulgarian strains. Using a bioassay guided isolation and purification procedure, different metabolites were isolated from the fungal culture filtrates. They were identified by spectroscopic methods as nectriapyrone, a pentaketide monoterpenoid, and altersolanols A and J and macrosporin, three octaketides anthracenones (EVIDENTE et al., 2011). These metabolites differed from foeniculoxin, that is a geranylkhydroquinone (EVIDENTE et al., 1994) and obviously from the exopolysaccharides (CORSARO et al., 1998) isolated from an Italian strain of the same fungus. Plant growth and development of

MAPs as well as the nature of secondary metabolites are affected by the physical environment, including light, temperature, rainfall, and soil properties (MÁTHÉ, 2010). The same circumstances could play a role in the secondary metabolite production of their pathogens illustrated by the fact that different isolates of same pathogen and host have produced different phytotoxic metabolites under ecological conditions in Italy and Bulgaria.

The ascomycetous fungus *Septoria carvi* Syd.was found as a dangerous pathogen of caraway in Poland (ZALEWSKA and MACHOWICZ-STEFANIAK, 2003). The fungus caused many small necrotic spots on petioles, leaves, stems and umbels. Infected plants could prematurely die. The pathogen was aggressive in pathogenicity tests. The disease occurred epidemically during warm and humid growing seasons. *S. carvi* was recorded in Czech Republic (ODSTRČILOVÁ et al., 2002), Germany (GABLER and MACHOWICZ-STEFANIAK, 2004), Austria (BEDLAN, 2005) and Lithuania (MAČKINAITĖ, 2012). In Bulgaria this pathogen has not been found yet (RODEVA and GABLER, 2009).

Mycocentrospora acerina (R. Hartig) Deighton, not previously recorded on caraway in Poland, has been found only recently (ZALEWSKA et al., 2015). Anthracnose caused by *M. acerina* has been considered as one of the major problems in caraway production in Netherlands for a long time (EVENHUIS et al., 1995). In Bulgaria *M.acerina* has been reported on wild growing caraway (Vanev 1988) but the anthracnose occurrence has never been observed on cultivated one (RODEVA and GABLER, 2009).

Two new diseases were found on coriander in Bulgaria caused by ascomycetous fungi. Wilting and root rot incited by *Macrophomina phaseolina* (Tassi) Goidanich led to the death of whole plants (RODEVA et al., 2010). The same fungus was also isolated from premature dead dill and caraway plants in the field (Rodeva, personal communication). In the laboratory experiments, the asexual morphs of the fungus were found either of microsclerotia or pycnidia but in the field only sclerotial form was observed. The microsclerotia served as units of long-term survival in soil. Their accumulation lead to an increase of the primary inoculum for root infection of coriander and many other host plants (more than 500 species) of the pathogen. The disease could be a threat for coriander and other Apiaceae hosts under conditions favorable for its development especially water deficit. Association of coriander with the host range of *M. phaseolina* has to be taken into consideration in crop rotations.

Phoma glomerata (Corda) Wollenweber and Hochapfel causing stem rot of coriander was found for the first time in Bulgaria and elsewhere (RODEVA et al., 2013). The identification was confirmed by PCR amplification, based on internal transcribed spacers and the 5.8 rDNA (ITS1-5.8-ITS2). The amplicon was sequenced and analyzed using BLASTn and showed a homology of 100 % with a corresponding sequence of *P. glomerata* (accession number DQ093699). The same pathogen was recorded as causal agent or crown rot diseases of fennel in Southern Italy (LAHOZ et al., 2007).

The basidiomycetous fungus *Itersonilia perplexans* Derx has been reported to be the causal agent of leaf blight of dill (*Anethum graveolens* L.) in several European countries, as Italy (MATTA and GARIBALDI, 1968), Germany (GEßNER, 1988; KUSTERER et al., 2002), Austria (BEDLAN, 1988), Swiss (USOLTSEVA and DAHL, 2006). Lately, it has been found in Bulgaria (RODEVA et al., 2009), UK (LAMBOURNE, 2011) and Cyprus (KANETIS et al., 2015). Serious infections by the pathogen were observed on different herbs including dill, coriander and parsley in Germany (Baden-Württemberg) in 2014 and 2015 (HINRICHS-BERGER, 2015). Since *I. perplexans* grows best at high relative humidity (>70 %.) and temperatures of 10-15 °C the way to diminish the disease appearance and development is to keep the dill plants under as dry conditions as possible. The initial symptoms of *I. perplexans* resembled those caused by lack of nutrient substances or water. For this reason it is very important to clear up the cause as the first symptoms appear and to take control measures. The infected plants have to be destroyed and do not put in the compost.

Lamiaceae hosts

Phomopsis sclarea Sarwar was isolated from sage (*Salvia officinalis* L.) in Poland for the first time in 2010 and recognized as the main cause of the necrotic spots on stem, peeling off and breaking the bark (Zimowska, 2010). In Germany, this pathogen was isolated from *S. officinalis* also for the first time in 2010. Additionally, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and *C. dematium* (Pers.) Grove were found but these pathogens were not predominant. The most serious damages were due to infections by the ascomycetous fungus *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley (syn. *Phoma exigua* var. *exigua* Desm.) and the downy mildew fungus *Peronospora salviae-officinalis* Y.J. Choi, Thines & H.D. Shin. Downy mildew fungi spread increasingly and are a considerable risk factor for many crops worldwide (Gabler, personal communication).

Boeremia strasseri (Moesz) Aveskamp, Gruyter & Verkley (syn. *Phoma strasseri* Moesz) was commonly isolated from the stems and the rhizomes of peppermint (*Mentha piperita* L.) showing symptoms of necrosis and tissue disintegration. The disease caused by the pathogen is called black stem and rhizome rot of peppermint (Zimowska, 2012). *Colletotrichum fuscum* Laubert, Gartenwelt has never been recorded on oregano (*Origanum vulgare* L.) anywhere in the world until recently. In Poland it was found as a new disease inciter causing characteristic symptoms on oregano leaves in the form of necrotic, rounded, concentrically zoned spots. This species, like other *Colletotrichum* species, had high thermal requirements and the optimum was 28 °C. Severe occurrence of anthracnose of oregano caused by *C. fuscum* must be therefore taken into account during the vegetation periods when the temperature is high (Zimowska, 2015). A severe outbreak of the black stem disease on *O. vulgare* subsp. *hirtum* (Link) Ietsw. (syn. *O. heracleoticum* Benth.) caused by *B. exigua* was reported in Germany (Saxonia-Anhalt) for the first time in 2002. The disease caused serious damages. The development of the pathogen (temperature optimum 20 °C) was favored by extreme cool and wet weather conditions in spring and early summer and by the high susceptibility of the predominantly cultivated cultivar (Gabler, 2004). A resistance screening method was developed for breeding of new cultivars with improved resistance (Gabler, 2006). *O. vulgare* subsp. *hirtum* was found as very susceptible host of *Puccinia menthae* Pers.in Bulgaria (Rodeva, personal communication).

A downy mildew disease of summer savory (*Satureja hortensis* L.) with a high damaging potential was observed in Germany for the first time in 2004. *Peronospora saturejae-hortensis* Osipyan was identified as causal agent with the help of molecular sequence analyses. The results demonstrated that the causal agent of this downy mildew was not *P. lamii* A. Braun as was often assumed (Gabler et al., 2012). The pathogen required for optimal development special weather conditions*:* leaf wet duration >6 h, air temperature <15 °C and relative air humidity >85 % at the same time. Such circumstances resulting in serious damages were given at the experimental site (Quedlinburg) only once (2009) within five years (2007-2011) (Gabler, 2013).

Conclusions

This paper included a non-exclusive list of new, emerging and re-emerging pathogens and was only an attempt to summarize some results obtained from an international research group bringing together the efforts of scientists from Bulgaria, Germany, Poland and Lithuania. The distribution and relative importance of the recorded pathogens differed significantly between years and localities depending on the specific geographical and environmental conditions. The pathogens considered as chronic-spreading and well known in one country could be new or emerging in another. Some changes in the pathogen populations affecting MAPs were observed in favor of the development of thermophilic pathogens (*Colletotrichum* spp., *Phomopsis* spp., *M. phaseolina*), which could be due to global climate warming. Because of selection pressure it is possible some newly emerged warm-temperature pathogens may become more important. Some other pathogens preferring cool and wet weather were more prevalent when such conditions occurred (*B. exigua*, *I. perplexans*, *P. saturejae-hortensis*). An integrated disease management should be applied to control the new, emerging and re-emerging diseases. There are not many chemicals registered for use on MAPs. Having in mind that MAPs are a source of natural therapeutic substances the use

of fungicides must be limited to a minimum number of treatments and dosage selecting highly effective, low-toxicity and low-residue products, which for organic cultivation are even prohibited. Monitoring of health and early detection of diseases in plants is critical issue. It is very important to focus on accurate diagnosis of new disease problems. Changes in disease occurrence impact disease management practices and also suggest a consideration of using resistant varieties to prevent these new diseases from becoming established on a large scale. To reduce the pathogen population a crop rotation is strongly advised avoiding usage of plant species susceptible to the same pathogens.

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CSL 1: Evaluation of parsley (*Petroselinum crispum*) focused to *Septoria petroselini* and *Plasmopara petroselini* causing *Septoria* blight and downy mildew

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Introduction

Parsley (*Petroselinum crispum* [Mill.] Nyman) is grown in temperate and subtropical climate worldwide and predominantly used as a pot herb. In Germany parsley is the most important spice plant, cultivated on more than 1,700 ha (Hoppe, 2006).

Evaluation of the species *P. crispum* for existence and availability of resistances to pathogens is of great interest. Widespread origins of tested accessions provide the opportunity for first-time characterization of existence, spreading and level of resistance / susceptibility to the economically important pathogens *Septoria petroselini* (Lib.) Desm. and *Plasmopara petroselini* Săvul. & O. Săvul. causing *Septoria* blight and downy mildew, respectively, throughout the species *P. crispum*.

Septoria blight (*S. petroselini*) is worldwide one of the most important pathogens for parsley. It is seed-borne (Tahvonen, 1978). Pycnidia are fixed permanently within the pericarp of the schizocarpic fruit, containing pycniospores for mass infection (Ferri, 1969).

In extensive tests for resistance to *S. petroselini* under climate chamber conditions with inoculation of fungus no accession was found to be without any symptom (Marthe and Scholze, 1996). Infection of resistant plants starts delayed and extent of lesions is considerably smaller.

Plasmopara petroselini was split from *Plasmopara nivea* by Săvulescu and Săvulescu (1951). Downy mildew on parsley was found several times in Germany at the end of 19th and the first half of 20th century (Brandenburger and Hagedorn, 2006). Damage caused by *P. petroselini* is new and of increasing significance. First reports came from Italy, *Plasmopara nivea*, seed-transmitted (D'Ercole, 1990), Germany, Pfalz, autumn 2000 (Leinhos et al., 2006), Belgium, d*uring the winter seasons of 2001 and 2002, Plasmopara petroselini (*Crepel and Inghelbrecht, 2003*), Sweden,* September 2004, Borgeby in southern Sweden (Amein et al., 2006), Germany, Quedlinburg at experimental field in 2005, and Turkey 2009 (Soylu et al., 2010). Together with *Septoria* blight they are currently the most relevant pathogens.

Results and discussion

A collection of 220 accessions of parsley (*Petroselinum crispum*) was evaluated at two experimental stations (Gatersleben and Quedlinburg, Germany) under natural infection. Widespread origins of tested accessions provide the opportunity for first-time characterization of existence, spreading and level of resistance / susceptibility to the economically important pathogens *Septoria petroselini* and *Plasmopara petroselini* throughout the species *P. crispum*. For each pathogen, accessions free or nearly free of symptoms were found: *S. petroselini*: free 1, nearly free 25, (Figure 1) *P. petroselini*: free 51, nearly free 22 (Figure 2). Nine accessions are free or nearly free of symptoms for both pathogens: PET16, PET36, PET169, PET172, PET177, PET178, PET192, PET212 and PET214.

To characterize level of resistance to *S. petroselini* for accessions free or nearly free of infection, additional climate chamber tests with inoculation are necessary.

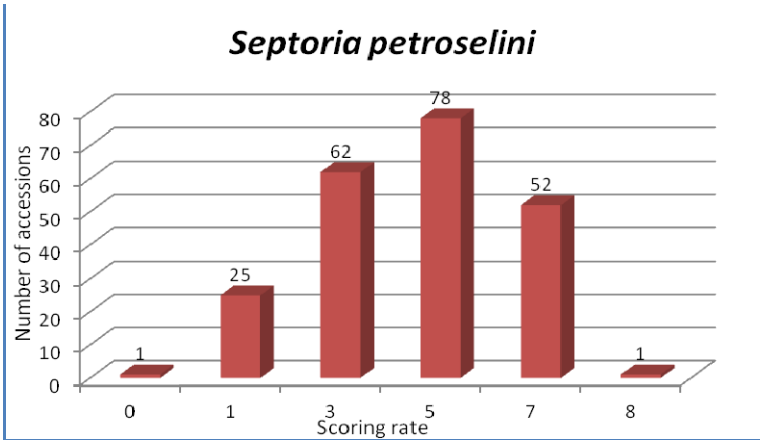

Fig. 1: Number of parsley accessions with scoring rates 0, 1: free or nearly free of symptoms; 3, 5: moderately susceptible; 7, 8: highly susceptible to natural infection by *Septoria petroselini*

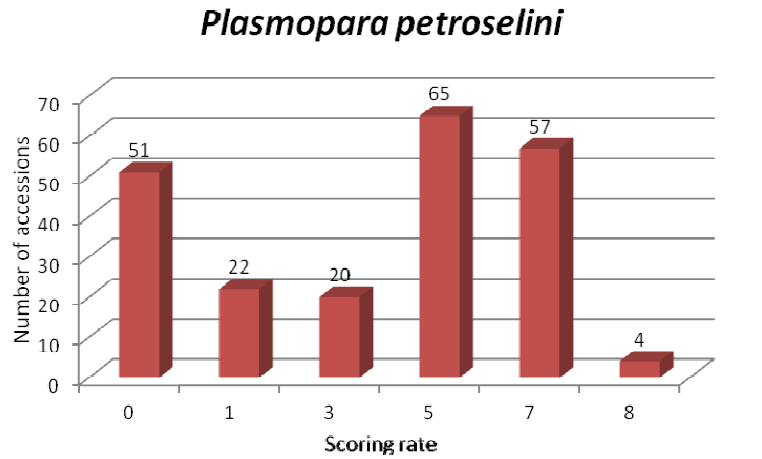

Fig. 2: Number of parsley accessions with scoring rates 0, 1: free or nearly free of symptoms; 3, 5: moderately susceptible; 7, 8: highly susceptible to natural infection by *Plasmopara petroselini*

For *P. petroselini* between both experimental places a good correlation was found of $r_s = 0.71$ for scoring results. From this, susceptibility is concluded for 146 out of 220 accessions with scoring rate of 3 and higher. The 73 accessions free or nearly free of symptoms of *P. petroselini* together with variety 'Felicia' tested free of symptoms out of nine varieties by Krauthausen and Leinhos (2007) are first candidates for intraspecific resistance in parsley. These candidates should be tested again for level of resistance. The accessions free of symptoms also open up for the first time the possibility to look for candidates for tests of race differences in *P. petroselini* because from many other downy mildew varieties a high number of different races are known.

The results from evaluation are essential for characterization of origin and domestication, but also for breeding cultivars with new resistances. The special importance of this evaluation comes from the combination of results for each accession. For practical use candidates with combinations of resistances can be detected and tested again (Marthe et al., 2013).

Keywords: resistance to phytopathogens, genetic resources, field conditions, natural infection

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CLS 2: Yield and quality affecting pathogens on Horseradish (*Armoracia rusticana*)

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Abstract

Horseradish (*Armoracia rusticana*) is cultivated in the major production area of Germany (Franconia) in a volume of about 1800 tons/year. A survey was conducted during 2012 to 2014 to assess the incidence and severity of diseases in horseradish fields of this area. Fungal leaf pathogens such as White Rust (*Albugo candida*) and various yield effecting leaf spot pathogens (*Alternaria* spp. *Colletotrichum* spp.) have been detected.

Quality losses are mainly due to the soil borne pathogen *Verticillium* spp., which causes discoloration of the vasculars. The problem of infections is that they can be evaluated only after harvest. They cause a critical influence on products as well as planting material. Fungicides are not effective and without authorization. For the identification of three of the main *Verticillium* species, a PCR assay was developed which is less time-consuming than the microscopic scoring of microsclerotia. A screening of affected fields revealed that the two most represented species are *V. dahliae* and V. *longisporum*, whereas *V. tricorpus* is of lower meaning. In a survey of storehouse diseases mycotoxigenic species such as *Penicillium* spp., *Aspergillus* spp. and *Fusarium* spp. have been detected additionally.

Furthermore, various aphids (e.g. *Mycus persicae*) are of evidence as vector of viral diseases such as Turnip mosaic virus (TuMV). Through meristem tip culture virus elimination has been carried out and propagation of virus-free plants were propagated by tissue culture in order to assess the effect on yield of TuMV-infections.

Moreover mycobiome analysis of horseradish were made. Beside the species *Verticillium* spp. also *Fusarium* spp. and a bacterial complex composed mainly of the three species *Stenotrophomonas* spp, *Burkholderia* spp. and *Serratia* spp. have been identified. Interestingly, in greenhouse experiments infections with the bacterial complex composed of these bacterias horseradish seem to be protected against fungal infections such as *Verticillium* spp. and *Fusarium* spp. Hence, using competitive, non pathogenic bacterial species are assumed to avoid fungal infections.

As registered varieties are not available in Germany, plants cultivated in Franconia originate of perennial plant propagation, which leads to a high risk of the accumulation of diseases. This points up breeding of horseradish cultivars with desirable characters such as resistance to plant pathogens would be necessary.

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CSL 3: First results of investigations into causes of diseases of cultivated chamomile (*Matricaria recutita* L.) in Germany

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Abstract

Diseases on cultivated chamomile have occurred in Germany since 2007, which have severely been affecting the crop yields. The causes of damage are very complex and have not been identified yet. Additionally to the damage in the stems caused by larvae, fungal pathogens are of relevance. Tests of the Julius Kühn-Institute first revealed that a new, not yet identified fungus is pathogenic to chamomile. Symptoms observed in infection tests like chlorosis, browning and black coloration of stems and leaflets were identical to those in the field. The fungus sporulated on diseased plant parts under the conditions of climatic chamber (20 °C to 22 °C and 12 hours of light, 122 µmol) from 17 days after inoculation (dai) and could be reisolated on agar plates. The identification, biology and epidemiology of the fungus as well as the specific harmful effect and interaction with other harmful factors, especially animal pests, are being studied presently in a project funded by the Agency for Renewable Resources (Fachagentur Nachwachsende Rohstoffe, FNR). The goal is to develop sustainable plant protection concepts based on the knowledge about the pathogens to enable a stable cultivation of chamomile in Germany.

Keywords: chamomile, *Matricaria recutita*, diseases, pathogen, pathogenicity

Introduction

Chamomile (*Matricaria recutita* L.) is one of the economically most important medicinal plants in Germany. The dried blossoms of chamomile are a plant-based source material for a great number of herbal medicines, cosmetics, and tea products for the food sector. Chamomile blossoms (*Matricaria flos*) are produced on a total area of 1.150 hectares in Germany, of which 1.030 hectares are located in Thuringia; whereas the rest of it is found in Hesse and Saxony (Plescher and Schmitz, 2012). 2007 first experienced the occurrence of disease symptoms in chamomile cultures in Thuringia, which deteriorated in the following years and have since been accompanied by a severe loss in yield. "New" fungal pathogens and not yet identified pests occur in addition to those which have been known. First investigations of the Julius Kühn-Institute and Pharmaplant GmbH have shown that the causes of the damage are very complex (Gärber et al., 2013). On the one hand, a species of fungus of the genus *Septoria* was found, on the other hand a fungus morphologically similar to *Entylomella trailii* was detected, however belonging to ascomycetes according to DNA-sequences. For the latter, it is still unclear whether it is two different fungi. Furthermore, damage caused by larvae was detected in the stems of chamomile, which is still to be identified. According to preliminary studies about the pathogenicity, the importance of certain harmful organisms for the disease can not be estimated yet. Since the beginning of 2016, the causes of the damage have been intensively studied in a project funded by the Agency for Renewable Resources (Fachagentur Nachwachsende Rohstoffe FNR) aiming to find out the significance of single harmful organisms for the disease process. Starting from the results of diagnosis, biology and epidemiology of the pathogens, first approaches for plant protection should be developed to enable a stable cultivation of chamomile in Germany with high yields and consistently high product quality. This paper will present both the general phytopathological problems in cultivated chamomile and first investigations into the pathogenicity of a not yet identified fungus.

Materials and Methods

Diseased plant samples from several fields in Thuringia were tested for harmful organism at the JKI in 2009 to 2015 resulting in isolation of several strains of a single, yet unknown fungus which is assumed to be a potential pathogen. The fungus belongs to the genus cf. *Rhexocercosporidium* according to the DNA sequence (Gärber et al., 2013). Aiming to find out the pathogenicity of this fungus, tests were performed in climatic chambers at temperatures of 20 °C to 22 °C and twelve hours of light (122 µmol). Seven-week-old chamomile plants were inoculated by spraying a conidial suspension (10^4 conidia/ml suspension, run off) and placed in growing chambers closed with a lid for three days to retain a high humidity. The fungus was incubated on chamomile-agar and MYP (Malt Yeast Peptone)-agar for 14 days at 20 °C and 14 hours of light (47 µmol) to produce inocula. The conidial slime was scrapped off the agar surface with a slide and transferred into destilled water. The density of conidia in the suspension was counted in a Thoma counting chamber.

Results

First symptoms like chlorosis as well as black coloration on stems and leaflets were visible from 17 days after inoculation (figures 1a and 1b).

Fig. 1a und 1b: Diesease symptoms on chamomile after inoculation with a conidial suspension (104 conidia/ ml suspension) of not yet identified fungus (17 dai)

Some days later, the symptoms occurred more severely on all inoculated plants. Single shoots died. The symptoms were identical to those in the field. The fungus could be first detected microscopically on several plants 20 days after inoculation (figure 2).

Fig. 2: Sporulating fungus on chamomile

The fungus could be reisolated, complying with Koch's postulates on the pathogenity of the fungus. As the disease progressed, plants browned more and more. At that time, first disease symptoms were found in the control variant of non-inoculated plants, which might indicate the spread of the fungus by air movement in the climatic chamber. The fungus could be detected microscopically on control plants four week later than on inoculated plants. The significance of the fungus in the disease process, its specific harmful effect and interaction with other harmful factors, especially animal pests, are to be identified in further studies. Fundamental investigations shall cover the study of the biology and epidemiology of the yet unknown fungus, including its identification. The knowledge of the phytopathogens should be used in the breeding process in a target-oriented way in order to improve the resistance of varieties. The aim is to develop sustainable plant protection concepts to ensure a stable cultivation of chamomile in Germany using the knowledge about the pathogens and first control approaches gained in the project.

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Session D: Improvement of organisms for bioreactors and photo bioreactors

DPL 1: Novel plant cell systems, vis-à-vis cultivation methodologies for the production of valuable phytochemicals

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Abstract

The bioreactor technologies are gaining importance as means of production of metabolites, food, pharmaceuticals, enzymes, bio-molecules and specialty chemicals. Though these technologies have been extensively adopted, the use of genetically modified cells/organisms and the problems related to their exploitation due to environmental concerns have laid emphasis on the contained-cultivation methods. Furthermore, utilization of solar energy and renewable energy systems as drivers of photo bioreactor systems is emerging as novel green technology. In order to enhance the efficiencies of the above mentioned systems, there is need for a two- pronged approach: firstly the engineering the organisms commensurate with the scale up technologies in bioreactors, and also the innovations of the scale up technologies, themselves.

With respect to the high value metabolites from medicinal plant systems, the aspects of pathway engineering to produce high quantities of the active principles is gaining attention. The production of desired metabolites using cell cultures, hairy root cultures, and genetically modified (GM) cells are of importance in controlled processes. To achieve this, they need to be integrated with bioreactors, immobilized cell culture systems and novel organisms (including cultivated transgenic plants as bioreactors), coupled to downstream processing.

The author's group at the Central Food Technological Research Institute at Mysore and presently at DSI, Bengaluru, has been engaged in the development of cell culture mediated production of secondary metabolites, design of novel bioreactor, immobilized cell cultures, and algal production in outdoor bioreactors and design of cells / organism through genetic engineering. The studies on cell culture / immobilized cell culture / hairy root culture mediated production of capsaicin, anthocyanin, betalains, *Withania* alkaloids , coupling them with elicitor mediated technologies for the enhancement of production capability; large scale production of metabolites of importance through algal technologies; pathway engineering of metabolites such as capsaicin, astaxanthin, gamma linolenic acid (GLA) were pursued.

We attempted novel methods of enhancing the metabolites by 2-3 folds, using microbial elicitors. The aqueous extracts from organisms viz., *Aspergillus niger*, *Rhizopus oligosporus*, were very effective in eliciting capsaicin in immobilized cell cultures of *Capsicum*. Similar results were obtained in the elicitation of several other secondary metabolites such as anthocyanin in the in vitro tissues of *Daucus carota* or annatto dye in *Bixa orellana*.

The elicitor technology has now been adopted in several industrial processes to enhance the metabolites of importance such as Shikonin in *Lithospermum erythrorhizon* and many more. The release of the metabolites to the exterior through permeabilization using agents appropriate to the system will be an added advantage for using the cell cultures in bioreactors in a continuous mode. In case of capsaicin, the molecule is naturally effluxed from cultured immobilized cells. These unit operations coupled to downstream processing technologies have been responsible for the enhancement of efficiency of the production process for the target compound(s).

Development of hairy root cultures of *Beta vulgaris* using *Agrobacterium rhizogenes*, as an alternative to the cell culture mediated process, resulted in enhanced productivity of betalains. Our studies have shown the advantage of using this system in terms of the enhanced production through nutritional stress, elicitors and also use of permeating agent for the release of betalains to the exterior in a continuous production mode.

The pathway engineering of carotenoids, especially astaxanthin, in algal systems was done in our laboratory in order to clone the genes for Betacarotene ketolase (BKT) and Betacarotene hydroxylase. The BKT cloning from the green algal form *Haematococcus pluvialis* was successfully done and introduced into *Dunaliella* sps. This opened up the possibility of production of astaxanthin in *Dunaliella* which otherwise is a known producer of Beta carotene only. Such examples will be of relevance in producing the genetically modified strains in bioreactors for high value compounds of importance as exemplified by our studies on pathway engineering of astaxanthin. Similarly we have successfully demonstrated cloning of cloning of gene for Delta-6 desaturase from the cyanobacterium *Spirulina* and transformed Soybean plants to produce a vegetable oil enriched with GLA.

Capsaicinoids are the pungency causing alkaloids synthesized in placental tissues of *Capsicum* fruits. Capsinoids are the non-pungent analogues also found to be synthesized in placental tissues of *Capsicum* fruits. Capsaicinoids, and Capsinoids are unique to *Capsicum* sp. pungency is regulated by involvement of either of two genes viz Pun1 (Acyl-transferase involved in condensation of C9-C11 Fatty acids with vanillylamine) & pAMT (Aminotransferase involved in vanillylamine synthesis). We have purified and characterized the pAMT protein and are now exploring the synthesis of various pAMT catalyzed pharmaceutically important compounds. For the first time we have functionally validated the involvement of pAMT in regulation of vanillylamine through Agrobacterium mediated genetic transformation experiments. We were able to show the synthesis of Vanillylamine in *Nicotiana* sp. using a binary vector sense construct, thereby proving the function of pAMT in alternate species. pAMT mutant species like CH-19 sweet pepper (*Capsicum* sps) produce vanillyl alcohol instead of vanillylamine. Capsinoids are fatty acid esters (C9-C11 fatty acids linked with vanillyl alcohol) in pAMT mutant plants.

It has now been possible to use this information to overproduce capsinoids. Capsinoids are recently explored compounds with a promising potential of anti-obesity properties. Capsinoids also exhibit chemopreventive and anticancer properties. They lack nociceptive responses and are most promising among vanilloid receptor agonists, hence safer for therapeutic usage.

The developments mentioned above will be presented to provide an overview of our attempts to produce novel compounds of importance through engineered cell systems to produce desired metabolites through scale up processes.

DSL 1: Cyanobacterial production of indole-3-acetic acid for use in agriculture

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Cyanobacteria or blue-green algae belong to the most primitive forms of life, yet they are of tremendous importance in many aquatic and terrestrial ecosystems. Besides marine and freshwater environments, cyanobacteria are common in the rhizosphere as well as epiphytes and symbionts.

Studies on the effects of cyanobacterial extracts and culture filtrates on the growth of different plants indicated the presence of phytohormones or phytohormon-like substances in some cyanobacteria strains. Finally, definite proof of auxins produced by cyanobacteria was provided by modern chromatographic methods combined with mass spectrometry.

The GMBU e.V. examined in cooperation with the Dr. Junghanns GmbH the potential of a free living, freshwater cyanobacterium to produce the phytohormone indole-3-acetic acid (IAA). To achieve this, crucial culture parameters influencing the IAA production were investigated. Based on these experiments a suitable cultivation process was developed in order to produce cyanobacterial biomass with a high content of IAA. Finally, the auxin-rich biomass was used to produce a plant growth stimulating formulation especially for applications in organic farming.

Session E: Ex situ and in situ genetic recourses – protection and use by colleting practice – cultivation of new species

EPL 1: Domestication and sustainable production of wild crafted plants with special reference to the Chilean Maqui berry (*Aristotelia chilensis*)

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Abstract

The principle threats for sustainable production of wild collected medicinal plants are related to ecological factors, such as endemism, and botanical factors critical for survival, such as the collection of roots or barks or slow growing species. The sustainable way to produce raw material on a large scale would be species specific management of the wild resources that guarantees conservation of biodiversity, or bringing the species under cultivation. A checklist proposed by WHO, UICN and WWF (1993) indicates that domestication of any medicinal plant concerns plant selection and breeding, studies about propagation, cultivation techniques, plant protection, time of harvest, among others. The different domestication steps are illustrated for the Chilean maqui (*Aristotelia chilensis*), a wild tree whose fruits are demanded in increasing volumes by the international market because of its high antioxidant capacity. High yielding plants with good fruit quality have been selected from wild populations and accessions have been cultivated under different environmental conditions to select the most suitable genotypes for the establishment of commercial orchards.

Keywords: Maqui, selection, vigor, fruit load

Introduction

Maqui (*Aristotelia chilensis* Mol., Elaeocarpaceae) is a dioecious, wintergreen tree or shrub native to Chile and the Patagonian forests of West Argentina (Damascos and Prado, 2001; Rodríguez, 2005). It is one of the sacred plants for the indigenous Mapuche people, a symbol of goodwill and peaceful intention (de Mösbach, 1992). The Spanish conquerers already described maqui as a food and medicinal plant to treat diarrhea, sore throat, intestial tumors, fiever or wounds (Muñoz et al., 1981; Montes et al., 1987; Hoffmann et al., 1992; Silva et al., 1995). Recent studies showed antioxidant capacity, anti-inflammatory, antidiabetic, antimicrobial, and cardio protective effects together with gastro protective activities (Miranda-Rottmann et al., 2002; Araya et al., 2006; Céspedes et al., 2008; Avello et al., 2009; Céspedes et al., 2010a and b; Mølgaard et al., 2011; Rojo et al., 2012; Fuentes et al., 2013). The large health benefits triggered in recent years an increasing demand for its fruit, all coming from the wild collection.

According to Vogel et al. (2014) cutting fruit-bearing branches from wild trees or shrubs to retrieve maqui berries is a practice that removes the reproductive buds that are already induced, and so threatens the fruit production for the next year. Also, an overexploitation of the wild resources would promote the genetic erosion by preferring the most productive individuals for wild crafting. To cover the future demand of processing industry for maqui berries as a raw material, we propose the domestication of this species.

Materials and Methods

Screening of wild populations

Nine wild populations of *A. chilensis* with more than 30 females each, distributed in Chile between latitudes 34 and 41° S, were studied in 2007. Fruit samples were taken from different trees and evaluated for the following characteristics: anthocyanin concentrations determined by pH-differential spectrophotometry, fruit weight, portion of fleshy parts and number of seeds per fruit. In each population plants with highest anthocyanin concentrations in its fruit were identified and vegetatively propagated by rooting cuttings.

Evaluation of pre-selected clones

In 2009, 68 so selected female clones were randomly distributed in a plot in the Experimental Station of Universidad de Talca (35°S). Three years later (2012), 45 of them, with early fruit production, including individuals of all provenances, were established randomly with 3-5 replicates in an experimental design at five different sites, distributed over 700 km in farms located between Curicó and Río Negro (Chile). In the northernmost locations first fruit production could be evaluated already during the second year, whereas the earliest clones cultivated in the southern locations started to produce fruit in the third year. Anthocyanin and polyphenol concentrations together with fruit size and yield per plant were determined.

Results

Results obtained during the 2nd and 3rd growing season indicate that the selections 'Luna Nueva' and 'Morena' start fruit production one year earlier in Central Chile than in South Chile. 'Luna Nueva' is a small, compact bush with early and very high fruit load. Its berries have high polyphenol content. They are firmly adhered and do not fall when ripening. 'Morena' is a relatively vigorous plant with a high number of berries per inflorescence and a large fruit producing zone on the branches. Fruit removes easily. The selection 'Perla Negra' is a compact bush, little tolerant to draught, but well adapted to the climate of southern Chile, where plants start their abundant production during the 3rd growing season. Its fruit is big and has high polyphenol and anthocyanin concentration.

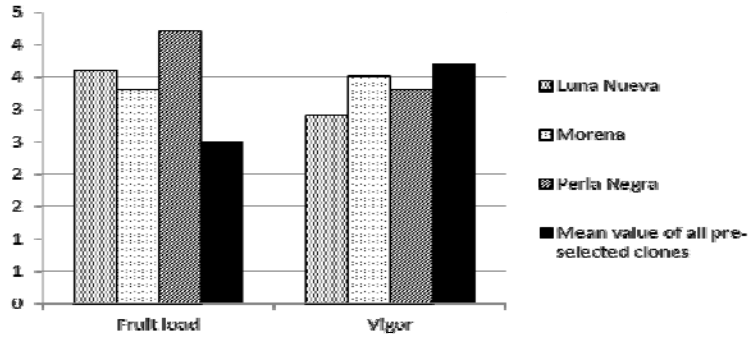

Fig. 1 Fruit load and vigor of three selected clones: 'Luna Nueva', 'Morena', and 'Perla Negra'. Scale ranging from 1 to 5 with 1 = very low and 5 = abundant fruit load / very vigorous growth

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ESL 1: Intraspecific taxonomy of plant genetic resources – Important for differentiation of medicinal and aromatic plants?

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Abstract

Taxonomy of plant genetic resources is an important input in characterising and evaluating cultivated plants and essential for identification and documentation of the diversity of genebank collections. In former times taxonomical determination was based only on morphological characters. Nowadays, new molecular and chemical methods and techniques are available for providing additional information. As examples, investigations of parsley (*Petroselinum crispum* [Mill.] Nyman, Apiaceae) and opium poppy (*Papaver somniferum* L., Papaveraceae) collections of the German genebank are demonstrated. In addition to morphological description, the molecular distance and the phylogenetic relationship of the accessions were performed with molecular marker analysis. Essential oil compound and content for parsley and the content of the five main alkaloids (morphine, codeine, thebaine, noscapine, papaverine) for opium poppy were measured with GC (gas chromatography) and HPLC (high pressure liquid chromatography), respectively. For parsley the results of the three methods support the existing taxonomy partly, a separation of root and leaf parsley was confirmed. However, the taxonomy of opium poppy should be revised because molecular and chemical data do not verify the morphological results. But nevertheless taxonomy of cultivated plants is an important tool to describe the variability of plant genetic resources.

Keywords: Parsley, plant genetic resources, opium poppy, taxonomy

Introduction

Taxonomy of plant genetic resources is an important input in characterising and evaluating cultivated plants. Especially, for large genebank collections it is necessary to know inter- and intraspecific taxonomy to describe the genebank's material. The German *ex situ* genebank is one of the ten largest genebanks worldwide. Nearly 150,000 accessions out of more than 3,000 species and 780 genera are maintained and reproduced at the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben (Börner, 2006). For such a large collection taxonomy is essential for identification and documentation the wide range of diversity in the assortment. It is a great source to describe the often enormous variability by various methods and techniques (Hanelt, 1988). In former times taxonomical determination was based only on morphological characters. Nowadays, new molecular and chemical methods and techniques are available for providing additional information. The aim of this work was to study two examples, parsley and opium poppy, with the intention of a clear intraspecific taxonomy with the help of molecular markers and chemical compounds. For both species complex morphological descriptions and intraspecific taxonomy containing subspecies, convarieties, botanical varieties and forms are available (Danert, 1958; 1959; Hammer, 1981; Hanelt & Hammer, 1987). But the question is if these new methods support or even improve the existing intraspecific taxonomy or if a revision is necessary.

Material and methods

Two crops with a known high intraspecific variability, parsley and opium poppy, were selected. The parsley collection contains 220 accessions including both morphological types, leaf parsley and root parsley, and on the other hand with modern and old cultivars as well as landraces. For the standardisation of the morphological characterisation a descriptor was applied with 15 morphological (growth type, leaf type, root type, etc.) traits (Lohwasser, 2009). For the molecular studies 88 RAPD- (Random Amplified Polymorphic DNA), 53 SRAP- (Sequence-Related Amplified Polymor-

phism) and 65 AFLP- (Amplified Fragment Length Polymorphism) markers were used. From the polymorphic bands of these 206 markers a binary matrix was compiled and a tree structure based on Nei & Li distances developed using the programme PAUP*4.0b10 (SWOFFORD, 2002). Essential oil contents were measured and the compositions of the oil were analysed by gas chromatography (LOHWASSER et al., 2010).

From the large opium poppy collection of the genebank 300 accessions were selected. Again modern cultivars, old cultivars and landraces were chosen and described morphologically based on a descriptor (DITTBRENNER et al., 2008). The AFLP fingerprint technique was used to produce a binary matrix out of 300 polymorphic markers from which a neighbor joining tree based on Nei & Li distances was generated (DITTBRENNER, 2009; DITTBRENNER et al., 2008) with the programme PAUP. *Papaver glaucum* Boiss. & Hausskn. was used for a clear separation within the opium poppy. For the phytochemical studies the content of the five main alkaloids morphine, codeine, thebaine, noscapine, and papaverine was measured with HPLC (high pressure liquid chromatography) based on a method described by DITTBRENNER (2009) and DITTBRENNER et al. (2009).

Results

As examples of the use of morphological, molecular and phytochemical data in order to verify existing classifications, investigations of parsley and opium poppy collections of the German genebank are demonstrated.

For parsley the morphological description has resulted in curled leaf, smooth leaf and root parsleys. These types can be separated quite well into two convarieties one for leaf parsleys and one for root parsleys. Accessions with a remarkable long petiole (Italian parsley) as discriminated by DANERT (1959) could not be identified definitely. The molecular studies show also two clusters, one for the leaf parsleys and a second one for the root parsleys together with some leaf parsleys (DECLERCQ, 2009). The morphological and molecular data fit very well with the targeted analysis of the essential oil content and compounds. High concentration of two monoterpenes, myrcene and β-phellandrene, can be correlated with root parsley and leaf parsley, respectively. For the volatile compounds two groups could be defined, one for all leaf parsleys without any difference of the leaf type and one for the cluster with the root parsleys (DECLERCQ, 2009). But a clear separation of the varieties and forms was possible neither with morphological traits nor with molecular or phytochemical data.

The intraspecific taxonomy of the opium poppy is based on a few morphological characters like setose buds, capsule dehiscence, shape of the stigmatic lobes and colour of flower and seeds. However, the classification is difficult because of different characters on one plant or due to the presence of variation within the accession. To summarize the results of the analysis of the morphological data, only a clear separation of the subspecies *setigerum* (DC.) Corb. by bud hairiness is possible. Both other subspecies and all varieties could not be determined definitely. The molecular analysis shows also only a clear separation of the subsp. *setigerum* but no further intraspecific structure within the opium poppies (fig. 1) which supports the morphological analysis. In addition, the analyses of the five main alkaloids present different compounds and contents of the accessions which do not fit with morphological and/or molecular results (DITTBRENNER et al., 2009). In conclusion, there is no clear intraspecific taxonomy of opium poppy in the range of the convarieties and varieties available neither by morphological characters nor by molecular or phytochemical data (LOHWASSER et al., 2010).

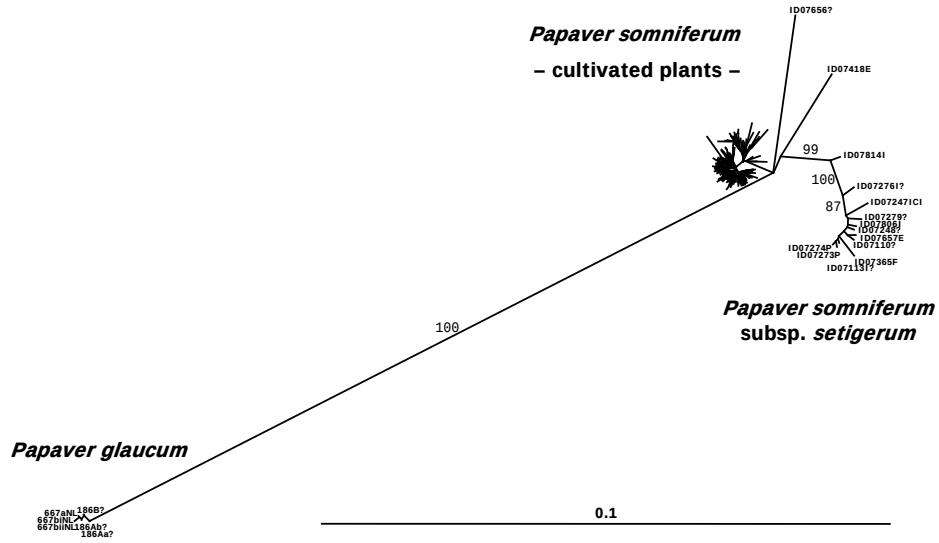

Figure 1: Neighbor joining tree based on Nei & Li distances from AFLP analysis

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ESL 2: Genetic resources of *Thymus vulgaris* L. and *T. vulgaris* x *T. Marschallianus* Willd. in the Czech Republic

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Abstract

Two varieties of *Thymus vulgaris* L. (´Krajový´ and ´Winter´) and three its hybrids with *T. Marschallianus* Will. (variety ´Mixta´ and two accessions of variety ´Lemona´) were evaluated according the Draft Descriptor List *Thymus vulgaris* L. and analysed for the essential oil content and composition in years 2014 and 2015. All evaluated accessions were found morphologically and/or chemically different. Varieties ´Krajový´, ´Winter´ and ´Mixta´ were assessed as a thymol type with the thymol (39.1 – 69.6 %), o-cymene (6.3 – 24.9 %) and γ-terpinene (2.75 – 13.8 %) as main oil components. The two accessions of ´Lemona´ variety were found significantly different each other: one of them (income No. 3239) was assessed as a terpineol type with the terpineol acetate (75.7 %) and α-terpineol (16.9 %) as main oil components and the other one (income No. 2757) as a geraniol type with the geranyl acetate (±42.4 %) and geraniol (±20.8 %) as main oil components. Only the accession with income No. 3239 was proved as a ´Lemona´ variety due its citral (±9.3 %) content, though even this content is too small compare to original ´Lemona´ where about 20 % of citral was declared.

Keywords: Thyme, descriptor list, essential oil, thymol, citral, terpineol acetate

Introduction

A wide breeding program for thyme was begun to work in the Czech Republic in the period 1951 – 1979. An old Czech origin variety of *Thymus vulgaris* L. ´Krajový´, which was made up as positive selection of wild populations and accepted as a variety in 1952, was a basic parental material. A new variety ´Aroma´ was made from ´Krajový´ by selection methods and then propagated vegetativelly to fixed high amount of essential oil (up to 2 %) and thymol content (about 60 %). ´Aroma´ was accepted as a variety in 1966 and then it became a new parental material for next breeding. It was (as well as the ´Krajový´ variety) fertile so the hybridization by plants from wild population of *Thymus marschallianus* Willd. was tested and focused on increasing of plant mass yield and possible mechanized harvesting. The two new varieties have been developed by this hybridization: ´Lemona´ (accepted in 1975) and ´Mixta´ (accepted in 1979). ´Lemona´ was characterized by untypical lemon aroma which comes from citral (about 20 % of essential oil) content. ´Mixta´ was chemically comparable to ´Aroma´ variety, but 3-4 days earlier, with high plant material yield and habitus suitable for mechanized harvesting. Both hybrid varieties are sterile, flowers have not stamens.

All thyme breeding program was carried out at Research and Breeding Institute of Vegetable growing in Olomouc but after 1994 when it was cancelled a lot of original plant material has got unfortunately lost or damaged. Later on, some materials were repatriated from other institutions, Czech as well as foreign, but it was not a case of ´Aroma´ variety.

Czech collection of genetic resources of medicinal and aromatic plants currently includes 3 accessions of *Thymus vulgaris* L. (varieties ´Krajový´, ´Winter´ and ´French Summer´) and 2 hybrid accessions *T. vulgaris* x *T. Marschallianus* Will. (´Lemona´ and ´Mixta´). Four of these materials were eval-

uated according the Draft Descriptor List *Thymus vulgaris* L. (ECP/GR, 2011) and analysed for the essential oil content and composition in years 2014 and 2015.

Materials and Methods

A 5 years old, well-developed overgrowths of five *Thymus vulgaris* and *T.vulgaris x T. Marschallianus accessions* (Tab. 1) were used for evaluation and comparison. The evaluation of morphological descriptors was made according the Draft Descriptor List *Thymus vulgaris* L. (ECP/GR, 2011). Dry thyme stems in full bloom were submitted to hydrodistillation with a Clevenger-type apparatus. The EO was co-distilled with 500 ml of distilled water for 4 h and collected and stored in glass vials in dark at 4 °C until the GC analysis. Hydrodistillation was performed one to four times for each sample and the mean values of the extraction yields are reported. GC-MS analyses were carried out on an Agilent 7890A (Agilent technologies) gas chromatograph equipped with an Agilent 5975C MS detector (Agilent technologies) and fitted with an HP-5MS capillary column (30 m, 0.25-mm ID, 0.25-µm film thickness, Hewlett-Packard). The injection port temperature was 200 °C and MS detector temperature was 150 °C. The column temperature ranged from 60 °C (15 min) to 180 °C at a rate of 3 °C min^{-1}. The samples were diluted 1:50 (hexane) and the injection volume of each sample was 1 µl. The samples were injected using a split automatic injector (split ratio 1:100) and with helium as a carrier gas at a flow rate of 1.0 $ml.min^{-1}$. The measurement of the peak areas was performed with an HP E.02.02 ChemStation.

Results

All evaluated accessions were found morphologically and/or chemically different. Varieties ´Krajový´, ´Winter´ and ´Mixta´were based on CG analysis of essential oil assessed as a thymol type with the thymol (39.1 – 69.6 %), o-cymene (6.3 – 24.9 %) and γ-terpinene (2.75 – 13.8 %) as main oil components. The two accessions of ´Lemona´ variety were found significantly different each other: one of them (income No. 3239) was assessed as a terpineol type with the terpineol acetate (75.7 %) and α-terpineol (16.9 %) as main oil components and the other one (income No. 2757) as a geraniol type with the geranyl acetate (±42.4 %) and geraniol (±20.8 %) as main oil components. Only the accession with income No. 3239 was proved as a ´Lemona´ variety due its citral (±9.3 %) content, though even this content is too small compare to original ´Lemona´ where about 20 % of citral was declared.

The essential oil profile of varieties ´Krajový´ and ´Winter´ (probably ´Deutscher Winter´) respond to result of Mewes et al. (2008) and both of these varieties as well as variety ´Mixta´ corresponds to the chemotypical requirement of the Pharmacopoea Bohemica MMIX (based on Pharmacopoeia Europaea) for Thymi herba where minimally 1.2 % of essential oil content and 40.0 % of thymol and carvacrol in the essential oil is required.

Morphological differences between the varieties with identical chemotype is possible to find for example in Plant height (´Krajový´ 292 mm (average of both evaluated years), ´Winter´ 204 mm, ´Mixta´ 309 mm), Flower length (´Krajový´ 7.3 mm, ´Winter´ 4.5 mm, ´Mixta´ 4.8 mm) and Male sterility (´Krajový´ absent, ´Winter´ and ´Mixta´ present).

Tab. 1 Plant material and the description of its origin

ECN / Income No.	Taxon	Origin, the year of inclusion in collection	Variety	Male sterility
A8900004 / 3420	T. vulgaris	2003, Seva-Flora Valtice, CZE	Krajový	no
A8900011 / 2194	T. vulgaris	1987, Royal Sluis, NDL	Winter	yes
A8900003 / 3238	T. vulgaris x T. Marschallianus	2001, UJEP Brno, Kraví hora, CZE	Mixta	yes
A8900002 / 2757	T. vulgaris x T. Marschallianus	1980, VŠÚZ Olomouc, CZE	Lemona	yes
A8900002 / 3239	T. vulgaris x T. Marschallianus	2001, UJEP Brno, Kraví hora, CZE	Lemona	yes

Tab. 2 Essential oil content and quality – the content of major compounds

Income No.		3420		2194		3238		2757	3239	
Year of analysis		2014	2015	2014	2015	2014	2015	2015	2014	2015
Number of analysis		1	4	1	3	1	2	4	1	2
Essential oil content (%)	Av.	0.84	1.80	0.80	1.74	0.80	2.70	3.41	1.36	2.33
	SD	-	0.12	-	0.36	-	0.43	0.48	-	0.11
o-Cymene (%)	Av.	**24.86**	**11.71**	**11.39**	**14.16**	6.26	**10.96**	0.23	-	0.15
	SD	-	0.82	-	1.42	-	0.68	0.05	-	0.02
γ-Terpinene (%)	Av.	4.61	**13.70**	5.85	**13.84**	2.75	7.58	0.77	-	0.20
	SD	-	0.43	-	0.43	-	0.42	0.20	-	0.09
α-Terpineol (%)	Av.	tr.	-	tr.	-	tr.	tr.	**16.93**	tr.	tr.
	SD	-	-	-	-	-	-	0.90	-	-
Geraniol (%)	Av.	-	-	-	-	-	-	-	**18.27**	**22.07**
	SD	-	-	-	-	-	-	-	-	1.60
Citral (%)	Av.	-	-	-	-	0.18	-	-	7.92	9.96
	SD	-	-	-	-	-	-	-	-	0.67
Thymol (%)	Av.	**52.13**	**61.92**	**58.70**	**55.95**	**69.60**	**39.13**	-	0.27	0.57
	SD	-	0.68	-	1.10	-	1.15	-	-	0.10
Terpineol acetate (%)	Av.	-	-	-	-	-	-	**75.74**	-	-
	SD	-	-	-	-	-	-	0.92	-	-
Geranyl acetate (%)	Av.	-	-	-	-	-	-	-	**43.42**	**41.95**
	SD	-	-	-	-	-	-	-	-	0.56

tr. = traces; the compounds exceeding 10 % are bold marked

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ESL 3: The pharmacological assay as a tool to medicinal plants domestication

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Abstract

In Brazil studies with native medicinal plants are usually performed using non-domesticated plants and as a result the genetic variability of wild species could express different levels of active principles changing their therapeutic effect. Based on that, the aim of this study was to demonstrate that extract of different half- sib families *Cordia verbenacea* (DC), widely used as medicinal plant in Brazil, have different efficacy in the Total Growth Inhibition (TGI) of 5 different human tumor cell lines. Data were statistically analyzed using ANOVA follow by Tuckey test and a heritability estimation of the plant families was performed. The results showed that TGI are different for each plant family according with each human tumor cell line. For instance, extracts obtained from families 3,11 and 12 were more effective to inhibit the U-251 and Ht-29 cell lines compared to the other families, while extracts obtained from the family 32 was more effective against thethe PC-3 line. The heritability coefficient indicated that plant population selection could promote a genetic improvement related to its active principle and their pharmacological effect and could provide the identification of the best families according to their pharmacological efficacy. In conclusion, this study suggests that the domestication of a wild medicinal plant should be better monitored by its pharmacological effect.

Keywords: medicinal plants, pharmacological effects, domestication

Introduction

In Brazil studies with native medicinal plants are usually performed using non-domesticated plants. However, the genetic differences among the wild population make difficult a better interpretation of chemical assays. Typical genetic variability frequently occurs in wild species that, as a result, will express different levels of active principles changing the efficacy of their therapeutic effect. Indeed the molecules with pharmaceutical interest are resulted from the secondary metabolism of plants that, in turn depends on genetic and ambient factors (Khanna & Shukla, 1991; Franz, 1996; Pank, 2006). Once the intrinsic value of a medicinal plant is its therapeutic effect, concerning its active principle, pharmacological assays could be used to determine the phenotype of an individual or a studied population. *Cordia verbenacea* DC. Boraginaceae is a medicinal plant widely used in Brazil. Their effects are popularly described as anti-inflammatory, anti-arthritic, analgesic and antiulcerogenic (Lorenzi and Matos, 2008). Based on that, the aim of this study was to demonstrate that extract of different families of the *Cordia verbenacea* have different efficacy in the Total Growth Inhibition (TGI) of 5 different human tumor cell lines.

Materials and Methods

Plants extracts samples are obtained from a field experiment involving 12 half-sib families of *Cordia verbenacea*. The experimental design was a complete randomized blocks with four replications. Each parcel contained 5 plants cultivated in a 100 x 200 cm distance. After 1 year, leaves were harvested, separated from their branches and dried in an oven at 40 °C. The dried leaves are grinded to prepare a dry extract. The extract was prepared with ethanol (96º GL) in the proportion of 1:5 (dried plant: solvent) with mechanical agitation for 1 ½ h (3 x) at room temperature. The extracts were then pooled, filtered and evaporated to dryness.

Human tumor cell lines used: U251 (glioma), MCF-7 (breast), NCI-H460 (lung, non-small cells), HT-29 (colon), PC-3 (prostate) were kindly provided by National Cancer Institute (Frederick, MA, USA).

Cell culture: stock cultures were grown in medium RPMI 1640 (GIBCO) supplemented with 5 % fetal bovine serum (FBS, GIBCO) and 10 U/mL penicillin, 10 µg/mL streptomycin at 37 °C in 5 % CO_2.Antiproliferative assay: cells in 96-well plates (100 µL cells/well) were exposed to SDE (0.25, 2.5, 25 and 250 µg/mL in DMSO/RPMI) at 37 °C, 5 % of CO_2 in air for 48 h. Doxorubicin (DOXO) was used as standard (0.025, 0.25, 2.5 and 25 µg/mL). Final dimethyl sulfoxide (DMSO) concentration did not affect cell viability (0.25 %). Before (T0 plate) and after sample addition (T1 plates), cells were fixed with 50 % trichloroacetic acid and cell growth determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B (SRB) assay (MONKS et al., 1991; SHOEMAKER, 2006). The TGI (concentration that produces total growth inhibition) was determined through non-linear regression analysis using the concentration-response curve for each cell line in the software ORIGIN 8.0® (OriginLab Corporation).

One-way ANOVA was performed to determine if the Total Growth Inhibition (TGI) were significant different ($p<0.05$) among treated groups. If so, the post hoc contrasts using the Tukey test were performed to determine the basis of the significant difference.

Results

As showed in table 1, different plant families had different magnitude of the TGI on human tumor cell lines, such as U251 (glioma), NCI-460 (lung non-small cell), PC-3 (prostate) or HT-29 (colon), but not in human tumor cell line MC-7 (breast). The effect analyzed by block was also significant; indicating that the complete randomized block experimental design choice was correct. The coefficient of variation indicates a good precision of the experiment, except concerning to the human tumor line MC-7. The heritability estimates indicates that selection in that population would promote genetic gains.

Tab. 1 Different plant families of half-sib familiesof Cordia verbenacea show differences in Total Growth Inhibition of human tumor cell lines.

		Mean Square				
Source of Variation	Freedom Degree	U251 µg/ml	MC-7 µg/ml	NCI-460 µg/ml	PC-3 µg/ml	HT-29 µg/ml
Block	3	526,4**	122,20	9547,4**	1684,4**	9088,8**
Half-sib Family	11	1209,6**	229,16	8662,1**	5930**	9371,7**
Error	33	60,7	105,90	87,9	104,6	242,9
Mean		62,4	44,3	114,2	83,3	110
Cv		12,5	23,20	8,2	12,3	14,2
h^2		0,73	0,36	0,75	0,74	0,73

The symbol (**) means different ($P<0.01$; ANOVA) among plant families related to Total Growth Inhibition (TGI) of U251 (glioma), MCF-7 (breast), NCI-H460 (lung, non-small cells), HT-29 (colon), PC-3 (prostate) cell lines. Cv = coefficient of variation; h^2 = heritability estimates

As shown in table 2, different families could be selected according to their effect in Total Growth Inhibition of the human tumor cell lines U-251, NCI-460, PC-3 e HT-29, but not MC-7 cell line. Plants extracts with lower TGI indicates greater concentration of the active principle or greater synergic effect. Therefore, as smaller is the mean as bigger is its TGI. Agreeing to these data it is possible to select the plant families that are more effective in each human tumor cell line tested. The statistical analysis shows (ANOVA follow by Tuckey post hoc test; $p<0.05$). The families 11, 3, 12 and 16 were more effective against U-251, NCI-460, PC-3 and HT-29 cell lines.The family 9 was more effective against NCI-460, PC-3 and HT-29 cell lines. The family 26 was more effective against HT-29 cell line. The family 32 was more effective against PC-3 cell line

Tab. 2 Concentration of plant extract necessary to achieve Total Growth Inhibition of different human tumor cell lines. Different letters means statistically differences (Tuckey test; $p<0.05$).

U-251	**Half-sib family**	21	32	9	24	2	33	10	26	16	12	3	11
	TGI mean (µg/ml)	123,3 a	87,2 b	71,2 bcd	68,9 bcd	67,6 bcd	66,2 bcde	58,9 bcde	48,4 cde	48,1 de	46,0 de	45,5 de	37,9 e
MCF-7	**Half-sib family**	33	32	9	26	10	24	11	16	3	21	2	12
	TGI mean (µg/ml)	67,1 a	56,0 a	51,4 a	51,2 a	46,7 a	44,7 a	41,7 a	37,9 a	36,3 a	31,7 a	30,5 a	30,3 a
NCI-460	**Half-sib family**	32	26	33	21	24	16	3	12	11	10	9	2
	TGI mean (µg/ml)	177,7 a	168,2 abc	163,9 abc	154,5 abc	150,5 abc	140,3 bcd	110,7 d	68,7 e	39,3 e	39,0 e	35,7 e	35,6 e
PC-3	**Half-sib family**	21	16	26	33	3	9	10	2	24	32	12	11
	TGI mean (µg/ml)	154,6 a	140,3 a	139,6 a	135,4 a	54,7 b	51,9 b	50,5 b	49,6 b	48,5 b	45,5 b	44,1 b	42,2 b
HT-29	**Half-sib family**	2	33	32	21	24	9	16	12	3	11	26	10
	TGI mean (µg/ml)	212,4 a	181,7 ab	169,4 abc	143,3 bc	121,5 cd	69,2 d	68,7 d	65,3 d	62,6 d	59,4 d	56,6 d	49,3 d

Conclusion

Take together, the data of this study suggest that the domestication of a wild medicinal plant should be initially monitored by its pharmacological effect instead by its chemical composition, once it is inconstant and depends on its genetic variability and ambient factors (phenotype)

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ESL 4: *Ex-situ* evaluation of morphological, agronomic and qualitative traits of a naturalized population of parsley (*Petroselinum crispum* (Mill) Nyman])

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Abstract

A naturalized population of parsley of the province of Trento, Italy, was evaluated *ex-situ* for its morphological and agronomic traits in a field trial in which it was compared with three commercial cultivars of the species. The naturalized population belongs to the smooth leaf type for the absence of curling, and differed from the other smooth leaf type accessions for the lower plant height, the smaller length of petiole and the prostrate attitude of the plant, all undesirable characteristics which make the harvest of plants more difficult. The yields of aerial parts and leaves were higher in the commercial cultivars, while the naturalized population had the highest yield and content of essential oil. Due to the high content and yield of essential oil, the naturalized population could be the object of an eventual breeding program aiming to improve the morphologic and agronomic undesirable characteristics.

Keywords: *Petroselinum crispum,* morphological traits, agronomic traits, essential oil.

Introduction

Parsley (*Petroselinum crispum* (Mill) Nyman, *Apiaceae*) is a biennial herbaceous plant naturalized in much of Europe and cultivated worldwide as an aromatic and edible plant. The origin of the species is uncertain, but perhaps South Est Europe or West Asia (Tutin *et al.,* 1976); in Italy it is present as cultivated or naturalized (Pignatti, 1982). A naturalized population of parsley is reported in the province of Trento, Italy, in the locality Cronil within the municipality of Ala (Prosser *et al.,* 2009). Its presence is known since long times by the people living in the neighbouring localities, who traditionally use to collect its seeds and to cultivate it in their homegardens. The aim of this study was to compare the morphological characteristics and the main agronomic traits of the parsley naturalized population with that of three commercial cultivars: information about the distinctness and productive capacity could provide the necessary cognitive basis for its improvement by the starting of a breeding program.

Materials and Methods

Seeds of the naturalized population were collected in their growing site in 2012, and plantlets obtained from them were cultivated *ex situ* and compared in a field trail with three cultivars, two of smooth leaf (Comune 2, Gigante di Napoli) and one curly leaf type (Nano Ricciuto 2). The trial was carried out during the years 2013-2014 at CREA-MPF of Trento, Italy, according to a block randomized design with four replications, consisting of forty plants per replication. Morphological traits were recorded twice, one day before starting the first and second harvest, on ten randomly chosen single plants per replicate, by the use of a modified CPVO technical protocol for the species (CPVO, 2007): nine characters, corresponding to qualitative characteristics, were determined by visual assessment, and ten, corresponding to quantitative characteristics, were measured. Plants were harvested two times, two and four months after transplant. Leaves were separated from petioles and yields of aerial part and leaves were recorded. Essential oils were extracted immediately after harvest from 1 kg of fresh leaves per replicate for the determination of the essential oil yield. For the determination of both morphologic and agronomic traits, average data of the two consecutive

harvests were subjected to ANOVA, being percentage data previously transformed in angular values, using XLSTAT software package.

Results

Morphological characterization

Results of the morphological characterization by qualitative traits are indicated in Table 1. The naturalized population belongs to the smooth leaf type for the absence of curling, but differed from the other two smooth leaf type accessions for a weaker undulation of the leaflet margin and for the attitude of leaves, prostrate instead of erect. Highly significant differences were detected among accessions for all the morphological quantitative traits (Table 2). In particular, the naturalized population parsley differed from the other two smooth leaf accessions for the lower height of plant and the smaller length of petiole. These characters, together with the prostrate attitude of plant, are undesirable for making the harvest of plants more difficult.

Tab. 1 Values of the morphological qualitative traits of the evaluated accessions.

Accession	CPVO N° and description						
	3	5	6	13	14	16	19
	Plant: density of foliage	Leaf: attitude	Leaf blade: curling	Leaf blade: intensity of green colour	Leaflet: shape	Leaflet: undulation of the margin	Petiole: anthocyanin coloration
Comune 2	medium	erect	absent	medium	narrow triangular	medium	absent or very weak
Gigante di Napoli	medium	erect	absent	medium	narrow triangular	medium	absent or very weak
Nano Ricciuto 2	dense	semi erect	present	medium	medium triangular	strong	absent or very weak
Naturalized population	medium	prostrate	absent	medium	narrow triangular	weak	absent or very weak

Tab. 2 Values of the morphological quantitative traits (mean ± standard deviation; n=4)*

Accession	CPVO N° and description								
	1	2	4	10	11	12	15	17	18
	Plant: height (cm)	Plant: width (cm)	Plant: number of leaves	Leaf blade: length (cm)	Leaf blade: width (cm)	Leaf blade: ratio length/ width	Leaf blade: distance between 1st and 2nd pair of leaflets (cm)	Petiole: length (cm)	Petiole: thickness (cm)
Comune 2	26.8 ± 3.4 A	42.2 ± 4.7 A	35.0 ± 21.5 A	18.3 ± 2.2 AB	13.1 ± 2.3 A	1.41 ± 0.20 A	8.4 ± 1.0 A	11.2 ± 1.1 A	3.9 ± 0.4 A
Gigante di Napoli	29.2 ± 2.7 A	39.4 ± 4.7 A	20.7 ±12.3 B	19.2 ± 2.1 A	13.9 ± 1.6 A	1.39 ± 0.10 A	8.3 ± 1.0 A	11.6 ± 1.5 A	4.3 ± 0.3 A
Nano Ricciuto 2	15.7 ± 1.4 B	29.7 ± 2.5 B	15.9 ± 6.0 B	11.3 ± 1.4 C	10.0 ± 0.9 B	1.13 ± 0.12 B	5.2 ± 0.4 B	6.7 ± 1.0 B	4.4 ± 0.4 A
Naturalized population	15.5 ± 2.9 B	39.0 ± 5.0 A	28.4 ± 18.5 A	15.9 ± 2.3 B	14.7 ± 2.2 A	1.09 ± 0.10 B	7.4 ± 1.0 A	7.0 ± 1.0 B	3.4 ± 0.4 B

* Mean values followed by different letters are significantly different at P<0.01 according to Tukey (HSD) test.

Yield and other agronomic traits

Cumulative yields of the two harvests of the investigated accessions are reported in Table 3. The naturalized population had the lowest yields both of aerial parts and leaves, and the highest essential oil yield, resulting statistically different from the other smooth leaf type accessions for the aerial part yield and from all the other accessions for the yields of leaves and essential oil.

Table 3 Agronomic traits of the evaluated accessions (mean ± standard deviation; n=4)*

Accession	Aerial part yield (g · m^{-2} dried weight)	Leaves yield (g · m^{-2} dried weight)	Essential oil yield (ml · m^{-2})	Essential oil content (% v/fresh weight)
Comune 2	410.8 ± 24.1 A	340.5 ± 14.1 A	1.93 ± 0.16 B	0.11 ± 0.04 B
Gigante di Napoli	394.3 ± 37.3 A	329.1 ± 23.2 A	1.54 ± 0.15 B	0.10 ± 0.04 B
Nano Ricciuto 2	343.3 ± 29.5 AB	320.7 ± 26.7 A	1.40 ± 0.24 B	0.10 ± 0.06 B
Naturalized population	277.5 ± 19.0 B	244.5 ± 19.3 B	2.92 ± 0.37 A	0.25 ± 0.06 A

* Mean values followed by different letters are significantly different at P<0.01 according to Tukey (HSD) test.

The naturalized population differed from the other accessions also for the essential oil content of the leaves calculated as a percentage of fresh weight, mean of the two harvests (Table 3), which was more than the double than the cultivars, and higher than those reported by the majority of previous authors, that is up to 0.16 % on the fresh weight basis (SIMON and QUINN 1988; PINO *et al.* 1997). In conclusion, the naturalized population differed from the smooth leaf type varieties evaluated in this study for some morphological characters that easily permit its identification. The small dimensions of the plant and especially its prostrate attitude are however two undesirable characters which could make more difficult its harvest. These characters, together with the low yields of leaves and aerial parts, could be improved in an eventual breeding program, which could be justified by the high yield of essential oil extracted from its leaves.

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ESL 5: Parasitic Angiosperms as medicinal plants

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Abstract

Parasitism is only found in eudicotyledonous Angiosperms in all continents and in one Gymnosperm from New Caledonia. At least eleven independent origins of parasitism in Angiosperms have been proposed.

The economic importance of parasites is generally considered as negative in connection with dangerous species for agriculture, horticulture and forestry (e.g., *Orobanche* spp. and *Striga* spp., Orobanchaceae, *Cuscuta* spp., Cuscutaceae, and *Arceuthobium* spp., Viscaceae). But there are a number of species useful as vegetables (*Melientha suavis*, Opiliaceae), sources for manufacturing cosmetics and soaps (*Santalum album*, Santalaceae) and fruit trees (*Ximenia americana*, Ximeniaceae). And there are numerous examples for the use of parasites as medicinal plants, often not yet confirmed by modern medical sciences.

In a recent survey, cultivated parasitic plants could be found in Olacaceae (1), Opiliaceae (1), Orobanchaceae (3), Santalaceae (6), Viscaceae (1) and Ximeniaceae (1, number of species in brackets). Among the species listed, there are also some medicinal plants. Most traditional under cultivation is *Santalum album* from tropical Asia (Santalaceae, hemiparasitic root parasite). An essential oil is extracted from the wood and roots; it is mostly used for oriental perfumes. Though their uses as medicinal plants may be rather old, some species have been taken into cultivation only towards the end of the last century, as *Viscum album* (Viscaceae, Europe and Asia, hemiparasitic on branches), *Cistanche deserticola* (China) and *Orobanche crenata* (Mediterranean, both Orobanchaceae, holoparasitic root parasites). For *Euphrasia officinalis* (Orobanchaceae, hemiparasitic root parasite) there are reports about cultivation in Europe in the past, but recent experiences are lacking.

The cultivation of parasites for medicinal uses is a challenge. But there are new prospects because of developing domestication and cultivation strategies.

Keywords: parasite, angyosperms, medicinal plants, cultivation, domestication

ESL 6: The controlled cultivation of *Cannabis sativa* at VitaPlant

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Abstract

Cannabis sativa (Cannabis) belongs -behind alcohol- to the world's most commonly used drugs. On the other hand positive effects in the therapeutic use of cannabis preparations were recorded in the last few years such as treatment of anorexia due to loss of appetite in HIV, for the relief of nausea and vomiting during chemotherapy, in the treatment of various pain disorders as well as for the reduction of symptoms associated with neurological disorders such as multiple sclerosis.

The law in Switzerland bans the cultivation and consumption as well as the trade of cannabis with a total content of THC (tetrahydrocannabinol) of over 1 %. Cannabis with THC levels above this level are considered as narcotics and the use is subject to a specific authorization requirement.

VitaPlant has an operating license from the canton of Thurgau and is required to apply for a special exemption from the Federal Office of Public Health (BAG) for every third party production. Research on cannabis has a long tradition at VitaPlant and several clones have been developed with specific active ingredients over the years. A main focus in the development of Cannabis at VitaPlant is the two cannabinoids: Δ9-THC (Δ9-tetrahydrocannabinol) and CBD (cannabidiol).

Currently VitaPlant is working on several projects for Swiss companies with respect to the production of cannabis in various grades. It is primarily concerned with the composition of the cannabinoids and the ratio of THC and CBD, but also with various kinds of harvesting. Two different forms of harvesting are used in the preparation of the drug: flowering branches (i.e. herba) or as pure flower (flos). In all cases the female flower is the main product. Depending on the harvested part the amount of THC and CBD differs considerably (Tab. 1). Hoewever, the relation between THC and THC-A or CBD and CBD-A is not stable. The time of harvesting and the maturity of flowers as well as the drying and processing of the raw material have an effect on the amount of both compounds.

Tab. 1 Content of THC and CBD in two selected clones depending on the harvested parts of the plant

Clones	Kind of harvest	CBD	CBD-A	Total CBD	THC	THC-A	Total THC
		[%]					
THC typ							
CAN 9#4	flos	0.0	0.0	0.0	1.6	7.4	9.0
CAN 9#4	herba	0.0	0.0	0.0	1.0	6.1	7.0
CBD typ							
CAN 11#3	Flos	0.3	12.8	13.2	0.0	0.4	0.4
CAN 11#3	herba	0.3	5.0	5.3	0.0	0.1	0.1

Session F: Plant breeding and plant analytics

FPL 1: Medicinal plant breeding in Poland: history and nowadays

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Abstract

Introducing of medicinal plants into cultivation and growing demands of phytopharma-ceutical producers have the influence on starting breeding research in Poland. In the Research Institute of Medicinal Plants of Poznan the breeding programs have been conducted from over fifty years. During these years 22 cultivars of medicinal plants were bred and successfully introduced into practice.

In Poland, medicinal plants were used from ages not only as a folk remedy but also as a drug in pharmacy. In the past, collecting in the wild was the main source of medicinal plant raw materials. Now, about 70 different species of medicinal plants are cultivated on app. 20 000 ha and Poland is recognized as the country of growing potential for the high quality raw material production, based on farmers long lasting experience and knowledge, but also based on the achievement of Polish medicinal plant breeding. The medicinal plant raw material, produced in Poland, mainly come from cultivation (10 000 - 20 000 t), but also from collection in the wild (3 000 - 5 000 t) (JAMBOR, 2001).

Chamomile (*Chamomilla recutita* (L.) Rausch.), thyme (*Thymus vulgare* L.), milk thistle (*Silybum marianum* (L.) Gaertn.), valerian (*Valeriana officinalis* L.), peppermint (*Mentha x piperita*), caraway (*Carum carvi* L.), lovage (*Levisticum officinale* Koch.), savory (*Satureja hortensis* L.), lemon balm (*Melissa officinalis* L.), sage (*Salvia officinalis* L.), evening primrose (*Oenothera paradoxa* Hudziok) and St.John's Wort (*Hypericum perforatum* L.) are the most commonly cultivated species in Poland . Different origin of raw materials and existence of medicinal plant chemotypes which are different in active substance content affect heterogeneous of raw material lots, what causes a lot of problems for producers. Following GMP demands, the drug production needs large, uniform consignment of the high quality raw material. The controlled cultivation of the medicinal plant cultivars provides the high yield of the raw materials, which contains a lot of active substances.

Establishing the Research Institute of Medicinal Plants in 1947 sped up the development of the breeding research in Poland. Prof. Waclaw Strazewicz, who gave rise to Institute, was the first medicinal plant breeder in Poland. He strongly pointed out the importance of medicinal plant breeding and he claimed that: "offering the high quality raw materials of medicinal plants from cultivation and developing breeding research is the only way for Poland to obtain the important position on the international market of medicinal plant raw materials" (STRAZEWICZ, 1948). Breeding research was also done in others Polish institutions as: University of Agriculture (SGGW-AR) in Warsaw (cultivars of chamomile and coriander), University of Agriculture in Wroclaw (evening primrose breeding) and some farmers (2 cultivars of valerian and mint).

The main directions of the medicinal plants breeding programs in the Institute are:

- Content of active substances
- Yield and its structure
- Adaptation to mechanical harvest
- Adaptation to abiotic (drought, winter hardiness) and biotic stress.

Medicinal plant breeders encounter considerable difficulties with:

1. Low income, because medicinal plants are cultivated on a small area

2. A large number of medicinal plant species (app. 170). Breeding programs usually cover species which are cultivated on large areas or are important for drug or food industries.

3. Most of medicinal plant species are perennial, so breeding programs are prolonged.

At the present time in the Institute, only the breeding program of lemon balm (*Melissa officinalis* L.) is performed to obtain the new, valuable cultivar.

In the Institute of Natural Fibres and Medicinal Plants 22 cultivars of medicinal plants were bred and introduced into cultivation:

- caraway (*Carum carvi* L.)	cvar. 'Kończewicki'
- caraway (*Carum carvi* L.)	cvar. 'Plewiski'
- chamomile (*Chamomilla recutita* (L.) Rausch.)	cvar. 'Zloty Lan'
- chamomile (*Chamomilla recutita* (L.) Rausch.)	cvar. 'Promyk'
- chamomile (*Chamomilla recutita* (L.) Rausch.)	cvar. 'Dukat'
- chamomile (*Chamomilla recutita* (L.) Rausch.)	cvar. 'Mastar'
- foxglove (*Digitalis lanata* Ehrh.)	cvar. 'Victoria'
- greater celandine (*Chelidonium maius* L.)	cvar. 'Cynober'
- hollyhock (*Althea rosea* Cav. var.nigra hort.)	cvar. 'Czarna Mańka'
- Jimsonweed (*Datura innoxia* Mill.)	cvar. 'Indianka'
- lovage (*Levisticum officinale* Koch.)	cvar. 'Amor'
- marjoram (*Origanum majorana* L.)	cvar. 'Miraz'
- milk thistle (*Silybum marianum* (L.) Gaertn.)	cvar. 'Silma'
- purple coneflower (*Echinacea purpurea* Moench.)	cvar. 'Ida'
- red pepper (*Capsicum annuum* L.)	cvar. 'Wulkan'
- sage (*Salvia officinalis* L.)	cvar. 'Bona'
- savory (*Satureja hortensis* L.)	cvar. 'Saturn'
- sweet basil (*Ocimum basilicum* L.)	cvar. 'Kasia'
- sweet basil (*Ocimum basilicum* L.)	cvar. 'Wala'
- St. John's Wort (*Hypericum perforatum* L.)	cvar. 'Topaz'
- thyme (*Thymus vulgaris* L.)	cvar. 'Sloneczko'
- valerian (*Valeriana officinalis* L.)	cvar. 'Polka'.

In the Breeding Laboratory of the Institute, the detailed rules of these cultivars maintenance breeding were elaborated and introduced into practice. Therefore, the basic seeds of cultivars are produced, then seeds for medicinal plant raw material producers (SEIDLER-LOZYKOWSKA , 2009).

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FPL 2: Alternatives to Chromatography in Plant Breeding

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Abstract

Wild plants were taken into cultivation because of special features. Usually, medicinal plants or spices show distinct secondary metabolites combined with a specific pattern of these compounds. Typically, chromatographic methods like gas chromatography (GC) or high performance liquid chromatography (HPLC) were applied as standard methods for a meaningful analysis of secondary metabolites. However, these methods are labor and time intensive. In the breeding process, usually numerous single plants have to be analyzed and therefore, high throughput methods are required. In this article, some examples for alternative strategies are given. Besides spectroscopic methods like near infrared (NIR), also biosensoric approaches should be considered. For instance, several enzymes can oxidize or hydrolyze secondary metabolites in dependence of their functional groups. Polyphenols can be determined by laccases. Polyphenols like catechins and flavonoids contribute to the bioactivity of many medicinal plants. Also cysteine sulfoxides, which are typical for *Allium* species like garlic and onions, can be enzymatically determined with high specificity. Finally, toxic cyanogenic glycosides can be quantified by the enzyme cyanidase.

Keywords: biosensors, polyphenols, cysteine sulfoxides, cyanogenic glycosides

Introduction

Selection processes are required for all types of breeding approaches. In a more classing setting, characters like growth or environmental resistance are used as selection criteria. In the case of vegetable plants, nutrients like carbon hydrates, fat or protein have to be analyzed. In terms of medicinal plants or spices, specific secondary metabolites have to be determined in a qualitative and quantitative manner.

This situation is rather clear for essential oil plants. The content of essential oil can be determined by standard methods like steam distillation and the pattern of individual compounds is usually analyzed by gas chromatography (GC). For all other compounds, analysis is more complicated; usually high performance liquid chromatography (HPLC) is used for this task. However, chromatographic conditions have to be adapted to each group of secondary metabolites as well as each plant species. During the breeding process, typically several hundreds of samples have to be analyzed in a rather narrow time frame and therefore, high throughput methods are required. However, chromatography is less suitable for such a high throughput approach.

Therefore, alternative strategies have to be worked out. Rather powerful are spectroscopic methods like near infrared spectroscopy (NIR). There are several examples that especially for essential oil plants a result can be archived within several minutes (SCHULZ AND BARANSKA, 2007). Additionally, Raman infrared spectroscopy in combination with microscopy allows identification of certain compounds in plant tissue. For instance, the distribution of rather unique thiopyrrols in the stems of the wild *Allium* species *A. jesdianum* BOISS. et BUHSE can be visualized (Fig. 1). These compounds are typical for wild onions in Middle Asia, which are highly estimated as vegetable and medicinal plant.

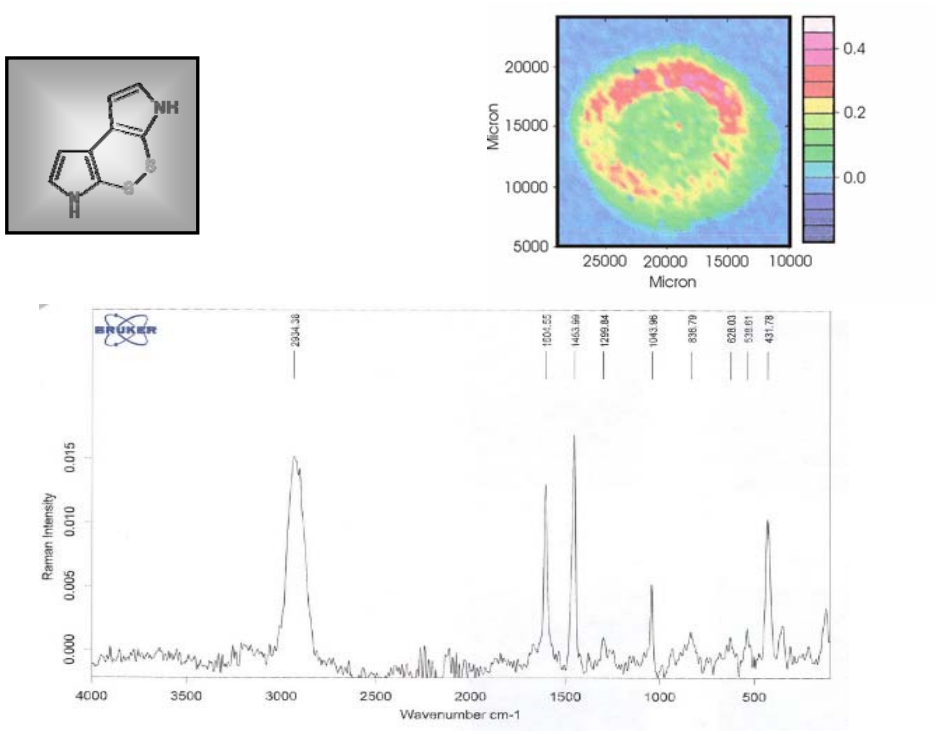

Fig. 1 Localization of thiopyrrols in stems of Allium jesdianum by IR-Raman-microscopy (scan on sharp and distinct peak at 1453 cm-1). The thiopyrrol seems to be located in the tissue surrounding vessels. On the left side box, the suggested structure of the thiopyrrol and the corresponding Raman IR is depicted below. Measurements were kindly provided by Prof. H. Schulz, JKI Quedlinburg.

Besides spectroscopic methods, also further alternative approaches should be considered. For instance, larger molecules can be recognized by specific antibodies. As a disadvantage, specific antibodies have to be designed firstly in a work intensive process. Also, antibodies show some cross specificity. As a further alternative, enzyme-based approaches are possible. Usually, degrading enzymes do not specifically act on individual secondary metabolites, but can be used for group determination. Most suitable are hydrolases and oxidoreductases. Hydrolases cause a slight pH shift of the sample solution, which can be detected by pH-sensitive devices, whereas oxidoreductases can be combined with amperometric sensors.

Combination of these enzymes with potentiometric electrodes, *e.g.*, pH electrodes or amperometric electrodes, can be utilized for the construction of biosensors (KEUSGEN, 2002). A biosensor is defined as a tight combination of a biological recognition element like enzymes or antibodies with a physical transducer like electrodes. The biological element is immobilized on the surface of the physical transducer (BARLEN et al., 2009); such a biosensor is reusable. With this type of sensors, several hundreds of measurements can be performed within one day. The sensor can be also miniaturized. In this article, three different approaches are described, which are suitable for plant breeding approaches as well as quality control of herbal products.

Materials and Methods

Key elements of the here presented biosensoric applications are the enzymes as mentioned in Fig. 2. Alliinase has been obtained from fresh or powdered garlic (*Allium sativum* L.; KREST AND KEUSGEN, 1999). Bacterial cyanidase has been used as recombinant enzyme (KEUSGEN et al., 2004). Laccases from *Trametes versicolor, Agaricus bisporus* and *Rhus vernificera* were obtained from Sigma-Aldrich.

Electrodes like depicted in Fig. 3 were bought from BVT Technologies (Brno, Czech Republic). Best results were archived with type AC1.W2.R1 (platinum working electrode). A mobile PalmSens potentiostat (Palm Instruments BV, Houten, Netherlands) at a potential of E=670 mV was used for measurements as shown in Fig. 4 and Fig. 5. Further details are given in SCHMIDT (2012).

For immobilization of the laccase, the platinum working electrode of the sensor as shown in Fig. 3 was treated four times with 0.25 µL of a 4-fluoro-3-nitrophenyl azide -(FNPA)-solution (10 mg/mL; good solubility in *tert*-butyl methyl ether) and irradiated for 1 h with UV light at λ=254 nm. Directly after irradiation, 0.5 µL of the laccase solution (35 mg/mL) was added and the sensor was dried at room temperature. The senor was rinsed with H_2O and stored in phosphate buffer (pH 7.0, 0.1 M) preserved with Thiomersal (0.05 %) before further use at +4 °C.

As a control, total phenol content has been determined according to Folin-Ciocalteu (SINGELTON AND ROSSI, 1965). Samples with a high content of phenolic compounds (> 1g/l) have to be diluted before measurement. From this aqueous test solution, 0.1 mL was taken and diluted by 8.4 mL of H_2O followed by 0.5 mL of the Folin-Ciocalteu reagent. After 5 min, 1.0 mL 0f saturated sodium carbonate solution was added and the absorption at λ=720 nm has been measured. For calibration, (-)-epicatechin was used; therefore all concentrations given in Fig. 4 and Fig. 5 are expressed in (-)-epictechin equivalents. For further details see SCHMIDT, 2012.

All real samples were obtained from local stores. Aronia and Cranberry juice could be directly used after filtration and dilution, whereas the samples of green tea were prepared as infusion. 12 g of tea leafs were given into 1 L of 90 °C hot, demineralized H_2O. After 5 min, the infusion has been filtrated and stored at +4 °C before measurement. Shortly before measurement, the sample has to be diluted in an appropriate manner (typically phosphate buffer pH 7.0). It has to be considered that phenols are easily oxidized by air oxygen and therefore samples should be tightly closed (best under inert gas atmosphere) as well as measurements should be performed as soon as possible after sample preparation.

Results and Discussion

Three different enzymes were tested as part of biosensor electrodes (Fig. 2). In all cases, the sensors were applied to chemical standards and to real plant samples. In Fig 2a, the so called 'laccase reaction' is shown. This enzyme is widely distributed in nature, especially in fungi like *Trametes versicolor* and *Agaricus bisporus* as well as in higher plants like *Rhus vernificera*. All three enzymes were tested with different standards and the laccase from *T. versicolor* was found to be most suitable for a biosensoric approach. The enzyme has been immobilized on the working electrode of a thick film electrode (TFE, Fig. 3) by different methods. Best results were archived by using the photo cross-linker 4-fluoro-3-nitrophenyl azide (FNPA). Alternatively, the enzyme can be immobilized on carbon nanotubes, entrapped into polylactic acid (PLA) or immobilized by covalent immobilization via amide binding. All these strategies are possible, but not superior over photo cross-linking by FNPA (SCHMIDT, 2012).

Laccase is a rather unspecific enzyme. Its function in nature is formation of radicals (Fig. 2a) followed by cross-linking like the formation of lignans. If polyphenols were used as educts, enzymatic products are typically of brown or black color; this reaction naturally occurs after tissue wounding in is easily visible by discoloration within seconds. A similar reaction can be catalyzed by phenoloxidases (SCHMIDT, 2012). Because of the low substrate specificity of laccase, a wide range of phenolic compounds can be determined by a biosensor based on this enzyme.

The obtained sensor has been calibrated with (-)-epicatechin in a concentration range between 10 and 100 µg/mL. The detection limit was determined to be at 7.7 µg epicatechin per mL. The pH of the test solution was adjusted at pH 7.0. In the used experimental set up, measuring time for a single sample was 5 min. In the next step, different real samples were analyzed and obtained results were compared with a standard reference method (Folin-Ciocalteu phenol determination). As shown in Fig. 4, the biosensor and the reference values gave a good correlation, but the bio-

sensor values are systematically about 20 % higher as results gained by the Folin-Ciocalteu method. There is no explanation for this phenomenon. However, it must be considered that the investigated samples contain complex mixtures of polyphenols, which are mainly catechin derivatives.

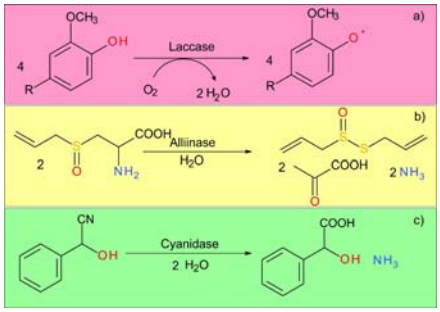

Fig. 2 Three different enzymatic reactions, which can be used for biosensor approaches. A) laccase reaction for the determination of phenolic compounds, b) alliinase reaction for the determination of cysteine sulfoxides and c) cyanidase reaction for the determination of cyanogenic compounds.

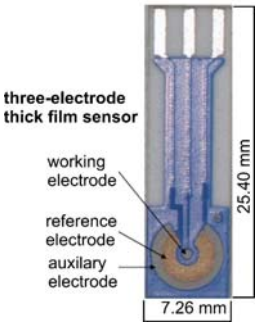

Fig. 3 Photo of a commercial thick film electrode (TFE; Schmidt, 2012). The enzyme has to be immobilized on top of the platinum working electrode.

The obtained sensor has been calibrated with (-)-epicatechin in a concentration range between 10 and 100 µg/mL. The detection limit was determined to be at 7.7 µg epicatechin per mL. The pH of the test solution was adjusted at pH 7.0. In the used experimental set up, measuring time for a single sample was 5 min. In the next step, different real samples were analyzed and obtained results were compared with a standard reference method (Folin-Ciocalteu phenol determination). As shown in Fig. 4, the biosensor and the reference values gave a good correlation, but the biosensor values are systematically about 20 % higher as results gained by the Folin-Ciocalteu method. There is no explanation for this phenomenon. However, it must be considered that the investigated samples contain complex mixtures of polyphenols, which are mainly catechin derivatives.

As depicted in Figure 5, two different types of juices have been investigated. Both juices showed a phenol content which is much higher as for the green tea. This requires a 100fold dilution of the samples before measurement. Surprisingly, the found amount of phenolic compounds for Aronia juices was 50 % lower as determined by the reference method. An explanation can be that Aronia is also rich in Vitamin C, which is also highly redox-active and can therefore interact with measurements by chemical reduction of formed radicals. This phenomena might lead to decreased polyphenol values by the here presented electrochemical method.

In the case of Cranberry juice, results of both methods were nearly identically. It has also to be considered that the calibration was performed with epicatechin, which is typical for tea but of less importance for fruit juices. Phenolic compounds in plants occur normally as a mixture of several compounds and therefore, calibration with only one compound is somewhat problematic. But nevertheless, the here presented method is well suitable for relative comparison of samples of a distinct plant species.

It also must be pointed out that results are more precise if the biosensor is placed inside a flow-through-analyzer (FIA). Such a FIA-approached has been published by KEUSGEN in 1998. However, the miniaturized sensor as depicted in Fig. 3 can be also used in the field because the used PalmSens potentiostat can be operated by batteries. Fresh plants can be analyzed by giving one drop of juice on the TFE sensor surface, but these measurements are less precise as using the FIA device.

Further on, some other secondary metabolites can be determined by enzymatic biosensors. As depicted in Fig. 2b, cysteine sulfoxides, which are typical for all onions, can be digested by the

enzyme alliinase. In this case, the alliinase was obtained from garlic. This reaction is rather interesting because it results in a slight pH-shift of the sample solution which can be detected by ph-sensitive semiconductor devices (KEUSGEN et al., 2003). Alternatively, the formed ammonia can be quantified by an ammonia electrode or a comparable method (KEUSGEN, 1998, MILKA et al., 1999). As a second alternative, the pyruvate might be enzymatically determined by conversion with lactate dehydrogenase into lactate (LDH; KREST AND KEUSGEN, 1999). The lactate formation correlates directly to the pungency of garlic and onions. The biosensoric method based on ammonia quantification could be successfully applied to several samples of garlic and wild onions (MILKA et al., 1999, KREST AND KEUSGEN, 2002, KEUSGEN et al., 2003). By using an automated FIA device, a single analysis needs 3 min.

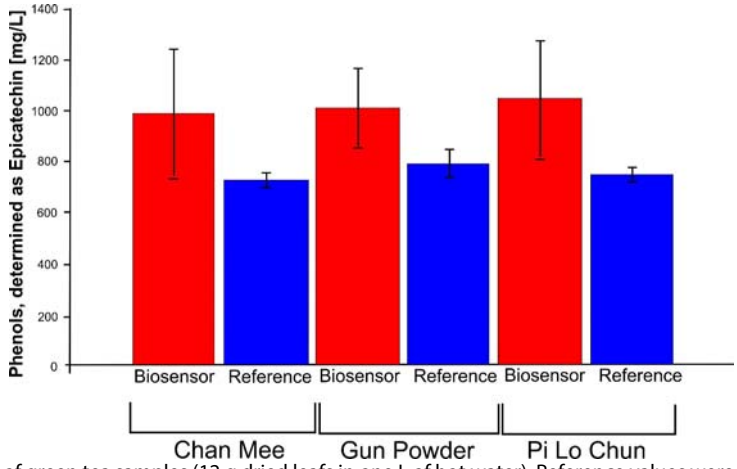

Fig. 4 Results of green tea samples (12 g dried leafs in one L of hot water). Reference values were obtained by the Folin-Ciocalteu method.

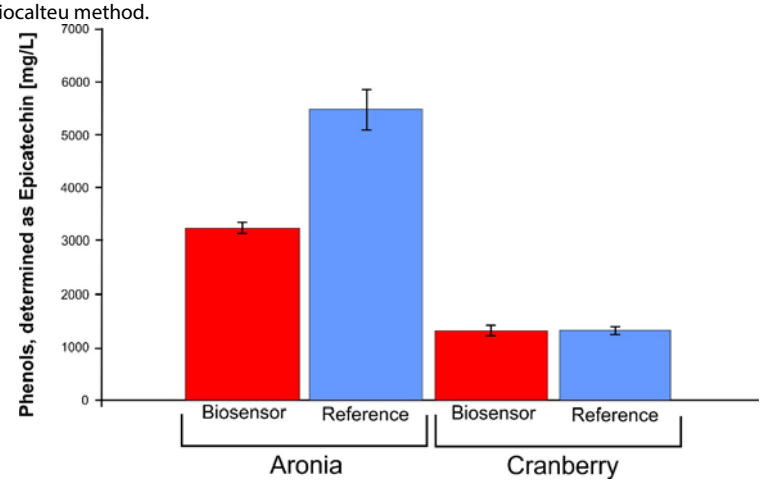

Fig. 5 Results for some commercially available juices. Reference values were obtained by the Folin-Ciocalteu method.

As a last example, cyanogenic glycosides can be determined by the enzyme cyanidase as shown in Fig. 2c (KETTERER, 2010). Again, ammonia is formed and the entire reaction causes a slight pH shift of the sample solution (KEUSGEN et al., 2004, TUREK et al., 2007, KETTERER AND KEUSGEN, 2010). Cyanogenic compounds are typical for the family *Rosaceae*, *Fabaceae* and the plants *Manihot esculenta* CRANTZ as well *Carica pap*aya L. On one hand, these compounds deliver an almond-like flavor, on the other hand, the liberated cyanide is highly toxic. The value of this biosensor is more

in the field of selecting species with a low amount of cyanogenic glycosides and decreased toxicity. The used recombinant cyanidase has been produced in *Escherichia coli* and is therefore available in reproducible quality and sufficient quantities (KEUSGEN et al., 2004).

In conclusion, it could be demonstrate that in some cases enzymatic biosensors can be considered as an alternative for chromatographic methods. Usually, not a distinct compound can be determined by theses sensors, but a class of secondary metabolites as shown by three examples. As a disadvantage, these sensors can be harmed by high concentrations of the analyte and improper storage conditions because of denaturation of the enzyme. But by experience it could be demonstrated that proper immobilization of the enzyme also increases stability of the enzyme. In any case, the sensor should be washed carefully with a suitable buffer after analysis and stored in a preserved buffer at +4 °C between measurements. This guarantees a lifetime of several months.

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FSL 1: Comparative investigation of 11 *Achillea collina* Becker accessions concerning phenological, morphological, productional features and active agent content

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Abstract

Eleven *Achillea collina* Becker accessions of different origin were tested in open field plots during three years for their phenological, morphological, productional features and active material content in Budapest, Hungary. Among the tested plant materials European selected cultivars, Hungarian cultivated stocks and populations from wild growing habitats were investigated.

Concerning flowering time, two types (early and late) were distinguished. Flowering time of the less abundant late type, represented by Hungarian variety 'Azulenka' and 'Gb22', started approximately 2 weeks later than that of the early flowering type. Plant height and length of flowering horizon varied only slightly among taxa, and increased after the first year of cultivation. The proportion of useful plant organs in the drug was stable. Biomass and drug production of the investigated genotypes was variable, late flowering types providing higher yields. Essential oil, proazulene, total phenolic and flavonoid content varied on a large scale among accessions and years.

Results demonstrate the high intraspecific variability of *A. collina* and also the role of valuable genotypes in drug production. Selected cultivars may provide stable and good yields and drug quality under particular environmental conditions, while genotypes of wild origin may be valuable sources of future breeding programs.

Keywords: *Achillea collina*, azulene, production, drug quality, essential oil, flowering horizon

Introduction

Yarrow (*Achillea*) species are widely known and popular medicinal plants all over the world. The most important active agent of the drug (*Millefolii herba*) is the essential oil with proazulenes, but further compounds are also present, like flavonoids, phenolics and non volatile sesquiterpenes. Numerous therapeutic effects of yarrow have been demonstrated, among others spasmolytic, analgesic, anti-inflammatory and digestive activities (Németh and Bernáth, 2008). Although the drug is officially listed in Ph. Eur., the drug quality seems to be often inadequate (Benedek et al., 2008). This can be traced back to the high chemical diversity of the collected natural populations and sometimes even the cultivated ones. Therefore in the present study, 11 different *A. collina* taxa were tested including registered cultivars, wild originating populations and cultivated stocks to gather more information about the morphological and productional features and the drug quality of yarrow.

Materials and Methods

The comparative investigation of the 11 accessions (Table 1) was carried out in open field for three years, from 2012 to 2014. The experiment was installed in perennial stands in small plots in 3 replications. Phenological and morphological characteristics of the populations were compared, plant height and length of flowering horizon (flowers of appr. 20-30 cm stems) in 6 replications, the proportion of useful plant organs in the drug and the drug yield were measured (3 replications). The essential oil content was detected by the method described in Ph.Hg.VII (*Achilleae herba*) and the proazulene content of the oil by the method recommended in Ph. Hg. VIII. (*Millefolii herba*), both in 3 replications. The total phenolic content was determined in 3 replications by the method

of SINGLETON and ROSSI (1967), while determination of total flavonoid content was carried out by the method recommended in PH. HG. VIII. (*Crataegi folium cum flore*) in 3 replications. Samples were taken in each year, but in 2014 only essential oil and proazulene content were measured. The trial was carried out at the experimental field and in the laboratory of the department.

Tab1 Origin and flowering time of the examined A. collina taxa.

Taxa name	Taxa code	Origin	Flowering time
'Azulenka'	T1	Hungarian variety	late
'Alba'	T2	Slovakian variety	early
'Proa'	T3	German variety	early
'Spak'	T4	Swiss variety	early
'Földes'	T5	cultivated stock from Földes, Hungary	early
'Gyula'	T6	cultivated stock from Gyula, Hungary	early
'Kál'	T7	cultivated stock from Kál, Hungary	early
'Gb9'	T8	wild originating population from Aszód, Hungary	late
'Gb10'	T9	wild originating population from Remeteszőlős, Hungary	early
'Gb22'	T10	wild originating population from Nagymaros, Hungary	early
'Gb47'	T11	Wild originating poulation from Mikóújfalu, Romania	early

Results

A fortnightly shift in flowering time was observed between the earlier and later ('Azulenka' and 'Gb22') genotypes (Table 1.). Morphological, productional features and active agent content of the taxa are presented in Table 2. The plant height was relatively stable among the taxa, only 'Gb47' reached higher values (67.5 cm in 2013) than others. Otherwise height was found to be higher from the 2nd year, when plants reached their full development. Flowering horizon of the plants varied on a larger extent (between 15.8 and 31.9 in 2013) among accessions and similarly to plant height, increased slightly after the first year by 6 cm in the average. The proportion of useful plant organs (flowers and leaves) in the drug was found to be stable (60.4-67.8 %) among years and accessions. Highest drug yields were measured in genotype 'Azulenka' both in 2012 and in 2013 (2.02 and 3.91 kg/10m^2, respectively). The yields increased after the 1st year of cultivation by 36-120 %, the production remained stable only in case of genotypes 'Alba' and 'Gb47'.

The essential oil content of the flowering horizon varied between 0.140 and 0.407 ml/100 g in d.w. In 2012 and 2013 the best results were achieved in accessions of wild origin: 'Gb 47' (0.395 and 0.290 ml/100 g), while in 2014 the highest content was measured in population 'Gyula' (0.407 ml/100 g). The proazulene content exceeded the requirements (0.02 % EO) of European Pharmacopoeia VII. in each population and showed maximum values in 'Gb22' (0,174 and 0.122 %) in 2012- 2013, and in 'Gyula' (0.150 %) in 2014 .

The phenolic content of the studied populations varied from 139 to 220 mg GAE/100 g. In 2012 highest value was determined in 'Proa' (220 mg GAE/100 g), while in 2013 'Gb22' (185 mg GAE/100 g) was found to be outstanding. Total flavonoid content of the taxa showed big differences, the values varied from 0.46 to 2.34 %, with highest concentrations in genotypes of wild origin: 'Gb 22' (2.06 %) and 'Gb47' (2.39 %). Both phenolic and flavonoid contents were unstable and their content changed inconsistently during the examined years.

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Tab2 Characteristics of the examined genotypes (average values and standard deviation of experimental years)

Taxa	Plant height (cm)	Length of flowering horizon (cm)	Drug yield (kg/10 m^2)	Essential oil content (mg/ 100 g)	Proazulene content (%)	Total phenolic content (mg GAE/100 g)	Total flavonoid content (%)
T1	47.3 +/-4.9	15.3+/- 0.9	2.97 +/- 1.33	0.277 +/- 0.029	0.099 +/- 0.016	181.5 +/- 36.1	1.57 +/- 0.19
T2	50.1 +/-5.8	19.3 +/-3.2	1.96 +/- 0.02	0.232 +/- 0.041	0.077 +/- 0.012	158.5 +/- 9.2	1.69 +/- 0.54
T3	48.4 +/-9.0	18.7 +/-3.0	1.70 +/- 0.55	0.219 +/- 0.047	0.114 +/- 0.018	188.0 +/- 45.3	1.78 +/- 0.61
T4	47.8 +/-11.2	18.6 +/-8.1	1.44 +/- 0.57	0.195 +/- 0.057	0.097 +/- 0.032	167.5 +/- 12.0	1.97 +/- 0.36
T5	48.6+/-8.4	23.1 +/-1.7	2.06 +/- 1.02	0.277 +/- 0.030	0.089 +/- 0.035	174.5 +/- 16.3	1.95 +/- 0.15
T6	45.5 +/-5.8	24.5 +/-7.1	2.14 +/- 0.98	0.302 +/- 0.092	0.102 +/- 0.043	154.0 +/- 11.3	0.98 +/- 0.74
T7	46.1 +/-5.8	19.0 +/-2.3	1.92 +/- 0.41	0.202 +/- 0.050	0.056 +/-0.30	171.0 +/- 43.8	1.44 +/- 0.44
T8	46.7 +/-5.2	20.7 +/-1.1	1.95 +/- 0.42	0.177 +/- 0.033	0.065 +/- 0.020	149.0 +/- 14.1	1.63 +/- 0.58
T9	47.4 +/-5.9	19.6 +/-4.1	2.09 +/- 0.86	0.203 +/- 0.013	0.082 +/- 0.016	175.5 +/- 29.0	0.91 +/- 0.42
T10	47.7 +/-4.3	14.3 +/-2.1	2.58 +/- 1.36	0.287 +/- 0.051	0.148 +/- 0.026	173.0 +/- 17.0	2.02 +/- 0.06
T11	56.8 +/-15.2	23.2 +/- 11.7	1.67 +/- 0.09	0.354 +/- 0.056	0.113 +/- 0.026	147.0 +/- 29.7	2.11 +/- 0.40

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FSL 2: ^{1}H NMR- based metabolite profiling of tropane alkaloids in *Duboisia* spec.

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Abstract

Duboisia R.Br. (*Solanaceae*) is the main source of the tropane alkaloid scopolamine, which is an important precursor of various active pharmaceutical ingredients due to its anticholinergic properties. As only little is known about the metabolite composition among the different species, NMR-based metabolic profiling was done in order to elucidate primary and secondary metabolism in *Duboisia* especially focusing on the tropane alkaloid pathway. For this purpose, plants of five different genotypes (*Duboisia myoporoides*, *D. leichardtii* and hybrids of *D. myoporoides* and *D. leichhardtii*) were cultivated under strictly controlled conditions in climate chambers, leaf and root extracts were prepared and measured via ^{1}H NMR. 14 different metabolites could be identified using 1D- and 2D-NMR techniques. Principal component analysis of the NMR data allowed a clear distinction between *Duboisia* hybrids and the wild types, which could be again subgrouped in *D. myoporoides* and *D. leichhardtii,* based on the metabolites identified.

Keywords: ^{1}H NMR, Metabolomics, Scopolamine, Alkaloids, *Duboisia*, *Solanaceae*

Introduction

Duboisia R.Br., which belongs to the family of *Solanaceae*, is indigenous to Australia and used as principal source of the active substance scopolamine (Barnard, 1952; Palazón et al., 2008). Until today, the synthetic production of scopolamine is expensive and no alkaloid levels competitive to field grown plants could be achieved by overexpressing biosynthetic genes in regenerated plants or by using cell culture systems (Foley, 2006; Hashimoto, 2003). Thus, the industrial production providing scopolamine is largely based on agricultural field plant cultivation (Foley, 2006).

For optimizing the breeding process and the plant cultivation, ^{1}H NMR-based metabolic profiling was applied as it allows a deeper insight into the primary and secondary metabolism of *Duboisia* with special focus on the tropane alkaloid biosynthesis. Therefore, samples of different species (three different wild types and two hybrids of *Duboisia myoporoides* and *D. leichhardtii*) were analysed choosing roots and leaves for extraction, as scopolamine is biosynthesized in the roots from where it is transported to the leaves, its main storage location (Wink, 1987).

Materials and Methods

Plant material

All plants under investigation belong to the genus *Duboisia* R.Br., family *Solanaceae,* and were supplied by Boehringer Ingelheim (Germany). Wild types of *Duboisia myoporoides* R.Br. (A) and *Duboisia leichhardtii* F.Muell. (B, C) and hybrids of *Duboisia myoporoides* R.Br. and *Duboisia leichhardtii* F.Muell. (D,E) were cultivated in climate chambers under strictly controlled conditions at 25 °C exposed to 12 h of light per day with an intensity of 110 µmol/m^2·s. After sampling, all plant material was frozen immediately using liquid nitrogen and stored at –80 °C. After grinding in liquid nitrogen and freeze-drying, all samples were extracted and subsequently measured via ^{1}H NMR.

Extraction

Samples were prepared according to a protocol published by Kim et al. with slight changes (Kim, 2010). 20 mg of the freeze-dried material was weighed into a 2 ml - centrifuge tube. 0.5 ml of CD_3OD and 0.5 ml of D_2O, containing 0.29 mM TSP-*d4*, buffered with KH_2PO_4 (90 mM) and adjusted to pH 6.0 using 1.0 M NaOD, were added. After vortexing 1 min at room temperature and 5 min of ultrasonication, the samples were centrifuged for another 5 min at 13,000 g at room temperature and 300 µl of supernatant were filtered into a 3 mm - NMR tube.

Measurements

The NMR experimental parameters were chosen based on the protocol of Kim et al. (Kim et al., 2010). ^{1}H NMR spectra were measured at 25 °C on a 600 MHz DMX-600 spectrometer operating at 600.13 MHz and equipped with a TCI cryoprobe and Z-gradient system. The resulting spectra were manually phased and baseline corrected with the help of TopSpin (ver. 3.1 Bruker).

Results

^{1}H NMR- based metabolite profiling

By using CD_3OD-KH_2PO_4 buffer in D_2O (1:1, v/v) for extraction, a wide range of polar metabolites was covered, including sugars, amino acids and secondary metabolites like flavonoids or tropane alkaloids. As the NMR-signals were often overlapping, it was first necessary to select the characteristic signals of possible metabolite-candidates. Subsequently, all metabolites were further verified by using 2D-NMR techniques like ^{1}H-^{1}H-correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC). All in all, 14 different primary and secondary metabolites could be identified in the leaves of *Duboisia* using NMR data. Regarding the root extracts, only sugars like glucose and sucrose and traces of scopolamine could be detected due to the very low concentration of single metabolites.

Comparison of different genotypes

Comparing the wild types of *Duboisia myoporoides* (A) and *Duboisia leichhardtii* (B, C) with the hybrids of *Duboisia myoporoides* and *D. leichhardtii* (D, E) by using principal component analysis (PCA), a separation into three different groups is possible based on the metabolites identified. Principal component 1 (PC1) divides the samples into wild types and hybrids, whereas principal component 2 (PC2) further subgroups the wild types into *Duboisia myoporoides* and *D. leichhardtii*.

The loading plot displays the identified metabolites, which are responsible for the group differentiation into wild types and hybrids according to PC1. Scopolamine and signals, which could be generally assigned to tropane alkaloids, were found in higher concentrations in *Duboisia* hybrids. Sugars like glucose or sucrose and amino acids like proline or threonine were more present in the wild types *Duboisia myoporoides* and *D. leichhardtii*. Hence, the tropane alkaloid biosynthesis seems to be enhanced in case of the hybrids, especially the last step of the biosynthesis, namely the conversion of hyoscyamine via 6β-hydroxy-hyoscyamine to scopolamine. Hyoscyamine was significantly increased in the wild types, whereas the hybrids of *Duboisia* contained higher amounts in scopolamine. This could be due to a higher expression level or activity of the responsible enzyme, the hyoscyamine 6β-hydroxylase (H6H). But additional proteomic and transcriptomic data will be needed for a final assessment.

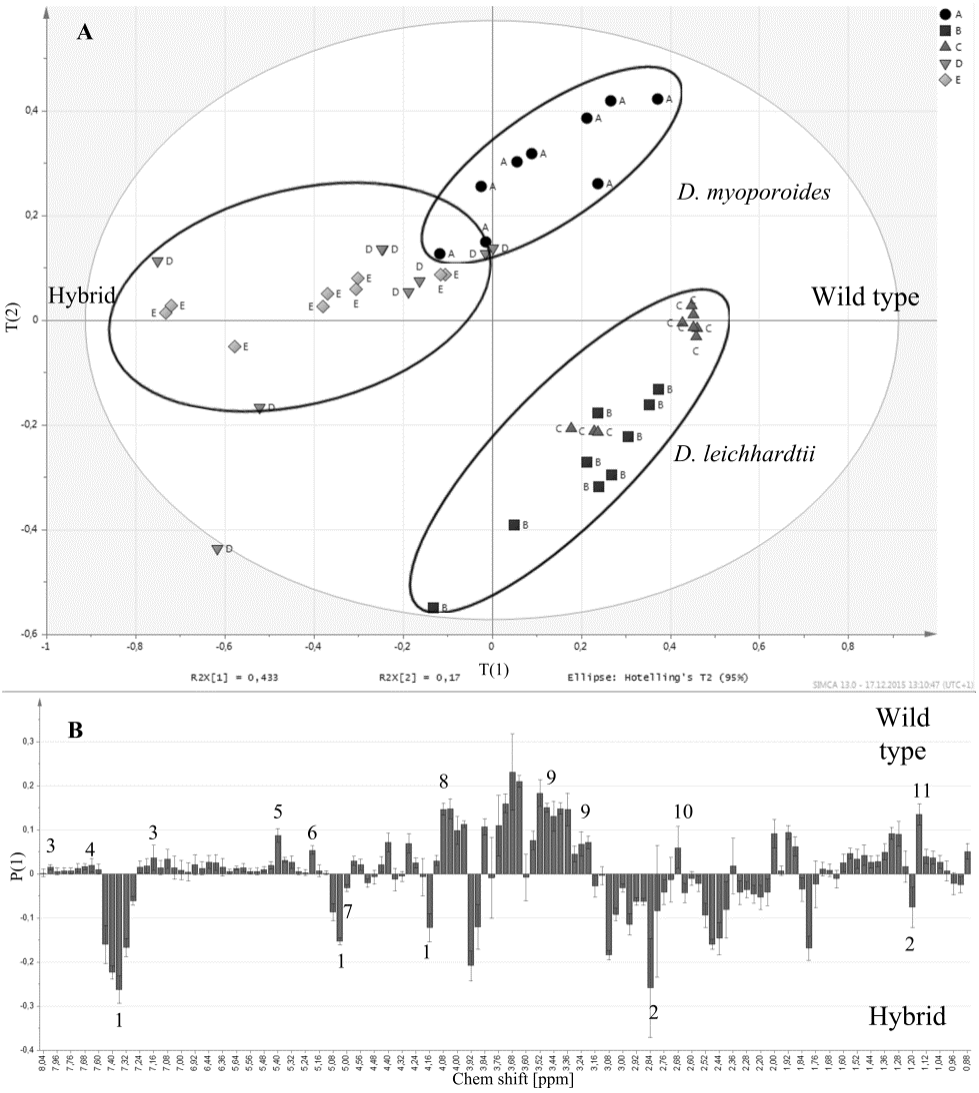

Figure 1 Score plot of PC1 and PC2 (A) and loading column plot of PC1 (B) as results of principal component analysis (PCA); data obtained by ^{1}H NMR comparing the leaf extracts of different genotypes (A-E) of *Duboisia*. Signals of: tropane alkaloids (1), scopolamine (2), scopoletin (3), quercetin (4), sucrose (5), glucose (6), 6β-hydroxy-hyoscyamine (7), proline (8), myo-inositol (9), hyoscyamine (10) and threonine (11).

This NMR analysis allows a fast and easy comparison of different samples of *Duboisia* by grouping them based on their metabolite composition. In addition, it can be applied in order to select promising genotypes and optimised cultivation conditions for the production of scopolamine.

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FSL 3: Recent achivements of the introduction and improvment of native medicinal plants in Iran

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Abstract

Iran is the country of different climates and rich genepool of different medicinal herbs. Both climate variation and available genetic resources, make possible the introduction and improvement of new plant varieties into agriculture. *Artemisia dracunculus* has been cultivated in different parts of Iran since unknown time. *Satureja rechingeri* is a wild endemic species growing in desert area of south west of Iran with annual rainfall of less than 250mm while, *Solidago virgaurea* and *Equisetum arvence* are native to north and northwest of Iran with more than 700 mm annual rainfall. Several experimets were conducted to introduce new varieties of these plants for economic and high quality plant material production in agricultural systems. Here some of the results are presented.

Keywords: Medicinal plants, Introduction, Tarragon, Wild Savory, Horsetail, Goldenrod

Introduction

In order to supply the demands of food and pharmaceutical industries with high quality plant material, improvement of *Artemisia dracunculus* and introduction of *Satureja rechingeri*, *Solidago virgaurea* and *Equisetum arvence* were performed.

Iran is the country of different climates with

French tarragon (*A. dracunculus* L.)

A. dracunculus, an important spice plant, is considered for its aromatic values in different food preparations or as a functional food or dietary supplement (Ribnicky et al., 2004). French tarragon is cultivated in different parts of Iran since unknown time for production of fresh herb, dried leaves or essential oils. An experiment was carried out during 2010-2015 to detect variability of morphological and volatile oil characters, rust resistance and DNA typing, and tolerance to salinity and drought stresses of cultivated accessions of Iranian *A. dracunculus*.

Wild Savory

Satureja rechingeri, an endemic herb of Iran, is characterized as a rich source of carvacrol and rosmarinic acid. In the framework of a domestication program, study of natural habitates, evaluation and selection of different genotypes and progeny testing of elite ones for general combining abilities of important traits were performed.

Goldenrod (Solidago virgaurea) and Horstail (Equisetum arvence)

Goldenrod, an herbaceous perennial plant, is widespread across Europe, North Africa and Asia. Herbal drugs of Goldenrod have been used to to increase the amount of urine and as adjuvant in treatment of minor urinary complaints. Morphological features and phytochemical quality of some Iranian natural populations of *S. virgaurea* were studied and compared with those of breeded European cultivars.

Horstail (Equisetum arvence)

Equisetum arvense, is a herbaceous perennial plant, native throughout the arctic and temperate regions of the northern hemisphere. Vegetative parts of *E. arvense* have been used in several cos-

metics and pharmaceutical preparations. Several *Equisetum* species are growing wild in Iran, of them *E. arvense* is normaly growing in higher altituede in north and northwest of Iran. Agromorphological and phytochemical charachteristics of some *E. arvense* Iranian populations were studied as a part of an introduction program.

Materials and Methods

Tarragon

Morphological, phytochemical, DNA (ISSR and SRAP) diversity and rust resistance of cultivated accessions of A. dracunculus was investigated.

Wild Savory (Satureja rechingeri)

A total of 85 samples of *S. rechingeri* were collected from seven natural populations in Iran and analyzed for morphological and phytochemical characters. Then, all populations were grown in the same environment, of them 58 talent and well stablished genotypes were selected and cloned. half-sib (HS) progenies of the 58 parent clones were obtained by polycross and were evaluated in a randomized complete block design (RCBD) with six replications in order to select the parents of a synthetic variety based on general combining abilities (GCA).

Goldenrod and Horstail

Diffrent populations of *S. virgaurea* and *E. arvense* were identified in the north of Iran and several individual plants were sampled to study their agro-morphological traits and accumulation of flavonoids and polyphenolic acids. Beside, two europian varieties of *S. virgaurea* were prepared and grown in the filed to study their adaptation and quality.

Results

Tarragon

High variability was recorded in plant height (37.22–59.62 cm), plant diameter (34.00 – 64.61 cm) and leaf area (0.39 – 1.78 cm^2). Leaf dry weight ranged between 7.93 g (for Qom2) and 27.19 g (in Yazd). The essential oil content ranged from 1.42 to 2.53 v/w. Analysis of the essential oil showed methyl chavicol (68.21 – 81.11 %), limonene (7.18 – 16.73 %) and terpinolene (0.01 – 7.68 %) as the main components. Both ISSR and SRAP methods were suitable for discriminating among accssions and the SRAP markers were more efficient. and preferable. The results of multiple regression analysis revealed statistically significant association between rust resistance and some molecular markers that they can provide clues for identification of the individuals with higher rust resistance. Results showed that all accessions of Abadeh, Neyshabour, Zarand, Esfahan1, Shahr-Rey, Yazd, Unknown2, Estahbanat, Ardestan, Tabriz, Dezfoul, Torbat-Heydarye and Shiraz did not show any infection. Total leaves, healthy leaves and infected leaves were numbered for each susceptible accession and then infected leaf percentage was estimated. Among susceptible accessions, Esfahan2 individual had the lowest infected leaf percentage (11.38 %) while Kermanshah showed the highest (74.79 %). Taragon accession also showed different susceptibility to both salinity and drought stresses.

Wild Savory (*S. rechingeri*)

Satureja rechingeri is growing in tropical and subtropical areas in the province of Khuzistan and Ilam, within longitude of 32 to 33 and latitude of 46 to 49 and altitude of 350 to 1100 m. It has been fully grows on rocky limestone and tissues. The highest coefficient of variation among all populations was obtained 43.01 % for leaf area. Lowest coefficient of variation was determined for

length and diameter sepal (16.93 % and 19.44 % respectively). Essential oil content was varied between 0.93 % to 6.2 % among populations. The essential oil composition was homogeneous as the main chemical component in oils of all the studied populations was carvacrol (89.2 – 96.2 %). Rosmarinicacid content of methanolic extracts had considerable variation varying from 0.54 to 7.29 % (w/w) of the dry matters based on qualitative and quantitative TLC analysis.

Evaluation of half-sib (HS) progenies of the 58 parent clones showed that highest narrow sense heritability was belong to plant diameter, plant height and main branches number while lowest value was obtained for the number of lateral branches. Additive variance was significant for main branch number, fresh weight, and dry weight, weight of leaves and flowers and plant height. By selection of 20 percent of the half-sib families, based on general combining ability for essential oil yield, families of F_{27}, Z_{12}, Z_{37}, F_{14}, E_{59}, E_{37}, Z_{26}, Z_{28}, K_{56} and G_{27} clones can be selected as parents of a synthetic variety.

Goldenrod and Horstail

It was possible to identify natural population of Goldenrod both in the jungles and in the rocky rangelands in the north of Iran. The growing sites were within altitude of 1300 to 1750 m. The plants were highly variable for their agromorphological traits. Plant heigh and dry weight were variable within 12 to 51 cm and 0.4 to 9.44 g/ plant, respectively. Flavonoids and polyphenolic acids were also variable among natural sites.

Populations of Horstail are growing within altitude of 600 - 2300 m. Height of horstail plants was variable from 15-39cm while plant dry weight was diffrent between 0.32 - 0.85 g/plant. Total phenolics, total flavonoids, Isoquercitroside content and silica percentage of diffrent populations were also variable giving the oppurtunity for selection.

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FSL 4: Agronomical and phytochemical evaluation of *Stevia rebaudiana* genotypes

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Abstract

The agronomical potential and the phytochemical variability of 18 genotypes of the Paraguayan plant *Stevia rebaudiana* have been investigated in Switzerland in order identify the best genotype for local cultivation. Over a two years period, yields in dry leaves ranged from 10 to 170 g m-2, with a percentage of leaves ranging from 53 to 75 %. HPLC analyses showed a notable variability in phytochemical composition, with stevioside content ranging from 0.3 to 7.9 % w/w and rebaudioside A from 0.3 to 6.5 % w/w. Cultivation of *S. rebaudiana* in Switzerland is feasible. With a density of 10 plants per m^2, the potential yields of dry matter are approximately 1-2 t ha^{-1}. The most productive genotypes (Pharmasaat, Hem Zaden, Stepa and Mediplant 3 and 11) will be submitted to the industry for organoleptic evaluation.

Keywords: stevioside, rebaudioside A, sweetener plant, Switzerland

Introduction

The Paraguayan shrub *Stevia rebaudiana* (Bertoni) Bertoni contains large amounts of calorie-free sweeteners that are up to 400 times sweeter than sucrose. The main ones are stevioside and rebaudioside A. Due to the high content of stevioside in some plants, *Stevia* also has a marked bitterness of licorice-like aftertaste that some manufacturers would like to avoid. The agronomic potential and the phytochemical variability of several genotypes have been investigated in Switzerland in order to identify the best genotype for local cultivation. The most productive genotypes will be submitted to the industry for organoleptic evaluation.

Materials and Methods

Plantlets of 21 genotypes (GAWI/Eustas, F/Eustas, Jelitto, Pharmasaat, Hem Zaden, Stepa and 15 Mediplant clones descending from seeds from the Botanical Garden in Ascunción, Paraguay) were planted in February 2013 in Conthey (480 masl, continental climate) in randomized blocks with a density of 10 plants per m^2. Dry matter yield and percentage of leaves were measured over three harvests (August 26 and October 18, 2013; October 10, 2015). Steviol glycoside content (stevioside and rebaudioside A) was estimated by UPLC based on the Waters Application Notes WA60128 and WA60129, with a detection at UV 200 nm.

Results

Dry leaf yield of all genotypes ranged from 10 to 170 g m-2, with a percentage of leaves ranging from 53 to 74 %. The genotypes Pharmasaat, Hem Zaden, Med1, Med3, Med11, Med15 and Med16 showed the best yields (Fig. 1). A great diversity of the steviol glycoside content was observed, with stevioside levels ranging from 0.3 to 7.9 % w/w, whereas rebaudioside A ranged from 0.3 to 6.5 % w/w (Fig. 2). The total steviol glycoside content was the highest in the genotypes Pharmasaat, Hem Zaden, Stepa and Mediplant 3 and 11. The stevioside-to-rebaudioside ratio was smaller than one for Hem Zaden, Stepa, Mediplant 6, 9, 11, 14 and 16, likely resulting in a less bitter after taste. In 2015, the genotypes GAWI and F from EUSTAS were compared to plants from seeds from Pharmasaat, showing that F has the lowest content in steviosid and the highest content in

rebaudiosid A, thus being much less bitter (Fig. 3). These results are comparable to previous studies (ANDOLFI *et al*., 2006; LANKES & MORA ZABALA, 2011).

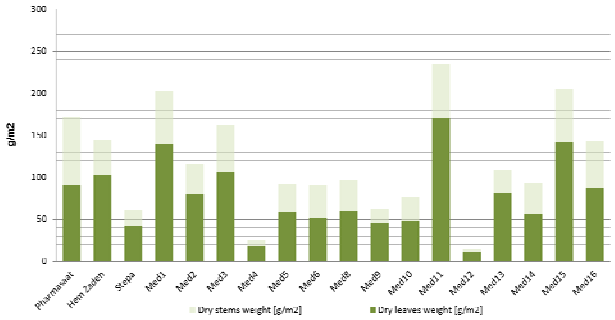

Fig. 1. Yields in dry stem and leaf matter [g/m2] for 18 genotypes of Stevia rebaudiana over two harvests. The most productive genotypes are Pharmasaat, Hem Zaden and Mediplant 1, 3, 11, 15 and 16.

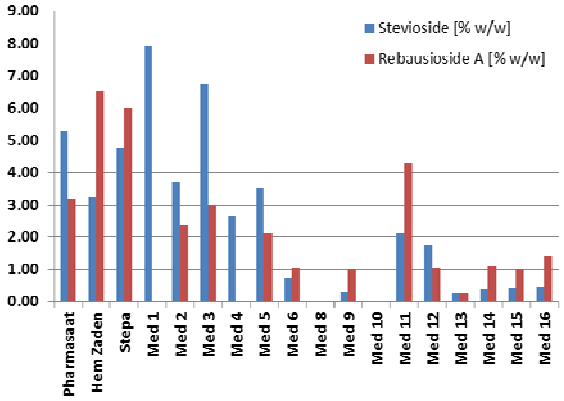

Fig. 2. Steviol glycosides content for 18 genotypes of Stevia rebaudiana. The global content in steviol glycosides was the highest in the genotypes Pharmasaat, Hem Zaden, Stepa and Mediplant 3 and 11. The stevioside/rebaudioside ratio was <1 for Hem Zaden, Stepa, Mediplant 6, 9, 11, 14, 15 and 16, likely resulting in a less bitter after taste.

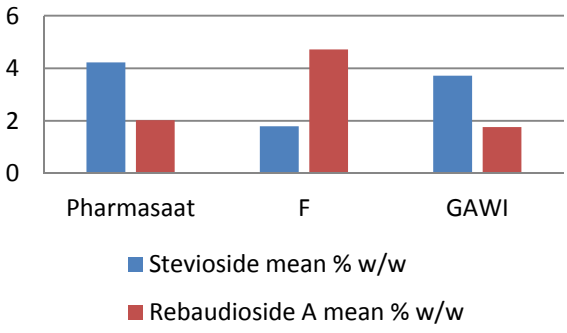

Fig. 3. Steviol glycosides content for 3 genotypes of Stevia rebaudiana selected in 2015, one harvest with four repetitions. Plants from Pharmasaat seeds and the GAWI clone from EUSTAS have more stevioside while the clone F from EUSTAS has more rebaudioside A.

Cultivation of *Stevia rebaudiana* is feasible in alpine areas. With a planting density of 10 plants/m^2, the potential yield of dry matter is 2.5 - 3.0 t ha^{-1} (leaves and stems) and 1 - 2 t ha^{-1} (leaves only) over two harvests (August 26 and October 18), which is much lower than the highest quantity of leaf dry matter of 3.6 t ha^{-1} estimated by Andolfi et al. (2006) for the most productive genotype in the first year in Central Italy. Under Swiss conditions in the first year, the theoretical yield range is 3-158 kg ha^{-1} of stevioside and 3-130 kg ha^{-1} of rebaudioside A. Pharmasaat, Hem Zaden, Stepa and Mediplant 3 and 11 showed the highest global content in steviol glycosides. The clone F from EUSTAS showed the lowest stevioside-to-rebaudioside A ratio, resulting in a less bitter after-taste.

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FSL 5: Temporal variation of essential oils in dried flower of two genotypes of Damask rose (*Rosa damascena* Mill.)

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Abstract

Damask rose (*Rosa damascena* Mill.) is the most important species in the production of rose water, perfumes and essential oils (EOs), perfumery and pharmaceutical industry applications. In order to exploit the most of this precious Iranian flower, research was conducted on two damask rose genotypes (Fars 1 and Fars 2) in six development stages in terms of EOs content and composition analysis. Results showed that all development stages of damask rose genotypes had significant differences in the EOs content. The highest EOs content was obtained from "Fars 2" genotype. It was found that the oil obtained from the dried petals of Fars 2 genotype contained a higher percentage of citronellol (C), geraniol (G) and phenylethyl alcohol (PEA) as well as a suitable ratio of C/G with the value of 2.464 as compared with Fars 1 genotype (5.546). In the last stages of flower bud development in both genotypes, the sum of the main hydrocarbons increased while citronellol content decreased, therefore essential oil quality dramatically declined. EO quantity was lower in the middle stages of flower bud development (stages 3 and 4) as compared to the final stages (stage 5 and 6), but its quality was superior in the former stages. Generally, it seems that "Fars 2" damask rose, as a promising genotype, can be used for EO production.

Keywords: Damask rose, Developmental stage, Genotype, Essential oils.

Introduction

Damask rose (*Rosa damascena* Mill.) is the most important aromatic species in the Rosaceae family (Baydar and Bayder, 2013). It is widely distributed throughout Syria, Morocco, Iran to Australia. Its fragrant flowers are used in the foods as rose-water, marmalade and pastry while its precious essential oil has a worldwide growing demand (Karami et al., 2012). Two major rose oil production areas familiar to the world are rose cultivations in Isparta of Turkey and Kazanlik of Bulgaria (Baydar and Bayder, 2013). Besides those regions, Fars province of Iran is another leading producer of damask rose which 8598 tons of flowers are produced annually in 6149 hectares of gardens (Iranian Ministry of Agriculture, 2012). Generally, essential oil amount of rose flowers is very little (ca. 0.03-0.04 %). Approximately 3500 kg of fresh top-quality rose flowers are harvested in the early morning of harvesting time which yield just 1 kg rose oil following hydro-distillation in the distilleries (Baydar and Bayder, 2013). Traditionally, the full-bloom stage is the most suitable stage of flowering for rose water or rose oil extraction. However, recent investigations indicated that rose oil yield distilled from rose buds was the same or higher for the same weight of flower material when compared to fully blown flowers (Rusanov et al., 2012).The objective of this research was to elucidate the effect of different genotypes of Fars damask roses and their floral development stages on quantity and quality of EO production.

Materials and Methods

Plant material

Flowers of two distinct genotypes of the Iranian damask rose were harvested during six stages of flower development from plants grown at the Estahban Agricultural Research Station in the Sothern of Iran. These two genotypes were recognized by genetic markers and other characterization as illustrated previously (Karami et al., 2013; Babaei et al., 2007). The descriptions of Picone et al. (2004) were used as a principle for each stage as temporally characteristics. The flowers of each

stage were dried in a dark room under room temperature conditions (25-30 °C) for a week and then packed in cardboard box and kept in a dark and cool place for further experiments.

Analysis of the oil

The aerial parts were air-dried at ambient temperature in the shade and hydrodistilled by using a Clevenger-type apparatus for 3 h. The oil was dissolved in n-hexane (Merck), dried over anhydrous sodium sulfate and stored at 4 °C ± 2 °C. GC analysis was performed using an Agilent gas chromatograph series 7890-A with a flame ionization detector (FID). GC-MS analysis was carried out by use of Agilent gas chromatograph equipped with fused silica capillary HP-5MS column coupled with 5975-C mass spectrometer. The constituents of the essential oil were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C8-C25) and the essential oil on a HP-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature (ADAMS, 2007).

Results

In general, the investigated damask rose flowering stage (Temporal Variation) and genotypes had a significant effect on the essential oils contents and composition as discussed more below.

Damask rose Genotypes

Essential oil content

The EO content of dried flowers of damask rose was extracted from two distinct genotypes of damask rose. Statistical analysis demonstrated that damask rose genotypes had significant differences in the EOs content. The highest EOs content (0.208 % (w/w)) was obtained from "Fars 2" genotype (Table 1).

Essential oil composition

The EOs of each genotype were collected for periods of 3 hrs and analyzed by GC (FID) and GC/MS analysis (Table 2). The qualitative and quantitative composition of the EOs from the dried flower of each genotypes elucidated that the EOs was auxiliary affected by genotypes. ANOVA analysis confirmed that damask rose genotypes had differences in the EOs composition at 5 % level of significance using LSD test (Data was not shown). However in both genotypes, the sum of the main hydrocarbons increased while citronellol content decreased, therefore essential oil quality dramatically diminished. It was found that the oil obtained from the dried petals of Fars 2 genotype was be qualified for the higher percentage of citronellol (C), geraniol (G) and phenylethyl alcohol (PEA) and as well as a suitable ratio of C/G with the value of 2.464 as compared with Fars 1 genotype (5.546). This result could be endorsed by the role of genotypes in the EOs qualification.

Temporal Variation

Essential oil content

The results revealed that the effects of flowering stage (Temporal Variation) on EO content were significant ($p \leq 0.01$) during flower development. The maximum oil content was obtained from stage 5 (0.354 % (w/w).

Essential oil composition

The composition of the EOs from the dried flower of both genotypes clarified that the EOs was affected by flower development. In the last stages of flower bud development in both genotypes

the sum of the main hydrocarbons increased while citronellol content decreased, therefore essential oil quality dramatically declined. Although EO quantity was lower in the middle stages of flower bud development (stages 3 and 4) as compared to the final stages (stage 5 and 6). But its quality was superior in the former stages. The results of the present investigation revealed significant differences in the essential oil profiles of Iranian Damask rose. The differences in the oil compositions could be attributed to their genetic variability and flower stage. Generally, it seems that "Fars 2" damask rose, as a promising genotype, can be used for EO production and they could be selected as a good genetic source in breeding programs.

Tab. 1 The relative percentage of essential oils from two genotypes (Fars1 and Fars2) of Rosa damascena at six flower developmental stages used in this study.

Components	RI*	Genotype	Flowering Stage					
			1	2	3	4	5	6
Phenyl ethyl alcohol	1091	Fars 1	-	-	-	-	-	-
		Fars 2	-	-	0.302	0.061	0.167	0.041
Citronellol	1210	Fars 1	0.229	3.832	1.809	0.840	0.675	0.536
		Fars 2	0.694	3.284	3.704	1.004	2.201	2.737
Geraniol	1230	Fars 1	0.090	-	-	0.351	0.301	0.213
		Fars 2	0.045	0.197	0.605	0.029	3.530	1.120
Methyl eugenol	1390	Fars 1	-	-	4.171	-	-	-
		Fars 2	0.032	0.493	0.957	0.118	0.301	0.198
Heptadecane	1692	Fars 1	0.217	1.604	2.361	1.067	0.524	0.481
		Fars 2	2.368	3.349	2.377	1.274	1.572	0.424
Nonadecane	1911	Fars 1	1.322	1.616	3.498	0.545	0.187	0.233
		Fars 2	12.099	6.951	27.208	16.416	3.794	5.222
Heneicosane	2126	Fars 1	0.686	0.930	1.718	1.260	8.168	5.815
		Fars 2	26.202	17.849	22.032	19.217	23.072	7.999

*RI: Retention indices analyzed on HP-5 column -: not detected

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FSL 6: Evaluation of lemon balm (*Melissa officinalis*) collections

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Abstract

Lemon balm (*Melissa officinalis* L.) is a well-known medicinal and aromatic plant and of increasing importance resulting in rising growth area in Germany. Because of its proven sedative, spasmolytic and antiviral effects, it is often used in watery or alcoholic extracts for self-medication or pharmaceutical and medical purposes. This therapeutic effect is due to the content of essential oil and phenolic carbon acids, like rosmarinic acid. Improved knowledge on the genome structure, number of chromosomes in connection with the taxonomical structure of balm is indispensable for improved new varieties. A set of 120 balm accessions was evaluated for the variability of essential oil content and composition as well as the content of rosmarinic acid. These accessions came from the Bavarian State Institute for Agriculture at Freising, Germany (LfL), the federal *ex-situ* collection of agricultural and horticultural plants of the Leibniz Institute of Plant Genetics and Crop Plant Research at Gatersleben, Germany (IPK) and the N.I. Vavilov Institute of Plant Industry at St. Petersburg, Russia (VIR). Out of these 120 accessions 40 balm accessions (*M. officinalis*) were characterized by flow cytometry and FISH (18/25S and 5S rDNA) to determine the chromosome number and ploidy level. Three different types were found: diploid genotypes with 2n = 2× = 32 chromosomes; tetraploid 2n = 4× = 64 chromosomes and triploid 2n = 3× = 48 chromosomes. Therefore a haploid base number of × = 16 chromosomes is likely. For the first time triploid accessions are described, which were sterile but cytologically and morphologically stable for many years. Triploids express better winter hardiness and regeneration after harvesting cuts as well as bigger leaves and internodes. We characterized three chemotypes (ct.) of essential oil: ct. citral, ct. germacrene D and ct. β-caryophyllene oxide. In addition autotetraploid material from diploid ct. citral was developed for this characterization and belongs also to ct. citral.

Keywords: Balm, chemotype, haplotype, FISH

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FSL 7: HPLC analysis of anthocyanins and flavonols and expressions of different copies of *F3´5´H* in grapevine transgenic lines

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Abstract

Transgenic lines of grapevine containing silencing constructs of *F3´5´H* based on ihpRNA mechanism were studied for their flavonoids and for the expression of *F3´5´H* gene copies. 83-89 % of transgenic lines showed various degrees of silencing, and increase of di-hydroxylated anthocyanins and kaempferol were observed in the leaves of transgenic lines.

Keywords: *Vitis vinifera*, anthocyanin, flavonol, *F3´5´H*, silencing

Introduction

Grape berries are valuable nutraceuticals due to the presence of different types of flavonoids such as anthocyanins and flavonols. Beside, flavor is an important popularity character of Grape berries and their byproducts. *F3´5´H* and *F3´H* are the major genes for flavonoid biosynthesis. In order to study *F3´5´H* and *F3´H* functions and their interaction in the grapevine flavonoids biosynthetic pathway, transgenic lines containing silencing constructs of *F3´H* and *F3´5´H*/*F3´H* were created using ihpRNA mechanism. In this paper the results of the flavonoids analysis and gene expression assays of *F3´5´H* regenerated transgenic lines are presented.

Materials and Methods

Among several grapevine regenerated lines, independent single copy lines were identified through southern blot analysis. The anthocyanin and flavonol compositions (HPLC analysis was performed according to Downey and Rochfort, 2008) as well as expressions of different copies of F3´5´H were studied in the leaves of transgenic lines in three replicates.

Results

Among studied *F3´5´H* copies in the leaves, the highest suppression was observed in *F3'5'H-f, F3'5'H-g, F3'5'H-j, F3'5'H-1*and *F3'5'H-p,* respectively. In all of *F3´5´H* copies, except *F3'5'H-p,* 83-89 % of transgenic lines showed various degrees of silencing (Fig 1). Increase of di- and decrease of trihydroxylated anthocyanins and increase of kaempferol and decrease of myricetin were observed in the leaves of transgenic lines in comparison to control plants (Tables 1 and 2). It seems that the biosynthetic pathway has shifted toward the increase of cyanidin, peonidin and caempherol due to inactivation of *F3´5´H*.

Table 1 Anthocyanin compositions (%) of *F3´5´H* transgenic lines and cv. Shiraz

Sample code	Delphinidin		Cyanidin		Petunidin		Peonidin		Malvidin	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
F2	1.68	0.04	0.00	0.00	0.00	0.00	94.65	0.15	3.67	0.19
F4	1.52	0.27	0.00	0.00	0.00	0.00	95.89	0.18	2.60	0.11
F6	0.35	0.02	0.16	0.00	0.00	0.00	92.89	0.11	6.61	0.12
F10	2.52	0.19	0.90	0.10	0.00	0.00	95.02	0.18	1.56	0.30
F13	4.17	0.33	0.85	0.07	0.00	0.00	94.29	0.31	0.69	0.10
F16	7.64	0.10	6.21	0.37	0.00	0.00	86.15	0.47	0.00	0.00
F17	0.76	0.18	0.38	0.04	0.00	0.00	91.41	0.24	7.46	0.02
F19	0.74	0.04	0.39	0.03	0.00	0.00	95.37	0.08	3.50	0.04
F24	4.64	0.62	7.52	0.07	0.00	0.00	87.84	0.68	0.00	0.00
F28	2.72	1.12	3.66	0.19	0.00	0.00	93.36	0.83	0.26	0.14
F29	7.44	0.92	1.17	0.09	0.00	0.00	91.39	0.96	0.00	0.00
F30	0.00	0.00	2.07	0.12	0.00	0.00	96.88	0.19	1.05	0.10
F35	9.14	0.32	0.93	0.10	0.00	0.00	89.92	0.42	0.00	0.00
F38	0.81	0.03	0.56	0.04	0.00	0.00	95.87	0.18	2.76	0.11
F41	0.61	0.01	0.30	0.02	0.00	0.00	95.53	0.03	3.56	0.02
F43	7.43	0.37	3.29	0.05	0.00	0.00	89.27	0.42	0.00	0.00
F44	6.15	0.17	4.12	0.14	0.00	0.00	89.74	0.22	0.00	0.00
F47	7.88	0.87	5.11	0.47	0.00	0.00	87.02	1.34	0.00	0.00
F21 (control)	6.75	0.16	0.59	0.06	0.87	0.15	55.67	1.15	36.13	1.17
Shiraz (control)	7.75	0.05	0.67	0.05	1.09	0.07	55.25	1.09	35.25	1.24

Table 1 Anthocyanin compositions (%) of *F3´5´H* transgenic lines and cv. Shiraz

Sample code	Dihydroxylated		Trihydroxylated		Glucoside		Acetylglucoside		Coumaroyl		Petunidin Caffeoylglucoside	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
F2	94.65	0.15	5.35	0.15	18.09	0.85	14.60	0.41	67.31	1.23	0.00	0.00
F4	95.89	0.18	4.11	0.18	23.57	1.35	13.02	0.39	63.41	1.74	0.00	0.00
F6	93.05	0.11	6.95	0.11	20.11	0.35	10.40	0.13	69.49	0.47	0.00	0.00
F10	95.92	0.12	4.08	0.12	25.65	0.71	12.47	0.45	61.88	1.03	0.00	0.00
F13	95.14	0.37	4.86	0.37	23.00	0.99	13.58	0.24	63.41	0.86	0.00	0.00
F16	92.36	0.10	7.64	0.10	43.92	1.88	13.91	0.41	42.17	2.26	0.00	0.00
F17	91.79	0.20	8.21	0.20	17.83	0.49	9.31	0.32	72.86	0.78	0.00	0.00
F19	95.76	0.06	4.24	0.06	23.33	0.39	10.52	0.08	66.15	0.43	0.00	0.00
F24	95.36	0.62	4.64	0.62	43.16	1.10	11.23	0.90	45.61	1.41	0.00	0.00
F28	97.02	0.99	2.98	0.99	37.61	0.79	11.32	1.09	51.07	1.67	0.00	0.00
F29	92.56	0.92	7.44	0.92	33.63	1.54	16.12	1.49	50.25	2.85	0.00	0.00
F30	98.95	0.10	1.05	0.10	35.98	0.72	7.33	0.25	56.69	0.57	0.00	0.00
F35	90.86	0.32	9.14	0.32	29.66	1.11	17.91	0.82	52.43	1.40	0.00	0.00
F38	96.43	0.15	3.57	0.15	18.82	0.32	12.02	0.19	69.16	0.29	0.00	0.00
F41	95.83	0.01	4.17	0.01	18.01	0.16	13.63	0.11	68.36	0.27	0.00	0.00
F43	92.57	0.37	7.43	0.37	26.74	0.64	16.93	0.82	56.33	1.44	0.00	0.00
F44	93.85	0.17	6.15	0.17	33.74	1.35	13.41	0.15	52.85	1.32	0.00	0.00
F47	92.12	0.87	7.88	0.87	33.16	0.73	18.21	1.63	48.63	1.20	0.00	0.00
F21 (control)	56.25	1.09	43.75	1.09	37.19	0.51	16.35	0.60	46.47	1.10	0.00	0.00
Shiraz (control)	55.92	1.14	44.08	1.14	40.28	0.48	17.48	0.03	42.24	0.45	0.00	0.00

Table 2 Flavonol compositions (%) of *F3´5´H* transgenic lines and cv. Shiraz

Sample code	Myricetin -3-O-glucoside		Quercetin -3-O-glucuronide		Quercetin -3-O-glucoside		Laricitrin -3-O-galactoside		Kaempferol -3-O-glucoside	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
F2	0.00	0.00	75.46	0.57	20.67	0.47	0.15	0.07	0.37	0.00
F4	0.00	0.00	70.68	0.56	25.10	0.38	0.33	0.03	0.30	0.05
F6	0.14	0.02	77.32	0.30	18.23	0.26	0.00	0.00	0.19	0.02
F10	0.00	0.00	66.15	0.51	29.56	0.46	0.00	0.00	0.65	0.05
F13	0.00	0.00	64.66	1.31	30.36	0.45	0.00	0.00	0.60	0.25
F16	0.09	0.01	62.43	0.55	34.54	0.53	0.00	0.00	0.28	0.00
F17	0.12	0.01	75.56	0.49	19.56	0.34	0.00	0.00	0.26	0.02
F19	0.00	0.00	74.21	0.47	22.28	0.41	0.00	0.00	0.20	0.00
F24	0.09	0.01	63.03	0.93	33.66	0.88	0.00	0.00	0.30	0.01
F28	0.07	0.01	55.68	0.61	41.03	0.47	0.00	0.00	0.37	0.02
F29	0.00	0.00	58.37	0.67	37.34	0.47	0.00	0.00	0.52	0.01
F30	0.10	0.01	54.17	0.76	41.31	0.44	0.00	0.00	0.54	0.00
F35	0.00	0.00	50.93	0.64	45.33	0.90	0.00	0.00	0.61	0.07
F38	0.11	0.01	64.44	0.46	31.43	0.26	0.61	0.38	0.32	0.02
F41	0.00	0.00	66.95	0.15	28.32	0.12	0.00	0.00	0.39	0.06
F43	0.00	0.00	55.34	0.25	42.50	0.24	0.00	0.00	0.23	0.01
F44	0.05	0.01	54.95	0.42	41.58	0.41	0.00	0.00	0.36	0.00
F47	0.15	0.02	56.49	0.70	39.38	0.34	0.00	0.00	0.49	0.01
F21 (control)	0.21	0.01	83.78	0.47	13.00	0.35	0.00	0.00	0.00	0.00
Shiraz (control)	0.68	0.05	67.86	0.37	29.73	0.41	0.00	0.00	0.17	0.00

Table 2 Flavonol compositions (%) of *F3´5´H* transgenic lines and cv. Shiraz

Sample code	Laricitirin -3-O-rhamnose -7-O-trihydroxycin-namicacid		Kaempferol-3-O-caffeoylate		Isorhamnetin -3-O-glucoside		Syringetin -3-O-galactoside	
	mean	SE	mean	SE	mean	SE	mean	SE
F2	0.00	0.00	1.49	0.03	1.87	0.02	0.00	0.00
F4	0.00	0.00	1.50	0.11	2.09	0.07	0.00	0.00
F6	0.00	0.00	1.31	0.03	2.81	0.02	0.00	0.00
F10	0.00	0.00	2.41	0.02	1.23	0.08	0.00	0.00
F13	0.00	0.00	3.21	0.56	1.16	0.26	0.00	0.00
F16	0.00	0.00	1.52	0.00	1.13	0.00	0.00	0.00
F17	0.00	0.00	1.66	0.17	2.85	0.02	0.00	0.00
F19	0.00	0.00	1.10	0.04	2.21	0.02	0.00	0.00
F24	0.00	0.00	1.71	0.03	1.21	0.00	0.00	0.00
F28	0.00	0.00	1.93	0.02	0.93	0.16	0.00	0.00
F29	0.00	0.00	2.75	0.02	1.02	0.19	0.00	0.00
F30	0.00	0.00	2.89	0.03	1.00	0.32	0.00	0.00
F35	0.00	0.00	2.40	1.01	0.73	0.04	0.00	0.00
F38	0.00	0.00	1.68	0.01	1.41	0.02	0.00	0.00
F41	0.00	0.00	2.51	0.03	1.83	0.01	0.00	0.00
F43	0.00	0.00	1.56	0.01	0.37	0.01	0.00	0.00
F44	0.00	0.00	1.95	0.01	1.12	0.00	0.00	0.00
F47	0.00	0.00	2.44	0.02	1.05	0.37	0.00	0.00
F21 (control)	0.00	0.00	0.39	0.09	2.63	0.04	0.00	0.00
Shiraz (control)	0.00	0.00	1.04	0.00	0.52	0.01	0.00	0.00

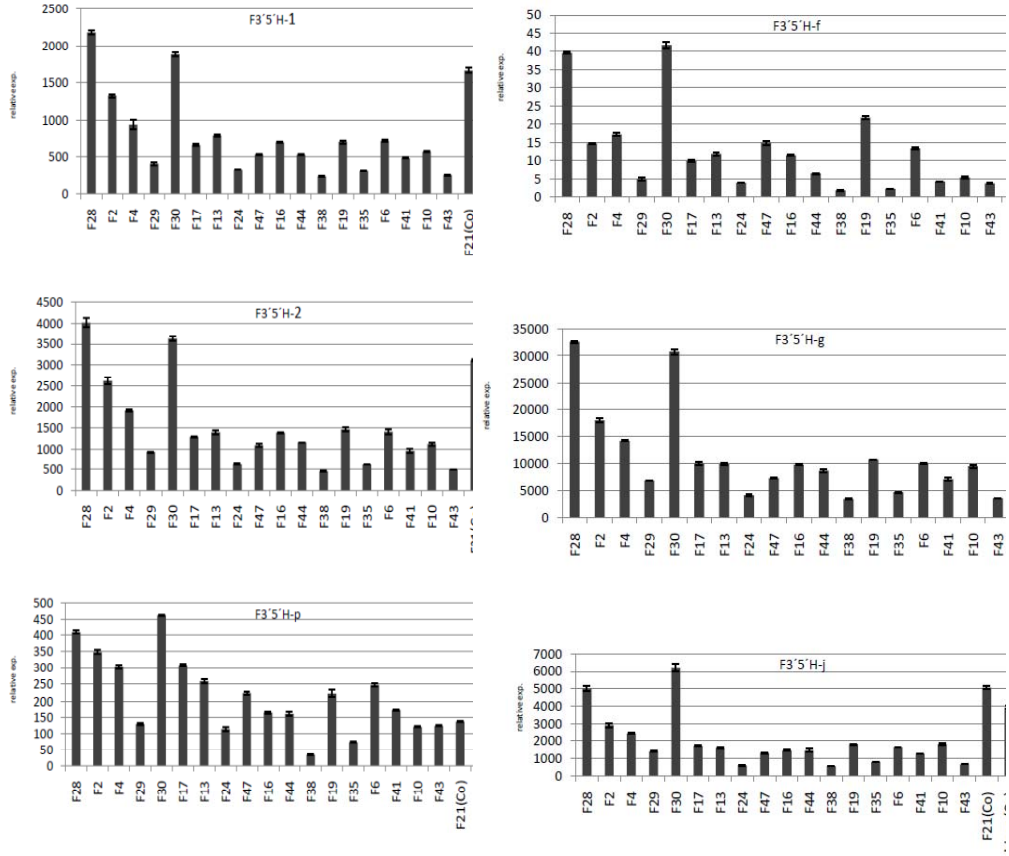

Fig1 Relative expression of different copies of *F3´5´H* studied in transgenic lines and cv.Shiraz

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Session G: CBD, Nagoya Protocol, EU regulations

GPL 1: Why and how did the Nagoya Protocol evolve?

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In 1992 the Convention on Biological Diversity (CBD) was adopted as the first international binding instrument on biodiversity, based on a new paradigm, i.e. national sovereignty over genetic resources. The concept of access and benefit-sharing was based on this new principle of sovereign rights. It assumed that access to genetic resources should be provided on conditions mutually agreed between the providing country and the recipient. The principle of sovereign rights was motivated by the increasing use of intellectual property rights to protect biodiversity-based products, mainly by users in developed countries.

In implementing the concept of access and benefit-sharing, provider countries soon discovered that it was difficult for them to follow up the use of the provided genetic resources upon their export to foreign countries, and hence it was impossible to monitor if users complied with the agreed obligations. This weakness was one of the reasons why the number of agreed international exchanges of genetic resources remained small, and access as well as benefit-sharing was not substantially realised, in conflict with the objectives of the CBD. Therefore, at the World Summit on Sustainable Development in Johannesburg in 2002 it was decided to negotiate within the framework of the CBD an international regime to promote and safeguard the fair and equitable sharing of benefits arising out of the utilization of genetic resources. In 2010 the Nagoya Protocol on Access and Benefit-sharing was adopted, creating a role for Parties to the Protocol to monitor compliance with agreed obligations by users of genetic resources in their countries, in addition to more explicit obligations of Parties to provide rules for access and for users to respect such rules. Furthermore, the Protocol contains similar provisions on accessing traditional knowledge associated with genetic resources as for the genetic resources themselves.

Whereas membership of the CBD is currently almost universal, the number of Parties to the Nagoya Protocol, in force since 12 October 2014, is rapidly increasing, now standing at 71. The Nagoya Protocol is binding to states, the Parties, whereas the EU Regulation 511/2014 is binding to all users in the European Union.

Workshop: Pyrrolizidine alkaloids – a problem?

WSL 1: Assessment of potential contamination of herbal medicinal products with PA: Activities of the German industry

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Abstract

Since July 2013 it has become evident that medicinal plant material may be contaminated by pyrrolizidine alkaloid (PA)-containing weeds e.g. *Senecio* species (BfR, 2013; Mulder et al. 2015). This is a big challenge for growers and manufacturers with regard to the precise qualitative and quantitative determination of the contaminants as well as to their reduction. Already at an early stage, the German herbal medicinal products industry has taken measures in order to avoid and/or reduce PA contamination as far as possible. These measures consist e.g. in the collection of data, participation in research projects and the adoption of a „Code of Practice" (BAH and BPI, 2015) that was elaborated together with herb growers. This document provides a framework for the implementation of individual measures in pharmaceutical companies along the entire process chain from cultivation up to control and release of the finished product.

The EMA Herbal Medicinal Products Committee (HMPC) had published its final „Public Statement" on the assessment of PA-containing herbal medicinal products in December 2014 (EMA/HMPC, 2014). It concludes that the exposure to PA should be kept as low as possible and sets a daily limit of 0.35 µg PA. E.g. in Germany, the health authority (BfArM, 2016) implemented a transitional limit of 1.0 µg PA daily, considering the fact that implementation of a lower limit for all medicinal plants did not seem realistic. Industry had argued that due to worldwide cultivation and season-dependent production processes, a short-term reduction of PA contamination at all sites is impossible.

The complexity of the problems requires an intensive co-operation of agriculture, industry, health authorities and scientific societies. In this respect, ongoing and new research projects on the occurrence and reduction of PA-containing weeds as well as the toxicological assessment of PA play an important role. The target is a continuous and sustainable further reduction of PA contamination and to guarantee further production of products which are safe and of high and consistent quality.

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Poster

P 1: Novel insights improve cryopreservation of the *Mentha* genebank collection

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Abstract

The IPK Gatersleben houses a mint collection of 286 accessions. More than 50 % of these accessions do not produce seeds and can only be maintained in the field, *in vitro* or in cryopreservation. Routine application of mint cryopreservation was started at IPK in 2006. This went along with the development of a simple droplet-vitrification protocol using *In vitro* plants as source material, the plant vitrification solution PVS 2 as cryoprotectant and aluminum strips as carrier material. Recently, the number of accessions exceeded 130, hence about 17 mint species are safely cryopreserved and show on an average 60 % regeneration after rewarming. Highest plant regrowth, up to 100 %, was achieved when *In vitro* plants coming from 10 °C cold storage were prepared for nodal culture and cultivated under changing temperatures at 25 °C/-1 °C. Under these conditions multiplication and hardening was realized in a short-term period of maximum 2 weeks. Genotype, incubation period of explants in loading solution (20 min-120 min) and in PVS2 for 20-40 min at room temperature had only a minor impact on overall regeneration. Therefore, factors determining successful cryopreservation are as important as high initial plant quality, precise shoot tip preparation, the avoidance of endophyte outbreak after rewarming by controlled climatic condition. The developed method is simple and applicable to all mint accessions.

P 2: The application of multi-shoots cultures in micropropagation of willow herb (*Chamaenerion angustifolium* (L.) Scop.)

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Abstract

Willow herb (*Chamaenerion angustifolium* (L.) Scop. syn. *Epilobium angustifolium* L.) from Onagraceae family is a valuable medicinal plant that has been used in the treatment of urogenital disorders including BPH (Benign Prostatic Hypertrophy). The raw material is a rich source of polyphenols as well as steroids, triterpenoids and fatty acids. The extracts show pharmacological activities: anti-androgen, anti-proliferative, anti-inflammatory, antioxidant, antibacterial and analgesic properties. Due to frequent interspecific hybridization, plants collected in the wild display a diverse and variable content of active compounds. This poses a challenge in obtaining high quality and homogenous raw material. Application of the *in vitro* cultures and micropropagation techniques may offer a solution for alternative methods of cultivation. This work presents preliminary results of the implementation of *Ch. angustifolium in vitro* cultures to obtain raw material for the first time. Sterile seedlings were donors of explants, which were used for induction of multi-shoots culture according to a modified Turker's protocol. Six different genotypes (lines) originating from root explants were chosen for clonal propagation. Efficiency of the elaborated method was 16 – 20 shoots per explants. Finally, over 3000 acclimatized plants were obtained and used for field crops.

Keywords: willow herb, *in vitro* culture, micropropagation, multi-shoots cultures.

Introduction

Willow herb (*Chamaenerion angustifolium* (L.) Scop. syn. *Epilobium angustifolium* L.) (Onagraceae family) can be found in Europe, Western Asia and North America. According to traditional folk medicine, its herb and roots were used in the treatment of gastrointestinal disorders, skin diseases and also of prostate, kidney and urinary tract disorders (Vogl et al., 2013). The raw material (*Epilobii herba*) is a rich source of polyphenols (flavonoids, phenolic acids, and tannins), steroids, triterpenoids and fatty acids (Granica et al., 2014). Oenothein B (macrocyclic ellagotanoid) is a major constituent of plant material (2 % - 14 %) and has been regarded as one of main active compounds. Anti-proliferative and antioxidant activity of the *Epilobium* extracts has been revealed (Vitalone et al., 2001, 2003; Kiss et al., 2006). The studies on human prostate cancer cell lines (LNCP) have confirmed the anti-proliferative and anti-cancer effect of oenothein B (Stolarczyk et al., 2013). The studies on willow herb extracts have induced growing interest in therapeutic potential of the *Epilobium* plants and their application in the treatment or prevention of BPH and other diseases. The great interest resulted in increasing demand on the raw material for the pharmaceutical and food industries. Nowadays, the crop plantations provide the majority of the raw material used for the medicinal or food products and only minority of them originate from the wild. It allows for obtaining large batches of high quality material and meeting the strict requirements for the medicinal products released on market. In case of *Ch. angustifolium* the raw material has been sourced from the wild. Due to frequent interspecific hybridization, the wild plants display a diverse and variable content of active compounds. Application of the *in vitro* cultures and micropropagation of selected genotypes offers an alternative way for traditional field cultivation. This work presents the preliminary results, where *in vitro* cultures and micropropagation technique were used for the first time in order to obtain willow herb raw material for the production of a dietary supplement used in the BPH prevention.

Materials and Methods

Ch. angustifolium seeds from the collection of Garden of Medicinal Plants of Institute of Natural Fibres and Medicinal Plants (INF&MP) were used for induction of *in vitro* cultures. Seeds were sterilized with use of 70 % ethanol and ACE® solution (2:1). Sterile seedlings were cuts for explants (roots, leaves, stem's segments and shoot tips) and placed on modified induction medium according to Turker (TURKER et al., 2008). Induction medium - MS medium (MURASHIGE and SKOOG 1962) supplemented with BAP (0.1 mg/L), IAA (0.5 mg/L) with vitamin C (0.1 g/L) and hydrolysate casein (0.5 g/L). After four weeks the obtained multiple-shoots were subcultured on fresh medium and the shoots were individually separated and transferred into vessels containing rooting medium: ½ MS with IAA (0.25 – 1.0 mg/L) with vitamin C (0.1 g/L). The number of shoots per explants was calculated from each ten multi-shoots individually separating shoots in three subsequent passages. Each seedling was marked by a number and multi-shoots and shoots derived from individual seedlings were represented by separated lines (genotypes) .The percentage of rooted plants was recorded after four weeks for 100 plants. All cultures were incubated in temperature 25 °C under the 16/8 h photoperiod (cool – white fluorescent lights 25 – 30 µmol m/l2s). The rooted plants were transferred into soil substrate and perlite (Kekkila Paperpot) and acclimatized in closed tunnels in the greenhouse conditions for two weeks. The plants were hardening in the open tunnels in temperature 16 °C for two weeks and for another two weeks in field conditions. All experiments were repeated three times.

Results

The best shoot regeneration was obtained from stem fragments (96 %) and root explants (60 %), which formed multi-shoots (table no 1). The rest of explants (leaves and shoot tips) only occasionally regenerated shoots, with a higher frequency of roots and callus formation. Browning of tissues, especially stem segments and leaves, leading to necrosis of explants was observed on induction medium in the subsequent passages.

Tab. 1 Shoot regeneration from explants: roots, stem's fragments, shoot tips and leaves on induction medium.

Explants	No of explants	**% explants forming shoots**	**% survived explants**
Roots	55	60 %	100 %
Stem's fragments	24	100 %	96 %
Leaves	123	10.7 %	83.7 %
Shoot's tips	15	25 %	80 %

Finally six different genotypes (lines) originated from root's explants were subcultured for over 6 months (passages numbers from 2 to 23). The numbers of shoots and multi-shoots (calculated for all lines) changed between single subcultures. The efficiency of shoot production was variable from passage to passage and depended on line. Average numbers of shoots calculated for explants varied between genotypes and oscillated between 16.1 and 20.4 (Figure 1).

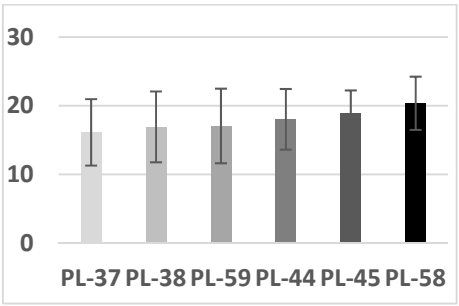

Fig. 1 Average number of shoots per explant of individual lines calculated in subsequent passages: 21, 22, 23. Error bars express SD.

The shoots were rooted on medium containing three different concentrations of IAA (0.25 mg/L; 0.5 mg/L and 1.0 mg/L). The optimal concentration of IAA was 0.5 mg/L and 98 % (±1.01) of healthy and rooted plants were recorded. The whole cycle lasted from 10 to 12 weeks from seedlings to acclimatized plants. The acclimatization resulted in high survival rate (98 %) of rooted and hardened plantlets. Finally, over 3 000 acclimatized plants were obtained and used for field crops.

The modified Turker's protocol is an efficient and rapid method, which allows for obtaining of a high number of shoots (16 - 20) per explant. Genotype dependent response of the explants was observed during regeneration. The major problem was browning of tissues and necrosis of the explants, what limited the number and quality of the regenerated shoots. The use of *Ch. angustifolium in vitro* cultures can contribute to the introduction of this valuable herb species for field crops and increase the availability of the raw material for food and the pharmaceutical industries.

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P 3: Resistance evaluation of parsley populations (*Petroselinum crispum*) for resistance to Septoria leaf spot (*Septoria petroselini*)

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Abstract

Septoria petroselini, the causal agent of Septoria leaf blight (SLB), causes economically significant yield losses in parsley. Due to fungicide resistance and the very low number of currently labeled fungicides, growing of SLB resistant cultivars is the most cost effective and environmentally friendly way to avoid losses. Therefore aims of this study are the characterization of virulence patterns of different *Septoria petroselini* isolates and the evaluation of parsley populations for resistance to *Septoria petroselini*.

In order to study virulence patterns isolates of pathogen have been collected from different locations in Germany. Phenotyping of parsley populations was conducted 2015 under controlled conditions in climate chamber (20 °C, 16 h light and 95 % air humidity) in order to characterize isolates. Genotypes were inoculated in order to calculate the area under the disease progress curve. Two resistant parsley genotypes and two susceptible were then inoculated with four isolates (ES 1, ES 3, ES 6, ES 14).

The average percentage leaf area diseased ranged significantly different from 9 % to 35 % between isolates / genotypes. The most aggressive isolate with regard to the infected leaf area was used further for the inoculation of 17 presumably resistant parsley breeding populations. Disease severity was, in a first step, estimated by scoring the percentage leaf area diseased 21 days post-inoculation (dpi). Leaf symptoms ranged between 1.7 % and 46.7 % (infected leaf area) between the accessions and within the populations. As one result, reduced percentage of infection with a delayed macroscopic visible infestation and reduced disease severity could be detected in 7 of these genotypes. In a next step, additionally to the visual quantification of the infection process, PTA-ELISA using the intracellular fluid from plants to detect fungi proteins has been performed. Using this sensitive approach the rate of pathogen proteins into the plant could be measured 3dpi and 21dpi. Using PTA-ELISA 3dpi serologically no pathogen could be detected whereas a positive correlation 21dpi between the results of PTA-ELISA (measured absorbance at 405 nm) and degree of infestation could be observed (rs = 0.94). Using the quantification of pathogen protein resistant parsley cultivars could be identified 21dpi so that the method is usable for an accelerated breeding process. This study is the basis for breeding new resistant parsley varieties and the detection of *Septoria petroselini*.

P 4: *Ex situ* regeneration of cross-pollinated MAP genetic resources in the Czech Republic

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Abstract

The multiplication and/or regeneration of germplasm of medicinal and aromatic plants (MAPs) is financially demanding, it requires space, time and well educated experienced staff. It is a group with very different demands on the cultivation and propagation. Many of these species are cross-pollinated and entomophilous, in some of them still remain some attributes of wild plants, other species produce compounds, which may cause skin and other problems in humans. Perennial species produce small amounts of seed and their germination capacity is mostly lower in comparison with other crops. The two types of technical solving of multiplication and/or regeneration of genetic resources of allogamous medicinal and aromatic plants in Olomouc, where both stationary and mobile isolation cages are used, are presented in this manuscript.

Keywords: multiplication, technical isolation, isolation cage, mesh house, controlled pollination

Introduction

Ex situ collections of plant genetic resources consist of seed genebanks, field genebanks and *in vitro* collections. Species with orthodox seeds are stored in seed genebanks, while the latter two methods are used mainly for vegetatively propagated crops and for species with recalcitrant seeds that cannot by dried and stored for long periods under cold conditions. Seed storage is the predominant form of plant genetic resources conservation, accounting for about 90 % of the total accessions held *ex situ* (FAO, 1996). Effective regeneration programmes are essential to maintain the viability and genetic integrity of *ex situ* seed collections of germplasm. 100 % germination rate and zero genetic change on regeneration both represent the ideal but neither of them is achievable (HAMILTON and CHORLTON, 1997).

The type of reproduction of the targeted species will determine regeneration conditions. An autogamous species can be multiplied and/or regenerated in the field. Allogamous species are preferably multiplied and/or regenerated in the glasshouse, mesh house or also in the field, but the land must be isolated and pollination strictly controlled. If the accessions comprise wild species, they can be multiplied and/or regenerated in furrows or plots in the field, or in the mesh house or glasshouse, depending on the quantity of available seed and on the species´ requirements (JARAMILLO and BAENA, 2002). The two types of technical solving of multiplication and/or regeneration of genetic resources of allogamous medicinal and aromatic plants in Olomouc are presented in this manuscript.

Materials and Methods

Multiplication and/or regeneration of genetic resources of allogamous medicinal and aromatic plants in Olomouc is carried out by two basic ways depending on persistence and/or pedigree status of accessions:

- Annual and/or culture/cultivated species (e.g. *Anethum graveolens* L., *Borago officinalis* L., *Calendula* L., *Cnicus benedictus* L., *Coriandrum sativum* L., *Foeniculum vulgare* Mill., *Malva* L., *Ocimum basilicum* L., *Oenothera biennis* L., *Origanum majorana* L., *Nigella sativa* L., *Satureja hortensis* L., *Silybum marianum* (L.) Gaertn. etc.)
- Perennial and/or wild species (e.g. *Agrimonia* Tourn. ex L., *Carum carvi* L., *Echinacea* Moench, *Hypericum* L., *Hyssopus officinalis* L., *Inula helenium* L., *Lavandula angustifolia* Mill., *Origanum*

vulgare L., *Polemonium caeruelum* L., *Ruta graveolens* L., *Salvia* spp., L., *Satureja montana* L., *Stachys officinalis* L. etc.)

Annual and/or culture/cultivated species are regenerated and/or multiplicated in stabile isolation cages (mesh houses). Stationary isolation cage (Fig. 1) have a dimension 5.15 x 2.85 m and height 1.50 – 2.00 m and its construction is made from profile 7 30/30/3 mm and closed profile (jäckel) 40/20/2 mm. Galvanized cage constructions are fix anchored on concrete socle and covered up with net hood before sowing or planting. Each stabile isolation cage is equipped by the drop irrigation installation directly to the plants.

Perennial and/or wild species of MAPs are regenerated and/or multiplicated in mobile isolation cages (Fig. 2). These mobile isolation cages have a dimension 2.00 x 3.00 x 1.70 m and they are constructed from closed profile (jäckel) 40/20/2 mm (roof part) and 3/4" tube with strength 2 mm. These isolation cages consist of demountable metal construction covered with the net hood which equipped with a wooden frame for its anchorage. Polyamide monofil textile is used to make the parts of isolation cages (the type No. 737968, vilament diameter 0.3 mm, mesh 0.6 – 0.8 mm, manufactured by TECHNOLEN, technical textile Ltd., Lomnice nad Popelkou.

Both types of isolation cages are equipped with incomplete honey-bee colonies (*Apis mellifera* L.) or bumble-bee (*Bombus terrestris* L., *Bombus lapidaries* L.) nests for plant pollination (Dušek et al., 2010).

Results

The regeneration of medicinal and aromatic plants is complicated by several factors. It is a category with very different demands on the cultivation and propagation. Many of these species are crosspollinated and entomophilous, in some of them still remain some attributes of wild species (e.g. covering by spines, which is typical for milk thistle, spiny rest harrow), other species produce the compounds, which cause skin and other problems in humans (e.g. common rue). In addition, perennial species produce relatively small amounts of seed (FAO, 1996) and its germination capacity is significantly lower in comparison with other crops.

Despite these complications the regeneration and/or multiplication of medicinal plants is successful in Olomouc. Stationary isolation cages are suited to most of MAPs due to a better ventilation and temperature regulation inside the cage compare to the glassed-in stationary cages, which are used for regeneration and/or multiplication of vegetable species in Olomouc. However, in rainy weather the plant stand inside the mesh cage is more subject to a pathogen damage, mainly to fungal diseases.

The isolation due to mobile isolation cages is suitable for field stands, especially for biennial and perennial cultures. When using portable isolation cages good results were observed mainly in regeneration of lavender, thyme, caraway, biennial coriander, digitalis, mallow, common rue, biennial as well as perennial savory etc. The advantage of this system is that the plants are isolated only for as long as the term of flowering and during the rest of the vegetation period they can grow in the conditions of the natural climate uncovered. Also the basic agronomic interventions (inter-row cultivation, prunning etc.) are possible to perform mechanized way before installation of isolation cages or after their removing. In addition to the simple installation there is also an advantage of possible multiple use of the constructions in the course of vegetation period when successive flowering of various plant species takes place from April until July. The folding construction allows an easy dismantling of vertical parts which helps for easy storage in non-flowering period.

The multiplication and/or regeneration of germplasm in both the stabile and/or mobile isolation cages is financially demanding, it requires technical equipment, time and well educated and experienced staff. Compare to the formerly used system, based on space isolation, it save the space and in case of regeneration of species which widely growing relatives could overcross the target genotypes, it is the only way to success.

Fig. 1 Stationary isolation cages

Fig. 2 Mobile isolation cages

Acknowledgement

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P 5: A descriptor list of *Silybum marianum* (L.) Gaertner – morphological and biological characters

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Abstract

Silybum marianum (L.) Gaertn (Milk thistle) is an important medicinal plant which fruits are used for treatment of various liver diseases. In an effort to utilize the genetic potential of cultivated plants in the best way, the breeding of new high-performance cultivars is underway all over the world. Genetic improvement in *Silybum* can only be, as with all other plants, achieved through a clear understanding of the plant´s behaviour and the amount of variability presented in wild populations. Surprisingly no descriptor list has been compiled up to now, which would permit an objective and easily repeatable description an evaluation of the different *Silybum* genotypes. The first part of such a descriptor list, which is intended mainly for evaluation of genotypes perspective for fruit production, is presented here and it contains both the morphological and biological characters.

Keywords: Milk thistle, evaluation descriptors, production of fruits

Introduction

Silybum marianum (L.) Gaertn (Milk thistle) is an important medicinal plant of which pharmaceutical importance rises once again and it is grown commercially almost all over the world. Its fruits (i.e. achenes), often referred to as seeds, have been valued for their medicinal properties (GAŽÁK et al., 2004) and currently the most important medicinal application of *Silybum* is its use as a hepatoprotectant and as supportive treatment of chronic inflammatory liver disease such as cirrhosis, hepatitis, and fatty infiltration due to alcohol and toxic chemicals. It has also been used in the treatment of liver damage by poisonous mushrooms (KVASNIČKA et al., 2003).

Despite the wide use and considerable volume of *Silybum* sales - 8,312,867 USD in 2005 (BLUMENTHAL et al., 2006) – there is lack of research efforts on the domestication and improvement of this plant (RAM et al., 2005) but the breeding of new high-performance cultivars is underway all over the world. The first step in breeding of wild or native populations is collection and description of genetic variation of plant populations for desired characters (SHOKRPOUR et al., 2011). Genetic improvement in *Silybum* can only be, as with all other plants, achieved through a clear understanding of the plant´s behaviour and the amount of variability presented in wild populations, including genotypes which may represent the maximum expression of adaptation capability to environmental conditions. The selection of an ideotype with desirable traits will facilitate agrotechnology and allows to maximal yield (GRESTA et al., 2006).

An objective and easily repeatable evaluation of many important crops is made possible by using descriptor lists. Surprisingly no descriptor list for different *Silybum* genotypes has been drawn up up to now and therefore the first part of such a descriptor list is presented here. It is intended mainly for evaluation of genotypes perspective for fruit production and it contains both the morphological and biological characters.

Materials and Methods

Presented descriptor list of *Silybum marianum* was created based on the references for the development of crop descriptor lists (BIOVERSITY INTERNATIONAL, 2007) and it comprises results and experiences of 5 years growing and evaluating of milk thistle genetic resources, which were collected in the Czech gene bank. Own measurements and evaluation, acquired in the period 2007-2009 in

locality Olomouc (CZE) and 2013-2014 in localities Olomouc and Znojmo (CZE), were confronted and supplemented by many literature references from scientific papers. The presented descriptor list is intended mainly for the evaluation of genotypes perspective for fruit production. In case of evaluation in order to other use (fodder plant, production of biomass, ornamental plant, weed etc.) some additional characters should be added.

Results

The first part of milk thistle descriptor list is presented in Tab. 1 and it contains both the morphological and biological characters. The economic characters, as the total silymarin and oil content and the content of its components, are processed.

Tab. 1 Descriptor list of Silybum marianum – Morphological and Biological characters

Descriptor	Scale	Values	Note
1. Morphological characters			
1.1 Plant			Average of 10 randomly selected plants.
1.1.1 Plant habit	1 erect		Recorded in terminal head full flowering stage.
	2 semi-erect		
1.1.2 Plant – height (cm)	3 low	< 110	Recorded in terminal head full flowering stage; from ground level to the top of terminal head.
	5 intermediate	110-140	
	7 high	> 140	
1.1.3 Plant – width (cm)	3 small	< 70	Recorded in terminal head full flowering stage.
	5 intermediate	70 - 90	
	7 great	> 90	
1.1.4 Plant - length of flowering stem (cm)	3 short	< 50	Length of stem from first branching point to the top of terminal head; recorded in terminal head full flowering stage.
	5 intermediate	50 - 70	
	7 long	> 70	
1.1.5 Plant - intensity of branching	3 low	< 10	Number of fertile branches per plant. Recorded at harvest time.
	5 intermediate	10 - 20	
	7 high	> 20	
1.2 Leaf		Average of 10 leaves, each leaf from different randomly selected plant.	
1.2.1 Rosette leaf – length (cm)	3 short	< 50	Fully developed leaves including petiole, in the beginning of stage of generative organ creation.
	5 intermediate	50 - 65	
	7 long	> 65	
1.2.2 Rosette leaf – width (cm)	3 narrow	< 25	Recorded in the widest point of fully developed leaves, in the beginning of stage of generative organ creation.
	5 intermediate	25 - 27	
	7 wide	> 27	
1.2.3 Rosette leaf - depth of incisions	3 pinnatilobed	< 1/3	Fig. 1; Deep of incisions of blade margin to the main vein.
	5 pinnatipart	1/3 - 1/2	
	7 pinnatifid	> 1/2	
1.2.4 Rosette leaf - degree of marbling	0 absent		Fig. 2
	3 low		
	5 medium		
	7 high		
1.3 Inflorescence			
1.3.2 Diameter of primary head (cm)	3 small	< 4,5	Average of 20 randomly selected plants. Recorded in terminal head full flowering stage.
	5 intermediate	4,5 - 5,5	
	7 great	> 5,5	
1.3.3 Inflorescence - number of heads per plant	3 small	< 6	Number of other flower heads per plant (except the primary head). Recorded in terminal head full flowering stage.
	5 intermediate	6 - 9	
	7 great	> 9	
1.3.4 Flower - colour	1 white		As present or using RHS colour chart.
	2 creamy		
	3 pinkish		
	4 light violet		
	5 dark violet		
	6 other		

Descriptor	Scale	Values	Note
1.4 Fruit			Recorded in full maturity stage.
1.4.1 Fruit – length (mm)	3 small	< 7	Average of 20 randomly selected fruits; achenes without crest.
	5 intermediate	7 - 8	
	7 great	> 8	
1.4.2 Fruit – width (mm)	3 small	< 3	Average of 20 randomly selected fruits; in the widest part of fruit.
	5 intermediate	3 - 4	
	7 great	> 4	
1.4.3 Fruit - colour	1 dark brown		The main colour of the seed is the colour with the largest area. As present or using RHS colour chart.
	2 brown black		
	3 other		
1.4.4 Number of fruits on primary flower head	3 low	< 80	Average of 10 random terminal flower heads.
	5 intermediate	80 - 120	
	7 high	> 120	
1.4.5 Fruit - Yield per plant (g)	3 low	< 15	Average of 10 random plants. Yield of all gradually harvested flower heads of plant. Example variety SILYB (CZE, 1988).
	5 intermediate	15 - 30	
	7 high	> 30	
1.4.6 Fruit - 1,000 fruits weight (g)	3 low	< 20	
	5 intermediate	20 - 25	
	7 high	> 25	
2. Biological characters			
2.1 Vegetation period			
2.1.1 Number of days from sowing to beginning of generative organs creating (days)	3 early	< 80	Beginning of generative organs creating = flower central head clearly visible between rosette leaves, vertical bract tips. Example variety SILYB (CZE, 1988).
	5 intermediate	80 - 90	
	7 late	> 90	
2.1.2 Number of days from sowing to beginning of terminal head flowering (days)	3 early	< 90	Beginning of terminal head flowering = the flower bud bends; first flower in blossom.
	5 intermediate	90 - 100	
	7 late	> 100	
2.1.3 Number of days from sowing to terminal head maturity (days)	3 early	< 100	Terminal head maturity = The bracts are dry. The head is opening and the pappus is visible and seed dispersal start.
	5 intermediate	100-115	
	7 late	> 115	
2.2 Biotic stress susceptibility			
In each case, it is important to state the origin of the infestation or infection, i.e. natural, field inoculation, laboratory. Record such information in descriptor 2.2 Notes. These are coded on a susceptibility scale from 1 to 9, viz.:			
	1 very low or no visible sign of susceptibility		
	3 low		
	5 intermediate		
	7 high		
	9 very high		
2.2.1 *Botrytis cinerea* - Botrytis head rot			
2.2.2 *Septoria silybi* - Septoria leaf spot			
2.2.3 *Alternaria silybi* - Alternaria leaf spot			
2.2.4 *Fusarium oxysporum* - Fusarium wilt			
2.2.5 *Golovinomyces cichoracearum* - Powdery mildew of milk thistle			
2.2. others			

Fig. 1 Depth of incisions of blade margin to the main vein (scale examples)

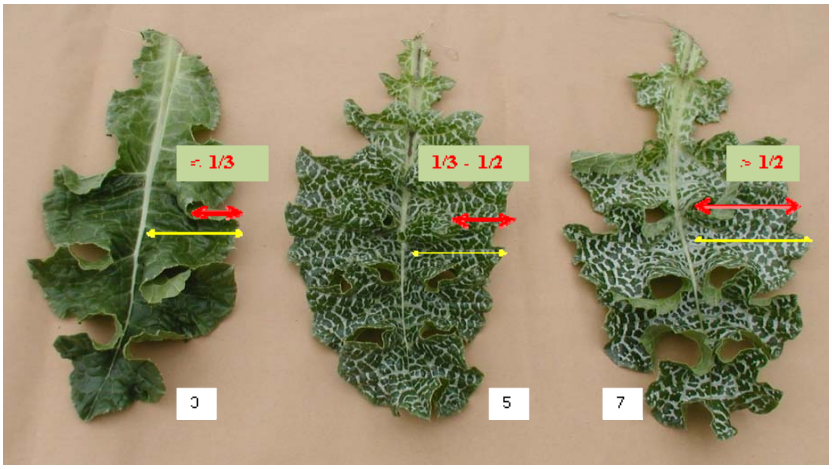

Fig. 2 Rosette leaf - degree of marbling (scale examples)

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P 6: Response of growth and wax production of jojoba (*Simmondsia chinensis* (Link) C.K. Schneid.) to the growing location in Egypt

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Abstract

Jojoba plant (Simmondsia Chinensis (Link) C.K. Schneid.) Or Ho-Ho-ba, Jojoba or goat nut is a shrub belongs to family Simmondsiaceae. It is well known as a useful medicinal plant and as a new industrial crop was of interest for many industrial countries at present. The present study was carried out at the two successive seasons of 2012 / 2013 and 2013 / 2014 at private jojoba farms located as following: El-Kassasin city, El-Ismailia, Marsa Matroh, El-Sharkia, Asuite and El-Khanka governorates, Egypt

The study focused on studying the effect of the different local growing site of Egypt on the growth and wax aiming to detect the best location for the suitable growing site to produce the best growth and wax yield.

Nine jojoba female shrubs were selected in each farm from the growing shrubs depending on its obvious morphological growth characters and the different seeds shape and then they were marked by labels for data measurements. The monthly temperature and relative humidity average during the study seasons of were taken and recorded. The Physical and chemical properties of the experimental soil were also determined and presented.

Growth and flowering characters of jojoba shrubs e.g. plant height and volume as well as flowering period and fruiting set were greatly altered due to the growing location. Since plant which were grown in Upper Egypt Asuite site tended to produce the best growth and the extended flowering. Moreover, plants which were cultivated in El Sharkia and Assiut sites gave the biggest yield of wax, compared to the other cultivation sites, while the lowest yield of wax was from plants cultivated in Marsa Matroh area. The best level of almost of the fatty acid content (palmitic, oleic, nervonic acids, gadoleic and erucic acid) was found in the wax extracted from seeds of shrubs grown in Ismailia growing site.

Key words: jojoba, growing site, wax, fatty acid, hohoba, growth, flowering

P 7: Variability of total flavonoid and mucilage content of wild growing chamomile (*Matricaria recutita* L.) populations

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Abstract

During our investigation 50 wild growing chamomile populations' active substance content, among them total flavonoid content and swelling index referring to mucilage content were examined in 2009 in the main chamomile collection areas of Hungary. Swelling index was determined according to the general and specified descriptions of *Althaeae folium* monograph of European Pharmacopoeia, while total flavonoid content was measured by the method described in the monograph of *Crataegi folium cum flore*. The 50 Hungarian wild growing chamomile populations proved to be very heterogeneous in terms of the examined features. The swelling index of their flower drug samples changed between 15.8 and 80.8 and their total flavonoid content varied from 0.94 to 2.28 %.

Significant correlation was also found between meteorological conditions and evaluated characteristics: there was medium strong positive correlation between spring total heat unit (sum of daily 10 °C higher average temperatures of the period lasted from 1st of March, 2009 until the day before flower collection) as well as total heat unit of 10 days before harvest and swelling index (r = 0.50-0.56), furthermore medium strong negative connection could be seen between total heat units and total flavonoid content (r = -0.60-0.65). Based on these findings it can be ascertained that raising temperature affects the mucilage accumulation positively, however, it has a negative effect on the amount of flavonoids.

Keywords: chamomile, environment, heat unit, mucilage, swelling index, total flavonoid content

Introduction

Chamomile is one of our most important medicinal plants. The dried flowers, the blue essential oil distilled from them and its different phytotherapeutic preparations are used in medicine mainly because of their anti-inflammatory, antispasmodic, skin regenerating and antiseptic effects.

Many studies have already dealt with the essential oil content and composition (chemotypes) of chamomile and according to most of them environmental factors can influence the accumulation level of essential oil and the amount of essential oil components significantly, but the typical essential oil composition (chemotype) is independent of the external environmental conditions (D'Andrea, 2002; Franz et al., 1986; Galambosi et al., 1988; Gasič et al., 1989; Gosztola et al., 2010; Letchamo, 1990; Šalamon and Hončariv, 1994).

However, in connection with the changeability and variability of other important active substances of chamomile responsible for its pharmacological effects, such as musilage or flavonoid content already much less information is available. The most important constituents of chamomile mucilage is glucose and glucuronic acid, but D-galacturonic acid, xylose (approx. 21 %), arabinose (approx. 10 %), galactose (approx. 15 %) and rhamnose (approx. 2 %) are also found in it (Janecke and Weisser, 1964). It accumulates in large mucilage containing cells of flowers and its amount changes between 3 and 17 % in the drug (Schilcher et al., 2005). The easiest and cheapest way to measure the mucilage content is to determine the swelling index of the drug according to Ph. Eur.

In relation to total flavonoid content we do not have a lot of research data, either. Roemisch (1960) measured 1.0-2.5 % accumulation levels in case of 102 commercially available samples, while Schilcher (1987) found 0.30-2.96 % total flavonoid content in the drug samples of 12 cultivated chamomile populations with different origin. Determination of total flavonoid content is carried

out by photometric method which has many advantages but the absolute values are actually about 20-30 % higher (SCHILCHER et al., 2005).

Materials and Methods

At the beginning of May, 2009 50 wild growing chamomile populations were involved in our study native to the Great Hungarian Plain of Hungary. For chemical analysis we collected representative amount of flowers by chamomile comb in full flowering stage in each population. Flowers were dried in natural way and after drying we removed the unnecessary, too long stem parts. After this we stored the samples on a dry place, protected from moisture, until the chemical measurements.

For the characterisation of mucilage content the swelling index was determined according to the general and specified descriptions (for *Althaeae folium*) of European Pharmacopoeia 8.0 by using 0.2 g dry, powdered drug. In case of the analysis of total flavonoid content measurements were carried out following the descriptions of method described in *Crataegi folium cum flore* monograph of European Pharmacopoeia 8.0. Examinations were performed in 5 repetitions in each case.

Climatic conditions were evaluated by using the data of nearest meteorological stations to the analysed chamomile populations. We counted the spring total heat unit by means of data (the sum of daily 10 °C higher average temperatures of the period lasted from 1st of March, 2009 until the day before flower collection) as well as the total heat unit of 10 days before harvest.

Results

Swelling index (SI)

The average swelling index referring to mucilage content changed between 15.8 and 80.8 in case of wild growing chamomile populations (Table 1). The relative st. dev. between populations was definite ($CV_{\%}$ = 41.2 %) and we also found significant differences between them ($p = 0.000$; $SD_{5\%}$ = 2.9). According to this it can be established that chamomile stands with different origin were very diverse in terms of their mucilage content. Most of the populations (60 %) had 20-40 SI values.

There were unambiguous connections between meteorological data and SI data of chamomile stands too. Plants' swelling index had medium strong, positive correlation with both spring total heat unit ($r = 0.56$) and total heat unit of 10 days before harvest ($r = 0.50$). Based on these findings it can be seen that SI referring to mucilage content is a very variable characteristic which is influenced by the temperature significantly. The higher temperature helps the accumulation of polysaccharide compounds.

Total flavonoid content

The average total flavonoid content of examined wild chamomile stands varied from 0.94 to 2.28 % (Table 1). According to this populations could be considered heterogeneous ($CV_{\%}$ = 22.2 %), between them significant differences also can be proved ($p = 0.000$; $SD_{5\%}$ = 0.09). More than half of the populations (60 %) had 1.20-1.80 % accumulation level.

Analyzing the meteorological data and populations' total flavonoid contents we could find clear connections again. There was a medium strong, negative correlation between amount of flavonoids and spring total heat unit ($r = -0.63$) as well as total heat unit of 10 days before harvest ($r = -0.60$). Based on this it seems that total flavonoid content – similarly to swelling index – significantly depends on the temperature. Warmer weather is unfavourable but cooler spring and lower average temperature before harvest can favour the accumulation of flavonoids.

Tab. 1 Swelling index and total flavonoid content of examined wild growing chamomile populations (2009)

Population code	Swelling index		Total flavonoid content (%)		Population code	Swelling index		Total flavonoid content (%)	
	Mean	St. Dev.	Mean	St. Dev.		Mean	St. Dev.	Mean	St. Dev.
1	16.7	1.4	2.24	0.03	**26**	34.2	1.4	1.28	0.01
2	19.2	1.4	1.86	0.03	**27**	45.0	0.0	1.72	0.13
3	25.0	2.5	1.71	0.05	**28**	54.2	3.8	1.26	0.02
4	29.2	1.4	2.05	0.04	**29**	56.7	1.4	1.52	0.05
5	25.8	1.4	1.62	0.08	**30**	31.7	1.4	1.48	0.00
6	28.3	1.4	1.91	0.05	**31**	56.7	1.4	1.07	0.03
7	29.2	1.4	1.55	0.04	**32**	27.5	2.5	1.50	0.03
8	24.2	1.4	1.43	0.03	**33**	26.7	1.4	1.45	0.08
9	26.7	1.4	1.43	0.09	**34**	27.5	0.0	1.45	0.04
10	15.8	1.4	1.70	0.04	**35**	42.5	2.5	1.44	0.03
11	22.5	2.5	2.06	0.01	**36**	40.0	2.5	1.56	0.00
12	25.8	1.4	1.48	0.10	**37**	35.0	2.5	1.25	0.02
13	38.3	1.4	1.82	0.03	**38**	40.0	0.0	1.07	0.05
14	15.8	1.4	1.98	0.04	**39**	33.3	1.4	1.21	0.01
15	15.8	1.4	1.89	0.01	**40**	44.2	1.4	1.14	0.02
16	17.5	2.5	2.12	0.05	**41**	24.2	1.4	1.48	0.05
17	26.7	1.4	2.28	0.02	**42**	26.7	1.4	1.60	0.03
18	52.5	0.0	0.98	0.01	**43**	25.8	1.4	1.50	0.02
19	38.3	1.4	1.15	0.03	**44**	58.3	1.4	1.10	0.05
20	33.3	1.4	1.62	0.02	**45**	22.5	2.5	1.21	0.03
21	36.7	1.4	1.25	0.05	**46**	19.2	1.4	1.15	0.02
22	31.7	1.4	1.02	0.07	**47**	35.0	0.0	1.83	0.01
23	22.5	2.5	1.75	0.00	**48**	80.8	1.4	1.31	0.01
24	45.0	0.0	1.35	0.03	**49**	41.7	2.9	1.29	0.06
25	47.5	2.5	0.94	0.02	**50**	67.5	2.5	1.67	0.03

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P 8: Productivity of different thyme varieties (*Thymus vulgaris* L.) in the condition of non chernozem-zone of Russian Federation

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Abstract

Creeping thyme (*Thymus serpillum* L.) is mostly used as a medicinal plant in the Russian Federation. It has much more winter hardiness then common thyme (*Thymus vulgaris* L.), which is similarly used in Europe. However, *T. vulgaris* is interesting as a plant in food industry and in medicine. *T. vulgaris* contains two to three times more essential oil (2 %) than *T. serpillum* L. and a higher content of thymol in its essential oil (up to 35-40 % depending on the genotype). The objectives of the present study are to determine the total essential oil content, the total polyphenol content, the total flavonoid content and the yield from 8 varieties of thyme during two years.

We found that the 'Colchida' and 'Lemon' varieties are characterized by high yields. The maximum amounts of essential oil were found in 'Deutscher Winter' (1.3 %), 'Médoc' and 'Lemon' (1.19 %), flavonoids content in plants was 1.27-2.87 %. Our studies have shown that *T. vulgaris* can be considered as a potential medicinal and aromatic plant for growing in the non-chernozem zone of the Russian Federation.

Keywords: essential oil, thymol, flavonoids

Introduction

Thymus L. is one of the most important genera regarding the number of species (more then 200) within the family Lamiaceae, but only a few species are of medicinal importance. Creeping thyme (*T. serpillum* L.) is mostly used as a medicinal plant in the Russian Federation. It has much more winter hardiness then common thyme (*T. vulgaris* L.), which is similarly used in Europe. *T. vulgaris* contains two to three times more essential oil (2 %) than *T. serpillum* L., and a higher content of thymol in its essential oil (up to 35-40 % depending on the genotype). *T. vulgaris* is more technological crop for growing in the field, because of its compact habitus and suitability for mechanized harvesting of raw material and row cultivation.

Materials and Methods

During two years the objectives of the present study are to determine the total essential oil content, the total polyphenol content and the yield from 8 varieties of thyme, including such popular ones as Duska tynuanova (Czech Republic, Sevaseed), 'Deutscher Winter' (Germany, Mayer's), *T. vulgaris* variety and genotypes from Germany (a country with similar climatic conditions of non-chernozem zone of the Russian Federation) and varieties of Russian and German firms: 'Lemon' (AF Aelita), 'Medoc' (AF Gavrish), 'Di Roma' (Germany), 'Colchida' (Company ZeDeK), 'Quedlinburger' (Germany), *T. vulgaris* (AF Gavrish).

The essential oil content was determined in dry herb of thyme by the distillation method presented in Pharmacopoeia. of Russian Federation. The total polyphenol content was determined colorimetrically using the method of Folin-Ciocalteu.

The amount of flavonoids was determined spectrophotometrically following the reaction with aluminum chloride. After sufficient mixing of the sample and the reagent, the mixture is incubated for 10 minutes at ambient temperature and the absorbance of the solution is read at 440 nm. Flavonoid content is expressed in mg/g of rutin.

Results

It is important that herbs must be harvested at the time of intense flowering for the preparation of medicinal raw material. During the study, thyme began to bloom in the first decade of June, although the length of the blooming is different. It was revealed that the varieties 'Duska tynuanova' (Czech Republic) and 'Deutscher Winter' (Germany) were characterized by a maximum height of 26.7±2.9 cm and 24.6±3.1 cm, respectively. The varieties 'Colchida' and 'Lemon' were characterized by high yields (84.0±9.6 g/plant, and 75.0±4.7 g/plant,, respectively). The maximum content of essential oil was found in `Deutscher Winter´ variety (1.3 % in dry raw materials) and the 'Médoc' and `Lemon´ (1.19 % in both samples).It is slightly lower than in the southern regions of Russia and in the European Union, but meeting the requirements of Russian Pharmacopoeia for a medicinal raw materials and requirements PhEur (12 ml / kg) for 'Deutscher Winter' variety. The highest content of flavonoids was detected in *T. vulgaris* of AF 'Gavrish' (2.87±0.11 %) and Quedlinburger (2.83±0.12 %).

Thus, *T. vulgaris* can be recommended as a potential medicinal and aromatic plant for the non-chernozem zone of the Russian Federation.

P 9: Identification of volatile components in two *Thymus* species from Iran and their antioxidant properties

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Abstract

Thymus species are well known to have significant amount of phenolic compounds and exhibit strong antioxidant activities.

This study is designed to analyze the essential oils of two Iranian *Thymus* species, (*T. kotschyanus* Boiss. et Hohen and *T. pubescense* Boiss. et Kotschy ex Celak) obtained by hydrodistillation of aerial part of this plants, using GC-FID and GC/MS and evaluate the *in-vitro* antioxidant activities in two quantitative methods (namely $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assay) to determine the total phenolic content of the species (assayed by colorimetric techniques) and to study the possible composition-antioxidant activity relationship.

The major aroma constitutes in the essential oil of *T. pubescense* were found to be thymol (38.7 %), γ-terpinene (7.5 %), *p*-cymene (5.5 %), α-terpenyl acetate (3.8 %) and β-bisabolene (3.7 %) while in the essential oil of *T. kotschyanus*, α-terpineol (16.9 %), 1,8-cineol (14.4 %), linalool (9.6 %), thymol (7.2 %) and geranyl acetate (5.4 %) were the main compounds.

Both of the tested essential oils exhibited concentration-dependent antioxidant activity. *T. pubescense* showed more activity in both $DPPH^{\cdot}$ [IC_{50}= 285.2 (236.5-344.0) µg/mL] and $ABTS^{\cdot+}$ methods [IC_{50}= 1.956 (1.810-2.113) µg/mL], as well as total phenolic content of *T. pubescence* [70254 ± 0.0049 µg/mg] was found to be slightly higher than *T. kotschyanus* [62933 ± 0.0026 µg/mg].

Keywords: *T. Pubescense; T. kotschyanus*; Antioxidant activity; Essential oil; Chemical composition; Labiateae.

Introduction

Thymus is an important genus of the Labiateae family, originated from the Mediterranean region. Among 300 to 400 species of this genus grown in the World, 14 species are distributed in the Iranian flora. Leaves and flowering part of *Thymus* species are commonly used in traditional medicine, as tonic and herbal tea, antiseptic, antitussive and carminative. In addition, *Thymus* essential oils are widely used in pharmaceutical, cosmetics and perfume industry, also for flavoring and preservation of several food products. Indeed, many species of this genus are well known for their health-benefit effects, including antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, antiparasitical and antispasmodical activity (Stahl-Biskup and Saez, 2002). In turn, these properties have been associated to phenolic composition of these plants.

The aim of the present work is to evaluate and compare the antioxidant activities of essential oils obtained from two Iranian *Thymus* species (*T. kotschyanus* and *T. pubescense*) by different methods. Because of the important role of the phenolics as potent antioxidants, the total amounts of the compounds are also determined.

Materials and Methods

Plant materials: The aerial parts of *T. kotschyanus* and *T. pubescense* were collected respectively from Azerbaijan and Khorasan provinces, in Iran, June 2013.

Isolation of the essential oils: The dried aerial parts of the plants were subjected to hydrodistillation for 3h using Clevenger-type apparatus to obtain the essential oils.

Quantitative Antioxidant assays

DPPH· Assay: The free radical scavenging abilities of the samples were measured using the stable radical DPPH· and IC_{50} were calculated for both essential oils and standards (NICKAVAR et al., 2007)

$ABTS^{\cdot+}$ assay: The antioxidant capacity of the samples were evaluated by a method based on the decolonization of radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) and IC_{50} were calculated for both essential oils and standards (NICKAVAR et al., 2008)

Qualitative Antioxidant assay: Bioautographical analysis (Both of essences were loaded on silica gel plate (TLC); after running, the plates were observed under UV254 light and then sprayed by DPPH· and $ABTS^{\cdot+}$ solutions and the results were compared)

Total phenolic content: The total phenolic contents (TPCs) of the extracts were determined spectrophotometrically by using Folin-Ciocalteu reagent and then calculated as gallic acid equivalents (NICKAVAR et al., 2008)

GC-FID and GC/MS analysis condition: Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the essential oils were identified by comparision of their mass spectra and retention indices published in the literature (ADAMS, 1995) and presented in the MS computer library (WILY275.L).

Results

Tab. 1 Antioxidant potency of the studied Thymus species, *(p>0.05)

Plant species/standard	IC_{50} (DPPH·) * (µg/mL)	IC_{50} ($ABTS^{\cdot+}$) * (µg/mL)
T. pubescens	246.7 (210.7-288.9)	1.859 (1.709-2.022)
T. kotschyanus	599.1 (570.3-629.3)	9.017 (8.197-9.919)
Vitamin C	2.016 (1.743-2.331)	0.5044 (0.4762-0.5343)
Thymol	38.76 (33.19-45.28)	1.178 (1.091-1.272)

Tab. 2 Total phenolic contents of the studied Thymus species, *(p>0.05)

Plant species	Essential oil density*(g/mL)	Total phenolic content* (µg gallic acid/mL essential oil)
T. pubescens	0.9508±0.0032	70254 (69920-70588)
T. kotschyanus	0.9224±0.0086	62933.25 (62636-63230)

Tab. 3 Top ten chemical composition of T. pubescens and T. kotschyanus essential oils, *RI (retention index) measured relative to n-alkanes (C9–C18) on the non-polar HP-5-DB-5 column

	T. pubescens			*T. kotschyanus*		
	Compound	**RI***	**Content (rel. %)**	**Compound**	**RI***	**Content (rel. %)**
1	Thymol	1302	38.67	α-Terpineol	1199	16.94
2	γ-Terpineol	1057	7.46	1,8-Cineol	1032	14.37
3	*p*-Cymene	1024	5.54	Linalool	1096	9.65
4	α-Terpinyl acetate	1352	3.78	Thymol	1293	7.16
5	β-Bisabolene	1508	3.71	Geranyl acetate	1382	5.36
6	Linalyl acetate	1276	3.54	Geraniol	1256	3.71
7	α-Pinene	935	3.54	Borneol	1166	3.59
8	Carvacrol	1311	3.07	Spatulenol	1586	3.40
9	α-Terpineol	1204	2.99	Terpinen-4-ol	1178	2.60
10	Linalool	1093	2.84	Carvacrol	1302	2.29

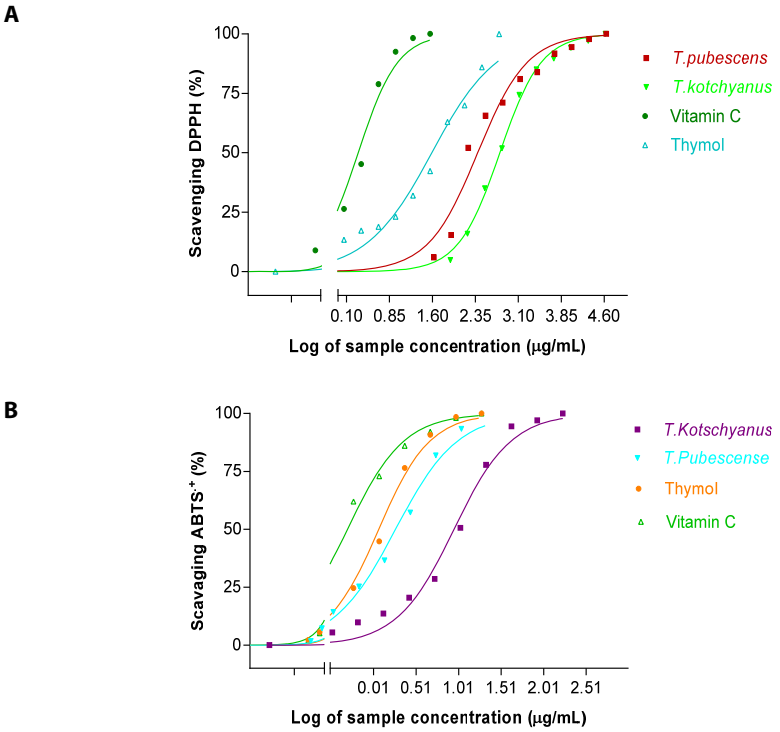

Fig. 1 Dose-dependent antioxidant activities of the studied Thymus extracts measured by using (A) the DPPH. Assay and (B) the ABTS.+ assay. Each point represents the mean of three experiments.

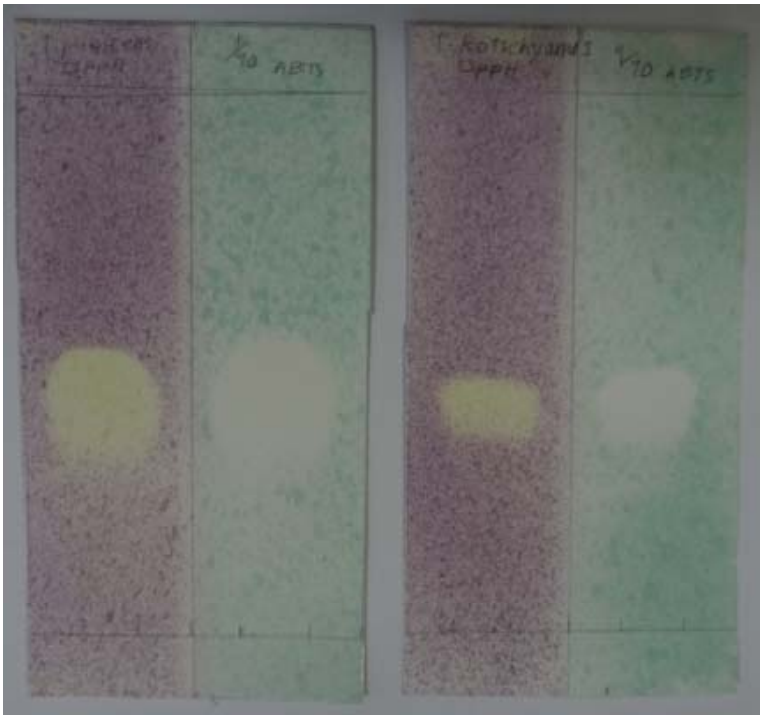

Fig. 2 Bioautographical analysis of T. pubescens and T. kotschyanus sprayed by DPPH. and ABTS.+ solutions.

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P 10: *Peucedanum ostruthium* (L.) Koch: Morphological and phytochemical variability of twelve accessions from the Swiss alpine region

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Abstract

Ostruthin, a natural bioactive compound mainly occurring in the roots of *Peucedanum ostruthium*, is the focus of this study. *P. ostruthium* was collected from twelve locations in the Swiss alpine region and reared in an experimental field, subdivided into twelve lots over two years. In the spring and fall, a portion of each of the twelve accessions was harvested and separated into above and below ground plant parts. The dried plants were then extracted with 60 % ethanol using accelerated solvent extraction (ASE) and analyzed using high pressure liquid chromatography (HPLC).The above and below ground plant parts were then analyzed concerning their dry matter yield (DMY), their ostruthin concentration and their ostruthin yield. Focusing on ostruthin, it was found that the below ground plant parts harvested in the fall rendered the highest ostruthin yield. Furthermore, a variability concerning ostruthin among the twelve accessions was found. This variability among the accessions is of interest with regards to a breeding program used to develop a cultivar with a high ostruthin yield.

Keywords: Ostruthin, *Peucedanum ostruthium*, ASE, HPLC, breeding program

Introduction

Natural products or derivatives make up about one third of all medications (ASIF, 2015). Ostruthin, a natural bioactive compound in the roots of *Peucedanum ostruthium*, has been found to have beneficial uses for a number of health related issues. The anti-proliferative activity of ostruthin could be of use in cardiovascular diseases (JOA et al., 2011); ostruthin's anti-mycobacterial activity could be of use in mycobacterial infections (SCHINKOVITZ et al., 2003). In addition, as an acetylcholinesterase inhibitor ostruthin could also be of use in the treatment of Alzheimer's disease (URBAIN et al., 2008).

The aim of this study was to analyze the differences between the spring and fall harvests of twelve accessions of *P. ostruthium*, with regards to DMY, ostruthin concentration as well as ostruthin yield of the above and below ground plant parts. The results of this study will allow for an identification of productive plants containing a high ostruthin yield to be used in a breeding program.

Materials and Methods

Plant Material

Twelve accessions of *P. ostruthium* from the Swiss alpine region were collected from the wild and reared for two years in an experimental field in Bruson (VS). Two harvests were conducted, one in the spring (May) and one in the fall (October) 2015. The whole plant was harvested and then separated into above and below ground plant parts, which were dried at 38 °C.

Accelerated Solvent Extraction and High Pressure Liquid Chromatography

The plant material was extracted with 60 % ethanol using an accelerated solvent extractor. The extracts were then analyzed qualitatively and quantitatively with high pressure liquid chromatog-

raphy. The target compounds of the extract were identified through their retention time and quantified by comparing them to external standards.

Results

Plant Parts

The above ground plant parts of *P. ostruthium* contained ostruthin in amounts below the defined quantification level. By contrast, the below ground plant parts were rich in ostruthin. The following data refers specifically to the below ground plant parts.

Harvesting

The mean DMY of the spring harvest was 429 g/m² and 1150 g/m² in the fall harvest (Fig. 1).The mean ostruthin concentration in the spring harvest was 1.75 g/100g dry matter (DM) compared with the mean of the fall harvest with 1.41 g/100g DM (Fig. 2). The mean ostruthin yield was 7.5 g/m² in the spring harvest and 16.4 g/m² in the fall harvest (Fig. 3.). Therefore, the best method for acquiring ostruthin seems to be harvesting the plant parts in the fall.

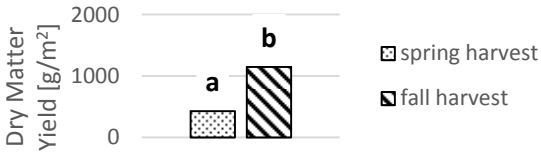

Fig. 1 Below ground plant parts: Mean dry matter yield of the spring and fall harvests for all accessions. ANOVA Tukey HSD p <0.001.

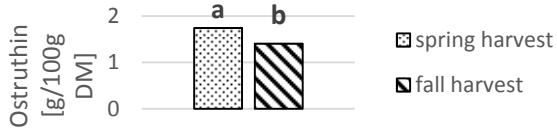

Fig. 2 Below ground plant parts: Mean ostruthin concentration of the spring and fall harvests for all accessions. ANOVA Tukey HSD p <0.001.

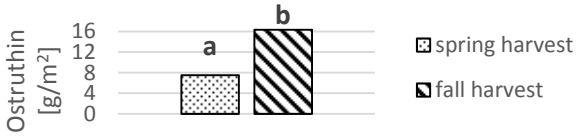

Fig. 3 Below ground plant parts: Mean ostruthin yield of the spring and fall harvests for all accessions. ANOVA Tukey HSD p <0.001.

Accessions

The DMY ranged from 277 – 630 g DM/m^2 in the spring harvest and from 773 – 1539 g DM/m^2 in the fall harvest. The ostruthin concentration varied from 0.87 – 2.27 g/100g DM in the spring harvest and from 0.94 – 1.94 g/100g DM in the fall harvest. The ostruthin yield ranged from 3 – 13 g/m^2 in the spring harvest and from 10 – 30 g/m^2 in the fall harvest. These results show that there is a variability in the accessions and as mentioned above, the fall harvest is the ostruthin rich harvest.

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P 11: Introduction of wild MAP species into the field culture

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Abstract

Althea officinalis L., *Dracocephalum moldavica* L., *Gentiana lutea* L., *Rhodiola rosea* L., and *Valeriana officinalis* L. are the species of wild medicinal plants which are not very commonly grown in field culture. The methods and practical experiences of their multiplication and growing in a field nursery in Olomouc (the Czech Republic) are explained and shown in the manuscript.

Keywords: field nursery, *Althea, Dracocephalum, Gentiana, Rhodiola, Valeriana*

Introduction

Medicinal and aromatic plants (MAPs) are a very heterogeneous group with very different pedigree status of species. Some of them are known, used and cultivated for a very long time but others are wild and still just collected by hand from natural fields. However in some cases also these wild species are useful or necessary to introduce into the field culture and to grow and propagate them in an organized way. An example of such introduction is *ex-situ* genetic resources maintenance where also minority and/or endangered species should be collected, grown and reproduced. The method of cultivation and propagation of some examples of such medicinal plants, that are in the care of the curators of collection of Czech MAPs genetic resources in Olomouc, are presented here. *Althea officinalis* L. (marsh mallow), *Dracocephalum moldavica* L. (Moldavian dragonhead), *Gentiana lutea* L. (great yellow gentian), *Rhodiola rosea* L. (golden root, rose root), and *Valeriana officinalis* (valerian) L. were selected for example.

Materials and Methods

A new species and/or genotypes of wild medicinal plants come to the MAPs collection usually as vegetative material or seed samples from the collection missions. Vegetative material (bunches, rhizomes, root sprouts etc.) together with ball of soil are picked up at original locality (at least 10 plants per accession) and then rooted to the *ex situ* collection immediately next day for founding of a field nursery. Seeds are dried in a drier with controlled air flow, 1/3 of seeds are saved in the seed bank as a security stock and the rest of seeds is sown depending on the species´ requirements (Dušek et al., 2010, Dušková et al., 2010). Then a preliminary multiplication (Jaramillo and Baena, 2002) of both types of original material follows.

Before the planting of original vegetative material and/or seedlings, carefull soil preparation has a crucial role in the founding of the field nursery. A lot of introduced species are perennial plants and will stay several years on the same spot. A rigorous weed clearance and optimal nutrient reserve in the soil is a basic precondition for a successful growing and introduction into the field culture.

The *ex situ* collection is organised in 50 m long rows or double rows in Olomouc, which enable mechanical cultivation and technical isolation by mobile isolation cages (see manuscript "*Ex situ* regeneration of cross-pollinated MAP genetic resources in the Czech Republic" in this issue). Single rows have 2 m space between them and double rows have 0.5 m distance between two neighbouring rows and 2 m between each row pair. The single row organization is preferred for sturdier species where the free access to plants is desirable. On the other hand double rows organization is suitable for small-growing species, where it safes cultivation area and helps in technical isolation by mobile isolation cages. In case of well growing species, the plants from double rows sometimes grow together into one huge row. During the whole vegetative season, a com-

mon mechanical cultivation is done by compact tractor between the rows and/or by hand between the plants in the row. Since all wild MAPs are planted "on water" (i.e. to the well watered holes), additional irrigation is usually not necessary or it is provided only for few months after planting. The pruning is practised in early spring, before the growing of new sprouts.

Besides the above mentioned universal practices also some individual species requirements should be announced here (Tab. 1). For *Althea officinalis* an every year low dose NPK fertilization and aphid chemical control can be recommended. The seeds of *Gentiana lutea* are sown to the boxes right after harvest and placed outside to the safety place where they winter. Such stratification is necessary for good seed germination and sprouting. The seedlings stay almost two years in the box up to planting to the field nursery because earlier manipulation causes plant damage according our experiences. The best results with *Rhodiola rosea* multiplication and growing were obtained when root sprouts were planted to containers in the spring and then they spend the summer in the shadow area. In the autumn or next spring, the well rooted young plants can be transplanted into the field nursery. *Valeriana officinalis* does not require any special procedures and the young plants can be situated to the field nursery in the same year when the seeds were sown. Only the aphid chemical control can be recommended for this crop.

Results

The best overview on successful introduction of presented MAP species is shown in Fig. 1-3. All mentioned species are as *ex situ* collection cultivated already several years in Olomouc and their generative and/or vegetative multiplication is fruitful.

Tab. 1 The multiplication and growing procedures recommended to the selected MAPs species

Species	Life form	Original material	Seed sowing	Planting	Field organization
Althea officinalis	perennial	rhizomes or seeds	mid March	March - April	single rows; 40 cm
Dracocephalum moldavica	annual	seeds	mid March	mid May	double rows; 40 cm
Gentiana lutea	perennial	seeds	promptly after seed harvest	mid September (2 years old plants)	single rows; 40 cm
Rhodiola rosea	perennial	root sprouts		rooted plants in the end of summer or in the spring	single or double rows; 40 cm
Valeriana officinalis	perennial	seeds	mid March	September	single rows; 40 cm

Acknowledgement

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Fig. 1 Althea officinalis L. and Gentiana lutea L. in field nursery in Olomouc

Fig. 2 Dracocephalum moldavica L. in compact double row planting

Fig. 3 Three years old plants of Rhodiola rosea L. in single row planting

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P 12: Conservation of medicinal and aromatic plants

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Abstract

The conservation of medicinal and aromatic plants includes *ex situ* and *in situ* methods. The genetic recourses of medicinal and aromatic plants are stored, studied and constantly maintained in the field collections of the Institute of Botany of Nature Research Centre, Kaunas Botanical Garden of Vytautas Magnus University and Aleksandras Stulginskis University of Agriculture. Presently seeds of 214 accessions representing 38 species of medicinal and aromatic plants are stored in a long-term storage in the Plant Gene Bank. The data about national genetic resources are collected and stored in the Central Database of the Plant Gene Bank.

Keywords: medicinal plants, aromatic plants, *ex situ*, *in situ*, conservation

Introduction

The gathering and utilization of medicinal plants is an age-old tradition in Lithuania, especially in forested areas of the country. Investigations into medicinal plants in Lithuania were commenced in the 18th century. The Botanical Garden of Vilnius University played a major role in medical practise of those days; medicinal plants were planted, distributed and studied. The first factory of processing medicinal plants was opened in Švenčionys in 1883 (BUDRIŪNAS, R., 1999). The researches of medicinal plants were commenced when the Department of Medicinal Plants at the University of Vytautas Magnus in Kaunas had been established by prof. K. Grybauskas in 1924 (RAGAŽINSKIENĖ, O. 2004). The investigations of medicinal plants at the Institute of Botany were started in 1959.

Within the framework of the National Plant Genetic Resources programme, the MAP working group was one of the crop-specific working groups, which consolidated researches of different institutions possessing collections. In 1995 Lithuania joined the European Plant Genetic Resources (PGR) conservation network.

Genetic biodiversity of medicinal and aromatic plants is under the threat of extinction as a result of urbanization of natural and agricultural areas, habitat loss due to harvesting of raw material and changes in land use and agricultural practice.

The aim of this paper is to give a brief review of the current status of the conservation of the medicinal and aromatic plants genetic resources in Lithuania.

Materials and Methods

The existing system of conservation of medicinal and aromatic plants includes *ex situ* and *in situ* methods. Preliminary assessment of genetic diversity has been carried out according to the morphometric variation of phenotype in wild populations. On the second stage the evaluation and selection of accessions has been made according to their morphological characters and chemical composition as well susceptibility to diseases. In the selection process of *in situ* conservation areas the following criteria were considered: ecological heterogeneity of the site, phenotypic diversity and concentration of the target species, economic value of the target species, the possibility of the site control, the location of the site with regard to protected areas.

The seeds of medicinal and aromatic plants are stored in a long-term storage in the Plant Gene Bank. Seed samples are cleaned of weed seeds, pests and diseases. A dehumidified drying chamber is used for seed drying. Seeds are dried for two–three months at temperature 15-20 °C and relative air humidity of 10 – 15 %. Seeds moisture content after drying reduces to 3 – 5 %, they are

packed in airtight aluminium foil bags and stored at -18 °C. Long-term storage conditions guarantee the seed survival for decades as only very limited metabolism can occur there.

Results

The Lithuanian flora contains 1334 plant species. There are more than 460 species, which are used in folk and traditional medicine in Lithuania (RADUŠIENĖ and JANULIS, 2004). The majority of medicinal and aromatic plants are still collected from the wild; the lack of advanced local varieties limits their cultivation. There are species which are difficult to cultivate and therefore vulnerable to harmful harvesting of wild populations**.** 33 species of medicinal plants are included in the Red Data Book of Lithuania**.** Generally, the conservation of wild plants species and their resources is regulated by the the Law on Wild Vegetation (1999), the Law on Protected Areas (1993, 2001), Law on National Plant Genetic Resources (2001) and supplementary legal acts. The protected areas account for 14.8 % of the total area of the country.

The existing system of conservation of medicinal and aromatic plants includes *ex situ* and *in situ* methods.

***Ex situ* conservation of medicinal and aromatic plants**

The recourses of medicinal and aromatic plants are stored, studied and constantly maintained in the field collections of the Institute of Botany of Nature Research Centre, Kaunas Botanical Garden of Vytautas Magnus University and the Aleksandras Stulginskis University.

Today the field collection of the Institute of Botany of the Nature Research Centre includes over 140 species of medicinal and aromatic plants and berry plants. About 90 % of the accessions are plants of wild origin native to Lithuania and neighbouring countries, collected mainly for the purposes of research and conservation. There are over 600 accessions of medicinal and small fruit plants. In Kaunas Botanical Garden the plants are classified by the pharmacognostic principle in respect to the biologically active compounds. The collection of medicinal plants consists of 400 species, the indigenous species comprise one fifth of the collection. The field collection of caraway, which vary in time of flowering, colour of inflorescence and the amount of essential oils are stored in Aleksandras Stulginskis University.

Long-term seed storage

The long-term seed storage was established in 1997 in the National Plant Genetic Resources Coordinating Centre. The Nordic Gene Bank provided all necessary facilities.

At the present time seeds of 214 accessions representing 38 species of medicinal and aromatic plants are put in long-term storage in the Plant Gene Bank. The majority of the accessions are of Lithuanian origin with rare exceptions of some foreign accessions of special value to Lithuanian growing conditions. The long-term seed storage is annually supplemented with new accessions.

***In situ* conservation**

Long-time observations have revealed that protection and certain management of natural populations *in situ* are required to ensure their survival and sustainable utilization (LABOKAS, 1999). In the selection process of *in situ* conservation areas the following criteria were considered: ecological heterogeneity of the site, phenotypic diversity and concentration of the target species, economic value of the target species, the possibility of the site control, the location of the site with regard to protected areas. Target species selected on the basis of socio-economic and scientific values are the following: *Acorus calamus* L, *Arnica montana* L., *Allium* spp., *Crataegus* L., *Origanum vulgare* L., *Thymus* spp., *Hypericum* spp., *Helichrysum arenarium* (L.) Moench., *Salvia* L., *Vaccinium* spp., and others. Among the most endangered are plant populations in the forest ecosystems because they are greatly subject to forestry activities. In most cases *in situ* conservation of medicinal and small fruits is more reliable within the already existing network of protected areas than outside them

(Labokas, 1999). Today the 21 areas for *in situ* conservation of medicinal plants and small fruits as well crop wild relatives in Lithuania are selected.

Status of national medicinal and aromatic genetic resources

Today the status of national genetic resources has been granted to 4 collections, 21 areas *in situ* and 207 accessions of medicinal and aromatic plants. The data about these national genetic resources are collected and stored in the Central Database of Plant Gene Bank.

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P 13: Evaluation of the phytochemical constituents total phenol, total flavonoid and anti-oxidant activity of *Delonix elata* flower extract

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Abstract

The objective of the present study was to evaluate the phytochemical constituents, total phenol, total flavonoid, anti-oxidant activity of flower extract of *Delonix elata.* Plants are widely used in pharmaceutical and food industries due to their biological importance. Among the plant parts, leaves, stem, roots and bark are widely studied for their biological properties. However, flowers are almost neglected and are not much probed for their importance. The present study was carried out to identify the phytochemicals and evaluate antioxidant activity of flowers of *D. elata*. The antioxidant activity was determined by the method of DPPH radical scavenging assay. The flower extract contain saponin, alkaloid, terpenoids, flavonoids, steroids, phenols, cardioglycosides, quinines, coumarins and tannins. Thus, clearly indicate that the flower extract of *D. elata* shows significant antioxidant activity which in turn greatly contribute in reducing the risk of many diseases including heart disease, cancer cell formation and cell physiological abnormalities.

P 14: Variability in essential oil of *Ducrosia anethifolia* (DC.) Boiss. growing wild in Fars province, Iran

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Abstract

Ducrosia anethifolia (DC.) Boiss. a traditional and popular herb grows wildly in several areas of Iran. The aerial parts, including flowers, were collected from *D. anethifolia* plant populations (Shiraz (1-2), Kazeroun, Noorabad, Farashband, Firoozabad, Ghir, Jahrom (1-2) and Darab) growing wild in Fars province, located in southwestern Iran. The essential oils (EOs) were subjected to hydro-distillation using a Clevenger-type apparatus.The essential oil yields ranged from 0.17 % to 0.93 % (w/w). Analysis of variance showed that geographic distinction had a significantly effect on the EO content in plants of different regions. Two populations, Jahrom 2 and Shiraz 2 engrossed the maximum EO yield. Seventy-five volatile compounds were identified by GC and GC–MS analysis. The main EO components were n-Decanal (1.493–45.062 %), Dodecanal (1.093–34.744 %), *cis*-Chrysanthenyl acetate (0–26.609 %), α-Pinene (0–16.539 %), n-Decanol (1.285–49.225 %), (2E)-Dodecenal (0.879–16.856 %), Decanoic acid (0–12.626 %), n-Nonanal (0.401–6.711 %), and Caryophyllene oxide (0.113–5.873 %). Analysis of the principal components based on the mean relative amounts of EO components led to the identification of four chemotypes: n-Decanal, Dodecanal, *cis*-Chrysanthenyl acetate, n-Decanol of which the n-Decanal chemotype is found more normally in different parts of Iran.

Keywords: Chemotypes, wild Population, essential oils

Introduction

Ducrosia anethifolia (DC.) Boiss. is a medicinal plant that belongs to Apiaceae family. It has been distributed mainly in Afghanistan, Iran, Iraq, Pakistan, and also to lesser extend can be found in Syria, Lebanon, and some of the Arabic and Persian Gulf countries. This species is used traditionally in animal nutrition, diseases eradication and cooking. It is also traditionally utilized to improve food taste and beverages. Their medical application ranged from cold therapy to cure stomach pain (Obaidi et al., 2012; Shooshtari et al., 2013). *D. anethiflia* is one of three species in this genus which grows wildly in several regions. *Ducrosia* is commonly known as Moshgak, Rishkag and Moshkbou in Iran (Mozaffarian, 2013). The aerial parts of plants are used as a pain killer and have analgesic activity which make them anti-headache, back pain, colic and colds. In some regions of Iran, it has been stated that they are effective against anxiety and insomnia (Obaidi et al., 2012).The antimicrobial, antibacterial and antianxiety activity of *D.anethifolia* has been previously defined in the available literature (Shahipour et al., 2012). In present study, the essential oil of D. *anethifolia* growing wild in Fars province, located in southwestern Iran, were investigated.

Materials and Methods

Plant material

Aerial parts of the plant were collected in April 2015 from *D. anethifolia* plant populations (Shiraz (1-2), Kazeroun, Noorabad, Farashband, Firoozabad, Ghir, Jahrom (1-2) and Darab) growing wild in Fars province, located in southwestern Iranat the time of flowering. The plant was identified by Fars Research Center for Agriculture. Voucher specimens were deposited at the Herbarium of Fars Research Center for Agriculture, Shiraz, Iran.

Analysis of the oil

The aerial parts were air-dried at ambient temperature in the shade and hydrodistilled by using a Clevenger-type apparatus for 3 h. The oil was dissolved in n-hexane (Merck), dried over anhydrous sodium sulfate and stored at 4 °C ± 2 °C. GC analysis was performed using an Agilent gas chromatograph series 7890-A with a flame ionization detector (FID). GC-MS analysis was carried out by use of Agilent gas chromatograph equipped with fused silica capillary HP-5MS column coupled with 5975-C mass spectrometer. The constituents of the essential oil were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C8-C25) and the essential oil on a HP-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature (ADAMS, 2007). For quantification purpose, relative area percentages obtained by FID were used without the use of correction factors.

Results

The aerial parts, including flowers, were collected from *D. anethifolia* plant populations (Shiraz (1-2), Kazeroun, Noorabad, Farashband, Firoozabad, Ghir, Jahrom (1-2) and Darab) growing wild in Fars province, located in southwestern Iran. The essential oils (EOs) were subjected to hydrodistillation using a Clevenger-type apparatus.The essential oil yields ranged from 0.17 % to 0.93 % (w/w). Analysis of variance showed that geographic distinction had a significantly effect on the EO content in plants of different regions. Two populations, Jahrom 2 and Shiraz 2 engrossed the maximum EO yield. The applied GC × GC/MS metabolite profiling resulted in the identification of a total of 33 compounds based on comparison with MS library, consisting of compounds from *D. anethifolia* EOs. The constituents of the EOs are represented in Table 1. Seventy-five volatile compounds were identified by GC and GC–MS analysis. The main EO components were n-Decanal (1.493–45.062 %), Dodecanal (1.093–34.744 %), *cis*-Chrysanthenyl acetate (0–26.609 %), α-Pinene (0–16.539 %), n-Decanol (1.285–49.225 %), (2E)-Dodecenal (0.879–16.856 %), Decanoic acid (0–12.626 %), n-Nonanal (0.401–6.711 %), and Caryophyllene oxide (0.113–5.873 %). Analysis of the principal components based on the mean relative amounts of EO components led to the identification of four chemotypes: n-Decanal, Dodecanal, *cis*-Chrysanthenyl acetate, n-Decanol of which the n-Decanal chemotype is found more normally in different parts of Iran. In the present study different chemical compositions of this species is reported. It is known that many factors influence the chemical constitution of *D. anethifolia* EOs. The differences in the EOs content and composition of the present and previous investigation may be dependent on the collection time, chemotypes, geographic and climatic factors, drying conditions and mode of distillation. The chemical variability could be endorsed to genetic and environmental factors as well as being helpful in the enhancement of *D. anethifolia* resources for food and pharmaceutical industries.

Tab. 1 The major essential oils of D. anethifolia plant populations growing wild in Fars province, located in southwestern Iran.

Compound	RI	Dar	Firz	Fash	Ghir	Jahr1	Jahr2	Shz1	Shz2	Kaz	Nor
α-Pinene	935	2.93	14.1	8.30	6.27	6.92	16.5	5.02	0	8.19	8.75
Limonene	1030	0.87	1.49	2.28	1.72	1.53	3.07	2.34	0.04	1.68	2.14
n-Nonanal	1104	1.27	0.75	0.59	0.69	0.54	1.25	2.51	6.71	0.44	0.40
n-Decanal	1202	24.5	20.2	43.9	41.4	45.0	1.49	44.8	18.8	35.8	29.4
cis-Chrysanthenyl acetate	1264	26.6	0	0.11	0.39	6.78	0	0.91	0.89	0.05	0.54
n-Decanol	1271	8.62	10.9	4.82	2.57	5.62	49.2	9.15	11.1	2.34	1.28
Undecanal	1305	2.56	1.42	0.87	1.87	0.66	4.80	0.82	2.51	0.54	0.45
Decanoic acid	1365	0.12	0	0	0.07	0	0.06	2.40	12.6	0	0
Dodecanal	1408	11.6	34.7	19.2	18.5	12.2	1.09	11.5	16.6	34.0	21.8
(2E)-Dodecenal	1467	1.73	0.87	2.99	6.13	1.51	1.52	5.75	1.99	4.77	16.8
Caryophyllene oxide	1579	1.97	0.66	0.26	1.73	0.40	1.28	2.69	5.87	0.11	0.11
n-Tetradecanol	1670	0.36	0.40	2.02	2.64	2.66	0	1.61	1.74	2.21	4.87

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P 15: Breeding of a high yielding chamomile variety (*Matricaria recutita* L.) with improved traits for machine harvesting

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Abstract

A more productive variety of chamomile (*Matricaria recutita* L.), which is more efficient in machine processing with consistent quality traits, will benefit the viability of german products in the global market. Breeding of an enhanced chamomile variety is part of a german multi-network project called KAMEL whose research aims on *Matricaria recutita* L., *Valeriana officinalis* L. and *Melissa officinalis* L. The agronomic and qualitative improvement of these speciality crops are the basis for further economic prosperity of medicinal and aromatic plant cultivation in Germany.

The main breeding goals of a new variety of chamomile are the increase of blossom product yield (*Matricariae flos*) to 6 dt/ha in up to three harvest stages through a homogenous flower horizon (pick height), an even flowering time, large flower heads and a high regeneration rate after each harvest stage. The upgrade of the content of essential oil content to a minimum of 0.8 % with its compostion according to Ph. Eur. and a chamazulene content of min. 25 % are further objectives of the breeding process. In addition to these quality traits, high tolerances against common fungal diseases are of particular interest. Development of an innovative chamomile variety is realized over nine years in three stages (2010 - 2019).

Keywords: medicinal and aromatic plants, chamomile, breeding, yield, quality, machine processing

Introduction

Chamomile (*Matricaria recutita* L.) is one of the most common medicinal plants worldwide. Its origin is the Near East and south and east Europe. It is to be found almost all over Europe, Asia Minor, North and South America, New Zealand and Australia (Franke et al. 2005). Chamomile has been used in herbal remedies for thousands of years, known in ancient Egypt, Greece, and Rome (Singh et al. 2011). Besides as a raw product for food industries it is one of the most important crops for pharmaceutical and cosmetic purposes in Germany (Wagner et al. 2005). The history of chamomile breeding in Germany started around the 1950´s with the development of the "Quedlinburger Großblütige" and the "Erfurter Kleinblütige" populations as commercial-used varieties (Hoppe et al. 2012). After this initiation, systematic breeding of chamomile in Germany started a rapid development with a widespread outcome. However, in other countries mainly in east Europe further breeding has resulted in improved populations and registered varieties.

Currently a large number of different varieties with agricultural use are known worldwide, which are mostly old cultivars, landraces or mixed populations without a current right protection. Only a few registered cultivars with high contents of α-bisabolol and chamazulene e.g. `Manzana´ (1986) and `Mabamille´ (1995) are dominating the german medicinal and aromatic plant cultivation.

The tetraploid variety `Bodegold´ (1962) which was bred in the 1950s in Quedlinburg is widespread too (Otto et al. 2015) not least because of its exemplary sensoric qualities. Other, mainly tetraploid (4x) and also diploid (2x) varieties are available in a large scale. In general they reveal deficits in agronomic traits regarding their harvest-ability. Parameters like an inhomogenous pick height or an uneven flowering time still need to be improved. Also their yield capability under machine harvest conditions is still unexhausted.

The aim of the project is breeding an open pollinating variety (non-hybrid) or a synthetic line (artificial population emerging of selected component lines) which is capable to exceed yield performance of current german cultivars (4.5 dt/ha) by use of machine processed harvesting.

Improved agronomic traits like a homogenous and small pick height, and even flowering time, large flower heads and a high regeneration after each harvest stage is intended. Another aim is the refinement of chamomile flower contents of essential oil to a minimum of 0.8 % and the consolidation of the content of matrizin/chamazulene to a minimum of 25 %. The general compostion of compounds must accomplish to Ph. Eur. Also breeding lines which are tolerant against common fungal diseases are intended to be found.

Material and methods

The breeding process is built of three stages which are arranged consecutively. (i) Characterizing known lines and development of source material (2010-2012), (ii) Development of different breeding lines and test of their combination ability (2014-2016), (iii) Selection of breeding lines (for cultivar registration), seed propagation of priority crossings and component lines, field trials under conditions of practice (2017-2019).

In the first stage, source material of 30 different origins was characterized in two years autumn and spring sown trials (Table 1). Yield performance was tested as hand-picking harvest within a plot-in-plot area of 1 m^2. Most important criteria of the evaluation were yield under hand-pick conditions, yield-distribution over different harvest stages, the arrangement of flowers at the plant (different pick heights of 6 cm from above, 15 cm, and under 15 cm), the flower size, pickability and flowering period as well as the content of quality determining constituents and the resistance against different diseases and pests. Of 30 characterized origins, eight lines were valued to be founder lines of single plant selections. Of these donors, isolated self-pollinated seeds were generated in the third year (I1).

Tab 1 Donor material for breeding a new chamomile (Matricaria recutita L.) variety

cultivar/ commercial variety	origin	ploidy level	cultivar/ commercial variety	origin	ploidy level
Argenmilla	RA	2x	Origin of USA	US	2x
Aromi	IT	4x	Origin of India	IN	2x
Bodegold	DE	4x/2x	Origin of Croatia	HR	2x
Bohemia	CZ	4x	Lazur	BG	4x
Bona	SK	2x	Lutea	SK	4x
Camoflora	DE	2x	Mabamille	DE	4x
Chamomilla organic B&T		4x	Manzana	DE	4x
Flortis company	IT	4x	Margaritar	RO	4x
GARAFARM company	HU	4x	Nosbona	SK	2x
Golden Line company	IT	4x	PNOS Polen	PL	4x
Klepenkeri company	DE	4x	Promyk	PL	2x
Ferme de Saint Marthe	FR	4x	Robumille Akk34	DE	4x
Germania	DE	2x	Robumille Typ046	DE	4x
Goral	SK	4x	wild type fr. Dover	GB	2x
Origin of Russia	RU	2x	Zloty Lan	PL	4x

In the second stage, self-pollinated I1-seeds of 60 selected lines were tested in field trials. Further limitation in this progeny was carried out to 30 lines and single plant selections were made for I2-seed production and analytics. The resulting set of I2-progeny was tested in 2015 in a large field trial with yield analysis (hand pick) and for reselection of single plants to strengthen homogeneity in relevant genotypes and to produce I3-seeds. In the next step, single plant progenies (I3-seeds) were chosen for test-crossings to evaluate the combination-ability in specific maturing- and height-groups. In addition, the component lines of test-crossings will be selected again to homogeneity and going to be multiplicated (I4-seeds) in 2016.

In the third stage (2017-2019) the hybridization-products will be tested in large scale field trials and will be evaluated for their combination ability. Component lines will be multiplicated and homogeneity of these parental lines will be improved. The project leads to the selection of poten-

tial candidates for cultivar registration. Field trials under conditions of practice are planned for validation.

Results

At the beginning of the first three-year stage of the project, 30 origins were characterized. Apparently none of these genotypes accomplished the breeding goals in every respect. This supports the necessity of an intercross- breeding scheme. Eight lines of the source material were scored as valuable. In the next step, overall 600 to 1000 plants of this valuable material were grown for selection. Overall 200 Single plants were selected to harvest a sample for analytics (first pick) and to generate self-pollinated I_1-seeds (second pick of isolated flowers). The breeding goal regarding essential oil content with 0.8 % were found in twelve single plant selections out of three different origins. The requested chamazulene content (min. 25 %) was found in 42 % of all analyzed samples. 83 % of all samples reached or surpassed the minimum content of Apigenin of 0.25 % of dry matter.
In the second stage, 60 I1-lines were tested in a plot-designed field trial resulting in a selection of 50 % of these lines for characterization and to generate isolated I2-seeds. Accordingly to their valuable components and performance they were grouped in yield-types (Y), essential-oil types (O), types with optimal pick-height (P) and types with resistances to common chamomile diseases (R). Whereas 19 single plant selections were identified as (Y)-types with general growth above average and a high flower yield. Seven lines (Y+O) could be exposed with high flower yield in combination with high contents of essential oil of 0.83 - 1.05 %. Other seven selected lines (P) provide the intended flower pick- height of 6 cm – 8 cm. The pick-height of other 24 lines (P) was in the range of 12 cm. The weather conditions in 2014 lead to a drastic infestation with powdery and downy mildew and facilitate the selection of resistant genotypes within the set. Nine selections (R) were free of any symptoms in this year. Monitoring of diseases is included in the further breeding process. The homogeneity of single breeding lines was still insufficient at this point. In 2015 a field trial with 50 selected I2-candidate lines of the different types (Y, O, and P) was carried out. It was aimed at the improvement of the homogenous growth through further single-plant selection within the breeding lines.The generation of I3-seeds as well as the selection of candidates for test-crosses were completed. In 2016 the I2-generation will be tested for agronomic parameters especially yield components in a single-trial. At the same time the I3-lines will be evaluated for all important breeding parameters. Besides these two trials, isolated nine test-crosses of selected lines are underway with simultaneous maintaining of their components at 49 isolated locations.

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P 16: Evaluation of agronomical and qualitative characteristics of Greek Oregano (*Origanum vulgare ssp. hirtum*) germplasm for breeding purposes.

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Abstract
Oregano (*Origanum vulgare*) is one of the most commercially valued species with remarkable biological properties, while its world trade and consumption is steadily increased. In order to identify a standardized plant material of Greek Oregano (*Origanum vulgare* ssp. *hirtum*), sixteen native populations collected from different regions of Greece, were evaluated for their essential oil yield and composition. A breeding program was initiated for the most effective populations, concerning the most desirable biochemical, agronomic and morphological characteristics, using pedigree method and honeycomb design for plant selection.

Keywords: *Origanum vulgare*, breeding, honeycomb, carvacrol, thymol

Introduction

Greek Oregano (*Origanum vulgare* ssp. *hirtum*) is widely grown and collected from native wild populations throughout Greece. In addition, essential oil from Greek Oregano has been suggested as one of the best quality worldwide, with high concentration of the major compounds: carvacrol and thymol, accompanied by *p*-cymene and *γ*-terpinene (KINTZIOS, 2002).

Despite the highly increasing consumption and the great commercial value of Oregano, the excessive and uncontrolled collection from wild has significantly reduced the native populations, which indeed are a natural source of biodiversity. CANTER et al. (2005) reported the growing concern about diminishing populations, loss of genetic diversity, local extinctions and habitat degradation. Therefore, bred populations and controlled cultivation instead of wild collection, has been proposed as a safe way to balance human demands and production, as well as to conserve biodiversity of Greek Oregano.

Last decades, efforts have been started in the area of domestication and systematic cultivation of Oregano (BERNÁTH, 1997, GOLIARIS et al. 2002). Taking into consideration the demands of growers, producers and consumers, it is necessary to develop oregano breeding programs which should be directed to the improvement of yield components (e.g. growth habit, leaf/stem ratio) and quality related parameters (e.g. essential oil content and composition) (FRANZ and NOVAK, 1996).

Therefore the aim of this study was the selection of efficient oregano genotypes, from native Greek populations, to use them further as starting material for breeding purposes, focused on the development of commercial oregano varieties.

Materials and Methods

Plant material and experimental design

Sixteen native populations of oregano (*Origanum vulgare* ssp. *hirtum*), collected from different geographical areas of Greece, were evaluated for oil content and the most characteristic chemical compounds (carvacrol and thymol). The populations indicating higher concentration of essential oil and carvacrol (No. 2, 3, 6, 7, 11, 12, 14, 15, 16) were propagated by seeds and cultivated in the experimental field of IPB&GR (40°34'35'' N 22°57'19'' E). These selected populations were evaluated under the same ecological conditions for two consequent growing seasons, for certain agronomi-

cal and biochemical characteristics. Among them, population No. 16, with desirable characteristics, was propagated with stem cuttings and seeds and was experimentally cultivated. Plants from the specific population, derived both from open (OP) and self pollination (SP), were used for the establishment of an R-13 honeycomb experimental design (Figure 1).

Figure 1. The R-13 honeycomb design used for the breeding program of *Origanum vulgare* ssp. *hirtum* (population No 16). Each entry is allocated in such a way that is always surrounded by plants of all the other twelve entries.

The principal breeding objectives were: a) qualitative characteristics; high essential oil and carvacrol, thymol yield, b) agronomical characteristics; early and uniform blooming and high biomass production and c) morphological characteristics; plants with straight and high stems and broad inflorescences.

Essential oil isolation

The Essential oil content was determined using the European Pharmacopoeia apparatus (Clevenger-type). The dried aerial parts (leaves and flowers) of oregano were subjected to hydrodistillation for 1.30 hours with a distillation rate of 3 to 3.5 mL min^{-1}. The oil content was estimated on the basis of dry weight plant material (mL 100 g^{-1} of dried leaves).

Analysis of essential oil

The essential oils were analyzed by GC-MS on a fused silica DB-5 column, using a Gas Chromatograph 17A Ver. 3 interfaced with a mass spectrometer Shimadzu QP-5050A supported by the GC/MS Solution Ver1.21 software, using the method described previously (Sarrou et al., 2013). The identification of the compounds was based on comparison of their retention indices (RI) relative to n-alkanes (C_7-C_{22}), with corresponding literature data and by matching their spectra with those of MS libraries (NIST 98, Willey) (Adams, 1995).

Results

Selection of genetic material

The essential oil content and composition varied among the 16 different native populations of Oregano (Figure 2). More specifically, four of them (No. 10, 11, 15, 16) presented high essential oil yield (> 7 %), while populations 12 and 16 exhibited the higher concentration in carvacrol (82.7 and 76.86 %) and thymol (2.7-4.3 %) (Figure 3).

Further evaluation of their morphological characteristics indicated that population 16 is the most desirable for starting breeding material, since it is characterized from high and straight stems, high leaf/stem ratio, broad inflorescences, early and uniform blooming (Figure 4).

Breeding methodology & Honeycomb design

Pedigree method was applied using individual plants from population No 16 in R13 honeycomb design (KOUTSOS, T. V. and M. KOUTSIKA-SOTIRIOU, 2001).The first year of plant's development, agronomical and morphological characteristics were determined (% area/plant, fresh and dry biomass production/plant, existence of blooming plants and seed production). The evaluation of these data in combination with the observations from the second year of experimentation, will determine the most preferable plant row material from this population.

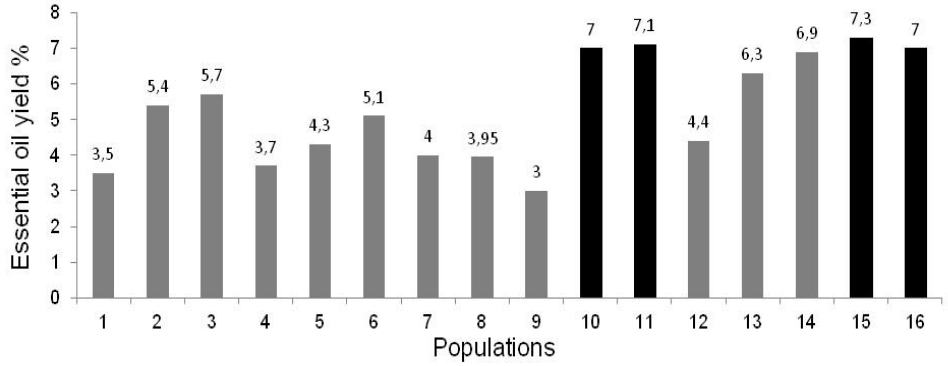

Fig. 2 Essential oil yield (%) of native populations of *Origanum vulgare* ssp. *hirtum,* collected from different geographical areas of Greece.

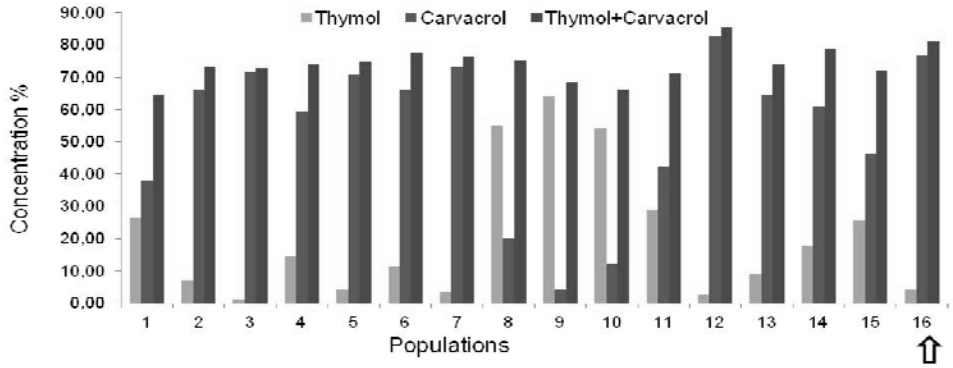

Fig. 3 (%) Composition of thymol and carvacrol of the essential oil from native populations of *Origanum vulgare* ssp. *hirtum* collected from different geographical areas of Greece.

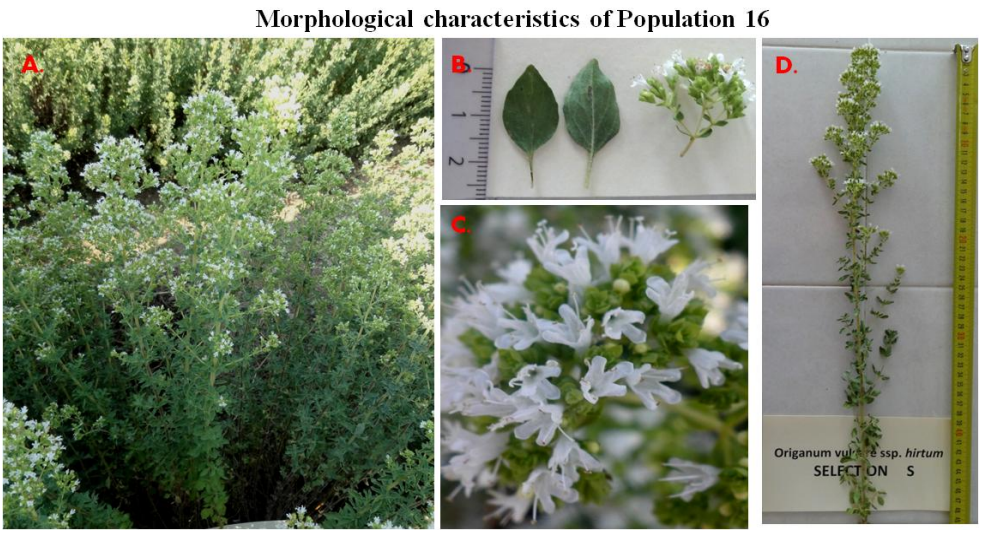

Fig. 3 Morphological characteristics of selected plants from population (No 16) of *Origanum vulgare* ssp. *hirtum*.

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P 17: ATR-FTIR Spectroscopy on intact dried leaves of sage (*Salvia officinalis* L.) – chemotaxonomic discrimination and essential oil composition

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Abstract

Sage (*Salvia officinalis* L.) is cultivated worldwide for its aromatic leaves which are used as herbal spice and for phytopharmaceutical applications. Fast analytical strategies for essential oil analysis, performed directly on plant material would reduce the delay between sampling and analytical results. This would enhance product quality by improving technical control of cultivation. The attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy method described here provides a reliable calibration model for quantification of essential oil components (EOC) and its main constituents (e.g. α-thujone and β-thujone) directly on dried, intact leaves of sage. Except for drying no further sample preparation is required for ATR-FTIR and the measurement time of less than 5 min per sample contrasts with the most common alternative of hydro-distillation followed by GC analysis which can take several hours per sample.

Keywords: infrared spectroscopy, essential oil, thujone, quantification, PLS, nondestructive, ATR-FTIR, *Salvia officinalis* L., chemometry

Introduction

Sage (*Salvia officinalis* L.) is a popular aromatic plant found on all continents except Antarctica and Australia (Al-Qudah et al., 2014; Ben Farhat et al., 2009; Grausgruber-Gröger et al., 2012; Lamien-Meda et al., 2010). The pharmaceutical uses and aromatic taste of sage are largely derived from the essential oil that is rich in thujone and camphor with α- and β-pinene, 1,8-cineole or camphene as minor constituents.

Today a large number of genetically different accessions of *S. officinalis* L. have been described that are characterized by varying content of essential oil and high diversity of the related chemical composition (Lamien-Meda et al., 2010). Furthermore, content and composition of essential oil depend on climatic and cultivation conditions, on developmental stage of the plant/leaf, origin of plant material and even on daytime of harvest. Hence, for both, plant breeding and cultivation, the exact content and composition of the essential oil is needed to ensure that the final products are of consistent and high quality (Pharmacopoeia, 2014).

Traditional analyses of essential oil based on gas chromatography (GC) are time-consuming and expensive and also require laboratory infrastructure as well as specially trained personnel. Hence, analytical methods directly applicable to intact plant material which are more efficient, faster and robust are demanded. With the ATR-FTIR technique, mono-layers of analytes can be investigated through direct contact between sample and the IR-crystal. Since the essential oil in *S. officinalis* L. is located in special glandular trichomes on the leaf surface, ATR-FTIR offers a promising alternative to GC.

Therefore, the aim of this study was to investigate the use of ATR-FTIR spectroscopy for the direct qualitative and quantitative analysis of the essential oil composition of dried intact leaves of sage and including the effect of different varieties, leaf age and date of harvest. Based on reference analysis of solvent extracts from the plant material by GC appropriate calibration models for es-

sential oil content and composition can be developed that include the chemical diversity of different sage accessions.

Materials and Methods

Seeds of 12 different sage (*Salvia officinalis* L.) accessions were obtained from the Leibniz Institute for Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germnay). Seeds of one additional commercial sage accession were obtained from Bingenheimer Saatgut GmbH (Echzell, Germany). Plants were cultivated at the experimental field of the Julius Kühn-Institute in Berlin (Germany) and leaf samples were taken for different developmental stages and at two different dates of harvest.

The ATR-FTIR spectra of intact dried sage leaves and pure standards were recorded with a portable ATR diamond crystal infrared spectrometer (Alpha, Bruker Optics GmbH, Ettlingen, Germany).

For estimating the variability of the samples, principal component analysis (PCA) was used based on the instrument software package OPUS 7.2 (Quant 2 module, Bruker Optik GmbH, Germany) and Unscrambler X (Camo, Norway).

Detailed botanical description, experimental information for solvent extraction, GC-FID analysis, infrared spectroscopic measurements and statistic are given in the appropriate literature (GUDI *et al.*, 2015).

Results

Sage is known to show a large variety in essential oil content and composition (GRAUSGRUBER-GRÖGER et al., 2012; LAMIEN-MEDA et al., 2010). Information about chemical heterogeneity of the accessions is highly relevant for the development of suitable calibration models with FTIR-ATR spectroscopy (where only a small amount of material is used with each measurement) since it determines an appropriate sampling and statistical evaluation.
Therefore, a representative number of plants (repetitions per accession) has to be incorporated and a greater degree of averaging ATR-FTIR spectra (summarizing variability by reduction of data points) has to be applied.
The use of spectra averaged across accessions leads to an improved prediction not only for the EOC yield and major constituents (α-thujone, β-thujone, camphor) but also for components with lower concentrations (in per mill range) such as α- or β-pinene and 1,8-cineole.
For all essential oil components, coefficients of determination (R^2) higher than 0.86 were obtained with low values for BIAS and RMSECV of less than 10 % of the corresponding mean values (GUDI et al., 2015).

Futhermore, the robustness of the validated models was investigated with an additional test-set validation of 12 independent samples (3 sets of 10 plants, old/young, H1/H2) which came from the commercial sage accession.

As shown by the values of R^2, RMSEP and BIAS given in Table 1, for essential oil components, a robust and reliable prediction model could be achieved with $R^2 = 0.98$. The relatively low prediction accuracy for individual metabolites could be explained by the genetic and chemotaxonomic diversity of the investigated sage acessions.

Tab.1 Statistical parameters for test-set validation of the developed prediction models of plant wise averaged spectra (n=48) with 12 independent samples from the commercial accession.

component	R^2	RMSEP*	BIAS*	Range*/mean*	LV**
EOC content	0.98	0.174	0.0371	2.223 - 5.514 / 3.869	7
α-thujone	0.74	0.124	0.061	0.547 - 1.268 / 0.908	8
β-thujone	0.80	0.0778	0.0375	0.085 - 0.593 / 0.339	5
camphor	0.74	0.09	-0.0768	0.253 - 0.895 / 0.574	7
1,8-cineole	0.87	0.0482	0.0241	0.142 - 0.598 / 0.37	6
α-pinene	0.55	0.0471	0.0184	0.056 - 0.312 / 0.184	7
β-pinene	0.84	0.0111	0.00267	0.02 - 0.103 / 0.0615	8

ml 100 g^{-1} DM, ** Number of Latent Variables (LV)

In the current ATR-FTIR quantification protocol plant material only needs to be dried and spectral measurements take only a few minutes. This allows rapid quality control of the material when it is first received for processing compared to the time-consuming analysis recommended in the Pharmacopoeia.

Transfer of the current analytical protocol to fresh material and application in the field is currently under investigation. This will undoubtedly improve growing and quality control for both plant breeding and cultivation and assist in evaluating the chemical variation of breeding populations and identifying optimal points for harvest.

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P 18: Discrimination of fennel chemotypes applying IR and Raman spectroscopy – discovery of a new γ-asarone chemotype

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Abstract

Various vibrational spectroscopy methods have been applied to classify different fennel chemotypes according to their individual profile of volatile substances.

Intact fennel fruits of different chemotypes could be successfully discriminated by Attenuated Total Reflectance Fourier transform Infrared (ATR-FTIR) and Near Infrared (NIR) spectroscopy. Solvent extracts (CCl4) of the considered fennel fruits showed characteristic fingerprints with marker bands related to the individual volatile components (*trans*-anethole, fenchone, estragole, piperitenone oxide, γ-asarone, limonene) for ATR-FTIR and FT-Raman spectroscopy.

Especially νC=C and νC=O absorption bands contribute to the different spectral profiles. Based on hierarchical cluster analysis, the considered fennel accessions were classified according to gas chromatographic (GC) and vibrational spectroscopic data. Furthermore, even a discrimination of "sweet" and "bitter" fennel fruits, both belonging to the *trans*-anethole chemotype, could be successfully performed. All vibrational spectroscopical techniques used in this study are rapid and easy to apply. Hence, they allow different fennel chemotypes to be reliably distinguished and can also be used for on-site measurement in free nature.

Keywords: ATR-FTIR, FT-Raman, NIRS, *Foeniculum*, chemometry, γ-asarone

Introduction

Fennel (*Foeniculum vulgare* Mill.) is cultivated worldwide for its characteristic aromatic leaves and fruits and represents the sole plant species in the genus *Foeniculum*.

The species shows a large diversity in morphology and chemical composition of essential oil (Bernath and Nemeth, 2007; Bernath et al., 1996; Chung et al., 1999; Gudi et al., 2014; Krüger and Hammer, 1999). Nevertheless, a distinct discrimination of different chemotypes only by visual evaluation is highly defective and represents the major challenge for goods receipt and quality control.

The application of GC/MS methods provides reliable information for the identification of the individual chemotype and the precise quantification of the related essential oil components. Due to the laborious and expensive extraction steps needed followed by chromatographic separation, gas chromatography is not suitable enough for large sample sets and routine screening.

Especially for breeding purposes, destructive analysis like GC would destroy the opportunity of subsequent cultivation. Hence, more efficient, rapid and robust analytical methods directly applicable to intact plant material like near infrared (NIRS) or mid infrared (IRS) spectroscopy are demanded.

Therefore, the aim of this study was to develop new vibrational spectroscopy methods for a fast and non-destructive classification of fennel fruits and related micro-extracts as well as a rapid determination of the most important volatile substances directly in the intact fennel fruit. The

results were already published as full paper in the Journal of Agricultural and Food Chemistry (DOI: 10.1021/jf405752x) (GUDI et al., 2014).

Materials and Methods

Fruits of different fennel accessions were provided by the Institute of Plant Genetics and Crop Plant Research in Gatersleben (IPK) and of the Federal Plant Variety Office in Dachwig (Germany). Further botanical information together with detailed experimental description for GC-FID, GC-MS, infrared and Raman spectroscopic measurements and nuclear magnetic resonance (NMR) spectroscopy are given in the appropriate literature (GUDI et al. 2014).

Solvent extracts of fennel fruits in CHCl4 were investigated by GC-MS for qualitative description of the volatile organic fraction and quantification of the individual components was performed by GC-FID.

Intact fennel fruits were analyzed by attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR, portable ATR diamond crystal infrared spectrometer Alpha, Bruker Optics GmbH, Ettlingen, Germany) according to GUDI et al. 2014.
Variability of the fennel samples was investigated by hierarchical cluster analysis (Ward's algorithm) and by principal component analysis (PCA) under usage of the instrument software OPUS 6.5 (Bruker Optics GmbH, Germany). Therefore, relevant spectral ranges were determined by comparison of sample spectra with those of appropriate standards.

Results

GC-MS/ GC-FID analyses of solvent extracts from the investigated fennel genotypes revealed highly diverse qualitative and quantitative composition of the volatile fractions. Except one accession (FOE 25), all considered types containdes between 6.29 and 35.08 % fenchone and are dominated by at least one phenylpropanoic structure as the main component. In contrast, FOE 25 is dominated by nearly 47 % of limonene and 29 % of piperitenone oxide and therefore, has to bee assigned to the piperitenone/ piperitenone oxide chemotype according to literature (BADOC et al., 1994).

Two other accessions (FOE 86 and 87) were dominated both by a phenylpropanoid but neither *trans*-anethole nor estragoe could be deteted. Based on ^{1}H- and ^{13}C-NMR spectroscopy in compbination with MS this structure could be identified as γ-asarone (Figure 1), a thitherto not described component in *Foeniculum*.

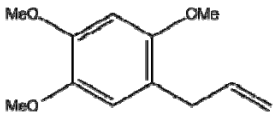

Fig. 1 Molecular structure of γ-asarone according to MS, 1H- and 13C-NMR spectroscopic analysis of volatile fractions from FOE 86 and FOE 87.

Different vibrational spectroscopy methods have been applied for intact fennel fruits and solvent extracts. ATR-FTIR showed to provide good separation of all different fennel chemotypes according to the appropriate GC-FID data. Figure 2 pesents the results of hierarchical cluster analysis for the 10 different fennel accessions based on GC-FID and ATR-FTIR of intact fennel fruits.

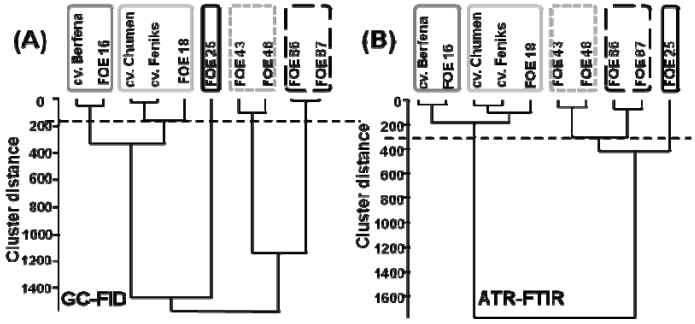

Fig. 2 Hierarchical cluster analysis of 10 different fennel accessions based on the GC-FID data (A) and ATR-FTIR spectra of intact fruits (B). The dashed lines mark the lowest level of complete discrimination of the individual chemotypes. Adapted to and reprint with permission from (Gudi et al., 2014). © 2014 American Chemical Society.

As shown in Figure 2 different fennel accessions could be successfully discriminated according to the chemical profile of the volatile fraction determined by GC-FID. The separation into chemotypes was based on the major component in the volatile fraction which is *trans*-anethole for FOE 16, FOE 18 and the cultivars 'Berfena', 'Chumen' and 'Feniks', whereas estragole represents the major component in FOE 43 and FOE 48. As decribed above, FOE 86 and FOE 87 are characterized by high amounts of γ-asarone in contrast to FOE 25, which mostly contained piperitenone derived structures and limonene.

Whereas the *trans*-anethole content resulted in discrimination of fennel cultivars conforming to EuPharm from those not consumable cultivars (separation into two big clusters at cluster distance level of 1700), the ratio of *trans*-anethole and fenchone was used for differentiation of sweet and bitter fennel (two clusters of 'Berfena'/FOE 16 and 'Feniks'/'Chumen'/FOE 18 at cluster distance level of 200). This differentiation by ATR-FTIR is accessable due to the characteristic absorptions for the C=O bond in fenchone and the conjugated C=C system in anethole.

In conclusion, ATR-IR offers for intact fennel fruits a tool for fast discrimination of different chemotypes according to their chemical profile of the volatile fraction, additionally enabling a fast differentiation of sweet and bitter fennel in between the trans-anethole-chemotype. A comprehensive overview about experimental data and complete description of the results can be found in Gudi et al. (2014).

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P 19: Characterization of the flower morphology of three *Duboisia* species

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Abstract

The tropane alkaloid scopolamine, an important precursor of active pharmaceutical ingredients due to its anticholinergic properties, can be extracted from leaf material of *Duboisia* R. Br., a member of the *Solanaceae* family. Robust and high-yielding *Duboisia* plants are a major requirement in cultivation because of challenges such as climatic changes and the rising demand of the active pharmaceutical ingredient. Therefore, breeding activities are carried out to improve *Duboisia* plants with regard to leaf and scopolamine yield maximization as well as a higher tolerance to environmental conditions.

Molecular genetic analysis of highly conserved plastid sequences was used to prove the identity of the species *Duboisia hopwoodii*, *D. myoporoides* and *D. leichhardtii*. To evaluate different breeding options various flower characteristics have been analyzed by means of stereo microscopy for the three species. For *D. hopwoodii* and *D. leichhardtii*, all characteristics occur stably with nearly no variation. In contrast, the number of flower organs such as petals and anthers varied for *D. myoporoides*.

Keywords: flower morphology, *Duboisia* sp., microscopy, molecular genetic analysis, *Solanaceae*

Introduction

The genus *Duboisia* R. Br. belongs to the tribe *Anthocercidiae* within the family *Solanaceae*. Four different species are comprised within this genus: *D. arenitensis* Craven et al., *D. hopwoodii* F. Muell., *D. myoporoides* R. Br. and *D. leichhardtii* F. Muell. (Craven et al., 1995). *Duboisia* species are woody plants that are distributed in Australia while *D. myoporoides* also occurs in New Caledonia (Craven et al., 1995). *D. arenitensis* is native to Arnhem Land in the Northern Territory (Craven et al., 1995) and *D. hopwoodii* to the arid interior region of Australia.

The distribution areas of *D. myoporoides* and *D. leichhardtii* in Australia are overlapping. In these areas naturally occurring interspecific hybrids exist (Barnard, 1952). Hybrids between these two species are used in large-scale production since the leaves of *D. leichhardtii* and *D. myoporoides* contain pharmaceutically important tropane alkaloids such as scopolamine (Foley, 2006) which is used as an active pharmaceutical ingredient to treat motion sickness and as a precursor for partially synthetic anticholinergics.

Conventional breeding approaches via crosses are used to optimize plants with regard to leaf and scopolamine yield maximization, resistance and tolerance against environmental influences. A detailed knowledge of flower biology is an important prerequisite for crosses and/or possible hybridization.

The aim of this study was the characterization of the flower morphology of the three different species *D. hopwoodii*, *D. myoporoides* and *D. leichhardtii*.

Materials and Methods

Plants were propagated via cuttings from material originally derived from Australia. Cultivation of *D. hopwoodii*, *D. myoporoides* and *D. leichhardtii* took place in the greenhouse at 20/24 °C heating / ventilation set points. Flowers for morphological studies were taken from about ten month old plants.

Selected sequences of three plastid genes, psbA (photosystem II protein D1), ndhF (NADH dehydrogenase F) and matK (maturase K) (SHAPCOTT ET AL., 2015), were amplified using Polymerase Chain Reaction (PCR) (primer sequences: psbA 5´ CTTCCCTCTAGACCTAGCTGCT, psbA 3´ CTCGCCTACTTACATTCCAT, ndhF 5´ TCTATTCAATATCTCTATGGGG, ndhF 3´ AATGAGTAAAATCAGCTAATCCTC, matK 5´ ACATTTATTACGATTCTTTCTCCAC, matK 3´ ACTCCCCACAAACTAGAAGAAGCT; PCR: 1 min/94 °C, [30 s/94 °C, 30 s/55 °C, 1 min/72 °C] x 40, 7 min/72 °C). After restriction enzyme digestion (*Dde*I, *Cac*8I and *Bam*HI; 90 min/37 °C) the fragments were separated on 2 % agarose gels.

For each species 30 flowers were analyzed regarding various flower characteristics. The flowers of *D. myoporoides* and *D. leichhardtii* were collected from three different inflorescences, one from the upper, middle and lower section of the plants. Due to a more growth habit of *D. hopwoodii*, 15 flowers each were taken from the upper and lower section.

After harvesting the plant material in the greenhouse, measurement and description of *Duboisia* flower characteristics was realized by means of stereo microscopy. The magnification was adjusted to the flower organs sizes and ranged between 6.5 and 50 fold.

Results

Sequences of three different plastid genes were used to verify species identity. The banding pattern that was received after restriction digestion correlated exactly with the banding pattern that was expected. Therefore, the three species could be distinguished and the identity of *D. hopwoodii*, *D. myoporoides* and *D. leichhardtii* was proven.

The analysis of flower morphology illustrated the variation in several flower characteristics of the investigated *Duboisia* species *D. hopwoodii*, *D. myoporoides* and *D. leichhardtii* (Fig. 1).

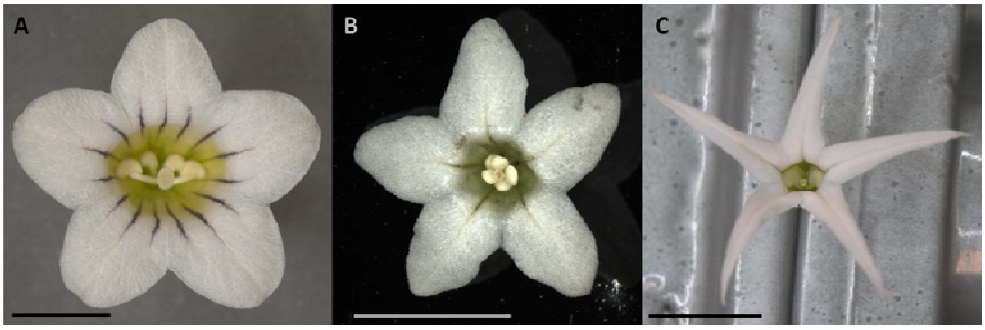

Fig. 1 Flower morphology of D. hopwoodii (A), D. myoporoides (B) and D. leichhardtii (C). Bar: 5 mm.

The flowers of *D. hopwoodii* appeared to be nearly consequently symmetric and widely open. Petals of the *D. myoporoides* and *D. leichhardtii* flowers more often differed in their orientation so that almost every flower had unique appearance. The pronounced star-like structure was typical for *D. leichhardtii*.

The data collected from flowers of the three species is shown in Tab 1.

Tab. 1 Flower characteristics of Duboisia spec. Means of observations of 30 flowers are presented.

Characteristic	*D. hopwoodii*	*D. myoporoides*	*D. leichhardtii*
Diameter of corolla [mm]	11.73	9.73	14.43
Number of petals	5 (100 %)	5 (87 %)	5 (100 %)
		6 (13 %)	
Number of anthers	4 (100 %)	4 (10 %)	4 (100 %)
		5 (77 %)	
		6 (13 %)	
Length of flower tubes [mm]	9.27	4.01	5.89
Diameter of flower tubes [mm]	4.62	2.43	2.54
Length of style [mm]	5.45	1.25	2.09

Regarding the diameter of corollas the data presented in this study is confirming the proportions published by CRAVEN ET AL. (1995). *D. myoporoides* showed the smallest corolla diameter. In contrast, *D. leichhardtii* offered the largest diameter due to its peaked corolla lobes. For *D. hopwoodii* and *D. leichhardtii* the number of petals and anthers was stable with five and four, respectively. Interestingly, *D. myoporoides* deviated from the two other species, because a small percentage (13 %) of flowers with six petals was recorded. Also, there was variation in the number of anthers in this species: while most flowers contained five anthers (77 %), there were some with four (10 %) or six (13 %) anthers. These unstable flower organ numbers were unique for *D. myoporoides*.

Measurements of the flower tubes emphasized the flowers of *D. hopwoodii*: in comparison to *D. myoporoides* and *D. leichhardtii*, *D. hopwoodii* had the longest and widest flower tubes. Also the longest styles were recorded for flowers of *D. hopwoodii*.

There was nearly no variation in the number of ovules (17 ± 1 ovule/ovary) for *D. myoporoides* and *D. leichhardtii* (data not shown).

In summary, for each *Duboisia* species typical flower characteristics were found. By their flower morphology, the three species *D. hopwoodii*, *D. myoporoides* and *D. leichhardtii* which had been confirmed by molecular markers can be easily distinguished.

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P 20: Intraspecific diversity of *Achillea collina* Becker evaluated by molecular genetic markers

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Abstract

Achillea species has been known and utilized worldwide in folk medicine and in up-to date phytotherapy as well. In our recent study we wanted to evaluate the intraspecific diversity of *A. collina* and to look for reliable, relatively simple molecular method for differentiation of different accessions. Five cultivated genotypes and six populations of wild origin were investigated. Besides, for comparison and control six other species were involved into the trial. The DNA samples were evaluated by RAPD (11 primers) and ISSR (12 primers) methods.

In the RAPD analysis 140 bands (97.14 % polymorph) were detected. They distinguished primarily among species and less characteristically among the *A. collina* populations. With ISSR primers we detected 188 bands (97.34 % polymorph). ISSR markers and combined RAPD and ISSR method enabled an informative intraspecific evaluation of *A. collina* accessions. The largest genetic distances were proven between *A. ptarmica* and the members of sect. *Achillea* (genetic distances 0.52 - 0.72). Similarity is highest (genetic distance 0.27) among the populations where geographical distances of the original locations are only 52-55 km. Nei's genetic distances of cultivated populations are also relatively low (0.23 - 0.36) and a common origin for the majority of these genotypes was assumed.

Keywords: *Achillea collina,* PCR, RAPD, ISSR

P 21: Study of self-pollination and capitula characteristics in globe artichoke (*Cynara cardunculus* var. *scolymus* Hayek L.) under different irrigation regimes

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Abstract

In order to estimate the drought effects on capitula characteristics and self-pollination of globe artichoke (*Cynara cardunculus* var. *scolymus* Hayek L.), the randomized complete block design was carried out with three irrigation regimes (20 %, 50 % and 80 % depletion of soil available water) and six replicates. The artichoke is mostly open-pollinated, however, after covering the buds and isolation of flowers to prevent cross pollination, 1.79 % self-pollination was observed and this amount was not affected by different irrigation regimes. In stress conditions (50 % and 80 % water depletion) as well as non-stress condition (20 % water depletion), plants with respectively one and two medium capitula and without small capitula had most relative frequencies in the population and drought stress increased these relative frequencies by reducing the number of medium and small capitula in plants. In addition, Capitula size and dry weight were significantly affected by water stress. Water shortage induced severe decrease in length and dry weight of all capitula including large, medium and small, although capitula width was less affected by water deficit and only slight decline in medium (12.5 %) and small capitula (23.7 %) was observed under severe stress condition.

Keywords: Artichoke, water stress, pollination, capitula characteristics

Introduction

Decreasing in water sources as a result of climate change, in combination with increasing population and increasing societal water demands lead to water shortage being one of the most important global issues. Drought negatively impacts plant growth and reduced crop yield more than other causes (Ings et al., 2013). Globe artichoke (*Cynara cardunculus* var. *scolymus* Hayek L.) is perennial, tall thistle-like plant of the Asteraceae family with edible and medical applications from Mediterranean origin. Buds of artichoke (capitulum) considered a healthy food due to low fat and cholesterol, while being a rich source of fiber, vitamins and minerals (Lattanzio et al., 2009).

The terminal, or top artichoke bud is the largest one in the stem, and others that formed below this terminal bud will be progressively smaller. Different irrigation conditions could influence capitula characteristics and change bud yield and quality, consequently (Green, 2013). Plant pollination system is very important since determine the crossing possibility and breeding method and also could be affected by different irrigation regimes (Chahal and Gosal, 2002).

Materials and Methods

The field experiment was designed as a randomized complete block with three irrigation regimes (three treatments) and six replicates in research farm of Fozveh that is located in west side of Isfahan (32°36'N; 51°26'E and 1612 m above the mean sea level), Iran. Irrigation regimes including 20 %, 50 % and 80 % depletion of soil available water were introduced as non-stress, moderate water stress and severe water stress conditions, respectively based on method of Allen et al. (2000). The irrigation time was adjusted through creating soil moisture curve obtained by time domain reflectometry (TDR) device (Model Sabta Barbara 6050X).

Sampling was conducted when the immature buds were firm and tightly closed and proper for use (marketing stage). Capitula length and width were measured with coulisse. For capitula dry weight determination, the samples were oven-dried at 80 °C for 72 h and then weighed. Pollination was excluded through the use of pollination bags.

Results

Drought stress significantly affected all capitula characteristics, although large capitula width and selfing percentage did not change significantly under different irrigation conditions (Table 1). The dry weights reduction in moderate and severe water stress regime were 25 %, 45.5 % in large, 16.6 %, 42.6 % in medium and 8.2 %, 39.7 % in small capitula, respectively (Fig. 1 a). So, the highest reduction in dry weight in both moderate and severe stress environments was happened in large buds and lowest reduction was observed in small ones. The length of large, medium and small capitula was decreased by drought intensification (Fig. 1 b). The width of large capitula was not affected significantly, and only in medium and small capitula under severe stress condition, significant change in capitula width was observed (Fig. 1 c). It was revealed that the reduction in size of artichoke buds (as it was confirmed with reduction in capitula dry weight) could be mostly attributed to reduction in buds length than width.

In respect to capitula numbers, all of artichoke plants had a one terminal bud in end of main stem that was the largest and the heaviest ones. The number of others buds (medium and small) varied (from zero to six) in different plants and under different irrigation regimes. One medium capitula plant with 40 %, 37.5 % and 46.8 % relative frequency in non-stress, moderate and severe stress had the highest frequency in the population and after that two medium capitula plant with 29.7 %, 32.1 % and 34.3 % relative frequency had the highest frequency in non-stress, moderate and severe stress condition (Fig. 1 d). Results showed that the moderate stress caused the reduction in one medium capitula frequency and elevation in frequency of two medium capitula plant in comparison with non-stress condition; however, severe water stress increased both one and two medium capitula frequency in comparison with non-stress condition by reduction in the number of medium buds (Fig. 1 d). Also plants without small capitula had the highest frequency with 58.3 %, 64.2 % and 75 % relative frequency in non-stress, moderate and severe drought stress, respectively. Similarly drought stress caused elevation in the plants without small capitula by reduction in the number of small buds (Fig. 1 e).

Variance analysis showed no significant difference between the percent of self-pollinated seeds under different irrigation regimes. The means of this trait was 1.79 %, and didn't change significantly by drought stress (Table 1). Artichoke is a cross pollinated plant that pollination can be done by insects (mainly by bee) or other vectors. Although, *C. scolymus* is self-compatible, reproduce viably via self-fertilization is very rare. The reason is that the flowers are protandrus and stigma is receptive 5-7 days after the pollen grains are released. Hence, the pollen grains remain alive only for 3 days in filed condition; therefore the selfing possibility is low. In addition, three genes for nuclear male-sterility have been reported, (ms1, ms2 and ms3) that might be another reason to avoid from inbreeding (Basnizki and Zohary, 1994). In overall, no change in the percent of self-pollinated seeds in different irrigation regimes could be attributed to the fact that pollination is mostly controlled by genetic factors and rarely affected by environmental factors.

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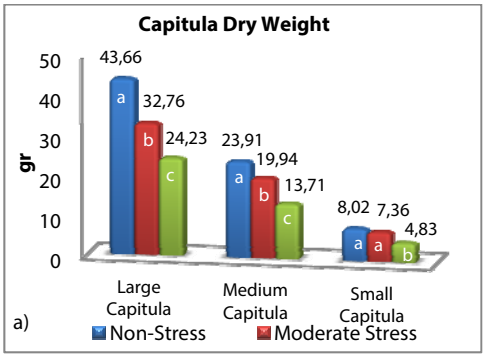
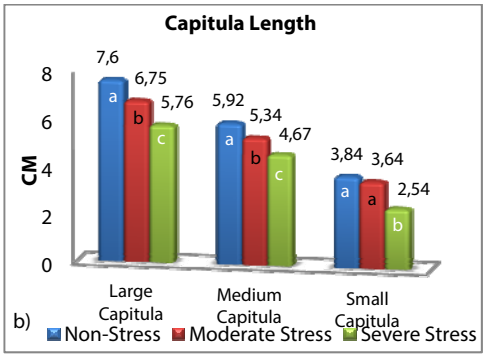
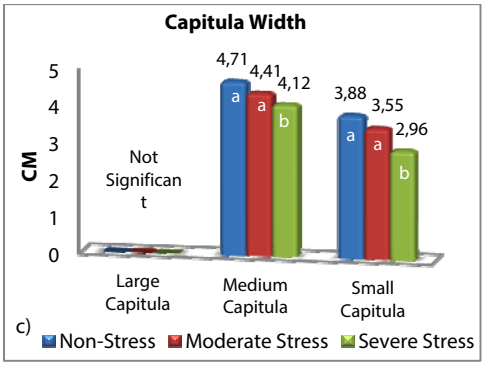
Tab. 1 variance analysis of artichoke capitula traits and selfing percentage at three levels of irrigation

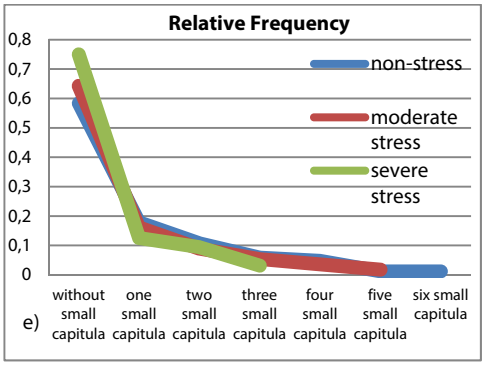
	Source of variation		
	Replication	**Irrigation treatment**	**Error**
df	5	2	10
Large Capitula Length	0.505^{*}	5.051^{**}	0.117
Large Capitula Width	0.092^{ns}	0.551^{ns}	0.291
Large Capitula Dry Weight	15.83^{ns}	569.08^{**}	12.05
Medium Capitula Length	0.676^{*}	2.347^{**}	0.139
Medium Capitula Width	0.205^{*}	0.325^{*}	0.064
Medium Capitula Dry Weight	18.59^{ns}	158.6^{**}	8.285
Small Capitula Length	0.563^{*}	2.931^{**}	0.141
Small Capitula Width	1.577^{ns}	1.282^{*}	0.19
Small Capitula Dry Weight	7.5^{ns}	17.05^{*}	2.98
Selfing	0.18^{ns}	0.13^{ns}	0.115

*, ** Significant at the 5 and 1 % levels of probability, respectively









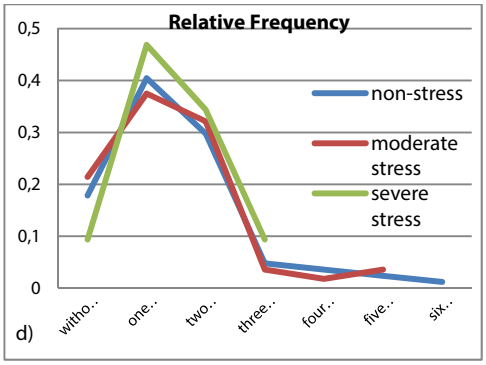


Fig. 1: a) Capitula dry weight b) capitula length c) capitula width d) relative frequency of plants with different number of medium capitula e) relative frequency of plants with different number of small capitula under differents irrigation regimes.

P 22: Hybrid-breeding of medicinally used valerian (*Valeriana officinalis* L. s.l.). A possible concept developing new varieties?

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Abstract

The aim of this work was to develop and verify a new concept for breeding new hybrid-varieties of valerian without a male sterility system. For this the cross-pollination rate and the performance of inbreeded plants must be determined.

Keywords: hybrid-variety, inbreed, inbred line, cross-pollination, open pollination

Introduction

The breeding program at the Bavarian State Research Center for Agriculture (LfL), started in 2008, aims to improve production profitability of the German medicinal plants production. Through selection and cross-breeding, coarser rooted valerian cultivars should be developed, so that high human and technical effort during the valerian harvest and postharvest process can be reduced. At the same time the quality requirements of the European Pharmacopoeia shall be safely kept.

The new hybrid-concept by valerian

The currently in cultivation used valerian varieties are populations from pure selective breeding (mass selection) or cross-breeding. In this context, detailed information about the extent of self-, respectively cross-pollination is not found in literature. The population varieties show usually unwanted high variability in many characteristics (e.g. ingredients contents in different years or morphological differences between plants of the same variety). Hybrid breeding has in addition to the aspect of heterosis, precisely the advantage of homogenization. Only varieties with reliably high homogeneity can achieve the above mentioned requirements.

For a reliable hybrid-system one condition have to be fulfilled, a hybrid-mechanism must exist. Manual crosses are not practical to produce a sufficient amount of hybrid-seeds, the use of gametocides seems not to be acceptable for medicinal plants and applicable male-sterility systems are not known for valerian. Therefore, a new approach has been developed. The parental components should produce seeds by open pollination (random mating) and thereby the hybrid-seed will be produced by using the occurring cross-pollination of valerian. Certainly, the harvested seed lot will contain a certain fraction of inbreed seeds. This fraction must be kept at a tolerable amount. It is assumed, that seeds respectively plants arisen by selfing usually show lower vitality and a reduced growth rate (Bernáth, 1997), and will be either suppressed or compensated by the hybrid plants in the established field crop.

The outstanding issues

The presented concept results in the following questions. A) What is the percentage of cross-pollinated seeds respectively inbreed seeds in cross-pollinated populations? B) Is for inbred lines an inbreeding depression identifiable or, in other words, exhibit inbreeded plants lower vitality?

Materials and Methods

A) Determine the cross-pollination rate

Non-systematic studies have been conducted, in which two valerian origins that are adjacent in the LfL-stock and blooming at the same time were incurred by Amplified-Fragment-Length-Polymorphism (AFLP). The AFLP analysis was conducted with young leaf material of all examined

existing plants and individuals from F1-population originated by open pollination (random mating) with more than 80 valerian populations of the LfL-stock. Molecular primers were applied, which have shown a variety of polymorphisms in a previous study with valerian (HEUBERGER et al. 2012). The AFLP analysis was carried out according to an established protocol for hops including a few modifications.

After these first findings, a more systematic study was started, of which the first results are presented here. 16 valerian plants (elites) were transferred in *in-vitro* for cloning using side shoots. At the same time the inbred lines were generated from the elite plants. The first generations (I1) were examined for homozygous present DNA-based marker bands (AFLP). Six elites could be found that differ in at least one homozygous band. The clones of the selected elites were used in different pair combinations based on their specific polymorphism characteristics.

The mating clones were planted together at two locations for two years. The planting corresponds to a poly-cross-system, so that an equal pollination (with foreign pollen) is possible. The seeds were taken separately from the plants in the center plot and purified according to the seed-treatment protocol of the LfL. One seed drilling was done by hand in multipot-culture-plates in the greenhouse. At the 3-4 leaf-stage of the plants, young leaf material of nearly 340 plants was sampled and analyzed using the AFLP-method as described by Heuberger et al (2012).

B) Estimate of inbreeding depression

As part of the breeding work, many inbred lines were developed. Thereby, single plants were selected from the inbred lines; these bloomed isolatedly, so that inbreed-seeds were produced. In several steps, such inbred lines with different grades of inbreeding (I1, I2, I3 and I4) could be generated. At each level of inbreeding a performance test was carried out on the field, were the characteristics e.g. vitality during germination and seedling cultivation, crop coverage and vitality in field, as well as rootstock weight was recorded.

Results

A) Determine the cross-pollination rate

Figure 1 shows the AFLP-result of the initial investigation on two valerian origins of the LfL-stock. The descendants were generated by cross-pollination for 100 % (F1-population A) and for 67 % (F1-population B).

From the poly-cross combinations of the second approach, the AFLP banding patterns of the F1 descendants from different combinations and two locations indicated different cross-pollination rates. Figure 2 shows the two evaluable combinations (combination A and B1/B2) with averaged cross-pollination rates of 93.4 % and 79.5 %. Remarkable are the rates of combination A at the location A. However, no significant differences were found (Welsh-t-Test p-value = 0,644).

B) Estimate of inbreeding depression

Inbred lines with different genetics and degree of inbreeding were analyzed for three years. The generation I3 shows poorer performances in the characteristics vitality during germination and seedling cultivation, crop coverage and vitality in field, as well as rootstock than inbred lines at the first generation (I1). Figure 3 shows this relationship for the example vitality during germination and seedling cultivation.

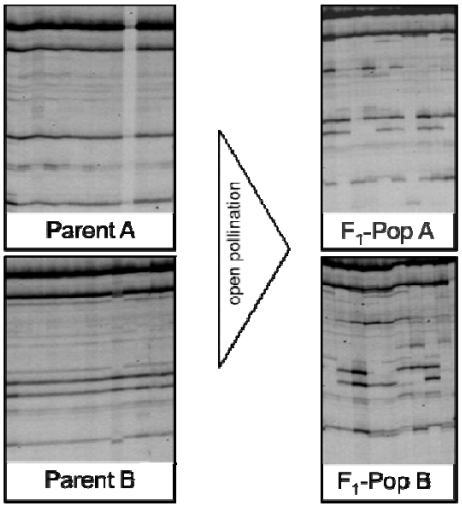

Fig. 1 Cross-pollination in open pollinated populations (Top-cross). Left: the AFLP band patterns of the seed bearer (Parents); Right: ALFP band patterns of the respective descendants (F1-populations).

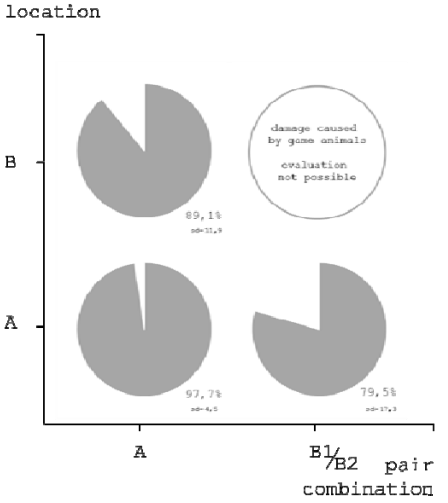

Fig. 2 Second approach of cross-pollination in open pollinated populations (Top-cross). The averaged cross-pollination rates from two locations (Y-axis) and two pair combinations (X-axis) are shown. (sd = standard deviation).

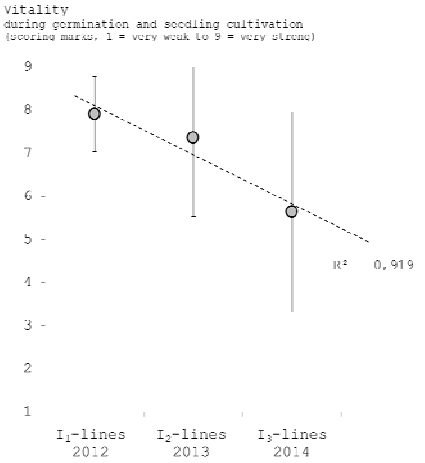

Fig. 3 Development of the vitality during germination and seedling cultivation of three inbred line generations. Vertical lines present the standard deviation, broken line present the trend line with the coefficient of determination (R^2).

Discussion and conclusion

As reflected in the introduction, the conditions for establishing hybrid varieties are a hybrid-mechanism and the lower performance of inbreed descendants. The high cross-pollination rates observed in the first experimental year could serve for this. Since wide variation in the cross-pollination rate occurred occasionally within the tested combinations, the second experimental year should be evaluated before final conclusions. It should be noted, that basically the validity applies only for the tested combinations. Most likely, other lines will behave similarly; however, in the end, the cross pollination rate of the developed hybrid variety must be verified. For this a working method using AFLP has now been established, after other approaches using isoenzyme polymorphisms have failed (PENZKOFER et al., 2014).

An occurring inbreeding depression is not surprising, but it was confirmed in valerian again. Thus, the second condition for the new concept would be also met, that is that a hybrid variety from open pollination, without male-sterility system will serve the needs of field cultivation. The study also supports previous information that the underlying pollination type of valerian is obligate-facultative cross-pollinated (HEEGER, 1956; BERNÁTH, 1997). This means, valerian prefers cross-pollination, however autogamy is possible. The latter is important for the development of hybrid varieties, because hybrid-breeding can only be exploited fully, if inbred lines are used (BECKER, 2011).

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P 23: Comparison of five *Perilla frutescens* (L.) Britt. genotypes in Hungary

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Abstract

In our research work five *Perilla frutescens* (L.) Britt. accessions (GB, J3, JTD3, PS3, 588P) of different origin were evaluated in an open-filed experiment in 2014. Fresh biomass, number of glandular hairs, essential oil content and composition were studied. The dried herb was hydrodistilled and the oil analysed by GC-MS. The components were identified by MS libraries and their LRIs.

Highest fresh biomass was produced by the accession JTD3 (864 g·plant^{-1}) while the lowest fresh weight was measured in the population of 588P (396 g·plant^{-1}). The highest dlandular hair density (1130 hairs·100 mm^{-2}) and essential oil content (1.4 ml·100 g^{-1} d.w.) was detected in the population 588P while the lowest result were detected in J3 (22 hairs·100 mm^{-2}, 0.14 ml·100 g d.w.$^{-1}$). Based on the essential oil composition the investigated populations represent different chempotypes. In PS3 and GB the main components of the essential oil was *ß-dehydro- elsholtzia ketone* (54-76 %), they are belong to the elsholtzia ketone (EK) chemotype. The main components of the essential oil in J3, JTD3, 588P population were *perilla aldehyde* (60-78%), *limonene* (6-15%) and *ß- caryophyllene* (5-6%) (PA – perillaaldehyde chemotype).

Keywords: perilla aldehyde, elsholtzia ketone, Lamiaceae, glandular hair

Introduction

Perilla (*Perilla frutescens* L.), so called Chinese basil, is used traditionally for medical and flavouring purposes. The leaves of the plant are applied against bronchitis or other problems of the respiratory system as well as in the treatment of skin allergic reactions (Habegger et al., 2004). Recent publications have also pointed out its antiallergic, anticancer and immunstimulant properties. Because of the morphological variety of Perilla it can also be used for ornamental purposes cultivated as an annual plant. In the last decade the interest about the plant is increasing however till now we have a lack of information about the cultivation and genetical diversity. In our research work five *Perilla frutescens* L. accessions with purple leaves of different origin were evaluated in an open-field experiment in 2014.

Materials and Methods

Plant material and growth conditions

Five *Perilla frutescens* accessions from different oigins were cultivated in the in the Experimental Field of the Faculty of Horticulture in Budapest, in 2014. Source of the accessions were: GB (Japan), PS3 (Gene Bank of the University), J3 (Jelitto Staudensamen GmbH), JTD3 (JTDSeeds catalog), 588P (Evergreenseeds catalog). Plants were planted into 50×30 cm spacing. All accessions were harvested at the beginning of flowering (23.09.2014.).

Measured parameters

After harvesting the plants, the fresh mass was measured immediately in 6 replications per accession. Leaf samples were collected from the 3rd internode from the tip of the shoots and circles from the leaf blade with 5.5 mm diameter were cut out. The number of peltate type glandular hairs on the abaxial surface was counted under a stereo- microscope (type BMS 74959).

Plant material was dried in shade, on frames. After the drying, the essential oil content was determined according to the method described in the VII. Hungarian Pharmacopoeia (3 repetition). The

main components of the essential oil were analysed with GC-MS method described formerly (SZABÓ et al. 2016).

The results were analysed with an IBM SPSS 22.0 statistical program. One-way ANOVA was applied, and for the pairwise comparisons of the variances, the Scheffe test was used with a confidence level of 5%.

Results

Signifficant differences were observed in the fresh biomass of the investigated *P. frutescens* accessions (Tab. 1.). Largest biomass production was measured in JTD3 while 588P produced only the half of that of this former one.

The studied genotypes showed extrem differences in the number of glandular hairs (Tab. 1.). In 100 mm^2 surface more than 50 fold larger number of glandular hairs was detected in the genotype 588P than in genotype J3. However, the number of glandular hairs of the other accessions varied on a large scale.

Highest essential oil content was detected in 588P while almos 10 fold lower essential oil content was measured in J3. Clear connection is visible between the glandular hairs and the essential oil content: higher number of hairs in the *Perilla frutescens* might result in higher essential oil content as well (Tab. 1.).

Two different chemotypes were identified among the 5 studied accessions. GB and PS3 belong to the *elsholtziaketone* chemotype while the other three accessories accumulated *perilla aldehyde* as the main essential oil component. The *elsholtziaketone* and *perilla aldehyde* chemotypes were formerly described by ITO and HONDA (1997).

Tab. 1 Effect of genotypes on the biomass production, glandular hair density, essential oil content and composition of Perilla frutescens (mean±standard deviation)

***P. frutescens* accession**	**Fresh weight ($g \cdot plant^{-1}$)**	**Glandular hair number ($pc \cdot 100\ mm^{-2}$)**	**Essential oil content ($ml \cdot 100\ g^{-1}$ dw.)**	**Main essential oil compound (area %)**
GB	523.00 ab ± 115.43	478.99 c ± 160.51	0.214 c ± 0.001	*ß-dehydro-elsholtzia ketone* (54%) *ß-caryophyllene* (10%) *elsholtzia ketone* (5%)
J3	501.34 b ± 190.89	21.80 d ± 22.66	0.144 c ± 0.031	*limonene* (6%) *perilla aldehyde* (78%) *ß-caryophyllene* (6%)
JTD3	864.67 a ± 349.80	162.72 d ± 149.71	0.412 b ± 0.031	*limonene* (13%) *perilla aldehyde* (70%) *ß-caryophyllene* (6%)
PS3	573.00 ab ± 84.52	661.37 b ± 196.74	0.359 b ± 0.041	*ß-dehydro-elsholtzia ketone* (76%) *elsholtzia ketone* (5%)
588P	396.67 b ± 115.43	1130.24 a ± 146.24	1.432 a ± 0.083	*limonene* (15%) *perilla aldehyde* (60%) *ß-caryophyllene* (5%)

Legends: Different letters in columns are for significantly different groups (α=0.05).

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P 24: Clone Selection for High Quality Types of Oregano (*Origanum dubium* Boiss.)

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Abstract

Origanum dubium Boiss. is one of the economically important wild oregano species and it is intensely collected from the natural flora of Antalya in Turkey. Carvacrol chemotype of *Origanum dubium* is used mainly for essential oil production due to its high essential oil content. In this study, chemical diversity of *Origanum dubium* was investigated in order to develop new cultivars with improved essential oil yield and carvacrol content using clone selection method under cultivated condition. Essential oils obtained by hydrodistillation of the aerial parts of *Origanum dubium* were analysed by GC-MS and 24 different components were identified. In these genotypes, carvacrol was the major component and followed by p-cymene, γ–terpinene, myrcene and α-thujene. Among the selected genotypes, essential oil yields were varied between 5.0% to 14.0%; carvacrol rates were varied between 73.76% to 88.21%; and p-cymene rates were varied between 3.16% to 9.10%.

Key words: *Origanum dubium,* essential oil, carvacrol, diversity

Introduction

Origanum dubium Boiss. is one of the economically important wild oregano species in Turkey. The most important characteristic of this oregano is high essential oil content (6-8%). It is intensely wild crafted from the upland of Alanya and Gazipasa towns of Antalya province and used for essential oil production. However, there are various genotypes and chemotypes within the wild populations such as high carvacrol and high linalool types. Also, natural populations have been decreased year after year (TURGUT et al., 2013). Therefore, cultivation of *Origanum dubium* seems to be the most convenient way for conservation of wild populations and production of stable drugs.

Origanum dubium Boiss. is grown in the wild flora of Turkey, Greece and Cyprus. Recently, Lukas *et al.* [2] reported a new basis about the taxonomic uncertainties concerning section *Majorana* (LUKAS et al., 2013). They assessed the taxonomic status of *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana* and discuss evolutionary relationships in section *Majorana* by considering molecular, morphological and phytochemical evidence. According to their results, "cymyl" chemotype of *Origanum majorana L*. is classified as *Origanum dubium* Boiss. The biological activities of essential oil of *Origanum dubium* Boiss. such as fungicidal (AHMAD et al., 2011), insecticidal (TANG et al., 2011) and antimicrobial (NOSTRO and PAPALIA 2012) properties associated with carvacrol content.

Recently, utilization of oregano oils has been increased significantly in various sectors (food, health, agriculture, cosmetics etc.). Thus, the yield of essential oil and carvacrol in oregano is very important due to its biological activity. *Origanum dubium* is used mainly for essential oil production not for culinary herb or herbal tea production because of high essential oil rate. Therefore, development of new genotypes with much higher essential oil yield and carvacrol content will be very valuable for the industry. The aim of the study was to improve chemical content of *Origanum dubium* Boiss. in order to develop new cultivars.

Materials and Methods

Plant material

In the preliminary studies, high essential oil and carvacrol type *Origanum dubium* Boiss. populations were identified and their seeds were collected from the natural flora of Gazipasa towns of Antalya province in Turkey (1372 m above sea level and N36 26.749 E32 28.266). Seeds were germinated in the greenhouse and then 2200 healthy seedlings were transferred to the experimental plot for individual plant selection. One hundred plants (genotypes) were selected clonally according to their agronomic features and essential oil yields. The study was conducted in Antalya located in Mediterranean Region of Turkey (33 m above sea level and 36 ° 53′ N; 30° 38′ E). This location was characterized by a Mediterranean climate with 1068 mm total rain fall, 19.7 °C mean air temperature, 13.6 ° C minimum air temperature and 24.2 maximum air temperature. Terra-rossa type soil characteristics of the experimental field were clay loam, very high in lime, low in salt, and light alkaline (pH 7.7). The layer of 0-30 cm soil had low concentrations of organic material and sufficient amount of nitrogen. Available phosphorus content of the soil was low and useful potassium content was high.

Essential oil isolation

Plant rows which consist of 10 plants (clones) were harvested separately and then aerial parts were dried at room temperature. For the study, 100 g of samples from each plant row were subjected to water distillation for 3 h using a Clevenger type apparatus. As a result, percentage of essential was measured by the volumetric method (v/w) for each sample.

Analysis of the essential oils

Samples were diluted 1:50 with hexane for analyses. GC-MS analyses were performed on a gas chromatography (Agilent 7890A)-mass detector (Agilent 5975C) GC-MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (250 °C). The identification of the components of *Origanum dubium* Boiss essential oil was confirmed by comparison of their relative retention times and mass spectra with OIL ADAMS, NIST and Wiley libraries. Retention indices of all the components were determined by the Kovats method.

Results

In this study, yield and composition of the essential oil from aerial parts of 100 selected *Origanum dubium* Boiss. genotypes were determined. In total, 24 different components were identified representing 99.68% of the essential oil by GC–MS analysis. Quantitatively, carvacrol was the major component and followed by p-cymene, γ-terpinene, myrcene and α-thujene. All compounds except carvacrol were found in much lower amounts. They were rich in the active monoterpene phenols such as carvacrol and monoterpene hydrocarbon precursors such as p-cymene and γ-terpinene. According to the results, essential oil yields were varied between 5% to 14%. Actually, 24 genotypes out of one hundred were produced more than 10.0% essential oil yield; 62 genotypes produced at least 8.0% essential oil yield. These essential oil yields were found to be much higher than earlier studies which were reported as 7.6% (Sarer et al., 1982) and 6.5 - 7.7% (Baser et al., 1993). Among the selected genotypes only one genotype produced extremely high (14%) essential oil yield with high carvacrol (84.65%) content.

The main constituent was carvacrol in all of the genotypes and carvacrol rates were varied between 73.76% to 88.21% with average rate of 83.29%. These results showed presence of significant variations in carvacrol content of *O. dubium*. Carvacrol contents of the genotypes were found to be higher than previous studies on *O. majorana* from Turkey 78.27 - 79.46% (Baser et al., 1993) and *O. dubium* from Cyprus 69.5 - 71.3% (Karioti et al., 2006). Carvacrol, linalool, linalool-carvacrol chemotypes as well as thymol chemotypes were found in *O. dubium*. Thymol, linalool and carvacrol

chemotypes were reported in Turkish *O. dubium* populations, while Cyprus ones revealed only carvacrol chemotype (FIGUÉRÉDO et al., 2006).

Second major constituent was appeared to be p-cymene in all samples and it varied between 3.16% to 9.10%. According to the results, the highest p-cymene (9.10%) rate was obtained from genotype G which had also the lowest carvacrol rate (73.76%). These results showed that carvacrol and p-cymene ratios were inversely correlated. In all samples, γ-terpinene was found to be third abundant component which varied from 1.14% to 5.17%.

In conclusion, considerable variations in essential oil yield and constituents were found within the population of *O. dubium* which were collected from the wild flora and then grown in the field. These variations proved that wild *O. dubium* populations are genetically and chemically heterogeneous. Selection and cultivation of oregano genotypes with high essential oil yield and carvacrol content are believed to be very important for essential oil producers and other related sectors.

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P 25: Breeding for downy mildew resistance in basil

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Abstract

Basil downy mildew is a recent, host specific disease. It appeared in all regions where basil is grown as a crop. Every year, complete crops loss occurred for some growers. Because this disease is spreading fast and has a strong destructive power, Iteipmai, in collaboration with several public and private research entities and growers, initiated the MILAROM project. This project uses a multifactorial approach to develop complementary solutions for field and greenhouse growers: improved agricultural practice, prediction modelling of disease epidemic, conventional and alternative phytosanitary products and breeding for resistant varieties.

Concerning the breeding aspects, a marker assisted backcrossing scheme has been applied. Resistance genes, identified within a collection of genetic resources of the *Ocimum* genus, were introgressed into a susceptible commercial variety and agronomy is improved by recurrent crosses. In order to run this breeding program, several methods had to be developed by iteipmai and its partners: crossing method, inoculum production, artificial inoculation, high-throughput screening method for disease resistance, molecular markers.

This project was started in 2010 and is still in progress in 2016. The progeny evaluation for downy mildew resistance indicates that several resistance genes, identified in twelve different sources, were successfully introgressed into the breeding material. The original donors of those resistances cannot be fully traced because open pollination was used at the beginning of the program. However, their inheritance in the progeny of crosses performed with the susceptible donors is showing that they probably are different from one source to another. Identified as dominant, some of those resistances are showing a non monogenic pattern.

The breeding work is still in progress and will lead to the creation of a synthetic variety composed of near isogenic lines of the commercial variety introgressed with the different resistance genes. This variety construction has been chosen in order to ensure the sustainability of its resistance. It should be available on the market in 2020.

P 26: Vegetative and generative maintenance of self-incompatibility in six accessions of German chamomile

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Self-incompatible (SI) plants are able to form ideal mother lines for hybrid crossing in hermaphroditic plants, assuring fertilization from the desired father line. To find out suitable ways to maintain SI was the aim of this study. Among 220 plants of German chamomile (*Matricaria recutita* (L.) Rauschert) within six accessions SI-genotypes were selected. SI was determined as staying seedless in three flower heads per plant. Initial SI-plants formed the basic paternal generation (P1) of i) maintaining the same genotypes over six months and repeating seed set analysis (P2) and of ii) conducting crossings in three versions (SIxSI, SIxNSI (not SI evaluated plants) and NSIxSI), thereby producing the F1 population. F1 exhibited 78 % SI and P2 62 % SI, indicating a higher environmental than genetic influence on SI. But heritability, calculated from the results of SIxSI crossings, showed high values (h^2 = 0.71). Within generative propagation, the influence of generation/crossing version was highly significant (p = 0.001) and the cultivar 'Degumille' explored the highest value of SI (86 %) after SIxNSI crossings. Therefore, the intra-cultivar combination of 'Degumille' SI mother plants crossed with NSI father plants can be recommended as the most promising version to maintain SI in chamomile.

P 27: Impact of targeted UPLC-MS/MS metabolomics on chemical and biochemical characterisation of MAPs

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Abstract

Analysis of natural product pattern (metabolites; metabolomics) and its formation (pathway; biosynthesis) in plants, especially in non-model or crop plants such as medicinal and aromatic plants (MAPs), is a research field with significant potential for breeders, growers and consumers. There is an increasing importance for constant and sustainable quality of MAPs final products. Polyphenols are one of the most important compounds for the antioxidant properties of MAPs and are often, if not identified as active principle, used as lead compounds in quality assessment of herbal drugs and related preparation (herbal tea, alcoholic extracts etc.). Therefore, offering an efficient, robust and reliable fast tool to determine these quality features of MAPs will guarantee the growers, industrial users and the consumers from possible frauds.

Keywords: metabolomics, secondary metabolites, polyphenols, MAPs

Introduction

Cultivation of MAPs is of growing interest to guarantee high quality products to be used in different industrial applications (food, beverages, phyto-pharmaceuticals, nutraceuticals), but also to protect natural habitats from overharvesting, due to increasing demand from consumers and the industry. This increased interest in MAPs is considerably associated with naturally occurring antioxidants and bioactive metabolites, such as polyphenols and various volatile organic compounds (VOCs) mainly of the class of terpenoids. Such metabolites are frequently used in food and pharmaceutical industry because of their potential in health promoting properties and disease prevention capability on one side and consumer acceptability on the other side. Sources of antioxidants (extracts or fractions), such as MAPs, have been extensively studied for their antioxidant activity and many indications/applications are known from traditional medicine, while their content and profil of bioactive metabolites as the main source of known, but also new pharmaceutical activities, is rather poorly described. The aim of the present study was the application of fast screening, targeted UPLC-MS/MS metabolomics, on chemical and biochemical characterization of several MAPs.

Materials and Methods

Chemicals

All reagents for LC-MS analysis were purchased from Sigma-Aldrich with LC-MS grade. Water used in sample preparation and analysis was purified by a Milli-Q water purification system. Specific standards e.g. hypericin, hyperforin, astilbin, amentoflavone, quercetin 3-*O*-xyloside, carnosol and carnosic acid were obtained from TransMIT PlantMetaChem (Giessen, Germany) and inserted in the analytical method described below.

Plant material

Aerial parts were collected from different accessions of *Salvia fruticosa*, *Sideritis sp*., *Mentha sp*., *Oregano sp*., *Satureja sp*., *Hypericum perforatum* and *Stachys iva,* cultivated in the experimental field

of IPB&GR (40°34'35'' N 22°57'19'' E) or from wild grown plants in different geographical areas of Greece. The fresh plant material was dried in a shady and dry place at ambient temperature for fifteen days. Commercial tea drugs were obtained from Alfred Galke GmbH (Bad Grund, Germany) and used as reference sample (Table 1).

Tab. 1 List of analysed plant material

Plant species	tissue	Treatment	Samples
Salvia fruticosa	leaves	different harvesting time	2 accessions; 7 time points
Sideritis sp.	herba	wild and cultivated	7 species; 13 accessions plus commercial tea drug
Mentha spicata, M. piperita	leaves, stem	stress and nutrition experiment	2 species; 5 treatments; 2 developmental stages
Origanum vulgare, O. vulgare spp. hirtum, O. onites	herba	wild and cultivated	3 species; 1, 6 and 1 accessions plus commercial tea drug
Satureja montana, S. thymbra	herba	wild and cultivated	2 species (3 and 2 accessions) plus commercial tea drug
Hypericum perforatum	herba, leaves, flowers	wild	3 accessions plus commercial tea drug
Stachys iva	herba	wild	3 accessions plus commercial tea drug

Sample preparation and extraction

100 mg dried tissue was weighed and transferred into a 15 mL falcon tube. A volume of 4 mL of methanol 80% or 5 ml hot water was added to each sample. The samples and solvent were mixed with the help of an orbital shaker for 3 h at room temperature and the extraction proceeded overnight. The resulting solutions where filtered on a 0.22 µm PFTE membrane into a glass vial and analysed with UPLC-MS/MS as described below. Water extracts were cooled down on ice after 10 minutes of incubation and directly filtered as described. Three biological replicates for each sample were done.

Metabolite analysis

The analysis of natural compounds was performed using advanced targeted UPLC-MS/MS method as described (Vrhovsek et al., 2012). Samples were directly injected after extraction. Chromatography, quantification and mass spectrometry conditions can be found in the literature referred to above. Data processing was performed using the Mass Lynx Target Lynx Application Manager (Waters).

Results

A recently established targeted UPLC-MS/MS-MRM method with a library actually consisting of more than 140 natural compounds of various chemical groups was used for the qualitative and quantitative analysis of methanolic and water extracts of different MAPs (Table 1). To further improve metabolic analyses, known and commercially available compounds found in *H. perforatum* and *Salvia sp*. (see Material and Method) were inserted to the database. Twenty eight (*Sideritis sp*.) and fifty five (*Hypericum perforatum*) metabolites were identified respectively, in different concentration in the methanolic and water extracts. This preliminary study revealed a high diversity in the

content and pattern of the secondary compounds in the different plant species. Beside more common derivatives of groups such as benzoic acids, phenylpropanoids, flavones, flavonols found in all extracts, although in different pattern and concentrations, stilbenes, coumarins, flavan-3-ols (including proanthocyanidins) and anthocyanins were only found in *H. perforatum*, carnosol only in *Salvia sp.* and dihydrochalcones in both of these plants. Additionally, in some extracts also intermediates (including their glycosides) of the general flavonoid pathway such as flavanones and dihydroflavonols were detectable.

These differences might due to factors such as: a) genetics (the species and genotype directly influence the content and composition), b) the type of plant tissue (leaves, stems, flowers) and the stage of development, c) environment (soil conditions, climate, altitude, humidity) and d) agronomic cultivation conditions (fertilizer and water supply, grow media, biotic stress etc). Therefore, a robust, fast and reliable metabolic method is needed to characterise different accessions, origins and treatments of MAPs and tissue specific extracts in scientific studies, but also for quality control for industrial applications. Furthermore, based on metabolite profiling comprehensive pathways schemes for identified metabolites can be proposed and together with transcriptomics approaches key genes for certain bioactive metabolites can be selected, for further genetic and biochemical analysis, but also for establishing molecular marker for fast screening of populations and use in fast breeding strategies.

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