

SEKCIJA 5: APLIKATIVNA BOTANIKA

SESSION 5: APPLIED BOTANY

Molecular genetic hop research in Slovenia

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Introduction

In Slovenia, hop growing has a tradition of more than 100 years and it has always been a significant agricultural production oriented towards export. The Slovene Institute of Hop Research and Brewing (SIHRB) has released eleven hop varieties, four of which occupy the majority of hop fields in Slovenia. Such a varietal structure emphasizes the importance of “regional” hop breeding and the adaptation of hop varieties to specific growing conditions. Traditional recombination and selection procedure have been successful in development of hop cultivars but there are ongoing needs in development of improved cultivars in terms of yield, high resin content and disease resistance. Hop genetic studies have been set up in order to assist the hop breeding program at the SIHRB. The main approach of genetic research is rational use of the genetic diversity available in the hop germplasm collections, and gene incorporation or introgression by means of hop genome mapping, marker-assisted selection and/or cloning and genetic transformation. Major effort is directed towards improving hop quality (resin content) and resistance to biotic stresses (*Verticillium* and hop Damson aphid). We present a review on our research work on the development of the hop molecular markers, assessment of hop genetic diversity, genome mapping and future research.

Development of molecular markers in hops

Different PCR-based molecular markers have enabled the fine-scale genetic characterization of the germplasm collection and they supply a large amount of data that can be used for germplasm management, genetic studies, and breeding purposes etc. RAPD and AFLP markers have mainly been used in hop in different studies and applications, mostly because of their simplicity, relatively high information value, and the lack of hop genomic information (Šuštar-Vozlič and Javornik, 1999; Jakše et al., 2001). We have used both these marker systems, but in order to gain more hop genomic information, the development of hop SSR markers has been undertaken in order to use them in genetic studies (Jakše and Javornik, 2001; Jakše et al., 2002; Štajner et al., 2005). At present, a large scale hop SSR development is underway. Developed molecular markers have been used for hop cultivar identification (Čerenak et al., 2003), testing homogeneity among hop plants of an introduced hop variety or in commercial samples (Jakše and Javornik, 1999), in management of redundancy in the hop gene bank (Jakše et al. 2000), identification of hop sex to eliminate male seedlings (Čerenak and Javornik, 1999), searching for markers linked to aphid resistance (Čerenak and Javornik, 2001), clarification of the origin of the accessions in the gene bank and for the identification of the *V. albo-atrum* pathotypes (Radišek et al, 2003, 2004)

Evaluation of hop genetic resources

The SIHRB maintains an extensive collection of worldwide hop cultivars and their own breeding lines and a fairly rich gene bank of wild hops. These genetic resources have been evaluated by morphological or biochemical descriptions and by molecular markers, thus providing more accurate and detailed information on the genetic diversity. The world collection of hop cultivars was assessed by RAPD (Šuštar-Vozlič and Javornik, 1999), AFLP and four microsatellite markers (Jakše et al., 2001). These studies have well documented the presence of two geographically distinct germplasms in hop cultivars – European (EU) germplasm characterised by its aroma and North American (NA) germplasm characterised by a distinct bitter taste. Within European germplasm, a distinct grouping of accessions was also established reflecting past hop breeding practices adopted in different European hop growing

regions. Work on hop genetic resources has been extended to studies of wild hops and male and female breeding lines by available SSR markers (Jakše et al., 2004) since wild hops have been the source of many important genes. The geographic classification of hop germplasm into European and North America has been confirmed not only by microsatellite allele distribution and the analysis of genetic distances, but also by notable differences in the structure of allele sequences. Molecular analysis failed to distinguish between female and male hops but results showed variability present within each of the hop geographic groups, indicating very high genetic variability within NA wild hops. Further studies (Javornik et al., in preparation) of genetic structure and diversity among hop genotypes using 25 new microsatellite markers and a set of 67 wild and cultivated hop accessions and model-based clustering placed the accessions into five gene pools that correspond to major breeding groups and to accessions showing mixed ancestries. A phylogenetic clustering shows good agreement with pedigree information and five gene pools, and further defined two additional gene pools. The above results are consistent with two biogeographically separated germplasms and with the known history of accessions and have so far given a most detailed insight into the structure and extent of genetic diversity present in collections, which is of prime importance for any breeding programme, in order to secure and enlarge the variability and to make a more informed choice of the breeding material used in the development of new cultivars.

Molecular characterization of phytopathogenic fungi *Verticillium albo-atrum*

Hop wilt, caused by *Verticillium albo-atrum* Reinke & Berthold or *V. dahliae* Klebahn is one of the most important diseases of hop and it can pose a serious threat to hop production unless wilt resistant cultivars are planted in hop gardens. In 1997 an outbreak of the lethal form of hop wilt was registered in the Slovene hop growing region and, over the next six years, new outbreaks of the lethal form were detected, seriously limiting hop production in the infected areas. The appearances of mild and lethal hop wilt forms in hop growing areas required the characterisation of field isolates. Pathogenicity tests and molecular analysis determined two groups of *V. albo-atrum* hop isolates, which indicates the appearance of a new, more virulent hop pathotype (Radišek et al., 2003). Molecular analysis identified pathotype linked markers which were successfully sequenced and converted into diagnostic markers and rapid PCR assays were developed for the identification and detection of *V. albo-atrum* hop pathotypes from Slovenia (Radišek et al., 2004). We extended the research on *V. albo-atrum* by the characterization of pathotypes proteomes by 2-D electrophoresis and peptide fingerprinting of differentially displayed proteins (Javornik et al., unpublished) and we envisage the proteomic analysis of xylem sap proteins induced after the infection of different hop genotypes by differently *Verticillium* pathotypes in order to identify known and novel proteins and thus to provide insight into plant-pathogen interaction in vascular wilt disease and to use protein information for hop breeding.

Hop genome mapping

Genetic maps are today essential tools in marker assisted selection (MAS) and genes isolation and have been constructed for many crops, in order to map monogenic traits and dissect polygenic traits. We started hop mapping project in order to develop hop linkage map(s) for the identification of QTL involved in alpha acid synthesis and markers linked to wilt resistance which can be directly applied to MAS in hop breeding. Both of the traits are the main objectives in breeding program. Maternal and paternal linkage maps using AFLP and microsatellite markers on the family segregating quantitatively for alpha-acid content were developed and data of repeated evaluations of alpha-acid content over three vegetation periods were used for mapping of QTLs. Quantitative analysis indicated four putative QTLs in three linkage groups of the female map, one of which overlapped with the AFLP marker and has a breeding value. Saturation of the maps will be carried out, particularly the putative QTL regions, using SSR markers, and the stability of the QTLs will be tested (Čerenak et al., 2006). Currently we are also mapping family segregating for wilt resistance using newly developed microsatellite markers.

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References

- Čerenak A. and Javornik B. (1999) In: Seigner E. (Ed.). Proceedings of the Scientific Commission [of the] International Hop Growers' Convention I.H.G.C., Pulawy, Poland, 27-30 July 1999. International Hop Growers' Convention: 39-42.
- Čerenak A. and Javornik B. (2001). Hmelj. bilt. 8 (2) 5-11.
- Čerenak A., Jakše J. and Javornik B. (2003). J. Am. Soc. Brew. Chem. 62 (1)1-7.
- Čerenak A., Satović Z. and Javornik B. (2006). Genome (accepted).
- Jakše J. and Javornik B. (1999). In: Seigner E. (Ed.). Proceedings of the Scientific Commission [of the] International Hop Growers' Convention I.H.G.C., Pulawy, Poland, 27-30 July 1999. International Hop Growers' Convention: 62-65.
- Jakše, J., Čerenak A. and Javornik, B. (2000). In: Tajnšek A. and Šantavec I. (Eds). Proceedings of symposium, [Moravske Toplice, 14. in 15. december 2000], (Novi izzivi v poljedelstvu). Ljubljana: Slovensko agronomsko društvo: 144-148.
- Jakše, J. and Javornik, B. (2001). Plant Mol. Biol. Report. 19:217-226.
- Jakše J., Kindlhofer K., Javornik B. (2001). Genome 44,773-782.
- Jakše, J., Bandelj Mavsar, D. and Javornik, B. (2002). Mol. Ecol. Notes (Print) 2(4): 544-546.
- Jakše, J., Satović, Z., Javornik, B. (2004). Genome 47, 889-899.
- Radišek, S., Jakše, J., Simončič, A. and Javornik, B. (2003). Plant Disease 87:633-638.
- Radišek S., Jakše J. and Javornik B. (2004). Plant Disease 88:1115-1122
- Šuštar-Vozlič J. And Javornik B. (1999). Plant breed. 118: 175-181.
- Štajner, N., Jakše, J., Kozjak, P., and Javornik, B. (2004): Plant Sci. 168, 213-221.

Slovenian Beans (*Phaseolus vulgaris* L.): From Diversity to Function

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Introduction

The germplasm collection at the Agricultural Institute of Slovenia is one of the three working collections at the Slovene gene bank. It maintains a fairly large *ex situ* collection that houses among others more than 1000 bean landraces from all parts of Slovenia. They are result of farmers selection of different populations adapted to growing conditions in different environments.

The genus *Phaseolus* includes numerous wild and cultivated species, originating in the New World. Only five species are important as cultivated food crops, *P. vulgaris*, *P. polyanthus* Greenm., *P. coccineus*, *P. lunatus* and *P. acutifolius* A. Gray. Because of its high nutritional value, the common bean (*P. vulgaris*) is by far the most important *Phaseolus* bean and is cultivated on all continents except Antarctica, under very diverse cultivation conditions (Singh, 1999).

Two major gene pools of common bean were first recognized in the wild form, Mesoamerican and Andean (Gepts, 1998). A third, genetically unique gene pool was later described in the Northern Andes (Debouck, 1999; Tohme et al., 1996). A fourth gene pool in Colombia might also exist, but it is still poorly understood (Debouck, 1999; Tohme et al., 1996). After domestication, the common bean spread between Mesoamerica and South America and, after the European discovery of the Americas, to Europe and Africa, where it was cultivated in diverse environments and agricultural conditions (Chacon et al., 2005).

There is no written evidence of the first introduction of common bean on Slovene territory, however, the naming of some accessions indicate that it could have come from France, Spain and Italy through trade and the exchange of material. In the first historical review of Slovene territory by Valvasor (1689), the cultivation on a larger scale of two bean species, common bean and runner bean (*P. coccineus*), is already described. Common bean has therefore been an important crop for human consumption in Slovenia for centuries and is still cultivated in almost every home garden.

The objectives of this work is to explain the genetic variability and the relationship among and within accessions of Slovene common bean landraces and cultivars, stored at the AIS Genebank, to relate their origin to the Mesoamerican and Andean gene pools and to introduce studies which are leading into explaining the involvement of different genes in common bean drought stress response.

Materials and methods

Phaseolus vulgaris germplasm is generally divided into two gene pools, Andean and Mesoamerican. Each pool is subdivided into several races based on morphological characteristics. Progress has been made in distinguishing them using molecular markers. From the Slovenian germplasm collection database of *Phaseolus* landraces a total of 139 *Phaseolus* accessions were evaluated for genetic diversity. Accessions were chosen according to multicrop data and morphological characteristics according to IPGRI descriptors. These accessions included nine Slovene common bean cultivars, 91 landraces collected in various parts of Slovenia and stored at AIS, 39 standard check accessions of which 27 were cultivars of known Andean and Mesoamerican origin, 10 wild accessions of *P. vulgaris* and two accessions of *P. vulgaris* related species, *P. coccineus* and *P. lunatus*. The molecular marker (RAPD, AFLP, microsatellite) and protein (phaseolin) analysis was carried out. Associations were displayed using cluster analyses.

Differentially expressed genes in common bean germplasm were identified using differential display RT-PCR technique (Liang and Pardee, 1994) and confirmed with the expression analysis using quantitative PCR. Common bean genotypes of both Mesoamerican and Andean origin were included in the study. Among them were drought-resistant and drought-sensitive cultivars. Identified up- and down-regulated genes were also verified in control- and drought-stressed plants of *Phaseolus coccineus*, *Phaseolus lunatus* and *Phaseolus acutifolius*.

Results and discussion

Slovenian accessions were divided using molecular and biochemical data and analysed using UPGMA cluster analysis into three groups. The majority of the standard cultivars clustered according to the gene pool of origin determined in previous studies.

Electrophoretic analysis of the phaseolin showed that Slovene common bean landraces and cultivars possess the three phaseolin patterns typical of the Mediterranean basin: "C", "T" and "S" (Gepts and Bliss, 1988). The preliminary RAPD analysis revealed a tight group of 15 accessions that didn't contain any Andean gene pool check lines. This suggests that these genotypes are significantly different from any *P. vulgaris* genotypes previously characterised with RAPD's, representing a unique set of germplasm that should be included in *Phaseolus* germplasm collections and used for further analysis. The same evidence of separate Slovene bean accessions with unique genetic diversity confirmed our preliminary results (Meglič et al., 1999). As suggested by Negri and Tosti (2002), Santalla et al. (2002) new introductions and gene exchange between Andean and Mesoamerican germplasm could have played a major role in the evolution of additional diversification centers for this species. Due to the long tradition of common bean cultivation in Slovenia, it could be hypothesized that a set of original genotypes of Andean origin has undergone an adaptive evolutionary process, resulting in today's new variations in this region.

Differential display reverse transcriptase PCR (DDRT-PCR) and quantitative relative PCR was used to study gene expression in *Phaseolus vulgaris* under drought stress. DDRT-PCR profiles on the agarose gels or on the ABI Prism 310 Genescan electropherograms showed negligible differences among different *P. vulgaris* genotypes for the same group of plants although differences in DDRT-PCR profiles have been observed among different *Phaseolus* species.

Conclusions

In conclusion, the results of several studies presented have provided the most detailed picture of genetic variation of Slovene common bean germplasm to date. Upregulated and downregulated genes were identified in the gene expression under drought study and belong to different gene families. The identified proteins were Chlorophyll a/b binding protein gene (LHC I) (down), small subunit of ribulose 1,5-bisphosphate (down), Ethylene-responsive transcription factor 9 (up).

The accumulated knowledge on the genetic structure of Slovene common bean genetic resources, will have a significant impact on further decisions regarding germplasm bank management, research programs and breeding activities at the AIS.

References

- Chacon, M.I., S.B. Pickersgill, and D.G. Debouck. (2005). Domestication patterns in common bean (*Phaseolus vulgaris* L.) and the origin of the Mesoamerican and Andean cultivated races. *Theor. Appl. Genet.* 110:432-444.
- Debouck, D.G. (1999). Diversity in *Phaseolus* species in relation to the common bean, p. 25-52. In: S.P. Singh (Ed.). *Common bean improvement in the twenty-first century*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Gepts, P. (1998). Origin and evolution of common bean: Past events and recent trends. *Hort Science* 33:1124-1130.
- Gepts, P. and F.A. Bliss. (1988). Dissemination paths of common bean (*Phaseolus vulgaris*, Fabaceae) deduced from phaseolin electrophoretic variability. II. Europe and Africa. *Econ. Bot.* 42:86-104.
- Gepts, P., T.C. Osborn, K. Rashka, and F.A. Bliss. (1986). Phaseolin-protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris*) - evidence for multiple centers of domestication. *Econ. Bot.* 40:451-468.
- Meglič, V., M.A. Johns, and J. Šuštar-Vozlič. (1999). Genetic diversity in a Slovenian bean germplasm collection as related to the Andean *Phaseolus* gene pool, p. 28. In: *World Seed Conference (1999): 6-8 September 1999*, Cambridge UK: ISTA 75th Anniversary: a unique event: programme & abstracts. Zürich .
- Negri, V. and N. Tosti. (2002). *Phaseolus* genetic diversity maintained on-farm in central Italy. *Genet. Resour. Crop Evol.* 49:511-520.
- Santalla, M., A.P. Rodino, and A.M. De Ron. (2002). Allozyme evidence supporting southwestern Europe as a secondary center of genetic diversity for the common bean. *Theor. Appl. Genet.* 104:934-944.
- Singh, S.P. (1999). Production and utilization, p. 1-24. In: S.P. Singh (Ed.). *Common bean improvement in the twenty-first century*, Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Tohme, J., D.O. Gonzalez, S. Beebe, and M.C. Duque. (1996). AFLP analysis of gene pools of a wild bean core collection. *Crop Sci.* 36:1375-1384.
- Valvasor, J.V. (1689). Die Ehre des Hertzogthums Crain. Reprinted in 1978, p. 65. Mladinska knjiga, Ljubljana, Slovenija.

Horse chestnut hairy roots as a source of aescin

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Introduction

Horse chestnut (*Aesculus hippocastanum* L.) seed extract is widely used in remedies for the treatment of peripheral vascular disorders (Bombardelli *et al.* 1996). It also exhibits powerful anti-oxidant (Wilkinson and Brown 1999), anti-inflammatory (Matsuda *et al.* 1997), anti-tumor and anti-viral (Yang *et al.* 1999) activities. An active component comprising several chemically related triterpene saponins is known as aescin. These compounds are found in many parts of the plant, but the seed cotyledons are the main site of its accumulation (Bombardelli *et al.* 1996). Seeds of horse chestnut are the sole industrial source of aescin. They contain up to 500 mg of pharmacologically active tissue and they are only shortly available during the year. Thus, it would be of great interest to obtain a highly productive and biochemically stable tissue for *in vitro* production of aescin. Hairy roots could be a good source, as they are highly productive, in addition to biochemical stability, and their nutritive demands are simple and inexpensive. Up to now, there has only been one report on the successful production of aescin in embryogenic calli and somatic embryos of *A. hippocastanum* (Profumo *et al.* 1991), but there is no further information on their application in a large-scale production. The aim of our study was to obtain stable *Agrobacterium rhizogenes*-mediated horse chestnut hairy root lines and to analyse their aescin content.

Materials and methods

Horse chestnut hairy root lines were obtained upon the infection of somatic embryos with *Agrobacterium rhizogenes* strain A4GUS, containing the TL- and TR-DNA. The acquired hairy root lines were maintained in MS liquid plant growth regulator (PGR)-free medium. To confirm the transformation and to determine the copy number of the transgenes, genomic DNAs from the hairy roots were digested either with *Bam*HI or *Eco*RI and subjected to Southern hybridisation with the 9.8 kb *Bam*HI fragment of the pLJ1 cosmid (Jouanin 1984, Zdravković-Korać *et al.* 2004). The presence of the *rolA*, *-B*, *-C* and *-D* sequences was confirmed with specific pairs of primers (Zdravković-Korać *et al.* 2003/04). Aescin analysis was conducted colorimetrically according to German Pharmacopoeia (Deutsches Arzneibuch 10). The amount of aescin was determined by measuring the absorbance at 540 nm after the conversion of saponins into a coloured molecule by ferrichloride/acetic acid/sulphuric acid reagent.

Results and discussion

Seventy-one horse chestnut hairy root lines were obtained from independent transformation events. Five lines preserved their growth capacity over a period of 7 years, while the other lines perished within a year. The five well growing lines have the capacity to increase their mass 6-8 fold within a 4-week culture period in a MS liquid PGR-free medium. Southern hybridisation confirmed the stable integration of the TL-DNA within the horse chestnut genome and revealed the copy number of the transgenes to be 2-4. PCR analysis revealed the presence of the four *rol* genes in all lines (Zdravković-Korać *et al.* 2004).

Aescin content differed significantly among the hairy root lines (Table 1.). Lines 47 and 39 produced the highest amount of aescin, although less than the zygotic embryos, but higher than leaves. These lines also exhibited the highest growth, long-term stability in growth (during 7 years) and very simple nutritive demands (Zdravković-Korać *et al.* 2003/04). Variations in aescin content were not correlated with the transgene copy number, as the line 31 (with the highest copy number of the TL-DNA) contained a substantially lower amount of aescin than lines 39 and 47. Thus, the differences in aescin content among the hairy root lines may be attributed to

the difference in the TL-DNA insertion site. This is in accordance with the results of Batra *et al.* (2004) and Chaudhuri *et al.* (2005). Batra *et al.* (2004) pointed out the importance of the TL-DNA presence for ajmalicine production in hairy roots of *Catharanthus roseus*. In a previous study, the presence of the whole *rolA*, *-B*, *-C* and *-D* cassette was demonstrated for all horse chestnut hairy roots (Zdravković-Korać *et al.* 2003/04). This is a prerequisite for high biomass productivity, as hairy roots containing the TR-DNA alone generally grow very slowly.

Table 1: Aescin content in horse chestnut hairy root lines grown in MS liquid PGR-free medium, expressed as percentages on the basis of dry weight. Means \pm SD of three independent experiments. All means are significantly different at a 0.05 probability level according to the LSD test.

	Aescin content X (%) \pm SD
Zygotic embryos	6.96 \pm 0.10
Leaves	3.05 \pm 0.02
Line 47	4.09 \pm 0.06
Line 39	3.57 \pm 0.03
Line 31	1.90 \pm 0.04
Line 43	1.16 \pm 0.01

Commercial production of a compound *in vitro* demands stable cell lines with high yields and growth potential. As suspension cultures often show insufficient yield and metabolic instability, it is better to use differentiated organs displaying genetic and biochemical stability (Flores *et al.* 1999). For this purpose, genetically transformed roots are particularly interesting due to their capacity for long-term growth (Flores *et al.* 1999). Therefore, hairy root cultures are widely used as a source of useful compounds (Oksman-Caldentey and Hiltunen 1996, Flores *et al.* 1999).

In conclusions, horse chestnut hairy root lines 39 and 47 produce a substantial amount of aescin and show high biomass productivity *in vitro*. Thus, we believe these lines could be good material for the further increasing of aescin content by applying elicitors, which will eventually lead to a large-scale aescin production.

References

- Batra J., Dutta A., Singh D., Kumar S. and Sen J. (2004). *Plant Cell Reports* 23 (3), 148-154.
- Bombardelli E., Morazzoni P. and Griffini A. (1996). *Fitoterapia* 67, 483-511.
- Chaudhuri K.N., Ghosh B., Tepfer D. and Jha S. (2005). *Plant Cell Reports* 24 (1), 25-35.
- Deutsches Arzneibuch 10 (1991), Amtliche Ausbauge, Band III. Hipocastani semen. Deutscher Apotheker Verlag Stuttgart Govi-Verlag GmbH Frankfurt.
- Flores H. E., Vivanco J. M. and Loyola-Vargas V. M. (1999). *Trends Plant Sci* 4 (6), 220-226.
- Jouanin L. (1984). *Plasmid* 12, 92-102.
- Matsuda H., Li Y., Murakami T., Ninimiya K., Araki N., Yoshikawa M. and Yamahara I. (1997). *Biol. Pharm. Bull.* 20, 1092-1095.
- Oksman-Caldentey K. M. and Hiltunen R. (1996). *Fiel Crops Res.* 45, 57-69.
- Profumo P., Caviglia A. M., Gastaldo P., Dameri R. M. (1991). *Planta med.* 57, 50-52.
- Wilkinson J. A. and Brown A. M. G. (1999). *Int. J. Cosmetic Sci.* 21, 437-447.
- Yang X. W., Zhao J. and Cui Y. X. (1999). *J. Nat. Prod.* 62, 1510-1513.
- Zdravković-Korać S., Čalić D., Druart Ph. and Radojević Lj. (2003/04). *Biol. Plant.* 47 (4), 487-491.
- Zdravković-Korać S., Muhovski Y., Druart Ph., Čalić D. and Radojević Lj. (2004). *Plant Cell Rep.* 22 (9), 698-704.

Genetic characterization of *Trifolium* species with notable agronomic value and its application in genebank accession management

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Introduction

The very large genus *Trifolium* L. comprises approximately 240 species (Zohary and Heller, 1984) belonging to the tribe Trifolieae, family of Leguminosae (Fabaceae). Despite the high agronomic value of the genus, nuclear DNA content has been reported for a surprisingly limited number of *Trifolium* species (Grime and Mowforth, 1982; Arumuganathan and Earle, 1991; Campbell et al., 1999). Other studies focused on *Trifolium* species were frequently based on various biochemical and DNA molecular marker techniques (Badr, 1995; Bullitta and Hayward, 1996). For instances, several Old World *Trifolium* species have been studied using analysis based on nucleotide sequence data of the internal transcribed spacer (ITS) of nuclear ribosomal DNA and chloroplast DNA restriction sites (Watson et al., 2000).

The aim of the research was to obtain basic genetic data on 31 *Trifolium* species with notable agronomic value, originating from Eurasian, African and American centers of divergence and belonging to seven major taxonomic sections of the genus *Trifolium* (*Lotoidea*, *Mistyllus*, *Vesicaria*, *Chronosemium*, *Trifolium*, *Trichocephalum* and *Involucrarium*). These sets of data including nuclear DNA content, chromosome number and ITS region analyses would be compared and discussed in relation to the proposed taxonomic classification. Furthermore, usefulness of measurements of nuclear DNA content of genebank accessions would be evaluated. We investigated, whether simple testing for relative nuclear DNA contents can be used as elementary marker data, specific of a species and indicating either variability of ploidy level or a mislabelling of an accession.

Materials and methods

Seeds of different accessions of 31 *Trifolium* species were obtained from five gene banks and were cultured in the greenhouse of the Biotechnical Faculty, University of Ljubljana.

The total DNA amount in the leaf nuclei was assessed by flow cytometry using *T. repens* L. cv. Milo ($2C = 2.07$) as an internal standard and *T. pratense* L. (genome size estimated using *T. repens* as standard was $2C = 0.85$ pg) as an intermediate standard. Samples were stained with PI (propidium iodide) according to a technique adapted from Doležel et al. (1989). For staining with DAPI (4,6-diamidino-2-phenylindole), the procedure was modified according to Otto (1988).

Somatic chromosome numbers were counted from root tips pretreated with icy water, fixed in Carnoy's solution 2 (alcohol–chloroform–acetic acid 6:3:1, v/v/v), hydrolyzed in 1N HCl and stained with Schiff's reagent and 2% acetocarmine.

Genomic DNA was extracted according to a modified CTAB procedure as described by Kump and Javornik (1996). The amplified DNA products were sequenced from both sides by Macrogen Inc. (Seoul, Korea). Nucleotide sequences were deposited in the GenBank database with the Accession numbers at DQ307456–DQ307486.

Results and discussion

In the presented study the nuclear DNA content of the majority of investigated *Trifolium* species was determined for the first time. Analyses revealed large interspecific variations in nuclear DNA content, since it ranged from 0.688 pg (*T. ligusticum*) to 7.375 pg (*T.*

burchellianum), exhibiting a 10.7-fold difference. 1Cx value of nuclear DNA content was calculated considering accessions ploidy level and resulted very informative in particular within *Lotoidea* sections. In this section, 1Cx values of six African and American species were twice those of the six Eurasian species of the same section. Clustering based on ITS polymorphisms showed high relationship with botanical sections with the only exemption in the section *Lotoidea*, which was divided mainly according to its origin (American, African or Eurasian). Nuclear DNA content and the ITS clustering were highly correlated in sections *Chronosemium*, *Involucrarium* and *Vesicaria* and varied approximately two-fold within sections *Trifolium*, *Lotoidea* (African origin) and *Lotoidea* (Eurasian origin).

Despite the high interspecific variability in nuclear DNA content, the intraspecific variability revealed marked constancy that could be useful for genebank accession management. Specifically, in majority of 23 analyzed *Trifolium* species presented intraspecific variability of relative DNA content lower than 5% if accessions with the same chromosome number were considered. Only 8 *Trifolium* species showed 1-2 atypical accessions with outstanding differences in relative DNA content, chromosome number and morphological features. The majority of these atypical accessions are taxonomically dubious and further molecular marker analyses are recommended. However the flow cytometry measurement was proposed as a first, quick and reliable method for testing accessions in genebanks collections.

Conclusions

It can be concluded that simultaneous analysis of genome size and ITS data is more informative than separate analysis, since a more general view of phylogenetic comparisons can be obtained in terms of the genetic variability at both “micro” and “macro” level. Furthermore, we are able to show that flow cytometry measurements of relative nuclear DNA content can be also used as a simple and reliable technique for the verification of integrity and genetic stability of *Trifolium* genebank accession.

Acknowledgement

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References

- Arumuganathan K., Earle E.D. (1991). Nuclear DNA content of some important plant species, *Plant Mol. Biol. Rep.* 9 (3), 208–218.
- Badr A. (1995). Electrophoretic studies of seed proteins in relation to chromosomal criteria and the relationships of some taxa of *Trifolium*, *Taxon* 44, 183–191.
- Bullitta S., Hayward M.D. (1996). Application of RAPD markers to a study of species relationships in the genus *Trifolium*. In: B. Pickersgill, J.M. Lock (Eds.), *Advance in Legume Systematics, Part 8: Legume of Economic Importance*, Royal Botanic Gardens, Kew: 127–135.
- Campbell B.D., Caradus J.R., Hunt C.L. (1999). Temperature responses and nuclear DNA amounts of seven white clover populations which differ in early spring growth rates, *N. Z. J. Agric. Res.* 42, 9–17.
- Doležel J., Binarova P., Lucretti S. (1989). Analysis of nuclear DNA content in plant cells by flow cytometry, *Biol. Plant.* 31, 113–120.
- Grime J.P., Mowforth M.A. (1982). Variation in genome size—an ecological interpretation, *Nature* 299, 151–153.
- Kump B., Javornik B. (1996). Evaluation of genetic variability among common buckwheat (*Fagopyrum esculentum* Moench.) populations by RAPD markers, *Plant Sci.* 114, 149–158.
- Leitch I.J., Bennett M.D. (2002). New insights into patterns of nuclear genome size evolution in plants, *Curr. Genomics* 3, 551–562.
- Otto F. (1988) High resolution DNA—flow cytometry using DAPI, Protocol 1, Partec Arlesheim, Munster.
- Taylor, N.L. (1990). The true clovers. In: Janick J. and Simon J.E. (Eds.), *Advances in new crops*. Timber Press, Portland, OR, USA: 177–182.
- Zohary M., Heller D. (1984). *The Genus Trifolium*, The Israel Academy of Science and Humanities, Jerusalem: 51 pp.
- Watson L.E., Sayed-Ahmed H., Badr A. (2000). Molecular phylogeny of Old World *Trifolium* (Fabaceae), based on plastid and nuclear markers, *Plant Syst. E* 224, 153–171.

Is it possible to distinguish provenances of Norway spruce with molecular markers?

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Introduction

Norway spruce (*Picea abies* L.(Karst.)) is one of the most economically important tree species in Europe. Because trees are generally best adapted to the ecological conditions of the region where they evolved (Pliura and Heuertz 2003) local populations are considered as superior sources of reproductive material for sowing or planting. To monitor gathering of reproductive material and further seed / seedling handling in nurseries, the application of molecular markers which enable differentiation among populations and comparison to the reference samples has been considered.

Material and methods

Three Norway spruce populations from 2 provenance regions and 3 different seed stands were analysed. Seed samples belonging to single trees were collected and sent to the Slovenian Forestry Institute upon collection. The provenances tested are shown in table 1.

Table 1: The origin and short description of provenances tested in the study.

Provenance	Leskov grm	Hrušica	Konačnik
Seed stand ID	6.0131	6.0137	2.0173
Provenance region	Dinaric	Dinaric	Pohorje
Origin	Non-autochthonous (2 nd generation)	Autochthonous	Unknown
No. of trees sampled	57	30	39

Total genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) from one seedling randomly selected among 5-10 seedlings belonging to each seed sample. Primer pairs (with Ta in °C) Sb17 (57), Sb42 (56), Sb51 (55), Sb58 (57), Sb70 (57) (Perry and Bousquet 1998), PA0002 (55) and PA0055 (55) (Schubert et al. 2001) in combination with restriction enzymes AluI, AfaI, HaeIII and DraI were used. PCR-RFLP products were separated by means of gel electrophoresis on 2% agarose gel and stained with ethidium bromide. Programmes TFPGA, GenePop, GSED and Fstat were used to analyse the data.

Results and discussion

Out of 7 markers, 5 were used for statistical analysis. Locus PA0002 was excluded due to unsatisfactory amplification rate, and locus Sb42 because of only approximately 10 bp difference between alleles, which was hard to detect on the agarose gel, and a high number of heteroduplexes formed. With the combination of the remaining 5 primer pairs 37 different genotypes were identified. Allele A3 on locus Sb17 was found only in the provenance Konačnik. Taking into account sample sizes, the alleles present in a population with a frequency equal to or higher than 0,05 in population Leskov grm, 0,09 in Hrušica and 0,07 in Konačnik were detected with probability 0,95. Allele A3 could be characteristic for the population Konačnik. However, other populations from Pohorje provenance region and whole Slovenia must be analysed to confirm this observation.

Fisher's exact test has shown departures from Hardy-Weinberg equilibrium in Konačnik population, which can be explained with low frequency of A3 allele found in the sample. FST of 0,003 shows no genetic differentiation between populations using the data from 5 analysed loci. Also a test for population differentiation (not assuming random mating within samples) based on genotype

randomisation among samples and log-likelihood G statistics did not detected any population differentiation (p-value over all loci was 0,2314). Low population differentiation is expected in species with continuous distribution and long ranging pollen flow such as Norway spruce. However, genetic distances calculated after Nei and Wright among populations show smaller distances between populations from the Dinaric provenance region than between a provenance from the Dinaric region and a provenance from the Pohorje region (Table 2). Božič et al. (2003) have previously used genetic distances based on isoenzyme data to show that Norway spruce populations from central Dinaric phytogeographic region need to be distinguished from populations belonging to Alpine phytogeographic region. Figure 1 shows an UPGMA tree based on Nei's genetic distance.

Table 2: Genetic distances according to Nei (1972) and Wright (1978).

	Nei						Wright
	Sb17	Sb51	Sb58	Sb70	PA0055	All loci	All Loci
Leskov grm - Hrušica	0,00	0,13	0,64	0,10	1,22	0,41	5,10
Leskov grm - Konačnik	0,87	0,04	3,22	0,61	2,55	1,51	9,74
Hrušica - Konačnik	0,79	0,32	0,98	0,22	0,24	0,56	5,87

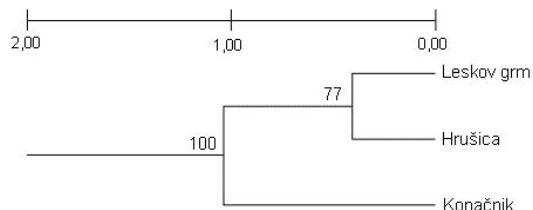


Figure 1: UPGMA tree based on Nei's (1972) genetic distances and 10.000 bootstrap iterations. The values on the nodes are the bootstrap values.

Conclusions

On the basis of STS and EST markers used in this study it is not possible to distinguish among the three Norway spruce provenances. However, on the basis of genetic distances it could be possible to assign a population to a provenance region. Analysis of more populations within and among provenances and possibly use of additional markers is needed to answer this question.

References

- Božič G., Konner M., Zupančič M. and Kraigher H. (2003). *Zbornik gozdarstva in lesarstva* 71, 19-40.
- Nei M. (1972). *Am. Nat.* 106, 283-292
- Wright S. (1978). *Evolution and the Genetics of Populations. Vol. 4: Variability within & among Natural Populations.* Univ of Chicago Press Chicago
- Perry D. J. and Bousquet J. (1998). *Genetics* 149, 1089-1098.
- Schubert R., Mueller-Strack G. and Riegel R. (2001). *Theor Appl Genet* 103, 1223-1231.
- Pliura A. and Heuertz M. (2003). EUFORGEN Technical Guidelines for genetic conservation and use for common ash (*Fraxinus excelsior*). IPGRI, 6 p.

Gene expression patterns of potato crops grown under different agricultural conditions

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Introduction

Commercial cultivation of genetically modified organisms (GMOs) has started in 1996. Since then the areas sown with GMOs and also the number of genetically modified traits have undergone an exponential increase globally. Before the release of new GMOs on the market extensive safety assessment of novel foods is required that comprises: (i) a molecular characterization of the introduced genetic fragment and the insertion site, (ii) analysis of key composition of relevant plant parts with respect to key nutrients and anti-nutrients, including allergens and toxins; (iii) potential gene transfer of the introduced trait, (iv) allergenicity of new gene products and (v) toxicological and nutritional assessment of the obtained data (Kok and Kuiper, 2003). Concerns that genetic modification could lead to unintended or unexpected side effects that could have an effect on human and animal health has led to development of new untargeted methods to detect changes in plant physiology in a larger extend. For this purpose high-throughput profiling techniques have been assessed to study the effect of genetic modifications at mRNA, proteome and metabolome levels (Kuiper et al. 2003; Kok et al. 2003). Microarray technique enables screening of expression of many thousand genes concurrently, but to be able to study unintended effect extensive data is needed about gene expression levels and their variability under different agricultural conditions. Our study will contribute to the understanding of gene expression changes in potato tubers grown under different input systems (organic or conventional), disease and fertility management.

Materials and methods

A nonredundant 4K potato microarray was prepared containing specific cDNA clones related to stress conditions, virus infection, tuber developmental stages and plant parts. Potato tuber samples from two experiments conducted at University of Newcastle were used as a model system: (1) *Phytophthora infestans* challenged potato plants of varieties Sante and Lady Balfour, subjected to different anti-fungal treatments and use of different fertilizers (14 treatments) and (2) potato tubers grown within conventional, 2 low input and organic farming systems in 2 different rotational contexts (8 treatments). RNA was isolated from potato tubers and hybridized to the 4K potato microarray. Each of the experimental conditions was repeated on the field in 4 biological replicates and additionally 2 technical replications of hybridization were performed yielding 176 hybridization experiments.

Results and discussion

A nonredundant microarray was designed and spotted to enable the study of gene expression in potato tubers containing 4006 spots. After hybridization of the mRNA from potato tuber samples quality check was performed for the obtained data and spots with low signal to noise ratio were removed from further analysis. The data was first normalized across all experiments to reduce differences caused by experimental procedure and to enable direct comparison of gene expression levels under different treatments. Principal component analysis was used as an unsupervised method to find patterns in the dataset and grouping of different treatments with similar expression patterns was observed. In parallel analysis of variance (ANOVA), using split-plot models to account for biological and technical factors, was performed for individual genes to find genes that show significant changes in expression among treatment

groups. The strategy to develop new safety approaches for the assessment of GMO-derived food and feed products as well as the initial results of array analysis will be presented.

Conclusions

Results of the microarray studies will be further confirmed using real-time PCR and biological relevance of gene expression variation to food safety will be assessed. The study of gene expression levels in potato tubers will provide us with an extended insight into the physiology of potato plants grown under different agricultural conditions and provide a background database of gene expression variation under field conditions that can be used in studies of genetically modified plants. The results will also be compared with proteome and metabolome analysis of the same sample set within the EU 6th framework project Safefoods.

References

- Kok E. J. and Kuiper H. A. (2003) *Trends in Biotechnology* 21 (10), 439 – 444.
Kok E. J., Kleter G. A., van Dijk J. P. (2003) *TemaNord report*, 558, 1-83.
Kuiper H. A., Kok E. J., Engel K. H. (2003). *Current Opinion in Biotechnology* 14 (2) : 238-43.

The influence of the applied auxins on the callus formation and development of *Prunus* 'GiSelA 5' leafy cuttings

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Introduction

The process of adventitious root formation is influenced by internal and external factors. Among internal factors, the most important role is ascribed to the auxins. IAA is generally regarded as the major auxin, universally found in higher plants, that plays a central role in adventitious rooting (De Klerk et al., 1999).

In difficult-to-root plant species, that suffer heavy losses due to the poor quality of their root systems (or shoots) as well as poor or slow rooting, exogenous auxin treatment is usually important. IBA is the most common exogenously applied plant growth regulator (De Klerk et al., 1999). It usually has greater ability to promote adventitious root formation than IAA and some evidence suggests that IBA acts as an auxin on its own, not only through its conversion to IAA (De Klerk et al., 1999; Ludwig-Müller, 2000).

The present work is about rooting of 'GiSelA 5', a commercially important cherry dwarfing rootstock. *Prunus* species are generally classified as difficult-to-root, therefore applied auxin is thought to be necessary for a better rooting. We investigated the difference in efficiency of two applied auxins, IAA and IBA in comparison with untreated cuttings and the influence of endogenous IAA levels on the quality of the new plantsprimerja.

Materials and methods

Experimental design

15 cm long terminal leafy cuttings of 'GiSelA 5' were prepared in June. The experiments were carried out in an unheated greenhouse under the fog system. Cuttings were put into the wet peat:sand substrate with slow-released fertilizers.

In the first experimental year IAA and IBA (4 g/l quick-dip solution) treatments in comparison with the control cuttings were analyzed. In the second experimental year only IBA (2.5 g/l quick-dip solution) treatment was compared to the control cuttings. At the end of the rooting season rooting rate, callus formation, number of main roots and shoot growth were measured.

Extraction and analysis of indole-3-acetic acid

Cuttings were collected immediately and 1, 2, 3, 4 and 5 days after excision. The basal 3 cm-portions of cuttings were stored at -80°C until analysis. IAA was extracted with K-phosphate buffer and purified on SPE, as described by Štefančič et al. (2005, 2006). The concentrated eluate was separated on HPLC by Varian Chromsep column and analyzed with fluorescence and UV-VIS detectors. The mobile phase with flow rate of 1 ml/min consisted of solvent A: 1% acetic acid and solvent B: MeOH. Gradient was linear from 10 to 55% of solvent B in 40 min. IAA was quantified by fluorimetry, comparing the peak areas with those of known amounts of IAA. Losses were evaluated by standards that had gone through the whole process of extraction and purification.

Statistical analyses

Statistical analyses were carried out with the programme Statgraphics Plus, using analysis of variance. Statistically significant differences between the treatments were tested with the LSD test at the 0.95 confidence level.

Results and discussion

Both auxin treatments had no effect on the final percent of the rooted cuttings (80 - 87%) neither on the survival of cuttings (45 - 63%), but they increased the percent of rooted cuttings without callus. As already established by other authors (De Klerk et al., 1999), IBA proved as the most efficient treatment. IBA was more successful than IAA in promoting earlier root development (data not shown) and it strongly hindered the callus formation (Figure 1, 2). That is an important fact, while callus formation impeded root

development, reducing the number of main roots; and the growth of the cuttings, reducing the average total length of shoots formed by individual cuttings (Table 1). Those parameters are very important for the quality and survival of the new plants and they are not the consequence of the higher IAA content in the rooting zones of cuttings in the first days of root development (Table 2). Endogenous IAA seems to be important only when present in high concentration at the time of preparing cuttings (. Exogenously applied auxins were not crucial for root formation, or their survival, however their application resulted in higher percent of more qualitative 'GiSela 5' leafy cuttings.

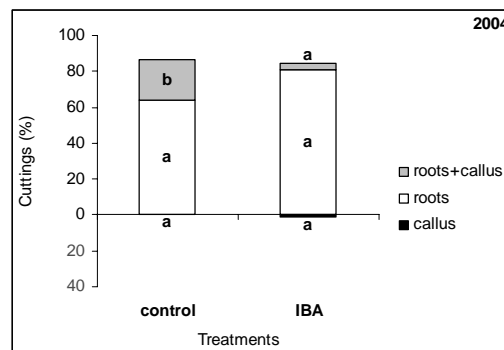
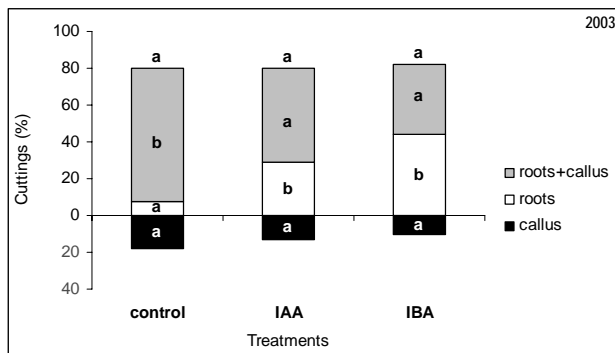


Figure 1, 2: Rooting and callus formation (%) of 'GiSela 5' leafy cuttings according to different treatments in 2 experimental years. Means for callus formation, root formation and callus with roots marked with the same letter are non-significant ($P=0.05$, LSD test), compared across the treatments.

Table 1: The influence of callus formation on number of main roots and shoot growth of all rooted leafy cuttings of 'GiSela 5' at the end of the growing seasons 2003 and 2004.

	Number of main roots		
	Roots		Roots+callus
2003	6.2	*	3.3
2004	10.8	*	6.1
	Shoot growth (cm)		
	Roots		Roots+callus
2003	45.4		14.4
2004	50.8	*	15.9

Table 2: Internal levels of IAA (ng/g of fresh weight) in stem bases of leafy cuttings of 'GiSela 5' at the time of establishment of cuttings and after 1st day.

		Day 0	Day 1 (the max. accumulation)
2003	Control		30.9
	IBA (4 g/l)	2.9	147.8
	IAA (4 g/l)		558.0
2004	Control		131.3
	IBA	40.7	182.8

References

- De Klerk G.-J., Van der Krieken W. and De Jong J.C. (1999). *In Vitro Cellular & Developmental Biology* — *Plant* 35, 189-199.
- Ludwig-Müller J. (2000). *Plant Growth Regulation* 32, 219-230.
- Štefančič M., Štampar F. and Osterc G. (2005). *Hort Science* 40(7), 2052-2055.
- Štefančič M., Štampar F. and Osterc G. (2006). *Journal of Horticultural Science & Biotechnology* 8 (3), 508-512.

Detection of ToMV (*Tomato mosaic virus*) in irrigation waters

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Introduction

Viral infections of economically important plants, such as beet (*Beta vulgaris*), carrot (*Daucus carrota*), carnation (*Dianthus caryophyllus*) and many others can cause development of disease symptoms and lead to crop loss. Several reports have been made where different plant viruses had been detected in environmental waters (Piazzolla *et al.*, 1986; Horvath *et al.*, 1999; Gosalves *et al.*, 2003). Irrigation waters can represent a source of infection for plants and consequently crop loss. Viruses present in water can infect plants through root system and cause the appearance of symptoms (Koenig, 1986). Sensitive molecular methods such as real-time PCR have been described that determine the presence of human viruses, such as enteric viruses (Monpoeho *et al.*, 2000), astroviruses (Le Cann *et al.*, 2004), and noroviruses (Beuret, 2004). Such method however has not yet been described for detection and quantification of plant pathogenic viruses.

Detection of human viruses from highly diluted water samples was performed after concentration of samples using different methods (ultracentrifugation steps, ultrafiltration and/or precipitation in polyethyleneglycol (PEG)). In case of diluted samples of measles and mumps viruses CIM[®] (convective interaction media) monolithic chromatographic supports were used for concentration of samples (Branovič, 2003). CIM also proved to be effective in concentrating *Tomato mosaic virus* (ToMV) virus particles (Kramberger *et al.*, 2004) diluted in tap water.

Sensitive detection method was developed for determining extremely low concentrations of ToMV. It was adapted for determining the presence of ToMV and its amount in environmental irrigation waters. We determined the viability of ToMV in original samples of irrigation water and observed morphology of virus particles after the concentration procedure using CIM.

Materials and methods

Real-time RT-PCR for detection of ToMV

Samples were initially heated at 90°C for 10 minutes and then diluted in buffer. 20 µl of sample was denatured at 90°C for 10 minutes to release viral RNA. RT reaction with cDNA Archive Kit (ABI, USA) followed and 4 µl of obtained cDNA was later multiplied in 20 µl real-time PCR reactions. The primers and MGB probe used were designed to be ToMV specific, aligning to the viral MP nucleotide sequence. Quantitative real-time PCR assay was also developed. Concentration of ToMV in sample was calculated according to calibration curves, obtained by diluting ToMV particles in buffer. Limit of detection (LOD) and limit of quantification (LOQ) were set by intra-assay comparison of 5 independent real-time PCR runs.

Analysis of irrigation water

After adaptation of ToMV specific real-time PCR detection procedure for testing of environmental waters and chromatographic fractions, CIM[®] monolithic chromatographic supports were included for the concentration of water samples. Concentrating procedure was optimised for environmental waters and detection scheme was proposed. Different irrigation water resources from different parts of Slovenia were tested for the presence of ToMV.

Infectivity testing and electron microscopy

Test plants *Nicotinana glutinosa* were mechanically inoculated with elution fraction and development of disease symptoms was observed for 3-4 weeks. Inoculated and symptomatic leaves were tested with ToMV specific real-time PCR. DAS-ELISA test was

later used to determine the presence and amount of ToMV in inoculated leaves with symptoms and non-inoculated leaves showing no disease symptoms.

Immuno-serological electron microscopy (ISEM) with ToMV specific antibodies was used to visualize virus particles from elution fraction after concentration of original irrigation water sample from gravel pit near Ivanci.

Original irrigation water sample from gravel pit near Ivanci (with ToMV concentration 10⁻⁶ mg/ml) was used to water propagation test plant *Nicotiana clevelandii* for 2 weeks. Elution chromatographic fraction obtained after the concentrating procedure was diluted to concentration 10⁻⁶ mgToMV/ml and the dilution was used to water the *N. clevelandii* plants. After 2 weeks plants were tested for the presence of ToMV with real-time PCR.

Results and discussion

The intact ToMV virus particles were observed in elution fraction using ISEM. The visualization of morphologically intact particles after the concentration procedure led to conclusion that intact viral particles had also been present in original irrigation water sample. Mechanical inoculation of *N. glutinosa* test plants with elution fraction resulted in development of disease symptoms (chlorosis). Using DAS-ELISA presence of ToMV could not be detected neither in inoculated and symptomatic nor in non-inoculated and non-symptomatic leaves. Further analysis of two inoculated and symptomatic leaves with ToMV specific real-time PCR showed that the estimated concentration of ToMV ranges from 10⁻⁸ mg/ml to 10⁻⁷ mg/ml. Upper, non-symptomatic leaf also tested positive for the presence of ToMV. Concentrations of ToMV were lower respectively reaching 10⁻⁹ mgToMV/ml. Sensitive and quantitative ToMV specific real-time PCR method proved to be successful in determining presence of ToMV in test plants. Appearance of disease symptoms and determination of presence of ToMV in mechanically inoculated test plants showed that virus particles remain infective even after the concentrating procedure suggesting that particles are present in such condition even in original irrigation water samples.

N. clevelandii propagation plants showed appearance of disease symptoms after 2 weeks of watering with ToMV positive water. Using ToMV specific real-time PCR the estimate concentration of ToMV in plants, watered with diluted elution fraction was 10⁻⁹ mg/ml. The Ct values in ToMV specific real-time PCR for plants watered with original irrigation water were at the limit of detection thus the concentration of ToMV in plant samples could not be determined. Exposing propagation plants to infected water for 2 weeks was in our experiment enough for the development of disease symptoms and transfer of ToMV to plants. This confirms previous results that ToMV particles are infective in irrigation water samples and also in elution fractions.

Conclusions

- ToMV specific, quantitative real-time PCR method can be used to monitor irrigation water and plants for the presence of the virus.
- ToMV is infective in original water samples and in chromatographic fractions after concentrating procedure.

References

- Beuret C. 2004. *Journal of Virological Methods* 115:1-8.
- Branović K., Forčić D., Ivančić J., Štrancar A., Barut M., Košutić-Gulija T., Zgorelec R., Mažuran R. 2003. *Journal of Virological Methods*, 110:163-171
- Gosalvez B., Navarro J.A., Lorca A., Botella F., Sánchez-Pina M.A., Pallas V. 2003. *Journal of Virological Methods*, 113:87-93.
- Horváth J., Pocsai E. Kazinczi G. 1999. V: *Zbornik predavanj in referatov. 4. Slovensko posvetovanje o varstvu rastlin*, Portorož, 3-4 mar. 1999. Maček J. (ed.). Ljubljana, Društvo za varstvo rastlin Slovenije: 353-356.
- Koenig R. 1986. *Advances in Virus Research*, 31:321-333.
- Kramberger P., Petrovič N., Štrancar A., Ravnikar M. 2004. *Journal of Virological Methods*, 120:51-57.
- Le Cann P., Ranarijaona S., Monpoeho S., Le Guyader F., Ferré V. 2004. *Research in Microbiology*, 155:11-15.
- Monpoeho S., Dehée A., Mignotte B., Schwartzbrod L., Marechal V., Nicolas J.-C., Billaudel S., Ferré V. 2000. *BioTechniques*, 1, 29:88-93.
- Piazzolla P., Castellano M.A., De Stradis A. 1986. *Journal of Phytopathology*, 116:244-246.

2025: European vision for plant genomics and biotechnology: “Plants for the future”

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Introduction

Technology platforms (TP) are a new instrument of developmental politics that was initiated by the European Commission (EC) to promote further investments into innovative technologies and knowledge, accompanied by an active role of the industry parties and the EU countries. The main task of TP is to include all interested parties, with the industry as a key holder of the initiative, in the preparation and realization of the further research and developmental politics of the EU, to increase private and improve public investments. The European TP "Plants for the future" launched in 2004 is a stakeholder forum on plant genomics and biotechnology, which rose from the joint initiative of industry and EC in the course of Lisbon strategy and preparation for FP7 (<http://www.epsoweb.org/Catalog/TP/index.htm>). The main purpose of the Slovene TP “Rastline za prihodnost” is to bind and coordinate the national and European, public and private partners in the area of plant production armed with means of modern biotechnology and breeding techniques.

Potentials and strategic goals of the technology platform "Plants for the future"

Today, plants are used not only as food for humans and feed for cattle, but as industrial materials. They are also valuable sources of vitamins, antioxidants, oils, fibres and carbohydrates. They provide ingredients for most drugs and are a major traditional energy resource. The economic importance of plants and plant-derived products in Europe is huge in terms of employment and trade. Plants are the basis of European industries with an annual turnover of 1 trillion euros. Genomics and biotechnology are the modern tools for understanding plants at various biological and environmental levels, as well as boosting classical breeding techniques. An array of novel techniques have emerged that are now permitting researchers to identify the genetic underpinnings of crop improvement, namely the genes that improve productivity and quality of modern crop varieties. There is an urgent need to make today’s chemical – intensive agriculture more sustainable while maintaining its productivity. In fact, we need to increase yields and simultaneously reduce or optimise the amount of fuel, fertilisers, pesticides and water used up in the process. A competitive global position for the EU in agriculture, biotechnology and food production will benefit employment and economic growth across the Union. Developing new technologies and agricultural products can help the environment and have a positive impact on rural development. In addition it would ensure a strong domestic and sustainable European food supply offering consumer a wide choice of healthy and diverse food.

"Plants for the future" - technological areas

The EC has prepared the “Plants for the future vision paper”. The goals set are very much in line with EU’s objective of becoming the most competitive and sustainable knowledge based economy by 2010. Stakeholders coming from companies, research institutions, funding bodies and regulatory authorities supporting the new launched platform “Plants for the future” were charged with drawing up a Strategic Research Agenda (SRA). In SRA, the EC recognizes, that Europe cannot afford to miss out on the benefits offered by plant genomics and biotechnology. Justified environmental and health concerns have to be balanced against tolerable risks, likely economic impacts and employing the new technology to help respond to major social and environmental challenges. The action plan will endeavour to address four main challenges:

Challenge one: Health, safe and sufficient food and feed

Challenge two: Sustainable agriculture, forestry and landscape

Challenge three: Green products

Challenge four: Competitiveness, consumer choice and governance

SRA is divided in three parts: Part I – summary, Part II – draft action plan 2010 and Part III – draft action plan 2006-2010 in which a concise summary of each of the four challenges, including deliverables and proposed research activities to address the challenges are presented. The foreseen advantages of following the goals stated in the SRA will bring many advantages: improve understanding of plant metabolism, secure a healthy, high quality food/feed supply, improve plant yield potential and security, increase the amount of useful plant matter, improve countryside biodiversity and genetic diversity of crop plants, reduce the environmental impact of agriculture, enhance crop monitoring, improve crop co-existence, develop renewable materials and more efficient biofuels.

Joining into European research consortiums

European TP “Plants for the future” is open for cooperation and all interested stakeholders are welcome. The most convenient way to participate in the TP is to join the national TP. In most of the EU countries TP were formed by means of national consultations. In Slovenia, the national consultations were organised by three research institutions dealing with plant science and in the first stage, the consultation with the European TP, were carried out through the Internet survey. The Slovene initiative “Rastline za prihodnost” brings new means of cooperation between all interested parties and takes an active role in policy making and realisation of the goals set by the EU in this particular area. Common advantages of the involved parties are acquiring guidelines for one's own developmental programmes, networking with other European companies and research institutions as well as potential partnerships in European projects (FP7). TP “Plants for the future” will collaborate directly with other TP: these may include Food for life, Sustainable chemistry, Innovative medicines, Farm animal breeding and Global animal health.

References

European technology platform “Plants for the future: <http://www.epsoweb.org/Catalog/TP/index.htm>

Stakeholders proposal for SRA, Part I - summary: <http://www.epsoweb.org/Catalog/TP/docs/SRA-I.PDF>

Stakeholders proposal for SRA, Part II – draft action plan 2010: <http://www.epsoweb.org/Catalog/TP/docs/SRA-II.PDF>

Stakeholders proposal for SRA, Part III – draft action plan 2006-2010: <http://www.epsoweb.org/Catalog/TP/docs/SRA-III.PDF>

Slovene TP “Rastline za prihodnost”: <http://www.kis.si>

Haploid induction of rocket (*Eruca sativa* Mill.) via androgenesis using microspore culture

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Introduction

Eruca sativa (rocket) is native to the Mediterranean region and North Africa (Fig. 1). It is popular in salad because of its piquancy and pungency. Its value increased in recent years mainly due to nutritional qualities (e.g. anticancer properties-due to glucosinolates, source of vitamin C and ferrous ions). Haploids are used in plant breeding to obtain pure lines that are homozygous. In the last two decades, remarkable progress in isolated microspore culture technology has been made in all major *Brassica* species, and especially in oilseed rape. Comprehensive utilization of this doubled-haploid production system has been involved in *Brassica* breeding programmes as well as in gene transfer, biochemical and physiological studies, and other manipulations. All these applications largely depend upon efficient microspore embryogenesis and embryo development (Gu et al., 2004). Microspores have the remarkable capacity to develop into haploid plants via embryogenesis *in vitro*. Isolated microspore culture has been successfully carried out with some *Brassica* vegetables such as cabbage, broccoli, oilseed rape, Chinese cabbage and pakchoi but the method has not been applied to rocket yet. The basic *Brassica* protocol is used with slight modifications depending on the species and genotype.

Materials and methods

Microspore cultures of four different rocket seed lots ("Valentin", "VMS", "Franchi", "Valli") were performed according to the common *Brassica* protocol. Optimization of determination of the microspore developmental stages in successive flower buds was performed using DAPI staining. Based on correlations between flower bud size and microspore developmental stage it was found that the appropriate bud length was from 4 to 5 mm, by which most of microspores were late uninucleate and some early binucleate. For each isolation 70 buds were harvested from young inflorescences and sterilised and after that washed three times in sterile water. Sterilised buds were macerated in liquid NLN-13 hormone free medium. A microspore suspension was obtained by filtration through two layers 45 µm nylon mesh. Suspension was centrifuged three times at 1000 rpm/min for 3 minutes each and resuspended in Petri-dishes at a density of 4×10^4 microspores/ml (Hansen, 2003). Cultures were incubated in the dark at 32 °C for 48 hours, and then maintained at 24 °C in the dark. After 10 days, the cultures were transferred to a gyratory shaker at 50 rpm.

Results and discussion

Haploid production through microspore culture involves the physical isolation of the microspores from plant buds and their regeneration into plants by *in vitro* culture. After five days of culturing cell divisions and cell clusters are visible in microspore suspension. The embryo number was assessed after 3-4 weeks of culture (Fig. 2). Results were expressed as number of embryos per Petri-dish (Table 1). Number of embryos of "Valentin" ranged from 51 to 404. Embryos were also obtained with the other three seed lots bud with more variable results. Number of embryos of "VMS" ranged



Fig. 1: Rocket (arugula) plants.

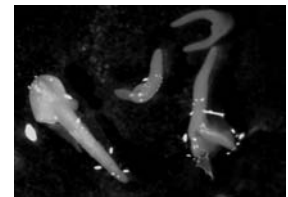


Fig. 2: Two weeks old embryos.

Table 1: Embryo yield of four different rocket seed lots

Rocket	Isolation	No. of Petri-dish	No. of embryos
"Valentin"	1	10	404
	2	11	264
	3	4	51
	4	5	149
"VMS"	1	3	526
	2	5	22
"Franchi"	1	16	41
	2	3	51
"Valli"	1	10	34

32 °C induces up to 40 % of isolated microspores that are cultured to into apparently normal mature pollen grains (Touraev et al., 1997). Variation was observed among embryo yields in successive microspore isolations from the same seed lot. This is in agreement with the large variability between plants. This variation might be due to variations in the manual selection of flower buds, as well as to physiological and genetic variation in the plant material. Seedling formation from the embryos on hormone-free B5 medium so far was accompanied with several abnormalities such as hyperhydricity and formation of secondary embryos (Fig. 3). In order to improve plant regeneration abscisic acid/dehydration embryo treatments are currently studied.

Conclusions

The results of the present study indicate possibility of obtaining doubled haploid (DH) lines of *Eruca sativa*. The microspore response is primarily affected by developmental stage of microspores. Genotype, donor plant physiology, developmental stage of microspores, pretreatments, medium constituents and culture conditions can influence microspore embryogenesis. The identification and optimization of these factors will result in the establishment of a reliable protocol for production of sufficient number of embryos required for a breeding programme. According to our knowledge this is the first report of microspore-derived embryo formation in *Eruca sativa*. With optimization of final steps the protocol can be used for efficient production of homozygous lines of rocket.

Acknowledgements

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References

- Duijs J.G., Voorrips R.E., Visser D.L., Custers J.B.M. (1992). *Euphytica*, 60: 45-55.
 Gu H.H., Hagberg P., Zhou W.J. (2004). *Plant Growth Regulation* 42: 137-143.
 Hansen M. (2003). In: *Doubled Haploid Production in Crop Plants. A Manual*. M. Maluszynski, K.J. Kasha, B.P. Forster, I. Szarejko (eds.), Kluwer Academic Publishers: 217-222.
 Touraev A., Vicente O., Heberle-Bors E. (1997). *Trends in Plant Science*, 2 (8): 297-302.

from 22 to 526. Number of embryos of "Franchi" ranged from 41 to 51 and number of embryos of "Valli" was 3. Further experiments are needed to determine whether variation is induced by genotype, donor plant conditions, or isolation procedure. As in *B. napus*, an initial high temperature treatment was shown to be necessary for embryogenesis in microspore culture (Duijs, 1992). *Brassica* microspores generally require a heat shock treatment (>30 °C) for 24-72 hours. Stress treatment acts as a trigger for inducing the sporophytic pathway, preventing the development of fertile pollen (gametophytic pathway). In *Brassica napus*, a heat shock treatment of 8 h at

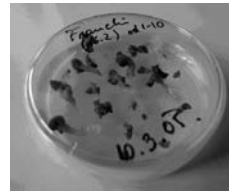


Fig. 3: Seedling formation from the embryos on hormone-free B5 medium expressing hyperhydricity and formation of secondary embryos.

Seasonal variation in secoiridoid and γ -piron compounds in *Gentiana lutea* L. leaves and roots

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Introduction

Yellow gentian (*Gentiana lutea* L.) is one of the most important medicinal plants with a long tradition, widely used in folk medicine as well as in pharmaceutical and food industry in many countries. *Gentianae* radix, the dried rhizomes and roots (underground parts) of *Gentiana lutea*, has been used in the treatment of anorexia and dyspepsia. There might be also some contra-indications: gastric or duodenal ulcers and hyperacidity (Escop monographs ..., 1997). In the pharmacopoeas only roots are described as an official drug, but lately interesting composition of aerial plant parts has been observed (Menković *et al.*, 2000). The amount of secondary metabolites, secoiridoids as well as γ -pirones, is changing during vegetation period (Franz *et al.*, 1985; Menković *et al.*, 2000). To utilize the resources of yellow gentian more effectively, it is necessary to optimise the harvesting time. Therefore, aims of the present study were to find out the seasonal variation of secoiridoids (gentiopicroside, amarogentin) and γ -pirones (mangiferin, homorientin and isovitexin) content in the leaves and roots and to determine the optimal harvesting time, to compare the differences between leaves' and roots' active compounds in order to suggest a better quality of raw materials for industrial use.

Materials and methods

Plant material (*Gentiana lutea* L. subsp. *symphyandra* Murb.) was harvested during vegetation period at the natural habitat Vremščica (Slovenia, 1027 m) in 2002. Air-dried samples of leaves and roots were ground and extracted according to Glatz *et al.* (2000). Identification of gentiopicroside, amarogentin, mangiferin, homoorientin and isovitexin in yellow gentian extracts was carried out by a Spectra Physics HPLC system using a gradient HPLC analysis (column Lichrochart 250x4 mm with precolumn Lichrochart 4x4 mm), according to the method developed by Menković *et al.* (2000). Authentic standards of gentiopicroside were purchased from Roth, amarogentin from Boehringer Ingelheim Pharma, mangiferin, homoorientin and isovitexin from LabService Analytica. All analyses run in triplicate and averaged. The results were presented as percentage of the dry weight \pm standard deviation. Analyses of variance were performed using the ANOVA. Differences between the means were determined using Duncan's multiple range test. *P* values < 0.05 were considered as significant.

Results and discussion

The content of secondary metabolites in yellow gentian leaves and roots varied depending on developmental stage. The dynamics in accumulation was due to the plant part observed, roots or leaves. Similar results for some of observed metabolites were obtained previously in investigations made by Menković *et al.* (2000) and Franz *et al.* (1985). The data showed (Table 1 and Table 2) differences in the profiles of compounds in leaves and roots. Tested secondary metabolites, gentiopicroside, amarogentin, mangiferin, homorientin and isovitexin, were identified in yellow gentian leaf extracts, while in root extracts only gentiopicroside and amarogentin were detected. The content of bitter secoiridoids, gentiopicroside and amarogentin, in roots was the highest in the leaf rosette stage (May). In the flowering stage (July) the contents of secoiridoids reached their minimum, but after flowering their contents increased again. In the leaves the contents of gentiopicroside, amarogentin and mangiferin reached their maximum in May

(leaf rosette developmental stage). Thereafter, the contents of metabolites decreased to the lowest content, determined in August. The highest content of homoorientin was detected in May, while that of isovitexin in a flowering developmental stage (July).

Table 1: Secondary metabolites content for secoiridoids in yellow gentian roots from Vremščica location (Slovenia) during the vegetation period of 2002. Means \pm SD are presented.

Secoiridoids in roots (%)		
Month	Gentiopicroside	Amarogentin
May	3.60 \pm 0.06 ^{c*}	0.41 \pm 0.02 ^d
June	2.47 \pm 0.13 ^b	0.16 \pm 0.05 ^b
July	1.84 \pm 0.15 ^a	0.10 \pm 0.02 ^a
August	2.03 \pm 0.19 ^{ab}	0.17 \pm 0.02 ^{bc}
September	2.45 \pm 0.09 ^b	0.22 \pm 0.01 ^c

*a-d: Different superscripts in the same column indicate significant difference (p<0.05).

Table 2: Secondary metabolites content for secoiridoids and γ -pirones in yellow gentian leaves from Vremščica location (Slovenia) during the vegetation period of 2002. Means \pm SD are presented.

Secoiridoids and γ -pirones in leaves (%)					
Month	Gentiopicroside	Amarogentin	Mangiferin	Homoorientin	Isovitexin
May	1.81 \pm 0.38 ^{c*}	0.20 \pm 0.02 ^c	0.44 \pm 0.09 ^c	0.22 \pm 0.05 ^c	0.04 \pm 0.00 ^b
June	1.54 \pm 0.55 ^b	0.17 \pm 0.07 ^b	0.25 \pm 0.10 ^b	0.11 \pm 0.05 ^b	0.03 \pm 0.01 ^{ab}
July	1.07 \pm 0.33 ^b	0.17 \pm 0.07 ^b	0.17 \pm 0.05 ^b	0.18 \pm 0.05 ^{bc}	0.05 \pm 0.02 ^b
August	0.34 \pm 0.07 ^a	0.05 \pm 0.03 ^a	0.02 \pm 0.01 ^a	0.02 \pm 0.01 ^a	0.01 \pm 0.00 ^a

*a-c: Different superscripts in the same column indicate significant difference (p<0.05).

Findings of the present study could represent an useful information for pharmaceutical and/or food industry that would select the most convenient time for harvesting plant material according to their quality needs. It should also be suggested on this place that aerial part (leaves) of yellow gentian could be used as a potential medicinal material - a source of γ -pirones and secoiridoids.

References

- ESCOP monographs on the medicinal uses of plant drugs. (1997) Exeter, ESCOP (the European Scientific Cooperative on Phytotherapy): 570 pp.
- Menković N., Šavikin-Fodulović K., Savin K. (2000). *Planta Medica* 66, 178–180.
- Franz Ch., Franz G. Fritz D., Schultze J. (1985) *Scientia Pharmaceutica* 53, 31–38.
- Glatz Z., Pospíšilová J., Musil P. (2000) *Journal of Liquid Chromatography and Related Technologies* 23 (12), 1831–1839.

Eradication of *Citrus tristeza virus* from cultivar Zorica Rana (*Citrus unshiu* Marc.)

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Introduction

Varieties of Satsuma mandarin are commercially the most important citrus crop in Croatia due to their relative frost tolerance. *Citrus tristeza virus* (CTV) is the causal agent of tristeza, the most devastating viral disease of citrus. CTV has been detected in most of the Satsuma mandarin (*Citrus unshiu* Marc.) varieties including different and even the newest clones of cv. Zorica Rana. The detailed procedure for CTV elimination from different citrus species has already been described and developed (Navarro *et al.*, 1975; Starrantino and Caruso, 1988; Singh, 2001) but not in case of Satsuma mandarin cv. Zorica Rana. Raising virus-free plants is an important aspect of citrus cultivation in Croatia.

Materials and methods

Two-year old Satsuma mandarin plants cv. Zorica Rana from collection orchard that were previously shown to be CTV positive (ELISA-test and IC/RT-PCR) were selected for virus elimination. After three months of thermotherapy plants of three clones were used as a source of explants for shoot-tip grafting (micrografting) on Troyer Citrange rootstock germinated *in vitro* following the method essentially described by Navarro *et al.* (1975). The original procedure was modified in performing grafting procedure ("L" incision) and nutrition media composition. Clones: 3/21-01, 5/19-01, 9/12-01 and 5/23-01 were also subjected to CTV biological detection on two-year old seedlings of Mexican lime (*Citrus aurantifolia* L.), CRC Grapefruit (*Citrus paradisi* Macf.), Sour Orange (*Citrus aurantium* L.) and Madame Vinous Sweet Orange [*Citrus sinensis* (L.) Osbeck]. Micrografted plants were tested for the virus by DAS-ELISA (*Double Antibody Sandwich Enzyme Linked Immunosorbent Assay*, Clark and Adams, 1977) using commercial antisera according to the manufacturers protocols (Agritest, Italy) and IC/RT-PCR (*Immunocapture Reverse Transcription Polymerase Chain Reaction*, Zemzami *et al.*, 2002) using primers for the CTV coat protein gene (Nolasco *et al.*, 1993). The same diagnostic methods were utilized for testing indicator plants.

Results and discussion

Observation of morphological changes and appearance of viral disease symptoms on citrus indicator hosts in comparison with positive and negative control plants had confirmed the presence of CTV in all tested clones of cv. Zorica Rana. Leaf cupping, chlorosis and vein enation were observed in Mexican lime seedlings only two months after grafting. Symptoms of stem-pitting (SP) started to appear on Mexican lime after seven months and, in the case of clone 5/23-01, in CRC Grapefruit seedlings, too. Lime reaction produced by CTV isolate from the mandarin clone 5/23-01 was much stronger in comparison with the other tested clones (growth retardation). No symptoms that could be attributed to CTV infection were observed in Sweet and Sour Orange indicators which suggests the presence of mild to moderate SP-CTV strains in 'Zorica Rana' clone 5/23-01. Biotest results imply biological diversity among detected CTV isolates. The presence of virus tristeza determined by biological indexing in tested clones has been positively correlated with CTV infection previously diagnosed by DAS-ELISA and IC/RT-PCR (Škorić *et al.*, 2005).

CTV-free plants of three 'Zorica Rana' clones were obtained by thermotherapy in combination with shoot-tip grafting *in vitro* from infected plants. Following this procedure, 20% of plants were tristeza-negative. Many factors influence the recovery of micrografted plants. The highest rate of successful grafts was obtained with 2-week old Troyer Citrange seedlings (Navarro, 1992), what is in agreement with our results. Degree of tissue differentiation of the rootstock, which is affected by light and age (Navarro, 1992) is also of great importance. The best results were obtained by grafting at the top of the decapitated epicotyl, placing the shoot-tip in contact with the vascular ring (Navarro *et al.* 1975). In our experiment, we placed shoot-tip in the "L" incision on the rootstock, which was also more practical to perform than inverse "T" incision commonly used in most laboratories (Singh, 2001). Additional pre-treatment with growth regulators before shoot-tip isolation could increase the incidence of successful grafts (Starrantino and Caruso, 1988).

After shoot-tip grafting, successfully cultured plants were established *in vivo*, this resulted in ninety percent survival and excellent subsequent growth.

Further sanitation of infected important cultivars of Satsuma mandarin, as well as other citrus species, is a prerequisite for obtaining high quality foundation plant material. Micrografting *in vitro* may also have an important role in basic research for graft compatibility, physiology of the graft union and plant aging.

Conclusions

The appearance of different symptoms in biological indicators suggests biological diversity among detected CTV isolates in investigated 'Zorica Rana' mandarin clones. *In vitro* grafting of small (0.3 mm) shoot tips on 2-week old Troyer Citrange rootstock seedlings enables successful grafts that were transplanted to soil with 90% survival rate. The efficiency of virus tristeza eradication from cv. Zorica Rana Satsuma mandarin was tested by using laboratory diagnostic methods DAS-ELISA and IC/RT-PCR. CTV elimination was confirmed in 20% of tested plants.

References

- Clark M.F. and Adams A.N. (1977) *J. Gen. Virol.* 34, 475 - 483.
- Navarro L., Roistacher C.N. and Murashige T. (1975) *J. Amer. Soc. Hort. Sci.* 100(5), 471-479.
- Navarro L. (1992) In: Bajaj YPS (Ed.) *Biotechnology in agriculture and forestry. High – Tech and Micropropagation II.* Springer, Berlin: 327 – 338.
- Nolasco G., Deblas C., Torres V. and Ponz F. (1993) *J. Virol. Meth.* 45(2), 201-218.
- Singh I. P. (2001) In: *Proc. 6th world Congress of the International society of citrus nurserymen, Brasil*, 128-130.
- Starrantino A. and Carruso A. (1988) *Acta Hort.* 227, 101 – 103.
- Škorić D., Krajačić M., Hartl D. and Gatin Ž. (2005) In: *Proc. MNCC, Options Méditerranéennes, Series B 43, CIHEAM/MAIB* (in press).
- Zemzami M., Soares C.M., Bailey A.M., Niblett C.L. and Nolasco G. (2002) In: *Proc. 15th IOCV Conf., Cyprus*, 8-12.

Gynogenic Haploid Induction in *Mimulus aurantiacus*

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Introduction

Mimulus aurantiacus is a self-fertile, hummingbird-pollinated perennial shrub with tubular hermaphroditic flowers of a wide range of colours. It belongs to the genus *Mimulus* (Scrophulariaceae) which has been widely used as a model flowering plant system for ecological and evolutionary genetics. It is a promising ornamental plant native from North America. The aim of our research was to establish an efficient method for gynogenic haploid induction. Genetically homozygous doubled haploid plants obtained directly or after chromosome doubling could be introduced in different breeding programs and genetic studies.

Material and methods

In order to stimulate gynogenic development from unfertilised female gametophytes several protocols has been tested on four different genotypes. Un-pollinated female reproductive organs (ovaries, placenta attached ovules, isolated ovules) from 1,237 flowers were excised and inoculated on different growth media. Three different basal media (MS, NLN, B5) enriched with plant hormones IAA, NAA, BAP, TDZ and 2,4-D in concentrations from 0.02 to 2 mg/l and 20 or 80 g/l sucrose were used. Chemical shocks were combined with temperature pre-treatment for a period of time from 1 to 8 days. Finally the explants were cultured on 23°C under 16h photoperiod.

In situ stimulation for gynogenic embryo formation was performed using pollination of 588 flowers with γ -irradiated pollen (600 Gy, Co⁶⁰) and subsequent *in vitro* culture of ovules containing embryos at different developmental stages. Ovules were excised 6 to 28 days after pollination and inoculated on three different basal media (MS, Nitsch & Nitsch, modified Rangaswamy media) supplemented with 0.1-1 mg/l of plant hormones kinetin, zeatin, IAA and 20-40 g/l sucrose. The excised ovules were incubated at 23°C in the dark or under 16h photoperiod. Some of the pollinated flowers were left on the plants until maturation of the seeds which were and inoculated on Nitsch & Nitsch media supplemented with 40 g/l sucrose at 23°C under 16h photoperiod.

Results

The results so far do not indicate any differences in gynogenic response among the evaluated genotypes. *In vitro* culture of un-pollinated reproductive tissues on different culture media resulted in swelling of ovules, callus formation and/or shoot and root regeneration. Ploidy analysis of tested regenerants revealed their diploid constitution. Considering the site of their origin and the results from flow cytometry we concluded that they probably originated from somatic tissues of the ovary, placenta or from callus cells.

Irradiation of pollen at 600 Gy did not prevent *in situ* germination of the pollen (Fig. 1). Maturation and germination of embryos obtained after pseudofertilisation with irradiated pollen was most successful on modified Rangaswamy media supplemented with IAA and lead to enlargements of ovaries and ovules which resemble processes of seed development. Cultures of such ovules (about 100,000) gave rise to 20 embryos (Fig. 2) after about six weeks. Some of them developed further and their ploidy levels were analysed. The flow cytometric analysis of plantlets showed that the majority of regenerants were diploids, one of them was haploid and one of them triploid. The haploid regenerant was transferred on fresh media and multiplied for further evaluations.

From 397 cultured seeds, 86 plants emerged and were analysed with the flow cytometer. All regenerants but one (which was triploid) were diploid.

Conclusions

This is the first report of gynogenic haploid induction in *Mimulus aurantiacus*. The results so far indicate that among approaches tested pseudofertilisation with irradiated pollen seems the most promising. The protocol of *in vitro* embryo maturation and germination has to be optimised and the homozygosity status of the putative doubled haploid regenerants needs further evaluation.

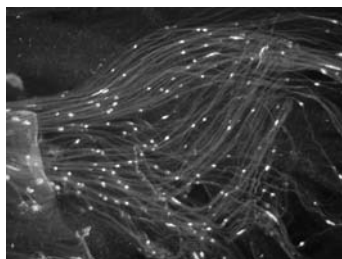


Fig 1 Pollen tubes germinating through the style of a pistil pollinated with irradiated pollen. The pistils were stained with 1 % aniline blue in 0.1N Na₃PO₄ for 1 h and observed under an epifluorescent microscope.



Fig 2 Embryo germinating *in vitro* from ovule culture. Ovules were isolated from pistils pollinated with irradiated pollen.

Acknowledgements

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References

- Bhojwani S.S. and Thomas T.D. (2001) In: Bhojwani S.S. and Soh W.Y. (Eds.) *Current Trends in the Embryology of Angiosperms*. Dordrecht, Kluwer Academic Publishers: 489-507.
- Hess D. and Wagner G. (1974). *Z. Pflanzenphysiol.* Bd. 72: 466-468.
- Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (Eds.) (2003). *Doubled Haploid Production in Crop Plants, A Manual*. Dordrecht, Kluwer Academic Publishers: 428 pp.
- Rangaswamy N.S. and Shivanna K.R. (1967). *Nature* 216: 937-939.

Effect of branch bending on chemical composition of ‘Williams’ pear fruits

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Introduction

Chemical composition of pear fruit, which determines its quality, has been less investigated in comparison with apples or peaches. Differences in compound amounts are not only consequence of species and cultivar; they are also depended on the maturity stage, environmental factors and technological measures applied in an orchard (Hudina and Štampar, 1999). Branch bending is a long established and widely used technological measure to reduce vegetative growth and induce fruiting of fruit trees (Luckwill, 1970). Ito et al. (2004) reported about influence of branch bending on sugar metabolism of lateral buds in Japanese pear. The aim of this study was to examine how bending affects sugars, organic acids and phenolics contents in pear fruits.

Materials and methods

The pear (*Pyrus communis* L.) trees cv ‘Williams’ planted in 1987 were researched. Three different treatments were done: (1) one five-year-old branch per tree was bent at an angle of 60° from the vertical to horizontal position in late summer 2003 or (2) in late spring 2004 or (3) was not bent - control tree. Fruits, which were harvested at commercial maturity, were analysed with high-performance liquid chromatography according to Dolenc and Stampar (1997) and Schieber et al. (2001) with minor modifications. All data were tested by one-way analysis of variance and means separated by Least Significance Difference (LSD) test at a $P < 0.05$.

Results and discussion

Fruits from summer bending branches had the lowest contents of individual sugars (Table 1). The highest contents of sugars, except of glucose, were observed in fruits from spring bending branches. Ito et al. (2004) reported branch reorientation had influence on increase in sorbitol and sucrose content of the central internode in Japanese pear.

Table 1: Average content of sugars (g kg⁻¹ of fresh weight - FW) in ‘Williams’ pears. N.S. – not significant (LSD test at $P < 0.05$).

	Bending treatment					
	Summer 2003		Spring 2004		Control	
Fructose	64.48	a	73.54	a	67.61	ab
Sorbitol	19.29	a	24.59	a	21.34	ab
Glucose	8.46	N.S.	9.01	N.S.	9.42	N.S.
Sucrose	6.25	N.S.	7.94	N.S.	7.28	N.S.

Fruits from the control had the highest content of citric acid, and the lowest malic and shikimic acid contents (Table 2). Fruits from the spring treatment had the lowest fumaric acid content. Malic, shikimic and fumaric acid contents were the highest in fruits from the summer treatment. However, there were no statistically significant differences in organic acids contents among the treatments.

Table 2: Average content of organic acids (g kg⁻¹ or mg kg⁻¹ FW) in ‘Williams’ pears.

	Bending treatment		
	Summer 2003	Spring 2004	Control
Citric acid (g kg ⁻¹)	2.87	2.89	3.05
Malic acid (g kg ⁻¹)	2.24	2.15	2.13
Shikimic acid (mg kg ⁻¹)	71.79	58.14	57.67
Fumaric acid (mg kg ⁻¹)	0.49	0.41	0.44

Fruits from summer bending branches had the lowest contents of all analysed phenolic compounds (Table 3). Contents of catechin, epicatechin, sinapic and syringic acid even significantly differed. The highest concentrations of catechin, epicatechin, syringic and caffeic acid were in fruits from the spring treatment. Chlorogenic, sinapic and vanillic acid had the highest values in the control.

Table 3: Average content of phenolic compounds (mg kg⁻¹ FW) in ‘Williams’ pears. N.S. – not significant (LSD test at $P < 0.05$).

	Bending treatment					
	Summer 2003		Spring 2004		Control	
Chlorogenic acid	280.86	N.S.	318.17	N.S.	357.34	N.S.
Syringic acid	95.46	a	131.32	b	120.66	ab
Epicatechin	46.55	a	83.09	b	69.82	ab
Catechin	25.67	a	44.81	b	36.50	ab
Vanillic acid	1.87	N.S.	2.52	N.S.	3.48	N.S.
Sinapic acid	0.83	a	1.18	ab	1.72	b
Caffeic acid	0.72	N.S.	1.04	N.S.	0.86	N.S.

Due to higher contents of most analysed compounds in fruits from the spring treatment and lower contents from the summer treatment, it is supposed bending altered the metabolism and influenced on fruit contents: spring bent branches saved the assimilates for fruits, but summer bent branches spent assimilates for the other tissues before fruit need them for its development and growth.

References

- Dolenc K. and Stampar F. (1997) An investigation of the application and conditions of analyses of HPLC methods for determining sugars and organic acids in fruits. *Research Reports of the Biotechnical Faculty University of Ljubljana* 69, 99–106.
- Hudina M. and Stampar F. (1999) Influence of water stress and assimilation area on the sugar content and organic acid during the growth period in the pear fruits (*Pyrus communis* L.) cv. ‘Williams’. *Phyton* 39, 107–111.
- Ito A., Yoshioka H., Hayama H. and Kashimura Y. (2004) Reorientation of shoots to the horizontal position influences the sugar metabolism of lateral buds and shoot internodes in Japanese pear (*Pyrus pyrifolia* (Burm.) Nak.). *Journal of Horticultural Science & Biotechnology* 79, 416–422.
- Luckwill L. C. (1970) In: Luckwill L. C. and Cutting C. V. (Ed.) *Physiology of Tree Crop*. Academic Press, London, New York: 237–254.
- Schieber A., Keller P. and Carle R. (2001) Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography A* 910, 265–273.

Production of pathogen-free seeds of some Slovenian common bean (*Phaseolus vulgaris* L.) cultivars by meristem culture

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Introduction

Production of common bean (*Phaseolus vulgaris* L.) cultivars Češnjevce, Jeruzalemski, Jabelski pisanec and Ptujski maslenec decline by years, because of bacterial and virus diseases transmitted by seeds. Meristem culture is a useful tool for elimination of viral pathogens, including seed-borne viral infection in legumes. 40% increase of yield was observed by Benedičič and Berljak (1996) after pathogen elimination from bean cv Zorin. In this study two developed procedures (Benedičič et al., 1997, Veltcheva and Svetleva, 2005) were used for pathogen-free seed production in above mentioned common bean cultivars.

Materials and methods

Dry seeds of four Slovenian cultivars of common bean (*Phaseolus vulgaris* L.), bush bean cv. Češnjevce, and climbing string beans cv. Jeruzalemski, cv. Jabelski pisanec and cv. Ptujski maslenec were obtained from company "Semenarna Ljubljana d.d.", Ljubljana, Slovenia. Seeds were dry heat-treated at 50°C for 3 days, than surface sterilized by submergence in a 70% ethanol for 20 seconds followed by immersion in 1% solution of sodium hypochlorite with a few drops of detergent "Tween 20" for 10 minutes, and finally rinsed three times with sterile distilled water. Only seeds with firm and glossy seed coat were selected for experiments and germination on water agar at room temperature. Sterile excised apical and lateral meristems of 6 to 10 days old seedlings were inoculated on the initial media. First initial medium consisted of MS salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg et al., 1968) supplemented with 1 μM 2iP. Differentiated meristems were subcultured according to procedure developed by Benedičič et al. (1997). Second initial medium was MS medium supplemented with 5 μM BA and developed meristems were further transferred according to procedure by Veltcheva and Svetleva (2005). Cultures were maintained in a growth chamber at 24°C with a 16/8 hrs light/dark photoperiod. Regenerated plantlets with well developed roots were acclimatized and transferred to glass house/screen house where they produced seeds.

Plants were tested on four important bean viruses (BCMV, BYMV, BCMNV, CMV) before flowering, and infected plants were removed out. Dry seeds from virus-free plants have been sowed to screen house for production of virus-free seeds.

Results and discussion

After surface sterilization approximately 30% of seeds of all four cultivars were suitable for experiments. Seedlings germinated on water-agar did not show any symptoms of bacterial infection. Excised meristems inoculated on both initial media, with 2iP and with BA, differentiated in green buds. Vitrification was occurred in some explants inoculated on medium supplemented with BA.

Comparison of two developed procedures for plant regeneration from meristem tissue of common bean it was evident that there are differences in meristem differentiation, shoot growth and plant regeneration requirements between used bean cultivars. Procedures describes by Benedičič et al. (1997) where cytokinin 2iP play the key role in meristem differentiation, and in combination with 0.1 μM NAA and 0.1 μM GA₃ enhanced bud elongation and shoot growth, was useful for regeneration of mature plants in cv. Češnjevce. Hormone BA had better effect on meristem differentiation and plant regeneration in three other bean cultivars Jeruzalemski, Jabelski pisanec and Ptujski maslenec. Shoot elongation was initiated by higher concentration of BA (22.2 μM) in combination with a low concentration of IAA (0.057 μM) during 3 weeks of culture. Plant regeneration with simultaneous leaf and

root development was obtained during four weeks on medium with lower concentration of BA (4.44 μM) supplemented with 0.58 μM GA₃.

We could not obtain root induction in shoots of cv. Jeruzalemski, cv. Jabelski pisanec and cv. Ptujski maslenec grown on half concentrated MS medium as was described in protocol of Benedičič et al. (1997). It was found that full-strength MS medium may be optimum for common bean plant regeneration. The same opinion described Eissa Ahmed et al. (2002). Shoots with roots were cultured for two weeks on MS medium without hormones for root elongation and hardening. Only plantlets with well developed roots were selected for in vivo acclimatization. After two weeks plants were transferred to insect protected glasshouse. Plants originated from meristem tissue have been developed to normal mature plants which produced normal seeds. Plants were tested on viruses.

Four important bean viruses were not confirmed in 50% of regenerated plants grown in screen house. In plants germinated from their seeds, virus infection was confirmed in 16 % of plants.

Conclusion

Production of pathogen-free seeds of common bean is possible by using plant tissue culture techniques, particularly meristem culture, until today. But, because common bean is recalcitrant species, each step of meristem development and plant regeneration should be carefully determined. Two procedures used for plant regeneration from common bean meristem in this work are welcome basis for searching of optimal procedures for plant regeneration in particular bean genotypes.

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References

- Benedičič D. and Berljak J. (1996) in: Šesek P (Ed.) *Novi izzivi v poljedelstvu '96: zbornik simpozija, Radenci*. 173-176.
- Benedičič D., Ravnika, M. and Gogala N. (1997) *Phyton* (Horn, Austria), 37 (1), 151-160.
- Eissa Ahmed E., Bisztray GY. D. and Velich I. (2002) *Acta Biologica Szegediensis*, 46(3-4), 27-28.
- Gamborg O. L., Miller R. A. and Ojima K. (1968) *Exp. Cell. Res.*, 50, 151-158.
- Grum M., Camloh M., Rudolph K. and Ravnika M. (1998) *Plant Cell, Tiss. Org. Cult.*, 52, 79-82.
- Murashige T. and Skoog F. (1962) *Physiologia Plantarum*, 15, 473-497.
- Veltcheva M. R. and Svetleva D. L. (2005) *Journal of Central European Agriculture*, 6 (1), 53-58.

Effect of the culture media on meristem differentiation, shoot and root development in grapevine

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Introduction

The grapevine is susceptible to numerous diseases which reduce productivity and quality of grapes. In vitro technology, particularly meristem culture is useful tools for production of virus free grapevine plants. Cultivars 'Modra frankinja' (red grape) and 'Šipon' (white grape) are very popular grapes in Slovenia, and it is very important to have healthy planting material for optimal grape production. The purpose of present work was to definite culture medium for meristem differentiation, shoot and root development in two grapevine (*Vitis vinifera* L.) cultivars 'Modra frankinja' and 'Šipon'. Results here presented are from different experiments.

Materials and methods

The two Slovenian cultivars of grapevine *Vitis vinifera* L., 'Modra Frankinja' and 'Šipon' were used in this work. The shoot tips were collected from 7 years old vine Modra frankinja from Gadova Peč, Dolenjska, and from 11 years old vine Šipon from Jeruzalem, Ormož vineyards. Leaves were removed before surface sterilization by dipping for 20 seconds in 70% ethanol and soaking for 10 minutes in solution of 1% Izosan G (hypochlorite) with a few drops of detergent Tween 20, then rinsed three times with sterile distilled water. Aseptically excised meristems with two primordias were inoculated on MS medium supplemented with different concentrations of cytokinin and/or auxin. Cultures were maintained under white fluorescent light with photoperiode 16 hrs light/8 hrs dark at temperature +24°C. Explants were regularly transferred on fresh medium every tree weeks. Well developed plantlets were rooted on medium supplemented with auxin IAA or IBA, acclimatized and transferred to the greenhouse.

Results and discussion

Meristems were excised from shoot tips of mature vines before panicle emergence, and inoculated on initial medium supplemented with cytokinin only or in combination with auxin (Table 1.). Meristems of cultivar 'Modra frankinja' inoculated on medium with 2 mg/l BA only had very low ability for differentiation (8%) while meristems of cultivar 'Šipon' differentiated in higher percentage (34%). Decreasing of BA concentration to 1.5 mg/l and addition of a low level of IAA (0.2 mg/l) stimulated differentiation in all inoculated meristems of cultivar 'Modra frankinja' (100%) and also increased a number of differentiated meristems in cultivar 'Šipon' (54%). After four weeks of culture differentiated meristems were transferred to MS medium with cytokinin for further development and elongation. Cytokinin level influenced on shoot development in both grape cultivar similarly (Table 2.). High percentage (96%) of developed shoots in cv. 'Modra frankinja' and 81% in cv. 'Šipon', were obtained on medium supplemented with 0.5 mg/l BA, while on medium with 1 mg/l BA in cv. 'Modra frankinja' only 24% of shoots developed, and in cv. 'Šipon' 53%. Well developed shoots were transferred on MS medium supplemented with auxins in order to root regeneration (Table 3.). Shoots subcultured on half concentration of MS salts medium supplemented with 0.2 mg/l IAA regenerated roots in very low percentage (8%) in cv. 'Šipon' and 31% in cv. 'Modra frankinja'. Better effect on rooting was obtained on full concentration of MS salts medium supplemented with 0.5 mg/l IBA in shoots of cv. 'Modra frankinja' (80%) and also in shoots of cv. 'Šipon' (78%). Results of different experiments showed differences in ability for meristem differentiation, shoot and root development in grape cultivars 'Modra frankinja' and 'Šipon'.

Table 1: Influence of initial medium on meristem differentiation; a - medium MS supplemented with 2 mg/l BA; b - medium MS supplemented with 1.5 mg/l BA and 0.2 mg/l IAA.

Cultivar	N° of inoculated meristems		N° of differentiated meristems		% of differentiated meristems	
	a	b	a	b	a	b
'Modra frankinja'	50	51	4	51	8	100
'Šipon'	130	65	44	35	34	54
Total	180	116	48	86	21	77

Table 2: Influence of cytokinin (BA) on shoot development; a - medium MS supplemented with 0.5 mg/l BA; b - medium MS supplemented with 1mg/l B.

Cultivar	N° of shoots in culture		N° of developed shoots		% of developed shoots	
	a	b	a	b	a	b
'Modra frankinja'	190	297	183	71	96	24
'Šipon'	90	256	73	135	81	53
Total	280	553	256	206	88.5	38.5

Table 3: Influence of auxins (IBA and IAA) on root induction; a - medium ½ MS supplemented with 0.2 mg/l IAA; b - medium MS supplemented with 0.5 mg/l IBA.

Cultivar	N° of shoots		N° of rooted shoots		% of rooted shoots	
	a	b	a	b	a	b
'Modra frankinja'	71	172	22	139	31	80
'Šipon'	135	45	11	35	8	78
Total	206	217	33	172	19.5	79

Conclusion

Application of meristem culture is very useful for production of virus-free grapevine planting material. Because this method is also very genotype depended, it is necessary to optimize contents of medium salts, vitamins, concentration and combination of growth regulators for meristem differentiation, shoot and root development.

Microbial contamination in tissue cultures of pyrethrum

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Introduction

Pyrethrum *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip. Asteraceae is a source of natural insecticides pyrethrins, an environmentally aware and efficient option of insect control and therefore interesting for in vitro biotechnological production (Hitmi et al., 2000). In plant cell and tissue cultures microbial contamination is one of serious problems. Of special importance are contaminations that are commonly not visible but influence tissue culture quality, as well as development of effective methods for contaminants elimination from established plant tissue and cell cultures (Herman, 2004). In callus cultures of pyrethrum microbial contaminants were observed routinely

(Figure 1) despite rigorous sterilization of plant tissues prior the establishment of tissue cultures.

The objective of our study was investigation of different isolation and cultivation procedures for microorganism in callus tissues and pyrethrum shoots and an introduction of a system for detection of contamination using classical and molecular techniques. In order to maintain aseptic cultures, the effect of antibiotic treatment on callus growth and microbial elimination was already investigated (Bergant et al., 2005).

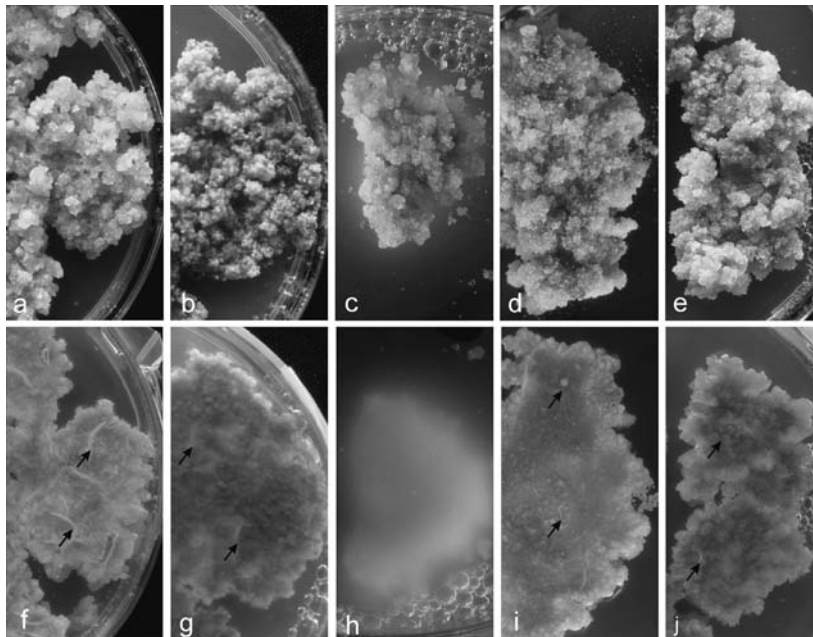


Figure 1: Different callus lines 3 weeks after subculture on the medium, upper side (a, b, c, d, e) and lower side (f, g, h, i, j); arrows indicate clearly visible contamination. Bar: 2 mm.

Materials and methods

Plant material, isolation and characterization of bacteria: Flower heads of pyrethrum *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip. originated from the Botanical Garden of Ljubljana, Slovenia and from Cres, Croatia, were used for tissue culture initiation. Callus cultures were maintained as previously described (Bergant et al., 2005). Microbial strains from callus lines were isolated and incubated following different procedures. DNA from pure cultures (Wozniak, 1997) and from calluses (Webster and Barker, 1994)

was isolated and purified as described. The 16S rDNA genes of both isolates were amplified with eubacterial primers 27f and 1495r and cut with *MnII* and *RsaI* (Bianciotto et al., 1996). Restriction fragment patterns of *MnII* and *RsaI* RFLPs of both 16S rDNA were compared.

Results and discussion

Microbial contamination in all callus lines became visible after few subcultures starting with callus initiation (Figure 1). Despite visible contamination, the growth of callus was not disturbed. The contamination appeared after stabilization of callus growth commonly seen as a white clouding or veils and was restricted strictly to the callus and the medium around it (Figure 1). Because media for plant tissue culture are not optimal for bacterial growth, they can remain undetected over several subcultures of shoot propagation or roots of *in vitro* plantlets (Keskitalo, 1998). In our study pyrethrum contamination could be observed in callus culture only and not in shoot culture. With different isolation procedures 21 strains of microorganisms from 6 out of 34 callus lines were isolated and cultured. None of them was isolated from more than two callus lines, indicating that they are not predominant population in callus cultures.

The presence of microorganisms was detected by direct amplification of eubacterial 16S rDNA gene (Bianciotto et al., 1996) in callus and pyrethrum plants *in vitro* and *in vivo*. Analysis of 16S rDNA restriction fragments determined the presence of similar bacterial groups in calluses and pyrethrum steams which differed from isolated bacteria (data not shown). Since it is known that with primers of Bianciotto et al. (1996) plant chloroplast DNA can sometimes be amplified (Dent et al., 2004, Cankar et al., 2005) it is necessary to clarify the origin of the amplified products in further studies.

In conclusion, this data indicate that microorganisms, which were observed in pyrethrum callus culture, were not cultivable with classical microbiological methods tested in our experiments. Although molecular methods are rapid and sensitive for detection of microbial contaminants the possible amplification of related plant-derived targets using PCR amplification of 16S rDNA has to be considered.

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References

- Bergant M., Ambrožič-Dolinšek J., Demšar T., Dreo T., Ravnikar M., Žel J., Camloh M. (2005) Effect of Antibiotic on Contaminated Callus Cultures of Pyrethrum. *Phyton* (Horn, Austria), 45 (1), 197-206.
- Bianciotto V., Bandi C., Minerdi D., Sironi M., Tichy H.V., Bonfante P. (1996) An obligately mycorrhizal fungus itself harbors obligately intracellular bacteria. *Applied and Environmental Microbiology*, 62(8), 3005-3010.
- Cankar K., Kraigher H., Ravnikar M., Rupnik M. (2005) Bacterial endophytes from seed of Norway spruce (*Picea abies* L. Karst), *FEMS Microbiology letters* 244, 341-345.
- Dent K.C., Stephen J.R., Finch-Savage W.E. (2004) Molecular profiling of microbial communities associated with seed of *Beta vulgaris* subsp. *Vulgaris* (sugar beet) *Journal of Microbiological Methods* 56, 17-26.
- Herman E.B. (2004) Recent Advances in Plant Tissue Culture VIII Microbial "Contaminants" in Plant Tissue Cultures: Solutions and Opportunities 1996-2003. Agritech Consultants, inc., Shrub Oak.
- Hitmi A., Coudret A., Barthomeuf C. (2000) The Production of pyrethrins by plant cell and tissue cultures of *Chrysanthemum cinerariaefolium* and *Tagetes* species. *Critical Reviews on Biochemistry and Molecular Biology*, 35 (5), 317-337.
- Keskitalo M.K., Pohto A., Savela M.L., Valkonen J.P.T., Simon J, Pehu E. (1998) Alteration in growth of tissue-cultured tansy (*Tanacetum vulgare* L.) treated with antibiotics. *Annals of Applied Biology*, 133: 281-294.
- Webster K.D., Barker H. (1994) Molecular biological techniques. Dundee, Scottish Crop Research Institute. 23-26.
- Wozniak G. (1997) Molecular methods for typing of *Clostridium difficile* strains. Graduation thesis, Ljubljana, University of Ljubljana, Biotechnical faculty: 12 pp.

Genetically modified oilseed rape - problem for coexistence and detection?

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Introduction

Oilseed rape (*Brassica napus* L.) belonging to the genus *Brassica* is a member of the Brassicaceae family. *Brassica* contains more than 100 species including *Brassica napus* and *Brassica rapa* L., important for production of oil as well as for tubers. In Europe, *Brassica napus* is sown mainly in autumn. Common used cultivars refer to open pollinating lines, different types of hybrids, and composite material. Oilseed rape (n=19) is an amphidiploid derivative of *B. oleracea* (n=9) and *B. rapa* (n=10). Crop is predominantly autogamous with different percentages of intraspecific cross-fertilization. Gene flow from crop to crop and to related weeds varies enormously depending on cultivar, size and design of the donor field as well as the acceptor field, distance between donor and acceptor, flowering period, pollen concentration in the air above rape field, availability of insect pollinators, wind mediated pollen dispersal and pollen viability (Eastham & Sweet, 2002; Förster & Diepenbrock, 2002). Moreover, oilseed rape has weedy traits which enable this crop to spread its genes independently in the agro-ecosystem.

That makes oilseed rape a rather problematic crop for the co-existence of genetically modified (GM) and non modified (non-GM) crops. To minimise gene escape in time and to enable co-existence it is important to be familiar with the biology of oilseed rape, and to adapt measures such as appropriate distance between GM and non-GM crops, weeds and volunteers control, suitable cropping and harvesting techniques and crop rotations. The presence of GM seeds in seed lots is also important source of adventitious presence of GM material in crops.

To control and evaluate measures and to monitor for adventitious presence of GM oilseed rape in non-GM one effective and reliable detection methods are necessary. As there are very few GM oilseed rape event specific methods currently available and because of high cost of detection of every transgenic line, methods based on screening elements are the methods of choice for detection of GM oilseed. Therefore screening system based on three screening elements was introduced and methods validated.

Materials and methods

RT73 reference material from AOCS (seeds) was homogenised using coffee grinder and liquid nitrogen. DNA from Ms8 x Rf3 reference material (flour) and homogenised RT73 reference material was isolated using NucleoSpin Food (Macherey Nagel). DNA of Topas 19/2 was obtained from Bayer CropScience as reference material. qPCR using Universal mastermix (Applied Biosystems) was performed on ABI 7900 HT instrument.

Results and discussion

Currently 6 lines are authorised in European Union (Table 1, http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). Since methods for detection by conventional PCR are time consuming and less sensitive, we implemented a system for screening with qPCR. Different screening systems are possible. Promotor, terminator, gene or construct specific sequences can be detected, but the choice should be made with the respect to the highest transgenic line coverage possible with the least number of analysis.

Table 1: Transgenic lines of rapeseed with inserted traits and introduced genetic elements

Transgenic line	Company	Trait	Gen	Promotor	Terminator	Detection
RT73	Monsanto	Herbicide tolerance	EPSPS	FMV 35S	pea rbcS E9	RT73
		Herbicide resistance	<i>goxv247</i>	FMV 35S	pea rbcS E9	
MS8xRF3	Bayer CropScience	Herbicide tolerance	<i>bar</i>	PSsuAra (<i>A. thaliana</i>)	Nos (A.t.)	NOS
		Male sterility	<i>barnase</i>	pTa 29 (Nicotiana tabacum)		
		Fertility restoration	<i>barstar</i>	pTa 29 (Nicotiana tabacum)		
MS1 x RF1	Bayer CropScience	Herbicide tolerance	<i>bar</i>	PSsuAra (<i>A. thaliana</i>)	Nos (A.t.)	NOS
		Male sterility	<i>barnase</i>	pTa 29 (Nicotiana tabacum)		
		Fertility restoration	<i>barstar</i>	pTa 29 (Nicotiana tabacum)		
MS1 x RF2	Bayer CropScience	Herbicide tolerance	<i>bar</i>	PSsuAra (<i>A. thaliana</i>)	Nos (A.t.)	NOS
		Male sterility	<i>barnase</i>	pTa 29 (Nicotiana tabacum)		
		Fertility restoration	<i>barstar</i>	pTa 29 (Nicotiana tabacum)		
TOPAS 19/2	Bayer CropScience	Herbicide tolerance	<i>pat</i>	CaMV 35S	CaMV 35S	35S
		Antibiotic resistance	<i>neo</i>	NOS (A.t.)	OCS (A.t.)	
T45	Bayer CropScience	Herbicide tolerance	<i>pat</i>	CaMV 35S	CaMV 35S	35S

For endogenous gene (necessary to confirm the successful isolation of DNA of good quantity and quality from the sample) we used gene for phosphoenolpyruvate carboxylase (PEP) (Zeitler et al, 2002), which can be also used for quantitative analysis. For detection the transgenic elements, three amplicons were used: the detection of 35S CaMV promoter (Pauli et al, 2001), detection of NOS terminator from *Agrobacterium tumefaciens* (Kuribara et al, 2002) and the sequence from the plant-insert border, which is specific for RT73 transgenic line (Monsanto, 2004).

We tested our screening system on Ms8 x Rf3 line for the detection of NOS terminator, on Topas 19/2 line for the detection of 35S promoter and on reference material for RT73 line for specific detection of RT73 line. We successfully detected oilseed rape lines in all three cases. Methods were validated and have high sensitivity and repeatability, which makes them suitable for routine detection. Additionally, using the assay for specific detection of RT73 line, quantification of this oilseed rape line in products was implemented. Reference materials for other GM oilseed rape lines are not available. However, since almost all transgenic lines with same promoter or terminator originate from the same developer we expect that our system would also detect other transgenic lines from same producer.

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References

- Eastham, K., J. Sweet (2002). Genetically modified organisms (GMOs): The significance of gene flow through pollen transfer. EEA.
- Förster, K., W. Diepenbrock (2002). Use of genetically modified plants - consequences for crop production. In: Schwellenwerte für Produkte aus gentechnisch veränderten Pflanzen. DFG Mitt. 7, 50-58.
- Kuribara H., Shindo Y., Matsouka T., Takubo K., Futo S., Aoki N., Hirao T., Akiyama H., Goda Y., Toyoda M., Hino A. (2002). Journal of AOAC international, 85 (5), 1077 – 1089.
- Pauli U., Liniger M., Schrott M., Schouwey B., Hübner P., Brodmann P., Eugster A. (2001). Mitt. Lebensm. Hyg. 92: 145-158.
- Zeitler R., Pietsch K., Waiblinger H.U. (2002). European Food Research and Technology 214 (4), 346 – 351.
- A recommended Procedure for Real-Time Quantitative TaqMan® PCR for Roundup Ready® Canola RT73; Monsanto Biotechnology Regulatory Sciences. <http://gmo-crl.jrc.it/detectionmethods/MON-Art47-pcrGT73rapeseed.pdf>.
- http://ec.europa.eu/food/dyna/gm_register/index_en.cfm.

Screening for gynogenic ability of *Impatiens* New Guinea

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Introduction

Impatiens New Guinea is a popular ornamental plant species since its introduction in early 80s. The original plants were collected in New Guinea, but the once we know today have been produced through inter or intra-specific hybridization with additional species from Java and the Celebes islands. The aim of our research was to screen for gynogenic ability of *Impatiens* New Guinea and to establish an efficient method for haploid induction, a promise of producing improved varieties with a reduced cost.

Material and methods

Three genotypes were evaluated and four approaches were used to induce gynogenesis: (1) *in vitro* culture of un-pollinated female reproductive organs, (2) *in vitro* culture of un-pollinated female reproductive organs stimulated by pollen, (3) embryo rescue from pollinated flowers treated with different inducers (2,4-D, TDZ, spermidine and putrescine mixture) one day after pollination and (4) embryo rescue from flowers pollinated with irradiated pollen. In all experiments, isolations of female organs were carried out under a stereomicroscope. Aseptic cultures were established with 15 min agitation of plant material in 2% dichloro-isocyanuric acid with few drops of Tween 20 and supplementing all plant media with antibiotics. In first three approaches, different types of female reproductive organs were cultured on 31 induction and 5 differentiation media composed of BDS, NLN, Nitsch & Nitsch, full and half strength MS or B5 basal media. Different plant hormones (BAP, TDZ, 2,4-D, NAA, IAA, zeatine) and sugars (sucrose, maltose and glucose) at various concentrations were supplemented to the basal media, also heat and cold-shock pre-treatment were used to induce gynogenic pathway. To establish a method for embryo rescue from flowers pollinated with γ -irradiated pollen (600 Gy) different periods of ovule excision (6 to 28 days after pollination) were tested. Ovules were placed on two basal media, MS or Nitsch & Nitsch supplemented with plant hormones (kinetin, zeatin or IAA) and incubated at 23°C in the dark or light (8/16h photoperiod).

Results

In vitro culture of 92 ovaries, 785 ovules and 12,843 placenta-attached ovules isolated from un-pollinated flowers and set on modified MS medium resulted in enlarged ovaries, ovules and callus formation on placenta. Adding pollen mixture to 1,248 ovules grown on the gynogenic induction medium did not have stimulating effect. Spraying flowers with 2,4-D (10 mg/l) or TDZ (10 mg/l) solution resulted in an evident ovary size increase, where the application of 0.5 mM spermidine and 10 mM putrescine mixture had no effect. Finally, 1,636 flowers were pollination with γ -irradiated pollen (Fig. 1), followed by *in vitro* embryo maturation and plant regeneration. Best results were obtained when excision of ovules was performed 9 days after pollination, followed by ovule culture on MS medium supplemented with 0,1 mg/l kinetin and 20 g/l sucrose (Fig. 2). Twenty-three embryos have developed following this procedure. Thirteen plantlets analyzed by flow cytometry, were diploid.

Table 1: Summary of four different approaches used for gynogenic haploid induction

Approach	No. of cultured female reproductive organs	Tissue culture results
1 st	12,945 flowers	Enlarged ovaries, ovules and callus formation on placenta
2 nd	1,248 ovules	No stimulating effect
3 rd	1,162 ovules	Enlarged ovules when 2,4-D or TDZ were tested, no effect when spermidine and putrescine mixture was used (Fig. 1)
4 th	1,636 flowers	23 embryos, 13 tested are diploid (Fig. 2)

Conclusions

Embryo rescue from flowers pollinated with γ -irradiated pollen (600Gy) proved to be the most promising approach toward haploid induction in *Impatiens New Guinea*. Further ploidy and homozygosity analysis of regenerants are needed for final evaluation. Additionally, wide hybridisation experiments will be tried as a possible stimulant of gynogenic pathway, which might lead to the production of putative haploid embryos of *New Guinea Impatiens*.

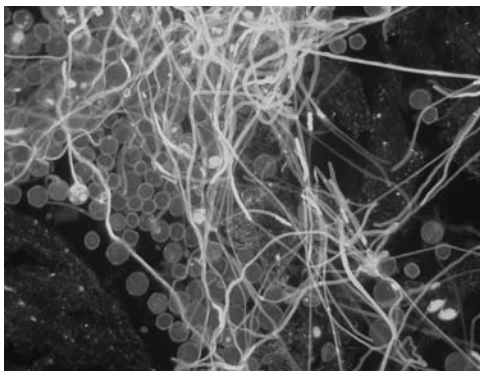


Fig. 1: Pollen tubes germinating through the style of a pistil pollinated with irradiated pollen. The pistils were stained with 1% aniline blue in 0,1N Na₃PO₄ for 1 h and observed under an epifluorescent microscope.



Fig.2: Putative haploid regenerants obtained from flowers pollinated with irradiated pollen

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Reference

- Bhojwani S.S. and Thomas T.D. (2001). In: Bhojwani S.S. and Soh W.Y. (Eds.). *Current trends in the Embryology of angiosperms, In vitro gynogenesis*. Dordrecht, Kluwer Academic Publishers: 489-507.
- Bohanec B., Jakše M., Ihan A. and Javornik B. (1995). *Plant Science* 104: 215-224.
- Gürel S.E. and Kaya Z. (2000). *Plant Cell Reports* 19: 151-159.
- Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. (2003). *Doubled Haploid Production in Crop Plants, A Manual*. Dordrecht, Kluwer Academic Publishers: 428 pp.

Sequence variation of the S-locus receptor kinase in inbreeding lines of cabbage (*Brassica oleracea* var. *capitata* L.)

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Introduction

To prevent inbreeding, self-incompatibility systems have evolved in many flowering plants. In the *Brassicaceae*, self-incompatibility is genetically controlled by a single polymorphic locus, termed the S-locus. Pollen rejection occurs when stigma and pollen share the same haplotype (S-haplotype). Molecular analyses revealed that the S-locus consists of at least two linked genes; both are expressed in stigma papillae (Boyes et al., 1993). One of the genes is the S-locus glycoprotein gene (SLG), which encodes a secreted glycoprotein (Nasrallah et al., 1987), and the other is the S-locus receptor kinase gene (SRK). S-haplotypes can be classified into two groups: class I and class II, where dominant S-haplotypes belong to class I and recessive S-haplotypes belong to class II (Nasrallah and Nasrallah, 1993). The aim of our study was to classify and identify various S-haplotypes of cabbage inbred lines with rapid and cost effective method.

Materials and methods

In this study we included twenty cabbage inbred lines (doublehaploids) produced via microspore culture from cross-breeds between local cultivar Varaždinsko and English hybrid cultivar Hawke F1. Genomic DNA was isolated from leaves using CTAB method. SRK was amplified using either class I specific primers (figure 1): KD5 and KD8 either class II specific primers (figure 1): KD4 and KD7 (Park et.al 2002). Polymerase chain reactions (PCR) were carried out in 20 µL volumes. Each reaction contained 10 pmol of each primer, 0.2 mM dNTP, 2 mM MgCl₂, 1x PCR buffer, 1 U of Biotools DNA polymerase and 50-100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by denaturation at 94 °C (1 min), annealing at 61 °C (KD4 and KD7) or 63 °C (KD5 and KD8) (2 min) and extension at 72 °C (1 min) repeated for 35 cycles.

Amplified DNA fragments were run on a 1.5% agarose gel and were isolated from the gel using Gel Extraction Spin Kit (Genomed). Approximately 100 ng of purified PCR product was used in cycle sequencing reactions following ABI PRISM BigDye Terminator protocols. Sequences were visualized on an ABI Prism 310 (Applied Biosystems) and aligned using the computer program BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

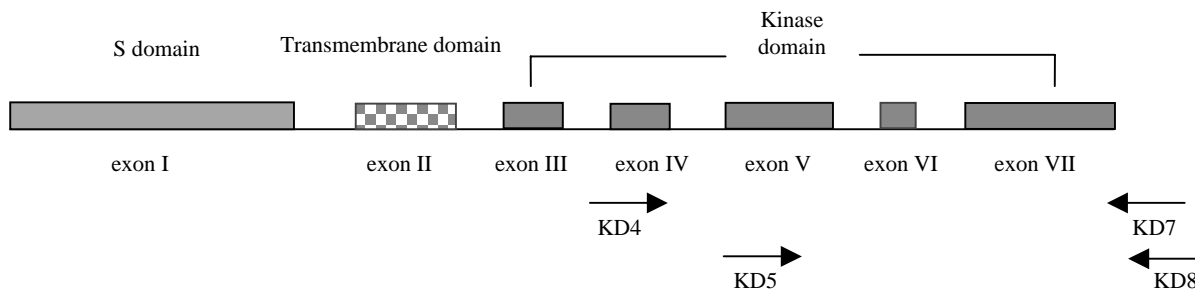


Figure 1: Primer sites for class I and class II specific amplification of SRK

Results and discussion

About 900 bp PCR products were generated using class I SRK specific primers KD5 and KD8 for eighteen cabbage inbred lines; for the remaining two lines about 1000 bp PCR products were generated using class II SRK specific primers KD4 and KD7. Therefore the majority of inbred lines belong to class I (Table 1).

Sequence alignment revealed five different S-haplotypes; four haplotypes (I, II, III and IV) within the class I, and the single haplotype (V) in the class II. A BLAST search showed some very similar sequences to our sequences (Table 1).

Table 1: S-haplotypes in twenty inbred cabbage lines analysed in this study

Class	S-haplotype (Group)	Cabbage line	Similarity to previously reported sequences (%)
Class I	I	278,546,502,556,578,712	96% (BoSRK-28; Acc.No. AB190355)
	II	564,365,924	92% (srk29; Acc.No. Z30211)
	III	490,667	97% (SRK46Bra; Acc.No. ABO13718)
	IV	929,608,917,547,706,441,207	90% (S30; Acc.No. AY534542)
Class II	V	885,70	94% (SRK-60; Acc.No. AB097116)

Sequence similarity among the four class I S-haplotypes was in the range from 77.6 (II and III) to 94.8% (I and III), while the similarity between class I and class II S-haplotypes was only about 36% (Table 2). Expectably, the majority of the variability was found in the intron regions; the sequences of exons were much more conserved at the DNA and the protein level.

Table 2: Similarity (%) between five S-haplotypes (I-V)

S-haplotype	I	II	III	IV	V
I	100	78.4	94.8	78.5	36.8
II		100	77.6	88.0	36.7
III			100	78.7	36.1
IV				100	35.6
V					100

Our results are in the agreement with the findings that the majority of S-haplotypes of Brassica oleracea belong to class I, and only some haplotypes belong to class II (Okazaki et al., 1999). Class II S-haplotypes express a low degree of self-incompatibility (Nasrallah et al., 1991) and they are recessive to pollen of the class I S-haplotypes. Therefore, parents with weak incompatibility system need to be excluded from the F1 hybrid production to avoid high frequency of selfed seeds.

References

- Boyes D.C., Chen C.H., Tantikanjana T., Esch J.J., Nasrallah J.B. (1993). *Mol. Genet.* 236, 369-373.
- Nasrallah J.B., Kao T.H., Chen C.H., Goldberg M.L., Nasrallah M.E. (1987). *Nature* 326, 617-619.
- Nasrallah J.B. and Nasrallah M.E. (1993). *Plant Cell* 5, 1325- 1335.
- Nasrallah J.B., Nishio T., Nasrallah M.E. (1991). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42, 393-422
- Okazaki K., Kusaba M., Ockendon D.J., Nishio T. (1999). *Theor. Appl. Genet.* 98, 1329-1334.
- Park J.I., Lee S.S., Watanabe M., Takahata Y., Nou I.S. (2002). *Plant Breeding* 121, 192-197.

Compatibility relationships within and between olive (*Olea europaea* L.) cultivars

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Introduction

The olive (*Olea europaea* L.) is one of the most important fruit species in the Adriatic part of Croatia. Growing region spreads from Oštro cape in the south to the river Mirna valley in the north of the country. In the old olive groves autochthonous cultivars are planted. Those orchards are mainly monovarietal and record low yield. 'Oblica' is the most widely planted autochthonous cultivar and accounts 80% of total production (Perica and Kovačević, 1994). Other valuable autochthonous cultivars are 'Drobnica', 'Lastovka', 'Levantinka' and 'Istarska bjelica'. In newly planted olive groves beside mentioned cultivars introduced ones are presented ('Frantoio', 'Leccino', 'Pendolino' etc.). Olive is a wind-pollinated species. The level of self-compatibility varies in different cultivars (Androulakis and Loupassaki, 1990). Thus, the orchard productivity depends upon combination of planted cultivars. Self-incompatibility often leads to the reduction of fruit set. The problem of self-incompatibility is mostly expressed in isolated old olive groves where single cultivar is planted. Simultaneous flowering period enables cross-pollination, fertilization and fruit set of different cultivars if compatibility between recipient and polleniser trees exists. There is a lack of knowledge about self- and cross-fertility of autochthonous and especially introduced cultivars under Dalmatian environmental conditions. The object of the research was to determine the duration of flowering period, self- and cross-compatibility within and between major autochthonous and introduced cultivars in conditions of Middle Dalmatia.

Materials and methods

The experiment was conducted in the Split area of Middle Dalmatia (Latitude: 43°52' N, Longitude: 16°51' E) during the 2005 flowering season. Twenty years old orchard received regular fertilization and summer irrigation. Four autochthonous cultivars 'Drobnica', 'Lastovka', 'Levantinka', 'Oblica', and two introduced cultivars 'Leccino' and 'Pendolino' were included in the study. The data about flowering period were obtained by visual examination of trees every three days according to Barranco et al. (1994). The duration of full bloom period was noticed. Three olive trees of uniform size per each cultivar were selected to test self-pollination, cross-pollination and free-pollination. 'Drobnica' and 'Pendolino' were observed only in the role of polleniser while the others were used as pollen acceptors as well. Four uniform shoots per tree, with twenty inflorescence each, were selected for pollination with each polleniser, for self-pollination and open-pollination. The shoots aimed for cross-pollination and self-pollination were bagged before anthesis until loss of stigma receptivity while those for free-pollination were marked but not isolated (Cuevas and Polito, 1997). Flowers within the bags were not emasculated to accomplish field conditions. Paper bags were used for isolation of the branches. The pollination was conducted during the full bloom. Cross-pollination was carried out by opening the bags, applying the appropriate pollen and enclosing the bags. Isolated branches aimed for self-pollination stayed enclosed to the end of anthesis. Final fruit set was determined 45 days after full bloom. Data for percentage of fruit set were arcsine transformed and then subjected to analysis of variance using proc GLM of the SAS-software. Mean separations were done by the LSD test at $P \leq 0.05$.

Results and discussion

The flowering period of studied cultivars lasted from May 24 to May 31. The beginning of anthesis was firstly noticed at cultivars 'Lastovka' and 'Levantinka' (May 24), and one day later at 'Drobnica' and 'Oblica'. The latest anthesis was noticed at 'Leccino' and 'Pendolino' (May 26). The full bloom of studied cultivars started three days after the beginning of the flowering and lasted two days. The significance of all pollination combinations showed that the final fruit set is influenced by efficiency in pollination of selected polleniser (Table 1).

Table 1. Final fruit set (%) of studied cultivars following self-pollination, cross-pollination and free-pollination

Pollen source	Final fruit set (%)			
	Lastovka	Leccino	Levantinka	Oblica
Lastovka	0.95 c*	5.07 bc	6.91 a	3.96 a
Leccino	4.57 b	1.38 d	3.08 bc	3.34 a
Levantinka	7.15 a	4.58 bc	2.16 c	2.56 a
Oblica	2.39 c	7.18 a	2.34 bc	1.11b
Drobnica	4.69 b	5.97 ab	2.99 bc	3.41 a
Pendolino	2.65 c	5.89 ab	3.62 abc	3.17 a
Free-pollination	3.09 b	2.43 cd	6.54 ab	2.86 a

*Different letters within column indicate significant differences at $P \leq 0.05$ by LSD test.

At 'Lastovka' the highest percentage of fruit set (7.15%) was noticed when 'Levantinka' was used as pollen source, while 'Lastovka' was the best polleniser (fruit set 6.91%) for 'Levantinka'. There was no significant difference in pollination efficiency of 'Oblica' by 'Drobnica', 'Lastovka', 'Leccino', 'Levantinka' and 'Pendolino'. In our study 'Leccino' has shown a high fruit set when it was pollinated with 'Drobnica', 'Oblica' and 'Pendolino'. Several clones of 'Leccino', one of the most widespread cultivar in Italy were described as self-compatible, even if this ability may vary according to year (Bartolini et al., 2002). In our study fruit set in self-pollination ranged from 0.95% ('Lastovka') to 2.16% ('Levantinka'). Compared to other tested cultivars 'Levantinka' was described as self-compatible (Vlašić, 1980).

Cross-pollination increases fruit set. 'Lastovka' and 'Levantinka', 'Drobnica' and 'Oblica', 'Leccino' and 'Pendolino' are compatible cultivars with simultaneous flowering period that ensure their successful cross-pollination. Such cultivars are recommended to be planted together in the orchards. Lower final fruit set in self-pollination was recorded at all cultivars comparing to almost all cross-pollination combination.

References

- Androulakis I. I. and Loupassaki M. H. (1990) Studies on the self-fertility of some olive cultivars in the area of Crete. *Acta Hort.* 286, 159 – 162.
- Barranco D., Milona G. and Rallo L. (1994) Épocas de floración de cultivares de olivo en Córdoba. *Investigación Agraria: Producción y Protección Vegetales* 9, 213 – 220.
- Bartolini S., Viti R. and Guerriero R. (2002) Observations on the fertilization process in self-pollinated flowers of cultivar "Leccino". *Acta Hort.* 586, 521 – 524.
- Cuevas J. and Polito V. S. (1997) Compatibility relationships in "Manzanillo" olive. *HortScience* 32(6), 1056 – 1058.
- Perica S. and Kovačević I. (1994) *Suvremeno maslinarstvo*. Avium d.o.o., Split.
- Vlašić A. (1980) Morfološki, citološki i fiziološki sterilitet sorta masline. NITP "Slobodna Dalmacija", Split.

Grapes (*Vitis vinifera* ssp. L.) from archaeological sites in Slovenia: wild or cultivated?

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Introduction

Wine crops probably originated in South Caucasus around the 4th millennium B. C., but viticulture was completely developed in Mesopotamia, Palestine and Egypt at the beginning of the 3rd millennium B. C., and in Greece in the 2nd millennium B. C. A Sumerian text on grapevine states that it was planed in Mesopotamia and watered on terraces, in Palestine vines grew on trees and sticks, while in Egypt royal vineyards grew on arches (Djoser's vineyards 2.700 years B. C.). After the harvest, wine was produced. The liquid was filtrated through flax linen and poured into ceramic amphorae that were closed by ceramic plugs, sealed and covered with resin, and kept in cold cellars. In the 1st millennium B. C. viticulture was the most important branch of economy in Greece. The production and exportation of wine was the very basis of Greek economy (along with the exportation of ceramic and metal vessels). As a result of Greek colonization viticulture was spread across the Mediterranean. The Romans intensified viticulture in Italy and in the provinces. They carefully selected resistant varieties of grape and favorable soil. Consumption of wine was widespread in the Roman Empire and viticulture had a particular place in the overall economy. In the 2nd century B. C. wine merchants sold Spanish wine in Gaul, Germania and Britain. Many varieties of grapevine that had been cultivated in the Rhine River basin, France and Spain in the Roman times disappeared in the period of the great migration (Jackson 2000).

The oldest seeds of grape (*Vitis vinifera* ssp. L.) found in Slovenian archaeological sites were found at Eneolithic pile-dwelling settlement Hočevarica, at Ljubljansko barje, Slovenia, dated to 36th century B.C. (Jeraj 2004). We carried out different biometric studies on grape seeds from Hočevarica, and from a Roman settlement in Vrhnika, Slovenia, dated to 1st century A.D. (Horvat, personal communication). The objective of the poster is to present the results of our ampelo-morphological measurements on archaeological grape seeds to find out whether our finds belong to wild or cultivated *Vitis vinifera*.

Material and methods

Representative samples of grape seeds from two archaeological sites in Slovenia, the Eneolithic pile-dwelling site and the Roman site, were investigated. Their identification to either wild or cultivated grapes was based on morphological features (after Jacquat and Martinoli 1998; Mangafa and Kotsakis 1996). The following measurements were taken: length (L), breadth (B), thickness (T), length of stalk (i.e. from the tip of the stalk to its base – edge of the seed corpus) (LS), placement of chalaza (i.e. the distance from the base of chalaza to the tip of the stalk) (PCH). Then, the following indices were calculated: breadth to length ($B/L \times 100$), thickness to breadth ($T/B \times 100$), thickness to length ($T/L \times 100$), length of stalk to total length ($LS/L \times 100$), placement of chalaza to total length ($PCH/L \times 100$).

Results and discussion

The identification of archaeological grape pips (*Vitis vinifera* ssp.) is problematic, and the morphological features generally used to distinguish the wild subspecies *V. vinifera* ssp. *sylvestris* from the cultivated subspecies *V. vinifera* ssp. *vinifera* are not satisfactory (Jaquat and Martinoli 1998).

Since the work of Stummer (1911) it has been generally accepted that domestication has caused changes in the dimensions and some other morphological characteristics of the seeds, so that in general it is possible to distinguish cultivated from wild vines. He described seeds of wild vines as small, robust, with a rounded outline, or cordate, with short stalks. They were almost flat ventrally with sharp angles and a strongly developed chalaza. On the other hand seeds of domesticated vines were described as large,

elongated, oval or pyriform, with a longer stalk. They were more rounded ventrally and less sharply sculptured. By calculating the breadth (B) : length (L) index (I.E. $B/L \times 100$) he suggested that indices from 44 to 53 characterize cultivated vines and indices from 76 to 83 wild vines. Seeds with indices from 54 to 75 could belong to either subspecies.

Our results of measuring archaeological grape seeds were compared to known phytogeographical and climatic data and to biometric results of measuring recent grape seeds. The results, as well as those from the modern experimental population of grape pips, were plotted as frequency graphs. We will present verification of the results by calculating Stummer's index to find out whether the seeds belong to cultivated or wild *Vitis*.

References

- Jackson R. S. (2000). Wine Science (2nd edition), A Harcourt Science and Technology Company, California, USA, 13-44.
Jacquat C., Martinoli D. (1999). *Vegetation History Archaeobotany* 8, 25-30.
Jeraj M. (2004). *Hočevarica*, ZRC SAZU, 56-64.
Mangafa M., Kostas K. (1996). *Journal of Archaeological Science* 23. 409-418.
Stummer A. (1911). *Mitteilungen der Anthropologischen Gesellschaft in Wien* 41, 283-296.