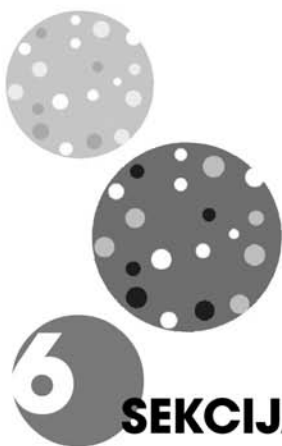

SEKCIJA 6: METODE V RASTLINSKI BIOLOGIJI

SESSION 6: METHODS IN PLANT BIOLOGY

Seeing plant biology in a new light

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Introduction

Light microscope has been one of the primary tools in biology research ever since Robert Hooke published his book *Micrographia* in the year 1664. He developed a compound microscope (composed of multiple lenses) that allowed him to see tiny chambers in cork, which he named "cells". More and more advanced designs of light microscopes followed in the next centuries, allowing scientists to study the morphology of organisms in increasing details. A few decades ago, when the optics became so improved, that the only limitation to the microscope resolution was the wavelength of light, it was thought that light microscopy reached its peak and the only possible improvement to see something new, could be using electrons instead of photons. However, with the use of molecular-biology methods, image analysis and novel physical principles, light microscopy entered its renaissance in the field of biology. In this paper I will present microscopy methods that we use in our research of plant biology.

Molecular-biology methods

In situ hybridization (ISH)

ISH is a method for localization of nucleic acids in organisms, tissues and cells. A labelled nucleic acid sequence (probe) is introduced into the tissue, where it binds to the complementary sequence in the tissue (hybridization). The conditions influencing nucleic acids pairing are concentration of monovalent cations (sodium), hybridization temperature, probe sequence length, GC content of probe and presence of duplex destabilizing agents, such as formamide. In our lab we have successfully used ISH to mRNA in plant tissue sections with RNA probes and commercial oligonucleotide probes.

We prepared RNA probes labelled with digoxigenin (DIG) using *in vitro* RNA transcription. The template was a PCR amplified fragment of the target gene, inserted into pGEM-T Easy plasmid vector. The length of RNA probes successfully used was 300-900 bases, where shorter probes were more specific with less background. Following hybridization, DIG-labelled probes are immunologically detected using anti-DIG antibodies, conjugated with the alkaline phosphatase enzyme (AP). AP catalyzes a redox reaction of a colourless substrate NBT/BCIP, resulting in a dark blue to brown water insoluble precipitate that marks the site of the probe binding to tissue mRNA. We used DIG-labelled RNA probes to detect mRNA for cell wall invertase INCW2 in maize caryopsis. The signal for INCW2 mRNA was located in the specialized transfer cells at the base of the endosperm.

Oligonucleotide probes are another type of probes that we used for detection of mRNA in tissue sections. Advantages of oligos over RNA probes are easier handling, since they are DNA molecules that are less prone to nuclease-mediated degradation than RNA, they are commercially synthesised and labelled with a variety of available markers. The oligos are also much shorter than RNA probes, thus allowing easier penetration in the tissue. However, their short length is also their drawback, since the complementary sequence of the target must be exposed for successful binding of the probe. Therefore, several different oligonucleotide probes targeted to different sections of the mRNA molecule should be tested in each experiment. We used oligonucleotide probes for detection of mRNA for LIN1, pathogenesis-related protein from flax. Best results were obtained using around 32 nucleotides long probes, labelled with DIG and detected using anti-DIG-AP and NBT/BCIP. We also tried shorter, fluorescently labelled probes, but their signal could not be distinguished from the strong autofluorescence of plant tissues. LIN1 mRNA was found to be constitutively expressed mainly in roots of young flax plants, and strongly induced by ethylene in stem apical meristem.

In situ detection of programmed cell death

During the process of programmed cell death, fragmentation of nuclear DNA often occurs as one of the steps of cell's content degradation. TUNEL reaction is a technique that employs an enzyme terminal deoxynucleotidyl transferase to incorporate labelled nucleotides into DNA strand breaks. The reaction is used to detect fragmented nuclear DNA in histological sections. We used TUNEL reaction in combination with DAPI staining. Fluorochrome DAPI stains all DNA, thus allowing us to see all the nuclei in the tissue section, whereas with the TUNEL reaction only the nuclei with fragmented DNA fluoresced due to incorporation of fluorescein-labelled nucleotides. With this method we analysed the process of programmed cell death, occurring early in maize caryopsis development. The cells of maternal tissue between vascular termini in the pedicel and the endosperm gradually degrade until only their cell walls remain, presumably to facilitate the transport of nutrients and water into the growing seed (Kladnik et al. 2004, Kladnik et al. 2005).

Novel physical principles - optical sectioning

The fluorescence microscopy of thicker specimens suffers from a problem of out-of-focus light emitted from the specimen molecules that were excited in layers below and above the focal plane. Several approaches exist to alleviate this problem of blurred fluorescence images. One is deconvolution, a software solution. The second is confocal microscopy, where laser is used to excite only a small region of the specimen, and out-of-focus emitted light is then eliminated with a confocal aperture. The third method is optical sectioning using structured illumination with a conventional fluorescence microscope (Neil et al. 1997). In our work we use Apotome (Carl Zeiss) that projects a grid onto the specimen, acquires three images and calculates an optical section. Many optical sections in sequential planes can be acquired and combined into a 3D image. An advantage of optical sectioning is also elimination of autofluorescence of tissues below or above the structures of interest.

Image analysis

Image analysis is a relatively new method that developed with the availability of powerful computers and cameras that are capable of digital image formation. A digital image is just another data matrix, which can be numerically analysed and transformed. If we calibrate the image analysis system, we can use the microscope as a measuring instrument. Quantitative measurements that we do in our lab are morphological and densitometric measurements. Morphological measurements are simple to perform, since the system only needs to be calibrated spatially. However, densitometric or any light intensity measurements require a complete control over microscope illumination and camera response to incoming light. Microscope illumination should be very stable and as uniform as possible. Camera response can be linear or logarithmic, the former type is preferred. We use micro-densitometry to measure DNA amount in meristem squashes or tissue sections stained with Feulgen reaction. Schiff's reagent stochiometrically binds to DNA, and the relative amount of DNA in nuclei is calculated from their optical density. We also linked this method with morphological measurements, to investigate a link between endopolyploidy and cell size (Vilhar et al. 2002, Kladnik et al. 2006).

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6-IL2

Measurement uncertainty in quantification of nucleic acids

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Introduction

Quantitative characterisation of nucleic acids is becoming a frequently used method in research as well as in routine analysis of biological samples. The quality of quantification analysis crucially depends on the properties of the sample itself, efficient homogenization of the sample, DNA extraction procedure and finally on the performance of the real-time PCR reaction. In food and feed analysis wide spectrum of different matrixes, ranging from simple ones like grains, flour, grout and soybean skins, to more complex ones, like flips, maize porridge, corn flakes, tofu, cookies, bread, lecithin and various feed mixtures are analysed (Lipp, 2005). In our previous study it was shown that isolation procedures and sample matrix can influence the Q PCR efficiency intensively. In this study the influence of the PCR efficiency variation on final result is demonstrated. GMO analyses are used as the model system.
The question of measuring true value and reliable expression of the result is very important, especially when compliance with regulations is needed. The uncertainty of measurement is a parameter associated with the result of a measurement, and characterises the dispersion of the values that could reasonably be attributed to the measurand (GUM, 1995). Practicability of the results obtained in defining compliance of results with regulations are presented using top down approach to calculate measurement uncertainty in routine testing of GMOs in food and feed samples.

Material and methods

Influence of Real-time PCR efficiency on the final GMO percent estimation
5 different extraction methods and 4 soya and corn matrixes, as well as 98 maize samples and 107 samples that contain soybean obtained by routine GMO detection of different sample matrices were used in experiments.
To demonstrate the influence of the PCR efficiency of the sample on GMO quantification we have performed a theoretical calculation for detection of genetically modified soybean (Table 1).

Estimation of Measurement uncertainty study
36 samples from routine analysis and proficiency tests of food and feed containing different concentrations of Roundup Ready® soybean were used. Measurement uncertainty was calculated according to Magnusson et al. (2004).

Results and discussion

Thresholds for adventitious or technically unavoidable presence of GMOs has been defined in many countries; in the European Union it was set to 0.9% in regard to a single plant component of the product in food and feed (Regulation (EC) 1892/03).

Influence of Real-time PCR efficiency on the final GMO percent estimation
In table 1 it is shown how different efficiency of amplification in the individual sample can influence the final quantitative result. Ratio between transgene and species specific gene is used for the expression of final result. The extend of effect of DNA extraction methods and food matrixes will be presented in the view of the effects on PCR efficiency and consequently on GMO quantification.

Table 1 – Effect of PCR efficiency differences on estimation of the GMO percentage.

The table shows the theoretical effect of the changes in real-time PCR efficiency in the sample on the estimation of the GMO percentage. The PCR efficiency of the standard curve for both amplicons was supposed to be 1. The calculations are performed supposing a 5% GM soybean sample is tested, using 100 ng of sample that contains 44250 copies of soybean genome and 2210 copies of transgene. (Eff. = efficiency, Ct= Cycle threshold)

SPECIES SPECIFIC GENE			TRANSGENE			GMO % ESTIMATE	
Eff. (amplicon)	**expected Ct**	**Estimation of the initial copy number**	**Eff. (amplicon)**	**expected Ct**	**Estimation of the initial copy number**	**% of GMO, transgene and species specific gene have the same efficiency**	**% of GMO, varying eff. of species specific gene only***
1.20	19.44	282095	**1.20**	23.23	20260	7.18	0.78
1.15	20.02	188227	**1.15**	23.93	12490	6.64	1.18
1.10	20.65	121215	**1.10**	24.69	7380	6.09	1.83
1.05	21.35	74962	**1.05**	25.52	4155	5.54	2.95
1.00	**22.11**	**44250**	**1.00**	**26.43**	**2213**	**5.00**	**5.00**
0.95	22.95	24752	**0.95**	27.43	1105	4.46	8.94
0.90	23.87	13004	**0.90**	28.54	512	3.94	17.01
0.85	24.91	6346	**0.85**	29.78	217	3.42	34.86
0.80	26.07	2838	**0.80**	31.17	83	2.92	77.96

* The % of GMO was calculated assuming that the PCR efficiency of transgene amplicon is equal to the efficiency of the standard curve (=1.0) and only the efficiency of the species specific gene is changing.

Estimation of Measurement uncertainty study

Calculation of measurement uncertainty on routine samples of RR soybean showed that the range near the threshold level, where doubt for labelling occurs, is from 0.73% - 1.17%, for the method applied. Samples with mean values below 0.73% need not be labelled, samples with mean values above 1.17% must be labelled for containment of GMO. Samples with mean values in-between are uncertain for labelling. Therefore final recommendations from regulatory authorities for consideration of MU are essential for synchronised decision making.

Acknowledgement

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6-SL1

3D MR imaging of diffuse porous and ring porous wood

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Introduction

Magnetic Resonance Imaging (MRI) is a non-destructive method for imaging of internal organs and tissues in living organisms. Because of its structure and high moisture content wood is a very appropriate material for NMR studies. NMR signal is proportional to moisture content in wood when imaging parameters are such that nuclear relaxation effects may be neglected (Hall and Rajanayagam 1986).
In the past, MRI was used for identification of wood structure at macroscopic and microscopic level, for wood defects (Coates *et al*. 1998, Morales *et al.* 2004), wounds and decay by fungi (Pearce *et al.* 1994, Barry *et al.* 2001) sapwood and heartwood and also for monitoring of wood drying kinetics and impregnation systems. Vast majority of the existing MRI studies were made by 2D MRI technique. The aim of our study was to asses and demonstrate potentials of 3D MRI technique for research of anatomy, water distribution and variability of moisture content within fine structures in wood and additionally to obtain spatial visualization of living tissues of diffuse porous beech (*Fagus sylvatica*) and ring porous pedunculate oak (*Quercus robur*).

Materials and methods

Two sample trees transplanted into portable pots were transported in the MRI laboratory of the Solid State Physics Department at the Jozef Stefan Institute. From each tree a branch was toped, clipped off segments were saved for anatomical investigations and living parts of branches were used for *in vivo* MR imaging. Experiments were performed on a TecMag NMR spectrometer with a 2.35 T horizontal superconducting magnet (Oxford, UK). MR images were acquired by the proton density weighted 3D spin-echo MR microscopy technique at parameters: FOV, 25 x 12.5 x 12.5 mm^3; imaging matrix, 256 x 128 x 128; repetition time, 600 ms; echo time, 2.4 ms and at 8 averages. Spatial resolution was 100 µm isotropic and the total imaging time was 22 hours. The MRI datasets were analyzed and processed by ImageJ (NIH, USA) image processing software.
The average MC was determined gravimetrically on parallel samples and the average pixel intensity value (PIV) was calculated from several intact successive cross-section slices. Quotient between the average MC and the average PIV was used as a correction factor by which was multiplied PIV to obtain normalized MC. Surface-rendered images were rendered from a 3D MRI dataset by ImageJ computer software. Conventional light microscopy technique was employed to verify individual structures recognized on planar MR images as well as on the 3D branch model.

Results and discussion

We compared MR cross and axial slices of the intact tissue with anatomical images. In both studied tree species we could clearly distinguish between the cambial zone with undifferentiated xylem and conducting phloem, and the pith (Figure 1). These structures appeared within in MR images as well. Although beech is a diffuse porous species, concentric layers of early wood vessels were prominent, indicating its conducting function and high moisture content. In MR cross-section images of beech, broad radial xylem rays extend from the pith trough all growing rings till the bark. Ring porosity and radial xylem rays that are relatively clear on anatomical oak cross-sections became less pronounced in MR images. On the contrary, radially oriented complexes of vessels and

vasicentric tracheids displayed high MR signal that reflects their vascular function. Bark had only low a MR signal. Maximal MR signal in both species was found in the pith and the cambial zone (CZ) with early wood vessels.

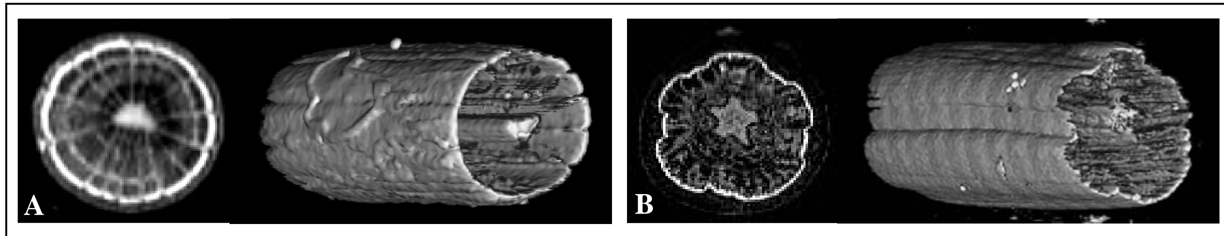

Figure 1: Planar cross-section MR images and 3D surface rendered water distribution images of: A- beech (*Fagus sylvatica*) and B- pedunculate oak (*Quercus robur*).

The average moisture content (MC) was 82 % in beech and 85 % in oak. Normalized MC displayed radial moisture distribution. At both species maximal MC (210 %) was detected in CZ together with last growing early wood vessels. The pith had MC about 170 %in beech and 130% in oak. From MR cross-section images surface plots of MC were drawn and they revealed high variability of MC within individual tissues. The highest moisture variability was detected in the cambial zone region and surrounding tissues of oak that reflect distribution of last early wood vessels. The 3D MRI technique enabled reconstruction of a 3D model of a spatial water distribution and revealed shapes of internal structures in the branches.

Conclusions

Three-dimensional Magnetic Resonance Imaging enables nondestructive visualization of internal structures in wood (pith, cambial zone with surroundings, xylem rays and complexes of early wood vessels). The method detects conducting function of all individual tissues with higher moisture content. It enables estimation of MC of any particular tissue in the branch. Finally, spatial visualization of internal structures of branches *in vivo* is feasible.

Acknowledgements

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6-SL2

Comparison of microarray and real-time PCR expression data of potato pathogenesis-related genes after PVY^{NTN} infection

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Introduction

Microarrays are a powerful tool in probing plant – pathogen interactions and the downstream signalling pathways (Wan et al., 2002). Nevertheless, the reliability of microarray experiments may sometimes be questionable since cross-hybridizations between cDNAs from representatives of members of gene families on cDNA microarray may lead to false interpretations (Gachon et al., 2004). Confirmation of microarray experiments by real-time PCR is one of the fastest growing applications of the technology. Benefits of this real-time PCR over conventional methods for measuring mRNA include its large dynamic range, sequence specificity and sensitivity (Wong and Medrano, 2005).
Potato virus Y^{NTN} (PVY^{NTN}), causing potato tuber ring necrosis disease, dramatically lowers the quantity and the quality of the potato yield all over the world. The cultivar Igor is one of the most susceptible cultivars, developing severe disease symptoms on plants as well as on tubers (Kus, 1995). Finding genes differentially expressed in the early response to infection, when the host response is more defense- than infection- related, could improve our understanding of the potato - PVY^{NTN} interaction.
The goal of our research was to study pathogenesis-related (PR) gene expression in the early response of potato cv. Igor following PVY^{NTN} infection by microarray and real-time PCR analysis and to compare the expression data obtained by both methods.

Materials and methods

Differential gene expression in early response of potato (*Solanum tuberosum* L.) cv. Igor plants to PVY^{NTN} infection was studied using cDNA-microarrays and real-time PCR. Expression was compared between mock inoculated and virus infected plants 30 minutes and 12 hours after virus inoculation, in three biological replicates.
Samples of cDNA, reverse transcribed from DNAse-digested total RNA, were hybridized to cDNA-microarrays containing clones of potato cDNA (TIGR 10K v3 potato arrays, The Institute of Genomics Research, USA). Hybridized cDNA was labeled with dendrimers of fluorescent Cy3 and Cy5 dyes (3DNA Array 350™ kit, Genisphere). After scanning, images were quantified and low quality spots were removed using ArrayPro® Analyzer 4.5 software (Media Cybernetics). Data for all arrays was combined and normalized in R (http://www.r-project.org/).
Same sample RNA was used for reverse transcription with High-Capacity cDNA Archive Kit (Applied Biosystems). cDNA was amplified by real-time PCR using SYBR Green chemistry and primers for 4 genes for PR proteins: PR-1, PR-10, proteinase inhibitor TR8 (PI TR8) and for potato cysteine proteinase inhibitor (PCPI) (Krečič-Stres, 2006).

Results and discussion

All observed PR genes showed differential expression in virus infected plants; with the strongest effect of virus infection observed for PCPI gene, 12h after inoculation (Fig. 1). Nevertheless, strong variability among biological replicates was observed. All clones of cDNA, spotted on microarray, coding for the same gene, had very similar expression profile. Except in the case of gene for PR-1, the

expression profile obtained by both methods was comparable. Generally, more pronounced differences in gene expression were measured by real-time PCR.

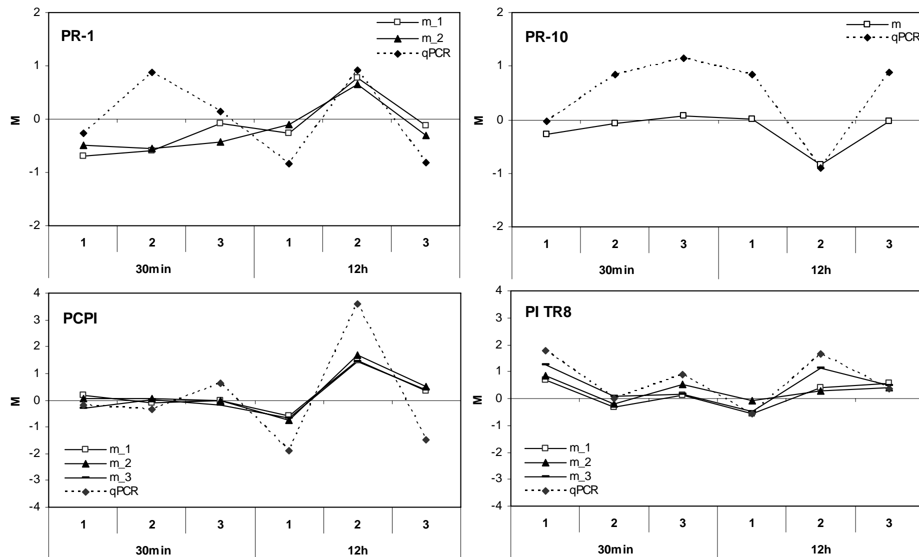

Figure 1: Expression profile of four genes for pathogenesis related proteins: PR-1, PR-10, proteinase inhibitor TR8 (PI TR8) and for potato cysteine proteinase inhibitor (PCPI) in potato cv. Igor 30 minutes and 12 hours following PVY^{NTN} inoculation as assessed by microarrays (solid lines) and real-time PCR (dashed line). M – log_2 of the ratio between the of expression in the infected and mock-inoculated plants, m_1,2,3 – cDNA clones of the same gene on the microarray, qPCR – real-time PCR.

Conclusions

- PR genes are involved in early response of potato cv. Igor to PVY^{NTN} infection.
- Real-time PCR was an efficient method for the confirmation of microarray results.

Acknowledgements

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6-SL3

Data mining as a tool for analyzing expression microarrays data – case study of gene silencing mechanism

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Introduction

Microarray technology is used for analyzing the expression of thousands of genes simultaneously. While there is much information to be gained from the data produced within a microarray experiment, data dimensions are very large. That is why statistical and data mining methods are used in order to draw significant and interpretable results from the data. This work presents an application of a recently introduced data mining methodology on potato microarray data where gene expression levels at different times that best distinguished resistant transgenic potato lines with gene silencing mechanism turned on from sensitive transgenic potato lines were determined.

Methods

Four transgenic potato lines cv. Igor transformed with viral coat protein gene were used in this experiment. Two of them are sensitive and two of them are resistant to the infection with potato virus PVY^{NTN}. Differences in gene expression levels were measured for virus infected versus mock inoculated plants from each transgenic line 8 hours and 12 hours after viral infection. Data preprocessing and filtration was done as described in Kralj et al, 2006.

The data mining task was to find differences in gene expression level that discriminate between virus sensitive and virus resistant potato transgenic plants. The results were expressed in the form of

IF Conditions THEN Class

where *Conditions* represented upregulation or downregulation of a gene 8 or 12 hours after the infection or their difference. *Class* was the target property of interest (Gamberger et al, 2004), in our case sensitive or resistant. The algorithm used is described in Kralj et al, 2006.

Results and discussion

None of the gene expression changes 8 hours after the infection seemed to be important to distinguish between resistant and sensitive transgenic lines. On the other hand, some of the gene expression levels 12 hours after the infection and the difference in gene expression levels between 12 and 8 hours after the infection seemed to distinguish well between sensitive and resistant classes for the transgenic lines tested. We could speculate that the difference in gene expression levels in the period between 8 and 12 hours post infection is crucial in determining resistance in transgenic potato lines, since this difference in gene expression levels (12-8) consisted most of the *Conditions* that discriminate between both classes. Genes that resulted as important for discriminating between sensitive and resistant transgenic lines were categorized in 6 different classes:

rec: genes, whose products are responsible for sensing the infections by viral pathogen (receptors) and whose products are part of the cell membrane

sig: genes, whose products are responsible for intracellular signaling transduction

TF: genes, whose products are regulating transcription in cell nucleus
def: genes, whose products are effectors of defense mechanism
hk: housekeeping genes, whose expression was historically accepted as constant regardless of the physiological state of the plant
uf: unknown function
The expression patterns are shown in Figure 1 A) and B). It seems that also some housekeeping genes, argued to remain unchanged regardless of the treatment given to the plant, are important for determining the resistance of samples tested.

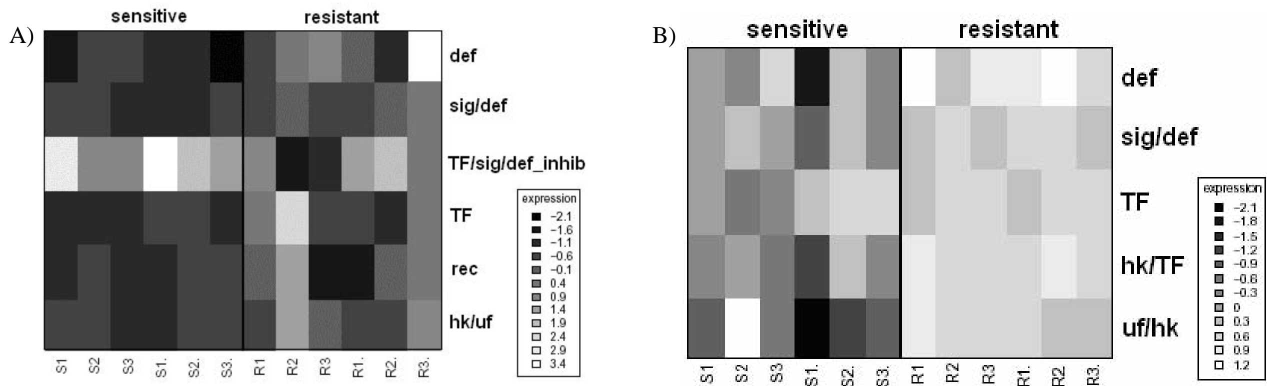

Figure 1: A) Heatmap for genes identified as important for sensitivity of transgenic lines. The heatmap shows that most of the genes of sensitive transgenic lines (marked with S, left side of the heatmap) were downregulated. Products of upregulated genes are inhibitors, important for signaling and defense pathways. B) Heatmap for genes identified as important for resistance of transgenic lines. The heatmap shows that genes that have been found to be important of determining resistance in sample tested were upregulated (marked with R, right side of the heatmap).

Conclusions

Using data mining tools can be useful for gaining insightful knowledge from large datasets. The algorithm used in our case resulted in 12 genes that determined sensitivity for samples analysed and 16 genes that determined resistance in samples analysed. The results are biologically meaningful and at the same time not difficult to interpret due to loss of redundancy in the number of genes. However, before any general conclusion can be drawn from the described experiment, careful validation of the results should be made by expanding the data dimensionality and applying the same algorithm. At later stages statistical methods and validation with molecular biology tools should be used.

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6-P1

Assessment of genotoxicity with the plant comet assay

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Introduction

Ecosystem degradation caused by anthropogenic pollution can be evaluated with chemical and biological analyses. Chemical analysis detects the presence and quantity of a contaminant in the ecosystem. On the other hand, bioassays evaluate the toxicity of substances to organisms (biological effects), as well as bioavailability and biotransformation of toxic substances. However, only a few standardised bioassays are available, and these mostly use animals or bacteria. Although plants constitute the basis of ecosystems and many plant species are also economically important in agriculture and forestry, there is a lack of plant bioassays for environmental monitoring. Evaluation of soil toxicity represents a particular problem, due to the complex composition of the soils.

One of the toxic effects of contaminating substances is DNA damage. Genotoxicity is evaluated with different techniques, e.g. the comet assay, the micronucleus assay, with evaluation of chromosome damage and somatic mutations. In this study, we used the comet assay (review in Collins, 2004) to test genotoxicity of different substances to potato plants (cultivar Desirée).

In the comet assay, the material exposed to a genotoxic factor can be the whole organism, an organ, a tissue, or a cell culture. Individual cells or nuclei are isolated and embedded in a gel layer on a microscope slide. The slides are then electrophoresed. During electrophoresis, the damaged DNA migrates from gel-embedded nuclei. Thus, the nuclear DNA of individual cells acquires the appearance of a comet, with a head (undamaged DNA) and a tail (damaged DNA). The amount of DNA in the tail correlates with the number of DNA strand breaks and thus indicates the extent of DNA damage.

Materials and methods

Potato plants (*Solanum tuberosum* L. cv. Desirée) were grown in stem-node culture on the MS-medium (Murashige and Skoog, 1962) in a growth chamber with a 16 hour light period (21 °C) and 8 hour dark period (19 °C). Five to eight week old plants were removed from the medium. Their roots were immersed in solutions containing different concentrations of genotoxic substances. After the treatment, nuclei were isolated from leaves and roots, and microscope slides for the comet assay were prepared. The alkaline comet assay protocol of Gichner et al. (2004) was followed with a few modifications. The parameter used to evaluate DNA damage in individual cells was the percentage of tail DNA, defined as the ratio between the amount of DNA in the comet tail and the amount of DNA in the whole comet.

Results and discussion

To optimise the comet assay conditions, we exposed the plants to different concentrations of ethyl metahanesulfonate (EMS). EMS is a direct acting alkylating compound, widely used in genotoxicity studies. When the plants were treated with EMS for 24 hours, the level of DNA damage in the leaf cells increased with increasing concentration of EMS (Fig. 1A). A concentration-dependent response was also observed after a shorter treatment (2 hours) with higher concentrations of EMS (Fig. 1B). DNA damage was higher in the roots than in the leaves. This organ-specific difference possibly arose because roots were in a direct contact with genotoxic solution, whereas the genotoxic effect in leaves presumably depended on transport of the genotoxic compound within the plant. The observed concentration-dependent response to a genotoxic substance demonstrates that potato plants of the cultivar Desirée are suitable for assessment of genotoxicity with the plant comet assay.

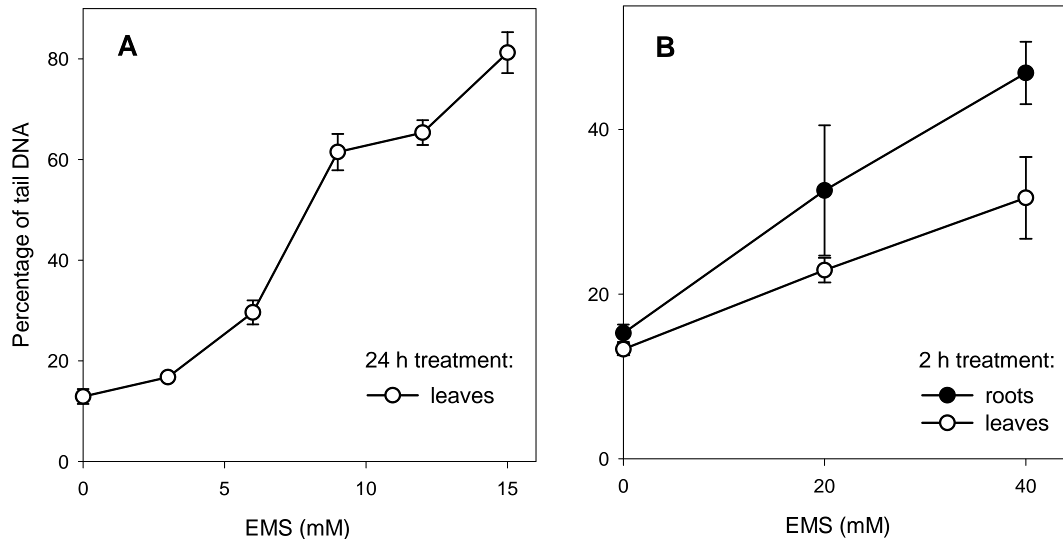

Figure 1: The nuclear DNA damage in potato plants treated with different concentrations of ethyl methanesulfonate (EMS). Plants were treated for 24 h (A) or for 2 h (B). After the treatment, the nuclei were isolated either from the leaves (open circle) or from the roots (closed circle). For each EMS concentration, the percentage of tail DNA was measured in two groups of 25 nuclei for each slide (two or three slides per treatment). For each group of nuclei, the median percentage of tail DNA was determined. The mean of the medians was calculated (the error bars represent the standard error of the mean).

Acknowledgements

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6-P2

Optimisation of temporal temperature gradient electrophoresis (TTGE) method for rapid assessment of fungal endophyte communities

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Introduction

Microbial community structures can be assessed in various ways, with clonal libraries being the mostly spread technique. Although cloning and sequencing results in high quality data of the environmental sample, it is relatively costly and time-consuming procedure (Kennedy and Clipson 2003). Advances in molecular biology led to DNA fingerprinting techniques that allow rapid assessment of the genetic structure of complex communities (Muyzer and Smalla 1998). In the current work, TTGE was chosen as the fingerprinting method because of its ability to provide a rapid visual indication of the fungal community structure, with possibility of the excision and sequencing of the individual bands in order to identify their origin (Smit et al. 1999). TTGE is a technique that works on the same basic principle as DGGE only without the requirement for a chemical denaturing gradient, thus producing more reproducible data (Yoshino et al. 1991).
Our aim was to develop and optimise a TTGE method based on ITS rDNA for fast and reliable analysis of fungal communities living in association with higher plants. Willow *Salix caprea* was used as a test object, as willows are known to form associations with a wide range of fungal endophytes: arbuscular mycorrhizal and ectomycorrhizal fungi as well as DSE (Trowbridge and Jumpponen, 2004).

Material and methods

Root samples for DNA extraction were collected from *S. caprea* plants along a secondary succession gradient in Žerjav, Slovenia. Fungal DNA was amplified using nested PCR approach with ITS1Ff-ITS4r and ITS3f-ITS4r set of primers. Primer ITS3f with a GC tail was used for TTGE analysis of the fungal ITS region on Dcode™ electrophoresis (BioRad). Individual bands from the TTGE gel were sequenced after cloning. For identification the sequences were subjected to a GenBank search and aligned with closest matches and additional representatives of the groups using neighbour-joining (NJ) analysis.

Results and discussion

Successful DNA extraction and amplification using ITS1Ff-ITS4r primers, followed by nested PCR resulted in a number of bands between 350 and 500 bp in size with varying intensity. A complex banding pattern that was reproducible for each sample between different PCR reactions and TTGE runs and yielded a total of 39 different ITS-TTGE bands with unique electrophoretic mobility. For elimination of repetitive steps of excision and PCR reamplification to obtain sufficient quality of PCR products for sequencing, which is frequently unsuccessful (Anderson et al., 2003), bands were cloned before sequencing. Sequencing of 34 ITS-TTGE bands yielded 27 different sequences showing similarities to many ascomycete or basidiomycete taxa (Table 1), with numerous representatives previously reported to form symbiotic associations with *Salix* species (Trowbridge and Jumpponen, 2004). TTGE bands with similar electrophoretic mobilities in different samples were confirmed to be identical (Figure 1). Sometimes bands with different electrophoretic mobilities were observed to have same phylogenetic affinities. This situation was also reported by other authors (Gadanho and Sampaio, 2004) and is probably caused by secondary structures that interfere with denaturation kinetics of the DNA molecule.

Table 1. Sequences from ITS-TGGE bands, with nearest matches from GeneBank and corresponding similarity percentages.

	Nearest match		
1	Unkn. fungus	AF461563	96%
2	*Alternaria* sp.	AY148445	99%
3	*Phialophora sp.*	AJ534703	98%
4	sordariaceous EM	AY916068	94%
5	Uncultured fungus	DQ093750	94%
6	*Phialophora finlandia*	AY578281	97%
7	*Alternaria* sp.	AY148445	93%
8	*Phialophora* sp.	AY578281	97%
9	Unkn. ascomycete	AY568066	93%
10	*Phialophora* sp.	AF083204	97%
11	sordariaceous EM	AY916067	99%
12	*Cylindrocarpon* sp.	AY295332	99%
13	*Phialophora finlandia*	AJ534703	98%
14	*Leptodontidium orchidicola*	AF486133	96%
15	Ukn. ascomycete	AY568066	95%
16	Uncultured ascomycete EM	AY833032	96%
17	*Exidiopsis* sp.	AY509549	91%
18	Uncultured fungus	AJ875393	98%
19	*Phialocephala fortinii*	AY078129	98%
20	*Hymenogaster* griseus	AF325636	94%
21	*Cortinarius* cf. *decipiens*	AJ889946	95%
22	*Phialophora finlandia*	AJ534703	98%
23	*Leptosphaeria korrae*	AF486626	93%
24	*Tumularia aquatica*	AY265337	92%
25	*Pseudomerulius aureus*	AY293153	98%
26	*Phialophora* sp.	AY578277	97%
27	*Phialophora* sp.	AF083204	96%

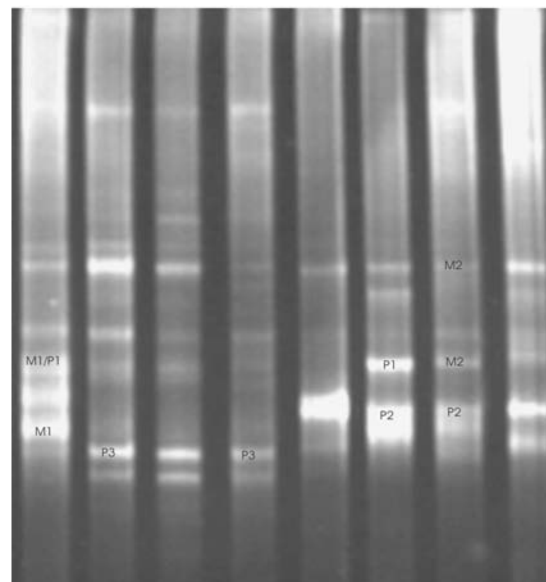

Figure 1. TTGE analysis of partial fungal ITS sequences amplified from root samples of *S. caprea*. Marked bands show some of the bands with similar position (P) on the gel that yielded similar sequences and bands with different electrophoretic mobility but with similar phylogenetic affinities (M).

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6-P3

Mycoremediation with contemporary use of plants for revitalising contaminated and sterile sites

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Introduction

Soils with unfavourable physical and chemical properties for the growth of plants are susceptible to wind and water erosion. This results in numerous damaging consequences, such as pollution of water ways, dust accumulation in surrounding areas, large areas of unproductive, unused soils, etc... Plant cover can reduce the erosion processes; however soils with suitable physical, chemical structure and appropriate microbial activity are then needed for plant growth. Natural succession course of different organisms in sterile or heavily contaminated soils is slow. The main reason for this is lack of organic matter. Material, which is rich with carbon, is wood. Organisms, which can successfully decompose all structural wood components, are wood decay fungi. This group of fungi can completely mineralize wood with their extracellular enzymes, although the end product of lignin decomposition is usually a partially decomposed, fragmented lignin. Such decomposition products and other fungal activities have an impact on the formation of organic soil components and can accelerate natural succession pattern. White rot fungi have especial capacity to break down numerous organopollutans and the exploitation of this fungal capacity was named mycoremediation (Humar and Pohleven, 2003). We introduced this term to the use of fungi for reconstruction of degraded surfaces. The aim of our work is to develop and test a procedure for mycoremediation system, which would economically and ecologically exceed traditionally used sanitation methods. The goal is to prepare organic poor and damaged surfaces for planting trees for biomass production by inoculating beech chips with white rot fungi.

Materials and methods

In May 2006 the first phase of planned experimental system was established. As contaminated site the thermoelectrical power plant ash dump (an abiotic surface, poor with organic matter and heavily contaminated with heavy metals) near Velenje was selected. Termoelektrarna Šoštanj (TEŠ) has prepared test field with 20 000 m^3 ash material, spreaded out on 900 m^2 area. In years 2005 and 2006 approximately 60 m^3 of wood chips were added on the selected areas. The dolomite cut slope of forest road on Paški Kozjak was chosen as sterile site, which is also poor with organic matter but without contamination. Approximately 200 m^2 surfaces were covered with 20 m^3 mixture of beech chips and sand. For water retention hydrogel was mixed into the substrate on both locations. Inoculation with wood chip spawn was executed in year 2006. There were different combinations of trees species planted at test fields (willow, alder, pine, robinia, poplar, maple, pear tree).

For preparation of wood chip spawn the fungal species *Pleurotus ostreatus* was selected. We have performed a quick screening among collected strains for the beech wood degrading ability with mini-block test as described by Pohleven and co-workers (2000). The strain with highest decay rate and primary isolation from the deciduous trees was used for further inoculum preparation. Fungal spawn was composed from beech chips of different granulation, wheat grains and mycelium of selected *P. ostreatus* strain. Spawn was prepared in polypropylene autoclavable bags with microporous filter.

Results and discussion

Test fields prepared in the year 2005 were not inoculated with fungal inoculum. Visual examinations after one year showed that planting at Paški Kozjak has been successful. Wood material at the control field at ash dump has started to decompose; mycelium and different moulds are seen on the beech chips.

The fungal species *Pleurotus ostreatus* was chosen for field inoculation, while it is easy to grow, thrives on a wide array of substrates, is highly adaptable and competitive with other fungi. *Pleurotus ostreatus* spawn was successfully prepared and a few days after inoculating test fields, mycelium expansion on surrounding wood chips can be observed. We expect spreading of *P. ostreatus* mycelium will be competitive with naturally appearing fungal species, will accelerate wood decay, contribute to succession and will accelerate the formation of organic soil components.

With planting trees on research areas the additional factor influencing soil alteration is present. Rhizosphere and the surrounding soils are distinguished in chemical, physical and biological characteristics. Rhizosphere is rich with organic substances, which are mainly root exudates, and with different organisms among which numerous interactions are present. This gives the rhizosphere exceptional complexity and the ability to enrich the soils with organic compounds, microbial communities, mycorrhiza and the ability to reduce different pollutants. Rhizosphere can be furthermore co-included in the wood break down.

Conclusions

With inoculation of white rot fungi to wood material we suspect the decay rate to be higher and otherwise slower succession of numerous organisms will be accelerated.

The development of mycorrhizal associations, succession of different rhizospheric organisms, the efficiency of experimental system and the possible use of planted trees for biomass production will be monitored in the mycoremediation system.

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6-P4

Detection of point mutations in selected genome regions from sporocarps of heavy metal exposed *Hydnum repandum* and *Clitocybe nebularis* as putative indicator species

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Introduction

Intensive army activities in terms of explosive and equipment testing can yield relatively high inputs of heavy metals and other explosive remains into the enviromnent. Heavy metals can subsequently enter the food chain of the local flora and fauna. Ectomycorrhizal fungi significantly enhance tolerance of mycorrhizal plants (Jentschke and Godbold 2000) but the direct influence to fungi at the genetic level is not always clear. The main objective of our work was to determine the influence of the army training polygone Poček on the content of heavy metals in sporocarps of selected and potentially indicative species and to assess possible point mutations in selected genomic regions using denaturing gradient electrophoresys as a mutation detection system.

Material and methods

Plots

Three plots were selected where sporocarps were collected in year 2005 for heavy metal and DGGE analysis. Rajhenavski Rog area and Počivalnik near Postojna were selected as comparative (control) plots with putative low heavy metal loads to be compared with the army polygone at Poček. The latter was divided into three subplots with regard to the distance from the central explosion point: Bile, Bukov vrh and Ivanji vrh. Allplots exhibit karst characteristics, mainly with a shallow soil layer. Poček is situated at the transition zone between Dinaric and Submediterranean phytogeographical region (dominant tree species: beech, spruce and oak) while controls belong to Dinaric beech and silver fir dominated Illyrean forests.

Mutation detection protocol using DGGE analysis

34 sporocarps of *Hydnum repandum* and 54 sporocarps of *Clitocybe nebularis* were analised. Each sporocarp was treated as a separate sample. Heavy metals (Pb, Cd, As and Hg) were detected as described in Al Sayegh Petkovšek et al. (2002). DNA extraction from sporocarps and the PCR of selected regions were performed as described previously in Kraigher at al. (1995) with modifications of annealing temperature according to characteristics of the used primers. Forward primers for amplification of ITS spacers (Gardes and Bruns 1993) and part of 28S rDNA were adapted with CG clamp (de Souza et al. 2004) and applied with regular reverse primer. Amplified products were first checked for successful amplification using modified primers on 2% agarose and subsequently separated in DGGE (Denaturing Gradient Gel Electrophoresys) on 6.5% acrilamide gel with 20%- 60% linear gradient of denaturant (urea and formamide). The presence of point mutations in the amplified regions was read manually from the ethidium bromide stained gels. Sporocarps exhibiting point mutations were correlated to the plot of origin.

Results and discussion

The analysis of heavy metals indicated increased concentrations of Pb and Cd in *C. nebularis* but not in *H. repandum* (which is known not to accumulate heavy metals) on all analysed plots at the army polygone Poček, but also at control plots, predominantly at

Počivalnik. Although the increased concentrations were not expected at Počivalnik, the plot was not excluded from the analysis. All measured concentrations in sporocarps are still below maximum allowed con centrations.

DGGE analysis of the amplified ITS regions and part of 28S rDNA regions showed very low frequency of point mutations. From 32 samples only two were recorded in *Hydnum repandum*, one from a sporocarp collected in Rajhenavski Rog (Fig. 1, lane 12) and another mutation in a sporocarp from Poček, Bukov vrh plot (Fig. 1, lane 13). The low presence is surprising since this species is known to be an indicator of pollution (Kraigher et al. 1996). No mutations were observed in the analysed sporocarps of *C. nebularis* (Fig. 1 lanes 1-10) despite higher concentrations of Cd and Pb (data not shown). DGGE is a useful tool for detection of point mutations in the selected regions. In the study of heavy metal polluted plot the low number of sporocarps and of observed mutations did not yield any significant correlation of mutations to pollution.

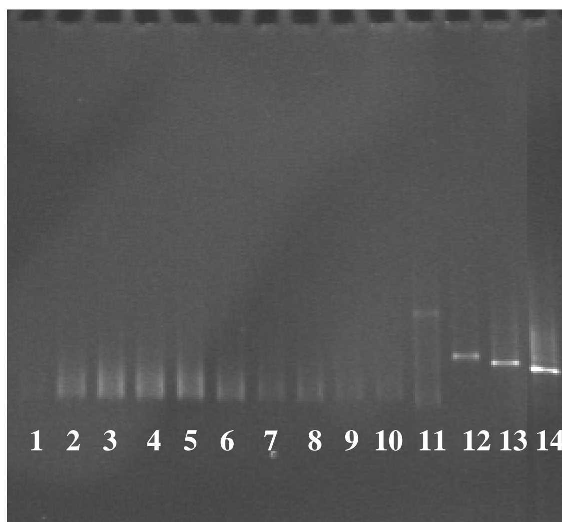

Figure 1: Amplified ITS regions for *Clitocybe nebularis* (lane 1-10), *Hydnum rufescens* (lane 11; outgroup) and *Hydnum repandum* (lane 12, 13 – mutant and lane 14 – type strain)

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6-P5

Induction of organogenesis and influence of selective treatments and antimicrobial substances on adventitious roots in *Coleus blumei* Benth.

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Introduction

The genetic transformation of plants requires selection and regeneration of transformed tissues. The purpose of this study was to determine the suitable conditions for shoots and roots regeneration and to examine effect of selective agents and antimicrobial substances on *Coleus blumei* organogenesis. Nodal, internodal and leaf explants were analyzed for the regeneration potential. Multiple shoots were induced on nodal explants cultivated on MS medium with different concentrations of indoleacetic acid (IAA) and 6-benzylaminopurine (BAP). Adventitious roots were induced on leaf explants cultivated on MS media with α-naphthaleneacetic acid (NAA). The organs formed directly from the explants without an intermediate callus phase. The sensitivity of non-transformed adventitious roots of *Coleus blumei* to selective agents (kanamycin, neomycin, geneticin, hygromycin and glufosinate ammonium) and antimicrobial substances commonly used to eliminate *Agrobacterium* from tissue culture (plant preservative mixture: PPM, carbenicillin: carb, cefotaxime: cef and vancomycin: van) was measured.

Materials and Methods

The leaves excised from *in vitro* growing *Coleus* plants, were cut into 1 cm^2 squares. Leaf explants were inoculated upside-down on MS medium supplemented with different NAA concentrations (0.01, 0.1, 0.5, 1 mg/l) and/or antimicrobial substances (500 mg/l carb, 250 mg/l cef, 100 mg/l van, 1ml/l PPM) and selective substances kanamycin (2 to 50 mg/l), neomycin (10 to 100 mg/l), geneticin (0.1 to 20 mg/l), hygromycin (1 to 20 mg/l) and glufosinate ammonium (0.01 to 5 mg/l). The plant stem was cut on internodal and nodal explants that were cultured on MS medium supplemented with different IAA and BAP concentrations. Leaf, internodal and nodal explants were cultured at 24 °C under fluorescent tube light (16 h photoperiod). Influence of different supplements on adventious organ induction was tested. Results were scored after 21 day. For each treatment 12 explants were analyzed.

Results and Discussion

Root induction

NAA induced root development on leaf explants. All explants cultured on MS medium supplemented with 0.5 mg/l NAA developed roots but number of root origin was higher (up to 30) on medium with 1 mg/l NAA (Fig. 1a). NAA concentrations higher then 1 mg/l blocked root elongation. Media with different concentrations of IAA and/or BAP, thidiazuron and N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) did not induced shoots on leaf and internodal explants. BAP alone, or in combination with IAA induced multiple shoots formation on nodal explants of *Coleus blumei* (Fig. 1b). The influence of antimicrobial substances used for *Agrobacterium* elimination after transformation was examinated. Leaf explants were cultured up side-down on MS medium supplemented with 1 mg/l NAA and appropriate antimicrobial or selective substance. Carbenicillin reduced root induction and caused sward root growth. Cefotaxime, vancomycin and PPM alone or in combination were suitable for bacteria elimination since they did not derange root induction and growth (Fig. 2). For the selection of transgenic tissue different selective agents are commonly in use. Their effect on root induction on leaf explant of *Coleus blumei* cultivated on MS medium with 1 mg/l NAA was examinated. Concentrations higher than 20 mg/l kanamycin, 70 mg/l neomycin, 2 mg/l geneticin, 10 mg/l hygromycin and 1 mg/l glufosinate ammonium completely stopped normal root induction on leaf explants of *Coleus blumei*.

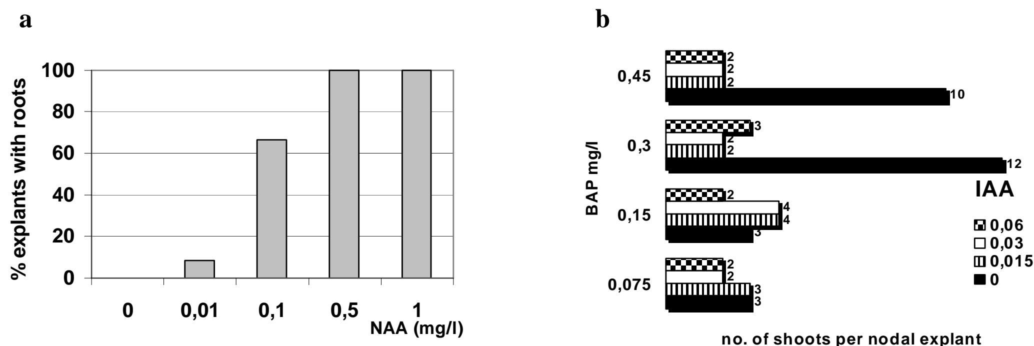

Figure 1. **a**) Effect of NAA on root induction on leaf explants of *Coleus blumei.* **b**) Effect of IAA and BAP on shoot induction on nodal explants of *Coleus blumei*.

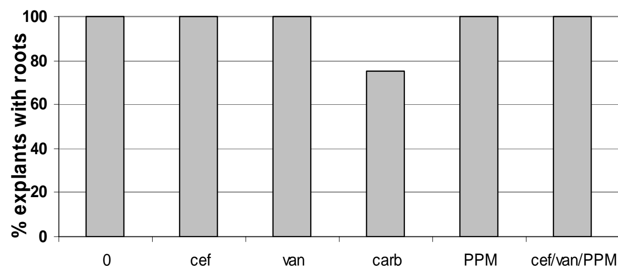

Figure 2. Root induction on leaf explants of *Coleus blumei.* Influence of antimicrobial substances (0: without substance; cef: 250 mg/l cefotaxime; van: 100 mg/l vancomycin; carb: 500 mg/l carbenicillin; PPM: 1 ml/l PPM) added to MS medium supplemented with 1 mg/l NAA.

Conclusions

Organogenesis of *Coleus blumei* leaf, nodal and internodal explants was analyzed. The highest number of roots, up to 30 per explant, developed on leaf explants on MS medium supplemented with 1 mg/l NAA. The highest number of multiple shoots was induced on nodal explants cultured on MS medium supplemented with 0.3 mg/l BAP. Leaf explants might be the best for incorporation into a transformation system where transgenic roots are expected. Antibacterial substances cefotaxime, vancomycine and plant preservative mixture did not influence root development and are good for bacteria elimination after transformation. Susceptibility of normal roots to different selective treatments was analyzed. Transgenic roots could be regenerated on media with 20 mg/l kanamycin, 70 mg/l neomycin, 2 mg/l geneticin, 10 mg/l hygromycin or 1 mg/l glufosinate ammonium.

6-P6

Alternative Real-time Q-PCR chemistries for GMO detection

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Introduction

Real-time polymerase chain reaction (PCR) is currently the most effective and widely used method for quantification of genetically modified (GM) components in food and feed. The method uses fluorescent reporters to detect the amount of product in each cycle of the reaction. Some flourogenic molecules can incorporate in double stranded DNA nonspecifically, while others are attached to different types of oligos, used as primers or probes for amplification (Kubista, 2006). Although more than thirty different chemistries have been developed, vast majority of laboratories use TaqMan and/or SYBR Green I only (Bustin, 2005). Here we have tested four alternative chemistries that have not yet been used for detection of GM organisms (GMO). We have compared their performance characteristics to TaqMan chemistry to check their appropriateness for research and routine analysis in GMO detection.

Materials and methods

DNA samples

Genomic DNA was obtained from reference flour samples containing 5% Mon810 GM maize. DNA was purified by Nucleospin Food Kit (Macherey-Nagel, Düren, Germany) as described by the manufacturer. The concentration of isolated DNA was determined by real-time PCR through comparison of the invertase gene content to the standard curve.

Chemistries for Real-time Q-PCR

Four different technologies were tested: Lux™ flourogenic primers (Invitrogen Corporation, Carlsbad, United States), Plexor™ (Promega, Madison, United States), Cycling Probe Technology™ (TaKaRa, Osaka, Japan) and LNA Probes (Sigma Proligo, Paris, France). All of them were compared to TaqMan probes (MWG Biotech AG, München, Germany) using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, United States).
Primers and probes were designed on the invertase as a reference gene and on event specific 5´ junction of the transgene.

Real-Time Q-PCR

The Real-time PCRs were carried out on ABI 7900 detection system (Applied Biosystems) according to recommended protocol for each of the chemistries. In the case of Lux™ primers and Plexor ™ dissociation curves were plotted. Further analysis was carried out on the comparison of threshold cycles (Ct).

Results

The TaqMan probe is located between the two PCR primers and labeled with a reporter dye on one side and with a quencher on another. After the probe hybridizes to its complementary sequence within the PCR target it is degraded due to the 5' > 3' exonuclease activity of *Taq* DNA polymerase. This separates the quencher from the reporter dye and the fluorescence intensity increases.
Lux™ and Plexor™ technologies do not employ a probe. Instead they use one flourogenic primer. In the case of Lux™ its fluorescence is quenched by a hairpin structure. During amplification the structure is opened, hence the signal is increased. Plexor™ on the other side has a high fluorescence at the beginning. The technology is based on a reporter primer modified with iso-dC which,

during amplification, binds only iso-dG from the reaction solution. Because this modified nucleotide is linked to the quencher, the signal decreases after the binding. Both technologies allow that dissociation curves are plotted additionally to monitor the specificity of the product.

We have established detection systems including the invertase and the transgene amplicons for both technologies. Looking at the efficiency and repeatability, the developed systems seem comparable to TaqMan in preliminary studies. However further experiments will be needed to check what are the benefits and drawbacks of each..

The Cycling Probe Technology™ (CPT) and Locked Nucleic Acid (LNA) probes are both hydrolysis probes and in this regard more similar to TaqMan. The CPT probe includes one modified RNA nucleotide forming a RNA-DNA duplex after hybridization on the target. The duplex is then recognized and cut by RNaseH into two parts resulting in separation of the quencher and increased fluorescence. LNA probe differs from TaqMan with only a few modified nucleotides. These nucleic acid analogues form methylene bridges after binding and lock the structure on the target DNA. Melting temperature of these probes is therefore significantly higher, hence they can be designed as much shorter.

Both CPT and LNA probes gave good results within the invertase region while for the transgene we had to redesign the CPT primers and probes and are not able to provide the results as yet. However in the case of the invertase we can so far conclude that the CPT is fast and has a low background. Despite a really good efficiency and level of quantification the method does not seem to exceed TaqMan due to lower sensitivity and much higher costs. LNA probes seem more promising. In general they are comparable to the TaqMan system in the terms of efficiency, repeatability, specificity and cost.

Discussion

Although each system has its own advantages LNA probes are the most promising of all four chemistries tested. Additionally they are easily transferred from widely used and certified TaqMan methods making them simple to use. Because LNA probes are much shorter they might be especially appropriate for the sequences where the design of a common TaqMan probe is difficult or even impossible, which often happens with junctions. They also have a prospect in single nucleotide polymorphism detection. CPT probes are unlikely to play a significant role in routine GMO detection because they are too expensive, while it is still early to say what could be the niche for Lux™ and Plexor™.

Conclusion

Four alternative chemistries have been compared to TaqMan for use in the research and routine GMO quantification. Successful detection was established for invertase and the transgene in all tested chemistries (excluding the redesigned CPT probe for the transgene). So far the LNA probe seems to be the most efficient so our next step will be accuracy studies. We will however conduct further studies to determine the potential of other three technologies.

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6-P7

Assessment of protein based method for detection of Roundup Ready® soybean in comparison to DNA based method

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Introduction

Roundup Ready® soybean is the most widespread genetically modified crop grown in different countries all around the world (Clive, 2006). According to European regulation all products containing more then 0.9% of genetically modified organism must be labelled. Therefore products containing soybean, from raw material like grains to more complex ones like feed and food, must be regularly tested for the presence of genetically modified organisms.

Currently available methods for detection of GMOs are based on detection of DNA or proteins (Hernández, 2005). Molecular methods based on DNA are very complex, but very precise and also allow for quantitative analysis. On the other hand protein based methods can be more simple to use, they can be cheaper, but they are available only for small number of GM constructs and immunological analysis is usually not suitable for processed material since proteins can be degraded and antibodies can not bind to specific protein.

In our research, we compared the strip test which detects the presence of specific protein indicating the presence of Roundup Ready® soybean and QPCR method which specifically detects the DNA sequence of the Roundup Ready® soybean construct. Three different kinds of samples – grains, food and feed were tested and suitability, cost and sensitivity were compared.

Materials and methods

Trait RUR Bulk Soybeans 5-Minute Test Kit (Strategic Diagnostics, Inc., Newark, DE USA) was used as strip immunological test. Kit is designed to detect EPSPS protein in grains of soybean at the level of 0.1 % of transgenic grains (1 out of 1000). Lateral flow strip assay uses double antibody sandwich format. Antibodies specific to the EPSPS protein are coupled to a colour reagent and incorporated into the lateral flow strip. In the presence of EPSPS protein binding occurs between the coupled antibody and the protein. One capture zone captures the bound EPSPS protein and the other captures colour reagent. These capture zones display a reddish colour when the sandwich and/or unreacted coloured reagents are captured in the specific zones on the membrane. The presence of only one line (control line) on the membrane indicates a negative sample and the presence of two lines indicates positive sample.

QPCR was used for detection of Roundup Ready® soybean construct specific sequence on DNA level (Schweizerische LebensMittelBuch-Methode 52B, 2000). Detection and if possible quantification of Roundup Ready® soybean was performed on series of samples obtained from routine testing for GMO.

Inhomogeneous samples were first homogenised, then DNA was isolated using Nucleospin Food kit (Macherey Nagel, Düren, Germany) and detection and quantification of Roundup Ready® soybean was performed on ABI 7900 HT real-time PCR. Positive samples were additionally tested with Trait RUR Bulk Soybeans 5-Minute Test Kit for the presence of Roundup Ready® Soybean. To 10g or 300 mg of sample 30 ml or 1 ml of distillate water was added and mixed well. 500 µl of mixture was transferred to microcentrifuge and Trait RUR strip was dipped to the liquid. After 90 seconds results were read. In case of negative result there was one coloured line and in case of positive sample there were two coloured lines. If no coloured line appeared, the test was not valid.

Results and discussion

Table 1: Sensitivity, price and suitability comparison

Sample type	Sensitivity		Cost		Suitability		No. of samples
	Trait RUR	**QPCR**	**Trait RUR**	**QPCR**	**Trait RUR**	**QPCR**	
Raw material	1%	<0,1%			Yes	Yes	27
Feed	/	<0,1%	Low	High	No	Yes	13
Food	/	<0,1%			No	Yes	24

With raw materials as soy skins or soy flour the detection of Roundup Ready® soybean above 1% is possible, while detection with QPCR is more sensitive and lower amounts can be detected in a sample.
For more complex matrices with lower content of soybean, lateral flow strip method is not appropriate, since it does not detect even very high levels of transgenic soybean, probably because of degradation of target protein EPSPS. Although in feed soybean is usually added as soy skins, method was not able to detect presence of EPSPS. Substances like fat can also disturb analysis and make it impossible although there is high percentage of Roundup Ready® Soybean in the sample.

Lateral flow strip tested in our experiments is therefore suitable only for detection of EPSPS protein in unprocessed samples like grains, flour or soy skins, at the levels of GMO close to or higher of the threshold for labelling the presence of GMO in EU. Because of its simple application and low cost it can be suitable for quick tests of large amount of raw materials already in the harbour or other entry point to the food chain on the border of European Union.

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6-P8

Usability of NASBA, an isothermal DNA amplification method, to detect GMOs

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Introduction

Real-time PCR DNA amplification is presently the most used and most powerful method for the detection and the quantification of GMOs in agricultural crops and plant-derived food products. However, the exponential increase of GMOs released on the market will severely limits the use of this technique for the detection of a single target because of the heavy handling and cost that may be generated. To avoid this problem, multiplex PCR is proposed to be a good relay especially when it is used in combination with the hybridization of its reaction products on micro-arrays. However, the use of many primers in a single tube reaction can be problematic.

We developed a non-PCR based method for the multiplex quantitative detection of GMOs. This method called NASBA (Nucleic Acid Sequenced-Based Amplification) is a sensitive, isothermal, transcription-based amplification system for the specific replication of nucleic acid *in-vitro*. It is widely used for RNA target amplification in virus and bacteria detection and competes with real-time PCR in terms of efficiency and convenience (Monis, P. T. *et al*.., 2006). In this work, we show how this system can be successfully adapted for multiplex DNA-based detection of GMOs, the product of the reaction being suitable for hybridization on DNA-chips.

Materials and methods

Test samples and DNA purification

Genomic DNA was purified from plant material and reference flour samples containing defined percentages of GMO material. DNA were purified using DNeasy Plant Mini Kit (QIagen, Hilden, Germany) as described by the manufacturer. Concentrations of DNA were determined by real-time PCR quantization by comparison of their IVR content with standard curves.

Multiplex DNA NASBA

Purified DNA was used in the amplification reactions. A three steps amplification approach was chosen. In a first amplification step "tailed" primers were used in a one cycle Taq polymerase amplification to get the appropriate template for NASBA bordered by "universal" regions. Universal primers corresponding to the universal borders in the template DNA were used for NASBA amplification based on the NucliSens® Basic Kit (bioMérieux bv, Boxtel, The Netherlands). Since NASBA main product is cRNA, a final reverse transcription was performed to obtain cDNA suitable for real-time PCR quantization.

Real-Time PCR

Amplification rates of NASBA method were measured by comparing Ct of target elements between NASBA amplified and control non-amplified samples.

Results

At first, we assayed the usability of NASBA method for DNA target amplification in GMOs material. The usual protocol for NASBA as proposed by NucliSens® Basic Kit manufacturer failed in amplifying GMOs targets. The optimisation of the procedure included the addition of a first amplification step to generate a suitable template prior to the NASBA reaction itself. An other improvement

was the use of “tailed” primers during this first step: these primers are composed of a specific sequence for the target to be amplified and, on their 5’-end, an “universal” region is added. Thus, in the following NASBA reaction, we used “universal” primers to avoid the problem of complex primers mix and allow the multiplexing of the procedure. Sequences of these “universal” regions were chosen such that their Tm values between 40°C and 45°C fit with the optimal temperature for NASBA (and the final reverse transcription). Finally, the crucial KCl concentration was optimised.

Several target elements from various GMOs were amplified in simplex NASBA reactions showing up-to a 22Ct-difference (more than 4.10^6 fold amplified) when compared to the control DNA on real-time PCR. The method was shown to be sensitive since less than 20ng of genomic DNA were needed to obtain solid amplification of the target DNA. Moreover, it was shown that the amplification is specific: only the target DNA is amplified.

After NASBA procedure was set for single target amplification, multiplex NASBA reactions were performed aiming specie-specific, construct-specific and event-specific target elements. Same amplification rates were found for each target element compared to simplex NASBA reaction. These results demonstrate the specificity of the first amplification step, and the non-discriminatory property of the “universal” primer set for any target element.

Discussion

NASBA is based on the activity of avian myeloblastosis virus (AMV) reverse transcriptase (RT), Escherichia coli RNase H and T7 RNA polymerase (Deiman *et al*., 2002). NASBA is an isothermal method requiring no particular material in opposite to PCR-based method. Moreover, since the multiplexing of the method was already demonstrated (Schneider, P. *et al.*, 2005), we chose to test the usability of NASBA for multiplexed amplification of GMO target elements in the goal to hybridize the reaction products on DNA-chips.

The optimisation of the NASBA procedure, including the use of a first amplification step, the design of “tailed” and “universal” primers and the determination of optimal KCl concentration leaded to an efficient protocol for GMO target amplification. The multiplexing, specificity and sensitivity of the method we developed, in addition with the strong DNA amplification rates, are in favour of the use of NASBA for GMO detection and quantification on micro-arrays, the main product being cRNA which is suitable for hybridization on oligo-arrays. Moreover, the use of molecular beacons makes this technique available for real-time detection of GMOs.

Conclusion

We show that due to its sensitivity, specificity, amplification efficiency and multiplexing, NASBA can be used for DNA target amplification in the goal of GMO detection. Products of the amplification reaction can potentially be used either for array-based detection, or for real-time detection.

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6-P9

Measurement of hydrogen peroxide in leaves using luminol dependent chemiluminescence reaction and microplate reader

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Introduction

Hydrogen peroxide can be produced as result of normal physiological state in plant such as Mehler reaction or it can be triggered by microbes, heavy metals and other stress. Because of its reactivity, cell enzymes which can degrade H_2O_2 and because of other components which can quench the chemiluminescence, its accurate detection and quantification in plants extracts is difficult to observe (Veljovic-Jovanovic *et al.*, 2002). For quantification of H_2O_2 in leaves we have modified luminol dependent chemiluminescence method described by Warm and Laties (1982). Luminol in the presence of catalyst and hydrogen peroxide is oxidized and light is emitted.

Materials and methods

S. tuberosum (cv. Désirée) leaves were frozen in liquid N_2 and ground to a fine powder. Approximately 1g of material was extracted with 6 ml 5% (w/v) trichloroacetic acid (TCA), homogenised and centrifuged for 10 min at 12000 x g. When polyvinylpolypyrrolidone (PVPP) was used for purification, 5% (w/v) of insoluble PVPP was added to homogenate before centrifugation. Supernatant (2 ml) was applied on a column (1.4 x 8 cm) containing approximately 1 g DEAE equilibrated with 15 ml 5% TCA and eluted with 7 ml 5% TCA. 25 µl of eluate was directly added to each microplate well containing 175 µl 0.2 M NH_4OH (pH 9.5) and 25 µl 0.5 mM luminol. When ascorbate oxidase (Sigma) was used to oxidize endogenous askorbate, eluate was neutralised with 5 M K_2CO_3, pH 6 and 10µl of ascorbate oxidase (1U) was added to 500 µl of neutralised eluate. Mixture was incubated for at least 10 min at room temperature. Reaction was initiated by addition of 2.5 µl 0.5 mM $K_3Fe(CN)_6$. The emitted photons were counted over 3 s integration time in 4 cycles using Tecan GENios microplate reader.

Results and discussion

Chemiluminescence decrease in time

One of the main problems in H_2O_2 quantification by luminol dependent chemiluminescence method was decrease of chemiluminescence with time (Fig. 1). The most important factor is time between addition of ferricyanide as catalyst and start of measurement. It is extremely important that luminescence is measured immediately. For addition of ferricyanide we used multichannel pipette and at most 4 wells were measured at time, so the differences between measurements of first and last well were at most 35 s.

Effects of purification of leaves extract on intensity of detected luminescence

The lumninescence emitted by luminol is quenched by coloured compounds (Warm and Laties, 1982) and endogenous ascorbate present in the leaves (Veljovic-Jovanovic *et al.*, 2002). Because of low amount of endogenous H_2O_2 in analysed tissue 2 ml 1 mM H_2O_2 was added to 2 ml of crude extract. If only PVPP was used for extract purification there was very little difference between crude samples and those purified with PVPP. When extract was passed through DEAE column, which removes coloured compounds, the significant increase in luminescence was achieved. The obtained results indicate that purification with DEAE and ascorbate

oxidase is most efficient but results are less reliable if PVPP is omitted, because H_2O_2 could be produced by phenolic compounds in leaf extracts without ascorbate (cit. in Veljovic-Jovanovic *et al*., 2002).
In conclusion our study indicates that a lot of different substances in leaves can interfere with endogenous H_2O_2 quantification. The reliable results can be obtained only by intensive purification.

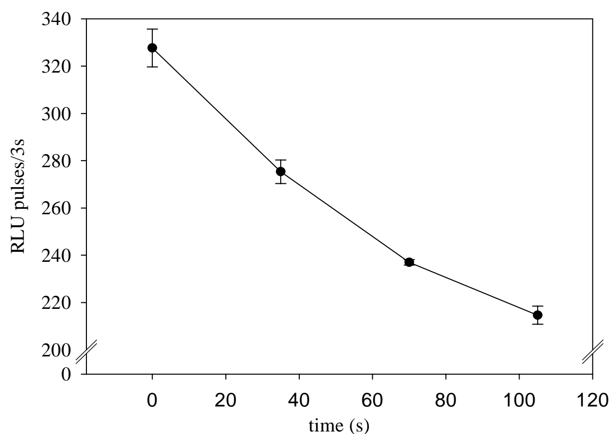

Figure 1: Chemiluminescence of H_2O_2 expressed in relative light units (RLU) measured at different time intervals after addition of $K_3Fe(CN)_6$ as catalyst.

Table 1: Comparison of used purification methods for measurement of H_2O_2 in potato leaves extracts. Quantity of H_2O_2 is expressed in relative light units (RLU).

Purification methods		
	- PVPP	+ PVPP
Extract	12.67 ± 1.76	14.00 ± 1.53
Extract + H_2O_2	55.67 ± 0.67	68.33 ± 8.95
Extract + H_2O_2 + DEAE	170.00 ± 5.69	286.67 ± 39.96
Extract+ H_2O_2 + DEAE + ascorbate oxidase	470.33 ± 32.54	440.00 ± 75.29

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