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## DIVISION OF ECOLOGY

**Head**      **František Nerud, PhD.**

A major focus in the environmental area in the *Division of Ecology* has been concentrated on physiology, biochemistry and enzymatic systems of white rot, brown rot and litter decomposing basidiomycetes, especially their ability to degrade ubiquitous recalcitrant compounds like lignocelluloses and ecologically important pollutants, oligocyclic aromatic hydrocarbons (PAH) and synthetic dyes. We have conducted both fundamental and applied studies on the elucidation of the enzymes involved in biodegradation pathways and possible use of either whole fungal cultures or isolated enzymes for the degradation of individual compounds. The effectiveness and the radical mechanisms of the degradation of priority pollutants by heterogeneous catalysts based on mixed iron oxides and polymer supported metal chelates have been determined. Another research line concerns the interactions between mycorrhizal fungi and organic matter. Besides this, the role of  $\gamma$ -globulin in noncentrosomal microtubule nucleation and organization has been followed.

The Division maintains the Collection of *Basidiomycetes* (see: <http://www.biomed.cas.cz/CCBAS/fungi.htm>) and part of this Collection significant for agriculture is now incorporated in the *National Program of Protection and Utilization of Genetic Resources of Economically Significant Plants and Microorganisms* (see: <http://www.vurv.cz/collections/vurv.exe/search>). This program was established to professionally and financially secure genetic resources of plants and microorganisms important for agricultural production and research.

Division members have been active in supervising and teaching graduate, undergraduate and high school students in research projects. Furthermore, the Division coordinates the *Center of Environmental Microbiology* and participates in the *Center of Functional Cell Organization*. In addition, staff members also cooperate with civic organizations in a project concerning the development and protection of the environment in the region Orlicko–Kladsko.

The research efforts are described in detail in the project summaries by each of the principal investigators.

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## BIOCHEMISTRY OF WOOD-ROTTING FUNGI

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### **Research field and principal results**

The research in the laboratory covers the ecology, physiology and biochemistry of saprotrophic fungi, ecology of microorganisms in the soil environment and the technological use of basidiomycetes and their extracellular enzymes along with the development of methods for the preservation of fungal cultures.

### **Cellulose and hemicellulose-degrading enzymes of wood-rotting basidiomycetes**

Polysaccharide hydrolases are indispensable for nutrient acquisition by wood-decaying fungi. We characterised the hydrolytic enzymes of a common brown-rot fungus *Piptoporus betulinus* that causes fast mass loss of birch wood. *Piptoporus betulinus* produced endo-1,4- $\beta$ -glucanase, endo-1,4- $\beta$ -xylanase, endo-1,4- $\beta$ -mannanase, 1,4- $\beta$ -glucosidase, 1,4- $\beta$ -xylos-

idase, 1,4- $\beta$ -mannosidase and cellobiohydrolase activities, but it was not able to efficiently degrade crystalline cellulose. The major glycosyl hydrolases, endoglucanase EG1 and  $\beta$ -glucosidase BG1, were purified. EG1 is a protein of 62 kDa and a pI of 2.6-2.8. It cleaves cellulose internally, produces cellobiose and glucose from cellulose and cellooligosaccharides and it also shows  $\beta$ -xylosidase and endoxylanase activities. BG1 is a protein of 36 kDa with a pI around 2.6. It is able to produce glucose from cellobiose and cellooligosaccharides but it also produces galactose, mannose and xylose from the respective oligosaccharides and shows some cellobiohydrolase activity. The fungus produces mainly  $\beta$ -glucosidase and  $\beta$ -mannosidase activity in its fruit bodies while higher activities of endoglucanase, endoxylanase and  $\beta$ -xylosidase were found in fungus-colonized wood. A significant part of hydrolytic enzymes of wood-decaying fungi *Pleurotus ostreatus*, *Trametes versicolor* and *P. betulinus* has been found in close association with fungal hyphae. The production of enzymes is largely affected by the presence of essential metal ions. During soil colonization by the saprotrophic basidiomycete *P. ostreatus*, enzyme production was affected by the presence of lignocellulose substrate. The fungus affected quantitative and qualitative composition of soil bacterial populations.

### **Degradation of xenobiotic compounds – biological and oxidative catalysis**

The screening of cultures of basidiomycetous fungi led to the identification of prospective fungal strains for the use in biotechnology. Based on the production of extracellular enzymes, hydrogen peroxide and on the potential to decolorize textile dyes, the species *Dichomitus squalens*, *Ischnoderma resinsum*, *Pleurotus calyptratus* and other species of the genus *Pleurotus* have been identified as the best degraders of xenobiotic compounds. In addition, basidiomycete fungi from forest litter have been found to combine an efficient degradation of xenobiotic compounds and the ability to grow under nonsterile conditions. Heterogeneous catalysts based on mixed iron oxides and polymer-supported metal chelates have also been found to efficiently degrade several xenobiotic compounds, e.g., oligocyclic aromatic hydrocarbons (PAHs), textile dyes and olive mill waste waters.

### **Maintenance and cryopreservation of fungi**

Cryopreservation using perlite as a carrier of fungal mycelium was successfully verified on a large number (442) of basidiomycete strains, 45 strains of *Ascomycota* (including 17 yeasts), 20 strains of *Zygomycota* and three strains of *Basidiomycota* counted among yeasts. The viability, growth and morphology of the strains were tested after 2 d and then after 1-year storage (in case of basidiomycete strains also after 2- and 3-year storage) under liquid nitrogen. In all surviving cultures no negative effects of cryopreservation by this method have been observed after storage in liquid nitrogen. The results indicate that the perlite protocol can be successfully used for cryopreservation of taxonomically different groups of fungi and also for fungi which failed to survive other routinely used preservation procedures. The cultures on perlite can be distributed directly without reinoculation and reused for successive inoculations. They also turned out to be a good substitute for agar cultures in long-term maintenance of fungi.

## Publications

- [1] Baborová P., Möder M., Baldrian P., Cajthamlova K., Cajthaml T.: Purification of a new manganese peroxidase of the white-rot fungus *Irpex lacteus*, and degradation of polycyclic aromatic hydrocarbons by the enzyme. *Res. Microbiol.* **157**, 248–253 (2006).
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- [10] Eichlerová I., Homolka L., Nerud F.: Ability of industrial dyes decolorization and ligninolytic enzymes production by different *Pleurotus* species with special attention on *Pleurotus calypratus*, strain CCBAS 461. *Process Biochem.* **41**, 941–946 (2006).
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- [14] Homolka L., Lisá L., Nerud F.: Basidiomycete cryopreservation on perlite: evaluation of a new method. *Cryobiology* **52**, 446–453 (2006).
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## EXPERIMENTAL MYCOLOGY

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### **Research field and principal results**

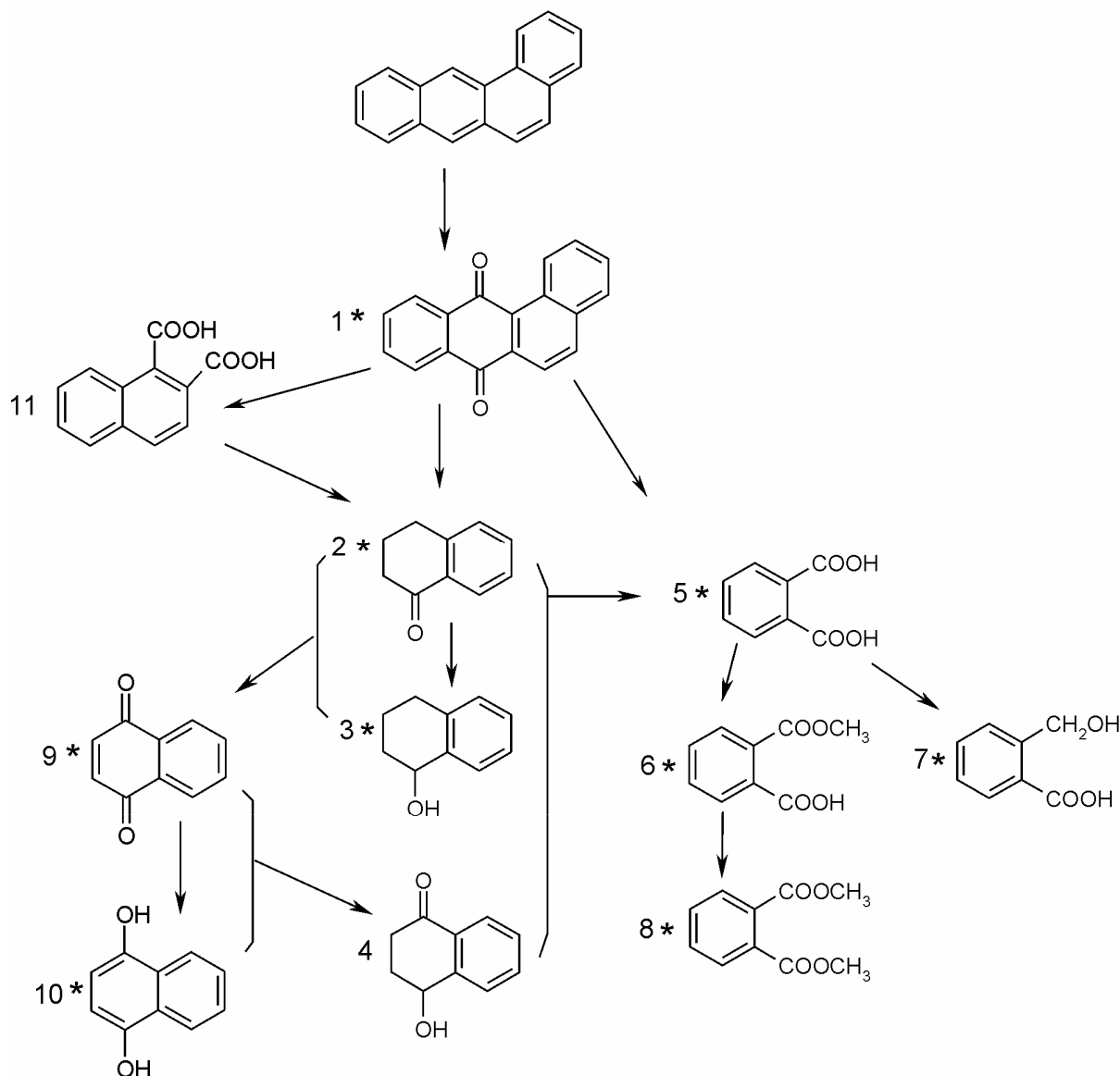
The research is mainly focused on the study of degradation of organic pollutants by ligninolytic fungi, composting and bacteria, and on exploration of mechanisms of degradation and practical utilization of microbial bioremediation technologies.

The plant determinants of symbiosis between nodule bacteria and host plants are studied in the model system *Pisum sativum*–*Rhizobium leguminosarum* bv. *viciae*.

### **Breakdown products on metabolic pathway of degradation of benz[*a*]anthracene by a ligninolytic fungus**

Benz[*a*]anthracene was metabolized by *Irpex lacteus* at the first step *via* a typical pathway of ligninolytic fungi producing benz[*a*]anthracene-7,12-dione (BaAQ). The product was further transformed in at least two ways. One of the pathways is similar to the anthracene metabolic pathway of the same fungus. Benz[*a*]anthracene-7,12-dione was degraded to 1,2-naphthalenedicarboxylic acid and phthalic acid, followed by the production of 2-hydroxymethyl-benzoic acid or monomethyl and dimethyl phthalates. Another degradation product of BaAQ was identified as 1-tetralone and its transformation *via* 1,4-naphthalene-

dione, 1,4-naphthalenediol and 1,2,3,4-tetrahydro-1-hydroxynaphthalene resulted finally again in phthalic acid. To our knowledge this is the first work reporting ring cleavage metabolites of benzo[*a*]anthracene by a ligninolytic fungus, with an elucidation of a new degradation pathway and identification of 11 new metabolites.



**Fig. 42.** Proposed pathway of benzo[*a*]anthracene degradation by the ligninolytic fungus *Irpex lacteus*. The structures labeled with asterisks were confirmed with chemical standards. The origin of some metabolites was not clarified and possible source compounds are bound with brackets.

#### Purification of a new manganese peroxidase by the white-rot fungus *Irpex lacteus*

Manganese peroxidase (MnP) from *Irpex lacteus* was purified using anion exchange and size exclusion chromatography. SDS-PAGE showed the purified MnP to be a monomeric protein of 37.5 kDa with an isoelectric point at 3.55. The pH optimum was relatively broad, from 4.0 to 7.0, with a peak at pH 5.5. Kinetic constants  $K_m$  were 8  $\mu\text{M}$  for  $\text{H}_2\text{O}_2$  and 12  $\mu\text{M}$

or 31  $\mu\text{M}$  for  $\text{Mn}^{2+}$  depending on the substrate. MnP was active at 5–70 °C with an optimum between 50–60 °C. At temperatures above 65 °C the enzyme rapidly lost activity. Degradation of four representatives of PAHs was tested and the enzyme showed the ability to degrade them *in vitro*. Major degradation products of anthracene were identified. The results confirm the role of MnP in PAH degradation by *I. lacteus* including the cleavage of the aromatic ring.

### **Application of supercritical fluid extraction (SFE) for predicting bioremediation efficacy**

Supercritical fluid extraction (SFE) with pure carbon dioxide was used to obtain desorption curves of oligocyclic aromatic hydrocarbons (PAHs) from four contaminated industrial soils. The desorption curves were fitted with a simple two-site model to determine the rapidly released fraction ( $F$ ) representing bioavailability of PAHs. The  $F$  data obtained under various SFE pressures were compared with degradation results of the composting method applied on the soils. The  $F$  values gave very good agreement with degradation efficiencies and the total regression coefficients ( $r^2$ ) ranged from 0.81 to 0.99. The results indicate that SFE could be a rapid test for predicting bioremediation results of composting of PAH-contaminated soils.

### **Biodegradation of industrial dyes by ligninolytic fungi**

Potential for removal of water-soluble organopollutants by ligninolytic fungi growing in liquid media and immobilized on solid support and the involvement of laccase (LAC) isoenzymes have been investigated. *Dichomitus squalens* and *Irpex lacteus* were used as model organisms.

Immobilization of *D. squalens* on pine wood (PW) improved LAC production, compared to liquid cultures and polyurethane foam-immobilized cultures. Two different chromatographical forms of LAC were detected that had identical molecular masses of 68 kDa and similar  $pI$ 's but differed in their catalytic properties such as pH dependence of the activity and ABTS oxidation rates. Different dye decolorization capacities of Lc1 and Lc2 were also demonstrated.

*I. lacteus* was used in cooperation with activated sludge (AS) cultures for the biodegradation and detoxification of various dye structures. A possible use of the fungus in combination with AS was demonstrated. Fungal treatment of a spectrum of model dyes as well as of three reactive dyes in real dyehouse wastewaters showed efficient decolorization without any re-colorization effect observed. A combined two-step scheme of treatment of textile wastewater was designed and examined, the dye being removed in the 1st step using fungal trickling filter and the rest of degradable organic carbon during the second step using an AS trickling filter.

### **Role of pea symbiotic genes in rhizobial symbiosis establishment**

Pea gene *SYM8* is a key determinant of nodule bacteria recognition. Its genomic sequence was identified as a homolog of the model legume *Medicago truncatula DM11* and its function was confirmed by the presence of a single nucleotide change in the mutant line Risnod27 and by its cosegregation with the asymbiotic phenotype in recurrent backcrosses. The unique location of the symbiotic mutation His309Tyr in the selectivity filter of the putative ionic channel coded by *SYM8* allows for a conclusion about the anionic specificity of the molecule.

## Publications

- [1] Baborová P., Möder M., Baldrian P., Cajthamlová K., Cajthaml T.: Purification of a new manganese peroxidase of the white-rot fungus *Irpex lacteus* and degradation of polycyclic aromatic hydrocarbons by the enzyme. *Res. Microbiol.* **157**, 248–253 (2006).
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## FUNGAL BIOLOGY

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## **Research field and principal results**

### **Interactions between mycorrhizal fungi and soil organic matter**

Arbuscular mycorrhizal fungi, an important component of soil microflora, are considered biotrophic organisms growing under full control of their host plants, and effects of soil environment including humic substances on mycorrhizal symbiosis are generally neglected. Our laboratory discovered strong positive effects of humic acid as well as fulvic acid (both constituting a considerable portion of soil humus) on the growth of the mycelium of arbuscular mycorrhizal fungus *Glomus claroideum* and mycorrhizal root colonization of maize. Application of soluble humic material strongly affects soil microflora in terms of abundance of saprotrophic fungi and bacteria. The addition of saprotrophic microorganisms able to utilize humic substances to the cultivation system further stimulated the mycorrhizal fungus. The results may help to improve cultivation of the inocula of arbuscular mycorrhizal fungi.

The effects of humic substances on ectomycorrhizal fungi are also currently studied with promising results. The quality and concentration of humic substances in the soil represents an important factor determining the composition of a cenosis of mycorrhizal fungi in the soil environment.



**Fig. 43.** *In vitro* culture of *Pinus sylvestris* inoculated with ectomycorrhizal fungus. The substrate contains humic acid stimulating the growth of fungal mycelium.

Our further works revealed the positive effects of organic fertilization on the development of arbuscular mycorrhizal symbiosis in a long-term field experiment. The results of root mycorrhizal colonization followed the same pattern as the length of hyphae of mycorrhizal fungi in soil samples and 16:1 $\omega$ 5 signature fatty acid, but did not correlate with spore counts. Our results indicate that the measurement of signature fatty acids in the soil is a useful tool for providing information in the characterization of soil microflora.

### Biological chlorination of humic substances

Humic substances are chlorinated in the soil resulting in a production of natural chlorinated organic compounds, the intermediates of humus degradation. Our results indicate that this process is mediated mainly by the activity of soil microorganisms. At the same time, composition of soil microbial cenosis is affected by the concentration of mineral chloride. Our research is directed at elucidating the effect of soil chlorine cycle on the stability of soil organic matter.

### Publications

- [1] Gryndler M., Hršelová H., Sudová R., Gryndlerová H., Řezáčová V., Merhautová V.: Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG23 is stimulated by humic substances. *Mycorrhiza* **15**, 483–488 (2005).
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## FUNCTIONAL CYTOLOGY

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## Research field and principal results

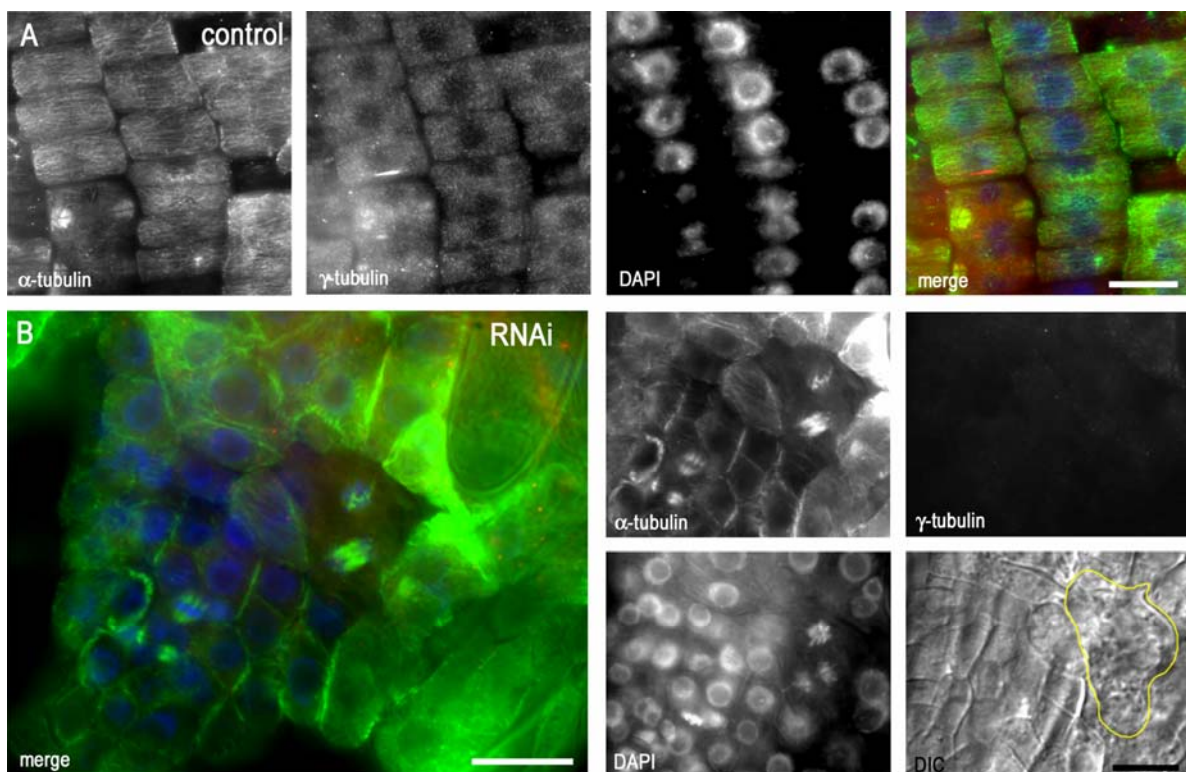
The overall aim of our research is to elucidate the mechanisms underlying noncentrosomal microtubule nucleation and organization.

### **$\gamma$ -Tubulin is essential for microtubule nucleation from dispersed sites**

It has recently emerged that microtubules nucleated independently of centrosomes play an important role in designing cytoskeleton architecture.  $\gamma$ -Tubulin is required for formation of microtubules at centrosomes, but its function in noncentrosomal microtubule nucleation is not understood.

We found that although  $\gamma$ -tubulin in acentrosomal plant cells is not associated with discrete polar centers, punctate staining on mitotic microtubules and in the polar region of dividing cells shows bipolarity from prophase to telophase (Fig. 44A). Study of the  $\gamma$ -tubulin distribution in cellular fractions of *Arabidopsis* using SDS/BN-PAGE revealed that membrane- and microtubule-associated  $\gamma$ -tubulin is present in the form of large protein complexes that are active in microtubule nucleation.

To further analyze the role of  $\gamma$ -tubulin, we conditionally downregulated  $\gamma$ -tubulin by inducible expression of RNAi constructs in *Arabidopsis*. After induction of RNAi,  $\gamma$ -tubulin was gradually depleted from all cellular locations. We found that  $\gamma$ -tubulin as a component of cortical nucleation templates guides cortical microtubules. The regrowth of microtubules from the perinuclear membrane rich region after drug-induced depolymerization was delayed in cells with reduced  $\gamma$ -tubulin levels. Similarly, immunodepletion of  $\gamma$ -tubulin from *Arabidopsis* extracts strongly compromised the *in vitro* polymerization of microtubules. An almost complete RNAi depletion of  $\gamma$ -tubulin led to the absence of microtubules. *In vivo* microscopical analysis revealed that GFP-microtubules were bundled and less dynamic in RNAi cells. In summary, we showed that  $\gamma$ -tubulin is essential for MTs nucleation from dispersed sites in acentrosomal plant cells.



**Fig. 44.** Aberant cytokinesis with misorientated cell division planes is the most common defect observed in *Arabidopsis* cells with reduced  $\gamma$ -tubulin. Compared to the precisely organized cell files of the control (**A**), patches of cells with defective arrangements of cell walls are observed in RNAi expressing plants with severely reduced  $\gamma$ -tubulin levels (**B**). Bars correspond to 10  $\mu$ m.

#### $\gamma$ -Tubulin is important for coordination of late mitotic events and cell polarity

Cells with decreased levels of  $\gamma$ -tubulin could progress through mitosis, but late mitotic events and cytokinesis were strongly affected. Particularly, we observed that polar distribution of  $\gamma$ -tubulin during late mitosis was disturbed and the phragmoplast formation failed. In contrast to the control cells where anaphase spindles were rearranged into the phragmoplast, long anaphase spindles persisted between separated nuclei in RNAi cells. The cell plate form-

ation sites were often misaligned (Fig. 44B). These discrepancies in late mitosis and cytokinesis often resulted in bi- or multi-nuclear cells and disruption of regular cell files. Ectopic root hair formation was observed in cells with randomized microtubules, anisotropic growth of root hairs was disturbed, formation of two growth axes was often observed. We suggest that some functions of  $\gamma$ -tubulin that are important for cytokinesis, cell specification and polar growth might be microtubule independent.

Co-immunoprecipitation experiments revealed several putative  $\gamma$ -tubulin interacting proteins. They were cloned using Gateway technology and inserted into selected target vectors including RNAi vectors and GFP/RFP fusion vectors for live cell imaging.

### **Oxidative mechanisms of lignocellulose breakdown by brown rot and litter-degrading basidiomycete fungi**

Pyranose dehydrogenase (PDH; discovered recently in our laboratory and assigned EC 1.1.99.29) of *Agaricus xanthoderma* is a monomeric glycoprotein containing a covalently bound FAD. The PDH polypeptide consists of 575 amino acids and has molar mass of 65.4 kDa as determined by MALDI MS. The homology model of PDH suggests a novel type of bi-covalent flavinylation in PDH, 9-S-cysteinyl, 8- $\alpha$ -N<sup>3</sup>-histidyl FAD. PDH from *A. meleagris* catalyzed transformations of a number of reducing sugars to new mono- and dicarbonyl sugar derivatives with an application potential.

A novel alcohol oxidase (AOX) purified from the brown rot basidiomycete *Gloeophyllum trabeum* was characterized as a homooctameric nonglycosylated protein containing non-covalently bonded FAD. The isolated AOX cDNA encoded a polypeptide of 651 amino acids displaying 51–53 % identity with other known fungal AOX sequences. Immunofluorescence and TEM immunogold labeling localized the enzyme in hyphal periplasmic space and extracellular membranous/slime structures secreted by hyphae in wood fiber lumina and within secondary cell walls of degraded wood fibers. The differences in AOX targeting compared with yeast peroxisomal localization were traced to a unique C-terminal sequence of the *G. trabeum* oxidase. These findings point to a possible role for AOX as a major source of H<sub>2</sub>O<sub>2</sub>, a component of Fenton's reagent implicated in the generally accepted mechanisms for brown rot through production of highly destructive hydroxyl radicals.

### **Publications**

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